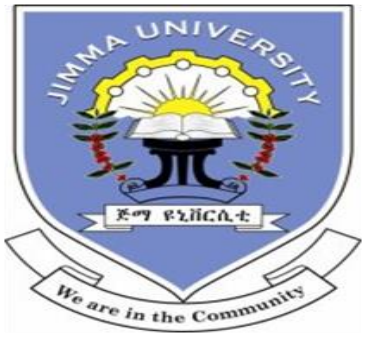

College of Natural Science

Department of Biology

Antimicrobial activities of indigenous trees associated rhisosphere Actinomycetes, southwest Ethiopia

BY: Abdurohman Mohammed

Advisor: Professor Ketema Bacha (PhD)

A thesis Submitted to the Department of Biology in Partial Fulfillment Requirement for Degree of Master of Science in Biology

JUNE, 2023

JIMMA, ETHIOPIA

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Approval sheet

As a thesis advisor, I certify that I have read and evaluated this thesis prepared under my guidance by Abdurohman Mohammed entitled Antimicrobial activities of indigenous trees associated rhisosphere Actinomycetes, southwest Ethiopia in Jimma city and I recommended the thesis paper to be submitted as fulfilling the requirements for the degree of master in Biology.

Approved by all listed below:

1. Ketema Bacha (prof) _____ _____

Name of advisor Signature Date

2. Shiferaw Demissie (PhD, Candidate) _____ _____

Name of Internal Examiner Signature Date

3._____ _____

Name of External Examiner Signature Date

Declaration

I declared that the work in this Thesis “in Jimma city” was performed by me in the Department of Biology Antimicrobial activities of indigenous trees associated rhisosphere Actinomycetes, southwest Ethiopia. This thesis has been submitted in partial fulfillment of the requirements for the award of a Master's Degree in Biology Jimma University.

Declared by:

Name: Abdurohman Mohammed

Signature: _____

Date: _____

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List of Abbreviations/Acronyms

AC Actinomycete Isolates

DAPA Diaminopimelic Acid

MM Millimeter

MRSA Methicillin Resistant *Staphylococcus aureus*

NA Nutrient Agar

PBP Penicillin-binding proteins

SCA Starch casein agar

SPP. Species

Rev/min Revolution per minute

Abstract

Actinomycetes are gram positive bacteria that widely serve as source of novel antimicrobial substance. This study was designed to evaluate antimicrobial activities of actinomycetes isolated from soil in Bada Buna. The study was conducted at Bada buna forest. Rhizospehre soil of indigenous plants of natural Bada Buna natural forest were selected purposively following purposive sampling technique. Then, twenty five soil samples were collected. For laboratory analysis, ten gram of each soil sample was taken in sterilized Erlenmeyer flask containing 90ml of normal saline solution (0.85%) and shaken on rotatory shaker (160rpm) for about5 minutes. The pure cultures were screened for antimicrobial activities by inoculating the pure culture into starch casein broth for fourteen days after which it was centrifuged at 10,000 rpm for 20 minutes. The supernatant was check for antimicrobial activities using disc diffusion and agar well diffusion method. In the present study, 60 actinomycetes were isolated out of which twelve (20%) showed antimicrobial activity against one or more human pathogenic test bacteria and fungus. Among a total of twelve isolates 6 (50%) actinomycetes showed antimicrobial activities both on gram positive and gram negative bacteria,5(41.66%) of the isolates showed antimicrobial activities against only gram negative bacteria and 1(8.33%) the isolate showed antimicrobial activities against gram positive bacteria. Four (33.3%) isolates showed antimicrobial activities on C.albicans (ATCC14053).Of the twelve isolates that displayed antimicrobial activity, 6 isolates were further screened for submerged fermentation based on their activities. Except isolate AC10, all the crude extracts of isolate exhibited antimicrobial activity against all the test organisms during secondary screening process. The isolates were identified and characterized by morphological, physiological, and biochemical characteristics. From the present study it could be conclude that rhizosphere soil could be potential source of antibiotic producing actinomycetes. Further work needs to carried out in details and the isolates identified up to species level by using molecular characterization.

Key word: *Actinomycetes, Antimicrobial, Drug resistance, Rhisosphere*

1. Introduction

Actinomycetes are group of prokaryotic that belong to phylum actinobacteria and order Actinomycetales usually grow with filament formation, free living and saprophytic bacteria (Silambarasan*et al.*, 2012). All members of this order are characterized by high % of guanine (G) +cytosine(C) in their DNA (Ventura *et al*., 2007). They are considered to be an intermediate group between bacteria and fungi, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria (Absan*et a*l., 2017). Actinomycetes are resembling fungi, as their filamentous growth forms mycelia colonies. For this reason they were long regarded as fungi. Due to the absences of chitin, cellulose in their cell wall, lack of nucleus and the presence of antibiotic sensitivity, these organisms were classified as bacteria (Silambarasan*et al*., 2012).

Actinomycetes are widely distributed in both terrestrial and aquatic ecosystems, mainly in soil, where they play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants, animals and fungal materials (Vijayakumar and Malathi, 2014). Actinomycetes are also widely distributed in other natural and man-made environments like manures composts, dusts, food products and colonizing plants (Newman and Gragg, 2007).Actinomycetes are of subject of great interest to scientists and industrialists due to their potential to produce an array of biologically active substances, such as antibiotics, vitamins and enzymes (McCarthy and Williams, 1992).

Actinomycetes species are known to produce different classes of secondary metabolites with diverse biological activity (Vengadesh*et al*., 2011) and are a major source of antibiotic agents, Although they are well known as a rich source of bioactive molecules including antibiotics (Silambarasan et al., 2012) They are also the most economically and biotechnologically valuable prokaryotes able to produce wide range of other bioactive secondary metabolites such as antitumor agents(Piericidins), immunosuppressive agents (Rapamycin), vitamins, nutritional materials, herbicides and pesticides (Ventura *et al*., 2007).

The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000 of which 10,000 are produced by actinomycetes, thus representing 45% of all bioactive microbial metabolites discovered(Ventura *et al*., 2007). Among actinomycetes, approximately 7,600 compounds are produced by Streptomyces species. Several of these secondary metabolites

are potent antibiotics. As a result of which Streptomyces has become the primary antibiotic-producing organism exploited by the pharmaceutical industry (Berdy, 2005). Antibiotics which are produced by actinomycetes have the ability to inhibit both fungi species and different pathogenic bacteria (including gram positive and gram negative bacteria) (Berdy, 2005).

Even though they can inhibit gram positive and gram positive bacteria, the majority of them show more activity against gram positive since gram negative bacteria have an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This structure makes the cell wall impermeable to lipophilic solutes. The Gram positive bacteria more susceptible by having only an outer peptidoglycan layer which is not an effective permeability barrier. Different scholars have affirmed as actinomycetes, particularly Streptomycetes can inhibit both fungi and pathogenic bacteria (Sharon et al., 2014).

The gram positive bacteria more susceptible by having only an outer peptidoglycan layer which is not an effective permeability barrier (Raja *et al*., 2010). Different scholars have affirmed as actinomycetes, particularly Streptomycetes can inhibit both fungi and pathogenic bacteria (Olano*et al.,* 2009). Actinomycetes are isolated from rhisospher soil and sediments of fresh water reservoir and all isolates were able to inhibit gram positive bacteria more effectively than gram negative (Brock, 1993).

In Ethiopia, some scholars have isolated actinomycetes from various areas for their antibiotic production and antimicrobial activities. For instance, Mulatu and Dawit (2016) isolated antifungal producing actinomycetes species from compost prepared for mushroom growth. Even though, some studies which are related to the current study were conducted in Ethiopia, still there is such report from rhizoshere soils of indigenous trees in Jimma Zone, So, this study was designed to evaluate antimicrobial activities of actinomycetes isolated from soil of Jimma areas**.** Therefore, there is an immense possibility to assess antimicrobial activities of actinomycetes in Jimma area.

1.1. Statement of the problems

Ethiopia is one of the sub Saharan African countries facing massive health problems (Rozman et al., 2020). Antibiotic resistant strain is the biggest threats to global health concern and Antibiotic resistance is rising to alarmingly high levels in all parts of the world (Sundaramoorthi*et al*.,

2011). Though there is continuous production of synthetic chemicals as antimicrobials, actinomycetes remain as relevant source of novel, natural antibiotics, which help to combat problems associated with rapid spread of antibiotic resistant pathogens causing life threatening infections (Sundaramoorthi*et al*., 2011).

As present there is also a series problem of multidrug resistant microorganisms that needs us emphasis to the issue. Bacterial pathogens that are resistant to multiple drugs represent a growing public health threat, since multiple drug resistant (MDR) infections are challenging and expensive to treat and few antimicrobial compounds, and still fewer antimicrobial agents using novel mechanisms of action, are in clinical development (Rozman et al., 2020)

Currently, the incidence of multidrug resistant organisms is increasing. As a result, there is a need for new potential drug sources and drugs which are effective again.st current antibiotic resistant pathogens, microorganisms such as, actinomycetes are among the potential source for isolation of bioactive antibiotics for multidrug resistance organisms (Chang *et al*., 2015).
Therefore isolation and characterization of indigenous soil Actinomycetes that have antimicrobial properties and evaluation of their antimicrobial activities has great value (Kuster, 1968). In bacteria no attention to resistance in bacteria and fungi. Therefore, this has been initiated to isolate and characterize antimicrobial activities of actinomycetes isolated from indigenous soil in Bada Buna forest.

1.2. Objective of the study

1.2.1. General objective

To evaluate antimicrobial activities of actinomycetes isolated from rhizosphere soil in Bada Buna forest.

