DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

IDENTIFICATION OF SOME INTERMEDIATE METABOLITE AND ENZYMES EFFICIENCY ON REGULATION OF VANCOMYCIN ANTIBIOTIC PRODUCTION BY AMYCOLATOPSIS ORIENTALIS

by
Hülya AYAR KAYALI

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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled "**Identification of Some Intermediate Metabolite and Enzymes Efficiency on regulation of vancomycin antibiotic production by Amycolatopsis orientalis**" completed by **Hulya AYAR KAYALI** under supervision of **Prof. Dr. Leman TARHAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

Prof.Dr.Leman Tarhan

Supervisor

Prof.Dr.Mustafa Toprak	Prof.Dr.Ayşegül Pala
Committee Member	Committee Member
Prof.Dr.Şebnem Harsa	Yrd.Doç.Dr.Nalan Tüzmen
Jury Member	Jury Member

Prof.Dr. Cahit HELVACI
Director
Graduate School of Natural and Applied Sciences

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Hülya AYAR KAYALI

IDENTIFICATION OF SOME INTERMEDIATE METABOLITES AND ENZYMES EFFICIENCY ON REGULATION OF VANCOMYCIN ANTIBIOTIC PRODUCTION BY AMYCOLATOPSIS ORIENTALIS

ABSTRACT

In order to identify the aspects of some metabolism factors relevant to vancomycin production, the correlation between vancomycin antibiotic production and intra-extracellular glycolytic and TCA intermediate products, α-ketoglutarate dehydrogenase, glucose kinase, pyruvate kinase, and glyoxalate shunt isocitrate lyase activities and glycogen levels as a storage metabolite were investigated depending on the glucose and glycerol concentration of *Amycolatopsis orientalis* medium with respect to incubation period. The intracellular levels of citrate, α-ketoglutarate, fumarate and malate, reached maximum on the 36th and 48th hours for 5 and 10-20 g/L of glucose while they reached their maximum on the 48th and 60th hours in glycerol. The levels of these intermediates increased with increasing glucose concentrations up to 12.5 and/or 15.0 g/L of glucose while these intermediates increased up to 10 and/or 15 g/L glycerol. Glucose kinase activities were showed positive correlation with the both carbon sources concentrations of the *A. orientalis* growth medium. The other glycolytic enzyme, pyruvate kinase activity was increased up to 15 g/L glucose while they rose significantly with glycerol concentration. The activity of α-ketoglutarat dehydrogenase, one of the main enzymes of TCA pathway, increased with increasing carbon sources concentrations up to 10 g/L of glucose and 15 g/L of glycerol, while the activity of the key enzyme of glyoxalate shunt, isocitrate lyase increased with increase in the carbon sources concentrations. In addition, intracellular glycogen levels showed positive correlation with concentrations of carbon sources. The increases in isocitrate lyase activity with decreases in TCA cycle intermediates after 48th hour is an indication of the correlation between TCA and glyoxalate cycles. The highest level of vancomycin production of *A. orientalis* was determined at 15 g/L of glucose and glycerol as 7.80 and 4.01 ppm on the 48th hour. Determination of maximum TCA intermediates levels and vancomycin production at the same carbon concentrations and incubation periods suggest that these metabolites are precursor of the antibiotic.

Keywords: Vancomycin, TCA and glycolytic intermediates and enzymes, glyoxalate shunt, glycogen, carbon metabolism

AMYCOLATOPSİS ORİENTALİS SUŞUNDA VANCOMYCİN ANTİBİYOTİK ÜRETİMİNİN REGÜLASYONUNDA ETKİN BAZI ENZİM VE ARA METABOLİT YOL İZLERİNİN AYDINLATILMASI

ÖZ

Amycolatopsis orientalis in vankomisin antibiyotiği üretiminde etkin bazı metabolik faktörleri aydınlatma amacıyla, antibiyotik üretim düzeyleri ile hücre-içi, -dışı glikolitik ve TCA çevrimi ara ürünlerinin yanısıra α-ketoglutarat dehidrogenaz, glukoz kinaz, pruvat kinaz ve glioksalat çevrimi izositrat liyaz enzimlerinin aktiviteleri ve ayrıca depo materyali glikojen seviyeleri arasındaki korelasyon besi ortamındaki glikoz veya gliserolün derişimine ve inkübasyon periyoduna bağımlı olarak incelenmiştir.

Hücre içi sitrat, α-ketoglutarat, fumarat ve malat seviyeleri genelde 5 g/l de 36. saatte, 10-20 g/L glukoz da ise 48. saatte, gliserolde ise aynı karbon kaynağı derişimlerinde sırasıyla 48. ve 60. saatlerde maksimuma ulaşmışlardır. Bu ara ürünler, glikoz derişiminin 10 ve/veya 12.5 g/L ye, gliserol derişiminin ise 10 ve/veya 15 g/L ye kadar artışı ile anlamlı düzeyde artışlar belirlenmiş olup 20 g/L karbon kaynağı derişiminde azalış eğilimine geçmiştir.

Glukoz kinaz aktivitesi ise *A. orientalis* besi ortamındaki her iki karbon kaynağı derişimleriyle pozitif korelasyon göstermiştir. Diğer bir glikolitik enzim olan pruvat kinaz aktivitesi, glikoz ve gliserolün sırasıyla 15 ve 20 g/L ye kadar artışı ile artışlar göstermiştir. TCA çevrimi enzimlerinden α-ketoglutarat dehidrogenaz, 10 g/L glukoz ve 15 g/L gliserol ortamında maksimuma ulaşırken glioksalat çevrimi anahtar enzimi olan izositrat liyaz hem glikoz hem de gliserol derişimine bağımlı anlamlı düzeyde artış göstermiştir. Glikojen seviyesi ile besi ortamındaki karbon derişimi arasında da pozitif korelasyon belirlenmiştir.

Gerçekleştirilen tez çalışmasında, TCA ara ürünlerinin azalış eğiliminde olduğu 48.saat sonrası izositrat liyaz aktivitesindeki artışlar bu iki çevrim arasındaki ilişkiyi göstermektedir. Vankomisin antibiyotiği ise, 48. saatte 15g/L glikoz ve 10 g/L gliserolde sırasıyla 7.80 ve 4.01 ppm olan maksimum düzeyine ulaşmıştır. TCA ara ürünlerinin maksimuma ulaştıkları karbon kaynağı derişimi ve inkübasyon periyodunda vankomisin antibiyotiğinin en yüksek üretim düzeyine ulaşması, bu metabolitlerin antibiyotiğin üretiminden sorumlu öncül maddeler olduğunu göstermektedir.

