DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EFFECTS OF GROWTH MEDIUM CONDITIONS ON ANTIBIOTIC PRODUCTION BY ACTINOMYCES SP.

by

Cihan Mehmet ALTINTAŞ

October, 2013

İZMİR

EFFECTS OF GROWTH MEDIUM CONDITIONS ON ANTIBIOTIC PRODUCTION BY ACTINOMYCES SP.

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by
Cihan Mehmet ALTINTAŞ

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İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"EFFECTS OF GROWTH MEDIUM CONDITIONS ON ANTIBIOTIC PRODUCTION BY ACTINOMYCES SP."** completed by **CİHAN MEHMET ALTINTAŞ** under supervision of **ASSOC. PROF. DR. HÜLYA AYAR KAYALI** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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EFFECTS OF GROWTH MEDIUM CONDITIONS ON ANTIBIOTIC PRODUCTION BY *ACTINOMYCES* SP.

ABSTRACT

Ramoplanin antibiotic has been shown to be the most effective to treat the bacteria that cause hospital infections even at low minimum inhibitory concentrations. However, the main obstacle for this antibiotic is its quite expensive cost. Due to preferred techniques in the antibiotic production require long-term optimizations, these techniques cause increasing production cost and period. To overcome shortcomings, metabolisms of industrially important microorganisms are examined by approaching to stoichiometric modeling and data analysis of integrated systems-based. For this purpose, it is started to research which is carried out computerized stoichiometric modeling that allows identification and regulation of metabolic flows within microorganism cells.

In this project, computerized stoichiometric modeling with MATLAB and COPASI software packages will be used for decrease to production cost of ramoplanin which is the most effective but very expensive in available antibiotics. In the thesis, in order to identify the aspects of intermediary metabolism relevant to ramoplanin production, the correlation between ramoplanin antibiotic production and intracellular tricarboxylic acid cycle (TCA) intermediate products were investigated depending on the glucose concentration and nitrogen sources of *Actinoplanes* sp. ATCC 33076 medium by using both the computerized stoichiometric modeling and experimental conditions. The flux balance analysis optimisation, using either growth or antibiotic production as objective function, did not correlate with experimental ramoplanin yields in fed processes since the maximum ramoplanin production of stoichiometric modeling and experimental conditions were determined at 5 and 10g g/L glucose, respectively. The diferences suggest that candidate process feed nutrients that might be expected to influence this network were used in process simulations and in silico predictions compared to experimental findings. Therefore, in this kind of study, experiments should be done and data of computerized stoichiometric modeling should be compared.

Keyword: Ramoplanin, *Actinoplanes* sp. ATCC 33076, metabolic engineering, metabolism, precursors.

AKTİNOMİSETLERDEN BESİ ORTAM KOŞULLARINA BAĞIMLI ANTİBİYOTİK ÜRETİMİNİN OPTİMİZASYONU

ÖZ

Günümüzde, ramoplanin antibiyotiğinin hastane enfeksiyonlarına neden olan bakterilerde çok düşük minimum inhibisyon konsantrasyonlarında (MIC) bile oldukça etkili olduğu gösterilmektedir. Antibiyotik üretiminde kullanılan tekniklerin uzun süreli optimizasyon çalışmalarına gereksinim duyması üretim süresinin ve maliyetin oldukça artmasına neden olmaktadır. Bu durum ramoplanin antibiyotiği için de geçerli olup ramoplaninin ticari satış fiyatı oldukça yüksektir. Günümüzde bu sorunların ortadan kaldırılması amacıyla stokiyometrik modelleme ve verilerin bütünleşik analizi yaklaşımıyla endüstriyel önemi olan mikroorganizmaların metabolizmaları sistem bazlı incelenmektedir. Bu amaçla, antibiyotik üretimine yönelik çalışmalarda mikroorganizmaların hücre içi metabolik akışların belirlenmesine ve düzenlenmesine imkân sağlayan bilgisayar destekli stokiyometrik modellemelerin gerçekleştirildiği çalışmalara başlanmıştır.

Bu nedenle hedeflenen projede, mevcut antibiyotikler içinde en etkili ancak çok pahalı olduğu belirlenen ramoplanin antibiyotiğinin üretim maliyetinin düşürülebilmesi MATLAB ve COPASI yazılım programları ile bilgisayar destekli stokiyometrik modelleme kullanılarak ramoplanin antibiyotiğinin prekürsörlerin ve metabolit seviyelerinin saptanması ve farklı glukoz derişiminde ve azot kaynaklarında büyütülen *Actinoplanes* sp. ATCC 33076 suş diyagramlarının belirlenmesinin ardından deneysel aşamaya geçilmiştir. Sonuçlar, deneysel ve stokiyometrik modelleme verilerinin korele olmadığını göstermekte olup maksimum ramoplanin üretimi deneyesel olarak ve modelleme de sırasıyla 10 ve 5 g/L glukoz ortamında elde edilmiştir. Bu sonuçların farklı bulunması biyokimyasal proseslerde pekçok faktörün etkili olması ve tüm bu değişenlerin bilgisayar modellemeye uygulamanın mümkün olmamasıyla açıklanabilir. Bu nedenle, bu tür modelleme çalışmalarının deneysel verilerle doğrulanması büyük önem arz etmektedir.

Anahtar sözcükler: Ramoplanin, *Actinoplanes* sp. ATCC 33076, metabolik mühendislik, metabolizma, öncüller.

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CHAPTER ONE
INTRODUCTION

1.1 Antibiotics

1.1.1 Antibiotic Definition

"Antibiotics" as a word originates from Greek and basically comprises of two words. The first part of the word that is "anti" refers to a Greek word which means "against". The last part “bios” means "life" in Greek.

Antibiotics are drugs that slow down the growth of or destroy bacteria causing illness such as syphilis, tuberculosis, salmonella, and some forms of meningitis (Nordqvis, 2007).

Virus related diseases such as influenza or cold can not be treated by antibiotics. A drug that kills viruses (which are smaller and simpler than bacteria) is called an antiviral, and a drug that kills parasites inside the body is an antiparasitic. Substances that kill bacteria and viruses outside the body, but that would be poisonous inside the body, are called antimicrobials, antibacterial, or disinfectants such as ordinary household bleach (sodium hypochlorite) (K. L. Lerner & B. W. Lerner, 2007).

In 1928, the discovery of antibiotics was achieved by Alexander Fleming. This found of antibiotics was a big breakthrough within medical science. The efficiency of treatment for many kinds of infections observed in humans or other types of livings has been improved by the antibiotics in the 1930s. Till the early 1930s, some of the illnesses such as tuberculosis, pneumonia and typhoid fever were not essentially treatable although some ways of fighting against microbial infections were already investigated.

1.1.2 Properties That an Antibiotic Should Have

A key prerequisite for any antibiotic is selective toxicity. An obvious way of achieving this is for a compound to direct its effect against a metabolic or physiological function found in microbial cells but not in the host. The nature and degree of this selectivity depends on two basics, non-toxicity and certain toxicity of given antibiotic to the host.

Practically used antibiotic for clinical purpose are all selectively toxic and the high level of this selectivity is dependant on the micorbial cell organisation which is different from the mammalian cells.

Antibiotics which inhibit the same process in host as in pathogen, or which cause harm to the host in some other way, are said to have side-effects. These may include directly toxic effects, hypersensitivity (allergic) reactions or adverse effects on the host's normal resident microflora (Hogg, 2005; Vardanyan & Hruby, 2006).

Antibiotics should not produce a hypersensitivity (allergic) reaction in the host. This is caused by an extreme response by the host immune system, and is not the same as toxicity.

Selective toxicity is the most important single attribute of an antibiotic, but ideally it should also have as many of the following properties as possible:

- Antibiotics need to be soluble in body fluids, in order to exert their effect by penetrating the body tissues. The compound must not be metabolised so quickly that it is excreted from the body before having a chance to act.

- If administered orally, it must not be inactivated by the acid environment of the stomach, and must be capable of being absorbed by the small intestine.

- Antibiotics should not have any significant effect on the resident microflora of the host.

- ❖ It should not be easy for the target pathogen to establish resistance against an antibiotic.

- ❖ It should be sufficiently stable to have a good shelf life, without special storage considerations.

- ❖ Side-effects such as allergic reactions should be minimal (Hogg, 2005).

1.1.3 Mechanism of Antibiotic Action

Antibiotics kill bacteria without harming other cells by attaching themselves to complex molecules called enzymes. Enzymes, which are found inside bacteria as well as on their outer shell, help chemical reactions to happen in the bacterial cell. Each enzyme participates in a different chemical reaction. By attaching to an enzyme, an antibiotic can stop the enzyme from doing its job.

Depending on which enzyme is targeted, the bacterial cell can be affected in several different ways. For instance, an antibiotic has impact on the ability of a bacterium by preventing its energy production from glucose. Another type of impact is observed on inhibition of bacterial cell wall construction with a specific antibiotic and correspondingly, bacteria die instead of reproducing.

Enzymes can be different in different cells, such as bacteria and body cells, and still do the same job. Antibiotics interfere only with the kinds of enzymes that bacteria use (K. L. Lerner & B. W. Lerner, 2007; Bayarski, 2006).

1.1.4 Spectrum of Antibiotic

Certain antibiotics, due to the mechanism of their action, are only effective against a few different pathogens, while others can be used successfully against many different kinds shown in Figure 1.1. They are said to have, respectively, a narrow spectrum and a broad spectrum of activity. On the face of it, all things being equal, you would expect your doctor to choose the antibiotic with the broadest possible

spectrum of activity, but this isn't always the wisest option. When the cause of an infection isn't known, it makes sense to hedge one's bets and prescribe a broad-spectrum antibiotic, but this policy is not without its dangers. The drug is likely to kill off many of the host's own resident microflora, which can lead to a superinfection, and the development of antibiotic-resistant strains is also made more likely. If the identity of the pathogen is suspected, an appropriate narrow-spectrum drug is to be preferred (Hogg, 2005; Bayarski, 2006).

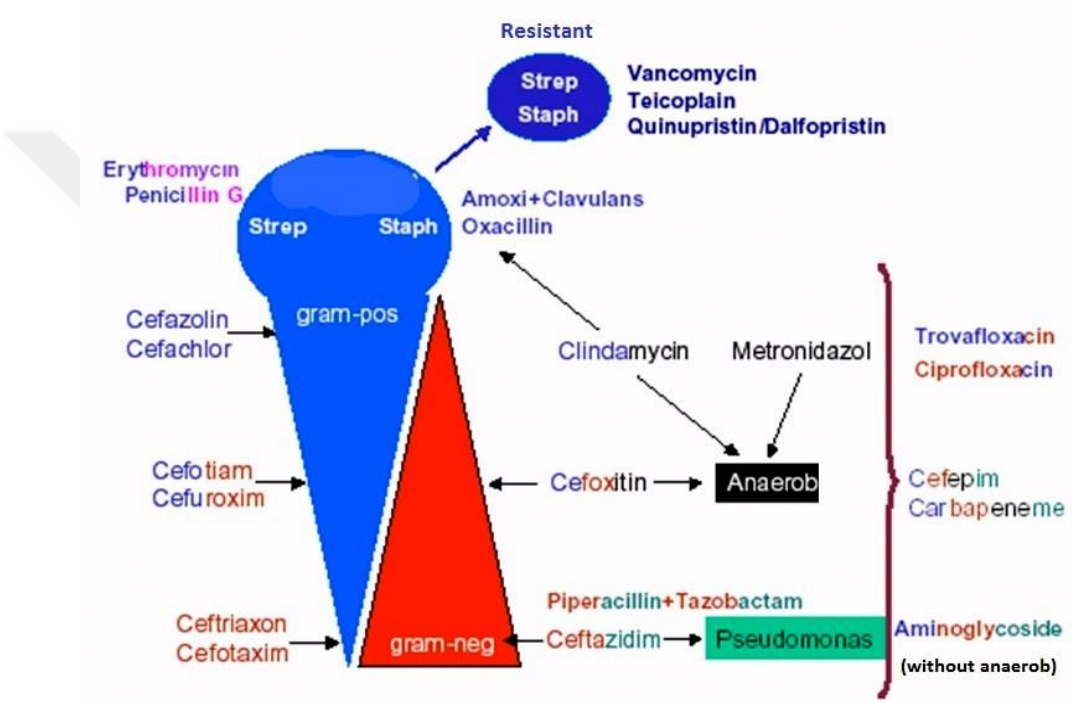

Figure 1.1 Spectrum of antibiotics.

Bacteria are single-celled life forms that can be either helpful or harmful. The human body needs many billions of the bacteria called *Escherichia coli* in the intestines to be healthy, but other bacteria, such as *Streptococci pyogenes*, can cause sickness or even death (K. L. Lerner & B. W. Lerner, 2007).

Effect of bacteria on the human race is too huge to fail to mention them completely. Some important examples about principal bacterial diseases of humans have been summarised in Table 1.1 (Hogg, 2005).

Table 1.1 Some bacterial diseases of humans.

	Genus	Disease
Gram -positive	*Staphylococcus*	Food poisoning, endocarditis, bronchitis, toxic shock syndrome
	Streptococcus	Pneumonia, pharyngitis, meningitis, scarlet fever, dental caries
	Enterococcus	Enteritis
	Listeria	Listeriosis
	Bacillus	Anthrax
	Clostridium	Tetanus, botulism, gangrene
	Corynebacterium	Diphtheria
	Mycobacterium	Leprosy, tuberculosis
	Propionibacterium	Acne
	Mycoplasma	Pneumonia, vaginosis
Gram-negative	*Salmonella*	Salmonellosis
	Escherichia	Gastroenteritis
	Shigella	Dysentery
	Neisseria	Gonorrhoea, meningitis
	Bordetella	Whooping cough
	Legionella	Legionnaires' disease
	Pseudomonas	Infections of burns
	Vibrio	Cholera
	Campylobacter	Gastroenteritis
	Helicobacter	Peptic ulcers
	Haemophilus	Bronchitis, pneumonia
	Treponema	Syphilis
	Chlamydia	Pneumonia, urethritis, trachoma

1.1.5 Activity and Resistance

1.1.5.1 Definitions

Activity: The activity of an antibiotic reflects its ability to inhibit microbial growth. While the concept of antibiotic activity appears simple and straightforward, the quantitative expression of antibiotic activity is complex and depends on the assay method and the conditions under which the test microorganism is grown (Lancini et al., 1995).

Resistance: Immunity developed within a specific type of bacteria via evolution against specific types of antibiotics by this means bacteria can resist under the therapeutic dose of antibiotic without showing any effects on the microorganism (Kumar & Lazarus, 2010).

Resistance of bacteria can be observed during the process of antibiotic use. This resistance can be classified by two signs: natural resistance and acquired resistance. Natural resistance is the genetic ability of bacteria that is coded in the chromosomes and spread to all lines of the given type of bacteria. In other words, bacteria are intrinsically resistant. For instance, there are genes responsible for resistance to their own antibiotic in *Streptomyces* and some bacteria don't have target or transport system for antibiotic (Todar, 2009).

The best known example related natural resistance is that *E. coli* strain, freeze-dried in 1946, was revived many years later, and found to have plasmid-encoded genes for resistance to streptomycin and tetracycline, neither of which was in clinical use until some years after the culture was preserved. It seems likely that bacteria possessed these genes to protect against naturally occurring antibiotics, an idea supported by the fact that R-plasmids have been found in non-pathogenic soil bacteria (Hogg, 2005).

Acquired resistance means that bacteria are usually sensitive to antibiotics, but liable to acquire the ability to oppose the given antibiotic. Acquired resistance is often caused by

- mutation of chromosomal genes,
- the acquisition of new mobile genetic elements such as plasmids, integrons and transposons
- a combination of these mechanisms (Vardanyan & Hruby, 2006; Giedraitienė at al., 2011).

1.1.5.2 Determination of Activity

Clinical microbiology laboratory has significant role on determining the characteristics of bacterial isolates such as antimicrobial susceptibility. Antimicrobial susceptibility testing is a kind of method for revealing the drug resistance in considered pathogens. It also aims to assure susceptibility to drugs of choice for specifically chosen infections. Rapid automated instrument and broth microdilution are used very commonly as testing methods. These methods use accessible equipments and materials in the market. Another testing method is the Manual Methods which include the gradient diffusion (for obtaining minimum inhibitory concentration - MIC) and disk diffusion in agar. These methods provide possible cost savings and flexibility in the test conditions. Each method which accurately tests the organisms has strengths and weaknesses. Some methods provide quantitative results for instance MIC, and all provide qualitative assessments using the categories susceptible, intermediate, or resistant. In general, current testing methods provide accurate detection of common types of antimicrobial resistance mechanisms. However, newer or emerging mechanisms of resistance require to be examined with high care depending on capacity of the test method to accurately detect resistance (Vardanyan & Hruby, 2006).

The diffusion in agar method includes some stages to determine qualitative inhibitory activity. A paper disc is used for providing antimicrobial drug - bacteria interaction. This is done by a specific amount of antimicrobial drug on the paper disc and covering that by agar which has a standard amount of bacteria. After waiting for incubation in a certain time period, the diameter of clean zones which refer to absence of the bacterial growth is measured and expressed in the form of categories of susceptibility to the considered drug (sensitive, intermediate, and resistant) by comparing with the standard diameters (Pucci, 1983; Jorgensen & Ferraro, 2009; Vardanyan & Hruby, 2006).

MIC in a liquid medium is determined by a method to reveal the qualitative sensitivity of an infection. For that purpose, a certain amount of microorganisms in a solution are exposed to a specific drug and serial two-fold dilutions of that mixture

are analysed. MIC refers to the minimum drug concentration which prevents the growth of bacteria following an overnight incubation and the measurements are expressed by an index of MIC. Lowest concentration of the drug which removes almost 99.9% bacterial content of the test condition refers to minimum bactericidal concentration (MBC). Drugs are usually regarded as bactericidal if the MBC is no more than four times the MIC. MBC is measured related to the amount of clean zones over the agar plates which were reincubated for one more night. The drug concentration available in the plasma, other tissues and fluids in the body should be correlated with the MIC and MBC (Pucci, 1983; Vardanyan & Hruby, 2006).

The antibiotics are divided into two types on the basis of their effect on the bacteria in other words, their aims on treatment. These are bactericidal which aims to destroy the bacteria and bacteriostatic that intends to prevent the bacterial growth. Preventing the growth of the bacteria by treating with bacteriostatic drugs allows neutrophils and other protective powers of the body to remove the pathogen. For bacteriostatic antibiotics, the MBC is considerably higher than the MIC. On the other hand, the bactericidal antibiotics have similar MBC and MIC (Bayarski, 2006; Vardanyan & Hruby, 2006).

The use of microbiological analytical techniques has grown tremendously in recent years. Controls of sterility have become ever more frequent, not only in the pharmaceutical and food industries, but also in chemical and electronic component industries. In addition, the preoccupying phenomenon of the spread of bacteria resistant to several antibiotics has caused an enormous increase in the number of requests for antibiograms made to the microbiological laboratories of hospitals. As a consequence, attempts have been made to develop microbiological techniques that would be less labor intensive and less costly. These attempts have developed in two directions: miniaturization and automation.

Miniaturization: Traditionally, tests have been carried out in 1-2 mL of medium, but at present tests are usually carried out in much lower volumes, e.g., 100 µL. Very common s the use of plastic plates, called microtiters, each containing 96 wells of

200 µL. The medium, the inoculum, and the antibiotic solutions can be distributed in the wells by appropriate multiple micro dilutors, thus saving a great deal of material and labor.

Automation: Automatic systems have been developed for reading inhibition halos or for counting colonies. They consist of optical readers coupled to computerized systems that record and analyze the data. The major development of automatic systems has been devoted to the determination of antibiograms in hospital laboratories. Nowadays, the most frequently used apertures based on systems for micro diluting samples in liquid medium (the volume varies from 0.02 to 0.1 mL). The antibiotics are generally contained, in a dry state, in appropriate tubes or plates. In some cases the antibiotic is absorbed on a paper disk from which it is eluted by contact with the culture medium. Bacterial growth is generally evaluated by automatically measuring the optical density at given time intervals. The elaboration and the interpretation of data are also performed automatically. The results are usually expressed in the form of categories of susceptibility (S= sensitive, I= intermediate, R= resistant), or, sometimes, as MIC (Lancini et al., 1995).

1.1.5.3 Biochemical Bases of Resistance

Bacteria have proved particularly adept at becoming resistant to each new antibiotic that is discovered in nature or synthesized by medicinal chemists. The biochemical mechanisms of natural and acquired resistances are identical and can be explained as the result of one of the following four reasons:

- **a.** Impermeable membrane,
- **b.** Multidrug resistance efflux pumps,
- **c.** Resistance mutations,
- **d.** Inactivation of antibiotic (Allen et al., 2010).

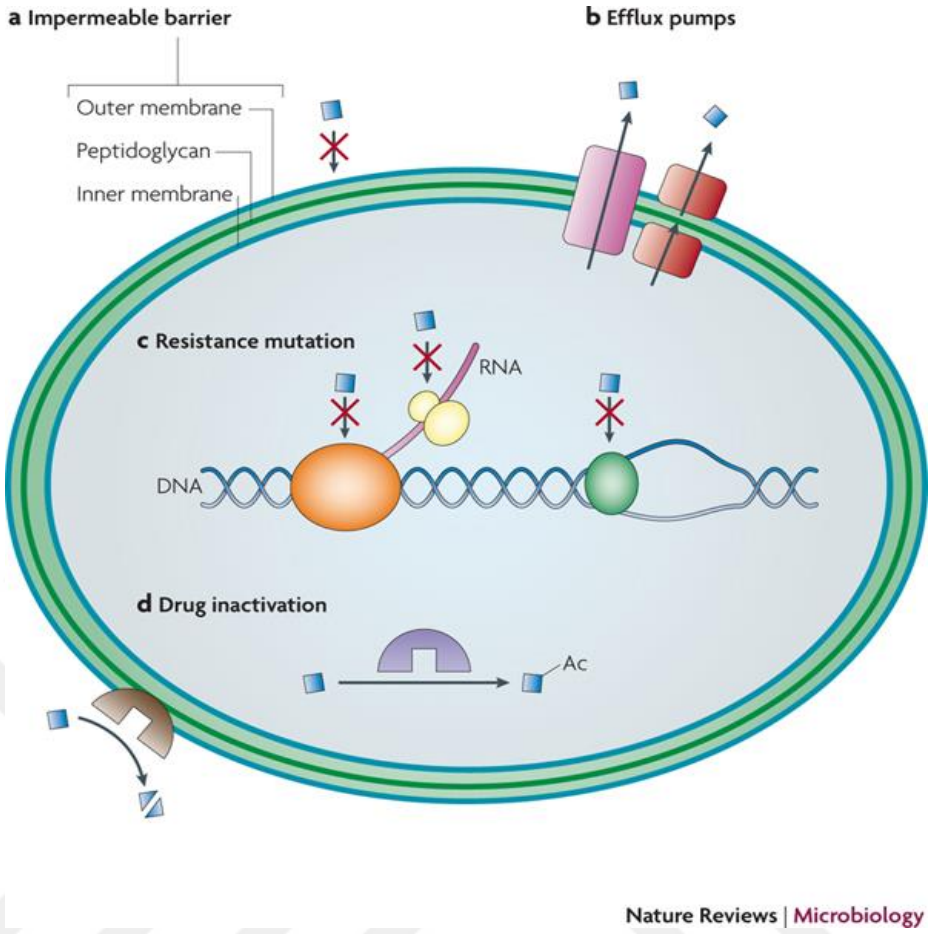

Figure 1.2 The biochemical mechanisms of antibiotic resistance.

*1.1.5.3.1 Impermeable Membrane***.** Some bacteria are able to resist certain antibiotic action by denying it entry to the cell because of having an impermeable membrane. Penicillin G for example is unable to penetrate the Gram negative cell wall (shown in blue squares in Figure 1.2). Formation of an impermeable barrier results in reduced antibiotic accumulation at target site (Hogg, 2005).

1.1.5.3.2 Multidrug Resistance Efflux Pumps. Some bacteria can transport the antibiotic out of the cell via efflux pump before it has had a chance to act, by means of enzymes called translocases; this is fairly non-specific, leading to multiple drug resistance. While some transporters, such as those of the resistance–nodulation–cell division family (pink), can pump antibiotics directly outside the cell, the others, such as those of the major facilitator superfamily (red), secrete them into the periplasm (Hogg, 2005; Allen et al., 2010).

Uptake of nutrients from the environment, release of cell signalling molecules and virulence factors as well as disposal of toxic compounds are essential for metabolic active microorganisms. In contrast to Gram-positive bacteria having a cytoplasmic membrane only, Gram-negative bacteria are surrounded by an additional outer membrane, which forms a strict barrier between the intracellular space and the environment. While lipophilic compounds can directly penetrate the membranes' lipid bilayers, hydrophilic molecules require transmembrane proteins forming water-filled pores for passive diffusion into the cell or energy-driven transmembrane pumps for active efflux.

Two mechanisms are operating alone or in concert to minimize the antibiotic concentration at the intracellular target site: Down regulation of the expression of the pore proteins also called porins, and upregulation of one or a set of several unspecific efflux pumps. However, the impact of these mechanisms on the resistance is low, since due to the essential function of porins for uptake of nutrients their reduction is limited and to avoid disturbances of membrane integrity due to extensive overproduction of mdr efflux pumps these are subjected a strict regulation (Heisig, 2008).

