# ADDIS ABABA UNIVERSITY

# SCHOOL OF GRAGUATE STUDIES


## ISOLATION AND SCREENING OF ANTIBIOTIC PRODUCING ACTINOMYCETES FROM SOIL

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*A Thesis Submitted to the School of Graduate studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Applied Microbiology*

**October, 2011**

## ACKNOWLEDGEMENTS

I would like to express my genuine gratitude to my esteemed advisor Dr. Fassil Assefa for his valuable advice, excellent guidance, willingness to offer constructive comment and encouragement throughout the study.

I would then like to extend my heartfelt thanks to Dr. Dawit Abate for his excellent guidance, support during laboratory work on extraction of antimicrobial compound and bioassay purification on chromatography test.

I am indebted to W/o Tigest Mengesha, her assistance in making avail laboratory materials and technical support. Besides, I am very grateful to W/t Zenebech Aytenew, laboratory technician of mycology laboratory, her support was also vital.

My sincere thanks also go to my fellow lab mates, Ato Mulisa and Ato Fekadu, for stimulating discussion and support.

Last but not least, I would like to pass my special thanks to my lovely family and friends for your all aspects of support throughout this study.

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**LIST OF SYMBOLES AND ABBREVATIONS**

| | |
|---|---|
| A.A.U. | Addis Ababa University |
| ATCC | American type culture collection |
| °C | Degrees Celsius |
| g/l | Gram per liter |
| hrs | Hours |
| Log cfu | Log of colony forming unit |
| mg | Milligram |
| min | Minute |
| ml | Milliliter |
| mm | Millimeter |
| mM | Milli molar |
| pH | Hydrogen ion potential |
| Rev/min | Revolution per minute |
| Rf | Retention factor |
| Rpm | Rotation per minute |
| TLC | Thin layer chromatography |
| UV | Ultra violate light |
| V/v | Volume/volume |
| W/v | Weight |

**ABSTRACT**

Antibiotics are secondary metabolites produced by microorganisms. Actinomycetes represent the top producer of antibiotics that are widely distributed in the different types of soils. The study was designed to isolate and screen antibiotic producing actinomycetes on samples obtained from soil and rhizosphere of plants. A total of 30 actinomycetes were isolated from Debrezit, Holeta and Arat Kilo campus. Out of the 30 actinomycetes, 18 (60%) showed antimicrobial activities of one of the tested microorganisms of which 9 isolates were selected for their wide spectrum of antibiotic activities. In this study, isolate AAUBA5 and AAUBA30 were found to inhibit all of the test organism, except *E.coli* ATCC 25922 and the latter was found to be the most resistant that was only inhibited by 3/18 ( 17 %) of the isolates. The isolates were identified and characterized by cultural, morphological, physiological and biochemical characteristics and found to be classified under the genus *Streptomycetes*. In order to isolate the active compounds of the extract, fermentation was carried out on nine isolates and the former was extracted by using ethyl acetate. The antimicrobial activity of the active compound of the isolates was studied using Disc Diffusion Assay method and found to inhibit the test microorganisms that could not during the preliminary screening. Finally, isolates AAUBA13 was selected for further study based on spectrum activity and inhibition zone diameter in the antimicrobial bioassay. Antimicrobial compound obtained from AAUBA13 isolate was separated and purified with thin layer chromatography (TLC), column chromatography and preparative chromatography. The purified compound found to be active against gram positive bacteria (*S.aureus* clinical isolate, *S.pneumoinae* ATCC49619 and *S.aureus* ATCC25923), gram negative bacteria (*E.coli* ATCC25922, *S.typhi* ATCC 6539 *Shigella boydii* clinical isolates and *P.aeruginosa* ATCC2585) and effective against *Candida albicans* ATCC62376. The data, in general, showed that the antimicrobial compounds obtained from AAUBA13 demonstrate broad spectrum and a remarkable antimicrobial activity against bacterial and *Candida albicans* ATCC62376. It is also suggested that the other isolates should be further processed to fully realize their antibiotic property on different test microorganisms.

**Key words:** Actinomycetes, Antibiotics, Antimicrobial bioassay, Antimicrobial compound, Disc Diffusion Assay

# 1. INTRODUCTION

Antibiotics are chemical substances produced by microorganisms that in small amount selectively inhibit or kill other microorganisms (Anejia, 2005; Tortora *et al*., 2010). They are secondary metabolites that inhibit other competing cells to give a competitive advantage for the microorganisms that produce them (Sanglier *et al.,* 1993).

Antimicrobial agents are natural products and produced by various types of bacteria and fungi. Hundreds of these natural products have been identified, and developed as therapeutic agents against many infectious diseases (Berdy, 2005). Microbial natural metabolites still appear as the most promising sources of antibiotic in the future (Fernando, 2006). Some of the important antibiotic producing microorganisms belong to *Streptomyces*, *Bacillus, Cephalosporium* and *Penicillium* that have been studied continuously for their ability to produce antibiotics (Brock and Madigan, 1991).

From all the known microbes, actinomycetes are the most important source of biologically active microbial products, including many medically and commercially important antibiotics (Dhanasekaran *et al*., 2009). The broad spectrum antibiotic, vancomycine the potent antimicrobial agent against metacillin resistant *Staphylococcus aureus* and rifampcin, the effective drug against tuberculosis and leprosy are derived from several species of actinomycetes (Berdy, 2005).

The genus *Streptomyces* has been widely recognized as industrially important microorganism because of its ability to produce and secrete a large variety of secondary metabolites (Kornwendisch and Kutzner, 1992; Saadoun and Gharaibeh, 2003; Pandey *et al*., 2004). These include aminoglcosides, macrolides, β lactams, peptides, polyenes, teteraclines, anthracycline antibiotics and nucleosides (Vijayakumar *et al*., 2007; Miyadoh, 1993). It is estimated that more than 80% of the antibiotics are obtained from *Streptomycetes* (Vijayakumar *et al*., 2007). *Micromonospora* is the runner up with less than one-tenth as many as *Streptomyces* (Saadoun and Gharaibeh, 2003; Arifuzzaman *et al*., 2010).

Although the introduction of antimicrobials helps to combat many diseases, large numbers of pathogenic bacteria and fungi causing different human disease have become resistant to antibiotics

in use (Livermore, 2003). *Staphylococcus aureus* a virulent pathogen that is responsible for a wide range of infections, including pimples, pneumonia, osteomyelitis, endocarditis and bacteremia, has developed resistance to most classes of antibiotics (Enright, 2003). The increase in antibiotic resistance has been attributed to a combination of microbial characteristics, the selective pressure of antibiotic use, social changes that enhance the transmission of resistant organisms (Okeke *et al*., 2005).

With increasing misuses of antibiotics and the evolution of emergent and reemerging antibiotic resistant disease is developing at an alarming rate (Demain, 1999). In recent years, new resistant strains emerge more quickly while the rate of discovery of new antibiotics is slowing down (Oskay *et al.,* 2004; Parungao *et al.,* 2007).

This necessitates the screening of microorganisms for antimicrobial activity for the production of new and novel drugs. Hence, intensive search for new antibiotics has become imperative worldwide especially from new actinomycetes (Oskay *et al.,* 2004; Parungao *et al.,* 2007).

It has been observed that on screening programs using highly selective procedures which allows detection and isolation of effective antibiotic producing microorganisms (Rondon *et al*., 2000; Oskay *et al*., 2004; Parungao *et al*., 2007) from soil, which is the largest source of microorganisms and a natural reservoir for microorganisms and their antimicrobial products (Dancer, 2004; Hackl *et al*., 2004).

Although soils have been screened by the pharmaceutical industry for about 50 years, only a small fraction of the surface of the earth has been sampled, and only a small fraction of actinomycetes taxa has been discovered ( Baltz, 2007). This requires the employment of several strategies to explore for new compounds from microorganism such as actinomycetes from different ecological niches that may yield novel compounds with diverse antimicrobial properties (Pandey *et al*., 2004; Ningthoujam *et al*., 2009).

In Ethiopia, a few investigators showed the existence of antibiotic producing microorganisms from different ecosystems. Biniam, (2008) isolated antimicrobial producing actinomycetes from southern part of Ethiopian Rift Valley alkaline lakes. The potential of a mushroom compost as a

good source for antibiotic producing thermophlic actinomycete was also reported by Moges, (2009). However, there is a further need for the exploration of indigenous actinomycetes for their potential to produce effective antibiotics from different sources in the country for the sustainable exploitation of actinomycetes to combat infectious diseases.

# 2. OBJECTIVES

## 2.1. General objective:

To isolate and characterize antibiotic producing actinomycetes from farm soil and the rhizosphere of plants and to evaluate their potential against some test microorganisms.

## 2.2. Specific objectives:

- To isolate antibiotic producing actinomycetes
- To screen antibiotic producing isolates.
- To characterize and evaluate the broad spectrum activities of the isolates against test microorganisms.

# 3. LITERATURE REVIEWS

## 3.1. General characteristics of actinomycetes

Actinomycetes are a group of prokaryotic organisms belonging to subdivision of the Gram-positive bacteria. Most of them are in subclass *Actinobacteridae*, order *Actinomycetales*. All members of this order are characterized by having high G+C content (>55 mol %) in their DNA (Goodfellow *et al*., 1992; Williams *et al*., 1989). 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 (Williams *et al*., 1989). For this reason they have been considered as fungi, as is reflected in their name, *akitino* means ray and *mykes* means mushroom/fungus, so actinomycete was called ray fungi (Takizawa *et al.,* 1993).

They 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 (Jensen and Fenical, 2000). The resemblance of actinomycetes to bacteria is because the actinomycetes species contain peptidoglycan in their cell walls. In addition, actinomycetes are sensitive to antibacterial antibiotics and not antifungal antibiotics. Actinomycetes differ from fungi in their cellular composition. They do not possess chitin and cellulose that is found in the cell wall of fungi. (Oakmi and Okazaki, 1978).

As the number of studies and scientific data accumulated, they were finally recognized as bacteria. Their morphology, however, varies among the different genera, from cocci and peomorphic rods to branched filaments that break down into spherical cells or aerial mycelium with long chains of spores. The spores are formed as a result of nutrient depletion and can survive prolonged desiccation until nutrients are again available. Their ability to sporulate is important for their survival in the environment (McCarthy and Williams, 1992).

They are diverse group of heterotrophic prokaryotes forming hyphae at some stage of their growth referred as filamentous prokaryotes (McCarthy and Williams 1992; Jensen and Fenical, 2000). Many of them are well known for the economic importance as producers of biologically active substances, such as antibiotics, vitamins and enzymes (McCarthy and Williams 1992; Sanglier *et al*., 1993).

Actinomycetes are the most widely distributed group of microorganisms in nature and are also well known as saprophytic soil inhabitants (Takizawa *et al.,* 1993). The soil actinomycetes produce a volatile compound called geosmin, which literally translates to ''earth smell'' (Gust *et al.*, 2003). 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. Some actinomycete genera such as *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora, Microbispora, Micromonospora, Nonomuraea*, which are often very difficult to isolate and cultivate due to their slowly growth, are called rare actinomycetes (Hayakawa, 2008).

## 3.2. Ecology of actinomycetes

Actinomycetes are ubiquitous and numerically abundant in soil, in which they play a crucial role in nutrient cycling and decomposition, as they can degrade an exceptionally wide range of natural and manmade polymers. These including members of the genera *Streptomyces*, *Saccharopolyspora* and *Amycolatopsis*, are adapted to survival in a highly variable and competitive soil environment. They are equipped with a wide array of enzymes for exploiting nutrients but also produce a broad range of bioactive metabolites of industrial and medical importance compounds with activity against fungal and bacterial competitors (Tepoele *et al.,* 2008).

The majority of Actinomycetes are free living saprophytic bacteria found widely distributed in soil, water and colonizing plants. They are occur in the environments rich organic matter in both mesophlic and termophlic environments. In general the optimal conditions for their growth are temperatures of 25-30°C (50°C for the thermo-Actinomycetes). Most are aerobic and neutrophilic (Erikson, 1949; Ponmurugan and Poomima, 2006).

Over the past decade, it has become evident that actinomycetes are also widely distributed in marine ecosystems. Besides known actinomycete genera such as *Micromonospora,* Amycolatopsis, *Marinophilus, Rhodococcus, Streptomyces* and *Williamsia* (Mincer *et al*., 2005; Kim *et al*., 2006), new genera like *Salinispora* have been discovered in marine environments, which have clearly diverged from their terrestrial counterparts (Maldonado *et al.,* 2005). These obligate marine actinomycetes have adapted to life in the ocean and require high salinity for growth (Mincer *et al*.,

2002). Their entirely different environmental conditions compared to those of their terrestrial relatives may have influenced the production of secondary metabolites; these marine actinomycetes may therefore represent an important source of novel secondary metabolites (Lam, 2006). For example, the genus *Salinispora*, which is widely distributed in tropical and subtropical marine sediments, produces novel secondary metabolites, and also represents a new source of rifamycin-like antibiotics outside the genus *Amycolatopsis* (Kim *et al*., 2006).

## 3.3. Actinomycetes in soil

The soil is a complex habitat for many microorganisms, such as bacteria, fungi, protozoa and algae. Bacteria are the most abundant, attending population in excess of one hundred million ($10^8$) to one hundred billon ($10^9$) individuals per gram of soil. Actinomycetes constitute a significant component of the microbial population in most soils, where they usually are present in numbers of $10^6$-$10^7$ colony forming units per gram of soil (Sylvia *et al*., 2005).

Majority of actinomycetes are found in various types of soils such as agricultural fields, tropical forests and natural caves. Soil is also the most prolific source of isolates, which include many found to produce antibiotics and other useful metabolites. Therefore, the most intensively studied habitat (Goodfellow & Williams, 1983).

Crawford *et al*., (1993) demonstrated number of actinomycetes in the rhizosphere can be found twice to thrice when compared with non rhizosphere. It is caused by root of plant producing exudates as nutrition for pathogenic and non pathogenic microorganism, but interaction of root plant and bacteria can be negatively by secretion of components of root exudates inhibiting growth of some bacteria. Diversity of soil actinomycetes has a positive impact for exploration of novel bioactive compounds which may biological activity. Goodfellow and Williams, (1983) explained that in the rhzosphere actinomycetes can protect plant roots from plant pathogenic fungi and promote plant growth. For plant root protection, the modes of action of actinomycetes include antibiosis, parasitism, the production of extracellular hydrolytic enzymes and competition for iron.

Actinomycetes population has been identified as one of the major groups of soil population, which may vary with the soil type .The number of actinomycetes in soil, is influenced by many environmental factors such as soil type, soil depth, vegetative type, PH, moisture content and

aeration (Lacey, 1973). Most soil actinomycetes also behave as neutrophiles in culture, growing between pH 5 and 9 with an optimum close to neutrality. As many soils are acidic, pH is clearly a major factor determining their distribution and activity; it has been known for many years that acidic soils yield low counts of neutrophilic actinomycetes Waksman, S. A. (1959) cited in (Goodfellow and Williams, 1983). Temperature is obviously an important factor; most actinomycetes behave as mesophiles in the laboratory, with optimum growth at 25°-30°C or 45°-55°C for thermophiles, with most colonies developing within 14 days (5 days for thermophiles). However, slower growing strains may be missed and incubation periods of 4-6 weeks have been recommended (Okami and Okazaki, 1978).

