DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PRODUCTION OF RAMOPLANIN ANTIBIOTIC BY SUBMERGED FERMENTATION

by
Deniz ERKAN

July, 2015
İZMİR

PRODUCTION OF RAMOPLANIN ANTIBIOTIC BY SUBMERGED FERMENTATION

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"PRODUCTION OF RAMOPLANIN ANTIBIOTIC BY SUBMERGED FERMENTATION"** completed by **DENİZ ERKAN** under supervision of **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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PRODUCTION OF RAMOPLANIN ANTIBIOTIC BY SUBMERGED FERMENTATION

ABSTRACT

Ramoplanin is a cell wall active lipoglycodepsipeptide antibiotic with high bactericidal activity against many clinically important multi-drug resistant bacteria. It has been fast tracked by US Food and Drug Administration, and is expected to be marketed in the coming years. However, its expensive cost due to the low production yield is the main obstacle for this antibiotic. Therefore optimization of fermentation conditions is required for high productivity. For this purpose, in this thesis, various industrial by-product nitrogen sources including soybean meal, meat-bone meal, poultry meal and fish meal, and different concentrations of trace metals including iron, potassium, magnesium, manganese and zinc were investigated for their influence on ramoplanin A2 production with respect to the incubation period. Ramoplanin A2 levels were determined by HPLC. The highest ramoplanin A2 level in nitrogen source containing media was determined as 406.805 mg/L in fish meal medium on seventh day, but the highest total ramoplanin A2 production was determined in poultry meal medium which is also lower priced. The highest ramoplanin A2 level in metal containing media was determined as 647.415 mg/L in 75 ppm zinc ion containing medium on seventh day. In addition, important parameters influencing antibiotic biosynthesis were also investigated. Total protein levels, total reducing sugar levels, protease, amylase and lipase activities were determined spectrofotometrically, whereas intracellular free amino acid levels were determined by HPLC. According to the results, it can be concluded that ramoplanin A2 production was generally stimulated with the increasing pH levels, limitation of carbon sources, and bioavailability of ramoplanin A2 precursors such as L-Leu, L-Thr, Gly L-Phe, D-Ser and D-Orn amino acids.

Keywords: Ramoplanin, *Actinoplanes* sp. ATCC 33076, industrial by-product nitrogen sources, trace metals.

RAMOPLANİN ANTİBİYOTİĞİNİN DERİN KÜLTÜR FERMENTASYONU İLE ÜRETİMİ

ÖZ

Ramoplanin hücre duvarına etki eden bir lipoglikodepsipeptit antibiyotik olup birçok klinik öneme sahip çoklu-ilaç direnci gösteren bakteriye karşı yüksek bakterisidal aktivite sergilemektedir. Amerikan Gıda ve İlaç Dairesi tarafından hızlandırılmış ilaçlar kategorisine alınmış olan ramoplaninin önümüzdeki yıllarda piyasaya sürülmesi beklenmektedir. Ancak, düşük üretim verimi nedeniyle maliyetinin yüksek olması büyük bir engel teşkil etmektedir. Bu nedenle bu antibiyotiğin yüksek verimlilikte üretimi için fermentasyon koşullarının optimize edilmesi gerekmektedir. Bu doğrultuda gerçekleştirilen tez kapsamında, soya küspesi, et-kemik unu, tavuk unu ve balık unu gibi çeşitli endüstriyel yan ürün azot kaynaklarının yanı sıra demir, potasyum, magnezyum, mangan ve çinko iz metallerinin farklı derişimlerinin inkübasyon süresine bağlı olarak ramoplanin A2 üretimine etkisi incelenmiştir. Ramoplanin A2 miktarları HPLC ile belirlenmiştir. En yüksek ramoplanin A2 üretimi, azot kaynakları içeren besi ortamlarından balık unu ortamında yedinci günde 406.805 mg/L olarak belirlenmiştir, ancak toplamda en yüksek ramoplanin A2 üretimi aynı zamanda daha düşük fiyatlı olan tavuk unu ortamında tespit edilmiştir. İz metal içeren besi ortamları arasında en yüksek ramoplanin A2 üretimi 75 ppm çinko iyonu içeren besi ortamında yedinci günde 647.415 mg/L olarak belirlenmiştir. Ayrıca, antibiyotik biyosentezini etkileyen önemli parametreler de incelenmiştir. Total protein seviyeleri, total indirgen şeker seviyeleri, proteaz, amilaz ve lipaz aktiviteleri spektrofotometrik olarak belirlenirken, hücre içi serbest amino asit seviyeleri ise HPLC ile belirlenmiştir. Sonuçlarımıza göre, ramoplanin A2 üretiminin genellikle artan pH seviyeleri, karbon kaynaklarının sınırlılığı ve ramoplanin öncülleri olan L-Leu, L-Thr, Gly L-Phe, D-Ser ve D-Orn amino asitlerinin biyobulunurluğu ile artış gösterdiği belirlenmiştir.

Anahtar kelimeler: Ramoplanin, *Actinoplanes* sp. ATCC 33076, endüstriyel yan ürün azot kaynakları, iz metaller.

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INTRODUCTION

1.1 Antibiotics

Antibiotics are complex chemical substances produced by a variety of bacteria and fungi as secondary metabolites, which are not essential for basic metabolic processes such as growth and reproduction. Antibiotics have the sole function of inhibiting or killing other microorganisms. Antibiotics which kill bacteria are termed as “bactericidal”, and which suppress bacterial growth are termed as “bacteriostatic”.

Antibiotic production is a self-preservation technique used by microorganisms, and is an ability that has been gained as a result of accumulation of mutations in their genetic materials. Antibiotics at very low concentrations act as growth stimulants (Demain, 1999), and provide an advantage to antibiotic producing microorganisms in competing for nutrients against the other microorganisms (Běhal, 2000).

Naturally produced antibiotics can be chemically modified to enhance their efficacy. Artificially modified antibiotics are termed **as “**semisynthetic antibiotics”.

Although the term antibiotic was initially used exclusively to describe those antibacterials of natural or semisynthetic origin, it has become extended in common usage to include antibacterial agents of purely synthetic origin as well (Dougherty & Pucci, 2012).

In addition, some compounds produced by plants or derived from plants have bacteriostatic or bactericidal activities. But antibiotic definition does not include plants and the compounds derived from them. This is because these materials are considered more as chemotherapeutic agents and thus are termed as “antimicrobials” (Awad et al., 2012).

The majority of antibiotics are either natural products of microorganisms or semisynthetic compounds derived from them. Therefore, microorganisms are still the major focus of the search for new antibiotics (Schaechter, 2009).

1.1.1 Attributes of an Ideal Antibiotic

Considering various characteristics of antibiotics and methods of determining microbial sensitivities to them, the characteristics of an ideal antibiotic can be listed as follows:

Antibiotics must dissolve in body fluids to be transported in the body, and must not bind too tightly to serum proteins in order to reach the infectious organisms.

Antibiotics must exhibit selective toxicity. They must display high toxicity to infectious microorganisms, without creating any injury to the host cell, or with only minimum toxicity to the host. Ideally, a great difference should exist between the low concentration that is toxic to microorganisms and the concentration that damages host cells.

Antibiotics should maintain a standard toxicity, and the toxicity should not change easily. Antibiotics should not be more or less toxic due to the interactions with foods, or other drugs, or any special conditions such as diabetes and kidney disease in the host.

Antibiotics should not elicit an allergic reaction in the host, and should not disturb the immune system such as cell defence action known as phagocytosis and the production of antibody.

Antibiotics should be sufficiently stable in body fluids, and should be degraded and excreted slowly to have therapeutic activity over many hours.

Microorganisms should not easily acquire resistance to a certain antibiotic.

Antibiotics should maintain their therapeutic properties over a long period of time with a minimum of special requirements such as refrigeration or shielding from light.

Antibiotics should be affordable to patients who need it (Najafpour, 2007; Black, 2012).

1.1.2 The Activity of Antibiotics

The activity of an antibiotic reflects its ability to inhibit microbial growth. Two different levels of growth can be distinguished: population growth and growth of a single cell. The activity of an antibiotic is normally defined as its ability to inhibit microbial population.

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).

1.1.3 The Spectrum of Antibiotic Activity

The spectrum of antibiotic activity shows the range of taxonomic group or groups of microorganisms that the antibiotic is against to.

Antibiotics, which are effective against a vast number of microorganisms from a wide range of taxonomic groups, including both Gram-positive and Gram-negative bacteria, are termed as “broad-spectrum antibiotics”. A broad-spectrum antibiotic, which increases the chance of susceptibility of the organism to it, is especially useful in case of serious infections caused by an unidentified organism.

Antibiotics, which are only effective against to a small number of microorganisms or a single taxonomic group, are termed as “narrow-spectrum antibiotics”. A narrow-spectrum antibiotic should be used in case of the infectious organism is identified.

Thus, besides the possibility of resistance development; the possibility of destruction of the host's microflora, which compete with and help to destroy infectious organisms, are minimized.

Antibiotics, which are artificially modified to be effective against additional types of bacteria (usually those that are Gram-negative), are termed as "extended-spectrum antibiotics".

The spectrum of activity of some major antibiotic classes and the range of taxonomic groups that they are effective against are shown in Figure 1.1 (Black, 2012).

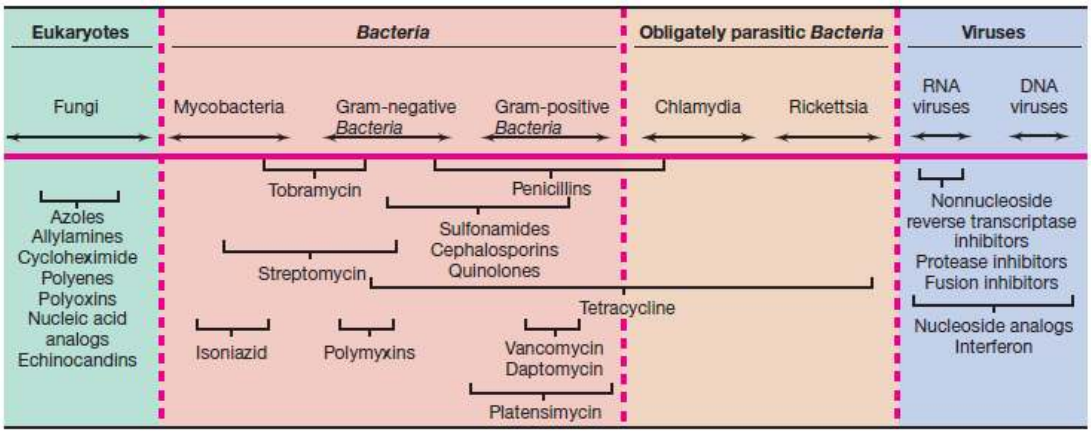

Figure 1.1 The spectrum of antibiotic activity

1.2 Classification of Antibiotics

Antibiotics can be classified according to their source (natural, synthetic or semisynthetic), bacterial spectrum (narrow, broad or extended), route of administration (oral, injectable or topical), type of activity (bactericidal or bacteriostatic), molecular structure or mechanism of action. Among these, the most useful classification is the one according to their mechanism of action, because certain antibiotics share a common mechanism of action, and similar patterns of effectiveness, toxicity, and allergic potential.

1.2.1 Mechanism of Action of Antibiotics

Antibiotics exert their effect according to one of the following methods:

- ✓ Inhibition of cell wall synthesis
- ✓ Inhibition of protein synthesis
- ✓ Inhibition of lipid synthesis
- ✓ Inhibition of nucleic acid metabolism
- ✓ Inhibition of folic acid metabolism
- ✓ Inhibition of cytoplasmic membrane structure

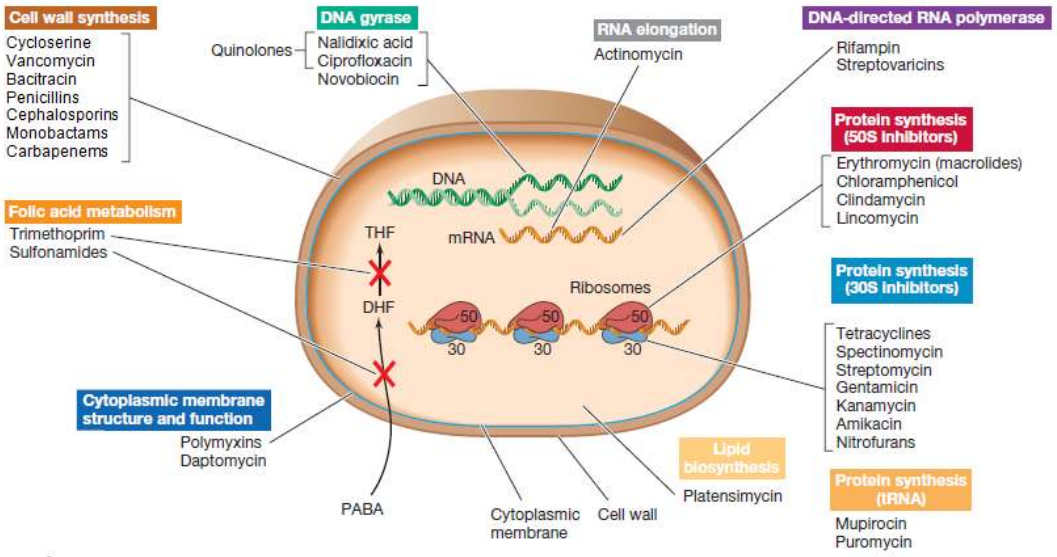

Figure 1.2 Mechanism of action of major antibiotic classes

In addition, major antibiotic classes according to their structures, subclasses, structural or biological basis and examples are given in Table 1.1 (Schaechter, 2009).

Table 1.1 Structural classes of antibiotics

Structural classes and subclasses	**Structural or biological basis**	**Examples**
β-Lactam	Azetidinone attached to pentacyclic or hexacyclic ring	Penicillin, cephalosporin
1. Penams	Saturated pentacyclic ring	Ampicillin, amoxicillin
2. Penems	Unsaturated pentacyclic ring	Fropenem
3. Cephems	Unsaturated hexacyclic ring	Cefaclor, cefixime
4. Carbapenems	Sulfur in pentacyclic ring replaced with carbon	Imipenem, meropenem
5. Monolactams	Azetidinone nucleus alone	Aztreonam, carumonam

Table 1.1 Structural classes of antibiotics (continued)

Aminoglycosides	Amino sugars	
1. Group I	Di- or tri-saccharides	αα-Trehalosamine
2. Group II	Streptomycin and derivatives	Streptomycin, bluensomycin
3. Group III-(A, B, C)	Structure of amino sugar	Apramycin, hygromycin, garamine
4. Group IV-(A, B)	4,5-di-2-deoxystreptamine	Ribostamycin, neomycin
5. Group V-(A, B, C, D)	4,6-di-2-deoxystreptamine	Kanamycin, gentamicin
6. Group VI-(A, B, C)	Presence or absence of amino sugar	Kasugamycin, spectinomycin
Macrolides	Macrocyclic antibiotics	
1. 14-Membered lactone ring	As the name indicates	Erythromycin
2. 16-Membered lactone ring	As the name indicates	Spiramycin
3. Aglycone alterations	As the name indicates	Azithromycin
Fluoroquinolone	4-Quinolones with or without fluorine atom at position 6	
1. Group I, II	Limited spectrum	Nalidixic acid, cinoxacin
2. Groups III, IV	Broad spectrum	Norfloxacin, ciprofloxacin, ofloxacin
3. Groups V, VI	Extended spectrum	Temafloxacin, levofloxacin
Peptide antibiotics	Peptides	
1. Glycopeptides	Contain sugar	Vancomycin
2. Lipoglycopeptides	Contain sugar and lipids or phospolipids	Ramoplanin, teicoplanin
3. Lipopeptides	Contain lipid chain	Polymyxins, daptomycin
Ansamycins	Aromatic ring with aliphatic side chain	Rifamycin
1. Group I	Naphthalene-type	No antibiotic known
2. Group II	Benzene-type	
Tetracyclines	Polycyclic structures of perhydronaphthacene carboxamides	Tetracycline, chlortetracycline, oxytetracycline
Different classes	Substitution in perhydronaphthacene ring	
Lincosamides	Proline substituted with 49-alkyl chain and a thiooctopyranoside; the whole unit is linked by amide bond	Lincomycin, clindamycin
Chloramphenicol (microbial and synthetic)	p-Nitrophenol connected to propanediol connected to dicholro acetamide	Chloramphenicol
Different classes	Modification of three components	
Benzylpyrimidines	Inhibitors of dihydrofolate reductase (DHFR)	
1. Class 1	Bycyclic derivatives-Anticancer drugs	Methotrexate
2. Class 2	Triazinopyrimiddines-Antiparasitic drugs	Pyrimethamine
3. Class 3	Benzylpyrimidines-Antibactierial drugs	Trimethoprim

Table 1.1 Structural classes of antibiotics (continued)

Sulfonamides	p-Aminobenzenesulfonamide	Sulfathiazole
Oxazolidinone		Zyvox (linezolide)

1.2.1.1 Inhibition of Cell Wall Synthesis

Many bacterial, fungal and plant cells have rigid external cell walls, whereas animal cells lack of them (Black, 2012). The cell wall is a structure that mechanically prevents the cell from swelling too much and undergoing osmotic lysis (Vogel & Todaro, 1996). The bacterial cell wall has a unique structure which consists mainly of a peptidoglycan layer located at the outside of the cytosolic membrane (de Kruijff et al., 2008). Bacterial cell walls are different from the cell walls of plants and fungi which are made of cellulose and chitin, respectively (Koch, 2003). Therefore, selective inhibition of cell wall synthesis is a target in the fight against bacteria. Cell wall active antibiotics exert their effect by attaching to the enzymes involved in the peptidoglycan biosynthesis. Consequently, fungi and archaea, whose cell walls lack peptidoglycan, are unaffected by these antibiotics (Black, 2012).

Cell wall active antibiotics are often considered to be bactericidal agents, because their action is so disruptive to the cell that viability is curtailed (Bush, 2012).

1.2.1.2 Peptidoglycan Biosynthesis

Biosynthesis of peptidoglycan, the major constituent of the bacterial cell wall, takes place in three stages that occurs at three different locations in the cell. The first stage begins in the cytoplasm, and the nucleotide sugar-linked precursors UDP-N-acetylmuramyl (UDP-MurNAc)-pentapeptide and UDP-N-acetylglucosamine (UDP-GlcNAc) are synthesized. The second stage takes place at the cytoplasmic membrane, and in this stage the UDP-MurNAc-pentapeptide precursor is linked to the transport lipid (undecaprenyl pyrophosphate), resulting in the formation of lipid I. The subsequent addition of GlcNAc from UDP-GlcNAc produces lipid II.

A peptide crossbridge is added at the third amino acid in species in which peptidoglycan is not directly cross-linked. Lipid II is then flipped to the external side of the cell membrane (most probably by FtsW proteins), where it is incorporated into nascent peptidoglycan by penicillin-binding proteins (PBPs). During the third stage, PBPs catalyse transglycosylation and transpeptidation reactions, resulting in the respective polymerization and cross-linking of the glycan strands via flexible peptides. PBPs can be classified according to their molecular masses and functional domains. High-molecular-mass (HMM) PBPs are divided into two subclasses according to their functional domains, which are class A and class B. Class A PBPs are bifunctional, having both transglycosylase and transpeptidase activities, whereas class B PBPs have only transpeptidase activity. Low-molecular-mass (HMM) PBPs have a penicillin-binding domain and are usually D,D-peptidases, although some, such as *S. aureus* PBP4, have transpeptidase activity (Pinho et al., 2013).

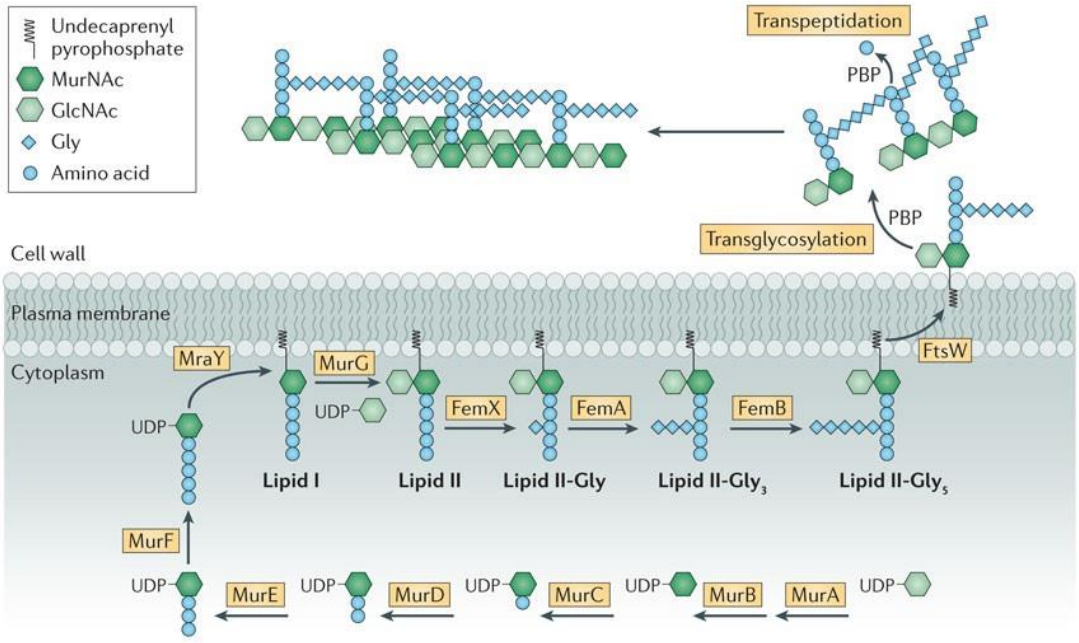

Figure 1.3 Peptidoglycan biosynthesis

1.2.1.3 Antibiotics That Target Cell Wall

Since all enzymes involved in peptidoglycan synthesis are known and various mechanistic aspects of the pathway are understood, this pathway forms a prime target

for antibiotics (de Kruijff et al., 2008). Antibiotics that target cell wall include β-lactams, peptides, glycopeptides, lipoglycopeptides, phosphonic acids and ionophores (Bush, 2012). The steps in the bacterial cell wall synthesis that inhibited by some medically important cell wall active antibiotics are shown in Figure 1.4 (de Kruijff et al., 2008).

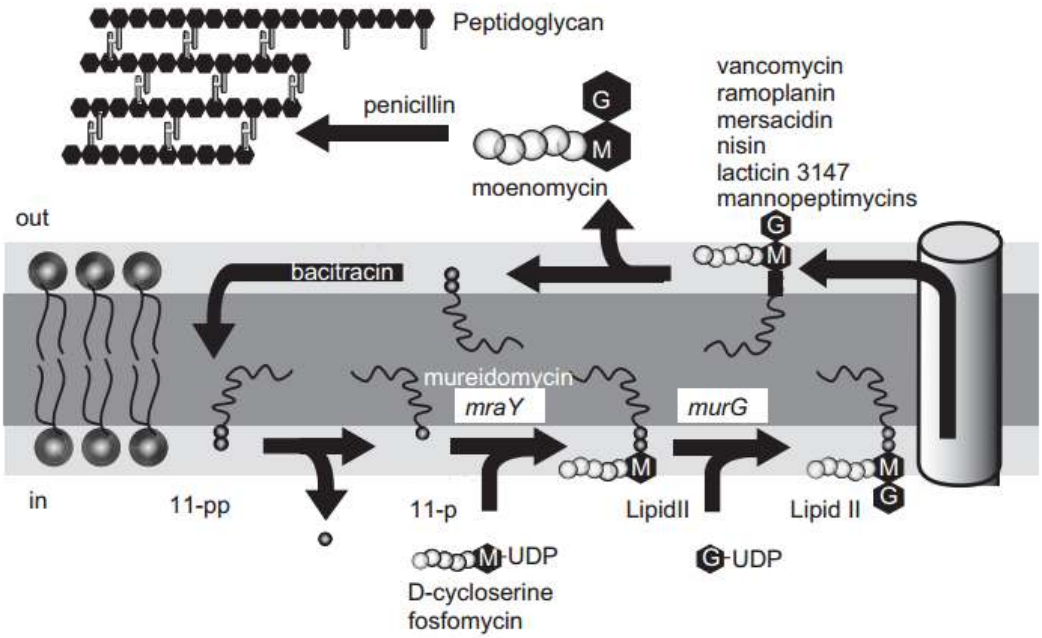

Figure 1.4 Cell wall active antibiotics and their target steps in the bacterial cell wall synthesis

β-lactam antibiotics include a β-lactam ring in their core structures. This group of antibiotics target specific PBPs involved in the bacterial cell wall biosynthesis and inhibit construction of the peptidoglycan chains. This, in turn, activates autolysins that degrade the cell wall, resulting in bacterial cell death (Murray et al., 2013).

β-lactam antibiotics include medically important penicillins, cephalosporins, cephamycins, carbapenems and monobactams. These antibiotics are usually administered with β-lactamase inhibitors because of the resistance against β-lactam antibiotics occurred by β-lactamase production in bacteria.

Peptide antibiotics have large molecular weight which contributes to a low bioavailability when these compounds are administered orally (Piscitelli et al., 2011).

Glycopeptide antibiotics, which impair cell wall synthesis in Gram-positive bacteria, consist of glycosylated cyclic or polycyclic nonribosomal peptides. The antimicrobial activity of glycopeptides is restricted to Gram-positive bacteria, due to the fact that they are extremely large molecules relative to other antibiotics, and not able to pass through porins in the outer membranes of Gram-negative bacteria because of their size (Hauser, 2007). Glycopeptides bind to the C-terminal D-alanyl-D-alanine terminus of the peptide side chain of precursor peptidoglycan subunits, and prevent the transglycosylation and transpeptidation reactions that are necessary for completion of the peptidoglycan chain, resulting in an incomplete cell wall and subsequent cell death (Bush, 2012).

Vancomycin, originally obtained from *Streptomyces orientalis*, is the first glycopeptide that introduced into clinical practice to treat infections caused by Gram-positive enterococci. It is active against nearly all streptococci and staphylococci, including methicillin-resistant staphylococci and strains of penicillin-resistant *S. pneumonia* (Hauser, 2007). Although, vancomycin resistance is widespread today, susceptibility among enterococci is variable.

Glycopeptides also have good activity against anaerobic Gram-positive bacteria such as *Clostridium difficile* (Murray et al., 2013).

Lipoglycopeptides are relatively a new group of antibiotics compared to glycopeptides, which have lipophilic side-chains linked to glycopeptides. This group of antibiotics include natural antibiotics such as ramoplanin, teicoplanin and parvocidin, as well as semisynthetic antibiotics such as parvocidin derivative dalbavancin, and vancomycin derivatives oritavancin and telavancin.

Ramoplanin will be discussed in Section 1.3.

Teicoplanin is widely used for the treatment of MRSA infections. It has a greater potencial than vancomycin against streptococci and enterococci, and has activity against some vancomycin-resistant enterococci.

Parvocidin exhibits lower activity against coagulase-negative staphylococci, but has three to four times longer half-life in mice, when compared with teicoplanin (Dougherty & Pucci, 2012).

Semisynthetic lipoglycopeptides were developed to maintain the activity against resistant strains such as MRSA and VRE, based on the idea of hydrophobic groups have a role in overcoming resistance mechanisms (Dougherty & Pucci, 2012).

Dalbavancin exhibits in vitro activity against a variety of Gram-positive pathogens including MRSA, VRSA and methicillin-resistant *Staphylococcus epidermidis* (MRSE) (Zhanel et al., 2010). Dalbavancin inhibits bacterial cell wall synthesis by formation of a complex with the C-terminal D-alanyl-D-alanine. It also dimerise and anchor its lipophilic side chain in the bacterial membranes (Chen et al., 2007).

Oritavancin has broad-spectrum activity against Gram-positive bacteria including both resistant and susceptible strains such as *S. aureus*, MRSA, enterococci, and streptococci. 4'-chlorobiphenylmethyl group of the molecule interfere with the cell membrane of the growing Gram-positive bacteria (Dougherty & Pucci, 2012). Oritavancin also disrupts membrane integrity and increase membrane permeability, and inhibits RNA synthesis (Zhanel et al., 2010).

Telavancin primarily exhibits activity against Gram-positive aerobic bacteria such as staphylococci, streptococci, and enterococci with lower MIC values than vancomycin or teicoplanin, and has limited activity against Gram-positive anaerobic bacteria, but has no activity against Gram-negative bacteria. Telavancin inhibits bacterial cell wall synthesis by binding to the N-acyl-D-Ala-D-Ala terminus of the peptidoglycan subunits. It also provides a second mechanism that leads to cell death by binding to lipid II that leads to membrane depolarisation and membrane disruption (Lunde et al., 2009).

Teixobactin, discovered in a screen of uncultured soil bacteria, is the first member of a new class of antibiotics, macrocyclic depsipeptide antibiotics. It inhibits cell wall synthesis by binding to peptidoglycan precursor lipid II and cell wall teichoic acid precursor lipid III. Teixobactin has activity against pathogenic Gram-positive bacteria that have developed resistance to available approved antibiotics including MRSA and VRE, and difficult-to-treat enterococci and *M. tuberculosis*, with *Clostridium difficile* and *Bacillus anthracis*. No resistance against teixobactin has been observed in vitro studies of treatment of *Staphylococcus aureus* or *Mycobacterium tuberculosis* (Ling et al., 2015).