Anahtar sözcükler: Vankomisin, TCA ve glikolitik ara ürün ve enzimler, glioksalat çevrimi glikojen, karbon metabolizması

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CHAPTER ONE
INTRODUCTION

The evaluation of fluxes through metabolic pathways is an important tool for the rational improvement of microorganisms to maximize the conversion of substrates into useful end-products. It is necessary to decipher and quantify the kinetic and regulatory structure of the metabolic pathways involved to optimize the bioprocesses performed by whole cells. In the past, such experimental data were seldom available and stoichiometric analysis of the bioprocess was considered a useful substitute (Varma et al., 1993). Attempts have been made to optimize metabolic dynamics, but progress appears to have been hampered by the lack of kinetic and regulatory information regarding the functioning of all enzymes in a particular cell. However, it is now possible to identify the optimal redirection of metabolic fluxes by means of mathematical tools (Stephanopoulos et al., 1998; Torres et al., 1997) whereas analytical tools have allowed the development of methodologies to reveal active metabolic pathways (Chassagnole et al., 2002; Yoon et al., 2003). Nowadays, modern experimental methods of genomics, proteomics, and metabolic profiling are rapidly changing the ‘‘playing field’’, and comprehensive quality data are being made available for integrative analysis and optimization. Although genes and proteins in simple organisms such as Escherichia coli have been identified, it is still impossible to predict how this microorganism will respond in untested environments and how its metabolism related to energy production, growth, and maintenance is linked to its secondary metabolism in certain biotechnological applications. Therefore, if whole cell bioprocess optimization is one of the aims, biocatalyst metabolism in different scenarios (reactor configurations, media, and physical conditions) must be understood before undertaking optimization strategies.

The present work is a first approach to understanding the link between the central carbon or primary metabolism and the production of vancomycin antibiotic as a secondary metabolite by *Amycolatopsis orientalis* which is important group of *Actinomyceteles*. To gain insight into the kinetics of the whole bioprocess and to

ascertain the connection between both types of metabolism, the activity of certain enzymes involved in the central metabolism, such as glucose kinase, pyruvate kinase (glycolysis), α- ketoglutarate dehydrogenase (Krebs cycle), isocitrate lyase (glyoxylate shunt) were followed.

Actinomycetes are important bacterial producers of secondary metabolites. There is a strong interest in the genetics of secondary metabolite biosynthesis, with most studies concentrating on these pathways and their control. Many secondary metabolites are initially derived from intermediates of the central pathways of primary metabolism. Little is currently known, however, about the enzymes and regulation of, for instance, glucose metabolism in actinomycetes. This is mostly because of a general lack of physiological studies on primary metabolism in actinomycetes (Hodgson, 1992).

1.1 Genera of *Actinomycetales*

Actinomycetes are a large group of diverse bacteria, having in common the characteristic (in some cases the simple tendency) to grow as filaments. They can be separated into two broad groups: the fermentative ones, generally living associated with man or animals, and the aerobic soil inhabitants.

The genus *Streptomyces* belongs to the order Actinomycetales, the filamentous bacteria. Streptomycetes are gram-positive, aerobic, and grow in mats of branched filaments called hyphae which constitute both a substrate and an aerial mycelium. Characteristic of the genus is the segmentation of the aerial hyphae into long chains of nonmotile spores.

The genus *Streptoverticillium* is mentioned here since it is almost indistinguishable in most aspects of its biology from the genus *Streptomyces.* The main difference is morphological: the aerial hyphae bear the spore chains in verticils or umbels, that is, several chains branch from a single point of a hypha.

These organisms are widespread in nature belying their classification as "rare actinos," which was related to being less easily detected than streptomycetes. The use of selective isolation techniques has demonstrated that some genera are not rare at all and that a number of strains can be found in any soil sample. No clear-cut habitat preference can be established for the different genera; however, some, such as *Micromonospora* or *Actinoplanes,* are more abundant in decaying plant material or muddy soils on freshwater shores. Thermophilic species are often found in natural warm habitats, such as compost or hay mounds.

1.2. Taxonomy of *Actinomycetes*

The genera of the order Actinomycetales are divided in group according to the composition of the cell wall. Within a group the morphology of the aerial mycelium, the spore arrangement, and chemical characters are used to define the genus. *Streptomyces* belongs to the group containing L-diaminopimelic acid in the cell wall (Ensing, 1992). As mentioned previously, the distinctive characteristic of the genus is the formation of chains of spores on the aerial mycelium. The spores are maintained in chains by a sheath which confers to them various types of surfaces: smooth, spiny, hairy, etc. The chains may be straight, flexuous, or may form open or closed spirals (Hirsch & McCann-McCormick, 1985). The shape of the chains and the appearance of the spores are elements used for species determination. Other characters considered are the color of the surface and reverse of colonies growth above or below 45°C, utilization of carbon compounds, and solubilization of specific substrates. The production of secondary metabolites, although of major practical importance, is not considered a determinant for speculation since it is well known that the same antibiotic can be produced by different microorganisms.

Although these criteria are widely accepted, species assignment within *Streptomyces* is complex problem (Goolieb & Shirling, 1967). In patents and in the scientific literature, over 3000 strains have been named as species. Obviously this

depends on different definitions of species and undoubtedly many of these are synonyms. Some order has been introduced by the International *Streptomyces* Project, a large cooperative study for the redescription of the species according to uniform criteria.

Distinctive features of *Streptoverticillium* are, besides the presence of verticils, the cottony appearance of the colonies and the "barbed wire" appearance of the mycelium when seen under moderate magnification.

The taxonomy of *Actinomycetates* is very complex and rapidly evolving as new concepts and criteria are adopted by specialist of discipline. Traditionally separation in genera has been based on morphological characteristic such as presence or absence of aerial mycelium, the presence and the arrangement of spores and spore-containing bodies and the spore motility and formation of specialized structure (O'Donnell, 1988). More recently, chemical properties have been accepted as valuable taxonomic characters. These include the cell membrane and wall composition and are especially useful in organizing the *Actinomycetales* in to groups, each comprising several related genera, roughly corresponding to the taxom "family", a term not universally used in *Actinomycetes* taxonomy.