## 3.4. Antimicrobial secondary metabolites production by actinomycetes

Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported. Over 10,000 bioactive secondary metabolites produced by actinomycetes. Among actinomycetes, around 7,600 compounds are produced by *Streptomyces* species, while the rare actinomycetes represent all together 2500 compounds. The representation of rare actinomycetes product in 1970 was only 5% in this group *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora* and *Streptosporangium* species (Berdy, 2005).

Many groups of microorganisms like Gram-positive, Gram-negative bacteria and fungi have the ability of synthesizing antimicrobial agents and the top cultivable antimicrobial agent producers present in soils are the actinomycetes (Pandey *et al*., 2002 cited in (Abo-Shadi *et al*.,2010**).** In the last decades actinomycetes became the most fruitful source for antibiotics (Moncheva *et al*., 2000). Making three quarters of all known naturally occurring antibiotics were derived from actinomycetes. Streptomycin, the first treatment for tuberculosis was derived from the largest genus of these bacteria *streptomycetes* , erythromycin and tetracycline are the two other example of common medicines that are highly useful pharmaceutical products derived from these microorganisms ( Lacey, 1973; Saadoun and Gharaibeh, 2003).

Antibiotics of actinomycetes origin evidence of a wide variety of chemical structure including aminoglycosides, anthracycline, β-lactams, nucleosides, peptides, polyene, actinomycine and tetracycline (Gonzales *et al*., 2005).

Considering the practically useful compounds, today about 130 to 140 microbial products and a similar number of derivatives (including semi-synthetic antibiotics) are applied in human medicine, mostly in chemotherapy, and veterinary medicine. Furthermore, some of 15 to 20 compounds are used in agriculture mainly as pesticides, plant protecting agents and food additives. The majority of these compounds, except fungal penicillins, cephalosporins and several bacterial peptides and few others, are also produced by actinomycetes (Moncheva *et al*., 2000).

After the discovery of gentamycin, originated from *Micromonospora*, the study of non-*streptomycete* actinomycetes received increasing attention. *Amycolatopsis* (formerly *Nocardia*) and *Actinomadura* species frequently produced vancomycin-type glycopeptides. The promising antitumor enediyne antibiotics were produced exclusively by rare actinomycetes. The members of new groups of macrolactam and naphtacene-quinone antibiotics were isolated from *Actinomadura*. The species from this genus frequently produced polyether antibiotics. *Micromonospora* and *Saccharopolyspora* strains were relatively rich sources of macrolides (Moncheva *et al*., 2000).

Soil actinomyctes, especially *Streptomyces* represent an important source of biologically active compounds with high commercial value and important applications in human, agriculture and livestock medicine (Berdy, 2005). They are also crucial importance in the environment because of their broad range of metabolic processes which allow biotransformation with degradation of the soluble remains of other organisms, such as lignocelluloses and chitin. Therefore, they have been the subject of genetic investigation focusing on the production of secondary metabolites (Paradkar *et al.,* 2003).

*Streptomycetes* are rich source of bioactive products and these organisms have been famous as producers of antibiotics. As antibiotics are typical secondary metabolites, they do not seem to play a central role in growth and catabolism of the organism and they tend to be produced after cell population ceases growth. Antibiotic production in *Streptomycets* species is regulated by a variety of physical and nutritional conditions and is co- ordinate with morphological development of the organism that include formation of aerial mycelia and sporulation; a process which is triggered by nutrient depilation (Glazer and Nikaido,1998).

The production of secondary metabolites in actinomycetes can be initiated or enhanced by the manipulation of fermentation conditions (Omura and Tanakn, 1984). The most common production methods involve liquid (submerged) and solid-state fermentations. Most of the biotechnology industries relied on submerged liquid fermentation process for the production of antibiotics, where the microorganisms are grown in liquid media using aeration (Gonzales *et al*., 2005). For actinomycetes shake flasks are commonly used with angular baffles, these indentations aid in the efficient mixing and in increased oxygen transfer (Katzer *et al*., 2001).

## 3.5. Screening for new secondary metabolites

Screening for the production of new metabolites with new isolates and/or new test methods, this is one way to obtain completely new class of substances. Screening can never be considered a routine activity, since the methods always adapt to the newest techniques and knowledge. The goal is always to detect and identify new substances of commercial interest (Crueger and Crueger, 1989).

There are no universal screening methods. The success of a screening program depends upon the selection of appropriate test method as well as appropriate microorganism to be tested. The choice of strain as a 30-40% influence on the outcome, the tested procedure a 60-70% influence (Crueger and Crueger, 1989).

In the search for novel microbial metabolites, a number of rational screens have provided an effective means in detecting secondary metabolic products. Targeted screens based upon mechanisms of action have detected metabolites with the desired bioactivity. These are either a known compound or have uncovered novel structural classes (Higashide, 1995).

The classical whole-cell agar diffusion assay has been the conventional approach used in the screening of secondary metabolites which has usually been conducted in a random fashion. Although incorporating new target organisms has lead to the discovery of new compounds (Higashide, 1995), in actinomycetes the rediscovery rate is 99 % (Zahner and Fiedler, 1995).

Alternative approaches to classical or modifications to secondary metabolites screening have identified a number of ways that could lead to the discovery of new compounds. These approaches include (i) re-evaluation and further development of secondary metabolites that have already been

commercially introduced; (ii) evaluation of known antibiotics not used for human therapy; (iii) searching new secondary metabolites using new test methods, novel microorganisms and varying culture conditions; (iv) focus on isolates from unusual or little-explored ecosystems; (v) directed evolution in accelerating enhanced enzymatic activity and broader substrate specificity (Zahner and Fiedler,1995; Bull *et al*., 2000).

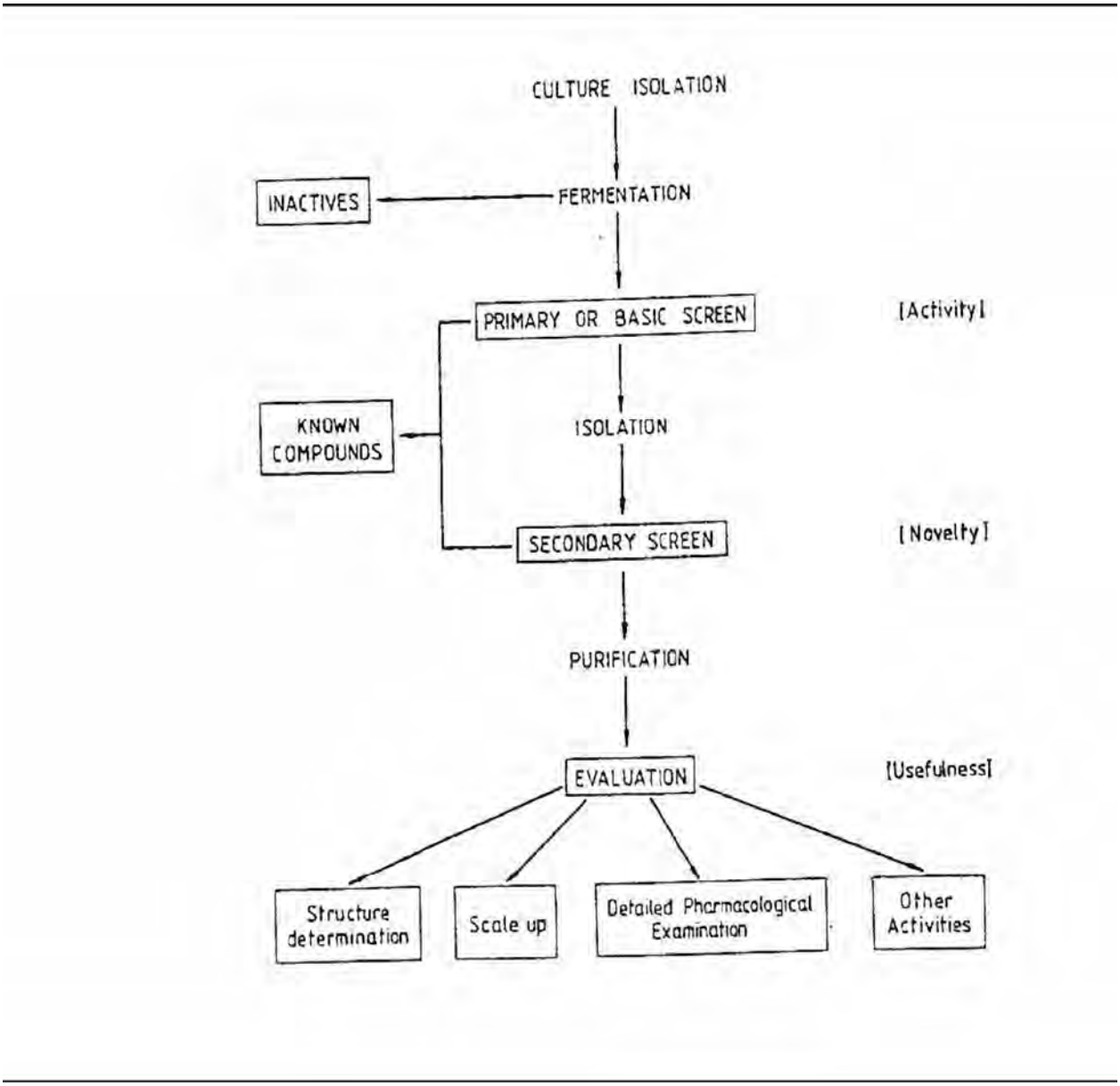


Fig 1. Screening process for new bioactive microbial metabolites (Berdy, 1989).

## 3.6. Factors affecting antibiotic production

Of primary consideration in submerged fermentation of secondary metabolites involving filamentous microorganisms such as fungi and actinomycetes is the fermentation vessel (Whitaker, 1992). Two forms of liquid media can be used in the production of secondary metabolites, these include chemically defined (synthetic) or undefined (natural, complex). In expensive complex media are usually employed in commercial fermentations which give higher fermentation yields at a lower cost (Dahod, 2000). Numerous studies on the nutritional requirement for production of antibiotics and other non-essential metabolites have demonstrated that there is a link between nutrient limitation and biosynthesis of secondary product. Changes in the nature and type of carbon, nitrogen or phosphate sources and trace elements have been reported to affect antibiotic biosynthesis during media formulation for fermentation (Stanbury *et al*., 1995).

Medium formulation consists of two factors as

1. Medium composition and
2. Fermentation conditions which may affect the antibiotic production.

### 3.6.1. Medium Composition

#### 3.6.1.1. Carbon Source, Nitrogen source, Inorganic Phosphate and an inorganic Salts

It is now recognized that the rate at which the carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. Fast growth due to high concentrations of rapidly metabolized sugars is often associated with low productivity of secondary metabolites (Stanbury *et al*., 1995)

Readily metabolized carbon sources, such as glucose, can suppress antibiotic production by preventing the synthesis of a key enzyme in the biosynthesis pathway. The phenomenon has been referred to as “catabolic repression”. The commonly used carbon sources in fermentations are glucose, maltose, sucrose, lactose, glycerol, corn steep liquor, molasses etc. The method of media preparation particularly sterilization, may affect the suitability of carbohydrates for individual fermentation may react with ammonium ions and amino acids to form black nitrogen containing

compound which will partially inhibit the growth of many microorganisms ( Iwai *et al*.,1981; Soliveri *et al*.1988 ; Stanbury *et al*.,1995 ; Gesheva *et al.,*2005).

It is well known that changes in the kind and concentration of nitrogen source influence greatly antibiotic production. Antibiotic production is inhibited by a rapidly utilized nitrogen source (NH4 +, NO3 -, certain amino acids, etc.). Antibiotic accumulation begins to increase in many cases only after the nitrogen source in the culture broth has been almost entirely consumed. In candihexin production, addition of a nitrogen source in the idiophase, returns the fermentation to the trophosphase and production is reduced (Stanbury *et al*., 1995; Gesheva *et al.,* 2005).

Recently, the presence of nitrogen regulation was revealed to the enzymatic level in fermentation of cephamycin and patulin. The inhibition of production by a nitrogen source can be usually avoided by selecting an adequate production medium with the proper kind of nitrogen source. The quantity of nitrogen source is chosen keeping in mind the quantity of carbon source present and this reflect C/N ratio. The use of complex nitrogen sources for antibiotic production has been common practice. They are thought to help create physiological conditions in the trophophase which favour antibiotic production in the idiophase. For example, in the production of polyene antibiotics, soyabean meal has been considered a good nitrogen source because of the balance of nutrients, the low phosphorous content and slow hydrolysis (Soliveri *et al*.1988 Stanbury *et al*., 1995; Gesheva *et al.,* 2005).

When adding a large amount of inorganic phosphate, consumption of carbon and nitrogen sources and respiration are accelerated resulting in good growth, but the production of antibiotics is usually reduced. Microorganisms have their optimal phosphate concentration for growth in a range of 0.3 to 300 mM, but the amount of inorganic phosphate adequate for the production of antibiotics is usually much lower than the amount required for growth (<10 mM), similar to the carbon or nitrogen source (Iwai *et al*.,1981;Stanbury *et al*., 1995).

As described above, the amount of inorganic phosphate strongly influences the production of antibiotics, but the precise mechanism has not been clarified. However, it is presumed that when the concentration of inorganic phosphate in the culture is high, the intracellular concentration of ATP is increased and the primary metabolism is accelerated. When the amount of inorganic phosphate is

lowered, the ATP concentration decreases; this decrease depresses metabolic conversions which are required for the production of antibiotics (Soliveri *et al*. 1988; Gesheva *et al.,* 2005).

It is well known for rather long time that addition of inorganic salts such as NaCl to antibiotic production media increases the production. But addition of larger amount of NaCl usually inhibits the production. The amount of trace metals required for the antibiotic production is larger than that required for growth: the concentrations added for growth are usually 10 to 100 fold greater. Addition of further amounts inhibits production ( Soliveri *et al*. 1988; Gesheva *et al.,* 2005).

## 3.6. 2. Fermentation Conditions

### 3.6.2.1. pH, Temperature, Oxygen and Others conditions

The pH of fermentation affects not only the growth but the production as well as does the medium constituents and the temperature. The inhibition of antibiotic production by glucose or $K_2HPO_4$ is not only to the above described regulatory controls but also to the effect on pH during fermentation (James *et al.,* 1991).

The control of pH may be extremely important if optimal productivity is to be achieved. A compound may be added to the medium to serve specifically as a buffer, or may also be used as a nutrient source. Many media are buffered at about pH 7.0 by the incorporation of calcium carbonate. The balanced use of the carbon and nitrogen sources will also form a basis for pH control as buffering capacity can be provided by the proteins, peptides and amino acids, such as in corn steep liquor. The pH may also be controlled externally by addition of ammonia or sodium hydroxide and sulfuric acid. Although pH of a medium is often noted after sterilization, but since the medium has been subjected to severe conditions of high pressure sterilization before use, the pH before sterilization is also important (James *et al.,* 1991; Stanbury *et al*., 1995).

