STRAIN IMPROVEMENT AND CHARACTERIZATION OF ANTIBIOTIC PRODUCING MICROORGANISMS FROM SOIL

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DECLARATION

I, **Khwathisi Adivhaho,** declare that this dissertation for Masters in Microbiology (MScMB) entitled “Strain improvement and Characterization of Antibiotic Producing Microorganisms from Soil” is my original work and has never existed before or written to any other institution for any degree. I declare that this is my own work in purpose and execution with no other exudation. In the case where some of the information is from an external source, all the sources that I have quoted, have been acknowledged by means of a complete reference list.

Signed (Student) ……  ……Date…**21 April 2021**……

DEDICATION

This work is dedicated to my son Ronewa, you have your Daddy's same kind heart and compassionate nature, hold on to that, even when life gets hard. There will be times in your life where you'd rather hide or run or bury your head in the sand than face whatever challenge is in your way. I know because I've been there many times. In those times, I want you to remember to be brave and show courage.

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LIST OF ABBREVIATION

%	Percentage
G	Gram
°C	Degree Celsius
H	Hour
µl	Microliter
DNA	Deoxyribonucleic acid
MRSA	Methicillin-resistant Staphylococcus aureus
MDR	Multidrug-resistant
rDNA	Ribosomal Deoxyribonucleic acid
PCR	Polymerase chain redaction
Spp.	Species
rRNA	Ribosomal ribonucleic acid
APS	Antibiotic-producing species
ZOI	Zone of inhibition
dH2O	Distilled Water
NMR	Nuclear magnetic resonance spectroscopy
bp	base pair
BLAST	Basic Local Alignment Search Tool
NaCl	Sodium chloride

SUMMARY

Since the discovery of Antibiotics in the 20th century, the idea of searching for antimicrobial compounds from natural sources came into existence. However natural products from microbial origin (especially soil microorganisms) have grasped a great devotion over the course of several decades. Recently, bacterial resistance have been observed against antibiotics of all classes, however it appears that the emergence of antimicrobial resistance is inevitable to almost every new drug.This necessitates carrying out studies that will generate new effective antibiotics. The present study is an attempt to identify, characterize and improve strains of bacteria for the ability to produce antibiotics.

About 12 soil samples were screened for antibiotic-producing bacteria against 4 pathogenic microorganisms including *Escherichia coli, Klebsiella pneumoniae Staphylococcus aureus* and *B. subtilis*. After preliminary screening, active isolates with secondary metabolites showing activity were selected for secondary screening by agar well diffusion method to identify antibiotic potency. VITEK 2 system was used for rapid identification of the active isolates. The amplification of the 16s rRNA by PCR followed by sequencing and sequence analysis was used for the molecular identification of these strains. Optimization of chemical and physical culture conditions was carried out by manipulation of fermentation parameter such as pH, Temperature and incubation period.

The results revealed 7 strains of antibiotic producing organisms. 4 bacterial strains demonstrated convincing growth inhibitory properties against pathogenic test organisms. Of these, were identified as Gram-positive cocci and 1 was identified as Gram-negative of the group of Rods and most of the isolates were active against the

Gram positive than gram negative pathogens. Phylogenetic analysis of amplified 16S rRNA gene showed the isolates shared sequence identities of 99.65% with known *Staphylococcus and Pseudomonas* species. TSH2 and TSP3 clustered together with a sequence identity of 99.68% with *Staphylococcus sciuri*. Isolate TSP1 sequence had a sequence identity of 100% with *Pseudomonas formosensis strain CC-CY503.* The production of antimicrobial substances started on the 4th day and went on increasing till it reached a maximum peak on the 7th day. The optimum growth conditions were pH 7.5, temperature at 35°C, and incubation period in 7 days.

In conclusion, the results of the present study indicate that soil contain great diversity of antibiotic producing organisms and the production of antimicrobial substances can be improved by manipulating the growth conditions.

Keywords: 16S rRNA, Antibiotics, antimicrobial resistance, Soil microbes, VITEK 2, Optimization

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Antibiotics, technically are defined as chemical substances of microbial origin known to possess antimicrobial activities (Berdy, 2005). They generally act at low concentrations and are deleterious to the growth or metabolic activities of other microorganisms, and have been an expenditure for centuries (Roszak and Colwell, 1987). Genetic and molecular techniques have been applied to demonstrate conclusively that microorganisms have the capacity synthesize a variety of antibiotics, even under field conditions, such as in endophytes and plants rhizosphere (Gaskins et al., 1985). Studies have shown that soil is rich in microorganisms capable of antibiotic synthesis, although the frequency with which synthesis occurs at ecologically significant levels in nature has been much less clear (Thomashow et al., 1997).

The massive mainstream of novel antibiotics has been detected by screening “wild isolates” obtained from soil and other natural habitats. Antibiotics are produced by bacteria, fungi, *actinomycetes*, algae, lichens and green plants (Korzybski et al., 2013). *Actinomycetes* are known to have the maximum ability to produce antibiotics. They are a group composed of branching unicellular microorganisms. Among *actinomycetes*, the *Streptomycetes* are dominant. Important species of bacteria known to produce a total of more than 5,000 compounds of antibiotics detected between 1945 and 1978 originated from this genus (Muhammad et al., 2009). However, only a few have been commercially used to treat human, animal and plant diseases.

Actinomycetes are Gram-positive bacteria with high guanine + cytosine content of over 55% in their DNA, which have been recognized as sources of several secondary metabolites, antibiotics, and bioactive compounds that affect microbial growth. *Actinomycetes* encompass a wide range of bacteria (Chaudhary et al., 2013). They have a universal occurrence and play an active part in the cycle of nature. *Actinomycetes* have filamentous nature, branching pattern, and conidia formation, which are similar to those of fungi. *Actinomycetes* produce branching mycelium which may be of two types, that is substrate mycelium and aerial mycelium. Around 23,000 bioactive secondary metabolites from microorganisms have been reported, and over 10,000 of these are from *actinomycetes*, constitute 45% of all bioactive microbial metabolites (Berdy, 2005).

A large number of *actinomycetes* have been isolated and screened from the soil in the past several decades, accounting for 70%-80% of relevant secondary metabolites available commercially (Khanna et al., 2011). *Actinomycetes* are a potential source of many bioactive compounds, which have diverse clinical effects and important applications in human medicine. It has been estimated that approximately one-third of the thousands of naturally occurring antibiotics have been obtained from *actinomycetes*. So, the searching for novel actinomycete constitutes an essential component of natural product-based drug discovery is appreciably in recent years (Sudha and Hemalatha, 2015).

Globally, drug resistance and re-emerging diseases have being a growing concern in the agriculture, food and medical industry resulting in the need for antimicrobial discovery and improved treatments of these infections. This even more relevant to the hospitals, where antibiotic resistance is immediately life-threatening, and is becoming a rapidly growing concern (Spellberg et al., 2008). Some bacteria are naturally

resistant to antibiotics, whereas in others resistance is acquired by different mechanisms such as mutation in the genes, acquisition of new genetic elements encoding for self-protective enzymes and protein (Jayaraman, 2009).

1.2 STUDY RATIONALE

Over the last decade, it has become clear that the widespread emergence of acquired resistance to antibiotics in bacteria constitutes a serious threat to public health. This is therefore threatening the capacity to treat regular diseases and raising a serious problem to the public health throughout the world (Sahoo, 2008). Recently, bacterial resistance have been observed against antibiotics of all classes, however it appears that the emergence of antimicrobial resistance is inevitable to almost every new drug (Kapoor et al., 2017). Henceforth the need to search for antibiotics with new modes of action, through natural products screening, mostly of soil microorganisms.

Soil has been known for a long time to be the source of antimicrobial compounds which form the backbone for the treatment of infectious diseases (Wellington et al., 2013). The treatment of infectious diseases caused by pathogenic bacterial and fungal strains is one of the most reoccurring problems in the clinical field. The resistance and adverse effects of the present antimicrobial compounds on some bacterial or fungal organisms persuaded the researchers to synthesize novel and more potent inhibitory compounds (like azoles and quinolones derivatives) (Moshafi et al., 2011).

Conversely, problems with rediscovery of known strains and compounds led to a decline in discovery efforts during the second half of the 20th century as the cost of screening increased, hence making it less attractive for industry. This encouraged investigators to modify the existing basic classes of antibiotics instead of searching for

new ones from nature(Tenson and Mankin, 2006). Though, most international drug companies complain on several issues. Namely, the lack of chemical diversity against targets, the difficulty of differentiating new antibiotics without embarking on huge trials, the financial risks of proving novelty and the habit of reserving truly new antibiotics by hospitals for emergencies (Okudoh, 2010).

The improvement of microbial strains is known to play a very imperative role in reducing production costs during industrial fermentation. The conventional method of “mutation and screening” has been widely used with great success, however it is very time-consuming and labor-intensive. Genetic engineering has been applied to improve the production level of useful compounds. Nevertheless, the use of genetic engineering to generate high-yield strains of a particular product has succeeded only in a few cases, and it is still a considerable challenge to improve the production yields of most industrial strains (Magocha et al., 2018). To date strain improvement of microorganisms to obtain high titers of secondary metabolites that are more suitable for industrial fermentations has depended largely on random mutagenesis and selection techniques. This study seeks to isolate, identify, improve the production, and further characterize the microorganism that are able to produce secondary metabolites with antibacterial properties isolated from soil.

1.3 OBJECTIVES OF THE STUDY

1.3.1 PRIMARY OBJECTIVE

• To isolate and characterize microorganisms from soil with the potential to produce antibiotics.

1.3.2 SECONDARY OBJECTIVES

- To isolate and identify microorganisms from soil using morphological and biochemical methods.
- To determine the spectrum of antimicrobial activity of the extracts obtained from the isolates against some clinically important bacteria.
- To characterize the isolated microorganisms that secrete compounds with antibacterial properties from soil using 16S rDNA genotyping.
- To optimize production of the antibiotic by manipulation of the fermentation parameters such as Temperature, pH and incubation period.
- To test the inhibitory efficacy of the isolates against selected pathogens using Agar well diffusion.

CHAPTER 2

LITERATURE REVIEW

2.1 SOIL MICROBES

Soil is a heterogeneous mixture of particles that are carrying different amounts of contaminants. It contains a high diversity of microorganisms (Nannipieri et al., 2003). The numbers and kinds of microorganisms present in the soil are dependent on many environmental factors such as soil type, nutrient availability, the degree of aeration, pH, temperature and soil moisture (Horwath, 2003). The soil is therefore regarded heterogeneous with respect to conditions for microbial growth and for the distribution of microorganisms and matrix substances.

In recent years, drug resistance and re-emerging diseases have been a growing concern globally, resulting in the necessity for antimicrobial discovery. However, several attempts made using traditional cultivation methods have failed to discover new antimicrobial products (Pandey et al., 2000). This is because isolate culture extracts often produced numerous previously described metabolites (Mincer et al., 2002) and the rate of the rediscovery of known antimicrobial has approached 99.9% (Silver, 2011).

It is reported that each gram of soil contains 10^6-10^8 bacteria, 10^4-10^6 *actinomycetes* spores, 10^2-10^4 fungal spores but only 1% of the greater than a million cells in each gram of soil seems able to form colonies on laboratory media implying that majority have eluded cultivation, therefore, their potential of producing antibiotics cannot be explored (Seneviratne et al., 2013).

2.2 ANTIBIOTICS

They are defined as "the complex chemical substances, the secondary metabolites which are produced by microorganisms and act against other microorganisms" (Croteau et al., 2000). In nature, there is a universal distribution of antibiosis among the microorganisms owing to which they are involved in antagonism. However, antibiotics produced by microorganisms have been very useful for the cure of certain human diseases caused by bacteria, fungi, and protozoa (Demain and Sanchez, 2009). Due to continuous endeavour made in this field, the number of antibiotics discovered at present is about 5,500. The total world production of antibiotics is more than one million tons per annum. This success has been possible only due to continuous research made during the last 4 decades.

Although generally, antibiotic refers to antibacterial, the term is loosely defined, and preferable to specify compounds as being antibacterial, antifungal and antiviral. However, this theory now is broadened to consist of any chemical compound of natural origin that has an effect against the growth of other organisms (Avise, 2012). Scientists distinguish this sort of antimicrobial agents from another group called chemotherapeutic agents which are chemically synthesized. Furthermore, a mixture of these two concepts now yields some antimicrobial compounds that are produced by organisms and chemically modified to obtain some desired properties (semi-synthetic antibiotics) (Koehn and Carter, 2005).

2.3 HISTORY OF ANTIBIOTIC DEVELOPMENT

The derivation of the term antibiotic can be traced back to 19th century. It was coined or first used as an antonym to symbiosis to describe the antagonistic action between

different microorganisms(Vuillemin, 1980). However it was later used to describe secondary metabolites which are produced by microorganisms that either has the capability to inhibit or kill other microorganisms. The term “antibiotic” is derived from two words: “anti” means against and “biota” means life. To date the term has a broader meaning, generally, antibiotic refers to antibacterial, the term is loosely defined, and preferable to specify compounds as being antibacterial, antifungal and antiviral(Page and Gautier, 2012). However, this theory now is broadened to consist of any chemical compound of natural origin that has an effect against the growth of other organisms. Scientists distinguish this sort of antimicrobial agents from another group called chemotherapeutic agents which are chemically synthesized(Wright, 2017). Furthermore, a mixture of these two concepts now yields some antimicrobial compounds that are produced by organisms and chemically modified to obtain some desired properties (semi-synthetic antibiotics).

