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par

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Sujet

Isolation, Screening and Characterization of Novel Antibiotic Producing Streptomycetes Strains from Soil Origin

Soutenue le 15 Décembre 2007, devant le Jury composé de:

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Dedication

To

The Memory of My Father

Rahimaho-Allah

The Merciful Source

My Mother

The Continuous Support

My Sisters and Brothers

The authentic Women

My Wife

The Flowers of my Life

My Children

Also to

My Dear Uncle Hocine and his Respectable Family

Rabah

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Contents

List of Abbreviations

ACN: Acetonitrile
ATP: Adenosine triphosphate
DIZ: Diameter of inhibition zone
DGGE: Denaturing Gradient Gel Electrophoresis
DNA: Deoxyribonucleic acid
ES: Egyptian strain
ESI-TOF: Electrospray time of flight
GYEA: Glucose-yeast extract agar
HPLC: High performance liquid chromatography
HRTOF: High resolution time of flight
ISP: International *Streptomyces* Project
KCN: Potassium Cyanide
LB: Lauria broth
L-DAP: L-diaminopimelic acid
LFRFA: Low-frequency restriction fragment analysis
MBA: Modified Bennett's Agar
MLST: Multilocus sequence typing
MS/MS: Tandem mass spectrometry
N HCL: Normal-Hydrochloric acid
PCR: Polymerase chain reactions
PDA: Potato dextrose agar
PFGE: Pulsed-field gel electrophoresis
RAPD: Randomly Amplified Polymorphic DNA
RPC18: Reverse phase carbon 18 column
RNA: Ribonucleic acid
TFA: Trifluoroacetic acid
TSB: Tryptic soy broth
TYG: Tryptone-yeast extract-glucose

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Introduction

The antibiotics have been useful in our battles against infectious bacteria and fungi for over 60 years. However, many antibiotics are used commercially, or are potentially useful, in medicine for activities other than their antibiotic action. They are used as antitumor agents, immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, and antiparasitic agents. A number of these products were first discovered as antibiotics which failed in their development as such, or as mycotoxins. In addition to the above alternative applications, new powerful antibiotics have been discovered and commercialized in recent years and others are in clinical testing at the moment.

With the seemingly exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, the need for discovering novel drugs is real. Among the different types of drugs prevailing in the

market, antifungal antibiotics are a very small but significant group of drugs and have an important role in the control of mycotic diseases. The need for new, safe and more effective antifungal antibiotics is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host as well as the Candidiasis. Candidiasis commonly called yeast infection or thrush is a fungal infection of any of the *Candida* species, of which *Candida albicans* is the most common.

The clinical manifestations may be acute, sub-acute or chronic to episodic. Involvement may be localized to the mouth, throat, skin, scalp, vagina, fingers, nails, bronchi, lungs, or the gastrointestinal tract, or become systemic as in septicaemia, endocarditis and meningitis. In healthy individuals, *Candida* infections are usually due to impaired epithelial barrier functions, and occur in all age groups, but are most common in the newborn and the elderly. They usually remain superficial and respond readily to treatment. Systemic candidiasis is usually seen in patients with cell-mediated immune deficiency, and those receiving aggressive cancer, immunosuppression, or transplantation therapy. Several species of *Candida* may be aetiological agents, most commonly, *Candida albicans* and rarely *C. tropicalis, C. krusei. C. parapsilosis, C. guilliermondii*, *C. kefyr (C. pseudotropicalis*) and *C. glabrata*. All are ubiquitous and occur naturally on humans.

For this reason, discovering or development of new antifungal agents, preferably with novel mechanisms of action, is an urgent medical need. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources. That is why; screening of microorganisms for the

production of novel antifungal agents or to find out new strains of actinomycetes able to produce these bioactive compounds continues to be the major interest by scientists over the world.

Aim of the present study

It has clearly seen from the introduction given that, screening of microorganisms from new sources; always encourage the attempt to find out clinically useful antibiotics or screen out new potent actinomycetes strains able to produce these bioactive compounds. It was therefore decided to do the following:

1- Isolation and purification of actinomycetes strains from different soils.
2- Screening of purified strains for their abilities to produce antifungal agents.
3- Selection of the most antifungal producing actinomycetes strains.
4- Determination of the optimal conditions for antifungal production.
5- Taxonomy and identification of the selected strains.
6- Extraction and purification of active substances.
7- Characterization of substances exhibited antifungal activity.

Chapter 1

Literature Review

1.1 The antibiotics

Everyone has heard about antibiotics, and most of people, at least in the developed world, have benefited from their curative powers. But how many from us know where they come from and how they developed into a cornerstone of medicine? The mold that famously contaminated Alexander Fleming's culture dish and eventually gave us penicillin is one of the icons of 20th century biology, but penicillin was just the first antibiotic to become a medicine. Dozens of important compounds followed, revolutionizing the treatment of infectious diseases. Most are made by a group of soil microbes, the actinomycetes, which were little known until their powers of antibiotic production, were revealed, starting some 60 years ago (Hopwood, 2007).

1.1.1 Definitions of antibiotics:

Waksman wrote in 1947 that antibiotics are chemical substances formed by microorganisms that can inhibit the growth of bacteria and other microorganisms or even destroy them. Antibiotic is a chemical substance produced by living organism that demonstrates inhibitory or germicidal activity toward microorganisms in vivo or in vitro (Samuel and Cecil, 1959). Navashin in 1970 defined antibiotics as fellow: (we think it reasonable to understand by antibiotics, those chemo-therapeutic substances that are derived from microorganisms or other natural sources, as well their synthetic analogues and derivatives that have the selective power to inhibit the growth in the body of the patient of, the causative agent of disease, and /or to arrest the development of malignant tumors (Egorov, 1985). Antibiotics were also defined by Dekker, (1975) and Egorov, (1978) as substances formed by micro-organisms which in low concentration can inhibit the growth of other micro-organisms or kill them. Whereas, Betina, (1983) and Pelczar *et al*., (1986) wrote that, antibiotics are chemical compounds produced by living organisms: bacteria, fungi and actinomycetes, which in small quantities can inhibit the growth of micro-organisms or even kill them.

1.1.2 The history of antibiotics:

For much of our history, humanity did not have a truly effective way of combating pathogenic microorganisms. Medical practice consisted largely of weak palliative measures aimed at alleviating the symptoms rather than attacking the source

of infectious diseases. This situation was radically altered with the invention of chemotherapy by Paul Ehrlich, a German physician. Ehrlich was impressed by the ability of certain dyes to stain specific parts of a cell in histological specimens. He reasoned that it might be possible to find a chemical that could specifically bind to a pathogenic microorganism but not to human cells. His effort to find such a "magic bullet" was finally rewarded when his assistant, Sahachiro Hata, discovered in 1910 that the 606^{th} compound they tested was active against the causative agent of syphilis, which is transmitted through sexual contact and causes devastating mental symptoms and death in its later stages. Although this compound, salvarsan had significant toxicity for humans; it was the first effective treatment for an infectious disease.

The next major advance in chemotherapy did not occur until nearly 30 years later. In the early 1930s, Gerhardt Domagk**,** working in a German company involved in the manufacture of dyes and perhaps influenced by Ehrlich's earlier ideas, tested various synthetic dyes as antimicrobial agents. He found that the red dye prontosil was effective in the treatment of experimental streptococcal infections in mice. A few years later, French workers discovered that the active moiety in Prontosil was the colorless compound sulfanilamide. This realization led to the development of various sulfanilamide derivatives, the so-called sulfa drugs.

The sulfa drugs were the first truly successful chemotherapeutic agents because they were active against most bacteria, many of them could be taken orally, and they were remarkably nontoxic. In 1940s, Donald, D. Woods concluded that, sulfa-drugs act by mimicking a natural compound, *p*-aminobenzoic acid, and thus by competitively inhibiting the enzyme dihydropteroate synthetase, which catalyzes a step in the de novo biosynthesis of the vitamin folic acid .The drug is not toxic to humans because we do not synthesize folic acid but rather acquire it from our diet. Thus the "selective toxicity" that was Paul Ehrlich's ultimate goal is due in this case to the different nutritional requirements of bacteria and human beings.

The tale of the discovery of the first antibiotic, penicillin, by Alexander Fleming is well known. Fleming is supposed to have kept a rather untidy laboratory. Tradition has it that he returned from vacation to find that mold had contaminated one of his bacterial cultures on a Petri dish and that the bacterial colonies adjacent to the

mold colony were lysed. This story is often cited as an example of the importance of serendipity in science. But it fails to take account of the fact that in an effort to find agents that could be used in the treatment of bacterial infections, Fleming had dedicated his entire career to the search for natural products that lyses bacterial cells. He had actually discovered the enzyme lysozyme several years earlier but then was disappointed to find that most human pathogens are intrinsically resistant to its lytic action .Because lysozyme is present in most of the tissues and body fluids of higher animals, pathogenic bacteria presumably have been subjected to evolutionary selection for such resistance.

A key aspect of Fleming's discovery of penicillin is that he tried to isolate the active substance and use it to treat infections. Dozens of scientists before Fleming had published reports of either lyses or growth inhibition of bacteria caused by products of molds and other microorganisms. Yet nothing came of those findings because the authors operated as classical "naturalists" reporting "curious" phenomena. The eventual mass production and practical application of penicillin came after great effort by many outstanding chemists and wartime collaboration among pharmaceutical companies. Seen in this light, Fleming's early efforts are all the more impressive: In his initial report of 1928, he showed the efficacy of the culture filtrate of his mold as a local therapeutic agent in an animal infection model.

Around 1938-1939, Florey and his collaborators undertook the reevaluation of the therapeutic possibilities of Fleming's penicillin at the sir William Dunn school of pathology at Oxford University, the outcome of this fortunate choice is now a milestone in the history of human kind. Dubos in 1939 described a series of microbial products (complex antibiotic) called tyrothroin produced by the soil bacterium, *Bacillus brevis*. Further investigations showed that this preparation contained two major materials, gramicidin and lyrocidine, both were found useful in treating humans infections. Similar investigations were made by Waksman, who made a systematic search for antimicrobial substances in a group of soil inhabiting *Streptomyces* (Hopwood 2007).

Waksman and his associates reported the isolation of actinomycin in 1940, streptothericin in 1942, streptomycin in 1943 and neomycin in 1949. The discovery of

streptomycin, which was found to be particularly useful in treating bacterial infection especially tuberculosis, greatly stimulated the search for useful antibiotics among *Streptomyces* (Waksman, 1969).

During the so-called “Golden Era” of antibiotic research, from the mid of 1940 to the mid of 1960, the rate of discovery of new microbial metabolites did not significantly change (Berdy, 1974). Subsequently, it accelerated slightly and there was a dramatic increase between 1972 and 1978, during such a period, the discovery rate jumped from 180 to 340 new antibiotic per year (Perlman, 1997). The isolation of 6-aminopenicilinic acid (6APA) in the late of 1950 opened the way to the semi synthetic penicillins and provided an example for development of semi synthetic antibiotics in general (Robinson, 1979; Vandame, 1980). Chemical derivation and bioconversion of natural compounds yielding semi synthetic antibiotics (Sebek, 1980; Vandame 1980) and use of immobilized cell technology (Vandame, 1983).

In the late 1950, a revival of antibiotic discovery occurred, owing to the application of novel screening programs, supersensitive test organisms, new antibiotic source, and the broadening of the search for novel microbial products (classically encompassing antiviral and antifungal agents) to include agents with pesticidial, anti tumor, insecticidal, herbicidal, anticoccidal, cytotoxic, anti helminthic, hormonal immunoregulatory food preserving, growth promoting and enzyme inhibiting activities as well as products with pharmacological activity (. ,, 1980; 1995; Demain, 1983; Miller *et al.*, 1983 ; Umezawa, 1983).

1.1.3 Origins of antibiotics:

As seen from Table 1.1, actinomycetes do indeed make some two-thirds of the known antibiotics that are produced by microorganisms (some definitions actually restrict the word “antibiotic” to microbial products), and amongst them nearly 80% are made by members of the genus *Streptomyces*, with other genera trailing numerically: *Micromonospora* is the runner-up with less than one-tenth as many as *Streptomyces*. If we include secondary metabolites with biological activities other than anti-microbial, actinomycetes are still out in front, with over 60% (again *Streptomyces* spp. account for 80% of these). However, Table 1.1 also brings out the fact that it is not the overall chemical versatility of the actinomycetes that is unparalleled – fungi and higher

eucaryotes beat them hands down – but the proportion of their out put that has a known biological effect.

Table 1.1: Approximate numbers of secondary metabolites produced by different groups of organisms as in Kieser *et al*., (2000).

Source	**Bioactive metabolites**			**"inactive" metabolites**
	Antibiotics	Others	Total	
Non-actinomycete bacteria	1400 (12%)	240 (9%)	1640 (11%)	2000-5000
Actinomycetes	7900[1] (66%)	1220[1] (40%)	9120[1] (61%)	8000-10,000
Fungi	2600 (22%)	1540 (51%)	4140 (28%)	15,000-25,000
Total mocroorganisms	11,900 (100%)	3000 (100%)	14,900 (100%)	25,000- 40,000
Lichens	150	200-500		¬ 1000
Algae	700	800-900		1000-2000
Higher plants	5000	25,000-35,000		500,000-800,000
Terrestrial animals	500	10,000-15,000		200,000-300,000
Marine animals	1200	1500-2000		2000-3000
Total higher Organisms	7500	35,000-50,000		> 1,000,000

[1] In each category, nearly 80% were found in *Streptomyces* and 20% in other actinomycetes.

1.1.4 Classification of antibiotics:

Although there are several classification schemes for antibiotics, based on bacterial spectrum, (broad versus narrow) Waksman and Lechevalier, (1962)**,** the type of activity (bactericidal versus bacteriostatic) according to Feingold, (1963); Simon *et al*., (1985), but the most useful is based on chemical structure. Antibiotics within a structural class will generally show similar patterns of effectiveness, toxicity and allergic potential (Berdy, 1974; Berdy *et al*., 1987).

1.1.4.1 According to their biological origin:

- **Antibiotics produced by representatives of the genus *Bacillus*:** e.g. Gramicidins produced by *B. brevis*, subtilin produced by *B. subtilis*, polymixiins produced by *B. polymyxa* and colistatin produced by *B. unidentified*.

- **Antibiotics produced by representatives of the genus *Pseudomonas*:** e.g. Pyocyanin produced by *Ps. aeruginosa* and viscosin produced by *Ps. viscosa* while coliformin is produced by *Escherichia coli*.

- **Antibiotics produced by representatives of genera *Micrococcus, Streptococcus, Diplococcus, Chromobacterium,* and *Proteus*:** e.g. Nisin produced by Str. lactis, diplomycin produced by *Diplococcus* X-5, prodigiosin produced by *Chramobacterium prodigiosum* (*Serratia marcescens*), and protaptins produced by *Pr. Vulgaris.*

- **Antibiotics produced by species of the genus *Streptomyces*:** e.g. Streptomycin *S. griseus*, tetracyclines produced by *S. aureofaciens* and *S. rimosus*, Novobiocin produced by *S. spheroides* and actinomycins produced by *S. antibioticus*, etc.

- **Antibiotics produced by fungi***:* Many antibiotics are produced by fungi, e.g. penicillin produced by *Pen. Chrysogenum*, griseofulvin produced by *Pen. Griseofulvum* and trichothecin produced by *Trichothecium roseum*, thermophillin produced by *Basidomycete thermophila*, lenzitin produced by *Lenzites sepiaria* and chetomin produced by *Chaetomiun cochloides* (ascomycete).

- **Antibiotics produced by lichens and algae***:* Few active compounds were found to be produced by lichens, e.g. Usinc (usninic) acid produced by Lichen and by Algae like Chlorellin produced by *Chlorella vulgaris.*

- **Antibiotics produced by higher plants***:* Antibiotics are also produced by higher plants, e.g. Allicin produced by *Allium sativum*, Raphanin produced by *Raphanus sativum*, Phytoalexins: Pisatin in peas (*Pisum sativum*) and phaseolin in true beans (Phaseolus vulgaris).

- **Antibiotics of animal origin:** These antibiotics are from animal sources, Lysozyme, Ecmolin, Cruzin (*Tripanosoma cruzi*) and Interferon.

1.1.4.2 According to their mechanism of biological action:

- **Antibiotics inhibiting synthesis of the cell wall:** These compounds, D-cycloserine, penicillins, cephalosporins, and some other antibiotics inhibit the synthesis of the cell wall of sensitive bacteria. D-cycloserine is produced by several streptomycetes (*Streptomyces roseochromogenes, Streptomyces lavendulae,* etc.). The antibiotics are an antagonist to D-alanine, the amino acid of peptideglycan D-cycloserine inhibits the activity of the enzymes alanine- racemase and D-aianine synthetase. In other words, D-cycloserine inhibits the enzymes which catalyze the incorporation of D-alanine in peptidoglycan (Hammes and Nehaus, 1974; Perkins and Nieto, 1974; Kuroda *et al*., 1980; Hong *et al*., 2002).

Penicillin G is highly active against some Gram-positive bacteria. It is only slightly active against some Gram-negative bacteria and is inactive against the cells of animals, higher plants, fungi, and protozoa.

As penicillin G acts on the sensitive microbes, the cell morphology is effected (the cells become elongated, swollen, distorted; long chains or spheres are formed; lyses occurs); the nature of biochemical processes and the routes of metabolism are also affected. The selective antimicrobial activity of penicillin presumably depends on the different composition of cells of Gram-positive and Gram-negative bacteria.

The cell wall of Gram-positive bacteria (2-3 %) of lipids produces some amino acids during hydrolysis. Aromatic or sulfur- containing amino acids rarely occur among them.

Cell walls of Gram-negative bacteria contain 18 to 20 per cent of liberate a greater number of amino acids during hydrolysis. Sulphur– containing amino acids are among them.

Cell walls of Gram-positive bacteria contain sugars, N-acetylmuramic acid (lactic ester and acetylglucosamine), amino sugars (glucosamine, galactosamine, and a

glucosamine derivative of muramic acid, glutamic acid, lysine, diaminopimelic acid). When exposed penicillin, cells of *Staphylococcus* accumulates three uridine nucleotides, for this reason, it was suggested that these nucleotides might act as carriers of peptides of acetylmuramic acid contained in the polymers of the cell wall.

The biological mechanism of action of cephalosporins is similar to that of penicillin. Thus, the inhibition of activity of two enzymes (transpeptidase of murein and D-alanine carboxyl peptidase), which are involved in the synthesis of the main polymer of the cell wall at its final stage, underlies the mechanism of the biological action of penicillins and cephalosporin.

- Antibiotics upsetting the membrane functions*:* Antibiotics substances acting on the cytoplasmic membrane can be divided into three groups:

i) **- Substances disorganizing membrane structures** (gramicidin S, polymyxins, polyenes). Bulgakova and Polin (1968) indicated that the biological action of gramicidin S is based on the disorganization of the state and function of cell membranes, which in turn depends on binding of the antibiotic which the membrane components. Gramicidin S could inhibit the energy metabolism, especially at its initial state i.e. dehydration.

Polymyxins cause the loss of low-molecular substances (phosphate, pentose) and decomposition of nucleic acids.

Polyenes, combine specifically which the cytoplasmic membrane of the fungal cell to affect its barrier function which ensures selective permeability.

ii) **- Antibiotics inhibiting membrane-bound protein (enzymes) which is involved in transport processes:** Oligomycin inhibits the mitochondrial adenosine triphosphatase (ATP-which is bound with bacterial membranes and is involved in the synthesis of ATP or its utilization, (Egorov, 1978).

iii) - **Antibiotic ionophores, changing specifically permeability of biological membranes:** Valinomycins, gramicidins (except for gramicidins S), and macrotetralides (actins) are ionophores and act as specific conductors of cations through the membrane, (Egorov, 1978).

- Antibiotics inhibiting synthesis of proteins*:* It was established that organisms synthesize proteins mainly in the cytoplasm on ribosomes. Ribosomes are ribonucleoprotein particles containing the high polymer RNA and structural protein. Ribosomes are normally present in the cell cytoplasm in the Free State. The biochemical role of ribosomes consists in the biosynthesis of proteins. This is probably their only faction. Amino acids are combined on ribosomes into polypeptides to form secondary and tertiary structures of proteins. The primary stage formation of the polypeptide chain of protein is activation of amino acid by adenosine triphosphate (ATP).

Streptomycin: is active against Gram-positive and Gram-negative bacteria. The biological activity of streptomycin increase which the pH of the medium. It was established that streptomycin produces strong varying effect on the metabolism of sensitive cells. The antimicrobial effect of this preparation depends on the inhibition of protein in the bacterial cell.

If streptomycin is added in different concentrations to the growing culture of *E. coli*, the growth is stopped immediately while the synthesis of nucleic acids is continued. It can be stated that streptomycin inhibits the synthesis of protein at final stages of this process (aminoacyl/ tRNA transport to ribosomes) without affecting the initial stage (i.e. the stage of activation of amino acids).

The antibiotic is attached to the ribosome to prevent the synthesis of protein. This is confirmed by the fact that streptomycin inhibits the incorporation of some amino acids (^{14}C) into the ribosome proteins to upset their function without affecting the structure and the physicochemical properties of ribosomes. In addition to the inhibition of protein synthesis, streptomycin can also inhibit oxidative processes for example, inhibits the enzymes succinic dehydrogenase and fumarase.

Tetracyclines: The chemical likeness of tetracycline, chlortetracycline, oxytetracycline, and other antibiotics of the tetracycline group suggests that the mechanisms of their biological action might be very similar as well**.**

Chlortetracycline and oxytetracycline inhibit phosphorylation processes e.g. they prevent inclusion of phosphorus into nucleic acids. The tetracycline inhibits some enzymes (peptidases, D-amylases, succinic dehydrogenases, fumarases, and tryptophanases). Sazykin (1965) revealed that oxytetracycline at 25 (mg/ml) inhibits selectively synthesis of protein by the cells of *S. aureus* whereas the synthesis of RNA and DNA still continues.

Chloramphenicol effects inhibition of protein synthesis. Chloramphenicol in low concentrations inhibits significantly the synthesis of protein in chloramphenicol – sensitive bacteria while high concentrations inhibit respiration, production and accumulation of glutamic acid and phenylalanine, and block the synthesis of nucleic acids. Chloramphenicol inhibits protein synthesis in bacteria at the stage of amino acid transfer from the aminoacyl tRNA to the ribosome. Hiroshi *et al*., (1999) reported that ferroverdins, inhibitors of cholesteryl ester transfer protein produced by *Streptomyces* sp. WK-5344.

- Antibiotics inhibiting synthesis of purines and pyrimidines:

Azaserine inhibits biosynthesis of purine by combining with the enzyme responsible for the conversion of formylglycinamide ribonucleotide into formyl glycianmidine ribonucleotide. The antibiotic acts mainly on cysteine, sarcomycin and blocks the synthesis of pyridine nucleotides and the inclusion of phosphorous in nucleotides and nucleic acid (Sadoff, 1972).

- Antibiotics inhibiting synthesis of nucleic acids:

Actinomycins: The power of actinomycins to inhibit the synthesis of protein and RNA without affecting formation of DNA has long attracted attention of many workers. Actinomycin D at a concentration of 5 (mg/ml) blocks completely the synthesis of protein and RNA in *Bacillus subtilis* without affecting DNA synthesis.

Mitomycins: They inhibit the growth of many bacteria, protozoa, and also slow down the growth of tumor cells. The mechanism of the biochemical action of mitomycins is to block the synthesis of DNA without practically affecting the synthesis of RNA and protein.

Daunomycin and rubomycins: (A, B_0, B_1 and $C_1$of which rubomycins B_1 and C_1 are most active) are formed by streptomycetes. Daunomycin is synthesized by *S. peuceticus* and rubomycins are derived from *S. coeruleorubidus*. These antibiotics have anti tumor action. It has been shown that the mechanism of the biological effect of daunomycin is due to its ability to set between pairs of DNA bases. The antibiotic is associated with those parts of DNA where the G.C. pairs are available. The chromophore of daunomycin sets the DNA pairs a part to find its place in between them.

- Antibiotics inhibiting respiration:

Actinomycins inhibit the oxidation of succinate, and inhibit the activity of succinate dehydrogenase, and acts on the component of the respiration chain common for succinate oxidase and NAD-H2 oxidase systems.

Oligomycins inhibit mitochondrial adenosine triphosphatase, but this is probably a secondary effect rather than the direct action. Oligomycins can be regarded as inhibitors of mitochondrial respiration because they are effective only against aerobic organisms. patulin it inhibits aerobic respiration of bacteria, fungi and phagocytes, (Egorov, 1978).

- Antibiotics inhibiting oxidative phosphorylation:

Valinomycin inhibits oxidative phosphorylation without practically affecting the oxygen consumption in the presence of ATP, hexokinase and glucose (Egorov, 1978).

1.1.4.3 According to their chemical structure:

The chemical structure of antibiotics is the most valuable classification according to Archer and Ronald, (2001); Diasio, (2003); Scott, (2004); Sherman, (2004); Dancer, (2004) and it is as the following:

- Penicillins: The penicillins are the oldest class of antibiotics and have a common chemical structure that they share with the cephalosporins. Classed as the betalactam antibiotics, the two groups are generally bacteriocidal, which means that they kill bacteria rather than simply inhibit its growth. The penicillins can be further

subdivided. The natural penicillins are based on the original penicillin G structure; penicillinase-resistant penicillins, notably methicillin and oxacillin, are active even in the presence of the bacterial enzyme that inactivates most natural penicillins. Aminopenicillins such as ampicillin and amoxicillin have an extended spectrum of action compared with the natural penicillins; extended spectrum penicillins are effective against a wider range of bacteria. These generally include coverage for *Pseudomonas aeruginosa.*

- Cephalosporins*:* Cephalosporins and the closely related cephamycins and carbapenems, like the penicillins, contain a beta-lactam chemical structure. Consequently, there are patterns of cross-resistance and cross-allergenicity among the drugs in these classes. The "cepha" drugs are among the most diverse classes of antibiotics and are themselves subdivided into first, second, and third generations. Each generation has a broader spectrum of activity than the one before. In addition, cefoxitin, a cephamycin, is highly active against anaerobic bacteria, which offers utility in treatment of abdominal infections. The third generation drugs, cefotaxime, ceftizoxime, ceftriaxone, and others, cross the blood-brain barrier and may be used to treat meningitis and encephalitis. Cephalosporins are the usually preferred agents for surgical prophylaxis.

-Fluoroquinolones: The fluoroquinolones are synthetic antibacterial agents and not derived from bacteria. They are included here because they can be readily interchanged with traditional antibiotics. An earlier, related class of antibacterial agents, the quinolines, drugs that were not well absorbed, could be used only to treat urinary tract infections. The fluoroquinolones, which are based on the older group, are broad-spectrum bacteriocidal drugs that are chemically unrelated to the penicillins or the cephalosporins. They are well distributed into bone tissue and so well absorbed that in general they are as effective by the oral route as by intravenous infusion.

-Tetracyclines: Tetracyclines got their name from the fact that they share a chemical structure that has four rings. They are derived from a species of *Streptomyces* bacteria. Broad-spectrum bacteriostatic agents, the tetracyclines may be effective against a wide variety of microorganisms, including rickettsia and amoebic parasites.

-Macrolides: The macrolide antibiotics are derived from *Streptomyces* bacteria. Erythromycin, the prototype of this class, has a spectrum and use similar to penicillin. Newer members of the group, azithromycin and clarithromycin, are particularly useful for their high level of lung penetration. Clarithromycin has been widely used to treat *Helicobacter pylori* infections, the cause of stomach ulcers. For people who are allergic to penicillin, erythromycin is a valuable alternative. But, unlike penicillin, erythromycin can be very irritating.

-Others: Other classes of antibiotics include the aminoglycosides, which are particularly useful for their effectiveness in treating *Pseudomonas aeruginosa* infections, and the lincosamide drugs clindamycin and lincomycin, which are highly active against anaerobic pathogens. There are other, individual drugs which may have utility in specific infections.

1.1.5 Formation of antibiotics in nature:

Antibiotics are produced by many microorganisms in various ecological conditions. Producers of biologically active substances can be found among representative of marine micro-flora, but the major part of microorganisms that can produce antibiotics inhabit soil. Many microorganisms pathogenic to man and animal, such as phytopathogenic fungi and bacteria are rapidly killed when they get into the soil, but if the soil is sterilized, the same microorganisms can live in it for a long time (Egorov, 1985). Egorov and others studied the relationships between bacteria and streptomycetes isolated from a restricted area near Moscow; they found that these relations are different (27 strains of *Streptomyces* and 39 strains of bacteria). It was established that bacteria induced or intensified the antibiotic activity of *Streptomyces* in 11 cases; in 8 cases the growth of streptomycetes was inhibited, in some cases the aerial mycelium disappeared and sporulation was delayed.

Some authors believed that antibiotics can be formed during the development of microorganisms directly in the soil where they display their biological activity. Others believed that antibiotics are not formed in the soil. Those who support the latter viewpoint derived their conclusion from the hypothesis that soils lack nutrient substances in amount sufficient for the normal growth of the microorganisms and

production of antibiotics. The study of antibiotics production in soil is difficult; the main difficulties are related to the determination of antibiotics in soil. Many antibiotics are easily adsorbed on the soil colloids and inactivated by the complex compounds contained in it (Egorov, 1985).

1.2 The Actinomycetes

Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but are now recognized as prokaryotic. The name 'Actinomycetes' was derived from Greek 'aktis' (a ray) and 'mykes' (fungus) and was given to these organisms form initial observation of their morphology. They have been recognized for over a hundred years primarily on morphological criteria. Actinomycetes are usually considered to be bacteria with the ability to form branching hyphae at some stage of their development (Goodfellow *et al*., 1983).

1.2.1 Actinomycetes and antibiotics

At the start of 20th century, medical bacteriology was a thriving study, after Louis Pasteur in France and Robert Koch in Germany had pioneered the germ theory of infectious disease in the preceding decades. Medical bacteriologist had clear objectives: to identify pathogenic bacteria and bring the diseases they caused under control. By comparison**,** soil bacteriology was a fragmented and unfashionable pursuit.

It was against this background that Selman Waksman began his career, investigating the biochemical capabilities of a previously obscure and poorly understood group of soil microbes and their contributions in agricultural fertility. As others had done before him, he grappled with the classification of this collection of apparently diverse organisms, described piecemeal from the 1870s onward. Halfway through Selman's career came his discovery that these organisms, the actinomycetes, are nature's most prolific producers of antibiotics. One, streptomycin, was found to cure perhaps the most feared human disease, tuberculosis (TB), caused by the tubercle bacillus that Robert Koch had identified in 1982. As a result, the actinomycetes were to become some of the most important players in applied microbiology as the basis, along with the molds that make penicillin and related compounds, of a multibillion dollar antibiotics industry (Hopwood, 2007).

