PRODUCTION OF ANTIMICROBIAL COMPOUNDS FROM A LOCAL MARINE BACTERIAL ISOLATE, *BACILLUS SP.* (S1A4)

By

LIM HUI MING

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

March 2007

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mother for her moral support and undying love because without it, I do not think I would have been able to accomplish anything.

My deepest gratitude and sincere thanks goes to Associate Professor Dr. Darah Ibrahim for her guidance and invaluable advice throughout these trying years. Her help and caring ways gave me strength to carry on when times looked bleak. A special thanks also goes out to Professor Haji Ibrahim Che Omar as his earlier teachings will always remain in my mind.

I would also like to thank everyone in the Fermentation and Enzyme Technology Lab, for they are really the greatest lab mates anyone could ever have. They unselfishly lent a helping hand to me when I most needed it, and for that, thank you so much. Also, I owe a great deal of gratitude to Mrs Falizah Rouse, Mr Patchamuthu, Miss Jamilah and Mr Johari for their willingness to aid me in areas which I knew nothing about. I must lastly thank USM for offering me the Graduate Assistancy Scheme.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

PRODUCTION OF ANTIMICROBIAL COMPOUNDS FROM A LOCAL MARINE BACTERIAL ISOLATE, *BACILLUS* SP. (S1A4)

ABSTRACT

A total of 160 strains of marine microorganisms were supplied by the Fisheries Research Institute, Penang. They were collected from samples all over the shores of Malaysia. Out of those, 134 were bacterial strains, 9 actinomycetes, 3 fungal and 14 yeast isolates. About 113 isolates were found to produce pigments. Non - pigmented strains (white colonies) were the most commonly found (26%) while the yellow coloured strains followed with a close second (25%), orange pigmented isolates (16%), and the rest were brown, beige, light green, dark blue, red, grey and transparent isolates in a small percentage. Among the marine isolates provided, only 88 isolates were tested for antimicrobial activity. 59.1% exhibited antimicrobial activity (52 isolates) of which, 50.0% (42 isolates) exhibited antibacterial activity, 10.3% (9 isolates) antifungal activity and 10.2% (9 isolates exhibited both antibacterial and antifungal activities. The distribution of the antimicrobial compounds in the three selected isolates showed that all of them produced both extracellular and cell – bound antimicrobial compounds. The isolate S1A4 was chosen for further experiments because it exhibited a wide spectrum of activity, was easily subcultured, and grew well in the cultivation medium. The isolate S1A4 was identified as the genus *Bacillus* sp. The production of antimicrobial compounds was enhanced by optimizing physical (culture conditions) and physiological (medium compositions) conditions. The optimized cultural conditions were: 150 rpm for the agitation speed, 4% (v/v) of 3×10^8 cells/ml of the inoculum size, initial pH medium of 7.3 and the incubation temperature was fixed at 37°C. About 50ml of natural filtered sea water was used in making the cultivation

medium which consisted of 0.40% (w/v) of starch, 0.50% (w/v) of peptone, 0.20% (w/v) of yeast extract, 0.01% (w/v) of ferric phosphate, 0.09% (w/v) of L-arginine and 0.05% of KCl. Even though the production of antimicrobial compounds started during its exponential growth phase, maximum production was achieved during the idiophase. After optimization using the shake flask system, there was an increase of 29.6 U/ml or 39.6% increment in antimicrobial compound production. After optimization in the shake flask system, a tubular airlift fermenter with a 2.0L capacity was used for scaling up and then all the parameters were again reoptimized. The optimized conditions were : 4 l/min of aeration, 4% (v/v) (3×10^8 cells per ml) of initial inoculum size and initial pH of 7.3. Filtered natural sea water was used in making the medium which consisted of : 0.5% (w/v) of starch, 0.6% (w/v) peptone, 0.2% (w/v) of yeast extract, 0.01% (w/v) ferric phosphate, 0.09% (w/v) of L-arginine and 0.05% of KCl. An increment of about 13.8% (4.1 U/ml) of antimicrobial compound production was obtained after optimization in a tubular airlift fermenter. Characterization of the crude extract of the antimicrobial compound found that it was thermostable in a temperature range between 35°C – 65°C, and pH stable between the pH values between 6 -10. The antimicrobial compounds exhibited bactericidal activity against the cells of *Staphylococcus aureus* .SEM and TEM micrographs showed that the antimicrobial compound lysed the cell wall of *S. aureus* besides interfering with the internal structure of the cells.

PENGHASILAN SEBATIAN ANTIMICROB DARIPADA PENCILAN MARIN TEMPATAN, *BACILLUS SP.* S1A4.

