Deanship of Graduate Studies

Al-Quds University

Antimicrobial activity of a Number of Streptomyces species

Ibtehal Mahmoud Rabie Ayyad

M.Sc. Thesis

Jerusalem - Palestine

1437 -2016

Antimicrobial activity of a Number of *Streptomyces* species

Prepared by:

Ibtehal Mahmoud Rabie Ayyad

B.Sc Degree in Biology from Alquds University palestine

Supervisor: Dr. Sameer A. Barghouthi

A thesis submitted in partial fulfillment of requirements for the degree of Master of Medical Laboratory Sciences

Department of Medical Laboratory Sciences / Al-Quds University .

1437 – 2016

Deanship of Graduate Studies

Al-Quds University

Medical Laboratory Science

Thesis Approval

Antimicrobial activity of a Number of *Streptomyces* species

Prepared by: Ibtehal Mahmoud Rabie Ayyad

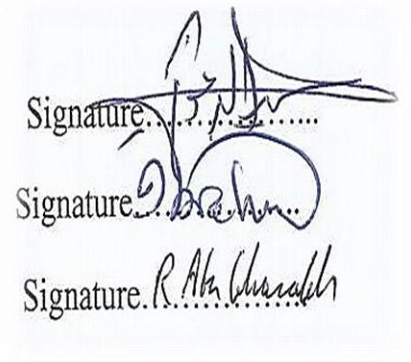
Registration No: 20913376

Supervisor: Dr. Sameer A. Barghouthi

Master Thesis Submission and acceptance date: 17/5/2016.

The names and signatures of examining committee members are as Follows:

1. Head of committee Dr. Sameer A. Barghouthi
2. The internal examiner Dr. Ibrahim Abassi
3. The external examiner Dr. Robin Abu Ghazaleh



Jerusalem – Palestine

1437- 2016

Dedication:

I dedicate my work to those dearest to me. To my husband, who faced a great deal of time and difficulties for the sake of my success, Ahmad I could not have done it without your support.

To my lovely daughters Zeinab, Jana and ro’aa.

And finally to my precious family and friends.

Ibtehal Mahmoud Rabie Ayyad.

Declaration

I certify that this thesis submitted for the degree of master, is the result of my own research, except where otherwise acknowledged, and that this study has not been submitted for a higher degree to any other university or institution.

Signed ابتهال عياد

Ibtehal Mahmoud Rabie Ayyad

Date: 17/5/2016.

Acknowledgments

I offer my deepest feelings of gratitude and acknowledgment to my supervisor Dr. Sameer A. Barghouthi for his sincere efforts in my training, supervision, and guidance. The study was conducted in the research lab of the faculty of health professions in Al-Quds University.

Abstract

Streptomyces a gram-positive filamentous bacteria, that is widely distributed in most soils and decaying vegetation. Most *Streptomycetes* reproduce by spores and distinct for their earthy odor that result from the production of a volatile terpene called geosmin which is one of the most famous metabolites that are produced by this genus. The discovery of streptothricin in 1942 was the starting point of history of antibiotics derived from *Streptomyces*, and then the discovery of streptomycin two years later, after which scientists intensified research's for antibiotics within the genus. Today, a large number of antibiotics are produced by the genus *Streptomyces*, producing antibacterial, antifungal, and antiparasitic drugs, and also a large number of other bioactive compounds, such as immunosuppressants. It is thought that the productions of the bioactive compounds produced by Streptomyces are initiated during the time coinciding with the aerial hyphal formation from the substrate mycelium.

Streptomycin and oleandomycin are examples of antimicrobial compounds against tuberculosis that are produced by *Streptomyces griseus and Streptomyces antibioticus* respectively. FK506 an immunosuppressant is another bioactive compound that is produced by *Streptomyces tsukubaensis*, while avermectin, bleomycin, and daunomycin are antitumor compounds that are produced by *Streptomyces avermitilis Streptomyces verticillus* and *Streptomyces peuceticus* respectively.

In this research, *Streptomyces pratensis* QUBC97 was isolated on CCG medium and identified by molecular methods. The isolate produced an antibacterial agent when grown in CCG broth that showed tow distinguishable compounds from fresh or old cultures. The freshly produced compound was extracted in n-butanol and showed an R*f* value of 0.86 when developed on silica gel TLC plates in n-butanol saturated with 1.7% ammonium acetate. The compound absorbed visible light at 426 and 440 nm. Putative identification of HPLC purified and concentrated compound using mass spectroscopy suggested the compound to be methoxy, 1-phenyl-, oxime.

This work is a step in the direction of developing our capacity in detection, isolation, and industrialization of antimicrobial agents.

Table of Contents

Table of abbreviations

Abbreviation	Full word
BLAST	*Basic Local Alignment Search Tool*
CCG *media*	*Sodium Citrate Casaminoacids Glycerol media*
CCCG media	Cyclohexamide *Sodium Citrate Casaminoacids Glycerol media*
cnBE	*Concentrated Butanol extract*
HEMW	*Hexane, Ethyl Acetate, Methanol, Water*
HPLC	*High Performance Liquid Chromatography*
ISP	*International Streptomyces Project*
MLSA	*Multi-locus Sequence Analysis*
MS	*Mass Spectroscopy*
NA	Nutrient Agar Media
nBAAC	n-Butanol Ammonium Acetate
PCR	Polymerase Chain Reaction
QUBC	Al-Quds University Bacterial Collection
spp.	Species
TLC	Thin Layer Chromatography
UV	Ultra Violet Light
V/V	Volume/ Volume

List of tables

List of figures

Chapter 1

Introduction

Streptomyces, is a genus of Gram-positive bacteria that belongs to the phylum Actinobacteria and forms branching filaments. They have high guanine and cytosine content in their DNA(Tsai et al. 2008). Historically, the Actinomycetes were thought to be true fungi, because of their formation of mycelia branching filaments (hyphae) (Osman, Fath Allah, and Abd El All (2011). The similarities to fungi are the result of adaptation of similar ecological environments and a saprophytic life. *Streptomyces* species are complex; they grow as branching mycellia, they reproduce in a mold-like manner by sending up aerial branches that turns into chains of spores. In soil, they are noted for their distinct earthy odor that results from production of a volatile metabolite, geosmin: an organic compound that has an earthy taste and aroma.

But unlike the true fungi, Actinomycetes have thin hyphae about 0.5-1.5 micrometers in diameter comparing them to fungi hyphae and they also have a prokaryotic ribosome. They are affected by antibacterial antibiotics as well.

Actinomycetes are known to have various shapes; the simplest is unicellular spheres and rods, others are filamentous. Some species of *Streptomyces* are plant and animal pathogens (Flardh and Buttner 2009). *Streptomyces* are an uncommon cause of infection in human, but they may cause

superficial disease after traumatic inoculation. They also cause mycetomas in the feet of farmers and perianal soft tissue infections (Chavez, Estrada, and Bonifaz 2002). In Human Iimmunodeficiency Virus (HIV) and immune compromised individuals uncommon visceral diseases are seen (Kapadia, Rolston, and Han 2007; Rose, Brown, and Fisher 2008).
Among the genera of Actinomycetes, *Streptomyces* is the largest genus that produces antibiotics, producing both antibacterial and antifungal, as well as a wide range of other bioactive compounds such as immunosuppressants (Watve et al. 2001).

Due to the emergence of antibiotic resistant bacteria due to intensive use of antibiotics within months and years, identification of new antimicrobial agents is very important. *Streptomyces* are important for the discovery of new antibiotics, mining of uncovered natural product biosynthetic clusters in bacterial genomes, and inhibitors of antibiotic resistance such as sulbactam clavunilic and dipicolinic acid (Spellberg et al. 2004).

This current research focused on one isolate of the *Streptomyces* QUBC97 and its antibacterial active compound.

1. Literature review

Antibiotic production by microbes

1.1 Actinomycetes

The full Lineage of *Streptomyces* as posted by ('NCBI')

Super kingdom: Bacteria

Phylum: Actinobacteria; contain a single class: Actinomycetes

Order: Streptomycetales;

Family: Streptomycetaceae;

Genus: *Streptomyces* which includes 778 species and 38 subspecies according to (Bacterionet). They are branching, prokaryotic Gram positive bacteria. The colonies look like a mass of mycelia with branching filament extensions that form spores. *Actinomycetes* are considered bacteria although they lack a defined nucleus. Most of its subspecies are aerobic but there is some species that could live under anaerobic conditions. They reproduce by spores and form radial colonies with pigments.

Genus *Streptomyces* is the most complex in the Order: Actinomycete. *Streptiomyces* Species are found almost everywhere, although they prefer alkaline and neutral conditions and the optimal pH ranges between 7 and 8. Most *Streptomyces* grow at temperatures between 15 and 30° C; while there are species that live at higher temperatures (Waksman 1953).

1.2 History of Streptomyces and antibiotics

In the 1940s, *Streptomyces* description was confirmed as an aerobic, spore forming genus that also produces antibiotic and bioactive compounds. Actinomycin was isolated by Waksman and woodruff from *Streptomyces antibiotica.* Since then, many investigators established isolation of antibiotics from many culture media of *Streptomyces* species. A rich period started in which many useful antibiotics were isolated and reported from Streptomycetes. The importance of *Streptomyces* is due to their ability to produce important bioactive metabolites such as antibiotics, antifungal,antitumar and antiparasitic agents; *Streptomyces avermitilus* produces the antiparasitic compound Ivermectin. (Osman, Fath Allah, and Abd El All 2011; Flardh and Buttner 2009).

1.3 Streptomyces and medicine

The genus *Streptomyces* is known for the production of a large number of antibacterial, antifungal, antitumor and antiparasitic agents, and also a wide range of other bioactive compounds, such as immune suppressants (Omura and Crump 2004). Members of the *Streptomyces* genus are the source for numerous antibacterial pharmaceutical agents; Chloramphenicol is an example of bacteriostatic antibiotic that is produced by *Streptomyces venezuelae*, while Streptomycin the first drug of choice used as tuberculosis treatment is produced by *Streptomyces griseus.* Tetracycline a broad spectrum antibiotic used to treat many bacterial infections is produced by *Streptomyces rimosus* and *Streptomyces aureofaciens*. There are more bioactive compounds such as avermectin, bleomycin, and daunomycin which are produced by *Streptomyces avermitilis* (Burg et al. 1979), *Streptomyces verticillus* (Umezawa et al. 1968), and *Streptomyces peuceticus* (Arcamone et al. 1969) respectively that are used as antitumor compounds, while tacrolimus (FK506) from *Streptomyces tsukubaensis* as an immunosuppressant that inhibit calcineurin (Kay 1996), and validamycin from *Streptomyces hygroscopicus* is used as a treatment of heath blight disease in rice (Watve et al. 2001).

