Isolation of Actinomycetes with Antifungal Activities

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Abstract- ***Three soil samples were taken from three different sites of dense foliage in Makogi, Ogun State. The total actinomycete population isolates from each of the sites was: site A, 2.4 x 104 cfu/g, site B, 2.0 x 105 cfu/g and site C, 1.8 x 103 cfu/g. Fourteen isolates were obtained and of the fourteen, four isolates identified as Streptomyces anulatus (Act 9), Streptomyces xanthophacus (Act 11), Streptomyces noursei (Act 13), and Streptomyces rimosus (Act 14), have antifungal activities. The fungal test strain used when known plants pathogens, Aspergillus niger, Aspergillus flavus, and Fusarium oxysporum. The antifungal zones of inhibition of the isolates ranged from 5 - 25mm. Streptomyces rimosus had the highest antifungal zone of the inhibition of 25mm against A. niger and also had the least zone of inhibition against F. oxysporum. Antifungal inhibition was also more prominent with A. niger and A. flavus than F. oxysporum. The isolate grew well on cellulose agar plates and had zones of clearing which indicated their ability to degrade cellulose.***

I. INTRODUCTION

The search for new drugs against fungal infections is a major challenge to current research in mycotic disease. Fungal phytopathogens pose serious problems worldwide and cause a number of plants and animal diseases such as ringworms athletic's foot, and several more serious diseases. Fungi cause a lot of diseases which include rusts, smuts, rots, and may cause severe damage to crops. Some species of fungi produce mycotoxins that are very toxic to humans. For example, the fungus *Claviceps purpurea* causes the ergot poisoning. An individuals infected with the mycotoxin experiences hallucination, gangrene, and blood flow restrictions in his limbs. Humans usually get infected with fungus after eating cereals grain contaminated with *C. purpurea* (Bauman, 2007). Excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide. Because of these problems in fungal disease control, a serious search is needed to identify alternate methods for plant protection, which are less dependent on chemicals and are more environmentally friendly. Microbial antagonists are widely used for the biocontrol of fungal diseases. Actinomycetes are the main source of bioactive antifungal agent hence, highly used pharmacologically and commercially. Actinomycetes have occupied a prominent position in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites including antibiotics with diverse chemical structure and biological activities (Tanaka and Omura, 1993). Among the different types of drugs prevailing in the market antifungal antibiotics are very small but significant group of drugs and have important role in the control of mycotic diseases. The need for new, safe and more effective antifungal is the major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immune compromised host. In recent years the microorganisms have become important in the study of novel microbial product exhibiting antimicrobial, antiviral, antitumor as well as anticoagulant and cardioactive properties. These active compounds may serve as a model system in discovery of new drugs. Thus, the proposed study is an effort to screen the actinomycetes from Makogi, Ogun state having antifungal activity. Makogi is largely unexplored, and is a newly developing area with untapped diversity. This diversity can be explored for isolation and characterization of native actinomycetes for antifungal metabolites *(Sharma et al., 2005)*

In order to screen a new antifungal, producer organism may be isolated and screened for, from different natural materials such as soils, which is based on the assumption that samples from new different locations and more highly to yield effective isolates and therefore hopefully effective metabolites.

In the present investigation, the ability of extracellular antifungal metabolites of actinomycetes against

Aspergillus niger, Aspergillus flavus, and Fusarium oxysporum has been reported. This study investigated the antifungal activity of the cell-free culture filtrate of these antagonists to determine secondary antifungal compounds. The antifungal potential of extracellular metabolites produced by soil borne actinomycetes could be exploited for its future use as an antifungal compound

II. LITERATURE REVIEW

1.1.1 ACTINOMYCETES

Actinomycetes (from Greek *''actis''* ray, beam and *''mykes''* mucus, fungus) is a genus of the actinobacteria class or bacteria. They are all Gram-positive. Actinomycetes are facultatively anaerobic (except *A. meyeri*, a strict anaerobe). All species grow under grow best under anaerobic conditions. Actinomycetes species noted for a filamentous and branching growth pattern that results, in most forms, in an extensive colony, or mycelium. The mycelium in some species may break apart to form rod or coccoid shaped forms. Many genera also form spores; the sporangia or spore cases, may be found on aerial hyphae, on the colony surface, or free within the environment. Motility, when present, is conferred by flagella do not form endospores, and, while individual bacteria are rod shaped, morphologically. The mycelium form of these colonies initially led to the (incorrect) assumption that the organism was a fungus and to the name "Actinomycetes" or ray fungus (Holt, 1994)

Actinomycetes reproduce via spores, which are round bodies that develop is single strand. From this strand bacterial body begins a first curl at the tip, and then to divide into segment within the strand. The wall of the strand thickens as the partition divide, and once these partitions mature the strand is ready to produce more spores to continue the division (Pandet *et al.*, 2004).

Actinomycetes are the most widely distributed group of micro-organisms in nature and are also well known as saprophytic oil inhabitant (*Takizawa et al.*, 1993). This soil actinomycete produce a volatile compound called geosmin which literally translates to "earth smell" (*Gust et al.*, 2003). This compound is responsible or a contributor to the strong odour that occurs the air when rain falls after a dry spell of weather. When dirt and soil are freshly dug, they release a distinct, earthly smell. Most bacteria in the dirt break substances down to their base compounds, but actinomycetes it which are saprophytic, are responsible for the decomposition of some of the toughest of these substances. Cellulose is found in the wall of cell plants, and chitin serves a similar purpose in the walls in the cell walls of the fungi. They aren't many bacteria that can break down these difficult substances, but actinomycetes are able to under the harshest of these conditions (Subba., 1999).