It is known that thermophilic actinomycetes such as thermoactinomycetes produce new antibiotics at temperature higher than 40$^{o}$C, but *Streptomyces* usually produces antibiotics at temperatures near 27$^{o}$C. Generally the range of a temperature supporting good growth is as wide as 25 degrees, but the temperature range adequate for good production of secondary metabolites is narrow i.e. 5 ~ 10 degrees centigrade . Usually, cultivation for antibiotic production is performed under one constant

temperature from the beginning to the end, but the temperature adequate for growth is not always the same as that for production. For example, *Streptomyces* species no.81 strain produces antibiotic M-81 at 27$^{o}$ C, forms cryomycin at the low cultivating temperature of 12$^{o}$C. Thus temperature must be considered separately for growth and for production. It would be of interest in antibiotic screening to use temperature shifts (James *et al.,* 1991; Gesheva *et al*., 2005).

Many antibiotic producing microorganisms require oxygen for growth. The water solubility of oxygen is very low and scale-up of antibiotic fermentation is based on dissolved oxygen in a cultivation medium. For determining the conditions for large scale cultivation of individual antibiotics, aeration and agitation conditions are selected depending on the optimal concentration of dissolved oxygen. The optimal level of dissolved oxygen may be different between growth and production. Therefore, it is necessary to cultivate the fermentation not under one aeration or agitation condition, but with shifts in this parameter (Iwai *et al*., 1981; Stanbury *et al*., 1995).

Besides the fermentation parameters described above, there are other factors that affect antibiotic production such as pressure, oxidation-reduction potential and light. Although agitation is usually considered only from the viewpoint of oxygen, it may have other effects. Cellular damage by agitation affects the production of maridomycin by *Streptomyces hygroscopicus*. In the bicyclomycin fermentation by *Streptomyces sappronensis*, the producing microorganism suffers a reduction in antibiotic production by increased agitation, concomitant with an absence of aerial mycelium (Stanbury *et al*., 1995).

## 3.7. Mechanisms of antibiotic action

For many antibiotics, the mechanism of action is not understood fully. However, it is known that antibiotics can act in the following ways: 1) inhibit cell wall synthesis, 2) inhibit protein synthesis and 3) inhibit nucleic acid synthesis (Kathleen *et al*., 1994).

The differences in cellular structure among bacterial species can lead to resistance to certain antibiotics. For example, Gram-negative bacteria exhibit high intrinsic resistance to many antibiotics because of the nature of their cell wall, which restricts absorption of many molecules to movements through openings called porins. Perhaps even more important, when β-lactumase are

present in the periplasmic space, the antibiotic remains outside the cell, where the enzyme, which is too large to enter even through an unmodified porin, can reach and inactivate it (Tortora *et al*., 2010).

Acquired resistance can arise either through mutation or horizontal gene transfer. Presence of the antibiotic in question leads to selection for resistant organisms, thereby shifting the population towards resistance. The major mechanisms of acquired resistance are the ability of the microorganisms to destroy or modify the drug, alter the drug target, reduce uptake or increase efflux of the drug, and replace the metabolic step targeted by the drug (Kathleen *et al*., 1994)

### 3.7.1. Antibacterial antibiotics and mode of action

#### 3.7.1.1. Inhibitors of cell wall synthesis

The cell wall of a bacterium consists of a macromolecular network called peptidoglycan. In Gram-positive organisms, the peptidoglycan layer is a 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 (Kathleen *et al*., 1994).

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. The β-lactams include the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall (Greenwood, 2000).

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 lipo teichoic acid (Greenwood, 2000). The mechanism of β-lactam resistance is via the action of the β-lactamases. These enzymes catalyze hydrolysis of the β-lactam ring and, thereby, inactivating these antibiotics and development of resistance by many bacteria (eg, *S.aureus, Neisseria gonorrhoeae, Pseudomonas sp, Bacteroides fragilis*, and some enteric Gram-negative bacilli) (Kathleen *et al*., 1994).

Many bacteria contain chromosomally encoded β-lactamases necessary for cell wall production and it is only through over-production of these enzymes that resistance occurs (Greenwood, 2000). β-lactamases encoded on plasmids or other transmissible elements can lead to such overproduction and, therefore, to resistance (Kathleen *et al*., 1994). There are also some bacteria that possess altered PBP's that result in reduced penicillin binding (Greenwood, 2000).

Since the discovery of penicillin and resistant bacteria, various new versions of the β-lactams have been used that have different spectrums of activity and different susceptibility to β-lactamases. Since the 1970s, several compounds, such as clavulanic acid, have been discovered that have the ability to bind irreversibly to β-lactamases and, thereby, inhibit their action. Combinations of these compounds with β-lactam drugs have been very successful in treatment of diseases (Bryan, 1984; Kathleen *et al*., 1994).

The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall. These drugs are large molecules that are excluded from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms. Glycopeptide resistance was long thought to be rare, but has recently been shown to be quite common (Bryan, 1984). Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug (Greenwood, 2000).

### 3.7.1.2. Inhibitors of protein synthesis

Protein synthesis is a common feature of all cells. Bacterial and eukaryotic ribosomes differ in both size and chemical composition. Eukaryotic cells have 80S (with 60S and 40S subunits) ribosomes; prokaryotic cells have 70S ribosomes. (The 70S ribosome is made up of a 50S and a 305 unit. Thus, antibiotics that affect protein synthesis can have a selective effect on sensitive bacteria without affecting human cells. Among the antibiotics that interfere with protein synthesis are chloramphenicol, erythromycin, streptomycin, and the tetracyclines (Kathleen *et al*., 1994).

There are many types of antibiotics that inhibit bacterial protein synthesis. These drugs take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes. The

aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins (Greenwood, 2000). The antibacterial activity of aminoglycosides is directed primarily against aerobic Gram-negative bacilli; there is little activity against anaerobes and Gram-positive bacteria (streptococci) (Kathleen *et al*., 1994; Greenwood, 2000).

Streptomycins act by binding to the 30S ribosomal subunit; kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (Greenwood, 2000). Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood (Bryan, 1984; Greenwood, 2000).

There are three mechanisms of amino glycoside resistance that have been identified to date. The first involves only streptomycin. Since streptomycin; binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high level resistance to the drug. The second mechanisms involve decreased uptake of the antibiotic and in one of these the cell membrane is altered, preventing active transport of the drug. In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport (Bryan, 1984).

Chloramphenicol is a broad spectrum antibiotic that is active against many gram positive and gram negative bacteria. Produced by chemical synthesis and although naturally occurring (Kathleen *et al*., 1994). Chloramphenicol inhibits peptide bond formation on 70S ribosomes (Bryan, 1984). This drug is especially useful in that it can penetrate eukaryotic cells and cerebrospinal fluid, making it a drug of choice for treatment of meningitis and intracellular bacterial infections such as those caused by Chlamydia (Greenwood, 2000).

Resistance to chloramphenicol is conferred by the enzyme chloramphenicol acetyl-transferase; a number of these enzymes have been discovered, each altering the chloramphenicol molecule to prevent binding to the bacterial ribosome (Greenwood, 2000). Chloramphenicol resistance in gram negative cells can also arise from alteration in outer membrane permeability that prevents the drug from entering the cell (Bryan, 1984).

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA (Kathleen *et al*., 1994). Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters and there are several genes encoding these pumps. Secondly, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring resistance. Third, a protein found only in *Bacteroides* spp., enzymatically inactivates tetracycline (Roberts, 1996).

The macrolides are a group of antibiotics commonly used to treat gram positive and intracellular bacterial pathogens. Erythromycin was the first of these and several other important macrolides have been discovered since, including clarithromycin and azithromycin. Azithromycin has a longer plasma half-life which allows treatment with a single dose for some pathogens or a once daily dose for others. Clarithromycin has enhanced absorption and causes less gastrointestinal discomfort (Gaynor, 2003).

It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research show several mechanisms are involved (Kathleen *et al*., 1994). The macrolides are now believed to promote dissociation of tRNA from the ribosome, inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation (Gaynor, 2003).

There are two major mechanisms of macrolide resistance. First, an efflux pump has been found that removes the drug from the cell. Second, modification of the ribosome can confer resistance; mutations at several sites of the ribosome can allosterically prevent macrolide (Gaynor, 2003).

### 3.7.1.3. Inhibitors of nucleic acid synthesis

The best-known derivative of the rifamycin family of antibiotics is rifampin. These drugs are structurally related to the macrolides and inhibit the synthesis of mRNA (Tortora *et al*., 2010). Microorganisms may develop resistance to rifampin rapidly in vitro as a one-step mutation; this also occurs in vivo. For this reason, rifampin should not be administered alone, except for short term chemoprophylaxis (Kathleen *et al*., 1994). This characteristic is probably an important

factor in its ant tubercular activity, because the tuberculosis pathogen is usually located inside tissues or macrophages (Tortora *et al*., 2010).

The quinolones are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhea and anthrax. Drugs in this class include nalidixic acid, norfloxacin, and ciprofloxacin (Kathleen *et al*., 1994). Quinolones inhibit bacterial growth by acting on DNA gyrase which are responsible for cutting DNA strands, thus preventing supercoiled DNA and topoisomerase IV. Although quinolones target both enzymes, in gram negative organisms the primary target is DNA gyrase and, in gram positive organisms, the primary target is topoisomerase IV (Greenwood, 2000). Resistance to some quinolones occurs with decreased expression of membrane porins, expression of efflux pumps in both gram negative and gram positive organisms and alteration of the target enzymes (Kathleen *et al*., 1994).

### 3.7.2. Antifungal antibiotics and mode of action

Eukaryotes, such as fungi, use the same mechanisms to synthesize proteins and nucleic acids as higher animals. Therefore, it is more difficult to find a point of selective toxicity in eukaryotes than in prokaryotes. Moreover, fungal infections are becoming more frequent because of their role as opportunistic infections in immunosuppressed individuals, especially those with AIDS (Tortora *et al*., 2010).

Many antifungal drugs target the sterols in the plasma membrane. In fungal membranes, the principal sterol is ergosterol. When the biosynthesis of ergosterol in a fungal membrane is interrupted, the membrane becomes excessively permeable, killing the cell. Inhibition of ergosterol biosynthesis is the basis for the selective toxicity of many antifungals, which include members of the polyene and azole groups (Tortora *et al*., 2010). Amphotericin B is the most commonly used member of the antifungal polyene antibiotics .For many years amphotericin B, produced by *Streptomyces* species of soil bacteria, and has been a mainstay of clinical treatment for systemic fungal diseases such as histoplasmosis, coccidioidomycosis, and blastomycosis. The drug's toxicity, particularly to the kidneys, is a strongly limiting factor in these uses. Administering the drug encapsulated in lipids (liposomes) appears to minimize toxicity. The first azoles were imidazoles, such as *clotrimazole* and *miconazole* which are now sold without a

prescription for topical application for treatment of coetaneous mycoses, such as athlete's foot and vaginal yeast infections. An important addition to this group was ketoconazole, which has an unusually broad spectrum of activity among fungi (Goldman and Green, 2009; Tortora *et al*., 2010).

The fungal cell wall contains compounds that are unique to these organisms. The first of a new class of antifungal drugs is the echinocandins, which inhibit the biosynthesis of glucans, resulting in an incomplete cell wall and cell lysis (Goldman and Green, 2009).

Flucytosine, an analog of the pyrimidine cytosine, interferes with the biosynthesis of RNA and therefore protein synthesis. Flucytosine has a narrow spectrum of activity, and toxicity to the kidneys (Tortora *et al*., 2010).

## 3.8. Antibiotic resistance

Antibiotic resistance is now well recognized as a major problem in the treatment of infections in hospitals and, with increasing and alarming frequency, in the community. Resistance can be active (i.e., the result of a specific evolutionary pressure to adapt a counterattack mechanism against an antibiotic or class of antibiotics) or passive (where resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the nonspecific barrier afforded by the outer membrane of Gram-negative bacteria). Bacteria achieve active drug resistance through three major mechanisms: (1) efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins; (2) modification of the antibiotic target (e.g., through mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways such as in resistance to the glycopeptides antibiotics); and (3) via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics. All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics. Therefore, bacterial cells expend a considerable amount of energy and genetic space to actively resist antibiotics (Wright, 2005).

# 4. MATERIALS AND METHODS

## 4.1. Soil Sample collection

A total of 15 soil samples were collected from the rhizosphere of plants and agricultural soils from field sites of Deberizit (5 samples) and Holeta (5 samples) agricultural soils and garden soil from the college of natural sciences (5 samples) in December 2010. The soil were excavated from depth of 5-15 cm by using sterile spatula and collected in clean, dry and sterile polyethylene bags. All samples were labeled, transported and stored in the refrigerator at 4°C for further investigations.

## 4.2. Media for the cultivation of actinomycetes

For isolation of Actinomycetes, the following two media were used, Starch Casein Agar medium (SCA) g/L: soluble starch 10, casein 0.3, $KNO_3$ 2, NaCl 2, $K_2HP0_4$ 2, $MgSO_4.7H_2O$ 0.05, and CaCO3 0.02, $FeSO_4.7H_2O$ 0.01, Agar 15 and pH was adjusted to 7.0±2 before sterilization (Arifuzzaman *et al.,* 2010**)** and Actinomycetes Isolation Agar (AIA) medium having the composition of g/L: Heart infusion broth 25.0,Casein hydrolysate 4.0,Yeast extract 5.0, Dextrose 5.0, Cysteine HCl 1.0, Soluble starch 1.0,Potassium phosphate 15.0, Ammonium phosphate 1.0, Magnesium sulphate 0.2, Calcium chloride 0.02 , Agar 20 were used and pH was adjusted to 7 before sterilization (Awad *et al.,* 2009). The two media were autoclaved with a temperature of 121$^{o}$C for 15 min, cooled up to 50$^{o}$C and supplemented with 50 µg/mL of Amphotericine B to minimize fungal growth, and 20- 25 ml of media was poured on sterile Petri dishes allowed to cool at room temperature .

## 4.3. Isolation, Maintenance and Designation of isolates

Actinomycetes were isolated by serial dilution plate technique (Arifuzzaman *et al*., 2010). About 1g of each soil sample was suspended in 10 ml of sterile normal saline (0.85%) and shaken on orbital shaker for about 30 min at 121rpm; the suspension was left for 30 min in the Laminar Air Flow hood. Consequently, 1ml of sample was taken and diluted 7 fold in 9 ml of sterile normal saline (0.85%). The suspensions were agitated with vortex and 0.1 ml of sample was taken from $10^{-3}$, $10^{-5}$, and $10^{-7}$ dilutions and was spread on each of the Starch Casein Agar medium and Actinomycetes Isolation Agar medium. The plates were incubated, at 30$^{0}$C for seven to ten days

(Dhanasekaran *et al*., 2009). All isolates were identified as actinomycetes based on colony morphology and color of mycelium (Williams and Cross, 1971). The isolates were further sub cultured to ensure their purity and maintained on Starch casein agar medium. The inoculated agar media or slant was incubated, at 30$^{o}$C for 7 days and then stored, at 4 $^{o}$C for further use. Code was assigned (such as AAUBA1) for every entity to confirm the source of the organism, the color of aerial mycelium.