- **Resistance Due to Multiple-Drug Resistance (mdr) Efflux**

Currently, five different molecular classes of mdr efflux pumps are known. While pumps of the ATP-binding cassette (ABC) transporter superfamily are driven by ATP hydrolysis, the other four superfamilies called resistance-nodulation-division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), and small multidrug resistance transporter (SMR) are driven by the proton-motive force across the cytoplasmic membrane. Usually a single pump protein is located within the cytoplasmic membrane. However, the RND-type pumps which are restricted to Gram-negative bacteria consist of two additional components, a periplasmic membrane fusion protein (MFP) which connects the efflux pump to an outer membrane porin. This architecture allows the disposition of the antibiotics outside the cell. Efflux is due to an enzymatic activity and therefore saturable. The

combined intrinsic activities of different efflux pumps play a major role for the intrinsic resistance of Gram-negative bacteria to macrolides and oxazolidinones as well as to the intrinsic resistance of *Pseudomonas aeruginosa* against a broad range of disinfectants and antibiotics. Acquired resistance has been observed by constitutive upregulation of mdr efflux pump expression due to a mutation inactivating a respective repressor or inducible, caused by molecules transiently inactivating repressor molecules upon binding. Depending upon the substrate spectra of the respective subset of efflux pumps upregulated, a multiple drug resistance phenotype is expressed, which in combination with a specific resistance mechanism can contribute to a clinically relevant level of resistance (Heisig, 2008).

- **Resistance to Tetracyclines Due to Specific Efflux Pumps**

For some naturally occurring antibiotics, like chlormaphenicol and tetracyclines, specific drug efflux pumps have been detected in antibiotic-producing bacteria. The tetracycline specific efflux pumps have been detected in many bacterial pathogens and are the major mechanism of resistance to these drugs. Several of the corresponding structural genes are inducible expressed: In the presence of tetracyclines in the growth medium a few drug molecules enter the cell by diffusion, bind to the pump repressor and, thus, induce tet efflux pump expression (Todar, 2009; Heisig, 2008).

1.1.5.3.3 Resistance Mutations. Mutations may occur which modify bacterial proteins in such a way that they are not affected by antibiotics, for example by disabling the antibiotic-binding site but not the cellular functionality of the protein intact. Specific examples include mutations in the gyrase (green), which cause floroquinolone resistance, in RNA polymerase subunit B (orange), which cause rifampicin resistance, and in the S12 rRNA of the 30S subunit (encoded by rpsL) (yellow), which cause streptomycin resistance (Gerald, 2011).

Target alterations usually result in the reduction of the affinity for a drug. This strategy enables the bacteria to respond to alterations in the selective pressure due to,

e.g. novel antibiotics within a short period of time. Genetic basis for a target alteration are either point mutations in the respective target gene or acquisition of a DNA fragment containing information for an altered less susceptible target. Alternatively, targets can be modified enzymatically (Springer et al., 2001).

❖ Target Alteration by Point Mutations

The major mechanism of resistance to fluoroquinolones is the acquisition of point mutations in the genes gyrA and parC encoding the respective A subunits of both targets, topoisomerases II and IV. Only a few codons are affected which are located in the so-called quinolone resistance-determining region (QRDR) between ala-67 and gln-106 (E. coli numbering of gyrA). To achieve a high level of resistance, at least two gyrA and one parC mutations are required.

Other examples include rifampin resistance due to point mutations in the rpoB gene encoding the β-subunit of RNA polymerase, or oxazolidinone resistance due to a G2576T mutation in the gene for the 23S rRNA as central part of the 50S large ribosomal subunit. Macrolide resistance is based upon the alteration of nucleotide A2058 by a point mutation.

❖ Target Modification by Acquisition of Genes Encoding Resistant Target

The targets of β-lactams, the penicillin binding proteins (PBPs) require several mutations in order to become resistant while simultaneously maintaining their viable function as cell wall transpeptidases/transglycosidases. Thus, in order to achieve clinically relevant resistance *Streptococcus pneumoniae* uses a unique strategy to rapidly accumulate several point mutations. Due to its natural competence for transformation during respiratory tract infections *S. pneumoniae* cells can acquire and insert into their chromosomes genetic material from closely related species, like viridians group streptococci. Since these cells carry genes for PBPs with reduced sensitivity to β-lactams due to the presence of several genetic variations, transformation/recombination generate so called mosaic genes encoding a β-lactam-

resistant variant protein, PBPX. *Staphylococcus aureus* cells can acquire large DNA fragments containing the mecA gene which encodes a complete new penicillin binding protein 2A (PBP 2A), as part of a transposon. PBP2A can substitute the natural set of penicillin-sensitive PBPs thereby mediating a complete cross resistance to all β-lactam antibiotics (Heisig, 2008; Penrose, 1998).

- **Target Alteration by Enzymatic Modification**

An alternative strategy is the alteration of the antibiotic target site by bacterial enzymes. Modification of nucleotide A2058 of the 23S rRNA is used by the majority of pathogens: the production of a specific methyltransferase which couples one or two methyl groups to the N6 amino group thereby reducing the affinity of macrolides to their target. Since this residue is also involved in the binding of other antibiotics, like streptogramin B and lincosamine, this mechanism is termed MLSB type. Another more sophisticated mechanism of enzymatic alteration of the target is the transfer of the complete regulon encoding a D-alanyl-D-lactate-ligase, like VanA, as basis for transferable resistance to glycopeptide resistance. In cooperation with some accessory factors the structure of the basic units of the bacterial cell wall, the disaccharide-pentapeptide with a C-terminal D-alanine, is transformed into a variant carrying a C-terminal D-lactate which prevents the binding of glycopeptide antibiotics at physiological concentrations and, thus, causes glycopeptide resistance. Such regulons reside on transposons allowing for the rapid exchange of the genetic information between different enterococcal species and even to staphylococci (Heisig, 2008).

1.1.5.3.4 Drug Inactivation. Many bacteria can secrete enzymes that modify or degrade antibiotics, causing them inactive. Inactivation can be the result of covalent modification of the antibiotic, such as that catalysed by acetyltransferases (purple) acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalysed by β-lactamases (brown) acting on β-lactam antibiotics (Allen et al., 2010).

The inactivation of a drug is an enzymatic process which results either in the cleavage of a chemical bond, e.g. by proteases or esterases, or the formation of a new chemical bond, e.g. by transferring phosphate (phosphotransferase), acetate (acetyltransferase), or nucleotidylate (nucleotidyltransferase) moieties to a functional group of the antibiotic. The cleavage causes the loss of function of the antibiotic. Chemical modification can eliminate a specific molecular interaction between drug and target or can alter the physic-chemical properties of a drug, e.g. electrical charge that has an impact on the ability of a drug to penetrate the bacterial membrane(s) (Todar, 2009).

❖ **Proteases:** β-lactamases are the proteases that hydrolyse a peptide bond in the essential β-lactam ring of penicillins, cephalosporins, carbapenems and monobactams and, thereby, irreversibly inactivate the drug. β-lactamases share this mechanism with the penicillin binding proteins (PBPs), which are essential enzymes catalyzing the biosynthesis of the bacterial cell wall. In contrast to the PBPs which irreversibly bind β-lactams to the active site serine, the analogous complex of the drug with β-lactamases is rapidly hydrolyzed regenerating the enzyme for inactivation of additional β-lactam molecules. According to their genetic relationship and their biochemical mechanism of action β-lactamases are divided into enzymes of the serine-protease type containing an active-site serine (molecular class A, C, and D enzymes) and those of the metallo-protease type (molecular class B enzymes), which contain a complex bound zinc ion (Miller et al., 2004).

❖ **Esterases:** Hydrolysis of macrolides by products of the ere genes detected in enterobacteria is only of scientific interest, while esterases VGB-A and VGB-B encoded by the vgb type genes mediate clinically relevant resistance in staphylococci to the B compound (quinupristin) of the streptogramin combination quinupristin–dalfopristin.

❖ **Acetyltransferases:** The preferred substrates of acetyltransferases are amino groups of antibiotics, like chloramphenicol, streptogramin derivatives, and the various aminoglycosides. The modification is believed to block a functional group

involved in the drug-target-interaction. All acetyltransferases use acetyl-coenzyme A as cofactor. The major mechanism of resistance to chloramphenicol is mediated by the chloramphenicol acetyltransferases (CAT enzymes) which transfer one or two acetyl groups to one molecule of chloramphenicol. While the CAT enzymes share a common mechanism, different molecular classes can be discriminated.

❖ **Nucleotidyltransferases, Phosphotransferases:** Beside AAC enzymes two different enzyme classes, nucleotidyltransferases (ANT enzymes), and phosphotransferases (APH enzymes) modify the hydroxyl groups of aminocyclitol–aminoglycoside antibiotics (Heisig, 2008).

1.1.5.4 How Does Antibiotic Resistance Arise?

In the real world, bacterial resistance does not evolve quite so simply. Nevertheless, it does evolve over time, and it is a serious medical problem (K.L. Lerner & B.W. Lerner, 2007).

Mutations occur spontaneously in bacteria, which render them resistant to one antibiotic or another. Usually the mutation leads to a change in a receptor or binding site such as those just described, rendering the antibiotic ineffective. The changes are usually brought about by point mutations occurring at very low frequency on chromosomal DNA. Bacteria can, however, become resistant much more rapidly by acquiring the mutant resistance-causing gene from another bacterium. This is called transmissible antibiotic resistance; it occurs mainly as a result of bacterial conjugation, and is the cause of most of the resistance problems we presently face. Transmissible resistance was first reported in Japan in the late 1950s, when multi-drug resistance in *Shigella* was shown to have been acquired by conjugation with resistant *E. coli* in a patient's large intestine. *E. coli* is known to transfer R (resistance) plasmids to several other gut bacteria including *Klebsiella*, *Salmonella* and *Enterobacter*, as well as *Shigella*. Whereas chromosomal mutations usually result in a modification to the drug's binding site, genes carried on plasmids code for enzymes which inactivate it, or lead to its exclusion from the cell (translocases) (Hogg, 2005).

There is a strong link between the use of a particular antibiotic in a locality and the incidence of resistant bacterial strains. This is because of selective pressure favouring the resistant forms of a bacterium. Fortunately this can, at least in part, be reversed, as several studies have shown, where a more restricted use of certain antibiotics over several years was followed by a reduction in the incidence of resistant bacteria forms (Hogg, 2005).

Resistance is also the reason why some scientists question the use of antibiotics in raising animals for food. Antibiotics are fed to cows, chickens, turkeys, and pigs in order to make them grow faster by killing off most of the bacteria in their bodies. The proportion of antibiotic production in United States reached 50 million pounds, while another 16 million pounds of antibiotics are transferred into livestock for a faster growth. However, many scientists think that dumping so many antibiotics into the environment may be helping antibiotic-resistant strains of bacteria to become more common (Barton, 2000; K.L. Lerner & B.W. Lerner, 2007).

1.1.5.5 Step by Step Development of Resistance to Available Antibiotics

For many years, antibiotics have been utilized successfully in the treatment of bacterial infection. However, the excessive use of broad-spectrum antibiotics which constitutes selective pressures on bacterial populations has resulted in evolution of resistance mechanisms.

Not long after penicillin was put into general use, strains of *Staphylococcus aureus* were found which did not respond to treatment, and by 1950 penicillin resistant *S. aureus* was a common cause of infections in hospitals. A decade later, a semi-synthetic form of penicillin, methicillin, was introduced; this was not affected by the β-lactamase enzymes that inactivated Penicillin G, and was used to treat resistant forms. Within years, infections caused by strains of *Staphylococcus aureus* couldn't be treated by using methicillin. The impact of methicillin resistant *S. aureus* (MRSA) has increased greatly since, and it represents the major source of nosocomial infections. In 1980, synthetic fluoroquinolones were introduced to counter the threat of MRSA, but within a year 80% of isolated strains had developed

resistance to these too. Vancomycin is regarded as a last-resort treatment for MRSA, for a number of reasons; it has a number of serious side-effects, its widespread use would encourage resistance against it, and it is extremely expensive (Hogg, 2005).

Using of new classes of antibiotics, for instance aminoglycosides, macrolides, glycopeptides, and also the chemical modification of available antibiotics allowed overcoming antibiotic resistance. Unfortunately, in recent years, antibiotic resistance has emerged in virtually all combinations of hospital-acquired pathogen–antibiotic drugs (McCafferty et al., 2002).

Increase in multiply resistant MRSA, VRE and FQRP over the last 30 years in US hospital intensive care units (ICU) was shown in Figure 1.3. The ICU is the last place we want to see very resistant pathogens, and yet this is where they seem to be the most frequent. In Europe, the situation is not much different. State of resistance in key pathogens isolated from bloodstream infections in Europe is shown in Figure 1.4 (Shlaes, 2010).

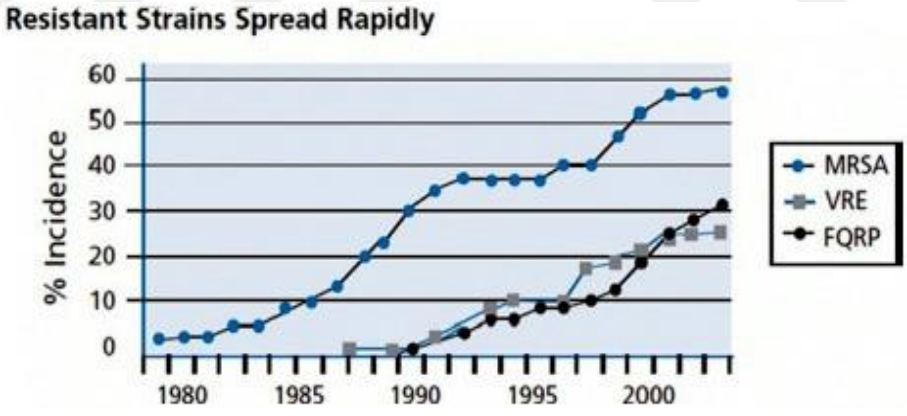

Figure 1.3 Resistance rates in US intensive care units over time. MRSA – methicillin-resistant *Staphylococcus aureus*. VRE – Vancomycin-resistant enterococcus. FQRP – Fluoroquinolone (ciprofloxacin) resistant *Pseudomonas aeruginosa*. From the CDC and the Infectious Diseases Society of America with permission.

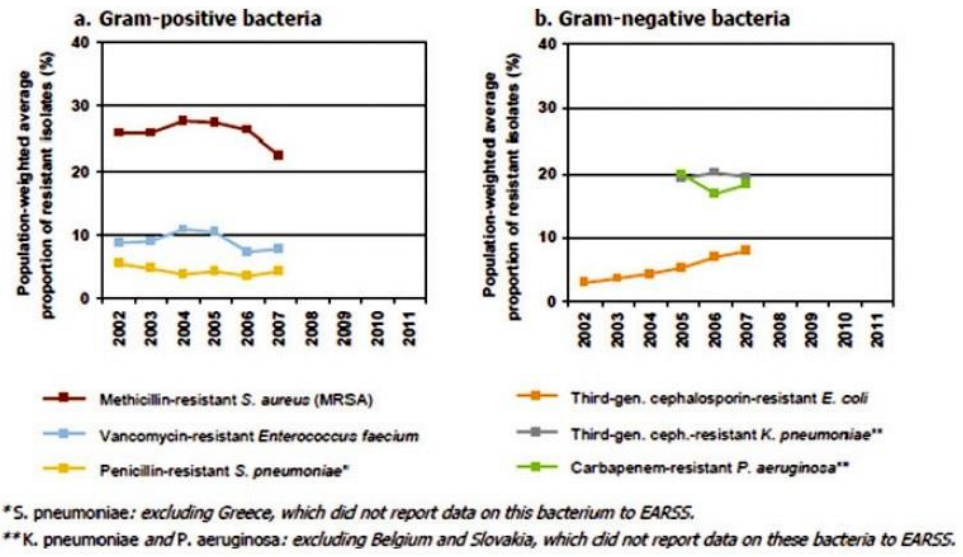

Figure 1.4 Population-weighted, average proportion of resistant isolates among blood isolates of bacteria frequently responsible for bloodstream infections, EU Member States, Iceland and Norway, 2002–2007. Taken from The Bacterial Challenge, a Time to React, published by the European Centre for Disease Prevention and Control and the European Medicines Agency.

The impact of certain antibiotics can be greatly reduced due to the development of resistance by target bacteria and these bacteria are called ‘superbugs’. This represents the greatest single challenge facing us in the fight against infectious diseases at the start of the 21st century (Hogg, 2005).

1.1.5.5.1 Glycopeptide Resistance. In 1996, vancomycin resistant *Staphylococcus aureus* (VRSA) was emerged in Japan; a few months later it had reached the USA. This represents a serious threat; some of these strains respond to treatment with a cocktail of antibiotics, but already people have died from untreatable VRSA infections. In 2003, a strain of VRSA was shown to have obtained its vancomycin resistance by cross-species transfer from a strain of *Enterococcus faecalis* (Hogg, 2005).

Glycopeptide antibiotic producing organisms are Gram-positive bacteria but also are intrinsically resistant to glycopeptide antibiotics. Resistance occurs through modification of the peptidoglycan D-Ala-D-Ala termini to D-Ala-D-Lac. This requires three enzymes and two regulatory proteins, encoded by the vanHAX and

vanRS clusters, respectively. VanS is a membrane-associated histidine kinase which upon activation will phosphorylate the response-regulator VanR, resulting in increased transcription of vanHAX. VanH converts pyruvate to D-Lac, VanA ligates D-Lac to D-Ala, and VanX cleaves the D-Ala-D-Ala bond. Glycopeptide antibiotics are rendered ineffective against the producing organism due to the modification o f peptidoglycan termini to D-Ala-D-Lac and concomitant loss of a hydrogen bond. A less prevalent resistance mechanism is modification of the D-Ala-D-Ala termini to D-alanyl-D-serine which also promotes resistance to glycopeptide antibiotics (Jovetic et al., 2010).

Clinically-relevant pathogens become resistant to glycopeptide antibiotics by altering peptidoglycan synthesis by either generating more D-Ala-D-Ala termini or modifying the D-Ala-D-Ala termini through acquisition of the resistance genes (i.e., vanRSHAX). The first mechanism has been observed in vancomycin-intermediate *S. aureus* (VISA) which produce a thickened peptidoglycan layer via longer glycan chains and less pentapeptide cross-linking, essentially producing more D-Ala-D-Ala termini to bind vancomycin and resulting in vancomycin tolerance. Recently, vancomycin-resistant *S. Aureus* (VRSA) has emerged and it was shown to be due to the transfer o f resistance genes from VRE to MRSA. Vancomycin and teicoplanin are generally thought o f as the “drugs o f last resort” but infections caused by these resistant pathogens can sometimes be treated with multiple antibiotics, antibiotics of a different class, or second-generation glycopeptide antibiotics (Lamb, 2007).

If the whole population is attacked with an antibiotic, resistant bacteria will survive. The next generation of bacteria will resemble the survivors, so when the population grows again, the same antibiotic will not work. Due to this reason scientists in both academia and industry are actively screening for new antibiotics which are active against resistant pathogenic bacteria in the hope of finding a new "drug of last resort". Ramoplanin is one of the promising candidates. Ramoplanin is a lipoglycodepsipeptide antibiotic that targets cell wall biosynthesis. It is active against VRE and MRSA strains and bacterial cells haven’t developed any resistance against it (K. L. Lerner & B. W.Lerner, 2007; Fang, 2008).

1.2 Classification of Antibiotics

1.2.1 Mechanism of Action

All antibiotics have the common property of interfering in some way with a normal, critical function of the target bacterial cell. The most commonly used antibiotics exert their effect by one of the following methods (Figure 1.5):

- ❖ Inhibition of Cell Wall Synthesis,
- ❖ Inhibition of Protein Synthesis,
- ❖ Inhibition of Replication and Transcription of Nucleic Acids,
- ❖ Inhibition of Folic Acid Metabolism
- ❖ Inhibition of Cytoplasmic Membrane Structure (Walsh, 2003).

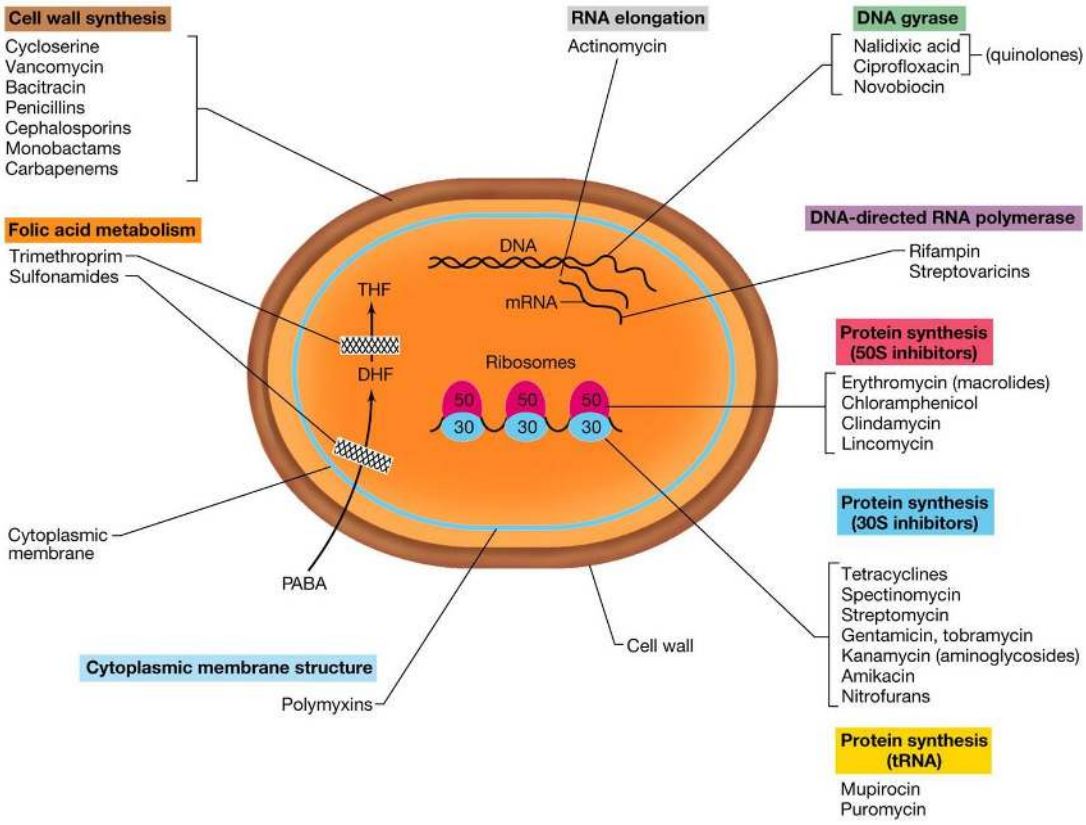

Figure 1.5 Mechanism of antibiotic action

1.2.1.1 Inhibition of Cell Wall Synthesis

The main groups which work in this way are the **β-lactam antibiotics**, so-called because they contain a β-lactam ring in their structure and **glycopeptides antibiotics**.

Target of β-lactams: Penicillin-binding proteins, PBP's, are a group of membrane-bound enzymes which act as transpeptidases in bacterial cell wall synthesis. Inhibition of the reticulation of the pentapeptidic chains of peptidoglycan precursors results in the death of bacteria (Goo & Sim, 2011; Bambeke et al., 2008).

An important factor in the strengthening of the bacterial peptidoglycan component is the cross-linking of chains by transpeptidation. It is this process which is acted on by the β-lactams; they bind irreversibly to the transpeptidase enzyme, forming covalent bonds with a serine residue within the enzyme's active site. The cell wall continues to form, but becomes progressively weaker as more new, unlinked, peptidoglycan is set down. Since bacteria are generally to be found in a hypotonic environment, as the wall weakens, water enters the cell, leading to swelling and then lysis (Brunton et al., 2010; Hogg, 2005).

β-lactam antibiotics have also a second mode of action. The β-lactams act by preventing the natural regulation of enzymes called autolysins. These enzymes function by breaking down peptidoglycan in a controlled fashion, causing breaks to allow for the addition of new peptidoglycan as the cell grows, and are normally regulated by naturally occurring inhibitors. The β-lactams neutralise the activity of these inhibitors, leading to further breakdown of the cell wall.

1.2.1.1.1 β-Lactam Antibiotics. Penicillins, cephalosporins, monobactams and carbapenems are included in this group. The penicillins are a group of antibiotics produced by *Penicillium* fungi including benzylpenicillin, procaine penicillin, benzathine penicillin, and penicillin V.

Benzylpenicillin, or penicillin-G is the first β-lactam antibiotic to be discovered. Action of this antibiotic is restricted to Gram-positive bacteria, because it is unable to

penetrate the Gram-negative cell wall. If it is administered intramuscularly, it is effective against Gram-positive bacteria. It is degraded rapidly by acidic environment of stomach so, it shouldn't be taken orally. Another naturally occurring penicillin, penicillin-V, represented an advance inasmuch as it is less acid-labile and can therefore be taken orally. All the penicillins are based on a core structure or nucleus called 6-amino-penicillanic acid (Figure 1.6); extensive research has led to the development of many variants of this, the so-called semisynthetic penicillins. These have attached to their nucleus novel side chains not encountered in nature, and have overcome some of the problems inherent in naturally occurring penicillins such as instability and narrow specificity (Cojocel, 2004).