1.2.2 Specific objectives

- ✓ To isolate and characterize antimicrobial producing actinomycetes from rhizosphere soil of indigenous plants of Bada Buna natural forest
- ✓ To evaluate the antimicrobial activities of the isolated actinomycetes against selected bacterial pathogens including *S.aureus (ATCC25923), E. coli (ATCC 25922), S. typhimurium (ATCC 13311)and Candida albicans (ATCC14053*)(fungus*).*
- ✓ To evaluate the stress tolerance of actinomycete isolates.

2. Literature Review

2.1. General Characteristics of Actinomycetes

General characteristics of actinomycetes are a group of prokaryotic organisms belonging to subdivision of the gram-positive bacteria phylum. Most of them are in subclass Actinobacteridae, order Actinomycetes. All members of this order are characterized in part by high guanine (G) +cytosine(C) content in their DNA (Sharma *et al*., 2014).

They are filamentous bacteria which produce two kinds of branching mycelium, aerial mycelium and substrate mycelium. The aerial mycelium is important as the part of the organism that produces spores. For this reason they have been considered as fungi, as is reflected in their name, akitino means ray and mykes means mushroom/fungus, so actinomycetes were called ray fungi. Actinomycetes are most abundant in soil and marine (Ventura *et al.*, 2007).

Actinomycetes are the most widely distributed group of microorganisms in nature and are also well known as saprophytic soil inhabitants. The soil actinomycetes produce a volatile compound called geosmin, which literally translate earth smell. This organic compound is responsible for a contributor to the strong odor that occurs in the air when rain falls after a dry spell of weather. In natural habitats, Streptomyces are common and are usually a major component of the total actinomycetes population (Sharma et al., 2014). Actinomycetes are originally considered to be an intermediate group between bacteria and fungi, but now are recognized as prokaryotic organisms having different morphological, cultural, biochemical and physiological characteristics (Budale*et al*., 2015).

Table 1. Similarity of actinomycetes with bacteria and fungi

Fungal characteristics of Actinomycetes	Bacterial characteristics of Actinomycetes
The production of thin slender hyphae	Prokaryotic in nature
Production of conidia	Dimension of cell and hyphae like bacteria
Powdery appearance of colonies	Presence of antibiotic sensitivity
Formation of filamentous	Contain peptidoglycan in cell wall

2.2. Taxonomy of actinomycetes

Bacteria represent one of the largest taxonomic units among the18 major lineages. According to the currently recognized lineages, it falls within the: Domain-Bacteria, Phylum-Actinobacteria, Class-Actinobacteria, Order-Actinomycetes, Family-Actinomycetacea and Genus Actinomycetes. Actinobacteria is divided into 6 classes, 16 order and have seven families, based on the hyphae and reproductive structures (Mulatu and Dawit, 2016).

2.2.1. Chemotaxonomic

Chemotaxonomy is the use of the distribution of chemical components to group organisms according to the similarities of their cellular chemistries (Mulatu and Dawit, 2016). The most commonly used chemical components in such systematics are cell wall amino acids, lipids, proteins, muramic acid types, sugars, and the base composition of DNA (Cummins and Harris, 1956). Presence of Diaminopimelic Acid (DAP) isomers is one in all the foremost necessary cell wall properties of gram positive bacterium and actinomycetes (Choudhary*et al*., 2013).

2.2.2. Classical taxonomic

Classical taxonomy for classification builds use of physiological, morphological and biochemical characters. The classical methodology delineated within the identification key by Nonomura 1974 and Bergey‟s Manual of Determinative Bacteriologyis very much useful in the identification of streptomycetes (Buchanan and Gibbons 1974)

2.2.3. Numerical Taxonomic

Numerical taxonomy involves examining several strains for a large number of characters prior to assigning the test organism to a cluster based on shared options. Numerical taxonomy was initially applied to Streptomyces (Silvestri*et al.*, 1962).

2.2.3. Ecology of Actinomycetes

Actinomycetes are widely distributed in nature, but primarily in soil. They constitute a significant component of the microbial populations in most soils. Their distribution in the soil is influenced by geographical location, temperature, soil type, pH, organic matter content, agricultural activities, aeration, nutrient availability, moisture content, and soil vegetation. Species distribution decreases as soil depth increases. These organisms play an important role in the recycling and mineralization of nutrients in the soil by degrading complex compounds which other organisms cannot degrade (Rinoy*et al*., 2012).

The ecological niche of most of the actinomycetes is probably the aerobic zone of soil where they live saprophytic ally at the expense of a wide variety of organic substrates. Their numbers have been reported to exceed one million/ gram of soil. Members of many genera have also been isolated from fresh and sea water sediments. Some Corynebacteria and Mycobacteria are important member of the normal microbial flora of the human skin. Actinomycetes may consider normal resident of gingival tissue of the mouth. Actinomycetes have some unique properties that may be related to their ability to survive in the soil. They are prolific producer of extracellular enzyme that degrades complex molecules substrates commonly found in soil (Kumar *et al.,* 2004).

2.2.4. Distribution of actinomycetes

Actinomycetes are widely distributed in different parts of the world. Because they can live in different environments this allows them to spread in different regions across the globe and compete with other organisms in their surroundings (Demain and Sánchez, 2009).

2.2.4.1. Soil

Actinomycetes constitute a huge extent of the microbial populace in many soils, and their suitable check frequently surpasses one million for each gram. The soil is likewise the most productive wellspring of segregates, huge numbers of which deliver antibiotics and other valuable metabolites in vitro. Delegates of more than 90% of actinomycetes genera have been isolated from soil (Brock, 1993). The soil is the widest habitat of actinomycetes which can be found in a wide range of soils. The actinomycetes in the soil become connected to surfaces, for example, to plant deposits or fungal hyphae (Mayfield *et al.,* 1972), and they may have a vital natural part in the degradation of litter in soil (Goodfellow and Simpson, 1987).

The existence of high population of actinomycetes was significantly correlated with organic matter and soil moisture contents. The predominant genus in all soil samples were *Streptomyces, Nocardia, Rhodococcu* and *Micromonospora* Actinomycetes (Wang *et al*., 1999).

2.2.4.2. Marine

Marine Actinobacteria dwelling in extremely different environment produce different types of bioactive compounds compared with terrestrial ones. Marine Actinobacteria also survive at temperature of 0- 8 °C on the deep sea floor to high acidic conditions at temperatures of over 8-100°C near hydrothermal vents at the mid-ocean ridge Members of the (Mukesh, 2014). *Rhodococcus* and *Streptomyces* have been designated as indigenous marine Actinobacteria and

the endospores of Thermoactinomyces can be transported very long distances by ocean currents (Hagedorn, 1976).

2.2.4.3. Freshwater

Some of the major types of Actinobacteria dwelling in freshwater include *Actinoplanes*, *Micromonospora, and Rhodococcusn and Streptomyces*. Actinoplanes are commonly found in soils, rivers, and lakes, and the spore vesicles of these organisms have the ability to withstand prolonged desiccation, but they release their motile spores for dispersal when rehydrated (Alexander, 1977). The zoospores are motile by means of a tuft of flagella exhibiting chemo taxis and require an exogenous energy source. Micromonospora are also considered to be a common freshwater Actinobacteria and found to be indigenous to such habitats where they turnover cellulose, chitin, and lignin. Numerous reports confirmed the presence of Micromonospora in streams, rivers, and considered them to be an integral part of the aquatic micro flora (Goodfellow*et al.*, 1987).

2.2.4.4. Root

Actinomycetes exist in the Rhizosphere of numerous plants, and Frankie strains are nitrogen fixing endophytes in root nodules of different non-leguminous bushes and trees. The entophytes are seen in the root nodules of non- leguminous plants as for some time suspected to be actinomycete, which was confirmed by its underlying isolation from *Comptonia* root nodules (Callaham*et al.*, 1978)**.**

2.2.4.5. Composts

Many hemophilic actinomycetes are active in compost in the initial stages of decomposition. However, the capacity for self-heating during decomposition provides ideal conditions for obligate or facultative thermophilic actinomycetes. Thermophilic actinomycetes grow well on animal manure. They have been active in fermentation of pig faces, straw and deodorization of pig faces. Thermomonospora species particularly grow during the second in door phase of preparation of mature for mushroom cultivation, whereas *Streptomyces diastaticusan* and *Thermoactinomyces vulgaris* predominate in the spent, steamed compost and its dust (Williams *et al*., 1983).

2.2.4.6. Air

The capacity of actinomycetes spores to go about as respiratory allergens have given the principal impulse to ponder on the recuperation of actinomycetes from the air. This connection

between actinomycetes spores and respiratory disease has produced a considerable measure of data on numbers and types of actinomycetes found in air (Lacey, 1988).

2.2.5. Economic Importance

2.2.5.1. Biomedical Use

Actinomycetes are produced many antibiotics, that are best recognized and most valuable. Like antibiotics amphotericin, nystatin, chloramphenicol, gentamycin, erythromycin, vancomycin, tetracycline, novobiocin and neomycin (Bernan*et al.,* 2004). In these antibiotics some are targeted bacterial ribosome and are used in treating respiratory infections, for example in treating the Legionnaires disease used tetracycline and erythromycin (Mukesh, 2014).

Vancomycin antibiotic are attacks on deadly organisms such as methicillin resistant staphylococcus aureus and bacterial cell walls. Rifamycins are useful for treating leprosy and tuberculosis, these targets bacterial RNA polymerase. Amphotericin is one of the minority antibiotics that attack fugal membranes (McCarthy and Williams, 1992*).* On the other hand actinomycetes metabolites for example Adriamycin, prevent DNA replication, because of this it is used in treating the cancer, although rapamymycin is used to repress the immune system to facilitate organ transplants (Kimura, 1980).