Actinomycetes grow in both cultivated and uncultivated soil. (Goodfellow and Williams, 1983) The actinomycetes are particularly important in areas where there is a lot of decaying plant matter, such as forests and grasslands. Without their ability to participate in the decay of plant and fungi, nutrients from the dead organisms would not be restored to the soil for living plants to absorb and use. Activated by high pH levels, a change in the soil's and nutrient level can deactivate the recycling process of these organisms and drastically impact the soil ecosystem. When the soil contains a low pH level, not only are the actinomycetes in active, but other soil decomposers, fungi, is activated. With this shift, soil condition change and can become favorable for a new set of plants, such as unwanted weeds and invasive species (Wollum, 1982; Basillio et al., 2003)

Actinomycetes are known for causing disease in humans, and for the important role they play in soil ecology. Some species of actinomycetes occurring in soil are important pathogens, and many others are beneficial source of antibiotics (David *et al*., 1998)
Actinobacteria are well known as secondary metabolite producers and hence of high pharmacological and commercial and agricultural interest and play an important role as a biocontrol agent (Bressan, 2003; Doumbou *et al*., 2002; Chamberlin and Crawford, 1999; Yuan and Crawford, 1995; Tahvonen and Avikainen, 1987). In 1940, Selman Waksman discovered that the soil bacteria he was studying was made actinomycin, a discovery which granted him a Nobel Prize. Since then, hundreds of naturally occurring antibiotics have been discovered in these terrestrial microorganisms, especially from the genus *Streptomyces* (Berdey, 2005; Watve *et al*., 2001).

The antagonistic activity of the actinomycetes to fungal pathogens is usually related to the production of antifungal compounds (Getha and Vikineswary, 2002; Ouhdouch *et al*., 1998) and extracellular hydrolytic enzymes (Prapagdee, 2008; Mukherjee and Sen, 2006; Valois *et al*., 1996). Other Actinobacteria inhabit plants and animals, including a few pathogens such as *Mycobaterium*, *Corynebaterium*, *Nocardia*, *Rhodococcus* and a few species of *Streptomyces*.

Genomes of 44 different strains of Actinobacteria from different genera are either already sequenced or underway (Sayers *et al*., 2001). Most members are aerobic but a few, such as *Actinomyces israeli*, can grow under anaerobic conditions. Unlike the Firmicutes, the other main group of Gram-positive bacteria, they have DNA with a high GC content and some Actinomycetes species produces external spores (Stackbrandt et al., 1997)

Some representative genera include
Actinomyces
Arthrobacter
Corynebacterium
Frankia
Micromonospora
Mycobaterium
Nocardia
Propionibacterium
Streptomyces
Streptomyces seem to be the easiest to cultivate (Sayers *et al*., 2011). Other genera like *Actinoplanes*, *Amycolatopsis*, *Catenuloplanes*, *Dactylosporangium*, *Kineospora*, *Microbispora*, *Micromonospora*, *Nonomuraea*, which are often very difficult to isolate and cultivate due to their slow growth are called actinomycetes rare (Hayakawa, 2008)

1.1.2. METABOLITES OF MICROORGANISMS.
Metabolites can be caused categorized into both primary and secondary. These metabolites can be used in industrial microbiology to obtain amino acid, develop vaccines and antibiotics, and isolate chemicals necessary for organic synthesis (Tanaka *et al.*, 1993)

1.1.2.1. PRIMARY METABOLITE.
Primary metabolites are involved in growth, development and reproduction of the organism. The primary metabolite is typically a key component in maintaining normal physiological processes; thus, it is often referred to as a central metabolite. Primary metabolites are typically formed during the growth phase as a result of energy metabolism, and are deemed essential for proper growth. Examples of primary metabolites include alcohols such as ethanol, lactic acid, and certain amino acids. Within the field of industrial microbiology, alcohol is one of the most common primary metabolites used for large scale production. Specifically, alcohol is used for processes involving fermentation which produce products like beer and wine. Additionally, primary metabolites such as amino acids including L-glutamate and L-lysine, which are commonly used as supplements, are isolated via the mass production of specific bacterial species, *Corynebacteria glutamicum*. Another example of a primary metabolite commonly used in industrial microbiology includes citric acid. Citric acid, produced by *Aspergillus niger*, is one of the most widely used ingredients in food production. It is commonly used in pharmaceutical and cosmetic industries as well.

1.1.2.2 SECONDARY METABOLITE.
Secondary metabolites are typically organic compound produced through the modification of primary metabolite synthases. Secondary metabolites do not play a role in growth, development and reproduction like primary metabolites do, and are typically formed during the end or near the stationary phase of growth. Many of the identified secondary metabolites have a role in ecological function, including defense mechanism(s), by serving as antibiotics and by producing pigment. Examples of secondary metabolite with importance in industrial microbiology include atropine and antibiotics such as erythromycin and bacitracin. Atropine, derived from various plants, is a secondary metabolite with important use in the clinic. Atropine is a competitive antagonist for acetycholin receptor, specifically those of the muscarinic type, which can be used in the treatment of bradycardia. Antibiotics such as erythromycin and bacitracin are also considered to be secondary metabolites. Erythromycin, derive from *Saccharopolyspora erythraea*, is commonly used antibiotics with a wide antimicrobial spectrum. It is mass produced and commonly administered orally. Lastly, another example of an antibiotic which is

classified as a secondary metabolite is bacitracin. Bacitracin, derived from organisms classified under *Bacillus subtilis*, is an antibiotic commonly used a topical drug. Bacitracin is synthesized in nature as a non-ribosomal peptide synthetase that can synthesize peptides; however, it is used in clinics as an antibiotic (Spann *et al*., 2004).

