



**ISOLATION AND CHARACTERASATION OF ANTIMICROBIAL PRODUCING
BACTERIA ASSOCIATED WITH THE SOIL**

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**A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES,
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**IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF
BACHELOR OF SCIENCE DEGREE (B. Sc.) IN MICROBIOLOGY**

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DECLARATION

I hereby declare that the project report was written under the supervision of Dr. M.A. Abiala and it is a product of my own research work. Information derived from various sources has been duly acknowledged in the text and the list of references provided. This researched project report has not been presented for the award of any degree or certificate.

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Date

CERTIFICATION

This is to certify that this research project titled “**ISOLATION AND CHARACTERASATION OF ANTIMICROBIAL PRODUCING BACTERIA ASSOCIATED WITH THE SOIL**” was carried out by **OLUWATOYIN, PRECIOUS**, with Matriculation number **18010101022**. This project meets the requirements governing the award of **Bachelor of Science (B.Sc.)** Degree in **Microbiology** from the Department of Biological Sciences of the Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This project is dedicated to the Divine, the giver of life, my support and strength and to my parents, Mr. and Mrs. Oluwatoyin for their constant prayers, advise and unending support.

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ABSTRACT

Antimicrobial agents or antibiotics are the most significant commercially available and utilized secondary metabolites, which are highly produced by the soil microbes which includes bacteria. In this study, bacteria were isolated from a local soil samples using serial dilution and pour plate technique. They were screened against known pathogenic bacteria using perpendicular method. After primary screening, out of ninety-eight (98) bacteria isolates two bacterial strains (wss8 and pfss11) exhibited antimicrobial activity against some common pathogenic bacteria namely, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella species*. To identify the antimicrobial producing bacteria (wss8 and pfss11), morphological identification was carried out and biochemical tests were performed and it was found that wss8 was *Micrococcus luteus* and pfss11 was *Bacillus subtilis*. The present work suggests that soil isolates, having antibiotic producing properties can be utilized commercially after proper standardization.

Keywords: Soil sample, Antibiotic producing microorganisms, antimicrobial activity, biochemical tests, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella*

CHAPTER ONE

INTRODUCTION

1.1 Background of study

Antibiotic resistance has recently emerged as a significant and alarming problem in the medical community. It is a result of the overuse or improper prescription of antibiotics, which has affected the empirical treatment of patients. Many pathogenic bacteria have developed resistance to common antibiotics by having the following traits: decreased cell permeability, efflux pump, and hydrolysis of the beta-lactam ring by beta-lactamases. One of the main methods for resistance to the current non-beta-lactam antibiotics is the ability of a pathogenic bacteria to create metallo-beta-lactamases. These demonstrate the urgent need for new antibiotics as well as other antimicrobial compounds that can be employed in a variety of settings, including hospitals, food preservation, and dairy products. (Procopio, *et al.*, 2012).

Antibiotics are natural compounds of biological, synthetic, and semi-synthetic origins that are secondary metabolites produced by microorganisms. Antibacterial drugs, usually known as antibiotics, work to stop or inhibit the development of germs. They are used to treat illnesses brought on by germs and include a number of strong medications. Viral illnesses including the common cold, the flu, and most coughs cannot be treated by antibiotics. To increase the number of antibiotics found, researchers are currently seeking for collections of microbes. This discovery will thereby boost the likelihood of identifying bacteria strains from soil samples that may have biochemical and pharmacological benefits. Since it is far simpler to isolate antibiotics from microorganisms than it is to chemically synthesize them, this method has led to the discovery of innumerable new antibiotics to date. (Shales, 2010)

Due to its heterogeneous character, soil is thought to be the home to numerous and diverse populations of microorganisms. The vast differences between the biotic and abiotic environments in soil force its microbial populations to adapt and create means of successful survival. The most successful methods for this adaptation are those that produce antimicrobials (Davies, 2015). Soil bacteria and fungi are responsible for producing antibiotics such -lactams, aminoglycosides,

streptomycins, and tetracyclines, among others. The majority of antibiotics currently in use are extracted from soil bacteria and used to produce other antibiotics. Because of the formation of resistant endospore and the synthesis of essential antibiotics like bacteriocin, *Bacillus* species, commonly found soil bacteria, have been proven to inhibit the growth of other organisms. Antibiotic-producing bacteria should be screened since they are simple to isolate, cultivate, maintain, and improve the value of their strains. However, the number of these microorganisms in the soil varies depending on a number of variables, including the soil's type, water activity, oxygen content, pressure, temperature, salt concentration, carbon sources, pH, and others. Recent studies have demonstrated that antimicrobial activity screening of soil has been done in many different locations of the world (Makut *et al.*, 2011). From this bioresource, numerous antibiotics with pharmaceutical importance have been discovered in the past. Examples include vancomycin, which was produced by the soil bacterium *Streptomyces orientalis* and isolated from a soil sample from Borneo (Griffith *et al.*, 2010), kanamycin, which was produced by the soil bacterium *Streptomyces kanamyceticus* (Umezewa, 1957), and erythromycin, which was first discovered in 1952 by Actinomycetes, Bacilli, and Pseudomonas are the bacterial taxa that have been identified thus far as a bio resource with a high likelihood of detecting chemicals of interest (Walsh, 2003). This study was conducted with the aim of isolating, characterizing, and identifying microorganisms that produce antibiotics from soil samples. Additionally, to evaluate the isolated bacteria's ability to suppress several human pathogenic microorganisms.