2.2.5.2. Use in Bioremediation

Actinomycetes digest complex carbohydrates like chitin, cellulose, hemicellulose etc. It also helps in the degradation of toxic compounds from the environment. Thus, it plays an essential role in the bioremediation of organic compounds. Actinomycetes can survive in a harsh environment like high temperature up to 50 degrees Celsius that is crucial for the composting process. (Adam *et al*., 1992)

2.2.5.3. Prevents Bio corrosion

Corrosion is a principal reason of pipe failure and high preservation costs in gas pipelines. Bio corrosion is defined as a caustic harm initiated or aggravated by the direct or indirect activities of microorganisms (Zue, 2007). A broad range of bacteria is present in most if not all areas of oil production and have been described from water injection plants, drilling mud, and live reservoir cores (Beal, 2000).Actinomycetes produce secondary metabolites, which act as AMSs (Antimicrobial substances). These antimicrobial substances attack pathogenic and phytopathogenic microorganisms, which can cause bio corrosion (Zen ova *et al*., 2011).

2.2.5.4. Use as Bio pesticide

As the environmental contamination by toxic chemicals increases, different approaches for controlling pest populations became analysis priorities. These have enclosed biological or ecological management strategies for limiting the harmful impacts of pest populations, particularly in agriculture. Actinomycetes play a significant role in the biological control of insects through the production of insecticidal active compounds against the house fly Muscadomestica (Hussain *et al*., 2002).

2.2.5.5. Plant growth hormone production

Actinomycetes have an extended tradition in the analysis of bioactive compounds. Several species manufacture a large form of secondary metabolites, including anti-tumor agents and majority of identified antibiotics. Free-living actinomycetes have additionally been concerned in the improvement of plant growth by production of plant growth-producing substances like auxins and gibberellin-like compounds. Indole-3-acetic acid (IAA) is the principal form of auxin, which regulates many basic cellular processes including cell division, elongation and differentiation (Mahmoud and Rehm, 1987). Actinomycetes produce agro active compounds as they are extensively present in the rhizospheric zone of the plant. Thus, they can actively colonize themselves with the plant roots and protect the plant from pathogenic fungi and other phytopathogens. Frankia is an example of actinomycetes, which acts as a "Symbiotic" that promote root nodule formation and thereby in nitrogen fixation (Fenton *et al*., 1992)

2.2.5.6. Enzymes

Amylases are starch-degrading enzymes that catalyze the hydrolysis of internal a-1, 4-O-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the α-amylases are metallo enzymes, which require calcium ions (Ca2+) for their activity, structural integrity and stability (Pandey*et al.,* 2000). Cellulase is a collection of hydrolytic enzymes which hydrolyze the glycoside bonds of cellulose and related cello-disaccharide derivatives (Schmid*et al*., 1998).

Lipase is produced from a variety of actinomycetes, bacteria, and fungi (Kulkarni and Grade, 2002). Lipases have broad applications in the detergent industries, foodstuff, and oleo chemical, diagnostic settings and also in industries of pharmaceutical fields (Schmid et al., 1998).

Xylanase is the second most abundant polysaccharide in nature. Xylanases have been reported from actinomycetes (Gold and Alic, 1993). The xylanolytic enzyme system is composed of an

array of hydrolytic enzymes, endo-1, 4-β-xylanase, α-glucosiduronase, α-larabinofuranosidase, and acetylxylan esterase (Barrow *et al*., 1997).

Proteases are also known as peptidyl-peptide hydrolases are important industrial enzymes and used in production of digestive aids and the recovery of silver from photographic film. Actinomycetes, particularly Streptomycetes are known to secrete multiple proteases in culture medium (Gold and Alic, 1993).

Keratinases are used for the bio degradation of keratin and produced only in the presence of keratin substrate. Some microbes have been reported to produce keratinases in the presence of keratin substrate. Keratinase producing microorganisms have ability to degrade chicken feathers and hairs (Pandey*et al*., 2000). L-asparaginase is used as therapeutic agent in the cure of certain human cancers, mostly in acute lymphoblastic leukemia (Floranceeet *al*., 1972).

Figure 1.Economic importance of actinomycetes (Mahmoud and Rehm, 1987).

2.2.6. Antimicrobial production of Actinomycetes

There are different soil microorganisms which have the ability to produce antimicrobial agents. These soil microorganisms are bacteria (both Gram positive and Gram negative), fungi and Actinomycetes. From three of these soil microorganisms, Actinomycetes are the major one in synthesizing antimicrobial agents (Barrow *et al*., 1997).

Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by Actinomycetes representing 45% of all bioactive microbial metabolites discovered (Kumar et al., 2011)**.** Actinomycetes are the most economically and biotechnologically valuable prokaryotes and are responsible for the production of about half of the discord bioactive secondary metabolites, antibiotics, anticancer agents and enzymes. Among actinomycetes, around 7,500 compounds are produced by Streptomyces species while the rare Actinomycetes represent all together 2500 compounds (Devi *et al*., 2013). Are the best common source of antibiotics, and provide approximately two third of naturally occurring antibiotics, including many of medically important discoveries. Antibiotics from microbial sources have yielded an impressive number of compounds over the past 50 years. The discovery of new actinomycetes taxa from diverse habitat with unique metabolic activity often led to the discovery of novel antimicrobial agent. Various antimicrobial agents have been isolated and characterized from actinomycetes including aminoglycosides, amthracyclines, glycopeptides, macrolides beta-lactams polumenes, phenazine, and tetracycline (Lacey and Crook, 1988).

In these antibiotics some are targeted bacterial ribosome's and are used in treating respiratory infections, for example in treating the legionaries" disease used tetracycline and erythromycin. Voncemycin antibiotic are attacks on deadly organisms such as methicillin resistant staphylococcus aurous (MRSA) (multiple drugs resistant) and bacterial cell walls, Rifamycins are useful for treating leprosy and tuberculosis, these targets bacterial RNA polymerase (Sharma *et al*., 2014).

2.2.7. Factors affecting microbial antibiotic production

It is well known that designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites through fermentation is influenced by various environmental factors, including nutrients, phosphorus, nitrogen source, oxygen pH, some precursors and carbon sources (Hasani*et al.,* 2014). Therefore, influences of medium components and environmental conditions are an initial and important step to improve metabolite production of the genus Streptomyces (Al-Ghazali and Omran, 2017).For the maximum production of antimetabolites the suitable factors must be optimized experimentally i.e. medium temperature, pH, agitation, carbon source, nitrogen source, and sodium chloride concentration (Sharon *et al,* 2014).

2.2.8. Mechanism of action of Antimicrobial Agents

2.2.8.1. Inhibition of microbial cell wall synthesis

A bacterium's cell wall is made up of a macromolecular network called peptidoglycan. In gram-positive organisms, the peptidoglycan layer is thick and may have a thin layer of teichoic acid outside the peptidoglycan. In contrast, gram-negative organisms have a thin single layer of peptidoglycan covered by a complex outer membrane layer composed of lipopolysaccharides, lipoproteins, and phospholipids (Glick, 1995).

There are two major groups of cell wall synthesis inhibitors, the β- lactams, and the glycopeptides antibiotics. As bacterial cell walls are wholly unlike the membranes of eukaryotes, they are an obvious target for selectively toxic antibiotics (Kimura, 1980).

The β-lactams include the penicillin, cephalosporin, and carbapenem. These agents bind to the penicillin-binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In Gram-negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured; in Gram-positive cells, autolysis is triggered by the release of lipoteichoic acid. The mechanism of β-lactam resistance is via the action of the β-lactamases. These enzymes catalyze the hydrolysis of the β-lactam ring and, thereby, inactivating these antibiotics and development of resistance by many bacteria (e.g. Neisseria gonorrhea, Pseudomonas spp., and some enteric Gram-negative bacilli) (Badale et al., 2015).

2.2.8.2. Inhibition of Microbial Protein Synthesis

Drugs that inhibit protein synthesis are aminoglycosides, macrolides, tetracyclines, lincosamides, ketolides, streptogranins, axazolidinones and chloramphenicol. Microbial protein synthesis is directed by ribosomes in conjunction with cytoplasmic factors, which transiently bind to particles during the initiation phase, elongation phase and termination phase (Sharon et al., 2014). Microbial ribosomes contain 70S particles comprising two subunits of 50S and 30S, which join at the initiation step in bacterial protein synthesis by interfering with the function of, wither the cytoplasmic factors or the ribosomes. Inhibitors that bind to the 30S ribosomal subunit interfere primarily with initiation, although some interfere with pairing of the mRNA codon with the tRNA anticodon, so impairing elongation. For examples Aminoglycosides act by binding to specific ribosomal subunits (Devi *et al*., 2013).

2.2.8.3. Inhibitors of microbial RNA synthesis

The inhibition of DNA synthesis by the refamycin class of semi-synthetic bactericidal antibiotics, similarly to the inhibition of DNA replication by quinolones, has a catastrophic effect on prokaryotic nucleic acid metabolism and is a potent means of inducing bacteria cell death. Rifamycins uniquely require RNA synthesis to not have progressed beyond the addition of two eibonucleotides; this is attributed to the ability of the drug molecule to satirically inhibit nascent RNA stand initialization (Kohansk*et al*., 2010).

2.2.8.4. Inhibition of microbial metabolic pathways

Trimethoprim and sulfonamides interfere with folic acid metabolism in the microbial cell by competitively blocking the biosynthesis of tetrahydrofolate, which acts as a carrier of one-carbon fragments and is necessary for the ultimate synthesis of DNA, RNA and bacterial cell wall proteins (New *et al*., 1996).