ABSTRAK

Sejumlah 160 pencilan mikroorganisma marin dibekalkan daripada Institut Penyelidikan Perikanan, Pulau Pinang. Daripada jumlah tersebut, 134 adalah pencilan bacteria, 9 aktinomiset, 3 kulat, dan 14 yis. Sebanyak 113 pencilan didapati menghasilkan pigmen. Pencilan yang tidak berpigmen (koloni putih) adalah yang paling banyak ditemui (26%), diikuti rapat dengan pencilan berpigmen kuning (25%), berpigmen jingga (16%), dan terlebihnya adalah perang, beige, hijau muda, biru gelap, merah, kelabu dan lutsinar dalam peratusan yang kecil. Daripada kesemua pencilan marin, hanya 88 pencilan sahaja yang diuji aktiviti antimicrobnya. Didapati sebanyak 59.1% (52 pencilan) menunjukkan aktiviti antimikrob, yang mana 50.0% (42 pencilan) menunjukkan aktiviti antibakteria, 10.3% (9 pencilan) menunjukkan aktiviti antikulat dan 10.2% (9 pencilan) menunjukkan kedua – dua aktiviti. Taburan sebatian antimikrob pada ketiga – tiga pencilan marin yang terpilih menunjukkan bahawa ketiga – tiganya menghasilkan sebatian antimikrob secara ekstrasel dan juga terikat pada sel. Pencilan S1A4 telah dipilih untuk kajian selanjutnya kerana ia mempamerkan spektrum aktiviti yang luas, tahan disubkultur, dan dapat tumbuh dengan baik di dalam medium pengkulturan. Pencilan S1A4 telah dikenalpasti sebagai *Bacillus* sp. Penghasilan sebatian antimikrob oleh *Bacillus* sp. S1A4 telah ditingkatkan dengan mengoptimumkan keadaan fizikal (keadaan pengkulturan) dan fisiology (komposisi medium) pengkulturannya. Keputusan pengoptimumman di dalam sistem kelalang goncangan yang didapati adalah; kelajuan goncangan sebanyak 150 psm, 4% (v/v)

daripada 3 x 10^8 sel/ml untuk saiz inokulum, pH awal medium sebanyak 7.3 dan suhu eraman pada 37°C. Sebanyak 50ml air laut semulajadi digunakan dalam pembuatan medium yang mengandungi 0.40% (b/i) of kanji, 0.50% (b/i) pepton, 0.20% (b/i) ekstrak yis, 0.01% (b/i) of ferum fostat, 0.09% (b/i) L-arginine dan 0.05% (b/i) KCl. Penghasilan sebatian antimikrob *Bacillus* sp. S1A4 dicapai pada peringkat idiofasa. Selepas pengoptimuman didalam sistem kelalang goncangan dilakukan, terdapat peningkatan dalam penghasilan sebatian antimikrob iaitu sebanyak 29.6 U/ml atau peningkatan sebanyak 39.6%. Selepas kajian pengoptimuman di dalam sistem kelalang goncangan lengkap dilakukan, maka pengoptimuman di dalam fermenter angkut udara tubular dengan keupayaan 2.0L dilakukan untuk skala besar. Keadaan optimum yang diperolehi adalah : pengudaraan sebanyak 4.0l/min, 4% (i/l; 3 x 10^8 sel/ml) saiz inokulum dan pH awal medium 7.3. Sebanyak 1.8L air laut semulajadi yang telah dituras digunakan dalam pembuatan medium yang mengandungi 0.50% (b/i) of kanji, 0.60% (b/i) pepton, 0.20% (b/i) ekstrak yis, 0.01% (b/i) of ferum fostat, 0.09% (b/i) L-arginine dan 0.05% (b/i) KCl. Peningkatan sebanyak 4.1 U/ml atau 13.8% dalam penghasilan sebatian antimikrob telah diperolehi. Pencirian ekstrak kasar sebatian ini menunjukkan bahawa sebatian ini bersifat termostabil pada julat suhu 35°C – 65°C dan stabil pH pada julat pH antara pH 6 -10. Sebatian aktif antimikrob ini menunjukkan kesan bakterisid pada sel *Staphylococcus aureus.* Mikrograf SEM dan TEM menunjukkan bahawa sebatian ini menglisiskan dinding sel *S. aureus* di samping mengganggu struktur dalaman sel.

1.0 INTRODUCTION

1.1 Bioactive secondary metabolites

The antibiotics belong to a group of substances referred to as secondary metabolic products, i.e. substances that are produced by living organisms, and that appear to be unrelated to the main processes of growth and reproduction. Flowers, plants, animals, all produce secondary metabolites in the form of colours, perfumes, essential oils, herbs, spices, alkaloids, and many more. These natural products have produced vast and interesting literatures. Table 1.1 shows a few classes of organic compounds which are produced during secondary metabolism. These complex novel substances have been regarded as arising by chance, perhaps as a result of the shunting of surplus products into metabolic sidelines. The metabolic pathways that are used are due to its genetic factor, but the biosynthetic pathways that are taken during antibiotic production are most probably activated during certain periods of growth and also during the lag phase. One can say it is during 'stressful' times like the attack of other organisms or other internally generated hazards that the production of these bioactive secondary metabolites swings into action (Calam, 1987). However it must be noted that secondary metabolites are not necessary for its survival. They do not serve vital roles in metabolism. It is unlikely that the complex systems of enzymes and genes required for this purpose exists without any plausible reason. Some of the possible roles include its ecological role in nature, as its acts in identification, attraction and repulsion of its own kind as well as others. Another possible function of secondary metabolism is to prevent the accumulation of primary metabolites during the resting phase that may prove harmful to the cell. Demain (1983) comments on the secondary metabolic

