CHAPTER ONE

INTRODUCTION AND OBJECTIVES

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1.1. INTRODUCTION

Resistance by pathogenic bacteria has become a major health concern. Many Gram-positive bacteria and Gram-negative opportunistic pathogens were becoming resistant to virtually every clinically available drug. The emergence of multi resistant pathogenic strains has caused a therapeutic problem of enormous proportions. For instance, they cause substantial morbidity and mortality especially among the elderly and immunocompromised patients. In response, there is a renewed interest in discovering novel classes of antibiotics that have different mechanisms of action (Ogunmwonyi, 2010).

Natural products have been regarded as important sources of antimicrobial compounds. There are great potential in bio-prospecting from the sea and marine natural products research has just started to bloom (Kiruthika *et al*., 2013).

Marine microorganisms have become an important source of novel microbial products (Haggag *et al*., 2014). Thus, the interest of investigators was directed towards marine habitat as unusual source to be explored for the development of new drugs. Significant part of this attention has been paid to marine microorganisms, which have become important in the study of novel compounds exhibiting antibacterial, antifungal, and antitumor (Kokare *et al*., 2004).

The dramatic increase in the prevalence of human pathogens resistant to most known antibiotics, and the emergence of new pathogens, stimulated the search for novel and potent antibiotics. Microorganisms in marine attract a great deal of attention, due to their adaptability to extreme environments. This allows the

organisms to produce different types of bioactive compounds including antibiotics with unique properties and applications (Mohan *et al*., 2013).

In the last decades members of Actinomycetes became almost the most important source for antibiotics. In the 60's and 70's of the 20th century 75 to 85% of all discovered antibiotics derived from the order Actinomycetales, mainly from *Streptomyces* species. The first antibiotic from Actinomycetes has been reported more than 50 years ago. Since that more than 4000 new bioactive compounds have been obtained. The search for new Actinomycetes of interest to biotechnology is still important (Sathiyaseelan and Stella, 2011).

Recently, true indigenous marine *Streptomyces* have been described, thus they drew special emphasis as a promising source of novel and unique metabolites (Kouadri, 2010).

Screening directed towards novel antibiotics from *Streptomyces* has been intensively pursued for many years by researchers. Each year screening of *Streptomyces* strains as source of new antimicrobial compounds are directed by many pharmaceutical companies. Although different bioactive compounds have been isolated from *Streptomyces*, these are thought to represent only a small fraction of the range of bioactive metabolites produced. About 50% of all *Streptomyces* isolated from different sources able to produce antibiotics (Ramazani *et al*., 2013).

1.2. Rationale

The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection (Baskaran *et al.*, 2011). New resistance mechanisms emerge and spread globally. This reduce the ability and existing antibiotics to treat common infectious diseases, resulting in death and disability of individuals who until recently could continue a normal course of life. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness, higher health care costs, and a greater risk of death. When infections become resistant to first-line drugs, more expensive therapies must be used. A longer duration of illness and treatment, often in hospitals, increases health care costs as well as the economic burden on families and societies (WHO, 2015).

According to literature, there are no previous studies carried out in Sudan to screen and isolate bioactive compounds from marine *Streptomyces* bacteria. This study was designed to screen Streptomyces in Red Sea soil sediments for antibiotic(s).

1.3. Objectives

General Objective

To isolate and characterize a novel antibiotic compounds from *Streptomyces* in various depths of the Red sea soil.

Specific Objectives

1. To isolate *Strepomyces* from various depths in the Red Sea soil.

2. To identify *Strepomyces* sp. by conventional and molecular methods.

3. To screen for antimicrobial compounds.

4. To optimize conditions for maximum production of antibiotic compounds.

5. To characterized antibiotic compounds by Gas Chromatography Mass Spectrometer (GC-MS).

CHAPTER TWO

LITERATURE REVIEW

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LITERATURE REVIEW

2.1. Antibiotic

2.1.1. Definition

Antibiotics are organic compounds with low molecular weight, produced by certain microorganisms (bacteria and fungi) that, at low concentrations, inhibit the growth of other microorganisms. As antibiotic can be synthesized chemically, the term antibiotic is expanded to include not only natural products but also the synthetic compounds (Peláez, 2006).

Natural antibiotics are secondary metabolites, they are not essential for life but enhance survival fitness, and are used against bacteria, fungi, and viruses (Nofiani *et al*., 2012). They are produced in response to certain conditions of environment stress like nutrients depletion and competence (Xu *et al*., 2007).

2.1.2. Historical background

The French word antibiose had been coined to describe antagonistic effects between microorganisms; it was the opposite of symbiosis, Selman Waksman in 1941, defined antibiotic as a "secondary metabolite, produced by microorganisms, which has the ability to inhibit the growth and even to destroy bacteria and other microorganisms, in a very low concentration" (Bentley, 2000).

Early in 1619, the use of plant extractions were used as antimicrobials, mercury then used to treat syphilis, when the era of true chemotherapy began (Gangle, 2005).

Paul Ehrlich hypothesized that dyes could be used as antimicrobial drugs in the early 1900s, based on their differential affinities for various tissues. In 1904, Ehrlich and Shiga discovered that a red dye called trypanrot was effective against trypanosomes (Molbak *et al.*, 1999).

The first truly effective class of antimicrobial drugs was the sulfonamides, discovered by Gerhard Domagk in 1935 (Greenwood, 2000).

Penicillin was first isolated from *Penicillium notatum* in 1928 by Alexander Fleming in 1929, but he was unable to isolate and purify enough drugs to be of any use. By 1941, Ernst Chain, Howard Florey, and Norman Heatley had shown the therapeutic value of penicillin, but they were also unable to produce enough penicillin for commercial use. Collaboration with Andrew Moyer and Robert Coghill at the (United States Department of Agriculture) USDA's Northern Regional Research Laboratory in Illinois led to much higher production yields of penicillin by 1943. After a worldwide search for *Penicillium* strains that could produce more penicillin, Raper and Fennel found a strain of *Penicillium chrysogenum* on a moldy cantaloupe at a local market that was capable of even higher yields of penicillin (Ogunmwonyi, 2010).

A series of different antibiotics were quickly discovered after penicillin came into use. In 1940, Selman Waksman began searching for antibiotic compounds produced by soil microorganisms (Greenwood, 2000).

Till the mid-eighties, almost all groups of important antibiotic were discovered: the antibacterial cephalosporin C, streptomycin, tetracyclines, erythromycin, vancomycin, the antifungal amphotericin B, imidazoles, griseofulvin, strobilurins, the antiviral acicluvir, vidarabine and many other compounds that play a role in therapeutics and agriculture (Gräfe, 2000).

2.1.3. Classification

Antibiotics are classified in several ways, including: 1) spectrum of activity, 2) effect on bacteria and 3) mode of action.

2.1.3.1. Spectrum of activity

Depending on the range of bacterial species susceptible to these agents, antibacterials are classified as broad-spectrum, intermediate-spectrum, or narrow-spectrum.

A) Broad spectrum antibiotics are active against both Gram-positive and Gram-negative bacteria. Examples include: tetracyclines, phenicols, fluoroquinolones, "third-generation" and "fourth-generation" cephalosporins.

B) Narrow spectrum antibiotics have limited activity and are primarily only useful against particular species of microorganisms. For example, glycopeptides and bacitracin are only effective against Gram-positive bacteria, whereas polymixins are usually only effective against Gram negative bacteria. Aminoglycosides and sulfonamides are only effective against aerobic organisms, while nitroimidazoles are generally only effective for anaerobes (Guardabassi and Courvalin, 2006).

2.1.3.2. Effect on bacteria

Because of differences in the mechanisms by which antibiotics affect bacteria, the clinical use of antibiotics may have different effects on bacterial agents, leading to an endpoint of either inactivation or actual death of the bacteria.

A) Bacteriocidal antibiotics are those that kill target organisms. Examples include aminoglycosides, cephalosporins, penicillins, and quinolones.

B) Bacteriostatic antibiotics inhibit or delay bacterial growth and replication. Examples of such include tetracyclines, sulfonamides, and macrolides.

Some antibiotics can be both bacteriostatic and bactericidal, depending on the dose, duration of exposure and the state of the invading bacteria. For example, aminoglycosides, fluoroquinolones, and metronidazole exert concentration-dependent killing characteristics; their rate of killing increases as the drug concentration increases (Guardabassi and Courvalin, 2006).

2.1.3.3. Mechanism of action of antibiotics

The molecular basis of this action is well understood and the main targets are well known. They are classified by the interaction of antibiotics targeting essential cellular functions, the fundamental principle to inhibit bacterial growth. This is a complex process that starts with the physical interaction of the molecule and its specific targets and involves biochemical, molecular, and structural changes, acting on multiple cellular targets such as: 1) DNA replication, 2) RNA synthesis, 3) cell wall synthesis, 4) protein synthesis and 5) cytoplasmic membrane (Chopra *et al.*, 2002; Nikaido, 2009).

A) DNA replication

DNA gyrase (topoisomerase) controls the topology of the DNA by catalyzing the cleavage pattern and DNA binding. This reaction is important for DNA synthesis and mRNA transcription, and the complex-quinolone topoisomerase-DNA cleavage prevents replication, leading to death of the bacteria (Chopra *et al.*, 2002; Nikaido, 2009).

B) Synthesis of RNA

The DNA-dependent RNA polymerase mediates the transcription process and is the main regulator of gene expression in prokaryotes. The enzymatic process is essential for cell growth, making it an attractive target for antibiotics. One example is rifamycin, which inhibits the synthesis of RNA by using a stable connection

with high affinity to the β-subunit in the RNA/DNA channel, separating the active site by inhibiting the initiation of transcription and blocking the path of ribonucleotide chain growth (Chopra *et al.*, 2002; Nikaido, 2009).

C) Cell wall synthesis

The bacterial cell wall consists of peptidoglycan, which helps maintain the osmotic pressure, conferring ability to survive in diverse environments. The peptidoglycan biosynthesis involves three stages: the first stage occurs in the cytoplasm, where low molecular weight precursors are synthesized. In the second stage, the cell wall syntheses is catalyzed by membrane-bound enzymes; and in the third stage the antibiotic acts by preventing the β-lactams and polymerization of the glycan synthesis of cell wall enzymes, acting on transpetidades (Procópio *et al.*, 2012).

D) Protein synthesis

The translation process of mRNA occurs in three phases: initiation, elongation, and termination involving cytoplasmic ribosomes and other components. The ribosome is composed of two subunits (50S and 30S), which are targets of the main antibiotic that inhibits protein synthesis. Macrolides act by blocking the 50S subunit, preventing the formation of the peptide chain: tetracycline in the 30S subunit acts by blocking the access of the aminoacyl tRNA-ribosome; spectinomycin interferes with the stability of the peptidyl-tRNA binding to the ribosome; and streptomycin, kanamycin, and gentamicin act in the 16S rRNA that is part of the 30S ribosome subunit (Procópio *et al.*, 2012).

E) Cytoplasmic membrane rapture

The cytoplasmic membrane acts as a diffusion barrier to water, ions, and nutrients. The transport systems are composed primarily of lipids, proteins, and lipoproteins. Daptomycin inserts into the cytoplasmic membrane of bacteria in a calcium-

dependent fashion, forming ion channels, triggering the release of intracellular potassium. Several antibiotics can cause disruption of the membrane. These agents can be divided into cationic, anionic, and neutral agents. The best known compounds are polymyxin B and colistemethate (polymyxin E). The polymyxins are not widely used because they are toxic to the kidney and to the nervous system.18–20 The latest antibiotic launched in 2006 by Merck (platensimycin) has different mechanism of action from the previous ones, since it acts by inhibiting the beta-ketoacyl synthases I / II (FabF / B), which are key enzymes in the production of fatty acids, necessary for bacterial cell membrane (Procópio *et al.*, 2012).

2.1.4. Source of antibiotics

There are three types of antibiotics these are natural, semi-synthetics and synthetics. The term ‘antibiotic’ has traditionally referred to natural metabolic products of fungi, and bacteria that kill or inhibit the growth of microorganisms. Antibiotic production has been particularly associated with soil microorganisms and, in the natural environment, is thought to provide a selective advantage for organisms in their competition for space and nutrients, although the majority of antibacterial agents in clinical use today are derived from natural products of fermentation (e.g. penicillin, cephalosporins) (Goering *et al.*, 2013).

Since 1960 few truly novel antibiotics have been discovered, although a surprising number of naturally occurring molecular variations on the penicillin structure have emerged. A more fruitful approach, especially with penicillins and cephalosporins, has been to modify existing agents chemically in order to derive semisynthetic compounds with improved properties.

Alongside developments in naturally occurring antibiotics, chemists and microbiologists have also been successful in seeking synthetic chemicals with antibacterial activity. Most have emerged through an indefinable mixture of biochemical know-how and luck rather than the rational targeting of vulnerable processes within the microbial cell. Commercially the most successful synthetic antibacterial agents have been the quinolones (Greenwood *et al.*, 2007).

2.1.5. Bacterial antibiotic resistance

According to Nikaido about (100, 000) tons of antibiotics are produced annually, which are used in agriculture, food, and health. Their use has impacted populations of bacteria, inducing antibiotics resistance. This resistance may be due to genetic changes such as mutation or acquisition of resistance genes through horizontal transfer, which most often occurs in organisms of different taxonomy (Procópio *et al*., 2012).

During the last decade, bacterial infections have increased and new pathogenic diseases have appeared, consequently 25% of deaths worldwide are caused by bacteria (Handelsman, 2005).

Even today, the available antibiotics cannot eradicate bacterial and fungal infections, which still exist especially in developing countries, where health hygiene conditions are poor. Furthermore, the prevalence of bacterial strains resistant to available antibiotics has increased (Hughes, 2003). Among them methicillin resistant *S. aureus* (MRSA) strains are the most common cause of nosocomial infections (hospital acquiring infections) and cause high mortality and morbidity rate (Hughes, 2003). The high prevalence of MRSA bacteria and their resistance to almost all available antibiotics except vancomycin make them a serious problem in hospitals (Khan *et al*., 2000). Recently the susceptibility to

vancomycin has decreased and vancomycin resistant strains have been reported (Hughes, 2003).

Drug resistant strains appeared in other types of bacteria including *Enterococcus, Pseudomonas, Klebsiella* and *M. tuberculosis (*Klein *et al*., 1998).

Considering that continuous and constant search for new antibiotics is an urgent need.

2.1.6. Need for new antibiotics

The world's demand for antibacterials (antibiotics) is steadily growing. Since their discovery in the 20th century, antibiotics have substantially reduced the threat of infectious diseases. The increasing resistance of pathogenic organisms, leading to severe forms of infection that are difficult to treat, has further complicated the situation, as in the case of Carbapenem-resistant *Klebsiella pneumoniae* and other microorganisms (Procópio *et al.*, 2012).

Infections caused by resistant bacteria do not respond to treatment, resulting in prolonged illness and greater risk of death. Treatment failures also lead to long periods of infectivity with high rates of resistance, which increase the number of infected people circulating in the community and thus expose the population to the risk of contracting a multidrug-resistant strain (MDR) (Costelloe *et al.*, 2010).

As bacteria become resistant to first generation antibiotics, treatment has to be changed to second or third generation drugs, which are often much more expensive and sometimes toxic. For example, the drug needed to treat multi-drug resistant *Streptococcus pneumoniae*, *Staphylococcus aureus*, *K. pneumoniae*, and *M. tuberculosis*, can cost 100 times more than first generation drugs used to treat non-resistant forms (Takesue *et al.*, 2010).

In the last decade the World Health Organization (WHO) launched the first global strategy to combat the serious problems caused by the emergence and spread of antimicrobial resistance. Known as the WHO Global Strategy for the Containment of Antimicrobial Resistance, 45 the strategy recognizes that antimicrobial resistance is a global problem that must be addressed in all countries. No nation, however effective, can close its border to resistant bacteria, thus proper control is required in all places (WHO, 2001).

2.1 Marine bacteria as a source of novel secondary metabolites

The oceans are massively complex and consist of diverse assemblages of life forms.

The water column of the oceans contains approximately 10^6 bacterial cells per ml (Hagström *et al*., 2002). Marine bacteria and other marine microorganisms develop unique metabolic and physiological capabilities. These capabilities enable them to survive in extreme habitats and to produce compounds that might not be produced by their terrestrial counterparts. Since 1990, the number of bioactive metabolites from marine bacteria has exponentially increased (Laatsch, 2005).

In the search for new bioactive compounds, microorganisms remain the best choice for natural compound production (Kelecom, 2002). Soil *Streptomyces* were extensively used for this purpose; they produce nearly 10,000 antibiotics and other biologically active compounds (Faulkner, 2002). With the decrease in the rate of discovery of new compounds from terrestrial sources and increased rate of re-isolation of known compounds, marine environment, the poorly explored source have proved to be a rich source for unique natural products. These products exhibited a broad spectrum of biological activities: antibacterial, antifungal, anticancer and anti-inflammatory activities (Kelecom, 2002).

The first antibiotic to be identified and characterized from marine bacteria was produced by *Pseudomonas bromoulitis* (Kouadri, 2010). Later on, several screening programs have led to the identification of many bioactive substances, some of which are under preclinical trials such as anticancer the thiocoraline from *Micromonospora* (Jensen *et al*., 2005a).

Since 1995, number of reports related to the bioactive compounds from marine microorganisms has increased dramatically, and nearly half of these marine natural products are new (Faulkner, 2002; Kelecom, 2002). Almost 250 marine bacterial compounds have been described since 2001. However, the search for marine natural products from microorganisms is still in beginning (Laatsch, 2005; Lam, 2006). Bioactive compounds have been reported from different marine bacteria such as *Vibrio*, *Pseudoalteromonas*, *Bacillus*, *Pseudomonas*, as well as *Streptomyces* (Kouadri, 2010).

Currently, there are three microbial phylogenetic hot spots known for the production of bioactive metabolites in marine environment (Kelecom, 2002; Wagner-Döbler *et al*., 2002).

A) The *Streptomyces*, a group of filamentous Gram- positive bacteria.

B) Cyanobacteria, photosynthetic bacteria known as blue green bacteria.

C) *Myxobacteria*, a distinct cluster within the γ- subclass of the proteobacteria.

2.3.1. Genus *Streptomyces*

The name *Streptomyces* was proposed by Waksman and Henrici in 1943 and classified in the family *Streptomycetaceae* on the basis of morphology and later on cell wall chemo type (Anderson and Wellington, 2001).

The bacteria is aerobic, Gram-stain-positive, non-acid-fast bacteria that form extensively branched substrate and aerial mycelia, chemoorganotrophic, having an oxidative type of metabolism. The vegetative hyphae (0.5–2.0 μm in diameter) rarely fragment. The aerial mycelium forms chains of three to many spores at maturity (Goodfellow *et al.*, 2012). *Streptomyces* is an enormous genus; there were nearly 600 species (Santhanam *et al.*, 2013). Members of the genus are strict aerobes, non-motile, spore forming and filamentous bacteria (Buchanan and Gibbons, 2002). *Streptomyces* species are characterized by their obvious odors resulting from volatiles, which act as chemical signals in bacterial ecology (Dickschata *et al*., 2005). Streptomycetes have a DNA G - C content of 69–78 mol% (Ceylan *et al*., 2008).

Growth occurs at the hyphal apices and is accompanied by branching, thus producing a complex tightly woven matrix of hyphae during the vegetative growth phase. As the colony ages, aerial mycelia (sporophores) are produced which develop into chains of spores (conidia) by the formation of crosswalls in the multinucleate aerial filaments. This is followed by separation of individual cells directly into spores (Anderson, and Wellington, 2001).

In general, this group of bacteria is widely distributed in nature, usually inhabits soil as saprophyte (1-20 % of cultural population). Cell wall contains LL.diaminopemilic acid (LL. DAP) in its peptidoglycan, which is a characteristic feature. Streptomycetes are well known for production of antibiotics such as streptomycin, chloromphenicol, and erythromycin (Kouadri, 2010).

Members of the genus *Streptomyces* undergo a complex life cycle, Strains belonging to the genus *Streptomyces* may produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia. In addition, colored

diffusible pigments may also be formed. Note that the production of pigments largely depends on the medium composition and cultivation conditions.

Many strains produce one or more antibiotics, the metabolism is oxidative and chemoorganotrophic. The catalase reaction is positive, and generally, nitrates are reduced to nitrites. Most representatives can degrade polymeric substrates like casein, gelatin, hypoxanthine, starch and also cellulose. In addition, a wide range of organic compounds is used as sole sources of carbon for energy and growth. The optimum temperature for most species is 25–35°C; however, several thermophilic and psychrophilic species are known. The optimum pH range for growth is 6.5–8.0 (Dworkin *et al.*, 2006).

2.3.2. Classification and identification

The classification of streptomycetes was originally based on morphological and biochemical characterisation, later on physiological tests (Saravanamuthu *et al*., 2010; Anderson and Wellington, 2001).

Serological methods, phage typing and protein profiling (Anderson, 2001) have also been used in their classification. The application of genetic methods, such as DNA-DNA re association (Lou *et al*., 2011) and 16S rRNA gene sequence analysis (Goodfellow *et al*., 2012; Stackebrandt *et al*., 1992; Anderson, 2001) has partly confirmed the phenotypic classification, but this approach has also provided new information (Rintala, 2003).

Numerous classifications were devised to accommodate the increasing number of *Streptomyces* species, most of them based on a few subjectively chosen morphological and pigmentation properties. Gause recognized 15 groups or series, distinguished by aerial and substrate mycelium color. Pridham defined 42 groups by sporophore morphology and aerial mycelium color. Shinbou describes 13

groups based on sporophore morphology melanin and nitrate production (Al-Gafadri *et al.*, 2007), while Waksman classified over 250 species into 16 series based on aerial and substrate mycelium color, spore chain morphology, melanin production and number of other properties including proteolysis (Odat, 2004).

