ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL METABOLITES FROM STREPTOMYCES SPECIES.

This report presented in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

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MAY 2015

DECLARATION

I hereby declare that, this project report is done by me under the supervision of **Dr. Zakia Sultana Sathi, Associate Professor,** Department of Pharmacy, Daffodil International University, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy. I am declaring that this Project is my original work. I also declare that neither this project nor any part thereof has been submitted elsewhere for the award of Bachelor or any degree.

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ACHKNOWLEDGEMENT

Praise to Almighty Allah who has given me strength, patience and ability to make this project possible and for his providence, grace and mercy upon my life.

It's my pleasure to express my heart-felt gratitude and indebtedness to my reverend teacher and research supervisor Dr. Zakia Sultana Sathi, Associate Professor, Department of Pharmacy, Daffodil International University for her helpful guidance, valuable comments, sympathetic advice and unparalleled encouragement, constructive suggestions and never-ending inspiration throughout the entire period of my research work.

I thankfully acknowledge indebtedness to Muhammad Arifur Rahman, Head of the department of pharmacy, Daffodil International University for his kind co-operation and encouragement.

I want to covey my special thanks and appreciations to Prof, Dr. Md. Selim Reza, Advisor, Department of Pharmacy, Daffodil International University.

I would like to acknowledge to Dr. Md. Zakir Sultan, Senior Scientist and Head, Drug Research Laboratory, Centre for Advanced Research in Sciences (CARS); University of Dhaka for giving facilities to isolated organism.

I offer my special indebtedness and gratitude to my teachers Professor Abdullah Faruque (Professor), Md. Al-Faruk (Senior Lecturer), Sharifa Sultana (Senior Lecturer), Md. Mustafezur Rahman (Senior Lecturer), Mrs. Farhana Israt Jahan (Senior Lecturer), KH Ahammad Uz Zaman (Lecturer) and Jesmin Akter (Teaching assistant), Department of Pharmacy, Daffodil International University, for valuable suggestions and inspiration during the course of my research work.

I would like to offer my thanks to other stuffs of Department of Pharmacy, Daffodil International University specially Md. Asaduzzaman (Lab assistant).

I convey my thanks to my all friends and well-wishers specially Md. Abdur Rahman Patwary, Md. Muyeen Nadim, Md. Rassel Hossain, Rummana Amin Sabana, Fatema Farah Tajin, Md. Fakhrul Islam, Md. Mohibul Alam and Suman Ahmed.

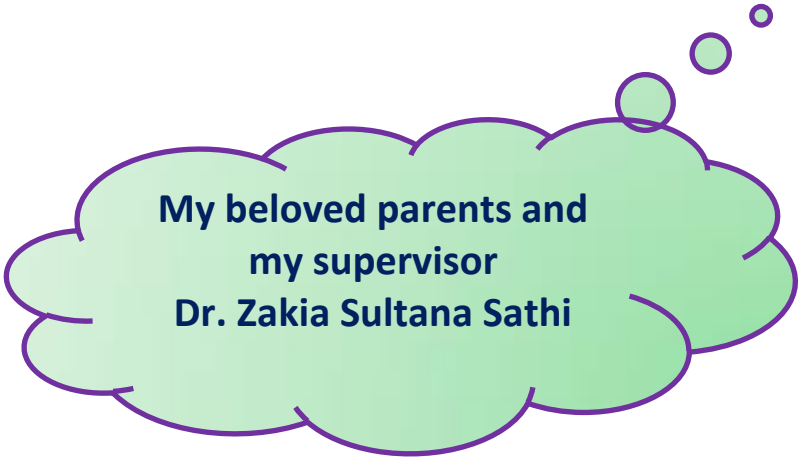
I also extend my thanks to my younger brother Abdul Alim, for his support in my dissertation.

Finally, but by no means least important, I am deeply grateful to my father Md. Abu Taher Shekh, my mother Mst. Nargis Begum, my uncle Md. Al-Amin Talukder and my brothers for their immeasurable sacrifice, continuous inspiration and financial assistance and blessing when they were needed most.

Md. Abu Naim Shekh

May 2015

Dedicated to



ABSTRACT

The focus of this study was the isolation of Streptomyces species which is antibiotic-producer from soil using a procedure for the selective growth of Streptomyces species. Actinomycetes are the most economically as well as biotechnologically valuable prokaryotes. An antagonistic microorganism (NN) was collected from soil from different fields in Dhaka and Munsigonj of Bangladesh. The microorganism was identified on the basis of its morphological characteristics as *Streptomyces* species. Bioactive metabolites were produced in the yeast-extract glucose broth media at 37.5°C temperature for 7 days.

Considerable antibacterial activity of ethyl acetate and chloroform extract of NN was against *Bacillus subtilis* and *Escherichia coli*. This organism (NN) showed positive antibacterial activity against both pathogenic test organisms.

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ABBREVIATIONS

g	: Gram
L	: Liter
mg	: milligram
ml	: milliliter
mg/dL	: milligram per deciliter
$CHCl_3$	: Chloroform
E. coli	*:Escherichia coli*

CHAPTER ONE

INTRODUCTION

1.1. General Introduction

Antibiotics or antibacterials are a type of antimicrobial used in the treatment and prevention of bacterial infection. They may either kill or inhibit the growth of bacteria. Several antibiotics are also effective against fungi and protozoans, and some are toxic to humans and animals, even when given in therapeutic dosage. Antibiotics are not effective against viruses such as the common cold or influenza, and may be harmful when taken inappropriately. Antibiotics revolutionized medicine in the 20th century, and have together with vaccination led to the near eradication of diseases such as tuberculosis in the developed world. Their effectiveness and easy access led to overuse, especially in live-stock raising, prompting bacteria to develop resistance [1].

Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. Staphylococcus aureus, for instance, a virulent pathogen that is responsible for a wide range of infections including pimples, pneumonia, osteomyelitis, endocarditis and bacteremia, has developed resistance to most classes of antibiotics [2]. The actinomycetes are Gram positive, organisms that tend to grow slowly as branching filaments. Many actinomycetes will grow on the common bacteriological media used in the laboratory, such as nutrient agar, trypticase soy agar, blood agar, and even brain-heart infusion agar. Actinomycetes encompass a wide range of bacteria. They have universal occurrence and play an active part in the cycle of nature [3, 4].

Antibiotics are the best known products of actinomycete. The morphology of an actinomycete growing on agar can provide useful and rapid clues to its identity, but viewing isolated colonies can give little worthwhile information. Morphological characters are still widely used for characterizing genera, for example, the presence or absence of spores on the substrate mycelium or the formation of zoospores in specialized spore vesicles or sporangia. The ability to produce motile spores is more widespread in the actinomycetes [5]. The present study is an attempt to produce antibiotics from actinomycetes, isolated from soil, by fermentation and the determination of their antimicrobial activity.

The soil microorganisms would provide a rich source of antibiotics. The number and kind of microorganisms present in soil depend on environmental factors such as amount and type of nutrient available, immune, degree of aeration, temperature, pH etc. [6]. In1977 Martin and Focht describe the relationship between number and kinds of microorganisms and the effect of pH on the number and kinds of microorganisms commonly found in soil. Table -1.1 represents the approximate number of organisms commonly found in soils. Table -1.2 represents number of bacteria and fungi in soil fertility plots at riverside's California, in relation to soil pH.

Table-1.1: Approximate numbers of organisms commonly found in soil. [7]

Organism	Estimated numbers/gm.
Bacteria(Other than actinomycetes)	3,000,000-500,000,000

Actinomycetes	1,000,000-20,000,000
Fungi(other than yeast)	5,000-900,000
Yeast	1,000-100,000
Algae	1,000-500,000
Protozoa	1,000-500,000
Nematodes	50-200

Table-1.2: Numbers of bacteria and fungi in soil fertility plots at riverside's California, in relation to soil pH [7].

Soil pH	Bacteria (millions/gm.)	Fungi (thousands/gm.)
7.5	95	180
7.2	58	190
6.9	57	235
4.7	41	966
3.7	3	280
3.4	1	200

Actinomycetes especially *Streptomyces* use a wide range of organic compounds as sole sources of carbon for energy and growth [8]**.** The optimum temperature is 25°C-35°C, some species grow at temperature within the psychrophilic and thermophilic range; the optimum pH range for growth is 6.5 - 8 .0 [9]**.** One gram of soil contains millions of different types of microorganisms. In order to isolate Actinomycetes, serial dilution technique [10] and enrichment culture techniques [11] are used.

With the emergence of new diseases, screening of microorganisms for the production of new antibiotics has rapidly increased during the last three decades. The majority of studies were focused on the exploitation of antibiotics from fungi and actinomycetes, which are capable of producing secondary metabolites with widely divergent chemical structures.

Parallel to the screening for new antibiotics, efforts have been focused in finding low molecular weight secondary metabolites with other biological activities. Among others, secondary

metabolites with activity as enzyme inhibitors, plant growth stimulators, herbicides, insecticides, antihelminthics and immunosuppressant's have been obtained.

Two main strategies have been used during the screening of microbes for the isolation secondary metabolites. The first strategy is to isolate the known secondary metabolites to evaluate the biological activities not mentioned in the literature and the second one is to isolate novel secondary metabolites with various biological activities.

Most of the secondary metabolites were used in the clinical practice as antibiotics. Some metabolites like mithramycin, bleomycin, daunomycin and adriamycin were used as antitumor compounds. Microbial secondary metabolites have specific and complex chemical structures, with fascinating array of diverse and unique functional groups. Studies on secondary metabolites revealed the fact that the microbial secondary metabolites have unique molecular skeleton which is not found in the chemical libraries which makes the chemist unable to synthesize more than 40% of the metabolites [12].