Codes of selected isolates: -

AAUBA1 Addis Ababa biology, Actinomycetes from Debrezit rhizospher, white
AAUBA5 Addis Ababa biology, Actinomycetes from Debrezit rhizospher, whitish brown
AAUBA6 Addis Ababa biology, Actinomycetes from Debrezit rhizospher, pink
AAUBA8 Addis Ababa biology, Actinomycetes from Debrezit rhizospher, grey
AAUBA10 Addis Ababa biology, Actinomycetes from Debrezit soil, brown
AAUBA13 Addis Ababa biology, Actinomycetes from Debrezit rhizospher, white
AAUBA19 Addis Ababa biology, Actinomycetes from Holeta rhizospher, dark grey
AAUBA30 Addis Ababa biology, Actinomycetes from Arat Killo rhizospher, yellowish white
AAUBA31 Addis Ababa biology, Actinomycetes from Arat Killo rhizospher, dark brown

## 4.4. Screening of antimicrobial activity of crude extracts of actinomycetes against test microorganisms

### 4.4.1. Test microorganisms

The test organisms used for antimicrobial activity of the isolates were; *Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 25853, *Staphylococcus aureus* (clinical isolate), *Shigella boydii* (clinical isolate), *Streptococcus pneumoniae* ATCC 49619, *Sallmonella typi* ATCC 6539 , *Candida albicans* ATCC 62376 and *Cryptococcus neoformance* (clinical isolate).

The test bacteria and fungi were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) and Biomedical Laboratory, Faculty of Life sciences, AAU.

### 4.4.2. Turbidity standard for inoculum preparation

Standardization of the inoculum density of isolates for susceptibility test was done by the method described in Lalitha, (2004). In order to determine the active phase of test organisms, each isolate was grown in 100 ml of Nutrient Broth for bacterial test microorganism and Sabourouds Broth for fungal test microorganism in 250 ml Erlenmeyer flask on a rotary shaker 120 r/min $^{-1}$, at 37 $^{O}$C. Samples were taken every 2hrs to measure optical density using spectrophotometer (JENWAY, London) at, 660 nm. The optical density values were extra plotted against time to determine the different phases of the growth curve.

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 $BaCl_2$ solution in to 99.5 ml of solution $H_2SO_4$ (Lalitha, 2004). The density of the turbidity standard was determined using spectrophotometer (JENWAY, London).

### 4.4.3. Primary screening

A total of 30 isolates were primarily screened for antimicrobial activity against five test microorganisms according to Egorov, (1985), (cited in Pandey *et al*., 2004). Seven day grown isolates were streaked as a straight line across diameter of Nutrient Agar plates (Oxiode) and incubated, at 30°C for 6 days. After 6 days, the test microorganisms namely *Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 25853, *Staphylococcus aureus* clinical isolate and *Shigella boydii* clinical isolate from overnight culture broth were streaked at right angle, but not touching to the streak and incubated at , 37$^{o}$C for 24 hours. Clearing zone formation between the antibiotic producing isolate and the test organisms was considered positive for antibiotic production. The isolates were then selected based on a wide spectrum activity against tested microorganisms for further studies.

### 4.4.4. Secondary screening

#### 4.4.4.1. Cultivation and extraction of cultures of actinomycetes for secondary metabolites

Nine isolates were selected for secondary screening in small scale submerged fermentation system. Two hundred milliliter of Starch Casein Broth was dispensed in to 500ml Erlenmeyer

Flask , to which a loop full of seven days grown isolates were inoculated and incubated on a platform shaker (New Brunswick Scientific), at 200 rpm at room temperature for 10 days according to (Remya and Vijayakumar, 2008; Dhanasekaran *et al*., 2009). After ten days of incubation, the content of incubation the flask content was filtered through Whatman No.1 filter paper. Equal volume of ethyl acetate (1:1) was then added to the culture filtrates and shaken vigorously for 1 hr and solvent phase that presumably contain antibiotics compound was separated from aqueous phase in a separatory funnel (Assistant, Germany). The ethyl acetate phase that contain antibiotic were evaporated and concentrated in vacuum rota-vapor (BUCHI-Germany) with 100 rev/min at temperature of 60 $^{o}$C (Fessenden, 1993; Remya and Vijayakumar, 2008). The dry crude extract was weighted using a balance (SCALTEC, Germany) and kept in small vials at 4 $^{o}$C for further test.

### 4.4.4.2. Disc Diffusion assay

Antibiotic activity of the crude extract from culture filtrates of each isolate was evaluated by using Disc diffusion Assay (Hassan *et al.,* 2001; Ningthoujam *et al.,* 2009). For this purpose , Nutrient Agar (NA) (Oxiod) for bacteria and Sabouraud Dextrose Agar (SA) (Oxiod) for yeast/fungi were inoculated with 0.2 ml overnight culture of each standardized test organism; *Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 25853, *Staphylococcus aureus* (clinical isolate), *Shigella boydii* clinical isolate, *Streptococcus pneumoniae* ATCC 49619, *Sallmonella typi* ATCC 6539, *Candida albicans* ATCC 62376 and *Cryptococcus neoformance* (clinical isolate).

About 20 µl of 50 µg /ml crude extract of each isolate was impregnated with sterile Whatman Antibiotic Assay Discs (6.0 mm), and placed on the inoculated agar plates. Blank discs impregnated with the solvent were also placed on inoculated plates to serve as control. This was done in duplicates. The Petri dishes were then kept in a refrigerator at 4 $^{o}$C for 2 hours to allow the diffusion of the extracts on the media. The Petri dishes were then incubated, at 37$^{o}$C for 24 hrs to detect and measure the inhibition diameter around the discs.

## 4.5. Bioassay guided purification of active compound from AAUBA13

The crude extract of AAUBA13 isolate was selected based on inhibition zone diameter and spectrum activity from antimicrobial assay test to detect the antimicrobial component of the crude extract using chromatographic methods (Al-Bari *et al*., 2006; Gurung *et al*., 2009).

### 4.5.1. Thin Layer Chromatography (TLC)

The TLC plate was cut in to 5cm x 10cm and the penal line was drawn on white side up using pencil and ruler. Consequentially, 10µl of the crude extract to be separated was spotted in a single small spot 1.5cm from the end of TLC plate using capillary tube. Before inserting the TLC plate the developing solvent was poured in to the solvent tank, to cover the bottom of the tank to a depth of 1.0 cm. When the spot was dried, the plate was immersed in to solvent tank containing a 1:9 solvent mixture of Chloroform: Methanol. The solvent was allowed going about 90% of the way up the plate was taken out of the jar with forcipes, and then the solvent front was marked with a pencil immediately; and allowed to dry. Spot were visualized with Ultraviolet (black) lamp at 254 and 366 nm (Fessenden, 1993; Gurung *et al*., 2009). Visualizion of the spot was also done with vanillin -sulfuric acid spray regent (Al-Bari *et al*., 2006; Selvameenal *et al*., 2009). The spot was circled with pencil. The distance of the spot moved up the plate and that of the solvent was measured in cm. The retention factor (Rf) values of the antimicrobial compound were calculated by dividing distance travelled by the spot to the distance traveled by the solvent (Fessenden, 1993).

$$Rf = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

In order to make sure that single spot from TLC plate active bioautography was done using Nutrient Agar plate inoculated with 0.2 ml ($3x10^6$) overnight culture of *Staphylococcus aureus* (clinical isolates). The spots from TLC plates were cut and placed down ward on the center of seeded plate. After 1 h at 4 $^oC$ in Refrigerator, the plate was incubated for 24 h at 37$^oC$. The inhibition of the test organism indicated the spot have antimicrobial activity (Pandy *et al*., 2004).

### 4.5.2. Column Chromatography

The single spot from TLC plate indeed active, column chromatography was performed to purify and detect the components of the antimicrobial compound based on their polarity with solvents. The column (Sorbisil 60 mesh; column dimensions 2.5 cm inner diameter x 30 cm length) was packed very tightly with silica gel as slurry with the desired solvent (ethyl acetate). One hundred (100mg) of the active crude extract was dissolved in a minimum amount of ethyl acetate and applied directly on the top of packed column. Then, 10ml of the eluting gradient chloroform: methanol solvent was added to the top of the column. The solvent mixture and the right proportion of crude extract to silica gel in just 1:30 ratio .The column was developed and the flow rate of the system was adjusted per minute. The elution process was conducted by adding different solvent namely; ethanol, petroleum ether, acetone and methanol in the middle of the process (Fessenden, 1993). Thirteen (13) different fractions of the compound were collected (each of 5ml) from the column chromatography.

### 4.5.3. Preparative Thin Layer Chromatography

Preparative TLC plate was prepared by suspending 160g of Merk silca gel GF 254 powder in 260 ml of de ionized water that was vigorously shaken for 45 seconds in rubber stopped 500ml of Erlenmeyer flask. The thickened slurry was poured in to 10x20cm plate and spread to prepare 1.0mm trailing edge. The plate was air dried until it turned white for 45 min and activated at 120°C for half an hour. Ten (10 µl) fraction was applied 1.5 cm above the lower edge of TLC plates and developed in solvent tank using chloroform: methanol system (1:9). The spots on the chromatography were visualized in UV chamber 245 and 366nm (Al-Bari *et al*., 2006). The obtained band was then scratched and dissolved with ethyl acetate and centrifuged, at 3000 rpm for 15 min. Supernatant was collected in a preweighed vial and kept for evaporation (Selvameenal *et al*., 2009).

### 4.5.4. In vitro antimicrobial activity of purified compound from AAUBA13

The antimicrobial potential of 13 fractions of the crude extract from AAUBA13 was tested based on disc diffusion techniques as described before in section (4.4.4.2).

## 4.6. Characterization and identification of selected isolates

Selected isolates, including AAUBA1, AAUBA5, AAUBA6, AAUBA8,AAUBA10, AAUBA13, AAUBA19,AAUBA30 and AAUBA31 were described by some cultural, morphological, physiological and biochemical characterization to identify the isolates at genus level based on Bergay's Manual of Systematic Bacteriology , Volume four (Williams *et al*., 1989).

### 4.6.1. Cultural (Macroscopic) characterization

Cultural characteristics of the isolates were investigated by growing the isolates on, Starch Casein Agar, Starch Nitrate Agar, and Glycerol Asparagine Agar, Glucose Asparagine Agar and (Actinomycetes Isolation Agar). A loop full of each isolate from 7 days old culture was taken and inoculated into each of the medium by streak plating technique and incubated at 30$^{o}$C for 7 days. The experiment was done in duplicates and colony morphology was noted with respect to color of aerial mycelium and substrate mycelium, colony diameter and diffusible pigment were examined (Williams and Cross, 1971; Remya and Vijayakumar, 2008) and the result was recorded.

### 4.6.2. Microscopic characterization

The microscopic examination was carried out by cover slip culture and Gram staining methods to study the morphology of the isolates.

Cover slip culture method was done according to (Williams and Cross, 1971; Kawato and Sinobu, 1959 cited in Tiwarty, 2009**)** by inserting sterile cover slip at an angle of 45$^{o}$ in the Starch Casein Agar medium. A loop full of isolates were taken from 7 day old culture and inoculated, at the insertion of the cover slip on the medium and incubated, at 30$^{o}$C for seven 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 minutes, and washed with tap water and flooded with crystal violet reagent for one minute followed by washing and blot drying. It was then examined through microscope (Wagtech, England) under magnification of X1000 in oil immersion. The morphology of spore chains and hyphae of substrate and aerial mycelia were observed and the picture was captured by U-LH100HG Florescent microscope (OLYMPUS BXSI, JAPAN) in the Applied Microbiology

Laboratory, Faculty of Life Science, AAU. The observed characteristics were compared with the actinomycetes morphology provided in Bergay's manual of Systematic Bacteriology (Williams *et al*., 1989).

### 4.6.3. Physiological characterization

Physiological characters of the isolates were studied on the basis of pH tolerance, temperature tolerance, resistance towards sodium chloride and utilization of carbon and nitrogen sources.

#### 4.6.3.1. pH tolerance

A loop full the test isolate from 7 days old culture was taken and serially diluted from $10^{-1}$- $10^{-6}$ in sterile distilled water, 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 level of 5, 6, 7, 8, 9, 10, 11, 12; the experiment was done in duplicates and colony was counted with log colony forming unit after incubating the isolates ,at 30$^{o}$C for 7 days and the result was recorded (Laidi *et al*., 2006).

#### 4.6.3.2. Temperature tolerance

Temperature tolerance of the isolates was determined on Nutrient Agar plates (Oxide). A loop full of the test isolate from 7 days old culture was taken and serially diluted from $10^{-1}$-$10^{6}$ in sterile distilled water; 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 with log colony forming unit after incubating the isolates at 15$^{o}$C, 25$^{o}$C, 30 $^{o}$C, 37$^{o}$C, and 45$^{o}$C and the result was recorded (Laidi *et al*.,2006).

#### 4.6.3.3. Growth with sodium chloride

The isolates were tested for the levels of tolerance to sodium chloride on Nutrient Agar (Oxide) supplemented with 5%, 7%, and 10% 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$^{o}$C for 7 days and observations were made to record highest concentration of salt that allows the growth (Santhi *et al*., 2010).

### 4.6.3.4. Utilization of carbon and nitrogen source

Utilization of different carbon and nitrogen source were tested, in the basal medium consisting of (g/l); K2HPO4, 1.0; MgSO4.7H2O, 0.5; $CaCl_2.2H_2O$, 0.04; $FeSO_4.7H_2O$, 0.005; $ZnSo_4.7H_2O$, 0.0005 and 15g agar and 1.0% of each of the carbon and nitrogen sources as D-Mannose, Sucrose, D-Galactose, D- Glucose, L-Arabinose, D-Mannitol, Cellobiose and D-Fructose was used. Nitrogen sources such as L-Arganine, yeast extract, peptone, $(NH_4)$ $SO_4$ and $(NH_4)$ $H_2PO_4$ and pH were adjusted to 7. The carbon and phosphate sources were sterilized separately and added just prior to inoculation. Each isolate from 7 days culture were inoculated with streak plate technique. The experiment was done in duplicates and the plates were incubated at 30$^{o}$C together with basal medium as negative control. The growth was read after 7, 14, 21 days and the result were recorded as Abundant (When growth on tested Carbon or Nitrogen in basal medium greater than growth on basal medium), Moderate (When growth on tested Carbon or Nitrogen in basal medium is significantly better than growth on basal medium) and Good **(**When growth on tested Carbon or Nitrogen in basal medium is less than growth on basal medium) (Shirling and Gottlieb, 1966; Oskay *et al.,* 2004; Pandy *et al*., 2005).

### 4.6.4. Biochemical characteristics

Biochemical characteristics of the isolates were studied. These include; gelatin hydrolysis, starch hydrolysis and esculin degradation.

### 4.6.4.1. Starch hydrolysis

Starch hydrolysis was done using Starch Agar plates having a composition of (soluble starch, 20; beef extract, 3; Peptone, 5; Agar 15 and distilled water 1L). The isolates were taken from 7 days old culture and streaked on the media and incubated, at 30$^{o}$C for 7 days together with un inoculated plates that serve as a control. The iodine solution was flooded on to the plates to see for the clear zone of hydrolysis around the colony (Aneja, 2005; Remya and Vijayakumar, 2008).