1.2.1.4 Resistance Against Cell Wall Active Antibiotics

Antibiotic resistance come out in bacteria with a variety of mechanisms, which are decreased penetration, increased efflux, target modification, enzymatic inactivation or modification, bypass pathways, and overproduction of target (Lewis, 2013). Since antibiotics have been used widely for the treatment of bacterial infections, many pathogenic bacteria have developed resistance against antibiotics through these mechanisms.

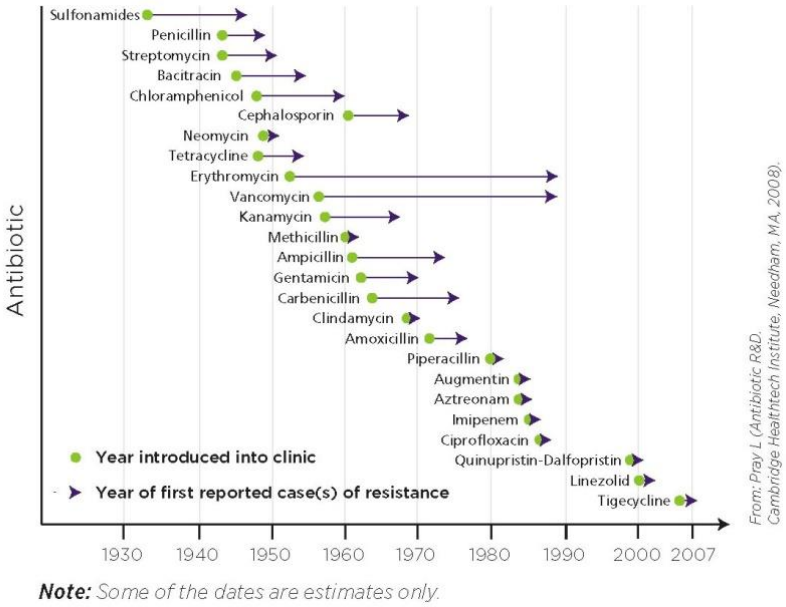

Figure 1.5 Years of antibiotics introduced into clinical practice, and years of first reported case of resistance

In Figure 1.5, years of some antibiotics began to be implemented in clinical practice, and years of first reported cases of resistance are shown. Currently, almost all pathogenic bacteria have been developed resistance to many clinically important antibiotics due to widespread use of antibiotics since 1950s. Penicillin and sulfa drugs, the first widely used antimicrobial agents, are not used as extensively today because many pathogens have acquired some resistance (Madigan et al., 2011). In recent years, antibiotic resistance especially takes place faster even against to the newly introduced antibiotics into clinical practice, and causes increasing cross-resistance.

In case of cell wall active antibiotic resistance, the first confronted resistance case is the one against to penicillin, which is one of the first widely used a β-lactam antibiotics. Two major resistance mechanisms against β-lactams are existed.

In Gram-positive bacteria, such as *Staphylococcus aureus*, resistance against β-lactams are either due to the presence of the PBP2a-encoding mecA gene that causes a PBP with low affinity for common β-lactams, or the production of penicillinase activity. Today, both mechanisms are existed in MRSA, but penicillinase production is remained unnecessary in the presence of the mecA gene.

Low affinity PBPs also have been demonstrated in the pneumococci and enterococci, too. Penicillin-resistant *Streptococcus pneumoniae* (PRSP) isolates display more restrictive β-lactam resistance than in *S. aureus* with a broad range of minimum inhibitory concentration (MIC) values for different β-lactams, which indicate that some PRSP isolates may be susceptible to certain cephalosporins on the contrary of MRSA. In addition, no β-lactamases have been reported in the streptococci, whereas enterococci were reported to produce a penicillinase, but which is now rarely seen in surveillance studies.

In Gram-negative bacteria, resistance occurs due to the production of β-lactamases which are the enzymes that produced in the presence of high levels of antibiotics. More than 1,000 unique β-lactamases with varying molecular and

functional properties, on the basis of their substrate preference and inhibitor profile, have been identified. In addition, efflux and modification or deletion of porin function can play a role in resistance to specific β-lactams (Bush, 2012).

Another important cell wall active antibiotic group is the glycopeptide antibiotics. Resistance against glycopeptide antibiotics is an emerging concern in clinic treatment of bacterial infections. Glycopeptide resistance is associated with structural modifications in the substrates for the enzymes that incorporate the final amino acids in the pentapeptide precursors. Modification is carried out by alterations in the structure of the D-alanyl-D-alanine dipeptide those resulted in either D-Ala-D-Lac or D-Ala-D-Ser depsipeptides (Bush, 2012). As the structure of the substrate is altered, glycopeptides are no longer able to recognize and bind to these precursors.

The transposon-related resistance against glycopeptides in enterococci was first reported in the late 1980s (Arthur & Courvalin, 1993). Glycopeptide resistance was initially characterised by phenotypes. Accordingly, VanA phenotype exhibits high-level of glycopeptide resistance, whereas vancomycin-resistant isolates with the VanB phenotypes are susceptible to teicoplanin and telavancin. Teicoplanin also has activity against VanC, VanE and VanG phenotypes. Full resistance to vancomycin in the staphylococci has been reported exceedingly rarely. It is microscopically identified that these strains display a thickened cell wall (Bush, 2012). In addition, glycopeptide resistance mechanism is found in some *S. aureus* strains, because the gene clusters that encode this activity in enterococci are transferable (Hauser, 2007).

The emerging antibiotic resistance indicate that the search for new antibiotics must continue in order to combat evolving pathogens, naturally resistant bacteria and fungi, and previously susceptible microbes that have developed resistance (Demain, 2007).

Ramoplanin is one of the most potent antibiotics that has attracted significant attention in recent years, due to its good in vivo and in vitro activity against Gram-

positive aerobic and anaerobic bacteria with a different mechanism of action than other peptide antibiotics.

1.3 Ramoplanin

Ramoplanin, also known as A-16686, is a novel lipoglycodepsipeptide antibiotic produced by strain *Actinoplanes* sp. ATCC 33076. Ramoplanin was discovered in an industrial drug discovery program organized in 1984 by Biosearch Italia, which was organized for identifying cell wall synthesis inhibitor novel antibiotics (McCafferty et al., 2002).

1.3.1 Chemical Structures of Ramoplanin Family of Antibiotics

Ramoplanin is a complex of three antibiotics, which consists of factors A1, A2 and A3. The yields of these factors were determined as 12, 72, and 16%, respectively (Cavalleri et al., 1984). Among these structures, besides being the most abundant one, factor A2 also exhibits the highest antimicrobial activity (Brunati et al., 2005). Therefore, factor A2 is the most being investigated for use as an antimicrobial agent, and is usually defined as ramoplanin (Farver et al., 2005).

The structural complexity of ramoplanin family antibiotics were enlightened by chemical degradation, MS and 2D NMR experiments (Ciabatti et al., 1989; Bionda et al., 2013).

As shown in Figure 1.6, factors A1, A2, and A3 all contain the same depsipeptide core carrying a dimannosyl group, but differ in the length of the Asn^1 N-terminal acyl chains, derived from C_8, C_9, and C_{10} fatty acids, respectively. All ramoplanin factors have 17 amino acid residues, including both D and L configurations and glycine. Among them, there are several nonproteinogenic amino acids, including ornithine (Orn), hydroxyphenylglycine (Hpg), chlorohydroxyphenylglycine (Chp), and β-hydroxy-asparagine (β-OH-Asn), because ramoplanin is a nonribosomal peptide synthesis product antibiotic just as many other peptide antibiotics produced

by bacteria and fungi. Hpg[11] is attached to the α-l,2-dimannosyl group. 10 of the remaining amino acids in the macrocyclic backbone, L-β-OH-Asn[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], are β-branched. The N- and C-termini of ramoplanin are linked by a lactone bond between the 17[th] residue 3-chloro-4-hydroxyphenylglycine (Chp[17]) and the β-hydroxyl of Asn[2], forming a 49-membered macrocyclic backbone which is essential for activity (McCafferty et al., 2002).

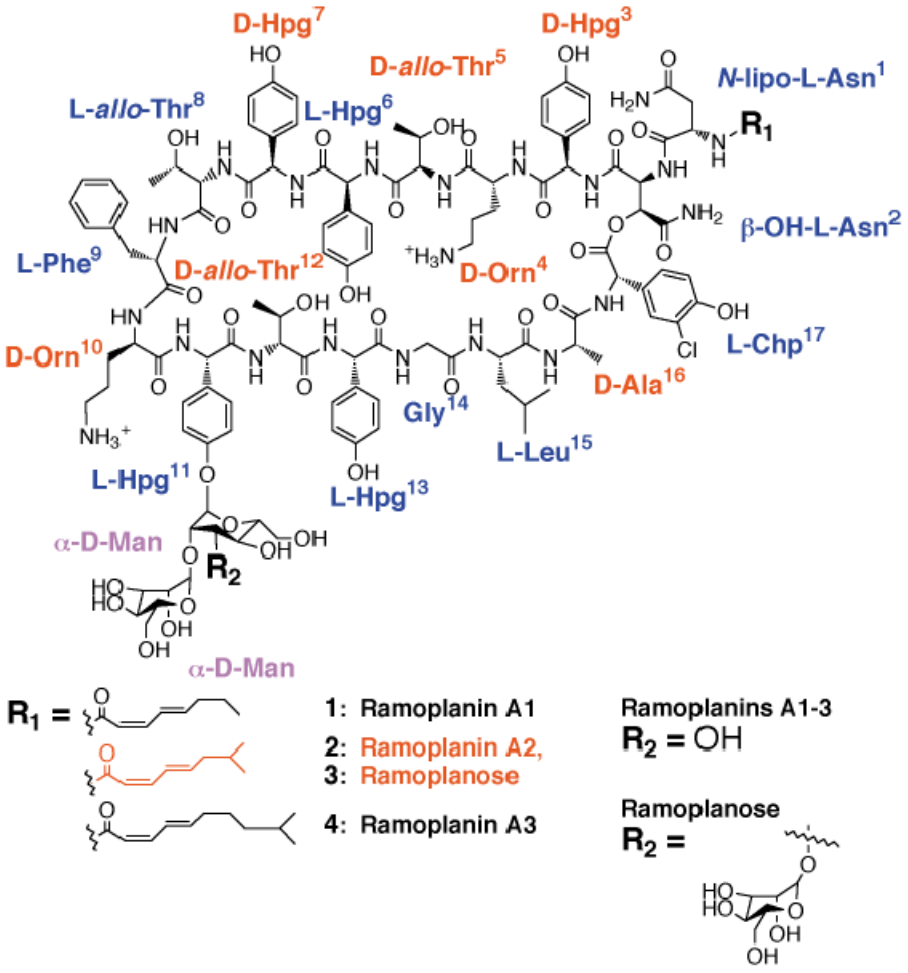

Figure 1.6 Chemical structures of members of ramoplanin family of antibiotics

Besides factors A1-A3, three more ramoplanin factors (A′1, A′2 and A′3) were also determined in small amounts in the fermentation broth of strain *Actinoplanes* sp. ATCC 33076 by Gastaldo et al. (1992). Factors A′1, A′2 and A′3 differ from the

factors A1-A3 by lacking one mannose unit from the glycosidic group (McCafferty et al., 2002).

In addition, there are some other structurally and functionally related antibiotics to ramoplanin, those are not produced by strain *Actinoplanes* sp. ATCC 33076. These antibiotics are ramoplanose and enduracidins (Figure 1.7).

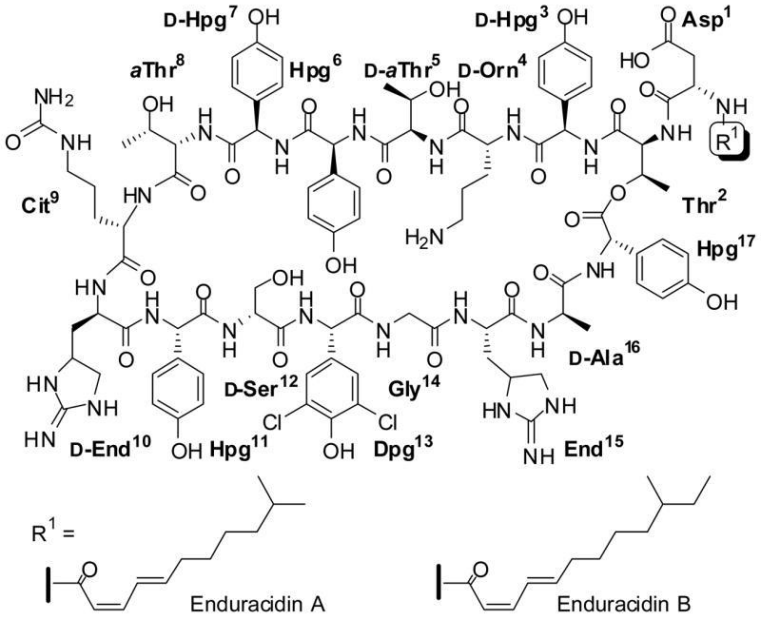

Figure 1.7 Chemical structures of ramoplanin family related antibiotic enduracidins

Lipodepsipeptide antibiotics enduracidin A and B are produced by *Streptomyces fungicidicus* B5477 (Hamburger et al., 2009), and both share a similar peptide core of 17 amino acids with chlorinated Hpg residues and differ mainly in the length of the acyl chain, and the presence of two D-mannose moieties attached to the Hpg^{11} by a hemiacetal bond in the ramoplanin molecule (Castiglione et al., 2005). In addition, the enduracidins differ from ramoplanin at three residues along the peptide sequence. The amino acids enduracididine (End^{10}), citrulline (Cit^{9}) and enduracididine (End^{15}) are found in positions that correspond to ornithine (Orn^{10}), phenylalanine (Phe^{9}) and leucine (Leu^{15}) (Castiglione et al., 2005).

1.3.2 Biosynthesis of Ramoplanin

The biosynthetic gene cluster of ramoplanin producing strain *Actinoplanes* sp. ATCC 33076 was sequenced by Farnet et al. (2002). The ramoplanin biosynthetic gene locus contains 33 genes responsible for a myriad of functions including amino acid (Orfs 4, 6, 7, 28, 30), fatty acid (Orfs 9, 16, 24, 25, 26, 27) and peptide biosynthesis (Orfs 11, 12, 13, 14, 15, 17), polypeptide tailoring (Orfs 10, 20), antibiotic export/producer resistance (Orfs 2, 8, 23, 31), and transcriptional regulation (Orfs 5, 21, 22, 33) (McCafferty et al., 2002).

Orfs 4, 6, 7, 28 and 30 are involved in Hpg biosynthetic pathway. Orfs 4, 6, 7, and 30 are resembling the Hpg biosynthetic machinery of chloroeremomycin and complestatin, whereas Orf 28, which exhibits high sequence similarity to chorismate mutase, appears to be involved in Hpg biosynthesis as an additional step. In addition, ramoplanin contains both D and L-Hpg residues. Because, there are no epimerization domains located in NRPS Orfs 13 and 14, either the enzymes of the Hpg biosynthetic pathway or the adenylation domains are D/L-enantioselective or intracellular epimerases assist in production of the alternate stereoisomer (McCafferty et al., 2002).

Ramoplanin's acyl chain is likely produced from the C_7 precursor. In analogy to fatty acid biosynthesis, Orfs 24 and 25 are putative FAD-dependent dehydrogenases (McCafferty et al., 2002), and Orf 16 is a NAD-dependent reductase with high similarity to NADH-dependent 3-oxoacyl acyl carrier protein reductases, which are involved in the degradation of fatty acids in primary metabolism (Marahiel et al. 1997; Hoertz et al., 2012). These enzymes likely work together to generate the unique cis-trans double bond regiochemistry found in the N-acyl chain. Although, there is no ketosynthase in the biosynthetic gene cluster, this activity is predicted to be borrowed from primary metabolism (McCafferty et al., 2002). The mature fatty acid is transferred via Orf 11, which is an acyl carrier protein (ACP) that shuttles activated fatty acid thioesters to Orf 12 for condensation with Asn^1 at the starter unit (Hoertz et al., 2012). In addition, Orf 27 is a putative ACP which is previously

thought to have the same function with Orf 11. In addition, Orfs 9 and 15, which are type II thioesterases, and Orf 26, which is an acyl-CoA ligase, are also found within the biosynthetic locus. Assembly of the acyl chain could involve the contribution of one or all of these gene products (McCafferty et al., 2002).

Orfs 12, 13, 14 and 17 within the ramoplanin biosynthetic gene cluster are the four putative NRPS peptide synthetases. NRPS have condensation, adenylation, and thiolation function domains for each amino acid in the peptide sequence, which are highly organized domains to recognize and incorporate the right amino acid into the peptide sequence. But there are 16 individual A domains that code for 16 of the 17 amino acids of ramoplanin, because Orf 12 is believed to be responsible for catalysing the incorporation of both Asn^{1} and β-OH-Asn^{2} into the peptide chain since this domain contains the sole A domain predicted to have specificity for asparagine (Hoertz et al., 2012). Orf 17 is responsible for the addition of the eighth amino acid in ramoplanin. L-allo-Thr or L-Thr are two candidate substrates for Orf 17. Hoertz et al. (2012) showed that Orf 17 preferentially activates L-Thr, although L-allo-Thr appears in the natural product. Therefore, it is concluded that the reason of L-allo-Thr incorporation in ramoplanin molecule might be attributed to the availability of the amino acid pool within the producer strain *Actinoplanes* sp. ATCC 33076.

Ramoplanin contains three covalently modified amino acids which are β-hydroxylated Asn^{2}, glycosylated Hpg^{11}, and chlorinated Hpg^{17}. However, only two ORFs that responsible for these modifications are involved in ramoplanin biosynthetic gene locus, which are Orf 10 and Orf 20. Orf 10 is thought to be a non-heme Fe-hydroxylase and responsible for β-hydroxylation of Asn^{2}. Orf 20, which exhibits high homology to known halogenases involved in the chlorination of secondary metabolites, thought to be responsible for chlorination of Chp^{17}. Moreover, ramoplanin is a potential substrate for cellular glycosyl transferases. But no glycosyl transferases were identified ramoplanin gene locus, and such a relationship has not yet been demonstrated (McCafferty et al., 2002).

In addition, functions of Orfs 1, 3, 18, 19, 29, and 32 are unknown due to their little homology to known proteins, but some of these proteins are predicted to contain transmembrane domains that might provide their involvement in transport, transcriptional regulation, or immunity (McCafferty et al., 2002).

1.3.3 Mechanism of Action of Ramoplanin

Ramoplanin inhibits bacterial cell wall biosynthesis by binding to the intermediates along the peptidoglycan formation, thus mechanically weakens cell wall and causes bacterial death by osmotic lysis. In this mechanism, ramoplanin is thought to be responsible for inhibition of two sequential enzymatic steps, which are the conversion of lipid I to lipid II by MurG, and transglycosylation of lipid II. Although mechanism of action of ramoplanin has been long studied, it was not clear which one of these two sequential steps ramoplanin preferentially targets. The early studies focused on investigation of permeabilized bacterial cells and membrane preparations by following the incorporation of radiolabel from a precursor into various intermediates along the pathway to peptidoglycan (Somner & Reynolds, 1990). These assays have a limitation such that if one enzymatic step is blocked, then no information can be obtained about subsequent steps (Lo et al., 2000). As a matter of fact, these studies concluded that ramoplanin inhibits peptidoglycan synthesis at the MurG step by binding to lipid I, which takes place inside the bacterial cell along, and preventing formation of lipid II from lipid I (Farver et al., 2005). But more recent researches indicate that ramoplanin primarily binds directly to the transglycosylation substrate lipid II on the external surface of the bacterial membrane, which is easier to reach than reaching to lipid I (Lo et al., 2000; Helm et al., 2002; Hu et al., 2003; Fang et al., 2006). Lo et al. (2000) showed by using synthetic analogue of lipid II that ramoplanin inhibits the polymerization of lipid II, and proposed that transglycosylation step is the primary target and binding occurs with a stoichiometry of 1:1 (ramoplanin:lipid II analogue). They also showed that ramoplanin undergoes a ligand-induced polymerization process in the presence of the synthetic analogue of lipid II. Ligand-induced polymerization causes a conformational change in molecular structure of ramoplanin, thereby ramoplanin

binds to proteins involved in transglycosylation. Helm et al. (2002) indicated that the inhibition of MurG by ramoplanin is too weak to explain the biological activity; furthermore, inhibition does not require binding to lipid I. Hu et al. (2003) showed that the stoichiometry of inhibition of bacterial transglycosylases by ramoplanin is 2:1 (ramoplanin:lipid II), and inhibition concentrations are consistent with reported MICs. Thus, it is proposed that the reason of bacterial cell death seems like transglycosylase inhibition. By using inhibition kinetics and binding assays, Fang et al. (2006) showed that ramoplanin and its related compound enduracidin preferentially inhibit the transglycosylation step of peptidoglycan biosynthesis compared with the MurG step. Hamburger at al. (2009) showed that ramoplanin forms an intimate and highly amphipathic dimer which has an impact on recognizing lipid II. Architecture of the ramoplanin dimer is shown in Figure 1.8.

Moreover, semisynthetic modification, total chemical synthesis, and functional studies indicate that the residues Hpg^{3} through Orn^{10} also play critical roles in lipid II recognition, and thereby in antimicrobial activity.

It is suggested that residues Hpg^{3} through Orn^{10} interact with the MurNAc carbohydrate and pyrophosphate moieties of the ligand. These residues may be capable of interacting with peptidoglycan precursors in both the monomeric and dimeric form (Hamburger et al., 2009).

Alanine scanning of ramoplanin revealed that mutations of these residues to alanine cause significant decreases in MIC values of ramoplanin. Mutations of Orn^{10}, Hpg^{3}, Hpg^{7}, Orn^{4}, Leu^{15} and Phe^{9} to alanine caused 540-fold, 74-fold, 53-fold, 44-fold, 40-fold and 8.6-fold decreases in MIC values of ramoplanin, respectively (Nam et al., 2007). These results indicate that Orn^{10}, which is situated at the interface between the hydrophilic and hydrophobic regions of the dimer structure, is perfectly positioned to capture the pyrophosphate of lipid II, since insertion of lipid II's undecaprenyl chain into the membrane outer leaflet will place the pyrophosphate moiety at the membrane interface. Also, Orn^{10} is expected to be responsible for the positive charge required for recognition of lipid II.

N-acyl chain of ramoplanin, which is essential for antibiotic activity, facilitates the interactions with the membrane by anchoring. That interaction between the membrane environment and ramoplanin does not cause a conformational change in molecular structure due to the fact that the conformation of ramoplanin is dictated principally by its sequence. Ramoplanin does not anchor to lipid II directly, but efficiently anchors to unilamellar vesicles composed of physiologically relevant diacyl phosphatidylethanolamine and phosphatidylglycerol phospholipids. Nonetheless, the membrane is thought to stabilize highly amphipathic ramoplanin dimer and position the dimer optimally for recognition of the lipid II target (Hamburger et al., 2009).

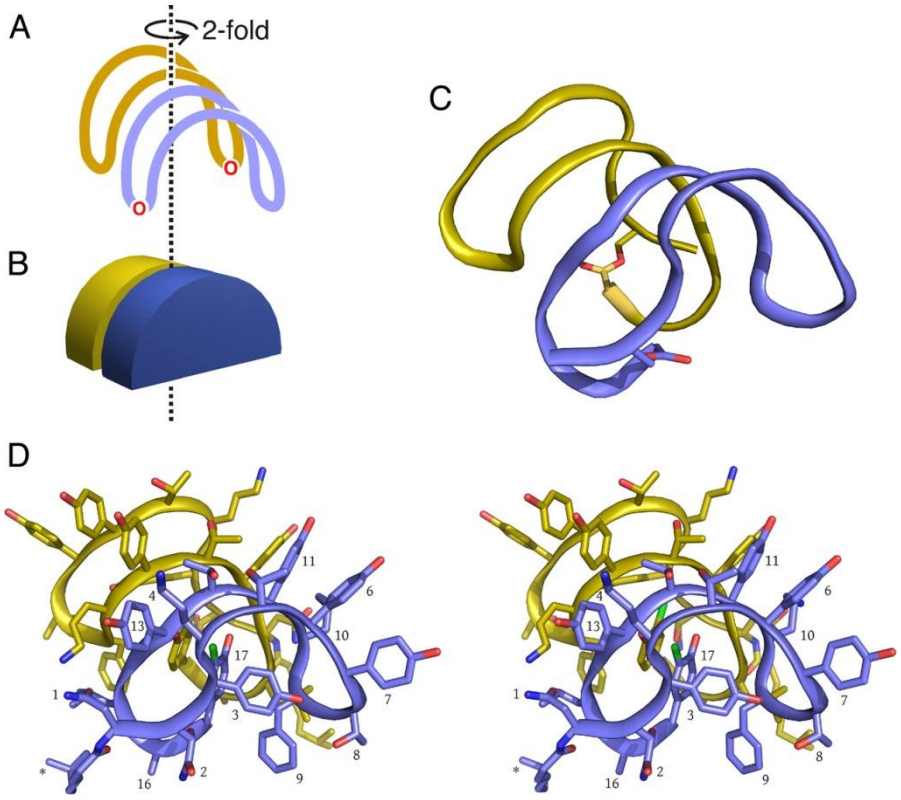

Figure 1.8 Architecture of the ramoplanin dimer. A schematic view of the dimer (A). The U-shaped character of each monomer and the hemidisk-like shape of the monomers (B). The actual structure of the dimer, showing the peptide backbone only (C). A stereoview of the complete dimer (D).

In conclusion, ramoplanin recognizes and binds to a peptidoglycan-binding site with a distinct molecular underpinning than the other glycopeptide antibiotics vancomycin and teicoplanin, and lipopeptide antibiotic daptomycin, which target the

D-alanyl-D-alanine residues terminus of the peptidoglycan subunits (Reynolds & Somner, 1990; Somner & Reynolds, 1990; Hamburger et al., 2009), and β-lactam antibiotics which target even further down the chain of enzymatic reactions involved in peptidoglycan biosynthesis (Farver et al., 2005).

1.3.4 Antimicrobial Activity of Ramoplanin and Its Clinical Use

The lipoglycodepsipolypeptide antibiotic ramoplanin, with its unique chemical structure and mechanism of action, is a promising candidate that has attracted significant attention in recent years to overcome multi-drug resistant bacterial infections. Ramoplanin exerts broad-spectrum *in vivo* and *in vitro* bactericidal activity against Gram-positive aerobic bacteria including *Staphylococcus, Enterococcus, Streptococcus, Bacillus, Pneumococcus, Listeria monocytogenes, Corynebacterium* sp., and Gram-positive anaerobic bacteria including *Clostridium difficile* (McCafferty et al., 2002), and other *Clostridium spp., Eubacterium spp., Lactobacillus spp., Peptostreptococcus spp., Propionibacterium spp., Prevotella spp.* (Farver et al., 2005). Ramoplanin is not effective against Gram-negative aerobic or anaerobic bacteria, atypical pathogens, or fungi (Farver et al., 2005).

Ramoplanin especially draws attention in treatment of vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*-associated diarrhea (CDAD), which are the infections that have high morbidity and mortality rates due to their multi-resistance to the current antibiotics such as vancomycin and penicillin G (Johnson et al., 1992), methicillin, ampicillin and/or erythromycin (Gastaldo et al., 1992), and oxacillin (Shonekan et al. 1992).

Ramoplanin has rapid and dose-dependent bactericidal activity against *Enterococci*, including those are resistant to antibiotics such as vancomycin, penicillin or ampicillin, without requirement of any aminoglycoside antibiotic (Johnson et al., 1992). Moreover, ramoplanin is effective against *Enterococci* at lower minimal inhibitory concentration (MIC) values than vancomycin, teicoplanin

and daptomycin. Ramoplanin displays bactericidal activity at concentrations close to its MIC values for most Gram-positive bacteria, whereas vancomycin just displays bacteriostatic activity at its MIC values (Bionda et al., 2013), although ramoplanin and vancomycin have similar spectrum of antibacterial activities (Jones & Barry, 1989). In addition, no cross-resistance was observed between ramoplanin and vancomycin, teicoplanin and daptomycin. These results are in conformance with the differences between their structures and mechanisms of action (Bionda et al., 2013).

MRSA is stubborn due to its resistance to currently used antibiotics including methicillin, oxacillin, penicillin and amoxicillin. Vancomycin has been used for treatment of MRSA for a long period of time, but widespread use of vancomycin caused it to become increasingly ineffective because of the emergence of vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) (Yin, 2014). Ramoplanin has bactericidal activity against susceptible *S. aureus* strains and MRSA with MIC values below 2.0 μg mL^{-1} (McCafferty et al., 2002). Ramoplanin was found more potent than both vancomycin and teicoplanin against coagulase-negative *Staphylococci* (Rolston et al., 1996).

CDAD is another refractory infection which has high morbidity and mortality rates in hospitals worldwide. The antibiotics used for the treatment of CDAD are metronidazole and oral vancomycin (Peláez et al., 2005). Most isolates are still susceptible to vancomycin and metronidazole, however transient and heteroresistance to metronidazole have been reported (Huang et al., 2009). Ramoplanin is the most potent antibiotic for the treatment of CDAD. The reported MIC_{50} and MIC_{90} values by Peláez et al. (2005) vary between 0.03-0.5 μg mL^{-1}, whereas MIC values reported by Mathur et al. (2013) are between 0.088-0.704 μg mL^{-1}, which are still lower than MIC values obtained for other antibiotics. Moreover, no resistance to ramoplanin has been reported in *C. difficile* (Bionda et al., 2013).