For the wellbeing of mankind one of the major contributions of science and research has been the isolation of variety of antibiotics from microorganism and their use as chemotherapeutic agents for the treatment of infectious disease. Most bacterial infections can successfully be controlled by means of the presently available antibiotic and chemotherapeutic agents. However, in course of time, more and more resistant bacterial strains have developed and spread under the selecting effect of various antibacterial drugs. The annihilation of these strains presents a serious problem and continuously requires new, more efficient antibiotics and chemotherapeutic agents. The outstanding successes that have been achieved in the chemotherapy of bacterial infection during the past few decades have, unfortunately, not been paralleled in virus infections, tuberculosis, AIDS and cancer. However, even in these fields the encouraging progress that has been made led to a further intensification of the search for active drugs.

Antibiotic research is the most important field in the microbial biotechnology to produce compounds against pathogenic microorganisms. Antibiotics represent greatest contribution of drug therapy [13]. The process of new drug discovery is driven largely by the desire to identify a structurally novel compound that possesses novel and potentially useful biological activity [14].

1.2 Aim of the work

The pathogenic microbes, with their determination to survive are gaining resistance by curious mechanisms and most of the effective antibiotics are becoming resistance due to indiscriminant use. As a result, microbial drug resistance is one of the most serious problems which the human-race is facing today. Beside this, new diseases like cancer and newer disease like AIDS are all around the world. So, medical scientists and researchers are searching for newer, safer

and more effective and improved antibiotics to combat against drug resistance mutants as well as to combat newer diseases. The present work is a part of such an effort.

Aim of the present study was isolation of marine actinomycetes as marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites.

More than 70% of our planet's surface is covered by oceans and marine environmental conditions are extremely different from terrestrial ones. Thus marine microorganism may have different characteristics and therefore might produce different types of bioactive compounds. A compilation of the microbial sources of antibiotics discovered in the United States and Japan between 1953 and 1970 revealed that approximately 85% of the antibiotics are produced by Actinomycetes. Based on this information my attempts in the present study are to find new Actinomycetes and to study its morphology, biochemical, antibacterial activities along with the isolation of antibiotic principle from them, subsequently the biological studies of the antibiotic.

1.3. The rationale for the study

The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals for the development of new therapeutic agents, and evaluation of the potential of lesser-known and/or new bacterial taxa [15]. There has been considerable progress on the detection and identification of soil microorganisms since they were reported to produce novel bioactive compounds [16, 17, 18]. It is necessary to continue to screen for new metabolites and evaluate the potential of less known and new bacterial taxa so that new and improved compounds for future use against drug-resistant bacteria or for chemical modification purposes may be developed [19].

One of the efficient ways of discovering novel bioactive metabolites is through isolating new microorganisms, especially actinomycetes which produce about 70% of the known bioactive metabolites [20]. Therefore, investigation of new ecosystems for isolation of actinomycetes is crucial for the discovery of novel actinomycetes and subsequently for natural product-based drug discovery. Recently, several studies reported the investigation of different habitats for isolation of novel actinomycetes as rich sources of bioactive compounds [21, 22], has estimated that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered, thus underestimating the actual potentials of this genus as sources of novel antibiotics.

Programmers' aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture. However, it soon became apparent during screening programs that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscoveringn known compounds [23].

Bangladesh is richly endowed with indigenous actinomycetes of which *Streptomyces* are most abundant. Hence, we hypothesize that the *Streptomyces* diversity in Bangladesh could be a potential source of novel antibacterial compounds. Extraction of these compounds, quantitative and qualitative screening of the bioactive compounds would lead to discovery of novel compound that can fight against organism pathogenic to humans. The discovery of this novel compound(s) would be useful to the pharmaceutical industry and medicine to compact prevailing human pathogens in Bangladesh and in the world at last.

1.4. Present study protocol

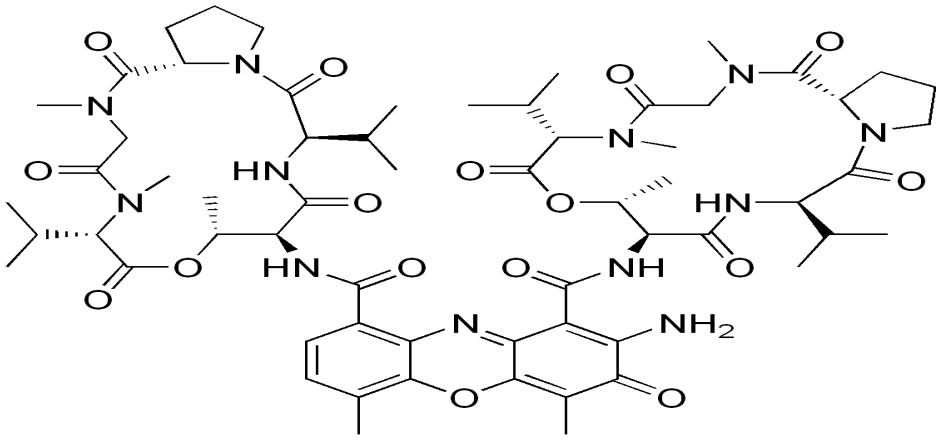
The present study protocol is designed as follows:

- ✓ Collection of soil samples from various parts of Dhaka and Munsigonj, Bangladesh.
- ✓ Isolation of antagonistic species from soil samples.
- ✓ Identification of the organisms on the basis of morphological study.
- ✓ Production of bioactive metabolites from the isolate.
- ✓ Extraction of the bioactive metabolites from the culture media.
- ✓ Susceptibility testing of metabolites
- ✓ Determination of cell cytotoxicity using brine shrimp lethality bioassay.

CHAPTER TWO

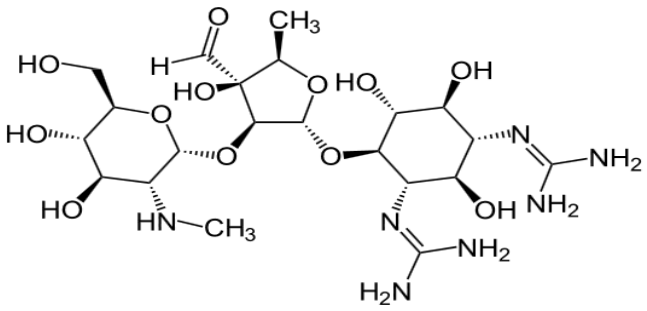
LITERATURE SURVEY

Waksman and woodruff [24] in 1940 first isolated dactinomycin, a crystalline antitumor antibiotic from the culture broth of *Streptomyces parvulus* in a nearly pure form. Farber [25] in 1966 found its clinical use in the treatment of rhabdomyosarcoma and Wilms tumor in children. He also reported that the survival rate of patients with pulmonary metastases increases with the use of dactinomycin. The chemistry of dactinomycin (I) has been determined largely through the efforts of Brockmann [26] and Johnson [27] and their co-workers. The ineffectiveness of penicillin-G in the treatment of infections due to gram-negative microorganisms was the primary stimulus for the search of antimicrobial agents effective against such bacteria. The development of streptomycin was the result of a well-planned scientific search for antibacterial substances.



Dactinomycin

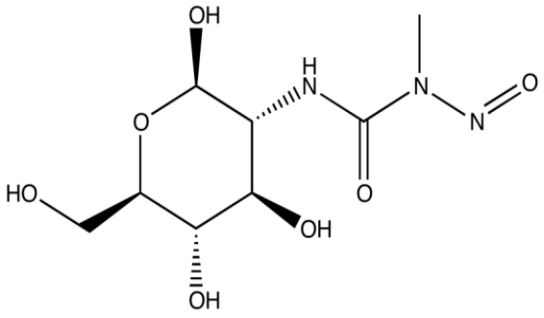
Waksman and co-workers examined a number of soil Actinomycetes between 1939 and 1943. In 1943, a strain of *Streptomyces griseus* was isolated which elaborated a potent antimicrobial substance. The discovery of this new antibiotic streptomycin by Schatz, Bugie and Waksman[28] early in 1944 and it was soon shown to inhibit the growth of the *Tubercle bacillus* and a number of gram-positive and gram-negative microorganisms *in vitro* and *in vivo.* Extensive bacteriological, chemical and pharmacological investigations of streptomycin had been carried out and its clinical usefulness was established [29].



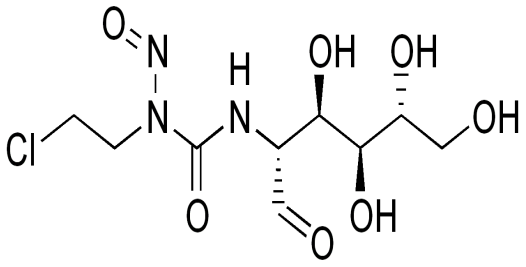
Streptomycin

Burkholder [30] in 1947 isolated **chloramphenicol** from the culture broth of *Streptomyces venezuelae.* Pharmacological studies in animals and man were soon undertaken by Smadel and Jackson. Later in 1947, the small amount of available chloramphenicol was employed in an outbreak of epidemic typhus in Bolivia with dramatic results. It was then tried with excellent success in cases of Scab typhus on the Malay Peninsula. By 1948, chloramphenicol was produced in amounts sufficient enough for general clinical use and was then found to be of value in the therapy of a variety of infections. By 1950, however, it became evident that the drug could cause serious and fatal blood dyscrasias.

Vanvra[31]**,** J; in I960isolated streptozacine from the *Streptomyces achromogenes.* Herts, R.R. established it to be the nitrosomethyl derivative of 2-deoxy glucose the diabetogenic effect of the drug makes it especially effective against malignant insulinomas.