1.2 Justification of Study

There are growing numbers of antibiotic resistant strains, mostly the acquired multi-drug resistant strains which is becoming fast developing concern and leads to serious public health problems worldwide. Soil is a complex and a diverse environment providing useful source of antibiotic producing bacteria. Thus, there is a need to isolate and characterize antimicrobial producing bacteria from the soil for the production of antibiotics.

1.3 Aim of the study

The aim of this research is to isolate, identify and characterize antimicrobial producing bacteria associated with the soil.

1.4 Objectives of the Study

The objectives of this research are as follows:

- i. Identification of antimicrobial producing bacteria associated with the soil using standard method
- ii. Culturing, colony counting and isolation of bacteria from the soil samples
- iii. Morphological and biochemical characterizations of the isolated bacteria
- iv. To characterize antimicrobial producing bacteria from the soil

CHAPTER TWO LITERATURE REVIEW

2.1 What are antimicrobial agents

An antimicrobial is a substance that either eliminates or inhibits the growth of microorganisms. Antimicrobial medications can be put together based on the germs that they are most effective against. Antibiotics are used to treat bacteria, while antifungals are used to treat fungi. They can also be categorized based on how they are used. Microbicides are substances that kill microorganisms, whereas bacteriostatic substances just prevent their development. Antimicrobial prophylaxis and antimicrobial chemotherapy are terms used to describe the use of antimicrobial treatments to treat and prevent infections, respectively. (Gottlieb, 2015).

Antibiotics, which eradicate microorganisms inside the body, disinfectants, which kill a wide range of microbes on inanimate surfaces to stop the spread of disease and, antiseptics, which are applied to living tissue and help prevent infection during surgery, are the three main categories of antimicrobial agents. Originally used to describe only preparations derived from living microorganisms, the term "antibiotic" is now also used to describe synthetic substances such as sulfonamides and fluoroquinolones. Bacteriostatic agents, which inhibit or stop bacterial growth, can be further divided into bactericidal agents, which kill bacteria. Further developments in antimicrobial technologies have led to systems that can do more than only prevent bacteria growth in response. Instead, specific kinds of porous media have been created to eradicate microorganisms. (Gottlieb, 2015).

2.1.1 What are antibiotics

An antibiotic is a type of antimicrobial drug that fights microorganisms. Antibiotic medications are frequently used in the treatment and prevention of bacterial infections since they are the most important form of antibacterial agent for doing so. They might stop the spread of bacteria or restrict it. Some antibiotics also have a little level of antiprotozoal action. Antibiotics should not be used to treat viral illnesses like the common cold or influenza; instead, antiviral medications or antivirals should be utilized (Gottlieb, 2015).

Occasionally, the term "antibiotic"—literally "opposing life," from the Greek roots "anti," "against," and "bios," "life"—is used to refer to any substance used against microbes, but in typical medical treatment, antibiotics—like penicillin—are those produced naturally (by one microorganism fighting another), whereas non-antibiotic antibacterial agents are entirely synthetic. Nevertheless, both types are present in antimicrobial chemotherapy and share the similar objectives of eliminating or inhibiting the growth of germs. In contrast to antibiotics, which are a significant class of antibacterial used more precisely in medicine and occasionally in livestock feed, "antibacterial" includes antiseptic medications, antibacterial soaps, and chemical disinfectants (Gottlieb, 2015).

2.1.1.1 History

Since ancient times, antibiotics have been utilized. Numerous references to the therapeutic benefits of moldy bread come from ancient Egypt, Nubia, China, Serbia, Greece, and Rome, among other cultures. John Parkinson was the first to formally record the use of mould to treat illnesses (1567–1650). In the 20th century, antibiotics changed medicine. Modern penicillin was discovered in 1928 by Alexander Fleming (1881–1955), and its widespread use was extremely helpful throughout the war. However, due to their efficiency and accessibility, antibiotics are sometimes overused, and some bacteria have developed resistance to them (Mensar, 2013). Antimicrobial resistance has been labeled a widespread hazard by the World Health Organization because it "is not a prediction for the future; it is happening right now in every region of the world and has the potential to affect everyone, of any age, in any country." In 2019, 1.27 million deaths were attributed to antimicrobial resistance worldwide (Murray *et al.*, 2022).

2.1.1.2 Classification

Antibiotics have been categorized using a variety of factors, including their method of action, generating organisms, and biosynthetic pathways (Berdy 1974; Queener et al. 1978). These requirements are problematic due to the fact that some microorganisms can produce a large number of antibiotics or that multiple modes of action can be active at once. Following that, antibiotics were divided into 13 classes based on their chemical makeup. This classification is the most often used since it can readily include the most recent antibiotic discoveries. Aminoglycosides (such as

kanamycin, neomycin, and streptomycin), ansamacrolides (such as rifamycin), beta-lactams (such as ampicillin, meropenem, penicillin G, ceftiofur, and cefotiam), chloramphenicol and its analogues, lincosaminides (such as lincomycin), macrolides (such as erythromycin, oleandomycin, tylosin), nucleosides (such as puromycin), oligosaccharides (such as puromycin), peptides (such as neomycin, bacitracin, avermectin), phenazines such as myxin), polyenes (such as amphotericin B), polyethers (such as nigericin, monensin, salinomycin), and tetracyclines (such as tetracycline, chlortetracycline, oxytetracycline) (Queener *et al.*, 1978).