1.1.3 ANTIMICROBIAL ACTIVITIES OF ACTINOMYCETES

Screening of microorganisms for the production of novelantibiotics has been intensively pursued for many years by scientists. *Actinomycetes* comprise an extensive and diverse group of gram positive, aerobic, mycelial bacteria that play an important ecological role in soil cycles. Many are well known for their economic importance as producers of biologically active substances, such as antibiotics, vitamins and enzymes (McCarthy and Williams 1992; Sanglier et al., Lazzarini et al., 2000). Approximately 80% of the world's antibiotics are produced by actinomycetes, mostly by the gene *Streptomyces* and *Micromonospora* (Pandey *et al*., 2004). The actinomycetes especially the strains of *Streptomyces* are the most common antibiotics producing microorganisms found in the soil. They have provided about two-third (more than 4000) of naturally occurring antibiotics discovered, including many of those in medicine, such as aminoglycoside, anthracyclines, chloramphenicol, β-lactams, macrolides and tetracyclines (Watve et al., 1988) *Micromonospora* is the runner up with less than tenth as many as *Streptomyces.* If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are out in front over 60%, *Streptomyces* species accounting for over 80% of these (Keiser, *et al*., 2000). In the late 60s, after the discovery of gentamycin, originated *Micromonospora*, from the study of non-streptomycete actinomycetes received increasing attention.

In general, it was observed that most of the rare actinomycetes products had already existed among *Streptomyces* metabolites. Nevertheless, there are certain types of structure which occurred more frequently in some rare actinomycetes species than in streptomycetes. *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 a new group of macrolactam and naphtacene-quinone antibiotics were isolated from *Actinomadura*. The species from this genus frequently produce polyether antibiotics. *Micromonospora* and *Saccharopolyspora* strains were relatively rich sources of macro macrolides. The productivity of *Streptomyces* strain as antibiotics producers remains unique amongst actinomycete strains. The relatively low occurrence of non-streptomycetes species as producers of secondary metabolite is due to the difficult technique required for the isolation (Takashi., 2003; Suzuki., 2000) of these strains from the environment. Another problem is their complicated preservation and cultivation methods, which frequently require some specific and unusual conditions. These are the main reasons for regarding these microbes as rare organisms and difficulties for investigations and manufacturing of their product (Berdy, 1995).

Some new screening programs have been already developed for discovering of new species or unknown bioactive substances. One of the modern approaches is the isolation and screening of microorganisms from relatively unknown and unstudied areas (Moncheva *et al*., 2002)

1.1.4 SOIL ACTINOMYCETES.

Actinomycetes, especially *Streptomyces* and *Micromonospora have* long been recognized as major producers of useful natural secondary metabolites (Miyadoh, 1993). Thus, the rate of discovery of new metabolite from this common actinomycetes has declined, thus the selection of improved methodologies for isolating rare actinomycetes is required to isolate new strains that produced bioactive metabolites and improve the quality of the natural products screened (Berdy, 2005; Takahashi and Omura, 2003; Lazzarini *et al.*, 2001). Various media and methods, including the technique that enhance the desirable actinomycetes in natural habitat sample (enrichment) or eliminate undesirable *Streptomyces* and other contaminated like fungi from the isolation plate media (pretreatment) for isolating noval actinomycetes from natural habitats, especially from

various types of soil, have been improved and developed.

1.1.5. ISOLATION OF ACTINOMYCETES.

Members of the actinomycetes especially *Streptomyces* and *Micromonospora* have long been recognized as a major producer of useful natural secondary metabolites (Miyadoh, 1993). Thus, the rate of discovery of new metabolite from these common actinomycetes has decline, so the selection of improved methodologies for isolating the uncommon and rare actinomycetes is required to avoid re-isolating strain that produce known bio active metabolites and to improve the quality of natural products screened (Berdy, 2005; Takahashi and Omura, 2003). Various media and methods including the technique that enhance the desirable actinomycetes in natural habitat sample (enrichment) or eliminate undesirable contamination from the isolation plate Media (pretreatment) for isolating noval actinomycetes from natural habitats, especially from various type of soil, were improved and developed (Lazarrini et al., 2001; Nakaew et al., 2009).

Actinomycetes are extremely slow growing, and require selective media for optimum growth. Growth typically takes 7 - 10 days when cultivating in the laboratory. Starch – casein agar medium is supplemented with nystatin (10 µg/ml) which prevent fungal growth and is used for actinomycetes isolation; the inoculated plates are incubated at 30^0 C for seven to ten days. Starch Casein Agar contains casein which is a source of nitrogen. Sodium propionate is a substrate used in anaerobic fermentation. Dipotassium phosphate provides buffering capability to maintain pH balance. Magnesium sulphate and phosphorus sulphate provides source of sulphate and metallic ions. Agar is a solidifying agent. Starch is a source of carbon (Leboffe et al., 2010; Hayakowa, 2008; Goodfellow et al., 1998).

1.1.6 FUNGI.

A fungus (plural: Fungi) is a member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds (British English: moulds), as well as the more familiar mushrooms (Novick, 1955). These organisms are classified as the Kingdom, Fungi, which is separate from plants, animals and bacteria. One major difference is that fungal cells have cell walls that contain chitin, unlike the cell wall of plant, which contains cellulose (Bowman et al., 2006). These and other differences show that the fungi form a single group of related organisms named the *Eumycota (true fungi or Eumycetes)*, that share a common ancestor. The fungal group is distinct from the structurally similar myyxomycetes (slime molds) and oomycetes (water molds). The discipline of biology devoted to the study of fungi is known as mycology (from the Greek μύκης, mukes, meaning ‘’fungus’’). Mycology has often been regarded as a branch of botany, even though it is a separate Kingdom in biological taxonomy. Genetic studies have shown that fungi are more closely related to animals than to plant (Hibbet et al., 2007).