1.3. *Nocardioform Actinomycetes*

This is a very heterogeneous group that includes the actinomycetes which form a mycelium breaking up into coccoid or rod-shaped fragments (Goodfellow, 1992). All of the organisms are gram-positive aerobes, but may differ in their chemical characteristics, except for the menaquinone structure which invariably contains eight or nine isoprene units. They are very common in soil, but some species are animal or plant pathogens.

Nocardia. Morphological traits include the presence of both vegetative and aerial hyphae, sometimes rudimentary, fragmenting into nonmotile elements. Chains of nonmotile spores may be formed. Cell wall components include *meso-diaminopimelic* acid, arabinose, and galactose. Mycolic acids are present, with chains of 46-60 carbon atoms. Membrane fatty acids are linear or bear a methyl group at position 10.

Nocardiae are mesophilic and grow on simple media containing ammonia, nitrates, or amino acids as N sources and glucose or acetate as C sources. Growth is slow, the doubling time being about 5 h, at least for the species tested. Typical of several strains is the capability of utilizing, as C sources, long-chain alkanes and even gaseous hydrocarbons.

A variety of antibiotics are produced by nocardiae; however, the strains producing the most important ones, rifamycins, vancomycin,and ristocetin, have been recently attributed to a different genus Vice versa, the B-Iactams cephamycin A, B**,** and C are produced by a strain originally considered a *Streptomyces* and latter classified as *Nocardia lactamdurans.* A strain of *N. lactamdurans* also produces the elfamycin antibiotic efrotomycin. Products some interest for their antitumor activity are the ansamytocins, ansamycins produced by *Nocardia* species (Parenti & Coronelli,1979). The nocardicins monobactam antibiotics isolated from *N. uniformis,* are also interesting. Formycin and coformycin are two nucleoside antibiotics produced by *N. interforma.* The latter has no antimicrobial activity, but is an inhibitor of the enzyme adenosine. Because of this property, coformycin potentiates the activity of vidarabine and other nucleoside antibiotics, by preventing their metabolic degradation. A similar activity is shown by 2-deoxycoformycin, a metabolite isolated from S. *antibioticus.*

Amycolatopsis. This genus has been recently proposed to accommodate species, formerly classified as *Nocardia,* which do not contain mycolic acids. The other taxonomic traits are similar to those of nocardiae, except for the presence of

branched-chain fatty acids in the *Amycolatopsis* cell membrane. Several species have been described which produce antibiotics belonging to different biosynthetic classes. Glycopeptides (dalbaheptides) are rather frequent and among them the therapeutically important vancomycin is produced by *A. orientalis* and ristocetin by *A. orientalis* subsp. *Lurida. A. orientalis* strains also produce an elfamycin antibiotic and the muraceins, muramyl peptide derivatives found to be inhibitors of angiotensinconverting enzyme.

A. mediterranei is the producer of the important ansamycins rifamycins. The original strain produces a complex of several components which rifamycin B was selected and used for the preparation of semisynthetic derivatives. Rifamycin SV was first prepared chemically from rifamycin B, but later was also isolated from a *Nocardia* speciesi A. *mediterranei* is one of the few actinomycetes whose genetics has been studied in some detail. Recombinants can be obtained with high frequency y conjugation of marked strains. By this technique a circular linkage map was constructed which, when compared with the much more complete S. *coelicolor* map, shows a good correspondence in the sequence of homologous markers (Queener & Day, 1986). In addition, a method for the formation an regeneration of protoplasts has been described.

Saccharopolyspora. Organisms of this genus, which includes only two validated species, differ from streptomycetes in two respects: the tendency of their substrate mycelium to fragment, and the composition of the cell wall, containing *meso*-diaminopimelic acid, galactose, and arabinose. The species of interest is S. *erythrea* (formerly *Streptomyces erythreus),* the producer of the well-known antibiotic erythromycin.

1.4. Growth Cycle of *Actinomycetes*

On solid substrates, streptomycetes grow in colonies whose life cycle begins either from a fragment of hypha or typically form a spore. Germination of the spores requires the presence of divalent ions, and may be hastened by a mild heat shock. It can be divided into three stages: darkening, swelling, and germ tube formation. The

germ tube gives rise to a network of branched septated hyphae constituting the substrate mycelium. Changes in the environment (or genetic programming) trigger the formation of aerial hyphae that grow at the expense of the substrate mycelium, which lyses releasing the nutrients necessary for aerial mycelium development. In several cases the involvement of a low-molecular-weight autoregulator, such as the well-known A-factor, has been demonstrated in this process. The next step is the segmentation of the hyphae into spores; this appears to be genetically programmed at a given stage of development rather than induced by external factors. Sporulation begins with the simultaneous formation of septa in the aerial hyphae at regular intervals of about 1μm. Both the wall and the septa thicken, and the spores assume their typical ellipsoid shape. Days later the sheath dissolves and spore dissemination completes the cycle.

The stages of development of streptomycetes growing in submerged cultures are not clearly defined. However, it is well known that many secondary metabolites are produced only at the end of vegetative growth, at a stage corresponding to the formation of aerial mycelium. Moreover, in some uncommon cases the formation of spores has been observed so. In submerged cultures, indicating some similarity with the life cycle on solid substrates.

1.5. The Genetics of Actinomycetes

One characteristic of the genus is the high G + C content of the DNA. The genome size is estimated at about 7500 kb, approximately two times that of *E. coli.* The rate of replication, as determined in S. *granaticolor* and in S. *hygroscopicus,* u600 bases per replication fork. This value is similar to that for *E. coli,* in agreement with the doubling time, which is around 1 ½ h at 28°C.

Members of these latter genera have very different morphological appearances, but the genera are so clustered because 16S RNA sequence has shown they are closely related and their cell walls are of the same chemotype (Goodfellow, 1989). Cell wall chemotype has proved a very useful means of taxonomic classification of

actinomycetes, which strongly correlated with sequence analysis-derived phylogenies.

1.6. The Ecological Niche of Actinomycetes

Actinomycetes are readily isolatable from soil, where their role appears to be to act as general saprophytes. They secrete extracellular enzymes and adsorb the soluble breakdown products of the interaction of those enzymes with insoluble polymers such as protein starch and cellulose. The source of most of this insoluble polymeric nutrient will be plants. It is clear, therefore, that the environment will be carbohydrate-rich but relatively nitrogen- and phosphate-poor. Being a member of the soil community, the *Actinomycetes* will be in competition with a very large number of other microbes, including bacteria, fungi and protists, and so we might expect that soil is a nutrient-poor or oligotrophic environment.