### 4.6.4.2. Gelatin hydrolysis

This test was done on sterile Nutrient Gelatin media having a composition of (beef extract, 3; peptone, 5 and gelatin, 120 and distilled water 1L). Each isolates were taken from 7 days old culture and stabbed into nutrient gelatin tubes with sterile needle. The tubes were incubated for 10 days at 30$^{o}$C together with un inoculated tube that should be used as a control. After incubation, the tubes were placed in to Refrigerator, at 4$^{o}$C for 15 minutes (Aneja, 2005; Sundaramoorthi *et al.,* 2011). The refrigerated gelatin tubes were examined to see whether the medium was liquid for positive test or solid to confirm negative test.

### 4.6.4.3. Esculin degradation

Esculin degradation was determined after Kutzner (1976) as cited from Tiwary, (2009). The isolates were taken from seven days old culture and streaked into Esculin Agar slants having a composition of (yeast extract, 0.3; ferric ammonium citrate, 0.05, agar 0.75, 0.1% of esculin and distilled water 50 ml) and incubated , at 30$^{o}$C for 7days. Observations were made between the periods of incubation to check the blackening of the medium. Positive tests were confirmed comparing to the control which was dark brown of substrate.

## 5. DATA ANALYSIS

Data analysis were made by Statistical Package for Social Sciences (SPSS) windows version 17 in terms of the mean of the growth inhibition zone value obtained from each of the seven bacterial test pathogens and two fungal test pathogens. The data on temperature and pH tolerance were analyzed by comparing the mean growth in log colony forming unit through ANOVAs. The mean for each group were compared using Tukey test (HSD).

# 6. RESULTS

## 6.1. Cultures of actinomycetes isolated from different habitats

A total of 30 different actinomycete isolates were recovered from rhizosphere of different plants and soil samples collected from Debrezit and Holeta farm lands and garden soils of Arat Kilo campus. Most (67%) of the actinomycetes were isolated from rhizospher of plants, whereas (33%) of the isolate were recovered from farm soil using Starch Casein Agar medium and Actinomyctes Isolation Agar medium supplemented with Amphotracine B (50 μg/ml) ( Table 1).

Table1. Culture collection of soil actinomycetes isolates

| Codes of isolates | Source of soil samples | | sites | Total number of isolates |
|---|---|---|---|---|
| | Rhizosphere | soil | | |
| AAUBA1 | + | - | Debrezit | 14 |
| AAUBA2 | - | + | Debrezit | |
| AAUBA3 | + | - | Debrezit | |
| AAUBA4 | - | + | Debrezit | |
| AAUBA5 | + | - | Debrezit | |
| AAUBA6 | + | - | Debrezit | |
| AAUBA7 | - | + | Debrezit | |
| AAUBA8 | + | - | Debrezit | |
| AAUBA9 | + | - | Debrezit | |
| AAUBA10 | - | + | Debrezit | |
| AAUBA11 | + | - | Debrezit | |
| AAUBA12 | + | - | Debrezit | |
| AAUBA13 | + | - | Debrezit | |
| AAUBA14 | - | + | Debrezit | |
| AAUBA15 | - | + | Holeta | 7 |
| AAUBA16 | - | + | Holeta | |
| AAUBA17 | - | + | Holeta | |
| AAUBA18 | - | + | Holeta | |
| AAUBA19 | + | - | Holeta | |
| AAUBA20 | - | + | Holeta | |
| AAUBA21 | + | - | Holeta | |
| AAUBA22 | + | - | Arat killo | 9 |
| AAUBA23 | + | - | Arat killo | |
| AAUBA24 | + | - | Arat killo | |
| AAUBA26 | + | - | Arat killo | |
| AAUBA27 | + | - | Arat killo | |
| AAUBA28 | + | - | Arat killo | |
| AAUBA29 | + | - | Arat killo | |
| AAUBA30 | + | - | Arat kllo | |
| AAUBA31 | + | - | Arat killo | |
| Total | 20 (67%) | 10 (33%) | | 30 |

## 6.2. Primary screening of the antimicrobial producing isolates

Out of the thirty isolates subjected for primary screening, 18(60%) crude extract showed varying levels of antimicrobial activities against the five test microorganisms (Table 2).

Table 2:- Primary screening of antimicrobial activity of crude extract actinomycetes isolates

| Isolates | Test microorganisms | | | | | Spectrum activity |
|---|---|---|---|---|---|---|
| | *S.aureus* ATCC 25923 | *E.coli* ATCC 25922 | *Pseudomonas aeruginosa* ATCC 25853 | *Shigella boydii* | *S.aureus* | |
| AAUBA1* | + | - | - | + | + | 3 |
| AAUBA3 | - | - | + | - | - | 1 |
| AAUBA5* | + | - | + | + | + | 4 |
| AAUBA6* | + | - | + | - | + | 3 |
| AAUBA8* | - | + | + | - | + | 3 |
| AAUBA9 | - | - | - | - | + | 1 |
| AAUBA10* | - | + | + | + | - | 3 |
| AAUBA11 | - | - | - | - | + | 1 |
| AAUBA12 | - | - | + | - | - | 1 |
| AAUBA13* | + | - | + | - | + | 3 |
| AAUBA14 | - | - | + | + | - | 2 |
| AAUBA19* | + | - | + | + | - | 3 |
| AAUBA21 | - | + | + | - | - | 2 |
| AAUBA22 | - | - | + | - | + | 2 |
| AAUBA26 | - | - | + | - | + | 2 |
| AAUBA28 | + | - | - | - | + | 2 |
| AAUBA30* | + | - | + | + | + | 4 |
| AAUBA31* | + | - | - | + | + | 3 |

Legend: + = active against test organism; - = inactive against test organism, *= Show broad spectrum activity

Out of the different actinomycetes isolates of crude extract screened for antibiosis, crude extract of two isolates (11%), AAUBA5 and AAUBA30, showed a wide spectrum of antibiosis against 4 test organisms except *E. coli* ATCC 25922. Likewise, the crude extracts of 7 (39%), (5) 28% and (4) 22% of isolates were found to inhibit any 3, 2, and 1 of the test organisms, respectively. The crude extracts of isolates that inhibited 3 test organisms were AAUBA1, AAUBA6, AAUBA8,

AAUBA10, AAUBA13, AAUBA19 and AAUBA31. Crude extracts of AAUBA14, AAUBA21, AAUBA22, AAUBA26 and AAUBA28, were found to inhibit 2 test organisms, and crude extracts of the rest isolate showed to inhibit 1 test organisms (Table 2).

The crude extracts of isolates also showed variations, in inhibiting the test organisms. Consequently, most of the crude extract of isolates 13(72%) inhibited *Pseudomonas aeruginosa* ATCC 25853, followed by crude extract of 12 isolates (67%), and crude extract of 8 isolates (44%) that showed inhibition on *S.aureus* (clinical isolate) and *S.aureus* ATCC25923, respectively. Likewise, crude extract of 7 isolates (39%) showed a pattern of suppression on *Shigella boydii* (clinical isolate). The most resistant test isolate was *E.coli* ATCC 25922 that was inhibited by only crude extract of 3 isolates (17%) namely; AAUBA8, AAUBA10 and AAUBA21 (Table 2).

Many crude extract of isolates (39%) also showed a wide pattern of dual inhibition on *Pseudomonas aeruginosa* ATCC 25853 and *S. aureus* (clinical isolate), and *S. aureus* ATCC25923 and *S. aureus* (clinical isolate); with a few crude extract of isolates (17%) that showed a dual inhibition capacity involving *E. coil* ATCC 25922. Most crude extract of isolates (28%) showed the capacity of multiple inhibitors of *S. aureus* ATCC25923 and *Pseudomonas aeruginosa* ATCC 25853), and *S. aureus* ATCC25923 and *Shigella boydii* (clinical isolate). Some crude extract of isolates (17%) had the capacity of multiple inhibition of *Pseudomonas aeruginosa* ATCC 25853 and *Shigella boydii* (clinical isolate), whereas with no crude extract of isolate that had a dual inhibition on *E. coli* ATCC 25922 except crude extract of isolate AAUBA8, AAUBA10 and AAUBA21 (Table 2).

From the products of nine isolates that showed a wide spectrum of activity, crude extract of 1 isolate (AAUBA10) was active against only gram negative microorganism and crude extract of 8 isolates (AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA13, AAUBA19, AAUBA30 and AAUBA31) were against both gram positive and gram negative microorganisms. Among the crude extract of 9 isolates, crude extract of 7 isolates (AAUBA5, AAUBA6, AAUBA8,AAUBA10,AAUBA13, AAUBA19, and AAUBA30) were active against *Pseudomonas aeruginosa* ATCC 25853, crude extract of 7 of the isolates (AAUBA1, AAUBA5, AAUBA6, AAUBA13, AAUBA19, AAUBA30 and AAUBA31) were active against

*Staphylococcusaureus*ATCC25923,andcrudeextractof7isolates(AAUBA1,AAUBA5,AAUBA6, AAUBA8,AAUBA10,AAUBA13,AAUBA30andAAUBA31)were active against *Staphylococcus aureus* ( clinical isolate), crude extract of 6 isolates (AAUBA1, AAUBA5, AAUBA10, AAUBA19, AAUBA30, and AAUBA31) were against *Shigella boydii* (clinical isolate) and crude extract of 2 isolates (AAUBA8 and AAUBA10) against *Escherichia coli* ATCC 25922 ( Table 2).

## 6.3. Secondary screening of selected actinomycetes crude extract by Disc Diffusion Assay

Based on the results of primary screening, 9 selectively effective actinomycetes isolates were selected for fermentation. The effective actinomycete isolates included AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19, AAUBA30 and AAUBA31.

The results of ethyl acetate crude extracts of the actinomycetes for antimicrobial activities are presented in Table 3. A total of 9 actinomycetes crude extracts were screened against 9 test microorganisms. These crude extracts showed activities against a minimum of 1 test organism and maximum of 8.

Out of the nine actinomycetes crude extract screened for antibiosis, two isolates crude extract of AAUBA5 and AAUBA13 showed activity against 8 test microorganisms with zone of inhibition ranging from 9mm to 22 mm except *Cryptococcus neoformance* (clinical isolate), whereas AAUBA6 crude extract showed activity against 7 test microorganisms with zones of inhibition ranging from 2mm to 19mm except *Candida albicanis* ATCC 62376 and *Cryptococcus neoformance* (clinical isolate) (Table 3). Likewise, crude extract of isolate AAUBA30, AAUBA8, AAUBA1, AAUBA10, AAUBA19 and AAUBA31 were showed activity against 6, 5, 4, 3, 1 and 1 test microorganisms, respectively with inhibition zones ranging from 3 to 15mm diameters (Table 3).

Isolate AAUBA30 crude extract was found to inhibit 6 test organisms except *Shigella boydii* (clinical isolate), *Candida albicans* ATCC62376 and *Cryptococcus neoformance* (clinical isolate). Isolate AAUBA8 crude extract inhibit 5 test organisms except *Pseudomonas aeruginosa*

ATCC25853, *S.typhi* ATCC 6539, *Shigella boydii* (clinical isolate), *C.albicans* ATCC62376 and *C. neoformance* (clinical isolates).

Table 3. Antimicrobial activities of fermentation products of the selected putative actinomycetes isolates

| Test microorganisms | Antimicrobial activity (zone of inhibition in mm) (Mean) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AAUB1 | AAUBA5 | AAUBA6 | AAUBA8 | AAUBA10 | AAUBA13 | AAUBA19 | AAUBA30 | AAUBA31 |
| *S.aureus* ATCC25923 | 11 | 9 | 9 | 14 | - | 19 | - | 14 | - |
| *E.coli* ATCC25922 | - | 13 | 11 | 10 | - | 20 | - | 8 | - |
| *P. aeruginosa* ATCC25853 | - | 18 | 8 | - | - | 18 | - | 10 | - |
| *S.pneumoniae*ATCC49 619 | 12 | 20 | 11 | 7 | 10 | 20 | - | 10 | - |
| *S.typhi* ATCC 6539 | 10 | 10 | 2 | - | 10 | 17 | - | 15 | - |
| *S.aureus* clinical isolate | 12 | 13 | 12 | 9 | 11 | 22 | 10 | 8 | 7 |
| *Shigella boydii* clinical isolate | - | 16 | 19 | 3 | - | 19 | - | - | - |
| *C.albicans* ATCC62376 | - | 17 | - | - | - | 17 | - | - | - |
| *C. neoformance* clinical isolates | - | - | - | - | - | - | - | - | - |

Legend: - , No inhibition zone

Isolate AAUBA1 crude extract was found to inhibit 4 test organisms except *E.coli* ATCC25922, *Pseudomonas aeruginosa* ATCC25853, *Shigella boydii* (clinical isolate), *C.albicans* ATCC62376 and *C.neoformance* (clinical isolates). Isolate AAUBA10 crude extract inhibit *S. pneumoniae* ATCC49619, *S.typhi* ATCC and *S.aureus* (clinical isolate). Isolate AAUBA19 and AAUBA31 crude extract have the capacity to inhibit only *S.aureus* (clinical isolate) (Table 3).

The crude extract of isolates showed variations in spectrum against test microorganisms. Consequently, 9 isolates (AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19, AAUBA30 and AAUBA31) crude extracts were found to inhibit gram positive bacteria *S.aureus* (clinical isolate), followed by crude extract of 7 isolates (AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13 and AAUBA30) and crude extract of 6 isolates

(AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA13 and AAUBA30) showed inhibition spectrum against gram positive S. *pneumoniae* ATCC49619 and *S.aureus* ATCC25923, respectively. Likewise, 6 isolates of crude extract (AAUBA1, AAUBA5, AAUBA6, AAUBA10, AAUBA13 and AAUBA30) showed a pattern of suppression on gram negative *S.typhi* ATCC 6539, 5 isolates of crude extract (AAUBA5, AAUBA6, AAUBA8, AAUBA13 and AAUBA30) and 4 isolates of crude extract (AAUBA5, AAUBA6, AAUBA13 and AAUBA30) showed inhibition on gram negative *E.coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC25853, respectively, 4 isolates of crude extract (AAUBA5, AAUBA6, AAUBA8 and AAUBA13) showed inhibition on gram negative *Shigella boydii* (clinical isolate). Two isolates of crude extract (AAUBA5 and AAUBA13) was found to inhibit *C.albicans* ATCC62376 (Table 3).

In the antimicrobial bioassay screening process (Table 3), larger inhibition zone diameter was observed by crude extract of isolate AAUBA13 (22mm) against *S.aureus* (clinical isolate) followed by *S. pneumoniae* ATCC49619 and *E.coli* ATCC25922 (20mm) and the least was shown by crude extract of isolate AAUBA6 (2mm) against *S.typhi* ATCC 6539. The resistance test isolates were *S.aureus* ATCC25923, *E.coli* ATCC25922, *Pseudomonas aeruginosa* ATCC25853, *S. pneumoniae* ATCC49619, *S.typhi* ATCC 6539 and *Shigella boydii* (clinical isolate) that were resistant to one of the antagonistic isolate. The most sensitive one were *S.aureus* (clinical isolate) that were inhibited by all isolates crude extract, they vary in inhibition zone diameter. However, the most resistant test isolate was *C. neoformance* (clinical isolates) against all antagonistic isolates, followed by *C.albicans* ATCC62376 except against AAUBA5 and AAUBA13 crude extract (Table 3).