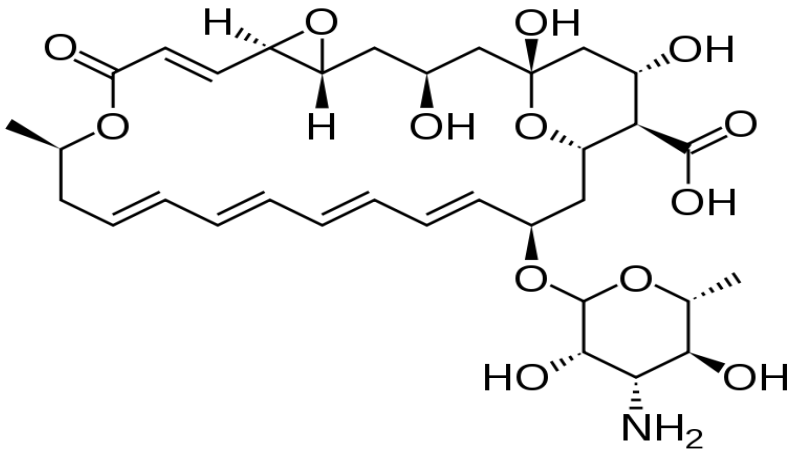


Streptozocin



Chlorozotocin

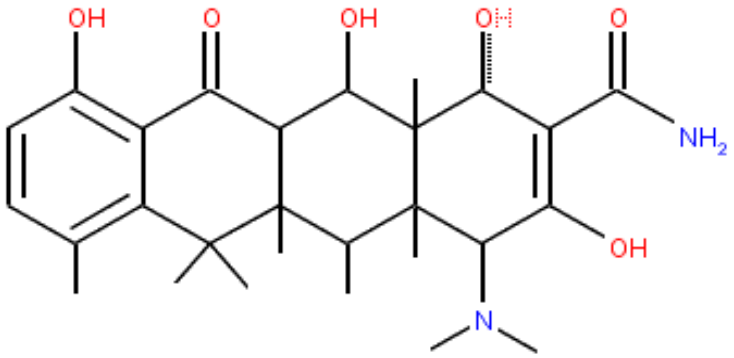
Bhuiyan[32]**.** B.K. in l965 reported nagalmycin, anthracyclin antibiotic with significant antitumor activity. It was obtained from *Streptomyce nogalater.*



Nagalmycin

Wallerslein[33] reported that of all the drugs that may be responsible for pancylopenia, chloramphenicol is the most common cause.

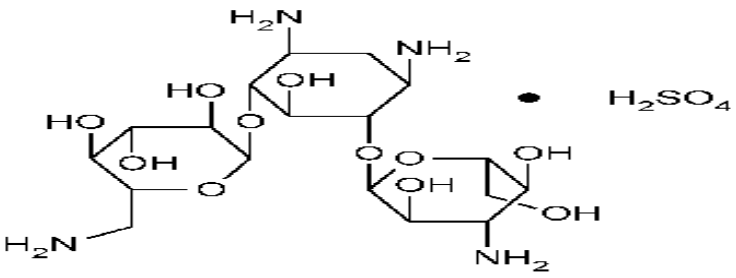
The first **tetracycline** antibiotic discovered by Duggar in 1948 was the result of, a systematic screening of soil specimens collected from many parts of the world for antibiotic producing microorganisms. The first of these compounds, chlortetracycline, was introduced in 1948.Two years later oxytetracycline became available. Chlortetracycline and oxytetracycline were obtained by *Streptomyces aureofaciens* and *Streptomyces rimosus* respectively. The antibiotics were produced in broth by deep-tank fermentation. Tetracycline was produced semi-synthetically from chlortetracycline. It was also obtained from a species of *Steptomyces* in 1952.



Tetracycline

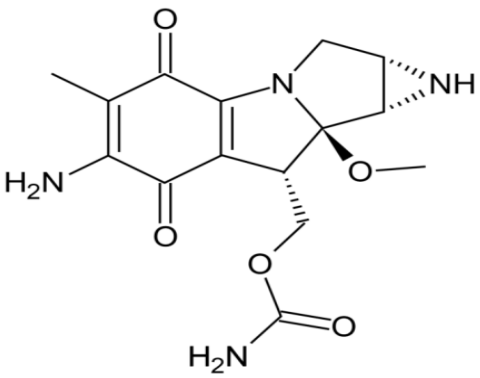
In 1949, Waksman and lechevalier isolated a soil organism, *Steptomyces fradiae,* which produced a group of antibacterial substances that were labeled "neomycin" One component of this group, neomycin B, is still utilized. However, it causes severe renal toxicity and ototoxicily when administered parentally. It should be employed only topically for its local effect on the bowel flora.

Kanamycin, an antibiotic produced by *Steptomyces kanamyceticus,* was first produced and isolated by Umezawa and co-workers at the Japanese National Institute of Health in 1957. It was found to be active against a variety of microorganisms. For several years it was an important antibiotic for the treatment of serious infections with gram-negative bacteria. Due to toxicity and the emergence of resistant microorganisms, kanamycin has largely been replaced by the newer aminoglycosides.

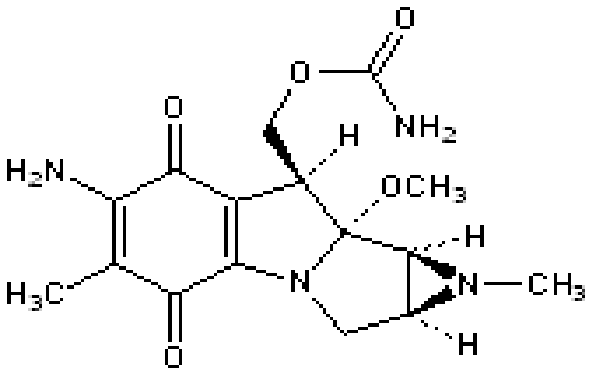


Kanamycin

Wakaki and associates (1958) isolated the aziridine ring containing antibiotic mitomycin from the culture broth of *Streptomyces csemptnsus*, Kersten[34] (1975) reported that **mitomycin** contains a urclhane and a quinone group in its structure, as well as an asiridine ring, which is essential for antineoplastic activity.

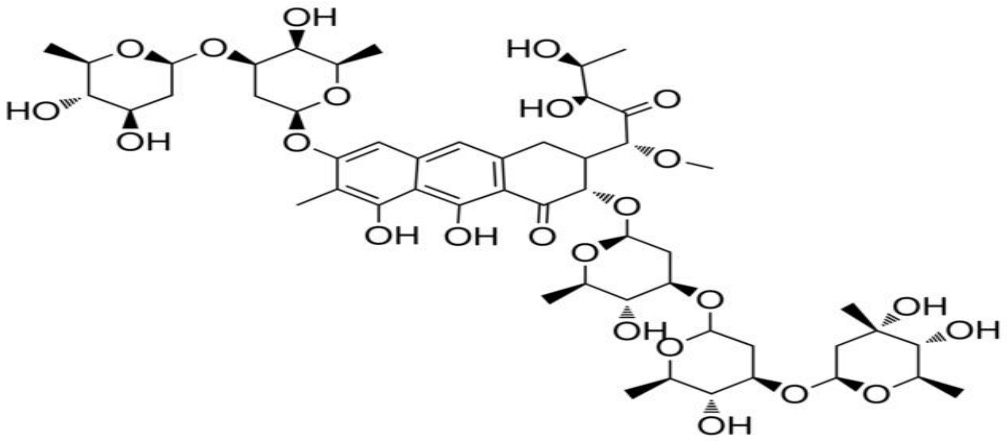


Mitomycin



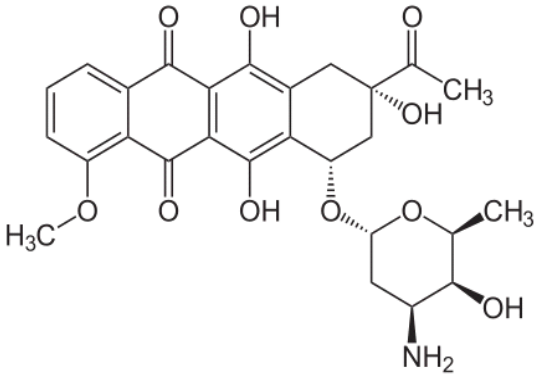
Porfiromycin

Rao and associates [35] isolated **mithramycin**, a cytotoxic antibiotic from the culture of *Streptomyces tanashiensis.* Bakhaera[71]**,** established the structure of mitfiramycin (4) and Umezawa [36] discussed the chemistry. Ream[37]. Kennedy[38], Hill[39] determined that mithramycin is beneficial to patients with disseminated testicular carcinoma, especially of the embryonal-cell type.

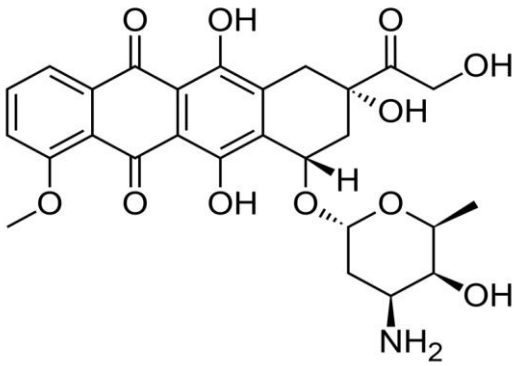


Mithramycin

Doxorubicin and Daunorubicin were the anthracycline, antibiotics produced by *Streptomyces peucetius* var. *Caesius*. Daunorubicin was isolated independently by Di Marcon and by Dubost and their colleagues in 1963. Doxorubicin was identified by Areamone and co-workers in 1969.



Daunorubicin



Doxorubicin

The clinical values of both the drugs are limited by their cardiomyopathy Dimarco[40], Samuels[41] reported the activity of Daumorubicin against solid tumors in children and lymphomas. Gottleib and Hill [42] reported that doxorbicin had activity in carcinomas of the endometrium, testes, prostrate, cervix etc.

Umezawa and co-workers[43] isolated bleomycin, a mixture of basic glycopeptides from the fermentation broth of *Streptomyces verticillus.*

Oppenheimer [44] determined the structure of bleomycin A_2 and B_2.williams and Einhorn [45] reported that bleomycin is effective in the treatment of testicular carcinomas. Bleomycin is also

useful in the palliative treatment of squamous cell carcinoma of the head, neck, esophagus, skin and the genitourinary tract, including the cervix, vulva, scrotum and penis. It is active in Hodgkin's disease and in other Lymphomas Calabresi [46]

Tastsuro Tanaka [47] isolated *Streptomyces* strain 35NT from soil which produced a new antibiotic active against *Staphylococcus aureus.* Strain 35NT had morphological and cultural characteristics resembling *Streptomyces celluloflavus.* It was active against gram-positive bacteria only and inhibited *the* growth of resistant *Staphylococcus aureus* at a concentration of 0.5 mg/ml.