Table 1.1 : Classes of Organic Compounds Which Secondary Metabolites Are Found (Zahner & Maes, 1972)

Amino sugars	Lactones	Pyrones
Anthocyanins	Macrolides	Pyrroles
Anthraquinones	Napthalenes	Pyrrolines
Aziridines	Napthoquinones	Pyrrolizines
Benzoquinones	Nucleosides	Quinolines
Coumarins	Oligopeptides	Quinolinols
Diazines	Phenazines	Quinones
Epoxides	Phenoazinones	Salicylates
Ergoline alkaloids	Phthaldehydes	Terpenoids
Flavonoids	Piperazines	Tetracyclines
Glutaramides	Polyacetylenes	Tetronic acids
Glycosides	Polyenes	Triazines
Hydroxylamines	Pyrazines	Tropolones
Indole derivatives	Pyridines	

products as antagonistic agents, symbiotic agents, sexual hormones, effectors of sporulation and germination, metal transporters, etc. that offer the producing organism an opportunity to survive in the competitive arena of nature. Their relation to differentiation is also stressed, as has been deduced by workers such as Bu'Lock (1975).

1.2 The Importance of Continuing the Search for Novel Antibiotics

Ever since the discovery of penicillin by a Scottish physician, Alexander Fleming in the year 1929, it quickly became a medical miracle, saving thousands by vanquishing the biggest wartime killer – infected wounds. Penicillin which was effective towards many microorganisms quickly became well known to be a 'cure for all' at that time, due to its effectiveness.

He actually stumbled upon it, as it was a fungal contaminant on a plate streaked with *Staphylococcus aureus.* He noticed a clear zone all around the contaminant fungus, and realized the importance of a metabolite that could control bacterial growth as he had devoted much of his career to finding methods to treating infections. The substance was named penicillin, after the fungus, which was found to be *Penicillium notatum.* However, he could not purify this compound because of its instability, and it was not until the period of the Second World War (1939-1945) that two other British scientists, Florey and Chain, working in the USA, managed to produce the antibiotic on an industrial scale for widespread use. All three scientists shared the Nobel Prize for this work, and rightly so - penicillin rapidly became the "wonder drug" which saved literally millions of lives. It is still a "front line" antibiotic, in common use for some bacterial infections although the development of penicillin-resistance in several pathogenic bacteria now

limits its effectiveness.

The emergence of penicillin soon led to the finding of other antibiotic producing microorganisms, especially from soil. It was no surprise when the next antibiotic, streptomycin was discovered, it was from a soil actinomycete, *Streptomyces griseus.* Actinomycetes, especially from the genus *Streptomyces* yielded many clinically important antibiotics. Some of them are shown in Table 1.2. Other bacteria, including *Bacillus* species, have yielded few useful antibiotics. Fungi also have yielded few useful antibiotics. Apart from penicillin, the most important antibiotics from fungi are the cephalosporins (beta-lactams with similar mode of action to penicillin, but with less allergenicity) and griseofulvin (from *Penicillium griseofulvum* and related species) which is used to treat althlete's foot and related fungal infections of the skin.

However, just after 4 years penicillin was mass produced, reports of microbes that was resistant to penicillin surfaced. The irony of it was that the first report of a microorganism that was resistant to penicillin was the very first microbe that led to the discovery of penicillin itself, *S. aureus.* This bacterium is often a harmless passenger in the human body, commonly carried on the skin and noses of healthy people, but it can cause illness, such as pneumonia or toxic shock syndrome, when it overgrows or produces a toxin.

It has become resistant toward methicillin, an antibiotic commonly used to treat staphylococcal infections, thus becoming MRSA (Methicillin-resistant *Staphylococcus aureus.*) The new strain has even become resistant to a few more antibiotics, and vancomycin, usually used as a last resort drug, had to be used (Lyon & Scurray, 1987). Even more disturbing,

Table 1.2 : Several Clinically Useful Antibiotics and their mode of actions

Antibiotic	Producer organism	Activity	Site or mode of action
Penicillin	*Penicillium chrysogenum*	Gram-positive bacteria	Inhibits the cell wall synthesis
Cephalosporin	*Cephalosporium acremonium*	Broad spectrum	Inhibits the cell wall synthesis
Griseofulvin	*Penicillium griseofulvum*	Dermatophytic fungi	Inhibits the microtubules
Bacitracin	*Bacillus subtilis*	Gram-positive bacteria	Inhibits the cell wall synthesis
Polymyxin B	*Bacillus polymyxa*	Gram-negative bacteria	Interfere the permeability of cell membrane
Amphotericin B	*Streptomyces nodosus*	Fungi	Interfere the permeability of cell membrane
Erythromycin	*Streptomyces erythreus*	Gram-positive bacteria	Inhibit protein synthesis
Neomycin	*Streptomyces fradiae*	Broad spectrum	Inhibit protein synthesis
Streptomycin	*Streptomyces griseus*	Gram-negative bacteria	Inhibit protein synthesis
Tetracycline	*Streptomyces rimosus*	Broad spectrum	Inhibit protein synthesis
Vancomycin	*Streptomyces orientalis*	Gram-positive bacteria	Inhibit protein synthesis
Gentamicin	*Micromonospora purpurea*	Broad spectrum	Inhibit protein synthesis
Rifamycin	*Streptomyces mediterranei*	Tuberculosis	Inhibit protein synthesis

