Isolation and Preliminary Characterization of Antimicrobial Agents from Marine Actinomycetes

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Submitted for partial fulfillment of
the Master Degree of Science
in
(Microbiology)

By

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(B.Sc. Microbiology, 2004)

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Approval Sheet

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عملية عزل وتمييز مبدئى لمضادات الميكروبات من أكتينومايسيتات بحرية

رساله مقدمه من
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كجزء من متطلبات الحصول على درجة الماجستير فى العلوم فى الميكروبيولوجى

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رساله الماجستير فى العلوم

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ثم
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ثم أتقدم بوافر الشكر والإمتنان لوالدى وإخوتى وزوجى وإلى كل من ساعدنى أثناء عملى لإخراج هذه الرساله فى أحسن صورة.
وجزيل الشكر الى قسم الميكروبيولوجى والى كل زملائى بالهيئه القومية للرقابة والبحوث الدوائية.

1-INTRODUCTION

The Actinomycete are Gram positive, free living, saprophytic bacteria found widely distributed in soil, water and colonizing many plants and can be found with greater or less frequency in most ecological niche **(Takahashi and Omura, 2003)**. They have universal occurrence and play an active role in nature **(Syker and Skinner, 1973 and Edward and Bergey, 1974)**. They produced numerous substances essential for health such as antibiotics **(Waksman, 1940)**, enzymes **(Bachmann and McCarthy, 1991)** and immunomodulators **(Iwami *et al.*, 1987)**. Around the world there are 23000 bioactive secondary metabolites produced by microorganisms have been reported and over 10000 of these compounds are produced by actinomycetes, 7600 derived from *Streptomyces* and 2500 from the so-called rare actinomycetes **(Berdy, 2005)**. Today about 130 to 140 microbial products and a similar number of derivatives (including semi synthetic antibiotics) are applied in human medicine, mostly in chemotherapy and veterinary medicine.

Recently the rate of discovery of new compounds from terrestrial actinomycetes has decreased whereas the rate of reisolation of known compounds has increased. Thus, it is excited that new groups of actinomycetes from unexplored or under exploited habitats be persued as sources of novel bioactive secondary metabolites **(Dania and Humanns, 2003)**.

There has been a growing awareness of the potential value of freshwater habitats as a source of actinomycetes that produce secondary metabolites of clinical importance **(Rifaat, 2003)**.

Aquatic microbes are particularly important because they have not been as extensively exploited as their terrestrial counterparts, and because of the high potency required for bioactive compounds to be effective in the aquatic environment, due to the diluting effect of water **(Zhang *et al.*, 2005)**. Little is known concerning the actinomycetes exhibiting antimicrobial properties from these habitats. The list of novel actinomycetes and products derived from poorly explored areas of the world stresses the importance of investigating new habitats **(Nolan and Cross, 1988).**

The emergence of antibiotic resistance increases the demands to discover new effective antibacterial agents, so actinomycetes from different habitats are needed to be screened for antimicrobial activity in hope of getting some actinomycetes strains that produce antibiotics which have not been discovered yet and active against drug resistant pathogens **(Kumar *et al.*, 2010)**.

AIM OF THE STUDY

The aim of this study was to find alternatives to conventional antibiotics by exploring actinomycetes in the marine environment.

OBJECTIVES

1. Isolation and identification of actinomycetes from marine habitats.
2. Screening for the antimicrobial activities of crude extract.
3. Partial purification of the antimicrobial substance by chromatography techniques
4. Studying the factors affecting the antimicrobial activity.
5. Optimizing culture conditions for antibiotic production.

2-Review of Literature

2.1-Actinomycetes as antibiotic producers:-

Actinomycetes are the most widely distributed groups of microorganisms in nature. The name "actinomycetes" was derived from Greek "atkis" (a ray) and "mykes" (fungus), and that has features of both bacteria and fungi **(Waksman, 1950; Kumar, 2001; Das *et al.*, 2008)**. They occur in a wide range of environments in which they have the ability to grow on most naturally occurring substrates **(Berdy, 1984; Goodfellow *et al.*, 1989)**. They are also well known as saprophytic soil inhabitants **(Takizawa *et al*., 1993)**. Soil actinomycetes produce a volatile compound called geosmin, which literally translates to "earth smell" **(Gust *et al.*, 2003)**.

Actinomycetes are prokaryotic organisms belonging to subdivision of filamentous, non-motile, free living, saprophytic Gram-positive bacteria with over 55 mol % of G+C content ratio in their DNA **(Williams *et al.*, 1993; Stackbrandt *et al.*, 1997; Xu *et al.*, 2007**). They are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA: rDNA pairing studies **(Goodfellow and Williams, 1983).** They comprise a group of branching unicellular microorganisms that produce two types of branching mycelium; substrate mycelium and aerial mycelium. Their cell wall consists of peptideglycanes but does not chitin or cellulose. They are usually placed in a separate

order, the Actinomycetales, which is said to be distinct from the Eubacteriales or the true bacteria **(Waksman, 1959).**

Actinomycetes have a cell wall structure characteristic of bacteria and frequently show the presence of lytic viruses (actinophages). **Cummins and Harris (1956)** established that actinomycetes have a cell wall composition of Diaminopimelic Acid (DAP) isomers. Presence of L-diaminopimelic acid (DAP) and glycine (type I), meso-DAP and glycine (type II), meso-DAP (type III), or meso-DAP, arabinose and galactose (Type IV) depends upon the type of cell wall.