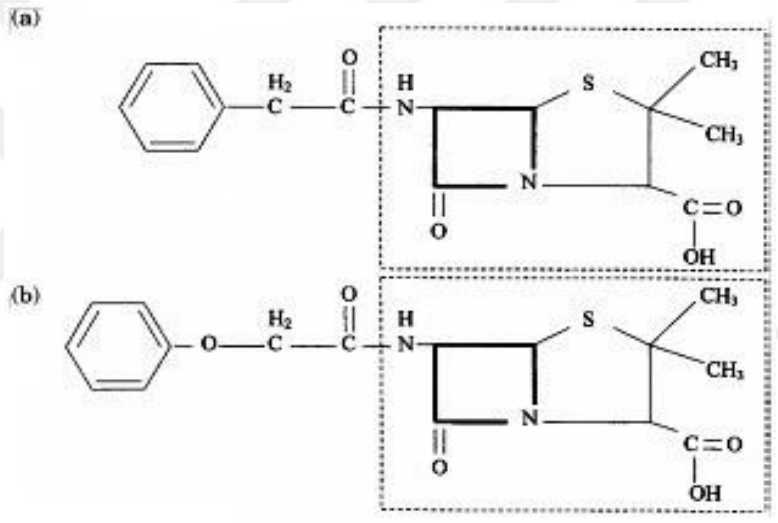

Figure 1.6 Naturally occurring penicillins. (a) PenicillinG (benzylpenicillin); (b) penicillin V. The dotted outline covers the 6-aminopenicillanic acid nucleus present in all variants of penicillin. The heavy outline denotes the β-lactam ring.

Ampicillin is semi-synthetic penicillin that has a broader specificity than Penicillin G; it is perceptibly more effective against Gram-negative bacteria for instance, *Salmonella* and *Escherichia coli*, its hydrophobic nature making it better able to penetrate their outer membrane. It has the additional benefit of being acid-stable and can therefore be taken orally (Hogg, 2005).

Another drawback to natural penicillins is that they are susceptible to naturally occurring bacterial β-lactamases (also called penicillinases), which breaks a bond in

the β-lactam core of the penicillin molecule (Figure 1.7). Sometimes, β-lactam antibiotics are taken in combination with a β-lactamase inhibitor such as clavulanic acid to overcome certain types of antibiotic resistance. Inhibitor binds to the β-lactamase with a high affinity, preventing it from acting on the antibiotic. Some semisynthetic penicillin such as methicillin and oxacillin are resistant to attack by the β-lactamases that can render certain bacteria resistant to their naturally occurring forms (Sharma et al., 2013).

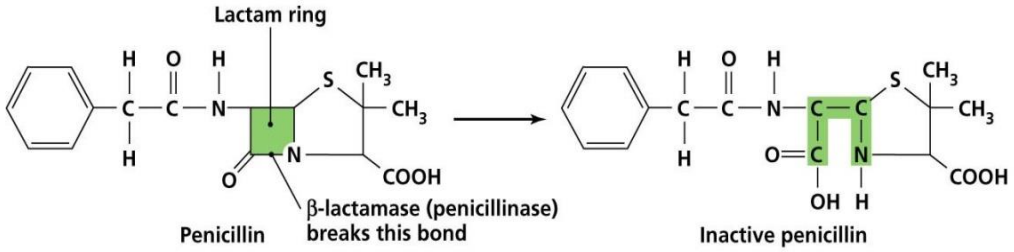

Figure 1.7 Action of β-lactamase on penicillin. A number of bacteria, especially staphylococci, possess the enzyme β-lactamase (penicillinase), which inactivates penicillin by cleavage of the β-lactam ring at the point marked by the arrow.

The cephalosporins, like the penicillins, have a structure based on a β-lactam ring. They also exert their effect on transpeptidases, but generally acts against a wide range of disease-causing bacteria and are more resistant to the action of β-lactamases. Ceftriaxone, for instance, is often used for the treatment of community-acquired or mild to moderate health care-associated pneumonia (in combination with macrolide and/or aminoglycoside antibiotics) and gonorrhoeal infections, caused by penicillin-resistant strains of *Neisseria gonorrhoeae*. In addition, patients who are allergic to penicillin are often treated with cephalosporins. Cephalosporins were first isolated in the late 1940s from a marine fungus called *Cephalosporium acremonium*, and came into general use in the 1960s.

Cephalosporins have been developed to widen the spectrum of activity to include many Gram-negative organisms, and to keep one step ahead of pathogens developing resistance to earlier versions. Cephalosporins are classified by five groups (generations) according to their antimicrobial properties:

- 1. Group: cefazedone, cephazolin, cefadroxil, cephalexin, cephradine
- 2. Group: cefaclor, cefamandole, cefprozil, cefuroxime
- 3. Group: cefcapene, cefdinir, cefditoren, cefixime, cefetamet
- 4. Group: cefepime, cefluprenam, cefozopran
- 5. Group: ceftobiprole, ceftaroline fosamil

Both penicillins and cephalosporins are also used prophylactically, that is, in the prevention of infections, prior to surgery in particularly vulnerable patients (Bayarski, 2006).

The monobactams are also a group of β-lactam antibiotics but differ from the other groups owing to the fact that β -lactam ring hasn't fused to another ring in structure (Figure 1.8). Aztreonam is the best known monobactam antibiotic and generally acts against a wide range of gram-negative bacteria (Hellinger & Brewer, 1999).

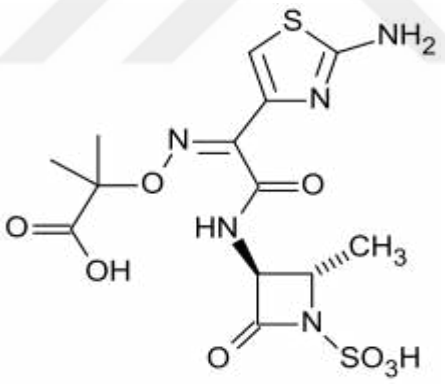

Figure 1.8 Structure of monobactams.

The carbapenems are a group of β-lactam antibiotics, as penicillins and cephalosporins. Carbapenems are produced naturally by a species of *Streptomyces*. A semisynthetic form, imipenem, is active against a wide range of Gram-positive and -negative bacteria, and is used when resistance to other β-lactams has developed (Hawkey & Livermore, 2012).

1.2.1.1.2 Glycopeptide Antibiotics. The chemical structure of these antibiotics consists of a chain of amino acids, often closed into a ring. They are produced by a great variety of microorganisms by biosynthetic pathways different from protein synthesis (Lancini et al., 1995).

These are linear heptapeptides, having at least five of the amino acids residues represented by benzene rings, which are connected together tı-o form diphenyl and triphenyl ether groups. These rings bear various substituents, like hydroxyl, chlorine atoms, and sugars. Their mechanism of action is related with the formation of a complex with the terminal D-alanyl-D-alanyl moiety of the intermediates in bacterial cell wall synthesis. Steric hindrance prevents the activity of transpeptidases and transglycosidases related with reticulation of peptidoglycan and it results in death of bacteria. Resistant mutants are very rare. The proposed name dalbaheptides derives from the two described characteristics (D-alanyl-D-alanyl-binding heptapeptide). The important members of this class are: vancomycin, produced by *Amycolatopsis orientalis*, and teicoplanin, a mixture of five very similar substances produced by *Actinoplanes teichomyceticus*, which are active against gram-positive bacteria, in particular staphylococci resistant to the β-lactams (Bambeke et al., 2004; Bambeke et al., 2008).

Bacitracin and vancomycin are two other antibiotics that exert their effect on the cell wall, but by a different mechanism. Bacitracin is derived from species of *Bacillus* and acts on bactoprenol pyrophosphate, the lipid carrier molecule responsible for transporting units of peptidoglycan across the cell membrane to their site of incorporation into the cell wall. Inhibition of the recycling of this lipid carrier results in death of bacteria. Its use is restricted to topical application, since its use internally can cause kidney damage. Vancomycin is a highly toxic antibiotic with a narrow spectrum of use against Gram-positive bacteria such as streptococci and staphylococci. It is particularly important in its use against infections caused by organisms resistant to methicillin and the cephalosporins, such as methicillin-resistant *Staphylococcus aureus.* It is not absorbed from the gastrointestinal tract and is therefore most commonly administered intravenously (Hogg, 2005).

1.2.1.1.3 Derivatives of Glycopeptide and Ramoplanin. Some of the basic therapeutic problems arise due to multiresistance ability in glycopeptide-resistant bacteria. So, new generation and more effective antibiotics are necessary to overcome resistance problems. The best known semisynthetic lipoglycopeptides are dalbavancin (derivative of teicolpanin), oritavancin and telavancin (derivative of vancomycin). These novel antibiotics can be efficiently used for treatment of infections of multi-drug-resistant gram-positive bacteria. The heptapeptide core, which is common for all glycopeptides, has also been located in these antibiotics to prevent transglycosylation and transpeptidation. Varied in vitro activities of three semisynthetic lipoglycopeptides are gained by heptapeptide core modifications. Lipophilic side chains in all three lipoglycopeptides are effective on several ways. Longer half-life for lipoglycopeptides is a major benefit of these chains. Also an increase in the activity of all three lipoglycopeptides against gram-positive cocci is provided with these side chains by insuring the position of agents on the cell membrane. In addition to these activities of lipoglycopeptides, telavancin and oritavancin have ability to disrupt bacterial membrane integrity and provide higher permeability of membrane; oritavancin is able to inhibit RNA synthesis (Zhanel et al., 2010).

Another such promising candidate is ramoplanin that has attracted significant attention in recent years. Ramoplanin produced by *Actinoplanes sp*. ATCC 33076, was first identified as peptidoglycan inhibitors in 1984 by Biosearch Italia in a screen for cell wall active antibiotics. This novel antibiotic also inhibits peptidoglycan (PG) biosynthesis at the extracellular stage. It is active against VRE and MRSA strains and bacterial cells have not developed any resistance against it (Fang, 2008).

As shown in Figure 1.9, ramoplanin is a lipoglycodepsipolypeptide that has a 49-member macrocyclic backbone joined by a lactone bond between the β-hydroxy group of Asn^2 and the carboxyl terminus of Chp^{17}. Also the structure of ramoplanin is composed of three closely related components 1-3, of which 2 is the most abundant, that differs only acyl group found on Asn^1 N-termus in the structure.

Among the 17 amino acid residues, nine are L amino acids, seven are D amino acids, and one is Glycine. As a nonribosomal peptide synthesis product, ramoplanin also contains several nonproteinogenic amino acids, such as ornithine (Orn), hydroxyphenylglycine (Hpg), chlorohydroxyphenylglycine (Chp), and β-hydroxyasparagine (β-OH-Asn). In addition, the phenol of amino acid 11 (Hpg^{11}) is attached to a disaccharide moiety, the α-l,2-dimannosyl group. Among the 16 amino acids in the ramoplanin macrocycle, 10 are β-branched: L-β-OHAsn^{2}, D-Hpg^{3}, D-*allo*-Thr^{5}, L-Hpg^{6}, D-Hpg^{7}, L-*allo*-Thr^{8}, L-Hpg^{11}, D-*allo*-Thr^{12}, L-Hpg^{13} and L-Chp^{17} (McCafferty et al., 2002).

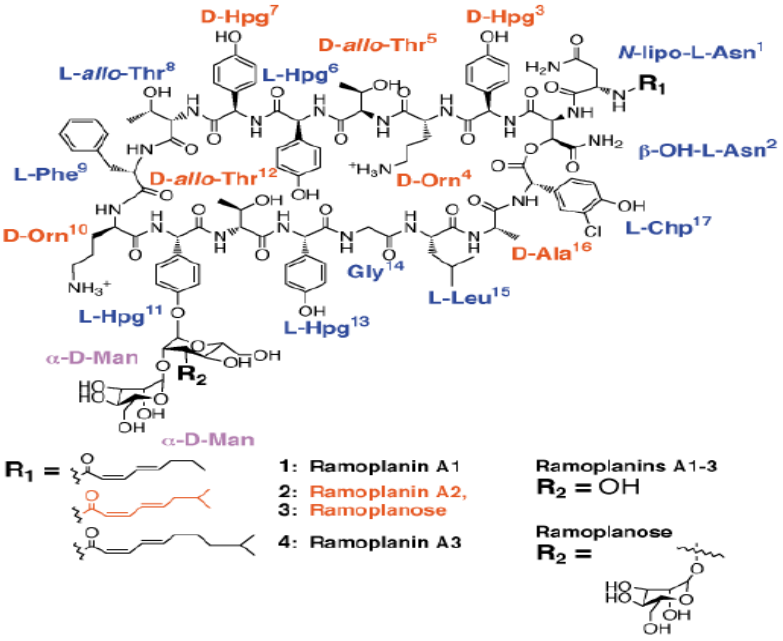

Figure 1.9 Structure of ramoplanin.

Ramoplanin exerts its effects by preventing the formation of peptidoglycan, an essential part of the bacterial cell wall as shown in Figure 1.10. The preceding steps involve the formation of peptidoglycan by the enzymatic conversion of lipid I to lipid II by MurG, resulting in the final polymerization with the cross-linking of lipid II. Initially, it was thought that ramoplanin blocked peptidoglycan biosynthesis by inhibiting the MurG-catalyzed conversion of lipid I to lipid II. However, further studies have shown that ramoplanin binds directly to lipid II, thereby resulting in inhibition of bacterial transglycosylases (Hu et al., 2003; Walker et al., 2005).

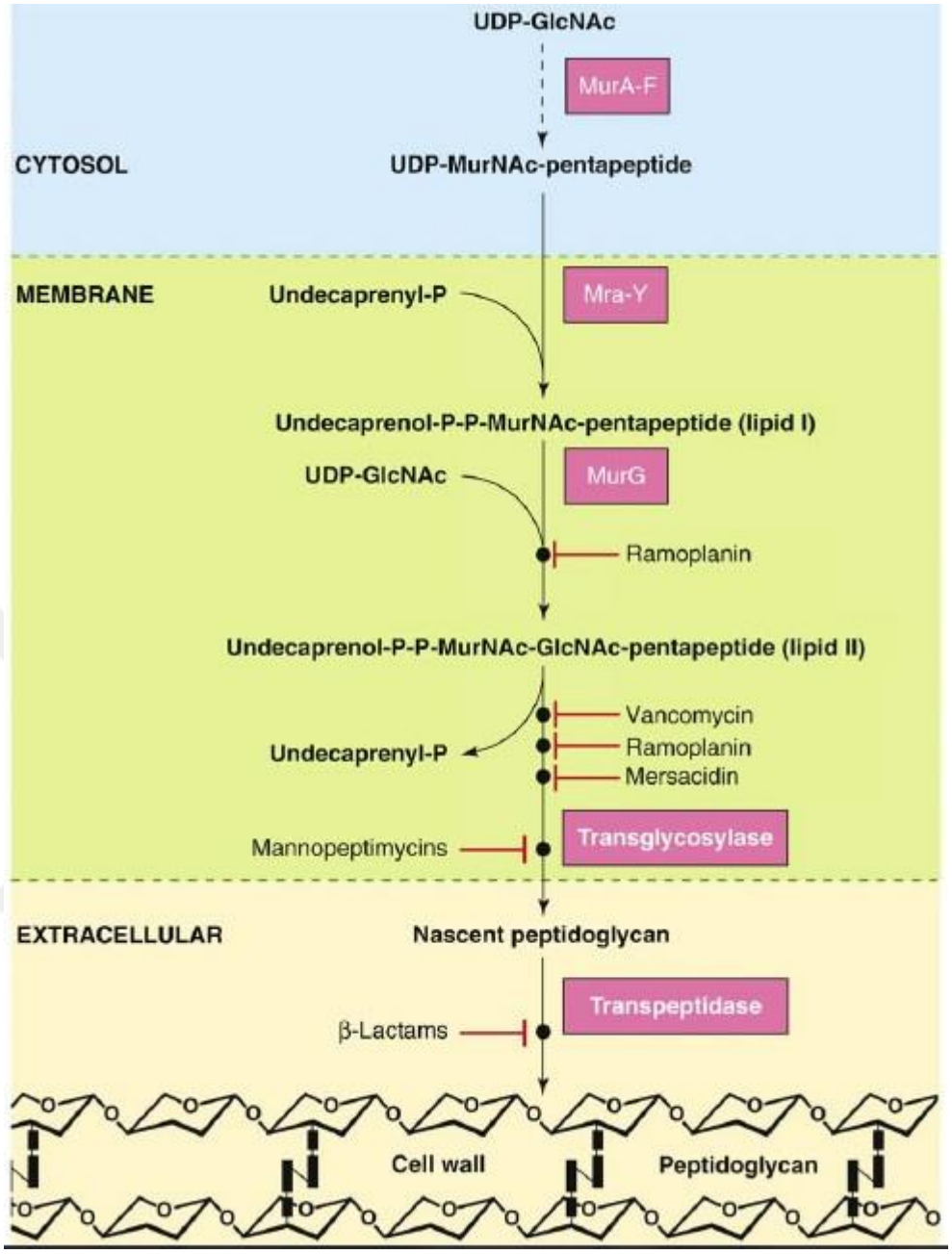

Figure 1.10 Mechanism of action of ramoplanin.

One proved ability of ramoplanin is that it is better in binding lipid intermediate II corresponding lipid intermediate I analogue. Ramoplanin is additionally able to bind MurG. It is not possible to inhibit MurG with externally used substrate which is not consistent with simple mechanism that involves substrate depletion. On the other hand, ramoplanin is not capable to bind lipid intermediate I. Yet, it can still inhibit MurG without showing any antimicrobial activity.

It is understood from the results that substrate recognition (lipid II > lipid I) while it is not needed for MurG inhibition, is effective in vivo antimicrobial activity. Another key point provided from these results is the relevance of extracellular transglycosidation inhibition (IC_{50}) 0.25 μM, MIC= 0.1 μM) with lipid II for exhibition of antimicrobial activity. The transpeptidase-catalyzed cross-linking reaction and a basic part of vancomycin action are preceded by these two major steps. Hence, it is not possible to observe mechanism-based cross resistance between vancomycin and ramoplanin. Also, in addition to its usage for topical infections, ramoplanin is a perfect candidate for wider clinical use (Lo et al., 2000).

Ramoplanin is currently in Phase III clinical trials for the treatment of *Clostridium difficile*-associated disease (CDAD), vancomycin-resistant *Enterococcus faecium* (VREF), and methicillin-resistant *Staphylococcus aureus* (MRSA) (Di-Palo et al., 2007; Nanotherapeutics, 2013).

Ramoplanin is effective against most types of Gram-positive bacteria in which many species of *Staphylococcus*, *Enterococcus*, and *Bacillus* are included. Comparing to vancomycin, ramoplanin was found more potent antibiotic on molar basis activity at several in vitro studies although the testing method and the conditions of testing process were not considered. Ramoplanin has some advantages over vancomycin as it is bactericidal at the concentration around its minimum inhibitory concentration level (MIC). On the other hand, vancomycin is bacteriostatic at the concentrations close to its MIC. Another significant point is that ramoplanin is active against vancomycin resistant *Enterococcus* (VRE) strains and MRSA strains. Related to these benefits of ramoplanin, it is promising agent for treatment of Gram-positive infection (Fang, 2008; Walker et al., 2005; Citron et al., 2003).

1.2.1.2 Inhibition of Protein Synthesis

Antibiotics that act by affecting protein synthesis generally have a relatively broad spectrum of action. Streptomycin was the first antibiotic that was shown to be effective against Gram-negative organisms. Its discovery in 1943 was particularly

welcome since such organisms were unaffected by penicillin or sulphonamides. It proved to be especially useful in the treatment of tuberculosis, the causative agent of which, *Mycobacterium tuberculosis*, is protected against the effects of penicillin by the waxy layer of mycolic acids in its cell wall (Hogg, 2005).

A protein synthesis inhibitor is a substance that stops or slows the growth or proliferation of cells by disrupting the processes that lead directly to the generation of new proteins (shown in Figure 1.11). It usually refers to substances, such as antibiotics, that act at the ribosome level like initiation, elongation (including aminoacyl tRNA entry, proofreading, peptidyl transfer, and ribosomal translocation), and termination. The substances take advantage of the major differences between prokaryotic and eukaryotic ribosome structures which differ in their size, sequence, structure, and the ratio of protein to RNA. The differences in structure allow some antibiotics to destroy bacteria by inhibiting their ribosomes, while leaving human ribosomes unaffected (Surhone et al., 2010).

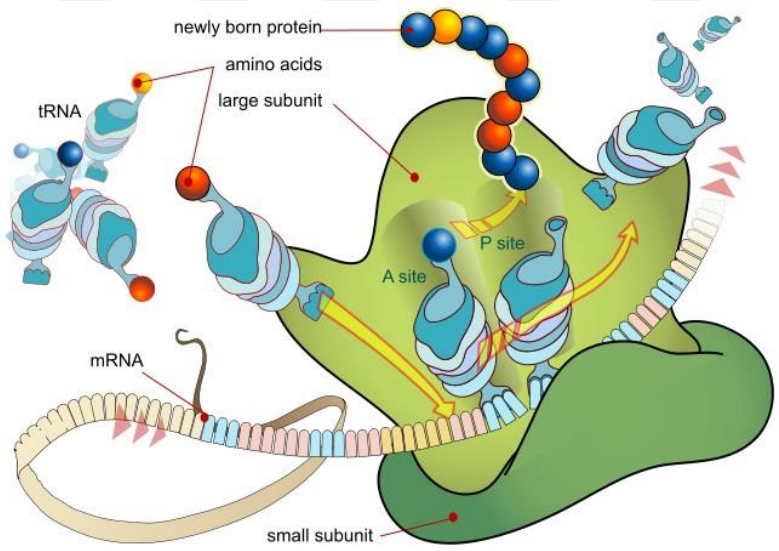

Figure 1.11 Translation of mRNA and the synthesis of proteins by a ribosome.

In general, protein synthesis inhibitors work at different stages of prokaryotic mRNA translation into proteins as shown in Figure 1.12. The following is a list of the stages which common antibiotics target;

a) By binding to the 30S subunit of the bacterial ribosome, aminoglycosides block the attachment of the 50S subunit. This prevents completion of the initiation complex, thus protein synthesis is inhibited.

b) Tetracyclines distort the shape of the 30S subunit, preventing the attachment of the appropriate aminoacyl tRNA.

c) Chloramphenicol inhibits peptidyltransferase and prevents formation of new peptide bonds.

d) Macrolides such as erythromycin bind to the 50S subunit, preventing elongation of the growing peptide chain (Hogg, 2005; Maguire, 2009).

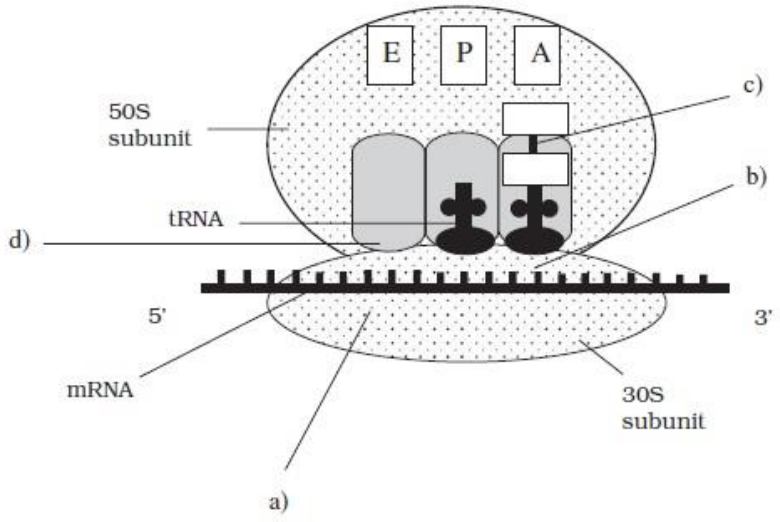

Figure 1.12 Inhibitions of protein synthesis.

In general, most well-known types of antibiotics related with inhibitions of protein synthesis are macrolides, aminoglycosides, and tetracyclines. However there are a lot of types of antibiotic included in this mode of action such as amphenicols, oxazolidinone, streptogramins, lincosamides, and glycylcycline.

1.2.1.2.1 Macrolides. This group of antibiotics inhibits to elongation step in protein synthesis by binding to 50S subunit of ribosome. Erythromycin, azithromycin, and clarithromycin are the best known of the macrolide group of antibiotics. Unlike chloramphenicol, it has a large hydrophobic molecule and is unable to gain access to most Gram-negative bacteria, thus restricting its spectrum of

activity. Erythromycin can be taken orally and its spectrum of activity is smilar to penicillin G; it is often used instead of penicillin in the treatment of staphylococcal and streptococcal infections in children because of causing little allergy problems compared to penicillins. It is particularly appropriate for this application as it is one of the least toxic of all commonly used antibiotics (Bambeke et al., 2008; Borisova, 2004).

1.2.1.2.2 Aminoglycosides. This group of antibiotics acts by binding to the 30S subunit of the bacterial ribosome, preventing attachment of the 50S subunit to the initiation complex. They can thus discriminate between prokaryotic (70S) and eukaryotic (80S) ribosomes, and consequently have a relatively high therapeutic index (although not as high as cell wall inhibitors). Streptomycin, kanamycin, amikacin, and gentamicin are the best known of aminoglycoside antibiotics. Like some other 'wonder drugs', streptomycin has proved to have undesirable side-effects which have led to it being replaced in most applications by safer alternatives. In addition, bacterial resistance to streptomycin is widespread, further diminishing its usefulness. Use of the aminoglycosides as a group has diminished since the development of later generation cephalosporins and the tetracyclines (Becker & Cooper, 2013; Hogg, 2005).