1.2.2 Discovery of the actinomycetes

The name *Actinomyces* goes back to 1877, when it was applied to a microbe responsible for a disease of cattle called "lumpy jaw." It causes proliferation and distortion of the bone, resulting in incurable swellings on the side of the face that can eventually make it hard for the animal to eat, (Hopwood 2007). A German botanist, Carl Otto Harz (1842-1906), working at the Royal Veterinary School in Munich, first described the causal agent in a lecture in May 1877 and wrote about it in the yearbook of the school. The bony lesions are roughly spherical and develop radial striations as they increase in size with growth of the organism. Long, thin filamentous are visible in the centre, while the outer layers show more regular ray-like structures that seem to end in club-shaped bodies. Harz interpreted these as "gonida" typical of the reproductive bodies of certain fungi, and the structure bearing them as fungal hyphae. He therefore described the micro-organism as a fungus and called it *Actinomyces bovis (Actinomyces* means "ray fungus"). Harz had to confine his study to a morphological description of what he saw in the animal tissues and could not obtain a pure culture (probably, hindsight, because the organism grows in the absence of oxygen and dies on prolonged contact with air). This was unfortunate, because otherwise he could doubtless have realized that the fine filamentous seen in the center of the lesions represent the microorganism, while the obvious structures on the outside are actually host cells. Hard was not the first to discover an organism that would eventually become known as an actinomycete. This happened in Norway just before Harz described his "ray fungus**".** Leprosy was common in Europe in the Middles Ages and then mysteriously declined. The last recorded leprosy patient was an old man who as admitted to the hospital in Bergen from one of the, small islands of the coast in 1962 or 1963 with gangrene of a toe. In the 19th century, leprosy was rife among the poor in the region around Bergen, which became a center of attempts to understand the disease. Armaeur Hansen (1841-1912), who joined the staff of the Bergen Leprosy Hospital in 1868 soon after completing his medical training, was the first person to identify the causal agent as a microorganism and describe it which is still called "Hansen's bacillus" today. One of the pioneers of 19th century bacteriology was Ferdinand Cohn (1828-1898), who published a treatise summarizing his observations on a whole range of microbes including one he called *Streptothrix foersteri* after a medical friend has supplied him with the material from infected human tear ducts in which he saw the

organism. The organism was present along with various typical spherical bacteria and Cohn could not separate it from them and grow it in pure culture. Nevertheless, his account of the organism is a milestone in the history of microbiology; because it is now recognized as the first description of a soil living actinomycete of the kind that Waksman would later spend his career studying. This organism we would now call a *Streptomyces* (Hopwood, 2007).

1.2.3 Ecology of actinomycetes

The majority of the actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population (Kutzner 1968), which may vary with the soil type (pisano *et al*., 1989). These organisms participate in the turn over of the soil components, especially in the transformation of organic compounds (Kuster, 1967; Kutzner 1968; Huntzens 1972). In soil they are involved in the decomposition and mineralization cycles with the production of extracellular enzymes, such as cellulases, chitinases, and lignin peroxidases. Since they can decompose complex mixtures of polymers in dead plant, animal and fungal material (Mc Carthy 1987; Crawford 1988), they have important role in soil biodegradation by recycling of nutrients associated with recalcitrant polymers (McCarthy and Williams, 1992).

This soil gram-positive group encompasses genera covering a wide range of morphology extending from the coccus (*Micrococcus*) and rod-cocccus cycle (e.g *Arthrobacter*), through fragmenting hyphal forms (e,g *Nocardia, Rothia*), to genera with a permanent and highly differentiated branched mycelium such *Micromonospora* and *Streptomyces* (Lacey, 1973;Goodfellow and Williams, 1983).

1.2.4 Methods used for isolation of actinomycetes from natural sources:

There is no single correct way to selectively isolate streptomycetes from environmental samples. Some pre-treatment methods have been used for the enrichment, such as heating or treating with ammonia and sodium hypochlorite, chloramines or CaCO3, this because streptomycetes spores are more resistant to these treatments than Gram-negative bacteria (Kutzner 1968, Goodfellow and Simpson 1987).

Further selectivity can be achieved by using selective nutrient sources in the cultivation media. In contrast to many other bacteria, streptomycetes are able to utilize many biopolymers and are satisfied with an inorganic nitrogen source like nitrate (Goodfellow and Simpson 1987, Williams *et al*., 1989). Isolation media containing starch or glycerol as the carbon source and nitrate, casein or arginine as the nitrogen source have proven to be the most effective growth media for selective isolation of streptomycetes (Kutzner 1981). The addition of antifungal agents to the isolation media suppresses the growth of fungal species on the plates; however antibacterial antibiotics cannot be used to reduce the amount of other bacterial isolates because they will also inhibit the growth of many streptomycetes. Cycloheximide (50-100 µg/ml) as well as pimaricin and nystatin (10-50 µg/ml) have been used (Kutzner, 1981).

Mesophilic streptomycetes are usually cultivated at temperatures range from 22 to 37 °C for 14 days and thermophilic species at 40 to 55 °C for 5 days (Kutzner, 1981; Goodfellow and Simpson 1987; Williams *et al*., 1989). Most streptomycetes are neutrophilic and the isolation media commonly have a neutral pH, thus, if acidophilic strains are to be isolated, the pH of the medium can be adjusted to 4.5, and for alkalophilic strains to pH 10-11. However, some species may also show remarkable adaptation to a wide pH range (Kutzner, 1981; Goodfellow and Simpson 1987).

To obtain as many actinomycetes cultures as possible from their habitat the following principles of enrichment and isolation have been successfully employed.

Enrichment

This can be performed within the substrate before isolation by addition of CaCO3 (Tsao *et al*., 1960). The hydrogen ion concentration of the soil after adding CaCO3 would be changed to favor the growth of the remaining actinomycete cultures and to inhibit or retard that of most fungi. Similar favorable results with this method were obtained by El-Nakeeb and lechevalier (1963).

Treatment

Treatment of the sample before isolation such drying storage of soil and heating. These methods take advantage of the relatively high resistance of the

arthrospores toward low moisture tension and dry heat (Nuesch, 1965). A detailed study has been carried out by Williams *et al*., (1972), who found that heat treatment of the soil (40 – 45 °C for 2 – 16 h) reduced the bacterial flora. The treatment of the sample before isolation by phenol claims successful elimination of bacteria and fungi without any detrimental effect on actinomycetes (Lawrence, 1956).

Inhibition

We can inhibit the accompanying flora by the incorporation of selective substances into the nutrient agar used for isolation. Antibiotics that selectively inhibit fungi have been used by various authors, cycloheximide 50-100 µg/ml (Dulaney *et al*., 1955.; Corke and Chase, 1956), and the polyene antibiotics pimaricin and nystatin, each 10-50 µg/ml (Porter *et al*., 1960). Polymyxin (5µg/ml**)** has been employed by Williams and Davies (1965). Unlike antifungal antibiotics, which have no effect on actinomycetes, antibacterial agents have to be used with care.

Besides

In addition to antibiotics, the following two agents are recommended: Na-propionate 4g/l (Crook *et al*., 1950) and Rose Bengal, 35 mg/ml (Ottow, 1972). These agents suppress the bacterial flora and reduce the spreading growth of fungi. Actinomycetes often grow more slowly than other bacteria and fungi, because they require a long incubation time. To eliminate undesirable bacteria and fungi and also to suppress bacterial and fungal growth on the nutrient medium, without adversely affecting actinomycetes growth, various pre-treatment methods have been developed (Hsu and Lockwood, 1975; Hayakawa and Nonomura, 1987; Hayakawa *et al*., 1991, 2004).

Humic acid-vitamin (HV) agar was a reliable means of selectively screening for actinomycetes from soils of various types, and most of the actinomycetes isolates grow well and sporulate abundantly on the medium (Lee and Hwang, 2002).

1.2.5 Taxonomy of Actinomycetes

The Gram-positive bacteria include two major branches: the low G+C organisms, containing genera such as *Bacillus, Clostridium, Staphylococcus* and

Streptococcus; and the high G+C organisms referred to as the actinomycetes. Many of the later develop a mycelial habit-originally regarded as the hallmark of the actinomycetes-at least at some stage in their life cycle, but others do not.

In 1943, Waksman and Henrici tried again to classify the actinomycetes Waksman and Henrici (1943). They proposed that the capacity to form branching cells was the hallmark of the actinomycetes and used the degree of branching to define three major groups, two of which they subdivided, giving five genera altogether (Figure 1.1).

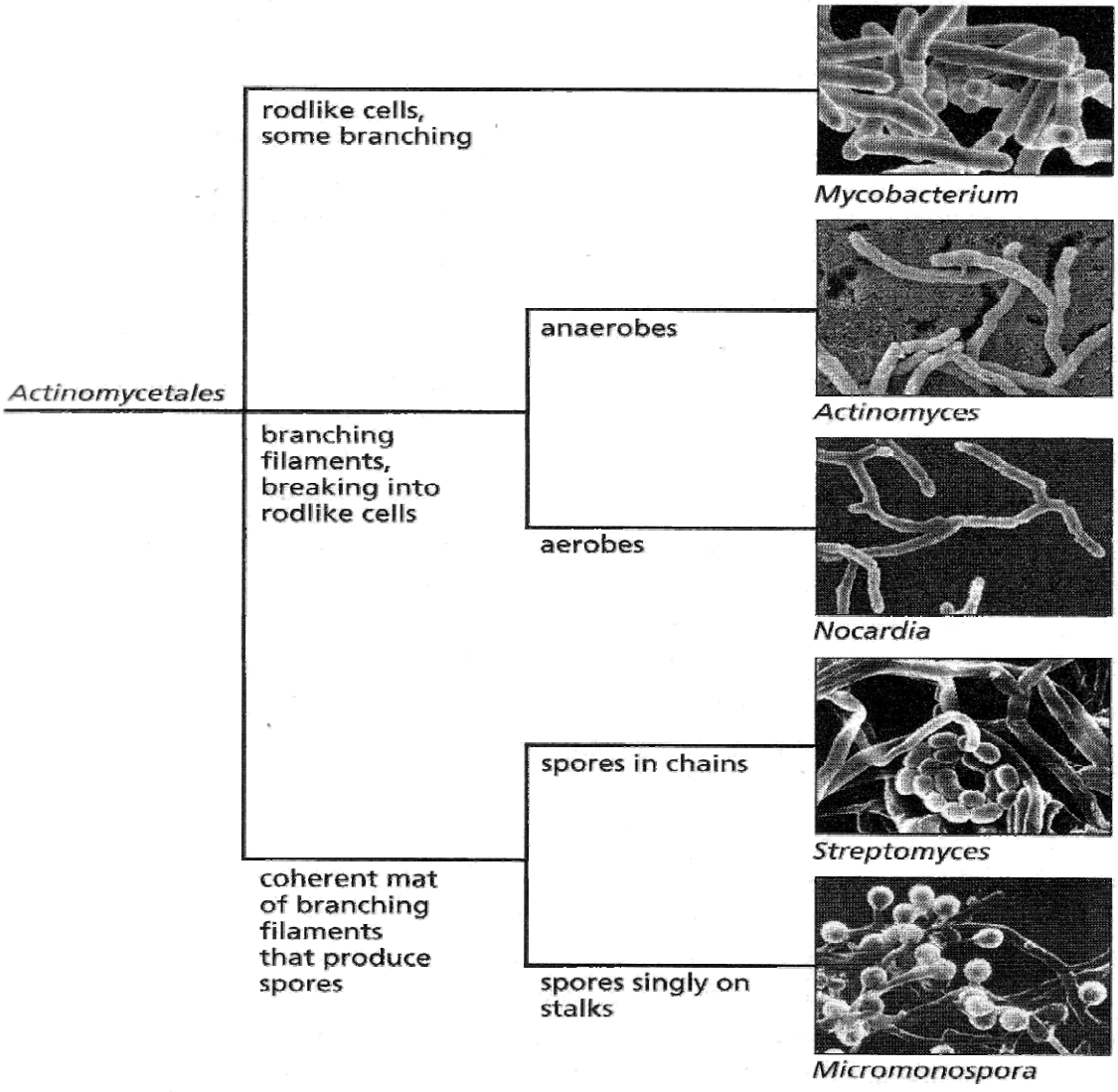

Figure 1.1: Waksman and Henrici's classification of the actinomycetes (Hopwood, 2007).

The first contained the leprosy and tubercle bacilli, which mostly grew as single rod-like cells but form time to time, formed side branches; the name *Mycobacterium*, given to the two pathogens by Karl Lehmann and Robert Neumann in

1896, (Lehmann and Neumann 1896) was retained for them. Organisms that developed a mass of branching filaments, which later broke up into individual cells, were classified in a second group. Some of these, like the lumpy-jaw organism, grew in the absence of oxygen: they were anaerobes. This was deemed to be an important characteristic, so these organisms were placed in a separate genus and the name *Actinomyces* was retained for them, while organisms that required oxygen aerobes were called *Nocardia*. This name had been used for various microbes since its introduction to honor a French microbiologist, Edmond Nocard, who had described the causal agent of a disease of cattle in Guadeloupe. The third and final group consisted of microbes that developed a dense mat of interconnected branching hyphae that remained intact and gave rise to specialized reproductive spores. Two genera were proposed for them, depending on whether the spores were produced in chains or singly, on stalks sprouting form the sides of the main hyphae. Cohn's *Streptothrix* fell into the former group, but it turned out that this use of the name was invalid because it had been applied earlier to a totally different microbe, so Waksman and Henrici invented a new one, *Streptomyces*. A previously used name, *Micromonospora* (meaning "small, single spores") was adopted for the second group (Hopwood, 2007).

Actinomycetes belong to the order Actinomycetales (Superkingdom: *Bacteria*, Phylum: *Firmicutes*, Class: *Actinobacteria*, Subclass: *Actinobacteridae*). According to Bergey's Manual actinomycetes are divided into eight diverse families: *Actinomycetaceae, Mycobacteriaceae, Actinoplanaceae, Frankiaceae, Dermatophilaceae, Nocardiaceae, Streptomycetaceae, Micromonosporaceae* (Holt *et al*., 1989) and comprise 63 genera (Nisbet and Fox, 1991).

Actinomycetes were originally classified according to their morphology. Subsequent analysis of chemotaxonomic markers has assisted in the detection of genera and differentiation of species in some groups. On the basis of cell wall analysis actinomycetes family has been divided into eight types (Table II). Actinomycetes from the types II-IV can be further distinguished by their whole organism sugar pattern (Table III). A whole array of taxonomic tools has been used to define genera and supergeneric groups of actinomycetes (Goodfellow and O'Donnell 1989), but partial sequence analysis of 16S rRNA is the most significant. Based on 16S rRNA classification system they have recently been grouped in ten suborders:

Actinomycineae, Corynebacterineae, Frankineae, Glycomycineae, Micrococineae, Micromonosporineae, Propionibacterineae, Pseudonocardineae, Streptomycineae and *Streptosporangineae* (Table IV) and a large members of actinomycetes are still remained to be grouped (www.ncbi.nlm.nih.).

Table 1.2: Cell wall chemotypes of Actinomycetes family

Chemotype	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
L-DAP	+							
Meso-DAP		+	+	+				
DABA							+	
Aspartic Acid						V[a]		
Glycine	+	+					+	
Lysine					+		V	
Ornithine					+			+
Arabinose				+				
Galactose				+		V		
	1	2	3	4	5	6	7	8

Note: 1 Streptomycetaceae, Nocardiaceae, 2 Micromonosporaceae, 3 Dermatophilaceae, Mycobacteriaceae, Thermomonosporaceae, Streptosporangiaceae, Frankiaceae, 4 Nocardiaceae, Mycobacteriaceae, Corynebacteriaceae, Pseudonocardiaceae, 5 Actinomycetacea, 6 Cellulomonadaceae, Micrococcaceae, Microbacteriaceae, Actinomycetaceae, 7 Microbacteriaceae, 8 Cellulomodaceae, Microbacteriaceae.
DAP: Diaminopimelic acid, DABA: Diaminobutyric acid, [a]V: Variable amount.

Table 1.3: Whole organism sugar pattern of Actinomycetes from cell wall Chemotypes II-IV

Pattern	Arabinose	Fructose	Galactose	Madurose	Xylose
A	+		+		
B				+	
C					
D	+				+
E		+			

Table 1.4: Taxonomic Classification of Order Actinomycetales

Suborder	Family	Genus
Micromonosporineae	Micromonosporaceae	*Micromonospora, Actinoplanes, Catellatospora, Couchioplanes, Catenuloplanes, Pilimelia Dactylosporangium*
Frankineae	Frankiaceae Sporichthyaceae Geodermatophilaceae Microsphaeraceae Acidothermaceae	*Frankia* *Sporichthya* *Geothermatophills, Blastococcus* *Microsphera* *Acidohermus*
Pseudonocardineae	Pseudonocardiaceae	*Pseudonocardia, Actinopolyspora, Actinosynnema, Amycolatopsis, Kibdelosporium, Kutzneria, Lentzea, Saccharomonospora, Saccharopolyspora, Saccarothrix, Streptoalloteichus, Thermocrispum.*
Streptomycineae	Streptomycetaceae	*Streptomyces*
Corynebacteriuam	Nocardiaceae Gordoniaceae Mycobacteriaceae Dietziaceae Tsukamurellaceae Corynebacteriaceae	*Nocardia, Rhodococcus.* *Gordonia* *Mycobacterium* *Dietzia* *Tsukamurella* *Corynebacterium, Turicella*
Micrococcineae	Micrococcaceae Brevibacteriaceae Cellulomondaceae Dermabacteraceae Intrasporangiaceae Jonesiaceae Microbacteriaceae Promicromonosporaceae	*Micrococcus, Arthrobacter, Kocuria, Nesterenkonia, Rorhia, Renibacterium, Stomatococcus* *Brevibacterium* *Cellulomonas, Oeskovia, Rarobacter* *Dermatobacter, Brachybacterium* *Intrasporangium, Sanguibacter, Terrabacter* *Jonesia* *Microbacterium, Agrococcus, Agromyces, Aureobacterium, Clavibacter, Curtobacterium, Rathaybacter* *Promicromonospora*
Actinomyineae	Actinomycetaceae	*Actinomyces, Mobiluncus, Arcanobacterium*
Propionibacterianeae	Propionibacteraceae	*Propionibacterium, Luteococcus, Microlunatus, Propioniferax*
Streptosporangineae	Streptosporangiaceae Thermomonosporaceae Nocardiopsaceae	*Streptosporangium, Herbidospora, Microbispora, Microtetraspora, Planobispora, Planomonospora* *Thermomonospora, Actinomadura, Spirillospora* *Nocardiopsis*
Glycomycineae	Glycomycetaceae	*Glycomyces*

1.3 The genus *Streptomyces*

The search and discovery of novel microorganisms that produce new secondary metabolites is, and can be expected to remain, significant in the race against new and emerging diseases and antibiotic resistant pathogens. The genus *Streptomyces* is well known as an excellent source of pharmacologically active compounds, especially antibiotics. Thus, detection and isolation of novel strains belonging to this genus are still of great interest over the world (Wael and Goodfellow 2007).

1.3.1 Streptomycetes as antibiotic producers:

Streptomycetes are industrially important bacteria renowned for their ability to produce a wide variety of bioactive compounds, including 80% of the antibiotics produced by actinomycetes (Berdy, 1974; Watve *et al*., 2001). Antibiotic production usually occurs late in growth, during late stages of the development of the aerial mycelium on solid medium and just before entry into stationary phase in liquid cultures (Bibb, 1996). Why *Streptomyces* produce so many kinds of antibiotics and bioactive compounds is not yet fully understood. "One of the answers is that *Streptomyces* strains have many gene clusters, which encode enzymes for many secondary pathways" (Omura *et al*., 2001).

As we have mentioned above, it is well known that members of the genus *Streptomyces* produce a great many antibiotics and other classes of biologically active secondary metabolites, but it is useful to put this statement in context. As seen from Table 1.1, actinomycetes do indeed make some two-thirds of the known antibiotics that are produced by microorganisms (some definitions actually restrict the word “antibiotic” to microbial products), and amongst them nearly 80% are made by members of the genus *Streptomyces*, with other genera trailing numerically: *Micromonospora* is the runner-up with less than one-tenth as many as *Streptomyces*. If we include secondary metabolites with biological activities other than anti-microbial, actinomycetes are still out in front, with over 60% (again *Streptomyces* spp. account for 80% of these). However, Table 1.1 also brings out the fact that it is not the overall chemical versatility of the actinomycetes that is unparalleled – fungi and higher eucaryotes beat them hands down – but the proportion of their out put that has a known biological effect. Table 1.2 describes a range of useful actinomycete antibiotics (Kieser *et al*., 2000).

Antifungal activity: *Streptomyces* produce a wide range of secondary metabolites, including antibiotics, many of which are of clinical importance in the treatment of infectious diseases or diseases caused by the proliferation of malignant cells **(**Pelezar *et al*., 1986; Innes & Allan, 2001).

Among the different types of drugs prevailing in the market, antifungal antibiotics are a very small but significant group of drugs and have an important role in the control of mycotic diseases. The need for new, safe and more effective antifungal antibiotics is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host as well as the Candidiasis. Candidiasis commonly called yeast infection or thrush is a fungal infection of any of the *Candida* species, of which *Candida albicans* is the most common. Several antifungal antibiotics were discovered during the two last decades. Nystatin is an antifungal agent produced by *Streptomyces noursei* and it is useful in the treatment of infections of the intestine, vagina, or oral cavity caused by *Candida albicans*. This antibiotic acts by changing the permeability of the cell membrane by combining with fungal sterols (Alcamo, 1986). Its antimicrobial activity was restricted to yeasts, *Aspergillus, Penicillium* and *Botrytis* species (Alcamo, 1986; Pelczar *et al*., 1986).

Phosmidosine which was isolated from broth of *Streptomyces* sp. PK-16 inhibits spore formation of *Botrytis cinerea,* the pathogenic fungus that causes grey mold disease of a variety of fruits and vegetables (Uramoto *et al*., 1991). Nisamycin was detected in the culture broth of *Streptomyces* sp K106. It was extracted by ethyl acetate and purified by silica gel and Sephadex LH-20 column chromatographies (Hayashi *et al*., 1994).

Phthoxazolins B, C and D antifungal antibiotics produced by *Streptomyces* sp. KO-7888 were found to be active against *Phytophthora parasitica* (Shiomi *et al*., 1995). The Kanchanamycins, a group of 36-membered polyol macrolide antibiotics were detected in the broth and mycelium of *Streptomyces olivaceus* TU4018, isolated from a soil sample collected in Thailand, exhibited antifungal activities against *Penicillium notatum,* (Fiedler *et al*., 1996).

An antifungal antibiotic UK-3A was obtained from the mycelial cake of *Streptomyces* sp. 517-02. The antifungal activity of UK-3A was relatively broad and its cytotoxic activity was weak (Ueki *et al*., 1997). Resormycin antifungal antibiotic exhibited antimicrobial activity against phytopathogenic fungi. This antibiotic was detected in the culture broth of *Streptomyces platensis* MJ953-SF5 (Igarashi *et al*., 1997).

Spirofungin which has a polyketide-spiroketal structure was detected in the culture filtrate of *Streptomyces violaceusniger* Tu 4113. This compound showed various antifungal activities, particularly against yeasts (Holtzel *et al*., 1998). The heptanes antibiotics 3874 H1 and H3 were discovered in the broth of *Streptomyces* sp. HAG003874 by Vertesy *et al*., (1998), exhibited broad antifungal activity against yeasts and filamentous fungi. In the same period, Ivanova *et al*., (1998) have isolated a natural niphimycin analog, N-methylniphimycin, from broth of *Streptomyces* sp. 57-13. It was also active against some yeasts and filamentous fungi.

A new antifungal antibiotic, HA-1-92, was isolated from the biomass of *Streptomyces* CDRIL-312, by extracting in butanol and further purified by silica gel column chromatography followed by preparative TLC. The antibiotic is presumed to be an oxohexaene macrolide and showed promising antifungal activity against yeasts and filamentous fungi including human and plant pathogens. It was found to be less toxic in mice than known oxohexaenes (Harindran *et al*., 1999).

Tetrin C is an antifungal antibiotic produced by *Streptomyces* sp. GK9244. It was active against *Mortierella ramannianus,* (Ryu *et al*., 1999). Whereas**,** the macrocyclic lactone antibiotic named mathemycin B, was isolated from broth of *Actinomyces* sp. Y-8620959. This compound exhibited activities against a variety of phytopathogenic organisms (Mukhopadhyay *et al*., 1999).

Antifungal metabolite active against plant pathogenic fungi (*Phytophthora capsici* and *Rizoctonia solani*) was produced by *Actinomadura roseola* Ao108 (Kim *et al.*, 2000). While *Streptomyces halstedii* K122 produced bafilomycin B1 and C1 compounds on solid substrates which inhibit the growth of fungi among Ascomycetes, Basidiomycetes, Deuteromycetes, Oomycetes and Zygomycetes. These compounds strongly affected hyphal branching and morphology (Frandberg *et al*., 2000).

A broad - spectrum antifungal antibiotic was produced by *Streptomyces aburaviensis* (IDA-28) isolated from a soil sample collected in India (Raytapadar and Paul, 2001). With the same characteristics, a novel phosphorous – containing antifungal antibiotic JU-2 was isolated from *Streptomyces kanamyceticus* M8 by (Datta *et al*., 2001). This antibiotic exhibited strong activity against various pathogenic and non-pathogenic fungi.

Watasemycins A and B two novel antibiotics were detected in the culture broth of *Streptomyces* sp. TP-A0597 isolated from the seawater sample collected in Toyama Bay, Japan. These new antibiotics exhibited significant activity against yeasts (Sasaki *et al*., 2002). Other researchers have isolated the antibiotic 2-methylheptylisonicotinate from the culture broth of *Streptomyces* sp. 201 (Bordoloi *et al*., 2002). The bioactive compound exhibited antifungal activity against phytopathogens (*Fusarium oxysporum, Fusarium moniliforme and Rhizoctonia solani*). In addition to the previous findings**,** Endophenazines A-D was isolated from the culture broth of *Streptomyces anulatus*. The new compound showed antifungal activities against some filamentous fungi (Gebhardt *et al*., 2002).

A new antibiotic TH818 was isolated and purified from the culture broth of *Streptomyces fulvoviolaceus* 818 by extraction and reversed- phase liquid chromatography. This antibiotic exhibited a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi (Rachev *et al*., 2003).

An active metabolite was produced by *Streptomyces rochei* AK 39 having strong activity against dermatophytes responsible for causing dermatophytoses in humans (Augustine *et al*., 2005).

1.3.2 Antibiosis in soil:

Recent evaluations of streptomycetes as potential antagonists of soil-borne plant pathogens, has focused on their ability to produce natural antifungal metabolites. *S. rochei* and *S. rimosus* from the chickpea rhizosphere were strong antagonists of *Fusarium oxysporum* f. sp. *ciceri* (Bashar and Rai, 1994). Rigorous model experiments on agar (Wiener 1996) have indicated that antibiotic production by streptomycetes colonies can play a significant role in preventing invasion by competing *Bacillus*

subtilis but it did not improve the ability of the antibiotic producer to invade and established population of sensitive *B. subtilis*, nor help competition between established streptomycete and bacillus population. This fits strikingly the idea (Chater and Merrick, 1979) that the major role of antibiotics is to defend the colony from the competitors at the time of development of the aerial mycelium in the lysing vegetative mycelium mat.

1.3.3 Physiology and regulation of antibiotic production:

Antibiotic production in streptomycetes is generally growth phase-dependent. In liquid cultures it begins as the culture enters the stationary phase. In agar-grown cultures, it coincides with the onset of morphological differentiation, and the occurrence of mutants deficient in both antibiotic production and the formation of aerial hayphae indicate at least some common elements of genetic control (Chater and Bibb, 1997). Most antibiotics are the products of complex biosynthetic pathways, with a cluster of genes (generally 20-30) dedicated to the synthesis of any one compound. These gene clusters usually contain pathway-specific regulatory genes with act as transcriptional activators, and which are themselves subject to control by pleiotropic regulatory genes. The onset of antibiotic biosynthesis is determined and influenced by a variety of physiological and environmental factors. These include growth rate, diffusible y-butyrolactone signaling molecules (Horinouchi and Beppu, 1994), imbalances in metabolism (Hood *et al*., 1992) and various physiological stresses (Hobbs *et al*., 1992).

In addition to these positive effectors of antibiotic production, antibiotic synthesis may also be subject to metabolite repression and /or inhibition by readily utilized sources of nitrogen (generally NH4 +), phosphate and /or glucose (Demain, 1992; Demain and Fang, 1995). The various factors that influence antibiotic production in streptomycetes are not readily accommodated in a simple unifying model. However, it seems reasonable to propose an overall regulatory influence of growth rate, with superimposed pathway-specific regulatory effects influencing the production of individual antibiotics. These effects may be elicited at the level of expression of the pleiotropic or pathway-specific regulators or the biosynthetic structural genes, and/or at the level of activity of the biosynthetic enzymes.

1.3.4 Ecology of *Streptomyces*:

Streptomycetes are ubiquitous in nature. Their ability to colonize the soil is greatly facilitated by growth as a vegetative hyphal mass which can differentiate into spores that assist in spread persistence. The spores are semi-dormant stage in the life cycle than can survive in soil for long periods (Ensign, 1978).The spores impart resistance to low nutrient and water availability, whereas the mycelial stage is sensitive to drought (Karagouni *et al*., 1993).

The relatively high numbers of streptomycetes in soil exist largely as inactive spores for most of the time. When laboratory spores were added to non-sterile soil, they exhibited very low germination efficiencies, probably because of competition with indigenous microorganisms, but pre-germinated spores grew for a short time and then re-sporulated (Lloyd, 1969).

Germination can be partially density-dependent, but the interaction did not cross species boundaries (Triger *et al*., 1991), suggesting special signaling factors between spores of the same strain, causing inhibition of germination above certain concentration. The advantage could be to limit the number of germinating propagules in accordance with available resources. Spore germination requires exogenous nutrients, water and Ca^{+2} (Ensign, 1978) and nutrient status of the germination site limits the extent of hyphal growth and the time of differentiation into aerial hyphae and eventually spores (Wellington *et al*., 1990).

Actinomycetes produce many extracellular enzymes in soil. By decomposing complex mixtures of polymers in dead plant, animal and fungal material (McCarthy, 1987; Crawford, 1988; Wang *et al*., 1989), they are important in soil biodegradation by the recycling of nutrients associated with recalcitrant polymers (McCarthy and Williams, 1992). Addition of chitin to acidic soil led to chitinolytic activity of acidophilic streptomycetes, with subsequent ammonification of the soil and eventual colonization by neutrophiles (Williams and Robinson, 1981).

In addition to their ability to colonize bulk soil, many streptomycetes successfully colonize the rhizosphere (Watson and Williams, 1974). This ability may be partly due to the antagonistic characteristics of streptomycetes in competition with

other rhizosphere bacteria such as pseudomonads and bacilli. An added advantage over Gram-negative soil bacteria is their ability to spread through the relatively dray soil via hyphal growth; in water soil, motile bacteria such as *Pseudomonas fluorescence* show more extensive colonization of rhizospheres than streptomycetes (Elliot-Juhnke *et al*., 1987; Karagouni *et al*., 1993; Milus and Rothrock, 1993).

1.3.5 Taxonomy of *Streptomyces* species:

The genus *Streptomyces* was proposed by Waksman and Henrici (1943) and classified in the family *Streptomycetaceae* for aerobic spore-forming actinomycetes to avoid confusion with the fermentative organisms, which were retained in the genus *Actinomyces.* This genus has been subjected to numerous systematic studies during the past 30 years but it is still difficult to identify unknown isolates. Many type species have been described, but there has been much over-speciation resulting from antibiotic patents and the consequent need to assign a name to the producing organism. Since the international Streptomyces Project in 1964, an attempt was made to produce valid species descriptions with at least a minimal number of standard phenotypic criteria. However, the criteria turned out to be too minimal and the proliferation of species continued, without any real attempt to compare species thoroughly with each other. The first study to do this relied on numerical phonetic techniques to define clusters of strains or species based on comparison of many phenotypic traits (Williams *et al*., 1983a).

Species of the genus *Streptomyces* form extensive branching substrate and aerial mycelia containing L-diaminopimelic acid (L-DAP) and glycine but no characteristic sugars in the cell wall, (Lechevalier, 1970; Lechevalier and Lechevalier 1970; Mikami *et al*., 1982; Iwami *et al*., 1985).

The isolation of actinomycin from a culture broth of *Streptomyces antibioticus,* (Waksman and Woodruff, 1940) stimulated extensive screening for bioactive compounds. In some cases the novelty of an antibiotic has frequently realize on the description of the producer as a new species. Between 1940 and 1957 more than 1000 *Streptomyces* species were described (Pridham *et al*., 1958).

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 which were rarely studied under standardized growth conditions (Atalan *et al*., 2000).