1.4 Culturing of *Streptomyces*

Media labeled as ISP 1, 2, or 4 are used for characterizing *Streptomyces* species according to the International *Streptomyces* Project (ISP). ISP media were developed by Difco Laboratories for the International *Streptomyces* Project (ISP) in order to introduce stable properties and reliable procedures for characterization of *Streptomyces* species. ISP Medium 1 contains Tryptone-Yeast Extract Broth; peptone and yeast extract provide nitrogen, vitamins, carbon and amino acids. ISP Medium 2 is also called Yeast Extract-Malt Extract Agar, Yeast extract and malt extract provide nitrogen, amino acids, and vitamins, while dextrose is the carbon source. ISP Medium 4 which also called Inorganic Salts-Starch Agar is composed of many inorganic salts like calcium chloride

and soluble starch to provide the essential nutrients for organism growth. Agar is the solidifying agent for all ISP media (Gottlieb and Shirling 1966).

Streptomyces needs carbon and nitrogen in order to grow, so new CCG medium was used to culture *Streptomyces* (Barghouthi and Qadi 2012). Glycerol is the main carbon source, while casein provides the nitrogen requirement under artificial conditions. Trace minerals such as NaCl, K_2HPO4, $MgSO_2 \bullet 7H_2O$, $CaCo_3$ and $FeSO_4.7H_2O$ are also necessary. In a few cases, antifungal are added to the culture in order to eliminate fungi and general bacteria from plates. Previous studies used copper to inhibit growth of algae, bacteria, fungi and other aquatic species that could be present in the samples. Incubation temperature is at 30 ^oC, and the pH 7.5 the media could be solid or broth media.

1.5 Secondary metabolites

Streptomyces spp. produces secondary metabolites including compounds that have important applications in medicine as antibacterial, antiviral, antiparasitic, and antifungal agent. Also, in agriculture these compounds act as growth promoters, agents of plant protection, and herbicides (Demain and Fang 1995).

The production of antibiotics by *Streptomyces* grown on agar or liquid media usually coincides with the early stages of morphological differentiation of the aerial hyphai (Waksman 1953).

One of the most important streptomycetes is *Streptomyces coelicolor*; many researches were done on it because it produces about five secondary metabolites, four of them have antibiotic activity; the colored and visually detected actinorhodin, undecylprodigiosin, methylenomycin, and the calcium dependent antibiotic (CDA) (Procopio et al. 2012). The aromatic polyketide actinorhodin is the best example of all *Streptomyces* antibiotics according to the change of color depending on the PH of the environment: blue in neutral and alkaline solutions and red in acidic.

1.6 Inhibitors of antibiotic resistance

Calvulanic acid, Sulbactam, Tazobactam, and Avibactam are compounds produced by microorganisms that play an important rule of protecting the familiar beta-lactam antibiotics from hydrolysis by penicillinases or broad-spectrum beta-lactamases, by adding those inhibitors to a safe and efficacious penicillin's or cephalosporin's for better results.

Some bacteria can produce extended spectrum β-lactamases (ESBLs) that breaks the beta- lactam ring that allows the antibiotic to work, which makes the infection more difficult to treat because of their resistance to penicillin's and cephalosporin's. Inhibitors of beta-lactamases acts by binding to the active site of the enzyme preventing it from binding to the lactam ring and damaging it (Drawz and Bonomo 2010).

1.7 Oxime

The oxime term refers to the combination between oxygen and imines. Oxime is an imine moleculewith a general formula $R^1R^2C{=}N\ O\ H$, where R^1 is an organic side-chain and R^2 may be hydrogen or any other organic group.it could have stereoisomers according to the both side chains of the molecule attached to the carbon and which side is closer to the carbon.

Bacterial drug resistance played an important role in the failure of treatment of many resistant bacterial infections. As bacterial resistance increased, the search for new antibacterial agents was important and urgent. Scientists started to study of oxime-ether derivatives in the recent years due to their anti-bacterial, antiprotozoan, antifungal, and antimicrobial activities. Different oxime-ether derivatives have also been reported to insecticidal activities. One example of oximes, 5-keto-5-oxime which is derivative of the anti helmentic and insecticidal milbemycins that exhibited high activity as anti-microfilariae in dogs. Oximes are produced in nature mostly, but not exclusively,

as intermediates of secondary metabolites by enzymic oxidation of an amine function (Rappoport and Liebman 2008)**.**

1.8. *Streptomyces pratensis*

Streptomyces flavogriseus IAF-45-CD (=ATCC 33331), a strain recently reclassified as *Streptomyces pratensis* which has been isolated recently from a wide region of North America in North Carolina, New York, Michigan, and Quebec. Despite different geographic origins, the genomes of the two strains are highly similar with 85.9% of genes present in the core genome and conservation of all natural product gene clusters. This strain includes a combination of two genes that codes for carbapenem which is used to treat infections caused by multidrug resistant (MDR) bacteria, and beta-lactamase inhibitor gene that codes for beta-lactamase inhibitor which bind to the active site of the enzyme that damages the lactam ring and prevents the antibiotic from working on bacteria, in addition to other genes that are required for spore development and antibiotic production. while other genes includes uncharacterized protein which may function as toxin-antitoxin system (Doroghazi and Buckley 2014).

The nucleotide divergence of multi-locus sequence analysis from strains of *Streptomyces pratensis* did not exceed 0.4% justifies their inclusion in a single species as *Steptomyces pratensis* showed a very high recombination. Recombination of interspecies among *Streptomyces* species is widespread; the high level of nucleotide similarity within different species provides evidence about the gene exchange (Berdy 2005).

1.9 Hypothesis

Streptomyces and other soil bacteria have to survive in harsh environment with changing variables and unstable conditions. The large number of *Streptomyces* species present in soils and other environments is a strong indicator of its survival strategies; some have not been classified yet

(Unclassified *Streptosporangineae* *sinosporangium*). This may encompass the production of antibiotic agents including antibacterial, antiviral, antifungal, antiparasitic, and anti protists, and other bioactive agents. The hypothesis is that several bioactive agents produced by *Streptomyces* have not been discovered yet and are awaiting discovery.

1.10 Problem statement

Months or years of misuse and bacterial exposure to antibiotics, select for antibiotic resistant bacteria which become dominant especially in health care centers. Identification of new antimicrobials and new antimicrobial targets is a continuous requirement in controlling pathogenic microbes. *Streptomyces* with its 778 spp and 38 subspecies represents a reservoir that has not been fully and systematically investigated, and as an important source of bioactive agents including antibacterial antibiotics. Therefore it is important to search for new antibiotics and inhibitors of antibiotic resistance amongst the *Streptomyces*.

1.11 Aim

The goal of this research is to establish and explore new methods that are necessary for detection, production, and enhancing production of new antibiotics and their possible applications. This project represents a starting point in that direction.

Chapter 2

Materials and methods

.

2.1 Isolation and culturing of *Streptomyces*

Soil and other samples were collected from various locations around the world to obtain representative samples and to reduce repetitive isolation of the same isolate or species. The soil samples (200-300 g) were collected from a depth of five centimeters in fresh plastic bags and stored at room temperature.

The samples were first cultured on CCCG selective agar medium (pH ~7.8 before autoclaving) for *Streptomyces* isolation, and the CCG medium was prepared as follows: Casamino acids (0.1%,

proteolytic hydrolysate of casein; Sigma Chemicals), 0.1% sodium citrate, 2% agar made in Jericho® bottled water as a source of trace elements, autoclaved and cooled to ~50º C. Both broth and solid (2% agar) media were autoclaved for 18 minutes; glycerol was aseptically added to other components after autoclaving. Addition of cyclohexamide (100µg/ml) was initially used to obtain pure isolates of *Streptomyces,* and hence the medium was labeled as CCCG; cyclohexamide was only used in the early stages of *Streptomyces* isolation. CCG was used both as broth or agar for the evaluation of antibacterial and antiyeast bioactivities of *Streptomyces* spp. Nutrient broth and agar were from BD Difco (United States). Plated samples were incubated at 28°C until colonies could be microscopically discerned. The colonies were subcultured on CCG medium and pure colonies were obtained after 5 days for repeated subculturing for more purification. Grown pure colonies were then inoculated to a liquid CCG culture medium in flasks on a shaker (120 rpm) at 28°Cfor 1-3 weeks. Direct microscopic examination was used at 4x and 10x objective lenses to distinguish fungi from putative *Streptomyces* for further identification.

2.2 Characterization of the isolates

The *Streptomyces* isolates were cultured on BD Difco ISP4 medium. This medium is recommended by ATCC to use in colony and pigment characterization according to the international *Streptomyces* project ISP. The phenotypic characteristics of the isolates were evaluated as QUBC97 in this study. Cultured samples were evaluated for the shape of the colonies, and the color of pigmentation of the colonies on ISP4. Shape of cells was studied under light microscope with 4x and 10x objectives, photographed with (3-12) Mpix digital camera (Exilim, Casio, Japan).

2.3 Manipulation of culture productivity of the antibacterial agent

First CCG liquid medium was prepared and inoculated with 1 loop-full of QUBC97 and incubated with shaking (120 rpm) at 28°C, then 1ml of the medium was collected every 24 hours for a total of 7 collections, centrifuged, then extracted with 0.5ml n-butanol, the extracted layer was tested for antimicrobial activity using sterile filter paper discs. This technique was repeated every day

over 7 days to study the production of the antibacterial agent by studying the diameter of the zone of inhibition. Three filter paper discs were impregnated with the extract and placed on the surface of NA coated with *Bacillus atrophaeus*, diameters of zones of inhibition were measured and recorded (Barghouthi and Salman 2009).