Ramoplanin has been fast-tracked by the US Food and Drug Administration (Fulco & Wenzel, 2006). Until now, twelve phase I studies, two phase II studies (one in CDAD and one in VRE) and one phase III study (in CDAD) have been conducted.

Currently a phase IIb study is under development in CDAD (Nanotherapeutics, 2014).

Ramoplanin was found safe and well tolerated at all tested doses in multiple phase I clinical trials. No detectable concentrations of ramoplanin were found in serum or urine after oral administration of a single oral dose of 200 mg (McCafferty et al., 2002). Oral ramoplanin was also found safe and well tolerated in two conducted phase II studies, although some adverse gastrointestinal effects, including nausea, diarrhea, abdominal pain, dyspepsia and flatulence, have been observed (Bionda et al., 2013). Ramoplanin is also found in high concentrations in feces (Farver et al., 2005). But, ramoplanin is poorly tolerated after intravenous or intramuscular administration. Also, ramoplanin is unstable in the bloodstream (Yin, 2014). In addition, limited data are available from the phase II and III trials to determine whether drug-drug or drug-food interactions will be of concern with ramoplanin administration (Farver et al., 2005).

1.3.5 Resistance Against Ramoplanin

Due to the fact that ramoplanin has a different mechanism of action than that of the glycopeptide antibiotics vancomycin and teicoplanin, lipopeptide antibiotic daptomycin and β-lactam antibiotics, no cross-resistance between ramoplanin and these antibiotics is theoretically expected (Farver et al., 2005, Pan et al., 2013). Therefore, ramoplanin is foreseen to be a potential lead for the generation of new antibiotics not susceptible to the emerging vancomycin resistance (Pan et al., 2013).

Although, no clinical resistance to ramoplanin has been reported yet; the first laboratory-generated *S. aureus* strain resistant to ramoplanin was reported by Schmidt et al. (2010). The resistant strain RRSA16 was achieved by serial passaging in the presence of increasing concentrations of the antibiotic. Observed alterations were that the strain RRSA16 displayed phenotypes including a thickened cell wall and reduced activity of autolytic enzymes. RRSA16 also gained the unanticipated acquisition of cross-resistance to vancomycin and nisin, although these antibiotics

have unsimilar chemical structures and different mechanism of actions. But, these results are beneficial to understand the molecular mechanism of ramoplanin resistance.

1.3.6 Semisynthetic Modifications of Ramoplanin

Antibiotics can be modified to enhance their spectrum of antimicrobial activity and stability in metabolism, or to decrease the side effects of the antibiotic, and prevent resistance formation.

In case of ramoplanin, semisynthetic modification studies generally have been conducted on decreasing the side effects of ramoplanin, since its excellent activity against a variety of Gram-positive bacteria clearly reveals the necessity of development of ramoplanin derivatives. Although, extensive chemical modification to generate novel derivatives with improved pharmacological activity has been hampered due to the molecule instability at acidic or alkaline pH and its structural complexity (Di Palo et al., 2007), some successful ramoplanin derivatives have been developed.

Ciabatti & Cavalleri (1992) prepared tetrahydroderivatives of ramoplanin factors by hydrogenation of the corresponding factors, and obtained aglycons of ramoplanin factors by selective hydrolysis (Ciabatti & Cavalleri, 1996). Ramoplanin tetrahydro derivatives are found active against Gram-positive bacteria, especially for topical treatment of wound infections and acne, whereas ramoplanin A2 aglycon is found equally or slightly more potent than the corresponding factor in antimicrobial assays.

Parenti et al. (2000) studied on injectable formulations of ramoplanin family of antibiotics suitable for intravenous administration which are well tolerated and effective in particular in severe Gram-positive infections.

Jiang et al. (2003) accomplished total chemical synthesis of ramoplanin A2 and ramoplanin aglycone. Chen et al. (2004) showed that the lactam analogue 2,

maintains the full biological activity of the natural product and is chemically stable, addressing the problem of hydrolytic instability caused by the natural lactone linkage.

Di Palo et al. (2007) enzymatically demannosylated ramoplanin and tetrahydroramoplanin, and obtained improved microbiological activity against some resistant staphylococci and streptococci.

Yin (2014) developed ramoplanin and rhamnolipid combinations for treatment of VRE, *Clostridium difficile*, or multidrug-resistant *Clostridium difficile*, and these combinations are found effective against MRSA/VISA and VRE.

1.3.7 Biological Production Studies of Ramoplanin

Recent studies indicate that the biological production of ramoplanin is essential for pharmaceutical production. Although, Jiang et al. (2003) accomplished the total chemical synthesis of ramoplanin A2 and ramoplanose aglycon, and ramoplanin A1 and A3 aglycons were totally synthesized by Shin et al. (2004), the common feature of these studies are those the overall yield was very low and this route is not competitive with the biological production (Di Palo et al., 2007). Therefore, biological production of ramoplanin needs to be developed to enhance productivity.

Cavalleri et al. (1984) studied on fermentation, isolation and preliminary physicochemical characteristics of ramoplanin, and studied on medium optimization for enhancing the production of factor A2 and co-produced factors A1 and A3.

Lancini et al. (1987) studied on selective increasement of the ratio of single major components of ramoplanin complex.

Restelli & Mainoli (1996) studied on different extraction processes to obtain high yield of ramoplanin.

Brunati et al. (2005) studied the influence of L-leucine and L-valine on ramoplanin production, which are two amino acids as biosynthetic precursors of the fatty acids in A2 and A3 factors. Addition of 5 g L^{-1} of L-leucine was found to improve production of both ramoplanin and factor A2, whereas addition of L-valine was found to modify the composition of the complex towards factor A3, but not improved total antibiotic productivity.

Wu et al. (2008) developed biochemically engineered *Actinoplanes* strains to acquire a high-yield strain that exhibits stable hereditary character. The mutant strain *Actinoplanes* sp. C6-78-14 produced 384.8 mg L^{-1} ramoplanin which is 4.13-fold higher than the amount of produced by the parent strain *Actinoplanes* sp. C2-9-45. They also studied the effect of some trace elements on ramoplanin production by *Actinoplanes* sp. C6-78-14, and determined that addition of 0.001% $FeSO_4.7H_2O$, 0.001% $CoCl_2.6H_2O$ and 0.001% $CuSO_4.5H_2O$ to fermentation medium enhanced the ramoplanin production 10%, 15% and 20% respectively, compared with the control group of strain.

Zhang et al. (2010) also developed a ramoplanin producing mutant strain W07-28 by treating UV, streptomycin-resistance, and microwave radiation. W07-28 was found to produce 59.3% more ramoplanin than the ramoplanin producing original strain RM05-55.

1.4 Biological Production of Antibiotics

Antibiotics constitute the most rapidly growing class of human therapeutics and the second largest class of drugs after vaccines (Smith, 2009). Microbial natural products are the origin of most antibiotics in the market today, and still the most promising source of novel antibiotics (Atta et al., 2010). Therefore, searching microorganisms still remains as a major area in discovery of new antibiotics.

Antibiotics of microbial origin are produced by batch fermentation process. This process consists of various growth phases shown in Figure 1.9 (Najafpour, 2007).

Nutritional considerations and growth rate of producer microorganism influence the antibiotic production. Generally, antibiotics are generated during the exponential phase of growth in a medium supporting slow growth, but in the stationary phase if the medium supports rapid growth (Umbreit, 1980).

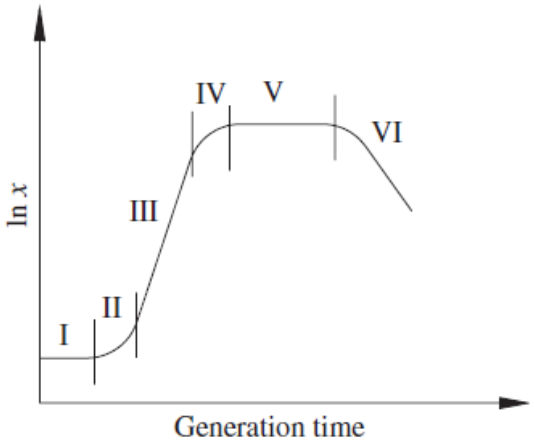

Figure 1.9 Batch growth curve of microorganisms. log phase (I); accelerating growth phase (II); exponential growth phase (III); declining growth phase (IV); stationary phase (V); death phase or lytic decline phase (VI).

1.4.1 Antibiotic Producing Microorganisms

Considering taxonomic classes of antibiotic producing microorganisms, it is obvious that antibiotics are mostly produced by filamentous microorganisms. Some of antibiotic producing genera are *Actinomycetes, Bacillus, Pseudomonas, Aspergillus, Penicillium,* and *Myxobacteria*. Among them, actinomycetes draw attention because they produce about 75% of all described antibiotics (Awad et al., 2012).

1.4.1.1 Genus Actinomycetes

Actinomycetes are a large group of phylogenetically related, filamentous aerobic Gram-positive bacteria (Madigan et al., 2011) with a high GC content more than 55% (Singleton & Sainsbury, 2006). They produce both aerial and submerged spores

and exhibit distinctive morphological features such as spore chain structures, which can be useful in characterization and identification (McNeil & Harvey, 2008).

Actinomycetes are the strongest antagonists among microorganisms (Ogunmwonyi et al., 2010). They are widespread in nature, and have been isolated from soil, freshwater, marine water, and organic matter. They can use recalcitrant compounds such as cellulose and chitin as a food source. Although the most of actinomycetes are free-living and saprotrophic, some form symbiotic associations and others are pathogenic in man, other animals, and plants (Singleton & Sainsbury, 2006). But, the vast majority of actinomycetes are not pathogenic (Goodfellow et al., 1984). More than 140 actinomycete genera have been described to date (Solecka et al., 2012).

Actinomycetes (order Actinomycetales) have great potency of producing industrially and medically important secondary metabolites including antibacterials, antitumours, antifungals, antiangals, antivirals, antiparasitics, antiprotozoals, anticholesterols, anti-ageings, antihelminths, immunesuppressants, enzyme inhibitors and diabetogenics for more than 70 years (Awad et al., 2012; Adegboye & Babalola, 2013; Weber et al., 2014). Therefore, actinomycetes routinely continue to be isolated from different habitats, and to be screened for their new bioactive metabolites.

To date, more than 10,000 bioactive metabolites have been isolated from different actinomycetes (Awad et al., 2012), and 80% of those compounds in practical use (Olano et al., 2008). *Streptomyces,* the best known actinomycete genus which contains over 500 species primarily inhabiting soil (Hogg, 2005; McNeil & Harvey, 2008), produce about 7,600 bioactive metabolites (Awad et al., 2012).

Rare actinomycetes (non-streptomycete actinomycetes) are usually regarded as the strains of actinomycetes whose isolation frequency is much lower than that of the streptomycete strains isolated by conventional methods. They are widely distributed in terrestrial and aquatic ecosystems. Environmental factors such as soil type, pH, humus content, and the characteristics of the humic acid content of the soil affect

their distribution. While rare actinomycetes may result in increased chances of discovering novel structures, their genetics and physiology are poorly known. To speed up their isolation process, the knowledge about distribution of such unexploited groups of microorganisms must be augmented (Tiwari & Gupta, 2012).

Since 1980s, rare actinomycetes have increased significantly up to a 25~30% share of all known antibiotics (Tiwari & Gupta, 2012). Today, 2,500 known bioactive metabolites are produced by rare actinomycetes (Solecka et al., 2012).

Some genera of rare actinomycetes are *Actinomadura, Actinoplanes, Amycolatopsis, Actinokineospora, Acrocarpospora, Actinosynnema, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kibdelosporangium, Kineosporia, Kutzneria, Microbiospora, Microtetraspora, Nocardia, Nonomuraea, Planomonospora, Planobispora, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Saccharothrix, Streptosporangium, Spirilliplanes, Thermomonospora, Thermobifida, and Virgosporangium* (Lazzarini et al., 2001). The ramoplanin producing strain *Actinoplanes* sp. ATCC 33076 used in this thesis is also a rare actinomycete.

Important classes of antibiotics produced by actinomycetes include: β-lactams, aminoglycosides, lipopeptides, glycopeptides, asamycins, anthracyclines, nucleosides, peptides, polyenes, polyethers, tetracyclines and macrolides. Table 1.2 shows important antibiotic classes produced by actinomycetes (Adegboye & Babalola, 2013). Many well-known antibiotics, such as tetracycline, erythromycin, vancomycin, and streptomycin, originate from the secondary metabolism of actinomycetes (Weber et al., 2014).

Table 1.2 Major antibiotic classes and producer actinomycetes

Antibiotic Class	**Producer Actinomycetes**
β-lactams	*Nocardia lactamdurans, Streptomyces clavuligerus, S. chartreusis, S. panayaensis, S. viridochromogenes, S. wadayamensis, S. jumonjinenesis, S. limanii*

Table 1.2 Major antibiotic classes and producer actinomycetes (continued)

Aminoglycosides	*Streptomyces, Streptosporangium, Saccharopolyspora, Streptoalloteichus, Micromonospora, Dactylosporangium, Frankia*
Glycopeptides	*S. orientalis, S. candidus, S. toyocaensis, Nocardia actinoides, Actinoplanes teichomyceticus, Amycolatopsis orientalis, Amy. balhimycina*
Anthracyclines	*S. peuceticus, S. galilaeus, S. nogalater, S. purpurascens , Micromonospora lupine*
Macrolides	*Saccharopolyspora erythraea, S. hydroscopicus, S. venezuelae*
Tetracyclines	*S. aureofaciens, S. rimosus, S. viridofaciens*
Polyenes	*S. nodosus, S. noursei, S. natalensis*

1.4.2 Antibiotic Production by Fermentation Process

Antibiotic production by batch fermentation process is carried out to overproduce the desired antibiotic, which is normally produced at very low quantities by producer microorganism in its natural habitat.

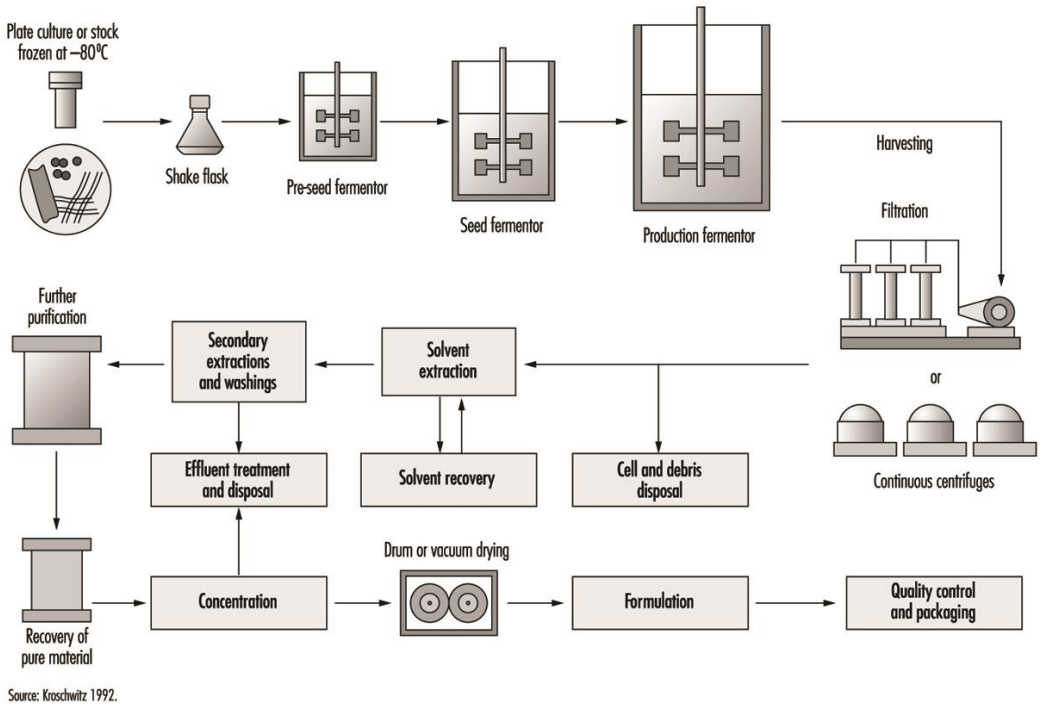

Figure 1.10 Diagram of a fermentation process

Fermentation process basically requires three steps, which are isolation of desired microorganism, providing the components of fermentation broth, and isolation of antibiotic product, those all must be carried out under sterile conditions. A diagram of fermentation process is shown in Figure 1.10 (Keith, 1998).

To initiate the fermentation process, sufficient cell number is required. Therefore, previously isolated and cold-stored antibiotic producing strain is first transferred to agar-containing plates, then step by step to shake flasks (250-500 mL), pre-seed fermenter (1-10 liters) and seed fermenter (300-3000 liters) containing appropriate growth components, as the sufficient cell number is obtained (Madigan et al., 2011). Usually after 24-48 hours of growth, the suspension in the seed fermenter is finally transferred to the large-scale industrial fermentation tank which is almost always constructed of stainless steel, with a capacity between 40.000-200.000 liters (Madigan et al., 2011). The process may take a few days to obtain an extractable amount of product (Smith, 2009).

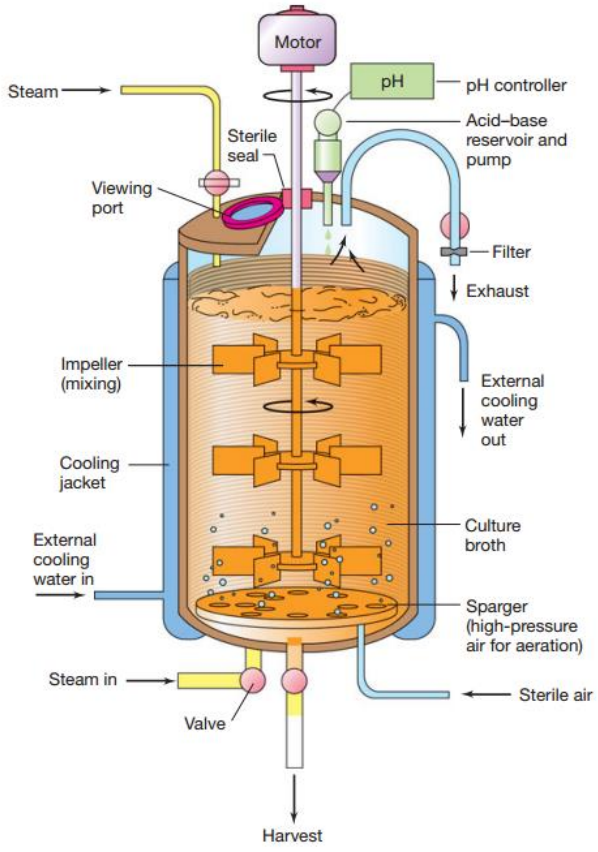

Figure 1.11 Diagram of an industrial fermenter

Mass production of antibiotics by fermentation requires development of an economically viable production process. At both laboratory scale and large scale antibiotic production, medium design and environmental variables including are two important parameters to optimize for yield improvement.

1.4.2.1 Nutritional Considerations in Antibiotic Production Medium Design and Optimization

The nutritional requirements for growth of the producer microorganism and production of the desired antibiotic are different due to the fact that the antibiotics are secondary metabolites those produced during the stationary phase of the growth cycle. Therefore, substantial microorganism growth is often carried out in "seed medium" which is then used to inoculate "production medium". Both contain carbon source(s), nitrogen source(s), and sources of trace nutrients such as magnesium, sodium, potassium, sulfur, copper, zinc, vitamins and amino acids. They may also contain some buffers, when used in the laboratory. The carbon and nitrogen sources used may be the same in both the seed and the production medium, but their concentrations can be different. Also, complex materials of plant and animal origin can be used in "complex" medium design. They are usually inexpensive, provide "unknown critical" ingredient, support good growth, and can be developed quickly. Their limitations are that the presence of the crude complex ingredients results in variable performance and pose difficulties in scale-up and may interfere in downstream processing. On the other hand, "defined media", which can be monitored and controlled easily in fermenters, results in consistent performance, ease of scale-up, and fewer problems in downstream processing. However, these media are costly and often fail to reproduce the high yields obtained with the complex media.

Carbon sources provide energy and building blocks for the microorganisms and their choice is very critical for the production of high levels of antibiotics. Monosaccharides like glucose and fructose, disaccharides like lactose and molasses, and polysaccharides like dextrins, starch and cellulose, polyols like glycerol, oils,

and alcohols are commonly used carbon sources in antibiotic production. Glucose can be obtained in cheap crude form as cerelose and corn-syrup. It is used essentially by all microorganisms. However, glucose can either cause excessive growth of microorganism which in turn causes of inhibition of production, or can cause catabolite repression of important enzymes involved in the antibiotic biosynthetic pathway. In addition, rapid depletion of glucose often results in a sharp decrease in pH with deleterious effects on production. These problems are usually solved by either using another carbon source which does not have the limitations of glucose, or addition of a slowly utilized carbon source that supports production whereas glucose supports rapid growth. The problem of reduction in pH can be avoided by the use of either inorganic buffers in the laboratory, and in fermenters by employing pH control or slow feeding of glucose.

Nitrogen sources are essential for the synthesis of cell components such as proteins, nucleic acids, and cell wall as well as primary and secondary metabolites. Ammonium salts, nitrates, urea, amino acids, protein hydrolysates, and proteins are commonly used nitrogen sources in antibiotic production. As carbon sources, nitrogen sources also can be of plant or animal origin. Animal proteins may cause difficulties with the regulatory agencies, whereas inorganic nitrogen sources are often used up rapidly, leading to reduction in pH and adverse effects on growth and/or production. Therefore, usually complex organic nitrogen sources are preferred. Amino acids, which are the precursors of the antibiotic of interest or play a regulatory role in the antibiotic biosynthesis, are also used as nitrogen sources, especially in defined media.

Phosphate is critical for the biosynthesis of nucleic acids and for energy metabolism. It is known that phosphate plays a regulatory role in the biosynthesis of many antibiotics including peptide antibiotics, polyene, macrolides, tetracyclines, and complex antibiotics, and phosphate concentrations above 10 mmol l^{-1} were found to inhibit the production of antibiotics. Although the mechanism of phosphate regulation is not fully understood, it has been suggested that either ATP concentration is the intracellular mediator of phosphate regulation, or adenylate

energy charge is involved in the regulation of synthesis, or PhoR–PhoP plays role in phosphate regulation. Regardless of the mechanism of phosphate regulation, it is important to be aware of the regulatory role of phosphate while designing a production medium. Phosphate also can be used as a buffering agent (Schaechter, 2009).

Trace elements including magnesium, copper, ferrous or ferric, cobalt, molybdenum, manganese, calcium, boron, zinc, sulfate, and chloride, are important nutrients for microorganisms that may contribute to both primary or secondary metabolite production (Schaechter, 2009; Vogel & Todaro, 1996). Iron and zinc have been found to influence antibiotic production. In addition, trace element concentration has an influence on antibiotic production. For example, $0.07x10^{-5}$ M manganese has positive effect on bacitracin production by *Bacillus licheniformis,* whereas 4.0 x 10^{-5} M manganese inhibits production, and $1.0x10^{-5}$ M of iron and $0.3x10^{-5}$ zinc is required for maximum growth of streptomycin producing strain *Streptomyces griseus*, whereas a zinc concentration of $20x10^{-5}$ M becomes an inhibitor (Vogel & Todaro, 1996).

1.4.2.2 Optimization of Environmental Variables

Besides nutritional supplements, the environment that the microorganism is grown in plays significant role on its metabolism. Therefore, optimization and control of environmental variables such as pH, temperature, aeration, and agitation is important for good productivity of desired antibiotic at all phases and scales of the process. These factors are are monitored and controlled in real time.

The pH of the seed and production media strongly affects the physiology of the producing culture. It has been observed that low or high pH values have a negative effect on yield. At the laboratory scale antibiotic production, the pH is maintained in the range of 5.50-7.00 with buffers such as MES and MOPS, whereas in the large-size fermenters, NaOH, KOH, H_2SO_4, or NH_3 gas which also act as a nitrogen

source, are used to control the pH. It is possible to adjust the pH for optimum growth and high yield production (Schaechter, 2009).

Process temperature is another important factor to be controlled for good production. Proper process temperature is maintained with the external cooling jackets. For sterilization of the culture medium, steam is transported through the external cooling jackets, whereas water is used for removal of heat (Madigan et al., 2011). For antibiotic production, a remarkable point about temperature is that the optimum growth temperature is not always conducive high productivity. Therefore, in cases which the process cannot be operated at multiple temperatures, a compromise value is selected (Schaechter, 2009).

Aeration and agitation are another vital factors of fermentation to maintain the high oxygen demand of the culture. Proper aeration and agitation is supplied with sterilized filtered air to provide transfer of oxygen throughout the growth medium and to mix the organisms through the medium to have uniform access to the nutrients (Madigan et al., 2011).

1.4.3 Isolation and Purification of Antibiotic

Antibiotic production is terminated before the death phase. The produced antibiotic undergoes a series of isolation and purification steps.

First, mycelial biomass is removed from fermentation broth by filtration or centrifugation. Then, antibiotic is isolated by solvent extraction or adsorption. Many antibiotics have excellent solubility in organic solvents and they are water immiscible. Extraction can provide concentrated and purified products. Butyl acetate, amyl acetate methyl isobutyl ketone and methyl ethyl ketone are the solvents used for penicillin extraction, whereas pentyl or amyl acetate are used erythromycin extraction (Najafpour, 2007). pH is an important consideration throughout the extraction process.

Purification of isolated antibiotic is either carried out by chromatographic methods such as adsorption chromatography, ion-exchange chromatography, affinity chromatography, size-exclusion chromatography and expanded beds, or precipitation method. Final step of purification and polishing is done by crystallization and drying (Crommelin et al., 2008; Schaechter, 2009).

After purification step, quantitative analysis of the extracted antibiotic is generally carried out by high-performance liquid chromatography (HPLC). In addition, an antibiogram test is used to determine the amount of antibiotic, whereas a bioassay is used to determine the activity unit of the bactericides (Najafpour, 2007; Smith, 2009).

The choice of the used processes depend on characteristics of the antibiotic (solubility, ionic charge, stability and sensitivity to pH and/or temperature), characteristics of the mycelium of the producer microorganism, the location of the antibiotic (extracellular or cell-associated), properties of the broth, purity of the starting material, quality of the final product required, economics, and other factors such as available equipment and infrastructure. Therefore, the order of the processes used, the number of times each is used, the types of adsorbents and eluents used, the types of stationary phases or the solvents used in chromatography are all different for each of the antibiotics, also they vary among manufacturers. Consequently, there is no definitive downstream process for any one of the antibiotics (Schaechter, 2009).

CHAPTER TWO
MATERIAL AND METHOD

2.1 Microorganism and Culture Conditions

The model microorganism used in this thesis is ramoplanin producing strain *Actinoplanes* sp. ATCC 33076, which was purchased from American Type Culture Collection (ATCC).

2.1.1 Maintenance of Strain Actinoplanes sp. ATCC 33076

Actinoplanes sp. ATCC 33076 was grown and maintained on oatmeal agar medium. The initial pH of medium was adjusted to 7.00 prior to sterilization. Medium was autoclaved at 121°C for 20 minutes, and 1 mL L^{-1} trace salts solution was added aseptically. Incubation was carried out at 30ºC for 8 days. The composition of oatmeal agar medium is shown in Table 2.1.

Table 2.1 The composition of oatmeal agar medium

Difco™ oatmeal agar	**Trace salts solution**
60 g oatmeal	0.1 g $FeSO_4.7H_2O$
12.5 g agar	0.1 g $MnCl_2.4H_2O$
1 mL trace salts solution	0.1 g $ZnSO_4.7H_2O$
1 L distilled water	100 mL distilled water

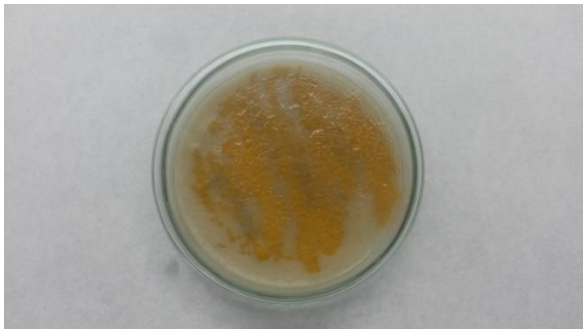

Figure 2.1 *Actinoplanes* sp. ATCC 33076 on oatmeal agar after 8 days

2.1.2 Seed Medium

The mycelium from oatmeal agar medium was used to inoculate the seed medium. The composition of the seed medium is shown in Table 2.2.

Table 2.2 The composition of seed medium

Seed medium
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

The initial pH of seed medium was adjusted between 7.00-7.20 prior to sterilization. Seed medium was autoclaved at 121°C for 20 minutes. 100 mL of seed medium in 500-mL erlenmeyer flasks were inoculated with 5 mL mycelium suspension (OD_{620}: 0.800). Flasks were incubated at 30°C for 96 hours on a rotary shaker set at 180 rpm (Cavalleri et al., 1984; Brunati et al., 2005).