This table was adapted from (Lancini & Lorenzetti, 1993) and Gross *et al.,* 1995.

intestinal bacteria, eg.enterococci that are resistant to vancomycin has been found. Another type of penicillin-resistant pneumonia, caused by *Streptococcus pneumoniae* and called pneumococcus, surfaced in a remote village in Papua New Guinea in the year 1967 and till now 25% of the strain has mutated to be resistant to multiple drugs. Resistance to fluoroquinolones, a newer class of drugs, also is on the rise.

Antibiotic resistance spreads fast. According to a report in the April 28, 1994, New England Journal of Medicine, researchers have identified bacteria in patient samples that resist all currently available antibiotic drugs. Antibiotic resistance is acquired through genes: bacterial mutation, transformation and plasmid transference. The later is found to be the most dangerous as a single plasmid can harbour a slew of resistances. In 1968, 12,500 people in Guatemala died in an epidemic of *Shigella* diarrhea. The microbe harboured a plasmid carrying resistances to four antibiotics. Besides natural occurrences, antibiotic resistance is also due to over use of antibiotics. Table 1.3 shows the different mechanisms employed by resistant bacteria towards various antibiotics.

When a person takes an antibiotic, the drug kills the defenseless bacteria, selecting those that can resist it. These renegade bacteria then multiply, increasing their numbers a million fold in a day, thus becoming the predominant microorganism. When that happens, it takes over as the main cause of infection. The antibiotic does not technically cause the resistance, but allows it to happen by creating a situation where an already existing variant can flourish. A patient can develop a drug-resistant infection either by contracting a resistant bug to begin with, or by having a resistant microbe emerge in the body once antibiotic treatment begins. Drug-resistant

Table 1.3 : Mechanisms of Resistance to Some Representative Antibiotics

(Lancini and Lorenzetti, 1993)

Antibiotic	Type of resistance	Location of genetic determinant	Description of resistance mechanism
β – lactam antibiotics	1. Inactivation	Extrachromosomal and Chromosomal	B – Lactamases that open the β – lactam ring. Some specific for penicillins or cephalosporins, others do not distinguish between the two antibiotics.
	2. Alteration of site of action	Chromosomal	Resistance to all the β – lactams as a result of modified penicillin binding proteins (PBP's).
	3. Permeability	Variable	Alteration of porins
	4. Tolerance	Unknown	Inhibition of growth by low antibiotic concentrations but no bactericidal effect even at high concentrations.
Chloramphenicol	1. Inactivation	Extrachromosomal	Acetylation by an inducible enzyme
	2. Modification of site of action	Chromosomal	Alteration of rRNA 23S
Aminoglycosides	1. Inactivation	Extrachromosomal	N-Acetylation, phosphorylation, adenylation related to various inactivating enzymes.
	2. Permeability	Chromosomal	Energy deficiency, modification of porins
	3. Modification of site of action	Chromosomal	Modification of RNA
Streptomycin	1. Modification of site of action	Chromosomal	Alteration of the S12 protein in the ribosomal
	2. Inactivation	Extrachromosomal	30S subunit and alteration of the 16 S rRNA
			Analogous to that of the other aminoglycosides
Kasugamycin	Modification of site of action	Chromosomal	Alteration of the 16 S RNA of the ribosomal 30 S subunit
Erythromycin	1. Modification of site of action	Chromosomal and extrachromosomal	Alteration of proteins of the ribosomal 50 S subunit and methylation of the RNA
	2. Inactivation	Extrachromosomal	Hydrolisis of lactone and consequent opening of the ring
Rifamycins	Modification of site of action	Chromosomal	Alteration in the β subunit of RNA polymerase
Cycloserine	Alteration of permeability	Chromosomal	Modification of the transport system of D-alanine and glycine, used by cycloserine
Tetracyclines	Alteration of permeability	Chromosomal	Decreased transport efficiency
Fosfomycin	1. Alteration of permeability	Chromosomal	Modification in the transport system of glycerophosphate or glucose-6-phosphate (used
	2. Inactivation	Extrachromosomal	to transport fosfomycin) Inactivating intracellular enzyme (reaction not yet identified)

infections increase risk of death, and are often associated with prolonged hospital stays, and sometimes complications (Liu & Chambers, 2003). Another concern is the over usage of antibiotics for feeding of livestock. This nowadays is common as farmers use antibiotics not to cure, but as a prevention of diseases. The very same antibiotics, used by humans, and if not, antibiotics with the same mode of action has been over used and is vulnerable a million fold as it can help the development of resistance, which could then be passed onto human pathogens easily by the method mentioned above.