2.1.1.3 *Mechanisms of Action*

Antibiotics are typically grouped according to how they work, their chemical makeup, or the range of activities they have. Most attack bacterial mechanisms of growth or function. Bactericidal actions are exhibited by those that attack the bacterial cell wall (penicillins and cephalosporins), the cell membrane (polymyxins), or the important bacterial enzymes (rifamycins, lipiarmycins, quinolones, and sulfonamides). Bacteriostatic agents include tetracyclines, lincosamides, and macrolides, which limit protein synthesis (with the exception of bactericidal aminoglycosides). Their target specificity is the basis for further categorization. Broad-spectrum antibiotics have an impact on a variety of bacteria, while "narrow-spectrum" antibiotics target particular types of bacteria, such as gram-negative or gram-positive bacteria. After a 40-year hiatus, classes of antibacterial chemicals have been discovered in the late 2000s and early 2010s, four new groups of antibiotics were made available for clinical use: cyclic lipopeptides, including daptomycin, glycylicyclines, including tigecycline, oxazolidinones, including linezolid, and lipiarmycins, including fidaxomicin (Shlaes 2010).

Table 2.1: Antibiotics produced by various microorganisms with discovery date

Antibiotic	Microorganism/source	Discovery year
Penicillin	<i>Penicillium notatum</i>	1929
Griseofulvin	<i>Penicillium griseojitvum</i>	1939
Tyrothricin	<i>Bacillus brevis</i>	1939
Streptomycin	<i>Streptomyces griseus</i>	1944
Bacitracin	<i>Bacillus subtilis</i>	1945
Cephalosporins	<i>Streptomyces clavuligerus</i>	1945
Chloramphenicol	<i>Streptomyces venezualae</i>	1947
Neomycin	<i>Streptomyces fradiae</i>	1949
Tetracyclines	<i>Streptomyces aureofaciens</i>	1952
Viomycin	<i>Streptomyces vinaceus</i>	1951
Vancomycin	<i>Streptomyces orientalis</i>	1956
Kanamycin	<i>Streptomyces kanamyceticus</i>	1957
Rifamycin	<i>Amycolatopsis mediterrane</i>	1957
Fusidic acid	<i>Fusidium coccineum</i>	1963
Gentamicin	<i>Micromonospora purpurea</i>	1963
Fosfomycin	<i>Streptomyces fradiae</i>	1969
Ribostamycin	<i>Streptomyces ribosidificus</i>	1970
Mupirocin	<i>Pseudomonas fluorescens</i>	1985
Biapenem	Actinomycete	2002
Doripenem	Actinomycete	2005
Retapamulin	Fungus	2007
Ceftobiprole Medocaril	Fungus	2008
Ceftaroline fosamil	Fungus	2010

Adapted from: Handbook of Antibiotics, edited by Baron (1950) (Butler and Cooper 2016)

2.1.1.4 Resistance

Antibiotic-resistant bacterial development is a well-known occurrence. Resistance development frequently reflects evolutionary processes that take place when taking antibiotics. The use of antibiotics may favor bacterial strains with physiologically or genetically improved resistance to high antibiotic dosages. Under certain circumstances, it may lead to the preferential growth of resistant bacteria, while the medication inhibits the growth of vulnerable germs (Levy, 2012). The Luria-Delbrück experiment, for instance, showed that antibacterial selection for strains that had previously acquired antibacterial-resistance genes occurred in 1943. (Luria *et al.*, 2015). Due to the growing resistance of many bacterial strains, antibiotics like penicillin and erythromycin, which once had a high efficacy against numerous bacterial species and strains, have lost some of their effectiveness. (Pearson, 2007).

Resistance may manifest as the biodegradation of pharmaceuticals, such as the introduction of sulfamethazine-degrading soil bacteria through medicated pig feces (Topp *et al.*, 2013). Bacterial survival frequently depends on an inherited resistance (Witte, 2004), although antibacterial resistance can also spread by horizontal gene transfer. The likelihood of horizontal spread is higher in areas where antibiotic usage is common (Dyer, 2003). In the absence of antibacterial chemicals, for example, antibacterial resistance may have a biological cost that lowers the fitness of resistant strains and prevents the spread of these bacteria. However, other mutations might offset this fitness cost and help these bacteria survive (Andersson, 2006). Antibiotics and antibiotic resistance are both old substances and mechanisms, according to paleontological evidence (D'Costa *et al.*, 2011). Antibiotic targets are those whose mutations impair bacterial survival or reproduction (Gladki *et al.*, 2013).

The rise of diseases that were once under control is now attributed to antibacterial-resistant strains and species, also known as "superbugs." For instance, emerging tuberculosis-causing bacterial strains that are resistant to earlier antibacterial therapies provide numerous therapeutic difficulties. Multidrug-resistant tuberculosis (MDR-TB) is anticipated to cause roughly 500,000 new cases each year (WHO). An *E. coli* "superbug" resistant to colistin, "the final line of defense" antibiotic, was discovered in the United States on May 26, 2016. (McGann, 2016; Moyer, 2016).

2.1.1.5 Applications of Antibiotics

Antibiotics have uses in aquaculture, agriculture, and medicine for both humans and animals (Kummerer, 2019). The class of antibiotics known as beta-lactams, which includes penicillins and cephalosporins, is the one that is most commonly used in human medicine worldwide. In addition to veterinary procedures, antibiotics are also employed in the case of animals for animal breeding and growth promotion (Gaskins *et al.*, 2012). Antibiotics like streptomycin and oxytetracycline are frequently used to stop infections in plants that produce crops, vegetables, and fruit. Numerous antibiotics, including oxytetracycline, sulphonamides, premix, sarafloxacin, erythromycin, and florfenicol, have been used in the farming of aquatic animals including fish and molluscs. (Cabello, 2004; Wolff, 2004).