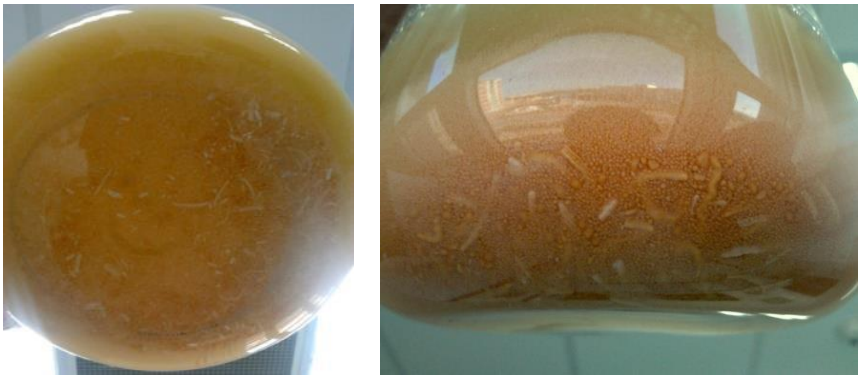

Figure 2.2 *Actinoplanes* sp. ATCC 33076 on seed medium after 4 days

2.1.3 Substrates

Soybean meal, meat-bone meal, poultry meal and fish meal (obtained from RDM Trade at Izmir, Turkey), which are by-products of agriculture and livestock industries, were used as nitrogen sources in this thesis.

By-product nitrogen sources were were milled, and then extracted with water at 90°C by mixing for 60 minutes. Extracted samples were autoclaved at 121°C for 20 minutes, cooled down to room temperature, and centrifuged at 5000 rpm for 10 minutes. Supernatants were used as protein extracts for preparation of ramoplanin production media.

2.1.4 Ramoplanin Production Media

The effects of different nitrogen sources on ramoplanin production were studied. The compositions of the ramoplanin production media are shown in Table 2.3.

Table 2.3 The compositions of nitrogen source media

Basal medium	**Meat-bone meal medium**	**Poultry meal medium**	**Fish meal medium**
4 g glucose	4 g glucose	4 g glucose	4 g glucose
4 g soluble starch	4 g soluble starch	4 g soluble starch	4 g soluble starch
20 g maltose	20 g maltose	20 g maltose	20 g maltose
20 g sucrose	20 g sucrose	20 g sucrose	20 g sucrose
20 g glycerol	20 g glycerol	20 g glycerol	20 g glycerol
30 g soybean meal	30 g chicken meat-bone meal	30 g poultry meal	30 g fish meal
6 g $CaCO_3$	6 g $CaCO_3$	6 g $CaCO_3$	6 g $CaCO_3$
1 L distilled water	1 L distilled water	1 L distilled water	1 L distilled water

The effects of different concentrations of various trace elements on ramoplanin production were also studied.

Table 2.4 Final concentrations of trace elements added to basal medium

Trace element	**Final concentrations of trace elements**		
$FeSO_4.7H_2O$	15 ppm	45 ppm	75 ppm
K_2HPO_4	15 ppm	45 ppm	75 ppm
$MgSO_4.7H_2O$	15 ppm	45 ppm	75 ppm
$MnCl_2.4H_2O$	15 ppm	45 ppm	75 ppm
$ZnSO_4.7H_2O$	15 ppm	45 ppm	75 ppm

In all experiments, the initial pH of all ramoplanin production media were adjusted to 7.00 prior to sterilization. Media were autoclaved at 121°C for 20 minutes. Trace metals were added aseptically. 20 mL of ramoplanin production medium in 250-mL Erlenmeyer flask was inoculated with 10 mL seed medium. Flasks were incubated at 30°C on a rotary shaker set at 180 rpm.

Flasks were harvested on 3rd, 5th, 7th and 9th days in order to determine ramoplanin production, pH levels, intracellular free amino acid levels, total protein levels, total reducing sugar levels, and protease, amylase and lipase activities. Data are reported as the mean values of at least three replicates for each experiment.

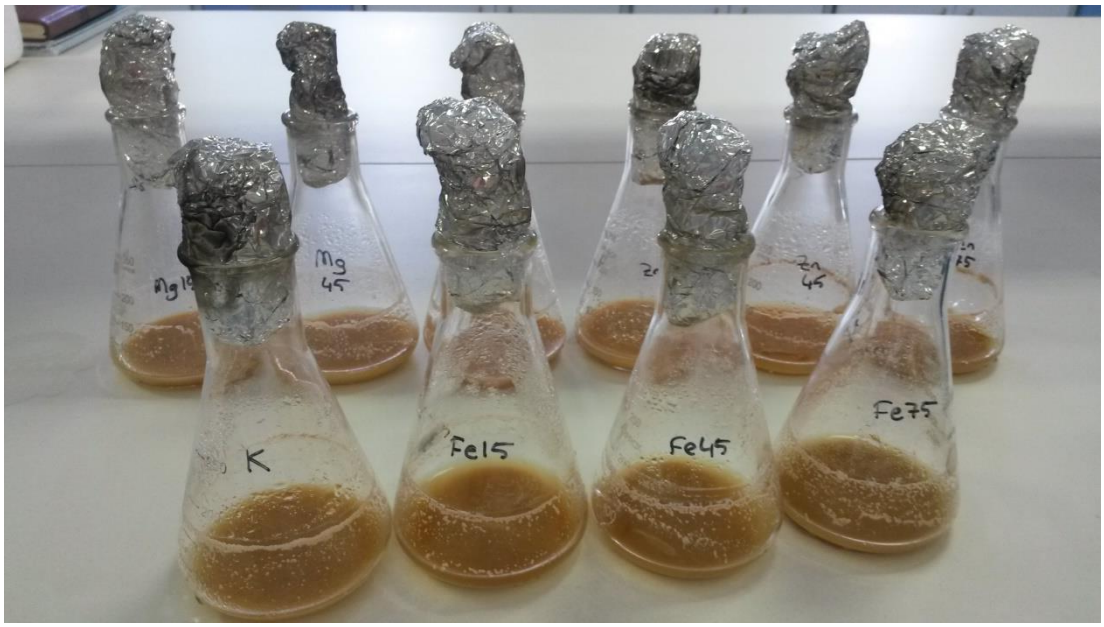

Figure 2.3 *Actinoplanes* sp. ATCC 33076 on some metal media after 5 days

2.2 Determination of pH

After flasks were harvested, 10 mL of the culture broth was kept for ramoplanin determination. The residual culture was centrifuged at 5000 rpm for 10 minutes at +4°C. The pH of culture supernatant was measured by using a pH-meter.

2.3 Determination of Ramoplanin

2.3.1 Ramoplanin Extraction

Ramoplanin extraction was carried out according to the method of Brunati et al. (2005) with slight modifications. 10 mL of culture were collected from the production flask, and mixed with 30 mL of acetone:water (2:1). pH was adjusted to 2.20 with concentrated HCl. Mixture was shaken for 30 min at room temperature and then filtered through paper filter. The filtered solution was extracted by an equal volume of ethyl acetate and centrifuged at 5000 rpm for 10 minutes. The water (lower) phase was frozen and then lyophilised. The lyophilized samples were resolved in acetonitrile:ultra-distilled water (2:8), centrifuged at 12000 rpm for 10 minutes at +4°C, and filtered through a 0.45 μm filter before injecting into the HPLC.

2.3.2 HPLC Conditions

Determination of ramoplanin by HPLC was performed according to the method of Restelli & Mainolli (1996) with slight modifications. The system was an Agilent HP 1100 HPLC system equipped with a photodiode detector (DAD). The column was a Thermo Hypersil ODS-2 (C_{18}) column (250x4.6 mm, 5 μm). The column was operated at 40°C with a flow rate of 1.2 mL min^{-1} using 25 mM NaH_2PO_4 (pH 3.50):CH_3CN (80:20, v/v) as phase A and 25 mM NaH_2PO_4 (pH 3.50):CH_3CN (20:80, v/v) as phase B. The gradient program was shown in Table 2.5. The injection volume was 20 μL, and the detection wavelength was set at 254 nm. An authentic sample of ramoplanin antibiotic (obtained from Sigma Aldrich) was used as internal standard.

Table 2.5 Gradient program for the elution of ramoplanin

Flow rate, mL min^{-1}	Time (min)	Eluents (%)	
		A	B
1.2	0	73	27
1.2	5	73	27
1.2	30	5	95

2.4 Preparation of Culture Samples

Culture samples were centrifuged at 12000 rpm for 10 min at +4°C. Cell free supernatants were used for determination of total protein, total reducing sugar and enzymatic activities.

2.5 Determination of Total Protein Concentration

Total protein concentration was determined according to the method of Bradford (1976). 100 µL of cell free culture supernatant was added to 900 µL of Bradford reagent in a quartz cuvette, and shaked gently. Absorbance was measured at 595 nm against a blank, after the mixture was incubated for 2 minutes at room temperature. The blank sample was prepared by using 100 µL of distilled water instead of the culture supernatant, and the same analysis procedure was followed. Bovine serum albumin (BSA) was used as standard.

2.6 Determination of Total Reducing Sugar Concentration

Reducing sugar concentration was determined according to the dinitrosalicyclic acid (DNS) method (Miller, 1959). 500 µL of cell free culture supernatant was added to 500 µL of DNS reagent, and mixed. Then the solution was boiled for 10 minutes and cooled for 1 minute in an ice bath. 5 mL of distilled water was added and mixed. The absorbance was measured at 546 nm against a blank. The blank sample was prepared by using 500 µL of distilled water instead of the culture supernatant and the same analysis procedure was followed. D-glucose was used as standard.

2.7 Determination of Protease Activity

Extracellular protease activity was determined by measuring the azo-dye formation from the hydrolysis of azocasein according to the method of Kembhavi et al. (1993) with slight modifications. The substrate solution was prepared by dissolving 0.5 g of azocasein in 100 mL of 50 mM Tris-HCl buffer (pH 7.00). The reaction mixture was prepared by adding 150 µL substrate solution to 90 µL of cell free culture supernatant. The reaction mixture was incubated for 30 minutes at 30°C. The reaction was terminated by adding 720 µL of 10% TCA, and then samples were centrifuged at 12000 rpm for 10 minutes at 4°C. 700 µL of 1 M NaOH was added to 600 µL of clear supernatant, and the absorbance was measured at 440 nm against a blank. The blank sample was prepared by using 90 µL of 50 mM Tris-HCl buffer (pH 7.00) instead of culture supernatant, and the same analysis procedure was followed. One unit protease activity was defined as the amount of the enzyme required to produce an increase in optical density of 0.001 per minute under the assay conditions.

2.8 Determination of Amylase Activity

Extracellular amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The substrate solution was prepared by dissolving 1 g soluble starch in 100 mL of 50 mM Tris-HCl buffer (pH 7.00). The reaction mixture was prepared by adding 250 µL of substrate solution to 250 µL of cell free culture supernatant. The reaction mixture was incubated for 30 minutes at 30°C. The amount of liberated reducing sugar was determined by DNS method as indicated in Section 2.6 (Miller, 1959). The blank samples were prepared individually for each sample by using 250 µL of 50 mM Tris-HCl buffer (pH 7.00) instead of substrate solution, and the same analysis procedure was followed. One unit amylase activity was equal to the amount of enzyme required to release 1 µmol of reducing end groups per minute under the assay conditions. D-maltose was used as standard.

2.9 Determination of Lipase Activity

Extracellular lipase activity was determined by measuring the formation of *p*-nitrophenol (*p*NP) from the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) according to the method of Grbavčić et al. (2011) with slight modifications. The substrate solution was prepared by adding 30 mg of pNPP in 10 mL of isopropanol to 90 mL of 50 mM Tris-HCl buffer (pH 7.00) supplemented with 100 mg of gum arabic and 0.4% Triton X-100. The reaction mixture was prepared by adding 900 µL of substrate solution to 100 µL of cell free culture supernatant. The reaction mixture was incubated at 30°C for 30 minutes, and the amount of liberated *p*NP was determined at 410 nm against a blank. The blank sample was prepared by using 100 µL of 50 mM Tris-HCl buffer (pH 7.00) instead of culture supernatant, and the same analysis procedure was followed. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol per minute under the assay conditions.

2.10 Determination of Free Amino Acids

Determination of free amino acids were carried out by HPLC after pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) according to the method of Jámbor & Molnár-Perl (2009) with slight modifications.

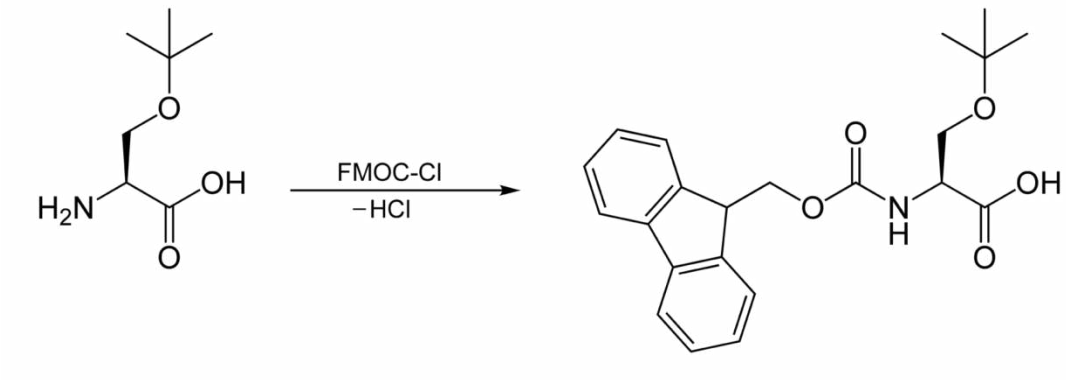

Figure 2.4 Scheme showing derivatization of an amino acid with FMOC-Cl

2.10.1 Extraction of Intracellular Free Amino Acids

Intracellular free amino acids were extracted from the wet cell pellet in boiling ultra distilled water 1:5 (w/v) by shaking for 15 min at 90°C in a water bath. The extracts were quickly cooled on ice, and centrifuged at 12000 rpm for 10 min at +4°C. Sample supernatants were used for determination of free amino acids.

2.10.2 Derivatization of Free Amino Acids with FMOC-Cl and HPLC Conditions

150 µL sample supernatant was mixed with 150 µL borate buffer (0.4 M, pH 9.00) and with 300 µL 0.1 mM FMOC-Cl reagent, dissolved in acetonitrile. After 2 minutes of incubation samples were filtered through a 0.45 µm filter, and injected into the HPLC.

The system was an Agilent HP 1100 HPLC system equipped with a fluorescence detector. The column was a Thermo ODS-2 Hypersil (C_{18}) column (150x4.6 mm, 5 µm). The column was operated at 50°C, in gradient mode. Phase A was 50 mM CH_3COONa, pH 7.20, phase B was 100 mM CH_3COONa:CH_3CN (23:22, v/v), pH 7.20, and phase C was CH_3CN. The gradient program was shown in Table 2.6. The injection volume was 20 µL, and the fluorescence excitation and emission wavelengths were set at 254 nm and 313 nm, respectively.

Table 2.6 Gradient program for the elution of free amino acids

Flow rate, mL min^{-1}	**Time (min)**	**Eluents (%)**		
		A	**B**	**C**
1.5	0	70	30	0
1.5	2	63	37	0
1.0	14	27	73	0
1.0	23	0	100	0
1.5	28	0	100	0
1.5	29	2	0	98
1.5	34	2	0	98
1.5	35	70	30	0
1.5	40	70	30	0

CHAPTER THREE
RESULTS AND DISCUSSION

The medium composition and cultivation conditions are critical parameters which influence the microbial growth and the antibiotic fermentation process of actinomycetes both in terms of diversity of antibiotic subtypes and their yields. Therefore, medium optimization by the selection of suitable carbon sources, nitrogen sources and trace metals are crucial to improve the production yield of the desired antibiotic subtype. Therefore, the influences of some nitrogen sources and trace metals were investigated in order to improve ramoplanin A2 production due to the fact that this factor exhibits better antimicrobial activity than factors A1 and A3 (Farver et al., 2005).

3.1 Variations in Metabolite Levels of *Actinoplanes* sp. ATCC 33076 Depending on Nitrogen Sources with Respect to Incubation Period

Generally complex media are preferred in industrial production processes, which are rich in ready to use nutrients those are not necessary to be synthesized by the microorganism itself. Complex media are also advantageous because they are inexpensive, and can be developed quickly. In this thesis, the influences of soybean meal, chicken meat-bone meal, poultry meal and fish meal on ramoplanin A2 production were investigated.

3.1.1 Variations in Ramoplanin Levels Depending on Nitrogen Source with Respect to Incubation Period

Ramoplanin A2 levels in all used animal by-product nitrogen sources containing media were found higher than those of basal medium. The ramoplanin A2 production by *Actinoplanes* sp. ATCC 33076 reached their maximum levels on 7^{th} day in all media, and then decreased significantly with the prolongation of the incubation period, although the yields were still higher than those obtained on 3^{rd} and 5^{th} days. The highest ramoplanin A2 level was determined as 406.805 mg L^{-1} in fish meal

medium on 7th day (4.89 fold of basal medium). On the other hand, although the maximum ramoplanin A2 yield was determined in fish meal medium on 7th day, ramoplanin A2 production in poultry meal medium was at a comparable level on 7th and 9th days. 374.218 mg L^{-1} ramoplanin A2 was produced in poultry meal medium on 7th day (4.50 fold of basal medium). Moreover, ramoplanin A2 yields of poultry meal medium were higher compared to fish meal medium on 3rd and 5th days. The highest ramoplanin A2 production in meat-bone meal medium was determined as 252.130 mg L^{-1} on 7th day (3.03 fold of basal medium).

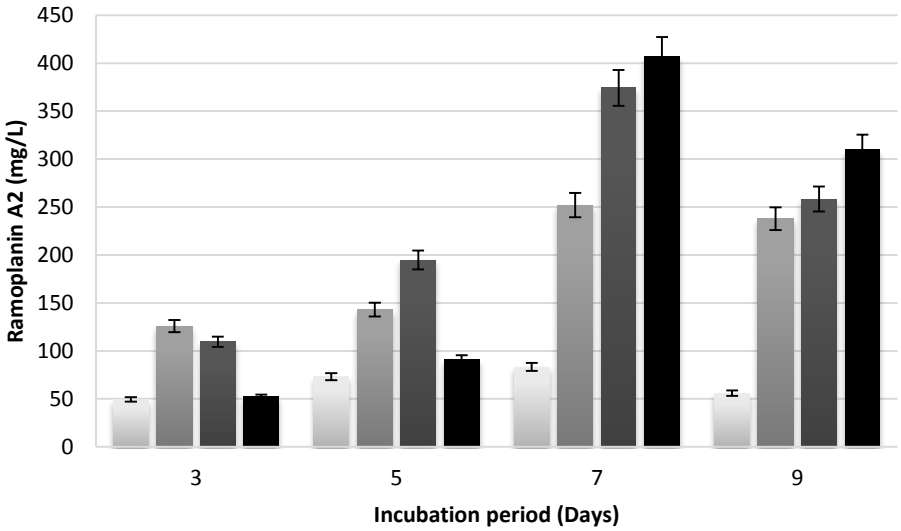

Figure 3.1 Ramoplanin production depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

The highest ramoplanin A2 level reached in this study was almost similar the amounts reported by Brunati et al. (2005) and Wu et al. (2008), respectively. Brunati et al. (2005) reported a maximum production of ramoplanin complex as 433 mg L^{-1} with addition of 5 g L^{-1} of L-leucine which was composed of 96% ramoplanin A2, whereas the maximum amount of ramoplanin complex obtained from the mutant strain *Actinoplanes* sp. C6-78-14 was 384.8 mg L^{-1} with a 87% ramoplanin A2 ratio (Wu et al., 2008). Although our results are similar previous findings, the results have importance due to the fact that we reached these levels by using low-cost nitrogen rich industrial by-products.

In addition, the growth of *Actinoplanes* sp. ATCC 33076 in basal medium was higher compared to the growth levels in the other media, while vice versa was determined for ramoplanin A2 production. These results indicated that the tested nitrogen sources, especially poultry meal medium and fish meal medium, supported ramoplanin A2 production instead of growth which could be mainly due to the increase of specific productivity (Brunati et al., 2005).

3.1.2 Variations in pH Levels of Mediums Depending on Nitrogen Source with Respect to Incubation Period

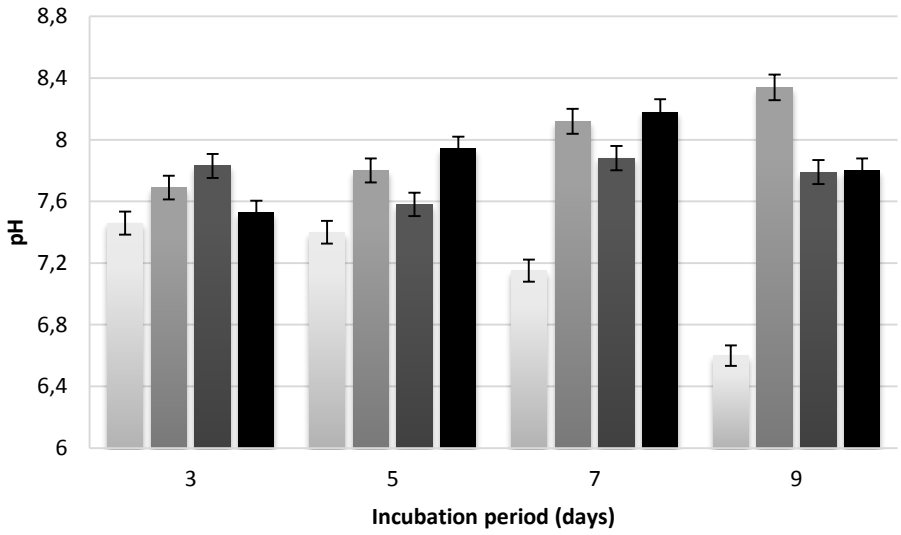

Figure 3.2 pH levels depending on nitrogen source containing growth medium conditions; basal medium (○), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

According to the results depicted in Figure 3.2, pH levels of basal medium were under 7.50 throughout the incubation period, and the minimum pH was measured as 6.60 on 9th day. This sharp decrease could be a result of rapid depletion of glucose depending on the prolonged incubation period (Figure 3.4), which could have caused deleterious effects on ramoplanin A2 production. pH levels measured in other three media, were above 7.50 throughout the incubation period. Considering the fact that higher ramoplanin A2 yields were obtained in the media other than basal medium, it can be thought that although the optimum pH level for growth of *Actinoplanes* sp. ATCC 33076 is between 7.00-7.20, the optimal pH level for ramoplanin production

may be higher than the optimum pH level required for growth. Moreover, although the growths of the strain were limited in meat-bone meal medium, poultry meal medium and fish meal medium, specific productivities enhanced. The increased productivities could have been due to increased ammonia levels leading the activation of biosynthesis of ramoplanin precursors such as ornithine.

3.1.3 Variations in Total Protein Levels of Mediums Depending on Nitrogen Source with Respect to Incubation Period

As can be seen from Figure 3.3, extracellular protein levels in all media increased up to 7th day, and then decreased.

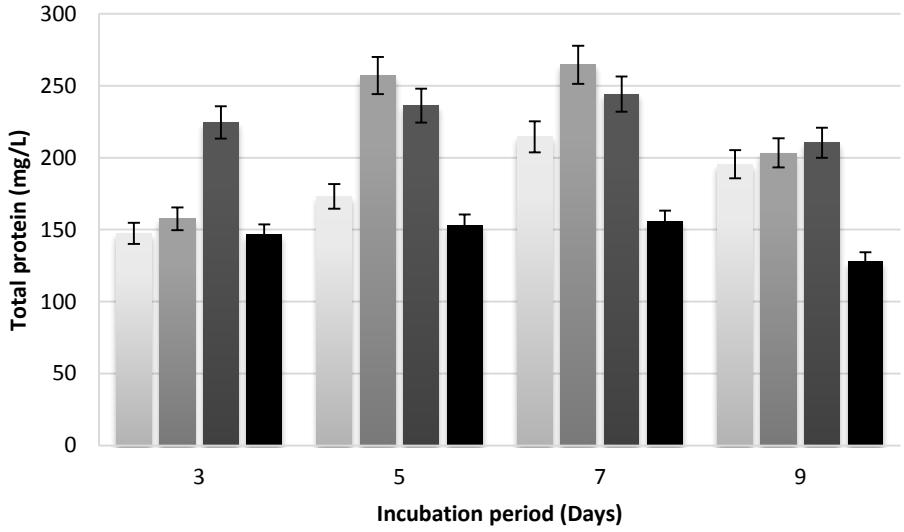

Figure 3.3 Total protein levels depending on nitrogen source containing growth medium conditions; basal medium (○), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

Extracellular protein levels in basal medium were generally very low compared to meat-bone meal medium and poultry meal medium. Low ramoplanin A2 yields of basal medium implied that degraded proteins were used in primary metabolism processes such as biomass production rather than ramoplanin A2 production.

Meat-bone meal medium resulted in high levels of extracellular protein. However, ramoplanin A2 production was not stimulated enough in meat-bone meal medium compared to poultry meal medium or fish meal medium.

Extracellular protein levels in poultry meal medium were remained stable throughout the incubation period. This medium resulted in increasing levels of ramoplanin A2 depending on the prolonged incubation period. However, high levels of extracellular protein did not stimulate the production in early days of the incubation period.

Similar to poultry meal medium, extracellular protein levels in fish meal medium were also remained stable throughout the incubation period, and the values were generally close to basal medium values until 5^{th} day. Ramoplanin A2 production in this medium was also limited and similar on 3^{rd} and 5^{th} days. However, independent from extracellular protein levels, ramoplanin A2 production was significantly stimulated in this medium depending on the prolonged incubation period.

When all these results are evaluated, no correlation between ramoplanin A2 production and protein levels of different nitrogen sources was observed, which indicated that the amino acid content of nitrogen source was more effective in ramoplanin A2 biosynthesis compared to extracellular protein levels.

3.1.4 Variations in Total Reducing Sugar Levels of Mediums Depending on Nitrogen Source with Respect to Incubation Period

As depicted in Figure 3.4, extracellular reducing sugar levels decreased in each medium as the incubation period was prolonged. High reduction of reducing sugars was observed in basal medium which was the medium that supported the maximum growth, but low ramoplanin A2 levels. On the other hand, moderate decreases were observed in reducing sugar levels of the other media compared to basal medium. According to the results, glucose in the culture medium was consumed continually during the incubation period. This situation is presumably by the results of an

increase in the intracellular glucose consumption rate in order to maintain energy metabolism (Ayar-Kayali & Tarhan, 2006). Also, ramoplanin A2 levels decreased significantly in all media after 7th day of the incubation period, which could be due to the significant decreases in total reducing sugar levels. These results indicated that the limitation of carbon sources have an important influence on ramoplanin A2 production. It is known that easily metabolized carbon sources favour growth and inhibit secondary metabolism (Oliveira et al., 2009).

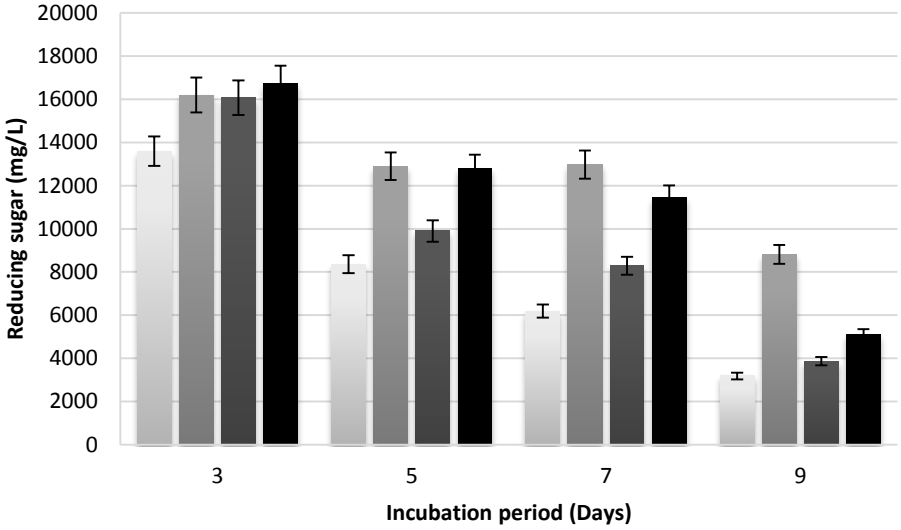

Figure 3.4 Total reducing sugar levels depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

3.1.5 Variations in Extracellular Protease Activities Depending on Nitrogen Source with Respect to Incubation Period

Proteolytic activities determined on each media throughout the incubation period, which indicated that all nitrogen sources could be degraded by *Actinoplanes* sp. ATCC 33076. Basal medium resulted in high proteolytic activities, and the highest protease activity was determined in this medium on 9th day (Figure 3.5), although protein levels did not change significantly (Figure 3.3). It seemed that degraded protein was sustained for primary metabolism processes such as biomass production rather than ramoplanin A2 production, which caused low protein levels in the

medium despite high proteolytic activities. On the contrary, high proteolytic activity supported erythromycin biosynthesis in recombinant *Saccharopolyspora erythraea* (Zou et al., 2011).