Thus, it is extremely imperative that the search for newer and better antibiotics should be continued considering the dangers at hand. Microorganisms have long met the demand but the search has been expanded to include an environment which holds a tremendous potential, and that is the marine environment, and it will make a huge impact on the drug discovery process.

1.3 Classification of Antibiotics

Antibiotics differ in sources of origin, chemical structures, physical properties, antimicrobial spectrum, mode of action, and at first glance may seem overwhelming to comprehend. Nevertheless, despite those differences, they may be categorized in different ways as well. Here the various ways of classifications, its methods and several drawbacks for each method will be reviewed thoroughly.

1.3.1 Classification of Antibiotics based on Biosynthesis Pathway

In terms of biogenesis, antibiotics are considered secondary metabolites. The study on the biosynthesis of antibiotics consists of identifying the enzymatic reactions by which one of more primary metabolites are converted into the antibiotic molecule. These processes include the steps leading from the nutrients that are supplied to the cell to its end products. The enzyme reactions that lead to synthesis of antibiotics do not differ fundamentally from those that lead to synthesis of primary metabolites. In fact, it is reasonable to hypothesize that the enzymes that are needed in the synthesis of special metabolites evolved from those of general metabolism. However there may be exceptional cases like in antibiotics which contain a nitro group, a function that is never found in primary metabolism and that is derived through a special pathway of amine oxidation. The biosynthetic pathways from which antibiotics are formed can be divided into 3 major categories:

a) Antibiotics derived from a single primary metabolite. The biosynthetic pathway consists of a series of reactions that modify the starting material in the same way as in synthesis of amino acids or nucleotides

b) Antibiotics derived from two or three different primary metabolites, which are modified and condensed to give a complex molecule. There are some analogous cases in primary metabolism in synthesis of certain coenzymes, such as folic acid or coenzyme A.

c) Antibiotics derived from polymerization of several metabolites to give a basal structure that can be further modified by additional enzymes reactions. The four classes of antibiotics which are derived from polymerization processes are :

- Polypeptide antibiotics – derived from a condensation of amino acids through a process similar to polyketide synthesis.
- Antibiotics built up of acetate – propionate units by polymerization mechanisms similar to those that give rise to fatty acids.
- Terpenoid antibiotics – derived from acetate units through isoprenoid synthesis.
- Aminoglycoside antibiotics – made thought condensation reactions similar to those that make polysaccharides.

However it should be emphasized that the basal structure obtained by polymerization is usually modified by further reactions, even by addition of molecules made through other biosynthetic pathways. Glycoside antibiotics made by condensation of one or more sugars onto a molecule biosynthesized by pathways are particularly common.

For the complete elucidation of a biosynthetic process, it ideally comprises of:

1. Identification of the 'building blocks, which is the primary metabolites from which the molecule is made

2. Isolation of intermediates of the pathway, whose structure may suggest a reasonable hypothesis as to the sequence of reactions.
3. Identification of the enzymes that catalyze the single reactions
4. Identification of the governing genes and determination of their sequence.

There are a few methods usually used to gain information about the biosynthetic processes. They are the usage of tracer techniques, the identification of intermediate metabolites, the identification of the enzymes produced, and using genetic and recombinant DNA techniques.

However, most microorganisms have a tendency to form a whole series of secondary metabolites of a certain type once the capacity for carrying out the reactions leading into a particular secondary branch has been developed. A few examples to illustrate this tendency toward multiplicity of related products are shown in Table 1.4.

- Actinomycins. Each actinomycin – producing organism forms several actinomycins, all of which have the same phenoxazone chromophoric group but which differ from each other in their peptide chains.
- Anthracyclines. *Streptomyces purpurascens* produces a large variety of rhodomycins, which differ from each other in the aglycone or in the sugar portion of the molecule. The same goes for *Streptomyces galileus*, which produces pyrromycins.
- Polymyxins. *Bacillus polymyxa* produces an almost irresolvable mixture of closely related peptide antibiotics.

Table 1.4: Antibiotics Which Do Not Conform to the Rule of Specificity

(Zähner & Maes, 1972)

Antibiotic	Producing organisms
Cephalosporin	*Cephalosporium* sp. *Streptomyces* sp.
Citrinin	*Penicillium* sp. *Streptomyces* sp.
Fusidic acid (Ramycin)	*Fusidium* sp. *Cephalosporium* sp. *Mucor ramannianus*
Bovinocidin (Beta – nitropropioopic acid)	*Streptomyces* sp. *Aspergillus* sp. Constituent of a glycoside in higher planes
Various phenazines	*Streptomyces* sp. *Pseudomonas iodinum*
Nebularin (9-(B-D-riboturanosyl)-purine)	*Afaricus nebularis*