Gauze *et al*., (1957), recognized 15 groups or series distinguished by aerial and substrate mycelial colour. Based on sporophores morphology and aerial mycelium colour**,** Pridham *et al*., (1958), identified 42 groups. Shinobu (1958), classified 13 groups on the basis of sporophores morphology, melanin production and nitrate reduction, while Waksman (1961), classified over 250 species into 16 series using aerial and substrate colour, spore chain morphology, melanin production and a number of other properties including proteolysis.

Some taxonomist's equated series with species, Pridham *et al*., (1965) reduced 400 *Streptomyces* species to 8 on the basis of spore surface and spore chain morphology. Pridham, (1976) recognized 10 species on the same grounds, however, in a previous work, Hutter, (1967) described 41 species using four criteria, sporophores morphology, aerial and substrate mycelial color and melanin pigment production. Biochemical, nutritional and physiological characters had also been used in streptomycetes taxonomy, but usually had been applied to only selected species (Kutzner *et al*., 1978).

In the early of 1960, it became increasingly clear that streptomycetes taxonomy had serious problems. In 1964, the International *Streptomyces* Project (ISP) was established to furnish reliable description of *Streptomyces* strains and related taxa, using a number of traditional tests applied under standard conditions (Shirling and Gottlieb, 1966; Gottlieb and Shirling, 1967). Over 450 species were described by Shirling and Gottlieb (1968a, b, 1969 and 1972). However many attempts had been made to compare and delineate *Streptomyces* species. Despite the limitation of the ISP description especially its narrow data base, the results were used by several workers to construct identification schemes (Gyllemberg, 1970, 1974, 1976).

1.3.5.1 Biochemical tests (Phenotypic studies)

a) - Morphological Characteristics:

Morphology has played a major role in distinguishing genus *Streptomyces* from other sporing actinomycetes and in the characterization of streptomycete species. The life cycle of a streptomycete offers three features for microscopic characterization:

1 - Vgetative (substrate) mycelium (on solid and in liquid medium).

2 - Aerial mycelium bearing chains of arthrospores (sometimes called “sporophores”).

3 - The arthrospores themselves (Kutzner, 1981 and Williams *et al*., 1989**).**

In the study of the morphology of actinomycetes, it is important not to distort or destroy the arrangement of hyphae and spores. As a consequence, actinomycetes should be studied microscopically by the methods used for the study of moulds. The examination of cultures growing on agar plates is helped by the use of long working distance condenser and objectives (Cross and Attwell, 1975).

The fine structure and development of aerial arthrospores have been examined by many workers and it is clear that they are formed by septation and disarticulation of pre-existing hyphal elements within a thin fibrous sheath (Locci and Sharples, 1984). The configuration of the spore chains (or sporophores) of streptomycetes has played a prominent role in species descriptions for many years. The chains are usually long and often contain over 50 arthrospores (Chater and Merrick, 1979). The range of spore chain morphology is extensive, and some workers have recognized many categories for example**,** Ettlinger *et al*., (1958), who grouped strains into 15 morphological types. A much simpler scheme was proposed by Pridham *et al.*, (1958) and this was adopted for the International *Streptomyces* Project (ISP); Shirling and Gottlieb (1966)**.** The three categories recognized were (a) straight to flexuous, (b) hooks and (c) spirals (Williams *et al*., 1989).

Electron microscopic studies on spores were carried out by many investigators (Kriss *et al.*, 1945; Vernon, 1955). They pointed out that the surface of spores may be smooth or rough, the latter showing, warty or hairy projections, the majority o spores are smooth. The occurrence of spiny or hairy spores is characteristic for particular

series or species and therefore, it is recommended as a useful taxonomic character (Preobrazhenskaya *et al*., 1960; Tresner *et al*., 1961; Dietz & Mathews, 1971). These ornaments, which are in fact borne on the spore sheath, were first detected from observations of spore chain by transmission electron microscopy (Ettlinger *et al*., 1958; Tresner *et al*., 1961).

b) - Cultural characteristics:

According to Pridham and Tresner, (1974**),** the majority of *Streptomyces* species produce colored aerial mycelium, which may be gray, blue, green, red, violet, white or yellow after 14 days. The formation of melanin pigment is characteristic for many *Streptomyces* species. Other pigments are often produced by many *Streptomyces* species (Gauze *et a*l., 1957; Waksman, 1959).

c) - Physiological characteristics:

Species of the genus *Streptomyces* represent fairly heterogeneous systems of variable physiological and biochemical activities. These differences are used together with other characters to determine the species of genus *Streptomyces* (Krassilnikov, 1981; Shirling and Gottleib, 1966; O'Donnell, 1986; Williams *et al*., 1989). Utilization of certain carbon sources by *Streptomyces* species are used as an aid for species identification (Shinobu, 1958; Pridham and Gottleib, 1948; Williams *et al*., 1989). The ability of some species to cause certain chemical changes, when growing in milk media (coagulation, peptonization, pH changes or formation of colour), or in gelatin media (liquification and formation of pigments), are also used for the identification of *Streptomyces* species (Gordon and Smith, 1955; Nonomura, 1974).

d) - Antagonistic characteristics:

The antagonistic properties of actinomycetes against bacteria, yeasts and filamentous fungi were reported by many investigators (Waksman, 1959; Slechta *et al*., 1982; Kobinata *et al*., 1993; Harindran *et al*., 1999; Sasaki *et al*., 2001a, b; Barakate *et al*., 2002; Saadoun and Gharaibeh, 2002). Clear elucidation of antagonistic properties was largely influenced by the composition of the medium and/or type of test organisms (Krassilnikov, 1950 and Waksman, 1959)**.** Certain species may fail to show inhibitory effects if cultivated on synthetic media (starch nitrate) but succeed to do so

on complex organic media such as fish meal extract, soybean meal broth, beef extract or casein medium and vice versa (Waksman, 1962; Raytapadar and Paul, 2001).

Some *Streptomyces* species inhibit the growth of organisms including bacteria, yeasts and fungi (Fiedler *et al*., 1996 and Ouhdouch *et al*., 2001), while others have specific antimicrobial activities and inhibit the growth of only one or few groups of microbes (Hara *et al*., 1991; Yon *et al*., 1995; Kim *et al*., 1999; El- Tarabily *et al*., 2000). The antimicrobial spectrum of antagonistic streptomycetes was used by Gauze *et al*., (1957); Krassilnikov (1957, 1960, 1970); Williams *et al*., (1983a, 1989) as criteria for species differentiation.

1.3.5.2 Chemomotaxonomy

The development of reliable, rapid and sensitive analytical methods such as electrophoresis, chromatography and spectroscopy has led to the development of chemotaxonomic methods for streptomycetes classification (Goodfellow and O'Donnell, 1989). Fatty acid analysis has been also used to distinguish between bacterial genera. Although fatty acid profiles were used to assign unknown streptomycetes to established taxa (Saddler *et al*., 1987), it is rather unreliable method for species identification within the genus *Streptomyces* and requires standardised conditions. In contrast, protein pattern profiles can be used for genus to species taxonomic refinements. In a study for the elucidation of taxonomy of plant pathogenic streptomycete isolates, DNA-DNA hybridisation showed similar results with protein profiles (Paradis *et al*., 1994). Whole cell analysis that involves non-oxidative thermal degradation of cells resulting in a fingerprint for the organism requires stringent standardisation for reproducibility (Dietz, 1986). Multilocus enzyme electrophoresis (MLEE) is to use variation that is accumulating very slowly in the population and that is likely to be selectively neutral, this method is useful for strain differentiation (Oh *et al*., 1996). Although only a small number of alleles can be identified within the population by using this type of variation, high levels of discrimination are achieved by analyzing many loci. This method has been used for both inter-and intraspesfic characterizations of streptomycetes (Ohno *et al*., 1996).

The use of antisera (ELISA) defined the cluster groups and showed genus to species specificity (Kirby and Rybick, 1986), however antisera are not

continuously produced and do not have the specificity of monoclonal antibodies (Wipat *et al*., 1991). Actinophages can be used for host-identification at the genus and the species level (Wellington and Williams, 1981). In general, streptomycetes phages are genus specific (Korn-Wendisch and Schneider, 1992), although some cross-reactivity has also been detected with other genera, including *Nocardia, Streptosporangium* and *Mycobacterium* (Bradley *et al*., 1961).

1.3.5.3 Molecular taxonomy

New advances in DNA technology have contributed considerably to bacterial taxonomy. DNA-DNA hybridizations of total chromosomal DNA have been used to determine species identity within streptomycetes. Total DNA homology studies have indicated genetic heterogeneity within some of the large phenotypic species groups defined by numerical taxonomy (Labeda, 1992). DNA fingerprints can be provided by restriction of the genome with infrequently cutting endonucleases, a technique called low-frequency restriction fragment analysis (LFRFA). The resulting fragments are examined using pulsed-field gel electrophoresis (PFGE) to provide relatedness (Beyazova and Lechevalier, 1993; Anderson and Wellington, 2001). The combination of these whole genome-oriented methods can determine relatedness from genus to strain. However, genome instability (amplifications, deletions) is an obstacle to the effectiveness of this approach (Rauland *et al*., 1995). Another method is the random amplified polymorphic DNA (RAPD) PCR analysis a rapid method by which streptomycetes can be screened for strain similarity but it does require stringent standardisation of the primers, annealing temperature and reaction mixture (Anzai *et al.*, 1994). PCR DNA fingerprinting using repetitive intergenic DNA sequences (rep-PCR) was used to differentiate between closely related strains of pathogenic *Streptomyces* such as *S. albidoflavus, S. scabies, S. acidiscabies* and *S. ipomoea* (Sadowsky *et al*., 1996). Multilocus sequence typing (MLST), were introduced by Maiden *et al.* (1998) who determined the sequences of fragments from 11 housekeeping genes in a reference set of isolates of *Neisseria meningitidis* from invasive disease and healthy carriers. This method has since been used for phylogenetic analysis of many other bacterial pathogens (Suerbaum *et al*., 1998, 2001; Suerbaum, 2000; Koehler *et al*., 2003; Muller-Graf *et al.*, 1999). MLST has been used to differentiate between synonymous *Streptomyces* species from ISP collection. Three species *S. krainskii* ISP 5321, *S. craterifer* ISP 5296, and *S. anulatus* ISP 5361, whose

names are usually referred to as synonymous, were shown to be different species (Filippova *et al*., 2000). The use of strain-specific small insert clones obtained by subtractive hybridization to target larger inserts for sequencing is an efficient, economical way to identify relevant interstrain differences and novel gene clusters. This method have been used to differentiate between *Thermotoga* sp. strain RQ2 from the closely related completely sequenced relative *Thermotoga maritime* strain MSB8 (Nesbø and Doolittle, 2003).

Sequence analysis of 16S and 23S rRNA is a particularly powerful tool in streptomycetes taxonomy. Comparisons of 16S rRNA and 23S rRNA sequences (Witt and Stackebrandt, 1990; Stackebrandt *et al.*, 1991; Embley and Stackebrandt, 1994) indicate that the *Streptoverticillium* genus should be unified with the *Streptomyces* genus, *Streptomyces* spp and *Streptoverticillium* spp.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Collection of Soil samples and Processing

Several soil samples were randomly collected from and around Cairo – Egypt, using an open-end soil borer (20 cm depth and 2.5 cm diameter) as described by Lee and Hwang, (2002). Soil samples were taken from depths of 10 - 20 cm bellow the soil surface, the top region (5 cm from the surface) was excluded. Samples were air-dried at room temperature for 10-14 days and then passed through a 0.8 mm mesh sieve to remove various contaminant materials, mixed thoroughly with $CaCO_3$ (10 % w/w), saturated with water and incubated at 28 °C for 10 days before use, (Tsao *et al*., 1960; El-nakeeb and Lechevalier, 1963). Samples (10 g) of treated soil were aseptically added to 90 ml sterile distilled water. The mixtures were shaken vigorously for about an hour and then allowed to settle for 30 minutes. Portions (1 ml) of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^1, 10^2, 10^3….. 10^6.

2.2 Isolation, screening and maintenance of actinomycetes

Actinomycetes strains were isolated by using the dilution agar plating technique (Johnson *et al*., 1959), on a basal salts agar medium (Kuster and willaims, 1964). The medium consisted of the following components (Gram per litre in distilled water): 20.0 starch; 2.0 potassium nitrate; 1.0 dipotassium hydrogen phosphate; 0.5 magnesium sulphate; 0.5 sodium chloride; 3.0 calcium carbonate and 0.01 ferrous sulphate, the pH was adjusted to 7. 2. Inocula consisted of adding aliquots (0.2 ml) of 10^3 to 10^6 dilutions to autoclaved starch casein or starch nitrate medium at 45 – 50 °C before pouring the plates and solidification. Three replicates were considered for each dilution. The plates were incubated at 28 °C for two weeks. From day 5 on, the developed colonies of actinomycetes, which were characterized by their sharp round edges, were picked up by a sterile needle and then re-inoculated on the same medium. Pure cultures were screened for their antimicrobial activities after incubation at 28 °C for 7 days. From good-grown streaks 6 mm agar discs of actinomycetes colony mass was prepared by using sterile cork borers. Disks were then aseptically transferred to LB or nutrient agar medium plates having fresh lawn cultures of bacterial and yeasts pathogen test organisms and potato dextrose agar (PDA) or C'zapeks agar plates having fresh lawn cultures of moulds isolates (Wu, 1984; Berdy, 1989). Controls included using plain disks from the same medium. The plates were then kept in a refrigerator for 2 – 4h to permit diffusion of the metabolites

before the growth of the test organisms takes place. Bioactivity was evaluated as diameter of inhibition zones (DIZ) and measured after incubation at 37 °C for 24 h for bacteria and yeasts, and at 28 °C for 48 - 72 h for filamentous fungi. The promising isolates were chosen for further studies. So they were maintained on (ISP-2) slants (International *Streptomyces* Project) at 4 ˚C and as spore suspensions, using yeast extract-malt extract (YEME) agar medium. Spore suspensions were prepared according to the method of Hopwood *et al*., (1985) by adding 20 % (v/v) glycerol onto a well-sporulated plates and scraping off the spores from the surface of each plate one by one. The suspensions were then filtrated through sterile non-absorbent cotton wool to remove mycelial remnants and stored at -20 ˚C before use.

2.3 Identification of the most active isolates

Isolates exhibited strong antimicrobial activity and looked promising were subjected to identification by conventional and molecular methods.

2.3.1 Morphological and cultural characteristics:

The morphological and cultural characteristics of the studied isolates were determined by naked eye examination of 7, 14 and 21 days old cultures grown on various International *Streptomyces* Project (ISP) media. These media were: Yeast extract - malt extract agar (ISP-2) medium (Shirling and Gottlieb, 1966), oatmeal agar (ISP-3) and inorganic salts-starch agar (ISP-4) medium (Kuster, 1959 a, b), glycerol-asparagine agar (ISP-5) medium (Pridham and Lyons, 1961) and starch nitrate agar medium (Tadashi, 1975). All of these media were sterilized at 1.5 atmospheres for 15 minutes. Media were cooled to about 50 ˚C and dispensed aseptically into sterile Petri dishes. The poured plates were left for a minimum of 24 hours at 37 ˚C to promote moderate drying and check sterility before inoculation. Six plates for each medium were used to be inoculated by one organism. Incubation was made at 28 ˚C, 2 plates were observed after7 days, 2 at the end of 14 days and the two others at the end of 21 days.

2.3.1.1 Color determination: The colour of mature sporulating aerial mycelium, the substrate mycelium (reverse side pigments) and the diffusible (soluble) pigments other than melanin were recorded. The plates that were prepared for morphological studies were also used for colours determination**.** The observations and records were performed according to the ISCC-NBS centroid colour charts (Kenneth, 1958).

2.3.1.2 Spore surface ornamentation: The spore chains and spore surface ornamentation were examined according to the method of Tresner *et al*., (1961). Grids with carbon films were gently pressed over the sporulating surface of yeast extract - malt extract agar (ISP-2) cultures of 7 days old, shadowed with chromium, and then examined and microphotographed using EM10 Karl-Zeiss electron microscope at the National Research Center, Cairo, Egypt.

2.3.2 Physiological characteristics:

2.3.2.1 Melanin production: The production of melanin pigment on peptone-yeast extract-iron-agar (ISP-6) and tyrosine agar (ISP-7) medium was tested**.** Each medium was inoculated with the tested isolates and examined after 2 - 4 days of incubation at 28 ˚C. Cultures that formed greyish brown to brown black or a distinct brown pigment were considered as positive and the absence of this colour was recorded as negative (Shirling and Gottlieb, 1964).

2.3.2.2 Carbon sources utilization: The ability of the isolates under study to utilize different carbon sources was examined by using carbon utilization agar medium (ISP-9) as recommended by Shirling and Gottlieb, (1966). The carbon sources used were: D-glucose (used as positive control) arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose, raffinose, (Nonomura, 1974) and lactose, maltose, galactose, cellubiose, sodium acetate, sodium citrate and starch, (Pridham and Gottlieb, 1948). For negative control, no carbon source was added to the basal medium.

The carbon sources were separately sterilized without heat. An appropriate weight of a carbon source was spread as a shallow layer in a pre-sterilized Erlenmeyer flask with a loose cotton plug. Ethyl ether was added so as to cover the carbohydrate. The ether was allowed to evaporate at room temperature. After a complete evaporation of the ether, sterile distilled water was added to make 10 % (w/v) solution of the used carbon source. The sterilized carbon source was added to basal medium to give the final concentration of 1.5 % (w/v).

2.3.2.3 Coagulation and peptonisation of skimmed milk: Skimmed milk was dispensed in 5.0 ml aliquots in test tubes. They were examined after 21 days of incubation at 28 ˚C according to the method described by Jensen, (1930).

2.3.2.4 Hydrogen sulphide Production: The production of hydrogen sulfide was detected by inserting sterile lead acetate filter paper strips into the mouth of a tube containing nutrient liquid medium inoculated with the isolates under study. Strips were hold in the cotton plug. The black colour of the filter paper strips at the end of incubation period was recorded as positive (Kuster and Williames, 1964).

2.3.2.5 Nitrate reduction: Reduction of nitrate was determined after 7 and 14 days of incubation at 28 ˚C, by addition of 0.2 ml of Griess-llsovay reagents (a) and (b). The distinct pink or red color indicates the presence of nitrate (Cowan, 1974).

2.3.2.6 Indole production: About 1 ml ether or xylol was added to a 48-hour culture in peptone water or nutrient broth medium, shaken well, and then 0.5 ml Ehrlich's reagent was run down the side of the tube. If a pink or red colour appears in the solvent indicates the production of indole (Cowan, 1974).

2.3.2.7 Degradation activity:

- Xanthine degradation:

The degradation of hypoxanthine and xanthine (1%, w/v) were detected in MBA (modified Bennett's agar medium). After 7, 14 and 21 days of incubation periods, the clear zones of degraded compounds under and around areas of growth were scored as positive result.

- Starch degradation:

Degradation of starch in the same basal medium (MBA) with the replacement of glycerol by starch, was detected after 7 days by flooding the plates with iodine solution (2 % w/v). Clear zones around growth indicated positive starch hydrolysis (Shirling and Gottlieb, 1964).

- Gelatine liquefaction:

Twenty grams of pure gelatine were dissolved in 100 ml of hot water, dispensed in 5 ml aliquots and sterilized. The solidified gelatine solution was then inoculated with studied isolates and incubated for 21 days at 28 ˚C. Cultures were investigated for signs of gelatine

liquefaction visually, and then restored at 2 – 4 ˚C for 24 h. If liquefaction was still present the test was consider positive (Jensen, 1930).

2.3.2.8 Enzymes and related biochemical activities:

-Protease, lecithinase and lipolytic activites:

The Proteolytic, lecithinase and lipolytic activities of the selected isolates were determined using egg-yolk medium according to the method of Nitsch and Kutzner (1969).

- Pectinolytic activity:

Pectinase and chitin decomposition was detected according to the methods of Hankin *et al*., (1971); Reid and Ogrydziak (1981). Zones of hydrolysis were detected after 4 and 6 days by flooding the plates with hexadecyltrimethyl ammonium bromide 91% (w/v) or iodine solution

- Catalase production:

The production of catalase was detected by adding few drops of H_2O_2 to 7 days old culture grown on a modified Bennett's agar medium (MBA), evolved oxygen was detected for positive results (Jones, 1949).

- Urease production:

The production of urease was tested on SSR (Stuart, Van Stratum and Rustigian, 1945) urea medium. The urease activity was examined daily up to 14 days; red colour was recorded as positive result (Cowan, 1974). Other lytic enzyme studies were performed according to the method of Pataraya *et al*., (1987).

2.3.2.9 Growth in the presence of inhibitory compounds

- Sensitivity to potassium cyanide:

Sensitivity was tested on potassium cyanide broth medium. The ingredient were dissolved in water, filtered through a sintered glass funnel, distributed in100 ml Screw-capped containers and sterilized at 115 °C for 15 minutes. Then, 1.5 ml of a freshly prepared 0.5 % KCN solution in sterile water was added to 1000 ml of basal medium, mixed and 1.0 ml was aseptically distributed in sterile 5 ml screw-capped bottles. KCN medium was inoculated with

1 ml of 48 hour broth culture of the studied organism. The cap of the bottles were tightly screw down and incubated for up to 48 -96h. Sensitivity to KCN was examined after 48, 72 and 96 h, where turbidity indicates a positive reaction (Cowan, 1974).

- Growth in other inhibitory compounds:

The effect of some inhibitory compounds such as (w/v): sodium azide 0.01 %; crystal violet 0.001, sodium chloride 1 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 %, 8 % and 9 %; (Gordon and Barnett, 1977; Athalye *et al.*, 1985) and the growth at pH range 5 to 9 and at temperature 42˚C were also investigated.

- Sensitivity to antibiotics:

The selected organisms were also tested for their abilities to grow on glucose-yeast extract agar (GYEA) medium supplemented with 5 different antibiotics, Chloramphenicol (25 mg/l), Erythromycin (10 mg/l), Gentamicin (5 mg/l), Oxytetracycline (25 mg/l) and Penicillin (25 mg/l) (Athalye *et al*., 1985).

2.3.3 Chemotaxonomy

2.3.3.1 Cell wall analysis:

The analysis was carried out using the methods described by Becker *et al*., (1964, 1965); Lechevalier and Lechevalier (1970). The isolates under study were grown on ISP-2 broth at 28 ˚C for 10 days. The biomass was collected by filtration, washed by water and ethyl alcohol and air-dried at room temperature.

2.3.3.2 Detection of diaminopimelic acid (LL-DAP) and sugars:

Ten milligrams of the dried biomass (mycelium) were hydrolyzed for 18 hours using 1 ml of 6 N HCL in sealed pyrex tube at 100 ˚C on sand bath. After cooling, the tube contents were filtrated through Whatman No. 1 filter paper. The sediment was washed with drops of distilled water, filtrated and dried three consecutive times on a steam bath to get rid of the HCL, the residue was taken up in 0.3 ml of 10 % isopropanol, then 20 µl of the liquid were spotted on Whatman No. 1 filter paper. Ten micro-litres of 0.01 M of a mixture of meso and LL-DAP were spotted on the paper to run alongside the sample to serve as a reference standard. Descending chromatography was carried out overnight using the solvent system (methanol - water -10 N HCL – pyridine) in the ration of (v/v), 80: 07.5: 2.5: 10. Amino acids were detected by spraying the papers with acetone – ninhydrine solution (0.1 % w/v) followed

by heating for 2 minutes at 100 ˚C. Spots of LL-DAP, exhibit an olive green fading to yellow colour. Other amino acids in the hydrolysis appear as purple spots (Hasegawa *et al*., 1983).

2.3.3.3 Detection of sugars:

Different sugars (400mg from each) were dissolved in 10 ml aqueous solution of isopropanol (10% w/v). The development solvent system for paper chromatography was: "n-butanol: acetic acid: water" in the ratio of (v/v), 5 : 3 : 3. The locating appearing reagent consisted of "aniline: diphenylamine: acetone: phosphoric 85% acid" in the ratio of 1 : 1 : 100 : 10 by volume. Diphenylamine phosphate may be precipitated from the mixture and re-dissolved by stirring or adding few drops of water. Whatman No. 1 filter paper was loaded with different sugars and the residue of our samples as mentioned before. The filter paper was dipped in locating reagent then air-dried and heated at 100 ˚C for few minutes. This reagent was satisfactory used to detect different sugars because of the variation in colours obtained with different sugars from green, blue to brown.

2.3.4 Molecular characterization

Because of the high resemblance of the two most active strains among the five potent isolates, in morphology and growth comportment as well as their antimicrobial activities, they were subjected to randomly amplified polymorphic DNA (RAPD) analysis to know, if they are two different strains or the same one.

2.3.4.1 Isolation of genomic DNA from *Streptomyces* strains:

Chromosomal DNAs were isolated from mycelia of the studied stains according to the protocol of Pospiech and Neumann, (1995) with some modifications. Mycelia (5 ml) grown in a LB broth shake culture were centrifuged, rinsed with TE and re-suspended in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added to a concentration of 1 mg ml $^{-1}$ and incubated at 37 °C for 0.5_1 h. Then 0.1 vol. 10 % SDS and 0.5 mg Proteinase K mg ml $^{-1}$ were added and incubated at 55 °C with occasional inversion for 2 h. One-third volume 5 M NaCl and 1 vol. chloroform were added and incubated at room temperature for 0.5 h with frequent inversion. The mixture was centrifuged at 4500 g for 15 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1 vol. 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70 % ethanol, dried under

vacuum and dissolved in a suitable volume (about 100 µl) of distilled water. The dissolved DNA was treated with 20 µg RNase A ml^{-1} at 37 °C for 1 h. Samples were extracted in the same volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and precipitated with 2.5 vols cold ethanol and 0.1 vols 3 M sodium acetate. The pellets were washed with 70 % ethanol, dried and dissolved in TE or distilled water. Whereas, *E. coli* DH5alpha was cultivated at 37 °C on Luria bacteria (LB) plates supplemented with ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (40 µg/ml) when appropriate (Sambrook *et al*., 1989). Cultures were stored at -20 °C.

2.3.4.2 Purification of DNA:

All the DNA purification solution system is kites of Puregene™, Gentra system, USA.

2.4 Randomly Amplified Polymorphic DNA (RAPD) PCR assays

RAPD analysis was performed according to Williams *et al*., (1990) with minor modifications. PCR reactions were carried out in 25 µl volumes containing 25 ng of total genomic DNA, 10 pmol primer, 200 µM dNTPs, 2 mM $MgCl_2$, 1X PCR buffer and 0.4 µl (2 units) Ampli Taq Polymerase (RTS Taq DNA polymerase). Amplification was performed in a Perkin-Elmer 9600 thermal cycler with the following profile: 94 °C for 5 min (initial denaturation), 94 °C for 1 min, 36 °C for 1 min, 72 °C for 90 sec for 40 cycles with a final extension at 72 °C for 7 min. Short oligonuclotides random primers from Oporon Technologies, USA were used. The RAPD-PCR products were analyzed directly on 1.5 % agarose gels in 1 x TAE buffer, visualized by staining with ethidium bromide and transilluminated under short-wave UV light. The five primers used were given in (Table 2.1).

No	Primers	Sequences
1-	O9	TCCCACGCAA
2-	K9	CCCTACCGAC
3-	N1	CTCACGTTGG
4	Q14	GGACGCTTCA
5-	K13	GGTTGTACCC

Table 2.1: Sequence of the five used primers

After distinguishing between the two similar isolates, they were subjected to 16S rRNA gene sequence analysis to be identified at species level according to the following steps.

2.5 Purification of PCR products

PCR products were run on a 0.8% agarose gel until all bands were clearly separated. The bands were excised and purified using Qiaquick gel extraction kit (QIAGEN) as per manufacturer's instructions.

2.6 Cloning of PCR products

2.6.1 Ligation of PCR products: The purified PCR products were cloned using the pGEM T-Easy vector system as per manufacturer's instructions.

2.6.2 Preparation and transformation of competent *E. coli* DH5α cells: 10 ml of LB was inoculated with a single *E. coli* DH5α colony from a 24 hr culture, and was incubated at 30°C for 24 hrs. 200 µl of this culture was used to inoculate 50 ml of LB which was incubated at 30°C for 3 hrs until OD_{600} = 0.4. After incubation the cells were placed on ice for 15 mins and then centrifuged for 15 mins at 3,000 g and the supernatant was discarded. The pellet was re-suspended in 1 ml of ice-cold 0.1 M $MgCl_2$ and centrifuged at 13,000 rpm for 1 min. The supernatant was discarded and the pellet re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and centrifuged at 13,000 rpm for mins. The pellet was again re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for at least 3 hrs prior to use. 50µl of competent cells were added to 2µl of ligation mixture in a 1 ml Eppendorf and incubated on ice for 2-5 mins. The cells were heat shocked at 42°C for 45 sec and immediately transferred to ice for 5 mins. 950µl of LB was added to the cells and incubated at 37°C for 1 h 30 mins. LB plates containing 100 µg/ml were pre-warmed and spread with 40µl of 2x X-gal and 10µl of 100mM IPTG. 100µl of cells were incubated on the pre-prepared LB plates and incubated overnight at 37°C.

2.6.3 Plasmid extraction: 10 ml of LB containing 100 µg/ml ampicillin was inoculated with the transformed *E. coli* cells and incubated for 24 hrs at 30°C. Plasmid DNA was extracted using the QIAGEN Mini-prep kit as per manufacturers' instructions.

2.6.4 Denaturing Gradient Gel Electrophoresis (DGGE): DGGE was done using the D Gene System (Bio Rad). GC clamped PCR products were generated using a primer pair where a GC rich sequence was attached to the 5' end of the forward primer (Table 2.3). A 10% polyacrylamide gel was used and a gradient of 20-70% was made using different volumes of 0 and 100% denaturant acrylamide solution as shown in (Table 2.2).

Gradient	Denaturant Concentration		Total
	0%	100%	
0%	20 ml	0 ml	20 ml
20%	16 ml	4 ml	20 ml
70%	6 ml	14 ml	20 ml

Table 2.2 Concentration and volumes of denaturant used to make DGGE gels

To each 20 ml of 0, 20 and 70% denaturant solution, 0.06% TEMED and 0.75% APS was added. The gel was cast so that there was a linear decrease of 70% denaturant to 0% denaturant from the bottom to the top of the gel. Upon cooling, the gel was inserted into the D Gene tank containing 0.5% TAE and electrophoresed for 6 hrs, 150V at 60 °C. The gel was stained by immersing into 0.5% TAE buffer containing ethidium bromide for 20-30 mins. The gel was washed once in SDW prior to visualizing.

2.7 Cloning and nucleotide sequence determination

PCR products of the 16S rDNAs of the selected strains were sub-cloned into pGEM-T Easy Vector for nucleotide sequence determination using an automated laser fluorescence sequencer (3100 genetic analyzer ABI PRISM, Applied Biosystem, HITCHI, USA). Sequencing reactions were carried out with the Big Dye termination kit (Applied Biosystems) according to the supplier's instructions. Nucleotide sequence of the 16S rDNAs of these strains were determined and the level of similarity was compared to the reference species of bacteria contained in genomic database banks, using the ''NCBI Blast'' available at http://www.ncbi-nlm-nih.gov (website).

2.8 Phylogenetic analysis

Phylogenetic and molecular evolutionary analysis were conducted using software included in MEGA version 3.0 (Kumar *et al*., 2004) package. The 16S rDNAs sequence of

the three strains were aligned using the CLUSTAL W program (Thompson *et al*., 1994) against corresponding nucleotide sequences of representatives of the genus *Streptomyces* retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and phylogenetic trees were inferred by the neighbour joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resembling of the neighbour joining data set.