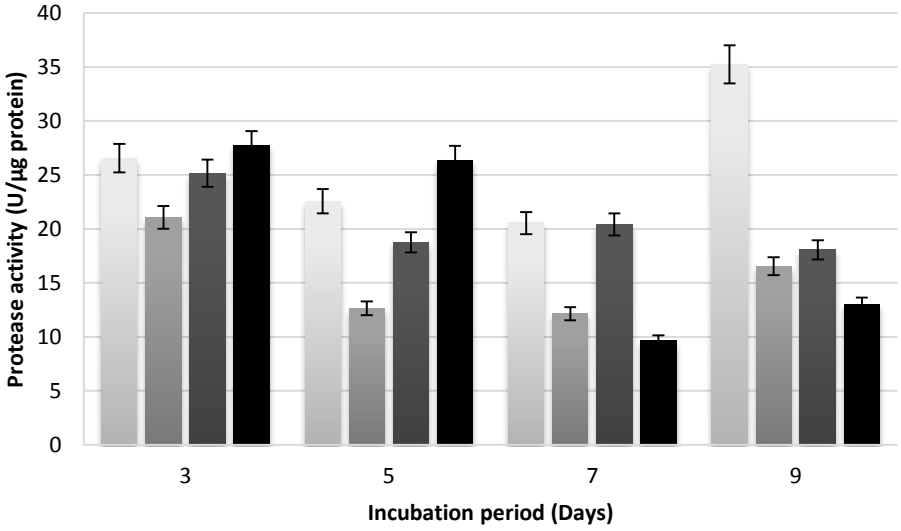

Figure 3.5 Extracellular protease activities depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

Considering both proteolytic activities and total protein levels, it can be implied that the protease activities were regulated by excessive protein levels especially in the culture media of meat-bone meal medium and poultry meal medium, which higher ramoplanin A2 levels were obtained.

3.1.6 Variations in Extracellular Amylase Activities Depending on Nitrogen Source with Respect to Incubation Period

According to the results depicted in Figure 3.6, extracellular amylase activities displayed varied profiles in different culture media. Amylase activities in basal medium and poultry meal medium increased depending on the prolonged incubation period, whereas it was vice versa in meat-bone meal medium. On the other hand, any significant changes were determined in fish meal medium.

The highest amylase activities were determined in basal medium. Considering that low ramoplanin A2 production, low reducing sugar levels, and high levels of growth were determined in basal medium, amylase activity results also supported that carbon sources were directed to primary metabolism rather than secondary metabolism. These results also indicated the importance limitation of carbon sources on antibiotic production. Similar to our results, Zou et al. (2011) determined the lowest amylase activity at the concentration of soybean flour that supported the highest erythromycin A production.

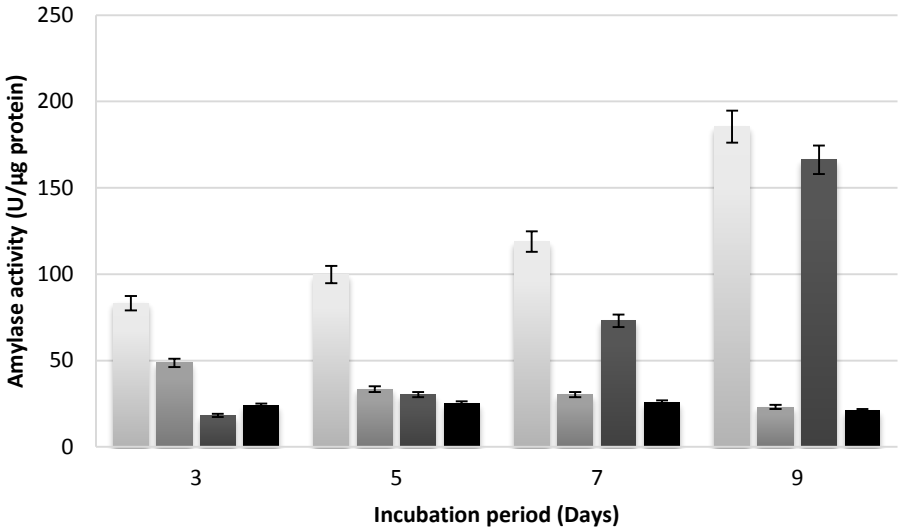

Figure 3.6 Extracellular amylase activities depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

3.1.7 Variations in Extracellular Lipase Activities Depending on Nitrogen Source with Respect to Incubation Period

As can be seen in Figure 3.7, ramoplanin producing strain *Actinoplanes* sp. ATCC 33076 displayed lipolytic activity. Extracellular lipase activities in basal medium remained stable after 3rd day. The maximal levels of lipase activities in meat-bone meal medium and poultry meal medium were determined on 7th day, and then lipase activities decreased on 9th day. Since lipid content of poultry meal medium was especially higher compared to the other media, it seemed that *Actinoplanes* sp.

ATCC 33076 used the lipids in this medium as the easily used carbon sources depleted. On contrary to meat-bone meal medium and poultry meal medium, lipase activities in fish meal medium decreased regularly as the incubation period was prolonged.

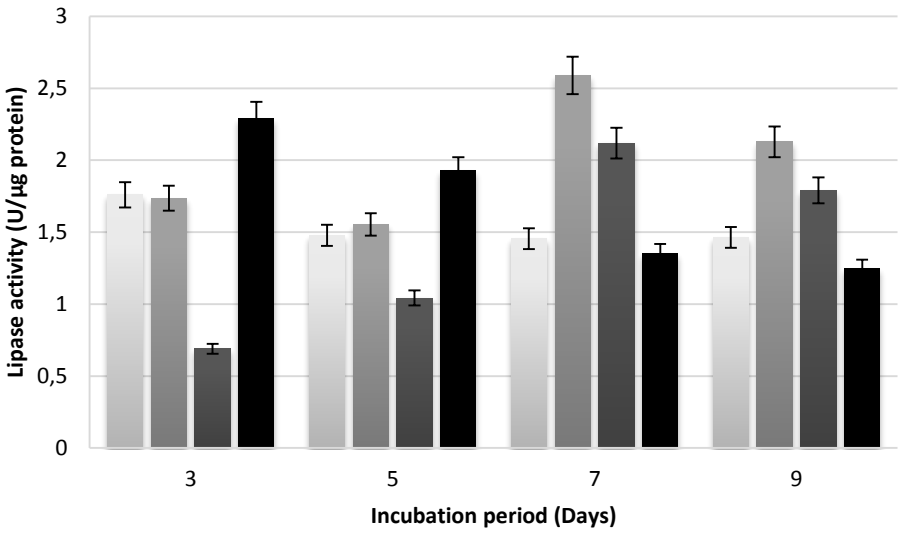

Figure 3.7 Extracellular lipase activities depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

3.1.8 Variations in Intracellular Free Amino Acid Levels Depending on Nitrogen Source with Respect to Incubation Period

Intracellular free amino acid levels were determined in order to investigate the influence of the amino acids presented in ramoplanin structure on biosynthesis of this antibiotic by *Actinoplanes* sp. ATCC 33076.

As shown in Figure 3.8, L-Glu levels were very high in all media compared to the other tested amino acids, and the highest L-Glu level was determined in meat-bone meal medium on 5th day. The low level of L-Glu in fish meal medium on 3rd day may be responsible for the low ramoplanin A2 level measured in this medium compared to those determined in the other media.

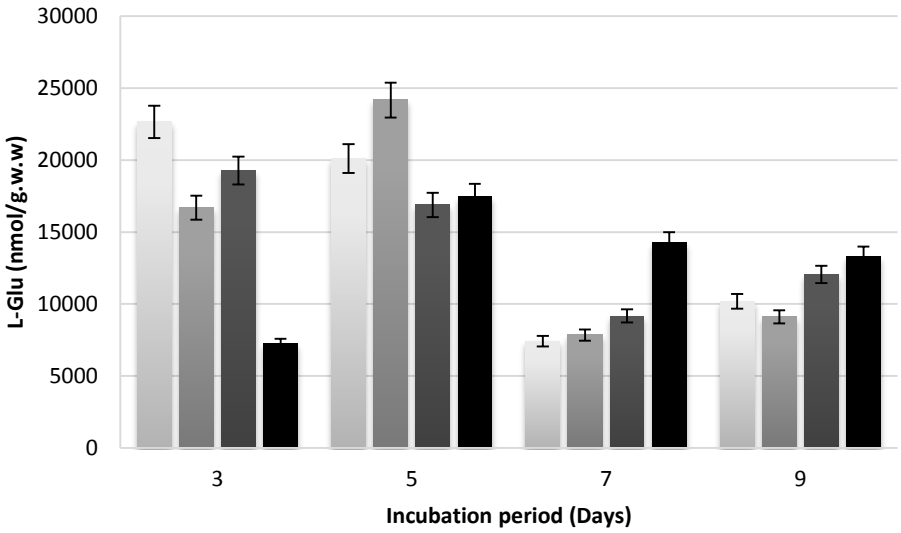

Figure 3.8 Intracellular levels of L-Glutamic acid depending on nitrogen source containing growth medium conditions; basal medium (●), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

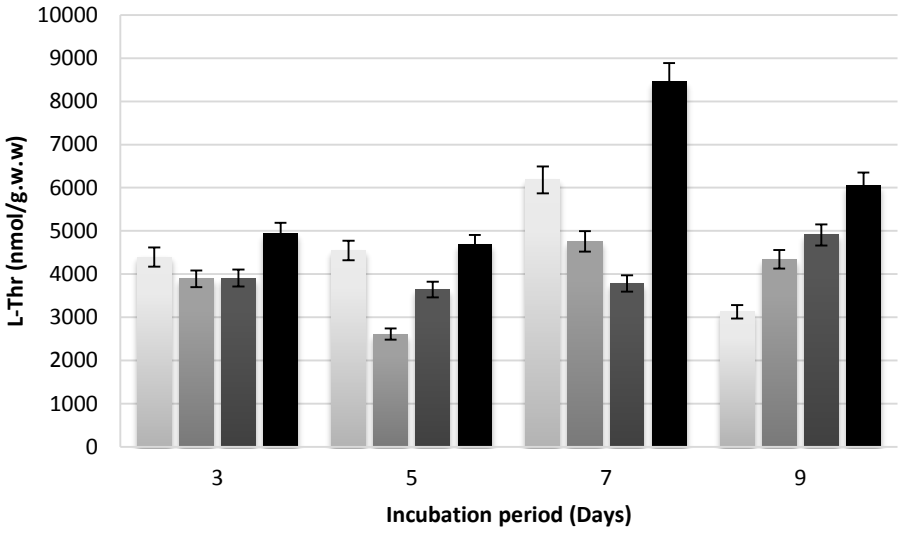

Figure 3.9 Intracellular levels of L-Threonine depending on nitrogen source containing growth medium conditions; basal medium (●), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

As shown in Figure 3.9, the second highest amino acid in *Actinoplanes* sp. ATCC 33076 was L-Thr in general. Throughout the incubation period, the intracellular L-Thr levels in basal medium were higher than those measured in other media.

However, low ramoplanin A2 levels obtained from basal medium indicated that L-Thr was not a limiting factor for ramoplanin A2 production.

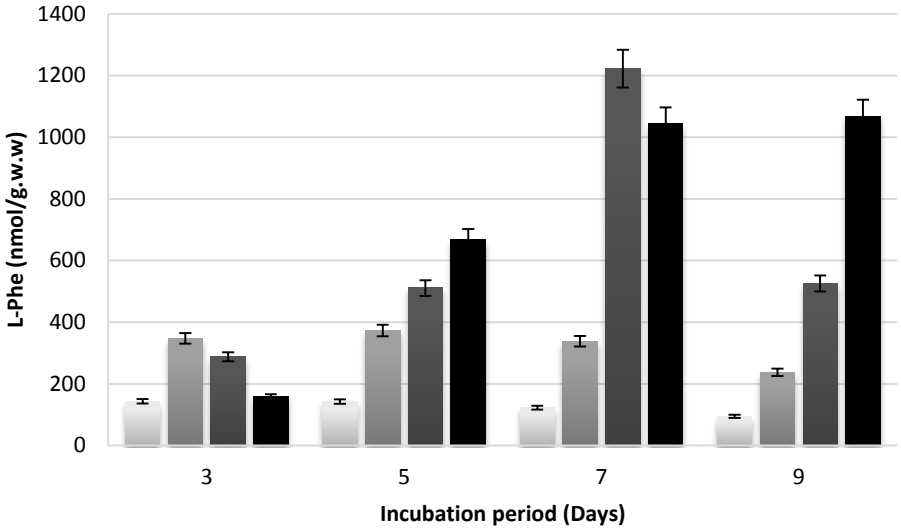

Figure 3.10 Intracellular levels of L-Phenylalanine depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

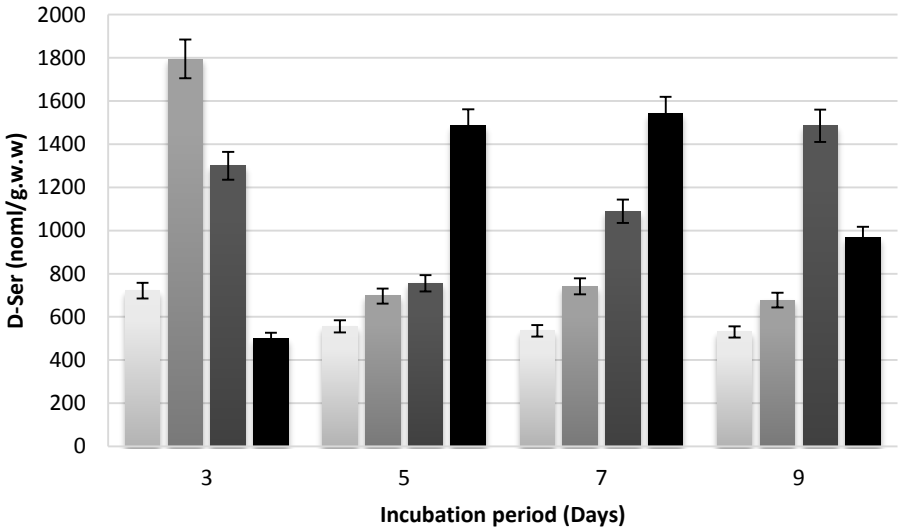

Figure 3.11 Intracellular levels of D-Serine depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

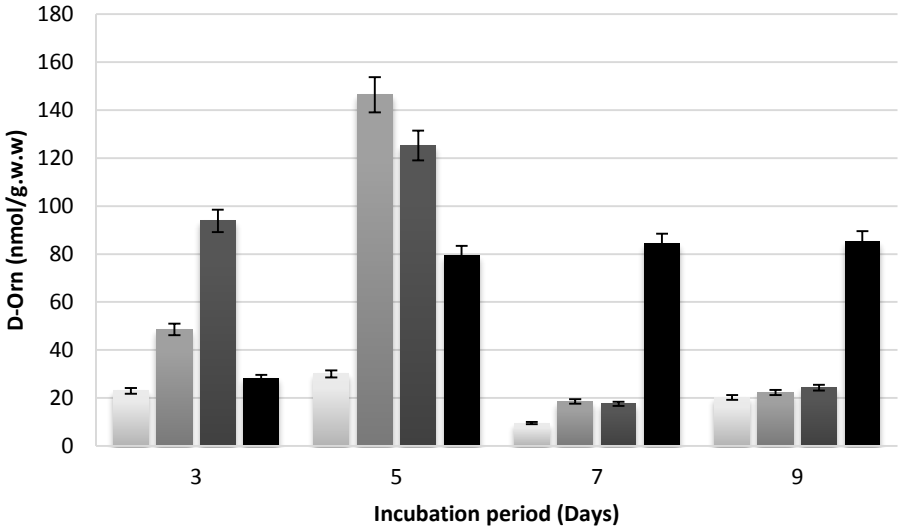

Figure 3.12 Intracellular levels of D-Ornithine depending on nitrogen source containing growth medium conditions; basal medium (●), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

On 3rd day, the intracellular L-Phe (Figure 3.10), D-Ser (Figure 3.11) and D-Orn (Figure 3.12) levels in meat-bone meal medium and poultry meal medium were found higher than those found in basal medium and fish meal medium. However, the levels of these amino acids generally increased in fish meal medium on the following days of incubation period. Moreover, ramoplanin A2 levels obtained from the culture broths of meat-bone meal medium and poultry meal medium were considerably higher than those of basal medium and fish meal medium by the end of 3rd day. It seemed that high intracellular levels of L-Phe, D-Ser and D-Orn amino acids could have supported ramoplanin A2 production despite the observed insignificant changes in intracellular levels of L-Thr (Figure 3.9), L-Leu (Figure 3.13) and Gly (Figure 3.14) amino acids. Parallel to the high D-Orn levels measured in meat-bone meal medium and poultry meal medium on 3rd day, and in fish meal medium on 5th day, it was also observed that pH levels increased (Figure 3.2). The changes in amino acid and pH levels are likely to have been caused by the induction of ornithine biosynthesis via α-ketoglutarate and glutamate formation due to the high ammonia levels. In clavulanic acid biosynthesis by *Streptomyces clavuligerus*, ornithine feeding was shown to have significant effects on production (Chen et al., 2003). Also, the remarkable increases in intracellular levels of L-Leu on 7th and 9th days

could have supported ramoplanin A2 production in fish meal medium. All these results indicated that in case of intracellular levels of L-Leu, L-Thr and Gly are sufficient, L-Phe, D-Ser and D-Orn amino acids play an inductive role on ramoplanin A2 production.

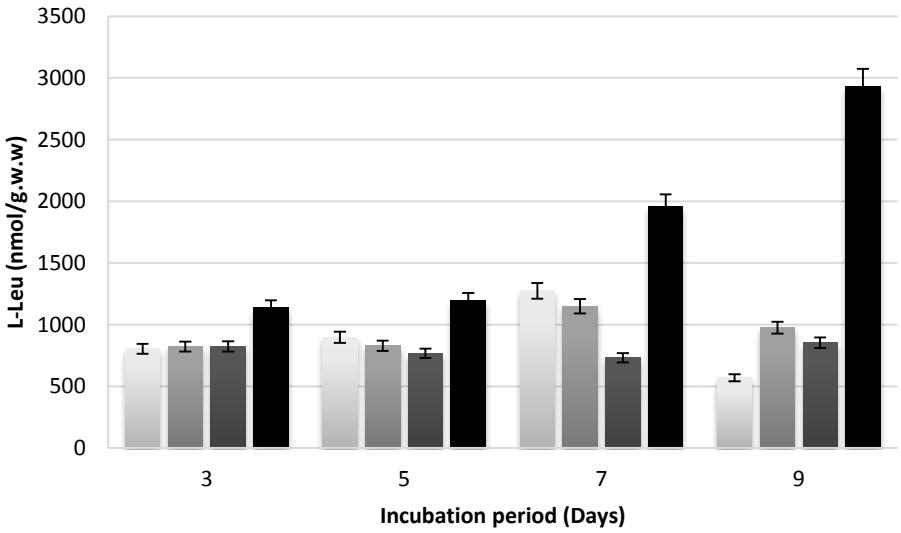

Figure 3.13 Intracellular levels of L-Leucine depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

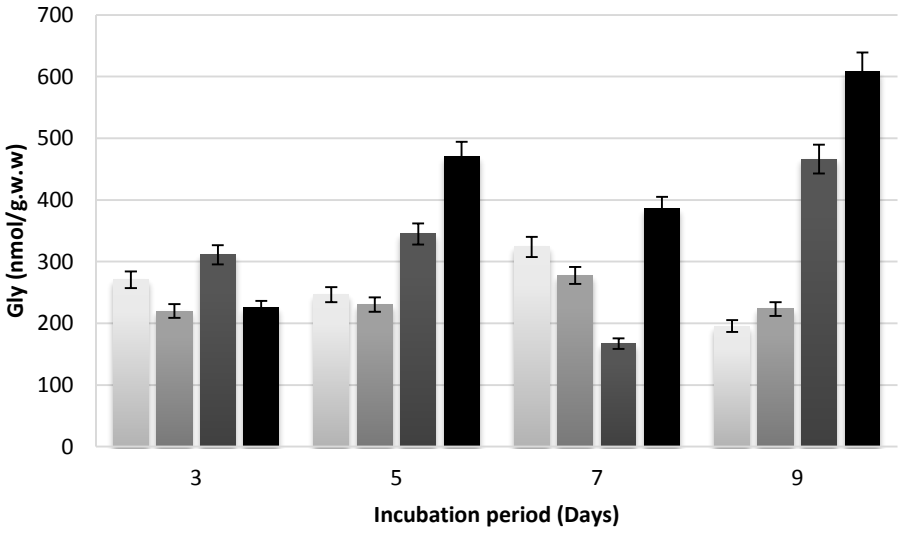

Figure 3.14 Intracellular levels of Glycine depending on nitrogen source containing growth medium conditions; basal medium ( ), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

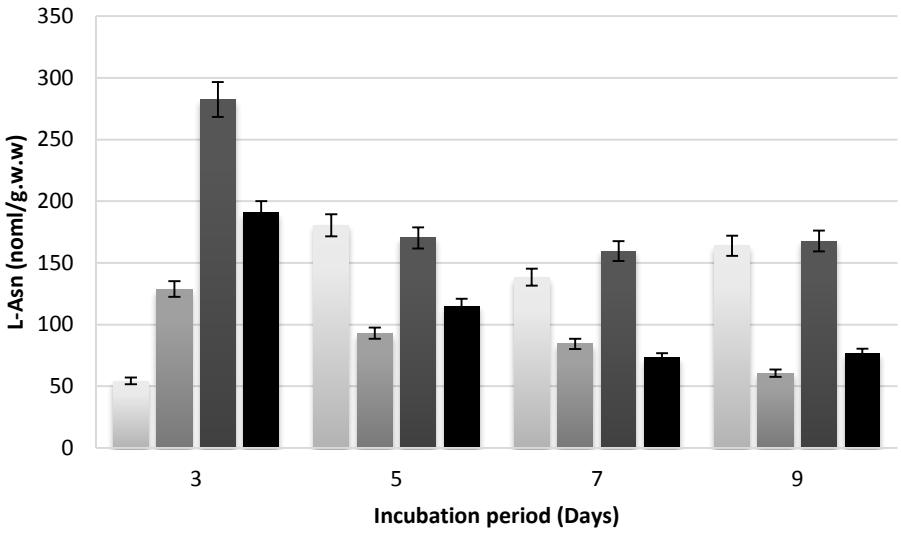

Figure 3.15 Intracellular L-Asparagine levels depending on nitrogen source containing growth medium conditions; basal medium (●), meat-bone meal medium (●), poultry meal medium (●), and fish meal medium (●).

On the other hand, throughout the incubation period, the intracellular L-Phe (Figure 3.10), D-Orn (Figure 3.12) and L-Asn (Figure 3.15) levels in basal medium was significantly low compared to the other media, which displayed correlation with the lowest ramoplanin A2 levels. These results also indicated the importance of both L-Phe and D-Orn amino acids on ramoplanin A2 production. But, no correlation was observed between the intracellular L-Asn levels and ramoplanin A2 levels, which implied that L-Asn was not an effective precursor for ramoplanin A2 production.

The correlation between the intracellular levels of L-Phe and L-Leu amino acids and ramoplanin A2 yields on 5th and 7th days indicated that these amino acids are both important precursors of ramoplanin A2. It has already been known that L-Leu is the precursor of ramoplanin A2 fatty acid, and the inductive effect of L-Leu on production of ramoplanin A2 was shown by Brunati et al. (2005). L-Leu was considered to be a limiting factor which increased the specific productivity of ramoplanin A2 by the producer strain. Similarly, leucine addition in fermentation medium of *Actinoplanes teichomyceticus* supported the formation of teicoplanin components teicoplanin A2-2, A2-5 and A2-4 (Borghi et al., 1991).

3.2 Variations in Metabolite Levels of *Actinoplanes* sp. ATCC 33076 Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

Trace metals are essential for microbial growth and secondary metabolism of actinobacteria as well as some other prokaryotic taxa and fungal groups due to their involvement in activation of some genes and/or enzymes of biosynthetic pathways (Běhal, 1986). Therefore, evaluation of the metabolic response of microorganisms to a diversity of trace metals has been a widely used approach to enhance production of secondary metabolites such as antibiotics. For example, it is known that zinc, iron and manganese are important trace elements for actinomycetes, whereas calcium and copper usually do not affect their secondary metabolism.

In this thesis, the effects of iron, potassium, manganese, magnesium and zinc metals on ramoplanin A2 production by *Actinoplanes* sp. ATCC 33076 were investigated.

3.2.1 Variations in Ramoplanin Levels Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

According to the results, ramoplanin A2 production increased until 7^{th} day of the incubation period, and then decreased in all experiments.

It has been shown that there is generally a linear relationship between iron concentration and secondary metabolite formation in actinomycetes (Shapiro, 1989). However, our results indicated that iron ion was not an important stimulating factor for ramoplanin A2 production although, some higher yields were obtained compared to basal medium. Low concentrations of iron ions did not have significant influences on ramoplanin A2 yields between 3^{rd} and 7^{th} days. As the incubation period was prolonged, higher yields were obtained in the presence of 45 and 75 ppm of Fe^{2+}, and yields were conspicuously higher compared to basal medium on the last day of the incubation period. The highest ramoplanin A2 levels compared to basal medium were obtained in 75 ppm Fe^{2+} containing medium throughout the incubation period.

The maximum ramoplanin A2 level was determined as 103.855 mg L^{-1} on 7th day (1.248 fold of basal medium) (Figure 3.16).

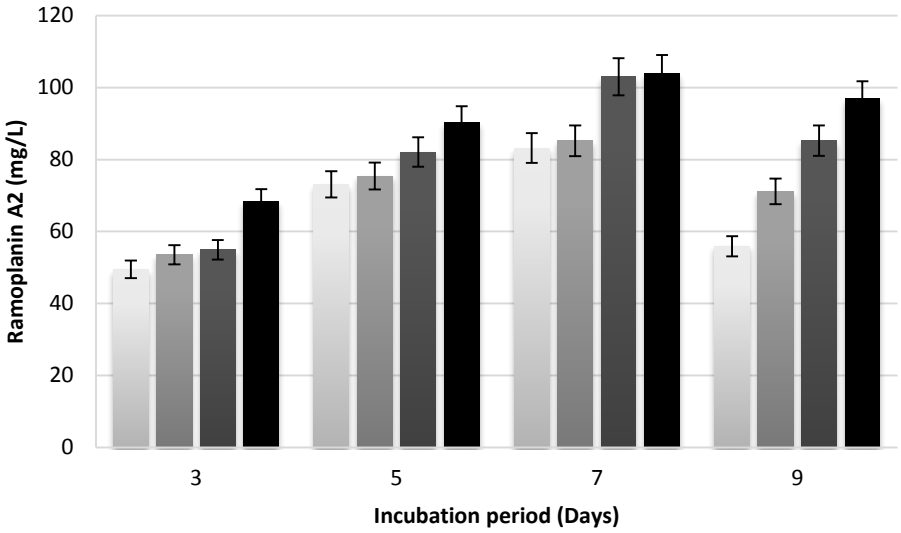

Figure 3.16 Ramoplanin production depending on iron metal containing growth medium conditions; basal medium ( ), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

Wu et al. (2008) reported that addition of 0.001% $FeSO_4.7H_2O$ to fermentation medium enhanced the ramoplanin production by mutant strain *Actinoplanes* sp. C6-78-14 10% compared with the control group of strain.

Similar to our results, Fe^{2+} did not substantially affect A40926 production by *Nonomuraea* sp. DP-13 (Chen et al., 2015). Depending on the iron concentration, our results were in accordance with the studies revealing that the increasing concentrations of iron enhanced the production of antibiotics zwittermicin A by *Bacillus cereus* UW85 (Milner et al., 1995) and surfactin by *Bacillus subtilis* ATCC 21332 (Wei et al., 2004). On the contrary, iron was shown to stimulate the production of various antibiotics including an antifungal antibiotic by *Streptomyces galbus* (Paul & Banerjee, 1983), the lipopeptide antibiotic iturin A by *Bacillus amyloliquefaciens* B128 (Lin et al., 2008), and the fusaricidin-type antifungal antibiotic by *Paenibacillus polymyxa* SQR-21 (Raza et al., 2009).