In a review Williams (1985) made the statement: Overall assessments of the rates of energy input into the soil mass and the biomass of bacteria maintained that soil is a grossly oligotrophic environment. This may seem surprising considering the constant input of material into soil. A most important point Williams makes in the article is that 'while there is a great deal of nutrient input' the soil biomass is so great that it is very rapidly exhausted. While in the laboratory under rich nutrient conditions streptomycetes can have generation times as short as one hour, in woodland soil the generation time for a streptomycete was estimated as 1.7 days (Williams, 1985). In his article Prof. Williams concluded that streptomycetes are facultative oligotrophs, i.e. they are capable of growing in both nutrient poor (oligotrophic) conditions and nutrient rich (copiotrophic) conditions. The following discussion of the control of streptomycete primary metabolism makes most sense if we consider streptomycetes as facultative oligotrophs.

1.7. Autotrophic Actinomycetes

Ware and Painter (1995) reported a filamentous bacterium capable of growing, on potassium cyanide as sole source of nitrogen and carbon on silica gel plates. Peptone and agar inhibited growth. The colonies formed aerial hyphae and small rod-shaped cells were released. This rather sounds like a nocardioform bacterium but, unfortunately, no further work on the strain was reported. Takamiya and Tubaki (1986) reported a streptomycete, S. *autotrophicus,* which was capable of growing on hydrogen, oxygen and carbon dioxide and simple salts medium. The organism has recently been transferred to a new genus, as *Amycolata autotrophica* (Lechevalier *et al.,* 1986).

Wainwright *et al.* (1984) reported the ability of a *Streptomyces* sp. to oxidize sulphur thiosulphate and tetrathionate to sulphate. However, this strain could not fix carbon dioxide but it could grow oligocarbophilically. No evidence was presented which confirmed that energy could be harvested from this oxidation. Yagi *et al.* (1971) reported that the majority of streptomycetes could oxidize sulphur to thiosulphate. It was shown that S. *virginiae* and S. *lavendulae* could not or could do so only poorly in the same minor cluster group (F61) and S. *erythreus* has since been transferred to the genus *Saccharopolyspora.* Again no evidençe- was presented that implying that the bacteria could gain useful energy from the process. The final product of keratin catabolism by a strain of *S.fradiae* (G68) was thiosuphate. Thiosulphate was also the product of a hydrogen sulphide-oxidizing enzyme purified from an uncharacterized streptomycete, *Streptomyces* sp. SH91, isolated from pig faeces compost.

A number of true autotrophic streptomycetes have been identified and characterized since the late 1980s. In all cases they gain ATP from oxidation of carbon monoxide (CO) and are moderate thermophiles. The first report was that of *Streptomyces* sp. G26 by BelI *et al.* (1988) and a very different streptomycete, S. *thermoautotrophicus*, was reported by Gadkari *et al.* (1992).

The taxonomic affiliation of *Streptomyces* sp. G26 has not yet been reported, but O'Donne11 *et al.* (1993) isolated and characterized the taxonomy of CO-utilizing s*treptomyces* and recently named two new species, *Streptomyces thermocarboxydovorans* and *Streptomyces them1Ocarboxydiis,* as representatives of the major cluster groups (Kim *et al.,* 1998). Surprisingly for a carboxydotrophic bacterium, *Streptomyces* sp. G26 could not grow on CO_2 as sole carbon and H_2 as sole energy source. However, more usually, and unlike S. *thermoautotrophicus,* it was a facultative chemolithotroph and could also grow on complex organic nutrient media. *Streptomyces* sp. G26 contains--the enzymes of the Calvin CO_2 fixation cycle and a CO oxidoreductase. The latter enzyme was purified to 95% homogeneity and found to have a 'uniquely low specificity towards its oxidising substrate' (BeII *et al.,* 1988).

It appears that S. *thermoautotrophicus* is unique in being an obligate carboxydotrophic chemolithotroph. The CO oxidoreductase of S. *thermoautotrophicus* is unusual in its electron acceptor specificity (Gadkari *et al.*1990) and its failure to show any homology to the homologous class of CO oxidoreductase found in both Gram-positive and Gram-negative bacteria (Hugendieck and Meyer, 1992). It was also reported that S. *thermoautotrophicus* could grow in the absence of any fixed nitrogen with a doubling-time of 10 h. and that ^{15}N2 could be incorporated into biomass (Gadkari *et a/.,* 1992). $^{15}N_2$ incorporation was inhibited by addition of ammonium. There was evidence that an entirely novel fixation pathway was involved: there was no acetylene reduction; fixation was not inhibited by the addition of acetylene, an inhibitor of nitrogenase; and there was no cross hybridization with the highly concerned nitrogenase structural genes *nifH.* and *nifKD.* However, there was evidence of hydrogen release during nitrogen fixation, which does occur during the action of nitrogenase. It was observed that the growth rate was not affected by growth on nitrogen or ammonium, although the growth yield was. This was a surprise, as nitrogen fixation requires a lot of ATP and reducing power, often limiting in autotrophs. Recently Ribbe *et- al.* (1997) reported further on the dinitrogenase enzyme Nitrogen fixation was coupled to CO oxidation by a rnolybdenum-containing CO dehydrogenase which generated superoxide anion (O^-_2).

The O^{-}_{2} was oxidized to O by a manganese-containing O^{-}_{2} oxidoreductase and the electrons transferred directly to a molybdenum-iron-sulphur-containing dinitrogenase. The latter enzyme is unique in requiring oxygen and being resistant to hydrogen peroxide, whereas all other nitrogenases are exquisitely sensitive to oxygen and hydrogen peroxide.

1.8. Biosynthetic Reactions and Pathways of *Actinomycetes*

For several years the opinion was widespread, among students of secondary metabolism, that the biosynthesis of antibiotics could involve patterns and reactions quite different from those of primary metabolism. This notion was based on the complexity of the structure of many metabolites, whose formation could not be easily interpreted on the basis of known microbial biochemistry. In addition, several secondary metabolites contain chemical groups, such as chlorinated moieties or nitro groups, absent in primary metabolites. However, as more biosynthetic pathways were elucidated, similarities in the two groups of reactions were observed. Today we see that there is a close relationship between primary and secondary metabolism. In fact, it is reasonable to hypothesize that the enzymes now deputed to the synthesis of special metabolites evolved from those of general metabolism. Confirmation of this hypothesis, which is so far based only on the formal similarity of a number of reactions, will probably be obtained in the future by comparison of the corresponding gene sequences. The limited data today available are in agreement with this idea.
Apparently even an esoteric reaction such as the chlorination of biological molecules can be associated with common reactions when the relevant enzymes are examined. It has been found, for instance, that the enzyme that catalyzes the chlorination step in chloramphenicol biosynthesis is a heme protein. This has bromoperoxidase and catalase activity, sharing several properties with conventional bacterial catalases such as the one from *Micrococcus luteus.*

Classification of antibiotics according to their biosynthesis is generally based on the primary metabolites from which they are derived. We thus have antibiotics derived from sugars, amino acids, acetate, nucleotides, and so on. Although useful, this classification is insufficient to order the biosynthetic pathways in a meaningful way, since several unrelated series of reactions can occur from the same metabolite. Moreover, many antibiotics are composed of intermediates derived from different primary metabolites.