Abundant worldwide, most fungi are in inconspicuous because of the small size of their structure, and their cryptic lifestyles in soil, on dead matter, and as symbionts of plants, animal, or other fungi. They may become noticeable when fruiting, either as mushroom or mold. Fungi perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling and exchange. They have been used as a direct source of food, such as mushroom and truffles, as leavening agent for bread, and in fermentation for various food products such as wine, beer and soy sauce. Since the 1940s, fungi have been used for the production of antibiotics, and more recently various enzymes produced by fungi are used industrially and in detergent. Fungi are also used as biological pesticides to control weed, plant diseases and insect pests. Many species produce bioactive compounds called mycotoxins such as alkaloids and polyketides that are toxic to animals, including humans (Tudzynski, 2005).

The fruiting structures of a new species contain psychotropic compounds and are consumed recreationally or in traditional spiritual ceremonies. Fungi can break down manufactured materials and buildings, and become significant pathogens of human and other animals. Losses of crop due to fungal diseases (e.g, rice, blast disease) or food spoilage can have a large impact on human food supplies and local economies (Mace et al., 1981; Joo, 2005).

Some species of fungi are very virulent and are causes of both plant and animal diseases. *Ustilago maydis* is

a pathogen plant fungus that causes smut disease in maize and teosinte. Plants have evolved efficient defence systems against pathogenic microbes. A rapid defense reaction after pathogen attack is the oxidative burst that includes the production of reactive oxygen species at the site at the site of the attempted invasion. As a pathogen, *Ustilago maydis* respond to such an oxidative burst by an oxidative stress response, regulated by the gene *yap 1*. This response protects *Ustilago maydis* from the host attack, and is necessary for the pathogen's virulence (Simon-Nobbe, et al., 2008). Furthermore, *Ustilago maydis* has a well-established recombinational DNA repair system that act during the mitosis and meiosis (Molina, et al., 2007). Recombinational repair during the mitosis and meiosis may assist the pathogen in surviving DNA damage rising from the host plant's oxidative defensive response to infection (Kojic., 2006).

Aspergillus niger causes black modes of onion. Infections of onion seedling by *Aspergillus niger* can be systematic, manifesting only when conditions are conducive. *Aspergillus niger* causes a postharvest disease of onion, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in the peanuts and in grapes (Samson et al., 2001). *Aspergillus niger* is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, *Aspergillus niger* can be deadly. This is due to the serious lung disease, aspergillosis, that can occur. Aspergillosis is, in particular frequent among horticultural workers that inhale peat dust, which can be rich in *Aspergillus* spores. It has been found in the mummies of the ancient Egypt tombs and can be inhaled when they are disturbed (Handwerk, 2005).

Aspergillus niger is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane (Abarca et al., 1994).

Aspergillus flavus is the primary cause of aflatoxin contamination of corn, cottonsees, and tree nuts (Diener et al., 1987). *Aspergillus flavus* population consist of individuals that vary in their ability to produce sclerotia or aflatoxin in vitro (Garber et al., 1997), and isolate are often grouped on the basis of quantities of aflatoxin and sclerotia produced.

The soil-borne vascular wilt fungus *Fusarium oxysporum* infects a wide variety of plant species by directly penetrating roots, invading the cortex and colonizing the vascular tissue (Handwerk., 2005).

1.2 STATEMENT OF PROBLEM

The application of agrochemicals for the control of plant diseases is an important method in agricultural practices, though it still has some problems. Such as environmental pollution and detrimental effect on non-target organisms. Actinomycetes produce the natural antibiotics within the microhabitat of the rhizosphere being less polluting and less stressful on indigenous microbes as compared with chemical fungicides. They also have the ability to colonize plant root surfaces protecting the plant from pressure of plant pathogens. These biological control agents compete for nutrients and space with plant pathogens; they also say synthesize extracellular enzymes that attack the phytopathogenic fungal, cell walls and have the ability to produce descant resistant spores to survive under water deficiency. All the property exhibited by actinomycetes, especially, those that belong to the genus, *Streptomyces*, as biological control agent or fungal phytopathogens, not only give us a better understanding of the environmental and ecological benefits, but also their impact as an attractive alternative for using agriculture. This environment friendly approach has recently received increased attention and has potential advantages over the prevailing use of chemicals to treat plant diseases, but it has not been incorporated into mainstream agriculture.

1.3 AIM AND OBJECTIVE OF THE STUDY.

1. To isolate actinomycetes from soil
2. To screen for production of antifungal agents by the actinomycetes
3. To screen for ability of the actinomycetes to produce cellulase.

III. MATERIALS AND METHOD

2.1 SAMPLE COLLECTION

Three samples were collected from farm areas located in Makogi, the village in Ibafo, Ogun state. Samples

were collected directly from surface and sub surface soil into sterile McCartney bottles and transported to the laboratory. Physico-chemical and microbiological analysis commenced immediately upon arrival.

2.2 STERILIZATION AND ASETIC TECHNIQUE

All glassware and bottles used were thoroughly washed with detergent, air dried and sterilizing in an oven of 170^0 Celsius for 2 hours. Media use were sterilized at 121^0 Celsius for 15 minutes in an autoclave. All inoculations, plate pouring, serial dilutions were carried out aseptically and in the inoculating room. The inoculating loop, inoculating needle where flame red hot with a bunsen burner before and after use. The mouths of Erlenmeyer flask were flamed before and after pouring media from them. Also mouth of tools were flamed before and after inoculation. The workbench was disinfected.