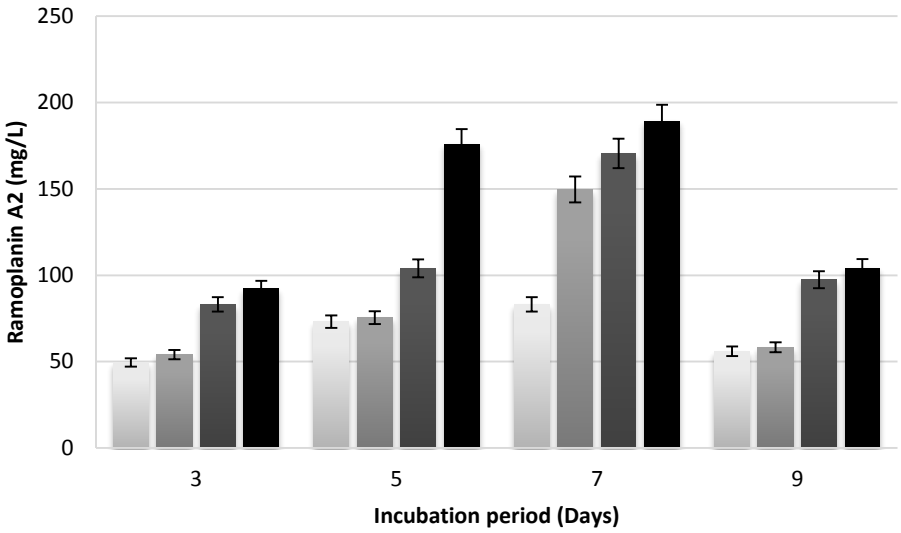

Figure 3.17 Ramoplanin production depending on potassium metal containing growth medium conditions; basal medium ( ), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

As shown in Figure 3.17, ramoplanin A2 production increased depending on the increasing potassium concentration during the incubation period. Remarkable increases in ramoplanin A2 production were observed on 5^{th} day in 75 ppm K_2HPO_4 containing medium, and on 7^{th} day in 15 and 45 ppm K_2HPO_4 containing media. Maximum ramoplanin A2 was determined in 75 ppm K_2HPO_4 containing medium on 7th day. Interestingly, ramoplanin A2 production was stimulated in 75 ppm K_2HPO_4 containing medium on 5^{th} day, and ramoplanin A2 levels almost remained stable on 7^{th} day. 175.894 mg L^{-1} (2.407 fold of basal medium) and 189.262 mg L^{-1} (2.275 fold of basal medium) ramoplanin A2 were produced on 5^{th} and 7^{th} days of the incubation period, respectively. After 7^{th} day, significant decreases were observed in all concentrations of potassium ion containing media.

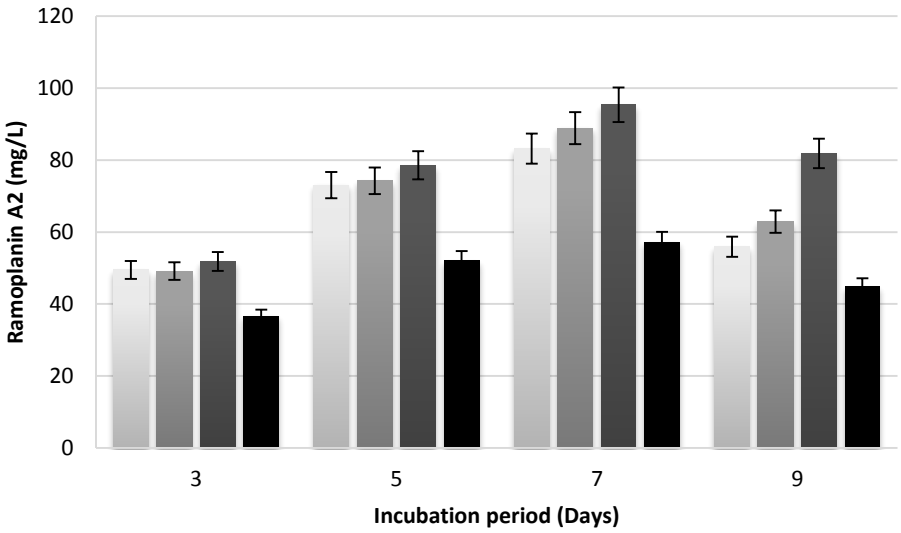

Figure 3.18 Ramoplanin production depending on magnesium metal containing growth medium conditions; basal medium ( ), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

Ramoplanin A2 production was not stimulated enough in the presence of magnesium ions (Figure 3.18), since magnesium ions are required for bioenergetic and metabolic processes such as pH homeostasis and ATP synthesis. In fact, magnesium ion stimulated growth of the strain. Ramoplanin A2 production was similar in the presence of 15 ppm Mg^{2+} on 3^{th} and 5^{th} days. The highest ramoplanin A2 levels were obtained in 45 ppm Mg^{2+} containing medium. Ramoplanin A2 was determined as 95.387 mg L^{-1} (1.146 fold of basal medium) on 7^{th} day in 45 ppm Mg^{2+} containing medium. In correlation with our results, 2 mM of $MgSO_4.7H_2O$ had no noticeable effect on iturin A production by *Bacillus amyloliquefaciens* B128 (Lin et al., 2008), whereas ansamitocin P-3 production by *Actinosynnema pretiosum* was improved 3 fold by addition optimal concentrations of magnesium (Jia & Zhong, 2011). Magnesium was also shown to enhance biomass production of *Streptomyces clavuligerus* DSM 738 in clavulanic acid biosynthesis (Hamedi & Goomeshi Nobary, 2013).

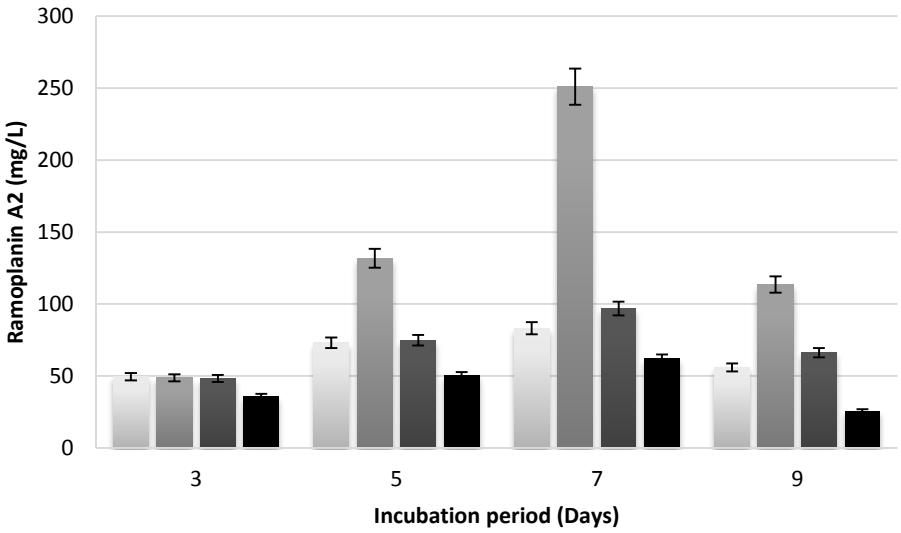

Figure 3.19 Ramoplanin production depending on manganese metal containing growth medium conditions; basal medium ( ), 15 ppm $MnCl_2.4H_2O$ (●), 45 ppm $MnCl_2.4H_2O$ (●), and 75 ppm $MnCl_2.4H_2O$ (●).

As can be seen in Figure 3.19, ramoplanin A2 production was induced in the presence of 15 ppm Mn^{2+}. Starting from 5th day, ramoplanin A2 levels of 15 ppm Mn^{2+} containing medium were significantly higher compared to basal medium. Ramoplanin A2 levels in 15 ppm Mn^{2+} containing medium were 131.728 mg L^{-1} (1.803 fold of basal medium), 251.073 mg L^{-1} (3.018 fold of basal medium), and 113.485 mg L^{-1} (2.029 fold of basal medium) on 5th, 7th and 9th days, respectively. On the other hand, ramoplanin A2 production was inhibited by the increasing concentrations of manganese ions. Ramoplanin A2 yields of 75 ppm Mn^{2+} containing medium and basal medium were on similar levels throughout the incubation period. Using 75 ppm Mn^{2+} resulted in significant inhibition of ramoplanin A2 biosynthesis. Moreover, manganese was the only trace metal that used in its chloride salt form in our experiments. Increasing concentrations of $MnCl_2$ did not affect A40926 production by *Nonomuraea* sp. DP-13 (Chen et al., 2015). Negative effect of metal chloride salts including $MnCl_2.4H_2O$ in clavulanic acid production by *Streptomyces clavuligerus* DSM 738 was shown by Hamedi & Goomeshi Nobary (2013). However, using 0.42 mg L^{-1} $MnSO_4.H_2O$ was shown to enhance production of clavulanic acid in the same study. Our results indicated that manganese ion was an important stimulating factor for ramoplanin A2 production, although we used it in its

chloride salt form. The promoting effects of manganese ions were also shown in the antifungal antibiotic production by *Streptomyces galbus* (Paul & Banerjee, 1983), and in ansamitocin P-3 production by *Actinosynnema pretiosum* (Jia & Zhong, 2011).

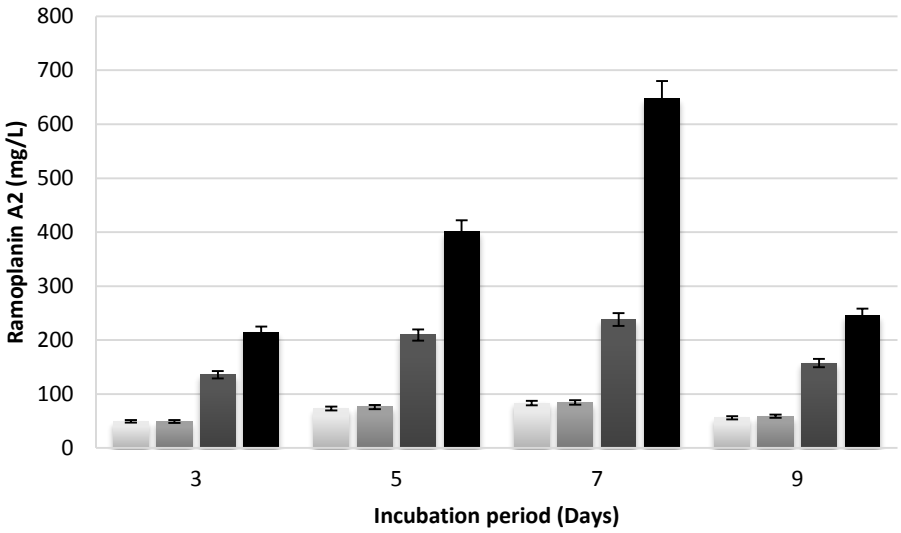

Figure 3.20 Ramoplanin production depending on zinc metal containing growth medium conditions; basal medium (○), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

The highest ramoplanin A2 production was obtained in zinc ion containing media among all media, and ramoplanin A2 production increased depending on the increasing zinc concentrations. As shown in Figure 3.20, 45 and 75 ppm Zn^{2+} containing media resulted in significantly stimulated ramoplanin A2 production. The most significant changes were observed in 75 ppm Zn^{2+} containing medium, and the maximum ramoplanin A2 was obtained on 7^{th} day as 647.415 mg L^{-1} ramoplanin A2 with a 6.782 fold increase compared to basal medium. Ramoplanin A2 levels in 75 ppm Zn^{2+} containing medium were 214.519 mg L^{-1} (4.334 fold), 401.843 mg L^{-1} (5.500 fold), and 245.940 mg L^{-1} (4.398 fold) on 3^{rd}, 5^{th}, and 9^{th} days, respectively. Increasing concentrations of zinc ion in the fermentation media could have increased ramoplanin A2 yields either by affecting the enzymes involved in its biosynthesis and pools of precursors, or by decreasing the ramoplanin A2 degradation. Zinc ion also enhanced the production of antifungal antibiotic by *Streptomyces galbus* (Paul & Banerjee, 1983). On the other hand, $ZnSO_4.7H_2O$ was shown to inhibit clavulanic

acid production by *Streptomyces clavuligerus* DSM 738 (Hamedi & Goomeshi Nobary, 2013), and $ZnSO_4$ did not cause significant changes in A40926 production by *Nonomuraea* sp. DP-13 (Chen et al., 2015).

Since *Actinoplanes* sp. ATCC 33076 take up manganase and zinc ions to meet the requirements for its secondary metabolism, optimization of the sources by feeding process may be one of the key points for successful production of ramoplanin A2.

3.2.2 Variations in pH Levels of Mediums Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

Culture pH is an important bioprocess parameter which affects a series of regulatory processes in different taxa of microorganisms, and has been reported to affect secondary metabolite production (Rao et al., 2006). Nitrogen sources and their depletion in culture medium play important role in regulating culture pH.

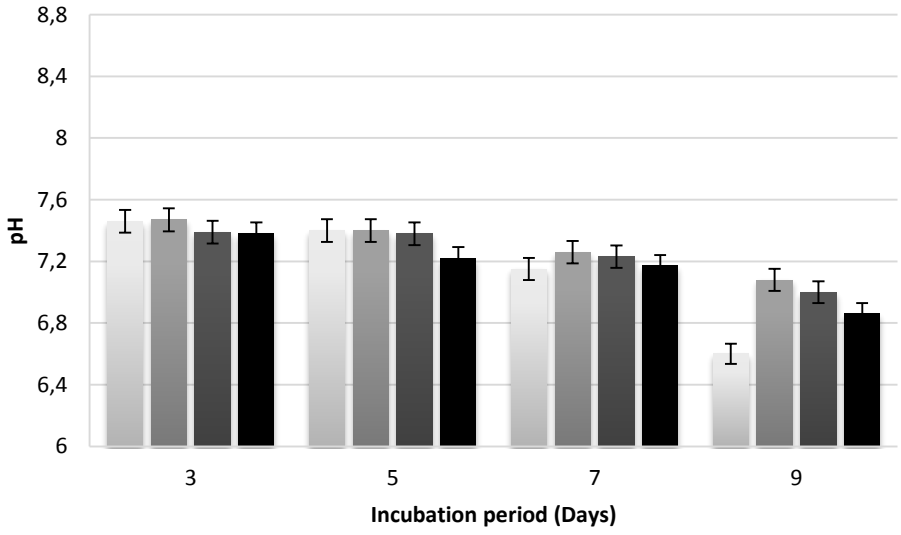

Figure 3.21 pH levels depending on iron metal containing growth medium conditions; basal medium ( ), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

According to the results depicted in Figure 3.21, pH levels of 15 ppm Fe^{2+} containing medium and basal medium were very close on 3rd day, and pH levels of these two media were the same on 5th day (7.40). Similarly, pH levels of 45 and 75

ppm Fe^{2+} containing media were very close on 3rd day. pH only changed 0.01 unit in 45 ppm Fe^{2+} containing medium on 5th day, therefore it was very close to pH levels of 15 ppm Fe^{2+} containing medium and basal medium. On the other hand, a 0.16 unit decrease was observed in 75 ppm Fe^{2+} containing medium on 5th day. On 7th and 9th days of the incubation period, pH levels decreased remarkably in all iron ion containing media. Moreover, it was observed that decreases in pH levels enhanced by the increasing iron ion concentration on 9th day. The results indicated that increasing iron ion concentration could have caused decreases in pH levels by the enhanced organic acid secretion (Ayar-Kayali & Tarhan, 2006).

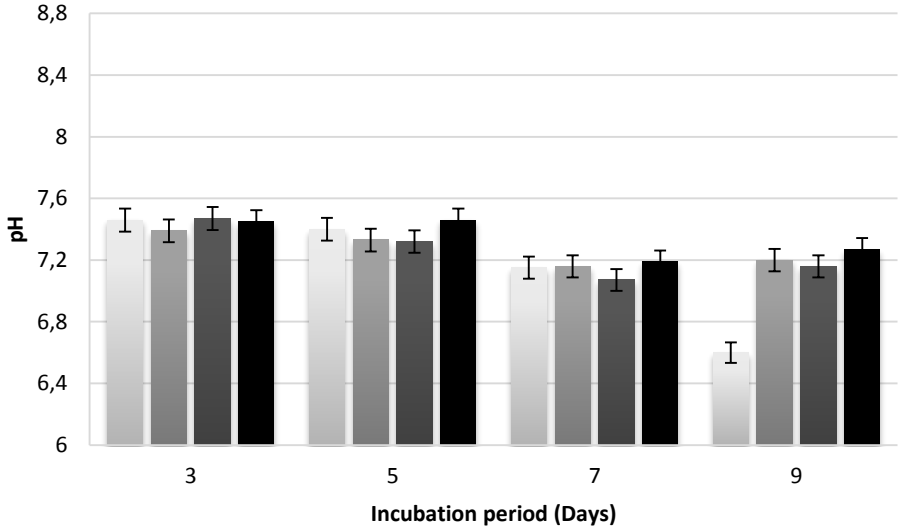

Figure 3.22 pH levels depending on potassium metal containing growth medium conditions; basal medium (○), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

pH levels of all concentrations of potassium ion containing media were quite parallel to pH levels of basal medium throughout the period, until 9th day (Figure 3.22). Slight increases lower than 0.10 unit in pH levels of all concentrations of K_2HPO_4 containing media were observed on 9th day. Decreases in pH levels of basal medium compared to K_2HPO_4 containing media on 9th day may suggest that the used potassium ion concentrations were important for the buffering of the medium (Bhunia et al., 2012).

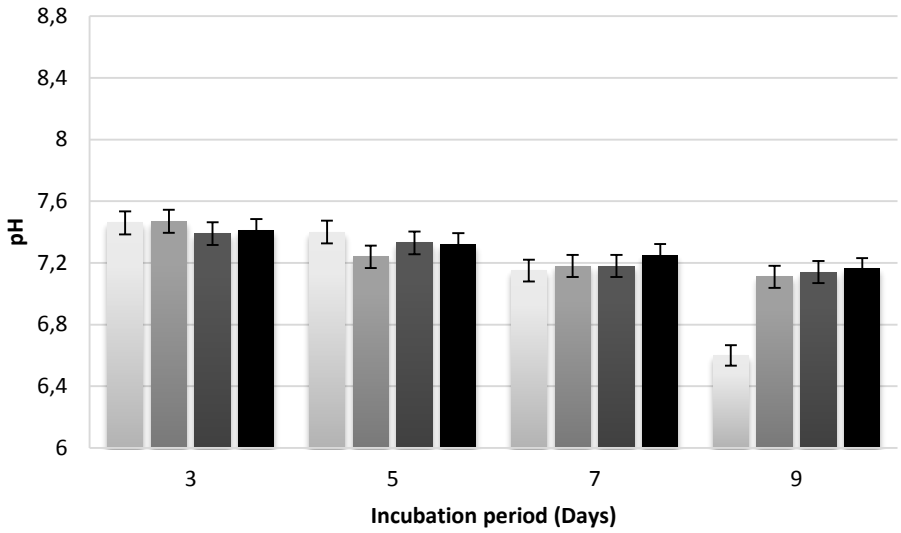

Figure 3.23 pH levels depending on magnesium metal containing growth medium conditions; basal medium (○), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

As shown in Figure 3.23, in contrast to sharper decreases observed in basal medium, smoother decreases were observed in all concentrations of magnesium ion containing media as the incubation period was prolonged. Moreover, changes in pH levels were quite similar. The results indicated that magnesium ions are required for metabolic processes of bacterium such as pH homeostasis (Bhunia et al., 2012).

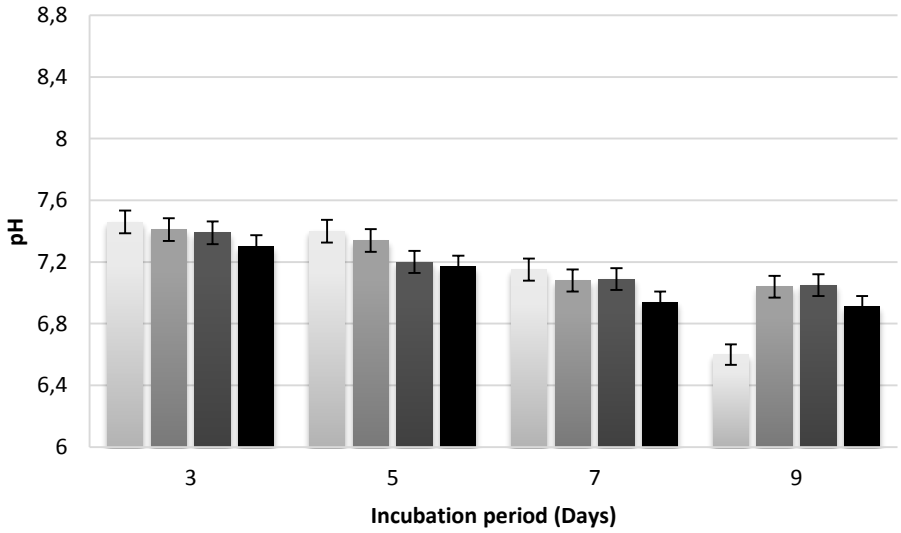

Figure 3.24 pH levels depending on manganese metal containing growth medium conditions; basal medium (○), 15 ppm $MnCl_2.4H_2O$ (●), 45 ppm $MnCl_2.4H_2O$ (●), and 75 ppm $MnCl_2.4H_2O$ (●).

pH levels of all concentrations of manganese ion containing media were lower than those of basal medium throughout the incubation period, except on 9th day. However, pH levels of all concentrations of manganese ion containing media almost remained stable after 7th day (Figure 3.24).

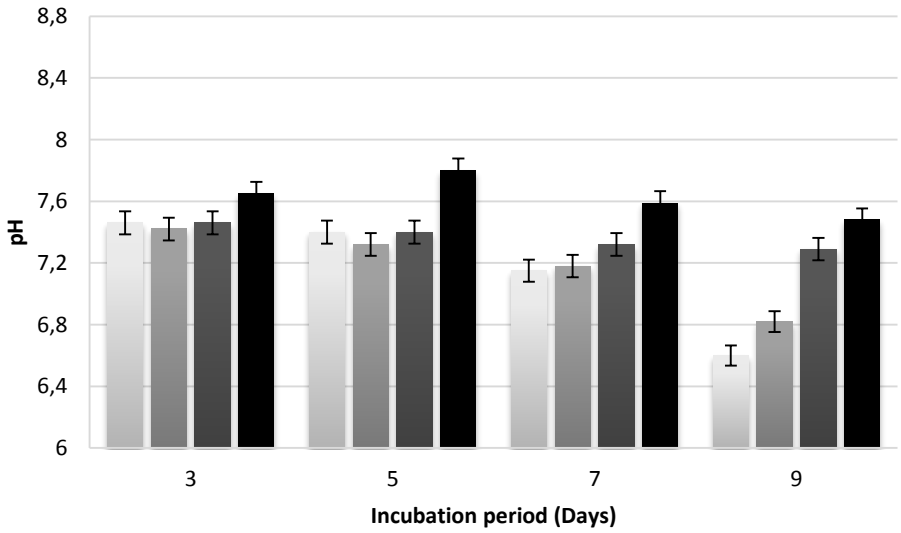

Figure 3.25 pH levels depending on zinc metal containing growth medium conditions; basal medium (○), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

According to the results depicted in Figure 3.25, pH levels increased depending on the increasing zinc ion concentrations, although the pH changes were generally very minor in 15 and 45 ppm Zn^{2+} containing media. pH levels of 15 ppm Zn^{2+} containing medium were generally very close to those of basal medium throughout the incubation period, except on 9th day (6.82 and 6.60 for 15 ppm Zn^{2+} containing medium and basal medium on 9th day, respectively). pH levels of 45 ppm Zn^{2+} containing medium and basal medium were interestingly the same on 3rd and 5th days, and pH levels of 45 ppm Zn^{2+} containing medium remained stable and higher than those of basal medium on following days of the incubation period. On the other hand, pH levels of 75 ppm Zn^{2+} containing medium were considerably higher than those of basal medium throughout the incubation period. Also, 75 ppm Zn^{2+} containing medium was the medium that provided the highest ramoplanin A2 yields. High levels of ammonia in this medium could have been contributed biosynthesis of ramoplanin precursors, and therefore enhancement of ramoplanin A2 production.

These results also supported the hypothesis that optimum pH level for ramoplanin A2 production is higher than the optimum pH level for growth of the strain which is between 7.00-7.20.

3.2.3 Variations in Total Protein Levels of Mediums Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

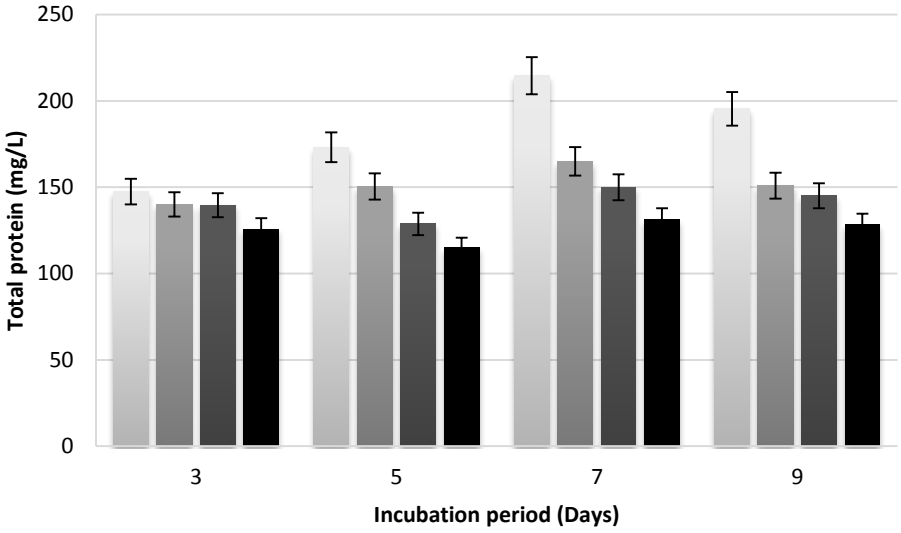

Figure 3.26 Total protein levels depending on iron metal containing growth medium conditions; basal medium (○), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

Extracellular protein levels in all concentrations of iron ion containing media were lower than those of basal medium throughout the incubation period, and extracellular protein levels decreased more and more depending on the increasing iron ion concentrations. Interestingly, the lowest values were determined in 75 ppm Fe^{2+} containing medium which was the medium that supported the highest ramoplanin A2 production (Figure 3.26).

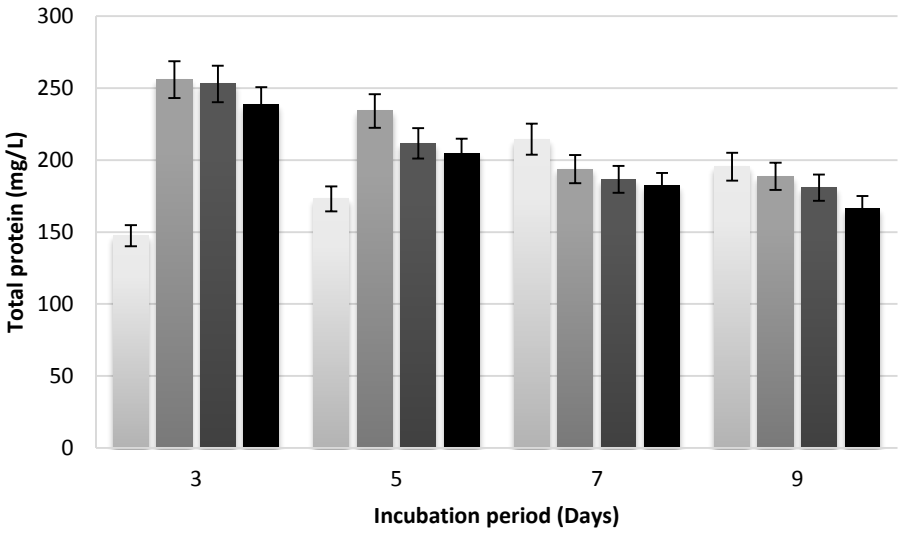

Figure 3.27 Total protein levels depending on potassium metal containing growth medium conditions; basal medium ( ), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

Extracellular protein levels in all concentrations of potassium ion containing media decreased as the incubation period was prolonged. On 3rd and 5th days, extracellular protein levels were significantly higher than those of basal medium, whereas it was vice versa on 7th and 9th days (Figure 3.27).

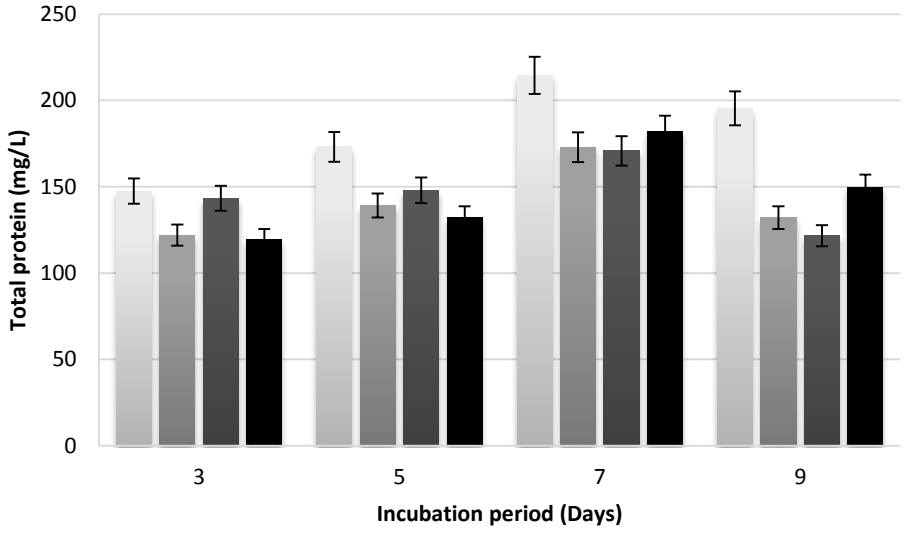

Figure 3.28 Total protein levels depending on magnesium metal containing growth medium conditions; basal medium ( ), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

Extracellular protein levels in all concentrations of magnessium ion containing media were lower than those of basal medium throughout the incubation period (Figure 3.28). Extracellular protein levels were very similar. On the other hand, both extracellular protein levels and ramoplanin A2 levels increased up to 7th day and then decreased in all concentrations of magnessium ion containing media, just as observed in basal medium. Condisering a high growth rate was observed in magnesium ion containing media, these results indicated that the degraded protein was preferentially used for primary metabolism activities like energy metabolism and biomass production.

