Faculty of Science
Microbiology department

Isolation, Characterization and Optimization of antimicrobial Activity of Actinomycetes of soil Origin

By
Ghada Khallaf Fath Allah

B.Sc. Microbiology
(1994)

Supervised By

Dr. Adel Ahmed El-Mahlawy
Ass. Prof. of Microbiology,
Faculty of Science,
Ain-Shams University

Dr. Samir Salah Ali El-Louboudy
Prof. of Microbiology,
Faculty of Science,
Al–Azhar University
Director Of Biotechnology and Applied Microbiology (Ferm-BAM) Centre

Dr. Housam Mohamed Mahmoud Ahmed Atta
Ass. Prof. of Microbiology,
Faculty of Science, Al-Azhar University

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إعداد

غادة خلاف فتح الله خلاف

بكالوريوس ميكروبيولوجي

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تحت إشراف

د. عادل أحمد رمضان المحلاوي

أستاذ مساعد بقسم الميكروبيولوجي

كلية العلوم – جامعة عين شمس

أ.د.سمير صلاح علي اللبودي

أستاذ الميكروبيولوجي – كلية العلوم

جامعة الأزهر بنين

مدير مركز بيوتكنولوجيا التخمرات و الميكروبيولوجيا التطبيقيه

د. حسام محمد محمود أحمد عطا

أستاذ مساعد بقسم الميكروبيولوجي

كلية العلوم – جامعة الأزهر بنين

Abstract

The isolation of antibiotics from microorganisms improved the discovery of novel antibiotics, which is relatively easy as compared to chemical synthesis of antimicrobial agents. The main goal of the present study is to isolate actinomycetes from soil habitat of Egypt then the most potent producer strains with broad activity against the tested human pathogenic bacteria and fungi were selected and identified. This study starts from isolation and purification of the antimicrobial producing actinomycetes followed by characterization. Two hundred thirty nine isolates were isolated from seven different soil samples collected from Egypt. Fifty five isolates could antagonize the selected human pathogenic bacteria and fungi with varying degrees of potency. Out of them, only two were found to have remarkable inhibitory effect on all of the tested human pathogenic bacteria and fungi. These two antimicrobial producing actinomycete isolates were identified as *Streptomyces graminofaciens* and *Streptomyces lavendulae*. The environmental and nutritional factors affecting the production of antimicrobial agents were studied against the test human pathogenic bacteria and fungi. The first antimicrobial agent produced by *S. graminofaciens* was extracted with ethyl acetate and the second one produced by *S. lavendulae* was extracted with chloroform and methanol

mixture ratio(1:1v/v). The active fractions were tested with the test human pathogenic bacteria and fungi. The purification of the antibiotic has been carried out by silica gel column chromatography and eluted stepwise with chloroform and methanol (3:1v/v) then the physical and chemical properties were studied to identify the antimicrobial agent. The elemental analysis was carried out at Microanalytical Center of Cairo University,Egypt. The elemental analytical data of the antimicrobial agent produced by *S. graminofaciens* showed the following: **C**= 68.78; **H**= 5.20; **N**= 7.86, **O** = 18.16 and **S=0.0** which indicates a suggested empirical formula of $\mathbf{C_{10}H_9NO_2}$ and the data of the antimicrobial agent produced by *S. lavendulae* showed the following: **C**=55.33; **H**=4.85; **N**= 4.09, **O** = 35.73 and **S=0.0** which indicates a suggested empirical formula of $\boldsymbol{C_{15}H_{15}NO_7}$**.** The spectroscopic analysis were determined at the National research center, for the purified antimicrobial agent produced by *S. graminofaciens***,** the **infrared (IR)** spectrum of showed characteristic band corresponding to 20 peaks, **ultraviolet (UV)** absorption spectrum recorded a maximum absorption peak at 296 nm and **Mass spectrum** showed that the molecular weight was at (191). For the purified antimicrobial agent produced by *S. lavendulae***, the infrared (IR)** spectrum showed characteristic band corresponding to 24 peaks, **ultraviolet (UV)** absorption spectrum of the antimicrobial agent recorded a maximum absorption peak at 330 nm and

Mass spectrum showed that the molecular weight was at (321)**.**

On the bases of the physical and chemical properties, it could be stated that the antibiotic is suggestive of being belonging to Abikoviromycin produced by *Streptomyces graminofaciens* and belonging to Reductiomycin produced by *Streptomyces lavendulae*.