2.3 PHYSIO-CHEMICAL ANALYSIS OF SOIL SAMPLES

The physio-chemical analysis of soil samples was carried out in the Department of Chemistry, University of Lagos. The total organic carbon, total organic matter. Moisture content, total hydrocarbon content, phosphate, nitrate and hydrogen ion concentration (pH), were determined.

2.31 MOISTURE CONTENT

The moisture content was determined by the gravimetric method with 5g of each soil sampled weighed into a crucible of known weight. The soil was then dried in the oven at 105^0 Celsius for 2 hours. The crucible and its contents were allowed to cool in a dessicator and the weight of the crucible+soil determined. This was repeated until a constant weight was achieved. The difference in weight of the cruciblei. alone and cubicle+soil was then used to calculate theii. moisture content of the soil.

2.32 TOTAL ORGANIC CARBON TOC AND TOTAL ORGANIC MATTER.

TOC was determined by the Walkley-Black Tree titrimetric method. About 0.3g of each choice sample was weighed into an Erlenmeyer flask with 10 ml of $K_2Cr_2O_7$ and 20ml concentration as to H_2SO_4 added. The mixture was gently swired until soil until soil and reagents were properly mixed and were allowed to stand for 30 minutes, 100ml of distilled water was added and the content titriated against standardized ferrous sulphate solution to a reddish-brown end point using ferroine as the indicator. The total organic matter was calculated from the value obtained from the total organic carbon (Walkley and Black 1934)

2.3.3 PH DETERMINATION

The pH was determined electrometrically by suspending the soil. In 1:2 (soil: 0.01 $MCaCL_2$ mixture. The suspension of the soil was allowed to stand for 30 minutes, with occasional stirring and the pH measured with a pH meter0 (Schofield and Taylor, 1955).

2.3.4 TOTAL HYDROCARBON CONTENT.

The total hydrocarbon content (THC) of the soils was determined by gravimetry. About 20g of each sample was refluxed in 100ml methanol containing 3.0g KOH. The suspension obtained was filtrated and methanolic extracts was cooled and extracted twice with n-hexane in volumetric flask. The n-hexane fraction was filtered through sodium sulfate into a clean Baker of known weight and evaporated. This was then re-weighed and the difference in weight was used to determine the THC level in the samples.

2.3.5 PHOSPHATE AND NITRATE.

Each sample 2g was accurately weighed and placed in a conical flask and 10ml conc. hydrochloric acid added. The mixture was heated gently for 2 hours and cooled. About 10ml of perchloric acid was then added and the heating continued for another 1 hour. The resulting mixture was cooled, transferred into a 50ml volumetric flask and made-up to mark with distilled water. This was then filtered through Whatman No 1 filter paper in acidic pre-wash polyethylene.

Phosphate

Each of the extract (0.5ml) was distilled in a standard flash and analyzed for its phosphate content using the asbcorbic acid reduction method. A prepared mixed reagent 8ml made-up of a mixture of sulphuric acid, ammonium molybate, antimonyl potassium tartarate abd ascorbic acid solutions was added to the 50ml diluted sample extracted obtain above. The mixture was left to stand for 10 minutes for color development and the absorbance, taking at 880 nm on a thermospectronic genesys uv/vis spectrophotometer. The concentration was then calculated from the

calibration graph of measured standard phosphate solutions (Murphy and Riley, 1962; APHA, 1995).

2.4 MICROBIOLOGICAL ANALYSIS.

The isolation of actinomycetes from the mixture microflora present in nature is complicated by the characteristic slow growth relative to that of other soil bacteria. Sample from each site were analyze, from each sample, 1g was weighed and agitated in 9ml of sterile distilled water for 10 minutes to dislodge the organisms from the soil particles. Using sterile micropipette tubes, 1ml aliquot was transferred serially. Spies serially into a series of test tube containing sterile distilled water to obtain tenfold serial dilution of 10^{-1} to 10^{-9}, after the soil sample, has been heated to about 50^{0} Celsius to kill other some other bacteria.

2.4.1 ENUMERATION OF TOTAL ACTINOMYCETES

To enumerate the total actinomycete colonies 0.1 ml aliquot of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} was inoculated by spread plate method onto starch casein agar plate in duplicate. This medium is selective for actinomycetes because of the complexity of the carbon source. The plates were incubated aerobically at 28^{0}C for 7 - 10 days. The resulting colonies were counted and recorded as colony forming units per gram cfu/g of soil sample (Chikere et al., 2009) our head all. 2009.

2.4.2 ISOLATION OF ACTINOMYCETES.

In order to obtain pure colonies of actinomycetes, individual, colonies were plated on starch casein agar and incubated at 28 degrees Celsius, for 7 – 10 days. Initial identification was carried out by gram staining of isolate

2.4.3. CULTURAL AND MORPHOLOGICAL CHARACTERISTICS.

Cultural characteristics of the isolates such as color or aerial mycelium, color of substrate mycelium and pigmentation of the selected actinomycetes isolates were recorded on starch, casein agar medium. Micromorphology of the actinomycetes isolates were examined by grams staining and slide culture method under the microscope

2.5 CHARACTERIZATION AND IDENTIFICATION OF ISOLATES.

2.5.1 GRAM STAINING TECHNIQUE.

The gram staining of isolate was carried out according to Cheesbrough (2006). A smear was prepared by using a sterile wire loop to pick up colonies and place on the grease free glass slide which had drop of sterile water. The smear was allowed to dry air, heat fixed by passing over the flame of a Bunsen burner. The smear was covered with crystal violet stain for 60 seconds and it was rapidly rinsed with clean running water. Water was tipped off and the smear covered with a lugol's iodine for 60 seconds. The iodine was raised with cleaning water. The smear was rapidly (few seconds) decolourized with acetone-alcohol and raised immediately and smear covered with neutral red for 2 minutes. The smear was rinsed with clean running water and allowed to air dry. Smear was examined first with 40x objective to check the staining and to see distribution of material, and then the oil immersion objective to report the bacteria and cells. Those cells that retained the primary stain (purple) were recorded as Gram - positive while those that picked up with counter stain (pink) were recorded as Gram - negative. The shape of these cells were observed and recorded.