The interest in antimicrobial chemotherapy was kindled as soon as the French chemist and bacteriologist Louis Pasteur contributed decisively to our understanding of the underlying causes of infectious diseases. In earlier times, Soil and plant products were sometimes used successfully in the treatment of disease (Hoitink et al., 1997), but neither doctors nor patients understood the basis for the mechanism of action of these therapeutic agents. The first antibiotic to be discovered from nature was mycophenolic acid which was isolated from *Penicillium glaucum (P. brevicompactum)* in 1893 by the Italian physician and microbiologist Bartolomeo Gosio. At that time it showed to inhibit the growth of *Bacillus anthracis* and then later it also showed to be effective or possess antiviral, antifungal, antitumor and anti-psoriasis properties. However, its discovery remained unnoticed until it was rediscovered in United States in 1913.

The very first commercially available antibacterial was Prontosil, a sulphonamide developed by the German biochemist Gerhard Domagk in the 1930s. Before this, in 1928, Fleming had discovered the first antibiotic, penicillin, but it took over a decade before penicillin was introduced as a treatment for bacterial infections. A series of different antibiotics were quickly discovered after penicillin came into use. Many discoveries were of drugs that were too toxic for human use, or that had already been discovered. In addition to soil, many of these drugs were discovered by isolating the producing microorganisms from interesting and unusual sources (Fenical and Jensen, 2006). Unfortunately, since the 1970's, only one new class of antibiotics has been introduced and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action(Fair and Tor, 2014).

2.4 CLASSIFICATION AND NOMENCLATURE OF ANTIBIOTICS

The classification of antibiotic involves distinct factors. Therefore, there are several methods of antibiotic classification that have been used by various authors. Some methods are based on the mode of action of compounds, e.g. whether they act on the cell wall or membrane or are inhibitors of protein or nucleic acid synthesis/functions, or interfere with the whole system of cellular metabolism. Antibiotics have also been classified according to the route of biosynthesis (Kudo and Eguchi, 2009). However, several different biosynthetic routes often have much commonality, making such classification difficult. The spectra of organisms killed/inhibited have also been used e.g. those affecting specifically bacteria, fungi, protozoa etc. However, some antibiotics belonging to a particular group may have a different spectrum from others in the same group. For instance, paromomycin is generally classified as antiprotozoal,

when its entire spectrum of activity and chemical structure is almost identical with that of other amino-glycosides (viz. neomycin, kanamycin) which are regarded as antibacterial antibiotics (Okudoh, 2001).

In some cases, antibiotics have been classified on the basis of the producing organism. However, some organisms may produce several antibiotics, e.g., the production of penicillin N and cephalosporin C by *Cephalosporium acremonium*. Alternatively, the same antibiotic may be produced by different organisms. For example, penicillin has now been shown to be produced by a wide range of organisms including species of the fungi *Aspergillus, Cephalosporium*, and *Trichophyton*, and some actinomycetes, such as *Streptomyces spp* (Kudo and Eguchi, 2009).

2.5 MECHANISM OF ANTIMICROBIAL ACTION

The antimicrobial potency of most classes of antibiotics are directed at some unique feature of the bacterial structure or their metabolic processes. However, it is important for a successful antibiotic to be selective in its mechanism of action, that is, it must have the ability to penetrate and concentrate in the microbial cell and should not interact with the cells of the host. The most common targets of antibiotics are illustrated in Figure 2.1. They should show suitable absorption and distribution properties within the host body (Schwartz et al., 1983).

Antimicrobial agents generally bring about microbial inhibition by interacting with specific cellular components and disordering cell metabolism. They block the growth of sensitive microorganisms by inhibiting the action of a macromolecule, such as enzymes or a nucleic acid essential to the function of the cell. This means that the

antibiotic molecule is able to bind to a specific site on the target macromolecule, forming a complex that is useless to the cell (Bergmann and Peppas, 2008).

Generally, Antibiotics essentially target bacterial structures or functions, such as cell wall biosynthesis (e.g., vancomycin), translation (e.g., streptomycin), RNA transcription (e.g., rifampicin), DNA replication and synthesis (e.g., novobiocin and metronidazole), membrane (polimyxins), and in general they inhibit bacterial growth (Figure 1)(Chifiriuc et al., 2016).

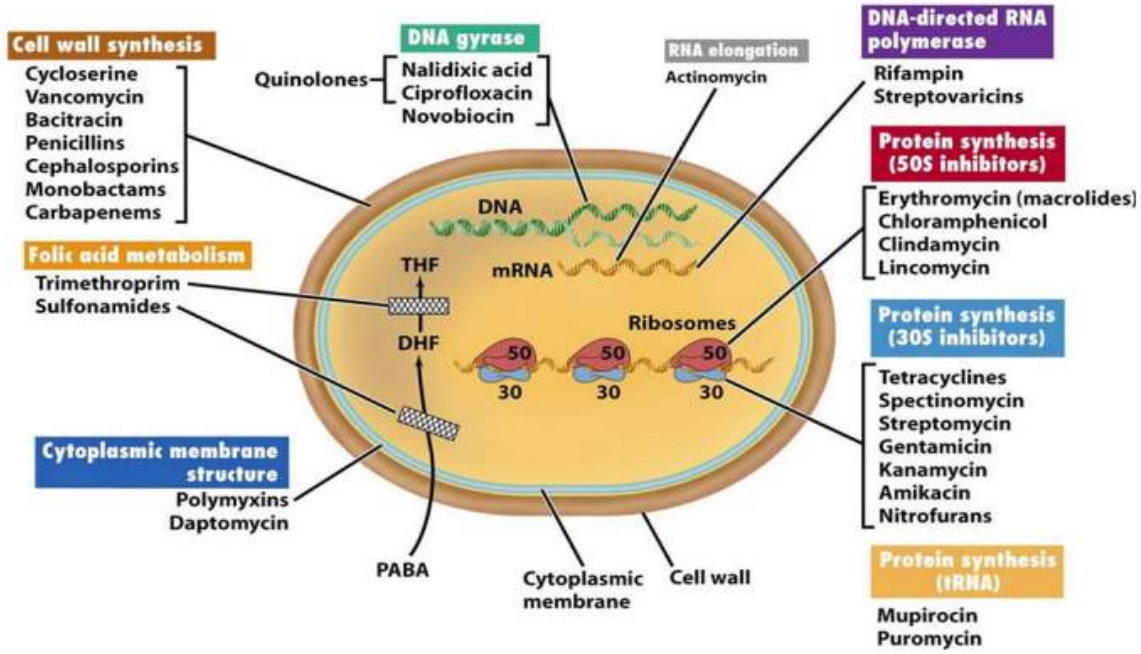


Figure 2.1: Schematic diagram showing principal antibiotic target sites (Madigan et al., 2006).

2.5.1 INHIBITORS OF CELL WALL SYNTHESIS

Examples include Penicillin, Cephalosporin, Cycloserine and Vancomycin (Hammond and Lambert, 1978). About 50 - 70% of the Gram - positive bacterial cell wall mass and to a lesser extent (10 - 15%) in Gram-negatives is composed of peptidoglycan (sometimes referred to as Murein or Mucopeptide). Its cross-linked structure provides a tough, fibrous fabric, giving strength and shape to the cell and enabling it to withstand a high internal osmotic pressure (Franklin and Snow, 2005).

Most of the antibiotics in this group affect peptidoglycan synthesis, while others interfere with the synthesis or assembly of other components of the wall, e.g. teichoic acid. β-Lactam antibiotics (penicillins, cephalosporins), so-called because of the presence of a cyclic amide forming a four-atom ring (lactam ring) in the molecule, all have a similar but not identical mechanism of action. They prevent peptidoglycan maturation by inhibiting the cross-linking of the linear peptidoglycan strands(Lancini and Parenti, 2013). Action of these antibiotics ultimately results in bacterial cell lysis.

2.5.2 INHIBITORS OF CELL MEMBRANE FUNCTIONS

Examples include polymyxin, tyrocidin, valinomycin and amphothericin B (Hammond and Lambert, 1978). Cell membranes have very similar constituents throughout the phylogenetic ladder from bacteria to mammalian cells. The only important difference is that there are no sterols in bacterial cell membranes, while zymosterol and ergosterol are present in the cell membranes of fungi and plants, and cholesterol in those of mammals. Some antibiotics in this group disorganize the super-molecular structure of the membrane, thus causing loss of cellular substance to the outside, while some act as carriers of specific ions (Ionophores) and cause an abnormal accumulation of ions inside the cell (Lancini and Parenti, 2013).

Polymixins and tryocidin are both cyclic polypeptide antibiotics. Their action disturbs membrane function by allowing leakage of cytoplasmic components (Ca2 + ions; Mg^{2+} ions) and uncoupling of oxidative phosphorylation (prevention of ATP generation during sugar oxidation). Valinomycin specifically drains the cell of potassium ions (K^{+}) and growth ceases because of the requirement for potassium in protein biosynthesis (Franklin and Snow, 2005)). Amphotericin B, a typical polyene antifungal antibiotic, creates instability in the membrane, by forming complexes with sterol components of the membrane that alter trans-membrane cation permeability. Most inhibitors of cell membrane functions are non-selective and consequently are too toxic to be given systemically, and are therefore used exclusively as antiseptics or topical agents(Schwartz et al., 1983).

2.5.3 INHIBITORS OF PROTEIN SYNTHESIS

Examples include streptomycin, tetracycline and chloramphenicol. Some antibiotics in this group affect amino acid activation and transfer reactions, while others interfere with the functions of the 30S or 50S ribosomal subunit (Hansen et al., 2003). Streptomycin and tetracycline distort the 30S ribosomal subunit enough to prevent normal bonding between the codon of mRNA and the anti-codon of tRNA. This causes mis-coding of the proteins, bringing normal protein synthesis to a halt (Hansen et al., 2003).

2.5.4 INHIBITORS OF TRANSCRIPTION AND REPLICATION OF GENETIC MATERIAL (NUCLEIC ACIDS)

This group include, rifamycin, actinomycin D and acridine dyes (Goldberg and Friedman, 1971). The synthesis of DNA and the various classes of RNA is an essential function of dividing and growing cells. Thus inhibition of DNA synthesis rapidly results in inhibition of cell division (McKenna and Davies, 1988). Rifamycins are enzyme inhibitors. They bind to, and inhibit the DNA dependant RNA polymerase of sensitive bacteria, but the precise mechanism of inhibition is still uncertain(Hansen et al., 2003).

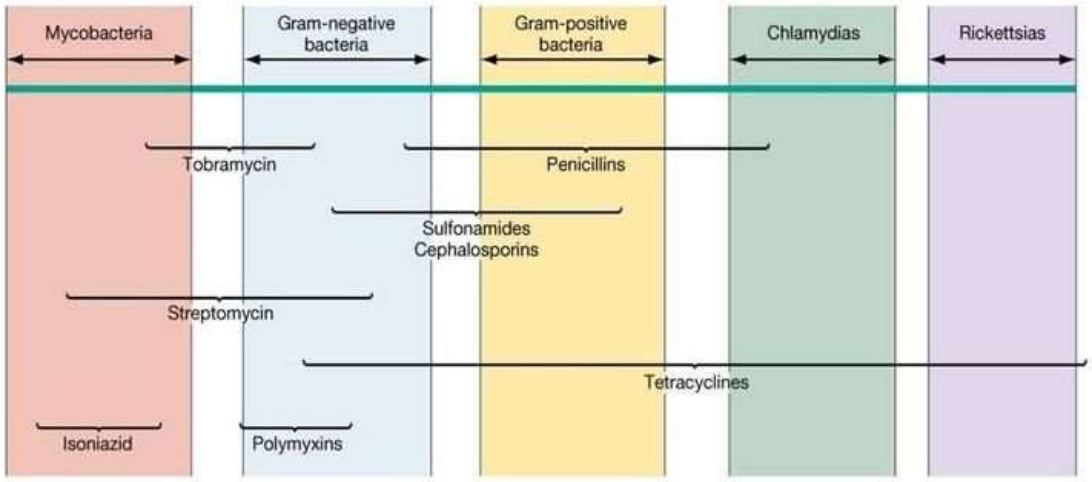
Actinomycin D molecules insert themselves into the smaller groove of the DNA double helix and form a reversible complex bound by hydrogen bonds. The complex does not permit RNA polymerase to travel along the DNA template thereby inhibiting synthesis of RNA (Kellett et al., 2019). Acridines show antimicrobial activity by distorting the sugar phosphate backbone of the DNA helix created by the intercalated dye molecules(Franklin and Snow, 2005). Most of the inhibitors of genetic material are used in cancer chemotherapy rather than as antimicrobial agents. Unfortunately, many of these do not show sufficient selectivity against the tumour cells and are too toxic to be used effectively(Mader and Hoskin, 2006).