Ito Yukio [48] isolated and purified new basic, water soluble antibiotic BD-12 and By-81 from the culture broths of *Streptomyces luteocolor* and *Streptomyces olivoreticuli* respectively. Both compounds were basic, Water-soluble and had activities similar to those of streptothricin. They were active in vitro against both gram positive and gram-negative bacteria.

Zbionvcky [49] reported a new basic, water-soluble antibiotic LL-AC541, produced by a strain *of Streptomyces hygroscopicus* (NRRL 3111). It was structurally related to streptothricin but devoid of beta-lysine.

Zhanovich [50] reported a pharmacologically active antibiotic glucoside used for the treatment of malignant neoplasm. The antibiotic was produced by incubating A*ctinomyces divovariabilis* on a nutrient medium.

Okuda [51] reported a new antitumor antibiotic YA-56, isolated from the culture filtrate *of Streptomyces humidus.* The hydrochloride salt of the antibiotic was effective against ascites sarcoma in mice.

Migamura [52] reported that an antitumor antibiotic 20798 RP was obtained from *Streptomyces* 31723 of *Streptomyces coerulearbidus.* It was found to be active against graftable tumors of mice, such as sarcoma 180 and leukemia LI210.

Henka [53] described a new antimetabolic antibiotic U-42126 was produced by *Streptomyces sviceus* grown in a mannitol media on a rotatory shaker at 32°C. Taxonomic studies of culture including microscopic, cultural and biochemical characteristics and carbon utilization tests, showed that the culture was different from *Streptomyces* species described in Bergay's Manual and other references. The *in vitro* antimicrobial activity of the antibiotic was limited to fungi, *E. coli* and *B. subtilis* were inhibited by U-42, 126 only when cultured on a synthetic medium. Al

400 mg/kg, it extended the life of mice injected with LI21O and P-388 leukaemias by 40% and 37% respectively.

Two Japanese researchers**, Ishida and Nakoa**[54] reported a new antibiotic having antineoplastic effect. The antibiotic TS-885 was produced by submerged aerobic fermentation of *Streptomyces phoricolorescons* var. *Yamashitaensis* S-885 ATCC 21956. The isolated compound had bactericidal and antiviral activity and inhibited the growth of ascites tumors in mice.

Shomura [55] isolated two new antibiotics SF-1306 A and B from the culture filtrate of *Streptomyces echinatus* and reported their structures, physical and chemical characteristics and cytotoxicity. The antibiotic A was decomposed at 170-178°C and was soluble in acetic acid and water. It had absorption maxima at 258 nm and was effective against HeLa cells. The antibiotic B was decomposed at 158-162°C and was soluble in water. It had also absorption maxima at 258 nm but did not have any effect against HeLa cells.

Nara, Takashi [56] isolated of a new antibiotic XK-49-I-B-2 from the culture filtrate of *Streptosporangium violaceachromogenes* ATCC 21807 grown in an aqueous nutrient medium at 30°C with aeration. The antitumor activity was found against Ehrlich ascites tumor cells and sarcoma 180 in mice.

Soeda and Momoe [57] reported a novel antitumor substance isolated from the culture filtrate Streptomyces *hachijoensis H2609*. The microbe was cultured aerobically at 30° for 7 days.

Bycroft[58] isolated a highly modified peptide antibiotic althiomycin form *Streptomyces* althioticus and *Streplomyces* matensis. The structure was determined by spectral and chemical methods.

Aoki-H [59] reported a new monocyclic antibiotic Nocardicin A obtained *from* the fermentation broth of a strain of Actinomycetes . The antibiotic producing organism, strain WS I57L was identified as *Nocardia uniformis suhsp. Tsuycimanensis ATCC* 21806. The antibiotic obtained as colorless crystals, exhibits moderate *in vitro* antibacterial activity against a broad spectrum of gram-negative bacteria including *proteas* and *pscudomonas.*

Uhmura [60] reported a new antileukaemic substance OS I 3256B. It was produced by *Streptomyces candidus*- var. *azaticus,* after culturing aerobically at 28°C for 2 days.

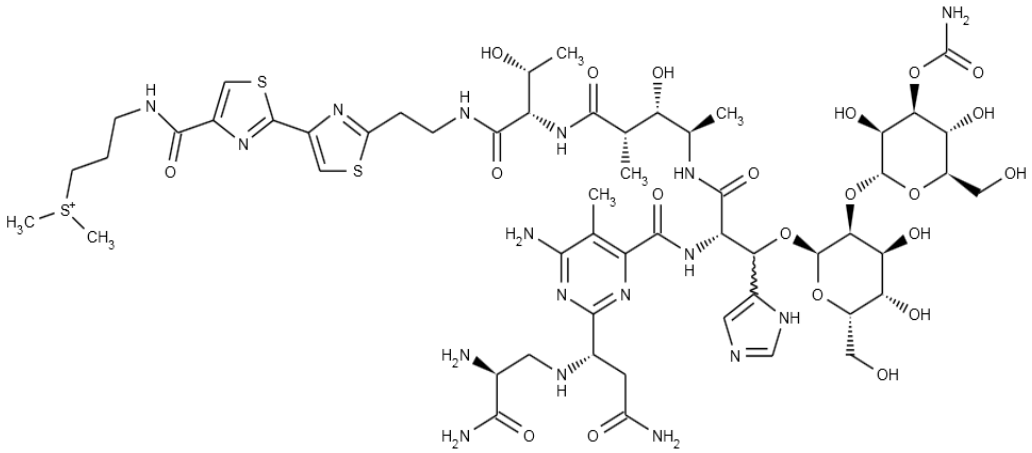
Omoto [61] described a new water-soluble antibiotic produced by *Streptomyces myxogenes*. It was purified over cellulose column chromatography and found that SFO1130 was an oligosaccharide composed of glucose. It showed antimicrobial activity mainly against gram-negative bacteria and was also active against sarcoma S-180.

Mori [62] isolated a new antibiotic FA-313 produced by *Streptomyces chromovarius* FA -313. The microbe was aerobically cultured at 28°C for 3-4 days on 35 liters medium (p^H 6.5). The antibiotic was effective against gram-positive, gram-negative and acidophillic bacteria and had an anticancer activity. The LD_{50} against mice was 1 mg/kg.

Sumiyama [63], reported a new antibiotic obtained from *Streptomyces* or *Actinomycetes* grown on a complex media. It showed antitumor activity *in vitro* against S-180, AH-66 and Sarcoma cells. It was soluble in water and insoluble in methanol, ethanol, acetone, ethyl acetate, benzene and chloroform.

Chan, wei [64] reported the isolation and characterization of a new antitumor antibiotic FCRC-53 produced by *Streptomyces griseus.* The antibiotic exhibited cytotoxic activity to both KB and P-388 cell lines' and inhibitory activity on P-388 ascites tumor. Chemically it closely resembled prumacetin -A.

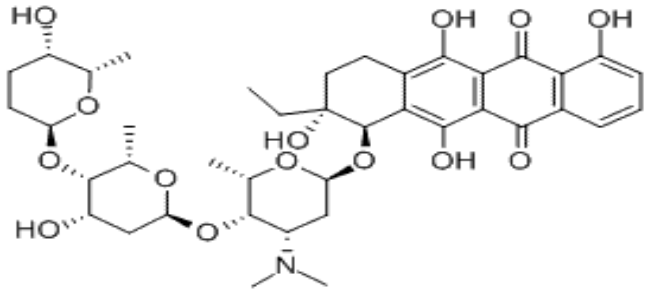
H. Umezawa [65] isolated an antibiotic PO-357 from the culture filtrate of *Streptosporangium* PO-357. The MIC was determined against 22 bacteria. The anticarinogenic activities were obtained against leukemia L-120, Ehrlich ascites cancer cells and sarcoma 180 in mice. Shomura [66] isolated a new antibiotic related to bleomycin, called SF-1961. It was isolated from the fermentation broth *of Streptomyces filamentosus.*



Bleomycin

Kohono –M [67] isolate an antitumor antibiotic TC-13 that was a protein -bound polysaccharide extracted from a rare actinomycetes and was tested on allogeneic and syngeneic tumors in mice. TC-13 showed antitumor activity against syngeneic tumors MM46 mammary carcinoma and MCS-8 meth Afibrosarcoma.

Audo, Toshihiko [68] isolated cosmomycin C and D from the mycelial extract of *Streptomyces cosmosus* TMF- 518 .The molecular structure of both the compounds were detected by physicochemical procedures including chromatography and NMR spectroscopy. Both the compounds were antitumor anthracycline antibiotics and both caused differentiations of Friend Leukemia cells.



Cosmomycin-C

Hussein [69] isolated a new antibiotic S. S. 20 from the fermentation broth of *Streptomyces saraceticus.* Fishmeal extract was used as the fermentative medium for antibiotic production. The antibiotic was extracted from dried broth by MeOH and purified by preparative paper chromatography.

Ivanova[70] isolated three antibiotic complexes from the culture fluid of a *Streptomyces species* from Bulgarian soils. The non-polynic macrolide AK-164 contained a guanidine moiety and its properties and biological actions resembled scopafungin, melanosporin, infymycin, azalomycin, copiamycin, and guanidofungins A and B. The complex A- 164 AB was identical with the antibiotic elaiphylin (azalomycin B). The complex IM-164 consisted of six components. The major component was identified as nigericin, whereas other components were probably nigericin derivatives.