Accordingly, crude extract of isolates AAUBA5 and AAUBA13 showed a wider spectrum of antimicrobial activities, AAUBA13 crude extract was found to show larger zone of inhibition when compared to the rest of isolates crude extract (Table 3).

The antimicrobial activity of the crude extract of isolate AAUBA13 against test pathogenic bacteria showed effective against all with variation in inhibition zone diameter ranging from ( 17mm to 22 mm) , among gram positives bacteria, *Staphylococcus aureus* clinical isolate found to be more sensitive followed by *S. pneumonia* ATCC49619 and *S.aureus* ATCC25923 in decreasing order. In case of gram negatives bacteria, *E.coli* ATCC25922, *Shigella boydii*

(clinical isolate), *Pseudomonas aeruginosa* ATCC25853, and *S.typhi* ATCC in decreasing order. However, no activity was observed against the test fungi *Cryptococcus neoformance* (clinical isolate) (Table 3).

## 6.4. Detection and purification of the active compound from AAUBA13

### 6.4.1. Thin layer chromatography and Column chromatography

The crude extract of AAUBA13 was run on a TLC plate and a single band (spot) with blue color was detected at Rf value 0.73. The column chromatography experiment showed 13 different fractions. This was already indicated by the color of the bands on the silica gel plate. Fractions were tested for antimicrobial activity. Accordingly, fractions 8 was identified as best active fraction and concentrated to be further characterized as given in Table 5.

### 6.4.2. Preparative Thin layer chromatography

Fraction 8 was further purified using preparative chromatography technique resulted in single visible band when visualized with UV light and up on scratching of these bands out from the glass plate a pure compound was obtained.

The antimicrobial form AAUBA13 showed Rf value 0.73 on chloroform: methanol (1:9), 0.48 ethanol: petroleum ether (1:1) solvent systems and 0.57 Chloroform: ethyl acetate (1:1) solvent system (Table 4).

Table 4. Rf values of the pure active fraction 8 in 3 solvent systems

| Solvent system | Rf values |
|---|---|
| Chloroform: methanol 1:9 | 0.73 |
| Chloroform: ethyl acetate 1:1 | 0.57 |
| Ethanol: petroleum ether 1:1 | 0.48 |

### 6.4.3. In vitro antimicrobial activities of the fractions

From the 13 fractions, only 3 fractions were found to be active against all tested microorganism (Table 5).

Table 5. Inhibition zone of the antimicrobial activity of fraction 4, 8 and 10

| Test microorganisms | Zone of inhibition in mm (Mean) | | |
|---|---|---|---|
| | F4 | F8 | F10 |
| *S.aureus* ATCC25923 | 6 | 7 | 5 |
| *E.coli* ATCC25922 | 4 | 8 | 4 |
| *P. aeruginosa* ATCC25853 | 6 | 5 | 0 |
| *S. pneumoniae*ATCC49619 | 13 | 14 | 13 |
| *S.typhi* ATCC 6539 | 15 | 16 | 12 |
| *S.aureus* clinical isolate | 11 | 24 | 5 |
| *Shigella boydii* clinical isolates | 12 | 8 | 10 |
| *C.albicans* ATCC62376 | 0 | 11 | 0 |
| *C. neoformance* clinical isolates | 0 | 0 | 0 |

Accordingly, fraction F8 displayed large inhibition zones than that of the fractions F4 and F10. The highest antimicrobial inhibition activity was observed against *Staphylococcus aureus* clinical isolate with a clear zone diameter of 24mm and the lowest was observed against *P.aeruginosa* ATCC25853 with the diameter of 5 mm. Fraction 4 showed activity against all test bacteria. Fraction F10 showed less activity against all test pathogens and this fraction has no activity against *P. aeruginosa* ATCC25853 when compared to fraction F4 and F8. Only F8 have activity against *C.albicans* ATCC 62376. However, all fractions did not show antifungal activity against *C. neoformance* (clinical isolates).

## 6. 5. Characterization and identification of selected isolates

### 6.5.1. Morphological characterization

Among the isolates, AAUBA5 and AAUBA10 formed a hook (retinaculiaperti) like structure, where as isolates AAUBA1, AAUBA6, AAUBA8, AAUBA13, AAUBA30 and AAUBA31 showed (rectiflexible) type of spore chains ( Table 6) with smooth spore chains of 3 or more and showed branched mycelium from the cover slip culture .

Table 6: Microscopic observation of selected isolates

| Characteristics | Isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AAUBA1 | AAUBA5 | AAUBA6 | AAUBA8 | AAUBA10 | AAUBA13 | AAUBA19 | AAUBA30 | AAUBA31 |
| **Spore chains** | RC | RT | RC | RC | RT | RC | RC | RC | RC |

Legend: RC= Rectiflexible, RT= Retinaculiaperti

## 6.5.2. Cultural characterization

The isolates were found to grow in all media with some variations. However, abundant growth of the isolates was observed on Starch Casein Agar medium, Moderate growth was observed on Actinomycetes Isolation Agar and good growth was observed on Glycerol Asparagine Agar medium. Diffusible pigment was observed only on isolate AAUBA6 on Starch Casein Agar medium and Glycerol Asparagine Agar medium. The isolates were characterized by different colony diameter on Starch Casein Agar medium at pH 7 (Table 7).

The color of the aerial and substrate mycelia varied depending on the type of the media used. Each isolates has different color series of aerial mycelium ranging from white, whitish brown, pink, grey, dark grey, yellowish white and dark brown. The color of substrate mycelium were also recorded (Table 7).

Table 7: - Cultural characteristics of isolates grown on different media

| Medium | Test isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AAUBA1 | AAUBA5 | AAUBA6 | AAUBA8 | AAUBA10 | AAUBA13 | AAUBA19 | AAUBA30 | AAUBA31 |
| **SCA** | | | | | | | | | |
| Aerial mycelium | White | Whitish brown | Pink | Grey | Brown | White | Dark grey | Yellowish white | Dark brown |
| Substrate mycelium | White | Brown | Reddish brown | Brownish grey | Brown | Light yellow | Dark grey | Yellow | Dark brown |
| Diffusible pigment | None | None | Red | None | None | None | None | None | None |
| Growth | + + + | + + + | + + + | + + + | + + + | + + + | + + + | + + + | + + + |
| Colony diameter ( mean Value) | 0.8mm | 1mm | 0.9mm | 3mm | 5mm | 5mm | 4mm | 6mm | 2mm |
| **SNA** | | | | | | | | | |
| Aerial mycelium | White | Whitish brown | Pink | Grey | Brown | White | Dark grey | Yellowish White | Dark brown |
| Substrate mycelium | White | Brown | Reddish brown | Brownish grey | Brown | Light yellow | Grey | Yellow | Dark brown |
| Diffusible pigment | None | None | None | None | None | None | None | None | None |
| Growth | + + | + + | + | + | + + | + + | + | + + | + + |
| **GAsA** | | | | | | | | | |
| Aerial mycelium | White | Brown | Pink | Grey | Brown | White | Dark grey | Yellowish white | Dark brown |
| Substrate mycelium | White | Brown | Pink | Dark grey | Brownish | Light yellow | Dark grey | Yellow | Brown |
| Diffusible pigment | None | None | None | None | None | None | None | None | None |
| Growth | + | + | + + | + | + | + | + + | + + | + |
| **GlyAsA** | | | | | | | | | |
| Aerial mycelium | White | brown | Pink | Pale grey | Brown | White | Dark | Yellow | Brown |
| Substrate mycelium | Yellow | Brown | Brown | Grey | Brownish | Light yellow | Dark grey | Yellow | Brown |
| Diffusible pigment | None | None | Red | None | None | None | None | None | None |
| Growth | + | + | + | + | + | + | + | + | + |
| AIA | | | | | | | | | |
| Aerial mycelium | White | Whitish brown | Pink | Grey | Brown | White | Dark grey | Yellowish white | Dark brown |
| Substrate mycelium | White | Brown | Reddish brown | Brownish grey | Brown | Light yellow | Dark grey | Yellow | Dark brown |
| Diffusible pigment | None | None | Red None | None | None | None | None | None | None |
| Growth | ++ | ++ | ++ | ++ | ++ | ++ | None | ++ | ++ |

Legend: - += good growth, + + = Moderate growth, + + += Abundant growth, SCA= Starch Casein Agar, SNA= Starch Nitrate Agar, GAsA= Glucose Asparagine Agar and GlyAsA= Glycerol Asparagine Agar

### 6.5.3. Physiological characteristics

#### 6.5.3.1. PH and Temperature tolerance

The isolates were found to grow well relatively in wider range of pH 5 to 12 in which maximum growth were observed at pH 7 and for isolate AAUBA1 and AAUBA13 pH 8. Growth tends to significantly decrease at the pH levels increase and decreases (Table 8). The isolates did grow at temperatures 15 to 37 $^{o}$C and 30$^{o}$C was found to be optimum temperature, in which number of colony and colony diameter was higher than the rest of temperature ranges. However, all isolates did not grow at temperature of 45$^{o}$C (Table 9).

Table 8. pH tolerance of selected isolates

| Growth with Log cfu | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| pH | AAUBA1 | AAUBA5 | AAUBA6 | AAUBA8 | AAUB10 | AAUBA13 | AAUBA19 | AAUBA30 | AAUBA31 |
| 5 | 4.32±0.005$^{f}$ | 5.26±0.005$^{f}$ | 4.95±0.000$^{e}$ | 4.97±0.010$^{f}$ | 5.53±0.005$^{e}$ | 5.78±0.005$^{f}$ | 4.02±0.005$^{g}$ | 6.22±0.005$^{e}$ | 5.24±0.005$^{ef}$ |
| 6 | 4.97±0.005$^{d}$ | 5.75±0.005$^{c}$ | 4.98±0.015$^{e}$ | 4.99±0.005$^{f}$ | 5.67±0.005$^{d}$ | 5.84±0.005$^{e}$ | 5.11±0.005$^{e}$ | 6.68±0.005$^{cd}$ | 5.55±0.005$^{d}$ |
| 7 | 5.23±0.005$^{ab}$ | 5.88±0.010$^{a}$ | 5.57±0.005$^{a}$ | 6.12±0.005$^{a}$ | 6.71±0.005$^{a}$ | 6.24±0.005$^{b}$ | 5.55±0.005$^{a}$ | 7.00±0.005$^{a}$ | 5.99±0.005$^{a}$ |
| 8 | 5.31±0.005$^{a}$ | 5.80±0.010$^{b}$ | 5.23±0.005$^{b}$ | 5.95±0.005$^{b}$ | 6.29±0.005$^{b}$ | 6.79±0.005$^{a}$ | 5.48±0.005$^{b}$ | 6.86±0.005$^{b}$ | 5.86±0.005$^{b}$ |
| 9 | 5.24±0.005$^{ab}$ | 5.72±0.005$^{c}$ | 5.15±0.005$^{c}$ | 5.67±0.005$^{c}$ | 5.78±0.005$^{c}$ | 5.99±0.005$^{c}$ | 5.37±0.005$^{c}$ | 6.79±0.005$^{c}$ | 5.77±0.005$^{c}$ |
| 10 | 5.16±0.005$^{c}$ | 5.68±0.005$^{d}$ | 5.02±0.005$^{d}$ | 5.58±0.005$^{d}$ | 5.49±0.005$^{f}$ | 5.89±0.005$^{d}$ | 5.32±0.005$^{d}$ | 6.69±0.005$^{cd}$ | 5.18±0.005$^{ef}$ |
| 11 | 5.05±0.055$^{cd}$ | 5.59±0.005$^{e}$ | 4.54±0.005$^{f}$ | 5.34±0.005$^{e}$ | 5.29±0.005$^{g}$ | 5.04±0.005$^{g}$ | 5.12±0.005$^{e}$ | 5.87±0.495$^{f}$ | 4.87±0.005$^{g}$ |
| 12 | 4.70±0.005$^{e}$ | 5.27±0.005$^{f}$ | 4.37±0.005$^{g}$ | 4.97±0.005$^{f}$ | 4.83±0.005$^{h}$ | 4.85±0.005$^{h}$ | 5.07±0.005$^{f}$ | 5.13±0.005$^{g}$ | 4.36±0.005$^{h}$ |

Means within the column under a pH parameter, having a common letter do not differ significantly ($p \leq 0.05$).

Table 9. Temperature tolerance of selected isolates

| Growth with Log cfu | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Temperature ($T^{o}C$) | AAUBA1 | AAUBA5 | AAUBA6 | AAUBA8 | AAUBA10 | AAUBA13 | AAUBA19 | AAUBA30 | AAUBA31 |
| 15 | 5.43±0.025$^{b}$ | 5.25±0.000$^{c}$ | 4.96± 0.005$^{d}$ | 5.38±0.005$^{b}$ | 4.92±0.005$^{d}$ | 5.30±0.005$^{d}$ | 5.16±0.005$^{c}$ | 5.28±0.005$^{d}$ | 4.99±0.005$^{d}$ |
| 25 | 5.49 ±0.005$^{b}$ | 5.42±0.005$^{b}$ | 5.31±0.005$^{b}$ | 5.39±0.005$^{b}$ | 5.21±0.005$^{c}$ | 5.48±0.005$^{c}$ | 5.25±0.005$^{b}$ | 5.39±0.005$^{c}$ | 5.60±0.005$^{c}$ |
| 30 | 5.59±0.005$^{a}$ | 5.57±0.005$^{a}$ | 5.53±0.005$^{a}$ | 5.49±0.005$^{a}$ | 5.48±0.005$^{a}$ | 5.84±0.005$^{a}$ | 5.66±0.005$^{a}$ | 5.67±0.005$^{a}$ | 5.92±0.005$^{a}$ |
| 37 | 5.33 ±0.005$^{c}$ | 5.41±0.005$^{b}$ | 5.23±0.005$^{c}$ | 5.32±0.010$^{c}$ | 5.26±0.005$^{b}$ | 5.57±0.005$^{b}$ | 5.27±0.005$^{b}$ | 5.45±0.005$^{b}$ | 5.79±0.005$^{b}$ |

Means within the column under a temperature parameter, having a common letter do not differ significantly ($p \leq 0.05$).

### 6.5.3.2. Growth with NaCl

All isolates were grown at a concentration of salt supplemented with 5% and 7% except isolate AAUBA8 and AAUBA19 (Table 10). However, all isolates did not grow at a concentration of salt supplemented on 10% sodium chloride. AAUBA31 did not grow at a concentration salt supplemented with 7% of sodium chloride (Table 10).

### 6.5.3.3. Utilization of Carbon and nitrogen sources

The isolates found to grow on a wider range of carbon and nitrogen sources used with little variations as shown below in (Table 10) this showed that the isolate can utilize different carbon and nitrogen source.