1.2.1.2.3 Tetracyclines. This group of antibiotics works by binding to the 30S ribosomal subunit, preventing the attachment of aminoacyl tRNA, and therefore extension of the peptide chain. They are yet another group of antibiotics produced by *Streptomyces* spp. Both natural and semisynthetic tetracyclines are easily absorbed from the intestine, allowing them to be taken orally. Coupled with their broad specificity (the broadest of any antibiotic), this led to inappropriately widespread use in the years following their discovery, sometimes resulting in complications caused by the destruction of the normal resident microflora. Tetracycline, chlortetracycline, minocycline, and doxycycline are the best known of aminoglycoside antibiotics. Tetracyclines are still used for a number of applications, notably to treat a variety of sexually transmitted diseases (Hogg, 2005; Chopra & Roberts, 2001).

1.2.1.3 Inhibition of Replication and Transcription of Nucleic Acids

In general, the main groups of antibiotics which inhibit replication and transcription of nucleic acids are; quinolones, RNA synthesis inhibitors, and anaerobic DNA inhibitors.

1.2.1.3.1 Quinolones. The quinolone group of synthetic antimicrobial drugs acts by disrupting DNA replication. This action occurs by inhibiting to the enzymes involved in DNA coiling such as DNA gyrase, topoisomerase IV and results in stabilization of enzyme-DNA complex and disruption of DNA replication. The most potent quinolones in use today, the fluoroquinolones, have fluorine at the C-6 atom, with substitutions elsewhere constituting the difference in their potency. Explosive research and development of fluoroquinolones starting in the 1980's and thereafter, brought about production of hundreds of derivatives and with each generation the derivatives become more potent and possess the ability to treat a wider spectrum of bacteria. Norfloxacin, enoxacin, lomefloxacin, ciprofloxacin, ofloxacin, levofloxacin, gatifloxacin, and moxifloxacin are eight examples of fluoroquinolones presently in use in the clinic. They are well absorbed in the tissues and have excellent oral bioavailability. These antibiotics currently are most potent against gram-negative pathogens. Ciprofloxacin is the most active fluoroquinolone against gram-negative bacteria, while levofloxacin, gatifloxacin, and moxifloxacin are most active against gram-positive bacteria (Lee, 2010; Tran & Jacoby, 2002).

1.2.1.3.2 RNA Synthesis Inhibitors. Rifamycins are a group of antibiotics which act on the inhibition of bacterial DNA-dependent RNA synthesis. Rifampin or rifampicin is the best known antibiotic in rifamycins group. It acts by inhibiting the enzyme RNApolymerase, thereby preventing the production of mRNA. Rifampin is used against the mycobacteria that cause tuberculosis, an application for which its ability to penetrate tissues makes it well suited. Unlike most antibiotics, rifampin interacts with other drugs, often reducing or nullifying their effect. When used in high doses, it has the unusual side effect of turning secretions such as tears, sweat and saliva, as well as the skin, an orangered colour (McClure & Cech, 1978; Hogg, 2005).

1.2.1.3.3 Anaerobic DNA Inhibitors. This group of inhibitors is divided two classes, nitrofurans and nitroimidazoles.

Nitrofurans with a NO_2 group bound to a nucleus furan have a bacteriostatic effect against both gram-positive and gram-negative bacteria. Bacteria reduce NO_2 groups faster than the human cells and only the reduced metabolites damage DNA. This explains why nitrofurans have few effects on human cells. However, their basic mechanism of action has not yet been clarified. They are used in the treatment of bacterial diarrhea. They have few adverse effects however they can cause different allergic manifestations. The nitrofuran indicated in the treatment of uncomplicated urinary bacterial infections is nitrofurantoin. Well absorbed by digestive tract, it is eliminated in high concentration in urine. It can cause some digestive adverse effects, nausea, diarrhea, hepatitis; blood disorders: leucopoenia, anaemia; nervous disorders: headache, dizziness. Nitrofurantoin can give a brownish colour to urines. Some nitrofurans are carcinogenic, and their future use is in doubt (Boothe, 2012).

Nitroimidazoles are active against anaerobic microorganisms like *Clostridium, Fusobacterium, and Streptococcus.* The nitrated group accepts electrons from flavoproteins and ferrodoxines of these microorganisms. The derivatives of nitro-5-imidazole having this spectrum of activity are metronidazole, ornidazole, tinidazole. Nitroimidazoles can give a disulfiram-like effect, or “antabuse” effect, when treated patients take alcohol. On prolonged therapy, they can give neutropenia and peripheral neuropathy. Metronidazole does not seem teratogenic in pregnant women (Lamp et al., 1999).

1.2.1.4 Inhibition of Folic Acid Metabolism

1.2.1.4.1 Antifolates. The human organism does not have the enzymes necessary for synthesizing folic acid which it needs. Folic acid must be supplied by the diet. Under these conditions, a pure inhibitor of the synthesis folic acid should not have many adverse effects in humans. Bacteria synthesize themselves folic acid and the inhibition of this synthesis inhibits their replication.

Folic acid can be regarded as constituted of a nucleus pterine, para-aminobenzoic acid (PABA), and one or more glutamate molecules. In absence of folate supply by the external medium, the microorganisms must synthesize it.

Sulfamethoxazole (sulphonamides) and trimethoprim (dihydrofolate reductase inhibitor) shown in Figure 1.13 are the best known of the antifolate antibiotics. Inhibitors of the synthesis of folic acid like sulfamethoxazole are structural analogs of PABA with which they enter in competition for the synthesis of folic acid that they disturb. They inhibit dihydropteroate synthase, a microbial enzyme responsible of the incorporation of PABA in dihydropteroic acid, precursor of folic acid. Trimethoprim is an inhibitor of DHFR, but resistance to its action develops quickly when it is used alone, so it is usually combined with another antibiotic (Golan et al., 2011).

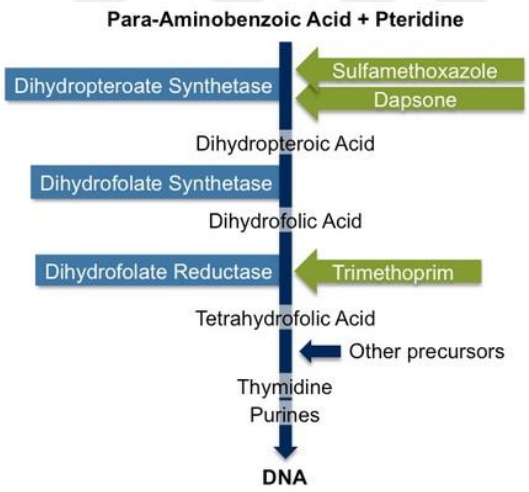

Figure 1.13 Inhibition of folate synthesis.

1.2.1.5 Inhibition of Cytoplasmic Membrane Structure

1.2.1.5.1 The Most Widely Used. Polymixins are a class of antibiotic that act by disrupting the phospholipids of the cytoplasmic membrane and causing leakage of cell contents. Produced naturally by a species of *Bacillus*, polymixins are effective against pseudomonad infections of wounds and burns, often in combination with bacitracin and neomycin (an inhibitor of protein synthesis). Their toxicity makes them unsuitable for internal use (Hogg, 2005).

Daptomycin is the other mostly used antibiotic. Daptomycin is a lipopeptide antibiotic that has been approved for the treatment of patients with MRSA bacteraemia and infective endocarditic, including cases caused by isolates exhibiting VRSA. However, daptomycin non-susceptibility, which has recently been observed in both recent clinical cases and in vitro studies, has led researchers to look for alternative therapeutic options. Combination therapy including dosing regimens consisting of daptomycin plus a β-lactam, has been shown, both clinically and in vitro to be able to produce bactericidal effects during a phenomenon known as the "see-saw effect". This phenomenon holds the potential to increase the susceptibility to β-lactams as the susceptibility to daptomycin decreases (Enoch et al., 2007).

The mechanism of action of daptomycin is distinct in that it inserts into the cell membrane in a phosphatidyl-glycerol dependent manner. Once inserted, it causes an alteration in the membrane curvature of the bacterial cell, which leads to the development of openings within the cell membrane. These openings result in rapid depolarization, which leads to a loss of membrane potential and inhibition of DNA, RNA and protein synthesis. In recent years, *in vitro* and clinical data have reported the emergence of reduced susceptibility to daptomycin, which has led the clinicians to search for other therapeutic options against daptomycin non-susceptible *Staphylococcus aureus* (DNSA). Daptomycin has been the typical source of treatment following vancomycin resistance (Bambeke et al., 2008; Mall, 2013).

1.3 Production of Antibiotics

1.3.1 Production of Biological

Biosynthesis is the production of a chemical compound by a living organism, as in metabolism.

Most antibiotics are produced by special bacteria or fungi. These bacteria or fungi are grown in large cultures; the substances they produce are then harvested and purified for medical use. Antibiotics produced in this way are called biosynthesized antibiotics. Bio means ‘‘life’’ and synthesize means ‘‘to make,’’ so biosynthesized

means made by living things. There are also synthetic antibiotics, which are chemicals made in factories without the help of living things. Most antibiotics are still biosynthesized (K. L. Lerner & B. W. Lerner, 2007).

One of the major breakthroughs in the treatment of infectious diseases was of course the discovery of naturally occurring antibiotics. These are metabolites produced by certain microorganisms, which inhibit the growth of certain other microorganisms. As we shall see, the definition has been extended to include semisynthetic derivatives of these naturally occurring molecules. Some commonly used antibiotics are listed in Table 1.2 (Hogg, 2005).

Table 1.2 Some antibiotics and their microbial source.

Antibiotics	**Microbial Source**
	Bacteria Gram positive
Bacitracin	*Bacillus subtilis*
Polymixin	*Bacillus polymixa*
	Actinomycetes
Gentamicin	*Micromonospora purpurea*
Erythromycin	*Streptomyces erythreus*
	Streptomyces
Ramoplanin	*Actinoplanes* sp. ATCC 33076
Tetracycline	*Streptomyces rimosus*
Vancomycin	*Streptomyces orientalis*

1.3.1.1 Antibiotic Producing Microorganisms (Taxonomic Position of Organisms)

1.3.1.1.1 Genus Bacillus. Bacillus species are aerobes or facultative anaerobes. They are chemoheterotrophs and usually motile by means of peritrichous flagella. Only a few species of *Bacillus* are pathogenic in humans, notably *B. anthracis*, the causative agent of anthrax. This is seen by many as a potential agent of bioterrorism, and here again the relative indestructibility of its spores is a crucial factor. Other species, conversely, are positively beneficial to humans; antibiotics such as

bacitracin and polymixin are produced by *Bacillus* species (Hogg, 2005; Slepecky & Hemphill, 2006).

1.3.1.1.2 Genus Pseudomonas. Members of this group of proteobacteria, the most important genus of which is *Pseudomonas*, are straight or curved rods with polar flagella. They are chemoheterotrophs that generally utilise the Entner–Doudoroff pathway rather than glycolysis for the oxidation of hexoses. They are differentiated from the enteric bacteria by being oxidase-positive and incapable of fermentation. A characteristic of many pseudomonads is the ability to utilise an extremely wide range of organic compounds (maybe over 100!) for carbon and energy, something that makes them very important in the recycling of carbon in the environment. Several species are significant pathogens of animals and plants; *Pseudomonas aeruginosa* is an effective coloniser of wounds and burns in humans, while *P. syringae* causes chlorosis (yellowing of leaves) in a range of plants. Because of their ability to grow at low temperatures, a number of pseudomonads are important in the spoilage of food (Moore et al., 2006).

Although most species carry out aerobic respiration with oxygen as the terminal electron acceptor, a few are capable of substituting nitrate (anaerobic respiration). Representative genera: *Pseudomonas, Burkholderia* (Hogg, 2005).

1.3.1.1.3 Genus Streptomyces. The best known actinomycete genus is *Streptomyces*, which contains some 500 species, all with a characteristically high GC content (69–73%). *Streptomyces* are very prevalent in soil, where they saprobically degrade a wide range of complex organic substrates by means of extracellular enzymes. Indeed, the characteristic musty smell of many soils is due to the production of a volatile organic compound called geosmin. A high proportion of therapeutically useful antibiotics derive from *Streptomyces* species, including well-known examples such as streptomycin, erythromycin and tetracycline. Most actinomycetes, including *Streptomyces*, are aerobic; however, members of the genus *Actinomyces* are facultative anaerobes (Ochi, 1995; Watve et al., 2001; Hogg, 2005).

1.3.1.1.4 Genera of Actinomycetales Other Than Streptomyces. The actinomycetes are aerobic, filamentous bacteria that form branching mycelia superficially similar to those of the Fungi. Remember, however, that the actinomycetes are prokaryotes and the fungi are eukaryotes, so the mycelia formed by the former are appreciably smaller. In some cases, the mycelium extends clear of the substratum and bears asexual conidiophores at the hyphal tips. These are produced by the formation of cross-walls and pinching off of spores, which are often coloured.

The coryneform bacteria are morphologically half way between single celled bacilli and the branching filamentous actinomycetes. They are rods that show rudimentary branching, giving rise to characteristic 'V' and 'Y' shapes. Among the genera in this group are Corynebacterium, Mycobacterium, Propionibacterium and Nocardia.

Corynebacterium species are common in soil, and are also found in the mouths of a variety of animals. *C. diphtheriae* is the causative agent of diphtheria; it only becomes pathogenic when it has been infected by a bacteriophage that carries the gene for the diphtheria exotoxin (Yassin et al., 1995).

Members of the genus Mycobacterium are characterised by their unusual cell wall structure; they include unusual complex lipids called mycolic acids. This causes the cells to be positive for the acid-fast staining technique, a useful way of identifying the presence of these bacteria. Mycobacteria are rod shaped, sometimes becoming filamentous; when filaments are formed, propagation is by means of fragmentation. *M. leprae* and *M. tuberculosis* cause, respectively, leprosy and tuberculosis in humans.

Propionibacterium species ferment lactic acid to propionic acid. Some species are important in the production of Swiss cheeses, whilst *P. acnes* is the main cause of acne in humans. Representative genera: Corynebacterium, Propionibacterium (Sykes and Skinner, 1973; Hogg, 2005).

1.3.1.1.5 Genus Aspergillus. Filamentous fungi are important to humans in both beneficial and harmful ways. From a beneficial viewpoint, they are important decomposers in the environment, serving to degrade complex organic and inorganic materials, which recycles carbon, nitrogen and other important elements for use by other organisms. They are also important in the food processing industry and in the manufacturing of certain drugs and antibiotics. In regards to their negative impact, fungi are a major cause of disease among plants and to a lesser extent, among animals. Fungi frequently infect economically valuable crops and many species cause serious illness in humans, in both healthy and immunocompromised individuals. Therefore, the study of filamentous fungi biology is warranted due to their tremendous impact on many aspects of human existence (Virtual Amrita Laboratories, n.d.).

Kingdom Fungi consist of one monophyletic group with four major branches: zygomycetes, ascomycetes, basidiomycetes and chytridomycetes. The most important genus in ascomycetes is genus *Aspergillus* which contains approximately 200 species. Many species of this genus cause disease in animals and humans. *Aspergillus* lacks zoospores and is classified within the Ascomycetes division. The most common species that causes invasive disease is *Aspergillus fumigatus*, followed by *Aspergillus flavus*. Other species that cause disease much more rarely include *A. niger, A. nidulans, A. terreus, A. glaucus* and *A. wentii*. More than 90% of all cases of invasive aspergillosis can be attributed to *A. Fumigates* (Wasylnka, 2002). Hundreds of such metabolites are produced by fungi. *Aspergillus* has been the source of a number of useful metabolites including antibiotics.

Aspergilli are filamentous fungi that grow as long, branched filaments of cells called hyphae. Extensive hyphal growth produces a tangled mass of cells known as a mycelium. The hyphae have cross-walls called septa that possess either single or multiple pores to allow the passage of nutrients between the cells. Fungal cells are characterized by an outer cell wall and inner plasma membrane and contain typical eukaryotic structures such as nuclei, endoplasmic reticulum and mitochondria (El-Ganiny, 2011).

In the laboratory, Aspergillus is usually grown on Czapek-Dox or Sabouraud dextrose agar and matures within 3 days at 37°C (192). The surface of the *Aspergillus* colony is initially white but then turns to green or black depending on the species (Wasylnka, 2002).

1.3.1.1.6 Genus Penicillium. Penicillium is classified as an anamorphic genus in the division *Ascomycota* (order *Eurotiales*, class *Eurotiomycetes*, family *Trichocomaceae*). The genus name is derived from the Latin word penicillus, which means little brush. *Penicillium expansum*, the common apple rot fungus, was selected to represent the type of the genus (Rivera, 2009).

Penicillin, which is produced by *P. chrysogenum*, was first discovered by Alexander Fleming in 1929 and found to inhibit the growth of gram negative bacteria. Little attention was paid to penicillin until Howard Florey in the late 1930's realized its potential as an antibiotic. Florey, along with Ernst Chain, purified and concentrated the compound. Penicillin saved the lives of many soldiers during World War II who were dying from infected wounds, earning Fleming, Florey and Chain the Nobel Prize in Medicine in 1949 (Lax, 2004).

Soil fungi, such as *Penicillium*, are particularly promising in pharmaceutical potential because their biocenotic competitiveness is often based on the production of antibiotics and other inhibitory substances. *Penicillium* species have also shown other commercial value. Some are used for the fermentation of foods, such as the production of Camembert, Roquefort, and Gorgonzola cheeses. Other species are valued for the uptake of heavy metals, their ability to inoculate soil as biofertilizers, transformation of organic compounds, and the production of organic compounds and enzymes (Leitao, 2009).

1.3.1.1.7 The Myxobacteria. Another group of bacteria that may be regarded as predatory are the *Myxobacteria* (shown in Figure 1.14). These are rod-shaped bacteria lacking flagella, which yet are motile by gliding along a solid surface, aided by the excretion of extracellular polysaccharides. For this reason they are sometimes

referred to as the gliding bacteria. They are heterotrophs, typically requiring complex organic nutrients, which they obtain by the lysis of other types of bacteria. Thus, unlike Bdellovibrio, they digest their prey before they ingest it. When a rich supply of nutrients is not available, many thousands of cells may aggregate to form fruiting bodies, inside which myxospores develop. These are able to resist drought and lack of nutrients for many years. *Myxobacteria* exhibit the most complex life cycles of any prokaryote so far studied. Representative genera: *Myxococcus*, *Chondromyces* (Gerth et al., 2003; Hogg, 2005).

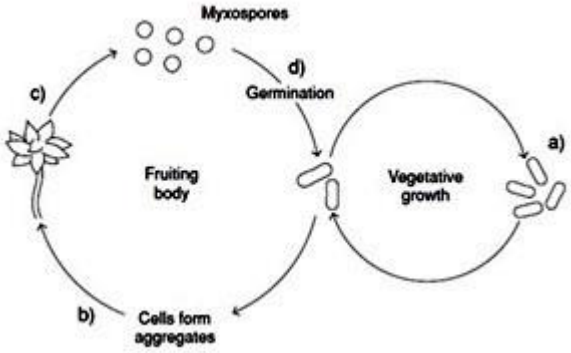

Figure 1.14 The Myxobacteria: a complex bacterial life cycle. When nutrients are in plentiful supply, myxobacteria divide by binary fission (a). On depletion of nutrients, they form aggregates of cells, which lead to the formation of a fruiting body (b). Within the fruiting body, some cells form myxospores, enclosed within a sporangium (c). Myxospores remain dormant until environmental conditions are favourable, and then germinate into vegetative cells (d).

1.3.1.2 The Biosynthesis Process of Antibiotics

Most of the antibiotics which are used clinically depend on natural products so biosynthesis of an antibiotic is very important. The commercial production of an antibiotic is a long and expensive process. First, the organisms which are capable of synthesizing the desired antibiotic compounds are identified. During this process, these species are monitored to determine which have antibacterial actions. Secondly, species that have antibacterial action are tested to see whether they are infected by well- known bacteria. Lastly, the organisms showing promising results are started to be grown on large scale so that the compounds that show antibiotic effects can be isolated (Stacey & Gale, 2002).

Production of an antibiotic microbially depends on a fermentation process in the large-scale shown in Figure 1.15. During fermentation, a great number of antibiotic-synthesizing organisms are required to obtain antibiotic material. The next stage is isolation of antibiotic. For a new antibiotic to be used as a drug, high yield and easy isolation are the only necessities. So that, extensive research is required to decide, whether the desired antibiotic can be commercially scaled up (Stacey & Gale, 2002; Behal, 2002).

In general, biosynthesis process of antibiotics is listed as;

a) Raw Materials

b) The Manufacturing

- ✓ Initiating the culture
- ✓ Fermentation
- ✓ Isolation and purification
- ✓ Refining

c) Quality Control

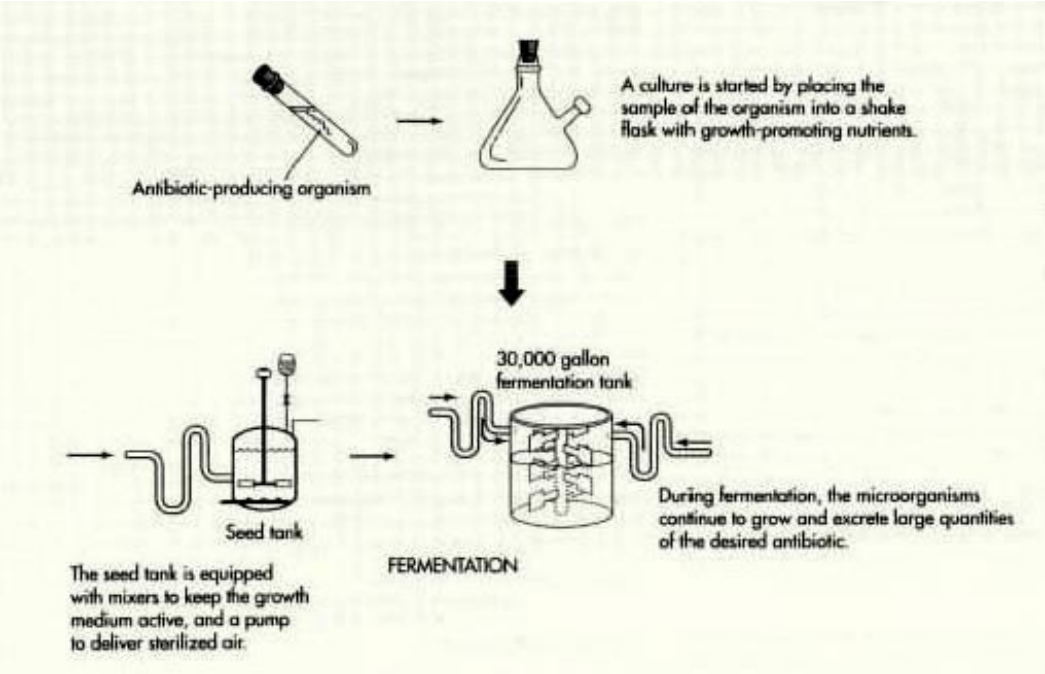

Figure 1.15 Scale-up at process of fermentation.

a) Raw Materials

The components of fermentation broth are called raw materials. Fermentation broth is an aqueous solution and contains lactose and glucose sources such as molasses or soy meal which are required for proliferation. Nitrogen is an another necessity for metabolic cycles so an ammonia salt is usually added to fermentation broth. Compounds that contain trace elements such as phosphorus, sulphur, magnesium, zinc, iron, and copper are another components of fermentation broth and they are important for the proper growth of the antibiotic-producing organisms. Lard oil, octadecanol, and silicones are used as anti-foaming agents so the component of fermentation broth does not foam during fermentation (Stacey & Gale, 2002).

b) The Manufacturing

Although most antibiotics occur in nature, they are not normally available in the Most of the antibiotics can be found in nature but the main problem is that they are in small quantities which makes it impossible for large-scale productions. Fermentation process is developed to get over this problem. This is three step-process which are;

1- Isolation of disred microorganism,
2- Providing the components of fermentation broth,
3- Isolation of antibiotic product.

To prevent contamination, all these steps must be carried out under sterile conditions (Stacey & Gale, 2002).

✓ Initiating the culture: Before the initiating the fermentation, the organism that produce the desired antibiotic must be isolated and duplicated to increase the number of it. To do this, the following steps must be followed:

1- Starting a culture by using previously isolated, cold-stored organism,
2- Transferring the sample organism to an agar-containing plate,

3- Putting the initial culture into shake flaks that contain all the components for growth.

After all these steps, the suspension can be transferred to seed tanks for further purposes shown in Figure 1.16 (Stacey & Gale, 2002).

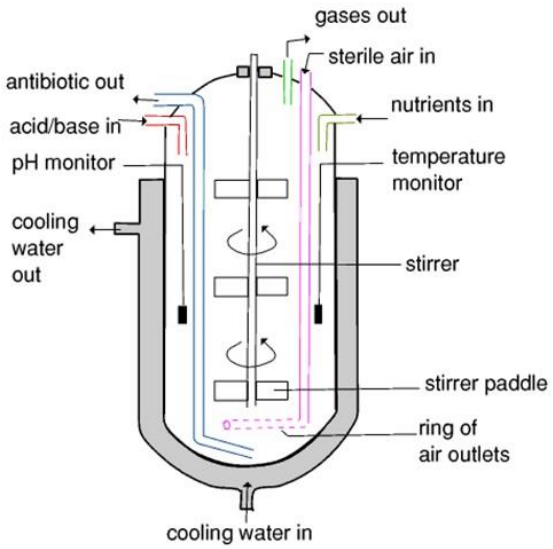

Figure 1.16 Seed tank.