2.6 SPECTRUM OF ANTIMICROBIAL ACTIVITY

Several studies have classified or group antibiotics based on their ability to act on different bacterial groups (Table 2.1). Spectrum of antimicrobial activity categorises antibiotics or antibacterial based on their target specification. The range of bacteria that an antibiotic affects can be divided into narrow spectrum and broad spectrum. Narrow spectrum antibiotics are active against a selected or limited group

of bacterial types, either Gram-positive or Gram-negative. For example, Clarithromycin which is used to treat a wide variety of bacterial infections, is known to be effective against bacteria responsible for upper respiratory infections. Narrow-spectrum antibiotics do not kill as many of the normal microorganisms in the body as the broad-spectrum antibiotics and thus have less ability to cause super-infection(Naveed et al., 2020). Additionally, the narrow-spectrum antibiotic will cause less resistance of the bacteria as it will deal with only specific bacteria.

Table 2.1: Antimicrobial activity by antibiotics. Table taken online at https://www.slideshare.net/AhmedOmara6/antibiotic-59860992)



A broad spectrum antibiotic acts against both Gram-positive and Gram-negative bacteria, in contrast to a narrow spectrum antibiotic, which is effective against specific families of bacteria (Figure 2.2). For example, ampicillin, a broad-spectrum antibiotic which is used to treat a wide variety of bacterial infections. Broad spectrum antibiotics are also used for drug resistant bacteria that do not respond to other, narrower spectrum antibiotics and in the case of super-infections, where there are multiple types

of bacteria causing illness, thus warranting either a broad-spectrum antibiotic or combination antibiotic therapy (Gill et al., 2015). Broad spectrum antibiotics are usually used when bacterial infection is suspected (based on the experience of the practitioner) prior to identification of the causative bacteria or when infection with multiple groups of bacteria is suspected (Bjarnsholt, 2013). Although powerful, broad-spectrum antibiotics pose specific risks, particularly the disruption of native, normal bacteria and the development of antimicrobial resistance by attacking indiscriminately both the pathological and the body's normal bacterial flora(Panda and Rath, 2012).

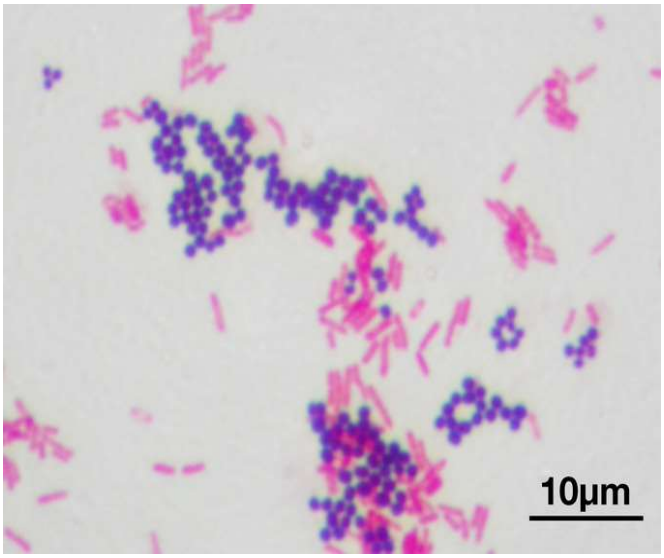


Figure 2.2: Image of a Gram stain of mixed Gram-positive cocci (*Staphylococcus aureus*, purple) and Gram-negative bacilli (*Escherichia coli*, red).

2.7 CLASSIFICATION OF ANTIBIOTICS

2.7.1 CLASSIFICATION BASED ON MODE OF ACTION

Generally, an antibacterial can be bacteriostatic or bactericidal when classified on the basis of mode of action. Bacteriostatic refers to antibacterial that are capable of inhibiting the growth or reproduction of bacteria. Although, the inhibition phenomenon of bacteriostatic agents involves inhibition of protein synthesis or some bacterial metabolic pathways, most bacteriostatic agents are able to kill bacteria, but at a lesser extent (Silva et al., 2016). For example, the first group of antibiotic to be discovered sulphonamides, their mechanism of action is to inhibit folate synthesis (which is important to several biological functions) at initial stage of the pathway (Figure 2.3).

Though sometimes it is difficult to mark a clear boundary between bacteriostatic and bactericidal, especially when high concentrations of some bacteriostatic agents are used then they may work as bactericidal (Silva et al., 2016). Bactericidal antibacterial agents are those which kills bacteria. However, bactericidal usually have different target sites which are crucial for the biosynthesis(Al-Humam, 2016). For example, gentamycin inhibits the protein synthesis, while ciprofloxacin(Kohanski et al., 2010) inhibits DNA replication by inhibiting bacterial DNA topoisomerase and DNA-gyrase (Figure 2.4).

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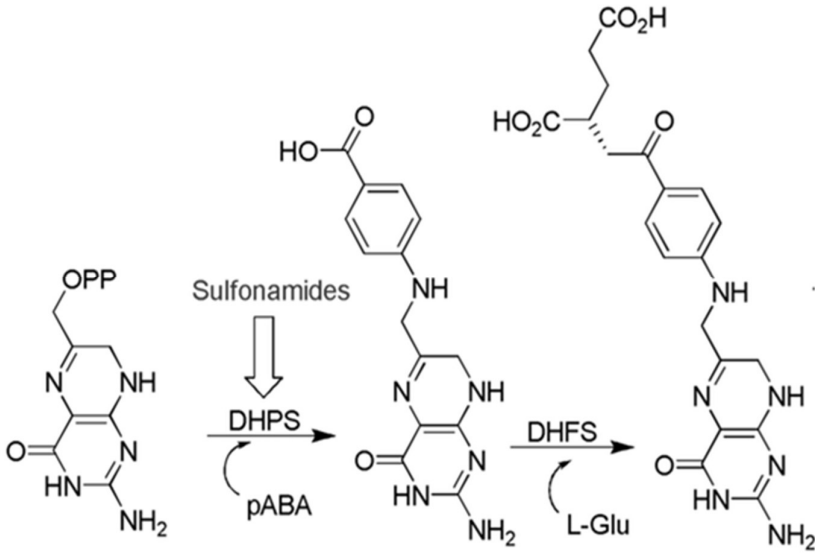

Figure 2.3: Key steps in the bacterial folate pathway. Enzyme targets of the sulfonamide drug indicated.

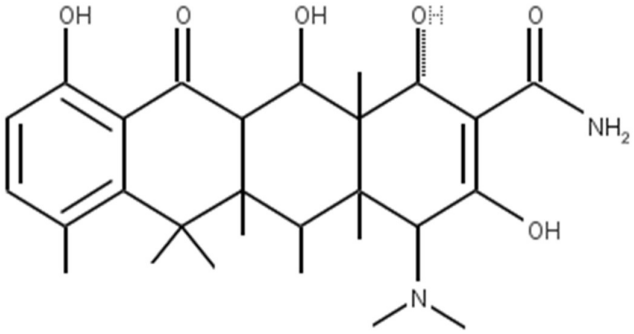

Figure 2.4: Structure of tetracycline, an example of Bacteriostatic antibacterial.

Although the advantages of bactericidal and bacteriostatic agents appear obvious (e.g., rapid elimination of bacteria and a decreased possibility of resistance development or infection recurrence), their activity could be undesirable in some clinical settings (Nazipi et al., 2021). For example, in CNS infection, the sudden lysis of bacteria by a these agents can possibly leads to a sudden increase in bacterial products such as lipopolysaccharide in gram-negative organisms or peptidoglycans

in gram-positive organisms that may stimulate cytokine production, causing potentially harmful inflammation(Arditi et al., 1989).

2.7.2 CLASSIFICATION BASED ON CHEMICAL STRUCTURE

Different chemical structures of antibacterial agents show different therapeutic behaviour; therefore, it is a decisive necessity to classify them on the basis of their chemical structure. This classification is also very key since similar structural units posses similar patterns of toxicity, effectiveness, and other related properties. Generally antibacterials have been classified into two groups, i.e β-lactams and aminoglycosides. However, in a more elaborated way, the antibacterials can be classified into β-lactams, β-lactam/β-lacta-mase inhibitor combinations, aminglycoside, macrolides, quinolones, and flouroquinolones(Ullah and Ali, 2017).

2.7.2.1 β-Lactams

β-Lactams are the most popular and widely used class of antibacterial agents. They are characterized by a four-membered lactam ring (Figure 2.5), known as β-lactam ring. However, they differ in side chain attached or additional cycles. Penicillin derivatives, cephalosporins, monobactams, and carbepenems, e.g. imipenems, all belong to this class. β-Lactams antibiotics are bactericidal agents that interrupt bacterial cell-wall formation as a result of covalent binding to essential penicillin-binding proteins (PBPs), enzymes that are involved in the terminal steps of peptidoglycan cross-linking in both Gram-negative and Grampositive bacteria(Ullah and Ali, 2017).

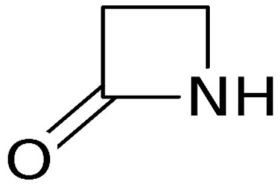

Figure 2.5: Basic structure of the β-lactam ring

2.7.2.2 Aminglycoside

Aminglycoside are broad-spectrum class of antibiotics that act through inhibition of protein synthesis. However they are mainly used in the treatment of aerobic gram-negative bacilli infections, although they are also effective against other gram negative bacteria (eg Staphylococci and Mycobacterium tuberculosis). In compounds of this group, two aminosugars joined by glycosidic bond to an aminocyclitol. Commonly used aminoglycosides are streptomycin, gentamicin, Apramycin, amikacin, neomycin B, and tobramycin (Jana and Deb, 2006). The structure of some of these is presented in Figure 2.6.

Although this class is known to be effective against a broad specturm of organisms, their resistance takes many different forms including enzymatic modification, target site modification via an enzyme or chromosomal mutation, and efflux(Mitsutomi et al., 2017). Each of these mechanisms has varying effects on different members of the class and often multiple mechanisms are involved in any given resistant isolate(Jana and Deb, 2006).

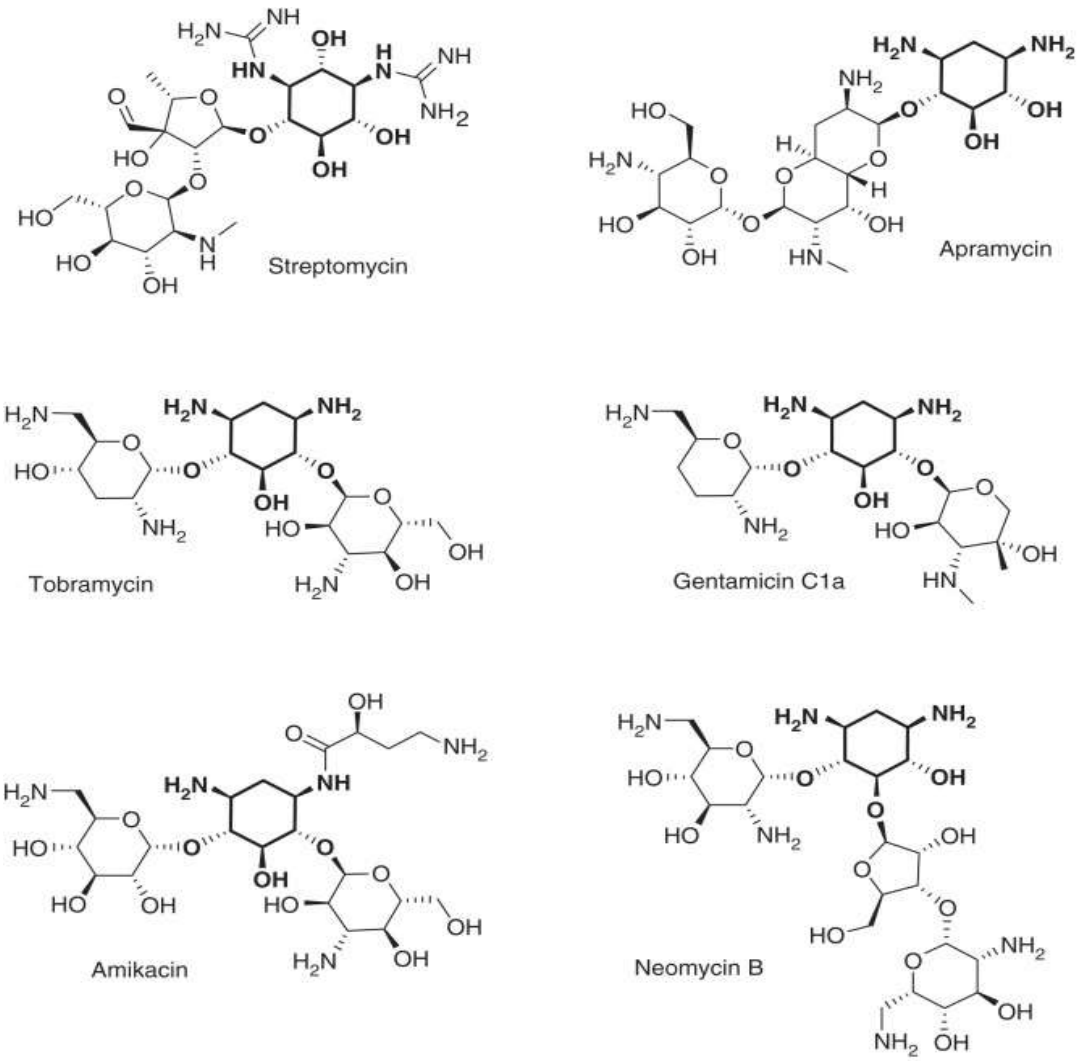

Figure 2.6: Structures of representative aminoglycosides

2.7.2.3 Macrolides

Macrolides are a class of natural products derived *from Saccharopolyspora erythraea* (originally called *Streptomyces erythreus*), a type of soil-borne bacteria. They are characterized by 14- to 16-membered macrocyclic lactone ring attached via glycosidic bonds (Figure 2.7), one or more sugars and are effective especially against gram-positive bacteria (such as staphylococci and streptococci). Macrolides are bacteriostatic antibiotics and inhibit protein synthesis in bacteria by reversibly binding to the P site of the 50S unit of the ribosome. Other commercially available macrolides

derived from erythromycin A include clarithromycin, dirithromycin, roxithromycin, and azithromycin(Vannuffel and Cocito, 1996).