2.4 Evaluation of production of antibacterial agent(s)

2.4.1 Agar plug diffusion test

Three quick and simple antibiotic production screening tests were devised. First Streptomyces isolates were allowed to grow for up to 4 weeks at 28° C. A glass Pasteur pipette was kept in 70% alcohol, drained, then flamed and allowed to cool, and then used to cut agar plugs near heavy zones of growth. Agar plugs were removed and placed on the top of a nutrient agar (NA) plate that has been coated with a layer of the test-organisms (*Bacillus atrophaeus* QUBC16 or the baker's yeast *Sacchromyces cerevisoae*). There plates were then incubated for 24 hours upside down at 30°C. A zone of inhibition surrounding the agar plug was considered as a positive indication of antimicrobial production by the isolated *Streptomyces*. The method was repeated for confirmation of results.

Aliquot of 1 ml was collected from each isolate that was inoculated into 50-ml liquid CCG medium in Erlenmeyer flasks and incubated on a shaker (120 rpm) at 28°C for one week and centrifuged to obtain a clear supernatant, which was removed to another 1.8 ml microfuge tube and extracted with 0.5 ml n-butanol and vigorous shacking followed by brief centrifugation. The butanol-antimicrobial agent top layer was removed for testing.

Another way of separation of antimicrobial agent was as follow: medium was adsorbed to a solid phase from talcum powder then eluted with a reduced volume (20-25% of the original volume) of methanol. Ten µl of extracted layer from both methods were used to impregnate a sterile filter paper disc, dried, and then placed on NA plates that have been coated with a layer of the test-organism. Plates were incubated overnight at 30°C and examined for production of inhibition zones.

2.4.2 Extraction of antibacterial agent(s)

Antimicrobial substance was recovered from liquid medium by two ways; 1-butanol extraction, and 2- solid phase using talcum powder.

Streptomyces QUBC 97 was grown in 2x 750 ml of CCG medium in 2-liter flasks with shaking (120 Strokes /min) for 2 weeks. After centrifugation of the culture medium in 6x30 ml tubes per each run, at 25,000 rpm for 15 min, supernatant was pooled and transferred to a separatory funnel, n-butanol was added in a ratio of 1:4 (v/v), vigorous shaking was applied to guarantee extraction. After 24h the upper layer was collected. A second washing step was performed to the culture medium in the separatory funnel; n-butanol was pooled from both extractions, and evaluated for antimicrobial activity using disc diffusion technique. This product was labeled crude n-butanol extract (cnBE). Concentration of cnBE was carried out to near dryness using a rotary evaporator at 50-60˚C. The dried substance obtained after evaporation was collected in a pre-weighed tube and weighed. The product was subjected to HPLC purity testing and further purification using preparative HPLC.

2.4.3 Magnesium silicate solid phase extraction

The second method was using talcum powder (magnesium silicate) as a solid phase to extract the antimicrobial substance. The talcum powder had been washed with highly pure sterile water (PCR water) several times to remove any soluble component before use. Culture supernatant (30 ml) obtained after centrifugation was mixed in a flask and stirred for 15 min. to ensure adsorption of the antibacterial substance to the silica, and then the talcum was allowed to settle for 24 h. The supernatant was removed and kept at room temperature. 10 ml of methanol was added to the precipitated powder, mixed, covered, and then left for the next day. The methanol layer was recovered and its antimicrobial activity was tested.

2.4.4 HPLC of cnBE

The dried material was dissolved in water and subjected to preparative HPLC purification. The water eluate was collected and tested for antibacterial activity by the disc diffusion method. The two positive fractions (50ml each) were pooled and re-extracted with n-butanol from the aqueous HPLC column fractions and concentrated by rotary evaporation. The dried material was re-suspended in 1 ml pure water for analysis by mass-spectroscopy for putative identification.

2.5 Testing the n-butanol extracted from QUBC 97 against tested microorganisms

The n-butanol extracted from a large scale (1.5 liter CCG broth culture) was subjected to testing against a number of bacterial species using disc impregnation and diffusion test. The dried discs were placed on lawns of NA plates of the following bacterial species *Staphylococcus epidermidis, S. aureus, Streptococcus viridians, S. pyogenes, S. aglactiae, Bacillus atrophaeus, Escherichia coli* (several isolates), *E. coli* HB101 laboratory strain, and several isolates of *Pseudomonas aeruginosa.* Baker's yeast was also used.

2.6 Thin layer chromatography (TLC)

Silica gel plates were cut to short strips (1x8 cm), the test compound was spotted about 0.5cm above the lower edge of the strip. Suitable volumes of n-butanol extract (5-15 µl) were spotted slowly allowing the formation of a small tight spot using a sterile clean 200- µl pipetting tip. After drying, the TLC strips were developed in different solvents. The solvent systems were prepared according to HEMW (Hexane, ethyl acetate, methanol, and water); Table A.1. Another solvent was used; n-butanol saturated with 1.7% ammonium acetate solution where the upper layer was used (Barghothi et al. 1991). The ratio of distance moved by the solvent and solute along the paper, from the origin baseline was measured to defined the R_f value

The developed strips were allowed to dry after marking the solvent front and studied under UV light. Antibiotic activity was determined by placing the TLC strips; face down, on a lawn of target organism spread on NA plate.

2.7 Identification of the isolate QUBC 97

It was important to identify the bacterium QUBC 97 for literature search, comparison purposes, and documentation. In addition to the Morphological appearance, 16S PCR with specific primers, superoxide primers specific for the genus *Streptomyces* and PCR product DNA sequencing followed by BLAST analysis to confirm and identify the QUBC 97 species. Primers used were *Streptomyces* specific primers with a general 16S primer see Table A.2.

Standard PCR method was optimized. The reaction conditions were as following: initial denaturation by hot start at 95 °C for 2 min, followed by 31 cycles of denaturation at 94° C for 90 s, annealing for 30 s at 60° C and 30 sec. at 58° C respectively and extension at 72°C for 105 s. A final extension at 72°C for 2 min. using commercial master mix from Promega Biochemical Company, which contains Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at (2X) concentrations. Reaction products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide and visualized and photographed under UV light. .

Selected amplicons were extracted using commercial Kit (NeucleoTrap, Mecherey Nagel, Germany) and sequenced (Bethlehem University, Bethlehem, Palestine) followed by BLAST alignment for species identification. Sequencing of ribosomal amplicon and Superoxide amplicon to compare sequencing based grouping to Multilocus Generated Clustering (Qadi 2012).

Chapter 3

Results

3.1 Isolation of *Streptomyces* species

When soil samples were cultured on NA plates and incubated for more than 3 days, filamentous colonies were observed. Microscopic examination of suspected colonies suggested *Streptomyces* nature due to fine filamentous mycelia that were not confused with fungal mycelia. Isolates were subcultured on CCG and ISP4 media for further macroscopic and microscopic monitoring figure (3.1).

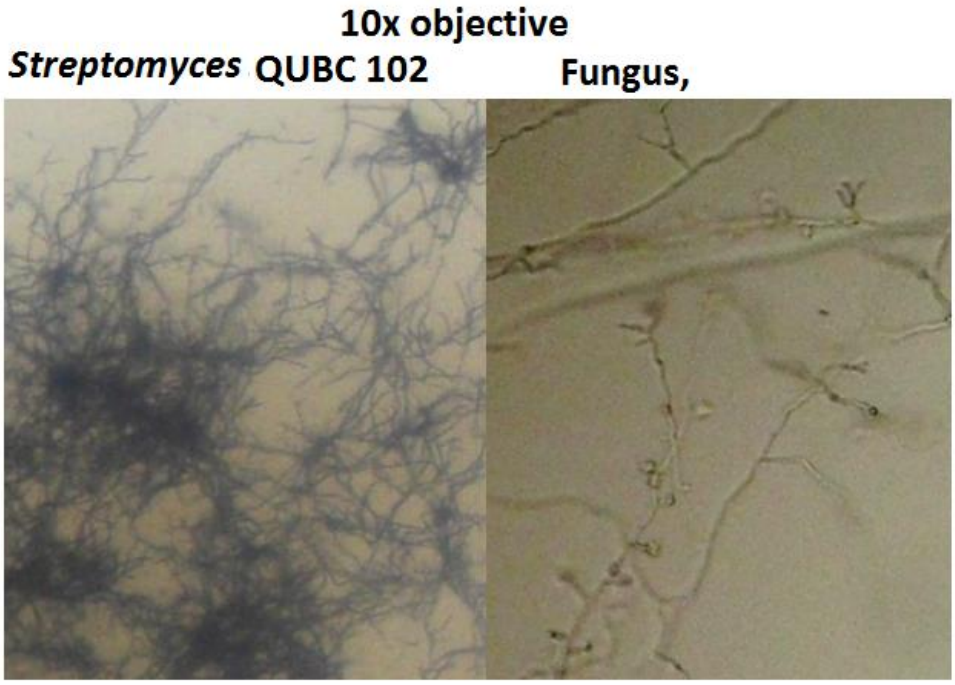


Figure 3.1: *Streptomyces* versus Fungus at 10x objective microscopic

3.2 Descriptions of *Streptomyces* isolates

Using the agar plug diffusion assay, QUBC97 was selected for further investigation due to its fast and stable growth, storage, and relative large zone of inhibition of *Bacillus atrophaeus* QUBC16 relative to other isolates; two other isolates were included, one of them was slow grower QUBC 293 (orange pigment producer on CCG) and the other QUBC 167 (blue ink diffused pigment producer on CCG) was inconsistent in producing antibacterial activity. Colonies of isolate QUBC97 were white colonies when grown on CCG Figure (3.2A) or ISP4 Figure (3.2C), yellow pigmentation of the lower side of colony and diffused yellow product on CCG Figure (3.2B) or ISP4 Figure (3.2D). The opaque nature of ISP4 due to its starch and calcium chloride content dimed the brightness of the yellow pigmentation relative to the transparent CCG agar. The low complexity of CCG medium allowed studying antimicrobial productivity and simplified agent

extraction with relative high purity, since there was little interference from the components of the medium.