Table 10. Utilization of Carbon and Nitrogen source and NaCl tolerance of selected isolates

| **Utilization of Carbon sources** | **Isolates** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **AAUBA1** | **AAUBA5** | **AAUBA6** | **AAUBA8** | **AAUBA10** | **AAUBA13** | **AAUBA19** | **AAUBA30** | **AAUBA31** |
| **D-Glucose** | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| **D-Galactose** | ++ | ++ | ++ | + | + | ++ | - | ++ | + |
| **D-Fructose** | + | ++ | + | - | - | + | + | ++ | - |
| **D-Mannose** | - | + | - | - | + | ++ | - | - | + |
| **D-Mannitol** | + | - | - | - | + | - | ++ | + | + |
| **L-Arabinose** | + | + | + | ++ | - | - | + | - | - |
| **Cellobiose** | - | - | + | + | ++ | + | - | + | ++ |
| **Sucrose** | + | - | ++ | - | - | + | - | + | - |
| **Utilization of Nitrogen sources** | | | | | | | | | |
| **L-Arganine** | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| **$(NH_4)H_2PO_4$** | + | - | ++ | - | - | + | - | ++ | + |
| **$(NH_4)_2SO_4$** | - | - | - | + | + | - | + | - | - |
| **Peptone** | - | + | + | ++ | + | ++ | - | - | + |
| **Yeast extract** | + | + | ++ | + | - | + | ++ | + | - |
| **Growth with NaCl** | | | | | | | | | |
| **5%** | + | + | + | - | + | + | - | + | + |
| **7%** | + | + | + | - | + | + | - | + | - |
| **10%** | - | - | - | - | - | - | - | - | - |

**Legend**: **Utilization of Carbon and Nitrogen sources**, + + = **Abundant** (When growth on tested Carbon or Nitrogen in basal medium greater than growth on basal medium), ++ = **Moderate** (When growth on tested Carbon or Nitrogen in basal medium is significantly better than growth on basal medium) and - = **Good (**When growth on tested Carbon or Nitrogen in basal medium is less than growth on basal medium). **Growth with NaCl, - =** No growth and + = Growth.

Consequently, all of the isolates showed profuse utilize D- Glucose and L- Arganine as a Carbon and Nitrogen source, respectively, followed by D- Galactose, D- Fructose, Cellobiose, D-Mannitol and L- Arabinose, and D- Mannose and Sucrose as a source of carbon in decreasing order; Yeast extract, Peptone, $(NH_4)$ $H_2PO_4$ and $(NH_4)_2SO_4$ as a source of Nitrogen in decreasing order.

Isolate AAUBA6 was found to utilize best both Carbon and Nitrogen source used, followed by isolate AAUBA13. The least utilization showed on isolates AAUBA10 and AAUBA31. Isolate AAUBA6, AAUBA13 and AAUBA30 were best in the utilization of the carbon source used (Table 10 ).

### 6.3.4. Biochemical test

All of the isolates showed clear zone when flooded with iodine solution indicating that they are capable of hydrolyze starch; they were also found to hydrolyze gelatin when chilling with Refrigerator before examination remain liquid. All isolates showed a dark brown to black complex on esculin medium which are a positive reaction to esculin degradation.

# 7. DISCUSSIONS

In this study, 30 actinomycete isolates were collected from rhizosphere of different plants and soil from three different sampling sites. Sixty seven (67%) actinomycete isolates were recovered from rhizosphere soil. Rouatt *et al*. (1951) also reported that greater percentages of actinomycetes are found in the rhizosphere soils which are under the influence of the exudates by plants.

Eighteen (18) out of the 30 isolates (60 %) showed antibiotic activity against the test organisms up on primary screening of which the 9 isolates were selected based up on spectrum activity against test organisms and this potent isolates were isolated from the rhizosphere soils. The present result in agreement with that of Abo-Shadi *et al.* (2010) reported that microorganisms isolated from rhizosphere soil could be an interesting source of antimicrobial bioactive substance. Ramakrishnan *et al.* (2009) also reported that rhizosphere soil can serve as an effective source of antimicrobial compounds. This ratio is much higher than the 22% and 34% reported by Abo-Shadi *et al* (2010) and Oskey *et al.* (2004), respectively. This difference may be attributed to the differences in inhibiting antibiotic resistant of test organisms and the genetic differences of the antibiotic producing isolates, and their capacity to produce more than one secondary metabolites.

In the identification of 9 actinomycetes isolates, the microscopic examination emphasized that the spore chain with rectiflexible and retinaculiaperti with smooth spore surfaces were typical characteristics of genus *Streptomycetes* (Williams *et al*., 1989). The cultural (Macroscopic) characteristics of aerial and substrate mycelium of isolates were ranging from white, grey, blue, yellow, brownish, pink and dark grey. No diffusible pigment was produced except in AAUBA6 isolate red diffusible pigment was observed. These results reveal that the actinomycetes isolates were related to the genus *Streptomyces* (Cross, 1989; Lechevalier, 1989; Locci, 1989). The biochemical tests and physiological properties of local isolates, as well as its carbon and nitrogen source utilization characteristics can be also compared to those of the actinomycetes described in Bergey's Manual of Determinative Bacteriology, showing these isolates found to be classified under genus *Streptomyces* (Lechevalier et *al*., 1989; Williams *et al*., 1989).

Cultural characteristics of the 9 isolates varied on the type of the media used most likely due to the nutritional versatility of the isolates. Concerning utilization of a variety of carbon sources by

this actinomycete isolates will help in adaptation to a variety of inoculation sites and wide soil types (Yadav *et al*. 2009). Concerning Utilization of a variety of Nitrogen source the isolates produce abundant aerial mycelium on organic nitrogen source medium and good growth was observed on inorganic nitrogen source this was also in agreement with the report by Petrova and Vlahov, (2007).

Investigation of the 9 antibiotic producing isolates through primary and secondary screening revealed different results. During primary screening , isolate AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19 and AAUBA30 (Table 2) crude extracts were found to inhibit *P. aeruginosa* ATCC 25853 that the crude extract of fermentation product could not inhibit ABA8, AAUBA10 and AAUBA19 ( Table 3). During secondary screening, the fermentation products of isolate AAUBA5, AAUBA6, AAUBA8, AAUBA13 and AAUBA30 were found to inhibit *E.coli* ATCC 25922 that primary screening could not inhibit AAUBA5, AAUBA6, AAUBA13 and AAUBA30 crude extract (Table 2). This difference might be due to the difference in the morphology of actinomycetes when grown in solid and liquid media as filamentous mycelia and fragmenting mycelia respectively ; present results in agreement with those obtained by Pandey *et al*. (2004 ) who reported that some of the active crude extract from primary screening didn't show the activity in the secondary screening while some showed little activity and some showed improved activity. Bushell , (1993) also reported that during the screening of the novel secondary metabolite, actinomycetes isolates are failed in their antibiotic activity in liquid culture.

Up on the result of primary and secondary screening methods it seems that from the 9 effective isolates , more isolates crude extract were active against Gram positive bacteria than Gram negative bacteria (Table 2 and 3). This might be due to the morphological differences on cell wall components between those two types of microorganisms. Gram negative bacteria have an outer lipopolysaccharide membrane, besides peptidoglican (Pandey *et al*., 2002); hence their cell wall is impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes (Nokaido and Vaara, 1985) to antimicrobial compound rather than positive bacteria.

From the result of antimicrobial bioassay method, the crude extract showed different diameter zone of inhibition, larger inhibition zone diameter observed by isolate AAUBA13 (22mm) crude

extract against *S.aureus* (clinical isolate) and the least was shown by isolate AAUBA6 (2mm) crude extract against *S. typhi* ATCC 6539. The differences in the ability to produce the clear zone were presumably dependent on the secondary metabolites that were produced by test isolates. This assumption was supported by Dharmawan *et al*., (2009) that stated the variation of clear zone diameter happen because every isolate produces different types of secondary metabolites. Different types of secondary metabolites have different chemical structure, compounds and also different in chemical concentration.

In this study, isolate AAUBA13 crude extract was found to be the best isolate from antimicrobial bioassay method shown by its broad spectrum activity with big zone of inhibition (22mm) than AAUBA5 crude extract and others; this isolate crude extract might produce more than one antimicrobial metabolites that made them effective inhibitor to gram positive and gram negative bacterial pathogen and also effective inhibitor to *Candida albicans* ATCC62376 fungal pathogen (Gurung *et al.,* 2009).

In this work, isolate AAUBA13 crude extract was further analyzed on thin layer chromatography and column chromatography. From the column chromatography different components of antimicrobial compound are obtained. Separation of the antimicrobial compound in to individual component with chromatography test confirms the crude extract was a mixture of different compound. The active fraction eluted at fraction 4, 8 and 10. This indicate the active components have polar and none polar nature. Fraction which move or eluted faster (F4), retained least on the adsorbed is most likely non polar and fraction that move slower (F10) retained more on the adsorbent silica gel is polar (Atta *et al*., 2009).

The antimicrobial activity of the purified fraction from isolate ABA13 crude extract showed variations in inhibition zone diameter from 5mm to 24mm against test microorganism. Maximum inhibition zone diameter for crude extract was 22mm against *Staphylococcus aurues* (clinical isolate) ( Table 3) , where as the purified fraction showed inhibition zone diameter of 24mm against *Staphylococcus aureus* clinical isolates (Table 5). This indicates, the activity of antimicrobial compound increases as the compound became pure this is agreed with the work of (Kavitha and Vijayalakshmi, 2007). However, like that of crude extracts, purified compound did

not show activity against *Cryptococcus neoformance*. Therefore, the antimicrobial compound obtained from AAUBA13 crude extract was not effective against *Cryptococcus neoformance*. On the other hand, the crude extract as well as purified compound obtained from AAUBA13 crude extract showed activity against *Candida albicans* ATCC62376.

The finding of this study showed that the antimicrobial compound obtained from AAUBA13 crude extract has an antibacterial activity and also an antifungal activity. Among the test, gram positive bacterial test pathogen, *Staphylococcus aureus* (clinical isolate) exhibited higher sensitivity followed by *Streptococcus pneumoniae* ATCC49619 and *Staphylococcus aureus* ATCC25923. In case of gram negative bacterial pathogen, *Salmonella typhi* ATCC6539 showed higher sensitivity followed by *Escherichia coli* ATCC25922, *Shigella boydii* (clinical isolate) and *Pseudomonas aeruginosa* ATCC25853 in decreasing order. Among fungal pathogen, *Candida albicans* ATCC62376 showed sensitivity. However, *Cryptococcus neoformance* (clinical isolate) showed resistant to the compound.

# 8. CONCLUSIONS AND RECOMMENDATIONS

## 8.1. CONCLUSIONS

The antimicrobial compound obtained from AAUBA13 demonstrates broad spectrum activity and a remarkable antimicrobial activity against bacteria and *Candida albicans* ATCC 62376.

In the antimicrobial bioassay testing of the crude extract as well as purified compound showed that the highest inhibition effect observed against gram positive bacteria.

The effective isolates of crude extract showed some difference in response to primary and secondary screening, there is a pattern of effectiveness with the fermentation product to inhibit the test microorganisms that could not during the preliminary screening.

Actinomycetes isolates recovered from rhizosphere samples showed the potential to produce antimicrobial bioactive compounds.

## 8.2. RECOMMENDATIONS

Based on the findings of this study the following recommendations are suggested:

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.

Further works have to be done on the purified antibiotic compound isolated from AAUBA13 showed significant and potent antibiotic activity. Efforts have to be establishing towards the structural elucidation and determine the physicochemical characteristics.

Further investigation should require for large scale production on potent antibiotic producing actinomycetes isolates.

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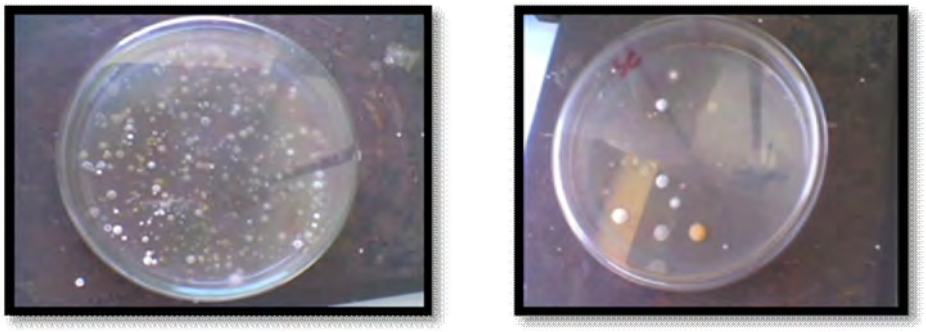
# 10. APPENDICES

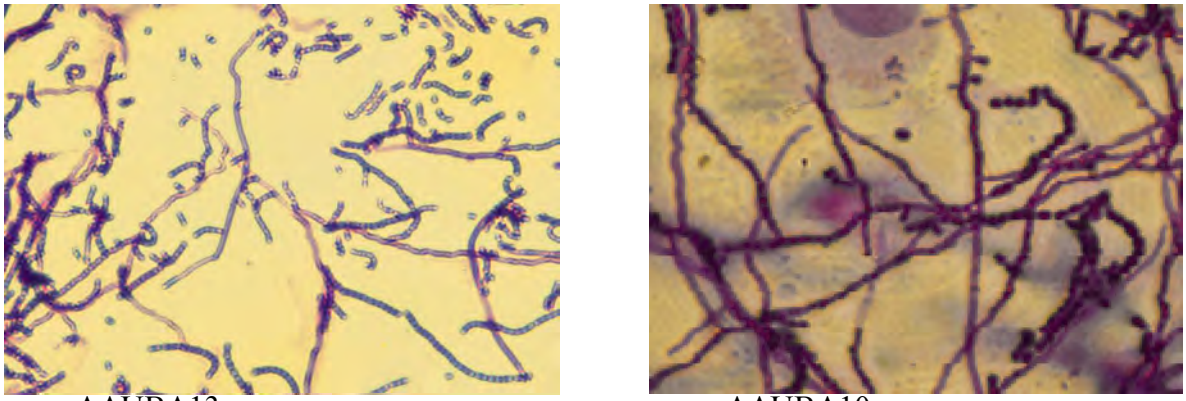


**Appendix 1.** Actinomycetes colonies on starch casein agar medium

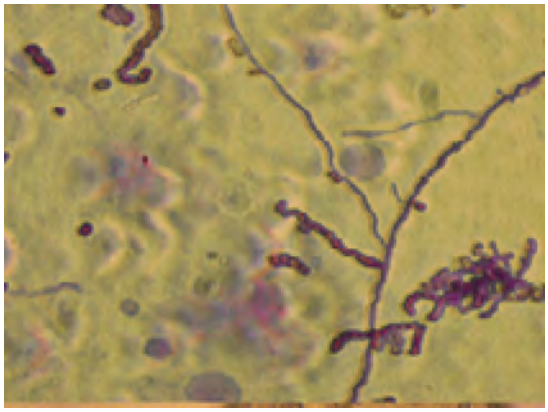
AAUBA13 AAUBA30 AAUBA21

**Appendix 2.** Primary screening of actinomycetes isolates

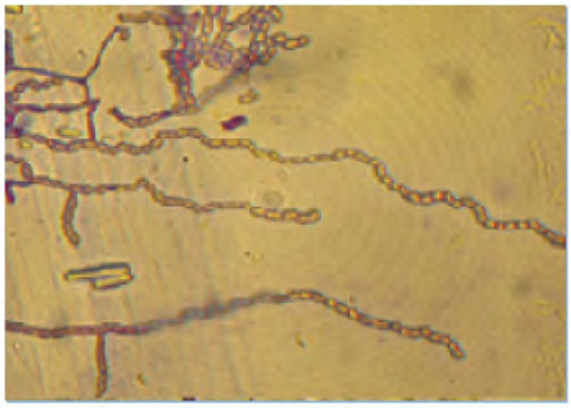
A. Slide culture for selected actinomycetes isolates