A common mechanism shared by bacteria for becoming resistant to these antimicrobial agents is the diminution of the affinity of the antibiotic for its target. This effect may result from enzymatic detoxification of the drug or, conversely, from target modification. Some bacteria, especially gram negative can diminish access to the target secondary to active efflux or decrease the uptake of the molecules(Harms et al., 2004).

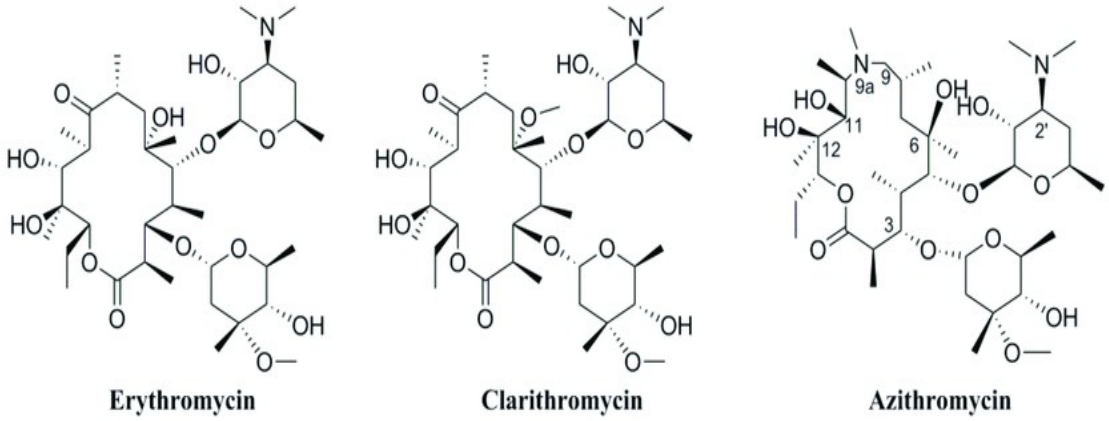

Figure 2.7: The chemical structure of macrolide antibiotics.

2.7.2.4 Quinolones and fluoroquinolones

Quinolones are a family of synthetic antibacterial agents that are derived from quinine structural units. However, an addition of fluorine at position 6 makes the compound fluoroquinolones (Figure 2.8). They are a class of broad-spectrum antibiotics that are active against bacteria of both Gram-negative and Gram-positive classes. Modification or structural alteration of the present known antibacterial agents has shown to lead to enhancement coverage and potency of antibacterial activity(Kvítek et al., 2008). Though, some of these modifications are associated with definite adverse effects, it

has added value to their use in certain infections, including some life-threatening ones, e.g. improved anti-Gram-positive activity of moxifloxacin and garenoxacin (Palanichamy et al., 2011).

Quinolones and fluoroquinolones exert their action by binding to the bacterial topoisomerase type II enzymes, interfering with the DNA synthesis pathway(Pham et al., 2019). Specifically, they inhibit the ligase activity of the type II topoisomerases, DNA gyrase and topoisomerase IV, which cut DNA to introduce supercoiling, while leaving nuclease activity unaffected. Thereby enabling these agents to be both specific and bactericidal. However, common mechanism shared by bacteria for becoming resistant to this antimicrobial agents typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV) and of changes in drug entry and efflux(Kvítek et al., 2008). Mutations (e.g. those that alter the target and those that reduce drug accumulation) are selected first in the more susceptible target: DNA gyrase, in gram-negative bacteria, or topoisomerase IV, in gram-positive bacteria.

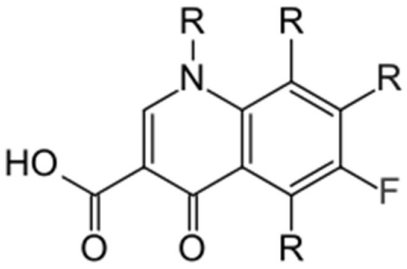

Figure 2.8: The basic structure of Quinolones

2.7.2.5 Sulphonamides

Sulphonamides are one of the important classes of synthetic organic compounds with great medicinal importance with a wide range of antimicrobial activity against both

gram-positive and gram-negative organisms(O'Shea and Moser, 2008). They are derived from *p*-aminobenzenesulfonamide which is a structural analogue of *p*-aminobenzoic acid, a factor required by bacteria for folic acid synthesis. Although some compounds belonging to this group are known to have antibacterial properties, sulfonamides are also active against *Toxoplasma gondii*, the causative organism of congenital toxoplasmosis(O'Shea and Moser, 2008).

Unlike other antimicrobial compounds, the antimicrobial action of sulphonamides is based on competition with the structurally similar *p*-aminobenzoic acid for the same enzyme, thus preventing normal utilization of *p*-aminobenzoic acid by microbes(Tačić et al., 2017). As a results, synthesis of folic acid is inhibited at the dihydropteroic acid step (Figure 2.9).

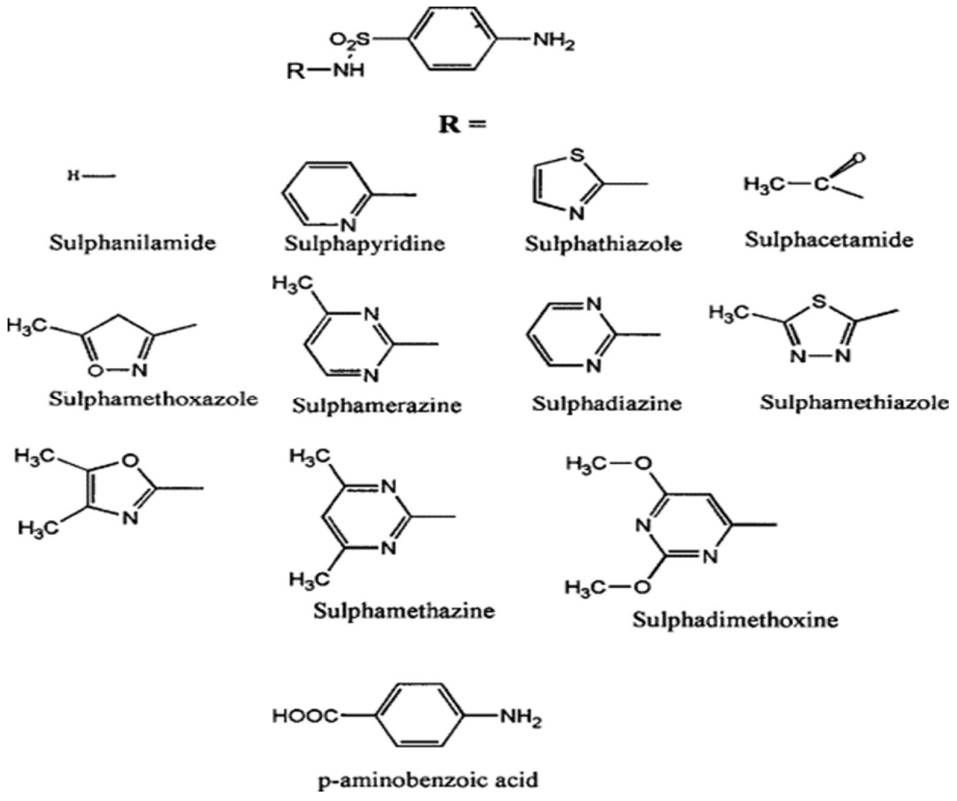

Figure 2.9: The various types of sulphonamides

The origin of sulfonamide resistance is disputed, but the evidence indicates that mutations occurring randomly give rise to resistant variants, which are then favored by selection in the presence of the drug. Although, resistance is more likely to develop if treatment is prolonged, the transfer of multiple drug resistance among different strains of bacteria has been responsible for the emergence of sulfonamide-resistant *Shigella* strains worldwide(Tačić et al., 2017).

2.7.2.6 Tetracyclines

Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria with four rings hydrocarbon containing compounds (Figure 2.10). These antimicrobial agents were originally derived from *Streptomyces* bacteria, but the newer derivatives are semi-synthetic. The antimicrobial properties and the absence of adverse effects of these agents has led to their extensive use in the therapy of human and animal infections(Ullah and Ali, 2017).

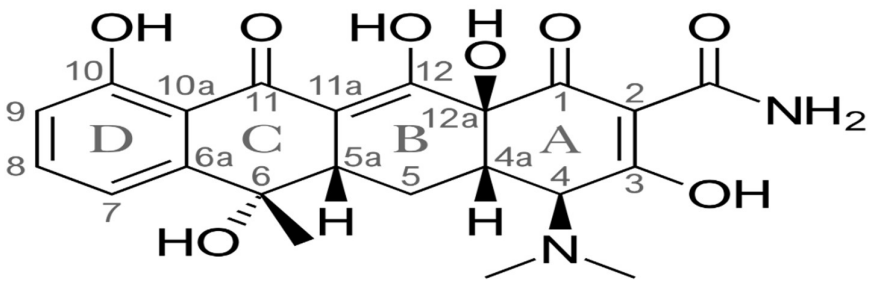

Figure 2.10: The typical structure of tetracyclines.

2.7.2.7 Nitroimidazoles

Nitroimidazoles are a group of compounds that contain a basic imidazole ring. The most commonly used example is metronidazole (Figure 2.11). Nitroimidazoles vary by the location of the nitro functional group. Most of the drugs of this class have their nitro group at position 6, such as metronidazole, and/or at position 2, such as benznidazole(Ullah and Ali, 2017).

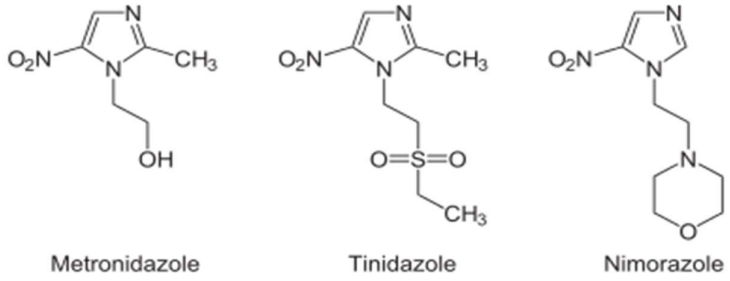

Figure 2.11: The various types of Nitroimidazoles

2.8 ANTIBIOTIC PRODUCING MICROORGANISMS

Most antibiotics are products of the secondary metabolism of three main groups of microorganisms: eubacteria, *actinomycetes* and filamentous fungi (Chandra and Chater, 2014). Many types of microorganisms such as molds, bacteria, protozoa and algae, all competing for limited nutrients in the soil, have to devise strategies to survive. There are numbers of bacteria having the potential to produce antibiotics, example of which is *Bacillus* species(Awais et al., 2007). The *actinomycetes* yielded about 70 % of these, and the remaining 30% are products of filamentous fungi and non-actinomycetes bacteria (Subramani and Aalbersberg, 2013).

2.8.1 ACTINOMYCETES

Actinomycetes represent a high proportion of the soil microbial biomass and are best known for their ability to produce antibiotics. According to a survey done in 1993, over 70% of the novel bioactive substances of microbial origin isolated in the decade 1984 - 1993 were derived from *actinomycetes*. It is believed that *actinomycetes* will continue to play a major role in providing novel bio-active substances in the next decade (Challis, 2008). Most *actinomycetes* are free-living, saprophytic bacteria found widely distributed in soil, water and colonizing plants.

2.8.2 STREPTOMYCETES

Streptomyces are Gram-positive bacteria belonging to the order of *actinomycetes*. They are aerobic bacteria, spore-forming and non-motile. In nature, *Streptomyces* is represented by the largest number of species and varieties, which differ greatly in their morphology, physiology and biochemical activities. *Streptomyces* species are multicellular organisms (Barka et al., 2016). They grow like fungi in terms of forming branching filaments of cells and their growth pattern led scientists to believe that these organisms were fungi when first discovered.