2.2.8.5. Disruption of cytoplasmic membrane

Most health works now believe that membranes are a lipid matrix with globular proteins randomly distributed to penetrate through the lipid bilayer. A number of antimicrobial agents can cause dis organization of the membrane. These agents can be divided into cationic anionic and neutral agents. The best-known compounds are polymyxin B and Polymyxin E (New et al., 1996).

3. Methods and Materials

3.1. Description study area and period

The study was conducted at Jimma town in south-western Ethiopia located in the Jimma Zone of the Oromia Regional state (Fig.2). Jimma is located at a latitude and longitude of 7˚40′26.01″N and 36̊50′8.85″E respectively, with an elevation between 1,500-2,400 meters above sea level characterized by temperate climate which is considered ideal for agriculture as well as human settlement. The town is generally characterized by warm climate with mean annual maximum temperature of 30°C and mean annual minimum temperature of 14°C. Jimma is found at a distance of 356 km to the south of the capital Addis Ababa. The area receives an average annual rainfall ranging between 1138 mm to 1690 mm. Maximum precipitation occurs during the three months period, June to August ,with minimum rainfall in December and January and evergreen almost throughout the year(Atsede, 2011).

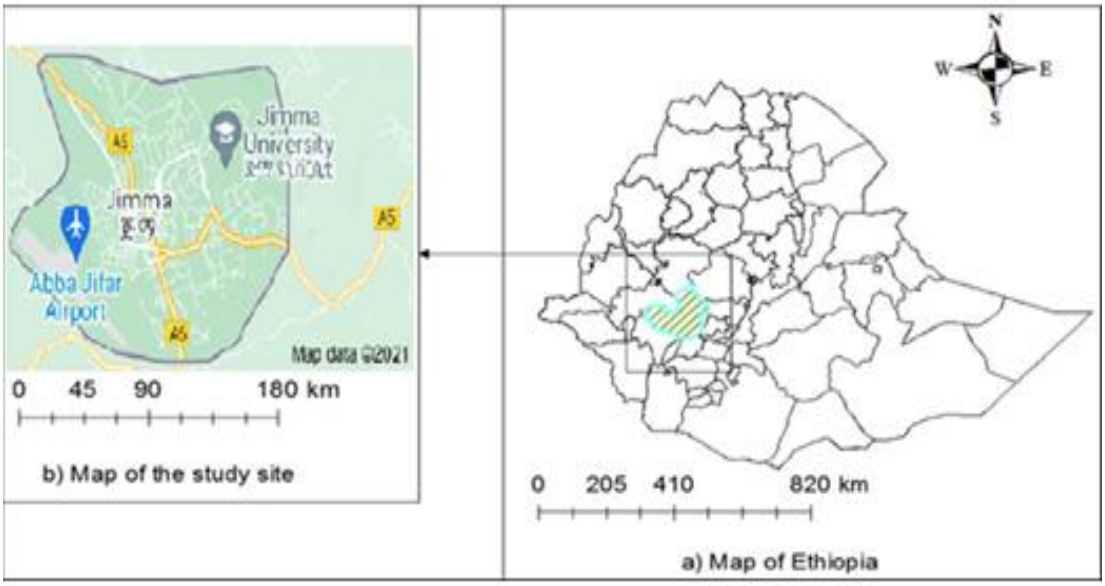


Figure 2. Map of Jimma

3.2. Study design

A cross sectional study design was used.

3.3. Sample size and Technique

Purposive sampling technique was applied to collect soil samples from rhizospehre soil of indigenous trees grown in the study area.

3.4. Sample collection

A total of 25 Soil samples were collected from rhizosphere soil of indigenous plants of Bada Buna natural forest of Jimma and rhizosphere soil of indigenous plants of Jimma University Campus. The soil samples were collected from a depth of 5cm by using sterile spatula and collected in sterilized polythene bags. The samples were collected aseptically, labeled and immediately soil samples were transported to the Research laboratory of Microbiology, Department of Biology, and Jimma University Campus. The samples were stored in refrigerator +4^{o}C until sample is prepared. Samples were prepared by using standard procedures. Accordingly, 10 gram of soil sample was suspended in 90 mL of sterile saline solution and shaking it. Then, 1ml of suspension was added into 9ml saline solution each test tube from one-fifth stepwise to a 10^{-5} dilution. A volume of 0.1 ml of suspension from 10^{-3} up to 10^{-5} dilutions spread plated on the surface of sterile starch casein agar (media composition (g/L): starch, 10; KNO3, 2; casein, 0.3; $NaCl_2$, 2; K_2HPO_4,2; $MgSO_4$.7H2O,0.05; CaCO3, 0.02; FeSO4.7H2O, 0.01; Agar ,15) , and yeast-Malt extract (media composition (g/L): Yeast extract,4; malt extract,10; dextrose ,4; Agar,15) pH of each medium was adjusted to 7.0+2 before sterilization. Amoxicillin (20µg/mL) and Griseoflavin (25µg/mL) were added in both media to inhibit bacterial and fungal contamination, respectively.

3.5. Isolation and Cultural characterization

Actinomycetes strains were isolated in its culture by using standard microbiological method or serial dilution plate technique. The plates were incubated aerobically at 30°C up to 7 days and observed intermittently during incubation. After incubation, actinomycetes on the plates were identified based on color of colonies. The identified colonies were purified by repeated streak plate method. After isolation of the pure colonies, each colony was further identified on the basis of its earthy like colonial morphology, color of hyphae and the presence or absence of aerial and substrate mycelium. Then, selected and identified colonies of actinomycetes were transferred from the plate to starch casein broth to starch casein agar slant and incubated at 30C°C for growth. After incubation, the slants containing pure isolated actinomycetes were preserved at 4°C and maintained longer by periodic subculture on starch casein agar.

3.6. Screening of isolates antimicrobial activities

3.6.1. Primary screening

Preliminary screenings for antibiotic activity of the isolates were conducted by inoculating the pure culture of in starch casein broth for fourteen days. After fourteen days the broth which contain the isolates were centrifuged at 10,000 rpm for 20 minutes. Then the supernatants were filtered by filter paper and the pellets were discarded. After that the collected supernatant was used to check whether the isolates produced antimicrobial activities or not by using disc diffusion method and inoculated into Muller Hinton agar at 30°C for 24 hours against pathogenic bacteria and 48 hours against pathogenic fungi and the zone of inhibition was measured using transparent ruler with a millimeter scale. The activity was done in duplicate. The after the isolates were selected based on their broad spectrum for the next activities.

3.6.2. Test microorganism

The standards test strains used for antimicrobial activity evaluation of the isolates were bacterial isolates including *Staphylococcus aureus (ATCC25923),Klebsiella pneumonia (ATCC7000603), Pseudomonas aeruginosa (ATTCC27853), Escherichia coli (ATCC 25922), and Salmonella typhimurium (ATCC 13311);* and the yeast *Candida albicans (ATCC14053).*They were obtained from Ethiopian Public Health Institute (EPHI).

3.6.3Turbidity standard for inoculum preparation

Standardization of the inoculum density of isolates for susceptibility test was done. In order to determine the active phase of test organisms, each isolate was grown in nutrient broth for bacteria and potato dextrose broth for fungi at 30°C for 24 hours and 48 hours respectively. Samples from the exponential phase were taken to adjust the inoculum density with 0.5 McFarland Turbidity Standard prepared by adding a 0.5 ml of BaCl2 solution into 99.5 ml of solution H2SO4. The density of the turbidity standard was determined using spectrophotometer.

3.6.4. Fermentation for Secondary Screening

Isolates that have potential antimicrobial activity were selected for secondary screening in Based on the zone of inhibition in primary screening, small scale submerged system. Starch casein broth was dispensed in to Erlenmeyer flask, to which a loop full of 7 days grown isolates were inoculated and incubated on a shaker at room temperature for 16 days. After 16 days of incubation, the flask content was filter through whatman No.1 filter paper. After that the filtrate was centrifugedat10, 000rev/min for 20 minutes. Equal volume of ethyl acetate (1:1) was then added to the centrifuged supernatant and shaken vigorously for one hour. The ethyl acetate phase

that contained antimicrobial was evaporated and concentrated in vacuum rota-vapor with 100 rev/min at temperature of 44°C. The dry crude extract was weighted using a balance and kept in small vials at 4°C for further test.

3.6.4.1. Agar well diffusion method

The test bacteria and fungi cultures were inoculated into prepared Muller Hinton agar plates (bacterial strains) and Muller Hinton agar plates (fungal strains)using sterile cotton swabs. Then, wells were made about 6 mm in diameter on the already inoculated plates using well cutter (cork borer) and each well was loaded with 50µl of supernatant from the centrifuged fermented broth. The plates contain bacterial strains were incubated at 30ºC for 24 hours and while the plates containing fungal strains were incubated at room temperature for 48 hours. After incubation, the zone of inhibition was measured and expressed as millimeter (mm) in diameter (Narendra *et al*., 2010).

3.7. Characterization of actinomycetes with antimicrobial activities

3.7.1. Morphological characteristics

Cultural characteristics on solid media: isolates were investigated by growing the isolate on starch casein agar and yeast extract malt extract. Briefly, a loop full of each isolate from 7 days old culture was taken and inoculated into each medium by streak plating technique and morphology was noted with respect to color of aerial mycelium and substrate mycelium, branch, and nature of colony was examined (Mulatu and Dawit,2016).