2.2 Soil as a source of microorganisms

The Earth's crust's topmost layer, known as soil, was created by the ongoing weathering of geological rocks. By providing nutrients and a place to anchor, this outer, loose portion of the lithosphere promotes the growth of soil organisms like bacteria and plants. Numerous biological interactions and reactions that underpin life occur on live, dynamic soil (Aminov, 2009; Alvarez Lerma *et al.*, 2010; Allen *et al.*, 2010). Additionally, soils are essential for preserving and cleaning up freshwater environments. Soil provides support for many different natural functions, including land for building, food production, obtaining raw materials for various manufacturing processes, and other natural resources. Consequently, soil has a crucial role for humans. Each form of soil has different properties depending on the parent rock type, geomorphological and land use history, climate, and soil flora and fauna. Soil is made up of solid, liquid, and gaseous phases. (Butler and Buss, 2006).

2.2.1 Components of the Soil

The elements that make up soil include minerals, organic materials, water, air, and soil organisms. The iron, aluminum, calcium, manganese, potassium, silicon, sulfur, phosphorus, and many other trace elements that make up soil's mineral or inorganic component come from the deterioration and corrosion of parent rock material. As a result, depending on the source rock, different soils have varying mineral contents and chemical compositions. The live, fecal, and dead remains of

plants and animals are the primary sources of the organic matter in the soil, which is mostly composed of carbon, nitrogen, and phosphate sources. Microorganisms and other soil inhabitants use organic matter as a source of energy. The amount of water in soil varies depending on the topography of the area and is influenced by the amount of rain, snow, and irrigation that is available. The atmospheric air that fills the pores between soil particles is made up of soil gases. For plants to properly absorb nutrients, thrive, and have a healthy metabolism, the soil has to have both water and air. (Baron, 2017; Martinez *et al.*, 2019).

2.2.2 Types of microorganisms in the soil

Microorganisms are tiny organisms that can be made up of a single cell or a colony of cells. Bacteria, fungi, protozoa, microalgae, and viruses can all be categorized among them. These organisms can be found in a variety of diverse and harsh habitats, including soils, water, food, and animal intestines. (Lancini and Parenti, 2015; Fischbach and Walsh, 2019).

2.2.2.1 Bacteria

Bacteria are the most prevalent microbes in soil and can flourish in a variety of habitats. Bacteria are unicellular, prokaryotic bacteria without chlorophyll. Cocci, which have a circular shape, Bacilli, which have a rod shape, and Spirilla (long wavy chains of cells). Out of them, Bacilli, Cocci, and Spirilla are most prevalent in soil. Some of the bacterial genera most frequently found in soil are *Pseudomonas*, *Arthrobacter*, *Achromobacter*, *Clostridium*, *Enterobacter*, *Sarcina*, *Micrococcus*, *Cyptophaga*, and *Chondrococcus*. Plants and other soil organisms benefit greatly from soil bacteria because they initiate the breakdown process and enhance nutrient availability. Making atmospheric nitrogen available to plants is made much easier by certain nitrogen-fixing, nitrifying, denitrifying, and ammonifying bacteria (Ruan, 2013).

2.2.2.2 Fungi

Another microorganism found in soil that possesses filamentous mycelium is a fungus. *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Cephalosporium*, *Chaetomium*, *Mucor*, *Monilia*, *Penicillium*, *Fusarium*, *Rhizopus*, *Gliocladium*, *Trichoderma*, *Pythium*, *Verticillium*, etc. are among the genera

with the most widespread soil fungi. Fungi are resistant to acidic soil conditions and aid in the first breakdown of new organic materials. The primary lignin decomposers are fungi, which are also significant in the breakdown of cellulose, hemicellulose, and pectin (Ruan, 2013).

2.2.2.3 Actinomycetes

These microbes have been put into a different group because they exhibit traits that are similar to both bacteria and fungi. Similar to bacteria, actinomycetes are unicellular and have hyphae and conidia like fungus. The most prevalent genera found in soil are *Actinoplanes*, *Nocardia*, *Streptomyces*, and *Micromonospora*. They also aid in the humus and organic wastes in the soil's decomposition (Ruan, 2013; Qin *et al.*, 2009; Koberl *et al.*, 2013).

2.2.2.4 Algae

In areas with lots of water and sunlight, such as marshes, paddy fields, depressions, or flooded terrain, algae can thrive. Algae, like plants, use sunshine to break down atmospheric carbon into sugar molecules that can be used to produce energy. They establish mutualistic relationships with other organisms and support soil fertility. (Martinez *et al.*, 2019).

2.2.2.5 Protozoa

Single-celled organisms known as protozoa move with the aid of a variety of organs, including flagella, cilia, and pseudopodia. They are secondary consumers that eat other protozoans, soil fungus and bacteria, and organic soil detritus. They support the soil's beneficial microbial community and aid in the release of nutrients from organic materials. (Martinez *et al.*, 2019).