2.9 Antimicrobial activity

To determine the antimicrobial activity of the studied isolates in solid agar culture, strains were grown on ISP-4 medium and starch-nitrate agar for 7 days at 28 °C and then, 6 mm diameter discs were cut out from these cultures under aseptic conditions using sterile cork borer and aseptically transferred to the surface of agar plates seeded with different test organisms. In case of liquid cultures, Erlenmeyer flasks (250 ml capacity) having 50 ml of ISP-4 and starch nitrate broth were inoculated with the experimented isolates and incubated at 28 °C on a rotary shaker at 200 rpm, for 5 days. Antimicrobial potentialities were assayed according to the method of Wu, (1984), 0.2 ml of the filtrate broth were transferred to 6 mm diameter wells cut in nutrient agar and Czapek's-Dox agar plates, which were previously seeded with the test organisms. The plates were then kept in a refrigerator for 2 – 4h to permit diffusion of the metabolites before the growth of the test organisms takes place. Inhibition zones were expressed as diameter and measured after incubation at 37 °C for 24 h for bacteria and yeasts, and at 28 °C for 48 - 72 h for filamentous fungi. The used target germs were: *Bacillus subtilis* (ATTC 6633), *Micrococcus luteus* (ATCC 9314) *Staphylococcus aureus* (ATCC 33591) and *E. coli* (ATCC 25922) in addition to yeasts and moulds from the culture collection of the Microbial Chemistry Laboratory, National Research Centre, Dokki - Cairo, Egypt, which were **(***Candida albicans* NRRLY-12983*, C. tropicalis* NRRLY-12968*, C. pseudotropicalis* NRRLY-8281*, Rhodotorula minuta* NRRLY-1589*, Aspergillus niger* NRRLA-326*, A. flavus* NRRLA-1957*, A. terreus* NRRLA-571*, Botrytis allii* NRRLA-2502*, Fusarium oxysporum* NRRLA-2018*, Helmenthosporium turcicum* NRRLA-1081*, Machrophomina phaseoli* NRRLA-62743*, Trichoderma viride* NRRLA-63065. Bacteria and yeasts were cultivated on nutrient agar whereas; filamentous fungi were grown on Czapek's Dox agar.

2.10 The optimization studies

To increase the production of bioactive compounds by the selected organisms, optimization was carried out in two steps.

2.10.1 Selection of suitable broth and correct culture conditions (first step): In favour of optimum formation of active compounds, a number of broth cultures media such as yeast-extract malt-extract glucose broth (ISP-2), Inorganic salts-starch broth (ISP-4), tryptone-yeast extract-glucose broth (TYG), tryptic soy broth (TSB) and starch nitrate broth were tried. After incubation at 28 ˚C for 144 h in New Brunswick Scientific Shaker at 200 rpm, antibacterial activities were assayed for each culture supernatant. After determination of the better culture broth, effects of different factors on the active compound formation were also investigated in the same culture conditions described above. Finally, and based on the obtained results, the effect of incubation periods (up to 144 hours) was also re-determined.

2.10.1.1 Effect of different carbon sources:

In the basal medium, starch was replaced by the following carbon sources (at the concentration of 2 g per cent): D-glucose, D-fructose, D-mannitol, mannose, glycerol, maltose and sucrose.

-Effect of different concentrations of starch:

Results of the previous experiment indicated that starch was the best carbon source. Thus, different concentrations (5, 10, 15, 20, 25, 30 and 35 g/L) of this compound were tested for their suitability to enhance antibiotic formation.

2.10.1.2 Effect of different nitrogen sources:

On the basis of the previously obtained results, the following nitrogen sources: potassium nitrate, sodium nitrate, ammonium nitrate, ammonium oxalate, ammonium sulfate, ammonium hydrogen phosphate, ammonium dihydrogen phosphate, triammonium phosphate peptone and soybean powder were tested in equivalent nitrogen weights for their ability to increase antibiotic formation by strain RAF10.

- Effect of different concentrations of ammonium sulphate:

As ammonium sulphate was the preferable nitrogen source, different concentrations of this compound (0.50, 1.0, 1.50, 2.0, 2.50, 3.0 and 3.5 g/L) were tested to augment antibiotic formation.

2.10.1.3 Effect of different phosphate sources:

Equivalent in phosphate weights of K_2HPO_4, KH_2PO_4, NaH_2PO_4, Na_2HPO_4, Na_3PO_4, $(NH_4)H_2PO4$, $(NH_4)_2HPO_4$ and $(NH_4)_3PO_4$ were tested for their ability to increase antibiotic formation by *Streptomyces* strain RAF10.

- Effect of different concentrations of dipotassium hydrogen phosphate:

Because dipotassium hydrogen phosphate was the most favourable, different concentrations (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 g/L) of K_2HPO_4 were tested for their ability to enhance antibiotic formation by *Streptomyces* strain RAF10.

2.10.1.4 Effect of incubation period:

Finally, based on the all obtained results, the 250 ml Erlenmeyer flasks containing 50 ml of optimized ISP-4 broth were inoculated with a standard inoculum of RAF10 strain and incubated at 28 ˚C on a rotary shaker at 200 rpm for 7 days. After every 24 h, the culture broth was analyzed for its antimicrobial activity by well diffusion method, in order to determine the suitable period for high active compound formation.

2.10.2 Optimization of antibiotic production using full factorial design (second study): After the preliminary studies on parameters influencing antibiotic production using a one-factor-at-a-time approach, the optimization process was carried out using a statistical experiment design to determine which nutrients and physical conditions affecting the production by applying a fractional two-level factorial design according to Plackett and Burman 1946.

For screening purposes, various medium components as well as environmental factors have been evaluated. The different factors were prepared in two levels: -1 for low level and +1 for high level, (Table 2.3), based on Plackett-Burman statistical design (Plackett and Burman, 1946). This is a two-level fraction factorial design that allows the investigation of *n-1*

variables in at least *n* experiments. Seven independent variables were screened in 8 combinations according to the design shown in (Table 2.4). All trials were done in 250 ml Erlenmeyer flask containing 50 ml of the broth. Each flask was inoculated with 2% v/v of the 48 hours seed culture grown on ISP-4 broth. Flasks were incubated for 96 hours at 28 ˚C in New Brunswick Scientific Shaker at 200 rpm. At the end, the culture filtrate was used as crude antibiotic.

Variable	code	high level (+1)	low level (-1)
Starch	X_1	1.5	1
Glucose	X_2	1	0.5
$(NH_4)_2SO_4$	X_3	0.25	0.15
Tryptone	X_4	1	0.5
K_2HPO_4	X_5	0.25	0.15
pH	X_6	7.5	6.5
Inoculum size	X_7	4	2

Table 2.3: Media components and test levels for Plackett-Burman experiment

The main effect and statistical t-value of each variable were calculated. The calculated effect can be positive, negative or neutral depending on the overall influence of the variable upon the measured response. Also, the variable significance was determined by using probability table depending on *P* value, the variables which have more significant effects can be used for further optimization. Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \Sigma \beta_i x_i \qquad (1)$$

where Y is the response (antibiotic production), β_0 is the model intercept at centre point and β_i is the variable coefficient for the independent variable and x_i are the independent variables. The values of the coefficients were calculated by data regression using Microsoft Excel 7.0 software. The model describes no interaction among factors and is used to screen and evaluate the important factors that influence antibiotic production. The variables whose confidence levels were higher than 95 % were considered to significantly influence the measured

responses. The Pareto plot best demonstrate results of Plackett-Burman design that illustrates the absolute relative significance of variables independent on their nature.

trials	X_1	X_2	X_3	X_4	X_5	X_6	X_7	DIZ (mm)
1	-	+	+	-	+	+	-	12
2	-	-	+	+	-	+	+	16
3	+	-	-	+	+	-	+	21
4	+	+	-	-	+	+	-	24
5	-	+	+	-	-	+	+	20
6	+	-	+	+	-	-	+	15
7	+	+	-	+	+	-	-	27
8	-	-	-	-	-	-	-	20

Table 2.4: Randomized Plackett-Burman experimental design for evaluating factors influencing antibiotic production

2.11 Antibiotic production in liquid shaken cultures:

Because it was impossible within the requirements of the present thesis to deal with the purification and characterization of the active compounds produced by the five chosen isolates, this work was restricted to the molecules produced by the most active one (based on its broad spectrum and largest zone of inhibition), namely *Streptomyces*. sp strain RAF10. The strain was cultivated for 7 days on ISP-4 agar plates at 28 ˚C. A loop full of spores from this culture was transferred to 250 ml Erlenmeyer flasks containing 50 ml of modified (optimized) ISP-4 broth and incubated at 28 ˚C in New Brunswick Scientific Shaker at 200 rpm for 48 h in order to prepare the inoculum. The same medium was used for large scale production using flasks of 1000 ml capacity; each contains 250 ml ISP-4 broth. 5 ml inoculum was used for each flask. The inoculated flasks were incubated on the same conditions mentioned above for 5 days.

2.12 Extraction of the active compounds produced by strain RAF10

Two different methods were used to purify the studied bioactive compounds: solvent-solvent extraction and precipitation.

2.12.1 Solvent-solvent extraction:

Solvent-solvent extraction involves the use of different solvents with different polarities for the extraction of active substances from broth. Solvents used in this study were petroleum ether, n-hexane, chloroform, diethyl ether, ethyl acetate, butyl acetate, benzene, and n-butanol. All solvents were immiscible with water, their ability to extract the active compounds from broth at different pH values (3, 7 and 10) were tested.

Broth was centrifuged at 7000 rpm for 10 min to remove the cells. The supernatant was divided into 7 portions, each was then divided into 3 parts, the first was acidified with acetic acid to pH 3; the second was kept neutral (pH 7) while the third one, was made alkaline by adding NH_4OH till pH 10. Equal volumes of the tested solvents were added to the supernatant broths, shaken vigorously in separating funnels and allowed to settle till the clear distinguishing of the two layers. The two layer were separated carefully, then each was tested for its antimicrobial activity using filter paper disks (6 mm), saturated previously with the solvent extract or water layer (50 -80 µl), dried then placed over the surface of Petri dishes pre-seeded with the test organisms. Inhibition zones were measured after 24 h for bacteria and yeasts and 48 -72 h for filamentous fungi.

2.12.2 Precipitation:

300 ml of broth was divided into three portions; the first portion was made alkaline with NH_4OH solution to pH 10, the second kept neutral, while the third was acidified with acetic acid to pH 3. Each portion was divided into two equal parts (50 ml), to each part equal volume of methyl alcohol or acetone were added. The formed precipitates were separated by centrifugation at 1000 rpm for 10 minutes, concentrated under vacuum till dryness, then dissolved in 5 ml sterile distilled water and tested for its antimicrobial potentiality using the test organisms *Micrococcus luteus*.

2.12.3 Purification of active compounds with HPLC:

An Agilent 1100 HPLC was used with a semi-preparative C-18 column (Agilent Zorbax Eclipse XDB C18, 100x21mm, with a guard column 10x21mm) and Agilent VWD detector set at 210nm. Mobile phase A is water with 0.1% TFA and B is acetonitrile with 0.1% TFA. A gradient was used as below:

Flow rate: 5ml/min:

0-5mins, 100% A

5-30mins, 100% A to 0%A

30-35mins, 0% A

35-37mins, 0%A to 100% A

37-50mins, 100%A

The active compounds were collected by precipitation after adding equal volume of methanol or acetone, or by raising pH to 10. This is then dried under reduced pressure at room temperature and reconstituted into small volume of water with 0.1% formic acid. After centrifugation for 10 mins at 16000g, supernatant was collected and applied to HPLC. All peaks were collected and checked by mass spectrometer (ESI-MicroTOF, Bruker).

The major peaks were dried under reduced pressure and reconstituted into small volume of distilled water and checked for activity.

- Mass spectrometry

Active fractions were subjected to further mass spec analysis. A Bruker HCT Plus used for tandem mass spec analysis, active compounds were fragmented in both positive and negative mode. A Bruker MicroTOF was used to generate formulas for interesting peaks; calibration was done with standard Bruker procedure. Both the HCT ion-trap and the MicroTOF were equipped with an ESI source.

Both of desferrioxamine E and B were identified through HRTOF analysis, right formulas were generated with less than 3ppm error, a distinguish iron isotopic pattern was also observed in these compounds.

- LC-MS analysis

An Agilent 1100 HPLC coupled with a Bruker HCT plus was used. An Agilent C18 column (100x4.6mm, with an Agilent guard cartridge) was used with a flow rate at 1ml/min. Mobile phases is the same as described above apart from 0.1% formic acid was added instead of TFA. A gradient from 5% B to 100% B in 30 minutes was applied.

Chapter 3

RESULTS

3 RESULTS

3.1 Isolation and screening of actinomycetes

In screening for actinomycetes having antifungal activity, 54 isolates were obtained, (35 actinomycete stains from Iraq and19 from Egyptian soil). All these strains were screened for their ability to produce antifungal agents using *Candida albicans* and *Aspergillus niger* as test organisms. Only five strains (14.28%) among the 35 obtained from El Madein (Baghdad-Iraq) soils were antifungal producers. The most active one among them which strongly inhibited the growth of Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram negative bacteria (*Esherichia coli* and *Pseudomonas aeriginosa*), yeasts (*Candida albicans* and *Sacharomyces cerevisiae*) and some filamentous fungi (*Aspergillus niger*, A. *flavus*, *A. terreus*, *Penecillium sp*. *Rhizopus sp*. *Fusarium* sp. *Rhizoctonia* sp. and *Mucor mecedo*) was designated RN+8. Morphological and chemical studies indicated that this strain belonged to the genus *Streptomyces*. Further cultural, physiological and enzyme activities compared to those of known species described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1989), suggested that this organism is close to *S. rochei.* Thus, strain RN+8 was given the name of this species to be *Streptomyces rochei* RN+8 (Forar *et al*., 2006a) see annex. Whereas, 13 out of the nineteen strains recovered in soils collected from and around Cairo – Egypt, showed appreciable antimicrobial activities against at least one among the five tested micro-organisms. Data in (Table 3.1) shows the following: ten (10) isolates (ES3, ES5, ES7, ES8, ES10, ES13, ES14, ES15 ES16 and ES19) were active against the Gram-positive bacteria *Staphylococcus aureus*, *Micrococcus luteus*, and Gram-negative bacterium, *Esherichia coli*. Among them, isolates (ES3, ES7, ES10, ES15 and 19) exhibited strong activity against all the tested microorganisms. Whereas, isolate ES4 exhibited antimicrobial activity only against *Micrococcus luteus* and *Esherichia coli* where isolate ES9 inhibited the growth of *E. coli* and *Aspergillus niger* only. Isolate ES1 was antifungal producer; it inhibited the growth of *Candida albicans* and *Aspergillus niger* without any antimicrobial activity against Gram positive or Gram negative bacteria. The other six isolates (ES2, ES6, ES11, ES12, ES17 and ES18) showed no antimicrobial activities.

Table 3.1: Antimicrobial activity of nineteen isolates of *Streptomyces* spp. against Gram positive and Gram negative bacteria, yeasts and filamentous fungi.

Isolate no.	*S. aureus*	*M. luteus*	*E. coli*	*C. albicans*	*A. niger*
ES1	-	-	-	+	+
ES2	-	-	-	-	-
ES3	+	+	+	-	-
ES4	-	+	+	-	-
ES5	+	+	+	-	-
ES6	-	-	-	-	-
ES7	+	+	+	+	+
ES8	+	+	+	-	-
ES9	-	-	+	-	+
ES10	+	+	+	+	+
ES11	-	-	-	-	-
ES12	-	-	-	-	-
ES13	+	+	+	-	-
ES14	+	+	+	-	-
ES15	+	+	-	+	+
ES16	+	+	+	-	-
ES17	-	-	-	-	-
ES18	-	-	-	-	-
ES19	+	+	+	+	+

+ Antibiosis - no effect

The Five isolates (no. ES3, ES7, ES10, ES15 and ES19) which were active against all the target pathogens in solid media (Table 3.1), they were retested for their activities in the same medium as culture broth. Tow similar promising isolates (ES10 and ES15), based on their broad spectrum of activity and largest zone of inhibition were selected for identification and designated strains SK4-6 and RAF10 respectively, results are given in Table.3.2.

Table 3.2: Antimicrobial activity of the supernatant of the five active isolates.

Isolate no.	*Staphylococcus aureus*	*Micrococcus luteus*	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
ES3	20*	16	13	22	17
ES7	18	21	20	15	17
ES10	25	23	24	22	23
ES15	22	20	23	25	22
ES19	23	22	22	18	18

*Inhibition zone diameter (mm) of the crude supernatant

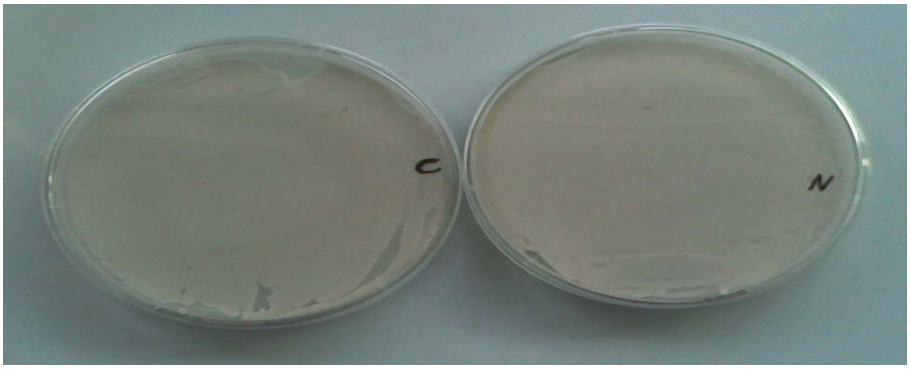

Figure 3.1a: The morphology semblance of strain SK4-6 (C) and strain RAF10 (N)

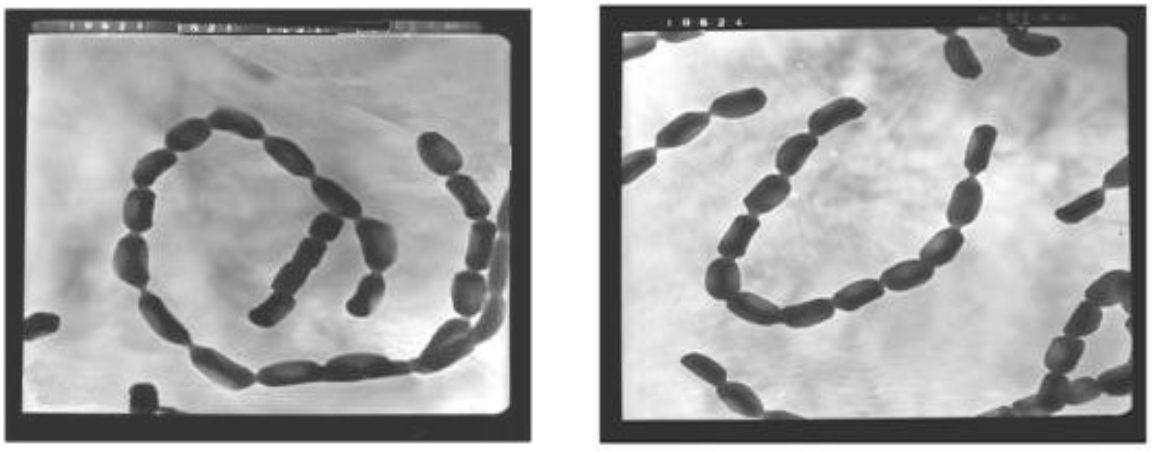

Figure 3.1b: Transmission electron micrograph of the two strains, SK4-6 and RAF10 respectively, shows smooth surface of spores (x10000).

Table 3.3: Physiological and biochemical characteristics of the two strains.

Physiological and biochemical tests	Strain SK4-6	Strain RAF10
Degradation activity		
Starch hydrolysis	+	+
Arbutin	+	+
Casein	+	+
Gelatine liquefaction	+	+
Nitrate reduction	-	+
H_2S production	+	_
KCN	+	+
Citrate	+	+
Nacl tolerance	7 %	7 %
Effect of inhibitory compounds		
Sodium azide 0.001 % (w/v)	-	-
Crystal violet 0.001 % (w/v)	-	-
Growth temperature	15 – 37	15 – 37
Melanin production		
Tyrosine agar medium	-	-
Peptone yeast extract iron agar medium	-	-
PH range	6 – 9	6 - 9
Utilization of carbohydrates		
Glucose	+	+
L- arabinose	+	+
Mannitol	+	+
Mannose	+	+
D-xylose	+	+
Inositol	+	+
Raffinose	-	-
Lactose	+	-
Galactose	-	-
Maltose	+	+
Sucrose	+	+
Enzyme activity		
Amylase	+	+
Gelatinase	+	+
Pectinase	+	+
Protease	+	+
Lipase	+	+
Lecithinase	+	-
Urease	+	+
Cellulase	+	+
Resistance to antibiotics		
Rifampicin (5 mg/l)	-	-
Penicillin (25 mg/l)	-	-
Streptomycin (10 mg/l)	-	-
Erythromycin (10 mg/l)	-	-
Gentamicin (5 mg/l)	-	-

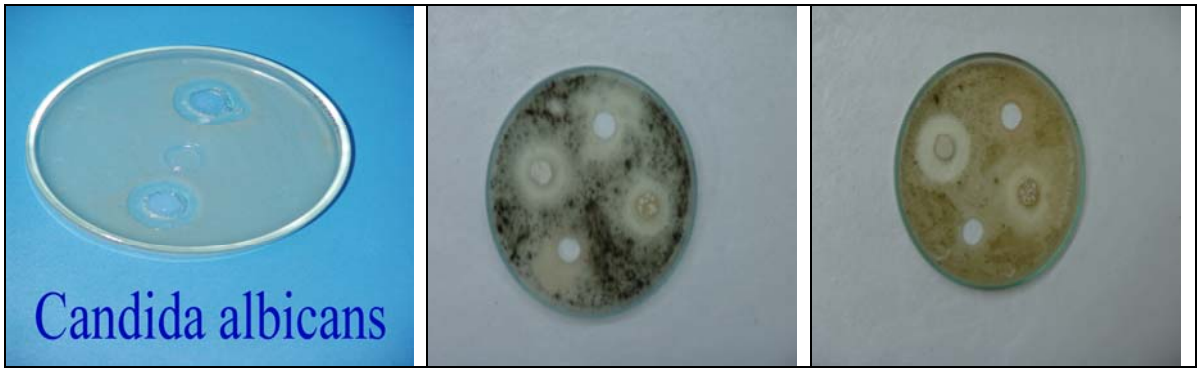

Figure. 3.2: Antifungal activity of the two most active strains against *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus***.**

Table 3.4: Broad antimicrobial spectrum of the two strains.

Test microorganism	Strain SK4-6	Strain RAF10
Bacillus cereus	26*	30
Bacillus subtilis	28	28
Staphylococcus aureus	22	28
Micrococcus luteus	20	25
Escherichia coli	23	28
Pseudomonas aeruginosa	18	21
Aspergillus flavus	20	20
Aspergillus niger	22	23
Aspergillus terreus	18	18
Botrytis allii	21	23
Diplodia oryzae	16	17
Fusarium oxysporum	18	20
Helmenthosporium turcicum	16	16
Machrophomina phaseoli	18	18
Trichoderma viride	20	20
Candida albicans	20	22
Candida tropicalis	23	24
Candida pseudotropicalis	22	22
Rhodotorula minuta	20	20

* Diameter of inhibition zone (mm).

3.2 Identification of strain SK4-6

3.2.1 Morphological characteristics:

The examination of strain SK4-6 grown on ISP-2 medium at 28 °C for 7 days revealed that sporophores are spiral. Transmission electron micrograph showed that spores are numerous, very fine and oval with smooth membranes (Fig. 3.2).

3.2.2 Cultural characteristics:

The aerial mycelium of strain SK4-6 was brown to grey on all the tested agar media except for glycerol asparagine agar where it was light brown grey and pink grey on malt extract agar. Substrate mycelium clearly varied in colors on the used agar media, it was light brown on starch nitrate agar, light grey yellowish brown on glycerol asparagine agar, light yellow on sucrose nitrate agar, brown yellowish grey on fish meal extract agar, slit brown on soybean meal, light earthy brown on oatmeal agar and medium yellowish brown on malt extract agar. This organism did not produce melanin pigment on ISP-6 and ISP-4 medium and no diffusible pigments were observed.

3.2.3 Physiological characteristics:

The strain SK4-6 hydrolyzed starch, degraded arbutin, casein, liquefied gelatine and peptonized milk, but it did not reduce nitrate nor produce H_2S. It utilized glucose, arabinose, mannose, mannitol, maltose, sucrose, xylose, inositol and sodium citrate however it could not utilize raffinose and galactose**.** As nitrogen sources, it utilized nitrates well and ammonium salts are either poorly utilized or not utilized at all. Strain SK4-6 was not able to grow on glucose-yeast extract agar (GYEA) medium supplemented with 5 different antibiotics, Chloramphenicol (25 mg/l), Erythromycin (10 mg/l), Gentamicin (5 mg/l), Oxytetracycline (25 mg/l) and Penicillin (25 mg/l). Also SK4-6 strain did not grow in the presence of sodium azide at 0.001 %, crystal violet at the same concentration and at 0.8 % sodium chloride. On the other hand, well growth was recorded at a temperature range of 15 to 37 °C and at pH range 6 to 9 as it is given in Table (3.3).

3.2.4 Chemotaxonomic analysis:

Analysis of the whole-cell hydrolysate of strain SK4-6 showed the presence of chemotype I cell wall characterized by LL-Diaminopimelic acid (LL-DAP), and no sugars

were detected. Chemotaxonomic investigations revealed that this strain has cell wall type I which is characteristic of the genus *Streptomyces* (lechevalier and Lechevalier (1970).

On the basis of the obtained morphological and chemical characteristics of strain SK4-6, and compared to those of known actinomycetes described in Berge's Manual of Determinative Bacteriology (Holt *et al*., 1989), it was classified in the genus *Streptomyces*.

3.3. Identification of strain RAF10

3.3.1 Morphological characteristics:

The examination of strain RAF10 grown on ISP-2 medium at 28 °C for 7 days revealed that sporophores are spiral. Transmission electron micrograph showed that spores are numerous, very fine and oval with smooth membranes too very similar to strain SK4-6 (Fig 3.2).

3.3.2 Cultural characteristics:

The cultures of strain RAF10 were brown, earthy-black or gray-black. The organism grew well to moderate on the tested organic and synthetic media. The colour of the aerial mycelium (AM) was lilac to pinkish lilac on most agar media; it varied depending on the type of used media. The brown substance was rarely produced on synthetic and organic media and stains them.

3.3.3 Physiological characteristics: The strain RAF10 hydrolyzed starch, degraded arbutin, casein, potassium cyanide reduced nitrate, liquefied gelatine and peptonized milk, but it did not produce H_2S. It utilized glucose, arabinose, mannose, mannitol, maltose, sucrose, xylose, inositol and sodium citrate, although it could not utilize lactose, raffinose, galactose, and sodium acetate**.** As nitrogen sources, it utilized nitrates well and ammonium salts are either poorly utilized or not utilized at all. Strain RAF10 was not able to grow on glucose-yeast extract agar (GYEA) medium supplemented with 5 different antibiotics, Chloramphenicol (25 mg/l), Erythromycin (10 mg/l), Gentamicin (5 mg/l), Oxytetracycline (25 mg/l) and Penicillin (25 mg/l). It did not grow at 0.001 % sodium azide and at 0.001 % crystal violet as well. Good growth was recorded at a temperature range of 15 to 37 °C and at pH range 6 to 9 and tolerated 0.7 % sodium chloride as it is shown in (Table 3.3).

3.3.4 Chemotaxonomic analysis: Analysis of the whole-cell hydrolysate of strain RAF10 showed the presence of chemo-type I cell wall characterized by LL-Diaminopimelic acid (LL-DAP), and no sugars were detected. Chemotaxonomic investigations revealed that this strain has cell wall type I which is characteristic of the genus *Streptomyces*.

Based on the obtained morphological and chemical characteristics of strain RAF10, and compared to those of known actinomycetes described in Berge's Manual of Determinative Bacteriology (Holt *et al.*, 1989), it was classified in the genus *Streptomyces* too.

3.4 Randomly Amplified Polymorphic DNA (RAPD) analysis

Because of the high resemblance of these organisms (SK4-6 and RAF10), in morphology (Figure 3.1) and growth comportment (Table 3.3) as well as antimicrobial activities (Table 3.4), they were subjected to Randomly Amplified Polymorphic DNA (RAPD) analysis, to know if they are different strains or the same one (Fig 3.3).

RAPD analysis was carried out using five pre-selected random decamer primers, namely OPO9, OPK9, OPN1, OPQ14 and OPK13. The size of the resulted amplification products ranged from 300 to 1600 bp. Out of 41 amplicons (bands) generated by PCR amplification using the aforementioned primers, 18 bands were polymorphic, i.e. the percentage of polymorphism is 43.9 %. Figure (3.3) shows the developed RAPD-based fingerprints of the two strains. From a glance at the presented RAPD banding pattern, it could be concluded that in spite of similarity in morphology and growth comportment of the two strains, they are genetically diverse. For instance, using the primer OPO9, polymorphic bands of (1500 bp) and (1200 bp and 500 bp) were detected and were unique to strains RAF10 and SK4-6, respectively. Similarly, using primer OPK9, a unique marker (600 bp) was detected in strain SK4-6 (Fig. 3.3). From the time when we knew that they are genetically diverse, we subjected them to 16S rRNA sequence analysis.

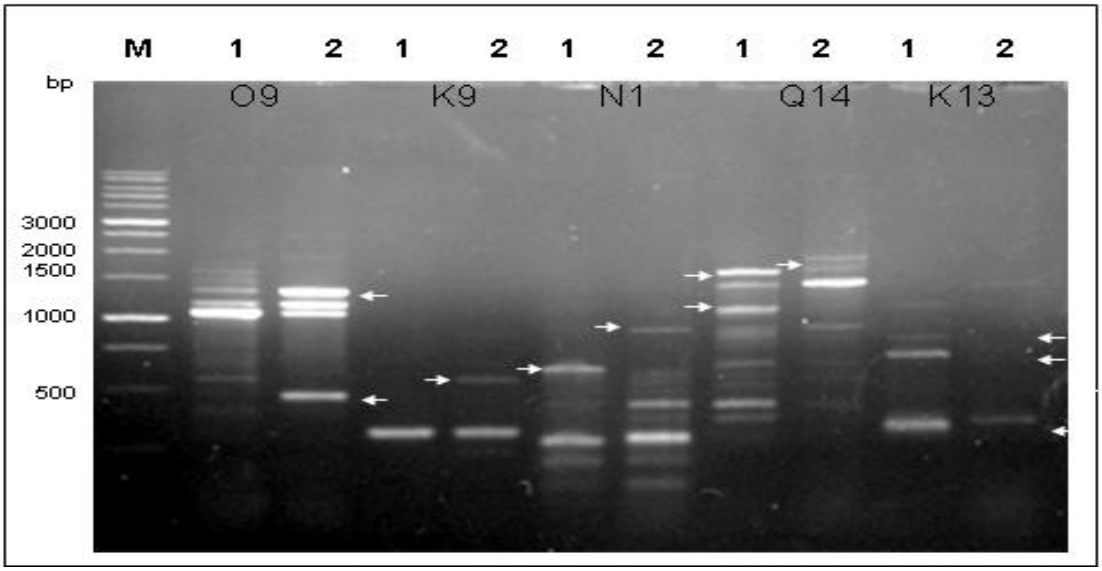

Figure 3.3: RAPD based DNA fingerprints of the two isolates 1 (strain SK4-6) and 2 (strain RAF10) generated by PCR amplification using five different random primers and electrophoresed using 1.5 % agarose gel in TAE buffer. M is kbp DNA Ladder of Bioron. Arrows point to polymorphic markers.