The microscopic examination was carried out by cover slip culture and gram staining method. Cover slip culture method was done by inserting sterile cover slip at an angle of 45° in the starch casein agar medium. A loop full of isolates was taken from 7-day old culture and inoculated, at the insertion of the cover slip on the medium and incubated at 30°C for 7 days. The cover slip was carefully removed using sterile forceps and placed upward on a glass slide. The growth on the cover slip was fixed with few drops of absolute methanol for 15 min and washed with tap water and flooded with crystal violet reagent for 1 min followed by washing and blot drying. It was then examined through microscope under magnification of x1000 in oil immersion. The morphology of spore chains and hyphae of substrate and aerial mycelia were observed and recorded for the isolates; predominant organisms were selected for the next activities (Mulatu and Dawit, 2016).

3.8. Physiological characterization

Physiological characters of actinomycetes were studied on the basis of pH tolerance, temperature tolerance and salt.

3.8.1. pH tolerance

A loop full of the test isolate from 7 days old culture was taken and serially diluted in sterile distilled water; it was agitated with vortex and about 0.1 ml of the suspension was taken and inoculated with spread plate technique on to starch casein agar media which was adjusted to pH levels of 5, 7, 9 and 11. The experiment was done in duplicates and colony was counted with log colony forming unit after incubating the isolates at 30°C for 7 days. The result was recorded (Laidi et al., 2006).

3.8.2. Temperature tolerance

Temperature tolerance of the isolates was determined on nutrient agar plates. A loop full of the test isolate from 7 days old culture was taken and serially diluted in sterile distilled water; it was agitated with vortex and 0.1 ml of the suspension was taken and inoculated with spread plate technique. The experiment was done in duplicate and colony was counted and expressed in log colony forming unit after incubating the isolates at 25, 30, 37 and 45°C.The result was recorded (Laidi et al., 2006).

3.8.3. Salt tolerance

The isolates were tested for their levels of tolerance to different concentrations of sodium chloride on nutrient agar supplemented with 4% 6%, 8% and10% sodium chloride. Agar plates were inoculated with test isolates with streak plate technique. The experiment was done in duplicates. The plates were incubated at 30°C for 7 days and observations were made to record the highest concentration of salt that allows the growth of isolate (Mulatu and Dawit, 2016).

3.9. Biochemical tests

Starch hydrolysis, nutrient gelatin liquefaction, simmon citrate test, urease production, esculin hydrolysis test and catalase test.

3.9.1. Starch hydrolysis

Starch hydrolysis test was conducted using starch agar plate [composition (g/l): soluble starch, 20; beef extract, 3; peptone, 5; agar 15 and distilled water 1L]. The isolate was taken from 7 days old culture and streaked on starch agar plate and incubated at 30^{o}C for two days together with un-inoculated plates that serve as control. After incubation, starch hydrolysis was determined by

the addition of Lugol's iodine solution. A clear zone around the line of growth after addition of iodine solution indicates that the organism has hydrolyzed starch

3.9.2. Gelatin Hydrolysis

This test was done on sterile nutrient gelatin medium (composition (g/l): (beef extract, 3: peptone, 5 and gelatin 120 with distilled 1L). Each isolate was taken form 7days old culture and stabbed into nutrient gelatin tubes with sterile needle. The gelatin hydrolysis test was observed by incubating the media contained isolate into the freezer for 1 hour. The test was positive if the media melted.

3.9. 3. Urease production

About 24.1g agar was suspended in 900ml distilled water, boiled to dissolve completely and autoclaved at 121^{o}C for 15 minutes. The sterilized agar was cooled to 50 ºC. About 100ml of filter-sterilized urea base was added to the cooled agar aseptically and mixed thoroughly. Then 4 to 5 ml of the mix was distributed sterile test tubes and tubes slanted before cooling. The development of bright-pink color indicates production of urease enzyme, hence degradation of urea by the isolate.

3.9.4. Simmon citrate test

About 23 gram simmon citrate was suspended in 1000ml distilled water and the medium was boiled for a few second to dissolved completely and autoclaved at 121°C for 15 minutes. Then 5 ml of the mix was distributed sterile test tubes and tubes were slanted before cooling. The change of color from green to bright-blue was indicated the positive result.

3.9.5. Esculin hydrolysis

Esculin hydrolysis was determined after Atsede (2011). The isolates were taken from 7 days old culture and streaked into Esculin agar slants having a composition of yeast extract, 0.3 g;ferric ammonium citrate, 0.05 g, agar, 0.75 g; 0.1% of esculin, and50 ml distilled water; they were incubated at 30°C for 7 days. Observations were made between the periods of incubation to check the blackening of the medium. Positive tests were confirmed compared to the control which was a dark brown substrate.

3.9.6. Catalase test

Loop full of isolates was put onto clean and dry slide. Then, 3% H_2O_2 were dropped onto the slide with isolate. Formation of gas bubbles were considered as positive test indicating the isolates capacity of producing catalase enzyme.

3.10. Data Analysis

Data analysis were conducted using Statistical Package for Social Sciences(SPSS) windows version 24 in terms of the mean of the growth inhibition zone value which was obtain from each the test organisms. The data on the temperature and pH tolerance were analyzed by comparing the mean growth in log colony forming unit through ANOVAs after duplicate of the experiment and presented by a table.

4. Results

From a total of 25rhizosphere soil samples, 60 different actinomycetes isolates were obtained at different depth of the soil. Out of these 60 isolates, 26(43%) were isolated from rhisosphere soil of Acaccia plants, 16(27%) from rhisosphere soil of Corton marostachyus, 10(17%) from rhisosphere soil of Cordian abyssinica and 8(13%) were isolated from rhisosphere soil of Olive spp. plants by using starch casein agar and Yeast malt extract agar medium.

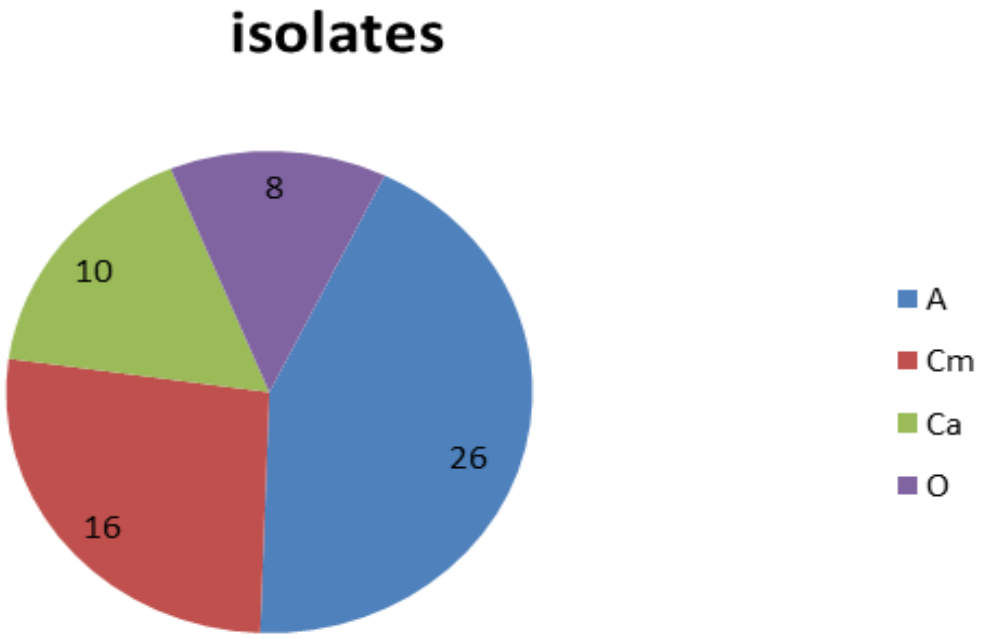


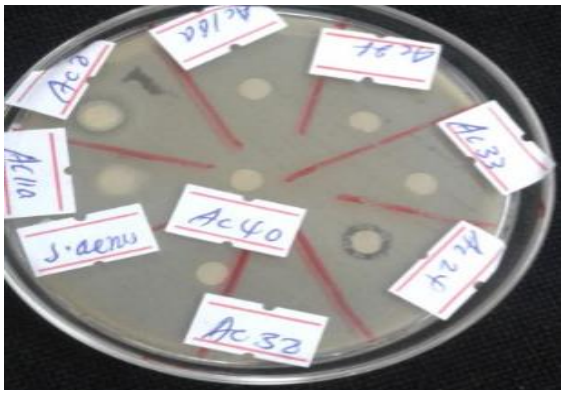
Figure 3. Isolated actinomycetes from various plants rhizosphere soil Where, A= Acaccia spp. Cm= Corton marostachyus; Ca= Cordian abyssinica O- Olive spp.