2.2.3 Importance of soil microorganisms

The majority of reactions and interactions in soil, which support all plants and, by extension, all animals, depend on soil bacteria. As was already said, the breakdown and decomposition of organic matter is predominantly carried out by soil bacteria. The recycling of elements that support life, such as carbon, nitrogen, and phosphorus, which are essential for the synthesis of

biomolecules, is greatly aided by soil microbes. By using a process called biological nitrogen fixation and ammonification, soil bacteria can fix atmospheric nitrogen. This fixed nitrogen is cycled by a number of other nitrifying and denitrifying bacteria. By boosting the availability of nutrients and the synthesis of growth hormones and antibiotics, interaction between plants and soil microbes is known to encourage plant growth. Beneficial microbes guard the roots from pathogenic bacteria and insect growth, protecting plants from disease. By enhancing soil porosity, forming soil aggregates, and enhancing water infiltration, soil microbes also maintain the structure of the soil. For the detoxification and recycling of trash, soil bacteria are also crucial. The biodegradation of oil, herbicides, insecticides, heavy metals, xenobiotic chemicals, and other harmful pollutants is carried out by a variety of naturally occurring bacteria. Numerous soil bacteria may also be useful in bioleaching, bioremediation, biocomposting, and biofertilizers. The creation of secondary metabolites like antibiotics, which have a significant impact on the development of plants, animals, and humans, is another major use of soil bacteria. (Falconer and Brown, 2019; Aminov, 2015; Baron, 2018; Baltz, 2018; Demain and Sanchez, 2019).

2.2.4 Antibiotic producing bacteria found in soil

2.2.4.1 *Bacillus species*

Aerobic rods that produce spores and stain gram positive or gram variable are known as *Bacillus spp.* Except for a small number of species, the vast majority are not pathogenic and have never been linked to disease in humans or other animals. The genus's members provide important microbiological purposes. Numerous *Bacillus* species produce peptide antibiotics that can be made using either a ribosomal or non-ribosomal method. (Turnbull *et al.*, 2016).

2.2.4.1.1 *Bacillus licheniformis*

A Gram-positive, spore-forming soil bacterium called *Bacillus licheniformis* is employed in the biotechnology sector to produce enzymes, antibiotics, biochemicals, and consumer goods. Being a facultative anaerobe, *B. licheniformis* might be able to flourish in more ecological niches. Additionally, certain strains of *B. licheniformis* are utilized to create peptide antibiotics like bacitracin and proticin. (Mahajan *et al.*, 2017).

2.2.4.1.2 *Bacillus polymyxa*

Gram-positive bacteria called *Paenibacillus polymyxa*, also referred to as *Bacillus polymyxa*, have the ability to fix nitrogen. It can be found in hot springs, marine sediments, plant tissues, and soil. Some *P. polymyxa* strains produce the antibiotics fusaricidin and polymyxins. (Shaheen *et al.*, 2011).

2.2.4.1.3 *Brevibacillus brevis*

Formerly known as *Bacillus brevis*, *Brevibacillus brevis* is a rod-shaped, Gram-positive, aerobic, motile, spore-forming bacteria that is typically found in soil, air, water, and decomposing waste. Rarely is it connected to infectious disorders. It is where gramicidin and tyrocidine, two antibiotics, were originally identified. (Abedon *et al.*, 2006).

2.2.4.1.4 *Bacillus subtilis*

A rod-shaped, gram-positive bacteria called *Bacillus subtilis* produces spores that can withstand heat. The soil is a typical place to find it. It is not infectious. The most well-studied Gram-positive bacterium is *B. subtilis*, which is also used as a model to research bacterial cell development and chromosome replication. Numerous *B. subtilis* strains have been reported to produce a few antibiotics, including subtilisin, surfactin, and bacilysin. (Hofemeister *et al.*, 2004).

2.2.4.2 *Streptomyces species*

Important subgroups of the actinomycetes family of soil bacteria are known as *streptomyces*. *Streptomyces* have a sophisticated secondary metabolism. Over two-thirds of the naturally occurring antibiotics that are clinically helpful are produced by them.

Numerous antibiotics are derived from members of the genus *Streptomyces*; the most significant of them are (Procopio *et al.*, 2012):

- Chloramphenicol from *S. venezuelae*
- Daptomycin from *S. roseosporus*

- Fosfomycin and Neomycin from *S. fradiae*
- Lincomycin from *S. lincolnensis*
- Puromycin from *S. alboniger*
- Streptomycin from *S. griseus*
- Tetracycline from *S. rimosus* and *S. aureofaciens*
- Oleandomycin, Mycangimycin and Boromycin from *S. antibioticus*
- Tunicamycin from *S. torulosus*

2.2.4.3 *Micrococcus species*

Water, dust, and dirt are just a few of the many settings where *Micrococcus can be found*. *Micrococci* often appear in tetrads and have spherical, Gram-positive cells that range in diameter from around 0.5 to 3 micrometers. Rare reports of its antibacterial efficacy against several infections. This bacterium is widespread in nature and is simple to find in undeveloped areas. (Willey *et al.*, 2008).

2.2.4.3.1 *Micrococcus luteus*

Micrococcus luteus is a member of the family *Micrococcaceae* and is a Gram-positive to Gram-variable, non-motile, coccus, tetrad-arranging, pigmented, saprotrophic bacterium. Both urease and catalase are present. *M. luteus*, an obligate aerobe, is a common component of the natural cutaneous microbiota of mammals and can be found in soil, dust, water, and air. Neoberninamycin is an example of an antibiotic generated by *M. luteus*. (Willey *et al.*, 2008)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The materials used includes; Distilled water, Sterile Petri dishes, Cotton wool, Alcohol (70% ethanol), Inoculating loop, Slides, Test tubes, Measuring cylinder, Conical flask, Durham tubes, Beaker, Aluminum Foil, Dropper, medium bottles, micropipette.