In the 1960s, a set of standardized methods for the description of *Streptomyces* cultures for taxonomic purposes was established as part of the International *Streptomyces* Project (ISP). Using these methods, a *Streptomyces* species can be described on the basis of spore color, spore chain and spore surface morphologies, coloration of substrate and aerial mycelia, production of soluble pigments, and carbohydrate utilization profile (Ritacco, 2007).

Streptomyces were divided into nine immune groups, but the sharing of multiple cytoplasmic antigens by numerous *Streptomyces*, which was documented by Taylor, made it difficult classifies these microorganisms by immunological methods (Odat, 2004).

Chemotaxonomic methods e.g. (analysis of whole cell fatty acid composition, the determination of diaminopimelic acid isomer in the cell wall, etc.) are those that determine relatedness or taxonomic grouping based on cellular phenotype. Typically, these methods are based on analysis of the chemical makeup of the bacterial cell. These chemotaxonomic techniques are important for distinguishing the *Streptomyces* form other genera but, not useful for identification of species within the genus (Anderson and Wellington, 2011).

Advances in molecular biology have revolutionizes the science of microbial taxonomy. Methods involving nucleic acid fingerprinting and gene sequence comparisons have become routine practice in species identification. DNA-DNA hybridization has long been used to determine genetic relatedness between

Streptomyces species, by measuring the percent homology of total chromosomal DNA between strains (Labeda, 1992).

Analysis of the 16S ribosomal RNA gene sequence (16S rRNA) originally championed by Carl Woese as a means for understanding microbial evolution and relationships has become a universal tool in Streptomycete taxonomy. Because this gene is highly conserved among bacteria, yet with distinctive variations, 16S rRNA sequence comparisons are widely used in genus, species, and strain identification. The complete 16S gene sequence can be easily amplified using polymerase chain reaction (PCR) and primers derived from highly conserved regions at both ends of the gene. While automated sequencing equipment can efficiently sequence the PCR product (Ritacco, 2007).

2.3.3. Colonial characteristics

Streptomycetes have many differential colonial features, such as pigmentation of spores, substrate mycelium, and diffusible exo-pigments, together with the morphology of colonies and the texture of the aerial mycelium. The production of different pigments has been widely used in classification and identification, but it is important to mention that colony morphology is too variable for use as a taxonomic character. One feature, widely used in streptomycete taxonomy, is spore mass color. *Streptomyces* species were assigned to seven color series: Blue, Gray, Green, Red, Violet, White, and Yellow. In a later survey, the series were extended to accommodate additional colors. The color of the spore mass is still useful, but its determination may be difficult, because the color can be influenced by factors such as the medium, growth regime, and age of the culture (Goodfellow *et al.*, 2012).

2.3.4. Pathogenesis

Members of *Streptomyces* are known for their low pathogenicity, *S. somaliensis* has been identified as one of the causal agents of actinomycetoma in different parts of the world (Datta *et al*., 2012).

Also the species *S. griseus*, *S. albus*, *S. rimosus*, *S. lavendulae*, *S. violaceoruber*, and *S. coelicolor*, have been isolated from clinical samples (Rintala, 2003; McNeil and Brown, 1994). Streptomycetes have been isolated from sputum, wounds, skin, blood, brain, tonsils, and dental caries (McNeil and Brown, 1994).

A serious pulmonary disease, hypersensitivity pneumonitis, or extrinsic allergic alveolitis, with farmer's lung disease being the most well-known example, can be caused by thermophilic actinomycetes, including some members of the genus *Streptomyces* (Rintala, 2003).

Skin reactions have also been reported in farmers who have become sensitized to these microorganisms (Spiewak *et al*., 2001).

2.3.5. Occurrence in aquatic and marine environment

Streptomycetes have been isolated from fresh water as well as marine environments, although, it has been a subject of debate, whether they are indigenous, or have been washed off from the surrounding soils (Rintala, 2003). (Moran *et al*., 1995) showed that in coastal marsh sediment, streptomycetes accounted for 2-5 % of the microbial community, and were an indigenous population.

Occasionally, streptomycetes also grow in drinking water reservoirs affecting the water quality by causing earthy odors, which are due to their production of volatile secondary metabolites (Rintala, 2003).

In contrast to terrestrial *Streptomyces*, marine and estuarine sediment counterparts have been ignored for a long time, due to the previous debates that these bacteria believed to be of terrestrial origin; they may have been transported (washed) into the sea where they remain dormant until isolation (Imasda, 2005).

Diversity of *Streptomyces* in the marine ecosystems such as coral reef, sediments, plants, and invertebrates have been studied. They have shown to form stable and persistent populations in marine environment and account for approximately 10 % of the bacteria colonizing marine aggregates (Mincer *et al*., 2002; Bull *et al*., 2005; Lam, 2006).

Beside the production of antibiotics, *Streptomyces* play an important role in the marine environment including formation of humic substances, transformation of aromatic pollutants and degradation of natural polymers (Moran *et al*., 1995, Bull *et al*., 2005).

They contribute to the breakdown and recycling of organic compounds. Their ability to degrade macromolecules such as starch, casein and protein, the production of antimicrobial metabolites, and the formation of spores which are heat and desiccation resistant make these bacteria a good candidate as potential probiotic strains to control vibriosis in plants (You *et al*., 2005).

2.3.6. Producing for secondary metabolites

Microbial metabolism can divide into two main categories: primary and secondary metabolism, which might be termed general and special metabolism, respectively. Primary metabolism, essentially, is identical in all living forms. It involves an interconnected series of enzyme-mediated catabolic and anabolic pathways, which provide biosynthesis intermediates, energy and convert precursors into essential macromolecules. The reaction of primary metabolism are finely balanced, and

metabolic intermediates other than those necessary for cell survival rarely accumulate (Gharaibeh, 2001).

According to one definition, microbial secondary metabolites are substances that are not needed for the growth or other essential processes in the cell. Secondary metabolites are mainly produced by microbial genera inhabiting soil and undergoing morphological differentiation, such as actinobacteria, bacilli and fungi (Rintala, 2003).

There are over 23 000 known microbial secondary metabolites, 42 % of which are produced by actinobacteria, 42 % by fungi, and 16 % by other bacteria (Lazzarini *et al*., 2000).

Microbial secondary metabolites are the low molecular mass products of secondary metabolism. They include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, antitumor agents and growth promoters of animals and plants. They have a major effect on the health, nutrition and economics of our society (Ogunmwonyi, 2010).

The antibiotic discovered in a *Streptomyces* species was in 1944 by Selmon Waksman who described the production of streptomycin by *Streptomyces griseus*. By the year 1948 Brwon and Hazan identified *Streptomyces norsei* and discovered the production of nystatin by this microorganism, which is the famous antifungal agent. Later, the rapamycin, an immunosupperessive drug produced by *Streptomyces hygroscopicus*, was discovered and trials began concerning the use of rapamycin as a potential drug against cancer, bacterial infections, and as immunosupperassive agent in organ transplantation (Odat, 2004).

The streptomycetes are very active producers of secondary metabolites. Out of the approximately 10000 known antibiotics, 45-55 % is produced by streptomycetes.

The secondary metabolites produced by them have a broad spectrum of biological activities; e.g. antibacterial (streptomycin, tetracycline, chloramphenicol), antifungal (nystatin), antiviral (tunicamycin), antiparasitic (avermectin), immunosuppressive (rapamycin), antitumor (actinomycin, mitomycin C, anthracyclines), enzyme inhibitory (clavulanic acid), diabetogenic (bafilomycin, streptozotocin) (Rintala, 2003).

The following are some secondary metabolites produced by marine *Streptomyes:*

A) Quinones

Streptomycetes were rich in biologically active quinones, and many marine quinones were described, such as the complex C-glycosides himalomycins A and B (Maskey *et al.*, 2003), anthraquinones with the rare fridamycin E chromophor, a precursor of the anthracycline antibiotics they were isolated from *Streptomyces* sp.

B) Polyenes

Polyene macrolides were typical domains of *Streptomyces* and rare actinomycetes. All compounds of this type were isolated from *Streptomyces* (Laatsch, 2005). Aureoverticillactam was a novel 22- atom macrocyclic lactam from marine *Streptomyces aureoverticillatus*; it showed anticancer activity (Mitchell *et al*., 2004).

C) Indoles

Three new indoles 15 – 17**,** were isolated from *Streptomyces sp.* from Mexican marine invertebrate; they exhibited cytotoxic activity (Sa'nchez Lo'pez *et al.*, 2003).

D) Polyketides

Two new polyketides, actinofuranones A (22) and B (23) were isolated from the culture broth of marine *Streptomyces sp*. (Cho *et al*., 2006). Additionally**,** SBR, 22 was antibacterial polyketide that exhibited activity against MRSA (Sujatha *et al.*, 2005).

E) Macrolide

Chalcomycin B (27a) was a new macrolide, isolated from marine *Streptomyces* sp. B7064, together with chalcomycin A (27b). They showed activity against *S. aureus*, *E. coli*, and *B. subtilis* (Maskey *et al*., 2002).

F) Piericidins

Two new piericidins C7 and C8 (24), which exhibited selective cytotoxicity were isolated from marine *Streptomyces* (Hayakawa *et al*., 2007).

2.3.7. Gene encoding for secondary metabolites

Genes encoding enzymes responsible for the synthesis of secondary metabolites are usually clustered on a contiguous piece of DNA. Genomic studies indicate that the genetic potential for producing secondary metabolites is not uniformly distributed within the bacterial world. In fact, most bacterial genomes lack any detectable gene cluster for secondary metabolism (Solanki *et al.*, 2008). On the other hand, *Streptomyces coelicolor* (Bentley *et al.*, 2002; Busti *et al.*, 2006) and *Streptomyces avermitilis* (Ikeda *et al.*, 2003; Omura *et al.*, 2001; Busti *et al.*, 2006), each possess more than 20 gene clusters devoted to the synthesis of secondary metabolites.

2.3.8. *Streptomyces* producing antibiotics

Actinomycetales were the most studied organisms by the scientists working in the field of natural products. Over 10000 compounds were isolated from this group, mainly from species of the genus *Streptomyces* and rare actinomycetes (AL-Zereini, 2006).

Despite the success of the discovery of antibiotics, and advances in the process of their production, infectious diseases still remain the second leading cause of death worldwide, and bacterial infections cause approximately 17 million deaths annually, affecting mainly children and the elderly. The history of antibiotics derived from *Streptomyces* began with the discovery of streptothricin in 1942, and with the discovery of streptomycin two years later, scientists intensified the search for antibiotics within the genus. Today, 80% of the antibiotics are sourced from the genus *Streptomyces*, actinomycetes being the most important (Watve *et al.*, 2001).

2.4. Biosynthetic pathways of bioactive compounds

Strpetomyces strains have a large linear chromosome (8.7 M b.p). The number of genes found in *S. coelicoler* is 7825 genes, which is twice the number of genes found in *E. coli*. The individual strain harbor multiple antibiotic gene clusters, the number of genes encoding bioactive compounds in *Streptomyces* exceeds the genome of smallest bacterium; this is responsible of the high degree of natural products diversity in these microorganisms (Weber *et al*., 2003).

These genes are responsible to encode a large number of medically important metabolites called polyketides and peptides, which are synthesized by polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs).

Polyketides are class of natural products with large structural diversity (macrolides, aromatics, polyethers and polyenes) that exhibit a wide range of activities

(Hopwood, 1997). Non-ribosomal produced peptides such as erythromycin and penicillin are synthesized by non-ribosomal peptide synthetases (NRPSs) (Kouadri, 2010).

2.5. 16S rDNA and molecular identification system

Ribosomes are indispensable components of the protein synthesis apparatus; they are large ribonucleoprotein structures that are responsible for the translation of the genetic code. Ribosomes are divided, both structurally and functionally, into large and small subunits, these subunits are defined by their apparent sedimentation coefficients, characterized by the Svedberg unit (S), which reflect the rate at which a molecule sediment in a solvent. Both subunits are composed of rRNA and proteins called (r- proteins). In eukaryotic cells the ribosomes (80s) is divided into large subunit (60s) and small subunit (40s), on the other side, bacterial (prokaryotic cell) ribosome (70s) is divided into large subunit (50s) and small subunit (30s). rRNA is encoded by what is called ribosomal RNA genes, or ribosomal DNA (rDNA) (Gharaibeh, 2001).

The 16S rRNA gene has been widely used for phylogenetic and diversity studies for several reasons. It consists of conserved and variable regions, which allows the development of primers and probes with variable levels of specificity. The conserved regions carry information about phylogenies at the higher taxonomic levels, since they have evolved slowly and are highly similar among the different taxa, whereas the variable regions have undergone more mutations during evolution, and are more useful for classification at the intraspecies level. The rRNA genes are essential, and therefore present in all organisms (Rintala, 2003). Comparisons of the sequences between different species suggest the degree to which they are related to each other, a relatively greater or lesser difference between two species suggests a relatively earlier or later time in which they shared

a common ancestor. The 16S rRNA has properties, which predestine it as a universal phylogenetic marker. There are regions on the 16S rRNA that are quite conserved and others, which are variable. Hence genotypic classification based on nucleotide sequence comparison of 16S rRNA genes has become available as additional taxonomic tool (Stackebrandt *et al*., 1991).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design

This study is carried out in Port Sudan, Sudan coast, and the experimental work was done in the Research Laboratory, College of Medical Laboratory Science, Sudan University of Science & Technology. The samples were collected from various depths and covered the site of collection. All samples were collected with sterile container and store at 4°C, two samples were taken for each depth.

3.2. Collection of samples

3.2.1. Description of sampling site

The Sudanese Red Sea coast (Fig 1) is about 750 km long. It is located at the central part of the Red Sea. The area is located on the western side of the central Red Sea, at Latitude 19º38 North and Longitude 37º13 East. It is particularly famous of its exceptionally unique and varied habitats with rich biological communities, especially coral reefs, its productive and highly sensitive coastal and marine habitats (coastal halophytes, mangroves, sea grasses / algal beds and coral reefs) (Rasul and Stewart, 2015).

Fig. 1. Sudan map shows the coast of Port Sudan

3.2.2. Site of collection and size of samples

Marine sediment samples were collected from Port Sudan coast (Falamingo area), Sudan (Lat.19° 39N, Long. 37° 14E) Fig (2).

Fig. 2. Site of samples collection (Falamingo area) coast, Port Sudan

Fifty (50) soil sediment samples were collected from several different depths ranged from 0.5 to 20 meter. Sterile containers were used for collection. All samples were air dried in laminar hood overnight and then ground lightly for preventing contaminations and ensuring a mass of spores, then store at 4°C until used (Mincer *et al*., 2002). The samples and its depths were presented in table (1).

3.3. Preparation for the primary isolation

3.3.1. Bacteriological culture media

The following culture media were used during this study; Starch casein agar (SCA), International Streptomyces Project No.7 (ISP No.7), Mueller Hinton Agar (MH), Nutrient Agar (NA), Soyabean Casein Digest Medium (Tryptone Soya Broth) (SCDM) and Nutrient broth (NB). All of them were obtained from Hi Media Lab. Pvt. Ltd. Mumbai, India. Appendix (1).

3.3.2. Sterilization

All culture media were sterilized by autoclave at 121 °C in 15 lbs. pressure for 15 min. Glass wares were sterilized using hot air oven at 160°C for 60 min.

3.4. Bacteriological techniques

All bacteriological techniques were done under aseptic condition in the cabinet under laminar flow near Bunsen burner.

3.5. 0.5 McFarland standard

This reagent was prepared according to Collee *et al*., (2007), as follows; 1 mol. of solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99% of water and mixed well. a 1.175 % w/v solution of barium chloride was prepared by dissolving 1.175g of dehydrated barium chloride in 100ml of Distilled Water 0.5 McFarland standard was prepared by adding 0.5ml of the 1.175% barium chloride solution to 99.5 of 1 mol. sulphuric acid solution, and mix well. The solution was kept in sealed container and stored in room temperature and in dark condition until used.

Small volume of the standard solution was transferred to screw-cap bottle and stored in dark place at 20-28 °C.

This standard was used in antibiotic sensitivity test for preparation of the inoculums and adjusting to the standard number of bacteria to (1.5×10^{8}).

3.6. Standard strains

The standard strains of (*S. aureus*) ATCC 25923 and (*E. coli*) ATCC 25922 were obtained from the National Laboratory for Public Health, Khartoum. They were used as indicator organisms throughout the study.

3.7. Preparation of soil samples

The soil samples were sieved to remove debris and then 1 g of sieved soil was add to 4 ml of sterile seawater, heated for 6 min at 55°C to reduce non-spore forming bacteria, shaken and diluted 1:4 in sterile seawater.

3.8. Preparation and inoculation of culture media

Inside laminar air hood from each prepared samples aliquots 1ml of the samples were transferred and spread by L-shape glass onto the surface of isolation media (Starch casein agar medium and International *Streptomyces* Project medium No.7 (ISP No.7) which prepared and sterilized at 121 °C in 15 lbs. pressure for 15 min). To prevent bacterial and fungal growth the autoclaved culture media were supplemented with rifampicin 5µg/ml and nystatin 25µg/ml near Benson burner and mix gently (Panchagnula and Terli, 2011). Plates were incubated at 30°C for 7 days (Mincer *et al*., 2002; Jensen *et al*., 2005b).

3.9. Culture Characteristics

All isolates were cultured on two agar media (CSA & ISP No.7) to observe the aerial mycelium growth & color of aerial mycelium.

Table 1. Sample codes and depths

No.	Sample Code	Depth in Meters
1.	PS 1	6
2.	PS 2	7
3.	PS 3	8
4.	PS 4	2
5.	PS 5	7
6.	PS 6	9
7.	PS 7	6
8.	PS 8	5
9.	PS 9	4
10.	PS 10	5
11.	PS 11	9
12.	PS 12	4
13.	PS 13	10
14.	PS 14	12
15.	PS 15	11
16.	PS 16	10
17.	PS 17	11
18.	PS 18	15
19.	PS 19	15
20.	PS 20	5
21.	PS 21	8
22.	PS 22	8
23.	PS 23	0.5
24.	PS 24	2
25.	PS 25	2
26.	PS 26	1
27.	PS 27	1
28.	PS 28	4
29.	PS 29	1.5
30.	PS 30	0.5
31.	PS 31	1.5
32.	PS 32	2
33.	PS 33	3
34.	PS 34	3
35.	PS 35	4
36.	PS 36	5

37.	PS 37	5
38.	PS 38	2
39.	PS 39	1
40.	PS 40	1
41.	PS 41	18
42.	PS 42	18
43.	PS 43	20
44.	PS 44	20
45.	Ps 45	20
46.	PS 46	8
47.	PS 47	8
48.	PS 48	12
49.	PS 49	12
50.	PS 50	5

3.10. Isolation of promising *Streptomyces* sp.

The obtained colonies were examined for antibacterial activity. This was done by Agar Overlay Technique. Five ml. of molten N.A (Temp. ~ 43°C) was inoculated by 0.1µl of bacterial suspension that prepared according to 0.5 McFarland Standard. The contents were mixed and poured over the colonies that isolated. Zones of inhibition around the colonies were recorded after incubation at 37°C for 24 hrs. (Srinu *et al.*, 2013). Only promising isolates with good inhibition zones were considered. Colonies of the promising isolates were picked up and sub-cultured on CSA and incubated at room temperature for 3 to 7days. The obtained growth was stored in refrigerator at 4°C for further investigations.

3.11. Maintenance of isolated *Streptomyces*

The cultures of *Streptomyces* isolates for routine work were maintained in starch casein agar slants at 4°C. The isolates were sub-cultured every three months. For long term storage, they were stored in the mixture of 80% NB and 20% glycerol at -20°C (Kouadri, 2010).

3.12. Identification of *Streptomyces*

3.12.1 Conventional methods

Promising isolates cultural characteristics including colony morphology and color of aerial mycelium were identified according to Bergey's manual of determinative bacteriology and recorded (Buchnan and Gibbons, 2002).

3.12.2. Molecular characterizations

3.12.2.1. DNA extraction

Streptomyces isolates were inoculated in SCDM prepared with 75% seawater and 25% distilled water (D.W), and incubated at 30°C for 72 hrs. Genomic DNA was extracted using Jena Bioscience bacterial DNA isolation and purification kit (Jena Bioscience Laboratories, GmbH, Germany) according to the manufacturer instructions.

3.12.2.2. Gel electrophoresis of extracted DNA

The purity of the extracted DNA was determined by running the DNA sample on 1.5% gel agarose (Sambrook *et al*., 1989).

3.12.2.3. Preparation of 10 X TBE buffer

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

3.12.2.4. Preparation of 1X TBE buffer

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

3.12.2.5. Preparation of ethidium bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

3.12.2.6. Preparation of DNA loading dye

Three ml of glycerol were added to 7ml of D.W and 2.5 g of bromophenol blue and dissolved into 100 ml D.W. The mixture was used as loading dye.

3.12.2.7. Preparation of agarose gel

Amount of 1.5 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer (AppliChem), then was cooled to 55°C in water bath, then, 5 µl of (10mg\ml) Ethidium bromides were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

3.12.2.8. Visualization of the DNA

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface, 5 µl of PCR products from each samples was mixed with 0.5 µl of loading dye and then added to wells of electrophoreses, 5 µl of DNA ladder (marker) was mixed with 0.5 µl of loading dye and were added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 100 V, 500 mA, UK). The electrophoresis was carried out at

75Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec – UK).

3.12.3. PCR-based analysis of *Streptomyces* genes

3.12.3.1. PCR primers

Two pairs of primers were used (StrepB "forward" and StepE "reverse") and (StrepB "forward" and StrepF "reverse") were designed according to the published data for the genus *Streptomyces* 16SrRNA gene. The primers were synthesized by (Macrogen, Korea) (Table 2).