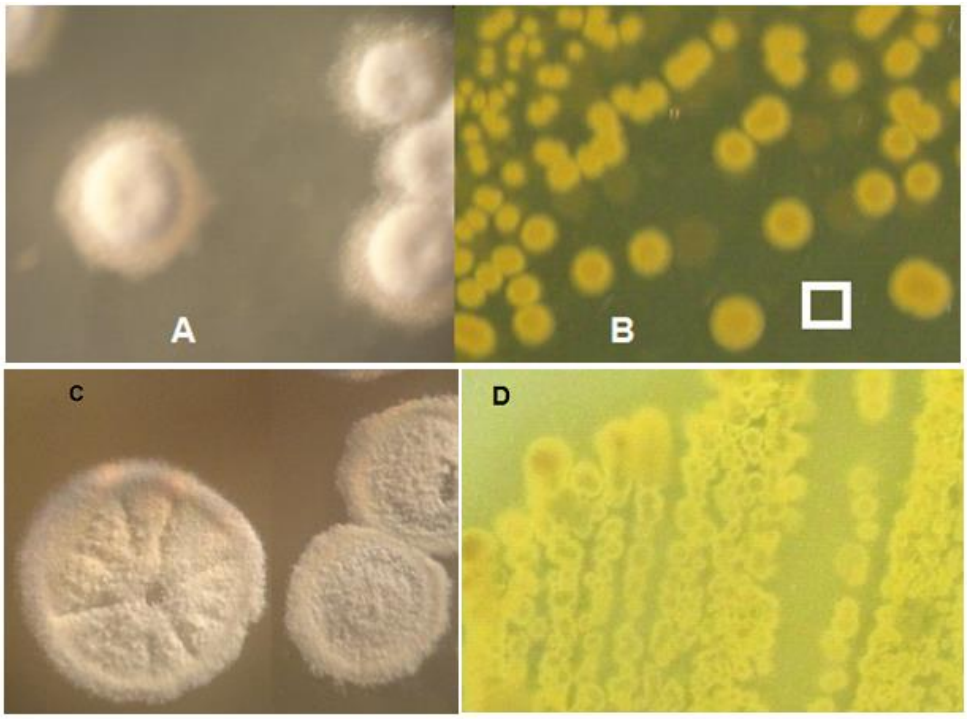


Figure 3.2: Isolate QUBC97, white colonies when grown on CCG (A) or ISP4(C), yellow pigmentation of the lower side of colony and diffused yellow product on CCG (B) or ISP4 (D). White square (B) indicates location of agar plugs (colony free areas close to colonies). The opaque nature of ISP4 due to its starch and calcium chloride content dimed the brightness of the yellow pigmentation relative to the transparent CCG agar.

3.3 Testing for antibiotic activity:

3.3.1 Agar plug diffusion test

Agar plugs were cut out from CCG agar plates after 14 days that showed good growth of pure cultures of putative *Streptomyces* isolates, antibiotic diffusion into the agar allows usage of agar as in disc sensitivity tests. The agar plugs were placed on lawns of *Bacillus atrophaeus* QUBC16 spread evenly on nutrient agar and incubated at 28°C, Figure (3.3) shows QUBC97 and two other isolates that produced antibacillus agents. QUBC97 was selected for further investigation due to its fast and stable growth, storage, and relative large zone of inhibition.

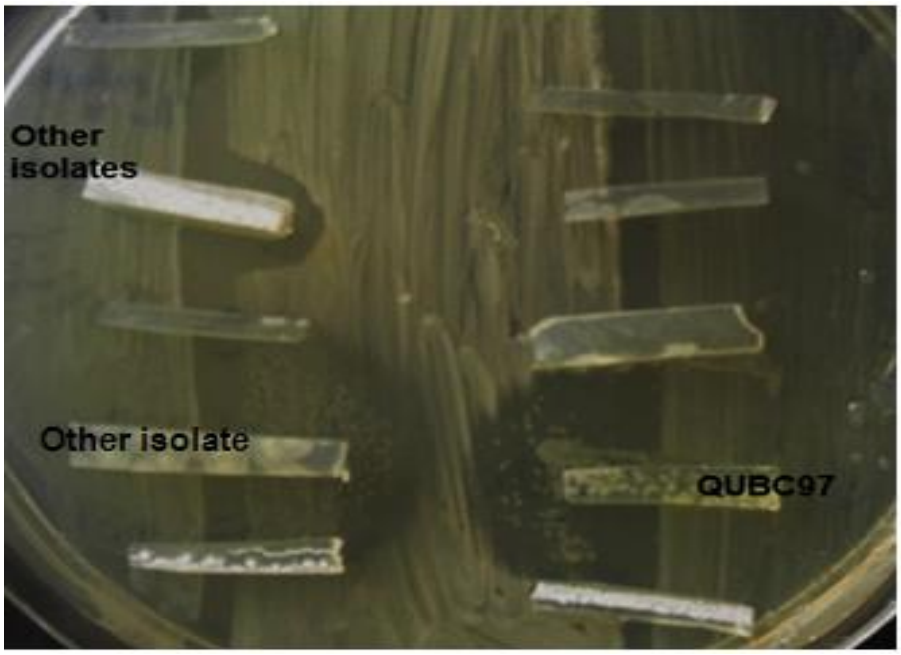
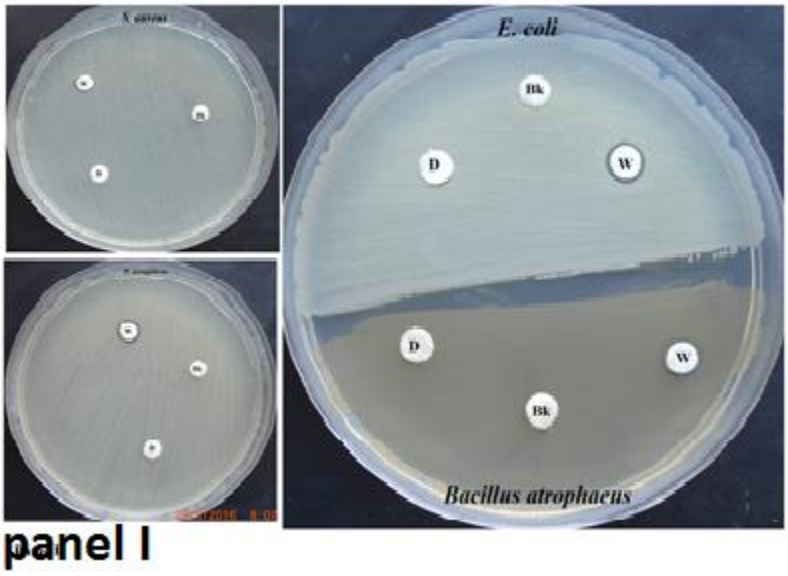


Figure 3.3: Three different *Streptomyces* isolates showing inhibition zones around agar plugs, against bacillus isolate *Bacillus* QUBC153 (unidentified) whereas *Bacillus* QUBC142 was not responsive to any agar plug. Including QUBC97 which was selected for further investigation.

3.3.2 Filter paper disc sensitivity testing

Organic extraction of QUBC97 cultures (from agar plates or broth media) was performed using n-butanol due to its miscibility with water and low volatility. N-butanol extract was used to impregnate sterile filter paper discs with (10 µl), after drying, sensitivity testing against test-organisms was carried out. The technique was more reliable than solid phase extraction (with talcum powder) due to time and cost saving and poor recovery of antibiotic agent. Diameters of zones of inhibition Figure (3.4).



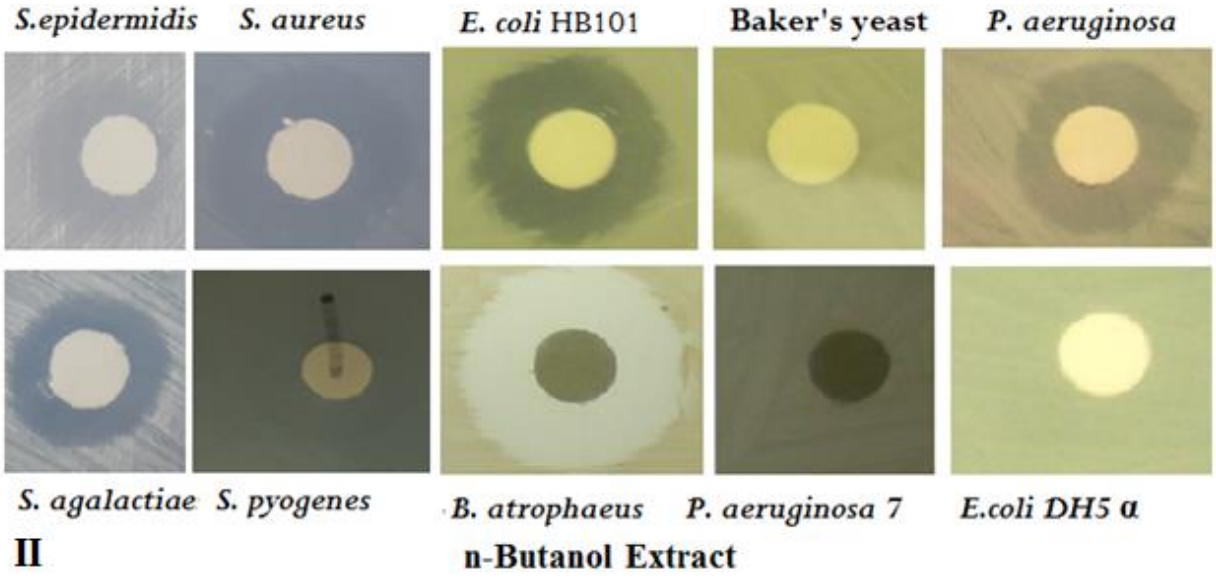


Figure3.4: Panel I; Control discs of blank (Bk), n-Butanol wet (W) (10 µl) or after air drying (D) on three bacterial species. **Panel II**; activity of n-butanol extracted antibiotic against different microorganisms, using impregnated filter disc diffusion technique. No inhibition of wild type *Pseudomonas* isolates, *Escherichia coli* DH5α, or baker's yeast, but was inhibitory to Gram positive bacteria and *E.coli* HB101. The antibiotic profiles for these bacteria were not determined.

Zones of inhibition using n-butanol extracts of QUBC97 are shown in figure (3.4) for *Bacillus atrophaeus, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus aglactiae, Escherichia coli* DH5α, and baker's yeast.