The MIC of the antibiotics were determined and the results showed that the minimum inhibitory concentration (MIC) of the antibiotic produced by *Streptomyces graminofaciens* for *Staphylococcus aureus*, NCTC 7447 and *Bacillus subtilis*, NCTC 1040 were (1.95 ug/ml) ; for *Escherichia coli*, NCTC 10416 & *Salmonella typhi*, NCIMB 9331 were (7.8 ug/ml) ; for *Candida albicans*, IMRU 3669 & *Aspergillus niger* IMI 31276 were (62.5 ug/ml) ; for *Aspergillus flavus* ,IMI 111023 & *Aspergillus fumigitus* were (31.25 ug/ml) and the minimum inhibitory concentration (MIC) of the antibiotic produced by *Streptomyces lavendulae*, for *Staphylococcus aureus*, NCTC 7447 & *Bacillus subtilis*, NCTC 1040 were(7.8 ug/ml) ; for *Escherichia coli*, NCTC 10416 & *Salmonella typhi*, NCIMB 9331 were (15.62 ug/ml) ; for *Candida albicans*, IMRU 3669 was (15.62 ug/ml) ; for *Aspergillus flavus* ,IMI 111023 & *Aspergillus niger* IMI 31276 were (62.5 ug/ml) and for *Aspergillus fumigitus* was (31.25 ug/ml).

رسالة الماجستير

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عنوان الرسالة

عربي عــزل وتعريــف الخصــائص والظــروف المثلــى لنشــاط مضــادات الميكروبات لأكتينوميسيتات التربة

إنجليزي Isolation, Characterization and optimization of antimicrobial activity of actinomycetes of soil origin.

لجنة الإشراف

د. عادل أحمد رمضان المحلاوي أستاذ مساعد بقسم الميكروبيولوجي – كلية العلوم – جامعة عين شمس

أ.د. سمير صلاح علي اللبودي أستاذ الميكروبيولوجي – كلية العلوم – جامعة الأزهر بنين ومدير مركز بيوتكنولوجيا التخمرات

د. حسام محمد محمود أحمد عطا أستاذ مساعد الميكروبيولوجي – كلية العلوم – جامعة الأزهر بنين

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Aim of The Work

- To isolate and identify the most potent antimicrobial producing actinomycetes from Egyptian soil.
- To study the environmental and nutritional factors affecting the production of antimicrobial agents of the most potent actinomycetes isolates.
- Optimization of the cultural condition of the most potent actinomycete isolates to obtain high yield of antimicrobial metabolites.
- Production of antimicrobial agents from the most potent actinomycete isolates using Bioflo 3000 fermentor .
- To extract, purify and identify these antimicrobial agents according to spectroscopic analysis and chemical tests.

Introduction

With the increasing misuses of antibiotics, the serious problem of antibiotic resistance is coming up very fast. Therefore, intensive search for new antibiotics is going on world wide **(Suetsuna & Osajima, 1990 and Haque *et al*., 1995)** from *Streptomyces* which is the greatest source of antibiotics. To make the production of the antibiotic feasible, it is necessary to develop the optimum production conditions. Several scientists have attributed considerably in this field**, (Korn-Wendisch & Kulzner, 1991**).

The occurrence of actinomycetes in different ecosystems points out their metabolic diversity and the evolution of specific mechanisms for dispersion and adaptability under different environmental conditions. This metabolic diversity allows these microorganisms to survive in saline, acid, and high temperature environments, indicating their good ability to adapt to adverse environmental conditions (**Araújo, 1998**).

The production of antimicrobial agents by actinomycetes is of great importance from the economic point of view for controlling both plant and human pathogenic bacteria and fungi. Actinomycetes are gram-positive filamentous bacteria with high G+C (55%) content in their DNA. These are wide spread in nature and can be found with greater or less

frequency in most ecological niche (**Takahashi & Omura, 2003**).

Actinomycetes are abundant in soils and act in the degradation of complex molecules as well as recalcitrant substances, especially cellulose, lignocellulose, xylan and lignin, that play an important role in soil organic matter decomposition processes (**Petrosyan *et al.*, 2003** and **Ding *et al.*, 2004**).

Among actinomycetes population from soils, *Streptomyces* species are reported to be the most abundant forms. They are the producers of most of the known bioactive metabolites. They include numerous potentially useful compounds providing the widest range and most promising array of pharmacologically and agriculturally active compounds. They are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics **(Bibb, 2005)**. The actinomycetes, mainly those belonging to the *Streptomyces* genus, make up an important group of soil bacteria from the actinobacteria class. Several species of the family *Streptomycetaceae* are widely studied because of their ample capacity for production of secondary metabolites, such as antibiotics and extracellular enzymes (**Inbar *et al.*, 2005).**

The nutritional source like carbon, nitrogen and

minerals, the environmental factors such as time, temperature and pH are found to have profound influence on antibiotic production by actinomycetes **(Sanchez &Demain , 2002 and Himabindu & Jetty, 2006).**