Table 2. Primers sequences used for detection of genes from *Streptomyces* isolates

Target Gene	Primer Name	Sequence 5′→ 3′	Position	Reference
16s rDNA	StrepB (F)	ACAAGCCCTGGAAACGGGGT	139–158	Malinova *et al*, 2014
16s rDNA	StrepE (R)	CACCAGGAATTCCGATCT	657–640	Malinova *et al*, 2014
16s rDNA	StrepF (R)	ACGTGTGCAGCCCAAGACA	1212–1194	Malinova *et al*, 2014

3.12.3.2. PCR amplification

The amplification was done using CONVERGYS® Ltd peltier thermal cycler (Germany). DNA amplifies of 16S rDNA gene was done using Maxime PCR PreMix kit (iNtRON, Korea).

DNA extracted from the promising *Streptomyces* with antibacterial activity was amplified using two pairs of primers specific for the genus *Streptomyces*. All of the strains formed an amplification product of 519 bp with the primers StrepB/ StrepE, and of 1074 bp with the pair StrepB/StrepF.

The primer pairs StrepB/StrepF and StrepB/StrepE amplified 1070 and 520 bp fragments, nucleotides 139 -1212. The PCR were programmed as follows: after the initial denaturation for 5 min at 98°C, 30 cycles of denaturation (1 min at 95°C), primer annealing 40 s at 57°C and primer extension (2 min at 72°C) were performed. A final extension at 72°C for 10 min followed. Multiplex PCR was done for the two primers as one reaction.

3.12.4. Sequence similarities and phylogenetic analysis

The PCR products obtained were sent to Macrogene (Korea) for standard sequence DNA analysis (Appendix 2). The same primers as above and an automated sequencer were used for this purpose. The sequence was compared for similarity level with the reference species of genus *Streptomyces* contained in genomic database banks, using the NCBI Blast available at the ncbi-nlm-nih.gov Web site. The phylogenetic tree of the 16S rRNA sequence was submitted to the NCBI bank data.

3.13. Screening for antibacterial activity of *Streptomyces*

3.13.1. Agar wells diffusion method

Antimibactial activity was determined using agar well diffusion method (Augustine *et al.*, 2005). Standard bacterial strain *S. aureus* and *E. coli* was used as indicator strains. *Streptomyces* isolates were cultured in CSDM prepared with 75% seawater & 25% D.W. The cultures were incubated on a rotary shaker for 7 days at 30 °C. By using sterile Millipore filter (Millipore Millex-HV Hydrophilic PVDF

0.45 μm) the broth medium was filtrate in sterile test tubes tubes. Test microorganism suspension, with a turbidity equivalent to that of 0.5 McFarland standards, were seeded on the MH agar plates. Wells were made in these plates using flaming sterilized cork-borer (7mm diameter), and filled with 50 μl of cell free filtrate. The plates were then incubated at 37 °C for 24 hours. The inhibition zones around the wells were measured (mm).

3.13.2. Perpendicular streak method

Antimicrobial activity was done by perpendicular streak method on nutrient agar plates. The test organism for primary screening used *S. aureus* and *E. coli*. Each of the isolates was streaked as a straight line on nutrient agar medium and incubated at 30 °C for 7 days. After the 7th day, test pathogenic bacteria were streaked at right angle, but not touching each other, and then incubated at 30-37 °C for 24 hrs. If the organism is susceptible to the antibiotic compound produced by the *Streptomyces*, then it will not grow near the *Streptomyces*. Those *Streptomyces* isolates showed maximum inhibition were selected for secondary screening (Rana and Salam, 2014).

3.14. Optimization of conditions

Streptomyces culture (PS 1 & PS 28) showed the high antibacterial activities were chosen to determine the optimal conditions for maximum production. Antimicrobial activity was carried out by disc diffusion method using nutrient agar medium and *S. aureus* and *E. coli* as indicator organisms.

3.14.1. Incubation period

100 ml of broth medium CSDM was prepared in 250 ml Erlenmeyer flasks with 75% seawater then inoculated with *Streptomyces* isolates PS1 and PS28, incubated for intervals 2, 4, 5, 6, 7, 8, 9, 10 and 12 days at 30 °C with shaking, and

antimicrobial activity was determined by agar well diffusion method at each interval.

3.14.2. pH

To determine the optimum pH, 250 ml Erlenmeyer flasks each containing 100 ml of broth medium was adjusted to pH values 6.5, 7, 7.5 & 8 using pH meter. Each flask was inoculated with *Streptomyces* isolates (PS1 and PS28). The flasks were incubated at 30 °C on a rotary shaker; antimicrobial activity was determined by agar well diffusion method for each pH value.

3.14.3. Effect of nitrogen source

Different soybean meal concentration (1.5, 2.5, 3.5 and 5 g/L) were added to 250 ml Erlenmeyer flasks each containing 100 ml of broth medium CSDM inoculated with *Streptomyces* isolates (PS1 and PS28) and incubate for 7 days at 30 °C and the antimicrobial activity was measured by agar well diffusion method for each concentration (Bhavana *et al*., 2014).

3.14.4. Effect of dark and light conditions

Two 250 ml Erlenmeyer flasks each containing CSDM medium was inoculated with *Streptomyces* isolates (PS1 and PS28), then incubate for 7 days 30 °C in dark and light conditions. Agar well diffusion method was done to test the antimicrobial activity for each light and dark condition.

3.14.5. Effect of temperature

Streptomyces isolates (PS1 and PS28) were inoculated in 250 ml Erlenmeyer flask containing 100 ml. of CSDM medium and incubate at different temperature degrees ranging from 20, 25, 30 and 40 ºC for 7 days and antimicrobial activity were examined by agar well diffusion method for each temperature degree.

3.14.6. Effect of trace elements

Different concentration of K_2HPO_4 (1, 1.5, 2.5 and 4 g/L) and $MgSO_4.7H_2O$ (0.5, 1, 1.5 and 2 g/L) were tested for optimum concentration in 250 ml Erlenmeyer flask containing 100 ml. of CSDM medium incubated for 7 days at 30°C in antimicrobial activity was tested by agar well diffusion method for each concentration (Bhavana *et al*., 2014).

3.15. Preparation of culture medium under optimum conditions

Several of 250 ml Erlenmeyer flask containing CSDM medium for each were inoculate with PS1 and PS 28 isolates according to previous cultural conditions and used for the extraction and identification of antibacterial compounds.

3.16 Antibacterial metabolites

Streptomyces PS 1 and PS 28 were selected for the extraction of active compounds since it showed the highest activity against *S. aureus* & *E. coli*. The selected antagonistic *Streptomyces* (SP 1 & SP 28) isolates were inoculated into CSDM, and incubated at 30ºC on a shaker (300 rpm) for seven days at 30°C. After fermentation filtrate was separated by centrifugation at 5000 rpm for 10 min. and the supernatant was filtrate through Millipore filter (Millipore Millex-HV Hydrophilic PVDF 0.45 μm) (Remya and Vijayakumar, 2008). The filtrate was transferred aseptically into a conical flask and stored at 4°C for further assay.

3.16.1. Extraction

The culture filtrate (200 ml) was extracted 3 times with solvent ethyl acetate. The solvent was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 20 minutes. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase using separating funnel. Ethyl acetate layer was concentrated by

evaporation to dryness at 40ºC and residue obtained was purified using methanol to give (0.8 g) of brown crude extract (Ahmed, 2007).

3.16.2. Determination of crude extracts activity

The crude extract of PS1 and PS 28 were dissolved with the ethyl acetate and were tested for antimicrobial activity using agar-well diffusion method by spreading 25µl of *S. aureus* and *E. coli* inoculate which match to McFarland standard solution on Muller-Hinton agar, the wells were dug by the help of sterile 6 mm cork-borer and loaded with the 100 µl of dissolving crude compound, then plates were left at 4 °C for 12-16 hours and incubated overnight at 37°C. Inhibition zone of test microorganisms around the wells were measured by ruler (Augustine *et al*., 2005; Khan and Patel, 2011).

3.17. Identification of antibacterial compounds

3.17.1. Thin layer chromatography (TLC)

To visualize the number of compounds present in the extract of isolates (PS 1 and PS 28), thin layer chromatography (TLC) was performed. Aluminum plates pre-coated with silica gel (20×20 cm, 0.25 mm Alugram® SIL G/UV 254, Macherey & Nagel, Duren) and two mobile phases (ethyl acetate : methanol 6:4 and Petroleum ether : Chloroform 1:1) (Attimarad *et al*., 2012).

Chromatograms were observed under UV light, fractions of the isolates retention factor (rf) value were measured and the ratio calculated.

3.17.2. GC-MS analysis

The active ethyl acetate crude was subjected to Gas Chromatography-Mass Spectrometer technique (Joel and Bhimba, 2012). The mass spectrum was recorded by using GC.MS-Shimadzu QP2010 Ultra, Japan (Appendix 3).

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

4.1. Isolation and identification

A total of fifty (50) soil sediment obtained from various depths were investigated for antibiotic-producing *Streptomyces*. The soil consistencies were varying from muddy to sandy and with color ranged from white to dark brown. Upon isolation, the colonies were small (1-10 mm diameter) creamy or leathery; initially with smooth surface but later developed a weft of characteristic branching aerial mycelia. The mycelia colors mostly were white followed with grey, yellow, brown and green.

Adoption of Agar Overlay Method showed that only 21 (42%) isolates were considered as promising organisms. Colonial zones of inhibition raged from 18 to 30 mm (Fig 3 & 4).

Genomic DNA was extracted from each of the promising isolates (n=21). Photometric method revealed large amount and good quality of genomic DNA in all isolates. PCR and Multiplex PCR techniques showed that nine (43%) out of the 21 isolates were *Streptomyces* species. The isolates showed positive results with 520 pb and 1070 pb in the two techniques (Fig 5 & 6). The nine *Streptomyces* were coded as follows; PS1, PS5, PS10, PS13, PS20, PS21, PS23, PS24, and PS28).

In this study the NCBI BLAST search analysis for the 16S rRNA gene sequences for the nine isolates (PS 1, PS 5, PS 10, PS 13, PS 20, PS 21, PS 23, PS 24, and PS 28) showed various results with the NCBI nucleotide database using BLAST (blastn), all isolates showed relation to the *Streptomyces* species (Table 3). According to blast results these isolates could be new strains.

Study on culture properties of the nine isolates on various culture media exhibited different colors in the aerial mycelia and spores (Table 4) and (Fig. 16). The antibacterial activity was done by agar wells diffusion method. The results showed that the isolates PS1 and PS28 were broad spectrum producers among the nine isolates (Table 5).

4.2 Optimization of nutritional and environmental conditions

The two isolates PS 1 & PS 28 were investigated to various nutritional and environmental conditions. These were pH, Light, Incubation period, temperature, Nitrogen source and Trace elements.

4.2.1 Effect of nitrogen source

Maximum antibacterial production was observed among several concentration of soyabean meal, and shown in (Fig. 18). The antibacterial activity was increasing continuously from 0.5g/L to 2.5g/L of soyabean meal concentration. Further increase in the soyabean meal concentration showed a gradual decrease in the production of antimicrobial compounds.

4.2.2. Effect of pH values

The strains of *Streptomyces* showed highly activity at pH with value between (7 to7.5) (Fig. 19).

4.2.3. Effect of temperature

The optimum activity of strains shows highly activity at 30°C, while the activity was decreased at lower temperature (Fig. 20).

4.2.4. Effect of incubation period

The activity of strains against pathogenic microorganisms was at high peak in the range of 5-7 days of incubation, while it is decreased with other periods (Fig. 21).

4.2.5. Effect of dark and light condition

The activity of antibacterial compounds exhibited there is no significant variance between dark and light environment.

4.2.6. Effect of $MgSO_4.7H_2O$ concentration

Optimum concentration of $MgSO_4.7H_2O$ required for the production of antimicrobial compounds was 1g/L. Further increase in $MgSO_4.7H_2O$ concentration showed a gradual decrease in the production of antimicrobial compounds (Fig. 22).

4.2.7. Effect of K_2HPO_4 concentration

Optimum K_2HPO_4 concentration required for the production of antimicrobial compounds was 2.0g/L. Further increase in the K_2HPO_4 concentration showed a gradual decrease in the production of antimicrobial compounds (Fig. 23).

4.3. Extraction of antibacterial compounds from isolates PS1 & PS28

Antimicrobial components purified by solvent extraction procedure of the isolates PS1 and PS28 gave brown gummy compounds were assessed for their antibacterial properties by agar well diffusion method.

4.4. Antibacterial activity of the PS1 & PS28 extract

The antibacterial activity against *S. aureus* and *E. coli* by agar well diffusion method of crude extracted was showed in (Table 6) and (Fig. 24 & 25).

4.5. Thin layer chromatography (TLC)

The Crude extracts by ethyl acetate were separated by TLC plates for the strain PS1 give three fractions (A,B & C), while the strain PS28 give two fractions (A&B) (Fig. 26 & 27). The Retention Factor (rf) value for the strain PS1=0.7cm, while the value for PS28=0.6cm.

4.6. Identification of antibacterial compounds

The GC-MS analysis showed that PS1 have 54 compounds, while the isolate PS28 have 96 compounds (Appendix 3). From these compounds PS1 have eight compounds is with antimicrobial activity from literature (Table 7), while PS28 compounds eleven with antimicrobial activity from literature (Table 8), chemical structure of these active compounds in (Appendix 4).

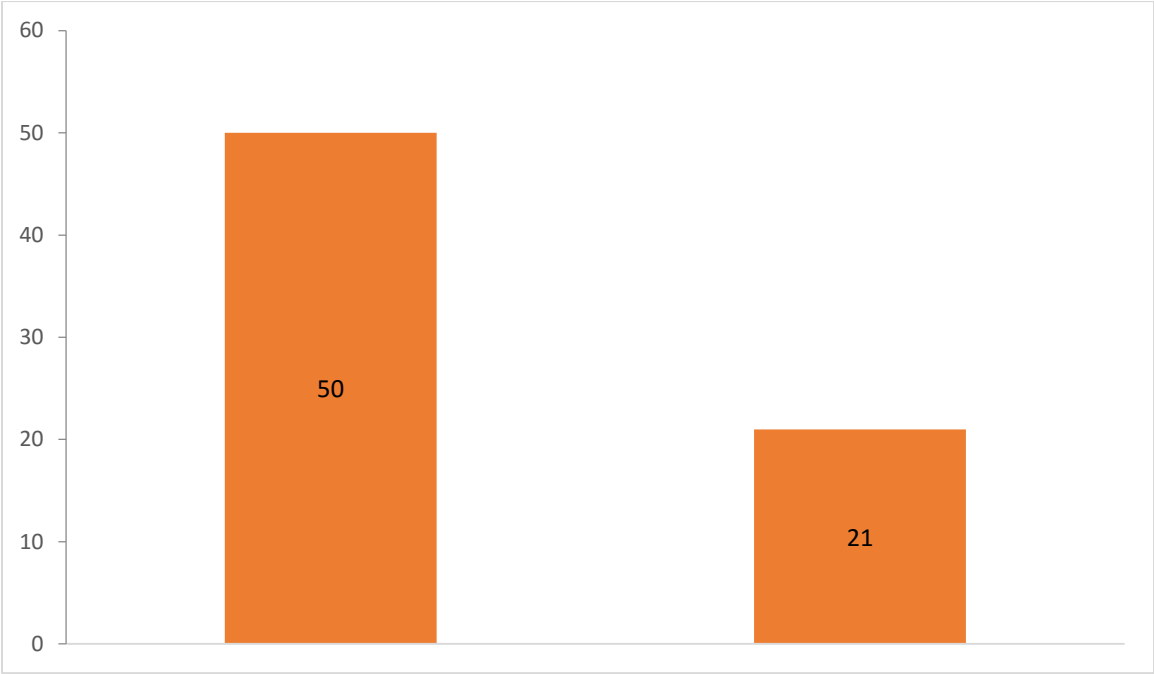

Fig. 3. Percentage of promising *Streptomyces* from sample's collected

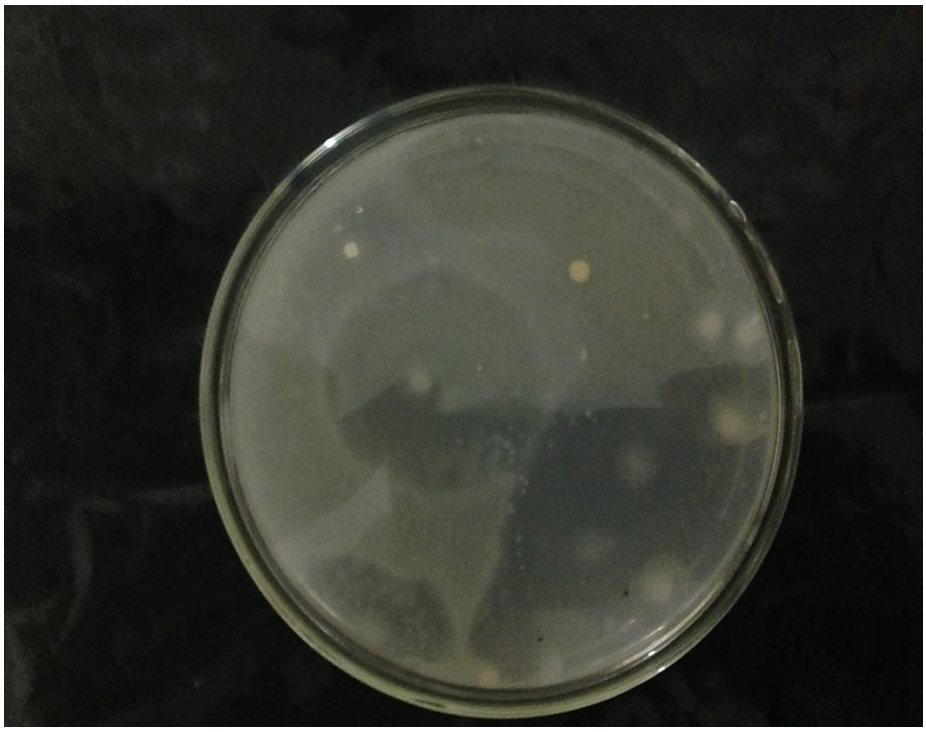

Fig. 4. Agar overlay plate show promising *Streptomyces* sp. producing antibiotic

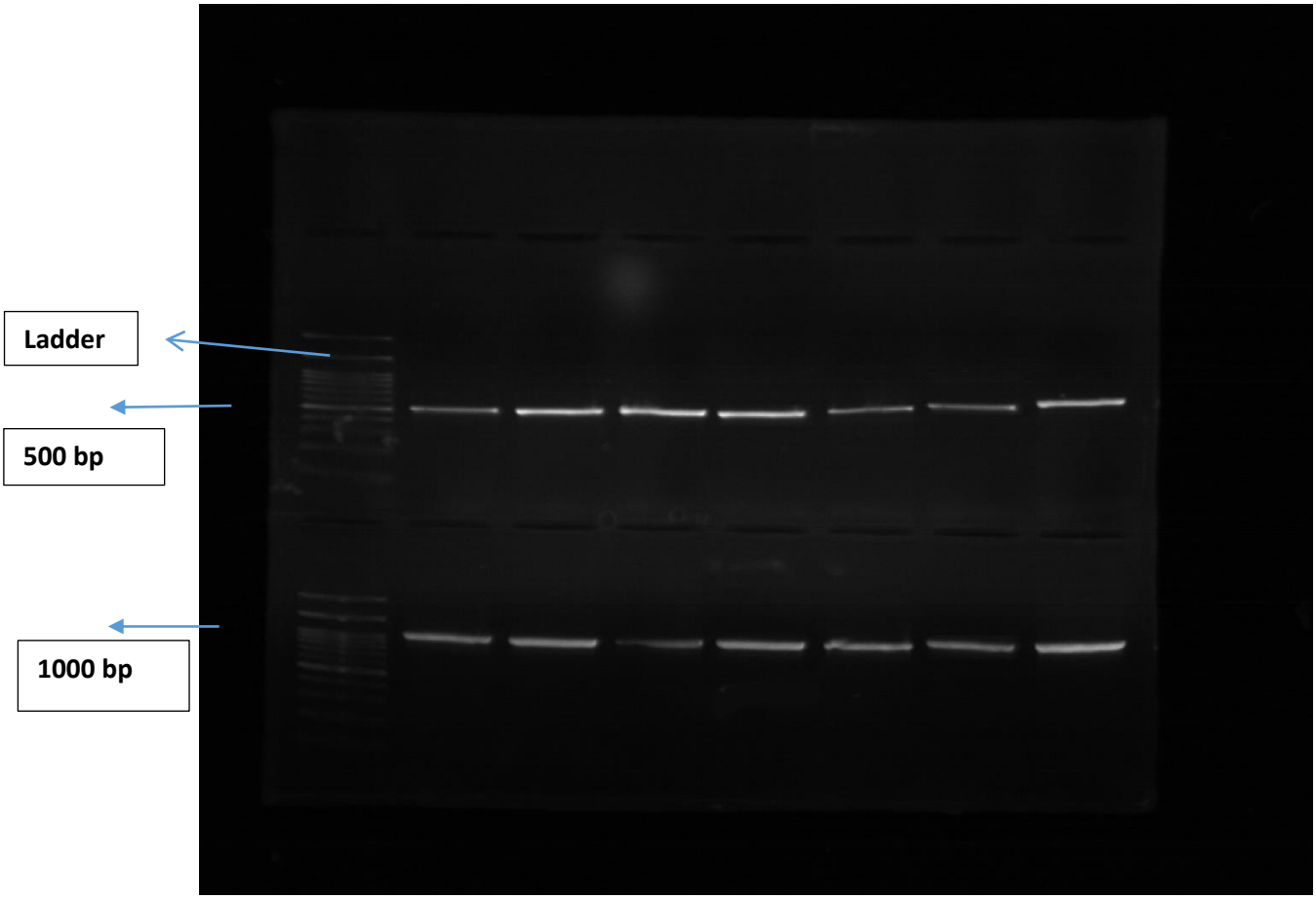

Fig. 5. Upper bands of Agarose gel electrophoresis of PCR amplification products of genomic DNA isolated from *Streptomyces* pure culture using strepB(F)/strepE(R) of 16S rDNA gene.