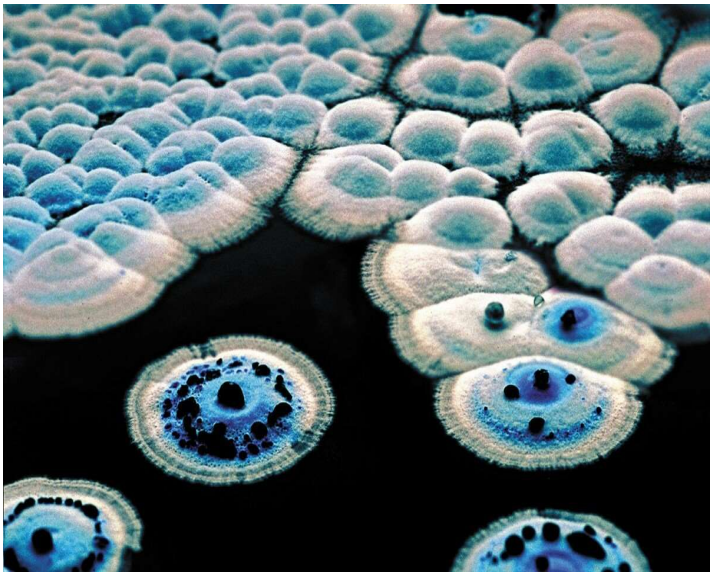

Figure 2.12: Images of actinomycetes (top) and streptomycetes (bottom).

2.9 BACKGROUND ON ANTIBIOTIC PRODUCTION

Antibiotic production is a feature of several kinds of soil bacteria and fungi and may represent a survival mechanism (Jamil et al., 2007). Naturally, Antibiotic producing microorganisms produces these antibiotics as defense mechenism due to an external force such as alteration of temp, pH or nutient supply. Conceivably, much of the chemical diversity of secondary metabolites produced by Antibiotic producing microorganims has evolved as a result of their interactions with other (micro)organisms in highly diverse environments. Antibiotic producing microorganims strains often produce only small amounts of antibiotic (µg/l), whereas production rates in the range of g/l are needed to set up a cost-effective production process. In order to increase the industrial yield of products, different strategies can be adopted such as mutagenesis, fementation conditions, media compositions and the use of precursors.

Random mutagenesis for the selection of overproducing mutants remains the preferred method when molecular genetic tools have not been developed for the producer microorganism. Although random mutagenesis and screening procedures have been widely used for genetic improvement of antibiotic production, there are certain disadvantages, such as the time necessary to obtain a favorable mutation. The knowledge-driven genetic manipulation can make the optimization of strains and conditions more efficient(Rowlands, 1984).

The tuning of media composition and fermentation conditions (carbon source, phosphate and nitrogen concentrations, pH, temperature) and the supply of specific precursors are the first approaches used in order to increase the yield in fermentation. Moreover, genetic manipulation of primary or secondary metabolism can be applied.

Regarding primary metabolism, mutations in pathways for amino acids or other molecules that are used as precursors in antibiotic biosynthesis or mutations in the ribosome can improve indirectly the yield of secondary metabolites (Parekh et al., 2000).

Regarding secondary metabolism, the over-expression of biosynthetic genes, such as the genes that codify for antibiotic specific precursors, the over-expression of pathway-specific positive regulators or the inactivation of pathway-specific negative regulators can result in an increase of antibiotic yield. Increasing self-resistance levels in producing organisms has been also used for improving production yields. Manipulation of pleiotropic regulators, involved in both primary and secondary metabolisms, was also successfully used to improve antibiotic yields(Parekh et al., 2000).

2.10 REVIEW OF METHODS USED FOR THE SCREENING OF MICROORGANISMS WITH ANTI-MICROBIAL PROPERTIES

2.10.1 GENERALIIES

2.10.1.1 Primary screening methods

General screening methods have not changed fundamentally in the past 40 years. Millions of different microorganisms, mostly actinomycete species originating from soils, were screened all over the world by means of the classical methods of (WAKSMAN and DUBOS, 1926).

2.10.1.2 The crowded plate method

This method is used to isolate organisms able to produce antibiotics active against other soil organisms. A heavy aqueous suspension (1: 10; 1:100) of soil is plated on

agar in such a way as to ensure the development of confluent growth. Colonies surrounded by clear zones are purified for further study. By altering the media used, different groups of organisms can be encouraged to develop (Harrigan and McCance, 2014).

2.10.1.3 Dilution plate method

This method is applied when the aim is to isolate antibiotics against a known organism or organisms. The procedure involves mixing a sample of soil at a suitable dilution with an appropriate melted agar and the mixture poured into plates. After the agar has solidified, the plate is inverted and incubated with the test spices until scattered colonies appear. The test species forms a lawn on the surface of the agar interspersed with clear zones of inhibition. Those colonies surrounded by clear zones are purified and transferred to an agar slant to be held for further studies (Chen et al., 2003).

2.10.2 PRIMARY TESTING OF ANTIBIOTIC PRODUCTION

2.10.2.1 The cross-streak method

This method is suitable for testing individual isolates. The purified isolate is streaked across the upper third of a plate containing a medium which supports its growth as well as that of the test organisms. A variety of media may be used for streaking the antibiotic producer. It is allowed to grow for up to 7 days, in which time, any antibiotic produced should have diffused a considerable distance from the streak (Gurung et al., 2009). Test organisms are streaked at right angles to the producer isolate and the extent of inhibition of the various test organisms observed/measured (Ahmed and Al Sani, 2013).

2.10.2.2 The agar plug method

This method is particularly useful for testing fungal isolates for antibiotic production, especially when the test organism(s) grow(s) poorly in the medium that supports the growth of the producer fungus. Plugs about 0.5 cm in diameter are made with a sterile cork borer from the fungus isolate plate. These plugs are then placed on plates seeded with different test organisms and incubated. Inhibition zones appear around those isolates producing antimicrobial compounds. This method may be used with *actinomycetes* (Harrigan and McCance, 2014).

2.10.3 REVIEW OF CULTIVATION METHODS

2.10.3.1 Cultivation-independent molecular approaches

Culture-independent studies mostly based on PCR amplification, cloning and 16S rRNA gene sequence analysis have revealed unexplored bacterial diversity that has previously not been cultured in the soil. However, these methods do not provide further information on the microbial physiology, abundance and ecological significance hence the need for cultivation-dependent approaches (Harrigan and McCance, 2014).

2.10.3.2 Cultivation dependent approaches

The diversity of soil microorganisms has been exploited for many years based on the cultivation and isolation of microbial species. Various unconventional culture media such as low nutrient media have been used in the recent past to isolate rarely isolated groups of bacteria in soil. Such media prevent the growth of fast-growing bacteria and substrate accelerated death of bacteria from low nutrients habitats (Harrigan and McCance, 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1 ETHICAL CLEARANCE

The study was approved by the research and ethics committee of the University of Venda.

3.2 STUDY AREA, DATA AND SAMPLE COLLECTION

Samples were collected at 12 different points at the land fill in Tshakhuma village under Makhado Municipality (-23.083019, 30.301499) at distance of varying proximity. The samples were collected from each site at depths of (0-20 cm) below the surface arbitrarily. Following collection, samples were placed in sterile plastic bags and stored, in the dark, at 4ºC and transferred to the Microbiology research laboratory, University of Venda where the entire research work was carried out. Processing was completed following receipt of samples within 24 h of collection.

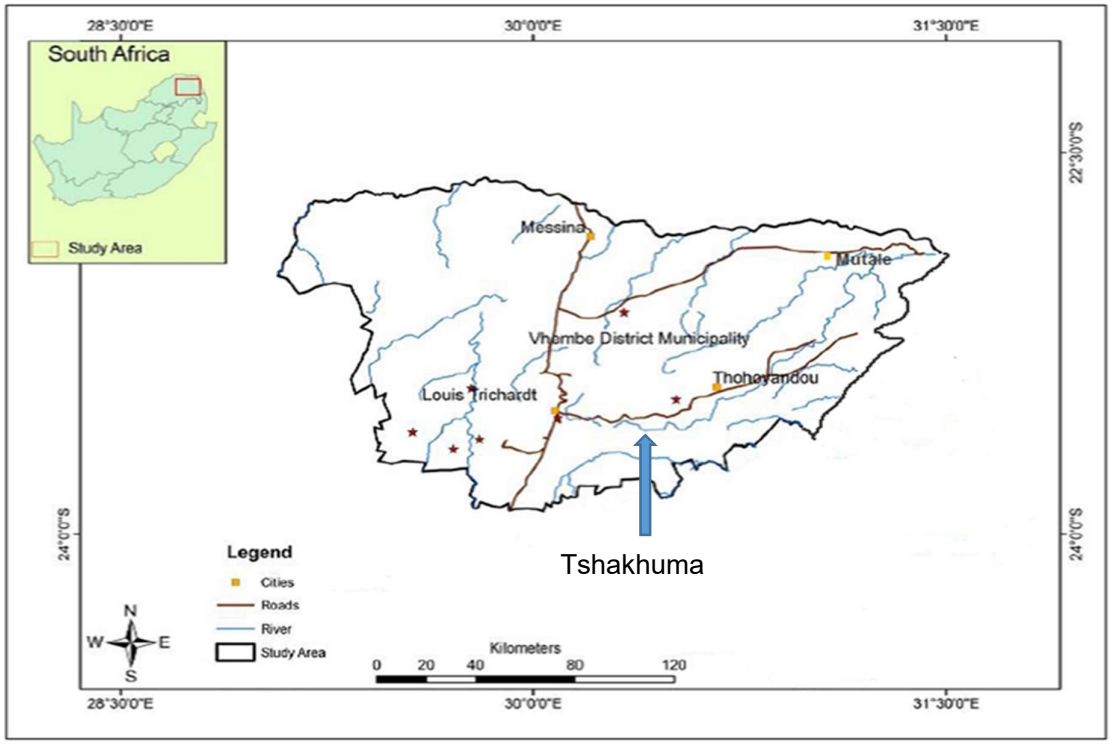

Figure 3.1: Map of the study area, Tshakhuma, Makhado municipality, Limpopo, South Africa.

3.3 SELECTION AND ISOLATION OF ANTIBIOTIC PRODUCERS FROM SOIL SAMPLES

In the present study, soil sprinkle technique was used to isolate antibiotic producing microorganism following the procedure described by (Jamil et al., 2007). For this purpose, Approximately 1 g of soil particles were sprinkled on the surface of non-selective nutrient agar plates seeded with the test organism, *E. coli* (ATCC 35218). The plates were aerobically incubated at 37°C for 24-72 hours. Antibiotic activity was checked by zone of inhibition, surrounding a colony. Colonies displaying clear zones of inhibition around them were picked and streaked on separate nutrient agar plates to get pure cultures. Each isolated soil microorganisms were assigned with specific codes and these isolates were used as the source of antibiotic producing microbes. All strains were stored at -20°C in 20 % glycerol Muller Hinton Broth (Merck, Kenilworth, NJ, USA) for further confirmation and characterization by PCR and sub cultured periodically.

3.4 SUB CULTURING

Sub-culturing was done by transferring some or all cells from a previous culture to fresh growth medium to revive them and use again(Lorenz et al., 2005). Antibiotic producing microorganisms were revived by picking bacterial colonies with clear margins and sub cultured into Mueller Hinton broth (Merck, Kenilworth, NJ, USA) for 2 hours at 37 ˚C. The bacteria were inoculated on fresh Muller Hinton Agar (Oxoid, Thermo Fisher Scientific, Basingstoke, Hampshire; UK) plates with sterile loop using streak plate method in laminar air flow followed by incubation for 24 h at 37 °C.

3.5 CHARACTERIZATION OF POTENTIAL ANTIBIOTIC PRODUCERS

3.5.1 MORPHOLOGICAL CHARACTERIZATION

Colony features obtained after 24-hour growth of the isolates in pure culture were examined and used in the presumptive identification of the organisms in Muller agar. Colony morphological characteristics including size, shape, elevation and margins were described and recorded.

3.5.2 IDENTIFICATION BY VITEK 2 SYSTEM.

VITEK 2 system was used for the phenotypic fingerprinting of the cultures. The cultures were analyzed based on their metabolic activities. VITEK 2 is an automated system, which contains 64 welled VITEK 2 GN and GP cards (France) having different substrates in each well. The bacterial culture (diluted in 0.45% (w/v) of NaCl) with the OD 0.5 measured by DensiCheck meter (bioMe'rieux) was used in an automated sampling system. The cards were incubated in the in-build incubator in the VITEK 2 machine.