1.3.2 Classification of Antibiotics based on Producer Organisms

As the name states, this style of classification are based on the organism that produces the metabolite. Many attempts have been made to find out the exact reason why a particular strain has the all the advantages while another poorer strain has virtually no ability to defend itself. However this type of classification does have its advantages, and among them, is that all the differing types of antibiotics can just be put together under the name of the same species. A few observations have been made so far. Firstly, most of the antibiotics derived are products of the secondary metabolism of 3 main groups of microorganisms: eubacteria, actinomycetes, and filamentous fungi. The actinomycetes produce far by the largest number as well as the greatest variety of known antibiotics. They have yielded more than 6000 substances. The lower fungi produce several kinds of secondary metabolites, which approximately 1500 types show antibiotic activity. The eubacteria (mainly bacilli and pseudomonads) too produce a fair number of antibiotics, which is around 1000. Another group, the myxobacteria, although little studied, too has revealed a high frequency of production.

A relationship seems to exist between the taxonomic group of the producing organism and the biosynthetic pathway for its antibiotics. However, further research is needed to confirm this relationship. Some biosynthetic pathways of secondary metabolism occur generally (e.g. the capacity to activate and to condense amino acids to produce polypeptide antibiotics is found in eubacteria, actinomycetes and lower fungi). There are some biosynthetic pathways only present in certain groups (e.g. practically all the known secondary metabolites originating from terpene synthesis are produced by fungi). Even within the same family, there seems to be

differentiation. In actinomycetes, there seems to be biosynthetic differentiation where the biosynthesis of aminocyclitol – containing antibiotics (aminoglycosides) is found much more frequently in the genera *Streptomyces* and *Micromonospora* than in other genera of the order Actinomycetales. However all these observations have merely statistical observations and not absolute value (Lancini & Parenti, 1995).

Nevertheless, its drawbacks are even heavier than its advantages. Many loopholes exist in this system, because a given strain can produce a group of structurally and biosynthetically related substances, and yet also produce unrelated antibiotics as well. Antibiotic production simply is not rigorously species – specific. A classic example is *Streptomyces griseus.* Different strains of the same species have the ability to produce completely different antibiotics. Streptomycin (an aminoglycoside), novobioicin (a glycoside with a complex aromatic moiety), cyclohexamide (aromatic structure derived from acetate), viridogrisein (a depsipeptide), griseoviridin (lactone), are just a few examples of the difference of structures of antibiotics which are produced. Table 1.4 further demonstrates the violation towards this 'rule of specificity'.

On the other hand, the same antibiotic molecule can be produced too by different taxonomical group. For example, cycloserine has been isolated from both a *Streptomyces* and a *Pseudomonas* strain and penicillin N is produced by both lower fungi *Cephalosporium* as well as streptomycetes.

1.5.5 Classification of Antibiotics based on Spectrum of Activity

An antibiotic is an inhibitor of microbial populations, and the way a particular compound inhibits another microorganism can be used as another way to classify antibiotics. Antibiotics inhibit growth either reversibly (bacteriostatic), that is, its blocks the ability of the cells to replicate and divide without killing them, or irreversibly (bactericidal), in which case the cells are killed. For an antibiotic to affect the metabolism of a microbial cell, it must first, enter the cell and reach the site of action. Secondly, it must bind physically or a cellular structure (target molecule) is involved in a process essential for maintenance of cell growth or homeostasis. Lastly, it must completely inhibit the process in which that structure is involved.

Antibiotics are classified according to the processes with which they interfere. They are commonly divided into the following groups.

1. Inhibitors of cell wall synthesis.

   The inhibitors can be divided into 2 major categories, those that inhibit peptidoglycan synthesis or disrupt the formation and elongation of the cell wall structure, and those that inhibit synthesis or assembly of other cell wall components which is effective towards Gram negative bacteria. The vast majority of known cell wall synthesis inhibitors belong to the former. For the antibiotics which work on the fungal cell wall synthesis, they work by inhibiting chitin synthesis or by inhibiting glucan synthesis. These two are major components in the make up of a fungal cell wall. However chitin is not a major component in a yeast cell wall, but it plays an important role in morphogenesis.

2. Inhibitors of the replication or transcription of genetic material.

   Under this category, there are many ways to inhibit another

microorganism. They are :

- Inhibitors of replication and transcription of nucleic acid
- Inhibitors of template functions of DNA
- Inhibitors of replication enzymes
- Inhibitors of RNA polymerase

3. Inhibitors of protein synthesis

   These types of inhibitors act by different mechanisms and also at different stages. They are a large and diverse group of substances, some of which have important clinical applications. They can be conveniently divided into 3 subgroups according to their site of action:

   - Inhibitors of aminoacyl – tRNA formation
   - Inhibitors of ribosomal functions
   - Inhibitors of extraribosomal factors

4. Inhibitors of cell membrane function.

   Both prokaryotic and eukaryotic cells are surrounded by a cell membrane that controls the bidirectional flow of substances. Antibiotics that act on the cell membrane can be divided into another two groups:

   - Inhibitors that disorganize the membrane structure, thus causing loss of cellular components
   - Inhibitors that act as carriers for specific ions, called ionophores, and cause either an abnormal accumulation or abnormal excretion of ions.