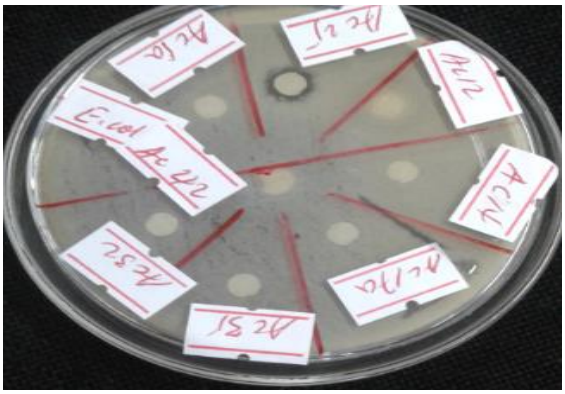
4.1. Screening of isolated actinomycetes for their antimicrobial activities

4.1.1 Primary screening

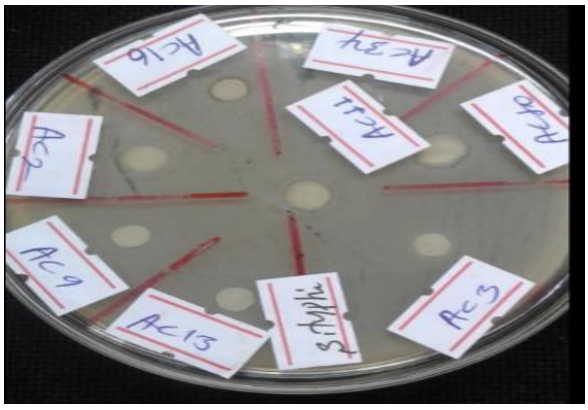
As the results of primary screening, twelve (20%) actinomycete were showed antimicrobial activity against one or more standard test bacteria and fungus. Of the twelve actinomycetes, six (AC2, AC8, AC13, AC20, AC24 and AC25) which accounted for 50% from rhisosphere soil of Corton marostachyus plants, four (AC3, AC10, AC11and AC11a) which accounted for 33.3%from rhisosphere soil of Cordian abyssinia plants, one (AC19) which accounted for 8.33% from rhisosphere soil of Acacia plants and one (AC42) which account for 8.33%rhisosphere soil of Olive plants were obtained. Among a total of twelve (Ac2, Ac8, Ac10, Ac11, Ac11a and Ac25) 6(50%) isolates showed antimicrobial activities both on gram positive and gram negative

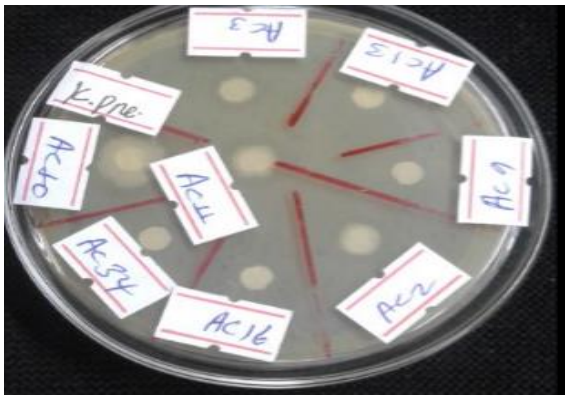
bacteria,5(41.66%) of the isolates showed antimicrobial activities only gram negative bacteria and 1(8.33%) the isolate showed antimicrobial activities against gram positive bacteria .Four (33.3%) isolates showed antimicrobial activities on *C.albicans*(ATCC14053) on which the highest zone of inhibition was recorded by isolateAC2(10mm) followed by the isolates AC8 and AC25(9mm) on this fungus. Out of twelve six isolates (AC, 2 Ac8, AC10, AC11, AC24 and AC25) showed broad spectrum by inhibiting three or more test organisms. The highest zone of inhibition recorded by the two isolates AC11and AC25 (15 and 16mm) respectively against *K.pnemonia* (ATCC7000603) and *S.typhimurium* (ATCC 13311) (Table 2).





S.aureus (ATCC2592) ***E.coli (ATCC25922)***





S.typhimuri (ATCC13) ***K.pnemonia (ATCC700603)***

Figure 4 .Some representative figure of primary screening

Table 2 .Primary screening of antimicrobial activities of actinomycetes isolates

Isolate Codes	Inhibition zone in mm					
	S.aureus (ATCC2593)	*K.pnemonia* (ATCC700603)	*P.aerginosa* (ATCC27853)	*E.coli* (ATCC2592)	*S.typhimurium* (ATCC13311)	*C.albica* (ATCC14053)
Ac2*	9	11	10	11	11	10
Ac3	-	9	-	-	-	-
Ac8*	10	10	11	8	14	9
Ac10*	10	10	-	11	10	-
Ac11*	10	15	-	10	11	-
Ac11a	-	12	-	8	10	-
Ac13	-	-	-	-	-	-
Ac19	-	9	10	-	-	-
Ac20	-	-	8	-	-	-
Ac24*	11	11	-	8	6	8
Ac25*	8	10	-	7	16	9
Ac42	9	8	-	-	-	-

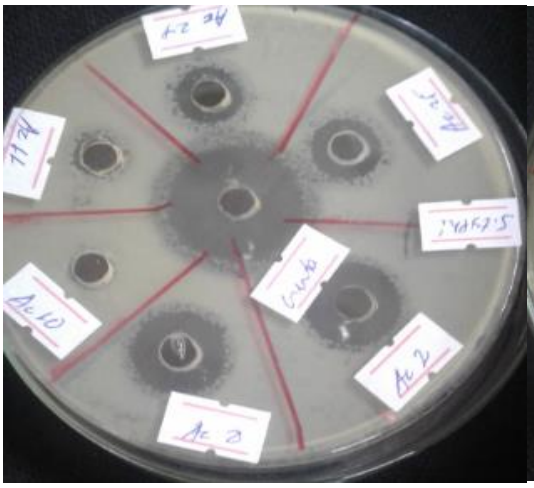
Legend: *On isolates code showed active inhibit three or more than test organisms

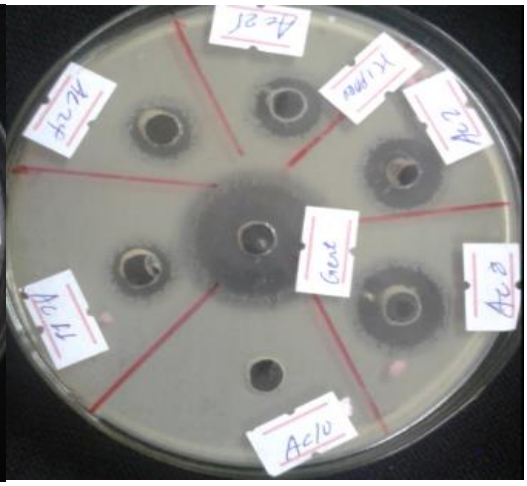
- = showed inactive against test organisms

4.1.2 Secondary screening for fermentation process

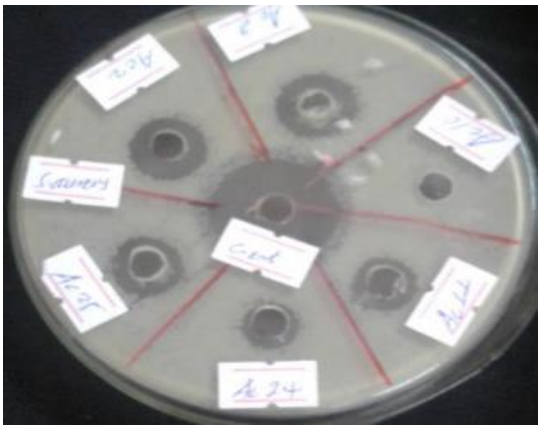
Based on the results of primary screening, 6 active actinomycetes isolates were selected for fermentation process depending on their effectiveness (Table3). The active actinomycete isolates include AC2, AC8, AC10, AC11, AC24 and AC25. Except isolate AC10, all the crude extracts of isolate exhibited against all of test organisms with the highest zone of inhibition 10-20mm during secondary screening process. Except isolate AC10, all the crude extract of isolates were found to inhibit gram positive bacteria *S.aureus(25923)* with the range zone of inhibition between11 to 18mm. The extracts of AC8 showed the highest zone of inhibition against gram

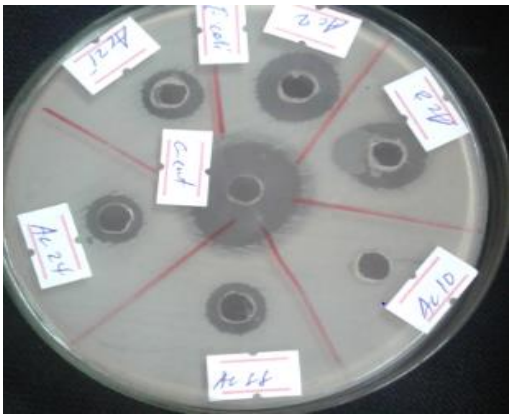
negative bacteria like *E.coli (ATCC 25922*) and *K.pneumonia (ATCC700603*) with 19 and 20mm zone of inhibition respectively. Two isolates (AC2 and AC8) also showed the highest inhibition zone against *E.coli (ATCC25922)* 20 and 19mm respectively. The isolates were showed antimicrobial activities against *C.albicans (ATCC14053*).