Saitoh, kyoichiro [71] reported the production, isolation and properties of boholmycin, a new aminoglycoside antibiotic from s*treptomyces hygroscopicus* HG17-25. It has a

pseudotetrasaccharide structure composed of a heptoses, 2 amino sugars and dicarbamoyl-scyllo-inositol. Intrinsic l activity of the antibiotic is weak but it exhibits broad-spectrum activity against gram-positive and gram-negative bacteria, including aminoglycoside resistant strains.

Elson [72] reported the production, isolation and properties of new quinone antibiotic of the granaticin type. Six antibiotics designated MM 44326, MM 44325, MM 44785, MM 44787, MM 44786, and MM 44788 were isolated from the culture filtrate of *Strptomyces lateritius* by extraction with chloroform followed by column chromatography on sephadex LH-20. The antibiotics were characterized by the mobilized on silica gel TLC and by their UV spectra. The compounds inhibited gram positive bacteria but were inactive against gram-negative organisms and *Candida albicans.*

Ajito, Kayo [73] reported the isolation of a new peptide antibiotic SF 2425 from the fermentation broth of *Streptomyces violaceus* SF 1425.The structure of the antibiotic was deduced to be I-N carbamimidoyleulicin from chemical and spectral studies. The antibiotic was active against some gram-positive and gram-negative bacteria and fungi.

Seaton[74] isolated four new colored components of *Streptomyces murayamansis,* the producer of the Kanamycin and determined the structures by a combination of mass spectral, high field NMR and biosynthetic technique.

Oishi –H[75] has produced thiolactomycin, a novel antibiotic ($C_{11}H_{14}O_2S$) containing a unique thiolactone moiety in its molecule .On the basis of taxonomic studies the Producing organism was identified as belonging to the genus Nocardia .The antibiotic exhibits a broad antibacterial spectrum and particularly potent activity against. *Salmonella, Serratia* and *Bacteroides.*

Mitscher –LA [76] isolate Siderochline a fermentation product of an unusual actinomycetes sp.

Me Alpine –JB [113] isolated novel macrolide antibiotics from a genetically manipulated actinomycetes. The major components produced have been isolated and identified as 2-norerythromycins A,B,C and D by mass spectrometry and extensive 1D and 2D NMR experiments.

Imai-H [77] isolated Okilactomycin, a novel antibiotic from the culture filtrate of *Streptomyces griseoflovus* subsp. Zamamiensis subsp. Nov. The antibiotic was obtained as colorless prisms from a dichloromethane solution .It exhibited weak antimicrobial activity against Ehrlich

ascites carcinoma *in vivo.* The apparent molecular formula of okilactmycin was determination as $C_{24}H_{32}O_6$. It is a new member of the lactone group antibiotics.

Dobashi-K [78] isolated two antifungal antibiotics octacosamicins A and B, from the culture broth of a strain of Actinomycetes, which were identified as a strain of Amycolatopsis, These antibiotics were isolated by resin adsorption and purified by preparative HPLC. Both antibiotics showed broad antifungal spectra.

Nakayama-M[79] isolated two novel antifungal antibiotic maniwamycins A and B from the culture broth of *Streptomyces prasinopilosus.* The antibiotics were isolated by resin absorption and extraction with EtOAc and purified by column chromatography. They showed broad antifungal spectra.

Volkalupova- OP and Makukho- LV[80] isolated antineoplastic antibiotics using a rapid method of evaluating their cytostatic effect on tumor cells .The method was applied to substances isolated from 600 culture of Actinomycetes, 35 antibiotics showed antitumor activity; 8 of them were studied in detail.

Kojiri –K [81] isolated a new antitumor substance BE-I3793C, produced by *Streptomycete.* The active principle was extracted from the mycelium of strain BA13793 with methanol and purified by Sephadex LH-20 column chromatography. BE-I3793C showed strong inhibitory activity against topoisomerases I and II inhibited the growth of doxorubicin resistant or vincristine resistant P 388 murine leukaemia cell lines, as well as their parent P388 cell line.

Karwowski-JP [82] isolated Danaimycins a new complex of Spiroketal 24- membered macrolides discovered in the fermentation broth of *Streptomyces diastatochromogenes* strains AB169IQ-32land ABI711j452.The dunamycins possess both immunosuppressive and antimicrobial activity.

Tomita-K[83] isolated new antibiotics Fluvircins Al, A2, B1 B2,B3, B4, and B5 that are active against influenza *A. virus.* Five strains of Actinomycetes were classified and designated as follows. Strains Q 464-31(fluvirucin AI producer), Microtetraspora tyrrhenii Sp. Nov. (Actinomadura Pusilla group) Strain L407-5 (fluirueim B2 producer) A maduromycete. Strain R 359-5 (fluvirueiv B2 producer): Microlelraspora pusilla (*Actmomadura pusilla* groups) strain R869-90 (fluvirucin A2 producer): Sacchharothrix mutabilis. Strain R516-16 (fluvirueins B2, B3, B4 and B5 producer): A maduromycete.

Takeuchi-M [84] isolated helvecardin A and B, novel glycopeptide antibiotics from culture broth of strain of actinomycete identified as *Pseudonocardia compacta* sub-sp. By affinity chromatography of D-alanyl-D-alanine and preparative HPLC. Though helvecardin A was structurally related to beta-avoparcin, it is clearly differed in the presence of an o- methyl moiety in its NMR spectrum. The physicochemical properties of helvecardin A and B are resembled to each other.

Papp-M [85] reported the anti-inflammatory effect of a proteinkinase C inhibitor (K-252a) on the development of the dextran-induced paw edema in the rat .The effect of a metabolite of Nocardiopsis Sp. as a proteinkinase C inhibitor from microbial origin was investigated on the onset and development of dextran-induced paw edema in the tat .The 1mg/kg close of K-252a retailed the appearance of the edema by 1 hour, the 3 mg/kg dose, however, prevented its onset for 4 hours.

Hashimoto-M, Hayashi-K [86] isolated WS9326A, a novel tachykinin antagonist isolated from *Streptomyces violaceusniger*. Tachykinin antagonists exhibit therapeutically useful anti-asthmatic activity. Here WS9326A binds comparatively to [3H] substance P (NK-1 receptor) binding sites on guinea-pig lung membranes (LC_{5o}=3.6x 10(-6) m), and acts as a tachykinin antagonist in various functional assays.

Nakanishi-S[87] isolated a novel compound, KS-505a from the culture broth of a strain identified as *Streptomyces argenteolus* A-2. The compound inhibited bovine brain Ca2+ and calmodulin-dependent cyclic-nucleotide phosphodiesterase with an LC_{5o} value (The concentration causing 50% inhibition) of 0.065 microM.

Takeueh-M[88] isolated Galacardins A and B, new glycopeptides antibiotics from a stain of Actinomycetes identified as *Saceharothix Sp,* SANK64289. They were structurally related to beta-avofoarein but differed from sugar composition only. They showed strong antimicrobial activity against Gram-positive bacteria and also showed excellent *in vivo* protective activity against *Staphylococcus aureus* infection in mice.

Tabata –N[89] isolated Diolmycins new anticoccidial agents produced by *Streptomyces* sp. The structure of diolmycins Al, A2, Bl and B2 here determined by spectroscopic analyses. Diolmycins A1 and A2 are stereoisomers with the structure of l-(3-indolyl)-4-(p-hydro-xyphenyl) 2, 3-butanediol. From the chemical synthesis of the erythro-isomer, of diolmycins

A1 and A2 were determined to be the erythro and threo-isomers, respectively. The stereoisomers, diolmy- cins Bland B2, were also deduced to be erythro and threo-1, 4-di- (p-hydroxyhenyl) -2, 3-butanediol, respectively.

Sing-MP [90] isolated bioxalomycins, a novel complex of broad-spectrum antibiotics, from fermentation of *Streptomyces viridodiastaticus* subsp, litoralis LL-3IF508. Bioxalomycin alpha2, the major component of this complex, exhibited antibacterial activity. The MICs ranged from 0.002 to 0.008 μg/ml for gram positive organism and from 0.50 to 4μg/m for gram-negative organisms. Bioxalomycin alpha-2 was found to be bactericidal and to inhibit bacterial DNA synthesis preferentially. Bioxalomycin alpha-2 protected mice from a lethal challenge with *Staphylococcus aureus* Smith.

Yasuzawa-T[91] isolated seven novel potent antitumor antibiotics, duocarmycins A (1), Cl(2), C2(3), D(4), Bl(5),B2(6), and SA(7), were isolated from three independently collected *Steptomyces* SP .The complete structure , including absolute stereo-chemistry, were determined by spectral and chemical studies of those duocarmycis and several derivatives . Duocarmycins A (1) and SA (7) possess a l, 2, 7, 7 a-tetrahydro cycloprop [1,2- c] indol-4-one subunit, a common pharmacophore with that of CC-1065(10) found from *Streptomyces zelensis.*

Ueki-M[92] isolated a novel antifungal antibiotic, UK- 3A, obtained from the mycelial cake of Streptomyces sp. 517-0.2. UK - 3A was very similar in structure to UK -2A, a structural relative of syntimycin A. The antifungal spectrum of UK- 3A was relatively broad (MICs for yeast and filamentous fungi: 1.56-6.25 and 0.39-1.56 µg/ml, respectively) .The cytotoxic activity of UK- 3A was weak (IC50: 18-100 µg / ml)

Aotani-Y[93] isolated Lympliostin (LK6-A), a novel immunosuppressant was isolated from the culture broth of Streptomyces sp KYII783. Lympliostin was determined by spectroscopic methods to be a novel tricyclic aromatic alkaloid. A key feature of the structure is the pyrrolo [4, 3, 2-de] quinoline moiety which is rate among natural products.

Bormann-C[94] produced novel nikkomycins *Lx* and Lz by genetically engineered *Streptomyces tendae* TU90 I.