3.3.3 Kinetics of antibiotic production

Longitudinal sampling of liquid culture medium every 24 hours for seven days followed by extraction with n-butanol, and disc diffusion assay against *B. atrophaeus* QUBC16 revealed varying zones of inhibition. Diameters of zones of inhibition were considered as a direct reflection of the concentration of antibacillus agent. For each time point 3 discs were prepared, and diameters of zones of inhibition were measured after 24 h incubation at 28°C. The results are presented in Table (3.1) and Figure (3.5).

The production of the antibiotic starts after 24 hours of incubation and increases with time until it reached the maximum diameter of zone of inhibition. Further study is needed to determine substances and conditions that may induce the productivity.

Table3.1: Kinetics of antibiotic produced by QUBC97 as assayed against *Bacillus atrophaeus* QUBC16

Day	Diameter of zone of inhibition in (mm)	2nd Diameter of zone of inhibition(mm)	3rd Diameter of zone of inhibition(mm)	Average +/- SD
1	8	8	9	8.3 ±0.0.57
2	15	15	15	15±0
3	18	18	17	17.7 ±0.57
4	20	20	20	20±0
5	21	20	21	20.7±0.57
6	21	21	21	21±0
7	21	21	21	21±0

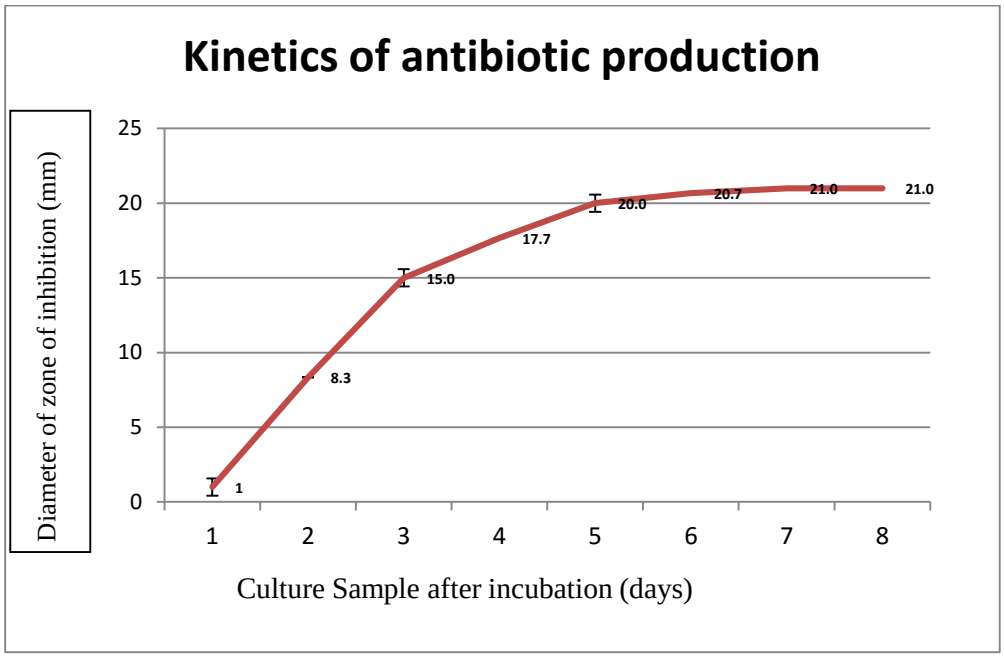


Figure 3.5: Kinetics of antibacillus production by QUBC97. Maximum production under indicated conditions can be achieved after ~130 h where it stabilized at 21 mm. Error bars indicate standard deviation from the mean.

3.4 TLC analysis of butanol extract

The recovered n-butanol extract from agar plates of QUBC97 was analyzed using chromatography on silica gel TLC plates. The results showed that two compounds can be separated into two bioactive spots when TLC was developed in n-butanol saturated with 1.7% ammonium acetate (nBAAc). In nBAAc, two spots were active R_f = 0.58 and 0.895. In water: methanol (1:1 v/v), R_f was 0.17 and another product showing as a streak along the TLC strip figure (3.6)

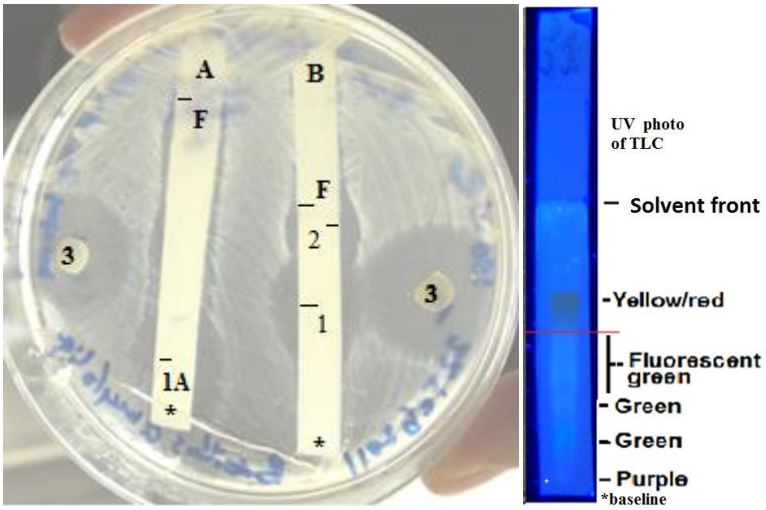


Figure 3.6: Agar plate of QUBC97 grown on CCG extracted with n-butanol. (**A**) Left strip of TLC was developed in methanol:water (1:1), spot 1A R*f*=0.17 and a long streak of inhibition. (**B**) Was developed in nBAAc, spot 1with R_f = 0.58 and spot 2 R*f*=0.895. The disc (3) diffusion test showing a diameter of 22mm of 5µl extract. TLC plate (UV photo) showed an orange/yellow spot that coincides with the slow moving activity at 22mm (B, spot 1) from TLC base line (*) suggesting the yellow diffused substance to be the active component or that co-purifies with the active component.

However when the bacterium QUBC97 was grown in liquid medium only one product could be extracted in the n-butanol. Therefore, liquid media provided a simple method to obtain one of the products for further analysis; two spots may due to an intermediate compound of the same antibiotic since it disappeared from liquid medium. In agar, modification of a product that diffuses away from the bacterium is not possible, which may explain the two compounds. Another possibility is that the two compounds are not related. Yet when liquid culture was kept for 6 months and reextracted with butanol, a single but a different compound with a different Rf was observed. Figure (3.7)

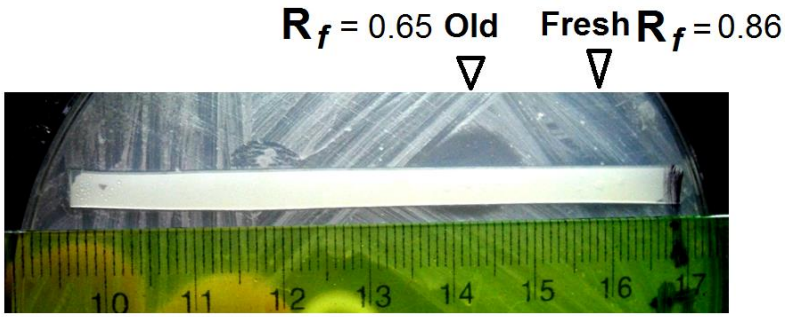


Figure3.7: Fresh and old n-butanol extracts were spotted separately (not shown) or co-spotted on the same TLC strip and developed in nBAAc to reveal complete separation of the two activities. Zone of inhibition depended on the amount of each fraction that was spotted on the TLC.

The n-butanol extract of liquid medium showed an R_f value of 0.3 when developed in a polar solvent (+6 a mixture of Hexane:Ethylacetate: methanol:water; 2:8:2:8, see Table (A.1) in appendix. Better mobility was obtained when TLC were developed in a less polar solvent (-2) which contained Hexane:Ethylacetate: methanol:water (7:3:5:5) R_f was 0.72. (liquid phase). The slower (old sample) with R_f =0.65 showed split absorption peak at λ426 and λ444 nm and faster (fresh sample) with R_f =0.86 as shown in Figure (3.8).

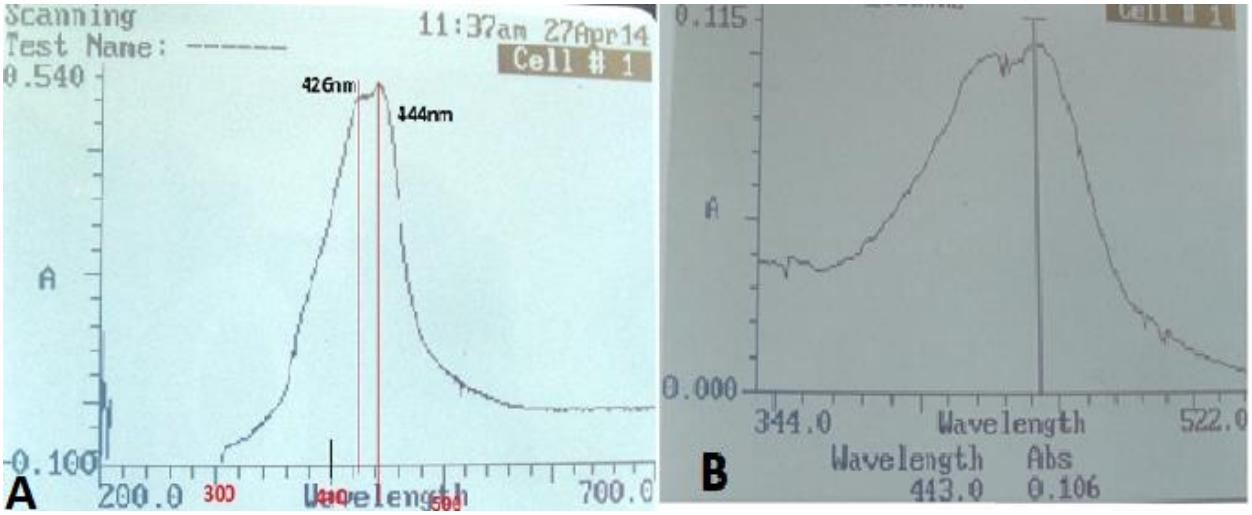


Figure 3.8: **(A)** Old Sample with slow mobility at 0.58 in nBAAc UV-Vis scan showing split peak absorption of blue light at 426nm and 444 nm. **(B)** Fresh sample with fast mobility at 0.86 in nBAAc UV-Vis scan showing split peak absorption of blue light at 428nm and 443nm. The complementary color for both peaks is yellow.