3.2 Culture media

The culture media used includes; Nutrient Broth, Simmon citrate, Methyl red Vogues Proskauer Agar, Nutrient Agar for the isolation of bacteria.

3.3 Equipment and Reagents

Equipment used includes; Oven, Incubator, Autoclave, Weighing Balance, water bath, Thermometer, Colony counter.

Reagents used include; Gram iodine, Kovacs Reagent, Crystal violet, Sodium Hydroxide (NaOH), Ethanol, Rhodamine B, Methyl red.

3.4 Collection of soil samples

Different soil sample were collected from agricultural and non-agricultural environment. Soil sample were collected in such way to get the soil of crust and depth of at least 6 inches with the help of sterile spatula and placed in sterile plastic bags for transportation to laboratory.

3.5 Preparation of nutrient agar

According to the manufacturer's instruction, Nutrient Agar medium was prepared 28g of nutrient agar was measured on a weighing balance into a sterile conical flask; 1000ml of distilled water was dispensed into the conical flask. Nutrient agar 7g was measured in 250 ml of water. Swirling

was done to the solution in the conical flask to dissolve the medium. The solution was then boiled in the water bath to ensure homogenization after mixing has been properly done. After boiling, the medium was autoclaved for 15mins at 121°C. Immediately after autoclaving, the medium was poured in the plates after serial dilution was done.

3.6 Isolation of microorganisms from soil sample

Initially, one gram of soil sample was taken and serially diluted up to 10^{-8} . From this, an aliquot of 0.1 mL of diluted sample factor 10^{-5} and 10^{-7} was taken and distribute the samples spread evenly over the surface of the plates and pouring the nutrient agar on it using the pour plate technique (Athalye, *et al.*, 1981). Subsequently, plates were incubated at 37°C for 24hours and were observed for the appearance of colonies. Afterwards, a loopful of distinct colonies were aseptically picked and further sub-cultured on freshly prepared nutrient agar for 24 h at 37°C to obtain pure cultures for further analysis.

3.7 Antimicrobial screenings

3.7.1 Primary screening

The antibacterial activity of pure isolates was determined by cross-streak method on nutrient agar (Lemos, *et al.*, 1985). The isolated bacteria strains were streaked as a parallel line on nutrient agar plates, *Escherichia coli*, were streaked at right angles to the original streak of bacteria and incubated at 37°C. The inhibition zone was measured after 48 hr.

3.7.2 Secondary screening

The isolated cultures were tested for their anti-bacterial activity against the test strains. The colonies taken were grown overnight in nutrient broth and were further screened for their inhibiting effect on known pathogenic microorganisms like pathogenic *E. coli*, *salmonella* and pathogenic *staphylococcus*. The isolates that produced the best zones of inhibition were taken and biochemical tests were performed to identify the antibiotic producers.

3.8 Morphological and biochemical identification of isolate

The antimicrobial positive isolate was identified using morphological and Biochemical characterizations. Morphological characterizations were done using colonial, cellular and pigment appearances on culture plates. Biochemical characterizations were done using Gram Staining, Oxidase test, Catalase test, Methyl red/ Voges Proskauer test, spore staining, coagulase test, Starch hydrolysis test, Sugar fermentation Test, Simmons Citrate test and Indole test.

3.8.1 Gram staining

The Gram stain is fundamental to the phenotypic characterization of bacteria. A smear was made on a glass slide and heat fixed. The crystal violet which is the primary stain was flooded on the fixed culture for 60 seconds; the stain was washed off with water. Iodine solution was added to the smear for 60seconds and was poured off; then was rinsed with water. A few drops of ethyl alcohol (decolorizer) were added and rinsed with water immediately after 5seconds and finally safranin which is the secondary stain was added for 60seconds and washed off, then the smear was left to air dry. After the drying of the slide, it was observed under the microscope. Gram staining was done to find reactions of the bacterial isolates to Gram reagents. Gram stain helps in distinguishing and classifying bacterial species into two large groups: gram-positive bacteria and gram-negative bacteria.

3.8.2 Catalase test

The catalase test is used to distinguish between Streptococci, which do not produce catalase enzyme, and Staphylococci, which do. In a test tube, 1 mL of hydrogen peroxide solution and a modest amount of bacteria growth were introduced. The appearance of air bubbles signified a successful outcome. (Cheesebrough, 2000).

3.8.3 Oxidase test

Utilizing an oxidase strip that has been soaked in a few drops of oxidase reagent, an oxidase test is conducted. Colonies from the 24-hour-old pure culture are collected using a sterile wire loop, and each colony is then smeared on the oxidase strip. A positive outcome is indicated by the

emergence of purple colour within 10 seconds, while a bad outcome is indicated by no color change. Two to three drops of oxidase reagents were applied to a strip. Using a glass spreader, a small amount of the organism was obtained and streaked across the paper's damp surface. The emergence of a deep purple colour indicated the presence of *Pseudomonas* (Hossain *et al.*, 2006).

3.8.4 Indole test

This is accomplished by inoculating the isolated organism in peptone water using a sterile wire loop. At 37°C, the tubes were incubated overnight. After the incubation period, a drop of the Kovacs reagent was added (Cheesebrough, 2006).