CHAPTER THREE

METHODS AND MATERIALS

3.1. Collection of soil sample

Soil is the major source of microorganisms. It has the appropriate conditions for microbial growth. However, condition of soil as well as its microbial content and type varies from place to place and with its depth. Thus for screening purposes soil samples were collected from a wide range of places like various parts like different parts of lake, playground, construction sites, grave yards, cultivated land of Dhaka and Munsigonj. Soils of depth ranging from 0.25 to 1.5 meter were collected. The soil samples were stored in small clean polyethene bags and were tested for antagonistic species by "Crowded plate technique".

3.2. Isolation of the antagonistic organism

3.2.1. Serial dilution technique of isolation

A pinch of soil contains millions of different types of microorganisms and it is impossible to identify a particular organism from there due to overlapping of growth. As isolation is necessary the numbers of microorganisms that grow on the plate were reduced by serial dilution technique. This was done by dilution of 1 gm of collected soil sample with 100 ml of distilled water in a 250 ml conical flask and shaken vigorously to form a uniform suspension of soil. This was named the stock suspension. One ml of this stock suspension was added to 99 ml of sterile distilled water to get a dilution of 1:10000. The flask was shaken vigorously and 1 ml of diluted was transferred by the same method to another 250ml conical flask 99 ml of sterile distilled water, thus a dilution of 1:1000000 was obtained.

3.2.2 Media for the isolation of the organism:

Soil is the most important source of a fascinating microbial world where different types of microorganisms are found and each type of organism requires specific growth condition and a source of energy, which is supplied by the media where they are grown.

Therefore, it is essential to try various types of media for the optimum growth of various organisms. My attention was concentrated on actinomycetes, as most of the effective antibiotics are isolated from these types of microorganisms. The following media were tested for the isolation of organisms from soil samples:

1. Yeast-Extract Glucose Agar
2. Tryptosen Soya Broth Media
3. Nutrient Broth Media

3.2.3. Crowded Plate Technique [95]

All the ingredients of Yeast- extract glucose agar medium were measured and dissolved in distilled water. It was then heated in water bath to give a transparent solution. Test tubes were marked as 10^2, 10^4, and 10^6 (three for each dilution) and 10 ml of the heated media after cooling to about 45°C was transferred to each of them. One ml of each soil dilutions as described in section 2.2.1 was added to the respective test tubes containing medium. The test tubes were agitated by rotation to give a uniform distribution. The contents of the test tubes were separately poured into sterile petridishes marked as 10^2, 10^4, and 10^6 (three for each). The petridishes were then rotated first clockwise and then anticlockwise for further uniform distribution of soil sample into the medium. Finally, they were allowed to solidify and incubated in inverted position at 37.5°C in an incubator.

3.2.4. Examination of the Plates:

After 3 days of incubation, the plates were checked both visually and microscopically to detect Actinomycetes species. Approximately 15 soil samples were collected from different places and depths and were tested for this purpose. One Actinomycetes species having clear zone around their colonies and those was microscopically identified as Actinomycetes species was then transferred and streaked by means of a sterile loop to the plates containing sterile Yeast- extract glucose agar medium. This was designated as NN. After 2 to 3 days of incubation, the streaking position turned into uniform growth of the organism without any contamination.

3.2.5. Pure Culture of the Identified Species by Serial Dilution:

In order to get a more pure culture of the identified species, serial dilution technique was followed. The procedure is described below:

i. 100 ml of distilled water was taken in each of six (250 ml) conical flasks, plugged with cotton, covered with paper, sterilized and cooled.
ii. A loop full of the identified organism were transferred to the first conical flask mixed well with the help of a sterile needle under a laminar air flow unit, the environment that was previously sterilized by the aid of UV lamp.
iii. 0.5 ml liquid from the first flask was transferred to the second flask by means of the sterile pipette and the same procedure was followed for the third flask.

iv. 0.5 ml liquid from each flask was removed, placed on to the surface of sterile yeast-extract glucose agar plates and spread evenly over the surface by means of a sterile bent glass rod.

v. The plates were incubated at 37°C for 10 days.

3.3. Suitable media for optimum growth of the isolated organism

All kinds of life, from microorganisms to human beings, share certain nutritional requirements for growth and functioning. These nutritional requirements vary from race to race and also from species to species. When a microbial medium confirms the optimum nutritional requirement then the growth of the organism is optimum. So, the isolated two organisms were inoculated in the following media to choose a suitable medium for optimum growth. The media are as follows:

1. Yeast-Extract Glucose agar
2. Tryptosen Soya Broth Media
3. Nutrient Broth Media

The compositions of the media are given in the appendix.

3.4. Examination of the antagonistic activity by diffusion assay method

The bacterial sensitivity can be measured *in vitro* by a number of methods among which the diffusion assay method is acceptable for preliminary evaluation of antimicrobial agents. The principle of this method is based on the ability of compound to diffuse from a confined source through the nutrient agar gel and create a concentration gradient. If the agar is seeded or streaked with a sensitive organism, a clear zone of inhibition will result around the place of application of the compound where the concentration of the compound exceeds the minimum inhibitory concentration (MIC) for that particular organism. To perform this test the following media and test organisms were used.

MEDIA:

i. Yeast- extracts glucose Agar

ii. Nutrient Agar

TEST ORGANISMS:

i. *Bacillus subtilis*

ii. *Escherichia coli*

PROCEDURE:

i. A streak of the antagonistic microorganism was made by means of a sterile loop in a plate containing sterile Yeast- extract glucose agar medium.

ii. The plate was incubated for 7 days to allow sufficient time for growth as well as concomitant production of antibiotic in the medium.

iii. Nutrient agar medium was prepared, poured into clean test tubes, plugged with cotton and then autoclaved.

iv. After cooling to about 45°C the medium in the test tubes were seeded with the test organisms by means of a sterile loop and rotated for uniform distribution.

v. The contents in the test tubes were poured into sterile petridishes and allowed to keep some time to solidify.

vi. A piece of solid agar medium containing the isolated organisms was transferred to these plates.

vii. The plates were kept overnight in a refrigerator for diffusion of antibiotic (which were produced by the organism) into the nutrient agar medium.

viii. After then the plates were incubated at 37.5°C for 24 hours.

ix. Clear zones in the petridishes around the isolated organism containing medium indicated the antagonistic activity of the organism.

3.5. Morphological studies and identification of the isolated organism

The following methods were successfully used for the morphological studies of the selected organisms:

3.5.1. Preparation of inoculums for visual observation

3.5.1.1 Colonial morphology

The strains were cultured in yeast-extract glucose agar plates, after 9 days colony characteristics such as elevation margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and the rate of growth were studied.

3.5.1.2 Appearance on agar slant

The selected organisms were transferred to agar slants. The modes of their growth on slants such as rhizoidal spreading adherent or shiny etc. were studied.

3.5.2 Preparation of Inoculum for Microscopic Observation

Cover slip culture technique was successfully used for the morphological studies of the selected strains.

3.5.3 Cover Slip Culture in Solid Media.

As sterile cover slips were inserted aseptically into the sterile solidified yeast extract glucose agar medium in a Petridis at an angle of 45° to 60° by means of a pair of sterile forceps. The organisms were inoculated by streaking method at the junction of the medium and the free space of the cover slip. The plate was then incubated at 37.5°C for different time interval. The hyphae of the organisms grew, spread on the cover slip and produced spores. The cover slips were then taken out, mounted on slides and observed under microscope using cotton blue and lactophenol.

3.5.4 Morphological Studies of the Organism:

In order to carry out the morphological studies the organism NN was streaked on Nutrient agar media and Yeast-extract-agar media. It was found that the sporulation of the organism was slower in nutrient agar medium than in other media, which is beneficial to observe the intermediate stages carefully. For the observation of the aero hyphae, the Yeast-extract-glucose-agar media was preferable to Nutrient agar media. The organism was grown on liquid medium to avoid the interference of the solidified agar during visualization. The well grown organisms were

transferred to clean glass-slides separately for the observation of both the aerial and vegetative hyphae. It was found that the organism sporulated within 3 days on almost all the media used and particularly within 40 hours on yeast-extract-glucose agar.

3.6. Production of antibacterial compound in liquid media

The selected isolate, NN is capable of producing antibacterial agent and as it is difficult to extract the component from the solid media. Since the antibacterial compound could easily be isolated from the liquid culture filtrate either by different organic solvent extraction or by other convenient chemical processes, the organism NN was tried to culture in liquid media.

3.6.1 Solvents Extracts:

I. Chloroform extract of NN
II. Ethyl acetate extract of NN

3.6.2 Test Organisms:

I. Bacillus subtilis
II. Escherichia coli

3.6.3 Procedure:

When the organism was grown in liquid medium, they secreted antimicrobial principle in the liquid medium. The cultures filtrate of NN was extracted with ethyl acetate and chloroform, respectively. After evaporation of the solvent, the extract was tested for their antimicrobial activity by disc-diffusion method.

3.7 Isolation of Antibacterial compound from the Culture Media

The organism NN were allowed to grow in the yeast-extract glucose broth medium at 37.5°C for 7-day in separate conical flasks. The medium became dark brown in color. The dark browns liquids were then separated from it mycelium mat and filtered through fresh cotton. The culture filtrate thus obtained was preserved for the extract of the antimicrobial component.

3.8 Extraction of Antibacterial compound from the Culture Filtrate

For extraction purpose the following procedure was followed.

3.8.1 Prerequisites:

i. Chloroform
ii. Ethyl acetate
iii. Separating funnel (250 ml)
iv. Measuring cylinder (25, 50 and 1000 ml)
v. Beakers (100, 500 and 1000 ml)

3.8.2 Procedure:

100 ml of culture filtrate of NN was taken separately in two separating funnels. These were then shaken with 3 x 30 ml chloroform or 3 x 30 ml ethyl acetate. The solvent was evaporated to get the mixtures of antimicrobial component.

3.9. Antimicrobial screening of chloroform and ethyl acetate

3.9.1. Introduction:

The extracts were screened against one gram positive and one gram negative pathogenic organism. The bacterial sensitivity can be measured *in vitro* by the diffusion assay [96] method.