3.6 PRIMARY AND SECONDARY SCREENING

3.6.1 PRIMARY SCREENING OF ANTIBIOTICS

Microorganisms isolated and identified from different soil samples were screened for their antimicrobial spectrum. The test bacteria that were used for screening were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC *35218*, *Klebsiella pneumonia* ATCC BAA-1705 and *Bacillus subtilis* ATCC 6051. Antimicrobial activities were assessed using Muller Hinton agar. Each plate was spread with the test organism and a small amount of the bacterial isolate was spotted on the test organism. Isolates

displaying clear zones of inhibition against the pathogens were sequestered onto plates and those displaying little or no inhibitory properties were retested and discarded if scarce antibiotic biosynthesis persisted. Purified microorganisms obtained were further tested for growth-inhibitory properties by using the agar well diffusion assay (Woappi et al., 2013), against all the test microorganisms. Isolates that showed inhibitory zones against one or more pathogen were further purified and maintained on Muller Hinton broth at 4°C.

3.6.2 SECONDARY SCREENING USING ANTIBIOTIC PRODUCTION ASSAY (APS)

Due to their potential industrial importance, the main aim is to thoroughly investigate antibiotic secreting abilities among the screened antibiotic producing species (APSs). Secondarily screened APSs and blank controls were grown in 50 ml of Muller Hinton broth at 37°C for 24 h. Cells were pelleted by centrifugation at 22°C, 10,000g for 10 min. The supernatant was collected and evaporated by sterile airflow incubation at 37°C. Desiccated supernatant were sterilized and reconstituted in 1 ml respective growth medium and stored at −20°C until analyzed for antibiotic properties through agar-plug assay.

3.7 AGAR-PLUG ASSAY

Agar-plug assay were conducted as described previously (Woappi et al., 2013). A 5-mm sterile pipette was utilized to plug wells on Muller Hinton agar plates lawned with the test organism. Each well was filled with 20 µl of reconstituted supernatant, respectively. The latter was incubated at 37°C and examined for zone of inhibition, a clear halo around the wells, after 24h. Isolates displaying remarkable inhibitory properties were selected and kept at -20 ˚C for further analysis. APS with little

inhibitory properties were retested and discarded if insufficient antibiotic biosynthesis persisted.

3.8 PHYLOGENETIC ANALYSIS

3.8.1 DNA EXTRACTION

DNA samples were extracted from 4 isolates using the boiling method as previously described (Dashti et al., 2009). About 500 µl culture isolates from preserved bacterial colonies were transferred to a 2 ml tube and centrifuged at 13,000 g (Eppendof, Hamburg, Germany) for 5 minutes and the supernatant was carefully removed from the tubes and the pellets were suspended in 500 µl of sterile distilled water. The samples were then boiled for 15 min in a water bath at 100°C. Subsequent cooling followed at -20°C for 10 min prior to centrifugation at 13,000 g for 5 min at 4°C. The DNA was kept frozen at -20°C until needed for amplification analysis.

3.8.2 PCR amplification

PCR amplification of the 16S rDNA was performed using the primers 27F (5'-AGAGTT TGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') as describe by (Chen et al., 2015). Each PCR reaction of 25 µl in total included 12.5 µl DreamTaq DNA polymerase mix (ThermoFisher Scientific, United States), 6.5 µl dH_2O, the final concentration 0.4 µM of each primer and 4 µl DNA template. The cycling conditions for the amplification of the 16S rDNA region were as follow: 5 min at 95 °C, 25 cycles at 94 °C for 40 s, at 55 °C for 30 s and 1 min at 72 °C, then followed by a final elongation step for 7 min at 72 °C.

3.9 MANIPULATION OF FERMENTATION PARAMETERS

3.9.1 EFFECT OF INCUBATION PERIOD

Shake–flask fermentations was conducted in 500 ml flask containing 100 ml of Muller-Hinton broth and was incubated at 37°C for optimum yields on a rotary shaker operating at 250 rpm for 7 days. At every 24 h interval, the flasks were harvested and antimicrobial metabolites production was determined in terms of their antimicrobial activity by employing agar diffusion method against the test organisms.

3.9.2 EFFECT OF PH AND TEMPERATURE ON THE PRODUCTION OF BIOACTIVE METABOLITES

The effects of pH and temperature on biomass and antimicrobial metabolites produced by the strain were studied by inoculating 48 h old seed culture in Muller-Hinton broth. Effects of different ranges of pH (5-9) and temperature (15-45 °C) on the production of biomass and antimicrobial metabolites were also be examined after 96 h of incubation and presented.

CHAPTER 4

RESULTS

4.1 Phenotypic and molecular characterisation of the isolates

4.1.1 Isolation of antibiotic producing bacteria

Isolation was done using soil sprinkle technique (Figure 4.1). The inoculated plates were incubated at 37 ˚C and observations were made after 72h. A total of 7 bacterial strains were found to be producing zone of inhibition, and they were picked and streaked on new Muller Hinton agar to get pure colonies, out of which 3 species were selected on the basis of maximum zone of inhibition (ZOI) for further analysis.

Figure 4.1: A photograph showing an *E. coli* medium culture plate with different colonies of potential antibiotic producers after soil sprinkle technique

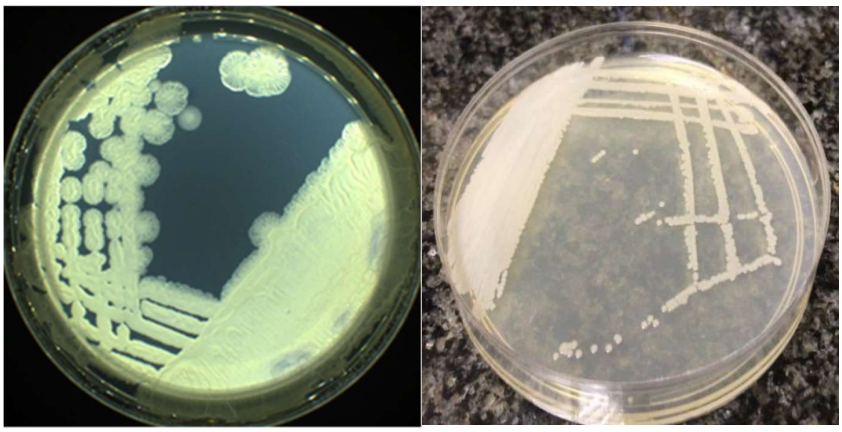

Figure 4.2: Colonies streaked as pure cultures as seen after 24 hours incubation at 37˚C

4.2 Characterization and Identification of bacterial Isolates

4. 2.1 Morphological characterization

Morphological characterization was based on observation of the bacterial colony (i.e. Colony colour, form, margin and elevation) and macroscopic techniques (i.e. shape, Cell arrangement and Gram reaction) of pure colonies (Figure 4.3). Most colonies were able to grow within 24h of incubation at 37 ºC. The colony morphology of the isolates obtained ranged from circular, entire, flat and filamentous. The morphology and size of the colonies varied between 1-10 mm in diameter with a relatively smooth surface at the beginning of the growth and the color ranged from white, creamy to light brown in pigmentation. About 70 % of the isolates were Gram positive while 30 % were Gram negative. The cells ranged from cocci; short rods while others were filamentous, results shown in Table 4.1.

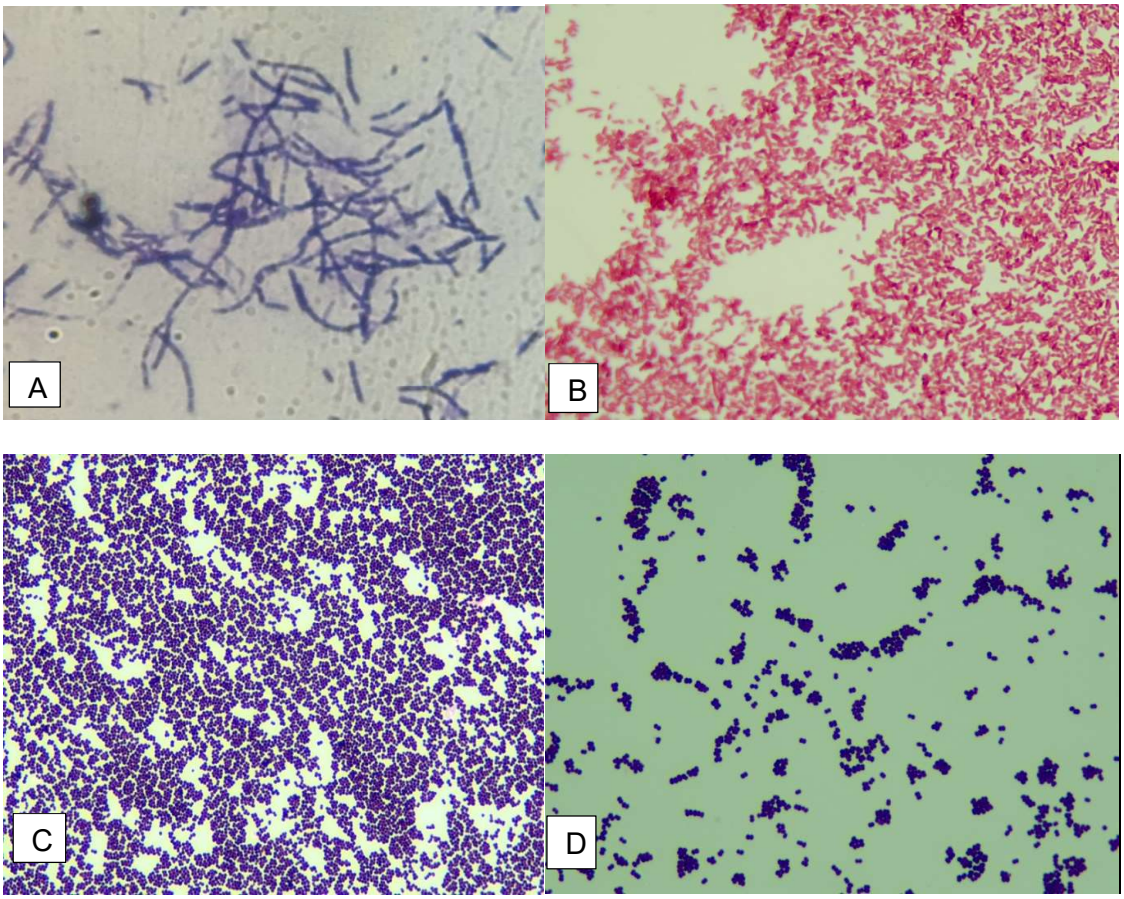

Figure 4.3: Gram stain images of isolates exhibiting antibacterial action. **A**= Gram+ Filamentous; **B**= Gram- Rods; **C**= Gram+ Cocci and **D**=Gram+ Cocci. Viewed under the light microscope (100x objectives).

Table 4.1. Morphological characteristics of the potential antibiotic producing isolates

Colony characterization					Cell characterization	
Strain	**Colony colour**	**Colony form**	**Colony elevation**	**Colony margin**	**Cell arrangement**	**Gram reaction**
TSH1	Cream	Irregular	Raised	Entire	Cocci	+
TSP1	White	Circular	Flat	Undulate	Rods	-
TSP3	Cream white	Circular	Flat	Undulate	Cocci	+
TSH2	Light brown	Circular	Convex	Entire	Cocci	+

Table 4.2: Identification of active isolates by VITEK 2 system

Isolates	**Identification**		
	Selected organism	***Probability %***	***Confidence***
TSP3	***Francisella tularensis***	**88%**	**Low discrimination**
TSH2	***Staphylococcus sciuri***	**92%**	**Good ID**
TSP1	***Bacillus cereus***	**89%**	**Good ID**
TSH1	**Not identified**		

4.2.3 Molecular characterization of bacterial isolates

4.2.3.1 DNA Extraction

Out of the 7 isolates only 4 which showed positive results for the production of antibiotics were selected for this analysis. Pure subcultures of the selected isolates were subjected to DNA extraction as described by (Chen et al., 2015). Presence of DNA yield was assessed using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Canada) for purity (Table 4.3). The genomic DNA was used as templates for subsequent PCR amplification.

4.2.3.2 PCR amplification of 16S rRNA gene

Total DNA from each isolate was used as a template for amplification in order to identify the isolates by 16S rDNA sequencing. A positive amplification is seen as a band at 1500 bp (Figure 4.4), which is the expected size of the 16S rRNA gene. An additional set of primes was used to amplify 16S rRNA from isolates that did not amplify at once. The samples that amplified the second time were also included in further sequence analysis.

Table 4.3: DNA yield from NanoDrop

Isolates	Concetration
TSH1	130ng/ul
TSP3	146ng/ul
TSP1	142ng/ul
TSH2	148ng/ul

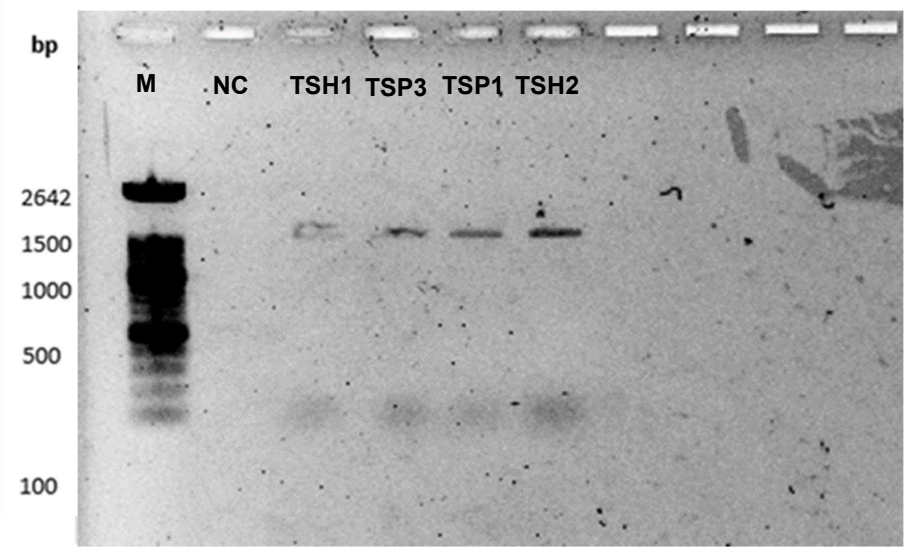

Figure 4.4: 1% Agarose gel picture showing the results obtained from PCR-amplification of rRNA. M -molecular marker (100bp XIV), Lane 1-10 DNA samples, NC- negative control.