Lower bands Agarose gel electrophoresis of PCR amplification products of genomic DNA isolated from *Streptomyces* pure culture using strepB (F)/strepF(R) of 16S rDNA gene.

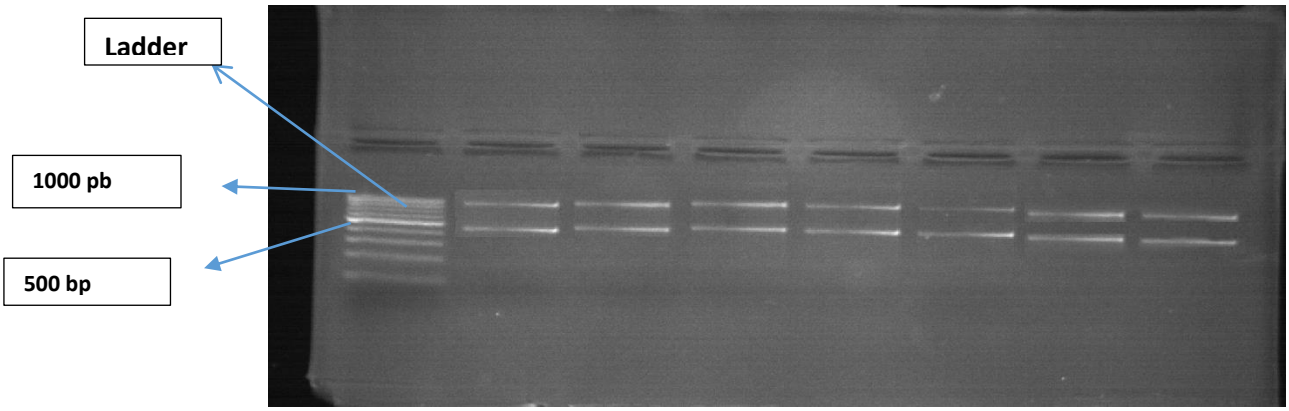

Fig. 6. Agarose gel electrophoresis of Multiplex PCR amplification products of genomic DNA isolated from *Streptomyces* pure culture using strepB(F)/strepE(R) & StrepB(F)/StrepF(R) of 16S rDNA gene.

Table 3. Shows five closest relative with *Streptomyces* species

Isolate Code	Closest relative	% Identity	Accession
PS 1	*Streptomyces afghaniensis*	95%	[GenBank:HG941978.1]
	Streptomyces fimbriatus	95%	[GenBank:EU841630.1]
	Streptomyces thermocarboxydovorans	95%	[GenBank:NR_112588.1]
	Streptomyces sp. C59 partial 16S rRNA gene, isolate SG 38	97%	[GenBank:EU551707.1]
	Streptomyces sp. 291 16S ribosomal RNA gene, partial sequence	97%	[GenBank:FJ754303.1]
PS 5	*Streptomyces griseostramineus*	99%	[GenBank:JQ955745.1]
	Streptomyces sp. SD 528 16S ribosomal RNA gene, partial sequence	99%	[GenBank:JN585736.1]
	Streptomyces vellosus	99%	[GenBank:AB184623.1]
	Streptomyces fimbriatus	98%	[GenBank:FJ486366.1]
	Streptomyces pseudogriseolus	98%	[GenBank:KT588652.1]
PS 10	*Streptomyces* sp. 4354 16S ribosomal RNA gene, partial sequence	98%	[GenBank:KC111835.1]
	Streptomyces afghaniensis	98%	[GenBank:HG941938.1]
	Streptomyces thermocarboxydovorans	98%	[GenBank:NR_112588.1]
	Streptomyces viridochromogenes	98%	[GenBank:AB184514.1]
	Streptomyces muensis	98%	[GenBank:JN560155.1]
PS 13	*Streptomyces capillispiralis*	99%	[GenBank:NR_041158.1]
	Streptomyces sp. SCAU5034 16S ribosomal RNA gene, partial sequence	99%	[GenBank:KR348890.1]
	Streptomyces gancidicus	99%	[GenBank:KP797911.1]
	Streptomyces sp. SXY37 16S ribosomal RNA gene, partial sequence	99%	[GenBank:GU045528.1]
	Streptomyces werraensis	99%	[GenBank:KT021807.1]
PS 20	*Streptomyces* sp. TDI-10 16S ribosomal RNA gene, partial sequence	99%	[GenBank:KT021816.1]
	Streptomyces rubiginosus	99%	[GenBank:LC034307.1]
	Streptomyces stramineus	99%	[GenBank:AY999907.1]
	Streptomyces caelestis	99%	[GenBank:KM081627.1]
	Streptomyces sp. 3482 16S ribosomal RNA gene, partial sequence	99%	[GenBank:DQ663172.1]
PS 21	*Streptomyces coelicolor*	95%	[GenBank:JN798179.1]
	Streptomyces caelestis	95%	[GenBank:KM081627.1]
	Streptomyces sp. MD16 16S ribosomal RNA	95%	[GenBank:JN896608.1]

	gene, partial sequence		
	Streptomyces werraensis	95%	[GenBank:KT021807.1]
	Streptomyces sp. MJN18 16S ribosomal RNA gene, partial sequence	95%	[GenBank:HM026269.1]
PS 23	*Streptomyces* sp. 13658E 16S ribosomal RNA gene, partial sequence	92%	[GenBank:EU741184.1]
	Streptomyces fimbriatus	92%	[GenBank:EU841630.1]
	Streptomyces pseudogriseolus	92%	[GenBank:KT588652.1]
	Streptomyces thermocarboxydovorans strain NBRC 16324 16S ribosomal RNA gene, partial sequence	92%	[GenBank:NR_112588.1]
	Streptomyces sp. 4354 16S ribosomal RNA gene, partial sequence	92%	[GenBank:KC111835.1]
PS 24	*Streptomyces* sp. 1A14 16S ribosomal RNA gene, partial sequence	96%	[GenBank:KT153618.1]
	Streptomyces radiopugnans	96%	[GenBank:KC570323.1]
	Streptomyces nanhaiensis	96%	[GenBank:KJ947850.1]
	Streptomyces fenghuangensis	96%	[GenBank:KJ947857.1]
	Streptomyces sp. HA12301 16S ribosomal RNA gene, partial sequence	96%	[GenBank:KJ419956.1]
PS 28	*Streptomyces muensis* strain MBRL 179 16S ribosomal RNA gene, partial sequence	98%	[GenBank:JN560155.1]
	Streptomyces afghaniensis	99%	[GenBank:HG941938.1]
	Streptomyces sp. DRL121 16S ribosomal RNA gene, partial sequence	98%	[GenBank:FJ853201.1]
	Streptomyces fimbriatus	99%	[GenBank:EU841630.1]
	Streptomyces sp. MWW159 16S ribosomal RNA gene, partial sequence	98%	[GenBank:HM588208.1]

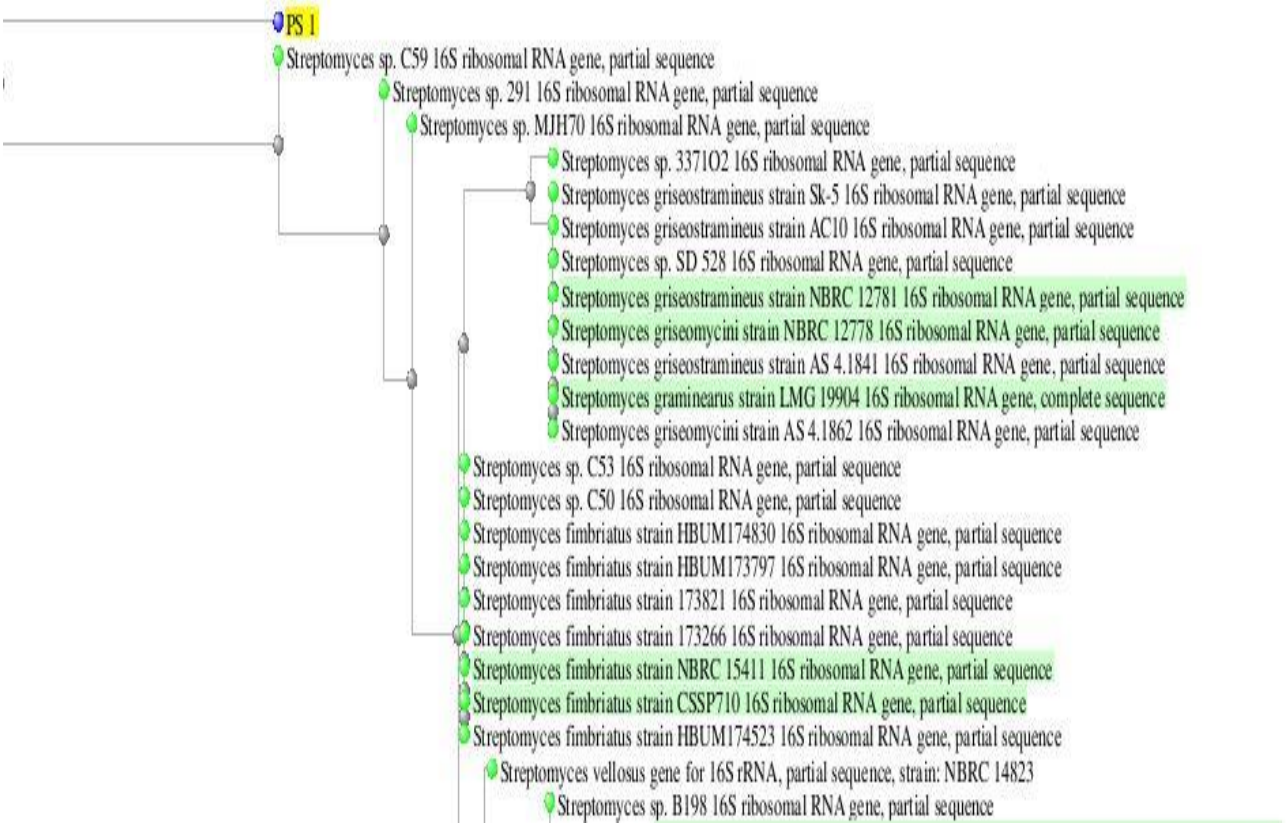

Fig. 7. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS1 and other sequence of *Streptomyces* species

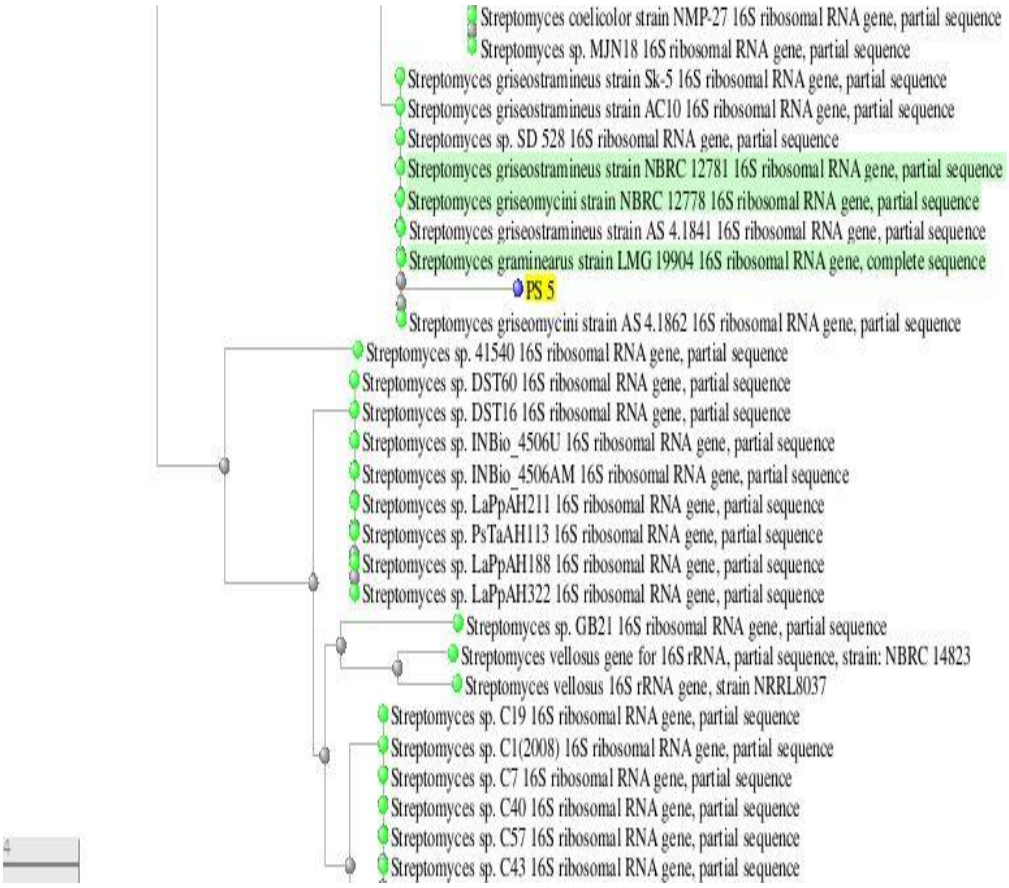

Fig. 8. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS5 and other sequence of *Streptomyces* species

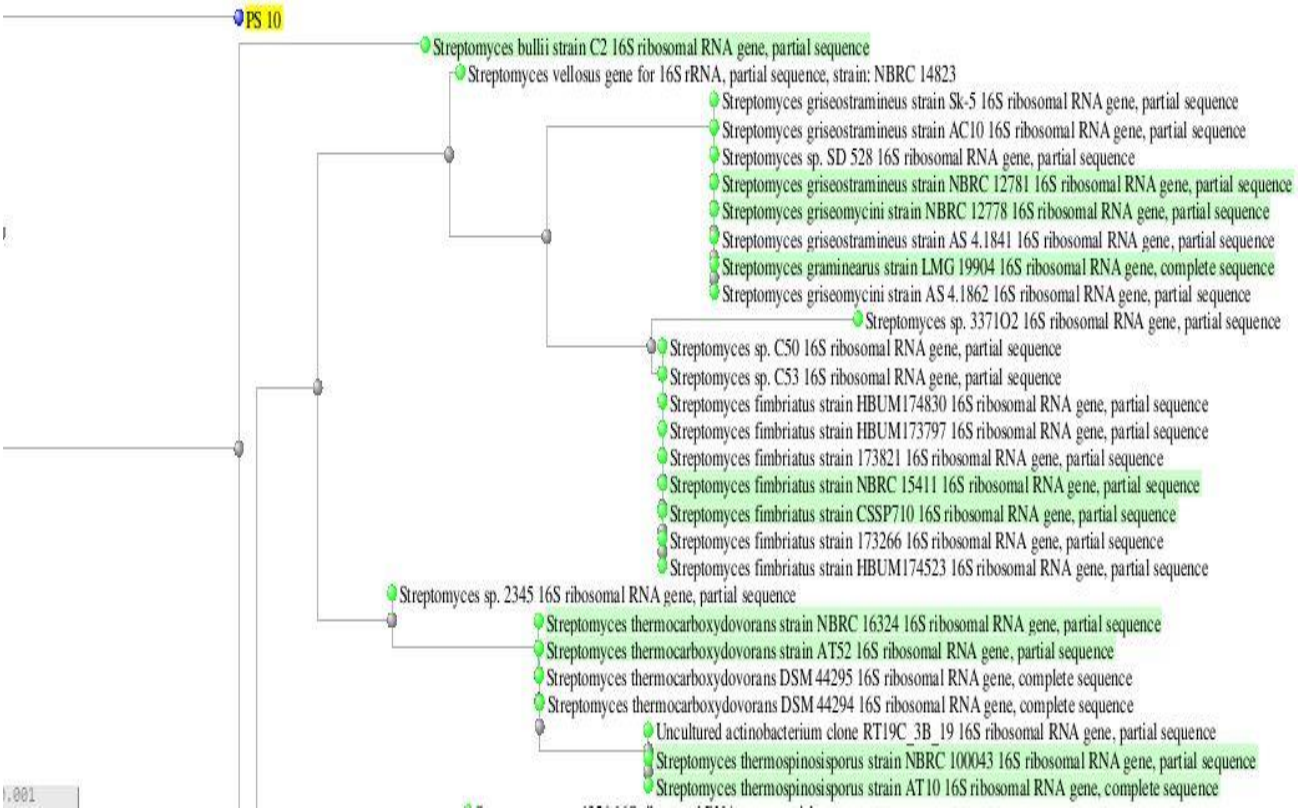

Fig. 9. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS10 and other sequence of *Streptomyces* species

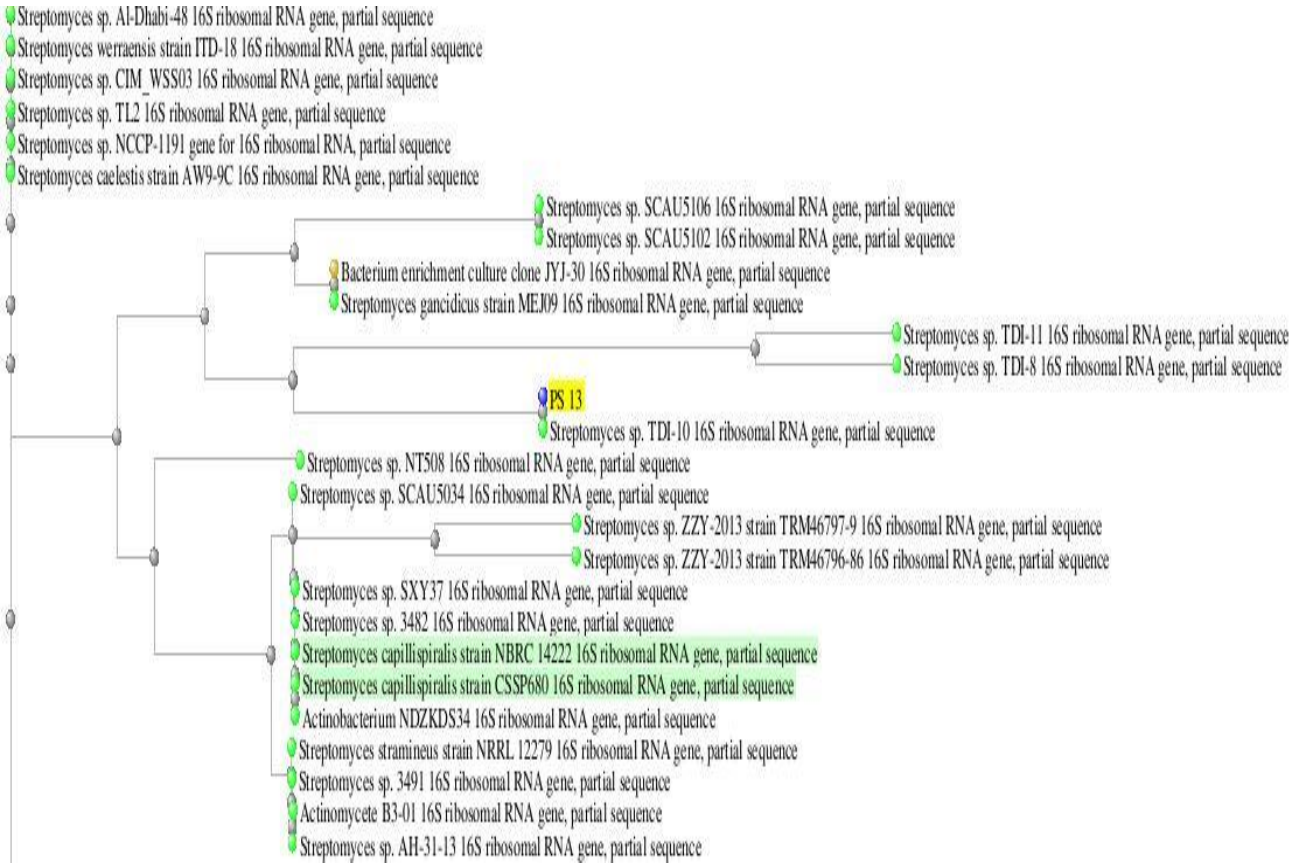

Fig. 10. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS13 and other sequence of *Streptomyces* species

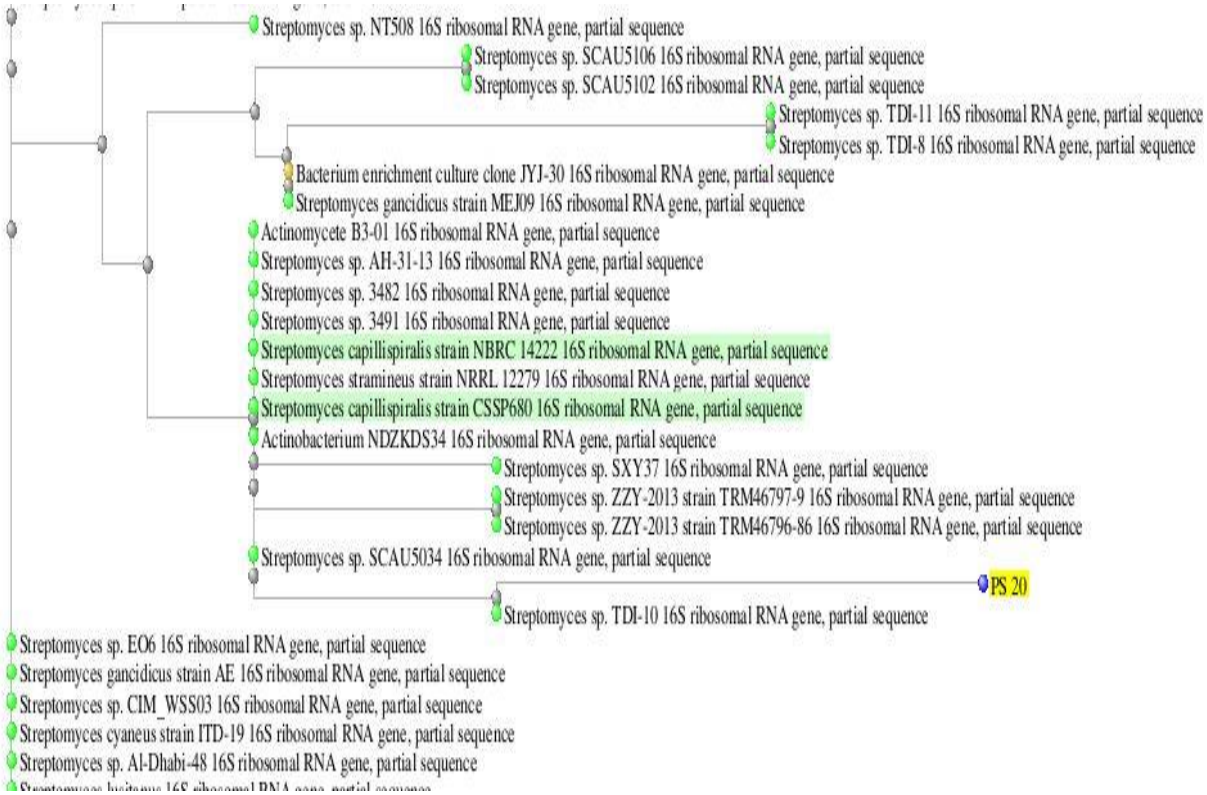

Fig. 11. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS20 and other sequence of *Streptomyces* species