3.9.2. Apparatus and Reagents:

i. Chloroform extract of NN.
ii. Ethyl acetate extract of NN
iii. Blank filter paper disc.
iv. Dimethyl Sulfoxide (DMSO).
v. Nutrient agar (D1FCO).
vi. Alcohol (95%).
vii. Standard disc (Kanamycin K-30).
viii. Test-tubes and petridishes.
ix. Sterile cotton.
x. Sterile forceps.
xi. Inoculating loop.
xii. Micropipette
xiii. Bunsen burner.
xiv. Laminar air flow unit
xv. Refrigerator

xvi. Autoclave

xvii. Incubator

3.9.3. Procedure:

The crude extracts were dissolved in DMSO (as it is nontoxic) in such a concentration that 25µl of it contained 200µg. Then 25µl of it was placed on sterile paper discs. Thus each disc contains 200µg. The discs were dried at low temperature. Nutrient agar medium was prepared according to the specification and transferred to test tubes in 15 ml quantity. The test tubes were plugged with fresh cotton and sterilized. After allowing sufficient time for cooling to about 45°C the different test pathogenic bacteria were inoculated in the media of the test tube, shaken vigorously for uniform distribution and then poured into sterilized petridishes. After solidification, the dried paper discs and standard discs were placed aseptically on the medium in the plates with a sterile forceps. The plates were then kept at low temperature (4°C) in a refrigerator for 24 hours to allow complete diffusion of the active component in the agar medium before any growth of the test organisms occurred. The petridishes were then incubated at 37.5°C for 12 hours. This resulted in a clear zone of inhibition around the disc, which could easily be determined because growth of microorganism made the medium opaque as compared to clear visible zone indicating the absence of growth. The diameters of the zones were measured with a transparent scale.

CHAPTER FOUR

RESULT AND DISCUSSION

4.1. Isolation of antagonistic species

The organism under investigation was obtained from the soil of various places of Dhaka and Munsigonj, Bangladesh at a depth of 0.25-1.5 meter. Among a number of culture media, yeast-extract glucose agar media was found to be the most suitable for the isolation purpose. Pure culture of the organism and its antibacterial activity was checked by disc diffusion assay [97] method against *Bacillus subtilis* and *Escherichia coli.*

4.2. Identification of the organism

For morphological study, the isolated organism NN was grown in yeast-extract glucose agar media. Then visual and microscopic observations were carried out.

4.2.1 Visual observations of NN:

i. Upper surface of the organism white (up to 3 days) then turn to brown.
ii. The surface was velvety.
iii. The background was reddish.
iv. The colony of the organism was almost round.

4.2.2 Microscopic observations:

The characteristics under microscope in yeast-extract glucose agar media were recorded at various time intervals as follows:

After 24 hours:

i. Vegetative mycelia were observed.
ii. There was no aerial mycelium.
iii. No sporulation.

After 48 hours:

i. Vegetative mycelia were branched.
ii. Insufficient aerial mycelium was observed.
iii. No spiral in aerial mycelia.

After 72 hours:

i. The spores were round.

ii. The vegetative mycelia were branched.

iii. The aerial mycelia were coiled or spiral.

iv. Abundant sporulation occurred.

v. Reddish diffusible pigment was observed.

4.3. Taxonomic Position of the Organism NN

On the basis of morphology, the organism (**NN**) may belong to the actinomycetales order, according to the Bergey's Manual of Determinative Bacteriology (9th edition) as-

- ✓ Mycelial filaments tend to remain intact and not fragmented.
- ✓ Spore chains are long.
- ✓ Aerial mycelium is abundant.

The organism (**NN**) may also belong to the family Streptomycetaceae as:-

- ✓ Presence of well-developed mycelia.
- ✓ The vegetative mycelium does not form cross wall.
- ✓ Non-motile aerial spores.
- ✓ Absence of sporangia like vesicle.

Beyond these the organisms characterized in the *Streptomyces* genus due to the following reasons:

- ✓ Spore chain morphology
- ✓ Spore wall ornamentation
- ✓ Ability to utilize particular carbon containing compounds for growth
- ✓ Ability to produce antimicrobial principle
- ✓ Color of the matured sporulated aerial mycelium

The Taxonomic Position of the Organism NN:

Order-Actinomycetales

Family- Strptomycetaceae

Genus- *Streptomyces*

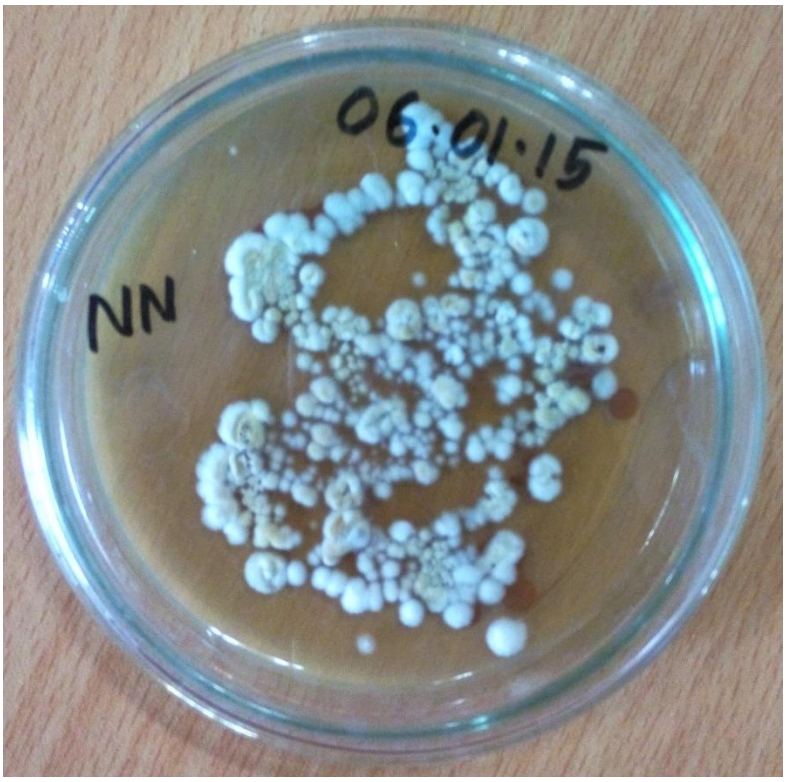


Figure-4.1: Visual observation of colony of the isolated NN (Front) after 7 day incubation.

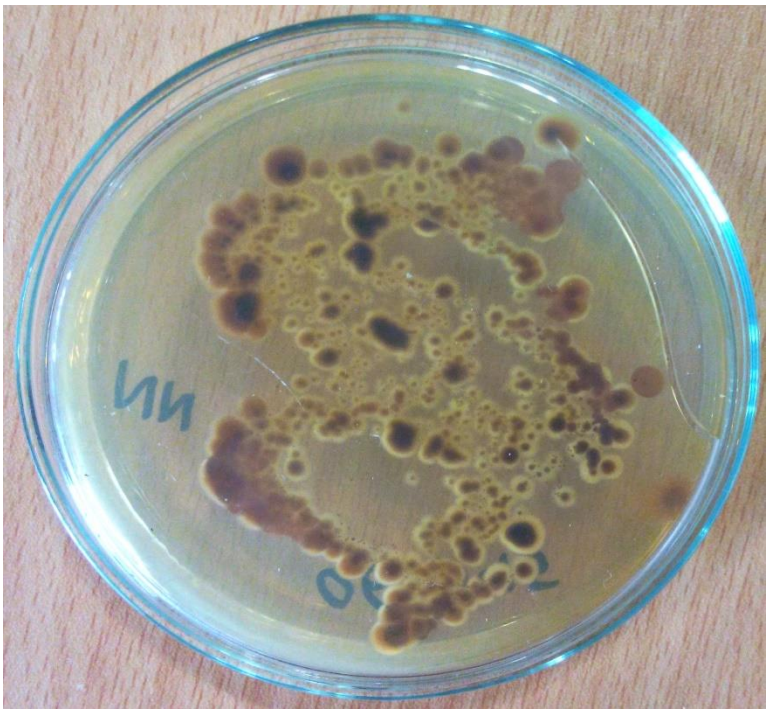


Figure-4.2: Visual observation of colony of the isolated organism NN after 7 day incubation

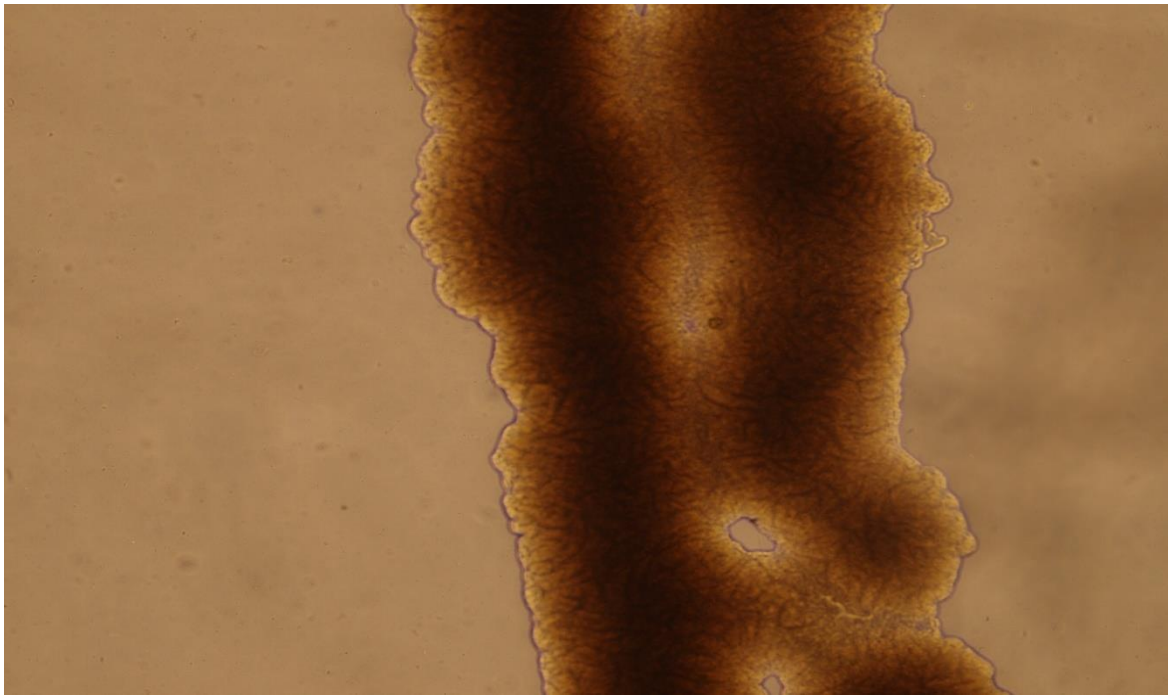


Figure-4.3: Microscopic view of the isolate, NN after 3 day incubation staining spores.