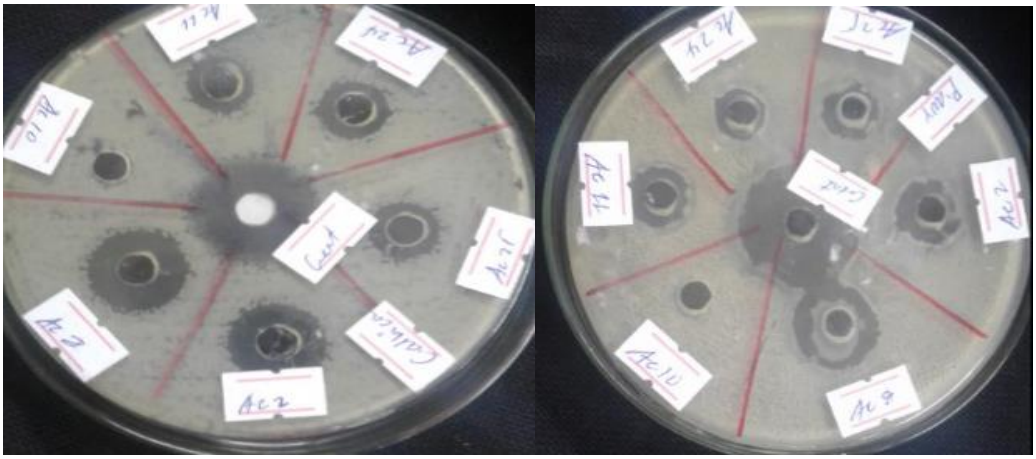


S.typhimurium (ATCC13311) ***K.pnemonia (ATCC700603)***





S.aureus (25923) ***E.coli (ATCC25922)***



C.albicans (ATCC14053) ***P.aerginosa (ATCC27853)***

Figure 5.Antimicrobial activities of crude extract of the selected isolates

Table 1.Antimicrobial activities of crude extract of the selected isolates

Test organisms	Isolates with their zone of inhibition(mm)						
	AC2	AC8	AC10	AC11	AC24	AC2	Gentamycin
S.aureus(25923)	17	18	0	12	11	15	23
k.pnemonia (ATCC700603)	18	20	0	13	14	16	25
E.coli (ATCC25922)	20	19	0	13	13	14	24
S.typhimurium (ATCC13311)	18	17	0	10	15	16	23
P.aerginosa (ATCC27853)	16	18	0	15	12	14	25
C.albicans (ATCC14053)	18	20	0	15	15	15	22

4.2 Characterization of selected isolates

4.2.1 Morphological characterization

4.2.2 Cultural characterization

The isolates were found to grow in all media with some variations. However, moderate growth of the isolates was observed on Malt-yeast agar and nutrient agar medium and also good growth was observed on Starch Casein Agar and medium. The color of the aerial and substrate mycelia varied depending on the type of the media used. Each isolates had different color series of aerial mycelium ranging from white, whitish brown; light yellow, Pink, grey brown, dark grey, Yellowish white, dark brown and none. The color of substrate mycelium white, brown, yellow, light yellow, white grey, Dark Yellow, Dark brown and light yellow.

Among the isolates,AC8, AC11, AC24and AC25 have a hook, loop or spiral (retinaculiaperti) like structure, whereas isolates AC2 and AC10 showed straight to flexuous (rectiflexible) spore chains with smooth and branched mycelium from the Microscope observation.

Table 4.Microscopic observation of selected isolates

	Characteristics			Isolates		
	AC2	AC8	AC10	AC11	AC24	AC25
Spore chains	RC	RT	RC	RT	RT	RT

Where, RT: Retinaculiaperti, RC: Recti flexible

4.3. Physiological characteristics

4.3.1. PH tolerance

The isolates were found to grow relatively in wider range of pH 5, 7, 9and 11in which maximum growth was observed at pH7 (Table 5). The isolate AC2 showed the maximum growth at pH7 compared to other isolates. However, the isolate AC8 showed the lowest growth at pH of 11 compared to other. The growth of actinomycetes on different pH tends to significantly decrease at the pH levels increase and decrease.

Table 5.pH tolerance of selected isolates

pH	**Log colony forming unit (Mean± SD)**					
	AC2	AC8	AC10	AC11	AC24	AC25
5	6.99±0.03	6.82±0.01	6.92±0.01	6.92±0.06	6.90±0.03	6.96±0.06
7	7.26±0.03	7.15±0.06	7.22±0.01	7 .21±0.03	7.24±0.01	7.24±0.04
9	6.86±0.03	6 .82±0.04	6.87±0.06	6.94±0.05	6.92±0.04	6.90±0.05
11	6.74±0.04	6.63±0.06	6.76±0.03	6.72±0.07	6 .78±0.03	6.80±0.05

4.3.2. Temperature tolerance

The isolates did grow at temperatures 25°C to 37°C (Table6). All the actinomycete isolates showed the highest growth at 30°C in which this temperature value was considered as the optimum temperature for all the actinomycete isolates. The isolate AC25 was recorded the maximum growth at this temperature and the isolate AC2 was shown the lowest growth.

Table 6 . Temperature tolerance of the isolates

T°	**Log colony forming unit (Mean±SD)**					
(°C)	AC2	AC8	AC10	AC11	AC24	AC25
25	6.90±0.07	6.77±0.04	6.78±0.08	6.81±0.06	7.76±0.11	6.28±0.06
30	7.27±0.06	7.35±0.03	7.30±0.05	7.20±0.1	7.86±0.08	7.99±0.006
37	6.63±0.02	6.72±0.10	6.65±0.10	6.77±0.02	6.68±0.04	6.65±0.07

4.3.3Salt tolerance of isolates

All the isolates grew in medium supplemented with salt at a concentration of 4%, 6%, 8% and all isolates did not grow at 10% salt concentration (Table 7).

Table7 . Salt tolerance of the isolates

Isolates	**Growth at different Salt Concentration**			
	4%	6%	8%	10%
AC2	+	+	+	-
AC8	+	+	+	-
AC10	+	+	+	-
AC11	+	+	+	-
AC24	+	+	+	-
AC25	+	+	+	-

Legend: +: positive

Table 8.Biochemical test of the isolates

Isolates Code	Starch hydrolysis	Simmon citrate	Gelatin Hydrolysis	Catalase Test	Esculin hydrolysis	Urease hdrolysis
AC2	+	+	+	+	+	+
AC8	+	+	+	+	+	+
AC8	+	+	+	+	+	+
AC11	+	+	+	+	+	+
AC24	+	+	+	+	+	+
AC25	+	+	+	+	+	+

Legend: +: positive

5. Discussion

Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. But now, because of the emergencies of multi-drug resistant pathogens, there are basic challenges for effective treatment of infectious diseases. Thus, due to the burden for high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antibiotics from soil actinomycetes in diversified ecological niches Cholarajan (2014). Present study, the soil samples were taken from rhizosphere for isolation of actinomycetes. Previous studies showed that selections of different potential are rhizosphere, industrial and public waste disposal areas (Wambui, 2017).

In the soil samples were an important activity for isolation of different types of potent antibiotic producing soil actinomycetes Cholarajan (2014).In the present study, among a total of 25 soil samples collected, 60 actinomycetes isolates were isolated on the basis of different colony characteristics from different rhisosphere soil of indigenous plants. As compared to the previous reports by Atsede (2011).

In present study of primary screening using disc diffusion methods indicated that, twelve (20%) out of 60 actinomycete isolates showed potential antimicrobial activity against one or more test bacteria and or fungus. This result (20%)is higher than18 .88%and less than 59.09%from previous reports by Atsede (2011).

According to the present study, the microscopic observation of the isolates displayed spore chains with Retinaculiaperti and Rectiflexible with smooth spore which are typical characteristics of the genus Streptomyces as per previously reported by Atsede (2018).

In this study, all the six isolates indicate positive result on starch hydrolysis, catalase test, Simmon citrate, gelatin hydrolysis urease hydrolysis and esculin hydrolysis. These isolates shared the features of genus *Streptomyces*(Atsede and Fassil 2018) have reported, the same result on the present study with positive on the above tests which may realize that as the isolates were belonged under with positive on the above tests which may realize that as the isolates were belonged under the genus Streptomycetes.

The extract of isolate AC10 did not inhibit the entire given test microorganisms during secondary screening but inhibited during the primary screening but all the isolates inhibited against *K. pneumonia (ATCC700603*) during secondary screening. Except isolate AC10, all the extracts of

isolates showed active against to *C. albicans (ATCC14053)* than primary screening. Isolate AC8 showed the highest zone of inhibition 20mm and this result disagreement with the study conducted by Mulatu and Dawit (2016) in which the maximum inhibition zone was 17.11mm.

From the present study result of antimicrobial activity by secondary screening the extract showed different diameter zone of inhibition. Larger inhibition zone diameter observed by isolate AC8 (20mm) extract against *C. albicans (ATCC14053)* which is greater than the result obtained by Atsede and Fassil (2018) and the least was shown by isolate AC24 (11mm) extract against *S.aureus(25923)*

6. Conclusion

Generally, in this study, antibiotic-producing bacteria were isolated, characterized, and identified from rhizosphere of indigenous trees based on laboratory investigation. From the present study, it can be summarized that 12 actinomycetes isolates possessed antibacterial activity against Gram-positive Bacteria and Gram-negative bacterial strains by using disk diffusion, and agar well diffusion methods. Actinomycetes isolates recovered from rhizosphere samples showed the potential to produce antimicrobial bioactive compounds. It is also suggested that the other isolates should be further processed to fully realize their antibiotic property on different test microorganisms. There is need for further studies to optimize the production conditions of the bioactive compounds from the potent actinomycetes isolates. The isolates were grown very well at the pH 7 and at 30°C. In terms of their cultural characterization starch casein agar medium is the best medium for its well growth. Rhisosphere soils of the indigenous plants are best habitat for isolation and characterization of antimicrobial producing actinomycetes.

6.1 Recommendation

The following recommendations are formulated based on the findings of this study:

- ✓ It is better to isolate antimicrobial producing actinomycetes from natural forest of soil.
- ✓ It is better to grow actinomycetes on starch casein agar medium for its antimicrobial activity.
- ✓ Although, the isolation, characterization, and identification of some actinomycetes bacteria, based on morphological, physiological, and some biochemical characterization, up to genus levels further study needs to characterized and identified isolates up to species levels by using molecular characterization.
- ✓ Other study should be needed to isolate actinomycetes for other secondary metabolites besides antimicrobial activities from the study area
- ✓ Further purification should be needed on the same isolates to realize their antimicrobial activity
- ✓ To get the exact compound that may be found in these isolates, further investigation should be needed.