The system can be refined if we consider, in addition to the starting molecules, the pattern of reactions, which can be ordered according to the classical biochemical pathways of primary metabolism.

We can consider three classes of biosynthetic reactions:

1. Class 1 reactions are those by which a primary metabolite is transformed into an intermediate of the biosynthesis. These reactions can be divided according to the general metabolic pathway to which they are related, such as amino acid synthesis and catabolism, nucleotide metabolism, sugar transformation, or coenzyme synthesis. The biosynthetic intermediates can: (a) be further modified, thus giving rise to antibiotics derived from a single primary metabolite; (b) be condensed with one or two other intermediates, thus giving rise to more complex molecules, in analogy with the synthesis of some coenzymes, such as folic acid, coenzyme A, or the prosthetic group of quinoproteins; (c) be condensed to several similar metabolites by class 11 reactions.

2. Class 2 reactions are polymerization reactions by which several similar units are linked together to form larger molecules. They are the key step in the synthesis of a vast number of antibiotics. Four major types of polymerization reactions are recognized: (a) condensation of acetate-malonate (sometimes propionate-methylmalonate) units, by a mechanism analogous to that of fatty acid synthesis, denoted polyketide synthesis; (b) condensation of amino acids (sometimes hydroxy acids) to form peptide or depsipeptide antibiotics; the mechanism involved, denoted thiotemplate mechanism, has some similarity with that of polyketide synthesis; (c)

condensation of isoprenoid units, as in the synthesis of terpenoids and sterols; (d) condensation of sugar units to give oligosaccharides. In addition, a few polypeptide antibiotics are synthesized by the normal transcription-translation system of protein biosynthesis.

3. Further modifications of the assembled molecule are performed by class III reactions. These normally fall into a few categories quite common in primary metabolism; for example, oxidations or reductions, methylations, glycosylations. The halogenation reaction, mentioned previously, may occur either as an early or as a late biosynthetic step. The production, commonly observed in many strains, of families of closely related substances is frequently the result of class III reactions. Thus, a single culture may produce several metabolites presenting a common general structure but differing for instance in the degree of methylation, in the level of oxidation, or in the length of acyl substituents. This is considered the effect of a low level of substrate specificity of the enzymes involved in secondary metabolism, in contrast to the specificity of those of primary metabolism. Provided that all of the different molecules eventually resulting from the reaction sequences were similar in their biological activity, it is conceivable that there was no selective pressure operating during evolution favoring one substrateenzyme relationship. It has to be remembered that, generally speaking, the enzyme specificity is not related to the entire molecular structure but to defined regions that fit in the enzyme active site. It is therefore likely that relatively large molecules, presenting some minor difference in their structure, may be substrates of the same enzymatic reaction, without implying a lack of enzymatic specificity.

In the following sections, to illustrate the variety of patterns by which secondary metabolites are made, a few examples of typical reactions of each class are given.

1.9. Metabolism of *Actinomycetes*

1.9.1. Primary Metabolism

Primary metabolism involves the catabolic and anabolic reactions that result in an increase in biomass; that is, the reactions that lead to the harnessing of energy and reducing power that in turn are used to synthesize the building blocks of proteins, nuc1eic acids, lipids and polysaccharide structural and storage materials. Regulation of primary metabolism is related with carbon and nitrogen metabolism of the organisms.

1.9.1.1. Carbon Metabolism

1.9.1.1.1. The Central Pathways of Glucose Catabolism

1.9.1.1.1.1. Glycolysis

A fermentation is an internally balanced oxidation –reduction reaction in which some atoms of the energy source (electron donor) become more reduced whereas others become more oxidized, and energy is produced by substrate-level phosphorylation. A common biochemical pathway for the fermentation of glucose is Glycolysis**,** also named the Embden-Meyerhof Pathway (EMP) for its major discoverers. Glycolysis can be divided into three major stages, each involving a series of individually catalyzed enzymatic reactions. Stage I of glycolysis is a series of preparatory rearrangements, reactions that do not involve oxidation-reduction and do not release energy but that lead to the production from glucose of two molecules of the key intermediate, glyceraldehyde 3-phosphate. In stage II, oxidation-reduction occurs, energy is conserved in the form of ATP, and two molecules of pyruvate are formed. In stage III, a second oxidation-reduction reaction occurs and fermentation products (for example, ethanol and CO_2, or lactic acid) are formed.

Some author showed the pathways of glucose catabolism in *Actinomyces*. Evidence was presented that supported the use of the EMP and hexose monophosphate plus pentose phosphate (Hmp) pathways in S. *coelieolor* (A1*A), S. griseus* (A 1 B), S. *scabies* (A3). Isotope studies implied, at least for S. *griseus* (A 1 B), that the former was the more important pathway. Work by Dekleva and Strohl (1988) expanded these observations to cover *Streptomyees* C5, S. *lividans (*A21) and S. *aureofaciens* (A14). Obanye *et al.* (1996) studied the EMP and Hmp pathways in S. c*oelieolor* A3 (2) (A21) and found the former to be active during exponential growth and the latter to be more active during the transition phase between exponential growth phase and stationary phase, when secondary metabolism was active.

Phosphofructokinase is, one of the main sites of regulation of the EMP pathway and enzyme and its gene have been studied in *Streptomyces coelicolor* A3(2) (A21) (Alves et al..1997). The enzyme is ATP-dependent in contrast to the enzyme from another actinomycete Am*ycolalopsis methanolica* which is pyrophosphate-dependent. The ATP-dependent enzyme was allosterrically inhibited by phosphoenolpyruvate (PEP). The stimulatory effect or otherwise of ADP and GDP was not reported.