However, generally these antibiotics are poorly selective, acting against both bacterial and eukaryotic cells, reflecting the considerable chemical and structural similarities of the cell membranes of different

organisms. As a consequence of this lack of specificity, they are usually too toxic to be given systemically and their use is limited to topical applications.

5. Antimetabolites

   The term antimetabolite refers to a group of natural and synthetic substances, with very heterogeneous chemical structures and mechanisms of action. Generally, but not always, their chemical structures are analogous to those of the metabolites they antagonize. Antimetabolites are divided into two large groups on the basis of their mechanisms of action:

   - Inhibitors that are incorporated into 'informational' polymers such as DNA, RNA and proteins, in place of natural monomers resulting in alteration of information content
   - Inhibitors that stops the formation of essential metabolites

In both cases, the antimetabolites generally have structures similar to those of natural metabolites, of which they are analogues, and interact with the enzyme site that normally recognizes the natural ligand. However the degree of inhibition depends on the relative affinity for the enzyme of the analog and the natural metabolite and on the ratio of their concentration.

However, this type of classification too has its share of problems. For example, the *in vitro* results may differ from the *in vivo* results from the antibiotic's mode of action. There are also compounds which have totally different chemical structures but yet exhibit a similar if not identical mode of action (e.g. macrolides (Erythromycin) and aminoglycosides (Streptomycin, Gentamicin) both inhibits protein synthesis by binding itself to ribosome. On the other hand, Gramicidin S has two types of mechanisms of inhibition, it

affects the membrane cell thus disrupting its selective permeability, and also disrupts the cells phosphorylative oxidation system.

1.3.4 Classification of Antibiotics Based on Chemical Structure

This attempt of classification tries to demonstrate the relationships that exist between their chemical structures and their biological properties, especially their anti - microbes activities, mechanisms of resistance and toxilogical profile. The study of relationships between chemical structure and biological activity consists of identifying the functional groups which will be used in the classification processes. This may involve examining the activity at the molecular level, for it is the basis of the structure – activity relationship (Lancini, 1982). However this classification can be unambiguously said to be the most rational type of them all.

The chemical structures of the antibiotics are one of the most diverse among natural products. They cover almost all types of organic molecules, and not to mention those that are chemically modified to enhance its biological activities and to make it more suitable for clinical usage. No other area of the natural product field has been confronted with such novelty, variety, and complexity of structures. Antibiotic chemistry has recently undergone explosive growth due to the advancement of various isolation and structural determination methods.

Antibiotics can be divided into a few large groups, and because it is so structurally diverse with chemical modifications coming into the picture and other novel metabolites, subdivisions within the group exists. The major groups according to Berdy (1980) are:

1.3.4.1 Amino acid and peptide antibiotics

This family of microbial antibiotics derived from amino acids represent the largest group of compounds, with over 1000 individual antibiotics recorded till then. However, this number includes numerous macromolecular antibiotics and the number of relatively simple, lower molecular weight peptide antibiotics is about 800. This family covers all of the amino acid groups, peptide, protein type compounds and those heterocyclic compounds in which its structures are actually derived from amino acids by a simple cyclization reaction, e.g., β – lactams, diketopiperazines, aspergillic acid, and thiostrepton compounds. A great majority of the antibiotics in this family are cyclic or linear oligo – or polypeptides, substituted peptides, and protein – type compounds. Simultaneously with the wide spreading of peptides and proteins in nature, a great variety of microorganisms are able to produce peptide antibiotics. They are produced by different species of actinomycetes, bacteria and fungi. It is remarkable that *Bacillus* species produce almost exclusively peptide antibiotics. It must be mentioned, that a great number of peptide antibiotics are fully utilized in the fields of human and veterinary medicine, agriculture and biochemical research. The first ever antibiotic to be mass produced, penicillin too is a β – lactam antibiotic. However, the general medical importance of other peptide compounds is much less than that of either aminoglycosides or tetracyclines, mainly because of their undesirable side reactions, particularly renal toxicity. Presently, in medical practice only polymyxins and some antitumor and antitubercular compounds (actinomycin, bleomycin, cycloserin) are in wide range use. The sub families of this particular group will be listed down in Table 1.5.

1.3.4.2 Carbohydrate antibiotics

This group too forms one of the most abundant groups of antibiotics and include many physiologically active and important substances. Their essential roles include the metabolism of the different types of cells. In nature they rarely occur in free form (sugars), more frequently in polymeric association (oligosaccharides, polysaccharides, homo or heteropolymers), and very often in association with other classes of compounds (glycoproteins, glycolipids, nucleotides, and various glycosides). These present compounds number around 500 to this date, including numerous very important antibiotics. In addition to the outstandingly important aminoglycoside antibiotics used in human therapy (streptomycin, gentamicin, kanamycin, neomycin, etc.) and aminocyclitols used in agriculture and veterinary medicine, there are numerous others important antibiotics in the family. All compounds in this family, except some polysaccharides, are produced by *Eubacteriales,* predominantly by *Actinomycetales* and to a lesser extend by *Bacillus* species. However, high frequency of allergic reactions has been reported, and by and by large numbers of resistant mutants too have been reported. Certain cases also cause deafness because it has a specific toxicity for the auricular apparatus that results in vestibular damage with alteration of equilibrium.