2.5.2. OXIDASE TEST.

The oxidized test is used in the identification of organisms that produce the enzyme cytochrome oxidase. Oxidase strips were used, a piece of glass rod was used to remove colonies of isolates and smear on the strips. The development of blue-purple colour within a few seconds indicated a positive result.

2.5.3 CATALYST TEST.

The test is used to differentiate those bacteria that produce the enzymes catalase from non-catalase producing bacteria (Cheesebrough, 2010). Catalase act as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. The isolates were tested for catalase production by bringing the organism into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. Smear of 5 - 7 days old cultures were made with sterile wire loop and hydrogen peroxide. The slides were observed for the evolution of gas bubbles caused by liberation of oxygen. Thus, liberation of oxygen indicates the presence of enzymes catalase.

2.6 PRODUCTION OF ANTIFUNGAL METABOLITES.

The secondary metabolites produced by the actinomycetes were extracted by the method of Ismet (2004). Pure cultures of the strains were transferred aseptically and individually into the fermentation medium; starch casein broth. The small-scale fermentation in 250ml flask containing 50ml of starch casein broth was carried out for 10 days with regular agitation. After this period, the flasks were harvested and the broth was extracted and centrifuged in sterile centrifuge tubes to obtain the supernatants free of cellular material and also filter sterilized, but containing metabolite. This extract obtained were used for antifungal activities against the test fungi *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum* using agar well diffusion method (Cappuccino and Sherman, 2004).

2.6.1. GROWTH OF TEST FUNGI AND SCREENING.

For the growth and screening of test fungi, Potato Dextrose Agar (PDA) medium were used. The fungi were grown on PDA for 72 hours and then aseptically inoculated into. Potato Dextrose Broth, for 48 hours. The fungi broth (0.1ml) was inoculated by the striking method on plates of Potato Dextrose Agar. Wells were created ascetically on the *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum* on the fungal plate using sterile cork borer. Micropipettes were used to deposit the antifungal metabolites from the actinomycetes isolates in the walls created in the fungal plates. Sterile distilled water was used as a control. The PDA plates were incubated at 30 degrees Celsius for 72 hours and the diameter of the inhibition zone of the test fungi around each well was measured.

2.7 SUGAR FERMENTATION USING API KIT

The API 20E (Biomerieux) strip contains 20 microtubules. Carbohydrate (sugar) test was determined using the API 20A kit ((Biomerieux, Marcyl'Etoile, France) according to manufacturer's instruction. All the API strips were incubated for 24 to 48 hours at 28 degrees Celsius and results were recorded.

API 20A is a standardized identification system associating 20 biochemical tests for the study of carbohydrate metabolism of microorganisms. The API 20A strip contains of 20 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives. The fermentation tests were inoculated in the API 20A medium which rehydrates the substrates. During incubation, fermentation were revealed by a color range in the tube caused by the anaerobic production of acid and detected by the pH indicator present in the medium.

Procedure: The purity of the isolates was checked using Gram staining and the isolates were grown on a starch casein agar. The actinomycete cultures were scooped from the culture plate using a sterile inoculating loop into the API 20A suspension medium; then 10ml of sterile distilled water were distributed into the honey combed wells of the tray to create a humid atmosphere after which the stripes were placed in the incubation tray. With a sterile micropipette and tips the API 20A medium suspension was dispensed in the 20 microtubes in the incubation tray by tilting the incubation bottle slightly forward. The tips were overlaid bromocresol purple which served as indicator while the indole sugar well was overlaid with mineral oil to prevent dryness or evaporation of the sugar. The stripes were incubated at 37^0C. For 24 - 48 hours. These stripes were read after incubation. A positive test corresponds to the acidification revealed by the bromocresol purple indicators contained in the medium, which changed to yellow while the esculin test change in color from red to black. All the positive and negative tests were recorded on the result sheet. The identification of the isolate was performed using the Bergey's Manual of Systematic Bacteriology (1975).

IV. RESULTS

3.1 DESCRIPTION OF SIGN OF SOIL SAMPLING SITE

The first two soil sites were loamy soils, which generally contain more nutrients, moisture and humus than sandy soils, have better drainage and infiltration of water and air than silty sandy soils, dark and rich with dead leaves. The third one was more sandy and light.

3.2 PHYSIO-CHEMICAL ANALYSIS OF SOIL

The result of soil sample analysis which were determined shows showed that the soil samples moisture content was varied. The total organic content of the soils also had different values as well as their total organic matter with 2.88. 6.62 and 5.0. and total phosphorus value of 0.16 mg/kg, 0.23 mg/kg and 0.19 mg/kg. The difference in value can also be seen in their total nitrates. All three samples had close pH ranges of 6.61, 6.21 and 5.59 respectively as shown in table 3.1 below.