4.2.3.3 Isolate sequences

The 16S rDNA nucleotide sequences from 4 of the isolates that inhibited growth of the tested bacteria were aligned with 3 references obtained from the gene bank. The remainder of the samples failed QC (no bands could be observed on the gel) and could not be sequenced. The sequences of the three isolates were used as references for the construction of phylogenic tree, NR118141, JQ246806 and MN788638 being the *Pseudomonas formosensis strain CC-CY503*, *Pseudomonas sp. XC1* and *Staphylococcus sciuri strain* respectively as reference sequence (Figure 4.5).

4.2.3.4 Phylogenetic analysis of the sequences

Sequence data was analyzed with Staden Package (http://www. staden.sourceforge.net). Alignments were checked and corrected manually where necessary. The sequences obtained were subjected to BLAST analysis and the partial sequence of 2 isolates with antibacterial activity showed to be closely affiliated with members of the genus *Staphylococcus* within the Firmicutes in the domain bacteria with similarities of 99.65% and 99.86%. One isolate belonged to the class Gammaproteobacteria in the phylum proteobacteria with similarities of 99.65%. Evolutionary analyses were conducted in MEGAX software. A phylogenetic tree was constructed using Maximum Likelihood and included sequences obtained from the GeneBank to show the phylogenetic position of each of the isolates studied (Figures 4.5). The isolates shared sequence identities of 99.65% with known *Staphylococcus and Pseudomonas* species. TSH2 and TSP3 clustered together with a sequence identity of 99.68% to *Staphylococcus sciuri strain*. Isolate TSP1 sequence with a sequence identity of 100% to *Pseudomonas formosensis strain CC-CY503* (Table 4.3).

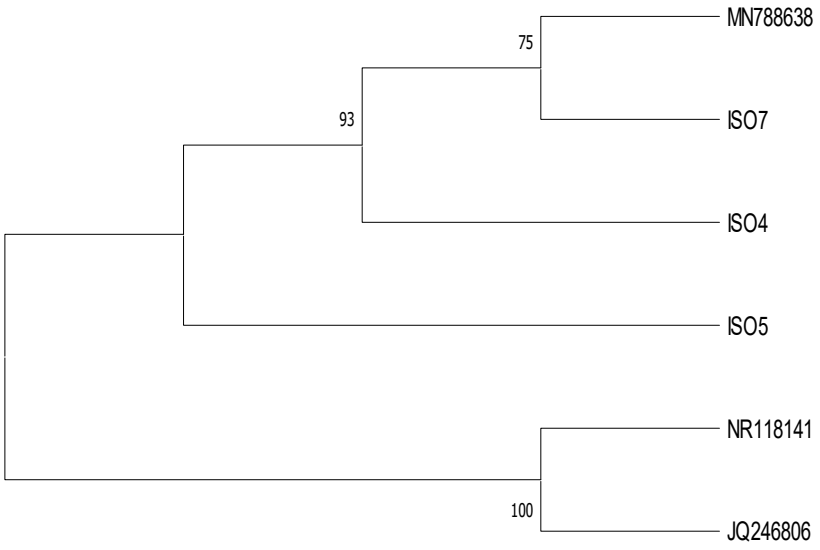

Figure 4.5. Molecular Phylogenetic analysis by Maximum Likelihood method of the sequences obtained from the active isolates.

Table 4.4. Isolates identified through BLAST search

Isolate	Length	Per Identity	Query cover	Description
TSH2	1428	99.65%	100%	*Staphylococcus sciuri strain SS*
TSP1	1422	99.65%	99%	*Pseudomonas Sp A84(2010)*
TSP3	1432	99.86%	99%	*Staphylococcus sciuri strain SS*

4.3 Evaluation of antimicrobial activity

4.3.1 Preliminary screening for antimicrobial activity.

For evaluation of antimicrobial activity, isolates were tested for their ability to produce inhibitory substance against pathogenic bacteria. However only 4 isolates were selected for the analysis based on the activity they showed from preliminary screening (Figure 4.6). The zone of inhibition of the isolates ranged from weak, moderate to good activity against target resistant bacteria (Table 4.5). The test organisms included 2 gram negative and 2 gram positive for secondary screening (Figure 4.7), the results showed good activity against both with a range from 4mm to 16mm with TSH2.

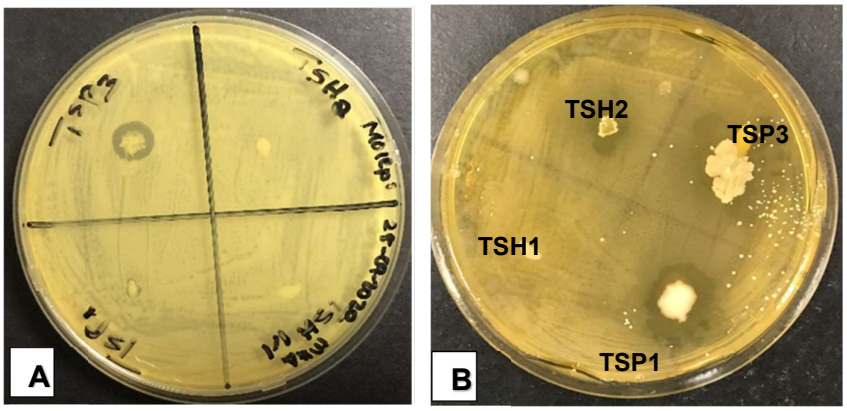

Figure 4.6: Preliminary screening of active isolates against *E. coli*: **A**, antimicrobial activity after 36 hours; **B,** activity after 96h of incubation at 37°.

TABLE 4.5: Inhibition Zone Shown By Different Bacterial Isolates against Test Organisms

Isolate Codes	Test organisms
	E. coli
TSH2	+
TSP3	++
TSP1	+++
TSH1/1	-

Note: +++: Good activity, ++ Moderate activity, Weak activity against the target bacteria, - means no activity.

4.3.2 Secondary screening for antimicrobial activity.

All the 4 isolates showed zone of inhibition against the test organisms in the secondary screening, as shown in figure 4.7. Of all the isolates 3 showed activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *E. coli* while 1 isolate showed activity against *Staphylococcus aureus* only.

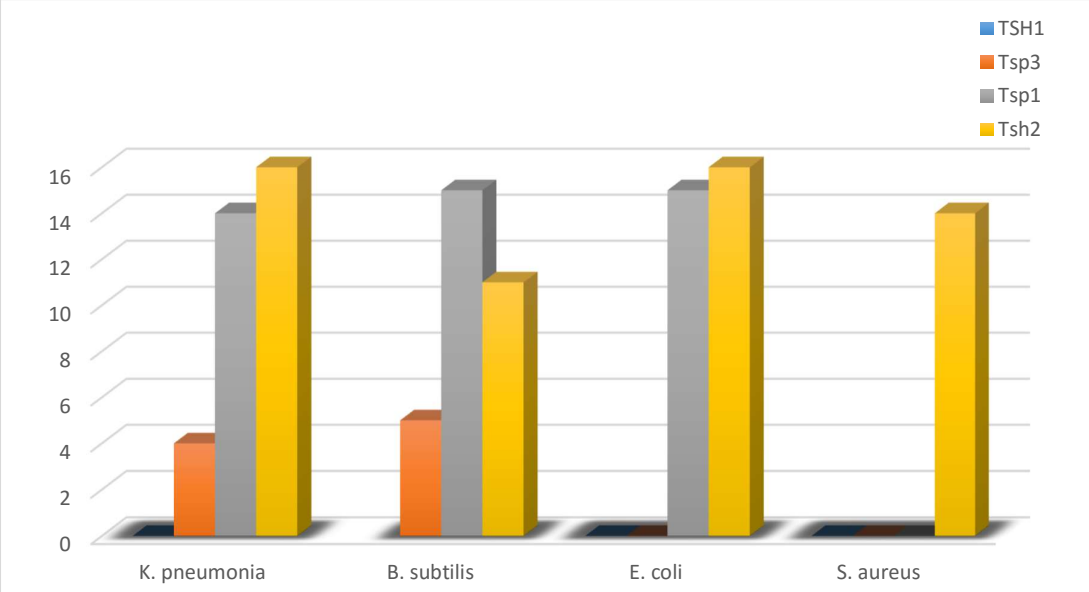

Figure 4.7: Schematic graph showing zone of inhibition of bacterial isolates in secondary screening of antimicrobial activity after 48h of incubation.

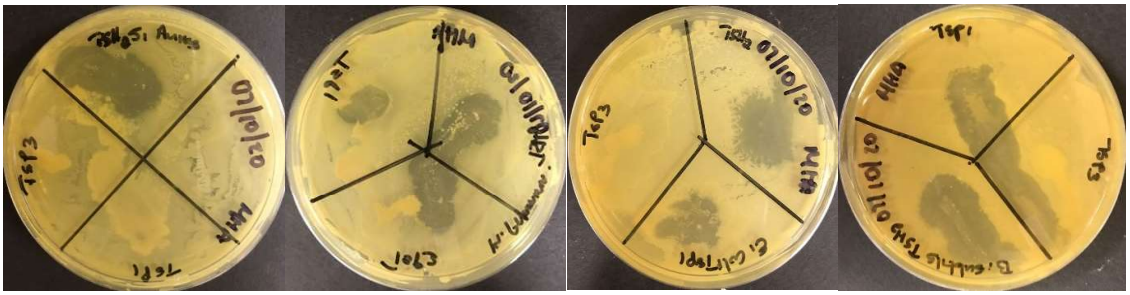

Figure 4.8: Antibacterial activity of bacterial isolates against selected bacterial strains. *K. pneumonia* ATCC BAA-1705, *S. Aureus* ATCC 25923, *B. subtilis* ATCC 6051 and *E. coli* ATCC 35218.

4.4 MANIPULATION OF FERMENTATION PARAMETERS

The manipulation of fermentation conditions was done using 3 parameters, including Temperature, pH and Incubation period. Our results revealed that antimicrobial metabolite production started at 20°C and reached the maximum at 35°C and thereafter its production gradually declined (Figure 4.9). Effects of pH on the production of antimicrobial compounds, showed to have a great impact on the antibacterial activity. The antibacterial activity increased with increasing initial pH from 6.5 to 7.5, but any further increase in its values resulted in decreased antibacterial activity (Figure 4.10). Effects of Incubation period showed a great impact on the production of secondary metabolites. Observation indicated that production of secondary metabolites started after 4 days of incubation and optimum activity was at high peak in the range of 5-7 days of incubation as seen in Figure 4.11. The inhibition rate reached the maximum at after 7 days incubation.