Another EMP pathway enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been studied in S. *arenae* (A 18) and S. *aureofaciens* (A 14). The enzyme was studied in S. *arenae* (A8) because there are two forms: one expressed during primary metabolism and one expressed as a resistance mechanism during the production of pentalenolactone an inhibitor of the former isozyme. The pentalenolactone-insensitive form has been sequenced and been shown to be similar to other Gram-positive GAPDs (Froehlich et *al.*. 1996). The gene for S. aureofaciens (A14) GAPDH was identified as linked to an RNA polymerase sigma factor. The gene was shown to be induced by glucose and, in the absence of glucose, at the time of aerial mycelium formation (Kormanec et *al.*. 1997).

One of the essential enzymes of the EMP pathway, phospoglycerate mutase has been purified and its gene cloned and sequenced from S. c*oelicolor* A3 (2) (A2l). This enzyme is responsible for the interconversion of 3-phosphoglycerate and 2-phosphoglycerate and had properties and sequence homology to the same enzyme from *Saccharomyces cerevisiae* (White *et al.,* 1992).

The origin of the glucose-6-phosphate from glucose in streptomycetes has also received some attention. In many bacteria, glucose is phosphorylated as it is transported into the cell via the phosphoenolpyruvate : carbohydrate : phosphotransferase system (PEP:PTS) (Saier, 1985). Sabater *el al.* (1972) reported their inability to find a PEP:PTS system in S. *violaccoruber* (A21); however, they did find an ATP-dependent glucose kinase with an apparent K_m of 0.3 mM. Novotna and Hostalek (1985) confirmed the absence of a PEP:PTS system in S. *aureofaciens* (A 14). Hodgson (1982) reported that mutants of S. *coelicolor* A3(2) S. *violaceoruber* (A21)) lacking glucose kinase enzyme activity could nonetheless transport glucose, implying that a glucose PEP:PTS system was not present. Ikeda *et al.* (1984) further characterized the glucose kinase from this strain, confirming the absence of a PEP:PTS system for glucose. The enzyme had an apparent K_m of 0.27 mM for glucose. An ATP-dependent glucokinase activity was also identified in S. *clavuligerus* (J71). This was surprising, as the strain could not use glucose as sole energy or carbon source. The presence of a polyphosphate-dependent glucose kinase was reported by Hostalek *et al.* (1976) in S. *aureofaciens* (A14). They found that the enzyme was present only after logarithmic growth, unlike the ATP-dependent kinase, which was present only during logarithmic growth. Recent work has confirmed the presence of the 'minimal' PEP:PTS system for transport and phosphorylation of fructose in streptomycetes.

1.9.1.1.1.2. The Tricarboxylic Acid Cycle

The early steps in the respiration of glucose involve the same biochemical steps as those of glycolysis. As noted, a key intermediate in glycolysis is pyruvate. Whereas in fermentation pyruvate is converted to fermentation products, in

respiration pyruvate is oxidized fully to CO_2 . One major pathway by which pyruvate is completely oxidized to CO_2 called the citric acid cycle.

Pyruvate is first decarboxylated, leading to the production of one molecule of NADH and acetyl molecule coupled to coenzyme A (acetyl-CoA). The acetyl group of acetyl-CoA combines with the four-carbon compound oxalacetate, leading to the formation of citric acid, a six-carbon organic acid, the energy of the high energy acetyl-CoA bond being used to drive this synthesis. Dehydration, decarboxylation, and oxidation reactions follow, and two additional CO_2 molecules are released. Ultimately, oxalacetate is regenerated and can fuction again as acetyl acceptor, thus completing the cycle.

Besides playing a key role in catabolic reactions, the citric acid cycle is important to the cell for biosynthetic reasons as well. This is because the cycle is composed of a number of key intermediates that can be drawn off for biosynthetic purposes when needed. Particularly important in this regard are the intermediates α-ketoglutarate and oxalacetate, which are the precursors of a number of amino acids, succinyl-CoA, needed to form the porphyrin ring of the cytochromes, chlorophyll, and several other tetrapyrrole compounds. Oxalacetate is also important because it can be converted to phosphoenolpyruvate, a precursor of glucose. In addition to these, acetyl-CoA provides the starting material for fatty acid biosynthesis. Thus, the citric acid cycle plays two major roles in the cell bioenergetics and biosynthetic. Much the same can be said about the glycolytic pathway, as intermediates from this pathway are drawn of for various biosynthetic needs as well.

A variety of organic acids can be used by microorganisms as carbon sources and electron donors. The acids of the citric acid cycle, such as citrate, malate, fumarate and succinat, are common natural products formed by plants and are also fermentation products of microorganisms. Because the citric acid cycle has major

biosynthetic as well as energetic functions, the complete cycle or major portions of it are nearly universal in microorganisms. Thus, it is not surprising that many microorganisms are able to use these acids as electron donors and carbon sources. Aerobic utilization of four-, five-, and six-carbon acids can be accomplished by means of enzymes of the citric acid cycle, with ATP formation by oxidative phosphorylation.

Anaerobic utilization of organic acids usually involves conversion to pyruvate followed by formation of acetate via acetyl phosphate with consequent ATP production by substrate-level phosphorylation.

The enzymes of the tricarboxylic acid (TCA) cycle have been studied in a number of streptomycetes because of their importance in the supply of precursors to secondary metabolism. The evidence that the TCA cycle is complete and active in a number of streptomycetes. Dekleva and Strohl (1988) demonstrated that a complete TCA cycle was alsa present in *Streptomyees* C5.

Hostalek and co-workers (1976) have devoted a lot of time to studying the enzymes of this cycle in S. *aureofaciens*. However, they did use improved strains in their studies. They reported (Hostalek *et al* 1969b) the presence of all the expected enzymes of the complete cycle and noted correlation of activity in different strains and abiIity to synthesize chlortetracycline antibiotic. Hostalek (1969a) reported some of the characteristics of the citrate synthase. Like the enzyme from *E. coli,* ATP inhibited the enzyme and AMP stimulated it. However, unlike *E. coli,* reduced NAD had no affect. One, perhaps surprising, result of their studies (Tinterova *et al.,* 1989) was the discovery of isozymes of malate dehydrogenase showing different responses to oxaloacetic acid and requirements for magnesium. The recent purification and characterization of imiIate dehydrogenase from S. *aureofadens* (AI4) by Mikulasova *et al.* (1998) revealed a single enzyme with a strong preference for NADH and a more effident back reaction, reduction of oxaloacetate, than forward reaction. Surprisingly, there was no evidence of product inhibition of malate oxidation; i.e. excess oxaloacetate did not inhibit.