Optimization of the cultural conditions is essential to get high yields of the antimicrobial metabolites. Actinomycetes have characteristic biological aspects such as mycelial forms of growth that accumulates in sporulation and the ability to produce an array of secondary metabolites many of which have antibacterial or antifungal properties **(Vasavada *et al*.,2006).** In facts, most antibiotics developed for human pharmaceutical use are actinomycetes secondary metabolites**, (Ponmurugan *et al.*, 2007)**

Microbial secondary metabolites; have the largest share in the production of antibiotics. Of the 520 new drugs discovered between 1983 and 1994; approximately, 39% were originated from microbial sources. Most of the antibiotics discovered up to now, are produced by members of the genus of *Streptomyces* and only 3% of these antibiotics are determined yet. During the classical investigation methods, some species not to grow under laboratory conditions and lack of relevant technologies. **(Oskay & Tamer, 2009).**

Literature Review

1-Screening for the antimicrobial compounds of microbial origin:

Antibiotics are useful in combating human, animal, and plant diseases. Micro-organisms provide a unique source of unexpected, helpful products and their natural products are the origin of most of the antibiotics on the market today. **(Vandame , 1984).**

Most of the actinomycetes commonly isolated from soil belong to the genus *Streptomyces* because they are more common in the environment and tend to have rapid growth rate and good sporulation compared to other actinomycetes (**Williams & Vickers, 1986**).

Actinomycetes are a particularly interesting group, because they represent a substantial fraction of producers of commercially important bioactive microbial compounds. Nearly 8000 actinomycetes antibiotics have been described, Soil-associated actinomycetes produce over 70 percent of naturally occurring antibiotics **(Okami & Hotta, 1988).**

It is significant in the development of antibiotics to mention the rapid increase over a short period of time. In 1955

only 500 antibiotics had been known twenty years later, in 1975, this number was already 500. Today, more than 13000 antibiotic natural products are known. The total number of semi-synthetic derivatives of natural antibiotics is close to 100000(**Bushell, 1989**).

As actinomycetes are one of the diverse groups of soil bacteria possessing commercially useful enzymes and therapeutically useful bioactive molecules, biochemical characterization of the individual isolates is of most importance to understand their basic physiology. 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, 1989).** They comprise 63 genera and are relatively well documented because of their value in drug discovery **(Nisbet & Fox ,1991).**

Some of actinomycetes secondary metabolites have employed as useful microbial compounds **(Prescott *et al.*, 1993).** Examples include streptomycin from *Streptomyces griseus* for treatment of tuberculosis caused by *Mycobacterium*

tuberculosis and the immunosuppress drug, tacrolimus (FK506) produced by *S. tsukubaensi.*

Nisamycin, a new manumycin group of antibiotics, was isolated from the culture broth of *Streptomyces* sp. K106. This strain was designated to genus *Streptomyces* by the taxonomic features. Nisamycin was purified by ethyl acetate extraction, silica gel column chromatography, Nisamycin is active against Gram-positive bacteria and fungi, and exhibits a cytotoxic activity (**Hayashi *et al.*, 1994).**

The results of extensive screenings have led to the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have been applied in human medicine, veterinary and agriculture. Most of them are produced from *Streptomyces* (**Hwang *et al.*, 1994**).

One reason for the slow progress compared to antibacterials is that, like mammalian cells, fungi are eukaryotes and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity to the host as well **(Georgopapadakou & Tkacz 1994).**

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).**

In 1995**, Kihara *et al.*,** isolated a new antibiotic liposilide A from a culture of *Streptomyces* species RS-28. The structure of liposilide A was inactive against Gram positive and Gram negative bacteria but showed inhibitory activity against some species of fungi.

The production of antibiotics by actinomycetes, may not be species-specific, but rather strain-specific. Antibiotics of actinomycete origin evidence a wide variety of chemical structures, including aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyketides, actinomycins, and tetracyclines **(Okami & Hotta, 1988 and Baltz, 1998).**

During the study on the oleandomycin production, A new oleandomycin derivative was purified.The new oleandomycin derivative has antibacterial activities similar to those of oleandomycin agaisnt *Enterococcus faecalis*, *Bacillus subtilis* and *Staphylococcus aureus*. Macrolides are a large and structurally diverse class of natural products that possess a wide range of biological activities useful for medicinal and agricultural fields as antimicrobials, immunosuppressants, antiparasitics, and anticancer agents **(Hopwood *et al.*, 1995).**

Since the discovery of streptomycin, an aminoglycoside antibiotic, from *Streptomyces*, a large number of antibiotics