3.5 16S rRNA sequence comparison

The alignment of the nucleotide sequence of strain SK4-6, (GenBank accession no. EF120466) scheme 1, (see annex) and strain RAF10 (GenBank accession no. EF 474464) scheme 2, (see annex) through matching with 16S rRNA reported genes sequences in the gene bank using the ‘‘NCBI Blast’’ available at http://www.ncbi-nlm-nih.gov (website), and compared to sequences of the reference species of bacteria contained in genomic database banks exhibited a high similarity, 99 %, with *Streptomyces qinlingensis* having the closest match. The phylogenetic tree obtained by applying the neighbour-joining method using BioEdit software (Hall, 1999) is illustrated in Fig 3.4. And in case of strain RAF10, the level of similarity ranged from 97.22 % to 98.37 % with *Streptomyces enissocaesilis* having the closest match. The phylogenetic tree obtained by applying the neighbour-joining method is illustrated in Fig 3.5.

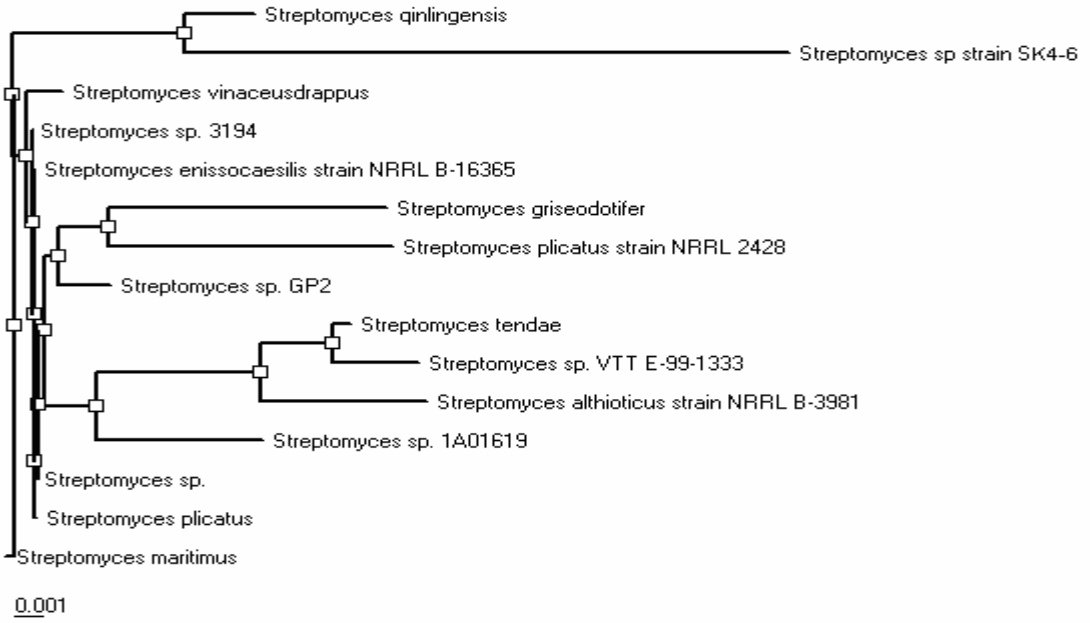

Figure 3.4: Neighbour-joining tree based on 16S rRNA sequences showing the relation between strain SK4-6 and the highest 14 similar *Streptomyces* species.

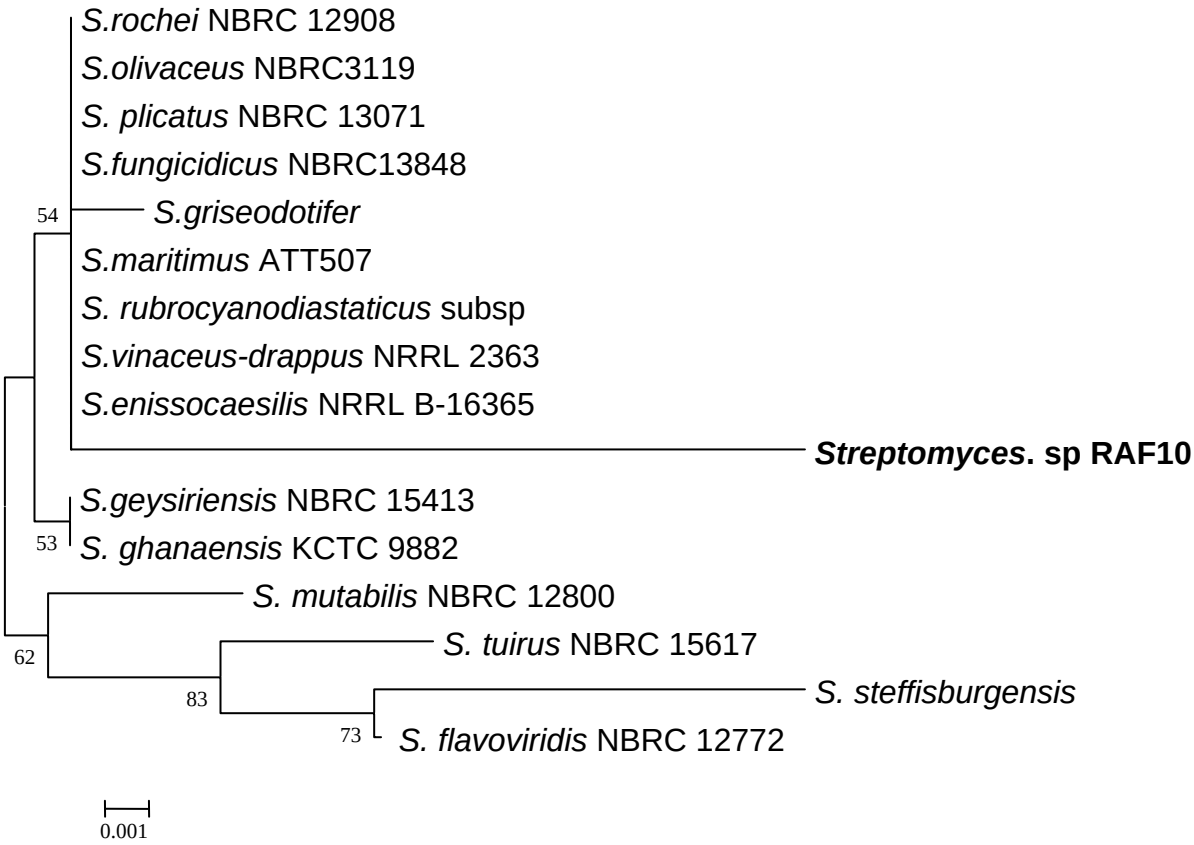

Figure 3.5: 16S rDNA tree showing the phylogenitic relationship neighbour-joining method between strain RAF 10 and other known sequences of *Streptomyces* sp.

3.6 Antimicrobial activity

Results in Table 3.4, show the broad antimicrobial spectrum of the two strains (SK4-6 and RAF10) against various test micro-organisms. They exhibited a good activity against Gram-positive bacteria (*Bacillus subtilis, B. cereus*), Gram-negative bacteria (*E. coli*), then filamentous fungi (*Aspergillus niger, Botrytis allii,* and *Trichoderma viride*) and yeasts (*Candida albicans, Rhodotorula minota and Candida tropicalis*).

3.7 The optimization studies

3.7.1 Parameters influencing antibiotic production by strain SK4-6 (first step):

On the basis of the preliminary study, using different broth cultures to determine the most suitable broth for antibiotic production by strain SK4-6, followed by the study of parameters affecting antibiotic production using one factor at-a -time approach, it was found that, (ISP-4) broth using, starch Table 3.5 and ammonium sulphate Table 3.7 at concentrations of 2.5 % Table 3.6 and 0.25 % Table 3.8 (w/v) as carbon and nitrogen sources respectively, and dipotassium phosphate Table 3.9 with concentration of 0.15% Table 3.10 for 96 hrs incubation Table 3.11 at 28 °C in orbital incubator with shacking at 200 rpm were the most suitable for antibiotic formation by this organism.

Table 3.5: Suitable carbon source for antibiotic production

Carbon source (w/v)	Inhibition zone diameter (mm)
D-glucose	27
Mannose	25
Maltose	24
D-mannitol	26
Sucrose	20
Starch (control)	30

Test organism: *Bacillus cereus*

Table 3.6: Suitable concentration of starch

Starch concentration (g/l)	Inhibition zone diameter (mm)
5	10
10	20
15	22
20	25
25	30
30	25

Test organism: *Bacillus cereus*

Table 3.7: Suitable nitrogen source

Nitrogen source (equimolar amounts)	Inhibition zone diameter(mm)
Ammonium sulphate (control)	34
Potassium nitrate	27
Sodium nitrate	22
Asparagine	22
L-cysteine	25
Peptone	28

Test organism: *Bacillus cereus*

Table 3.8: Suitable concentration of ammonium sulphate

Ammonium sulphate concentrations (g/l)	Inhibition zone diameter (mm)
0.5	17
1.0	20
1.5	27
2.0	33
2.5	35
3.0	35

Test organism: *Bacillus cereus*

Table 3.9: Suitable phosphate salt for antibiotic production

Phosphate salt (equimolar amounts)	Inhibition zone diameter (mm)
Dipotassium hydrogen phosphate (control)	32
Potassium dihydrogen phosphate	25
Ammonium dihydrogen phosphate	24
Diammonium hydrogen phosphate	23
Sodium dihydrogen phosphate	22
Disodium hydrogen phosphate	19

Test organism: *Bacillus cereus*

Table 3.10: Suitable concentration of dipotassium hydrogen phosphate

Dipotassium hydrogen phosphate concentrations (g/l)	Inhibition zone diameter (mm)
0.50	20
0.75	24
1.00	30
1.25	33
1.50	36
1.75	32
2.00	28

Test organism: *Bacillus cereus*

Table 3.11: Suitable incubation period for antibiotic production

Incubation periods (hours)	Inhibition zone diameter (mm)
24	18
48	26
72	32
96	38
120	38
144	30
168	25

Test organism: *Bacillus cereus*

3.7.2 Optimization using full factorial design (SK4-6, second step):

In this step, evaluation of the factors affecting antibiotic production by strain SK4-6 was carried out using Plackett-Burman statistical design to reflect the relative importance of various fermentation factors. Seven different factors (variables) including fermentation conditions and medium constitution, were chosen to perform this optimization process. Antimicrobial activity was measured after 96 h of incubation. The independent variables examined and their settings are shown in Table 2.3. The design plan and the averages of antimicrobial activity for the different trials were given in mm of inhibition zone and shown in Table 2.4.

The main effect of each variable upon antimicrobial activity was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor. The data in Table 2.4 show a wide variation from 0 to 29 mm of inhibition zone. This variation reflects the importance of medium optimization to attain higher production. The analysis of the data from the Plackett-Burman experiments involved a first order (main effects) model. The main effects of the examined factors on the antimicrobial activity were calculated and presented graphically in Figure (3.6).

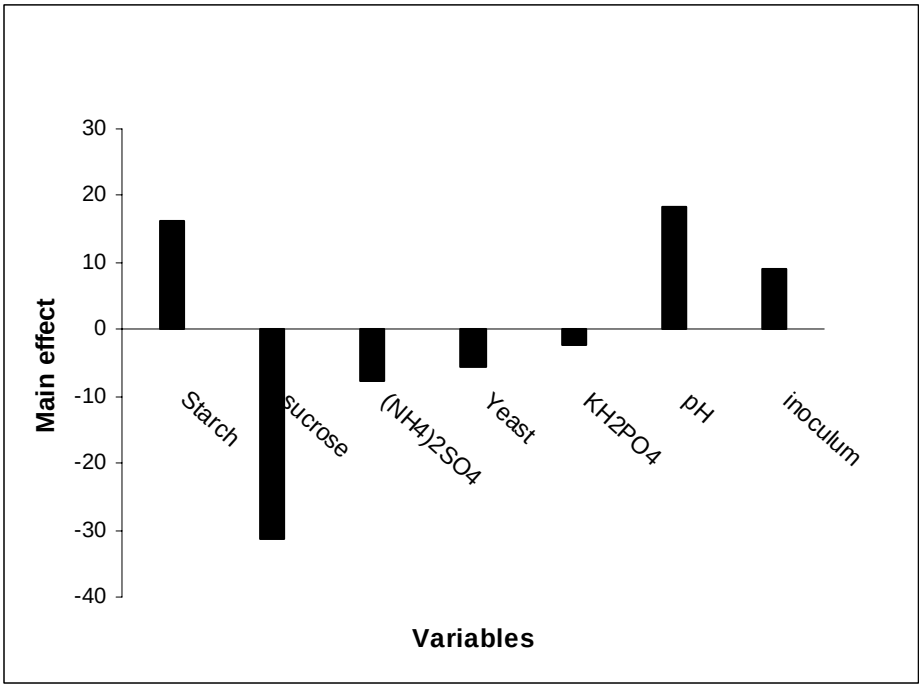

Figure 3.6: Effect of different factors on the active compounds production by strain SK4-6.

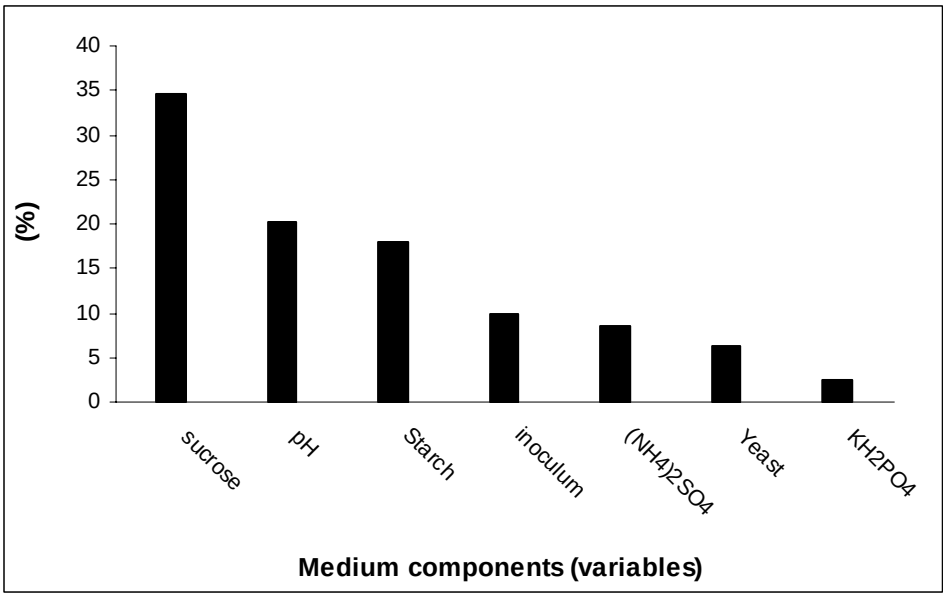

Figure 3.7: Pareto chart rationalizing the effect of each variable on the active compounds production by strain SK4-6.

Variables	Main effect	T-statistic	P-value
Starch	16.33	4.949747	0.126908
sucrose	-30.33	-9.49543	0.066799
$(NH_4)_2SO_4$	-7.66	-2.32335	0.258752
Yeast	-5.66	-1.71726	0.335703
KH_2PO_4	-2.33	-0.70711	0.608173
pH	18.33	5.555839	0.113372
Inoculum size	9	0.926842	0.389768

Table 3.12: Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-values of each variable

3.7.3 Parameters influencing antibiotic production by strain RAF10

The optimization studies for maximum production of active compounds by strain RAF10 were determined. Results obtained indicated that, (ISP-4) broth using, glucose Figure 3.8, ammonium sulphate Figure 3.10, at concentrations of 1.25 % and 0.25 % (w/v) Figures 3.9 and Figure 3.11, as carbon and nitrogen sources respectively, and dipotassium phospshate Figure 3.12, at concentration of 0.15 % Figure 3.13, for 120 h incubation Figure 3.14, at 28 °C in orbital incubator with shacking at 200 rpm were the most suitable for active substances production by this bacterium.

- **Suitable carbon source**

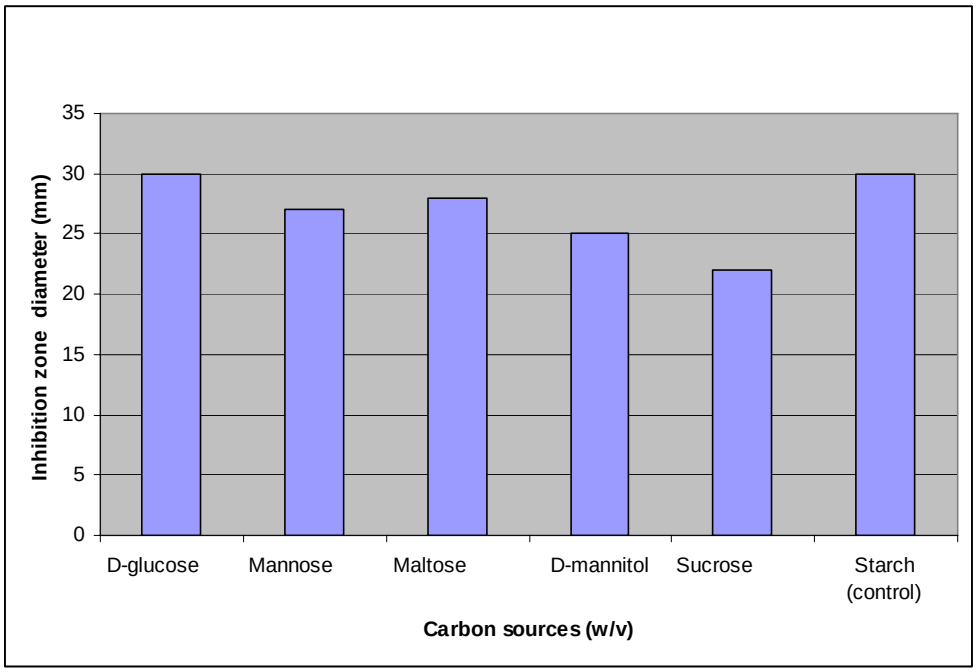

Figure 3.8: Suitable carbon source for antibiotic production

The data given in Figure 3.8 show that, among the 6 different carbon sources tested, glucose and starch were the most suitable for active compounds production by strain RAF10 where inhibition zone were 30 mm with both of them. They were followed by maltose then mannose which were also good sources for antibiotic production. After that we found mannitol

and sucrose with considerable affect. This gives an idea about the ability of this strain to use different carbon sources for growth and active compounds formation.

- Suitable concentration of glucose

Glucose was preferable for high antibiotic formation by strain RAF10 so it was selected for further investigation as sole carbon source in this step. The effect of its different concentrations (5 to 15 g/l) on the production of the active compounds was also determined. Results given in Figure 3.9, show that antimicrobial activity was increased gradually by increasing the concentration of glucose until reaching its maximum at 12.5 g/l and then it declined at concentration of 15 g/l.

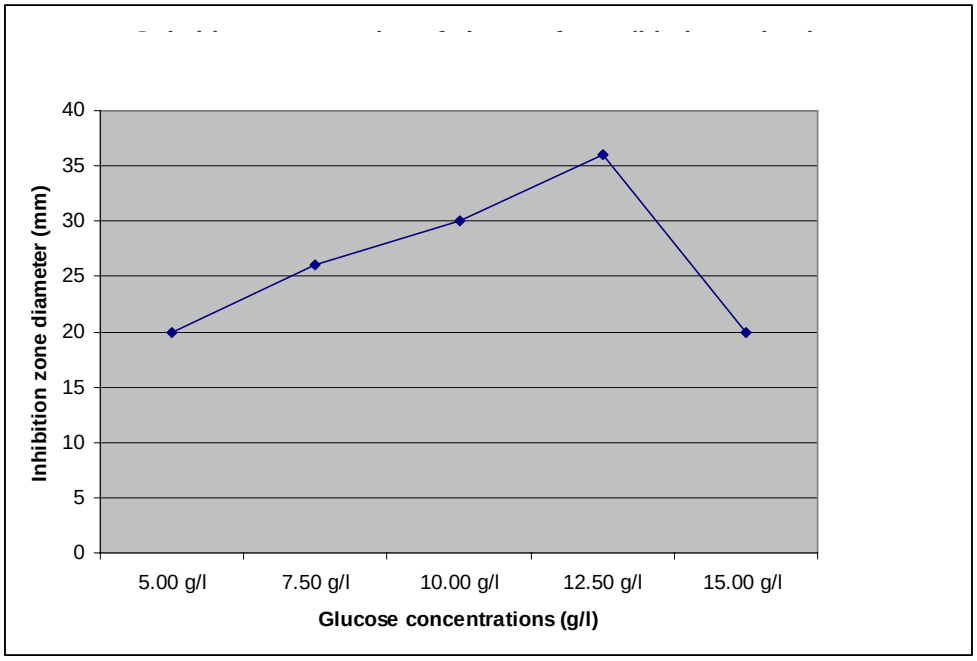

Figure 3.9: Suitable concentration of glucose for antibiotic production

- Suitable nitrogen source

Effect of different nitrogen sources including, ammonium sulphate, potassium nitrate and sodium nitrate as inorganic sources and asparagine, cysteine and peptone as organic sources on antibiotic production was also studied. The obtained results as it is clear in Figure 3.10 indicated that, highest level of production was supported by ammonium sulphate among the three inorganic nitrogen sources tried followed by sodium nitrate then potassium nitrate.

Amongst the organic nitrogen sources studied, peptone was the most suitable pursued by the amino acid cysteine then asparagine.

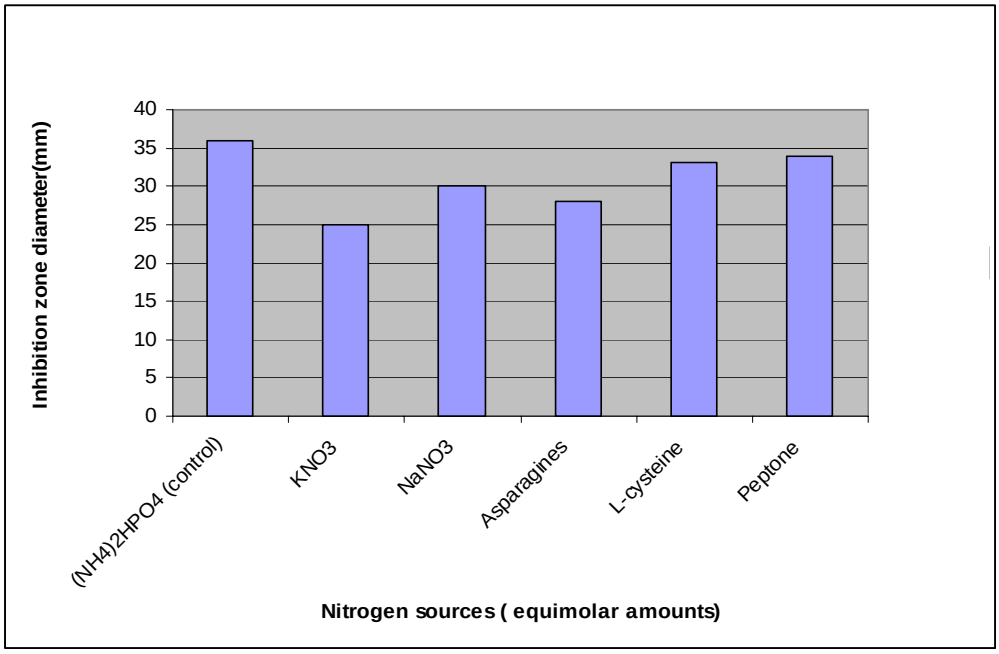

Figure 3.10: Suitable nitrogen source for antibiotic production

Suitable concentration of nitrogen source

Results given in Figure 3.11 show that, the most favourable ammonium sulphate concentration is 2.5 g/l, for high antibiotic production by strain RAF10. On increasing the concentration of ammonium sulphate, a gradual drop in the production of this active compound was recorded. All nitrogen sources tried support growth and antibiotic production with different inhibition zones.

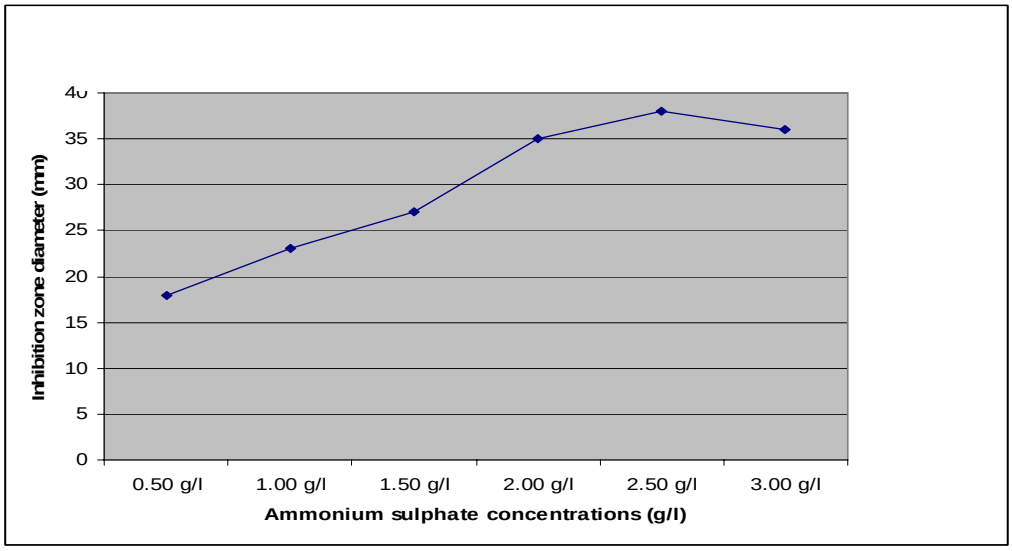

Figure 3.11: Suitable concentration of ammonium sulphate for antibiotic production

- Suitable phosphate salt

In this experiment, equimolecular amounts of phosphorus supplied from different phosphate sources were used. The optimal culture conditions determined from the previous experiments were maintained in this study.

The results given in Figure 3.12 indicated that, dipotassium hydrogen phosphate proved to be the most satisfactory phosphate source for getting the highest yield of active compounds by strain RAF10. The other phosphate salt sources exhibited remarkable affects on the active compounds production.

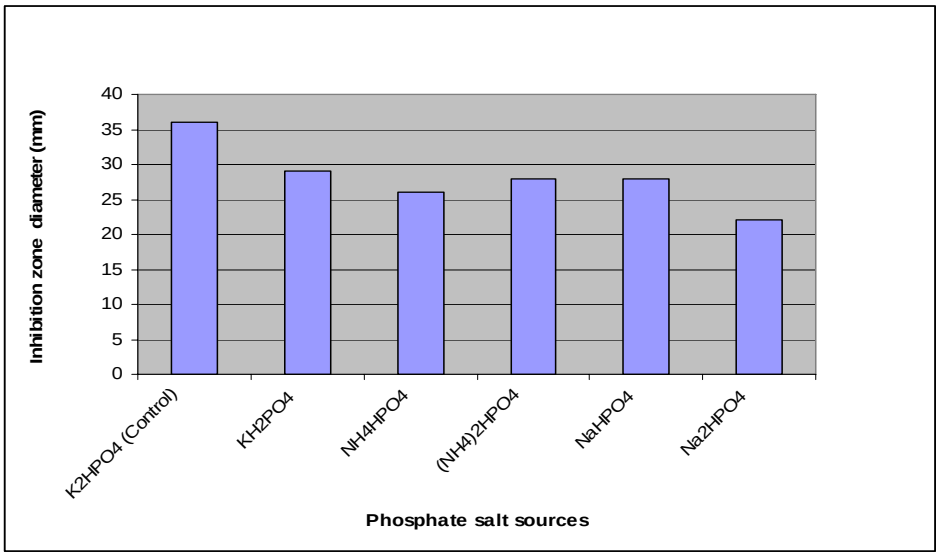

Figure 3.12: Suitable phosphate salt for antibiotic production

- Suitable concentration of phosphate salt

The optimized broth was supplemented with different concentrations of dipotassium phosphate to determine the suitable amount for best production. The other medium ingredients were added in their optimum levels. Figure 3.13 show that, the maximum antibiotic productivity was attained on culture broth supplemented with 1.5 g/l dipotassium phosphate.

Generally speaking, the antimicrobial activity gradually increased with increased concentration of dipotassium phosphate up to 1.5 g/l, higher than this concentration, the antimicrobial activity evidently declined. This indicates that antibiotic production by strain RAF10 is greatly affected by the concentration of dipotassium phosphate.

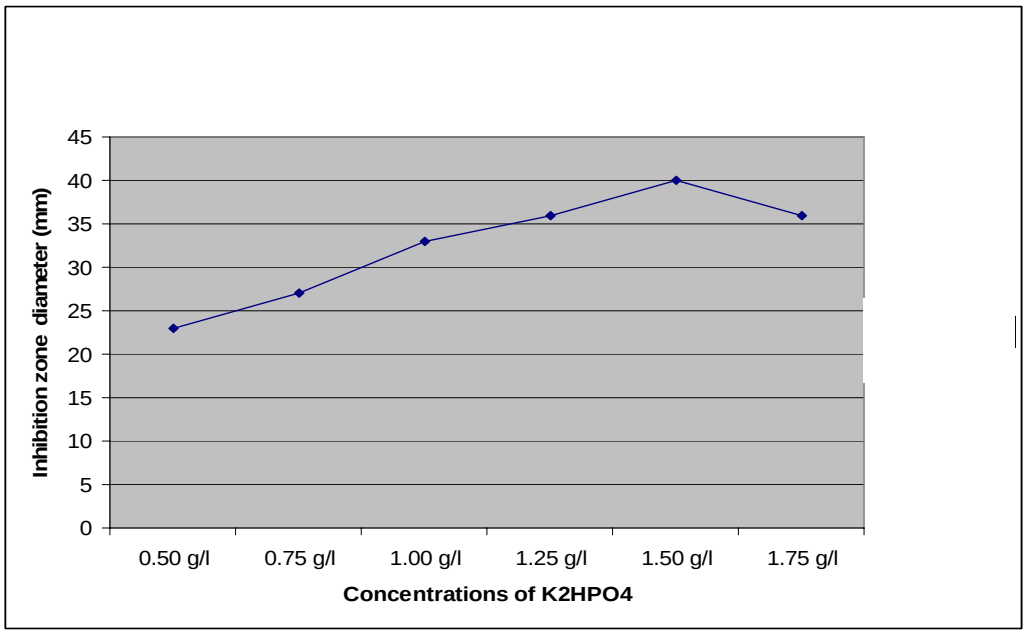

Figure 3.13: Suitable concentration of dipotassium phosphate for antibiotic production

After determination of the better culture broth, the suitable carbon and nitrogen sources and the most favourable phosphate salt on the antibiotic production by strain RAF10, the effect of incubation periods (up to 168 hours) was also determined using the above ingredients at their optimum levels.

Results given in Figure 3.14 which show that, the antimicrobial activity is observed after 48 hrs of incubation and reached its maximum after 120 hrs and remained constant. This means that these active compounds are secondary metabolites.