The seed tanks are made of steel and they offer the optimal environment for growing the organisms. They contain all the things that the organism needs such as warm water, carbon and nitrogen sources and growth factors which are needed for survival and development of the organism. The growth medium is moved with mixers and a pump supplies sterilized filtered air. The material in the seed tanks is transferred to the primary fermentation tanks usually after 24-48 hours (Stacey & Gale, 2002).

✓ Fermentation: The fermentation tank is larger type of steel seed tank and can hold 30,000 gallons approximately. It contains the same growth media in the seed tank and induces the growth. In the fermentation tans, the microorganism grows and multiplies so that large amounts of desired antibiotic are synthesized. For optimum temperature, tanks are cooled. Anti-foaming agents are added periodically to prevent

constant agitation. To provide optimum pH, acids or bases are added when needed (Stacey & Gale, 2002; Hook, 2006).

✓ Isolation and purification: after three to nine days, the maximum amount of antibiotic will have been produced and the isolation process can begin. The purification depends on the specific antibiotic produced. While ion-exchange method is suitable for purification of water soluble antibiotic compounds, solvent extraction method can be used to isolate oil-soluble antibiotics such as penicillin. At the end of these steps, a purified powdered form of antibiotic is gained that can be used in different product types. To isolate an oil-soluble antibiotic such as penicillin, a solvent extraction method is used. In this method, the broth is treated with organic solvents such as butyl acetate or methyl isobutyl ketone, which can specifically dissolve the antibiotic. The dissolved antibiotic is then recovered using various organic chemical means. At the end of this step, the manufacturer is typically left with a purified powdered form of the antibiotic, which can be further refined into different product types (Stacey & Gale, 2002; Behal, 2002).

✓ Refining: antibiotics can be delivered on many different forms depending on the final form. In intravenous bags and syringes, they are in aqueous form in which the crystalline antibiotic is dissolved in a solution. For gel capsules, the powdered antibiotic is physically filled into the bottom half of a capsule then the top half is mechanically put in place. When used in topical ointments, the antibiotic is mixed into the ointment. (Stacey & Gale, 2002).

From this point, the antibiotic product is transported to the final packaging stations. Here, the products are stacked and put in boxes. They are loaded up on trucks and transported to various distributors, hospitals, and pharmacies. The entire process of fermentation, recovery, and processing can take anywhere from five to eight days. The whole process was shown briefly in Figure 1.17 (Behal, 2002; Hook, 2006).

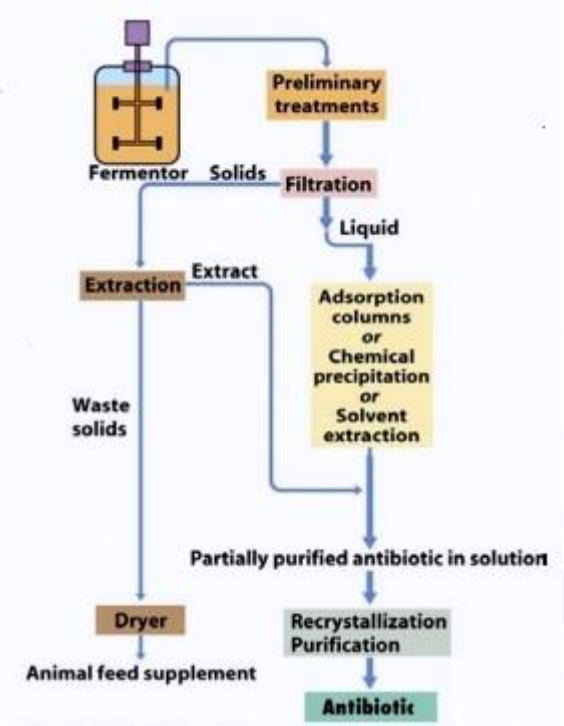

Figure 1.17 Isolation and purification process.

c) Quality Control

Quality control is all about fermentation process and very important for the production of antibiotics. It must make certain that no contamination is introduced in any step production. To ensure that, the medium and all processing equipment are sterilized by steam. Of particular importance are frequent checks of the condition of the microorganism culture during fermentation. The usage of various chromatography techniques are used to be successful. Various physical and chemical of the final product such as pH, melting point, and moisture content is also controlled (Stacey & Gale, 2002).

In the United States, antibiotic production is controlled by the Food and Drug Administration (FDA). Antibiotic type and the application determine whether more or less tests must be applied. In some cases, for certain antibiotics each batch must be checked by the FDA to ensure their purity and effectiveness. This is a very important step, because uncertified batch cannot be sold for general consumption (Stacey & Gale, 2002).

1.4 Introduction and Approaches for Improvement in Antibiotic Production

1.4.1 Strain Improvement and Process Development

Due to high research or development costs of a novel drug, pharmaceutical companies have done a few researches in recent years. However, spread of bacteria resistant to several antibiotics has spurred a revived interest in the development of novel antibiotics. If novel antibiotics are not discovered or improvements are not made on the available ones, this resistant issue could have serious medical problems on the world's public health. This challenging problem will be the focus of research for many years to come.

Recently scientists are constantly looking for novel antibiotics to stay one jump ahead of the ever-evolving, disease-causing bacteria of the world. One method of producing new antibiotics is to try to genetically engineer antibiotic-making bacteria to actually change the bacteria's genes to force them to make new antibiotics. The goal is to create new antibiotics faster than bacteria can evolve resistance to them (K. L. Lerner & B.W. Lerner, 2007).

1.4.2 Computer Modelling

Numerous chemical reactions take place in the microorganisms. Most of these reactions occur repeatedly in the systems. The combined effect of all the chemical reactions in the microorganism is referred to as metabolism which is important to growth and produce primer and seconder metabolites.

A sequence of biochemical reaction steps which are observable and feasible is called as metabolic pathway. Scientists study these pathways to understand the functioning of cells, and eventually the complete system. Enzymes catalyze each step in a cellular pathway, so determining the specific enzymes participating in a reaction is important. Metabolic engineering involves discovering and analyzing such metabolic pathways to better understand metabolic processes that can be altered to benefit mankind (Khannapho, 2006).

Microorganisms and single-cell creatures are much simpler than their multi-cellular counterparts and have less genetic material and fewer biochemical reactions to be investigated. Consequently, researchers are more successful in engineering metabolic pathways in microorganisms. Thus metabolic engineering helped create several beneficial bioproducts-products derived through living systems. Also it is helpful in finding cures for diseases and ecofriendly ways for cleaning up the environment, for the mass production of antibiotics, and to enable effective means of energy production (K. L. Lerner & B.W. Lerner, 2007).

1.4.2.1 Metabolic Flux Analysis

It is aimed to improve the properties and productivity of microorganism which have a significant role industrially by modifying the physiology of the organism, improving pathway flux, and by increased understanding of the underlying mechanisms.

Metabolic pathway analysis (MPA) and metabolic flux analysis (MFA) are the most important branches of metabolic engineering. It is included a specified set of input and output metabolites. The metabolic flux is the rate at which a metabolite is processed through a metabolic pathway. The determination of metabolic fluxes in vivo has been increasingly undertaken by researchers worldwide and has been termed metabolic flux analysis (Khannapho, 2006).

Determination of metabolite fluxes is related with a metabolic network, particularly in the steady-state condition. MFA is based on mass balances of metabolites, which assumes that when the cell is in a steady-state condition the fluxes of input substrates (precursors) will be equal to the fluxes of output products (metabolites). There are lots of enzyme reactions which may occur in living cells including glycolysis, the pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCA), and biosyntheses of amino acids, etc. A stoichiometric model is set from the reactions of relevant microorganism in the study.

By definition, when cells reach steady-state the carbon balance is assumed to be 100%. This means the rate of carbon consumed from substrate is equal to the sum of the rates of carbon compounds produced products, e. g. antibiotic production. Using mass balancing technique along with the constructed stoichiometric model and the constraints placed upon the system, the intracellular carbon fluxes can then be determined once the rates of these input and outputs are measured (Khannapho, 2006).

1.4.2.2 The Solution to System of Equations

Firstly, the reactions of biological system are taken to the stoichiometric matrix layout. Tools of MFA have used ordinary differential equations (ODE) to simule time-flow profile of metabolite levels. So achieved stoichiometric model is taken to the ODE form to be solven by ODE solver such as COPASI as shown in Figure 1.18.

MFA uses mass balancing of metabolites which can be represented as the following equations;

$$\frac{dx}{dt} = S.v - b$$

Where; $\frac{dx}{dt} = the\ accumulation\ rate\ of\ metabolites$

S = the stoichiometric matrix containing the stoichiometric coefficients of the reactions in the network

v = the vector of metabolic fluxes

b = the vector of measured rates

The concentrations of intracellular metabolites are constant under the assumed steady-state, therefore, dx/dt = 0 and hence the system can be expressed by the equation below S.v = b. If the system at steady state, this equation reduced to S.v = 0 (Kauffman et al., 2003; Llaneras & Pico, 2008).

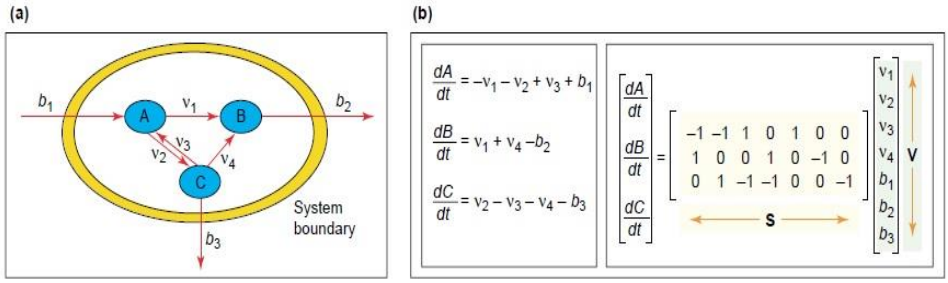

Figure 1.18 Construction of an exemplary kinetic model; (a) A model system comprising three metabolites (A, B and C) with three reactions (internal fluxes, vi, including one reversible reaction) and three exchange fluxes (bi). (b) Mass balance equations accounting for all reactions and transport mechanisms are written for each species. These equations are then rewritten in matrix form. At steady state, this reduced to S.V = 0.

1.4.2.3 COPASI, Program Used in This Field

The updated version of the software is always advantageous for biochemical analysis. COPASI 4.8 is the recently updated version of GEPASI. COPASI has a graphical user interface that bases on menu/contact approach and contains the functions of arrangement and creating of the graphical results and the model (Figure 1.19). Besides this, COPASI includes complex analysis methods and has ability to determine sensitive analysis which depends on certain parameters (Mendes et al., 2009; Hoops et al., 2006).

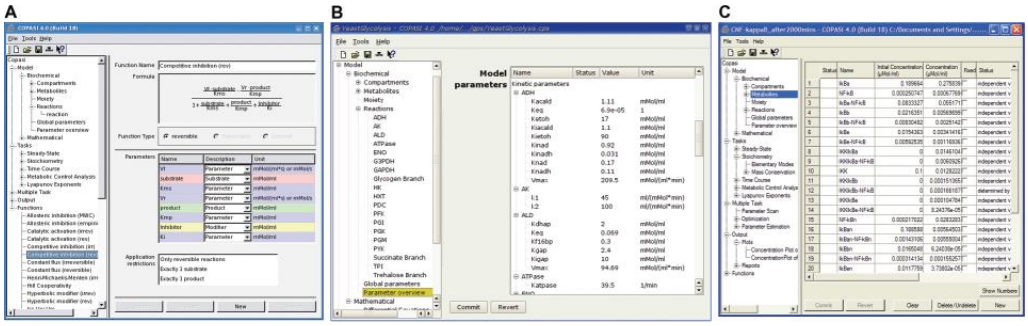

Figure 1.19 User interface of COPASI; left panel (A) includes all potential applications. On the other hand, right panels (B-C) change according to the choices within the control system.

Graphical interface enables to gain both biochemical perspective and mathematical appearance. These two alternative visualities provide some advantages

for the users with different experiences. Another significant advantage is that graphical interface is able to determine the units of kinetic functions automatically by analysis, which is not seen in other softwares (Hoops et al., 2006).

The most important feature of COPASI is that it has optimization and fitting. COPASI is capable of optimizing a group of criteria contains some metrics such as eigen values, temporary times and variety of others. Hence, this capacity of COPASI makes it highly flexible and applicable to the optimization problems. (Sauro & Bergmann, 2009)

COPASI is quite easy to use as it needs few parameters for the analysis. These parameters are the stoichiometric equations system that describes the model, the preliminary concentrations of all reaction compounds and the preliminary measurements of all speed constants. In addition, windows based software and easy setup stages are some other advantages for widely usage of COPASI. (Sauro & Bergmann, 2009)

COPASI's file format bases on XML. This feature allows other tools to read or to write the files created by COPASI. Also, as it is the same for SimBiology software, COPASI is able to read the SBML file format and this enables gathering models from lots of simulation tools, model and metabolic way data banks (Liangzhi et al., 2007; Kim et al., 2004). COPASI has a capacity of writing ordinary differential equations in a simple manner as C and Berkeley Madonna's file formats (Hoops et al., 2006).

CHAPTER TWO
MATERIAL AND METHOD

2.1 Microorganism and Culture Conditions

The strain of *Actinoplanes* sp. obtained from American Type Culture Collection as ATCC 33076 in the form of lyophilized was used as model microorganism in researching aimed at ramoplanin antibiotic.

The content of lyophilised vial stored at 4°C was suspended in physiological solution shown in Table 2.1. Then this suspension was used to inoculate different types of solid mediums (ATCC, n.d.).

Table 2.1 The composition of physiological solution.

ATCC #1877 Broth (ISP Medium 1)	
3 g yeast extract, 5 g tryptone,	15 g agar (if necessary), 1 L distilled water.
❖ **Adjust medium for final pH 7.0 - 7.2.** ❖ **Autoclave at 121°C for 15 minutes.**	
Inoculation Procedure: ➢ Using a single tube (5 to 6 mL), of #1877 Broth withdraw approximately 0.5 to 1.0 mL with a glass pipet. ➢ Rehydrate the pellet in vial. ➢ Transfer this aliquot back into the broth tube and mix well. ➢ Use several drops of the suspension to inoculate a second tube of #1877 Broth and petri dishes.	

Actinoplanes sp. ATCC 33076 was propagated in three different solid mediums called as Difco™ ISP Medium 2, Difco™ Oatmeal Agar and ATCC #551 Oatmeal Agar. The composition of these mediums was shown in Table 2.2. Incubation was carried out at 30°C for 8 days. The best grown of *Actinoplanes* sp. ATCC 33076 was obtained on Difco™ Oatmeal Agar shown in Figure 2.1 (ATCC, n.d.; Zimbro & Power, 2009).

Table 2.2 The composition of solid mediums.

Difco™ ISP Medium 2	**Difco™ Oatmeal Agar**	**ATCC #551 Oatmeal Agar (ISP Medium 3)**
4 g glucose, 4 g yeast extract, 10 g malt extract, 20 g agar, 1 L distilled water.	60 g oatmeal, 12.5 g agar, 1 mL trace salts solution, 1 L distilled water.	20 g oatmeal, 18 g agar, 1 mL trace salts solution, 1 L distilled water.
❖ **Autoclave at 121°C for 15 minutes.** ❖ **Aseptically add 1 mL Trace Salts Solution.**		
Trace Salts Solution: ➢ 0.1 g $FeSO_4.7H_2O$, ➢ 0.1 g $MnCl_2.4H_2O$, ➢ 0.1 g $ZnSO_4.7H_2O$, ➢ 100 mL distilled water.		

Figure 2.1 *Actinoplanes* sp. ATCC 33076 on Difco™ Oatmeal Agar after 8 days.

The mycelium from solid medium of Difco™ Oatmeal Agar was used to inoculate 100 mL of seed (VM) and vegetative medium (VB) in 500-mL erlenmeyer flasks containing 95 mL liquid medium and 5 mL mycelium suspension. The composition of these mediums was shown in Table 2.3. Flasks were incubated at 30°C for 96 hours on a rotary shaker set at 200 rpm. The best culture of *Actinoplanes* sp. ATCC 33076 was obtained on seed medium (VM) shown in Figure 2.2 (Cavalleri et al., 1984; Brunati et al., 2005).

Table 2.3 The composition of liquid mediums.

Seed Medium (VM)	Vegetative Medium (VB)
3 g meat extract, 5 g yeast extract, 5 g tyrptone, 1 g glucose, 24 g soluble starch, 4 g $CaCO_3$, 1 L distilled water.	13 g soybean meal, 12 g glucose, 13 g soluble starch, 4 g $CaCO_3$, 1 L distilled water.
❖ **Adjust medium for final pH 7.0 - 7.2.** ❖ **Autoclave at 121°C for 15 minutes.**	
Inoculation Procedure: ➢ Distilled water was added to agar medium to prepare a mycelium suspension. ➢ Inoculate 5 mL from mycelium suspension ($OD_{620:}$ 0.800) to the liquid medium.	

Figure 2.2 *Actinoplanes* sp. ATCC 33076 on seed medium after 96 hours.

10% of seed medium was used to inoculate 30 mL of production medium (PB) incubated in 250-mL erlenmeyer flasks at 30°C on a rotary shaker set at 200 rpm. The composition of production medium was shown in Table 2.4 (Brunati et al., 2005).

Table 2.4 The composition of production medium (PB).

Production Medium (PB) - Basal Medium	
4 g glucose, 4 g soluble starch, 20 g maltose, 20 g sucrose,	20 g glycerol, 30 g soybean meal, 6 g $CaCO_3$, 1 L distilled water.
❖ **Autoclave at 121°C for 15 minutes.**	

Production medium (Basal medium) was optimized for increasing to the yield of ramoplanin production. In parallel with this purpose, glucose as carbon source, leucine, tyrosine, glycine as amino acids and meat-bone meal, and feather meal as food waste material (obtained from Abalioglu Yem-Soya ve Tekstil A.S) were added to the medium in variable amounts. The compositions of basal and optimized production mediums were shown in Table 2.5. Optimized mediums were incubated under the same conditions as for the production medium.

Table 2.5 The compositions of basal and optimized production mediums.

Basal Medium	**Meat-bone Meal**	**Feather Meal**
4 g glucose, 4 g soluble starch, 20 g maltose, 20 g sucrose, 20 g glycerol, **30 g soybean meal,** 6 g $CaCO_3$, 1 L distilled water.	4 g glucose, 4 g soluble starch, 20 g maltose, 20 g sucrose, 20 g glycerol, **30 g meat-bone meal**, 6 g $CaCO_3$, 1 L distilled water.	4 g glucose, 4 g soluble starch, 20 g maltose, 20 g sucrose, 20 g glycerol, **30 g feather meal,** 6 g $CaCO_3$, 1 L distilled water.

Leucine Medium	**Tyrosine Medium**
4 g glucose, 4 g soluble starch, 20 g maltose, 20 g sucrose, 20 g glycerol, **30 g soybean meal,** 6 g $CaCO_3$, **5 g leucine,** 1 L distilled water.	4 g glucose, 4 g soluble starch, 20 g maltose, 20 g sucrose, 20 g glycerol, **30 g soybean meal,** 6 g $CaCO_3$, **5 g tyrosine,** 1 L distilled water.

5% Glucose	**10% Glucose**	**20% Glucose**	**20% Glucose + 2% Glycerol**
50 g glucose, 30 g soybean meal, 6 g $CaCO_3$, 1 L distilled water.	100 g glucose, 30 g soybean meal, 6 g $CaCO_3$, 1 L distilled water.	200 g glucose, 30 g soybean meal, 6 g $CaCO_3$, 1 L distilled water.	200 g glucose, 20 g glycerol, 30 g soybean meal, 6 g $CaCO_3$, 1 L distilled water.

Every 24 hours two flasks as shown in Figure 2.3 were harvested and extracted to measure optic density, pH and antibiotic production. The amount of ramoplanin in production medium was measured by HPLC determination. Data are reported as the mean values of at least two replicates for each experiment.

Figure 2.3 *Actinoplanes* sp. ATCC 33076 on optimized medium after 120 hours.

2.2 Determination of Ramoplanin

2.2.1 Extraction

The method described by Brunati et al. (2005) was modified as: 10 mL of culture were collected from the production flask, centrifuged at 3000 rpm for 5 min for the determination of optic density and pH. 10 mL of flask culture was mixed with 30 mL of acetone:water 2:1 brought to pH 2.2 with 1N HCl, shaken for 20 min at room temperature and then filtered through paper filter. All of the filtered solution were extracted by an equal volume of ethyl acetate and centrifuged at 5000 rpm for 10 minutes. The organic phase was eliminated and the residual water (lower) phase was transferred to the lyophilisation vials. Samples in vials were lyophilised. The lyophilized samples were resolved in approximately 1 mL of ultra distilled water. Prepared samples were analyzed by HPLC for ramoplanin determination (Brunati et al., 2005).

2.2.2 HPLC Conditions

The method described by Restelli et. Al, (1996) was modified as: HPLC analyses were performed on a Hypersil ODS-2 (C_{18}) column (5 µm, 4.6x250 mm) and eluted at 1.2 mL/min flow rate with a 30-min linear gradient elution mode as time (B%): 0 (27), 5 (27), 30 (95). Phase A was 25 mM NaH_2PO_4:CH_3CN 80:20 (v/v) and Phase B was 25 mM NaH_2PO_4:CH_3CN 20:80 (v/v) mixtures. The column temperature was operated at 40°C and injection volume was 20 µL. The chromatography was performed with a HP 1100 Series HPLC system equipped with a photodiode array detector (DAD) and detection wavelength was set at 254 nm. An authentic sample of ramoplanin antibiotic (obtained from Sigma Aldrich) was used as internal standard (Restelli & Mainolli, 1996).

2.3 Determination of Organic Acids

2.3.1 Extraction

The different extraction procedures were studied as: boiling ethanol, 50% (v/v) boiling ethanol, boiling water. Extraction in boiling water was chosen as the best procedure.

The samples were prepared by using a bit modification of the method described by Ganzera et al. (2006). Chilled intracellular metabolites from the cell pellet were extracted in a solution of boiling water 1:5 (w/v) for 15 min at 90^{o}C in a water bath. Solution of extraction was quickly freezed and thawed then centrifuged at 12000 rpm for 10 min at +4^{o}C. The supernatant was quickly lyophilized. The lyophilized samples were resolved in 200 µL of ultra distilled water and then filtered through a 0.45 µm filter before injecting into the HPLC (Ganzera et al., 2006).

2.3.2 HPLC Conditions

HPLC analyses were performed on an Alltech IOA-1000 column (9 µm, 7.8x300 mm) and eluted at 0.4 mL/min flow rate with a 30-min isocratic elution mode using

mobile phase $9x10^{-3}$ M H_2SO_4. The column temperature was operated at 42°C and injection volume was 20 µL. The chromatography was performed with a HP 1100 Series HPLC system equipped with a photodiode array detector (DAD) and detection wavelength was set at 210 nm. Standards of oxalacetic acid, citrate, α-ketoglutarate, and succinate, (obtained from Sigma Aldrich) were prepared and calibration curves were created (Kayalı, 2005).

2.4 Determination of Total Protein

Total protein amounts in the samples were determined by the method of Bradford. Bovine serum albumin as a standard was used.

In the total protein assay, 900 µL reagent was added to cuvette then 100 µL sample was added and shaked gently. Absorbance value was read at the end of two minutes against blank (100 µL buffer instead of sample).

BSA was used as a protein standard. 1000 ppm stock BSA solution was diluted to 10, 25, 50, 75 and 100 ppm in the 20 mM potassium phosphate buffer (pH 7.4). The protein amounts were determined by using calibration curve drawn between known concentration of BSA standards and their absorbance values at 595 nm (Bradford, 1976).

2.5 Determination of Reducing Sugar Concentration

Production medium was centrifuged and supernatant was used for analysis of reducing sugar content by the dinitrosalicyclic acid (DNS) method (Miller, 1959). The solution used for DNS method was prepared by first making solutions A and B as follows:

Solution A: 1g of dinitrosalicyclic acid was dissolved in 20 ml of 2N NaOH solution.

Solution B: 30 g of sodium-potassium tartarate was dissolved in 50 ml of distilled water.

Solution B was added to A while stirring and the total medium was covered to 100 mL with distilled water. The final solution (DNS) was kept in the dark. The first step of the analysis method was to mix 500 µL of supernatant with 500 µL of DNS solution. The mixture was then boiled for ten minutes and cooled for one minute in an ice bath. 5 ml of distilled water was added and mixed. The absorbance was measured at 546 nm against a reference sample. The reference sample was prepared by using 500 µL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curves were prepared in the range of 0-165.1 µg of glucose per milliliter of liquid, and the same procedure was performed. (Miller, 1959)

CHAPTER THREE
RESULTS AND DISCUSSION

Ramoplanin produced by *Actinoplanes sp*. ATCC 33076, is a lipoglycodepsipeptide antibiotic (Fang, 2008). Its structure consists in a cyclic peptide formed from sixteen amino acids and one side branch amino acid, which is acylated by a diunsaturated fatty acid. A disaccharide residue (d-mannosyld-mannose) is attached to the cyclic peptide (McCafferty et al., 2002).