3.5 Identification of QUBC97 *Streptomyces* species

Figure 3.9 shows the PCR amplicons and superoxide dismutase as well as other *Streptomyces* specific amplicons for two isolates; QUBC188 and QUBC97 with different patterns. Sequencing of both Superoxide amplicon (333bp) and the 16S amplicon and BLAST analysis were used to determine and confirm the identity of putative QUBC 97 isolate. BLAST showed that QUBC97 was most likely identical to *Streptomyces pratensis* ATCC 33331. Morphologically, the isolate

was found to agree with Waksman's description of *S. flavogresius;* which was recently renamed as *Streptomyces pratensis* (Waksman 1953*)*. BLAST Sequence alignment showed 100% identity of the PCR amplicon to *Streptomyces pratensis*.

StmyF.Rn3 and SOXD PCR amplicons of QUBC97 were sequenced using automated sequencing services (Bethlehem University, Genetics Lab, Bethlehem, Palestine), the sequences were manually edited Figure (3.9) and assembled then analyzed using online Nucleotide BLAST services. BLAST alignment of 16S ribosomal amplicon suggested that QUBC97 closely matched *Streptomyces pratensis* ATCC 33331.

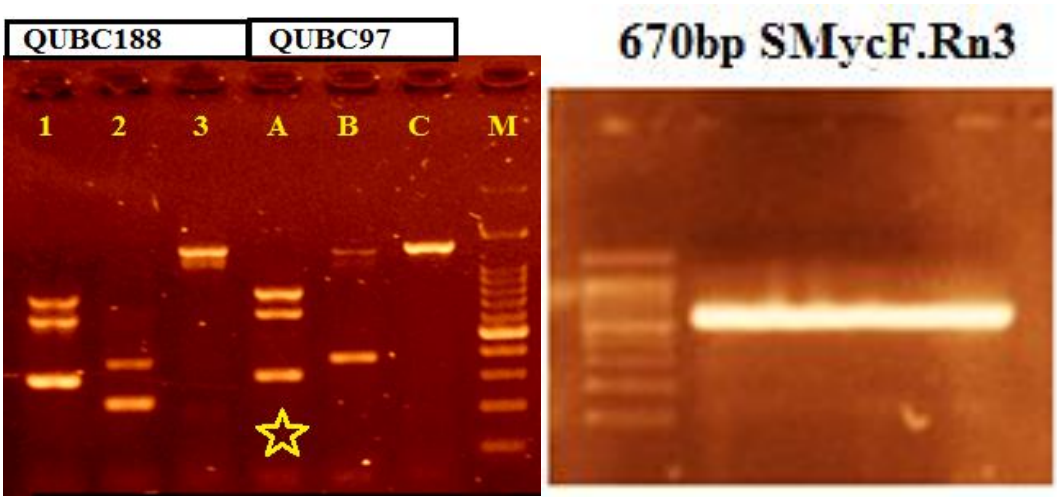


Figure 3.9 (A): Gel electrophoresis (1.6% agarose) showing multiple bands generated for two separate Streptomyces isolates ; the control isolate QUBC188 (lanes 1,2,and3) and the test isolate QUBC97 (lanes A,B, and C); the band above the asterisk is the Superoxide dismutase 333 bp which appears in both lanes 1 and A.

The second band in lane A is a 640bp amplicon of 16S generated by QUGPFn6 and the Streptomyces specific Reverse primer (SMycR);. Another 670bp band was generated with SMycF and QUGPRn3 for sequencing (right side gel, Figure 3.9A).

SMycF.Rn3 amplicon:

ATGCTGCAACAGAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCT

GACGACAGCCATGCACCACCTGTATACCGACCACAAGGGGGGCACCATCTCTGATGCTTTCC

GGTATATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCGTCGAATTAAGCCACATGCTCCGCTG

CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGG

AACTTAATGCGTTAGCTGCGGCACCGACGACGTGGAATGTCGCCAACACCTAGTTCCCAACGT

TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGT

CAGTAATGGCCCAGAGATCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACC

GCTACACCAGGAATTCCGATCTCCCCTACCACACTCTAGCTAGCCCGTATCGAATGCAGACCC

GGGGTTAAGCCCCGGGCTTTCACATCCGACGTGAACAAGCCCGGCCCTACCGAGCTCTTTAC

GCCCAATAATTCCGGAACAACGCTTTGCCGCCCCTAACGTATTACCGCGGGCTTGCTGGCACG

TAGTTACGCCGGCGCTTCTTTCTGCAAGGTACCGTCATCTTTT

Superoxide dismutase amplicon:

Figure 3.9 (B): Nucleotide sequences edited and assembled from both forward and reverse sequences of the SMycesF and QUGP Rn3**.**

Streptomyces flavogriseus strain ATCC 33331 16S ribosomal RNA gene, complete sequence
Sequence ID: ref|NR_074559.1|Length: 1514Number of Matches: 1
Range 1: 193 to 694 GenBank Graphics Next Match Previous Match (Matched many spp., not specific)

```
Query  1    AAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGC  59
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  AAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGC  252
Query  60   CTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGA  119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  CTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGA  312
Query  120  CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCT  179
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCT  372
Query  180  GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG  239
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  373  GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG  432
Query  240  AAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCG  299
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  433  AAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCG  492
Query  300  CGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCG  359
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  493  CGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCG  552
Query  360  GCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGC  419
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553  GCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGC  612
Query  420  TAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT  479
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  613  TAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT  672
Query  480  CAGGAGGAACACCGGTGGCGAA  501
           ||||||||||||||||||||||
Sbjct  673  CAGGAGGAACACCGGTGGCGAA  694
```

Figure 3.9 (C): Nucleotide sequence BLAST identified *Streptomyces flavogriseus* strain ATCC 33331with 99.4% 16S identity; the bacterium was recently renamed *Streptomyces pratensis*.

3.5 HPLC purification

HPLC separated one peak in about 100ml pure water eluate due to the polarity of the relative low polarity of the product, three fractions were tested for antibacterial activity after being re-extracted in n-Butanol. The rextracted layer was collected and 10µl from each fraction were spotted slowly on silica gel strips then allowed to dry and were developed in solvents -2 and -6 to give Rf=0.762 and Rf=0.30 respectively.

The eluted peaks was concentrated by rotary evaporator and then analyzed by Mass spectroscopy for putative identification of the antibacterial agent. The analysis suggested that the product is m**ethoxy,1-phenyl-,oxime** Figure (3.10)

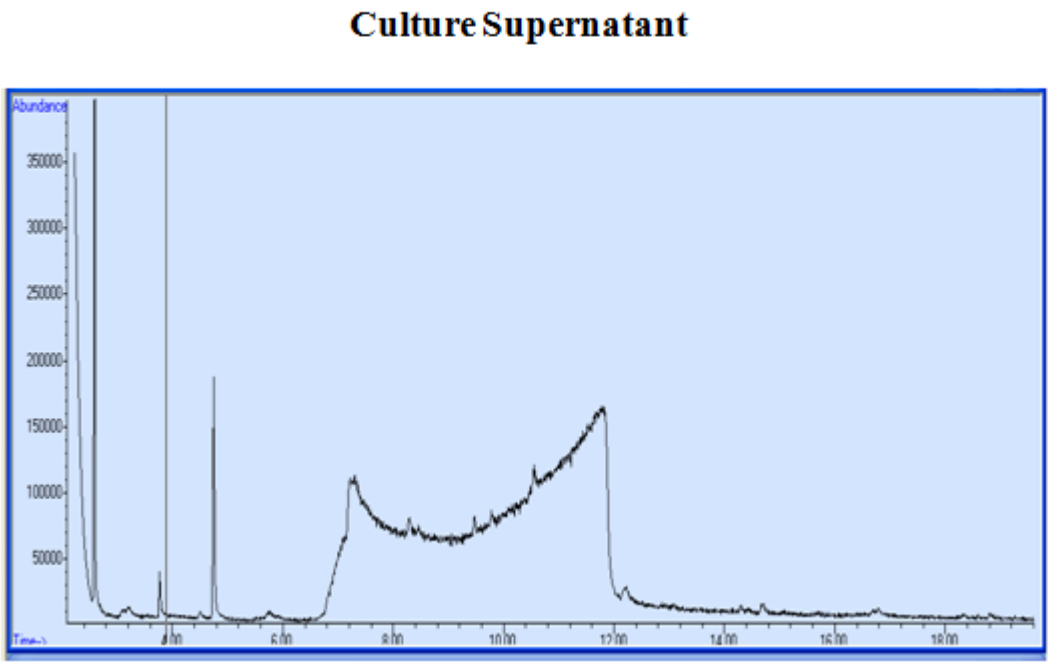


Figure 3.10 (A): Culture supernatant HPLC analysis showing a large number of peaks.

nButanol extract showed a major peak only.