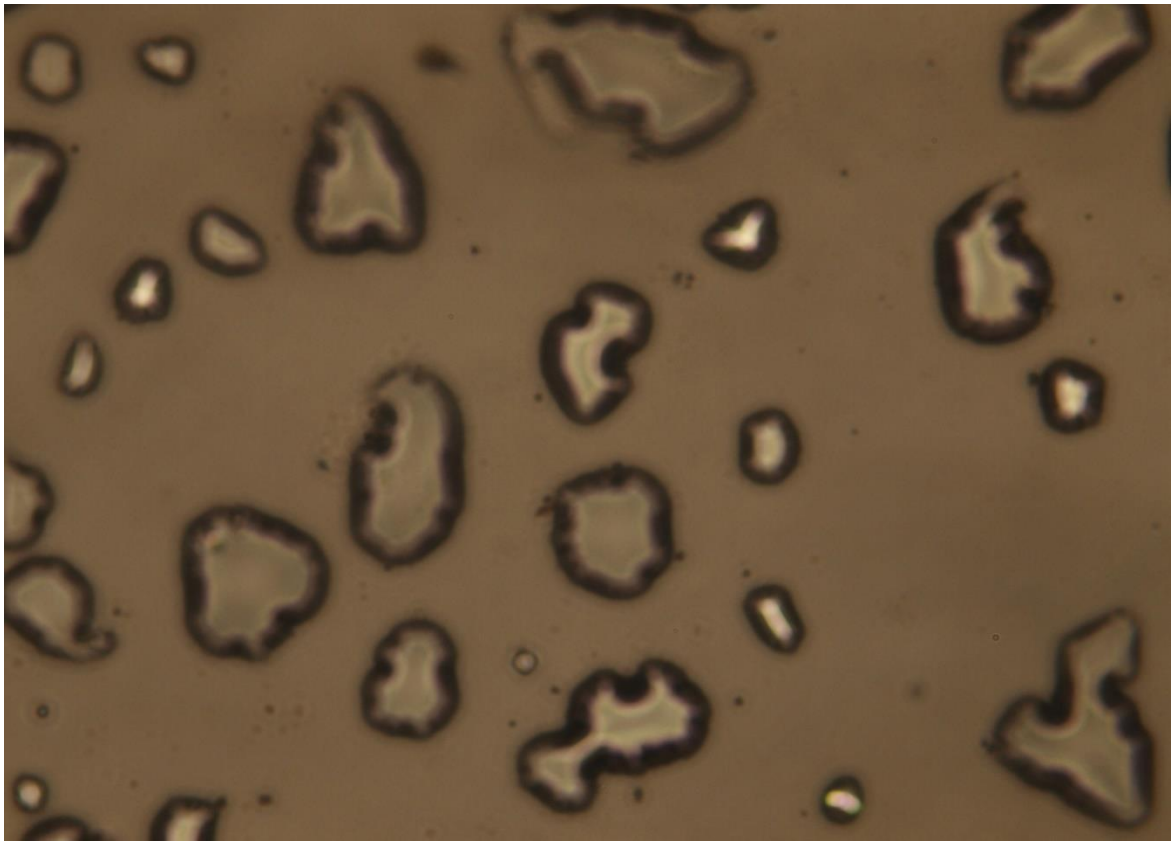


Figure-4.4: Microscopic view of spore of the isolate, NN on 15 day incubation staining spores.

The isolate, NN was grown in the yeast extract glucose broth media and the broth medium was separated using the cotton filtration. Half of the filtrate was then extracted with ethyl acetate while the rest half was extracted with chloroform.

Table-4.1: Antibacterial screening of chloroform extracts of NN.

Test Bacteria	**Diameter of zone of inhibition (mm)**		
	Chloroform extract 200µg/disc	Kanamycin 30µg/disc	Ethyl acetate extract 200µg/disc
Bacillus subtilis	25	30	28
Escherichia coli	27	33	27

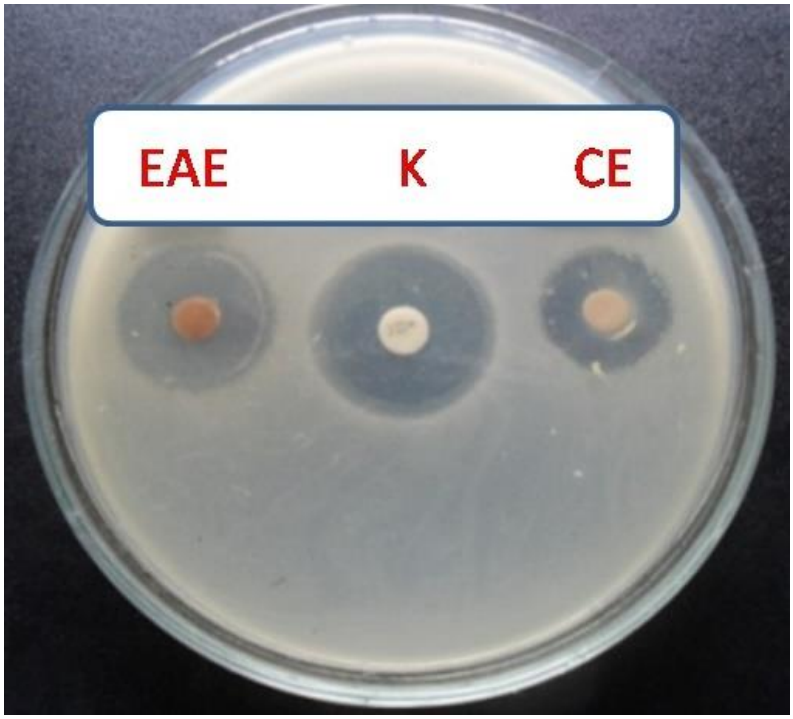


Figure-4.5: Ethyl acetate extracts (EAE) and chloroform extracts (CE) of NN and Kanamycin (30µg/disc) against *Bacillus subtilis.*

Figure-4.6: Ethyl acetate extracts (EAE) and chloroform extracts (CE) of NN and Kanamycin (30µg/disc) against *E coli.*

CHAPTER FIVE

SUMMARY AND CONCLUSION

Actinomycete is a group of microorganisms intermediate in properties between bacteria and fungi. They are gram-positive, free-living, saprophytic bacteria, widely distributed in soil, water, and colonizing plants. Soil is the most important source of all kinds of fascinating microorganisms. Specially the Soil of Bangladesh and its temperature are suitable for growth of microorganisms. As a part of such an effort, soil samples were collected from different depths and places from the Dhaka and Munsigonj of Bangladesh for the isolation of the antagonistic Actinomycetes. It was found that the soil samples collected from a depth of about 0.25-1.5m contained the highest percentage of Actinomycetes. Different organisms were collected and finally one of them was selected for their highest antimicrobial activities. The organisms have been designated as NN. It was purified and the whole work was carried out with this organism. Pure cultures of the organism were grown in Yeast-extract-glucose agar medium. For microscopic examination, cover slip culture of pure strain of the organisms were made in Yeast-extract-glucose agar medium and observed after without staining. The visual and microscopic examination revealed that the isolated organism, NN was order of Actinomycetes and genus of *Streptomyces*.

In order to obtain maximum yields of metabolites from the selected organisms a number of broth culture media was tried for the production of antibiotics. The Yeast-extract-glucose broth media was found to be the best for the organism NN. Hence, the organism NN was grown in Yeast-Extract-Glucose broth media for the production of antimicrobial metabolites. Chloroform and ethyl acetate was used for the isolation of the antimicrobial metabolites.

The antibacterial activities of the chloroform and ethyl acetate extract was tested against a gram positive and a gram negative bacteria by disc diffusion method at a concentration of 200 µgm/disc and Kanamycin (k-30) was used as standard Both of the test organisms were found to be sensitive to the crude extract. Then this organism secreted the secondary metabolites which possess antimicrobial property.

Finally, soil collected from the Dhaka and Munsigonj of Bangladesh has been shown to possess an antibacterial. The organism is currently undergoing detailed investigations with the objective of isolating biologically active molecules along with the search for novel compounds.

Furthermore, the encouraging biological activities observed in this study to conclude that the Bangladeshi soils are a potential source of variety of organisms worthy of further investigation.

APPENDIX

COMPOSITION OF CULTURE MEDIA USED

Table-6.1: Yeast-extract glucose agar (1000 ml)

Ingredient	Amount
Yeast extract	1gm
Glucose	10gm
Potassium nitrate (KNO_3)	1gm
K_2HPO_4	0.1gm
Agar	15gm
Distilled water q.s.to	1000ml

Table-6.2: Tryptosen Soya Broth Media (100 ml)

Ingredient	Amount
Nutrient agar	2.0gm
Tryptosen Soya	3.0gm
Distilled water q.s.to	100ml

Table-6.3: Nutrient Agar Media (100 ml)

Ingredient	Amount
Yeast extract	1.0gm
Peptone	0.5gm
NaCl	0.5gm
Agar	2.0gm
Distilled water q.s.to	100ml

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