3.8.5 Simmons citrate test

In order to completely dissolve the medium, a 2.14g aliquot of Simmons citrate agar was gently homogenized in 500ml of distilled water using a magnetic stirrer. The medium was then autoclaved at 121°C for 15 minutes, let cool at 50°C, and then put into sterile test tubes. A loopful of each isolate was then poked into each test tube, and the tubes were then moved to the incubator and cultured at 37°C for 24 hours. The tubes were seen after 24 hours (Olutiola *et al.*, 2000)

3.8.6 Methyl Red/Voges Proskauer (MRVP) test

Distilled 500 ml of water were gently homogenized with 8.5 g of MRVP broth to completely dissolve the medium. Each test tube received 10ml of the broth, which was then sealed with corks, autoclaved for 15 minutes to sterilize it, and let to cool at room temperature for another 28 minutes. Each test tube was inoculated with a different isolate while being appropriately labeled. After 24 hours, the tubes were inspected after being incubated at 37°C. Each solution was then given 5 drops of methyl red solution. Positive reactions are indicated by the appearance of the color red, whilst negative reactions are shown by the appearance of the color yellow (Olutiola *et al.*, 2000)

3.8.7 Sugar fermentation test

A conical flask was filled with a weight of 5 g peptone, 0.5 g NaCl, 5 g fermentable sugar (glucose, sucrose, maltose, lactose, and galactose), and 1 pint of bromocresol purple. 500 ml of distilled water was then added, homogenized, and divided among 19 test tubes. Each test tube contained an inverted Durham tube, which was sealed with a cork and sterilized for 15 minutes. Each isolate was then individually injected into each test tube and incubated at 37°C. The results were examined and reacted to after 24 hours (Olutiola *et al.*, 2000).

3.8.8 Starch hydrolysis test

Each sterile petri dish had 20ml of molten starch agar added aseptically, allowed to set, and then it was inverted in a 37°C incubator. The organism was streaked across the plate's surface and was then incubated for 24-48 hours at 37°C. The plates were then saturated with a certain amount of Gram's Iodine, and results were seen (Olutiola *et al.*, 2000).

3.8.9 Spore staining

The heat helps a heat-fixed smear to pass through the spore when it is steamed after being saturated with the primary stain, an aqueous malachite green solution. Heating serves as a mordant in this method. The vegetative cells can be easily decolorized with water, but the endospore is resistant to decolorization once it has absorbed the stain (leaving the vegetative cells colorless). The endospores look green when counterstained with safranin, whereas the vegetative cells take on the color of safranin and appear red or pink (Olutiola *et al.*, 2000).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Collection of samples

This study was carried out to screen the antibiotic producing bacteria collected from soil sample of three (3) different sites of the Ogun state, Nigeria, using standard microbiological techniques.

4.2 Isolation of microorganisms from soil sample

After the serial dilution (dilution factor 10^5 and 10^7) of 15 soil sample 5 each from each sites, a total of 98 isolates were cultured in a freshly prepared nutrient agar plate and incubated for 24hrs at 37°C to obtain pure cultures for further analysis. The differences in the bacteria distribution could be related to the different activities been carried in the sampling points and the soil type as seen in waste and pig farm soil sample than in just street soil. Besides, the organic constituents of the sampling point can as well influence the population of microorganisms in that soil and might also affect the synthesis of antibiotic production. (Table 4.1, 4.2 and 4.3)

4.3 Primary screening

After the proper subculturing of the 98 isolates to get pure culture of the isolates, pure cultured isolates were used to streak lines on the nutrient Agar (NA) which was incubated for 2–3 days on 28°C . During the primary screening, these streaked lines of bacteria were screened against pathogenic *E. coli* by drawing a perpendicular line to the previous line of bacteria. Results showing the zone of inhibition were observed within 24 hours after proper incubation at 37°C , and the zone of inhibition was recorded in the 98 isolates, only 2 isolates wss8 and pfss11 showed zone of inhibition (ZOI) 1cm and 1.2cm respectively (Table 4.4)

4.4 Secondary screening

The two positive isolates wss8 and pfss11 were further screened for their inhibiting effect on known pathogenic microorganisms like pathogenic *Escherichia coli*, *salmonella* and pathogenic *staphylococcus aureus* and results were recorded in Table 4.5

4.5 Morphological and biochemical characterizations of positive isolate

From the various morphological and biochemical test performed wss8 and pfss11 was identified to be *Micrococcus luteus* and *Bacillus subtilis* respectively. (Table 4.6 and 4.7)

Table 4.1: Colony count for soil sample (Mountain Top University)

Samples	10^{-5} (cfu/cm ³)	10^{-7} (cfu/cm ³)	Isolates
<i>Sss1</i>	80	37	5
<i>Sss2</i>	70	40	4
<i>Sss3</i>	85	20	7
<i>Sss4</i>	80	40	5
<i>Sss5</i>	75	50	5

Table 4.2: Colony count for soil sample (Dumpsite)

Samples	10^{-5} (cfu/cm ³)	10^{-7} (cfu/cm ³)	Isolates
<i>Wss6</i>	70	50	5
<i>Wss7</i>	100	53	10
<i>Wss8</i>	76	20	6
<i>Wss9</i>	65	20	6
<i>Wss10</i>	81	43	4

Table 2.3: Colony count for soil sample (Pig farm)