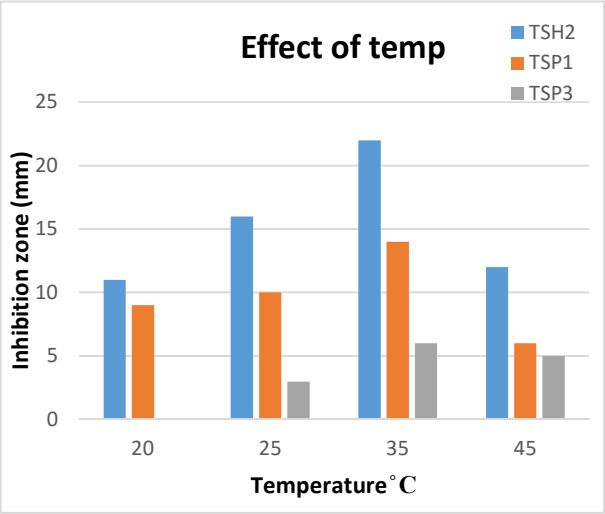

Figure 4.9: Schematic graph showing the Effects of temperature

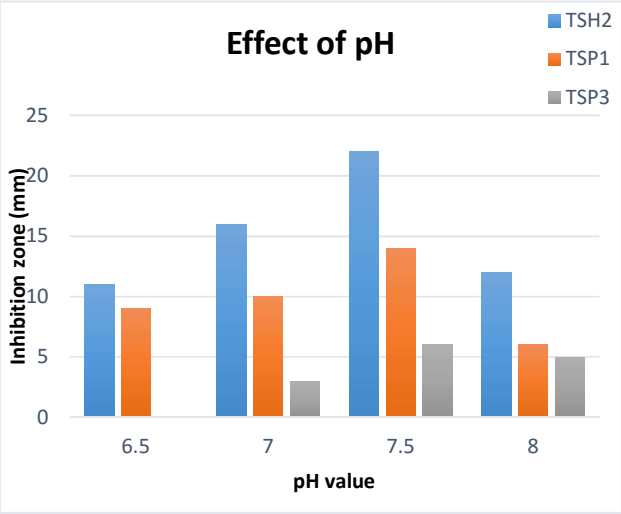

Figure 4.10: Schematic graph showing the Effects of pH

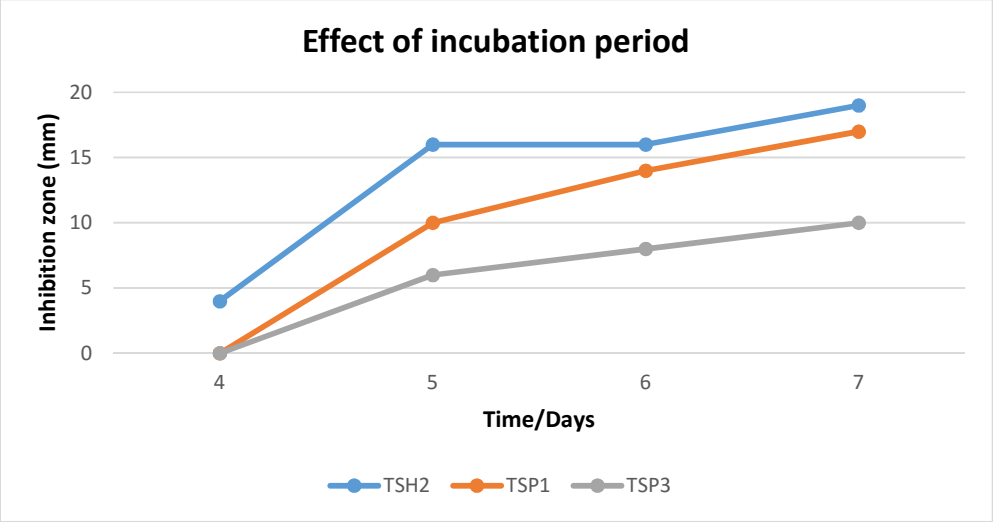

Figure 4.11: Schematic graph showing the Effects of Incubation period

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 Discussion

Many soil microorganisms are known to be potential producers of secondary metabolites that have antimicrobial properties with the ability to treat infectious diseases. This might be due to complexity and microbial diversity of the environment they inhabit. However, their capability to produce these antimicrobial compounds is not a static property and it can be significantly affected by several factors. Studies have been conducted from different sources in nature such as medicinal plants, marine and terrestrial organisms (Watts et al., 2017, Doss et al., 2017, Hayashi et al., 2013) to combat the tide of antibiotic resistance. Additionally, natural products from microbial origin have grasped a great devotion over the course of several decades as it is more renewable, and maybe reproducible source than plants or animals (Lam et al., 2007). The present study was primarily focused on strain improvement and characterization of antibiotic producing soil microorganisms.

The traditional identification of bacteria is based on the phenotypic characteristics such as morphological , cultural and biochemical characteristics (Marzan et al., 2017). In the present study, the selected isolates were subjected to colony, cellular, biochemical and molecular characterization. Cultural characteristics displayed by isolates on agar media, and the results from the colony and cellular characterization showed that the isolates were mostly cream-white, cocci-shaped, Gram-positive bacteria. However, one cannot conclude based on cultural and morphological characterisation because many microorganisms have similar characteristics, we can therefore only speculate that these isolates were members of *Streptomyces* genus.

Another study done by Gautham et al., (2012) who isolated microorganisms from soil samples from Western Ghats revealed that Streptomyces have varied features, with respect to the environment they inhabit. However, a detailed morphological characteristic of *Streptomyces* has been reviewed by Chandra and Chater (2014). The authors documented that *Streptomyces* is a gram positive white-colony bacteria that range from simple cocci to the various complex mycelial forms. In addition, the emergence of morphological complexity is usually generated during physiologically stressful growth transitions (Chandra and Chater, 2014). In another study conducted in Kenya, they isolated bacteria from oil contaminated soil, using morphological characteristics they identified one of the isolates as *Klebsiella pneumonia* (Mwaura et al, 2018).

In the current study, isolates were identified by the VITEK 2 system as *Francisella tularensis*, *Staphylococcus sciuri* and *Bacillus cereus*. In a previous study, (Al-Humam, 2016) reported that VITEK 2 provides reliable identification of *Bacillus* species and members of related genera. However, (Elbendary et al., 2018) working on soil isolates from Egypt identified *Kocuria kristinae, Kocuria rosea, Streptomyces griseus, Streptomyces flaveolus* and *Actinomycetes* using VITEK 2 system. The difference could be due to environmental conditions which could influence genetic divergence or due to the occurrence of atypical phenotypic characteristics in some microorganisms. While the similarities among closely related species may also lead to misidentification or using phenotypic characteristics.

The 16S rRNA gene sequencing remains the gold standard approach for determining the identity of organisms as well as the phylogeny based on the assumption that sequence diversity is purely due to evolutionary change and that the 16S rRNA gene is not influenced by horizontal gene transfer (McInnes et al., 2020). In this study, we

amplified 16S rRNA gene from the four active isolates using the universal primers. However, only 3 isolates were successfully sequenced. The identities of the 3 antibiotic-producing bacterial isolates were determined by comparing the sequences obtained to the available 16S rRNA sequences found in Genbank (NCIB). BLAST similarity scores ranged from 99.65% to 99.99%, where 3 matches of 16S rRNA gene sequence were included in the analysis. Using BLAST search, we found that 2 isolates belonged to *Staphylococcus* species within the Firmicutes in the domain bacteria and 1 isolate strain belonged to *Pseudomonas* species**.**

Although *staphylococcus* species are known to be mostly pathogenic, our results reveal that these species also have a potential to produce antimicrobial compounds (Nazipi et al., 2021). Similar results were documented from a study done by Nazipi *et al*., (2021), who isolated a bacterial strain with the potential to produce antimicrobial compounds from the surface of the African social spider and their results indicated that the rRNA gene sequence had 100% similarity to *Staphylococcus* sciuri. Generally, antibiotic producing genes can be shared and acquired by transposition environmentally from one microorganism to another and this evolutionary adaptation is perpetual within microbial rich soil. Another study done by Mitsutomi *et al*., (2017) reported the phylogenetics of 29 antibiotic-producing strains isolated from soil. The sequence comparison showed 98-99% identical similarity with 16S rRNA gene sequence of *Pseudomonas sp*. However, Abbas et al., (2014) reported that soil bacteria with potential to produce antibiotics showed to be 100% similarity with *Streptomyces, Bordetella* and *Achromobacter*.

Preliminary screening for antimicrobial activity was carried out at 37°C against *E. coli* which was achieved by the sprinkle technique. Maximum inhibition was observed with TSP1 against *E. coli* and Minimum inhibitory activity was observed with TSH1/1. A

study done by Sethi *et al*., (2013) demonstrated isolation of antibiotic producing microbes from soil using sprinkle technique and reported similar results that *Streptomyces* metabolites had maximum antimicrobial activity against *Escherichia coli.* In the present study, the use of *E. coli* as an indicator was beneficial as this process excluded the possibility of isolating non-producing organisms from the soil sample. The drawback of using indicator is that potential antibiotic producer producing antibiotic to which *E. coli* will be resistant might have remained undetected.

Microorganisms use antibiotics as signaling molecules and as a means of communication between cells in natural environments (Brameyer et al., 2015). Their production also represents a mechanism of survival against competing microorganisms in the natural environment living in their vicinity (Koehler et al., 2013). There are many factors that affect antibiotic production, such as carbon and nitrogen source, pH and most importantly temperature (Zong et al., 2015). From our study, the preliminary and secondary screening results clearly showed that TSP1, TSP3 and TSH2 possessed a very good antimicrobial activity against a variety of organisms (Gram positive and gram negative) under normal conditions, but exhibited better activity against gram positive test organisms. This might be due to the characteristics of cell wall. Gram positive bacteria are characterized by the presence of a very thick peptidoglycan layer which is known to be responsible for the rigidity of bacterial cell wall and cell shape and it is known not be effective to the permeability of antimicrobial agents. While Gram negative bacteria are known to have the outer membrane, which provides a formidable barrier that is impermeable to most antimicrobial agents. The finding from our study are in contrast with the findings reported by Uzair et al., (2018) demonstrating that *Pseudomonas* species have the highest antimicrobial activities against both gram positive and gram negative bacteria. Another study by Haba et al.,

(2003) indicated that *Pseudomonas aeruginosa* has excellent antimicrobial properties against both gram positive and gram negative.

Highest zone of inhibition of 16mm was observed against *E. coli* by TSH2 and lowest zone of inhibition of 4mm was noted against *K. pneumonia* by TSP3 in the current study. In a similar study conducted in India, bacterial strains isolated from soil showed antibiotic activity under normal growth condition and were found to have Maximum inhibition diameter 19.8mm against *E. coli* and Minimum zone of inhibition of 8.2mm against *K. pneumonia* (Sethi et al., 2013). Another study done in Saudi Arabia, Isolated Bacillus species from soil samples and showed highest zone of inhibition of 26mm against *E. coli* and lowest zone of inhibition of 6 against *S. aureus* (Al-Humam, 2016).

According to O'sullivan et al., 2002 , the ability of microorganisms to produce antimicrobial compounds can the improved or deteriorated under different culture conditions. In the present study, antimicrobial metabolites production was evaluated by manipulation of fermentation parameters such as temperature, pH and incubation period. Temperature is considered as an important physical factor that affects the growth of microorganisms along with production of secondary metabolites. In this study, high temperature (45°C) were not suitable for the production of antimicrobial compounds while 35°C was an optimum temperature for production of antimicrobial metabolites. Our results are in accordance with the study conducted by Palanichamy *et al* (2011) in which the optimization of cultivation parameters for growth and production of biological pigments, temperature range appeared to be effective with the optimum temperature range being 28-30°C. The current study also correlate with findings by Berdy, (2005) who reported maximum titer of antimicrobial metabolites at 35°C. In another study conducted in India, they documented that the strain showed

maximum production of antimicrobial metabolites and biomass levels when it was incubated at 35 °C (Kadiri and Yarla, 2016).

Maximum production for antimicrobial compounds in the present was found suitable near neutral pH value. Another study explored the fermentation conditions to enhanced antibacterial metabolites from a strain isolated from cassava rhizosphere showed that the growth of the strain was outlined within a pH range of 6–8 with optimum growth at pH 7 (Yun et al., 2018). Another study done in India, isolated a fungal strain from soil, and examined the effect of different fermentation parameters. The results showed maximum biomass and antimicrobial activity were at pH level of 3 (Pandey et al., 2018). Although studies have investigated the effects of growth parameters for the maximum production of antimicrobial compounds (Zong et al., 2015, Yang et al., 2018, Bundale et al., 2015), the results vary with the particular type of microorganism and their initial habitat of which they were isolated from, with the new environmental condition (Laboratory), consequently the differences can affect production of antibiotics.

Another study explored the optimum time at which the maximum antimicrobial production activity occurred, and revealed that the maximum antimicrobial production varies with the particular species of bacteria, because different species have different metabolic pathways (Demirkan *et al.,* 2013). Our results revealed that antimicrobial metabolite production started after 48h of incubation and reached the maximum at 120h and thereafter its production gradually declined. Another study done by Atta, (2015) indicated that maximum inhibition zones of produced antibiotic against tested microorganisms was reached at after 5 days of incubation. The current study results also correlate with another study done by Awais *et al.,* (2007) who also reported maximum inhibition after 3-5 days of incubation of bacterial isolates from soil samples.

5.2. Conclusion.

The results of the present study indicate that soil contain great diversity of antibiotic producing organisms. The isolated strain TSH2 exhibited narrow spectrum of antimicrobial activity as it showed great activity against gram positive bacteria. The results of the antibacterial test indicated that 1 of the 4 isolates showed consistency of inhibitory activity against pathogenic bacteria. Strain improvement of this bacteria may yield better results in fighting against β-lactam resistant *E. coli*. In addition, the findings suggest that the antimicrobial compound(s) produced by these isolates might be a novel anti-bacterial substance and there is a chance to develop other industrially important metabolites from them. In the future, there is need to do purification and the characterization antimicrobial compounds using NMR.

5.3. Limitations: Although this study has provided us with novel information in the field of antibiotics discovery research. The use of indicator microorganism has limited the scope of our study since the potential antibiotic producer which *E. coli* was resistant to would remain undetected.

5.4. Recommendation:

- Further research on purification and characterization of the specific antimicrobial compounds produced by these microorganisms is of great importance. This will help to elucidate the structures and biochemical characteristics of any novel bioactive metabolites detected.
- Further analysis of bacteria is necessary for complete characterization and identification of strains by carrying out full genome sequencing.
- Modification of protocols to allow the isolation of more diverse genera.

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