Ramoplanin is an inhibitor of bacterial cell wall biosynthesis and exerts its effects by inhibiting peptidoglycan transglycosylases (Walker et al., 2005). It is not reported that there is no cross-resistance between beta-lactams or glycopeptides such as vancomycin (Lo et al., 2000).

Structure of the antibiotic is composed of three closely related components A1-3, of which 2 is the most abundant, that differ in the length and branching of the di-unsaturated fatty acid residue (McCafferty et al., 2002). Ramoplanin A2 exhibits activity against clinically-important Gram-positive bacteria including vancomycin-resistant *Enterococcus* sp. (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-intermediate resistant *Clostridium difficile*.

Ramoplanin component A2 (so far indicated as ramoplanin) is currently in Phase III clinical trials for the treatment of *Clostridium difficile*-associated disease (CDAD), vancomycin-resistant *Enterococcus faecium* (VREF), and methicillin-resistant *Staphylococcus aureus* (MRSA) (Di-Palo et al., 2007; Nanotherapeutics, 2013).

The interest in obtaining higher yields of the ramoplanin produced by *Actinoplanes* sp. ATCC 33076 has led to several studies on the physiology, and biochemistry of the pathways involved in the antibiotic biosynthesis in particular, and in secondary metabolism, in general. The generic status of the antibiotic provides an incentive to explore generally-applicable approaches to increasing product yield

by rational culture design. Metabolic flux analysis has been applied to bioprocesses for penicillin, vitamins, amino acids, proteins and recombinant DNA for gene therapy. In addition, fluxes through amino acid biosynthetic reactions have been analysed with regard to the production of transglutaminase by *Streptoverticillium mobaraense* and serine alkaline protease by *Bacillus licheniformis*. However, few studies have applied metabolic flux analysis to the design of mutants for enhanced antibiotic production (Bushell et al., 2006).

Metabolic flux analysis differs from FBA in that optimisation algorithms are not employed, the approach relying on experimentally determined flux estimates to reduce the underdetermined nature of the network (Stephanopoulos et al., 1998). This has been applied successfully to process design, using small metabolic networks, to a number of bioproducts including production of recombinant DNA for gene therapy (Rozkov et al., 2004) and antibiotic yield optimisation (Bushell et al., 2006). This is surprising as the technique facilitates the identification of the pathway fluxes that are significantly correlated with the synthesis of bioproducts of interest, providing the potential for rational strain or process design for maximum product yield.

3.1 Computer Modelling

The concentrations of antibiotics produced by non-industrial strains are comparatively low and, therefore, carbon fluxes through amino acid precursor pools will be proportionately lower than those observed in protein bioproduct synthesis. In this study, we propose that MFA in antibiotic-producing cultures can, nevertheless, be employed to identify those intermediary metabolites whose production rates are critical to attainment of maximum yields.

We constructed a metabolic network of about 100 reactions created by Simbiology Toolbox 4.2 of MATLAB software for the primary and secondary metabolism of *Actinoplanes* sp. ATCC 33076 and computational metabolic flux analysis was used to investigate some of the factors affecting production of ramoplanin (Figure 3.1). COPASI 4.10 (Complex Pathway SImulation) software was

used for flux analysis. In deterministic modeling, the program solves differential equations using the routine LSODE (Livermore Solver of Ordinary Differential Equation).

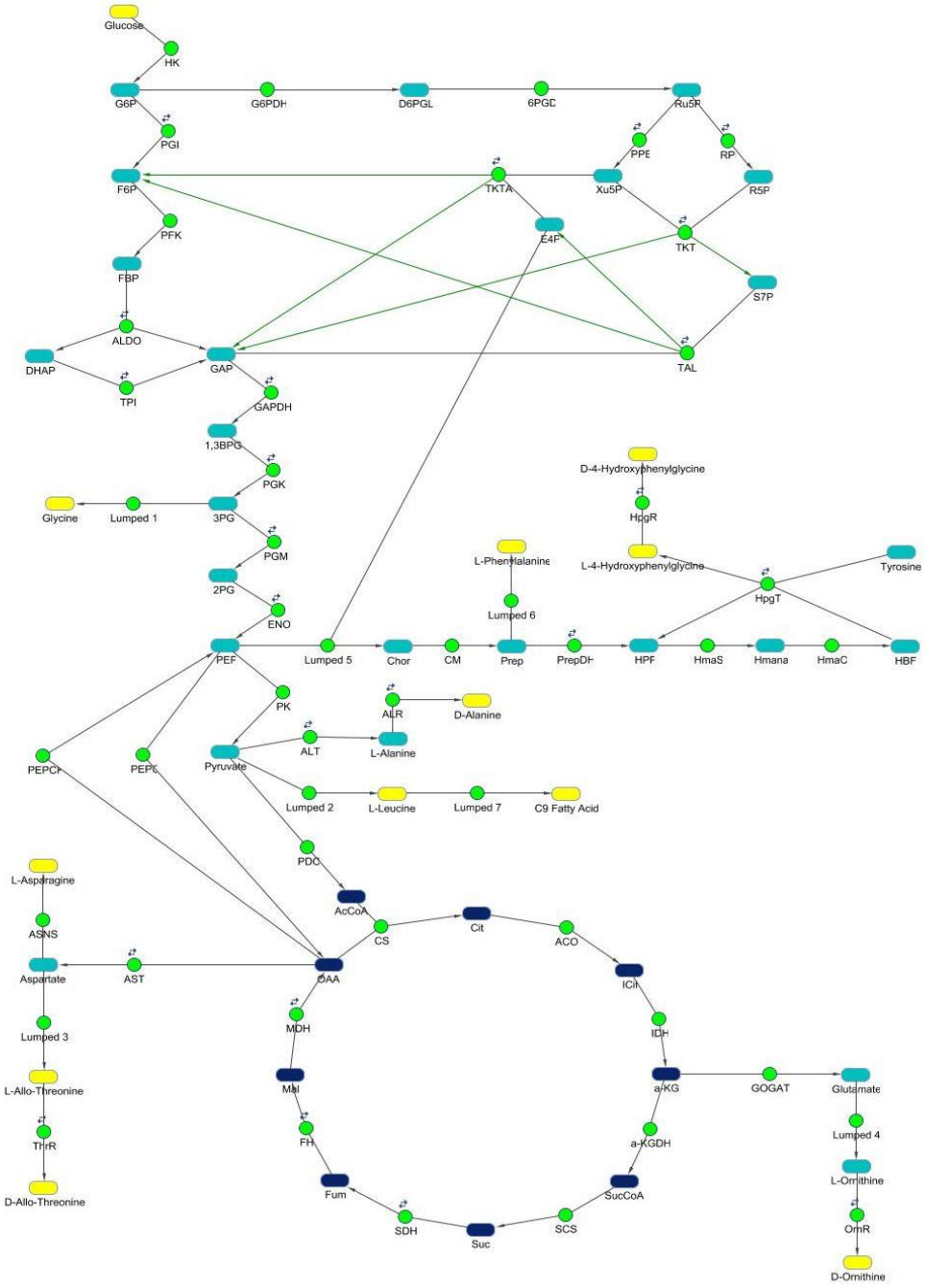

Figure 3.1 A view of biologic pathway related with *Actinoplanes* sp. (created by SimBiology)

Cellular metabolism is very complex and functions of fluxes. To study the metabolism, the fluxes need to be evaluated. Biosynthesis mechanism of ramoplanin antibiotic is largely known but kinetic parameters of enzymes in *Actinoplanes* sp. ATCC 33076 are lacking. The model set up is based on using combination models of Hynne and *E. coli*, and also some estimated kinetic parameters. The kinetic model consisted of Michaelis-Menten and mass action for enzyme kinetics. We fitted a comprehensive model of ramoplanin biosynthesis including glycolysis, tricarboxylic acid, pentose phosphate pathways, and synthesis of some amino acids in strain of *Actinoplanes* sp. ATCC 33076.

It was found that due to large number of unknown parameter that govern the differential equations problems as differential equation stability and multiple optima was encountered when attempting to solve all differential equations simultaneously. Thus the complex network was decomposed into smaller subnetworks.

It is potentially more interesting to model of ramoplanin biosytnhesis in *Actinoplanes* because of the biotechnological importance of ramoplanin. A good description of this model could also increase our understanding of the cell synchronization observed experimentally in suspensions of *Actinoplanes* strains.

Computational results indicated that the ramoplanin production was related with leucine and increasing concentration of glucose. Metabolic flux distributions computed for various phases of the batch culture indicated that the amount of ramoplanin production was affected by pentose phosphate pathway, shikimate biosynthesis, and leucine fluxes. Consequently, leucine is an important factor in ramoplanin production because of being precursor of fatty acid. Hexokinase is inhibited at high glucose concentrations so ramoplanin production is limited.

270 mmol/L (5%) glucose was added to the growth medium and model was analysed. When leucine (38 mmol/L) was added to growth medium, production of ramoplanin was increased to 6.11 mmol/L from 2.36 mmol/L shown in Figure 3.2 and Figure 3.3.

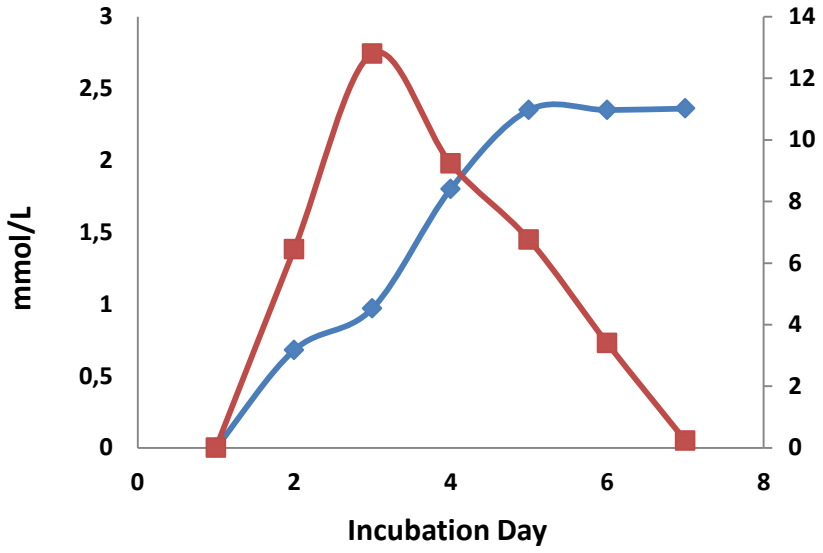

Figure 3.2 Effect of leucine upon production of ramoplanin without addition of leucine into the growth medium; ramoplanin (—♦—) and leucine (—■—).

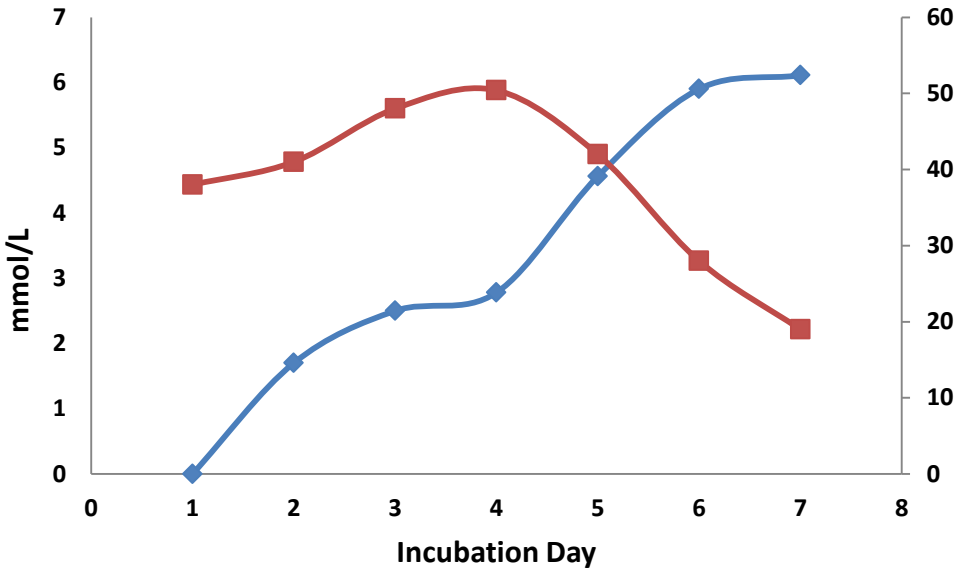

Figure 3.3 Effect of leucine upon production of ramoplanin with addition of 38 mmol/L leucine into the growth medium; ramoplanin (—♦—) and leucine (—■—).

Ramoplanin production decreased with respect to increases in glucose concentration since high glucose concentrations inhibit to the hexokinase enzyme which leads to decreases in glycolysis metabolite levels that are important precursor of ramoplanin (Figure 3.4).

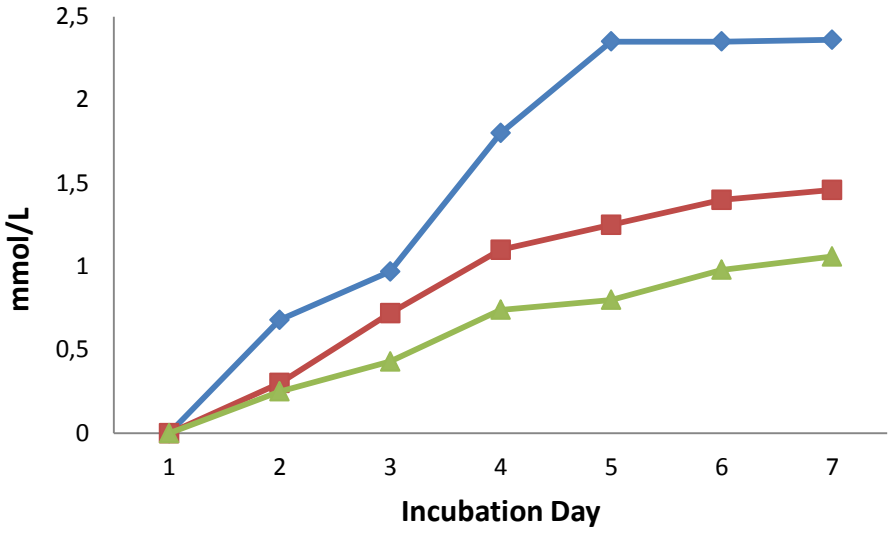

Figure 3.4 Effect of glucose concentration upon production of ramoplanin in the growth medium; 5% (—♦—), 10% (—■—) and 20% (—▲—) of glucose.

3.2 Variations in the Growth and Metabolite Levels of *Actinoplanes* sp. ATCC 33076 Depending on Glucose Concentration

Glucose primarily metabolized via glycolysis pathway is a common constituent of media for antibiotic production and has also been reported to stimulate the production until it causes inhibitory effect. The inhibitory effect of glucose may be due to the low pH created by its metabolism. In a medium containing glucose plus a more slowly utilized carbon source, glucose usually is used first in the absence of antibiotic production. After glucose is depleted, the second carbon source is then used for antibiotic biosynthesis. Therefore in the thesis, 5% - 20 glucose concentrations were added to the medium to determine of the glucose influence upon production of ramoplanin antibiotic.

Secondary metabolites as antibiotics are synthesized by a greater variety of pathways than are primary metabolites. So it is important to control the pathways related with antibiotic production. The production of ramoplanin by *Actinoplanes* sp. ATCC 333076 is closely related with glucose medium and it is generally believed that the producing organisms initiate the synthesis of antibiotics towards the end of the exponential phase of growth.

3.2.1 Variations in the Growth Curve of Actinoplanes sp. ATCC 33076 Depending on Glucose Concentration in Respect to Incubation Period

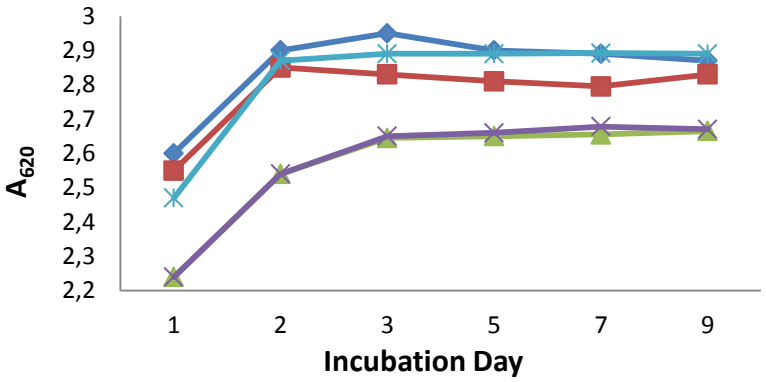

Figure 3.5 The optic density depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% glucose (—▲—), 20% glucose plus 2% glycerol (—X—) and basal medium (—*—).

As shown in Figure 3.5, *Actinoplanes* sp. ATCC 33076 displayed logarithmic growth during the first 24–48 h for all studied glucose concentrations after which it entered into their stationary phases. The growth rate decreased with respect to increases in glucose concentrations which indicate that glucose was no longer a growth limiting factor for the final biomass.

3.2.2 Variations in the Extracellular pH Level of Mediums Depending on Glucose Concentration in Respect to Incubation Period

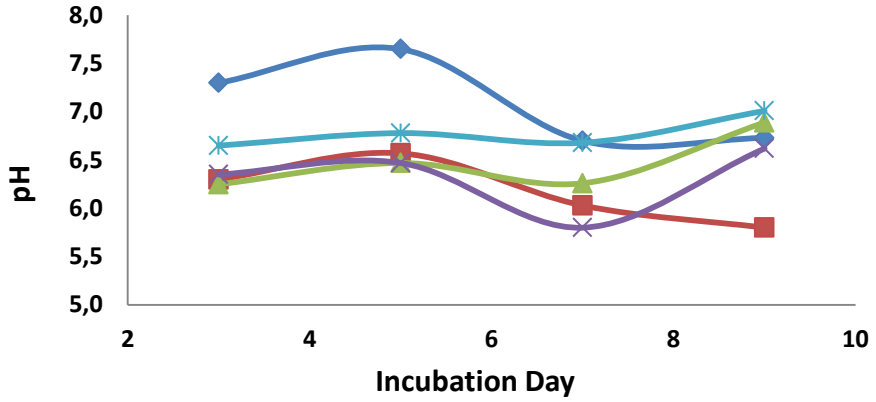

Figure 3.6 The pH levels depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—) and basal medium (—*—).

According to the results, pH levels of *Actinoplanes* sp. ATCC 33076 increased up to 5th day for all glucose concentrations we tested and then decreased continuingly until 7th day (Figure 3.6). The pH values increased more at 5% glucose concentrations in the culture medium. The biggest pH rise was observed at 5% glucose as 0.35 units. In the subsequent incubation periods, pH values slightly increased. The insignificant changes of pH level for basal medium can be due to the availability of diffirent carbon sources besides glucose to support to steady state carbon sources concentration in the metabolism.

3.2.3 Variations in the Reducing Sugar Content of Mediums Depending on Glucose Concentration in Respect to Incubation Period

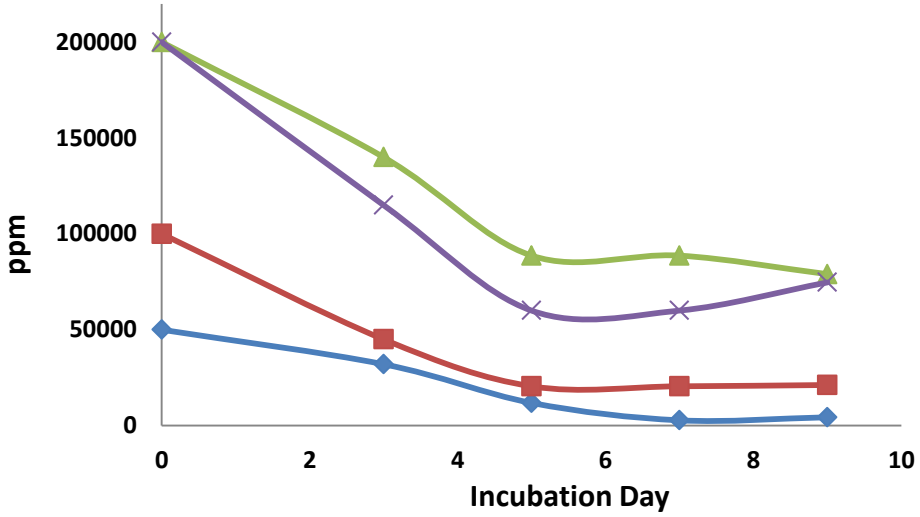

Figure 3.7 The variations of reducing sugar content depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—).

As can be seen from Figure 3.7, extracellular glucose levels increased with increasing glucose concentrations of the growth medium. According to the results, glucose in the growth medium was consumed rapidly during the log phase of the incubation period. This situation is presumably the result of an increase in the glucose consumption rate in order to maintain energy metabolism during log phase. After the 5th day of incubation, the extracellular glucose levels did not change significantly.

3.2.4 Variations in the Ramoplanin Production Level of Actinoplanes sp. ATCC 33076 Depending on Glucose Concentration in Respect to Incubation Period

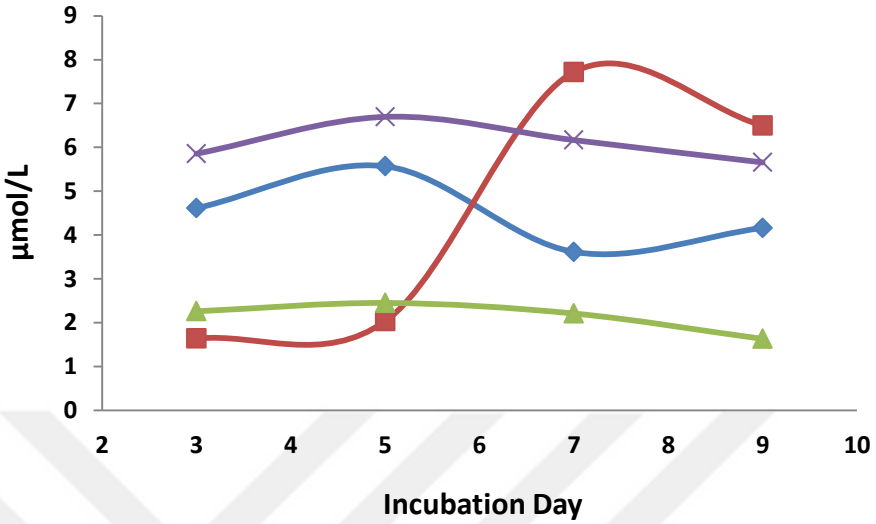

Figure 3.8 The production on ramoplanin depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—).

Depending on the biosynthetic pathways involved, production of antibiotic has been known to be effected by media components and cultural conditions, such as carbon resource, nitrogen resource, aeration, agitation, pH and temperature. In order to screen a suitable carbon source, *Actinoplanes* sp. ATCC 33076 was incubated in the basal medium containing various glucose concentrations. Glucose, usually an excellent carbon source for growth but it can interfere with the biosynthesis of secondary metabolites.

Each glucose concentration was added into the basal medium in the range of 5 - 20% (Figure 3.8). The medium including 10% glucose gave the maximum antibiotic production. Glucose concentration above 10% negatively affected the synthesis of ramoplanin in *Actinoplanes* sp. ATCC 33076. This result may be due to the limitation of oxygen diffusion, which has an important effect on antibiotic production. In addition, it can be suggested that this effect is due to the phenomenon of catabolic repression by glucose.

3.2.5 Variations in the Intracellular Tricarboxylic Acid Cycle Metabolite Levels of Actinoplanes sp. ATCC 33076 Depending on Glucose Concentration in Respect to Incubation Period

Most bacteria employ the tricarboxylic acid (TCA) not only for generating the energy required for growth but also for producing the precursors of the secondary metabolites. A higher antibiotic production yield can be achieved by increasing the availability of these precursors which can be regulated by changing growth conditions. Glucose has been widely used as a substrate for the production of a large number of antibiotics and other secondary metabolites. Therefore, varied glucose concentration in the range of 5-20% was added to each medium to determine of glucose influence.

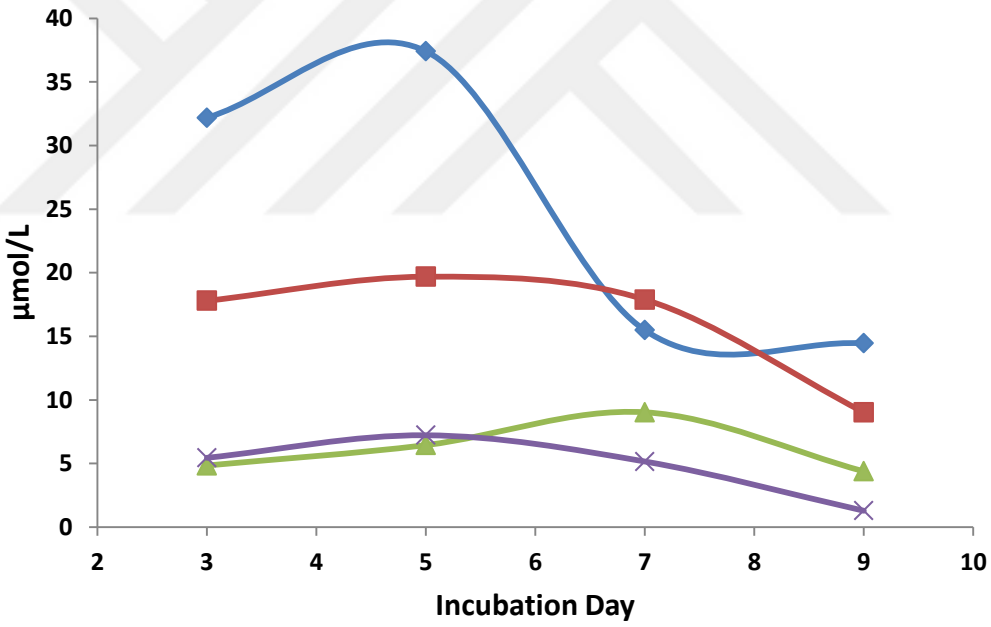

Figure 3.9 The variations of oxaloacetate levels depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—).