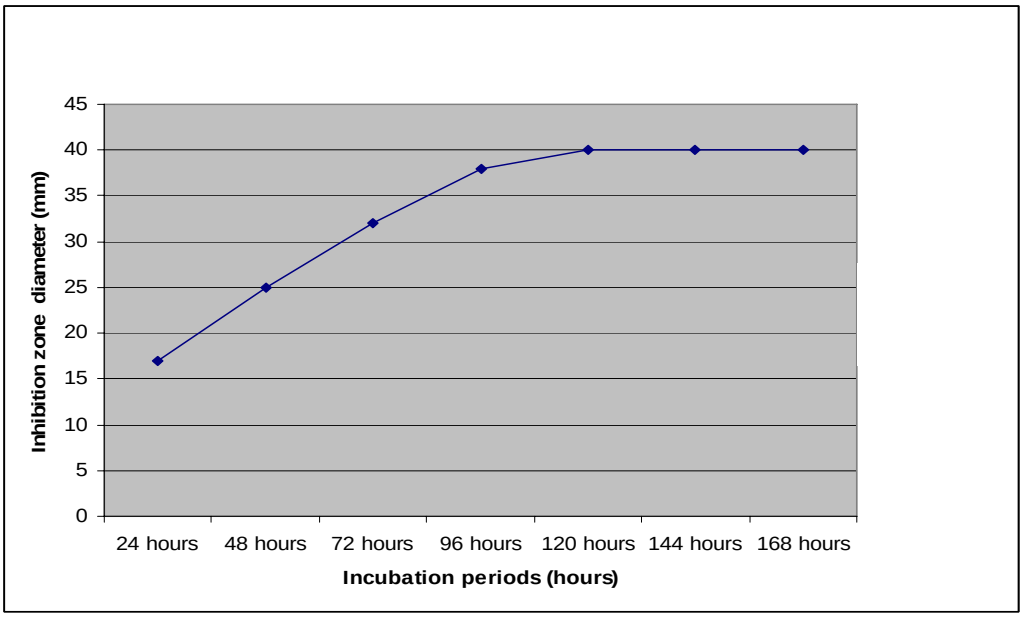

Figure 3.14: Suitable incubation period for antibiotic production

3.7.4 Optimization using full factorial design (second step)

Plackett-Burman statistical design was applied to reflect the relative importance of various fermentation factors. Seven different variables including fermentation conditions and medium constitution were chosen to perform this optimization process. Antimicrobial activity was measured after 120h of incubation. The independent variables examined and their settings are shown in Table 3.13. The design plan and the averages of antimicrobial activity for the different trials are given in mm of inhibition zone and shown in Table 3.14.

The main effect of each variable upon antimicrobial activity was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor. The data in Table 3.14 show a wide variation from 12 to 27 mm of inhibition zone. This variation reflects the importance of medium optimization to attain higher productivity. The analysis of the data from the Plackett-Burman experiments involved a first order (main effects) model. The main effects of the examined factors on the antimicrobial activity were calculated and presented graphically in Figure 3.15

Table 3.13: Media components and test levels for Plackett-Burman experiment.

Variable	code	high level (+1)	low level (-1)
Starch	X_1	3	2
Glucose	X_2	2.5	1.5
$(NH_4)_2SO_4$	X_3	2.5	1.5
Tryptone	X_4	0.5	0.2
KH_2PO_4	X_5	0.2	0.1
Inoculum size	X_6	7.5	6.5
pH	X_7	2.5	1.5

Table 3.14: Randomized Plackett-Burman experimental design for evaluating factors influencing antibiotic production

trails	Starch	Glucose	$(NH_4)_2SO_4$	Tryptone	K_2HPO_4	Inoculm.size	pH	DIZ
1	-1	1	1	-1	1	-1	1	12
2	-1	-1	1	1	-1	1	1	16
3	1	-1	-1	1	1	1	-1	21
4	1	1	-1	-1	1	-1	1	24
5	-1	1	1	-1	-1	1	1	20
6	1	-1	1	1	-1	1	-1	15
7	1	1	-1	1	1	-1	-1	**27**
8	-1	-1	-1	-1	-1	-1	-1	20

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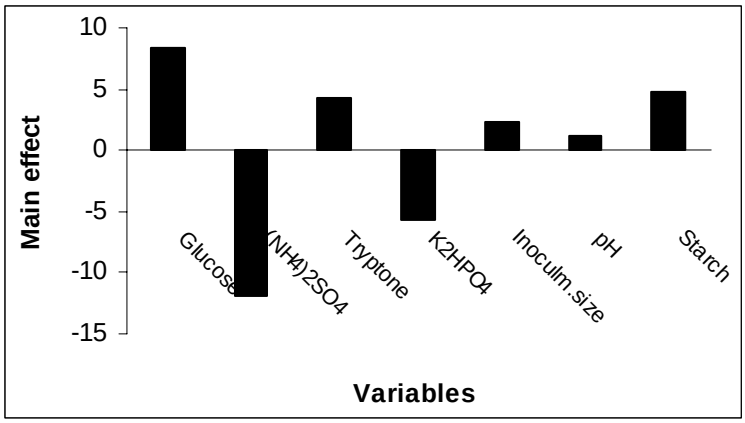

Figure 3.15: Effect of different factors on the antibiotic production by strain RAF10.

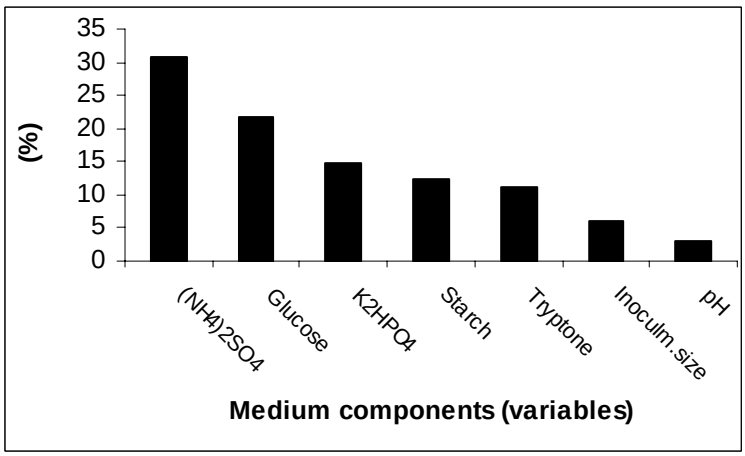

Figure 3.16: Pareto chart rationalizing the effect of each variable on the antibiotic production by strain RAF10.

Table 3.15: Statistical analysis of Plackett-Burman design showing coefficient values, t- and P-values of each variable.

Variables	main effect	t-value	p-value
Starch	4.75	1.48516082	0.18804238
Glucose	8.33	35.3553391	0.01800153
(NH4)2SO4	-11.83	-50.2045815	0.01267883
Tryptone	4.33	18.3847763	0.03459346
K2HPO4	-5.66	-24.0416306	0.02646464
Inoculm.size	2.33	9.89949494	0.0640909
pH	1.16	4.94974747	0.12690837

3.8 Production, extraction and purification of active compounds

3.8.1 Extraction of the active compounds produced by strain RAF10:

The active compounds were not soluble in organic solvents. Thus, they were precipitated by acetone from the culture supernatant. The precipitate was centrifuged at 10.000 rpm for 10 minutes and concentrated under vacuum till dryness. The obtained powder was dissolved in suitable volume of 0.1% H_2CO_2 and subjected to HPLC for purification. Different peaks were collected separately, drained and bio-assayed for their antimicrobial activities. On the basis of the bioassay results, fractions were collected and pooled together for further analysis. The ethanol extract of the biomass showed no antimicrobial activity. This shows the extra-cellular nature of active substances. Mostly antibiotics are extra-cellular, (Hacene *et al*., 2000; Augustine *et al*., 2005). Results are given in Figure 3.18.

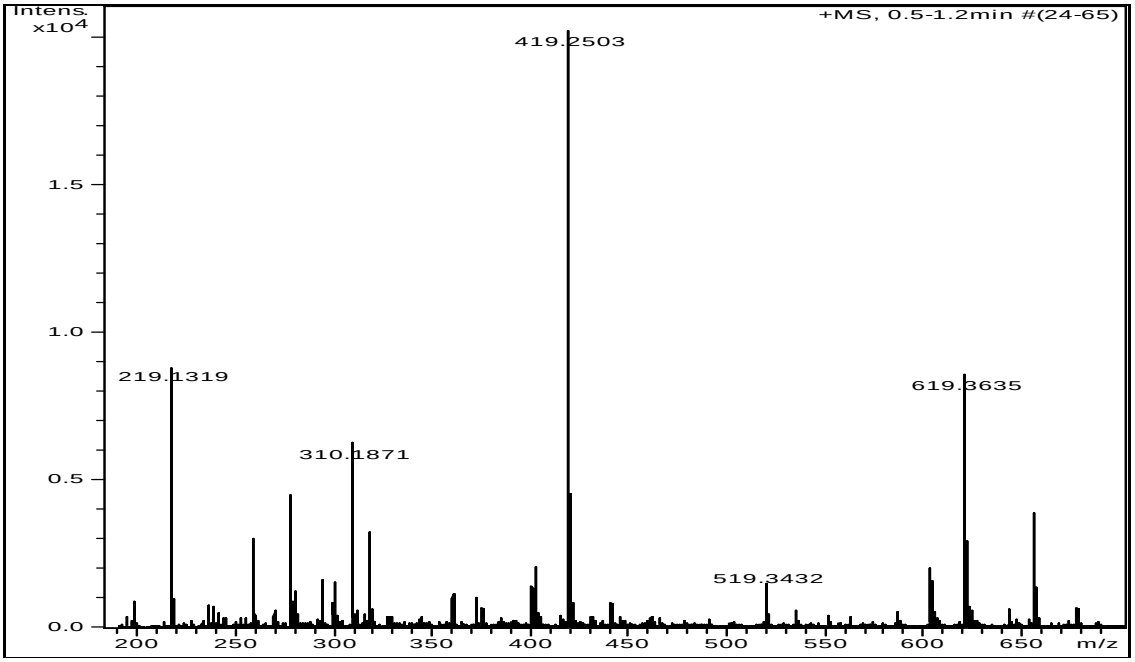

Figure 3.17: HRTOF analysis of HPLC fractions Rt 16-21min

This figure show one of the active fractions from HPLC separation, high resolution mass spectrum analysis indicated the presence of some of the known compounds, such as Des E (m/z 654), as well as some new compounds, m/z 619, 419. The peaks at 310 and 219 are from fragmentation of 419 peak, which was confirmed during tandem mass spectrum study of the compound 419.

3. 8.2 Purification

Mass spectrum analysis: Precipitate of spent culture was first separated with an RP-C18 column, (Zorbox 100x21mm) with a gradient of 0-100% ACN with 0.1% TFA in 30 minutes. Fractions were collected and pooled together based on bioassay results.

Desferrioxamine B and E are well known produced by large number of *Streptomyces* species (Kazuki *et al*., 2005; Francisco *et al*., 2006). Formula generated from HRTOF (Peak m/z 654, Figure 3.19) confirmed that, these are identical with known compounds, schema 3.3. These results are in agreement with those reported by (Kazuki *et al*., 2005; Francisco *et al*., 2006).

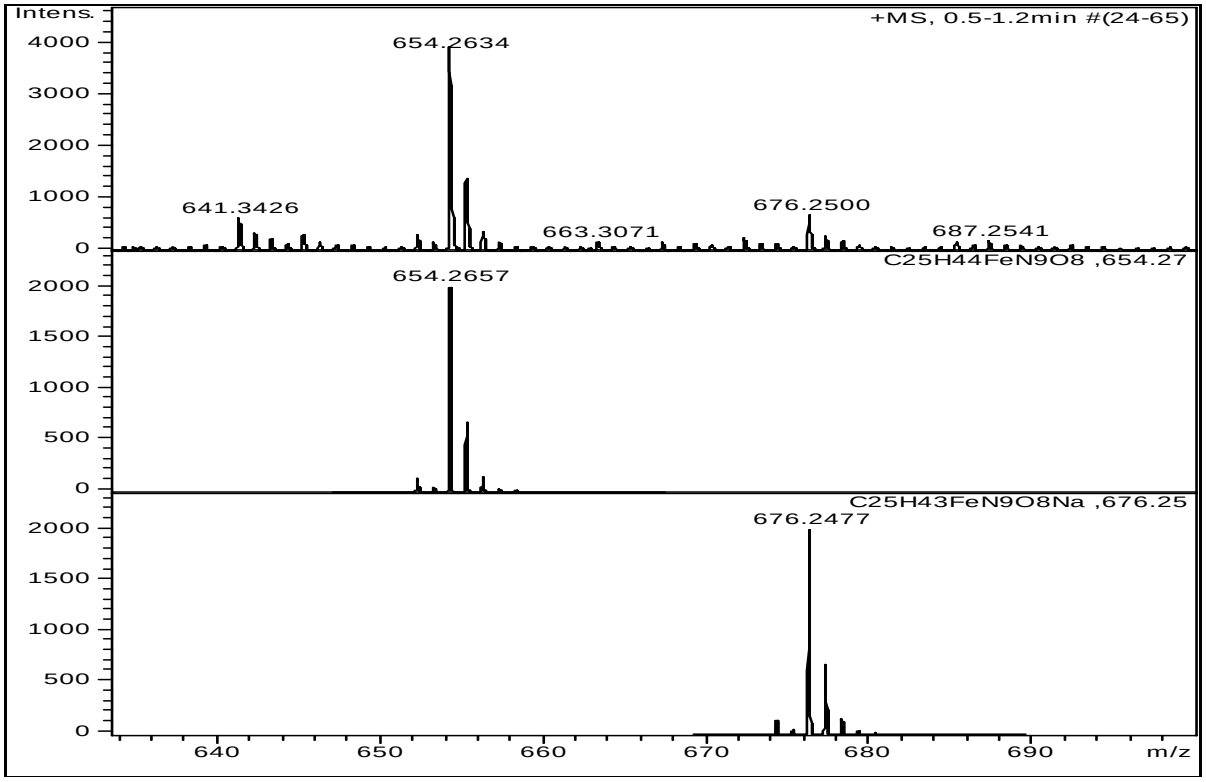

Figure 3.18: Peak m/z 654, shows mass spectrum of desferrioxamine B and E.

Peak 619 (formula C30H55FeN6O4 with less than 1 ppm error) is very interesting, it has same molecular weight as ferriocoelichelin (Figure 3.20), but the molecular formula generated indicated this is probably a different structure, further analysis is needed.

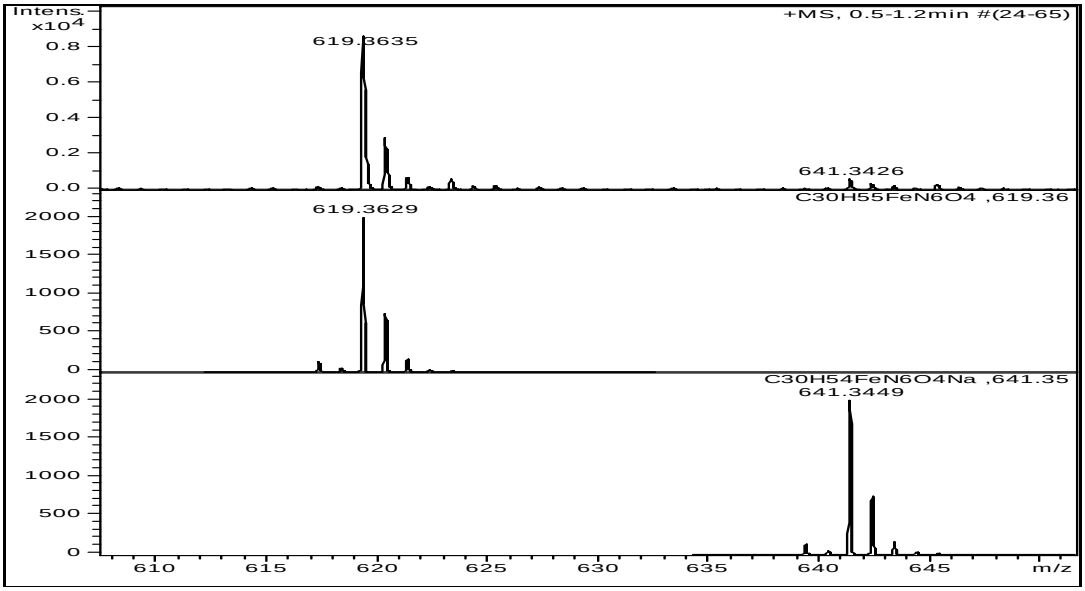

Figure 3.19: Peak m/z 619, shows mass spectrum of ferriocoelichelin.

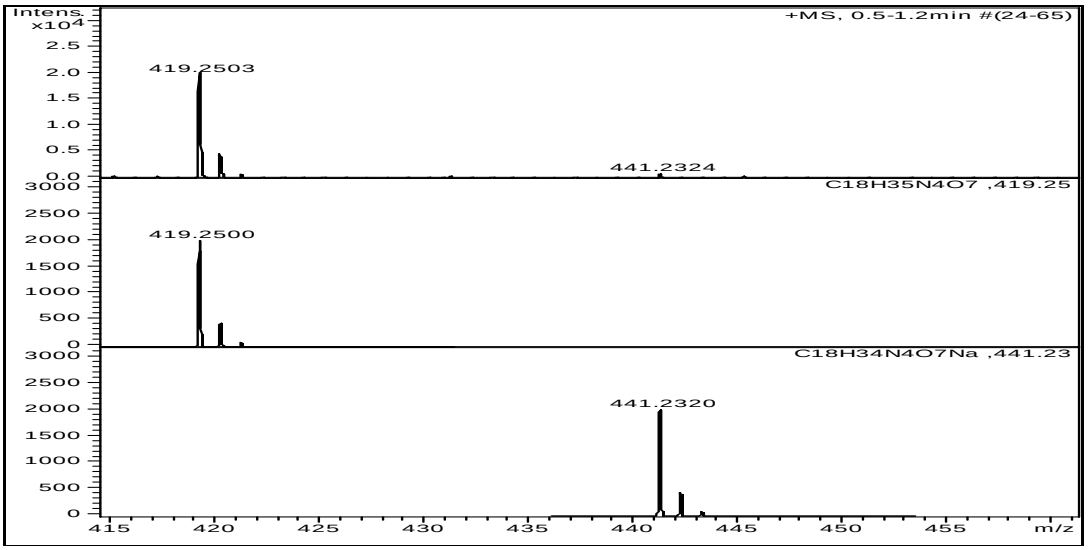

Figure 3.20: Peak419 shows mass spectrum of the two new compounds.

HRTOF analysis gave two formulas as C18H35N4O7 and C19H31N8O3 with less than 3ppm error, a search in SciFinder indicated these are not known.

Tandem mass spec analysis

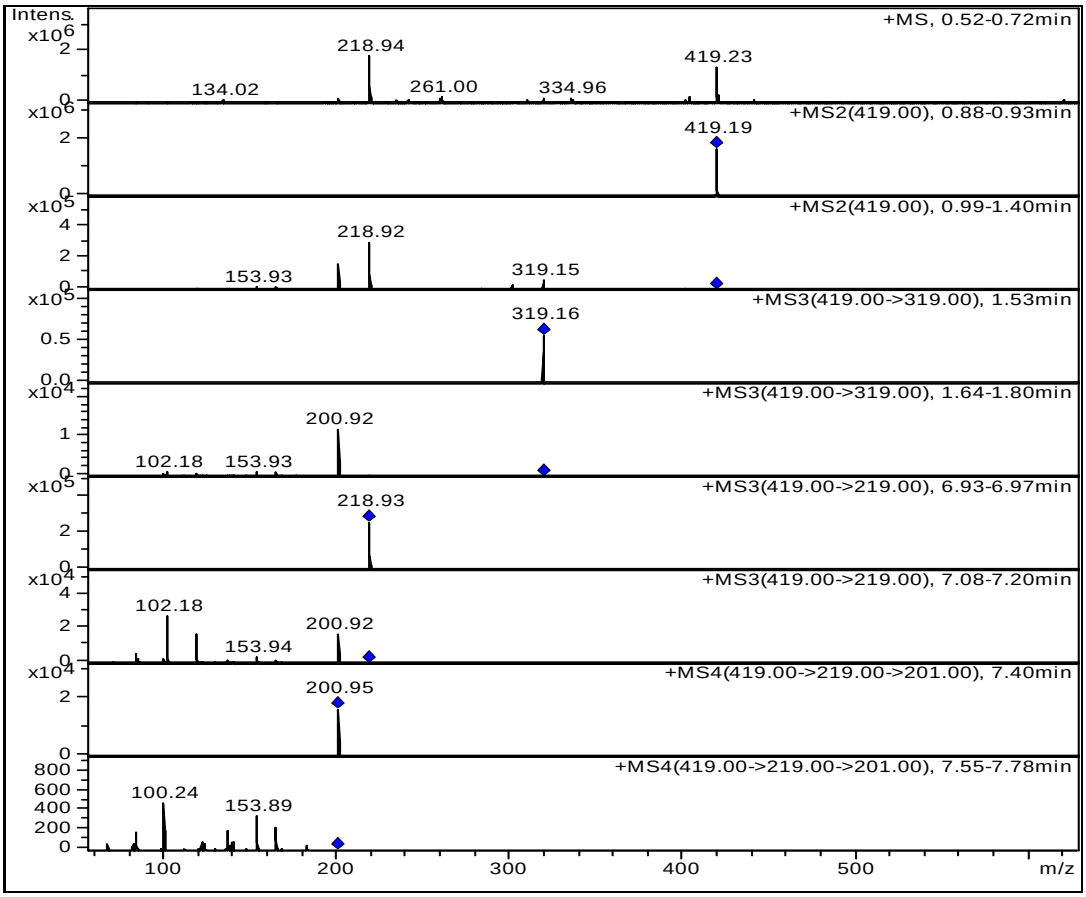

Figure 3.21: MS/MS of 419 peak in positive mode

MS/MS data showed a big neutral loss (200Da) in positive mode, the compounds fragmented during ESI-TOF analysis also indicated it is not very stable. Based on its poor solubility in any organic solvent and TOF analysis, this probably is a dipeptide. Further NMR analysis is needed to figure out the structure.

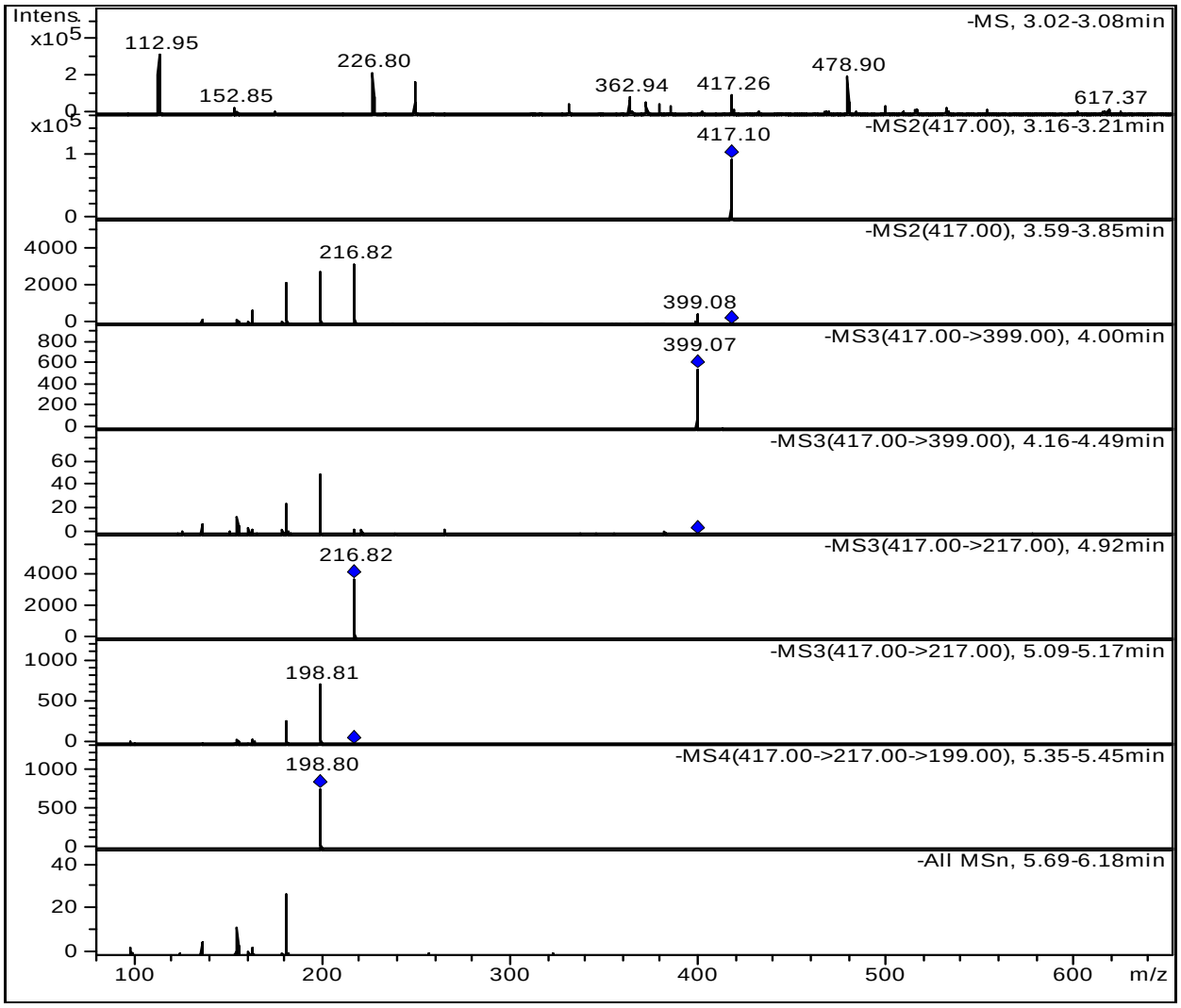

Figure 3.22: MS/MS of 419 peak in negative mode

Chapter 4

DISCUSSION

4. DISCUSSION

Streptomycetes are Gram-positive, aerobic actinomycetes with a high G+C ratio in the range of 69 to 78 % (Williams *et al*., 1983a). The cell wall is type I sensu (Lechevalier, 1970; Lechevalier and Lechevalier, 1970) because it does not contain any characteristic sugars and contains LL diaminopimelic acid and glycine. The ability of *Streptomyces* to produce many valuable bioactive compounds led to over-speciation of members of the genus. Producers of novel natural products were described as new species and patented; however, many of these strains were synonyms. In 1964 the International *Streptomyces* Project (ISP) was initiated to solve this problem. Shirling and Gottlieb (1968 a, b) described standard tests that were used for the identification and classification studies. Such tests involve spore chain morphology, spore surface ornamentation, spore colour, substrate mycelium and soluble pigments, production of melanin pigment and the utilisation of a range of carbon sources. This led to the re-analysis of many isolates and designation of type strains that were subsequently deposited into culture collections. Williams *et al.* (1983b) incorporated these tests in a numerical taxonomic study. A probabilistic data matrix for the identification of *Streptomyces* was constructed using information from the numerical taxonomic database (Williams *et al*., 1983a). This study forms the basis of the classification in Bergey's Manual of Systematic Bacteriology, (Williams *et al*., 1989). A second numerical analysis was made by Kämpher *et al*. (1991) which include more species and more than one strain of each species also it took advance in genetic characterisation into account as well as phenetic data. Additional chemotaxonomic and molecular methods were also used in order to refine the species relatedness within the *Streptomyces* genus.

The development of reliable, rapid and sensitive analytical methods such as electrophoresis, chromatography and spectroscopy has led to the development of chemotaxonomic methods for streptomycetes classification (Goodfellow and O'Donnell, 1989).

The present work involved the isolation and characterization of new actinomycetes strains able to produce natural bioactive compounds effective against Gram positive bacteria including methecilin resistance *Staphylococcus aureus and Micrococcus luteus* in addition to *the* causative agents of Candidiasis and aspergillosis, *Candida albicans* and *Aspergillus*

species, respectively. Five isolates belong to the grey colour, out of 19 *Streptomyces* isolates obtained from Egyptian soils, showed antimicrobial activity against all the tested micro-organisms. The two most active organisms based on their largest zone of inhibition were selected for further studies and designated strain SK4-6 and strain RAF10.

On the basis of their morphological and chemical properties, they were classified in the genus *Streptomyces.* These strains showed high resemblance in morphology, growth comportment and antimicrobial activity as well. In order to differentiate between them, they were subjected to Random Amplified Polymorphic DNA (RAPD) PCR analysis. The new advances in DNA technology have contributed considerably to bacterial taxonomy. In a study for the elucidation of taxonomy of plant pathogenic streptomycete isolates, DNA-DNA hybridisation showed similar results with protein profiles (Paradis *et al*., 1994). Several other methods were successfully used to differentiate between similar strains. Multilocus enzyme electrophoresis (MLEE) is to use variation that is accumulating very slowly in the population and that is likely to be selectively neutral, this method is useful for strain differentiation (Ohno *et al*., 1996). Random Amplified Polymorphic DNA (RAPD)-PCR assays (Williams *et al*., 1990) use single primers with arbitrary nucleotide sequences to amplify DNA, using a low annealing temperature so that polymorphisms can be detected. The resulting pattern of PCR products provides a characteristic fingerprint that enables chromosomal differences between isolates to be detected without having any prior knowledge of the chromosomal sequence.

RAPD-PCR is a rapid method by which streptomycetes can be screened for strain similarity. It does, however, require stringent standardization of the reaction parameters. These include primer sequence, annealing temperatures, buffer components, concentration and quality of template DNA (Anzai *et al.,* 1994; Mehling *et al*., 1995).

Thus, RAPD analysis was used to differentiate between strain SK4-6 and RAF10. The size of the resulted amplification products ranged from 300 to 1600 bp. Out of 41 amplicons (bands) generated by PCR amplification using the aforementioned primers, 18 bands were polymorphic, i.e. the percentage of polymorphism is 43.9 %. Figure 3.4 shows the developed RAPD-based fingerprints of the two strains. From a glance at the presented RAPD banding pattern, it could be concluded that in spite of similarity in morphology and growth comportment of the two strains, they are genetically diverse. For instance, using the primer OPO9, polymorphic bands of (1500 bp) and (1200 bp and 500 bp) were detected and were

unique to strains SK4-6 and RAF10, respectively. Similarly, using primer OPK9, a unique marker (600 bp) was detected in strain SK4-6 (Figure 3.4). This method was used to differentiate between closely related strains of pathogenic *Streptomyces* such as *S. albidoflavus*, *S. scabies, S. acidiscabies* and *S. ipomoea* (Sadowsky *et al*., 1996).

Anzai *et al*., (1994) demonstrated the variation in fingerprints obtained when a single base was substituted on the arbitrary primer; 11 primers were investigated and banding patterns ranged from zero to 20, the most significant differences were observed when the sequence at the 3' end was altered. In the same study, Anzai *et al*., (1994) investigated the relationship of *Streptomyces virginiae* strains to *Streptomyces lavendulae* strains by RAPD-PCR. It had previously been proposed that *Streptomyces virginiae* was a synonym of *Streptomyces lavendulae* (Williams *et al*., 1983a). RAPD-PCR results were compared with the results for DNA±DNA hybridization, LFRFA, cultural and physiological tests. Consistent results were obtained using all these methods after RAPD PCR optimization. However, the inter-specific relationship of *Streptomyces lavendulae* and *Streptomyces virginiae* could not be sufficiently resolved, though it was possible to distinguish between duplicate strains.

The taxonomic characters for colonial growth were confined to pigmentation of spore mass, substrate mycelium and diffusible pigments as well as intensity of growth, all of which were markedly influenced by the type of growth media, temperature, pH and age of culture. Since species of *Streptomyces* differ greatly in their nutritional requirements, it was difficult to propose a common medium that suffices all nutrients for all species and serves as a universal for the description of all *Streptomyces* species. The diversity of media applied by different specialists lead to incomparable descriptions of cultures, thus, the International Committee of *Streptomyces* (ISP), recommended about 14 different media to be applied to admit comparison possible. In addition to facilitate comparison, the obtained colours of the examined strains were matched with those of chart of colours, (Kenneth, 1958). The colours of both aerial and substrate mycelia of the isolated strains were similar or closely related to certain reference species and in agreement with those described in the ISP, (Shirling and Gottlieb, 1964, 1966, 1968a, 1968b; Nonomura, 1974).

Concerning the physiological and biochemical activities, the isolated *Streptomyces* strains showed different reactions which were affected absolutely by the nutritional and environmental conditions under which the species are grown. These biochemical activities are

essential to give adequate identification and to ascertain species classification of newly isolated strains as recommended by Buchanan and Gibbons (1974) and Bergey's Manual in Systematic Bacteriology (Holt *et al*., 1989).