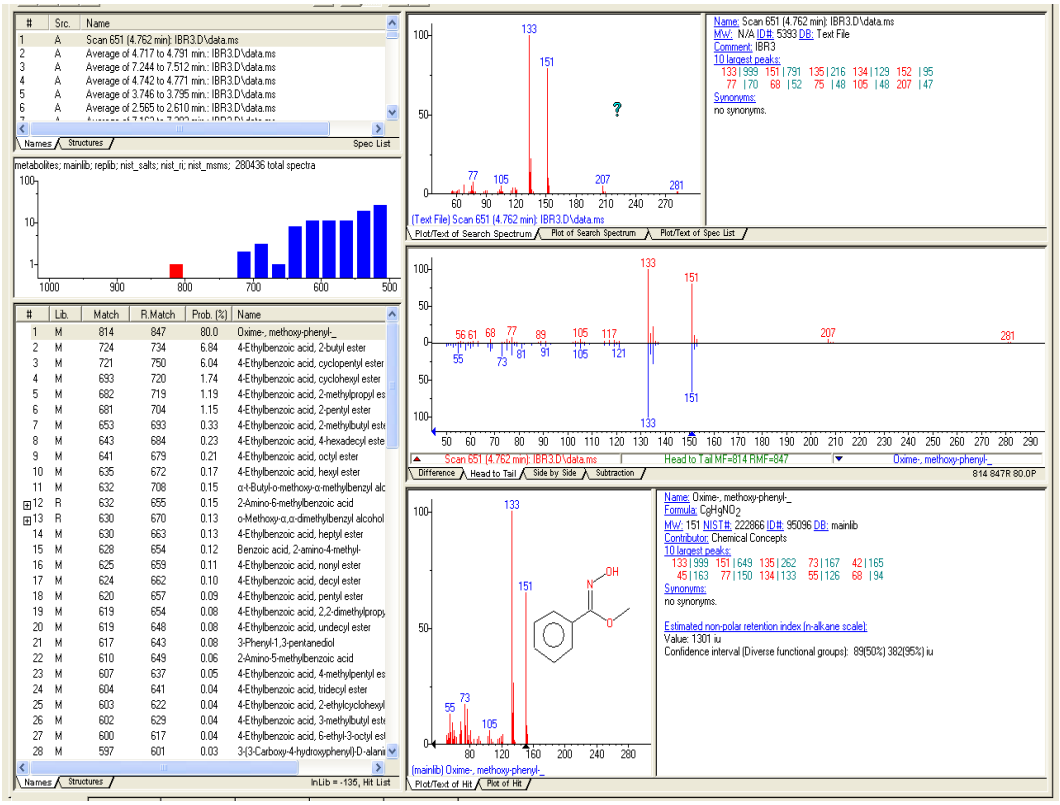
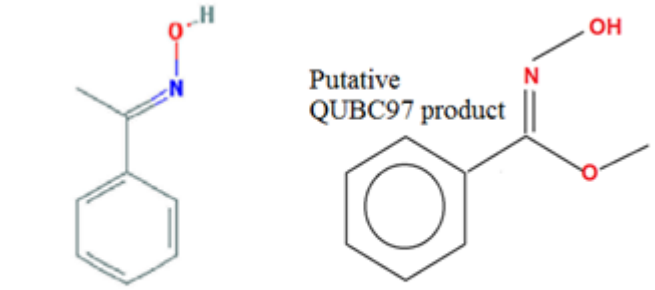


Figure 3.10 (B): Masspectroscopy analysis showing the major breakdown products that were indivative of **Methoxy,1-phenyl-,oxime**



Ethanone, 1-phenyl-, oxime ,oxime

Product Methoxy,1-phenyl- ,oxime

Figure 3.10 (C): The chemical structure of MOPO(right) and a related compound included for comparison (left).

Chapter 4

Discussion

The isolate QUBC97 was examined macroscopically and microscopically and putatively identified as *Streptomyces*. Similar and stable morphology appeared on both CCG Figure (3.2A) andISP4 Figure (3.2C) media. The two fold advantages of using CCG, was transparency compared to the opaque ISP4 medium, a property that allowed direct examination of agar plates with 10X objective lens, and second, its suitability to study growth factors. Identification of QUBC97 as *S. pratensis* was supported by Doroghazi and Buckley, 2014 since they explained the frequent chromosomal recombination which causes some variations and makes taxonomy more challenging. The other alternative identification was made as *S. griseus.* However PCR amplified an amylase amplicon from QUBC97 which on BLAST analysis did not pick any of the *S. griseus,* so the best identification would be as *S. pratensis*..

The utilization of agar plugs was utilized as a quick and sensitive method for following up antibacterial production. An added advantage was observed, byproducts by QUBC97 were accumulated in the agar plug to reflect fresh and older products relative to liquid cultures which showed one type of byproduct when fresh (<3 weeks) and another type when old (>2 months). Diffusion assay showed the consistent production of antibacterial activity from this isolate, which was the reason for selecting QUBC97 for further investigations.

Extraction of the antibiotic produced by QUBC97 was better with n-butanol than solid phase extraction using talcum powder followed by elution with methanol, due to the lower cost and short time. Extraction with butanol needs mixing of broth with butanol or overlaying agar plates with a thin film of n-butanol, the process can be repeated for second or third round of extraction if needed. Solid phase purification is more demanding and cannot be applied to agar media, hence it was not a viable choice with QUBC97.

Disc diffusion technique was used in semi-quantitative analysis when kinetic studies were conducted at different time points of culturing QUBC97 in liquid CCG. This was possible since n-butanol extraction was standardized over the seven-day follow up period. Disc diffusion conducted on n-butanol extracts was consistent and produced expected results. Zones of inhibition were quantitatively consistent and reliably reflected the increased concentration of antibacterial agent from older (aged) culture as shown in Figure (3.4). Although the conducted experiments were semi-quantitative, they were suitable to provide information on the kinetics of [putative Oxime] production, although quantitative tests should be done on Mueller-Hinton agar to compare Oxime production in different media and conditions for mass production in future.

Putative oxime was sufficiently stable in crude n-butanol extract for periods longer than 3 years; the same extract was kept at room temperature with no noticeable change in its anti-bacillus activity. Disc diffusion results consistently produced zones of inhibition ~21mm regardless of the age of product. This important aspect should allow pooling of batches produced within a set frame of time. For example batches produced within 3 -6 months that have been extracted into n-butanol can be pooled and analyzed, and this property is industrially advantageous.

There was a noticeable difference between antibacterial activities extracted from agar plates (showing two spots with different R*f* values of silica TLC developed in n-butanol/ammonium acetate solvent system) and liquid culture media. When the bacterium QUBC97 was grown in liquid medium only one stable product could be extracted in the n-butanol phase. Therefore, liquid media provided a simple method to obtain one of the two products for further analysis; young cultures produced a fast moving spot on TLC whereas a slower spot was produced when cultures were aged. Possible explanation is that one of the products (the fast moving spot) was an intermediary compound that became modified to produce the slower moving product; this explains the disappearance of the fast moving compound (FastA) from the medium and the domination of

the slow compound (SlowB). In agar, modification of the product was nearly impossible since its diffusion away from the bacterial colonies which are confined in place unlike those in shaking liquid cultures allows it to escape modification causing the two compounds to co-purify in n-butanol. Another possibility is that the two compounds are not related, although no evidence exist that hint or support such a possibility. Absorbance of the crude extract from old and fresh extracts showed similar profiles of split peak absorption at 426 and 444 nm which support the compound modification (fast A to slow B) rather than two independent compounds. The split peak may represent some form of isomerization of the side chain double bond present in the putative Oxime figure (A.1).

The report shows similar peak split of oxime compounds for oxime and iso-oxime at similar wavelengths (Chem 1984).

The activity is more hydrophobic since it can be extracted in n-butanol from aqueous medium. On TLC plates the activity moved faster when developed in a less polar solvent system such (mixture -2: Hexane: Ethylacetate: Methanol: water ;(7:3:5:5)) with R*f* = 0.72 than when developed in a polar solvent; R*f* =0.3 (+6; 2:8:2:8) table (A.1).

TLC plate showed an orange /yellow spot that coincides with the slow moving activity at 22mm from TLC base line, suggesting the yellow diffused substance to be the active component or that co-purifies with the active component. Yellow color production on plates intensified and accumulated as plates aged and become completely yellow within 3 weeks, which is consistent with kinetics of antibacterial production from liquid medium assayed by disc diffusion Figure (3.4) figure (A.2). (Blogspot)

The compound was putatively identified as methoxy-phenyl,oxime as suggested by mass spectroscopy, although this needs to be confirmed by other analytical methods to unveil the true identity of the anti-bacillus agent.

Although most Gram negative bacteria and yeast were resistant to the putative Oxime, E.coli HB101 was responsive and showed a clear zone of inhibition, which can be explained based on

permeability of the [Oxime] into the laboratory strain HB101 but not into wild type Gram negative bacteria. The responsiveness of MRSA Staphylococcus aureus to [oxime] is consistent with oxime being different from β-lactam antibiotics that are usually not effective against MRSA.

Recommendations

Continuation of this study and utilization of this bioactivity at industrial scale can be of important value, especially as oximes have been patented (Quinazolin-oxime derivatives as Hsp90 inhibitors (Jackson SE,2013;328:155-240. doi: 10.1007/128_2012_356) as inhibitors of Hsp90, a molecular chaperone protein, involved in several microbial and cancer diseases, which makes oxime an important compound to study.

Refrences

Arcamone, F., G. Cassinelli, G. Fantini, A. Grein, P. Orezzi, C. Pol, and C. Spalla. 1969. 'Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius', *Biotechnol Bioeng*, 11: 1101-10.

Barghothi, S., S.M. Payne, J.E. Arceneaux, and B.R. Byers. 1991. 'Cloning, mutagenesis, and nucleotide sequence of a siderophore biosynthetic gene (amoA) from Aeromonas hydrophila.', *Journal of bacteriology*, 173: 5121–28.

Barghouthi, S., and M. Qadi. 2012. 'Multi-locus Typing of Streptomyces spp', *The Seventh Palestinian Conference of Laboratory Medicine*.

Berdy, J. 2005. 'Bioactive microbial metabolites', *J Antibiot (Tokyo)*, 58: 1-26.

Burg, R.W., B.M. Miller, E.E. Baker, J. Birnbaum, S.A. Currie, R. Hartman, Y. Kong, R.L. Monaghan, and G. Olson. 1979. 'Avermectins, New Family of Potent Anthelmintic Agents: Producing Organism and Fermentation', *Antimicrob Agents Chemother*, 15: 361-67.

Chavez, G., R. Estrada, and A. Bonifaz. 2002. 'Perianal actinomycetoma experience of 20 cases', *Int J Dermatol*, 41: 491-3.

Chem, J. 1984. 'phynelacetones from o-methyl bromoacetone oxime', *rhodium*: 795.

Demain, L., and A Fang. 1995. 'Emerging concepts of secondary metabolism in actinomycetes', *Actinomycetologica*, 9: 98-117.

Doroghazi, J. R., and D. H. Buckley. 2014. 'Intraspecies comparison of Streptomyces pratensis genomes reveals high levels of recombination and gene conservation between strains of disparate geographic origin', *BMC Genomics*, 15: 970.

Drawz, S. M., and R. A. Bonomo. 2010. 'Three decades of beta-lactamase inhibitors', *Clin Microbiol Rev*, 23: 160-201.