As can be seen in Figure 3.9 highest level of oxaloacetate of *Actinoplanes* sp. ATCC 33076 was in %5 glucose medium. The level of oxaloacetate decreased with increases in glucose concentrations of culture medium.

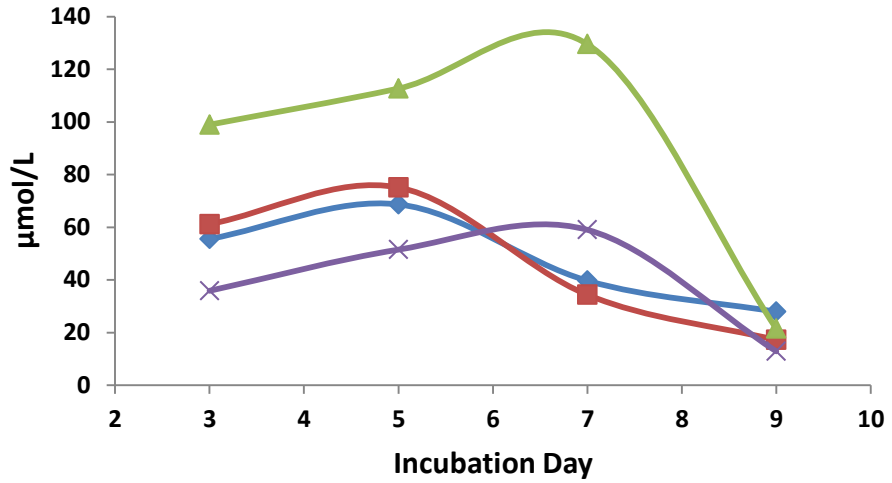

Figure 3.10 The variations of citrate levels depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—).

Similar changes in citrate and α-ketoglutarate levels variations were observed when *Actinoplanes* sp. ATCC 33076 growth in different glucose concentrations. According to the results the highest level of citrate was determined in medium containing 20% glucose and it determined as 0.13 mmol/L ppm. The detemined citrate levels for %5 and 10 glucose concentration were smiliar and the levels were 3.6 fold lower compared to the level of 20% glucose medium as shown in Figure 3.10.

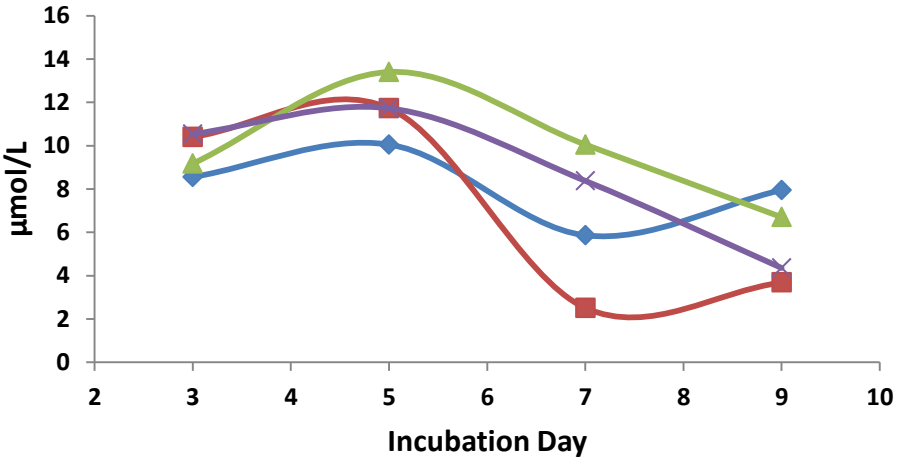

Figure 3.11 The variations of α-ketoglutarate levels depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—).

According to the results in Figure 3.11, in contrast to oxaloacetate patterns, levels of α-ketoglutarate increased with respect to increasing in glucose concentrations of the growth medium. Nevertheless, α-ketoglutarate levels for all investigated glucose concentrations reached their maximum levels during the initial stationary phase and the α-ketoglutarate levels decreased in the subsequent incubation periods, where was indicative of the end stationary growth phase.

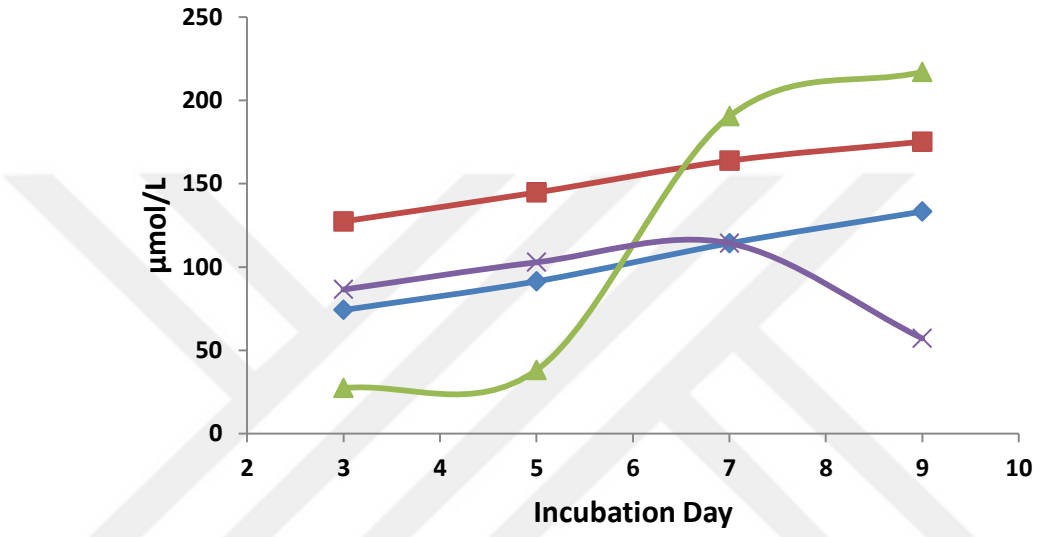

Figure 3.12 The variations of succinate levels depending on the growth medium conditions containing 5% (—♦—), 10% (—■—), 20% (—▲—) glucose and 20% glucose plus 2% glycerol (—X—).

As shown in Figure 3.12 the highest level of succinate was determined in 20% glucose medium. In addition to that, levels of succinate increased with respect to the increasing glucose concentration.

Increases were observed for α-ketoglutarate, citrate and succinate levels of *Actinoplanes* sp. ATCC 33076 towards 20% from 5% glucose concentrations. This result indicates that the glycolysis levels increased which lead increases in tricarboxylic acid cycles. The increases in these cycles can be explained by increasing glucose levels and retarded pentose phosphate cycle as a result of inhibition of glucose-6-phosphate dehydrogenase enzyme. Any decrease in ramoplanin antibiotic level can arise, related to the retarded pentose phosphate, from decreasing concentration of Hpg, tyrosine and phenylalanine amino acids which are

precursors for ramoplanin antibiotic and synthesised in this cycle. In addition, decreases in ramoplanin antibiotics within the 20% glucose concentration can be due to the decreases in oxaloacetate levels and correspondingly related to the decreasing asparagine and threonine amino acids which are precursors for ramoplanin antibiotic.

3.3 Variations in the Growth and Metabolite Levels of *Actinoplanes sp.* ATCC 33076 Depending on Nitrogen Source in Respect to Incubation Period

A critical physiological parameter that influences the level of production of antibiotics by microorganisms is carbon flux from primary to secondary metabolism. In order to increase production levels of specific metabolites, it is essential to determine inefficient steps in the pathway which may be targeted and improved through antibiotic production. Therefore in the thesis food waste materials as soybean, meat-bone and feather meals were added to the each medium to increase production of ramoplanin antibiotic. Due to high cost production of ramoplanin, it is important to decrease the cost of medium composition by using food waste materials to consider the economic efficiency. Average prices of the food waste materials for soybean meal, meat and bone meal, and feather meal are ordered as; 505 $ per ton, 440 $ per ton, 640 $ per ton, respectively (The U.S. Department of Agriculture's Agricultural Marketing Service, 2013). Also the prices of leucine and tyrosine are higher than these food waste materials.

3.3.1 Variations in the Growth Curve of Actinoplanes sp. ATCC 33076 Depending on Nitrogen Source in Respect to Incubation Period

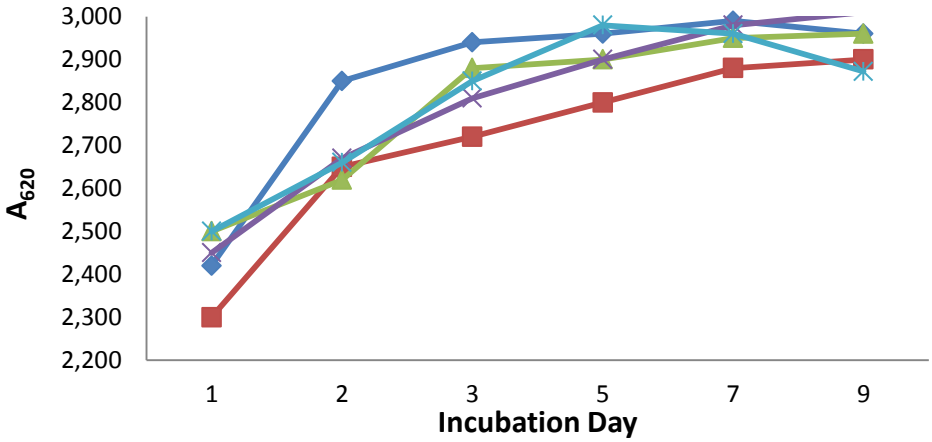

Figure 3.13 The optic density depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—) and feather meal (—*—).

As shown in Figure 3.13, the optimum incubation time for growth of *Actinoplanes* sp. ATCC 33076 varied with respect to nitrogen sources. The maximum growth rate of *Actinoplanes sp.* ATCC 33076 occurred at the 5th day for both basal and feather meal mediums and similar levels were also observed for the other nitrogen sources mediums at 7th day.

3.3.2 Variations in the Extracellular pH Level of Mediums Depending on Nitrogen Source in Respect to Incubation Period

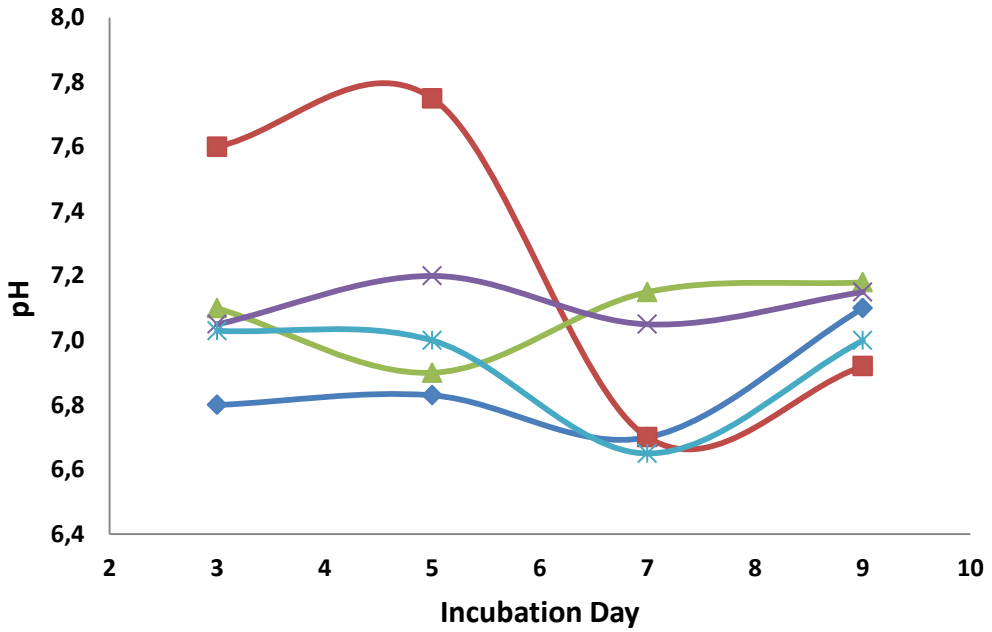

Figure 3.14 The pH levels depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

The Figure 3.14 shows that extracellular pH levels of *Actinoplanes sp.* ATCC 33076 increased in the 3-5th days of incubation period. The maximum pH levels in all nitrogen sources we tested occurred at leucine medium on 5th day except for tyrosine medium for which the maximum value was measured at the 7th day.

3.3.3 Variations in the Reducing Sugar Content of Mediums Depending Nitrogen Source in Respect to Incubation Period

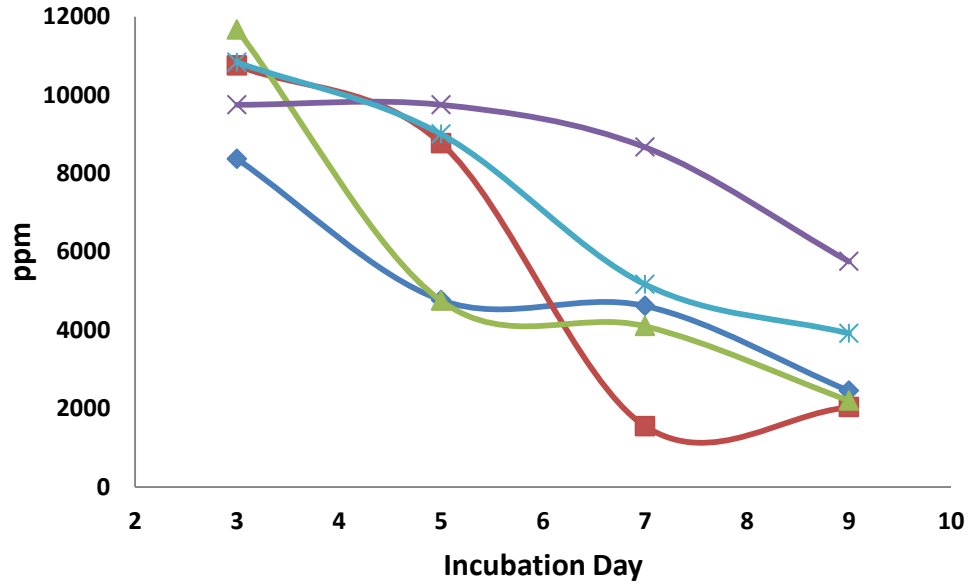

Figure 3.15 The variations of the reducing sugar content depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

According to our results, extracellular glucose decreased significantly with respect to the incubation period. As can be seen from Figure 3.15, the decreases in extracellular glucose levels in *Actinoplanes* sp. ATCC 33076 grown in leucine, tyrosine, meat-bone meal, and feather meal mediums were significantly lower than the basal medium during the 5 day of incubation. In subsequent incubation, the consumption rate of glucose increased for leucine medium.

3.3.4 Variations in the Ramoplanin Production Level of Actinoplanes sp. ATCC 33076 Depending on Nitrogen Source in Respect to Incubation Period

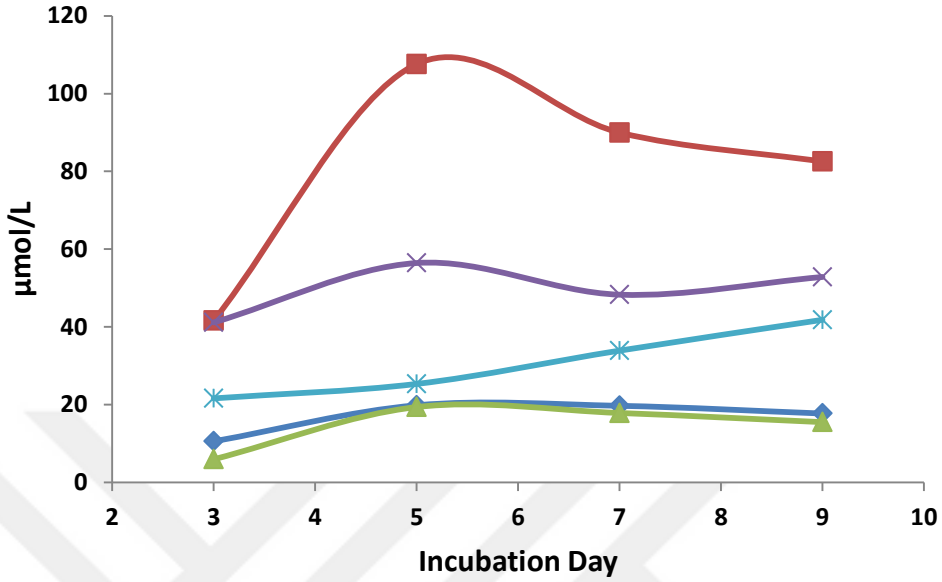

Figure 3.16 The production on ramoplanin depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

Various nitrogen sources such as leucine, tyrosine, meat-bone meal, and feather meal were added at a concentration of 0.5%, 0.5, 3, and 3 into the basal medium, respectively (Figure 3.16). The highest antibiotic production was reached after addition of leucine. Leucine might be incorporated in biosynthetic paths for the formation of lypoglycodepsipeptide antibiotic and might be also served as precursor of the lypoglycodepsipeptide antibiotic production. Tyrosine allowed significant growth of *Actinoplanes* sp. ATCC 33076 although ramoplanin values were low. To consider the economic efficiency, the cost of leucine is higher than that of meat-bone meal, feather meal, and soybean meal. In comparison with basal medium, meat-bone meal and feather meal sources gave relatively higher antibiotic production. As a result, due to economic efficiency and production yield, the meat-bone meal is the most efficient food waste material in comparison with soybean meal and feather meal.

The results also show that ramoplanin production of *Actinoplanes* sp. ATCC 33076 growths in all tested nitrogen sources started with logaritmic phase and reached maximum at the onset of stationary phase. Further increases in incubation period did not enhance ramoplanin production probably due to the depletion of precursor of ramoplanin. On the other hand, ramoplanin production continued to increase until 5th of incubation in leucine medium. This result shows that the feeding of *Actinoplanes* sp. ATCC 33076 with leucine besides soybean could maintain cell metabolism, and thereby enhance effectively the production of the precursors of the ramoplanin such as amino acids and fatty acids.

3.3.5 Variations in the Intracellular Tricarboxylic Acid Cycle Metabolite Levels of Actinoplanes sp. ATCC 33076 Depending on Nitrogen Source in Respect to Incubation Period

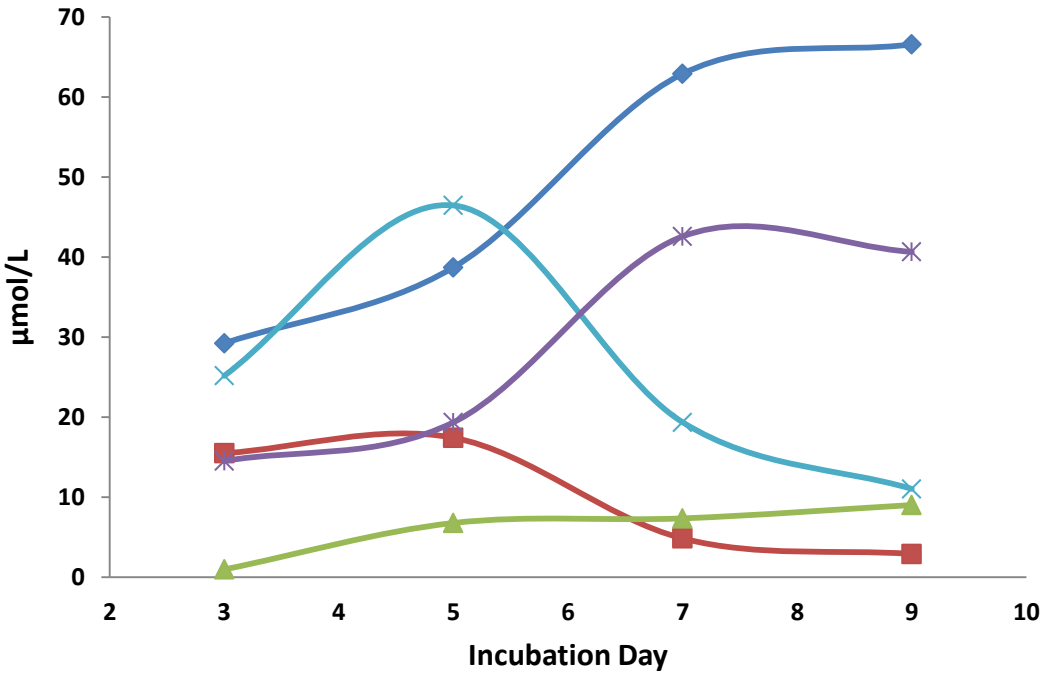

Figure 3.17 The variations of oxaloacetate levels depending on the growth medium conditions; basal medeium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

As shown in Figure 3.17, oxaloacetate level increased with respect to incubation period and the maximum level was detemineded as 0.067 mmol/L in the basal medium. However oxaloacetate level of *Actinoplanes* sp. ATCC 33076 in leucine medium decreased 3.8 fold and it was 0.019 mmol/L. In addition oxaloacetate levels for both of meat-bone and feather meal were significantly lower than basal medium but higher than leucine medium. According to the results oxaloacetate level decreased during exponantial growth phase. The decreases in the oxaloacetate level for meat-bone meal and feather meal during the exponantial phase probably are due to the depletion of necessary metabolite for oxaloacetate level. On the other hand the increases in oxaloacetate level for leucine and basal mediums can be probably the efffect of soybean meal to supply to the metabolite.

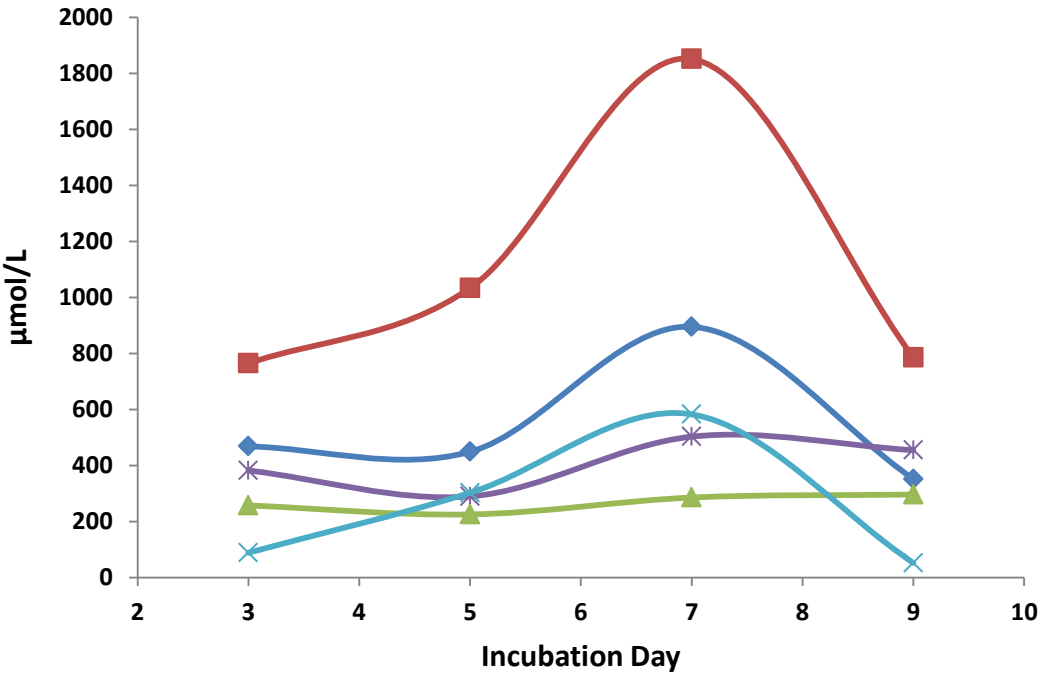

Figure 3.18 The variations of citrate levels depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

Figure 3.18 shows that the highest level of citrate determined in leucine medium and the level orders were followed as basal, feather meal and meat-bone meal mediums.