The fact that a large number of *Streptomyces* species have capability to produce substances inhibiting the growth of various other organisms suggested that this ability may be of potential diagnostic species differ greatly in their inhibiting activity. Some are of wide spectrum, antagonizing the growth of many different types of bacteria, yeast and filamentous fungi, while others are of limited spectrum, causing death of only one or two groups of the sensitive micro-organisms.

Further cultural, physiological and enzyme activities of the strain SK4-6 were compared to those of known species described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1989) suggested that *Streptomyces qinlingensis* was the nearest species. Whereas, the cultural and physiological characteristics of the strain RAF10 showed that *Streptomyces enissocaesilis* was the closest species. This species was identified as *Actinomyces enissocaesilis* INMI 40-31, when it was first isolated and described by Krassilnikov 1970 (Gauze *et al*., 1983).

Modern *Streptomyces* identification systems are based on 16S rRNA sequence data, which have provided invaluable information about streptomycetes systematic, and then have been used to identify several newly isolated *Streptomyces* (Pineau *et al.*, 2003; Lee *et al*., 2005; Kim *et al*., 2006; Groth *et al*., 2007; Forar *et al*., 2007; Wael and Godfellow, 2007; Zhang *et al*., 2007).

Molecular methods are now used together with numerical and chemotaxonomic techniques to improve the understanding of species relatedness. Woese and Fox (1977) used molecular systematic analysis of ribosomal RNA (rRNA) molecules to provide an evolutionary classification of organisms. All 16S rRNAs have conserved primary structures (Woese, 1987; Kim *et al.,* 1991) which allowed Edwards *et al.* (1989), to design universal primers to amplify the entire 16S rRNA gene. Analysis of the 16S rRNA gene revealed regions that are genus specific (Salama *et al.,* 1991) and more variable regions that can be used to infer relationships to a lower taxonomic order. Stackebrandt *et al.* (1991 and 1992) identified three regions within the 16S rRNA gene that show variation; α-region (nt 982-998)

(*S. ambofaciens* nomenclature) and β-region (nt 1102-1122) which be used to resolve species to the genus level. The most variable region is the γ-region (nt 150-200) and this region is species specific.

The 16S rRNA sequence of strain SK4-6 was compared to those of other known *Streptomyces* species; it showed a high sequence similarity, 99 %, with *S. qinlingensis* the most closely related species. To our knowledge there is no detailed publication about *Streptomyces qinlingensis* to compare with our results; this may suggest the novelty of strain SK4-6. On the other hand, the broad antimicrobial spectrum which produces this organism, especially against *Micrococcus luteus, Staphylococcus aureus* (MRSA), *Candida albicans* and *Aspergillus* species which cause serious mycotic infections, made it a promising bacterium which allowed us to consider this organism as a new strain of *S. qinlingensis*, and designated S .*qinlingensis* strain SK4-6 (Gen bank Accession No, EF120466) scheme 3.1.

Table 4.1: Some interesting differences between SK4-6 and *S. qinlingensis*

Characteristic	Strain SK4-6	*S. qinlingensis**
Aerial mass color	Brown-grey	grey
Spore shape	ovule	ovule
Spore ornamentation	smooth	smooth
Melanin production	-	+
Nitrate reduction	-	ND
Carbon sources utilization		
Arabinose	+	-
Fructose	+	-
Raffinose	-	ND
Galactose	-	ND
Antimicrobial activity		
Bacillus subtilis	+	+
B. ciralus	ND	+
Staphylococcus aureus	+	+
Micrococcus luteus	+	ND
Gram - bacteria		
Escherichia coli	+	+
Pseudomonas aeruginosa	-	+
Salmonella typhi	ND	+
Yeasts		
Candida albicans	+	ND
Molds		
Aspergillus niger	+	ND

(+) active (-) not active ND= not determined

*Data from Wu *et al*., (2006).

The 16S rDNA sequence of strain RAF10 was also compared with sequences of the reference species of bacteria contained in genomic database banks. The similarity level ranged from 97.22 % to 98.37 % with *Streptomyces enissocaesilis* having the closest match. However, it is clear from phylogenetic analysis that, strain RAF10 did cluster with neither S. *enissocaesilis* nor any of *Streptomyces* species and represented a distinct phyletic line suggesting a new genomic species. This may suggest the novelty of this bacterium.

On the other hand, the antagonism of S. *enissocaesilis* is manifested poorly towards individual species of Gram-positive bacteria (*Staphylococci* and *Mycobacteria*), they do not suppress the growth of Gram-negative bacteria, yeasts and moulds (Krassilnikov**,** 1981)**,** while strain RAF10 showed greater potency against both of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. Thus, we considered this bacterium as a new strain too and designated *Streptomuyces*. sp RAF10 (Genbank Accession No EF 474464).

Table 4.2: Antibiosis of strain RAF 10 and *S. enissocaesilis* towards various test organisms.

Test organisms	Strain RAF10	*S. enissocaesilis*
Gram + bacteria		
Bacillus cereus	+	ND
B. subtilis	+	ND
Staphylococcus aureus	+	+
Micrococcus luteus	+	ND
Mycobacterium. sp	+	+
Gram - bacteria		Inactive*
Escherichia coli	+	-
Pseudomonas aeruginosa	+	-
Yeasts		Inactive*
Candida albicans	+	-
C. tropicalis	+	-
C. pseudotropicalis	+	-
Rhodotorula minuta	+	-
Molds		Inactive*
Aspergillus niger	+	-
A. flavus	+	-
A.terreus	+	-
Botrytis allii	+	-
Diplodia oryzae	+	-
Fusarium oxysporum	+	-
Helmenthosporium turcicum	+	-
Trichoderma viride	+	-

ND= not determined (+) active (-) not active

* Data from Krassilnikov, (1981)

It is well known that, many factors affect antibiotic production from different micro-organisms, especially the three independent variables namely concentration of carbon and nitrogen sources and temperature of incubation (Jonsbu, *et al*., 2000; Gupte and Kulkarni, 2002). Accordingly, after preliminary studies on parameters influencing antibiotic production by strain SK4-6, using a one-factor-at-a-time approach as first optimization, it was found that, (ISP-4) broth using, starch and ammonium sulphate at concentrations of 2.5 % and 0.25 % (w/v) as carbon and nitrogen sources respectively, for 96 hrs of incubation at 28 °C in orbital incubator with shacking at 200 rpm, were the most suitable for the production of active compounds produced by strain SK4-6. Then a second optimization process was carried out using a statistical experiment design to determine which nutrients and physical conditions affecting the production by applying a fractional two-level factorial design according to Plackett and Burman (1946).

On the basis of the analysis of the regression coefficients of the 7 variables after 96h of incubation: starch, pH and inoculum size showed positive effect on antibiotic production. However, sucrose, $(NH_4)_2SO_4$ and yeast extract repressed antimicrobial agent production, KH_2PO_4 exhibited a slight effect. Figure 3.8 shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and it is a convenient way to view the results of a Plackett-Burman design (Strobel and Sullivan, 1999).

The polynomial model describing the correlation between the 7 factors and the antibiotic production could be presented as follows:

$$Y_{activity} = 16.25 + 8.16\,X_1\ 15.66\,X_2 - 3.83\,X_3 - 2.83\,X_4 - 1.16\,X_5 + 9.16\,X_6 + 4.5\,X_7$$

Based on the calculated t-values and confidence level (%) starch, pH and inoculum size, were found to be the most significant variables affecting antibiotic production. Some variables of negative significant effect can not be included in the next optimization experiment, but instead can be used in all trials at their (-1) level.

Optimization was also done to increase the production of active compounds produced by strain RAF10. Results obtained showed similarly data with some differences, (ISP-4) was also the appropriate broth using, glucose and ammonium sulphate at concentrations of 1.25 % and 0.25 % (w/v) as carbon and nitrogen sources respectively. The incubation period was fairly

longer, it was 120 hrs for strain RAF10 instead of 96 hrs as it was for strain SK4-6, but the temperature of incubation and shacking speed were the same with both of them.

The Plackett-Burman statistical design was also applied to reflect the relative importance of various fermentation factors as described in above. The analysis of the regression coefficients of the 7 variables after 120 hrs of incubation indicated that: glucose, starch, tryptone, pH and inoculum size showed optimistic effect on antibiotic production. However; $(NH_4)_2SO_4$ and K_2HPO_4 repressed antimicrobial agent production. Fig. 3.2 shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and it is a convenient way to view the results of a Plackett-Burman design (Strobel and Sullivan, 1999).

The polynomial model describing the correlation between the 7 factors and the antibiotic production could be presented as follows:

$$Y_{activity} = 19.37 + 2.37\,X_1 + 4.16\,X_2 - 5.91\,X_3 + 2.16\,X_4 - 2.83\,X_5 + 1.16\,X_6 + 0.58\,X_7$$

Based on the calculated t-values and confidence level (%) glucose, starch and starch, were found to be the most significant variables affecting antibiotic production. Some variables of negative significant effect can not be included in the next optimization experiment, but instead can be used in all trials at their (-1) level.

The carbon catabolic effect of starch and glucose (strain SK4-6 and strainRaF10) respectively, are obvious since the moderate quantity of antimicrobial was synthesized when starch or glucose was applied as a carbon sources. In contrast, the highest level of antimicrobial production was supported by starch which was followed by glucose then mannitol in case of SK4-6 and glucose which was followed by maltose and then mannose in case of strain RAF10. The most suitable concentration of starch was 2.5% whereas, glucose was 1.25% with a marked reduction in antibiotic production or antimicrobial activity with lower or higher concentrations, this phenomenon was introduced by Inoue *et al*., (1982); Mellouli *et al*., (2003).

Generally, ammonium sulphate at concentration of 0.25 % was the superior as a nitrogen source compared to sodium nitrate or other nitrogen sources, whereas lowest level of production was recorded on using potassium nitrate. Young *et al.*, (1985) found that while ammonium and phosphate salts were required for growth, they had negative effects on

antibiotic synthesis, whereas, Farid *et al*., (2000) confirmed that only ammonium sulphate, sodium nitrate or beef extract were the suitable nitrogen sources in supporting natamycin production by *Streptomyces natalensis.* All the tested phosphate sources except K_2HPO_4 gave a moderate quantity of the antibiotic; the highest antibiotic production was obtained in a cultivation medium containing 1.5 g/l of dipotassium hydrogen phosphate. These results are in agreement with those reported by Inoue *et al*., (1982); Nurettin, (2003).

In fact, it has been shown that the nature of carbon and nitrogen sources, temperature, pH and incubation period, strongly affect active metabolite production in different organisms, **(**Vilshes *et al*., 1990; Holmalahti *et al*., 1998; Auguestine, *et al*., 2004).
Results obtained match with what was reported by Chattopadhyay, and. Sen, (1997); Yasser, (2007).

The antibiotic production and /or antimicrobial activity is observed after 48 hrs of incubation and reached its maximum on the 4^{th} day (strain SK-6) and declined progressively after that, this may indicates that this compound is a primary metabolite which could be transferred to other compounds during the fermentation process. While in case of strain RAF10, the active compounds reached their maximum on the 5th day of incubation and remained constant. This stability is significance that these compounds are secondary metabolites. The majority of antibiotics is secondary metabolites and remains constant (Augustine 2005). The decline of other active compounds indicates their accumulation after a certain period of streptomycetal growth, because they are primary metabolites. In explanation for this trend, the observation of Raytapadar and Paul, (2001) who found that antibiotic production by *Streptomyces aburaviensis* in synthetic media reached the maximum on day 5th of incubation after which it was decline till the end of the experiment.

The active compounds produced by strain RAF10 showed different potentialities for antagonizing the tested micro-organisms. The antagonistic criteria is influenced by many factors such as physico-chemical properties of the production media, type of the antagonized or non-antagonized organisms and other circumstance applied in the production of the antibiotic.

These active substances were found to be insoluble in organic solvents. All trials to extract these materials by organic solvents were unsuccessful whereas, they were precipitated easily by acetone, methanol and by raising pH to 10. Therefore they were precipitated by

acetone from the culture supernatant, whereas the ethanol extract of the biomass showed no antimicrobial activity. This shows the extra-cellular nature of active substances. Mostly antibiotics are extra-cellular (Hacene *et al*., 2000; Augustine *et al*., 2005).

The semi purified active compound**s** produced by *Streptomyces*. sp RAF10 showed relatively high antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus* species and *Micrococcus luteus* but it was not active against Gram-negative bacteria such *E. coli* and some filamentous fungi. Whereas, it was very active against yeasts such as *Candida albicans*, *Candida pseudotropicalis*, *Rhodotorula minuta* and *Saccharomyces cerevisiae* and moulds as well.

Antifungal agents are specific natural products of microbial metabolites with high physiological activities against individual groups of micro-organisms that selectively slow down or completely inhibit their growth (Egorov, 1985, Augustine *et al.,* 2005). Discovery of new active natural compounds produced by streptomycetes still continues and generally based on screening of naturally occurring streptomycetes and on biotechnological manipulation of known antibiotic producing strains (Okami and Hotta, 1988, Hacene *et al*., 2000; Augustine *et al.,* 2005).

Three out of 5 compounds produced by strain RAF10 were well known. These molecules are: desferrioxamine B and E and coelichelin and were produced by a number of *Streptomyces* species such as *Streptomyces coelicolor* A3 (2) and *Streptomyces ambofaciens* ATCC 23877 (Challis and Ravel, 2001; Barona *et al*., 2006). While desferrioxamine E was discovered in the culture broth of *Streptomyces griseus* (Yamanaka *et al*., 2005). The formulas generated from HRTOF (Peak m/z 654, Figure 3.19) in this investigation have confirmed that, these are identical with known compounds. Results obtained are in agreement with those reported by Beppu and Ueda, (2005); Yamanaka *et al*., (2005); Barona *et al*., (2006). Whereas, Peak 619 (formula C30H55FeN6O4 with less than 1 ppm error) is very interesting, it has same molecular weight as ferriocoelichelin **(**Challis and Ravel, 2001), but the molecular formula generated indicated this is probably a different structure, so this molecule needs further analysis. Which concern the two others, HRTOF analysis gave two possible formulas as C18H35N4O7 and C19H31N8O3 with less than 3ppm error, a search in SciFinder indicated these are not known, this is a significance that these two compounds are knew. So they were kept for further investigation.

Conclusion

Actinomycetes are industrially important bacteria well-known for their ability to produce a wide variety of bioactive compounds, counting 80% of the antibiotics produced by these microorganisms. Among this famous group of Gram-positive bacteria inhabit soil; *Streptomyces* genus is renowned as a rich source of useful secondary metabolites including antibiotics and active compounds value in human and veterinary medicine, agriculture and unique biochemical tools. Structural diversity is observed in these secondary metabolites that encompasses not only antibacterial, antifungal, antiviral, and antitumor compounds, but also metabolism with immunosuppressant, antihypertensive and antihypercholesterolemic properties. Thus, the present work involved the isolation and characterization of new actinomycetes strains able to produce natural bioactive compounds effective against Gram-positive bacteria including methecilin resistance *Staphylococcus aureus* (MRSA) and the causative agents of Candidiasis and aspergillosis, *Candida albicans* and *Aspergillus* species, respectively.

About 54 actinomycetes were isolated from soil samples; these strains were screened for their ability to produce antimicrobial activity. Three isolates identified later as *Streptomyces* strain RN+8, *Streptomyces qinlingensis* SK4-6 and *Streptomyces* strain RAF10 showed maximum crude antimicrobial activity against Gram-positive such as (*Staphylococcus aureus*, *Microoccus luteus*), Gram-negative bacteria (*Escherichia colic*, *Pseudomonas aeruginosa*), yeasts (*Candida albicans*) and filamentous fungi (*Aspergillus niger*) compared to other actinomycete isolates. Thus, they were selected for further studies on parameters influencing the production of active compounds using two methods. In preliminary studies using a one-factor-at-a-time approach, after that, an attempt was made to use a more practical, full factorial method. Extraction, purification and characterization of active compounds produced in the fermentation broth were also investigated.

In view of all the previous characteristics of the two strains SK4-6 and RAF10, it could be stated that, they are new strains belong to the genus *Streptomyces.* They were designated *Streptomyces qinlingensis* SK4-6 and *Streptomyces* sp RAF10 respectively. Strain RAF10 produces 5 different active compounds. Three out of them were previously discovered and well known such as desferrioxamine B and E and ferriocoelichelin, in addition to two

other unknown active compounds. These organisms are potential sources of active compounds hence merit further investigations.

Future studies and recommendations

1- Confirmation of the novelty of strain RAF10 and SK4-6 at species level using DNA-DNA hybridation.
2- Production of the active compounds at large scale in order to purify the two new compounds produced by strain RAF10, using NMR to generate their formula structures.
3- Continue the search in this field (Isolation and screening of novel actinomycetes strains or species) in order to extract novel active compounds.

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عزل و انتقاء وتشخيص سلالات جديدة من الستربتوميسيتات منتجة لمضادات حيوية

تناولت هذه الدراسة عزل واختبار الفعالية الضد ميكروبية لأكتينومايستات معزولة من التربة (35 عزلة من تربة عراقية- ضواحي بغداد و 19 عزلة من تربة مصرية- ضواحي القاهرة) مع توصيف السلالات المنتجة لمواد مثبطة لنمو بعض المكروبات الممرضة من كائنات الاختبار التالية: باسيلس سيرس، ستافيلوكوكس أورس، ميكروكوكس ليتس، كانديدا ألبكنس و أسبرجللس نيجر.

كان اهتمامنا مركزا على تلك التي تنتج موادا مثبطة لنمو الفطريات خاصة فطر الخميرة كانديدا ألبيكنس والأنواع التابعة لجنس أسبرجللس فانتقينا 3 سلالات من بين تلك التي عزلت من التربة العراقية و 13 سلاله من بين 19 عزلة تم الحصول عليها من التربة المصرية، و التي ظهرت بألوان مختلفة غلب عليها اللون الرمادي يليه اللون البني ثم الوردي فالأصفر المبيض.
و من بين 13 عزلة نشطة، 5 سلالات كانت قادرة على تثبيط نمو جميع كائنات الاختبار على الأوساط الصلبة. ثم قمنا بدراسة الفعالية الضد ميكروبية لهذه العزلات (5) على الأوساط السائلة.

تم تعريف هذه العزلات بناءا على الشكل المورفولوجى لسطح الأبواغ ولون الميسليوم الهوائى و الميسليوم الأساسى، وإنتاج صبغات الميلانين و بعض الاختبارات الفسيولوجية الأخرى. ولقد تم إختيار ثلاث عزلات أبدت فعالية ضد ميكروبية عالية ضد بكتريا سالبة وموجبة لصبغة غرام ، إضافة إلى فطر الأسبرجللس نيجر و الكانديدا ألبيكنس.

أنشط سلالتين بدا بينهما تشابه كبير من حيث المظهر وسلوك النمو و القدرة التثبيطية. لذا قمنا بدراسة وراثية للتأكد من كونها مختلفتين أو أنهما نفس السلالة. أظهرت النتائج أنه رغم التشابه المورفولوجي الكبير إلا أنهما وراثيا مختلفتين. تم التعريف على مستوى النوع باستخدام التتابع النيوكليوتيدي للحمض النووي. كما تم تحديد الظروف المثلى لإنتاج المواد الفعالة للسلالتين المتشابهتين ثم استخلاص و محاولة تنقية المركبات النشطة للسلالة الجديدة **راف 10.**

ويمكن تلخيص النتائج كالأتي:

1- أظهرت الدراسة أن جميع العزلات تعود إلى جنس ستربتومايسز

2- تم تعريف أنشط سلالتين بناءا على التتابع النيوكليوتيدي لهما كالأتى: ستربتوميسس كنلنجنسز **س ك 4-6** و ستربتوميسس إينسيكوسيليس **راف 10**.

3- لهاتين السلالتين قدرة عالية على إنتاج مواد تثبيط نمو بعض الأنواع من البكتريا الموجبة و السالبة لصبغة غرام و بعض أنواع الخمائر و الفطريات الخيطية.

4- وجد أن أنسب مصدر كربون لإنتاج المضاد الحيوي بواسطة هاتين السلالتين هو النشا و الجلوكوز وذلك بتركيز 20 و 12.5 غرام في اللتر على الترتيب.

5- كما وجد أن أنسب مصدر نيتروجين هو كبريتات الأمونيوم بتركيز 2.5 جرام في اللتر لكليهما.

6- يبدأ إنتاج المضاد الحيوي في اليوم الثاني من التحضين ويزداد تدريجيا إلى أن يصل إلى أعلى معدل في اليوم الرابع من التحضين بالنسبة للعزلة **س ك 4-6** و اليوم الخامس بالنسبة للعزلة **راف 10**.

7- وجد أن الأسيتون هو أقدر ماده على ترسيب المواد الفعالة من وسط التخمر يليه الميثانول بالنسبة للعزلة **راف 10**.

8- المواد المنتجة غير قابلة للذوبان في المذيبات العضوية و لكنها تترسب بالأسيتون و الميثانول و كذلك برفع رقم درجة الحموضة إلى 10 .

9- تم فصل 5 مركبات نشطة مختلفة، 3 منها معروفة و هي:

desferrioxamine E and B و coelichelin .

10- أما فيما يخص المركبين الآخرين فقد قمنا بإجراء بحث **مقارنة من خلال (SciFinder)**، فكانت النتيجة أنهما غير معروفين أي غير مسجلين بالموقع مما يشير إلى كونهما جديدين. لذا نحتاج إلى مزيد من البحث لتحديد الصيغة الكيميائية لهما.

النتائج التي تحصلنا عليها خلال هذا العمل مشجعة و تحتاج إلى مزيد من الدراسة و البحث.

Résume en Francais

Dans le cadre d'un programme de criblage d'actinomycètes productrices de nouveaux antibiotiques, 35 souches ont été isolées à partir du sol Irakien et 19 souches à partir du sol Egyptien. 13 d'entre ceux d'origine Egyptien ont montré une activité contre au moins un des germes-cibles suivants : *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Candida albicans* et *Aspergillus niger*. Cinq souches ont montré une activité contre tous les germes utilisées. Deux des souches les plus intéressantes sur le plan du spectre et de l'intensité d'action (souches SK4-6 et RAF10) ont été sélectionnées pour identification. D'après les caractéristiques morphologiques, biochimiques et physiologiques des deux souches, elles ont été rattachées au genre *Streptomyces*. Du fait de la grande ressemblance entre les 2 souches retenues, nous avons utilisé une méthode rapide (la RAPD-analysis) pour savoir s'il s'agit de la même espèce de *Streptomyces*. Les résultats obtenus ont montré qu'elles sont génétiquement différentes. Ensuite, en nous basant sur la comparaison de séquences nucléotidiques de l'ARN 16S des deux souches avec celles existantes au niveau des banques de données, il a été possible de constater que les souches SK4-6 et RAF 10 sont très proches de *Streptomyces qinlingensis* (avec une similitude de 99 %) et *Streptomyces enissocaesilis* (similitude de 98 %) respectivement. Les essais d'optimisation du milieu de culture ont montré que les sources de carbone : amidon (2%) et glucose (1,25%) puis le sulfate d'ammonium a raison de 0.25% constituent le meilleur milieu pour une production maximale d'antibiotique. En ce qui concerne la purification des antibiotiques synthétisés, la souche SK4-6 élabore un antibiotique du type butyrolactone alors que la souche RAF10 produit 5 substances actives différentes. Trois molécules ont été caractérisées. Il s'agit de la desferrioxamine E, B et de la coelichelin déjà connues pour être élaborées par des *Streptomyces*. Par contre, pour les 2 autres, nous les avons comparées par rapport aux données de la littérature et des informations disponibles dans les banques de données (SciFinder). Celles-ci ont abouti à la conclusion qu'il s'agit de nouvelles molécules qui nécessitent un travail plus approfondi pour établir leur structure chimique de façon précise.

Abstract

In screening program for actinomycetes having antifungal activity, 35 isolates from Iraqi soils and 19 others from Egyptian soils were obtained. These isolates were purified and screened for their ability to produce antifungal-agents. Thirteen out of them showed appreciable antimicrobial activities against at least one target among the followed test pathogen microorganisms, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. Five isolates inhibited the growth of all the tested microorganisms. Two promising isolates, based on their largest zone of inhibition and broad antimicrobial spectrum were selected for further studies and designated SK4-6 and RAF10. Morphological and chemical studies indicated that these strains belong to the genus *Streptomyces*. Because of their high resemblance in morphology, growth comportment and antimicrobial activity as well, they were subjected to randomly amplified polymorphic DNA (RAPD) analysis to differentiate between them. The developed RAPD banding patterns revealed incidence of polymorphism among the two isolates, although there were some common amplification bands. Phylogenetic analysis based on 16S rDNA sequence of these organisms exhibited high 16S rDNA similarity with their closest neighbors, *Streptomyces qinlingensis* (99%), and *Streptomyces enissocaesilis* (98%) respectively. Optimization studies showed that, ISP-4 broth using, starch and glucose as carbon sources and ammonium sulphate as nitrogen source were the most suitable for active compounds production by the two strains (SK4-6 and RAF10) respectively. Purification of the active molecules produced by strain RAF10 exhibited that, three out of five active substances discovered were known compounds, these are desferrioxamine E and B and coelichelin. Whereas, the two other compounds are new, hence merit further investigation to generate their formula structures.

The media used in this investigation

All the media used were from (Difco laboratories, Detroit Mi, USA)

Trace salts solution

$FeSO_4$. $7H_2O$ …….0.1 g
$MnCL_2$. $4H_2O$……..0.1 g
$ZnSO_4$. $7H_2O$…….....0.1 g
Distilled water……100.0 ml

Tryptone-yeast extract broth (ISP-1)

Bacto-Tryptone …….……5.0 g
Bacto-Yeast extract ……..…...……..3.0 g
Distilled water…….....1.0 liter

The medium was adjusted to pH 7.0 to 7.2 before autoclaving.

Yeast extract-malt extract agar medium (ISP-2)

Yeast extract…….4.0 g
Malt extract…….10.0 g
Dextrose……4.0 g
Distilled water……1.0 liter

The medium was adjusted to pH 7.3 before sterilization, and then agar was added…
Agar……20.0 g

Oatmeal agar (ISP-3)

Oatmeal……20.0 g
Agar…….18.0 g

The oatmeal was boiled in 1000 ml distilled water for 20 minutes, filtered through cheese cloth then, distilled water was added to restore volume filtrate to 1000 ml. After that we add.
Trace salt solution…….1.0 ml
Adjust to pH 7.2 with NaOH before adding agar. Liquefy by hot at 100°C for 15-20 minutes.

Inorganic salts-starch agar medium (ISP-4)

Solution I: Soluble starch 10**.**0g. Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 ml.

Solution II:

K_2HPO_4 (anhydrous basis)……1.0 g
$MgSO_4.7H_2O$……1.0 g
NaCl……..1.0 g
$(NH_4)_2SO_4$……2.0 g
$CaCO_3$ ……..2.0 g
Distilled water…….500.0 ml
Trace salt solution……1.0 ml
pH should be between 7.0 and 7.4. Do not adjust if it is within this range.
Mix starch suspension and salts solution.
Add agar……20.0 g

Liquefy agar by steaming at 100 °C for 15-20 minutes**.**

Starch-nitrate agar medium

Soluble starch.....20.0 g
$NaNO_3$…….2.0 g
K_2HPO_4……1.0 g
$MgSO_4.7H_2O$.....0.5 g
$CaCO_3$ .....2.0 g
Agar.....20.0 g
Distilled water.....1000.0 ml

The pH of the medium was adjusted to pH 7 – 7.4 before sterilization.

Glycerol-asparagine agar (ISP-5)

Glycerol.....10.0 g
L-asparagine..... .....1.0 g
K_2HPO_4.....1.0 g
Distilled water.....1.0 liter
Trace salt solution.....1.0 ml
The pH of this solution is about 7-7.4. Do not adjust if it is within this range.
Agar…….....20.0 g

Liquefy agar by steaming at 100 °C for 15-20 minutes**.**

Peptone-yeast extract iron agar medium (ISP-6)

Bacto-Peptone.....15.0 g
Protease Peptone.....5.0 g
Ferric Ammonium Citrate.....0.5 g
K_2HPO_4.....1.0 g
Sodium Thiosulfate.....0.08 g
Bacto-yeast extract.....1.0 g
Agar.....15.0 g
Distilled water.....1.0 liter

The medium was adjusted to pH 7-7.2 before autoclaving.

Tyrosine agar (ISP-7)

Glycerol.....15.0 g
L-tyrosine…….0.5 g
L-asparagine.....1.0 g
K_2HPO_4 (andhydrous basis) .....0.5 g
$MgSO_4.7H_2O$.....0.5 g
NaCl.....0.5 g
$FeSO_4.7H_2O$.....0.01 g
Distilled water.....1.0 litre
Trace salts solution.....1.0 ml
Adjust pH to 7.2 - 7.4.
Bacto – Agar.....20.0 g

Liquefy agar by steaming at 100 °C for 15-20 minutes**.**

Nitrate broth (ISP-8)

Beef extract.....3.0 g
Peptone.....5.0 g
NaCl.....5.0 g
KNO_3.....1.0 g
Distilled water……...1000.0 ml.

The medium was adjusted to pH 6.5-7.0 before sterilization.

Carbon utilization medium (ISP -9)

The ability of the strains under study to utilize different carbon sources was examined using carbon utilization medium (ISP- 9) as recommended by Shirling and Gottlieb (1966) modified from Pridham and Gottlieb (1948).

The carbon sources used are D-Glucose (used as positive control), Maltose, L-arabinose, sucrose, D-xylose, I-inositol, D-mannitol, D-Fructose, Rhamnose, Raffinose and cellulose. In addition to other carbon sources (Williams *et al*., 1983). All of these carbon sources are chemically pure.

The carbon sources were separately sterilized without heat. An appropriate weight of a carbon source was spread as a shallow layer in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Ethyl ether was added so as to cover the carbohydrate. The ether was allowed to evaporate at room temperature. After a complete evaporation of the ether, sterile distilled water was added to make 10% (w/v) solution of the used carbon source. The sterilized carbon source was added to the basal medium to give a final concentration of 1% (w/v).

The content of the basal medium is as follows:

$(NH_4)_2SO_4$.....2.64 g
KH_2PO_4.....2.38 g
$K_2HPO_4.7H_2O$.....5.64 g
$MgSO_4.7H_2O$.....1.0 g
Trace salt solution.....1.0 ml
Agar..... .....20.0 g
Distilled water.....1000.0 ml

The pH was adjusted to 7 before sterilization.

After autoclaving, the basal medium was cooled at 60 °C, and then the sterile carbon sources were aseptically added to give the final concentration required. The medium was agitated and 20 ml of the medium was poured in each dish. A duplicate plate for each culture was prepared. The plates were left for solidification and drying for about 4 hours. The inocula from the organisms under study were streaked on the surface of the plates. Only one culture per plate was used to avoid false positive due to cross feeding. Results were recorded after 7, 14 and 21 days. After incubation at 30 °C, the growth was observed and compared with the positive control (the basal medium was supplied with glucose) and for negative control (the basal medium without any carbon source).

Strain RAF10 has given the impression of being a new species, so it was selected for further investigation.