1.9.1.1.1.3. Glyoxylate Cycle

Utilization of two- or three-carbon acids as carbon sources cannot occur by means of the citric acid cycle alone. This cycle can continue to operate only if the acceptor molecule, the four carbon acid *oxalacetate,* is regenerated at each turn of the cycle; any removal of carbon compounds for biosynthetic reactions would prevent completion of the cycle. When acetate is used, the oxalacetate needed to continue the cycle is produced through the glyoxylate cycle which is important anaplerotic reactions, so called because glyoxylate is a key intermediate. This cycle is composed of the citric acid cycle reactions plus two additional enzymes; isocitrate lyase, which splits isocitrate to succinate and glyoxylate, and malate synthase, which converts glyoxylate and acetyl-CoA to malate. Besides the glyoxalate cycle; carboxylation of pyruvate by pyruyate carboxylase or carboxylation of PEP by PEP carboxykinase or PEP carboxylase to produce oxaloacetate are anaplerotic reactions.

Biosynthesis through glyoxylate cycle occurs as follows. The splitting of isocitrate into succinate and glyoxylate allows to succinate molecule (or another citric acid cycle intermediate derived from it) to be drawn off for biosynthesis. Because glyoxylate combines with acetyl-CoA to yield malate. Malate can be converted to oxalacetate to maintain the cyclic nature of the citric acid cycle despite the fact that a C_4 intermediate (succinate) has been drawn off. The succinate molecule can be used directly in the production of porphyrins, be oxidized to oxalacetate and serve as a carbon skeleton for C_4 aminoacids, or be converted (via oxalacetate and phosphoenolpyruvate) to glucose.

In S. *aureofaciens* (A14), malate synthase was found but not isocitrate lyase (Hostalek *et al.,* 1969b). Dekleva and Strohl (1988) reported that *Streptomyces* C5, S. *lividans* (A21) and S. *aureofaciens* (A14) used acetate poorly and that the enzymes isocitrate lyase and malate synthase were not present in the author's laboratory, S. *lividans* (A21) and S. *coelicolor* A3(2) (A21) grow on acetate, albeit poorly. However, these strains did grow well on Tween, a source of fatty acids, which,

following fatty acid catabolism, is the same as growing on C2 compounds. The relatively poor growth on acetate is probably related to weak acid poisoning. Isocitrate lyase and malate synthase were identified in S. *lividans* (A21) and S. *coelicolor* A3(2) (A21) during growth on Tween, but only the former when growing on acetate (Han and Reynolds, 1997). Malate synthase has been identified in S. *arenae* (A18) grown on acetate or ethanol (Huettner *et al.,* 1997) and S. *clavuligerus* (J71) exposed to acetate . In neither case was there a report of isocitrate lyase activity.

The consistent absence of isocitrate lyase activity in *Actinomycetes* growing on C2 compounds has led to the suggestion of an alternative route to glyoxylate in *Actinomycetes.* When S. *collinus (AI8)* was grown on acetate no isocitrate lyase was present. However, the enzyme was present when the streptomycete was grown on Tween. The proposed pathway of glyoxylate synthesis arose from the discovery of two genes that were essential for acetate utilization: ccr, which encodes crotonyl-coenzyme A (CoA) reductase, and *meaA,* which encodes a novel vitamin B_{12}-dependent mutase (Han and Reynolds, 1997). The authors proposed two pathways by which glyoxylate could be generated from acetyl-CoA. Both pathways would require propionyl-CoA carboxylase, which has been identified in S. *coelicolor* A3(2) (A21) (Bramwell *et al.,* 1996). The authors also demonstrated the activity of isobutyryl-CoA mutase and a methylmalonyl-CoA mutase and propose d an anaplerotic path-way whereby condensation of two acetyl-CoAs would lead to the formation of succinyl-CoA and hence succinate.

If either or both the proposed S. *collinus* (A 18) glyoxylate-generating pathways exist and are common to streptomycetes, it would explain why malate synthase was present during acetate growth but not isocitrate lyase during C2 growth. The implication would be that growth on aliphatic compounds. such as fatty acids, involves the more common glyoxylate cycle with malate synthase and isocitrate lyase.

1.9.1.1.1.4. Production of Fermentation Products

During the formation of two molecules of 1,3-bisphosphoglyceric acid, two molecules of NAD^+ are reduced to NADH. However, a cell contains only a small amount of NAD^+, and if all of it were converted to NADH, the oxidation of glucose would stop; the continued oxidation of glyceraldehyde 3-phosphate can proceed only if there is a molecule of NAD^+ present to accept released electrons. This "roadblock" is overcome in fermentation by the oxidation of NADH back to NAD^+ through reactions involving the reduction of pyruvate to any of a variety of fermentation products (in the case of yeast, pyruvate is reduced to ethanol with the release of CO_2, in lactic acid bacteria, pyruvate is reduced to lactate). Many routes of pyruvate reduction in various fermentative prokaryotes are known, but the net results is the same; NADH must be returned to the oxidized form, NAD^+, for the energy-yielding reaction of fermentation to continue. As a diffusible coenzyme, NADH can move away from glyceraldehyde 3-phosphate dehydrogenase**,** attach to an enzyme that reduces pyruvate to lactic acid (lactate dehydrogenase), and diffuse away once gain following the oxidation of NADH to NAD^+ to repeat the cycle all over again.

In energy-yielding process, oxidation must balance reduction and there must be an electron acceptor for each electron removed. In this case, the reduction of NAD^+ at one enzymatic step in glycolysis is balanced with its oxidation at another. The final product(s) must also be in oxidation-reduction balance with the starting substrate, glucose.

1.9.1.1.1.5. Acid Secretion

Actinomycetes are usually classified as obligatory aerobic. Some strains have been reported to be able to grow microaerophically. There are two reports of dissimilatory nitrate and nitrite reduction in *Actinomycetes.* The implication is that anaerobic respiration is possible with nitrate or nitrite as electron acceptor(Albrecht *et al.,*1997

Shoun *et al.,* 1998). Albrecht *et al.* (1997) demonstrated that growth on nitrate under an aerobic conditions was impossible. So, the fact remains that *Actinomycetes* seem to need oxygen. The majority of bacteria that use the EMP pathway or the hexosemonophosphate shunt can grow anaerobically on glucose and related sugars. They can harvest energy through substrate phosphorylation and regenerate reduced NAD by reduction of pyruvate to ethanol or lactate. Hockenhull *et al.* (1984) demonstrated that S. *griseus* (A1B) produced low levels of lactate during growth on glucose under a restricted aeration regime, which would imply that lactate dehydrogenase was present. Perhaps *Actinomycetes* with lactate dehydrogenase, and/or pyruvate decarboxylase plus ethanol dehydrogenase, are sensitive to these waste products. Alternatively there could be a requirement for oxygen in other essential process.