Table 3.1: Physico-Chemical Parameters of Soil Samples

SOIL SAMPLE	pH	MOISTURE CONTENT	TOTAL ORGANIC CARBON %	TOTAL HYDROCARBON CONTENT mg/kg	TOTAL PHOSPHORUS Mg/kg	TOTAL NITRATE Mg/kg
Sample A	6.61	4.56	2.88	346.48	0.16	1.62
Sample B	6.21	3.93	6.92	166.67	0.23	2.73
Sample C	5.59	22.23	5.0	34.15	0.19	1.69

3.3 ISOLATION OF ACTINOMYCETE POPULATION OF SOIL SAMPLE.

Actinomycete were isolated from soil samples. The color on plates on the colonies range from white to cream, brown, orange and red. Some isolates had an earthly smell and powdery appearance, while some had a shiny appearance with slimy consistency; others had a shiny appearance, but were not slimy. The actinomycete population of the soil samples varied from 2.4. x 10^4 cfu/g to 1.8 x 10^3 cfu/g. Table 3.2 shows the actinomycete population in the soil samples.

Table 3.2: Actinomycete population of soil samples

SAMPLES	TOTAL COLONY FORMING UNITS PER GRAM
SAMPLE 1	2.4. x 10^4 cfu/g
SAMPLE 2	2.0 x 10^5 cfu/g
SAMPLE 3	1.8 x 10^3 cfu/g

3.3.1. INITIAL IDENTIFICATION OF ACTINOMYCETES ISOLATES.

Colonial examination of the isolates was carried out to determine the type of shape elevation, and pigmentation pattern that isolates exhibited. The morphological characteristics of the isolates are shown in Table 3.3.

Table 3.3: Morphological Characteristics of Isolates

ISOLATES	COLONIAL CHARACTERISTICS
Acts 1	Cream to pale yellow, dry and hard
Acts 2	Cream, moist and shiny
Acts 3	Brownish, with powdery tops
Acts 4	Egg yellow and dry
Acts 5	Whitish and powdery
Acts 6	Cream and shiny
Acts 7	Cream and dry
Acts 8	Peach to Orange, round and hard
Acts 9	Greenish – grey colonies, with cream edges
Acts 10	Cream colonies growing into the medium
Acts 11	Greyish with cream surround and an earthy smell
Acts 12	Creamy and shiny
Acts 13	Whitish colonies, round and susty, releasing yellow colour into the media
Acts 14	Whitish brown and leathery, powdery with an earthly smell, colouring medium beige

3.3.2 GRAM STAINING OF ACTINOMYCETES.
Microscopic examination of the gram-stained smell of the isolates on slide showed that Acts 9 and Acts 13 were Gram positive and filamentous, and Act 11 was Gram positive, filamentous cocci. The figures (Fig 3.1, 3.2 and 3.3) show the gram stained isolates on slides under the microscope.

3.3.2 GRAM STAINING OF ACTINOMYCETES
Microscopic examination of the Gram-stained smear of these isolates on slide that showed that Act 9 and Act 13 were Gram positive and filamentous, and Act 11 Gram positive, filamentous cocci. The figures (Fig 3.1, 3.2 and 3.3) show the gram-stained isolates on slides under the microscope.

3.3.3 OXIDASE TEST
All isolates were positive for oxidase tests with the strips changing from ash to purple

3.3.4 CATALASE TEST
All the isolates reacted to the hydrogen peroxide to produce gas bubbles in the starch casing agar plate.

3.4 ANTIFUNGI ABILITIES TO IDENTIFIED ISOLATES.
Of the 14 actinomycetes isolated, five showed until antifungal activity to varying degrees on all three fungal tests organisms. *A. niger, A. flavus, and F. oxysporum.* The antifungal zones of inhibition ranged from 3 to 20mm (zone of ambition). Antifungal inhibition was also more prominent with *A. niger* and *A. flavus* than *F. oxysporum*. The isolates were grown in triplicates with 3 phytopathogenic fungi, cultural controls of the fungi on PDA plates substantial growth without any form of inhibition while the average of the distances of clearing zones were recorded in the Fig. 3.4, 3.5, and 3.6.

All other isolates showed negligible or no noticeable zones, and as such were not selected for further analysis. Controls show 100% growth of the fungi. Table 3.4 below shows the different zones in of inhibition.

All other isolates showed negligible or no noticeable zones, and as such were not selected for further analysis. Controls show 100% growth of the fungi. Table 3.4 below shows the different zones in of inhibition.

Table 3.4: Fungi Inhibition by Actinomycetes' Diffusible Compounds

Test Fungi	Distance of Inhibition zones (mm)				
	Acts 3	Act 9	Act 11	Act 13	Act 14
A. niger,	8	18	20	20	25
A. flavus	-	19	22	10	22
F. oxysporum	-	10	15	18	5

3.5. FINAL IDENTIFICATION OF ISOLATES.
3.51 MORPHOLOGICAL CHARACTERISTICS.
Most of the isolates had an earthly smell and some of them colored the media yellow, brown or cream. Table 3.5 below shows their morphological characteristics.

Table 3.5: Morphological Characteristics of the Actinomycete Isolates with Antifungal Activities

Isolates	Characteristics on SCA	Characteristics after Gram staining
Act 9	Greenish – grey colonies, with cream edges	Gram positive cocci/rods
Act 11	Grey center and cream surround dry colonies with an Earthy smell	Gram positive filamentous cocci
Act 13	Whitish colonies, round and dusty, releasing yellowish colour into the media	Gram positive tiny rods
Act 14	Whitish – brown and leathery colony with an earthy smell	Gram positive rods filamentous

	colouring media beige	

3.6 SUGAR FERMENTATION TEST

The results of the fermentation of sugar of the organisms conducted using API 20A kit (Biomerieux, Maroyl'Etoile, France) were recorded. The fermentation of Sucrose, Lactose, Mannose, Maltose, Xylose and Glucose were also done in the lab and the results were also recorded. Most tube had colour change after incubation of 37^0 C after 24 hours from red to yellow, while some tube produced gas by a displacement of fluid in the Durham tube after 48 hours. The absence of colour change in some in some showed that there was no fermentation.