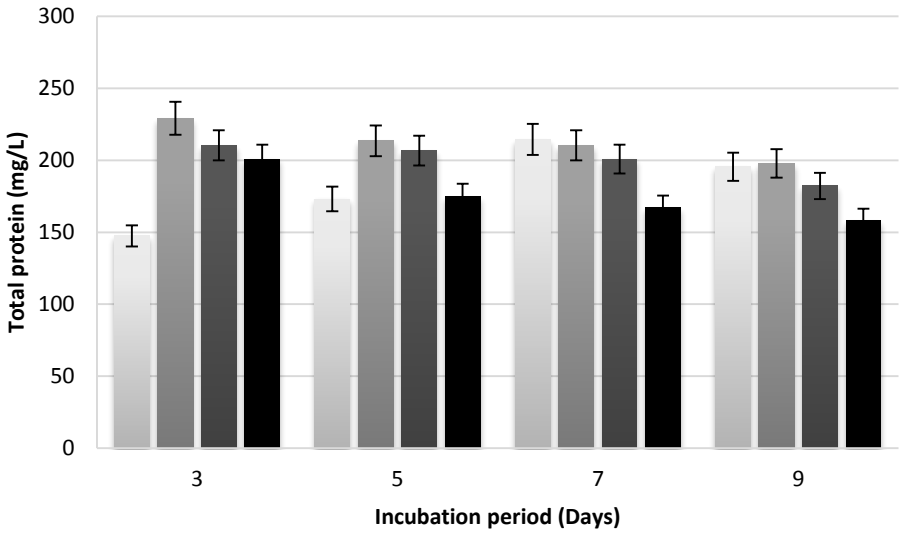

Figure 3.29 Total protein levels depending on manganese metal containing growth medium conditions; basal medium ( ), 15 ppm $MnCl_2.4H_2O$ (●), 45 ppm $MnCl_2.4H_2O$ (●), and 75 ppm $MnCl_2.4H_2O$ (●).

Extracellular protein levels decreased by the increasing manganese ion concentrations (Figure 3.29). Extracellular protein levels in 15 and 45 ppm Mn^{2+} containing media were high compared to basal medium on 3rd and 5th days. On the other hand, extracellular protein levels of basal medium were generally high compared to manganese ion containing media on 7th and 9th days. Considering that the highest ramoplanin A2 production was observed in 15 ppm Mn^{2+} containing medium, slightly high extracellular protein levels of this medium in comparison with

basal medium could have enhanced ramoplanin A2 production due to the limited intracellular uptake of protein source and the subsequent nitrogen starvation.

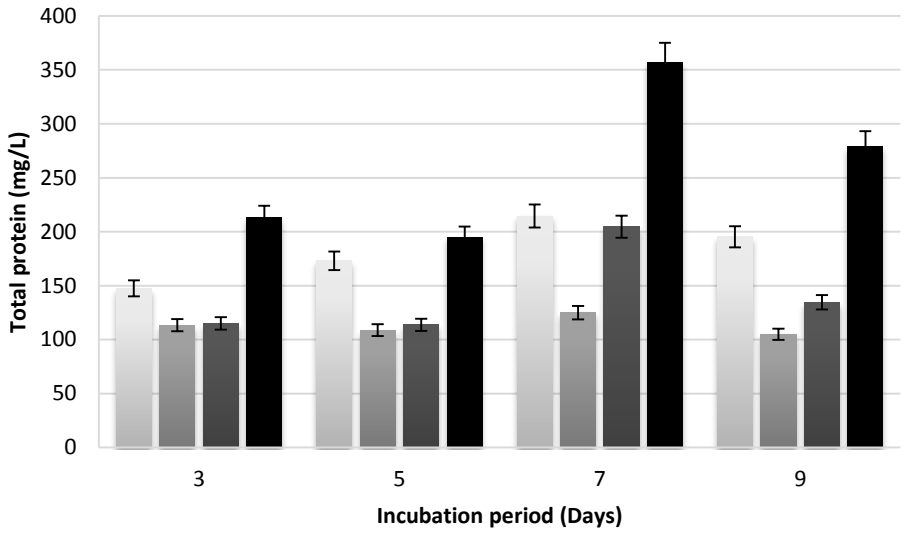

Figure 3.30 Total protein levels depending on zinc metal containing growth medium conditions; basal medium (○), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

Extracellular protein levels increased in correlation with the increasing zinc ion concentrations (Figure 3.30). Extracellular protein levels of 15 and 45 ppm Zn^{2+} containing media were significantly low compared to basal medium. It is notable that ramoplanin A2 production was slightly inhibited in 15 ppm Zn^{2+} containing medium. However, ramoplanin A2 levels were higher in 45 ppm Zn^{2+} containing medium compared to basal medium. The most remarkable results were obtained in 75 ppm Zn^{2+} containing medium. This medium was the one that provided the highest ramoplanin A2 production, and high levels of protein in this medium could have led the activation of ramoplanin A2 production due to the limited intracellular uptake of protein source and the subsequent nitrogen starvation.

3.2.4 Variations in Total Reducing Sugar Levels of Mediums Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

Availability of different carbon and energy sources have to be converted into metabolites of the central metabolism and then serve as fuel for energy conservation

or as precursors for the synthesis of secondary metabolites (Ayar-Kayali & Tarhan, 2006).

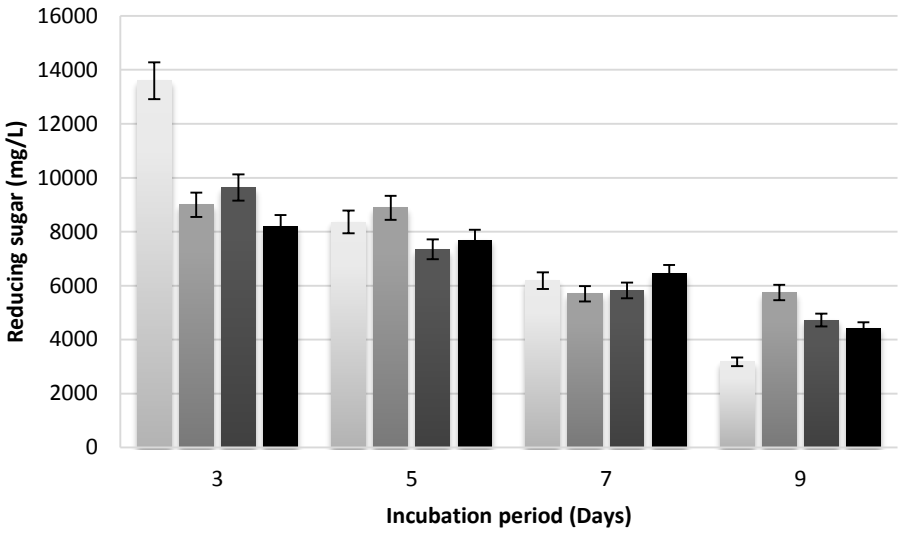

Figure 3.31 Total reducing sugar levels depending on iron metal containing growth medium conditions; basal medium ( ), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

As can be seen from Figure 3.31, extracellular reducing sugar levels of iron ion containing media decreased depending on the prolonged incubation period, and the values were similar regardless from iron ion concentrations. It seemed that carbon sources were consumed rapidly in the early days of log phase, and consumption almost remained stable after then.

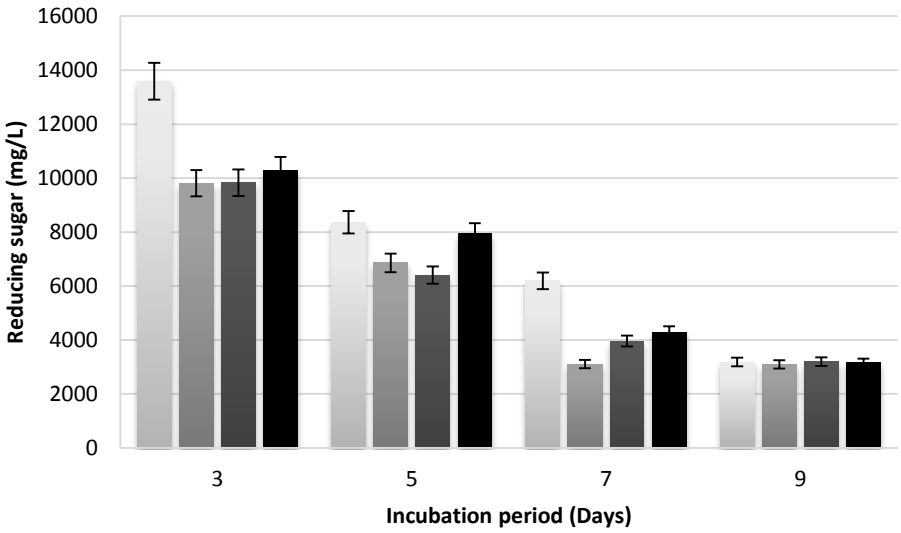

Figure 3.32 Total reducing sugar levels depending on potassium metal containing growth medium conditions; basal medium ( ), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

Extracellular reducing sugar levels of different concentrations of potassium ion containing media were lower than those of basal medium throughout the incubation period. Moreover, values were considerably low compared to basal medium on 3rd day, just as observed in iron ion containing medium (Figure 3.32).

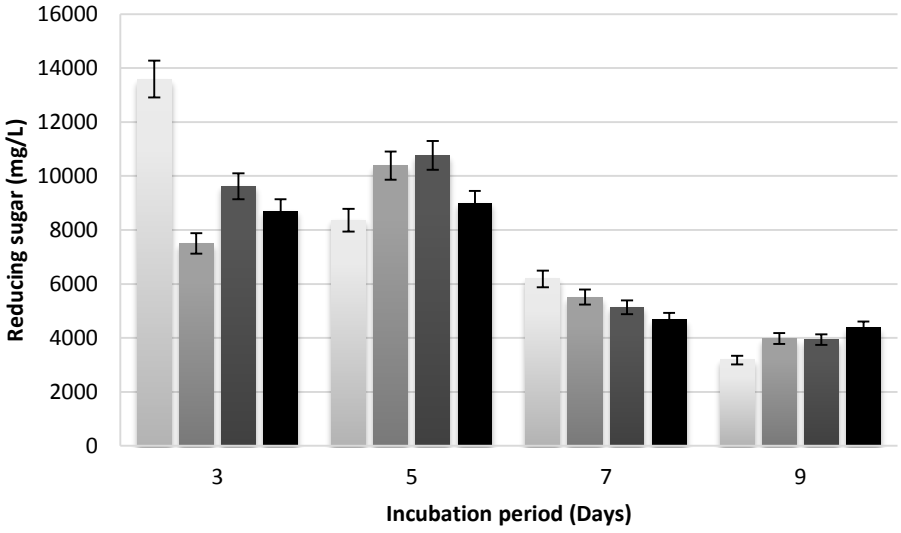

Figure 3.33 Total reducing sugar levels depending on magnesium metal containing growth medium conditions; basal medium ( ), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

Extracellular reducing sugar levels of different concentrations of magnesium ion containing media were lower than basal medium values on 3rd and 5th days. However, increases in extracellular reducing sugar levels of all magnesium ion containing media were observed on 5th day, and dramatic decreases were observed on 7th day (Figure 3.33). Decreasing levels of extracellular reducing sugar levels in magnesium ion containing media depending on the prolonged incubation period could have supported biomass production.

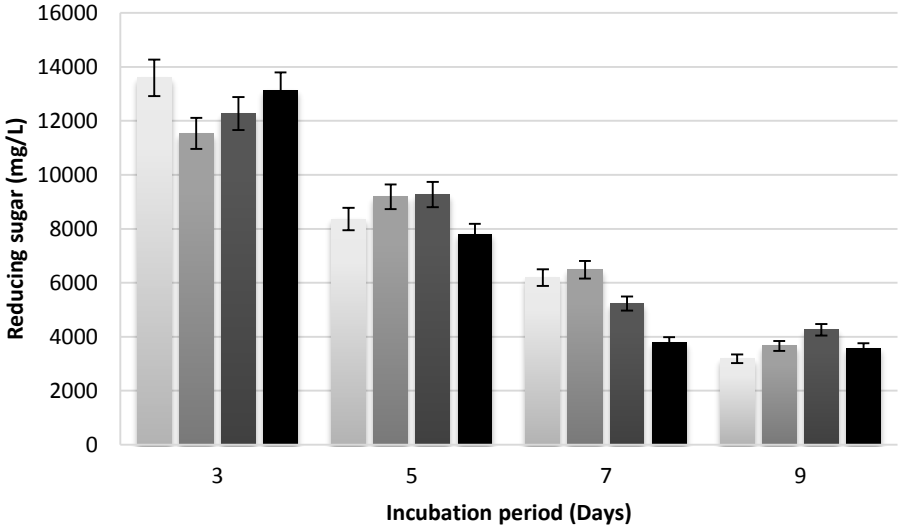

Figure 3.34 Total reducing sugar levels depending on manganese metal containing growth medium conditions; basal medium ( ), 15 ppm $MnCl_2.4H_2O$ (●), 45 ppm $MnCl_2.4H_2O$ (●), and 75 ppm $MnCl_2.4H_2O$ (●).

Extracellular reducing sugar levels of different concentrations of manganese ion containing media were high compared to basal medium on 3rd day. On the following days of the incubation period, extracellular reducing sugar levels were similar to those of basal medium, except some significantly low values in 45 and 75 ppm Mn^{2+} containing media were observed on 7th day (Figure 3.34).

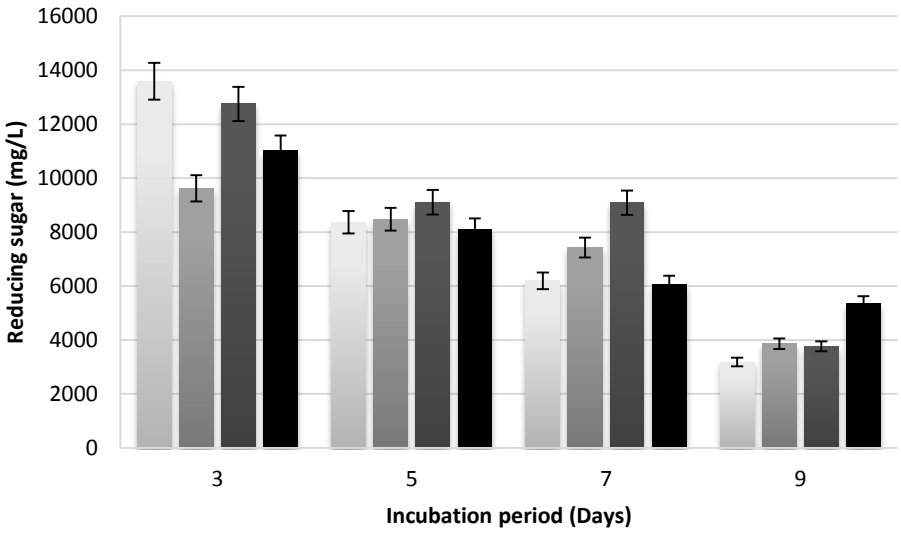

Figure 3.35 Total reducing sugar levels depending on zinc metal containing growth medium conditions; basal medium ( ), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

Extracellular reducing sugar levels in different concentrations of zinc ion containing media were lower than basal medium on 3rd day, just as observed in iron, potassium and magnesium ion containing media (Figure 3.35). Dramatic decreases were observed in 45 and 75 ppm Zn^{2+} containing media. These results indicated that the limitation of carbon sources could have induced secondary metabolism.

3.2.5 Variations in Extracellular Protease Activities Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

Variations in protease activities were determined depending on the trace metal ion concentrations throughout the incubation period to evaluate the effects of trace metals on degradation of nitrogen source soybean meal.

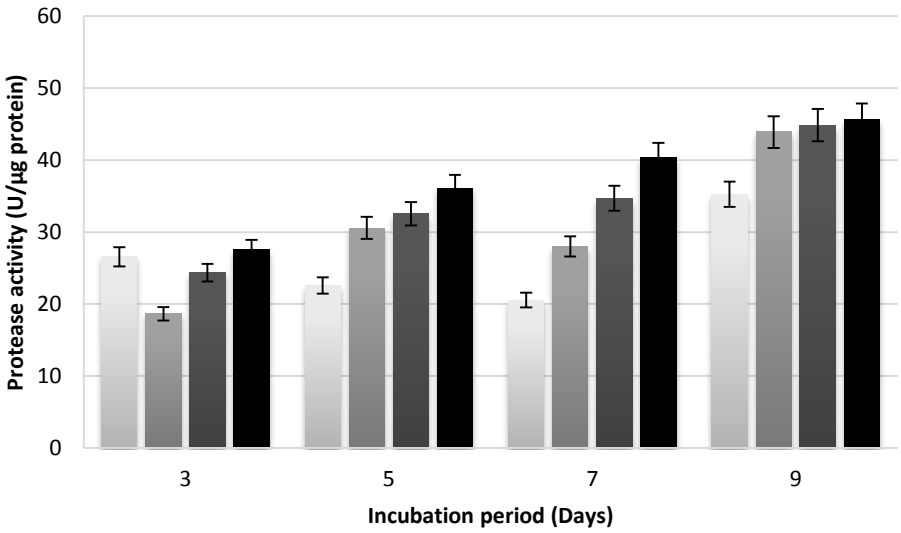

Figure 3.36 Extracellular protease activities depending on iron metal containing growth medium conditions; basal medium ( ), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

Extracellular protease activities stimulated in presence of increasing concentrations of iron ions (Figure 3.36). Extracellular protease activities enhanced as the incubation period was prolonged, and maximal protease activities in different concentrations of iron ion containing media were determined on 9th day. Protease activities were very similar regardless from iron ion concentrations on 9th day. According to these results, it may be concluded that iron ion plays a role as cofactor of *Actinoplanes* sp. ATCC 33076 extracellular protease. Contrary to our results, 30% inhibition of keratinolytic serine alkaline proteinase from *Streptomyces* sp. AB1 by 5 mM of $FeSO_4$ was reported by Jaouadi et al. (2010).

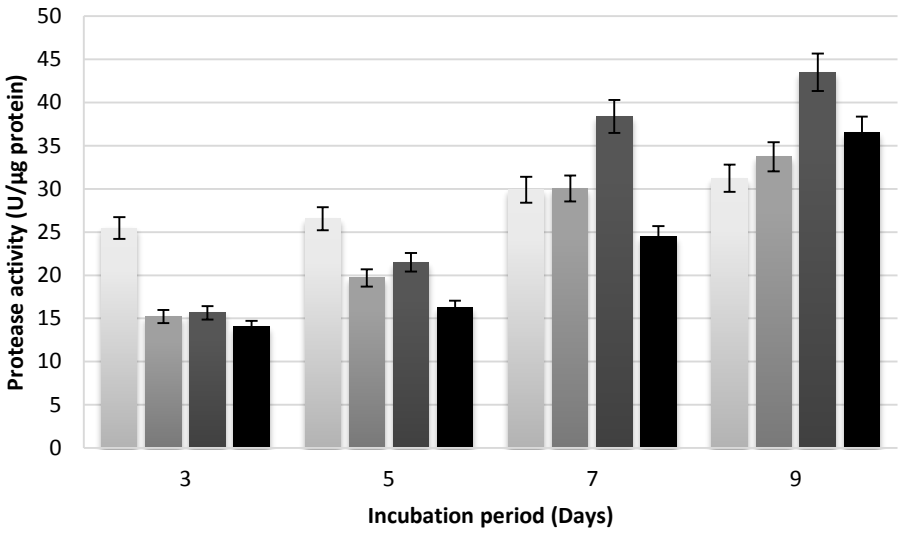

Figure 3.37 Extracellular protease activities depending on potassium metal containing growth medium conditions; basal medium ( ), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

Extracellular protease activities increased depending on the increasing potassium ion concentration, as well as the prolonged incubation period (Figure 3.37). On 3rd day, protease activities in potassium ion containing media were similar, and considerably lower than the extracellular protease activity determined in basal medium. The highest activity was determined in 45 ppm K_2HPO_4 containing medium on 9th day, and all concentrations of potassium ion containing media resulted in increased extracellular protease activities higher than that of basal medium on 9th day. Also, it is notable that extracellular protease activities of 75 ppm K_2HPO_4 containing medium, which supported the highest ramoplanin A2 production, was the lowest one. Owing to buffering action, the stabilization of the fermentation medium pH (pH homeostasis) could have indirectly favoured protease synthesis.

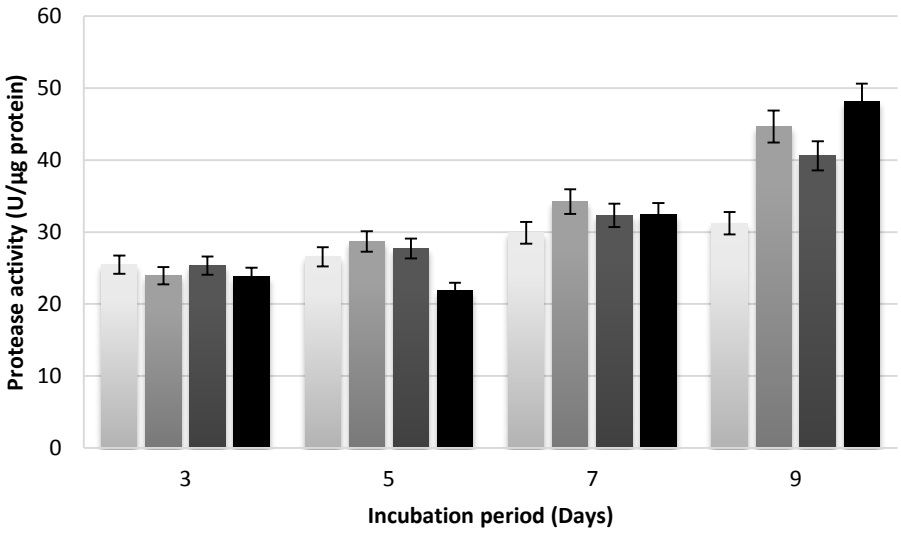

Figure 3.38 Extracellular protease activities depending on magnesium metal containing growth medium conditions; basal medium ( ), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

Extracellular protease activities were usually similar among the different concentrations of magnesium ion containing media throughout the incubation period. Moreover, protease activities were generally very close to those of basal medium, except magnesium ions led activation of extracellular protease activities on 9th day. Moreover, increases in both extracellular protein levels and ramoplanin A2 production were correlated with the increasing extracellular protease activities. The highest protease activity was determined in 75 ppm Mg^{2+} containing medium on 9th day (Figure 3.38). Jaouadi et al. (2010) reported that the addition of different concentrations of $MgSO_4$ between 1-10 mM enhanced *Streptomyces* sp. AB1 protease activity. Also, magnesium ions were found to improve activity and stability of proteases from *Bacillus pumilus* CBS (Jaouadi et al., 2008) and *Bacillus mojavensis* A21 (Haddar et al., 2009). 2 mM $MgCl_2$ caused sligh activation (3%) of fibrinolytic protease from *Streptomyces* sp. CS684 (Simkhada et al., 2010).

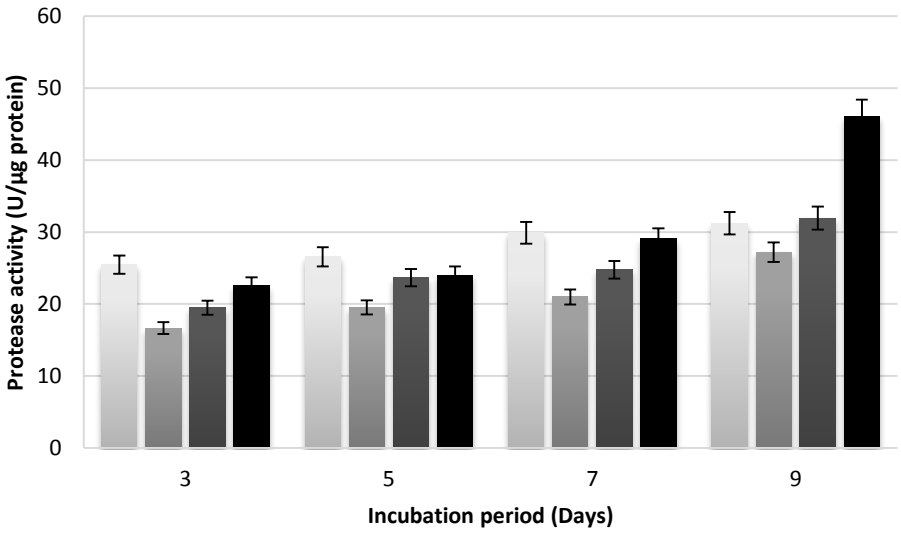

Figure 3.39 Extracellular protease activities depending on manganese metal containing growth medium conditions; basal medium (○), 15 ppm $MnCl_2.4H_2O$ (●), 45 ppm $MnCl_2.4H_2O$ (●), and 75 ppm $MnCl_2.4H_2O$ (●).

Extracellular protease activities of different concentrations of manganese ion containing media were generally lower than basal medium values throughout the incubation period. Only 45 ppm Mn^{2+} containing medium resulted in a slightly high activity, and 75 ppm Mn^{2+} containing medium resulted in a notably high activity compared to that of basal medium on 9^{th} day. It is conspicuous that 15 ppm Mn^{2+} containing medium, which supported the highest ramoplanin A2 production in manganese ion containing media, resulted in the lowest extracellular protease activities (Figure 3.39). 5 mM of $MnSO_4$ caused slight activation (11%) of keratinolytic serine alkaline proteinase from *Streptomyces* sp. AB1 (Jaouadi et al., 2010). On the other hand, $MnCl_2$ caused a slight reduction (5-6%) in extracellular protease activity of *Streptomyces* sp. DP2 (Bajaj & Sharma, 2011).

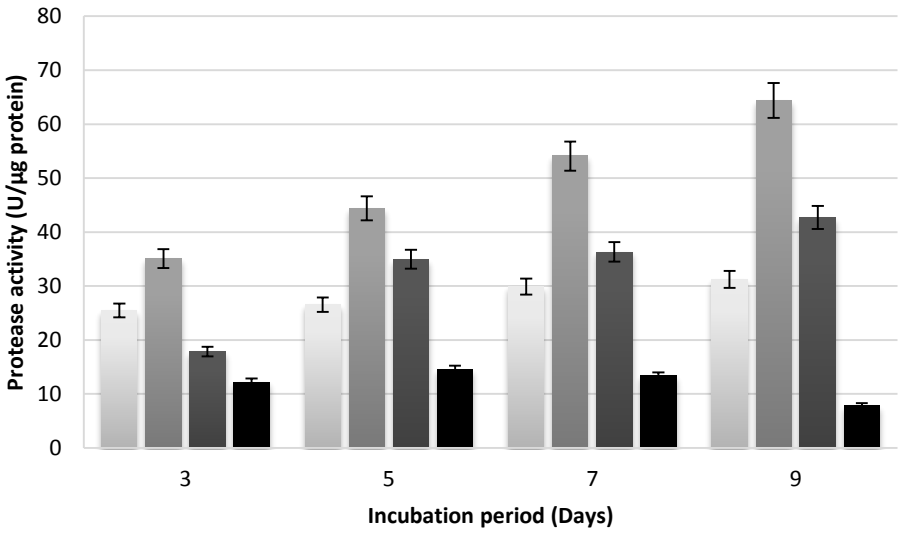

Figure 3.40 Extracellular protease activities depending on zinc metal containing growth medium conditions; basal medium ( ), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

As shown in Figure 3.40, increasing zinc ion concentrations caused significant decreases in protease activities. The results indicated that 15 ppm Zn^{2+} stimulated protease activity. Protease activities in 15 ppm Zn^{2+} containing medium were higher than both those of basal medium and higher zinc ion concentrations containing media in a considerable extent, and activities increased regularly throughout the incubation period. High protease activities and low extracellular protein levels determined in 15 ppm Zn^{2+} containing medium indicated that zinc ions could have been facilitated the intracellular uptake of hydrolysed protein and their direction to primary metabolism. Lower ramoplanin A2 levels of 15 ppm Zn^{2+} containing medium compared to basal medium supports this hypothesis. On the other hand, decreased protease activities were associated with the increased protein levels in both 45 and 75 ppm Zn^{2+} containing media, and ramoplanin A2 production were parallel to the increased zinc concentrations. The protease activity was suppressed in 75 ppm Zn^{2+} containing medium. The unavailability of protein sources due to the inhibited protease activities depending on the increasing zinc ion concentrations could have led to cellular stress and thereby stimulation of ramoplanin A2 production.

The increases in protease activities with respect to the incubation period showed negative correlation with the decreases in pH levels ranged between 7.40 to 6.80. The results were in coherrance with the other studies which showed that maximum protease activity was determined around 6.50. In contrast to our results, 5 mM $ZnSO_4$ did not noticeably affect proteinase activity of *Streptomyces* sp. AB1 (Jaouadi et al., 2010). 2 mM $ZnCl_2$ led 65% suppresion of the activity of fibrinolytic protease from *Streptomyces* sp. CS684 (Simkhada et al., 2010).