Table 1.5 : Classification of Antibiotic Compounds According to its Chemical Structure (Berdy, 1982)

Main family of antibiotics	Subfamily	Important representatives
Amino acids and peptides	Amino acids derivatives	Cycloserine Alanosin Penicillin Glitoxin Chaetocin
	Homopeptides	Netropsin Negamycin Gramicidin Bacitracin Viomycin
	Heteromer peptides	Polymyxin Amphomycin Thiostrepton Bleomycin Sideromycin
	Peptolides	Actinomycin Surfactin Etamycin Telomycin Valinomycin
	Macromolecular peptides	Nisin Pacibilin Bacteriocins
Carbohydrates	Pure saccharides	Streptozotocin Soedomycin Glucans
	Aminoglycosides	Streptomycins Bluensomycin Neomycin Gentamicin Validamycin Fortimicin Sorbistin
	Other glycosides	Streptolin Vancomycin Chromomycin
	Sugar derivatives	Everninomicin Lincomycin Moenomycin

Table 1.5 : Classification of Antibiotic Compounds According to its Chemical Structure (Con't.)(Berdy, 1982)

Main family of antibiotics	Subfamily	Important representatives
Macrocyclic lactone (lactam)	Macrolides	Erythromycin Leucomycin Tylosin Borrelidin
	Polyene	Mycotrienine Nystatin Rimocidin Eurocidin Candihexin Dermostatin
	Macrocyclic lactones	Oligomycin Primycin Boromycin Chlorothricin
	Macrolactams	Rifamycin Maytansin Viridenomycin
Quinone and similar antibiotics	Tetracyclics	Tetracycline Rhodomycin Ayamycin
	Napthoquinones	Javanicin Granaticin Rubromycin
	Benzoquinones	Spinulosin Mitomycin
	Quinone-like compounds	Maytenin Epoxidon
Heterocyclics	Nitrogen containing heterocyclic compounds	
	• Single heterocycles	Pyrrolnitrin Blasticidins
	• Condensed heterocycles	Pyocyanine Anthramycin Tubercidin
Heterocyclics	Oxygen-containing heterocyclic compounds	
	• Small lactones	Acetomycin Penicillic acid Actinobolin
	• Polyether	Monensin Nigerisin

Table 1.5 : Classification of Antibiotic Compounds According to its Chemical Structure (Con't.) (Berdy, 1982)

Main family of antibiotics	Subfamily	Important representatives
Alicyclic antibiotics	Cycloalkane	Sarcomycin Fumagillin Ketomycin Streptimidone
	Small terpenes	Coriolin Vernolepin
	Oligoterpenes	Fusidic acid Saponins Trichotecin
Aromatic antibiotics	Benzene derivatives	Flavipin Chloramphenicol Xanthocyllin
	Condensed aromatic compounds	Griseofulvin Gossypol Orchinol
	Non benzoid aromatic compounds	Puberulic acid Lactaroviolin
	Other aromatic derivatives	Zinninol Novobiocin Nidulin
Aliphatic antibiotics	Alkane derivatives	Elaiomycin
	Carboxylic acid derivatives	Mycomycin Eulicin
	Sulfur and phosphor containing aliphatic compounds	Fluopsin Phosphonomycin Allcin

1.3.4.3 Macrocyclic lactone (lactam) antibiotics

Macrocyclic compounds are frequently occurring structural types among antibiotics. They can be classified as derivatives of long – chain aliphatic hydroxyl acids, forming internal lactone. Their specific and unique chemical and biological properties and their great importance require that they be discussed as a distinct group of antibiotics. Beside macrocyclic lactones and lactams, numerous large – ring cyclopeptides, cyclodepsipeptides, and a few carboxylic terpene derivatives are found in this group of natural products. Overall, there are to date about 1000 antibiotic compounds found in this group. Among the microbial bioactive metabolites, macrocyclic lactone family are relatively abundant, as compared to other natural products. Among the macrocyclic lactone family, they have antifungal, antimicrobial, cytotoxic, cation – active biological activities. The compounds in this family are produced almost exclusively by various *Actinomycetes*, mainly *Streptomyces* species. Only some macrocyclic lactone derivatives (cytochalasins, brefeldin, etc.) are produced by fungi. So far, none of the bacterial species have been reported to produce this type of antibiotic. The maytansine type and lactams are produced by higher plants and *Nocardia* species. One of the positive side to this antibiotic is that the side effects are much less and usually slight. But, the principal problems with this class of antibiotics seem to be unsatisfactory absorption and the spread of resistant strains.

1.3.4.4 Quinone and similar antibiotics

Chemically quinones are diketones derived from dihydroaromatic compounds. Quinoid compounds are widely distributed in nature. Various