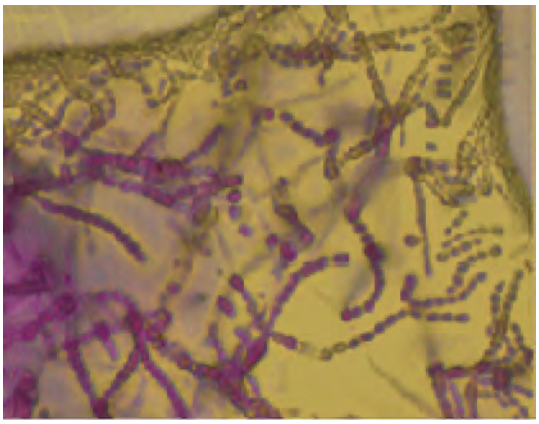
AAUBA13 AAUBA10



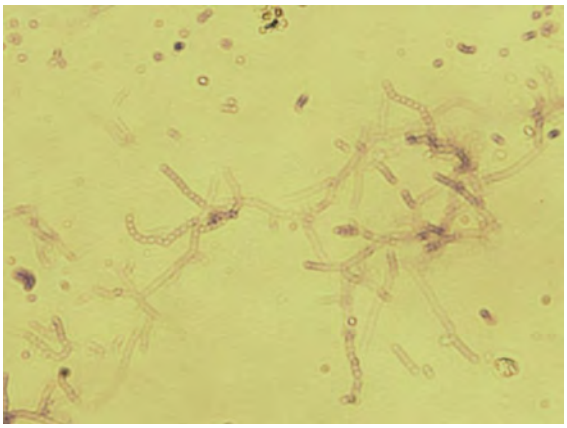
AAUBA5



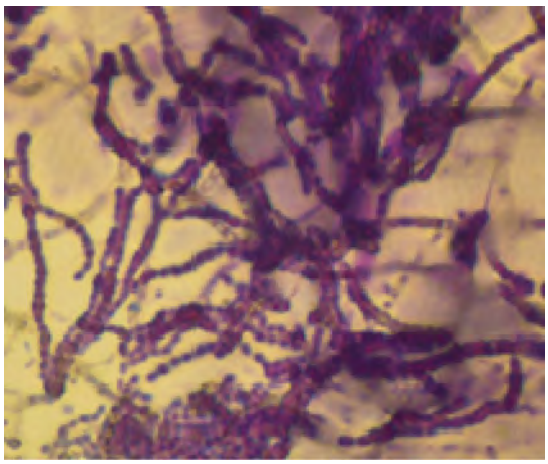
ABA30



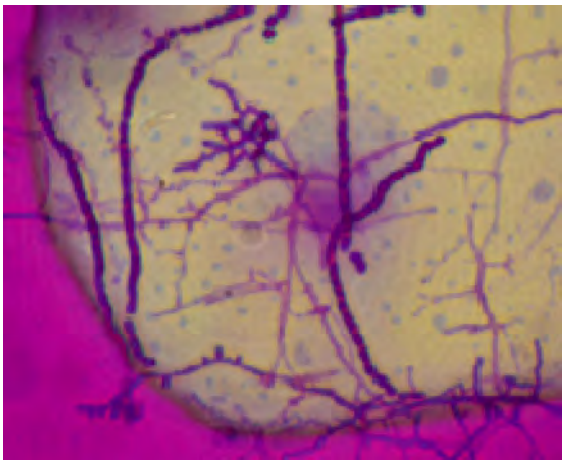
AAUBA8



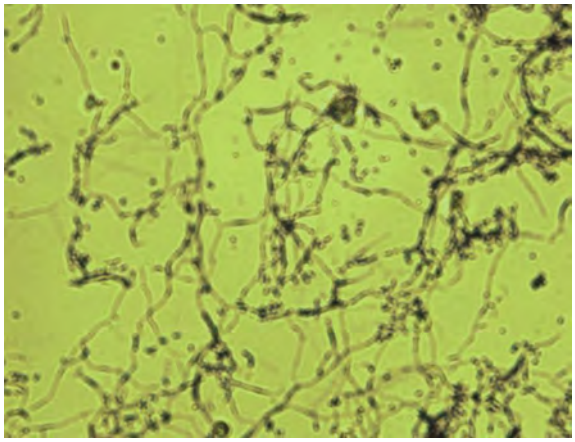
AAUBA1



AAUBA6



AAUBA31



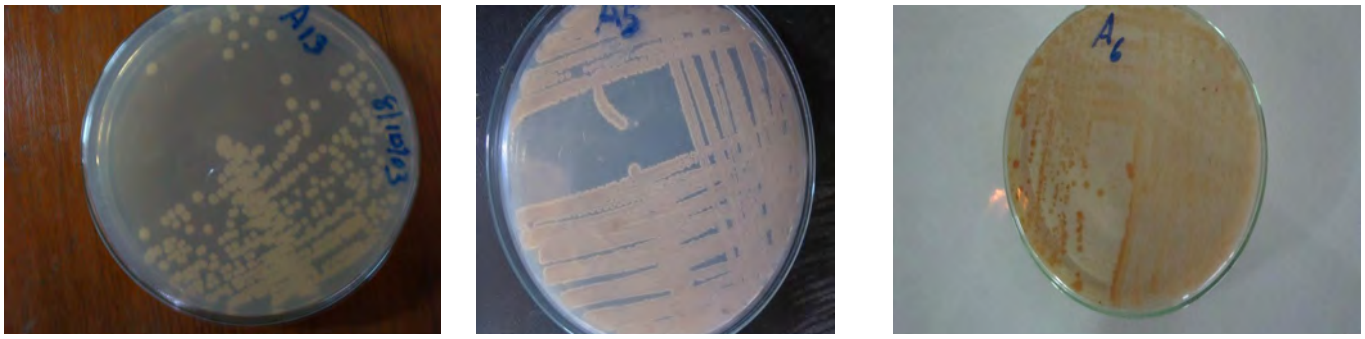
AAUB19

**Appendix 3.** Cover slip culture for isolate (AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19, AAUBA30, and AAUBA31) showed with aerial, substrate mycelium and spore chain under florescent microscope at (1000x).

B. Cultural characteristics of actinomycetes isolates

AAUBA13 AAUBA5 AAUBA6

**Appendix 4**. Actinomycetes with aerial mycelium on starch casein medium

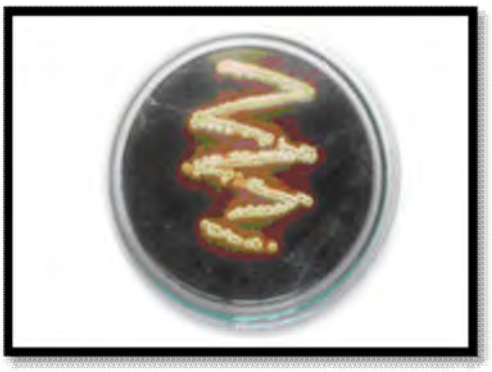


AAUBA13 AAUBA5 AAUBA6

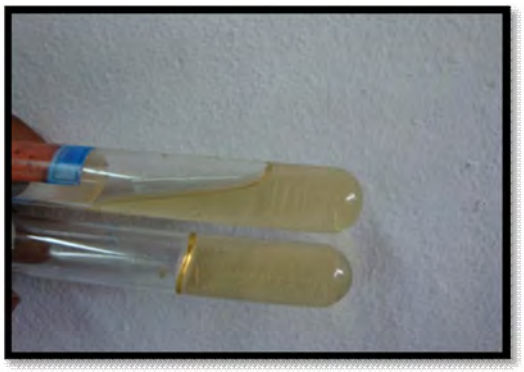
**Appendix 5**. Actinomycetes with substrate mycelium on starch casein medium

## C. **Biochemical test**

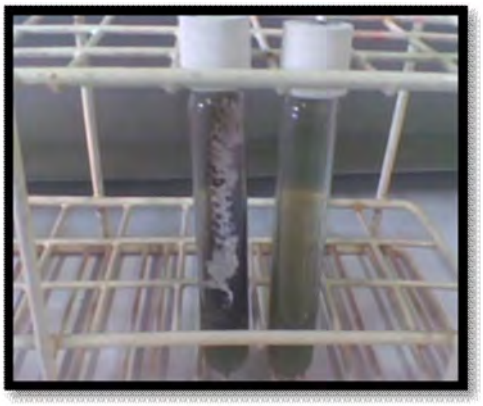
.



**Appendix 6.** Starch hydrolysis of isolate AAUBA13



**Appendix 7.** Gelatin hydrolysis of isolate AAUBA13



**Appendix 8.** Esculin hydrolysis of isolateAAUBA13

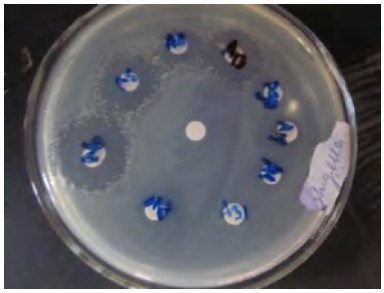
D. Extraction process

**Appendix 9.** Extraction in flask

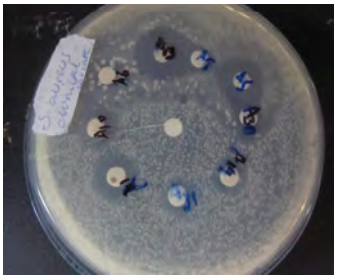
**Appendix 10.** Separatory funnel

**Appendix 11.** BUCHI vacuum rota vapor for extraction setup of Antimicrobial compound

E. Disc Diffusion Assay of crude extract

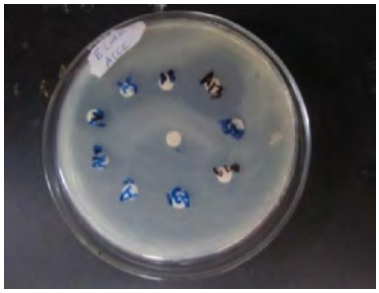


*Shigella boydii*



*S.aureus clinical isolate*

*S.pneumoniae*



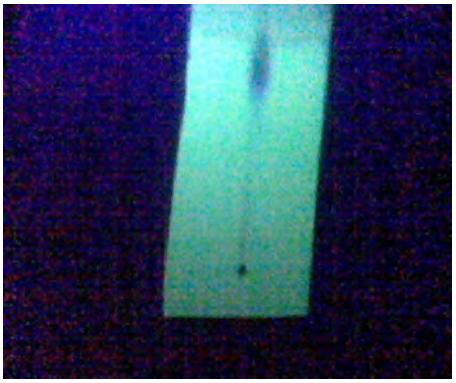
*E.coli*

*S.typhi*

*C. albicains*

**Appendix 12.** Antimicrobial assay of crude extract from actinomycetes isolates against test microorganisms

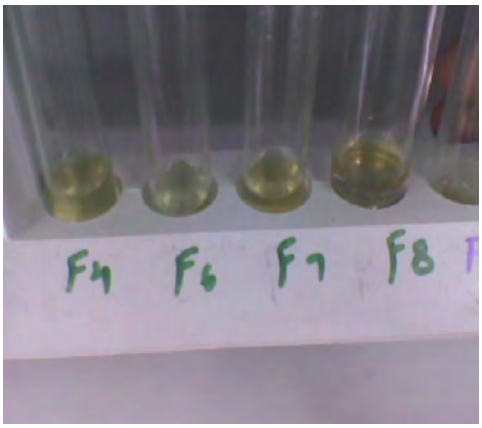
F. Purification process of crude extract from AAUBA13



**Appendix 13.** TLC plate showing spots at 366nm

**Appendix 14.** Bioautography of the spot From TLC plate

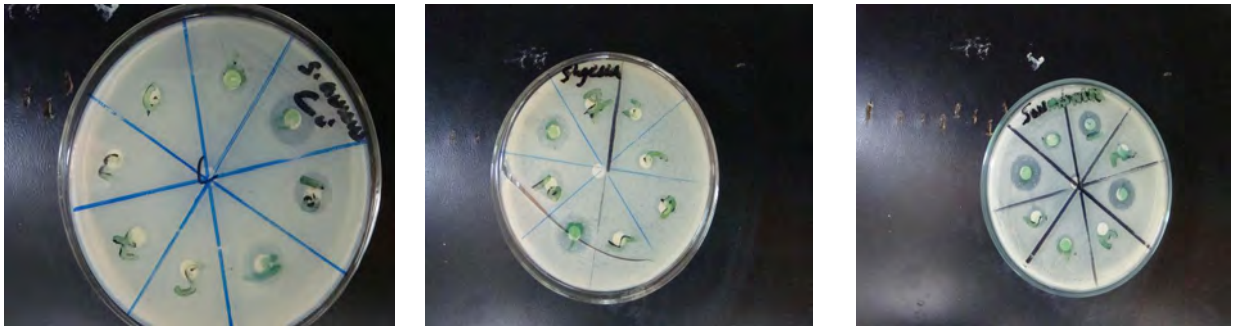
**Appendix 15.** Column chromatography Separation




**Appendix 16.** Fractions obtained from Chromatography

**Appendix 17.** Preparative TLC plate of the fraction eight with the developed Solvent chloroform: Methanol (1:9).

G. Disc Diffusion Assay of pure extracts



Appendix 18. Antimicrobial assay of pure extract from AAUBA13

Table 12. Inoculums size of test microorganisms

| Test microorganisms | Inoculums size from 0.5 McFarland standard in cfu/ml |
|---|---|
| *S.aureus* ATCC25923 | $2.98x10^6$ |
| *E.coli* ATCC25922 | $2.4x10^6$ |
| *P. aeruginosa* ATCC25853 | $2.6x10^6$ |
| *S. pneumoniae*ATCC49619 | $1.7x10^6$ |
| *S.typhi* ATCC 6539 | $2.5x10^6$ |
| *S.aureus* clinical isolate | $3x10^6$ |
| *Shigella boydii* clinical isolates | $3.1x10^6$ |
| *C.albicans* | $1x10^6$ |
| *C.neoformance* | $1.67x10^6$ |

0.5 McFarland Standard

$H_2SO_4$ (1% v/v) ……1ml concentrated $H_2SO_4$ in 99ml distilled water
Barium chloride (1.175% w/v) ……2.35g $BaCl_2$ in 200ml distilled water