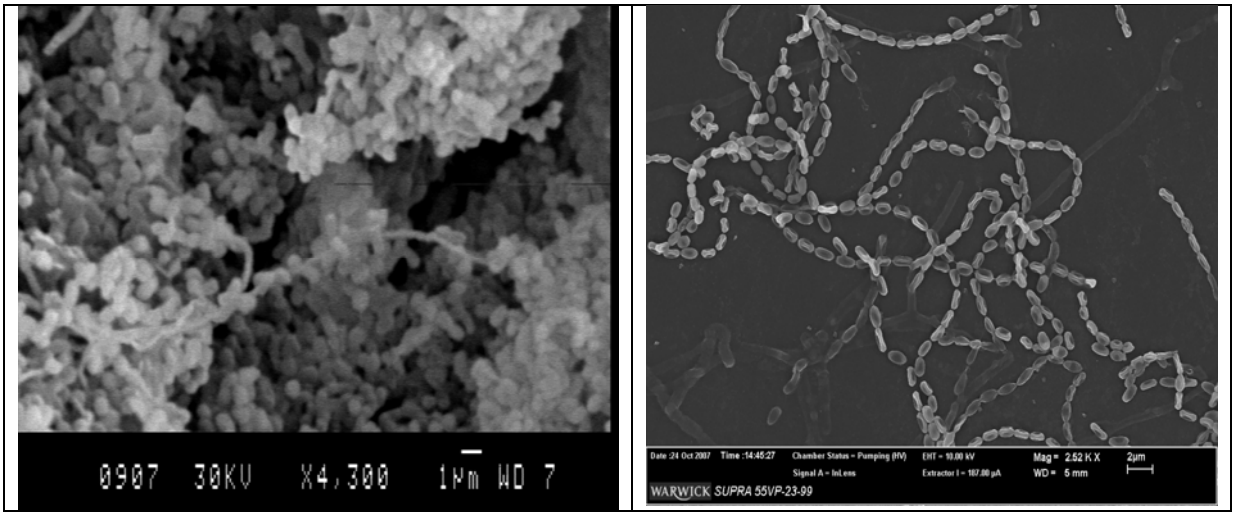


Figure 1a: Scanning electron micrograph of strain RAF10, showing aerial mycelium bearing numerous spores chains on the left and the smooth surfaces of the very fine spores on the right.

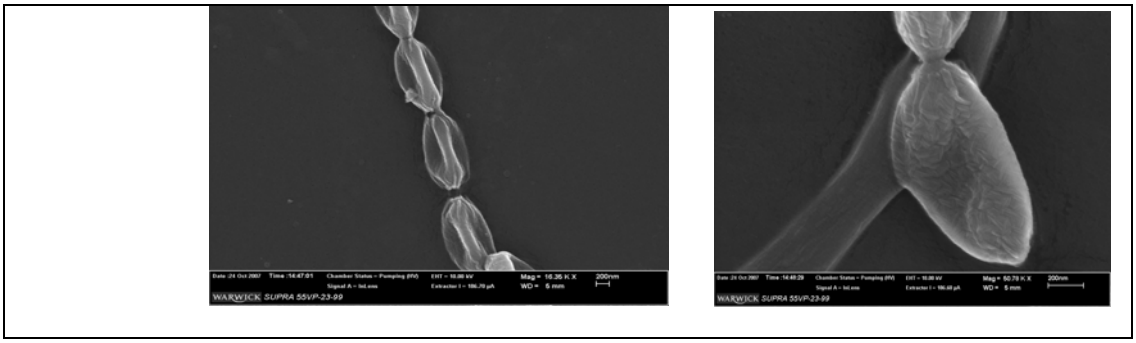


Figure 1b: Spore surface ornamentation of strain RAF10.

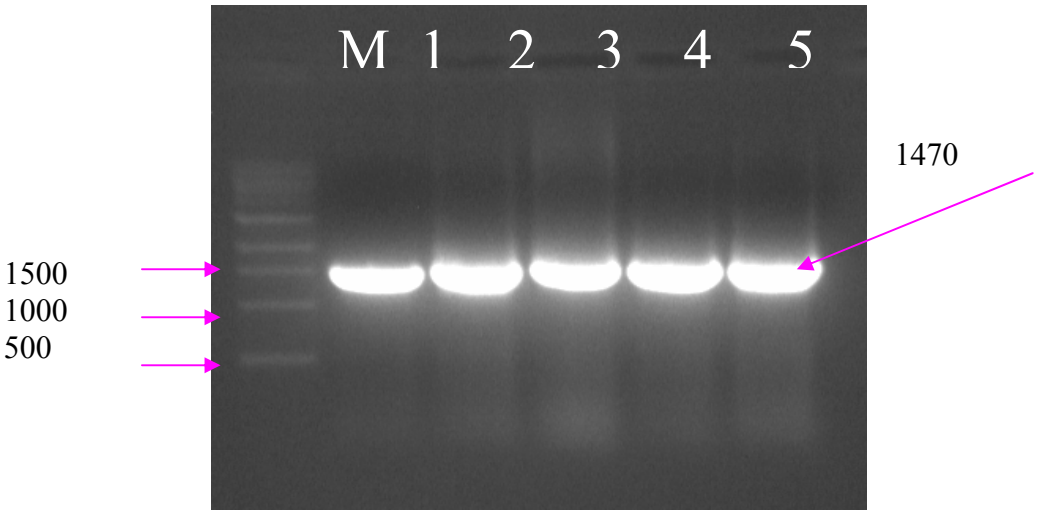


Figure 2: Specific PCR products, using forward and reverse specific primers to amplify 1470 bp of 16S r DNA from 5 selected isolates.

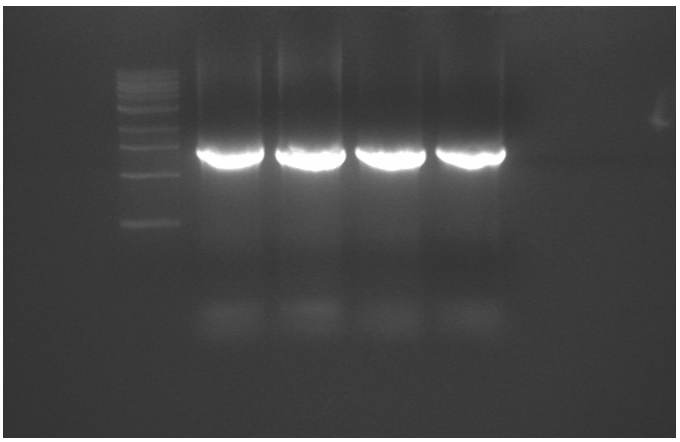
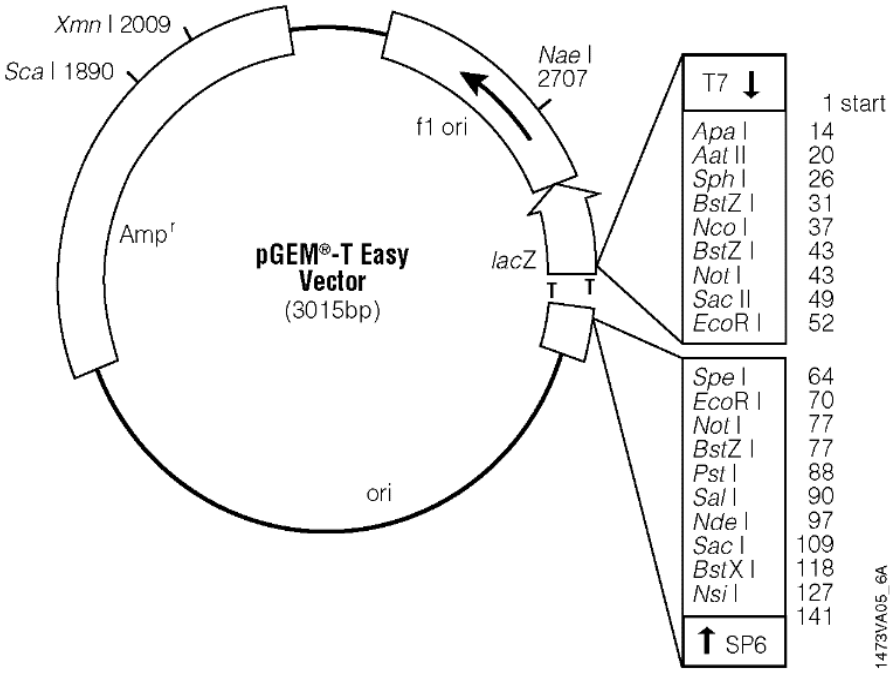


Figure 3: Plasmid PCR after cloning.

Various DNA fragments were sub cloned or sequenced directly using an automated laser fluorescence sequencer (3100 genetic analyzer ABI PRISM, Applied Bio-system, HITCHI, USA). Sequencing reactions were carried out with the Big Dye termination kit (Applied Bio-systems) according to the supplier's instructions.



a) **Figure 4:** Restriction map and general specification of the cloning vector pGEM-T Easy vector, the sequence of the multiple cloning sites is shown with a PCR product inserted by TA cloning strategy, the inserted PCR product is flanked on each side by EcoR1 site. The arrow indicates the start of the transcription of the T7 RNA polymerase.

Figure 5: The three most active strains

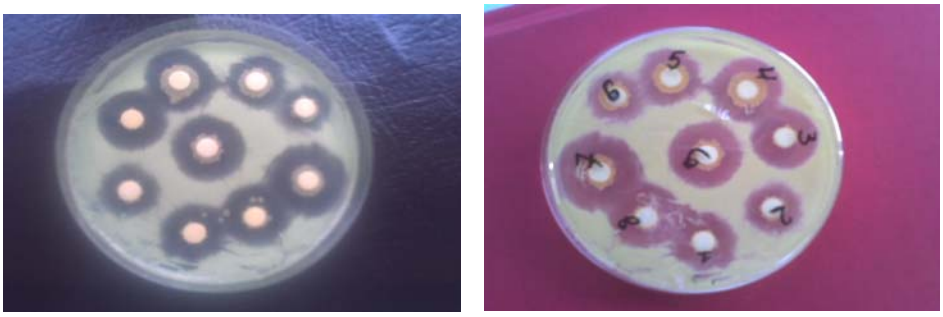


Figure 6: Plackett-Burman statistical design was applied to reflect the relative importance of seven different variables in 8 trails to perform the optimization process. Number 9 is the previously optimized broth. Antimicrobial activity was measured after 120 h of incubation at 28 °C. Inhibition diameter zones are given in mm, using disk paper diffusion method.

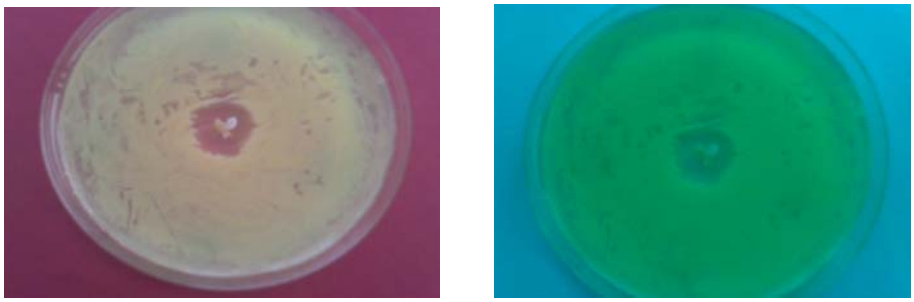


Figure 7: Antimicrobial activity of the crude powder of strain RAF10.

Figure 8: Antimicrobial activity of the 5 active compounds discovered in the culture broth of strain RAF10. The obtained crude powder was dissolved in suitable volume of 0.1% H_2O_2 and subjected to HPLC for purification. Different peaks were collected separately, drained and bioassyed for their antimicrobial activities.

a. GenBank flat file: Scheme 1 (strain SK4-6).

LOCUS EF120466 700 bp DNA linear BCT 08-JAN-2007

DEFINITION

Streptomyces qinlingensis strain SK4-6 16S ribosomal RNA gene, partial sequence.

ACCESSION EF120466

VERSION EF120466

SOURCE Streptomyces qinlingensis

ORGANISM Streptomyces qinlingensis

Bacteria; Actinobacteria; Actinobacteridae;

Actinomycetales;

Streptomycineae; Streptomycetaceae; Streptomyces.

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 tagtggcgaa
      121 cgggtgagta acacgtgggc aatctgccct gcactctggg acaagccctg
 gaaacggggt
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 ggcggtgcag
      241 gatgagcccg cggcctatca gctagttggc gaggtaacgg ctcaccaagg
 cgacgacggg
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 gactcctacg
      361 ggaggcagca gtggggaata ttgcacaatg ggcgaaagcc tgatgcagcg
 acgccgcgtg
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 attccggtag
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//
```

GenBank flat file: Scheme 2 (strain RAF10).

LOCUS EF474464 1489 bp DNA linear BCT
13-APR-2007
DEFINITION
Streptomyces sp. RAF 10 16S ribosomal RNA gene, partial sequence.
ACCESSION EF474464
VERSION EF474464
SOURCE Streptomyces sp. RAF 10
ORGANISM Streptomyces sp. RAF 10
Bacteria; Actinobacteria; Actinobacteridae;
Actinomycetales;
Streptomycineae; Streptomycetaceae; Streptomyces.

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 ggacaagccc
     121 tggaaacggg gtctaatacc ggatactgat cctcgcaggc atctgcgagg
 ttcgaaagct
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 ggctcaccaa
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tcgcagatca
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gtcacgaaag
    1381 tcggtaacac ccgaagccgg tggcccaacc ccttgtggga gggagctgtc
gaaggtggga
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//
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Screening, isolation and identification of antifungal producing actinomycete, *Streptomyces* strain RN+8

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3- Industrial Chemistry Department, Faculty of Sciences, University of Sciences and Technology, Oran - Algeria.

ABSTRACT

Thirty five different actinomycetes strains were isolated from soil samples collected from and around El Madain- Baghdad. These isolates were purified and screened for their antifungal activity against some pathogenic fungi. Out of these, 14.28% of the isolates exhibited activity against tested fungi. One promising strain, RN+8 which showed strong antifungal activity against pathogenic fungi was selected for further studies. The spore morphology and cell wall chemotype indicated that strain RN+8 belongs to the genus *Streptomyces.* Further cultural, physiological, biochemical characteristics, biological properties and enzyme activities suggested that strain RN+8 is identical to *Streptomyces rochei*. Therefore, it was designated *Streptomyces rochei* RN+8. This organism produces antifungal metabolite, which is active *in vitro* against the target micro-organisms, yeasts (*Candida albicans, Candida pseudotropicalis, Candida tropicalis*, and *Rhodotorula minuta*) and molds (*Asprgillus flavus, Aspergillus niger, Aspergillus terreus, Botrytis allii, Diplodia oryzae, Fusarium oxysporum, Helmenthosporium turcicum, Machrophomina phaseoli* and *Trichoderma viride*). Yeasts were most sensitive to the antifungal metabolite followed by *Aspergillus* species and then the other molds.

Key words: *Streptomyces,* morphological characteristics, physiological characteristics, antifungal agents, cell wall analysis**.**

INTRODUCTION

The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (Bevan *et al.,* 1995). This involves the screening of micro-organisms and plant extracts (Shadomy. 1987). The `strike-back' of pathogens has revitalized the search for new drugs (Lemonick, 1994 and Jaroff, 1994). Fungi (molds and yeasts) are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism without exhibiting any toxicity to humans (Berdy, 1989). Candida species responsible for causing candidiasis in humans have acquired resistance to certain antimycotic drugs as well as dermatophytes the responsible group of causing dermatophytoses in humans, and there is evidence that they have acquired resistance to certain antimycotic drugs too. Thus, there is lack of effective and safe antifungal antibiotics (Macura, 1993 and Gupte and Kulkarni 2002). During the last 15 years, many investigations were carried out in screening programs to isolate new potent actinomycetes strains or to discover new active compounds to combat pathogenic micro-organisms (Chattopadhyay & Sen, 1997; Raytapadar & Paul, 2001; Auguestine *et al*., 2004)

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 which were rarely studied under standardized growth conditions (Atalan *et al*., 2000). Biochemical, nutritional and physiological characters had also been

used in streptomycetes taxonomy, but usually had been applied to only selected species (Kutzner *et al*., 1989; Schlegel, 1992). Moreover, the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966) and numerical identification (Williams *et al.,* 1983) (Kampfer *et al*., 1991) are still applied. The genus *Streptomyces* was classified in the family Streptomycetaceae that includes also a number of other taxa (Waksman, 1961). Several investigations have been done to screen out new isolates of actinomycetes able to produce antifungal agents (Auguestine *et al.,* 2005; Lee *et al.*, 2005). *Streptomyces* have been the most fruitful source of micro-organisms for all types of bioactive metabolites that have important applications in human medicine as anti-viral and anti-cancer compounds and in agriculture fields as herbicides, insecticides and anti-parasitic compounds (Watve *et al.*, 2001).

In the present work, an isolate RN+8, capable of producing a broad-spectrum antifungal agents against yeast and fungi, was tested for its taxonomic profile. This included the study of some morphological, cultural, physiological, biochemical and biological properties as well as enzymatic activities and the cell wall chemotype. Investigation of active molecule is now in progress.

MATERIALS AND METHODS

Soil sampling

Several soil samples randomly were collected from different localities in Baghdad, using an open-end soil borer (20 cm depth and 2.5 cm diameter) at depths between 10-20 cm then air-dried, mixed thoroughly with $CaCO_3$ (10% w/w), and incubated at 28°C for 10 days, according to Lee and Hwang, (2002).

Isolation of *Streptomyces* spp.

Plates containing basal salts agar medium (Waksman, 1961) adjusted to pH 7.0 were used for isolation using the serial soil dilution technique (Johnson *et al.*, 1959). The medium contained the following components (g/l): 20.0, starch; 2.0, potassium nitrate; 1.0, dipotassium hydrogen phosphate; 0.5, magnesium sulphate; 0.5, sodium chloride; 3.0, calcium carbonate and 0.01, ferrous sulphate. It was inoculated with a soil suspension (0.1% w/v) and incubated at 28°C for 7 days. Various *Streptomyces* isolates, based on their special morphological characteristics as different colors of aerial mycelium with sitting colonies, were selected and purified by streaking (3-5 times) on agar plates. Long term preservation of isolates was achieved in soil cultures (Pridham *et al.*, 1973).

Screening of *Streptomyces* isolates:

Isolates were screened for their ability to produce antifungal agents against yeasts and filamentous fungi by agar diffusion method (Wu, 1984). The most active isolate was selected for identification and designated strain RN+8.

Identification of strain RN+8

Morphological characterization

The spore chain morphology of the selected organism of 7 days old was examined by the electron microscope. The spore surface was examined using spore print technique (Tresner *et al*., 1961) to prepare materials for transmission electron microscopy using Em10 Karl-Zeiss electron microscope. The spore chains and the ornamentation of spores were also detected**.**

Cultural characterization

The cultural characteristics of the strain RN+8 were tested on the bases of the observations made on International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb, (1966). The colors of mature sporulating aerial mycelium and substrate mycelium were monitored for the 7, 14 and 21 days old cultures grown on the

following agar media: starch nitrate, oat meal, glycerol asparagines, yeast malt extract, fish meal extract, soybean, Czapect-dox and nutrient (Difco laboratories, Detroit Mi, USA). Diffusible pigments were detected on glycerol asparagine agar medium. Color determination was carried out using ISCC-NBS color charts (Kenneth, 1958).

Physiological and biochemical characteristics

The physiological and biochemical characteristics were determined according to the methods of Shirling and Gottlieb (1966), Waksman (1967) and Holt *et al*., (1989). Cultures were incubated at 28°C and examined after 7-14 days, except for gelatin liquefaction as it was tested during growth after 2, 4 and 7 days.

Utilization of carbon sources was investigated using the procedure of Pridham and Gottlieb, (1948). Carbon sources were added to the basal salt medium at 1.0% (w/v). Growth and fermentation were recorded after 7, 14 and 21 days.

Enzyme activities

Proteolytic, lipolytic and lecithinase activities of the strain RN+8 were performed according to the method of (Nitsch and Kutzner, 1969). Pectinase and chitin decomposition was detected according to (Hankin *et al.,* 1971 and Reid and Ogrydziak, 1981). Other lytic enzymes were performed according to (Pataraya *et al*., 1987).

Cell hydrolysate analysis

Cell-wall analysis, to detect the characteristic amino acids (mureine diamino acids), of strain RN+8 and determination of the whole-cell sugars were performed according to Hasegawa *et al*., (1983).

Antifungal activities:

The antifungal activities of the bacterium culture broth were examined by the agar diffusion method and expressed as diameter of the inhibition zone according to the agar plate diffusion method (Wu, 1984). The tested micro-organisms (Table. 1) were obtained from MIRCEN Cairo, Faculty of Agriculture, Ain-Shams University, Egypt. Yeasts and molds were cultivated on agar media of nutrient and Czapek's Dox respectively (Difco laboratories, Detroit Mi, USA). Erlenmeyer flasks (250 ml) each containing 50 ml of the liquid basal salts medium (Waksman, 1961) were inoculated with a disk of 0.4 cm diameter taken from 7 days old culture plates of the tested isolates. Flasks were incubated on a rotary shaker (180 rpm) at 28˚C and initial pH 7.2 for 5 days. The culture broth was centrifuged at 5000 rpm at 4˚C in order to separate the microbial cells and the supernatant was tested for its antifungal activity.

RESULTS

Screening of *Streptomyces* isolates

Thirty five isolates of *Streptomyces* spp were obtained. Twenty four of them showed noticeable antimicrobial activities against Gram +ve and Gram-ve bacteria, yeast and fungi. Five isolates (no. 8, 10, 22, 29 and 35) showed high activities against the tested micro-organisms (Table. 1). These isolates were tested for their antifungal activities expressed as the inhibition zone diameter (Table 2). The most active strain was selected for identification and it was designated strain RN+8.

Morphological characteristics

The microscopic examination of the selected organism, RN+8 revealed that aerial mycelia were morphologically related to section spiral. Mature spore mass is belonging to grey color series with spiny surface (Fig. 1). No distinctive color of substrate mycelium was recorded.

Cultural characteristics

Cultural properties of the selected strain on various media are presented in Table 3. The organism grew well to moderate on the tested organic and synthetic media. The color of aerial and substrate mycelia varied depending on the type of the media used.

Physiological and biochemical characteristics

The physiological characteristics studies revealed that strain RN+8 did not produce melanin pigments on peptone-yeast extract-iron agar and tyrosine agar. It hydrolyzed starch, liquefied gelatine, reduced nitrate and relatively produces H_2S. It utilized citrate and tolerated 6%NaCl. Well growth was recorded at a temperature range of 15 to 42^{o}C and at pH range of 5 to 9 as it is well obvious in Table. 4. The organism could utilize all the carbon and nitrogen sources except L- arabinose and L-valine which were doubtful as it is very clear in Tables 5 and 6 respectively.

Enzyme activities

The strain RN+8 showed moderate activity of some enzymes as α-amylase, gelatinase, protease, lecithinase, pictinase and urease. Cellulase and lipase activities were not detected as shown in Table. 7.

Cell hydrolysate analysis

Analysis of the whole-cell hydrolysate of strain RN+8 showed the presence of a chemotype I cell wall characterized by L-DAP as it was reported by Lechevalier and Lechevalier (1970). No diagnostic sugars were detected.

Antifungal activity

Results in Table 8 showed a broad spectrum antifungal activity of strain RN+8 against yeasts (*Candida albicans, Candida pseudotropicalis, Candida tropicalis* and *Rhodotorula minuta*) and fungi (*Asprgillus flavus, Aspergillus niger, Aspergillus terreus, Botrytis allii, Diplodia oryzae, Fusarium oxysporium, Helmenthosporum turcicum, Machrophomina phaseoli* and *Trichoderma viride*).

Discussion

The search for new antifungal antibiotics continues to be of extreme importance in research programs around the world because of the increase of resistant pathogens and toxicity of some used antibiotics (Kim *et al.,* 2006). Actinomycetes have been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man. They have provided about two-thirds (more than 4000) of the naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, *b*- lactams, macrolides, tetracyclines etc. (Gupte *et al.,* 2000). Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism without toxicity to humans (Glazer and Nikaido 1995; Dasgupta, 1998). Fungal infections have been gaining prime importance because of the morbidity of hospitalized patients (Beck- Sague and Jarvis, 1993; Gupte *et al.,* 2002). Although synthetic drugs contribute to a major proportion of the antifungals used, natural antifungal-agents have their own place in the antimycotic market (Cragg *et al.,* 1997). The classical method, still used today, was followed in the present course of research program, which includes the whole cell bioassay technique, e.g. using susceptible and resistant species of *Aspergillus* and *Candida* species.

In an attempt to recover actinomycetes having antifungal activity, 35 isolates were obtained from soil samples collected in El-Madain – Baghdad. They were screened and from which 5 isolates showed activity against at least two fungal pathogens. The most active one of these isolates was selected for taxonomy and identification. The organism was characterized by methods and techniques described by the collaborations of the International *Streptomyces* Project (ISP) for the identification of *Streptomyces* species (shrilling and Gottlieb 1966), the classification key of Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1989). Globally, strain RN+8 grew well to moderate depending on the type of the media ingredients , the colonies were elevated and spreading,

the colors of vegetative and aerial mycelia were generally grey and white grey to grey yellowish.

The comparison of these data and of cell wall hydrolysate, with those of the known species of actinomycetes described in Bergye's Manual of Determinative Bacteriology (Holt *et al*.**,** 1989), and the obtained morphological properties suggested strongly that strain RN+8 belongs to genus *Streptomyces*. The characteristic of *Streptomyces* species is essentially based on the color of aerial and substrate mycelia and of soluble pigment. The ornamentation of spore surface has been considered of great bearing for taxonomic purpose because of its stability, however, distinction between certain sporal topography is not so easy, on the other hand this difficult, was solved by the application of transmission electron microscope. The ability for melanin production is also considered as one of the essential criteria in species characterization (Shirling and Gottlieb, 1968). For adequate identification, some physiological characters such as temperature range growth, the degradation of starch, gelatine, inositol and rhamnose, some additional tests relative to the use of arabinose, glycerol, galactose and mannitol were carried out to ascertain species classification of a newly isolates strains as recommended by Buchanan and Gibbons**,** (1974)**;** Holt *et al*., (1989).

Comparison of the results obtained of the strain RN+8 with those of *Streptomyces* known species indicated that *Streptomyces rochei* was the nearest species. The two strains have the same aerial and strain substrate mycelia colors, spore shape and ornamentation, absence of melanin production. They have the same range of growth temperature and nitrate reduction; the present data are in agreement with those reported by (Auguestine *et al*., 2005). Both strains have similar patterns of carbohydrate utilization, enzyme production and gelatine liquefaction. However, arabinose was doubtful in case of strain RN+8 and not utilized by *Streptomyces rochei*. These results confirmed those obtained by Nonomura *et al.,* 1974 and Ludmila *et al.,* 1991. Consequently, RN+8 was designated *Streptomuyces rochei* RN+8. The antimicrobial activities of secondary metabolites produced by *Streptomyces rochei* were previously mentioned by (Miguel *et al*., 1998, Usanee, *et al*., 2004 and Auguestine *et al.,* 2005), this is in agreement with the broad spectra of antifungal activity detected in the culture broth of strain RN+8. These metabolites are active against yeasts and filamentous fungi. The antifungal antibiotics produced by novel *Streptomyces* spp. were also reported by Pineau *et al.* (2003) and Lee *et al*. (2005).

Conclusion

Consequently, strain RN+8 was designated *as Streptomyces rochei* RN+8. It is a potential source of active secondary metabolites with antimicrobial properties. Results obtained from the present work are promising and encouraged us to continue our investigation. Determination of the optimal conditions for antifungal production, extraction, purification and characterization of the active metabolite are now in progress.

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Table 1: Antimicrobial activity of thirty five isolates of *Streptomyces* spp. against G+ve and G-ve bacteria, yeasts and filamentous fungi

Isolate no.	*Staphylococcus aureus*	*Bacillus cereus*	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
MS1	-	+	+	-	-
MS2	+	+	-	-	-
MS3	-	-	+	-	-
MS4	-	+	+	-	-
MS5	+	+	+	-	-
MS6	-	-	-	-	-
MS7	-	+	+	-	-
MS8	+	+	+	+	+
MS9	-	-	+	-	+
MS10	+	+	+	+	+
MS11	-	-	-	-	-
MS12	-	-	-	-	-
MS13	-	-	-	-	-
MS14	+	+	+	-	-
MS15	-	-	+	-	-
MS16	+	+	-	-	-
MS17	-	-	-	-	-
MS18	-	-	-	-	-
MS19	+	+	+	-	-
MS20	-	+	+	-	-
MS21	+	-	-	-	-
MS22	+	+	+	+	+
MS23	-	+	+	-	-
MS24	-	-	-	-	-
MS25	-	-	-	-	-
MS26	-	-	-	-	-
MS27	+	+	+	-	+
MS28	+	-	+	-	-
MS29	+	+	+	+	+
MS30	-	-	-	-	-
MS31	-	+	+	-	-
MS32	-	-	-	-	-
MS33	+	-	-	-	-
MS34	-	-	+	-	+
MS35	+	+	+	+	+

+ Antibiosis - no effect MS = Medain strain

Table 2: Supernatant antimicrobial activity of the five selected isolates.

Isolate no.	*Staphylococcus aureus*	*Bacillus cereus*	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
MS8*	20*	25	23	24	20
MS10	22	26	23	18	21
MS22	19	22	20	18	17
MS29	28	30	28	25	22
MS35	18	20	20	17	15

* Inhibition zone diameter (mm)

Table 3: Cultural characteristics of strain RN+8.

Media	Growth	Arial mycelium	Substrate mycelium	Diffusible pigment
Oat meal agar	Moderate	Y.gray. 93	gy.y 90	gy.y 90
Yeast -malt extract agar	Well	y.gray 93	gy.y 90	m.y 87
Glycerol asparagine agar	Well	l .01 Br 94	m .01 Br 94	gy.y 90
Fish meal extract agar	Moderate	y.white 92	gy.y 90	m.y 87
Soybean agar	Well	l .01 Br 94	l .01 Br 94	—
Czapeck-dox agar	Moderate	l .01 Br 94	l .01 Br 94	l .01 Br 94
Starch nitrate agar	Well	Med.gray 264	d.gray 93	1-01gy112

Y = Yellow, Br = Brown, M = Medium, G = Gray, D = Dark, GY = Gray yellowish. Numbers (see code number in colors charts of Kneeth, 1958)

Table 4: Some physiological and biochemical characteristics of strain RN+8

Reaction	Response
Starch hydrolysis	+
Gelatin liquefaction	+
Nitrate reduction	-
H_2S production	+
Utilization of :	
- Citrate	+
- KCN	+
Nacl tolerance	6% (w/v)
Growth inhibitors :	
- Na azide 0.01%	+
- Crystal violet 0.001%	-
Growth temperature	15-42°C
Melanin formation :	
- Tyrosine agar medium	-
- Peptone yeast extract iron agar medium	-
pH range	5 - 9

+ Good response - none

Table 5: Utilization and fermentation of carbon sources by strain RN+8

Carbon source 1% (w/v)	Growth	Fermentation
No carbon (control)	-	-
Glucose	+	+
Raffinose	+	-
D-xylose	+	+
I-inositol	+	-
D-mannitpl	+	-
Fructose	+	+
L-rhamnose	+	-
Sucrose	+	-
L-arabinose	-	-
Cellubiose	-	-

+ Well utilized - Not utilized

Table 6: Utilization of nitrogen sources by strain RN+8.

Carbon source 1% (w/v)	Growth
Alanine	+
Asparagine	+
Cysteine	+
Histidine	+
L-arginine	+
L-serine	+
L-valine	-
Methionine	+
Phenylalanine	+
Tryptophan	+
Therionine	+

+ Well utilized - Not utilized

Table 7: Enzymes activities of strain RN+8

Enzymes	Reaction
Amylase	+
Gelatinase	+
Pectinase	+
Protease	+
Lipase	-
Lecithinase	+
Urease	+
Cellulase	-

+ Well utilized - Not utilized

Table 8: Antibiosis of strain RN+8 towards various yeasts and fungi.

Microorganisms	Inhibition zone diameter (mm)
Aspergillus niger	38
Asprgillus flavus	35
Aspergillus terreus	33
Botrytis allii	30
Diplodia oryzae	25
Fusarium oxysporium	29
Helmenthosporum turcicum	32
Machrophomina phaseoli	26
Trichoderma viride	27
Candida albicans	42
Candida tropicalis	40
Candida pseudotropicalis	41
Rhodotorula minuta	38

Figure 1: Transmission electron micrograph showing spiny spore surface of strain RN+8.