Samples	10^{-5} (cfu/cm ³)	10^{-7} (cfu/cm ³)	Isolates
<i>Pfss11</i>	90	61	10
<i>Pfss12</i>	70	43	8
<i>Pfss13</i>	76	45	7
<i>Pfss14</i>	89	56	8
<i>Pfss15</i>	70	35	8

Table 4.4: Result for primary screening

<i>Isolates</i>	<i>Status of zone of inhibition</i>
<i>Sss1</i>	No zone of inhibition
<i>Sss2</i>	No zone of inhibition
<i>Sss3</i>	No zone of inhibition
<i>Sss4</i>	No zone of inhibition
<i>Sss5</i>	No zone of inhibition
<i>Wss6</i>	No zone of inhibition
<i>Wss7</i>	No zone of inhibition
<i>Wss8</i>	Zone of inhibition (1cm)
<i>Wss9</i>	No zone of inhibition
<i>Wss10</i>	No zone of inhibition
<i>Pfss11</i>	Zone of inhibition (1.2cm)
<i>Pfss12</i>	No zone of inhibition
<i>Pfss13</i>	No zone of inhibition
<i>Pfss14</i>	No zone of inhibition
<i>Pfss15</i>	No zone of inhibition

Table 4.5: Result for secondary screening

Positive isolates

	ZOI (<i>E. coli</i>)	ZOI (<i>Salmonella</i>)	ZOI (<i>path. Staph.</i>)
<i>Wss8</i>	1	0.9	0.7
<i>Pfss11</i>	1.2	1.1	1.4

Table 4.6: Morphological identifications of isolates

<i>characteristics</i>	<i>Wss8</i>	<i>Pfss11</i>
<i>Shape</i>	Circular	Irregular
<i>Size</i>	Large	Small
<i>Color on nutrient agar</i>	Yellow	Milky
<i>Opacity</i>	Opaque	Opaque
<i>Elevation</i>	Raised	Flat
<i>Surface</i>	Smooth and glittery	Rough and dull
<i>Edge</i>	Entire	Entire
<i>Consistency/Texture</i>	Viscoid	Butyrous
<i>Emulsifiability</i>	Emulsifiable	Emulsifiable

Table 4.7: Biochemical characterization of isolates

Biochemical tests	Wss8	Pfss11
Gram staining	+ve cocci	+ve rod
Indole	-ve	-ve
Methyl red	-ve	-ve
Voges Proskauer	+ve	+ve
Starch hydrolysis	-ve	+ve
Sugar fermentation: glucose	-ve	+ve
Sucrose	-ve	+ve
Inositol	-ve	+ve
Fructose	-ve	+ve
Lactose	-ve	-ve
Maltose	-ve	+ve
Spore staining	-ve	+ve
Catalase	+ve	+ve
Citrate	+ve	+ve
Oxidase	+ve	+ve
Probable bacteria	<i>Micrococcus luteus</i>	<i>Bacillus subtilis</i>

-ve = negative, +ve = positive

4.6 Discussion

Microorganisms have been screened for antibiotic synthesis using relatively rapid and easy procedures while looking for novel antibiotics. Many different types of soil bacteria produce antibiotics as a primary characteristic, which may be a survival strategy. The generation of antibiotics may also be impacted by temperature variations. The soil-isolated bacteria exhibit antibiotic action in conditions conducive to normal growth. In present study, bacteria were isolated from soil samples (agriculture and non-agricultural environments). The selected culture strains of bacteria were then identified by techniques, like gram staining, spore staining and biochemical characterization tests. The isolates were subjected to test microorganism (*E. coli*, *S. aureus*, *Salmonella species*) in order to check their ability to produce antibiotics using perpendicular method. In our study, results indicate that the two positive isolates wss8 and pfss11 were *Micrococcus luteus* and *Bacillus subtilis* respectively.

The test bacterium with the highest zone of inhibition is *Bacillus subtilis*. When it comes to displaying antibiotic activity against *S. aureus* and *E. coli*, *Bacillus subtilis* was dominant. These findings support that of Ahmed *et al.* (2013), who examined soil microorganisms for the generation of antibiotics and found that all studied microorganisms were susceptible to the antibacterial effects of *Bacillus* species. The ability of *B. subtilis* to manufacture antibiotics has been known for the past 50 years (Akbar *et al.*, 2016) *B. subtilis* is a rhizobacterium that produces endospores (Sonenshein *et al.*, 2002). Most industrial soils include *Bacillus subtilis*, which is important for the synthesis of antibiotics with uses in medicine, agriculture, and veterinary care (Akbar *et al.*, 2016; Schallmeyer *et al.*, 2003)

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The present study was an attempt to identify and characterize useful strains of bacteria and to check their ability for antibiotic production. Two bacterial isolate were found producing clear zone of inhibition against the test microorganisms i.e., *S. aureus*, *Salmonella* and *E. coli*. The two potential antibiotic producer bacterial species identified include *Micrococcus luteus* and *Bacillus subtilis* respectively.

5.2 Recommendations

These bacteria's ability to produce antimicrobial compounds has the potential to be helpful for a variety of applications and needs to be further investigated. Therefore, it is advised that additional research be done about characterization employing molecular techniques for their identification, as well as protein electrophoresis and mass spectrometry, which may aid to reveal and the protein's structure. To increase the strain's activity, mutagenic agents should be used to improve the strain. The manufacturing of pure antibiotics can be done using extraction and purification techniques.

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