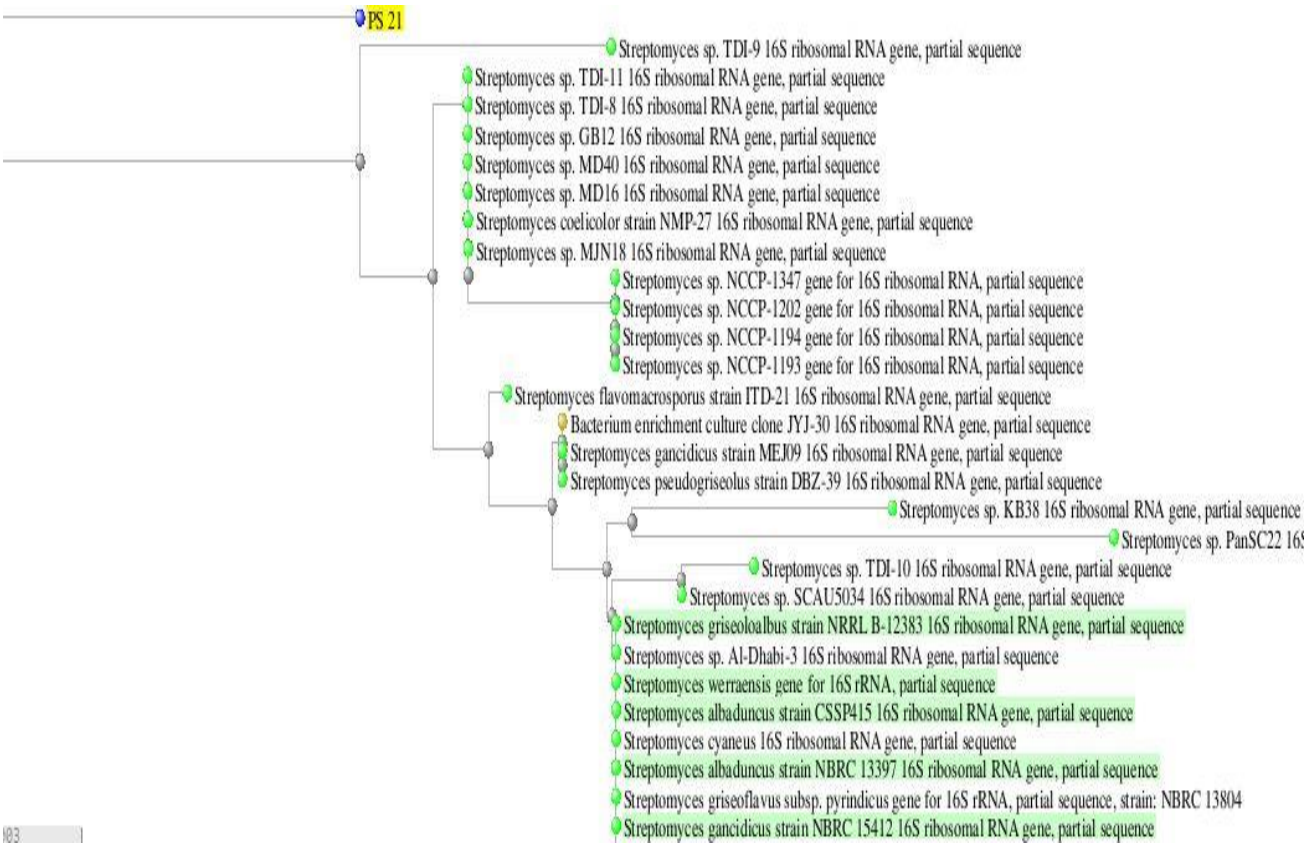

Fig. 12. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS21 and other sequence of *Streptomyces* species

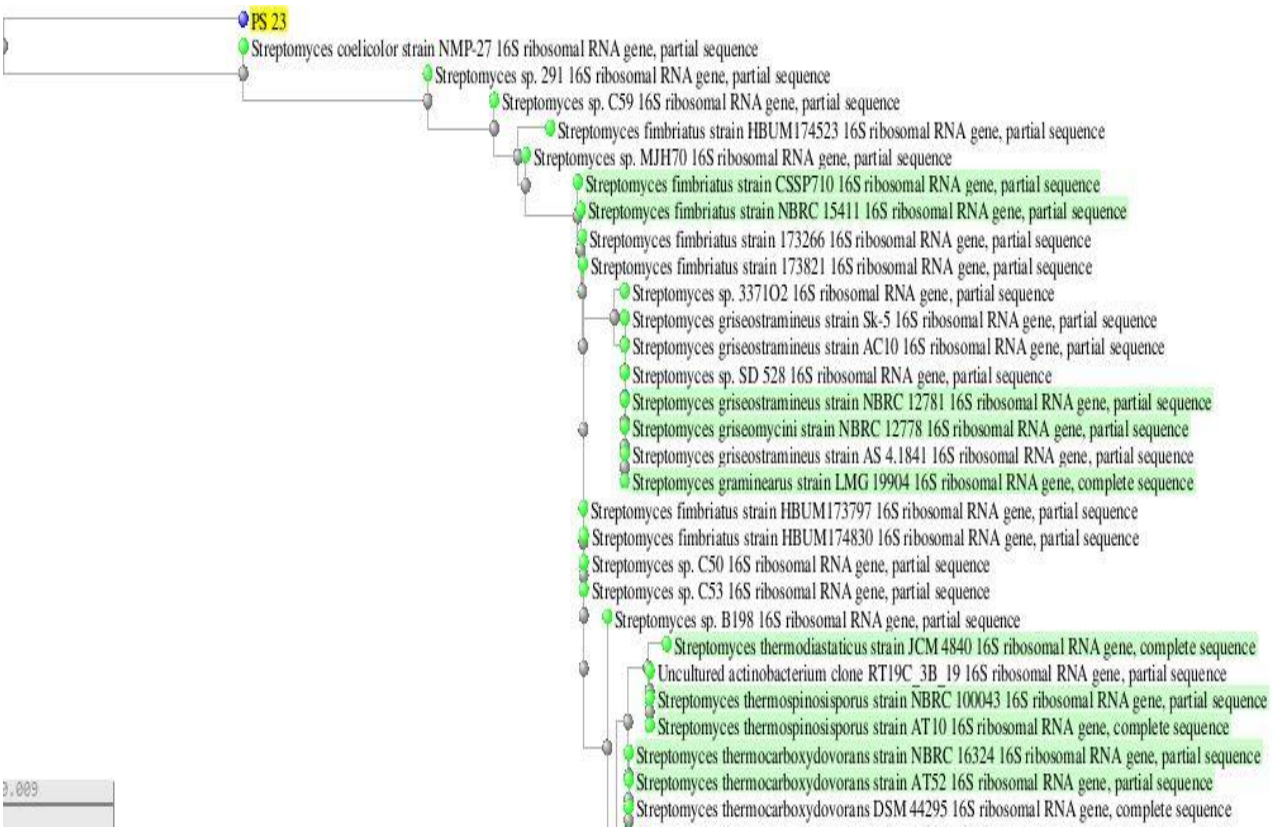

Fig. 13. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS23 and other sequence of *Streptomyces* species

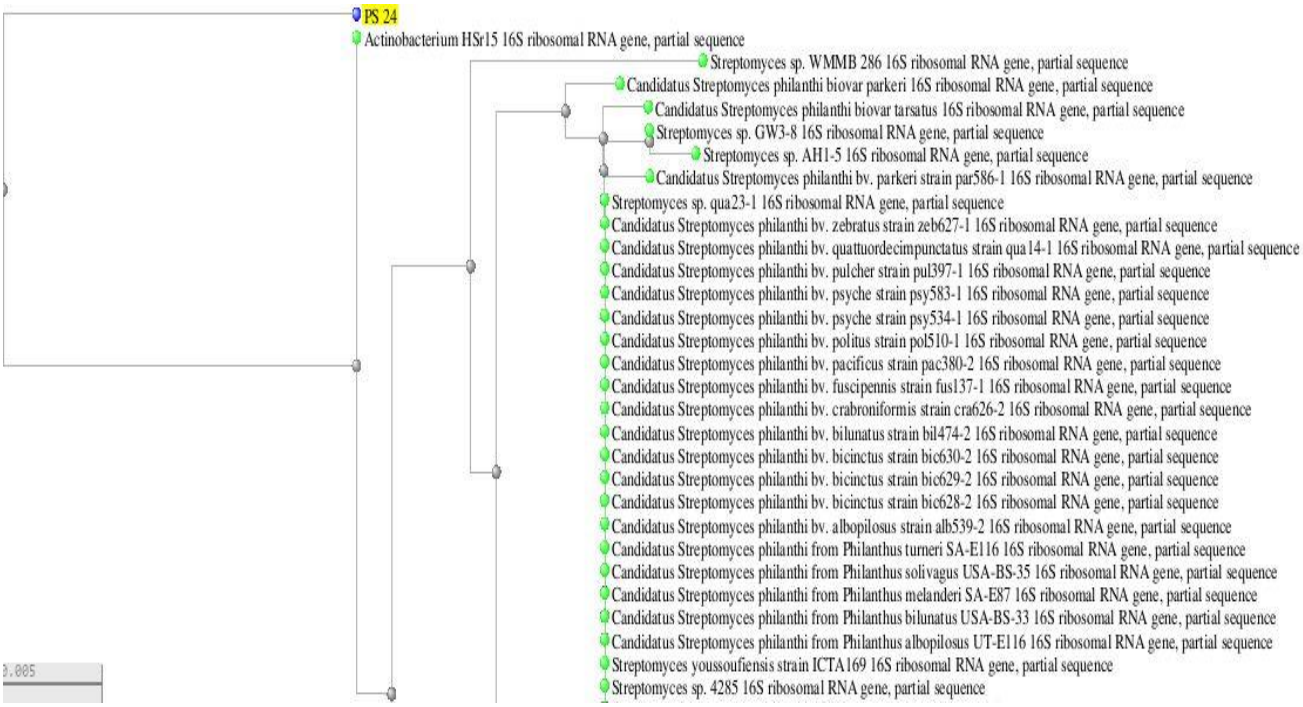

Fig. 14. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS24 and other sequence of *Streptomyces* species

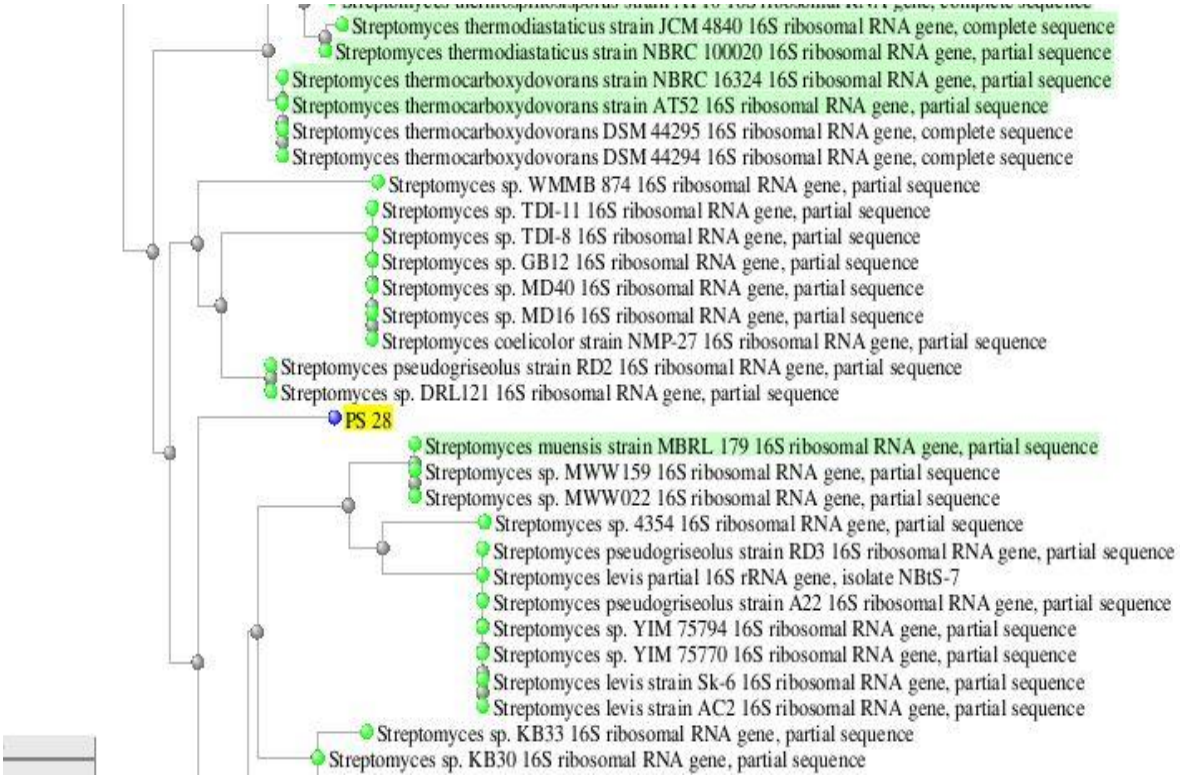

Fig. 15. 16s rRNA gene tree showing the phylogenetic relationship neighbor-joining method between PS28 and other sequence of *Streptomyces* species

Table 4. Culture characteristics of the *Streptomyces* species

Isolate Code	Medium	Growth	Aerial Mycelium Color
PS 1	ISP No.7	Well	Dark Grey
	CSA	Well	Yellow
PS 5	ISP No.7	Well	Green
	CSA	Well	Creamy
PS 10	ISP No.7	Well	Grey
	CSA	Well	White
PS 13	ISP No.7	Well	White
	CSA	Well	Yellow
PS 20	ISP No.7	Well	Creamy
	CSA	Well	White
PS 21	ISP No.7	Well	White
	CSA	Well	White
PS 23	ISP No.7	Well	Brown
	CSA	Well	Grey
PS 24	ISP No.7	Well	Yellow
	CSA	Well	Yellow
PS 28	ISP No.7	Well	Yellow
	CSA	Well	Yellow

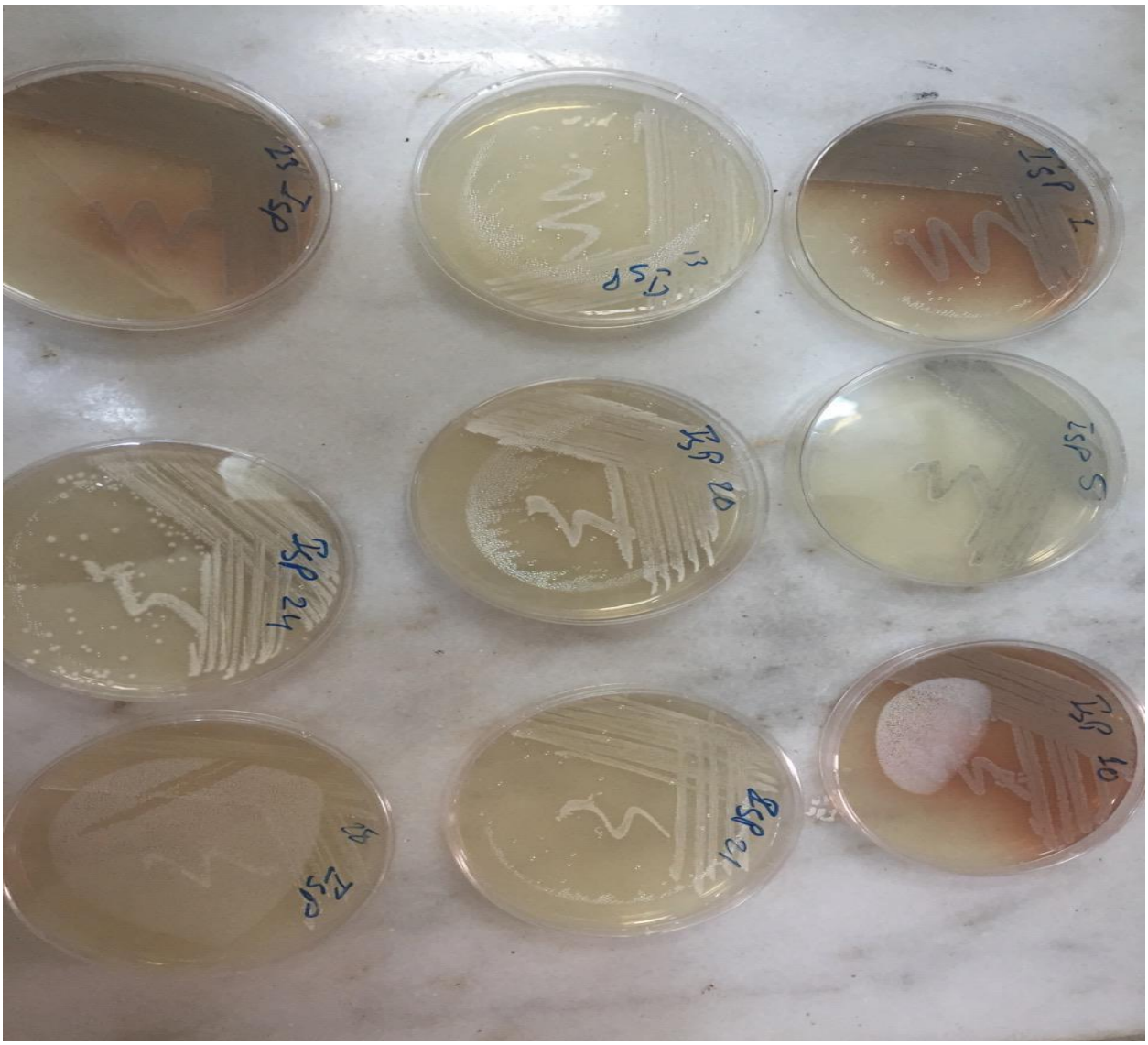

Fig. 16. Growth of the isolates shows aerial mycelium color on ISP No.7 medium

Table 5. The isolates activities against standard bacterial strains

Isolate Code	Inhibition zone (mm) against	
	S. aureus ATCC 25923	***E. coli*** ATCC 25922
PS 1	18	14
PS 5	13	6
PS10	10	-
PS 13	12	-
PS 20	11	7
PS 21	14	9
PS 23	14	-
PS 24	15	8
PS 28	20	14

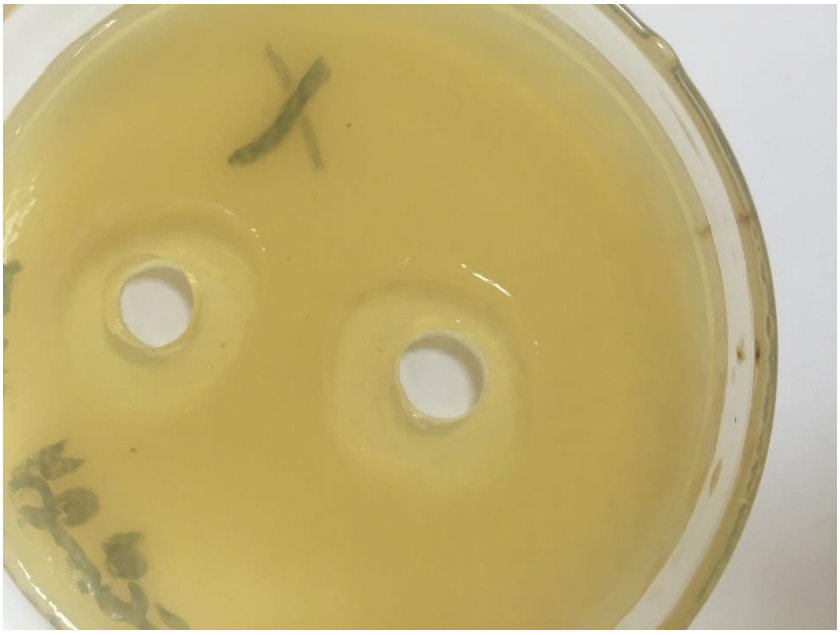

Fig. 17. PS1 & PS28 zone inhibition.