3.2.6 Variations in Extracellular Amylase Activities Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

Variations in amylase activities were determined depending on trace metal ion concentrations throughout the incubation period to evaluate the effects of trace metals on depletion of carbon sources.

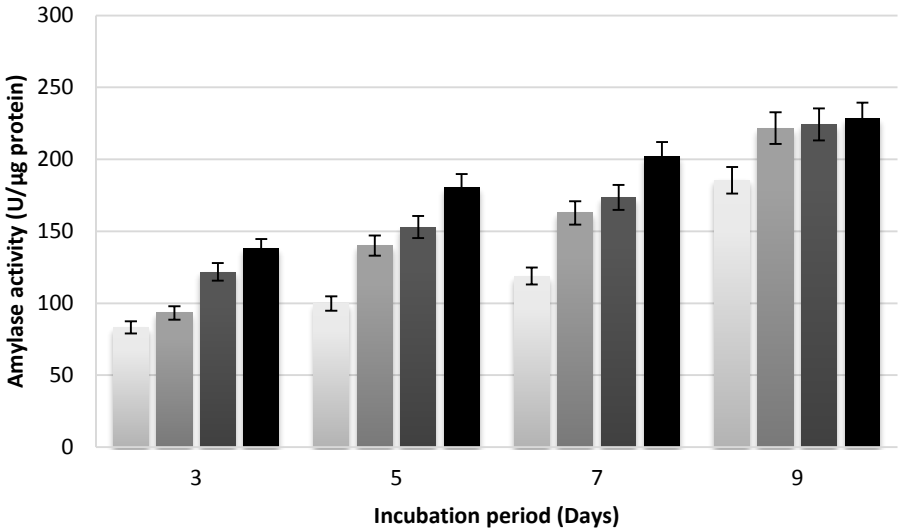

Figure 3.41 Extracellular amylase activities depending on iron metal containing growth medium conditions; basal medium ( ), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

As depicted in Figure 3.41, extracellular amylase activities in iron ion containing media were higher than basal medium values throughout the incubation period, and enzyme activities increased both depending on the increasing iron ion concentration

and the prolonged incubation period. Amylase activities in all concentrations of iron ion containing media were similar on 9th day. These results indicated that iron ions could have play a role as cofactor of *Actinoplanes* sp. ATCC 33076 extracellular amylase independent from the studied iron ion concentrations. As shown in Figure 3.31, extracellular reducing sugar levels were also low compared to basal medium. High levels of extracellular amylase activities in iron ion containing media could have led rapid depletion of carbon sources, and subsequent inhibition of ramoplanin A2 production. Contrary to our results, iron ions caused strong inhibition of α-amylases produced by *Streptomyces* sp. D1 (Chakraborty et al., 2009), and *Saccharopolyspora* sp. A9 (Chakraborty et al., 2011). The inhibition enhanced by the increased iron ion concentrations in both study.

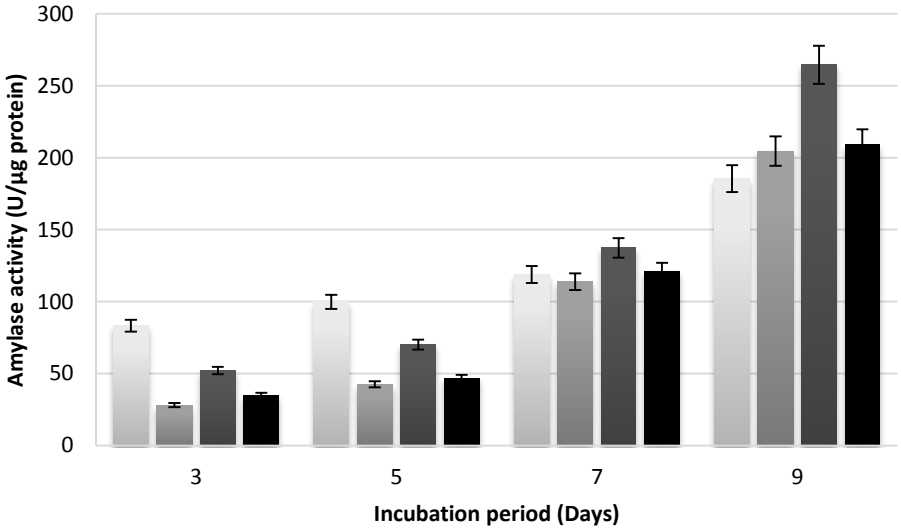

Figure 3.42 Extracellular amylase activities depending on potassium metal containing growth medium conditions; basal medium (○), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

Extracellular amylase activities in potassium ion containing media increased depending on the prolonged incubation period. 15 ppm and 75 ppm K_2HPO_4 containing media resulted in similar extracellular amylase activities, and the values were both lower than the extracellular amylase activities of 45 ppm K_2HPO_4 containing medium and basal medium, although some minor increases compared to basal medium were observed on 9th day. It seemed that 45 ppm K_2HPO_4 containing medium stimulated extracellular amylase activity. Also, it is notable that

extracellular amylase activities of 75 ppm K_2HPO_4 containing medium, which supported the highest ramoplanin A2 production in potassium ion containing media, was generally low (Figure 3.42). Limitation of carbon sources could have had a positive effect on ramoplanin A2 production. Similar to our results, potassium ions had insignificant effects on α-amylases from *Streptomyces* sp. D1 and *Saccharopolyspor*a sp. A9 (Chakraborty et al., 2009; 2011).

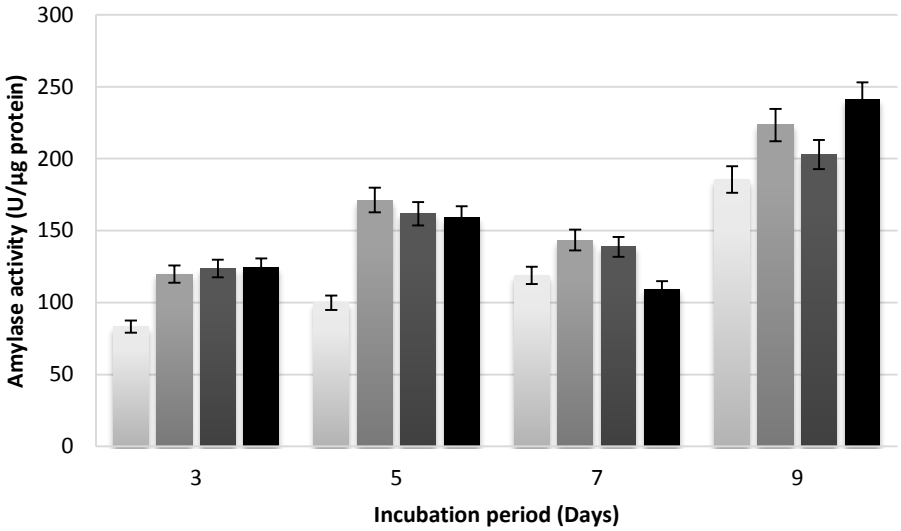

Figure 3.43 Extracellular amylase activities depending on magnesium metal containing growth medium conditions; basal medium ( ), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

Extracellular amylase activities in magnesium ion containing media were usually high in comparison with those of basal medium (Figure 3.43). Although some slight inhibitions were observed on 7th day, the highest activities were determined on 9th day. Extracellular amylase activities in magnesium ion containing media could have been high to meet the requirements of induced cell growth, thereby inhibition of ramoplanin A2 production. Similar to our results, enhancement of the enzyme activity with the increasing magnesium ion concentration was reported for *Streptomyces* sp. D1 α-amylase (Chakraborty et al., 2009).

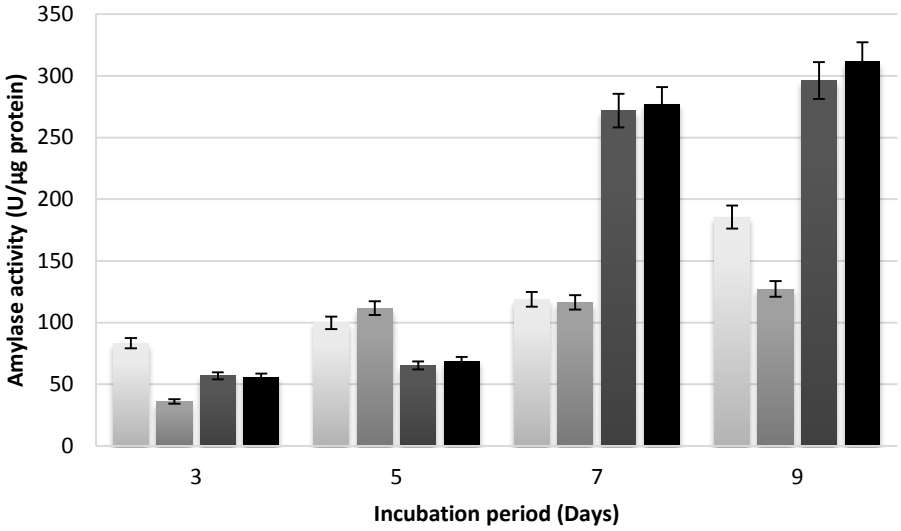

Figure 3.44 Extracellular amylase activities depending on manganese metal containing growth medium conditions; basal medium (●), 15 ppm $MnCl_2.4H_2O$ (●), 45 ppm $MnCl_2.4H_2O$ (●), and 75 ppm $MnCl_2.4H_2O$ (●).

The highest ramoplanin A2 production among different concentrations of manganese ion containing media was obtained in 15 ppm Mn^{2+} containing medium (Figure 3.19). As can be seen from Figure 3.44, extracellular amylase activities of this medium was usually low, although values increased depending on the prolonged incubation period, but not changed much after 5th day. On the other hand, it seemed that the extracellular amylase activities were stimulated both in 45 and 75 ppm Mn^{2+} containing media starting from 7th day. Extracellular amylase activities of these two media were very similar throughout the incubation period. Chakraborty et al. (2011) reported that the increasing concentrations of manganese ions potentiate the activity of α-amylase produced by *Saccharopolyspora* sp. A9. On the contrary, 1 mM $MnCl_2$ caused 55% inhibition of the activity of α-amylase from *Streptomyces megaspores* SD12 (Dey & Agarwal, 1999).

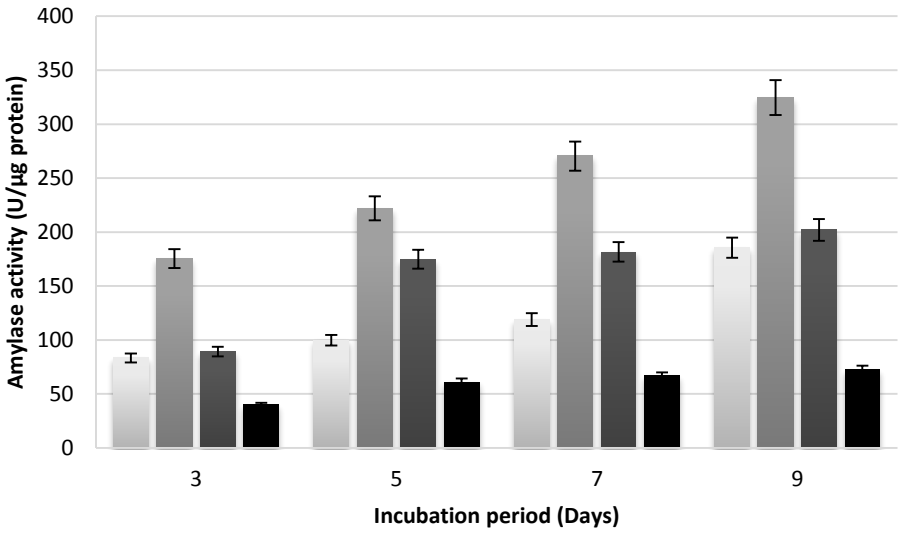

Figure 3.45 Extracellular amylase activities depending on zinc metal containing growth medium conditions; basal medium ( ), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

As depicted in Figure 3.45, extracellular amylase activities in zinc ion containing media increased depending on the prolonged incubation period. 15 and 45 ppm Zn^{2+} containing media resulted in high extracellular amylase activities compared to basal medium. The highest extracellular amylase activities determined in 15 ppm Zn^{2+} containing medium, which was the medium that ramoplanin A2 production was very close to those of basal medium. But the increasing zinc ion concentrations caused inhibition of extracellular amylase activities, and 75 ppm Zn^{2+} containing medium, which supported the highest ramoplanin A2 production, resulted in the lowest extracellular amylase activities. The values of 75 ppm Zn^{2+} containing medium were even lower than those of basal medium. Inhibition of amylase activities could have led the limitation of carbon sources and thereby activation of ramoplanin A2 production related with cellular stress. Strong inhibition of α-amylases by zinc ions were previously reported (Dey & Agarwal, 1999; Prakash et al., 2009; Chakraborty et al., 2011).

3.2.7 Variations in Extracellular Lipase Activities Depending on Trace Metal Types and Concentrations with Respect to Incubation Period

Variations in lipase activities in trace metal ions containing media were also determined in order to evaluate whether trace metals have effect on the lipolytic activity of *Actinoplanes* sp. ATCC 33076.

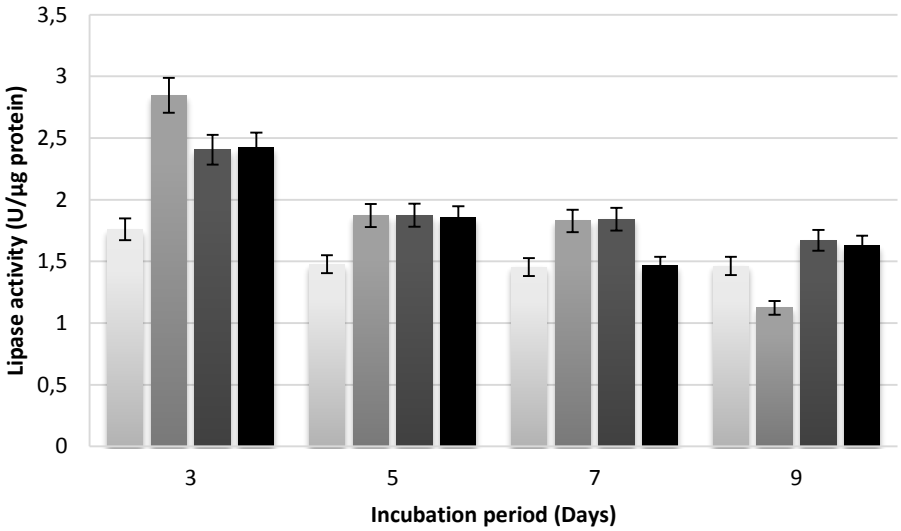

Figure 3.46 Extracellular lipase activities depending on iron metal containing growth medium conditions; basal medium ( ), 15 ppm $FeSO_4.7H_2O$ (●), 45 ppm $FeSO_4.7H_2O$ (●), and 75 ppm $FeSO_4.7H_2O$ (●).

As can be seen from Figure 3.46, extracellular lipase activities enhanced in the presence of iron ions. The highest lipase activity was determined in 15 ppm Fe^{2+} containing medium on 3rd day. A regular decrease was observed in lipase activity of 75 ppm Fe^{2+} containing medium until 7th day, and a slight increase was detected on 9th day. Mander et al. (2012) reported that increasing concentrations of Fe^{2+} ions caused inhibition of lipase activity of *Streptomyces* sp. CS133. Similar results were reported for neutral lipase from *Streptomyces* sp. CS326 (Cho et al., 2012).

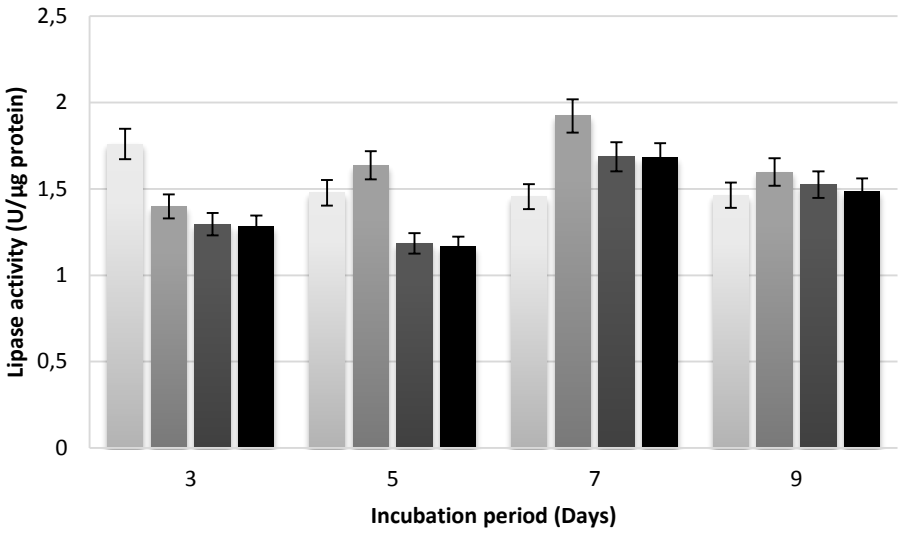

Figure 3.47 Extracellular lipase activities depending on potassium metal containing growth medium conditions; basal medium (○), 15 ppm K_2HPO_4 (●), 45 ppm K_2HPO_4 (●), and 75 ppm K_2HPO_4 (●).

According to our results, extracellular lipase activities were stimulated in 15 ppm K_2HPO_4 containing medium compared to both 45 and 75 ppm K_2HPO_4 containing media (Figure 3.47). On the other hand, extracellular lipase activities were similar in 45 and 75 ppm K_2HPO_4 containing media on the same days of the incubation period, which indicated that higher concentrations of potassium did not cause any significant changes in lipase activities. Moreover, extracellular lipase activities in all potassium ion containing media were slightly high compared to those of basal medium on 7^{th} and 9^{th} days. Zhang et al. (2008) reported that potassium ion had no significant effect on the activity of lipase from *Streptomyces fradiae* var. k11. Similarly, 2 mM K^+ neither activated nor inhibited the activity of *Streptomyces* sp. CS326 neutral lipase (Cho et al., 2012).

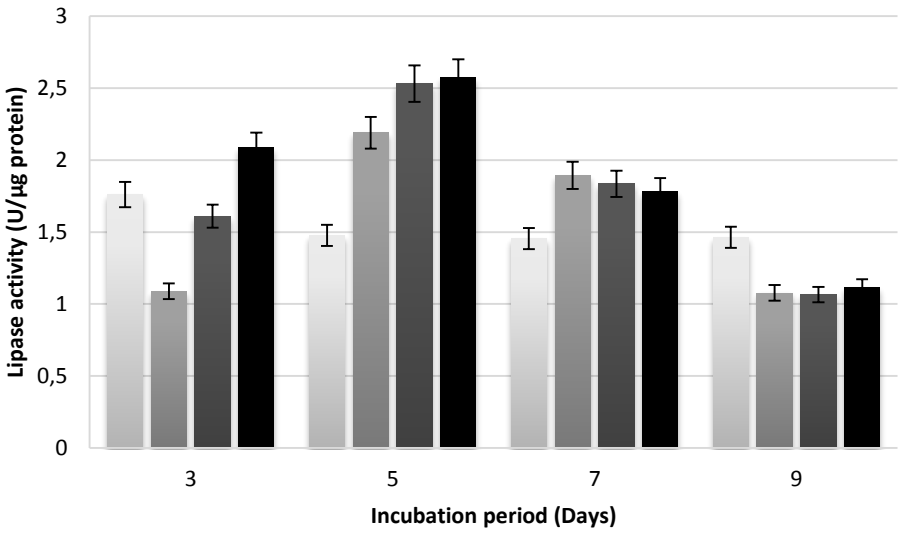

Figure 3.48 Extracellular lipase activities depending on magnesium metal containing growth medium conditions; basal medium ( ), 15 ppm $MgSO_4.7H_2O$ (●), 45 ppm $MgSO_4.7H_2O$ (●), and 75 ppm $MgSO_4.7H_2O$ (●).

As can be seen from Figure 3.48, extracellular lipase activities increased depending on the increasing magnesium ion concentrations in the early days of the incubation period. However, activities decreased after 5th day, and did not significantly change among the different concentrations of magnesium ion containing media on 7th and 9th days. Slight activation of *Streptomyces fradiae* var. k11 lipase by 1 mM Mg^{2+} was reported by Zhang et al. (2008). On the other hand, activities of *Streptomyces coelicolor* A3(2) lipases were slightly inhibited in the presence of magnesium ions, independent from the increasing ion concentrations (Côté & Shareck, 2008). Magnesium is also known as an inducer of lipase production by a variety of microorganisms. Pokorny et al. (1994) reported that lipase production by *Aspergillus niger* was enhanced in the presence of magnesium ions. Janssen et al. (1994) reported several fold of increase in lipase production by a thermophilic *Bacillus* sp. in magnesium, iron, and calcium added production medium. Extracellular lipase production by *Acinetobacter calcoaceticus* BD 413 was also enhanced when the medium was supplemented with Mg^{2+}, Ca^{2+}, Cu^{2+}, and Co^{2+} (Kok et al., 1995).

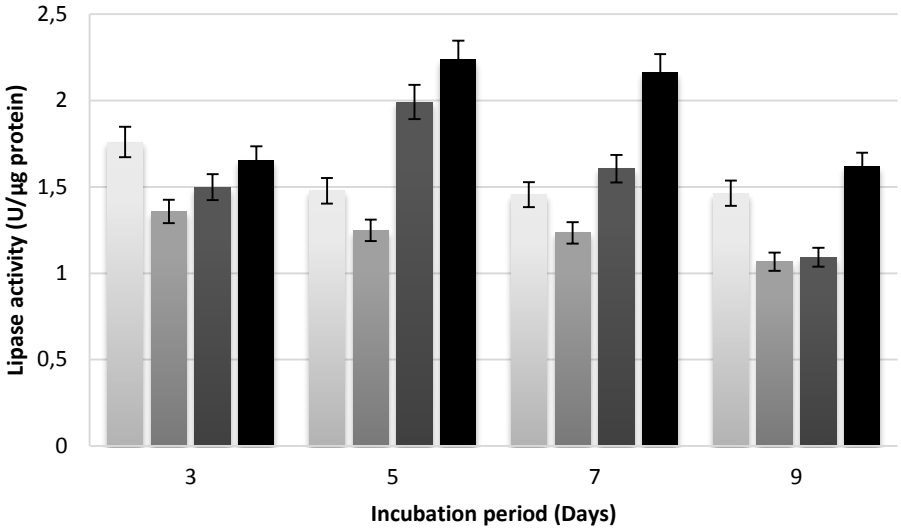

Figure 3.49 Extracellular lipase activities depending on manganese metal growth medium conditions; basal medium ( ), 15 ppm Mn^{2+} (●), 45 ppm Mn^{2+} (●), and 75 ppm Mn^{2+} (●).

According to our results, manganese ion was an important activator of *Actinoplanes* sp. ATCC 33076 extracellular lipase (Figure 3.49). Increases in lipase activities were parallel with the increasing manganese ion concentrations. In contrast to our results, the activity of *Streptomyces fradiae* var. k11 lipase (lipS221) was 35.1% inhibited in the presence of 1 mM of manganese ion (Zhang et al., 2008).

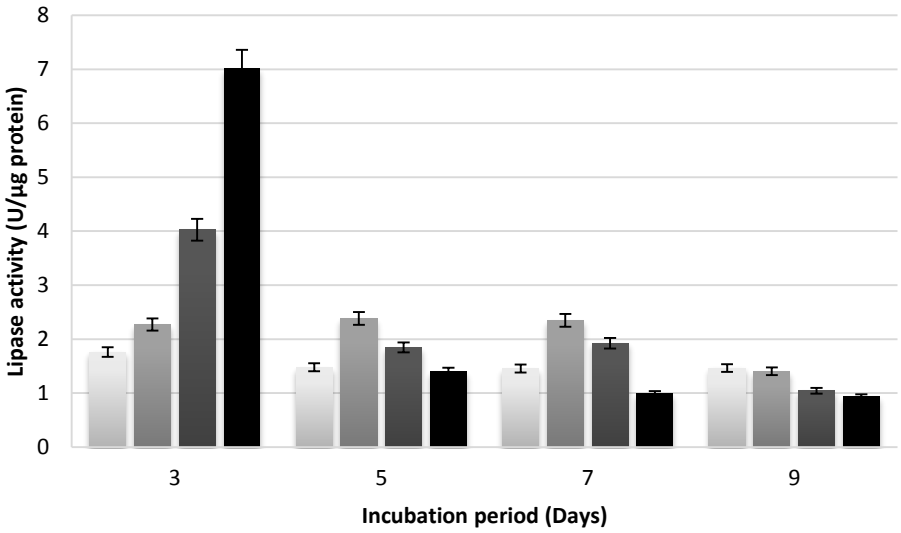

Figure 3.50 Extracellular lipase activities depending on zinc metal containing growth medium conditions; basal medium ( ), 15 ppm $ZnSO_4.7H_2O$ (●), 45 ppm $ZnSO_4.7H_2O$ (●), and 75 ppm $ZnSO_4.7H_2O$ (●).

Similar to the results obtained in magnesium ion containing media, extracellular lipase activities in zinc ion containing media increased in coherence with the increasing zinc ion concentrations on 3rd day. High extracellular lipase activity in 75 ppm Zn^{2+} containing medium could have enhanced the uptake of required lipid sources for membran lipids and ramoplanin A2 biosynthesis. However, the relationship between extracellular lipase activities and zinc ion concentrations were vice versa on the following days. 15 ppm Zn^{2+} containing medium resulted in a stable lipase activity profile compared to 45 and 75 ppm Zn^{2+} containing media (Figure 3.50). Mander et al. (2012) reported that the increasing concentrations of zinc ions had noticeable inhibitory effects on the activity of *Streptomyces* sp. CS133 lipase. 2 mM of $ZnSO_4$ also 58% inhibited lipase activity of *Streptomyces* sp. CS326 (Cho et al., 2012). The inhibitory activity of transition metal cations (Fe, Cu, and Zn and Co) at higher concentrations may be due to the direct inhibition of the catalytic site and/or formation of complexes between metal ions and ionized fatty acids (Chakraborty & Paulraj, 2009).

CHAPTER FOUR
CONCLUSION

Ramoplanin is a cell wall active lipoglycodepsipeptide antibiotic with a different mechanism of action than other cell wall active antibiotics such as vancomycin, teicoplanin and β-lactams. It has an excellent in vivo and in vitro activity against Gram-positive bacteria at even very low minimum inhibition concentrations, and it is the most potent antibiotic for the treatment of high morbidity and mortality hospital infections including vancomycin-resistant *Enterococcus* sp., methicillin-resistant *Staphylococcus aureus*, and vancomycin intermediate-resistant *Clostridium difficile*. Ramoplanin has been fast tracked by US Food and Drug Administration, and is expected to be placed on the market in the coming years. However, ramoplanin is the most potent antibiotic, it is an expensive antibiotic due to its high fermentation cost, although ramoplanin production by fermentation is the only way since its total synthesis yield is very low. Therefore, optimization of fermentation conditions is required for the mass production of this antibiotic.

It has been known that the choice of nutritional considerations such as carbon and nitrogen sources, and trace metals influence antibiotic production both in the extent of total yield and the composition of antibiotic subtypes. The aim of this study was to investigate the effects of different nitrogen sources and different concentrations of various trace metals on ramoplanin A2 production with respect to the incubation period. As well as ramoplanin A2 production, important parameters influencing antibiotic biosynthesis such as pH levels, total protein levels, total reducing sugar levels, protease, amylase and lipase activities, and intracellular free amino acid levels were also investigated.

Soybean meal, meat-bone meal, poultry meal and fish meal were used as nitrogen sources. According to the results, the maximum ramoplanin A2 production was determined as 406.805 mg L^{-1} in fish meal medium on 7^{th} day. This amount was 3.89 fold higher compared to that found for basal medium at the same day. On the other hand, although 374.218 mg L^{-1} ramoplanin A2 was determined in poultry meal

medium, total yield of ramoplanin A2 in this medium was higher compared to fish meal medium. The highest ramoplanin A2 production in meat-bone meal medium was determined as 252.130 mg L^{-1} on 7^{th} day (3.03 fold of basal medium). Considering the prices of fish meal and poultry meal, economic production of ramoplanin A2 can be carried out by using poultry meal.

The effects of different concentrations of iron, potassium, magnesium, manganese and zinc metal ions on ramoplanin A2 production were also investigated with respect to the incubation period. Ramoplanin A2 production was stimulated in the presence of zinc, manganese and potassium metals. 647.415 mg L^{-1} ramoplanin A2 in 75 ppm Zn^{2+} containing medium, 251.073 mg L^{-1} ramoplanin A2 in 15 ppm Mn^{2+} containing medium, and 189.262 ramoplanin A2 in 75 ppm K_2HPO_4 containing medium were determined on 7^{th} day. These amounts were 7.782 fold, 3.018 fold, and 2.275 fold of basal medium at the same day for zinc, manganese and potassium ions containing media, respectively. However, iron and magnesium ions did not significantly stimulate ramoplanin A2 production.

Considering that both nitrogen sources such as poultry meal and fish meal, and zinc, manganese and potassium metal ions stimulated ramoplanin A2 biosynthesis, biological production of this antibiotic could be enhanced by optimization of complex media.

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