Actinomycetes are a large heterogeneous group of microorganisms, comprising several genera and numerous species. They vary greatly in their morphology, physiology and biochemical activities. They play an important role in nature by bringing about the decomposition of complex plant and animal residues and the liberation of a continuous stream of available elements, notably carbon and nitrogen essential for fresh plant growth.

Actinomycetes, especially *Streptomyces* sp., are recognized as the producers of many bioactive metabolites that are useful to humans in medicine, such as antibiotics **(Waksman, 1961; Lacey, 1973; McCarthy and Williams, 1990; Ouhdouch *et al.*, 2001; Saadoun and Gharaibeh, 2003; Blunt and Prinsep, 2006)** anti-tumor agents, immuno-

suppressive agents **(Mann, 2001)** and enzymes (**Strohl, 2004; Berdy, 2005; Cragg *et al*., 2005)**.

The total number of discovered compounds was only 10-20 in 1940, 300-400 in 1950, approximately 800-1, 000 in 1960 and 2, 500 antibiotics were already known in 1970. From that time the total number of known bioactive microbial metabolites has almost doubled in every ten years. About 5,000 in 1980, 10,000 in 1990 and almost 20,000 antibiotic compounds were known in 2000. Over 22,000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature by the end of 2002 **(Berdy, 2005)**.

Almost 80% of total antibiotic products are produced by *Streptomyces* that originated in the soil **(Imada and Okami, 1998; Lazzarini *et al*., 2000; Kim and Garson, 2005; Bull and Stach, 2005)**. According to **Strohl, 2004**, one strain often produces different compounds. For example, *Streptomyces antibioticus, Streptomyces griseus,* and *Streptomyces hygroscopicus* produce 13, 32 and 46 different compounds respectively; some strains of *Streptomyces hygroscopicus* produce bialophos, hygromycin, spectinomycin, rapamycin, and others.

Antibiotics of actinomycete origin evidence a wide variety of chemical structures including aminoglycosides, macrolides, glycopeptides, lincosamides, lipopeptides,

polypeptides, tetracyclines and chloramphenicol **(Waksman, 1968; Jeffrey *et al*., 2007)**.

Search for new antibiotics generally involve screening of naturally occurring actinomycetes and/or biotechnological manipulations of known antibiotic producing strains. Efforts have been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased. Thus, it is crucial that new groups of actinomycetes from unexplored or underexploited habitats be pursued as sources of novel bioactive secondary metabolites. The above condition can be improved by the continued improvement of the objectivity and efficiency of the isolation procedures **(Fenical *et al*., 1999)**.

2.2-Exploring the diversity of marine actinomycetes:-

Although the diversity of life in the terrestrial environment is extraordinary, the greatest biodiversity is in the oceans **(Dania and Humanns, 2003)**.More recent surveys of actinomycetes in aquatic environments are directed at their potential value as producers of novel enzymes and metabolites (**Cross, 1982; Takizawa *et al*., 1993; Jiang and Xu, 1996)**.

As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds. The living conditions to which marine actinomycetes had to adapt during evolution range from extremely high pressure, anaerobic conditions, low temperatures and high acidic conditions **(Bull *et al*., 2005; Stach *et al*., 2003)**. It is likely that this is reflected in the genetic and metabolic diversity of marine actinomycetes, which remains largely unknown.

The number of marine natural products continues to rise with more than 200 new metabolites reported annually **(Blunt *et al*., 2003; Blunt *et al*., 2004; Blunt *et al*., 2005; Blunt *et al*., 2006; Blunt *et al*., 2007; Taylor *et al*., 2007; Blunt *et al*., 2008)**. These compounds have shown promise in treating inflammation, cancer as well as microbial and viral infections **(Newman and Cragg 2004; Fenical, 2006; Newman, 2008)**. Indeed, the marine environment is a virtually untapped source of novel actinomycete diversity and, therefore, of new metabolites **(Jensen *et al*., 2005; Magarvey *et al*., 2004)**.

2.3-Genus *Streptomyces*:-

The genus *Streptomyces* has received considerable attention for its importance as a source of antibiotics **(Sanglier *et al*., 1993; Young, 1993; Lazzarini *et al*., 2000; Habib *et al*., 2001; Kokare *et al*.; 2004; Choi *et al*., 2005; Boudjella *et al*., 2006 and Volka and Furkert, 2006)**.

Streptomycetes are among the most numerous and ubiquitous soil bacteria, where they play a central role in carbon recycling. Streptomycetes exhibit complex multi-cellular development with branching, filamentous vegetative growth gives rise to aerial hyphae bearing long chains of reproductive spores **(Kieser *et al*., 2000 and Bentley *et al*., 2002)**.

They belong to the order Actinomycetales, which includes, besides a large number of antibiotic-producing species, also major pathogens like *Mycobacterium tuberculosis*, *Mycobacterium leprea* and *Corynebacterium diphtheria* reviewed in (**Embley and Stackebrandt, 1994**).

Species of the genus *Streptomyces* are the source of several useful antibiotics that are used not only in the treatment of various human and animal diseases but also in agriculture and biochemistry **(Martin, 1982; Demain *et al*., 1983; Ubukata *et al*., 1995; Hayakawa *et al*., 1996 and Jones, 2000)**. At least 70 of the approximately 100 marketed antibiotics used for the