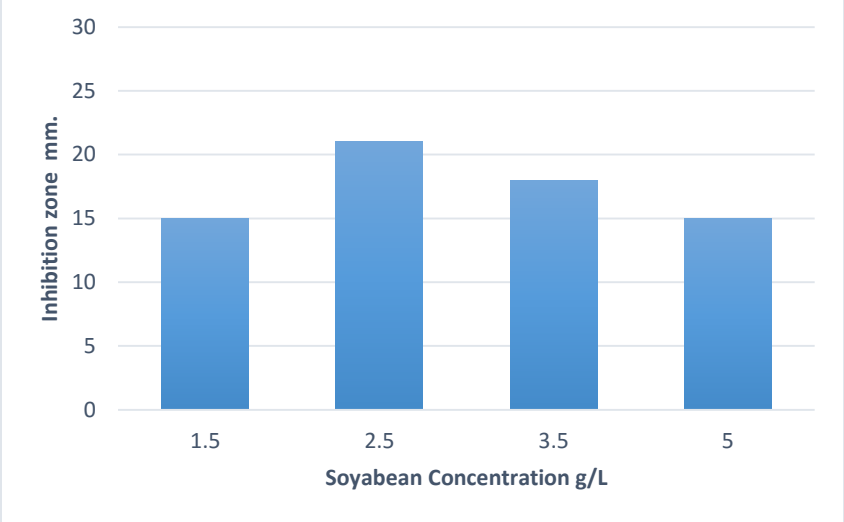

Fig. 18. Effect of nitrogen source concentration on growth and antimicrobial compound production of PS1 & PS28

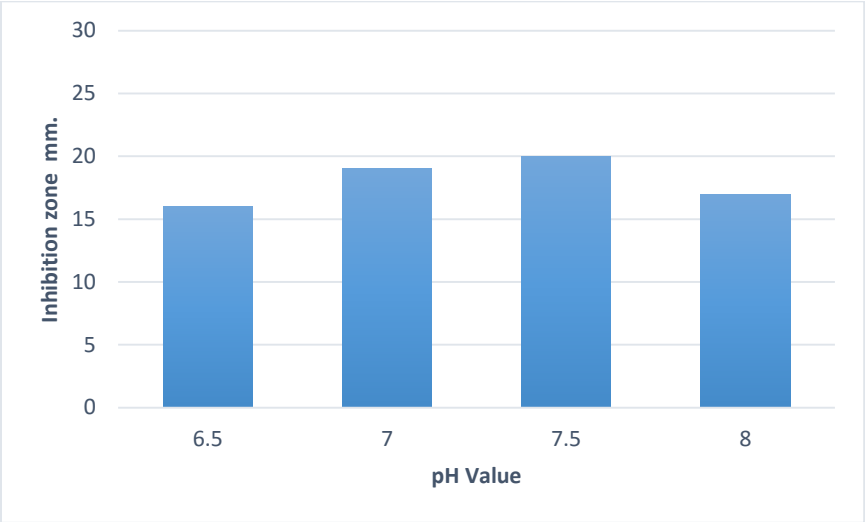

Fig. 19. Effect of pH value on growth and antimicrobial compound production of PS1 & PS28

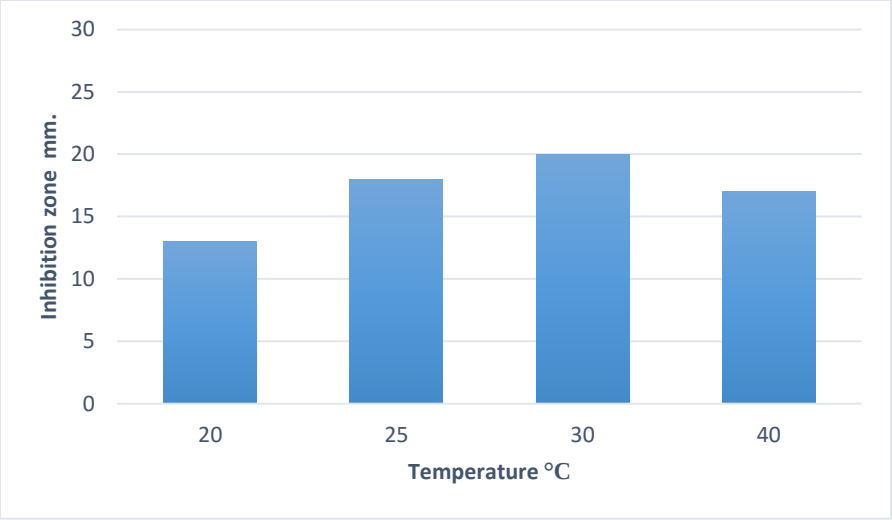

Fig. 20. Effect of temperature on growth and antimicrobial compound production of PS1 & PS28

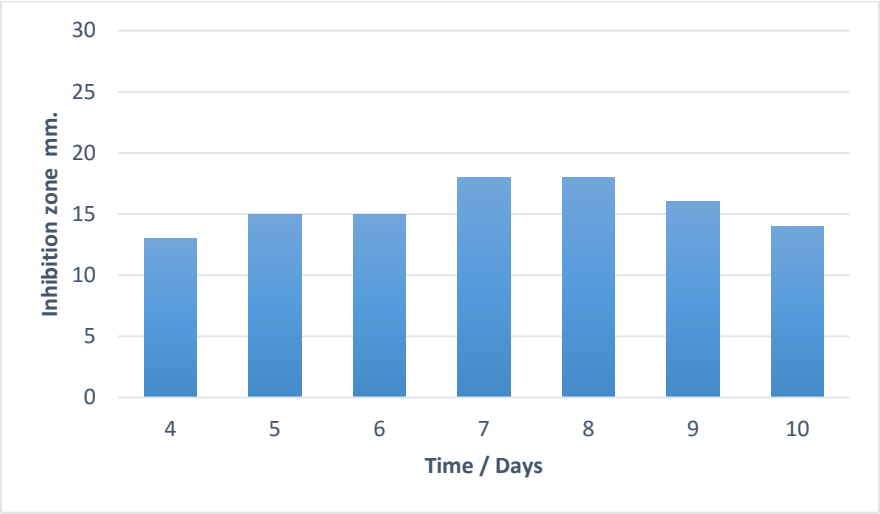

Fig. 21. Effect of incubation period on growth and antimicrobial compound production of PS1 & PS28

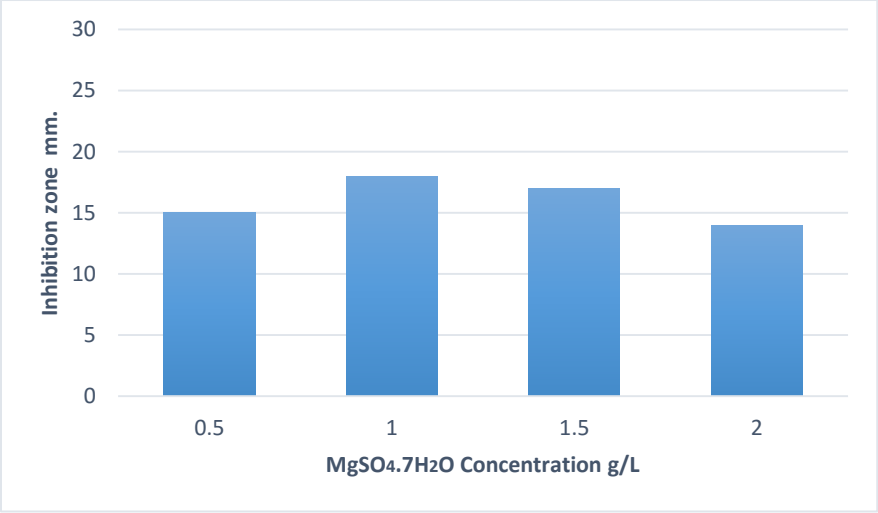

Fig. 22. Effect of $MgSO_4.7H_2O$ concentration on growth and antimicrobial compound production of PS1 & PS28

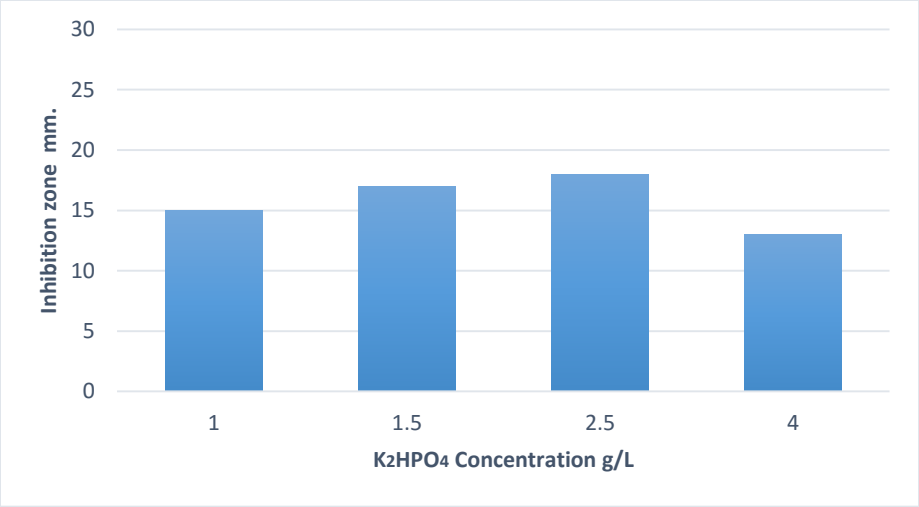

Fig. 23. Effect of K_2HPO_4 concentration on growth and antimicrobial compound production of PS1 & PS28

Table 6. Antibacterial activity of PS1 & PS28

Isolate Code	Inhibition zone (mm) against	
	S.aureus **ATCC 25923**	***E. coli*** **ATCC 25922**
PS 1	20	16
PS 28	22	16

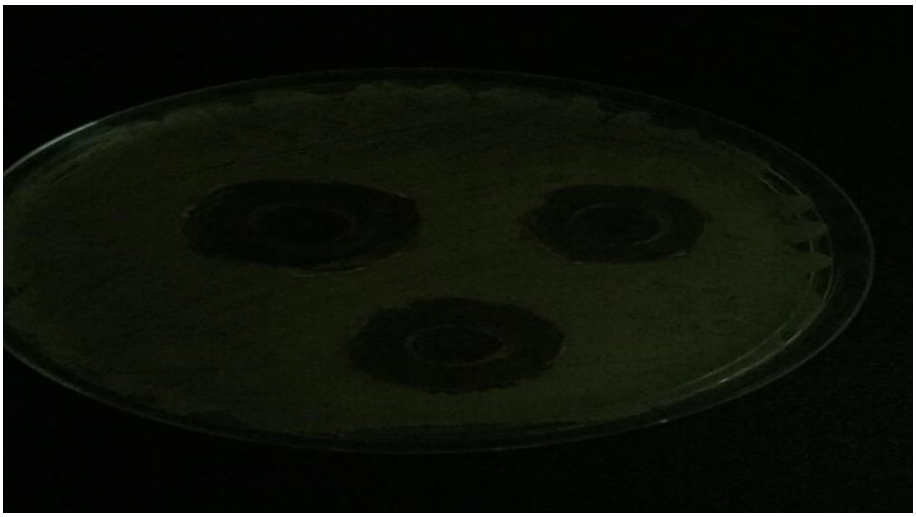

Fig. 24. Antibacterial activity of PS1 against *S. aureus*

Fig. 25. Antibacterial activity of PS28 against *S. aureus*

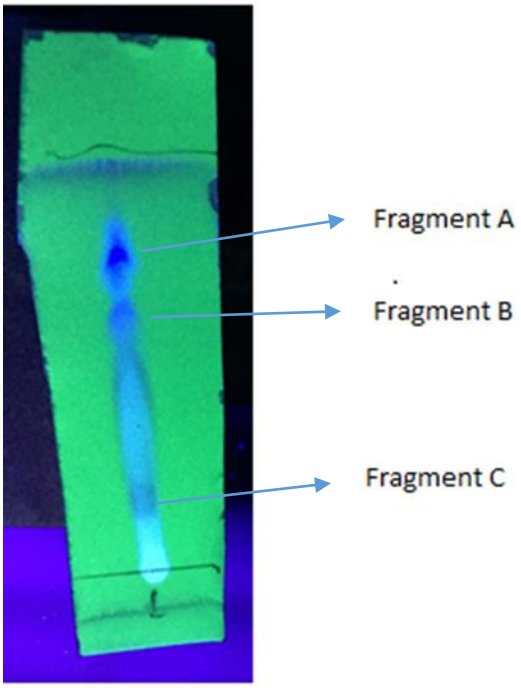

Fig. 26 TLC plate of PS1 show fragments

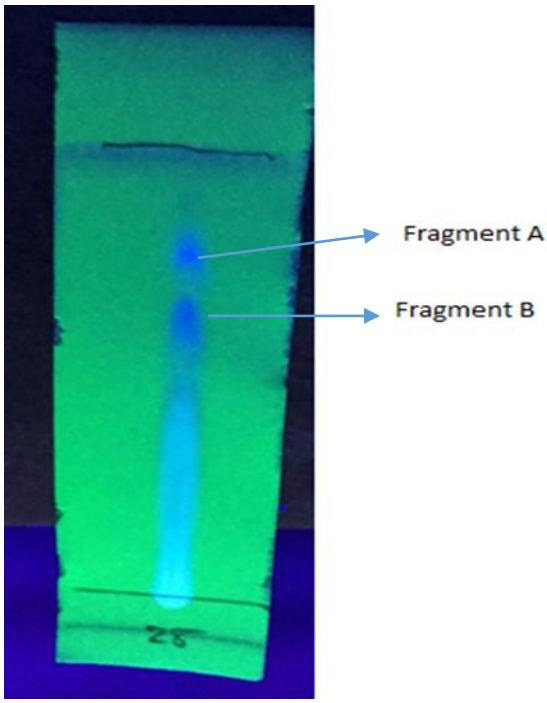

Fig. 27 TLC plate of PS28 show fragments

Table 7. Compounds with antibacterial activities produced by PS1 isolate

Compound No.	Compound name	R-Time	Similarity %	Area %	Compound formula	Molecular weight
I	4-Vinyl-imidazole	6.727	96	2.52	C5H6N2	94
II	Ornithine	8.595	95	5.2	C5H12N2O2	132
III	4-Methyleneproline	13.055	96	15.02	C6H9NO2	127
IV	Pyrimidinone, 6-amino-2-methyl	15.712	95	0.82	C5H6N4O2	154
V	2,6-Dibuty1-4-methylpiperidine	15.879	95	2.70	C14H29N	211
VI	Actinomycin C2	15.809	99	0.46	C63H88N12O16	1268
VII	n-3-imidazol-1-yl-propyl-n-4-isopropyl-phenyl-oxalamide	20.024	98	2.14	C17H22N4O2	314
VIII	5-nitroso-2,4,6-triaminopyrimidine	22.817	98	5.79	C4H6N6O	154

Table 8. Compounds with antibacterial activities produced by PS28 isolate

Compound No.	Compound name	R-Time	Similarity %	Area %	Compound formula	Molecular weight
IX	pyrrolidine n 3-methyl-3- butenyl	3.870	97	0.67	C9H17N	139
X	Piperidine, 2,3-dimethyl	4.875	96	0.14	C7H15N	113
XI	Cyclohexanamine, N-2-propenyl	7.793	96	0.17	C9H17N	139
XII	alpha-campholenal	8.329	96	1.0	C10H16O	152
XIII	Norvaline	15.072	99	6.07	C26H47NO4	437
XIV	2-Piperidinone	15.415	98	0.51	C10H16N2O	180
XV	l-leucine, n-cyclopropylocarbonyl- pentadecyl ester	15.783	99	5.76	C25H47NO3	409
XVI	5,10-diethoxy-2,3,7,8-tetrahydro-1h,6h-dipyrrolo[1,2-a;1,2-d]pyrazine	16.059	97	6.21	C14H22N2O2	250
XVII	n(1) 3-methyl-1,2,4-oxadiazole	17.737	95	0.55	C9H15N5O	209
VII	n-3-imidazol-1-yl-propyl-n-4-isopropyl-phenyl-oxalamide	20.062	99	5.65	C17H22N4O2	314
VIII	5-nitroso-2,4,6-triaminopyrimidine	22.914	98	7.23	C4H6N6O	154

CHAPTER FIVE

DISCUSSION

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DISCUSSION

5.1. Discussion

Since the discovery of penicillin in 1928, antibiotics have been used effectively to control emerging pathogens, resulting in progress affecting against these pathogens and preventing life threating (Omulu *et al*., 2015). The search for new antibacterial compounds is become a global issue due to problem of bacterial resistance to existing antibacterial agents (Martins *et al*., 2015). This resistance has become a major health concern. Many opportunistic pathogens were becoming resistant to virtually every available antibacterial agent. The emergence of multi-resistant pathogenic bacterial strains has caused a therapeutic problem of enormous proportions. In response, there is interest in discovering novel classes of antimicrobial agents that have different mechanisms of action (Greenberg, 2003). Natural products have been regarded as important sources of their compounds. Seas and marines were considered as major niches of microorganisms promising for natural products (Gulve and Deshmukh, 2012; Kiruthika *et al*., 2013). Thus, the researches were directed towards such habitats as unusual sources to be explored for the development of new drugs. Significant part of this attention has been paid to marine microorganisms, which have become important in the study of novel compounds exhibiting antibacterial activities (Kokare *et al*., 2004). Recently, true indigenous marine *Streptomyces* have been described; hence they drew special attention as a promising source of novel and unique metabolites (Maldonado *et al*., 2005; Moore *et al*., 2005).

Since the marine environment in Sudan is still unexplored and unexploited, this study was performed to make an attempt to characterized antibacterial compounds from *Streptomyces* sp. isolated from marine environment, and seem as thought to be the first one.

During the course of study 9 (43%) *Streptomyces* species were isolated from marine soil sediments. This result is similar to that reported by Kokare and his colleagues (2004) who isolated 43% from West Coast of India, but more than reported by Al-Hulu, (2013) from terrestrial samples from Iraqi soil are only 9%. This percentage shows that the habitant of Port Sudan is promising area for detection for this organism. It is evident that the percentage of the actinomycetes in marine sediments varied greatly from one region to another. Bull *et al*. (2005) confirmed that the percentage of actinomycetes was very low (0.9%), while Pathom-aree (2006) reported high proportion of actinomycetes from western Pacific Ocean (60%). Laatsch (2005) reported that actinomycetes are rare in free water column and present in marine sediment. It is obvious that they are not as abundant in marine environment as they are in terrestrial samples.

Our isolated *Streptomyces* strains morphological colors were vary from white to green, while the most of them are white, then the yellow color, gray and finally green; this result is similar to study done by Koudari, (2010), who isolated *Streptomyces* strains from Red Sea, Jordan. The differences in color series dominance and pigment production might be also due to the environmental factors and habitats.

The identified *Streptomyces* nine (n=9) have an activities against both Gram-positive and Gram-negative pathogenic bacteria. Similar result (17%) was obtained by Mohan *et al*., (2013). Moreover; Bizuye *et al*., (2013) showed that 26% of the isolates exhibited activity against pathogenic bacteria which is more than that

reported in the present study. Of the broad spectrum *Streptomyces* isolates, only PS1 & PS28 showed high activities against *S. aureus* and *E. coli*. These results are similar to that obtained by Reddy *et al.,* (2011) from India marine sediments.

The differences in sensitivity to antibiotics between Gram-positive and Gram-negative bacteria may be attributed to morphological differences between these microorganisms; Gram-negative bacteria have an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic compounds; while the Gram-positive bacteria is more susceptible as they have only an outer peptidoglycan layer which is not affect permeability of cell wall (Singh *et al*., 2014).

In the present study all culture media were prepared by sea water instead of distilled water, this can be an influence factor for the growth and production of antibiotics, Saha *et al.,* (2004) revealed that seawater enhanced antibiotic production. Similarly, Sunga *et al*., (2008) revealed that the production of antibiotic lipoxazolidinones by a novel marine actinomycete strain NPS8920 could only be detected in the seawater-based media. All culture media prepared in this study was supplemented with sea water regarding the composition of sea water with micro elements were required for the production of secondary metabolites as Kouadri, (2010) report of the study was done to soil samples collected from Aqaba Gulf, Jordan.

The identification of the *Streptomyces* sp. isolated in this study was done by the 16s rRNA gene sequence. Analysis of 16s rRNA gene sequence analysis has become a common method for the accurate identification of bacterial isolates (Drancourt *et al*., 2004). The use of 16s rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in

almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda and Abbott, 2007).

Secondary metabolites produced by bacteria still interested, due to their complicated chemical structures and highly specific antimicrobial activities. The soil bacteria resembling to the genus *Streptomyces* are rich sources of large number of bioactive natural products; they are wide used as antimicrobials. *Streptomyces* species produce about 75% of useful antibiotics (Kumar *et al*., 2014). Al-Zahrani, 2007 reported that incubation conditions influence the qualitative production of *Streptomyces* species secondary metabolites, Kathiresan *et al*., (2005) reported that ability of bacteria to produce antimicrobial agents can be increased or lost under different culture conditions. Maximum antibiotic production was observed with soyabean meal as a source of nitrogen compared to other compounds i.e. yeast extract, peptone or beef extract so soyabean was used in this study to influence the growth and production of antibacterial compounds, the pH values affect the cellular metabolisms and biosynthesis of secondary metabolites in *Streptomyces* species (Bhavana *et al*., 2014). Ripa *et al.*, (2009) reported that extreme pH is unfavorable for production of secondary metabolites. Kokare *et al*., (2007) showed that 28°C is the optimum temperature and it's lower than our study, but (Kokare *et al*., 2007) reported that 28°C is the optimum temperature and this is lower than degree from our study. Kathiresan *et al*., (2005) reported that 5 days of incubation gave the broad activity of secondary metabolites and this shorter period to this study. (Ghadin *et al*., 2008) reported that the extraction of secondary metabolites by ethyl acetate showed the obvious activity against pathogenic bacteria than other

solvent and this the same solvent used in this study. Optimization of culture media for maximum antibiotic production is extremely important in pharmaceutical industry for economic reasons. In recent study six conditions were adjusted (nitrogen source, pH, temperature, dark & light, duration and trace elements), and the antibiotic production is greater than basal media.