Flardh, K., and M. J. Buttner. 2009. 'Streptomyces morphogenetics: dissecting differentiation in a filamentous bacterium', *Nat Rev Microbiol*, 7: 36-49.

Gottlieb, D., and E.B. Shirling. 1966. 'Methods for characterization of Streptomyces species', *Int J Syst Evol Microbiol*, 16: 313-40.

Kapadia, M., K. V. Rolston, and X. Y. Han. 2007. 'Invasive Streptomyces infections: six cases and literature review', *Am J Clin Pathol*, 127: 619-24.

Kay, J. E. 1996. 'Structure-function relationships in the FK506-binding protein (FKBP) family of peptidylprolyl cis-trans isomerases', *Biochem J*, 314 (Pt 2): 361-85.

'NCBI'.

Omura, S., and A. Crump. 2004. 'The life and times of ivermectin - a success story', *Nat Rev Microbiol*, 2: 984-9.

Osman, M., H. Fath Allah, and W. Abd El All. 2011. 'Antibiotic Production from Local Streptomyces Isolates from Egyptian Soil at Wadey El-Natron

II: Novel Antibiotic Identification Produced by Streptomyces plicatus', *Australian Journal of Basic and Applied Sciences*: 1906-10.

Procopio, R. E., I. R. Silva, M. K. Martins, J. L. Azevedo, and J. M. Araujo. 2012. 'Antibiotics produced by Streptomyces', *Braz J Infect Dis*, 16: 466-71.

Qadi, Mohammad. 2012. ' Phylogenetic Typing of Streptomyces Species Using Multilocus PCR Analysis', Al-Quds University

Rappoport, Z., and J Liebman. 2008. *The chemistry of hydroxylamine, oximes and hydroxamin acids* (Wiley).

Rose, C. E., 3rd, J. M. Brown, and J. F. Fisher. 2008. 'Brain abscess caused by Streptomyces infection following penetration trauma: case report and results of susceptibility analysis of 92 isolates of Streptomyces species submitted to the CDC from 2000 to 2004', *J Clin Microbiol*, 46: 821-3.

Spellberg, B., J. H. Powers, E. P. Brass, L. G. Miller, and J. E. Edwards, Jr. 2004. 'Trends in antimicrobial drug development: implications for the future', *Clin Infect Dis*, 38: 1279-86.

Tsai, H., C. Huang, A. Lin, and C. Chen. 2008. 'Terminal proteins of Streptomyces chromosome can target DNA into eukaryotic nuclei', *Nucleic Acids Res*, 36.

Umezawa, H., M. Ishizaka, K. Kimura, J. Iwanaga, and T. Takeuchi. 1968. 'Biological studies on individual bleomycins', *J Antibiot (Tokyo)*, 21: 592-602.

Waksman, S. A. 1953. 'Streptomycin: background, isolation, properties, and utilization', *Int Rec Med Gen Pract Clin*, 166: 267-80.

Watve, M. G., R. Tickoo, M. M. Jog, and B. D. Bhole. 2001. 'How many antibiotics are produced by the genus Streptomyces?', *Arch Microbiol*, 176: 386-90.

Electronic refrences

1. Bacterionet http://www.bacterio.net/Streptomycesa.html last access in 15/4/2016
2. Bioedit software http://www.mbio.ncsu.edu/BioEdit/page2.html
3. Blogspot http://hofeldtstudentexperience.blogspot.com/2015/10/one-fish-two-fish-red-sunset-blue-sky.html
4. Chem, J. (1984). "phynelacetones from o-methyl bromoacetone oxime." rhodium: J Chemical Socciety Chemical Communication 795. https://www.thevespiary.org/rhodium/Rhodium/chemistry/p2p.br-acetone.o-me-oxime.html
5. Liquid http://www.theliquidphase.org/images/thumb/b/bc/HEMWatfull.jpg/300px-HEMWatfull.jpg
6. NCBI:http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=1916&lvl=3&lin=f&keep=1&srchmode=1&unlock
7. Oxime isomers http://www.eie.gr/nhrf/institutes/tpci/researchteams/cctmp/cctmp-chemsensors-en.html

Appendix

Table A.1: thin layer chromatography liquid phase table. (Liquid phase)

HEMWat system #	relative proportions of solvents			
	hexane	ethyl acetate	methanol	water
-8	10	0	10	0
-7	9	1	9	1
-6	8	2	8	2
-5	7	3	7	3
-4	7	3	6	4
-3	6	4	6	4
-2	7	3	5	5
-1	6	4	5	5
0	5	5	5	5
+1	4	6	5	5
+2	3	7	5	5
+3	4	6	4	6
+4	3	7	4	6
+5	3	7	3	7
+6	2	8	2	8
+7	1	9	1	9
+8	0	10	0	10

Table A.2: Primers used in detection and identification of QUBC 97(Qadi 2012)

Primer Name (F/R)	Target Gene	Sequence	Tm °C	Comment/ Reference
SMyces F	16S ribosomal gene	**5'-GGTCGAAAGCTCCGGCGGTGAA-3'**	60	
SMycesF2	16S ribosomal gene	5'-CCCTTCACTCTGGGACAAGCCC-3'	61	
QUGP 3R	16S ribosomal gene	5′-CAGGATTAGATACCCTGGTAGTCC-3′	65	
SOXD-F	Superoxide dismutase	***5'-GCAGGTAGAAGGCGTGCTCCCA-3'***	63	
SOXD-R	Superoxide dismutase	**5'-CCTCGCGTTCCACCTCTCCGG-3'**	63	

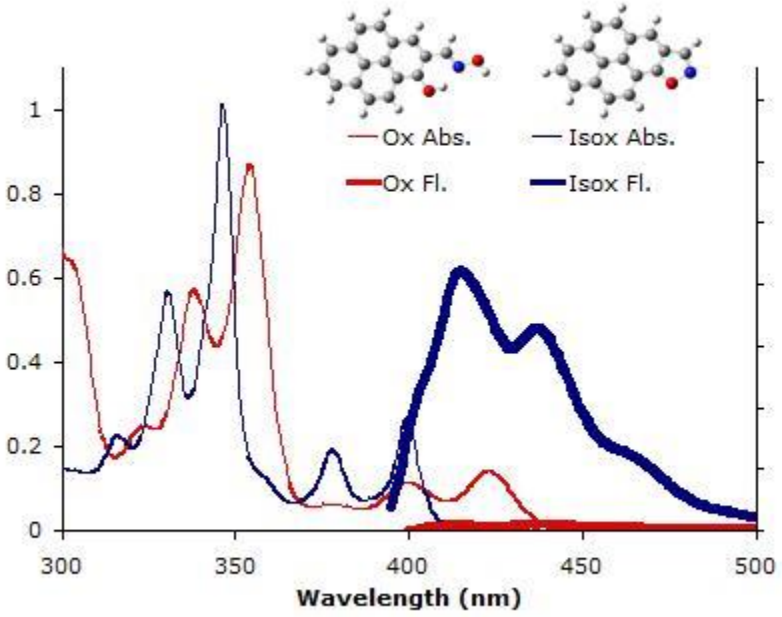


Figure 4.1: oxime isomeration. (oxime isomers)

Figure A.2: a chart of wavelengths of the different colors throughout the spectrum (Blogspot).

في هذا البحث, تم عزل بكتيريا ستريبتومايسز براتنسز (*Streptomyces pratensis)* على وسط زراعة جديد (CCG) والتي سميت بالبحث (QUBC97) ومن ثم التعرف عليها بطرق جزيئية (molecular). تميزت هذه البكتيريا بقدرتها على انتاج مضادات حيوية عند زراعتها على هذا الوسط والتي اظهرت مركبين مميزين من عينات قديمة وحديثة مزروعة. العينات المزروعة حديثاً تم استخلاص هذه المركبات منها عن طريق مذيب البيوتانول(n-butanol) وعند عمل فحص استشراب على طبقة سيليكا رقيقة

(silica gel TLC) اعطى نتيجة R_f=0.86 . المركب امتص الضوء المرئي على درجة 426 و 440 نانوميتر وتم (عمل HPLC) و (mass spectroscopy) لتحديد هذا المركب الذي تبين لاحقا انه

methoxy, 1-phenyl-, oxime

نشاط المضادات الحيوية لعدد من أنواع الستريبتومايسز

اعداد: ابتهال محمود ربيع عياد

اشراف: الدكتور سمير عبد اللطيف البرغوثي

الملخص

تعد البكتيريا من فصيلة ستريبتومايسز (*Streptomyces* spp.) ذات أهميه كبيرة في المجالين الطبي والزراعي، اذ انها تنتج العديد من المواد الي تصنف كمضادات حيوية بما فيها مضادات البكتيريا،الفطريات، الطفيليات، والفيروسات. كما انها تنتج العديد من المضادات للافات الزراعية ومضادات للخلايا السرطانية.

ان ظهور الطفرات في بعض انواع البكتيريا التي تقاوم انواع عديدة من المضادات الحيوية اثارت الكثير من المشاكل الصحية، ولذلك كان و مازال من المهم ايجاد بدائل لها لتسد الفراغات و تحل هذه المشكلة.

في 1942 تم اكتشاف الستربتومايسن وهو اول مضاد حيوي اكتشف ويصنع من هذه البكتيريا. و منذ ذلك الوقت بدأ الباحثون بدراسة الستريبتومايسز واستخراج المضادات الحيوية والمواد الفعالة التي تنتجها واعتبرت على انها اكثر فصيلة منتجة للمضادات الحيوية.

هذه الدراسة تهدف الى استخراج احد المواد الحيوية الفعالة من بكتيريا التي تم عزلها من مسحوق الكاكاو التجاري وتم تصنيفها وفقا لبرنامج الستريبتومايسز العالمي على انها ستريبتومايسز وسميت في هذه الدراسة (QUBC97) وبعد ذلك تم دراسة تأثير المضاد الحيوي الذي تنتجه على اكثر من نوع من البكتيريا عن طريق اخذ قطعة من الاجر الذي نمت عليه هذه البكتيريا و انتشر مضادها الحيوي فيها ووضع هذه القطع على سطح مزروع عليه انواع مختلفة من البكتيريا و دراسة منطقة التثبيط حول هذه القطع.