7. Reference

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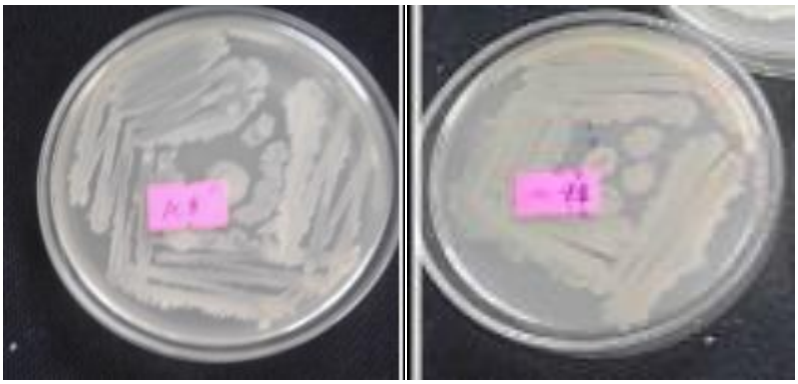
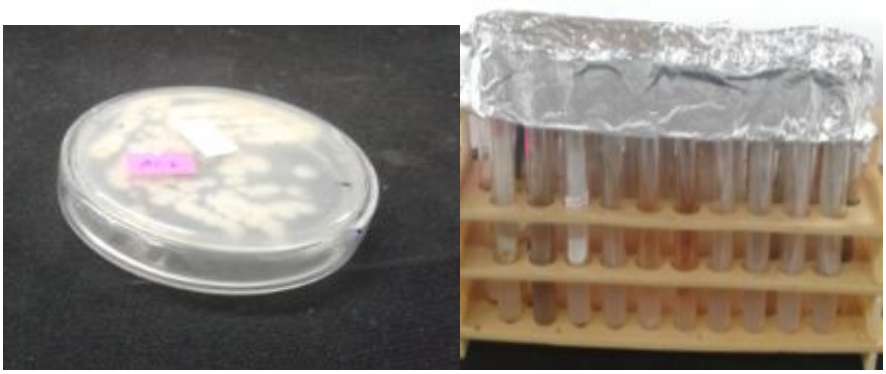
8. Appendixes

Appendix 1 One of soil sampling site

Appendix 2 Some of the isolates grown in starch casein agar

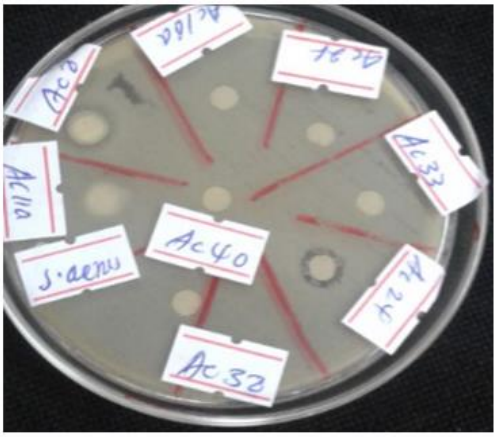


Appendix 3 Cultures in plates and slants partially

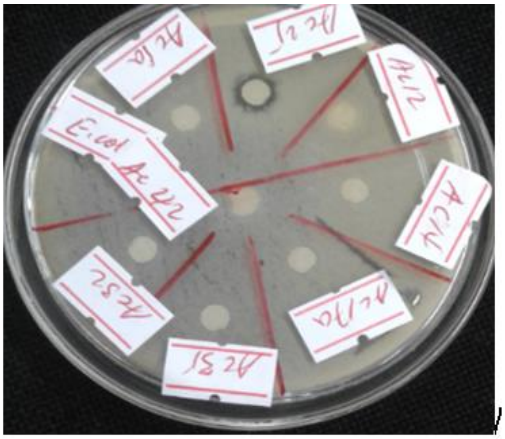


Appendix 4 Substrate preparation

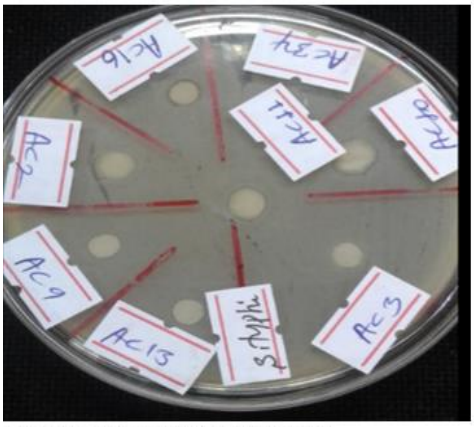
Appendix 5 primary screening of the isolates



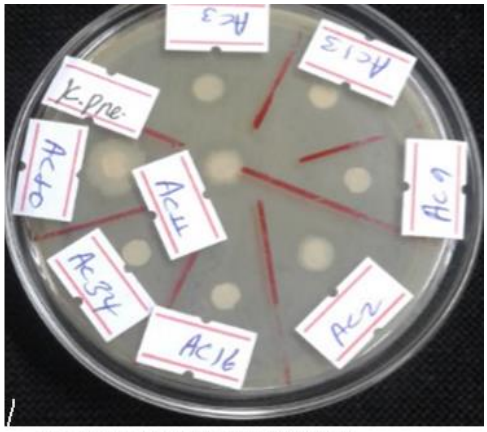
S.aureus (ATCC25923)



E.coli (ATCC25922)

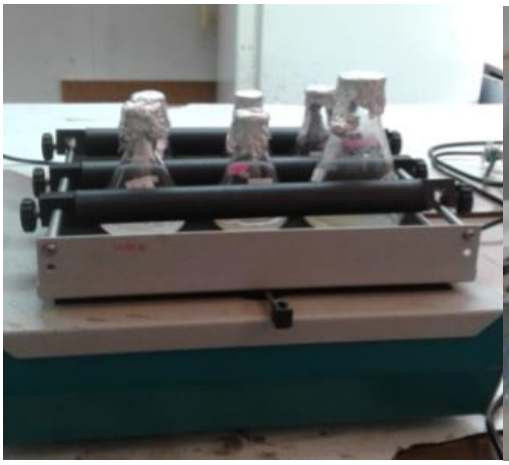


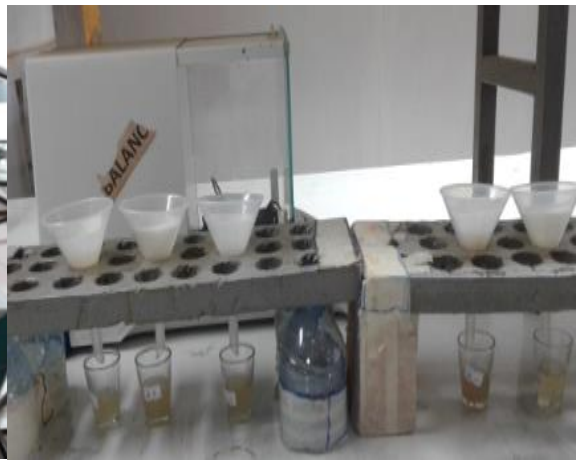
S.typhimurium (ATCC13311)



K.pnemonia (ATCC700603)

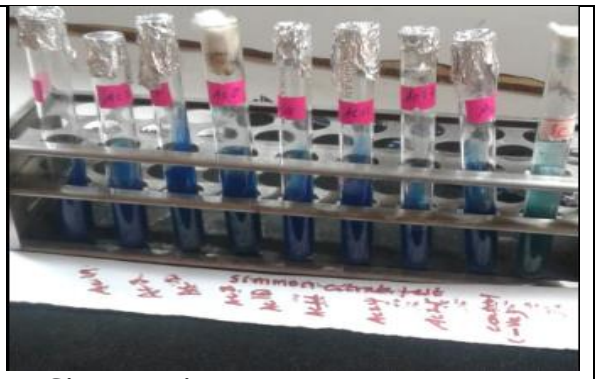
Appendix 6 Fermentation on orbital shaker and Filtration



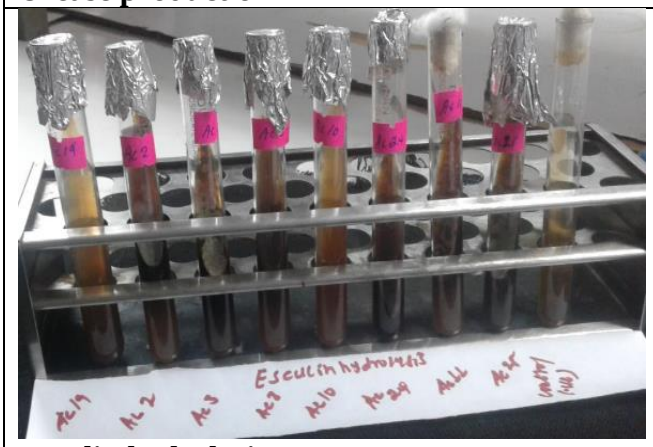


Appendix 7 Biochemical test

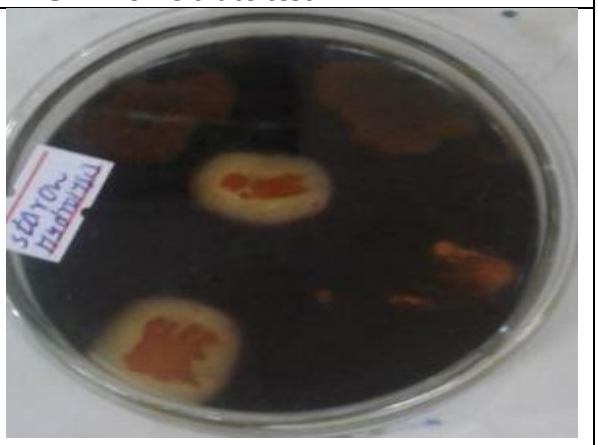
Urease production



Simmon citrate test



Esculin hydrolysis



Starch hydrolysis