The GC-MS analysis showed a variety of compounds from the extract of PS1 & PS28, Jalaluldeen *et al.*, (2015) revealed that 77 compounds were separated by GC-MS analysis this is higher than PS1 strain compounds and lower than PS28 strain compounds. Many of these compound are responsible for the antibacterial activity that shown in this study. The GC-MS analysis technique is the most accurate and raoid for the identification of crude marine metabolites (Krishnakumar *et al*., 2013).

5.2. Conclusions

This study provides recent information about bioactivity of marine *Streptomyces* isolates from the Red Sea, this environment (especially coast of Sudan) has not been greatly studied and have proved to be a promising source for antibacterial compounds. These compounds exhibited inhibitory activity against pathogenic bacteria (*S. aureus* & *E. coli*).

The percentage of identified of *Streptomyces* strain is comparable with terrestrial and marine sources, revealed the promising site especially if considered the long of coast and covering the depths of the sea. This could lead to discover new species and strains of this organism as a result of high possibility to extract new secondary metabolites (antimicrobial drugs). According to sequencing similarities these isolates have big chance to be isolated for first time as a novel strains.

5.3. Recommendations

The microbiology of the Red Sea, Sudan environment has to be further explored and investigate for microorganisms with ability to produce bioactive compounds. Further studies are required to purify and identify bioactive compounds from *Streptomyces* isolated from the Port Sudan Coast with different activities such as anticancer, antiviral, and antifungal along with studying the genes encoding these compounds. Other future studies may include further characterization of antimicrobial products for therapeutic index.

The use of sea water is an important supplement for the culture media in the approach to make the environment in the laboratory close to natural habitat, also important to optimize cultural conditions for this reason.

It is highly recommended to amplify the whole genome of the organism in order to discover new species and this can lead to discover new antibiotic.

The researcher recommended in future studies to work as a team in order to economic reason and to get faster results and prefer the team workers include microbiologist and pharmaceutical microbiologist researcher.

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APPENDIXES

APPENDIX 1

Culture Media

1. Starch casein agar (SCA)

Casein powder, 0.3 g; Starch, 10.0 g; KNO3 2; NaCl 2; K2HPO4 2; MgSO4 7 H2O 0.05; CaCO3 0.02; FeSO4 7H2O 0.01; Agar, 18.0 g; Distilled water, 1000 ml; pH, 7.2.

2. International Streptomyces Project No.7 (ISP No.7)

L-Asparagine, 1.0 g; L-Tyrosine, 0.5 g; Dipotassium phosphate, 0.5 g; Magnesium sulphate. 7H2O, 0.5 g; Sodium chloride, 0.5 g; Trace salt solution (ml), 1.0 g; Agar, 20.0 g; Ferrous sulphate, 7H2O, 1.369 mg; Copper chloride, 2H2O, 0.027 mg; Cobalt chloride, 6H2O, 0.040 mg; Sodium molybdate, 2H2O, 0.025 mg; Zinc chloride, 0.020 mg; Boric acid, 2.850 mg; Manganese chloride, 4H2O, 1.800 mg; Sodium tartarate, 1.770 mg; Distilled water, 1000 ml; pH, 7.3.

3. Mueller Hinton Agar (MH)

Beef, infusion from, 300.0 g; Casein acid hydrolysate, 17.5 g; Starch, 1.5 g; Agar, 17.0 g; Distilled water, 1000 ml; pH, 7.3.

4. Nutrient Agar (NA)

Peptic digest of animal tissue, 5.0 g; Sodium chloride, 5.0 g; Beef extract, 1.5 g; Yeast extract, 1.5 g; Agar, 15.0 g; Distilled water, 1000 ml; pH, 7.4.

5. **Soyabean Casein Digest Medium (Tryptone Soya Broth) (SCDM)**

Pancreatic digest of casein, 17.0 g; Papaic digest of soyabean meal, 3.0 g; Sodium chloride, 5.0 g; Dextrose(Glucose), 2.5 g; Dipotassium hydrogen phosphate, 2.5 g; distilled water, 1000 ml; pH, 7.3.

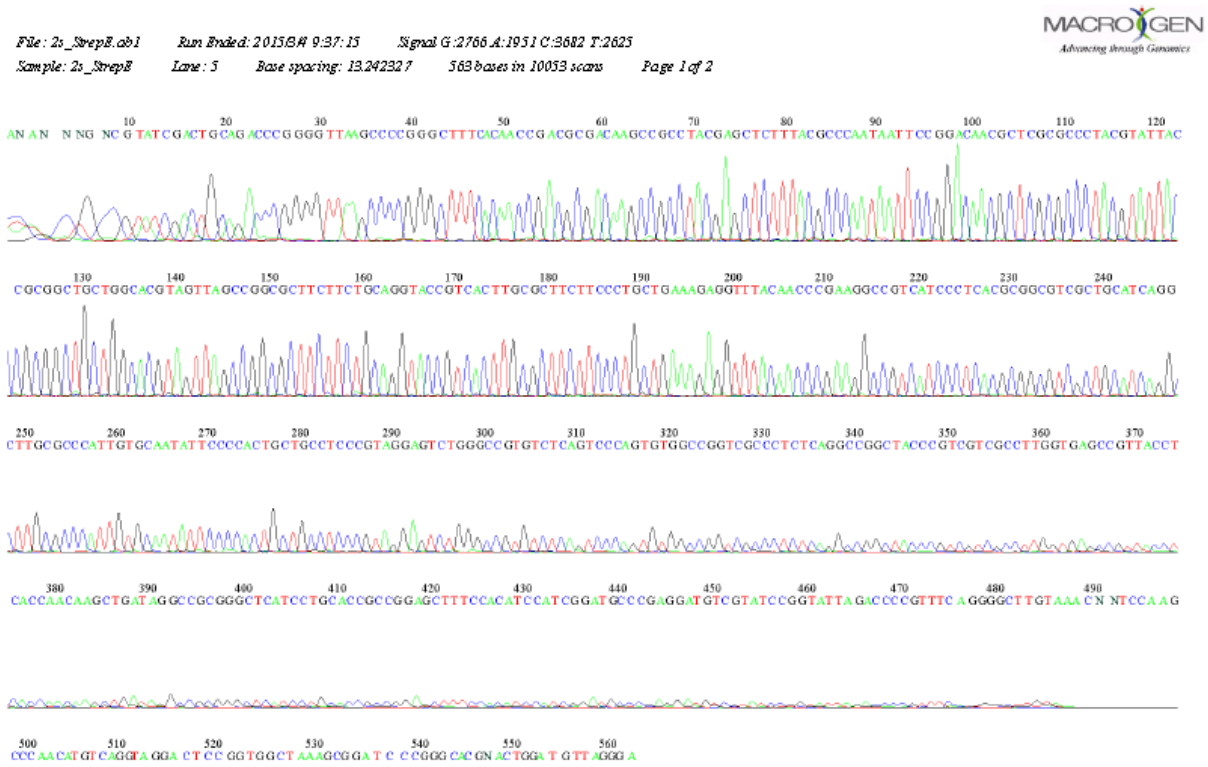
6. **Nutrient Broth (NB)**

Peptone, 10.0 g; Beef extract, 10.0 g; Sodium chloride, 5.0 g; Distilled water, 1000 ml; pH, 7.3.

APPENDIX 2

Sequencing analysis

PS1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MACROGEN
Advancing through Genomics

PS5


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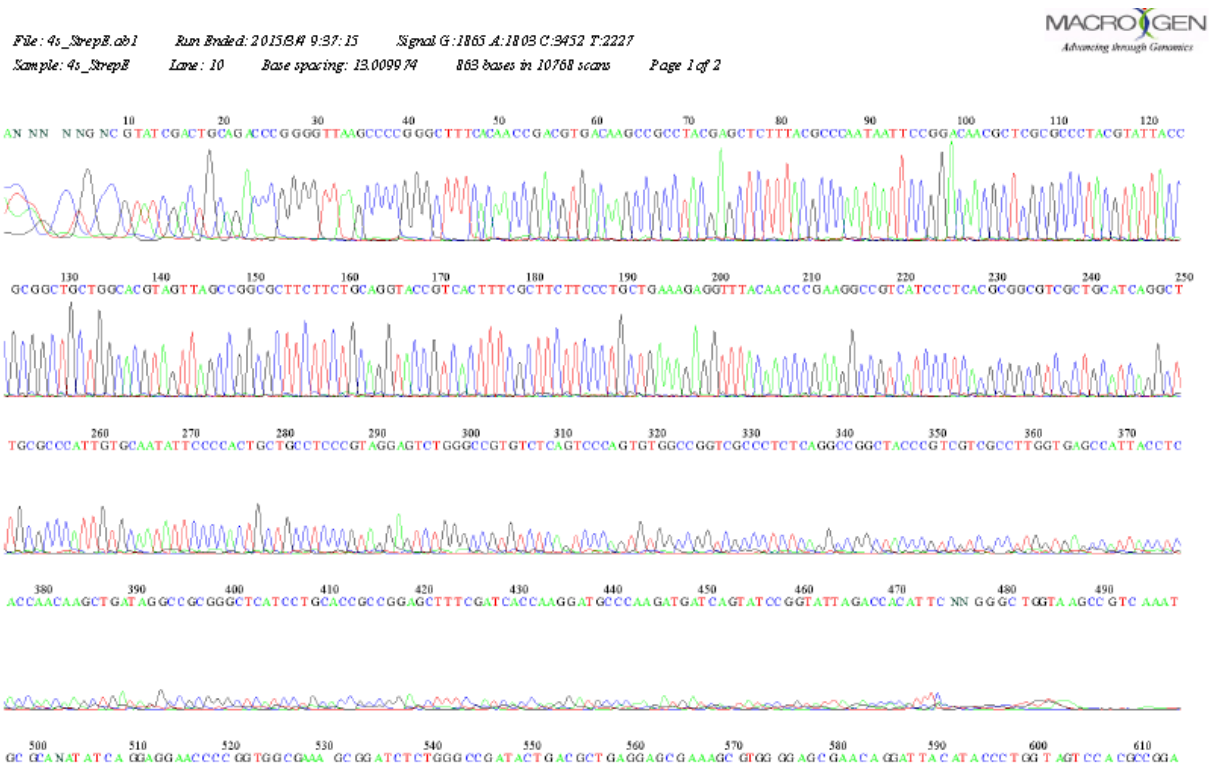
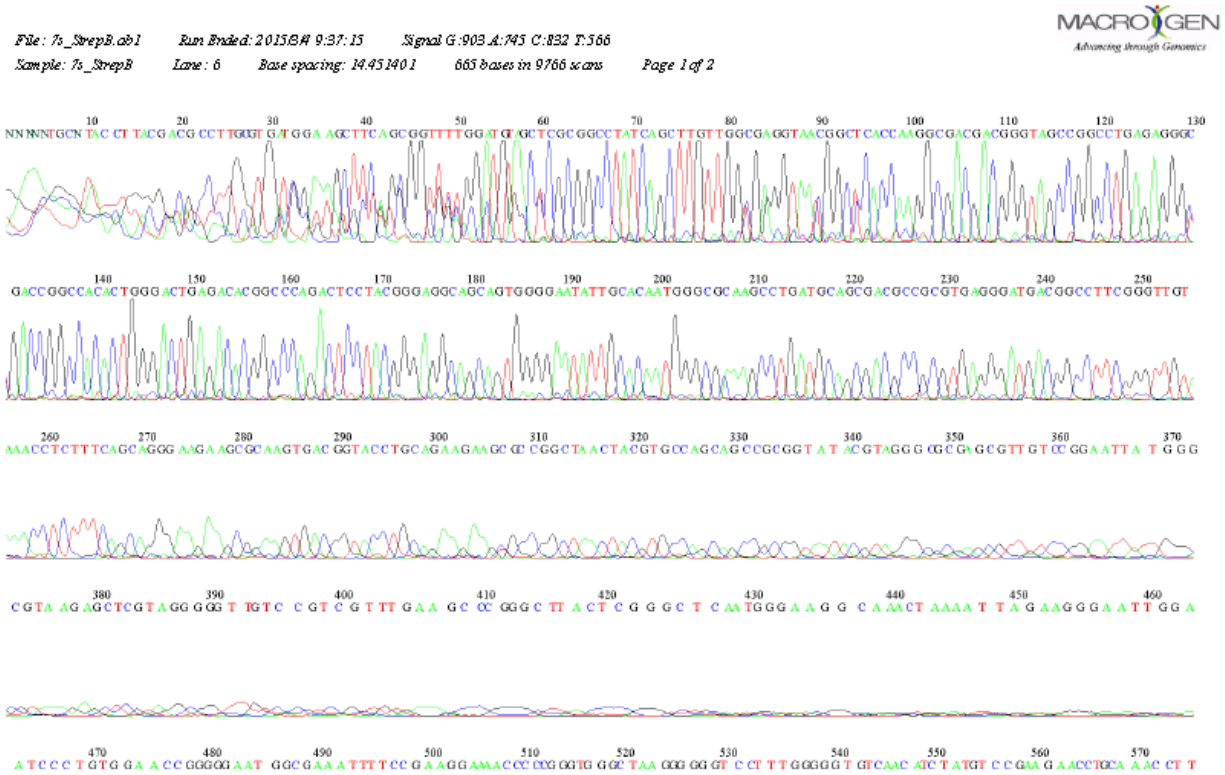
MACROGEN
Advancing through Genomics

PS10


File : 4s_Strep8.ab1 Run Ended: 2015/8/4 9:37:15 Signal G:1865 A:1803 C:3452 T:2227
Sample: 4s_Strep8 Lane : 10 Base spacing: 13.009974 863 bases in 10768 scans Page 1 of 2
MACROGEN
Advancing through Genomics

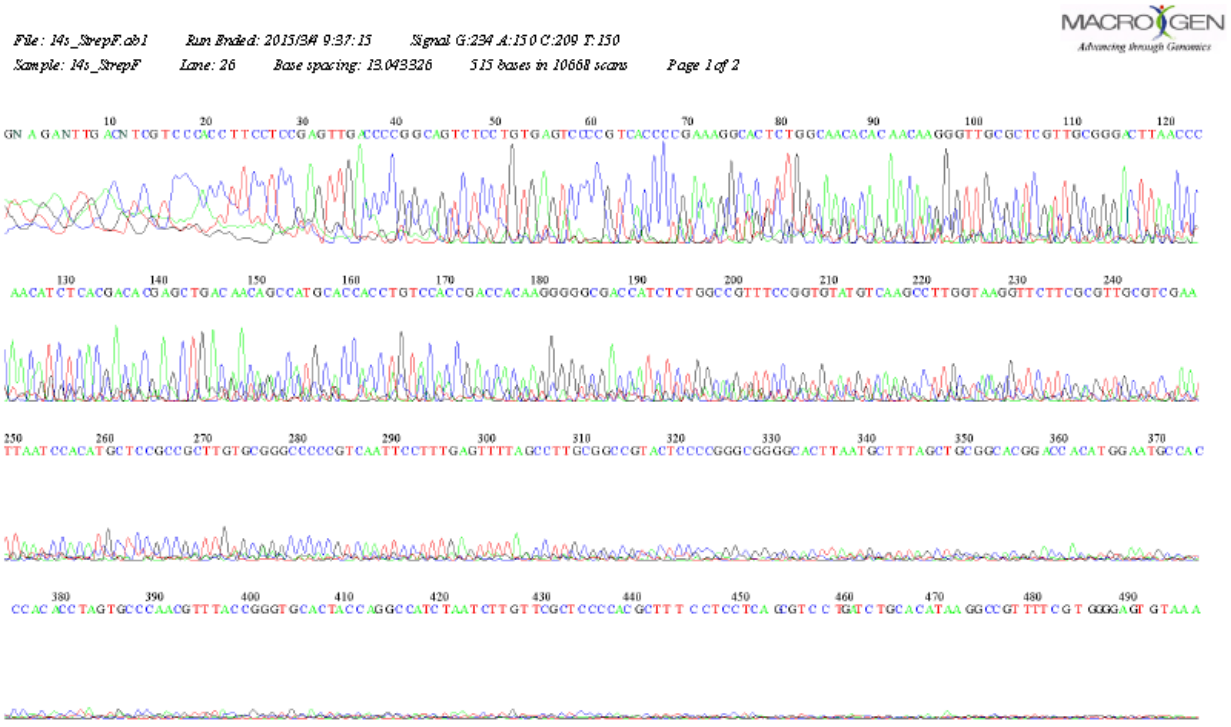
PS13


File : 7s_StrepB.ab1 Run Ended: 2015/8/4 9:37:15 Signal G:903 A:745 C:832 T:566
Sample: 7s_StrepB Lane : 6 Base spacing: 14.451401 665 bases in 9766 scans Page 1 of 2
MACROGEN
Advancing through Genomics

PS20


File : 13s_StrepB.ab1 Run Ended: 2015/3/4 9:37:15 Signal G:2157 A:2562 C:3336 T:2146
Sample: 13s_StrepB Lane: 19 Base spacing: 13.031735 672 bases in 10382 scans Page 1 of 2

PS23


File: 14s_StrepF.ab1 Run Ended: 2015/3/4 9:37:15 Signal G:234 A:150 C:209 T:150
Sample: 14s_StrepF Lane: 26 Base spacing: 13.043326 515 bases in 10668 scans Page 1 of 2
MACROGEN
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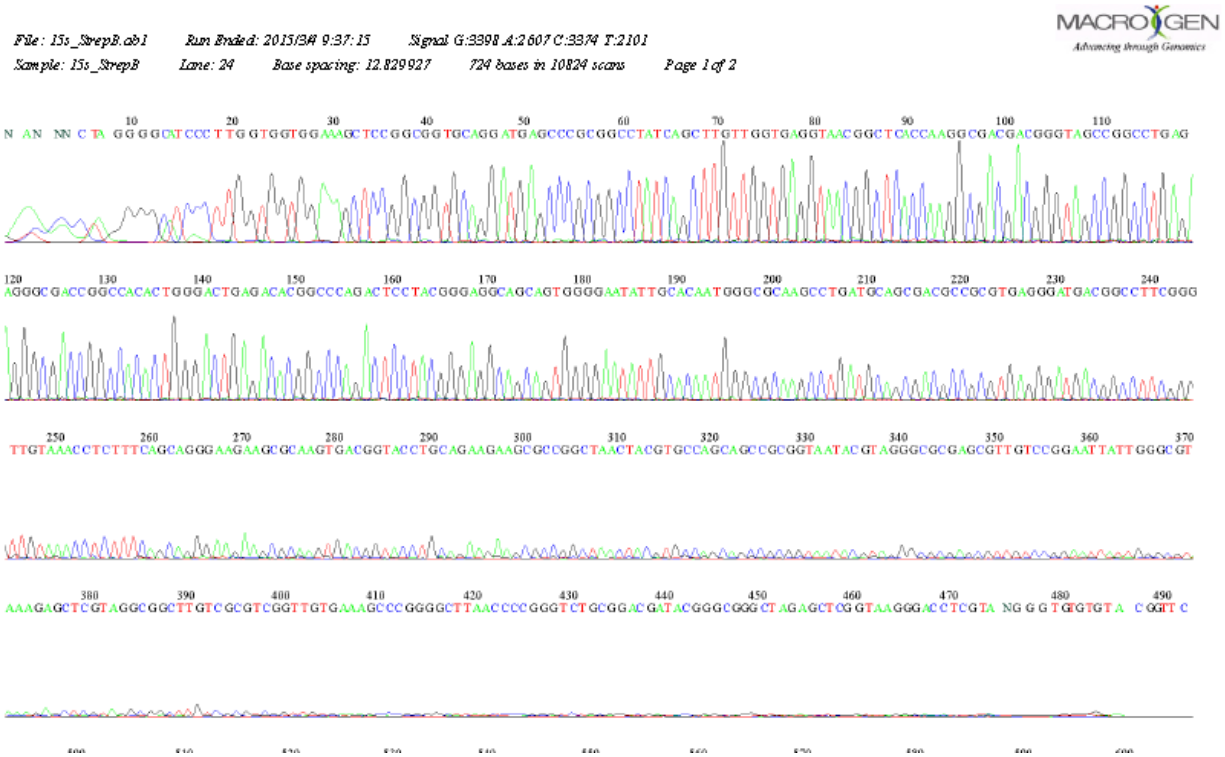
PS24


File : 15s_StrepB.ab1 Run Ended: 2015/3/4 9:37:15 Signal: G:3398 A:2607 C:3374 T:2101
Sample: 15s_StrepB Lane: 24 Base spacing: 12.829927 724 bases in 10824 scans Page 1 of 2
MACROGEN
Advancing through Genomics