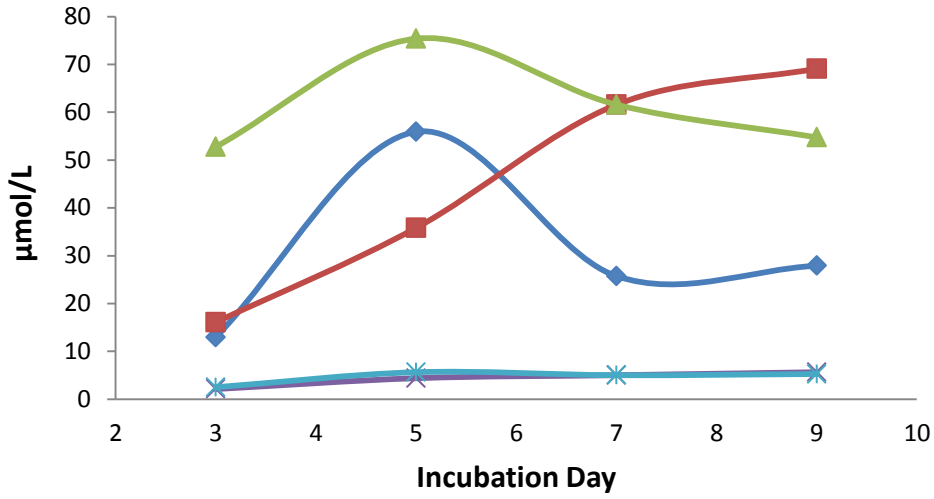

Figure 3.19 The variations of α-ketoglutarate levels depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

As can be seen in Figure 3.19 the variations of α-ketoglutarate levels showed similartiy with citrate levels and the highest α-ketoglutarate level detemined in leucine medium during the stationary phase while it was highest for tyrosine medium during the exponential phase of *Actinoplanes* sp. ATCC 33076. Nevertheless α-ketoglutarate levels were lower approximately 10 folds in meat-bone meal and feather meal mediums compared with basal medium.

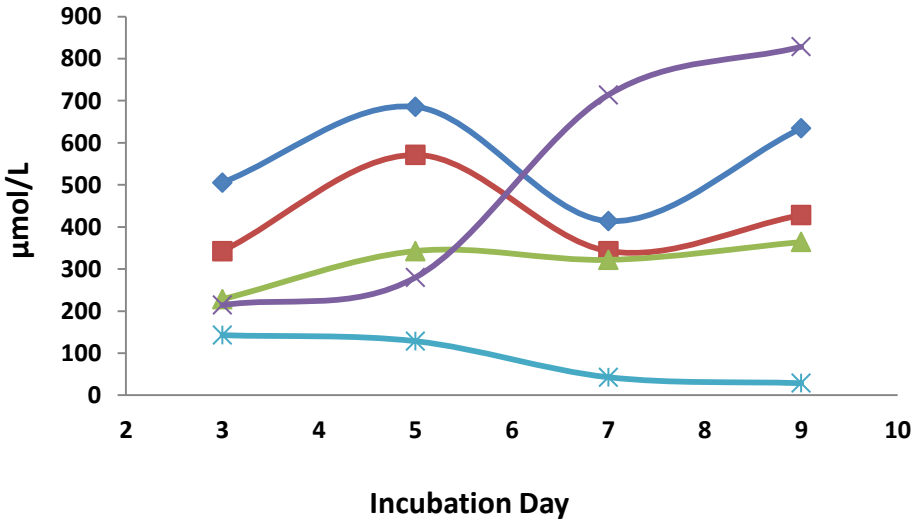

Figure 3.20 The variations of succinate levels depending on the growth medium conditions; basal medium (—♦—), leucine medium (—■—), tyrosine medium (—▲—), meat-bone meal (—X—), feather meal (—*—).

Figure 3.20 indicates that the optimum time of incubation for maximal succinate production varried with growth medium. According to our results succinate levels for basal and leucine mediums rose up to the 5th day and then decreased until 7th day. Nevertheless, succinate level increased continuingly for meat-bone meal while it decreased for feather meal medium depending on incubation periods.

The increased citrate and decreased oxaloacetate levels in leucine medium compared to basal medium can be explained by increased Acetyl CoA levels during leucine degredation. Under conditions of leucine supplemention of the growth medium, increased ramoplanin production accompany with decreased oxaloacetate levels is probably due to the oxaloacetate converted asparagine, threonine and phosphoenolpyruvate which are precursors of ramoplanin. As it is known, in the metabolism, phosphoenolpyruvate plays an important role for synthesis of alanine, phenylalanine and also Hpg which are derived from erythrose 4-phosphate besides phosphoenolpyruvate. In addition, the parallel increases in α-ketoglutarate level with ramoplanin production in leucine medium may be explained by the synthesis of glutamate and ornithine from α-ketoglutarate to supply ramoplanin precursor. In addition, succinate and fumarate levels did not change significantly.

The addition of tyrosine into the basal medium repressed the biosynthesis of ramoplanin. The reason of this can be due to the three reasons: 1- The decreases in investigated tricarboxylic acid cycle organic acids metabolites except for α-ketoglutarate that can provide the antibiotic biosynthesis. 2- The addition of tyrosine directed some metabolic flux to melanin which leads to more diversity and high-coloured medium. 3- Addition of tyrosine to the basal medium caused the inhibitory effect on the biosytnthesis of phenylalanine which is precursor of ramoplanin. Reason of high levels of α-ketoglutarate can be explained by tyrosine in the medium converted to the L-dopa and accumulation of this metabolite leaded to flux to proline amino acid that is precursor of α-ketoglutarate.

The reason for the decline of the α-ketoglutarate in growth medium containing meat-bone meal may be due to the high threonine content of the meat-bone meals

compared to the basal medium. Because citrate synthase is allosterically inhibited by succinyl-CoA which is derived from threonine. Therefore, both citrate and a-ketoglutarate were lower for the medium. The increases in succinate levels in the medium also supported the increases in succinyl-CoA level in the medium contained meat-bone meal probably due to high threonine level of the medium. The another possible reason for the low α-ketoglutarate level in the meat-bone meal mediums, which have the high amino acid content, is the conversion of α-ketoglutarate to glutamate and ornithine in order to eliminate ammonia released during deamination of the amino acids. In this medium, the determination of the high level of ramoplanin may also support the previous results that meat-bone meal medium provides leucine, phenylalanine, arginine, lysine as well as being highly ornithine which are precursors of the antibiotic.

Altough similar results in terms of investigated organic acids determined in the feather meal medium compared to meat-bone growth medium, determination of low ramoplanin level may be explained by deficieny of required amino acids contents of the feather meal for the production of ramoplanin.

In conclusion, glucose concentrations and nitrogen sources play an essential role on the metabolites levels related with the ramoplanin antibiotic biosynthesis of *Actinoplanes* sp. ATCC 33076. Although detemination of the highest ramoplanin production in leucine medium, the relatively high level of ramoplanin production in meat-bone meal indicated that it is more suitable companent of the medium to decrease the production cost of ramoplanin economically.

It is certain that computer modeling results can not be used since complex metabolism of living systems and also not avaible kinetic parameters to define the complex system. In addition, the presention of only certain strain such as *E. Coli, S. cerevisiae*, *Lactococcus lactis* kinetic parameters for computer analysis cause to determination of false data for the examined metabolic flow when different organism is used. Therefore, the prediction models obtained by computational tools can not be used and therefore they must be validated by experimental data.

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APPENDICES

APPENDIX A - LIST OF REACTIONS USED IN METABOLIC MODEL

Glycolysis

1) Glucose + ATP → Glucose 6-phosphate + ADP
(Hexokinase - HK)

2) Glucose 6-phosphate ↔ Fructose 6-phosphate
(Phosphoglucose isomerase - PGI)

3) Fructose 6-phosphate + ATP → Fructose 1,6-bisphosphate + ADP
(Phosphofructokinase - PFK)

4) Fructose 1,6-bisphosphate ↔ Glyceraldehyde 3-phosphate + Dihydroxyacetone phosphate
(Aldolase - ALDO)

5) Dihydroxyacetone phosphate ↔ Glyceraldehyde 3-phosphate
(Triosephosphate isomerase - TPI)

6) Glyceraldehyde 3-phosphate + NAD ↔ 1,3-Bisphosphoglycerate + NADH
(Glyceraldehyde 3-phosphate dehydrogenase - GAPDH)

7) 1,3-Bisphosphoglycerate + ADP ↔ 3-Phosphoglycerate + ATP
(Phosphoglycerate kinase - PGK)

8) 3-Phosphoglycerate ↔ 2-Phosphoglycerate
(Phosphoglycerate mutase - PGM)

9) 2-Phosphoglycerate ↔ Phosphoenolpyruvate
(Enolase - ENO)

10) Phosphoenolpyruvate + ADP → Pyruvate + ATP
(Pyruvate kinase - PK)

Gluconeogenesis

11) Pyruvate + ATP → Oxaloacetate + ADP
(Pyruvate carboxylase - PC)

12) Oxaloacetate + GTP → Phosphoenolpyruvate + GDP
(Phosphoenolpyruvate carboxykinase - PEPCK)

13) Phosphoenolpyruvate ↔ 2-Phosphoglycerate
(Enolase - ENO)

14) 2-Phosphoglycerate ↔ 3-Phosphoglycerate
(Phosphoglycerate mutase - PGM)

15) 3-Phosphoglycerate + ATP ↔ 1,3-Bisphosphoglycerate + ADP
(Phosphoglycerate kinase - PGK)

16) 1,3-Bisphosphoglycerate + NADH ↔ Glyceraldehyde 3-phosphate + NAD **(Glyceraldehyde 3-phosphate dehydrogenase - GAPDH)**

17) Glyceraldehyde 3-phosphate ↔ Dihydroxyacetone phosphate
(Triosephosphate isomerase - TPI)

18) Dihydroxyacetone phosphate + Glyceraldehyde 3-phosphate ↔ Fructose 1,6-bisphosphate
(Aldolase - ALDO)

19) Fructose 1,6-bisphosphate → Fructose 6-phosphate + Pi
(Fructose 1,6-bisphosphatase)

20) Fructose 6-phosphate ↔ Glucose 6-phosphate
(Phosphoglucose isomerase - PGI)

21) Glucose 6-phosphate → Glucose + Pi
(Glucose 6-phosphatase)

TCA Cycle

1) Pyruvate + NAD + CoA-SH → Acetyl CoA + NADH + CO_2
(Pyruvate dehydrogenase complex - PDC)

2) Oxaloacetate + Acetyl CoA → Citrate + CoA-SH
(Citrate synthase - CS)

3) Citrate → Isocitrate
(Aconitase - ACO)

4) Isocitrate + NAD → α-Ketoglutarate + NADH + CO_2
(Isocitrate dehydrogenase - IDH)

5) α-Ketoglutarate + NAD + CoA-SH → Succinyl CoA + NADH + CO_2
(α-Ketoglutarate dehydrogenase - α-KGDH)

6) Succinyl CoA + GDP ↔ Succinate + GTP + CoA-SH
(Succinyl CoA synthetase - Succinate thiokinase - SCS)

7) Succinate + FAD ↔ Fumarate + $FADH_2$
(Succinate dehydrogenase - SDH)

8) Fumarate ↔ Malate
(Fumarase - FH)

9) Malate + NAD ↔ Oxaloacetate + NADH
(Malate dehydrogenase - MDH)

Pentose Phosphate Pathway

1) Glucose 6-phosphate + NADP → 6-Phosphogluconolactone + NADPH
(Glucose 6-phosphate dehydrogenase - G6PDH)

2) 6-Phosphogluconolactone → 6-Phosphogluconate
(6-Phosphogluconolactonase - PGLS)

3) 6-Phosphogluconate + NADP → Ribulose 5-phosphate + NADPH + CO_2
(6-Phosphogluconate dehydrogenase – 6PGD)

4) Ribulose 5-phosphate ↔ Xylulose 5-phosphate
(Ribulose phosphate 3-epimerase - PPE)

5) Ribulose 5-phosphate ↔ Ribose 5-phosphate
(Ribose 5-phosphate isomerase - RPI)

6) Ribose 5-phosphate + Xylulose 5-phosphate ↔ Sedoheptulose 7-phosphate + Glyceraldehyde 3-phosphate
(Transketolase - TKT)

7) Sedoheptulose 7-phosphate + Glyceraldehyde 3-phosphate ↔ Fructose 6-phosphate + Erythrose 4-phosphate
(Transaldolase - TAL)

8) Xylulose 5-phosphate + Erythrose 4-phosphate ↔ Fructose 6-phosphate + Glyceraldehyde 3-phosphate
(Transketolase I - TKTA)

Others

1) 3-Phosphoglycerate → Glycine **(Lumped)**

2) 2 Pyruvate → L-Leucine **(Lumped)**

3) Aspartate → L-Allo-Threonine **(Lumped)**

4) Glutamate → L-Ornithine **(Lumped)**

5) 2 Phosphoenolpyruvate + Erythrose 4-phosphate → Chorismate **(Lumped)**

6) Prephenate + Glutamate → L-Phenylalanine + α- Ketoglutarate **(Lumped)**

7) Chorismate ↔ Prephenate
(Chorismate Mutase - CM)

8) Prephenate ↔ 4-Hydroxyphenylpyruvate
(Prephenate dehydrogenase - PrepDH)

9) 4-Hydroxyphenylpyruvate → 4-Hydroxymandelic acid
(4-hydroxymandelic acid synthase - HmaS)

10) 4-Hydroxymandelic acid → 4-Hydroxybenzoylformate
(4-hydroxymandelic acid oxidase - HmaO)

11) 4-Hydroxybenzoylformate + tyrosine ↔ 4-Hydroxyphenylpyruvate + 4-Hydroxyphenylglycine

(L-p-hydroxyphenylglycine transaminase - HpgT)

12) Pyruvate + Glutamate ↔ L-Alanin + α- Ketoglutarate

(Alanine transaminase - ALT)

13) α- Ketoglutarate + Glutamine + NADPH → Glutamate + NADP

(Glutamate synthase - GOGAT)

14) Oxaloacetate + Glutamate ↔ Aspartate + α- Ketoglutarate

(Aspartate transaminase - AST)

15) Aspartate + Glutamine + ATP → L-Asparagine + Glutamate + AMP

(Asparagine synthetase - ASNS)

Racemization Reactions

1) L-Alanine ↔ D-Alanin

(Alanine racemase - ALR)

2) L-Ornithine ↔ D-Ornithine

(Ornithine racemase - OrnR)

3) L-Allo-Threonine ↔ D- Allo-Threonine

(Allo-Threonine racemase - ThrR)

4) L-4-Hydroxyphenylglycine ↔ D-4-Hydroxyphenylglycine

(Hydroxyphenylglycine racemase - HpgR)

Serine & Glycine Biosynthesis

1) 3PG + NAD → NADH + PHP

(3-Phosphoglycerate dehydrogenase)

2) PHP + GLU → AKG + 3PSER

(Phosphoserine transaminase)

3) 3PSER → PI + SER

(Phosphoserine phosphatase)

4) THF + SER → GLY + METTHF

(Glycine hydroxymethyltransferase)

Leucine Biosynthesis

1) 2 Pyruvate + NADPH → NADP + α- Keto-isovalerate

2) α- Keto-isovalerate + Glutamate + Acetyl CoA + NAD → Leucine + NADH + α- Ketoglutarate

Threonine Biosynthesis

1) ASP + ATP ↔ ADP + BASP

(Aspartate kinase)

2) BASP + NADPH ↔ NADP + Pi + ASPSA

(Aspartate semialdehyde dehydrogenase)

3) ASPSA + NADPH ↔ NADP + HSER

(Homoserine dehydrogenase)

4) HSER + ATP → ADP + PHSER

(Homoserine kinase)

5) PHSER → Pi + THR

(Threonine synthase)

Ornithine Biosynthesis

1) Glutamate + ATP → Glutamate 5-phosphate + ADP

(Glutamyl kinase)

2) Glutamate 5-phosphate +NADPH → Glutamate 5-semialdehyde + ADP

(Dehydrogenase)

3) Glutamate 5-semialdehyde + Glutamate → Ornithine + α- Ketoglutarate

(Ornithine transaminase)

Phenylalanine Biosynthesis

1) Prephenate → Phenylpyruvate

(Prephenate dehydratase)

2) Phenylpyruvate + Glutamate → Phenylalanine + α- Ketoglutarate

(Amino transferase)

APPENDIX B - ABBREVIATIONS

Abbreviations of metabolites

Glc	Glucose
G6P	Glucose 6-phosphate
F6P	Fructose 6-phosphate
FBP	Fructose 1,6-bisphosphate
GAP	Glyceraldehyde 3-phosphate
DHAP	Dihydroxyacetone phosphate
1,3BPG	1,3-Bisphosphoglycerate
3PG	3-Phosphoglycerate
2PG	2-Phosphoglycerate
PEP	Phosphoenolpyruvate
Pyr	Pyruvate
OAA	Oxaloacetate
AcCoA	Acetyl CoA
Cit	Citrate
ICit	Isocitrate
α-KG	α-Ketoglutarate
SucCoA	Succinyl CoA
Suc	Succinate
Fum	Fumarate
Mal	Malate
D6PGL	6-Phosphogluconolactone
D6PGC	6-Phosphogluconate
Ru5P	Ribulose 5-phosphate
Xu5P	Xylulose 5-phosphate
R5P	Ribose 5-phosphate
S7P	Sedoheptulose 7-phosphate
E4P	Erythrose 4-phosphate
Leu	Leucine
Ala	Alanin
Gly	Glycine
Orn	Ornithine
Allo-Thr	Allo-Threonine
Chor	Chorismate
Prep	Prephenate
Phe	Phenylalanine
HPP	4-Hydroxyphenylpyruvate
Hmana	4-Hydroxymandelic acid
HBF	4-Hydroxybenzoylformate
Tyr	Tyrosine
Hpg	4-Hydroxyphenylglycine
Asn	Asparagine
Asp	Aspartate
Gln	Glutamine
Glu	Glutamate

AcO^-	Acetate
FA	Fatty Acid

Abbreviations of enzymes

HK	Hexokinase
PGI	Phosphoglucose isomerase
PFK	Phosphofructokinase
ALDO	Aldolase
TPI	Triosephosphate isomerase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
ENO	Enolase
PK	Pyruvate kinase
PC	Pyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PDC	Pyruvate dehydrogenase complex
CS	Citrate synthase
ACO	Aconitase
IDH	Isocitrate dehydrogenase
α-KGDH	α-Ketoglutarate dehydrogenase
SCS	Succinyl CoA synthetase - Succinate thiokinase
SDH	Succinate dehydrogenase
FH	Fumarase
MDH	Malate dehydrogenase
G6PDH	Glucose 6-phosphate dehydrogenase
PGLS	6-Phosphogluconolactonase
6PGD	6-Phosphogluconate dehydrogenase
PPE	Ribulose phosphate 3-epimerase
RPI	Ribose 5-phosphate isomerase
TKT	Transketolase
TAL	Transaldolase
TKTA	Transketolase I
Lumped Glycine	Biosynthesis of glycine
Lumped Leucine	Biosynthesis of leucine
Lumped Allo-Threonine	Biosynthesis of allo-threonine
Lumped Ornithine	Biosynthesis of ornithine
Lumped Chorismate	Biosynthesis of chorismate
Lumped Phenylalanine	Biosynthesis of phenylalanine
CM	Chorismate mutase
PrepDH	Prephenate dehydrogenase
HmaS	4-Hydroxymandelic acid synthase
HmaO	4-hydroxymandelic acid oxidase
HpgT	L-p-hydroxyphenylglycine transaminase
ALT	Alanine transaminase
GOGAT	Glutamate synthase
AST	Aspartate transaminase
ASNS	Asparagine synthetase

ALR	Alanine racemase
OrnR	Ornithine racemase
ThrR	Threonine racemase
HpgR	Hydroxyphenylglycine racemase

Symbols

k	Rate canstant
K - K_m	Michaelis Menten constant
K_{eq}	Equilibrium constant
V_{max}	Maximum reaction rate

No	Enzyme	Reaction	Rate Law	Kinetic Value	Ref
1	01 - HK (Hexokinase)	Glucose -> G6P	HMM	K_m=0,37 mmol/L, V_{max}=51 mmol/L.min	Hynne modeli
2	02 - PGI (Phosphoglucose isomerase)	G6P = F6P	HMM	K_m=0,80 mmol/L, V_{max}=496 mmol/L.min	Hynne modeli
3	03 - Lumped 1	G6P -> E4P	HMM	K_m=4,53 mmol/L, V_{max}=496 mmol/L.min	Estimation
4	04 - PFK (Phosphofructokinase)	F6P -> F16BP	HMM	K_m=0,15 mmol/L, V_{max}=45,4 mmol/L.min	Hynne modeli
5	05 - ALDO (Aldolase)	F16BP -> GAP + DHAP	HMM	K_m=0,30 mmol/L, V_{max}=2207 mmol/L.min	Hynne modeli
6	06 - TPI (Triosephosphate isomerase)	DHAP -> GAP	HMM	K_m=1,23 mmol/L, V_{max}=116,3 mmol/L.min	Hynne modeli
7	07 - Lumped 2	GAP -> 3PG	HMM	Km=0,60 mmol/L, Vmax=833 mmol/L.min	Estimation
8	08 - Lumped 3	3PG -> Glycine	HMM	K_m=5,90 mmol/L, V_{max}=343 mmol/L.min	Estimation
9	09 - Lumped 4	3PG -> PEP	HMM	K_m=0,20 mmol/L, V_{max}=343 mmol/L.min	Estimation
10	10 - PK (Pyruvate kinase)	PEP -> PYR	HMM	K_m=0,20 mmol/L, V_{max}=343 mmol/L.min	Hynne modeli
11	11 - Lumped 5	2 * PEP + E4P -> Prep	Multisubstrate MM for Stoichiometry	For PEP, K_m=0,74 mmol/L, For E4P, K_m=0,10 mmol/L, V_{max}=343 mmol/L.min	Estimation
12	12 - PDC (Pyruvate dehydrogenase complex)	PYR -> AcCoA	HMM	K_m=2,95 mmol/L, V_{max}=259 mmol/L.min	Estimation
13	13 - PC (Pyruvate carboxylase)	PYR -> OAA	HMM	K_m=2,60 mmol/L, V_{max}=259 mmol/L.min	Estimation
14	14 - Lumped 6	2 * PYR -> Leucine	Bi (irreversible)	For PYR, K_m=2,40 mmol/L, For PYR, K_m=2,40 mmol/L, V_{max}=259 mmol/L.min	
15	15 - ALT (Alanine transaminase)	PYR -> Alanine	HMM	K_m=42 mmol/L, V_{max}=259 mmol/L.min	Estimation
16	16 - Lumped 7	Prep -> Phenyalanine	HMM	K_m=0,80 mmol/L, V_{max}=15 mmol/L.min	Estimation
17	17 - Lumped 8	Prep -> Hpg	HMM	K_m=0,083 mmol/L, V_{max}=15 mmol/L.min	Estimation
18	18 - CS (Citrate synthase)	OAA + AcCoA -> Cit	Multisubstrate MM for Stoichiometry	For OAA, K_m=0,07 mmol/L, For AcCoA, K_m=0,03 mmol/L, V_{max}=91,2 mmol/L.min	E. coli model

19	19 - ACO (Aconitase)	Cit -> ICıt	HMM	K_m=1,70 mmol/L, V_{max}=91,2 mmol/L.min	E. coli model
20	20 - IDH (Isocitrate dehydrogenase)	ICıt -> a-KG	HMM	K_m=0,008 mmol/L, V_{max}=14,72 mmol/L.min	E. coli model
21	21 - a-KGDH (α-Ketoglutarate dehydrogenase)	a-KG -> SucCoA	HMM	K_m=0,10 mmol/L, V_{max}=35,84 mmol/L.min	E. coli model
22	22 - Lumped 9	a-KG -> Ornithine	HMM	K_m=1,40 mmol/L, V_{max}=35,84 mmol/L.min	Estimation
23	23 - SCS (Succinyl CoA synthetase)	SucCoA -> Suc	HMM	K_m=0,02 mmol/L, V_{max}=35 mmol/L.min	Estimation
24	24 - SDH (Succinate dehydrogenase)	Suc -> Fum	HMM	K_m=0,02 mmol/L, V_{max}=7,38 mmol/L.min	E. coli model
25	25 - FH (Fumarase)	Fum -> Mal	HMM	K_m=0,15 mmol/L, V_{max}=44,64 mmol/L.min	E. coli model
26	26 - MDH (Malate dehydrogenase)	Mal -> OAA	HMM	K_m=2,60 mmol/L, V_{max}=356,64 mmol/L.min	E. coli model
27	27 - AST (Aspartate transaminase)	OAA -> Aspartate	HMM	K_m=0,10 mmol/L, V_{max}=1,05 mmol/L.min	Estimation
28	28 - ASNS (Asparagine synthetase)	Aspartate -> Asparagine	HMM	K_m=0,40 mmol/L, V_{max}=5 mmol/L.min	Estimation
29	29 - Lumped 10	Aspartate -> Threonine	HMM	K_m=0,25 mmol/L, V_{max}=5 mmol/L.min	Estimation
30	30 - Ramoplanin	Glycine + Alanine + Phenyalanine + 2 * Ornithine + 3 * Threonine + 6 * Hpg + 2 * Asparagine + Leucine -> Ramoplanin	Mass action (irreversible)	K_m=0,10 L^16/(mmol^16.min)	Estimation

Hyne Model; Hynne, F., Dano, S., & Sorensen, P. G. (2001). Full-scale model of glycolysis in *Saccharomyces cerevisiae*. *Biophysical Chemistry, 94,*121–163.

E.coli Model; Singh, V. K., & Grosh, I. (2006). Kinetic modeling of tricarboxylic acid cycle and glyoxylate bypass in *Mycobacterium tuberculosis*, and its application to assessment of drug targets. *Theoretical Biology and Medical Modelling, 3,* 27.