Final identification of the isolates was done using the Bergey's manual. Table 3.6 below shows the identified isolates.

Table 3.6: Confirmatory Identification of Isolates with Antifungal Activities

Act 9	+	+	+	+	+		+	+	+	+	-	*Streptomyces anulatus*
Act 11	+	+	+	+	+		+	-	-	-	-	
Act 13	+	+	+	+	+		+	+	+	+	+	
Act 14	+	-	+	+	+		-	+	+	+	-	

3.7 PRODUCTION OF CELL WALL DEGRADING ENZYMES.

3.7.1 CELLULOSE PRODUCTION.

The isolate grew well on the cellulose Agar plate and showed zones of clearing, indicating their ability to degrade cellulose, except Act 3 which did not show visible growth.

3.7.2. CARBOXYLMETHYLCELLULASE PRODUCTION

The optical densities of the organisms in carboxylmethylcellulase were recorded every 2 days for 10 days and a growth curve was plotted to show their ability to utilize CMC as their sole carbon source due to their production of the enzyme CMC-ase. The growth curve showed that the organisms underwent an initial Lag phase in which they were adapting to the growth medium though this did not take up to 24 hours. There was an exponential phase of about 4 days in the isolate Act 13 and Act 14 with little or no noticeable stationary phase, while the isolates Act 11 had the sharpest exponential phase rise. Act 9 and 14 enter into the into the decline phase of growth by the 5^{th} day, while Act 11 and Act 13 enter into the decline phase. After the 6^{th} day. Act 14 had the sharpest decline of all the isolates.

V. DISCUSSION

Out of the 16 isolates from the soil samples, 5 isolates exhibited broad spectrum antimicrobial activity against *Aspergillus niger, Aspergillus flavus and Fusarium oxysporum* using the agar well diffusion method. The 5 isolates were used for further tests, and 4 isolates exhibited a wide spectrum antifungal antagonistic effect against *Aspergillus niger, Aspergillus flavus and Fusarium oxysporum* using the same method. Isolate 3 showed inhibitory activity only against *Aspergillus niger*. Similar work reported by Aghighi et al., (2004), showed that a small number of actinomycetes had the capability to produce broad spectrum antifungal compounds. The biological activities of the antifungal agent revealed that the antibiotics are active against filamentous (phytopathogentic) fungi such as *Aspergillus niger, Aspergillus flavus and Fusarium oxysporum*. These actions may be survival adaptation resulting from different plant producing different types of secondary metabolites and some of these chemical compounds being toxic to the soil microorganisms including actinomycetes, has cause adaptation in the actinomycetes, hence the production of their own

secondary metabolites. These secondary metabolites are of great economic importance.

Act 3, which was only active against *Aspergillus niger* showed that their inhibitory properties are a combination of factors, which the other four isolates had, and Act 3 lacked. Therefore, it may not be a good candidate for further research work because it has a very narrow spectrum of action. Enzyme assay for the isolate, show that all isolates can be utilized as sole carbon source and grow on cellulose agar except Act 3 which did not show visible growth on cellulose agar plates. Act 3's inability to grow on cellulose agar plate is of significance importance, as it will not harm the plant by degrading its cell walls (El-Tarabily et al., 2000). Mechanism of action of antagonistic actinomycetes is based on the production of bio active metabolite such as cell wall degrading enzymes, antibiotics, competition for nutrients (dual culture bioassay) (El-Tarabily and Sivassithamparam, 2006)
The three different places chosen for soil sampling had different soil characteristics. Since water logged soils and soils with low pH are unfavourable to the growth of actinomycetes, (Sykes 1973, and Cambell 1882) in this study, only one isolate could be gotten from the third soil sample C, which had the moisture content of 22.23 and a pH of 5.59. This means it is it may not be possible to use the live organisms as biocontrol agent in the farm like rice paddies and for other plants that require maximum moisture.

The foregoing research has shown that these actinomycetes produced these secondary metabolites in quantities that are sufficiently toxic to these fungi, which are known plant pathogens. The zones of inhibitions were very wide and could be clearly seen. These metabolites can also be adapted as biocontrol agents, in agriculture. The challenge is harvesting them in insufficient quantities preservation and the safe utilization of these metabolites.

CONCLUSION

The focus of this study was on the isolation of actinomycetes with antifungal activities. The four actinomycetes isolates derived from Makogi subsoil in Ogun state, were unable to inhibit growth of some phytopathogenic fungi used in this study through some antifungal activities when the agar well diffusion method was used. This suggests that the extract can penetrate through semi – solid matter and be effective. The enzymes assay showed their ability to utilize cellulose and its derivative (CMC) as sole carbon sources with adequate production of antifungal compounds. This means they have a potential for the future use as biological control agents of fungi that affects plants. These bioactive molecules can also be exploited for further research including other biological assay such as anticancer (cytotoxicity studies), antiviral and anti-inflammatory activities. These investigations will throw more light on the metabolite's activity. For identification of the active compounds in these metabolites, structural elucidation using mainly one dimensional (1D) or two-dimensional (2D) NMR techniques and spectroscopy of the pure active compound using gas chromatography and mass spectrophotometer (GC – MS) analysis

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