PS28

APPENDIX 3

GC-MS analysis of isolates PS1

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	3.104	20529	0.11	Oxime-, methoxy-phenyl-_
2	3.302	52913	0.29	2-Methyl-1-vinylimidazole
3	3.534	74461	0.41	Nanofin
4	4.487	80234	0.45	2-Dodecylcyclobutanone
5	4.740	57398	0.32	1-Butanamine, 2-methyl-N-(2-methylbutyli
6	4.855	113400	0.63	Piperidine, 4-methyl-
7	4.996	59695	0.33	Bicyclo[2.2.2]oct-2-ene, 1-methylamino-
8	5.607	111754	0.62	Propanoic acid, 3-(methylthio)-
9	5.877	58813	0.33	Piperidine, 5-ethyl-2-methyl-
10	6.727	453303	2.52	4-Vinyl-imidazole
11	7.620	126254	0.70	N1,N1-Diethyl-N2-[1,2,4]triazolo[4,3-a]pyr
12	8.448	77303	0.43	Indole
13	8.595	935254	5.21	Ornithine
14	8.950	105827	0.59	Cyclohexanone, 3-ethenyl-
15	9.822	254560	1.42	2H-Azepin-2-one, 3-aminohexahydro-
16	9.872	200135	1.11	Pyrrolidne-1-acetonitrile,2,5-dioxo-
17	10.473	356492	1.99	Acetamide,N-(5-methylisoxazol-3-yl)-2-mor
18	10.577	210764	1.17	4H-Pyran-4-one, 2-methoxy-6-methyl-
19	10.766	20747	0.12	2H-indol-2-one,1,3-dihydro-
20	10.842	169211	0.94	4,5-Pyrimidinediamine, 6-methyl-
21	11.269	240852	1.34	Piperidine, 1-(1-pentenyl)-
22	11.519	73741	0.41	1,8-Diazabicyclo{5,4,0}undec-7-en-11-one
23	11.700	253763	1.41	5-Isopropyl-2,4-imidazolidinedione
24	11.974	118039	0.66	1,2:5,6-Di-O-ethylborandiyl-D-glucohexodi
25	12.105	94840	0.53	Quinoline, 2-ethyl-
26	12.482	47918	0.27	2(equat)-Methyl-trans-decahydroquinol-4-
27	12.590	31981	0.18	2,5-Piperazinedione, 3-methyl-6-(1-methyl
28	12.705	145492	0.81	2,5-Piperazinedione, 3-methyl-6-(1-methyl
29	12.798	15337	0.09	Thiazolidin-4-one,5-(2-ethyl-2H-pyrazol-3-
30	13.055	2696396	15.02	4-Methyleneproline
31	13.305	24447	0.14	Glycyl-L-glutamic acid
32	13.579	673037	3.75	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-
33	13.714	121822	0.68	dl-Alanyl-l-leucine
34	13.846	330955	1.84	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-
35	14.070	1240601	6.91	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahyd
36	14.192	47639	0.27	Propanamide,N-(2-fluorophenyl)-3-(4-morp
37	14.534	29158	0.16	2-Benzyl-3-hydroxypropanoic acid, ethyl es
38	14.721	497060	2.77	2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4,-
39	14.985	796322	4.44	L-Proline, N-valeryl-, tetradecyl ester
40	15.712	147024	0.82	4(1H)-Pyrimidinone, 6-amino-2-methyl-5-n
41	15.809	81910	0.46	Actinomycin C2
42	15.879	484838	2.70	(2S,6R)-2,6-Dibutyl-4-methylpiperidine
43	15.974	572915	3.19	Bromocriptine
44	16.031	881060	4.91	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-di
45	16.163	220674	1.23	Bicyclo[2.2.1]heptan-2-ol, 7-cyclohexyl-, (e
46	17.039	25561	0.14	[1,2,4]Triazolo[1,5-b]pyridazin-6-ol, 8-met
47	17.122	125643	0.70	[1,2,4]Triazolo[1,5-a]pyrimidin-7-ol, 5-met
48	18.767	140343	0.78	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enor
49	18.896	177771	0.99	4,4-Dimethyl-2,5-dioxo-1-vinylimidazolidin

Peak#	R.Time	Area	Area%	Name
50	19.012	698717	3.89	3-[1-Aziridyl]butyraldehyde azine
51	19.661	438299	2.44	Ergotaman-3',6',18-trione, 9,10-dihydro-12
52	20.024	383951	2.14	4-Ethoxybenzene, 4,4'-dithiobis-
53	22.503	1518360	8.46	Sebacic acid, 2,6-dimethoxyphenyl ethyl es
54	22.817	1039478	5.79	5-Nitroso-2,4,6-triaminopyrimidine
		17954991	100.00	

GC-MS analysis of isolates PS28

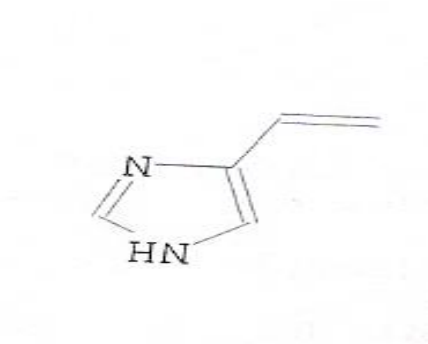
Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	3.119	43688	0.02	1-Butylpyrrolidine
2	3.190	434374	0.21	2-Cyclobutyl-2-propanol
3	3.250	88234	0.04	Vigabatrin
4	3.276	478796	0.24	2-Methylcyclohexylamine
5	3.348	297870	0.15	2-Bicyclo[2.1.1]hexanamine, 3-iodo-, trans-
6	3.514	462897	0.23	N-(2-Methylbutylidene)isobutylami
7	3.552	519433	0.26	Nanofin
8	3.595	312423	0.15	1H-Azepine, hexahydro-3,3,5-trimethyl-
9	3.870	1363339	0.67	1-Propanamine, 3-(methylthio)-
10	4.276	185605	0.09	2-Pyrrolidinemethanol,2-methyl-,(S)-
11	4.459	667386	0.33	Pyrrolidine, N-(3-methyl-3-butenyl)-
12	4.575	170704	0.08	Benzylamine
13	4.745	885640	0.44	1-Butanamine, 2-methyl-N-(2-methylbutyli
14	4.857	278379	0.14	Piperidine, 2,3-dimethyl-
15	4.915	163723	0.08	2-Pyrrolidinone, 1-methyl-
16	4.941	703052	0.35	Bicyclo[2.2.2]oct-2-ene, 1-methylamino-
17	5.245	376131	0.19	2-Pyrrolidinone
18	5.705	2242089	1.10	Amphetamine
19	5.878	65095	0.03	2-Cyclopenten-1-one,3-hydroxy-2-methyl-
20	6.150	1243693	0.61	Succinimide
21	6.401	166548	0.08	7-Azabicyclo[4.1.0]heptane, 1-methyl-
22	6.440	229342	0.11	(S)-6,6-Dimethyl-2-azaspiro{4.4}non-1-ene
23	6.601	14819	0.01	1,2-Dioxolan-3-one, 5-methyl-4-methylene-
24	6.649	59741	0.03	2,4(1H,3H)-Pyrimidinedione, 5-(1,1-dimeth
25	6.697	180323	0.09	Pyrrole, 1-methyl-3-(1,1-dimethylethyl)-
26	6.753	157892	0.08	5-Vinyl-pyrazole
27	7.014	194522	0.10	Pyridine, 1-acetyl-1,2,3,4-tetrahydro-
28	7.251	69759	0.03	4-Quimolinamine,decahydro-1-methyl-
29	7.302	50660	0.02	2-(1,2,3,6-Tetrahydro-1-pyridinyl)-1-propa
30	7.369	124015	0.06	3,7-Diazabicyclo{3,3,1}nonan-9-one,3,7-dir
31	7.428	36020	0.02	Tricyclo[4.3.1.1(2,5)]undec-3-en-10-one, 5-r
32	7.467	34220	0.02	Amantadine
33	7.793	338369	0.17	Cyclohexanamine, N-2-propenyl-
34	8.035	273180	0.13	Naphthalene.1,2,3,4,4a,5,8,8a-octahydro-4a
35	8.329	2039626	1.00	.alpha.-Campholenal
36	8.450	244946	0.12	Indole
37	9.112	139634	0.07	Piperidine, 4-methyl-
38	9.304	228121	0.11	Cycloheptano{d}imidazolidin.1,3-dihydrox
39	9.471	382857	0.19	DL-Proline, 5-oxo-, methyl ester
40	9.538	265070	0.13	2,5-Pyrrolidinedione,1-butyl-
41	9.916	5729734	2.82	l-Alpha-amino-epsilon-caprolactam
42	10.668	1199831	0.59	Pyridine, 3-phenyl-
43	10.737	171511	0.08	9-Borabicyclo{3,3.1}nonan-9-amine,N-met
44	10.792	231129	0.11	Phenol,2-(ethylamino)-4-methy-
45	10.944	506838	0.25	Pyrrolizin-1-one, 7-propyl-
46	11.236	140649	0.07	[1,1'-Biphenyl]-2-amine
47	11.290	365808	0.18	3-Butylindolizidine
48	11.550	308897	0.15	1,8-Diazabicyclo[5.4.0]undec-7-en-11-one
49	11.604	245108	0.12	(3S,5R,8aR)-3-(Hex-5-en-1-yl)-5-(pent-4-en

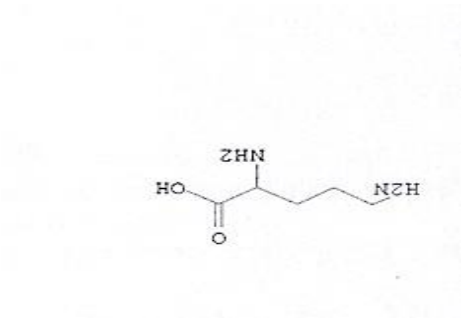
Peak#	R.Time	Area	Area%	Name
50	11.704	349732	0.17	Glutaric acid, ethyl 4-methyl-3-nitrobenzyl
51	11.932	811415	0.40	3-Pyrrolidin-2-yl-propionic acid
52	12.105	173090	0.09	3-Methyl-4-phenyl-1H-pyrrole
53	12.430	788808	0.39	DL-Isoleucine
54	12.769	447973	0.22	Propanamide, 2-amino-3-phenyl
55	13.160	1139975	0.56	Gephyrotoxin 207a
56	13.221	324403	0.16	N,N-Dimethyl-8-(1H-1,2,3-triazol-1-yl)-1-n
57	13.362	472532	0.23	Varenicline
58	13.649	7208989	3.55	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-
59	13.900	10543328	5.19	(3S,6S)-3-Butyl-6-methylpiperazine-2,5-dio
60	14.143	2971103	1.46	Pipradrol
61	14.200	6832444	3.37	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahyd
62	14.336	410023	0.20	Oxalic acid,monomorpholide,undecyl ester
63	14.811	16625684	8.19	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahyd
64	14.883	798123	0.39	3,6-Diisopropylpiperazin-2,5-dione
65	15.072	12327314	6.07	l-Norvaline, n-propargyloxycarbonyl-, hep
66	15.178	218102	0.11	N-{4-Cyclooctylaminobutyl}aziridine
67	15.415	1039754	0.51	2-Piperidinone, 1-(3,4,5,6-tetrahydro-2-pyr
68	15.526	244931	0.12	1,7-Trimethylene-2,3,5-trimethylindole
69	15.783	11699969	5.76	l-Leucine, N-cyclopropylcarbonyl-, pentad
70	15.954	11386177	5.61	4(1H)-Pyrimidinone, 6-amino-2-methyl-5-n
71	16.059	12602267	6.21	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-di
72	16.108	4582966	2.26	2-Ethyl-1,3,4-trimethyl-3-pyrazolin-5-one
73	16.390	416926	0.21	9H-Pyrido[3,4-b]indole, 1-methyl-
74	16.460	447181	0.22	9H-Pyrido[3,4-b]indole
75	17.506	494536	0.24	4,6-Disila-dispiro[3.1.3.1]decane
76	17.593	949938	0.47	Diethyl-.alpha.-naphthylamine
77	17.737	1110521	0.55	N(1)-(3-Methyl-1,2,4-oxadiazol-5-yl)-1-pipe
78	18.127	352961	0.17	4(3H)-Pteridinone, 2,7-dimethyl-
79	18.186	333153	0.16	Sebacic acid, (2-(cyclohexenyl-3)-1-phenyl)
80	18.374	379480	0.19	2,5-Piperazinedione, 3-(phenylmethyl)-
81	18.491	1308096	0.64	4(3H)-Pyrimidinone, 2-ethyl-3,6-dimethyl-
82	18.798	3363133	1.66	Octahydro-1H-pyrido(1,2-c)pyrimidin-1-on
83	18.843	343568	0.17	Quinoline, 4-styryl-
84	18.933	2601368	1.28	4,4-Dimethyl-2,5-dioxo-1-vinylimidazolidin
85	19.709	9776896	4.82	Ergotaman-3',6',18-trione, 9,10-dihydro-12
86	19.842	724712	0.36	2,4-Imidazolidinedione, 5-(4-hydroxybutyl)
87	20.062	11471528	5.65	N-(3-Imidazol-1-yl-propyl)-N'-(4-isopropyl
88	20.397	159211	0.08	1,3,5,2-Oxadiazaborine, 2,2-diethyl-1,2,5,6-
89	20.607	265082	0.13	Succinic acid, diamide, N,N'-diethyl-N,N'-d
90	20.711	342743	0.17	1,3-Benzenediol, O-(3-cyclopentylpropiony
91	21.138	515114	0.25	1,2,3,4,6,7,8,9-Octahydrodipyrido[1,2-a:4,3
92	21.320	403088	0.20	3H-Quinazolin-5-one, 2-isopropylimino-7,7
93	21.416	221811	0.11	4-(2-t-Butyl-5-oxooxazolidine-3-carbonyl)-N
94	22.039	258878	0.13	2-Methyl-5-ethoxymethylenimino-1,2,4-tria
95	22.687	22950314	11.31	Sebacic acid, 2,6-dimethoxyphenyl ethyl es
96	22.914	14679938	7.23	5-Nitroso-2,4,6-triaminopyrimidine
		202980998	100.00	

APPENDIX 4

Chemical structure of these active compounds



No. I



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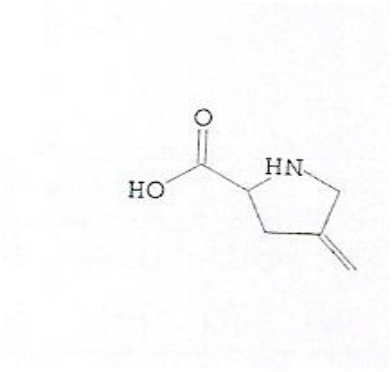

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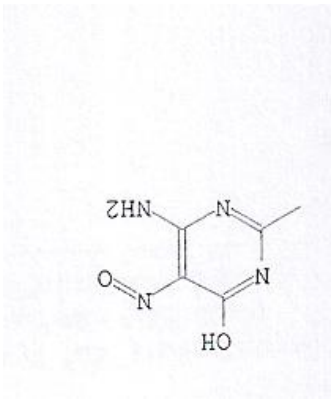

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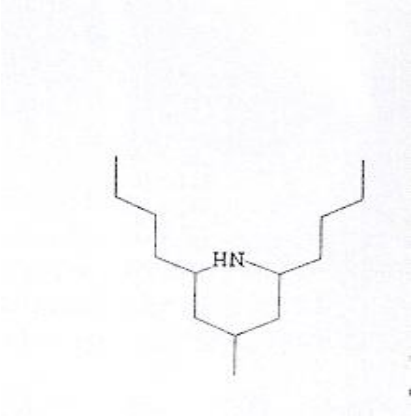

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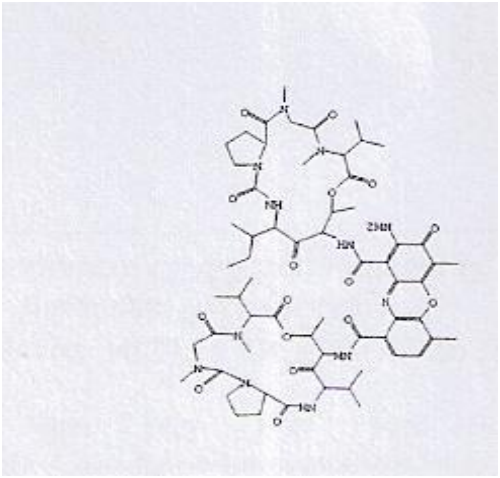

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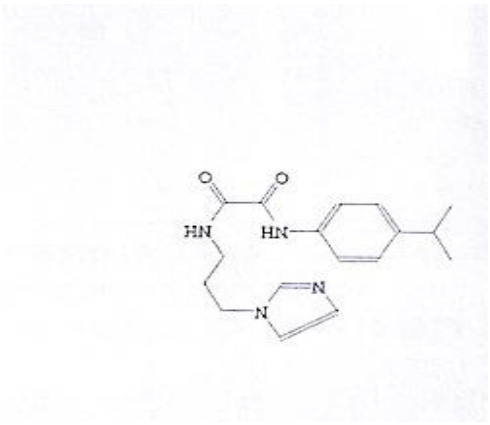

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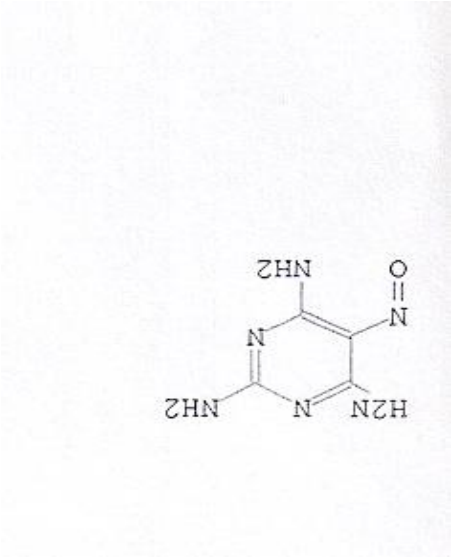

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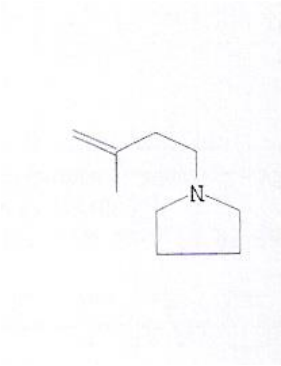

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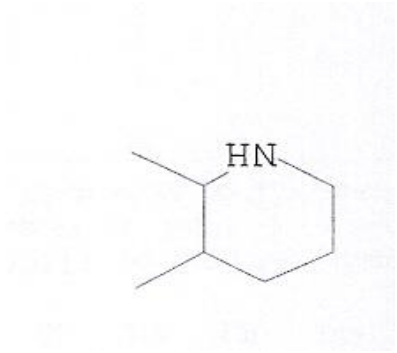

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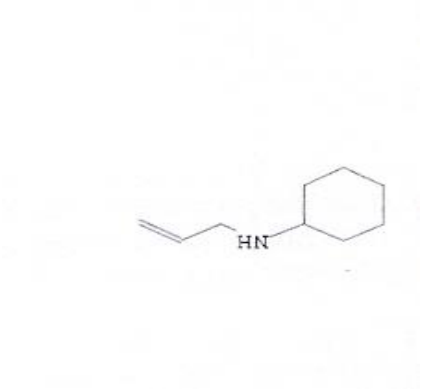

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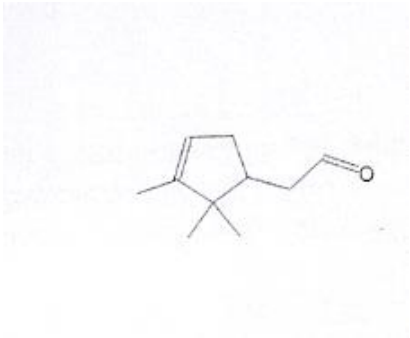
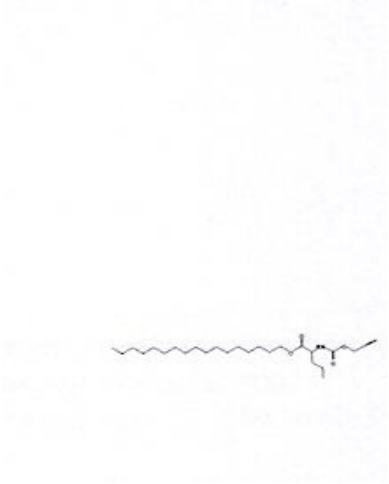


No. XII



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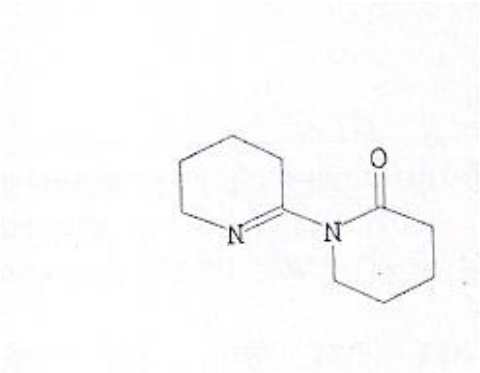

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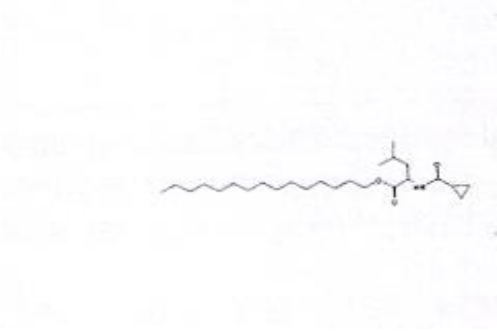

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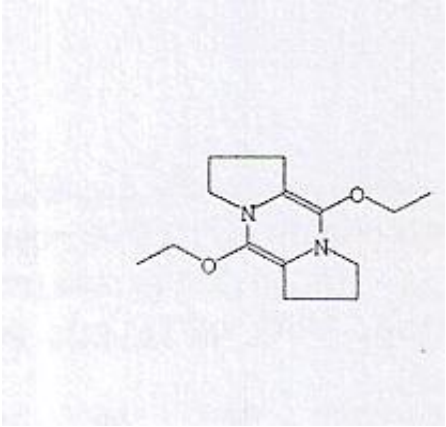
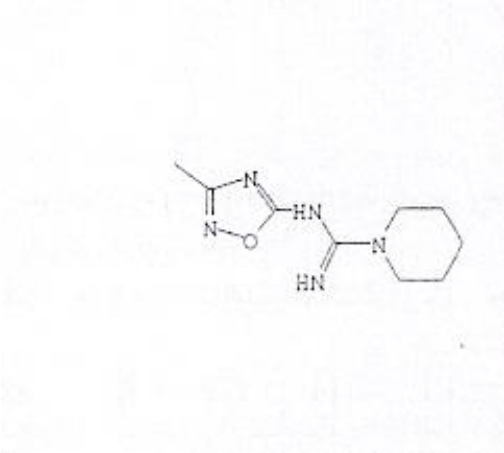


No. XVI



No. XVII