Whilst studying lactate production by S. *griseus* (A 1 B), Hockenhull *et al.* (1984) noted that during exponential growth pyruvate was excreted into the medium. Kannan and Rehacek (1970) reported the excretion of TCA intermediates into the medium by S. *antibioticus* (A31). Since these initial reports quite a number of papers have been published on this phenomenon. Ahmed *et al.* (1984) repoi1ed acid secretion by older mycelium during nitrogen-limited growth in S. *venezuelae* (A6). They reported that during the transition to acid excretion the rate of glycolysis remained constant but there was loss of α-ketoglutarate dehydrogenase activity. They isolated a mutant that no longer excreted acid and in which the enzyme remained active in older mycelium. Unlike the case of S. *aureofaciens* (A14), the acids were not reabsorbed into the cell and used later, presumably because the α-ketoglutarate dehydrogenase activity remained low.

Surowitz and Pfister (1985) examined acid secretion in S. *alboniger* (A1B) cells growing on glucose. This strain had been shown to secrete organic acids during growth on glucose. These acids inhibited aerial mycelium formation, but this inhibition could be reversed by the addition of adenine. Surowitz and Pfister showed that the acid produced was pyruvate. They also showed, contrary to the observation

in S. *venezuelae.,* that the enzymes of the TCA cycle were not affected by growth 011 glucose; however, the rate of glycolysis increased. The lack of balance between glycolysis and the TCA cycle was responsible for the acid excretion. This imbalance was corrected by the addition of adenine. The authors stated that they had not achieved conditions that led to reabsorption and use of the acid at a later stage.

It was reported the acidogenesis of S. *peuceticus.* In contradiction to all previous reports, this strain produced large amounts of acid only in the stationary phase, and then only in some cases and not others. All cultures produced low levels of pyruvate and α-ketoglutarate, however. Again, the excess acid was reabsorbed for reuse. These authors also reported that S. *lividans* and S. *coelicolor* A3(2) (A21) did not produce acid under their conditions. Conditions have been found for excretion of pyruvate and α-ketoglutarate and reabsorption of the former in S. *coelicolor* A3(2) (A21) and S. *lividans* (A21) (Madden *et al.,* 1996).

In at least some cases, it is clear that acid secretion is a result of imbalance between glycolysis and the TCA cycle. Some strains appear to shut down the TCA cycle, which may be a consequence of ageing, possibly a step in the shift from primary to secondary metabolism. The inability of some strains to stimulate the TCA cycle whilst stimulating glycolysis might be a consequence of the mode of gene regulation in that organism. Acidogenesis might be a stopgap method of dealing with excess glucose. Energy could be gained by substrate phosphorylation, and the product of glycolysis, in this case pyruvate, could be secreted into the medium for use later when the glucose supply was exhausted. Presumably more energy could be trapped and reduced NAD reoxidized by the action of the terminal respiratory pathway.

1.9.1.2. Control of Primary Metabolism in Actinomycetes

1.9.1.2.1. Carbohydrate- Uptake

Once complex polysaccharides have been broken down by the extracellular enzymes into oligo-, di- and monosaccharides, they can be taken into the cell. In some cases, oligo- and disaccharidases may be present inside and outside the cell. One imagines that it would be to the streptomycetes' advantage to be able to transport as complex a carbon and energy source as possible into the cell so as to deny them to any competitor.

Attempts to find a PEP-coupled phosphotransferase system (PEP-PTS) for glucose transport and phosphorylation in streptomycetes have proved unsuccessfull. However, Titgemeyer *et al.* (1995) have presented evidence for the presence of a PEP-PTS for fructose transport in S. *lividans* (A2l), S. *coelicolor* A3(2) (A21) and S. *griseofuscus* (A12). The systems were inducible by fructose in the first two strains and constitutive in the third. Specific fructose transport was not assayed, but membrane-bound PEP-dependent phosphorylation of fructose was observed in addition to a soluble ATP-dependent fructose phosphorylation in S. *lividans* and S. *coelicolor* A3(2). There was already overwhelming evidence of an ATP-dependent fructose kinase in the closely related S. *violaceoruber* (A21) (Sabater *et al.,* 1972b). The constitutive PEP-dependent and ATP-dependent fructose phosphorylation activities found in S. *griseofuscus* were lower than in the other two streptomycetes, and there was only weak evidence or a membrane-bound PEP-dependent phosphorylation of fructose in this streptomycete. No PEP-dependent glucose phosphorylation activity was found in any of the streptomycetes, nor were the HPr (Ser) kinase or HPr (Ser-P) phosphatase activities found. The latter activities are central to PEP-PTS control of catabolite repression in low GC Gram-positive bacteria. Therefore, that the PEP-PTS fructose transport system has no role in catabolite repression in streptomycetes.

A number of carbohydrate uptake systems have been identified and studied in streptomycetes. They are classified into two types: inducible and the constitutive. The inducible carbohydrate transport systems were generally induced by the substrate. Glucose repressed a number of inducible transport systems and inhibited a number of constitutive transport systems, which raises the possibility that the mode of action of glucose repression is via inducer exclusion; i.e. glucose inhibits the transport of the inducer into the cell. In a number of cases, 'constitutive' uptake systems were found only in mycelia but not in spores. Glucose transport systems were present in S. *violaceoruber* (A21) (Sabater and Sebastiane, 1972) and S. *antibioticus* (A31) (Salas et al., 1984) spores. In the case of S. *violaceoruber* (A21) the germination process induces the 'constitutive' fructose and mannose.

Where substrate affinity *(K_m)* of streptomycete carbohydrate uptake systems has been reported, a dichotomy is apparent. The inducible uptake systems show affinities similar to those seen for carbohydrate uptake in most other bacteria; i.e. within the μM range. However, the constitutive uptake systems have substrate affinities that are a thousand-fold, or more, lower in affinity. It is a pleasing congruence that the theoretical calculation of the K_m for glucose transport in S. *coelicolor* A3(2) (A21), 6.1 mM, agreed with the experimentally determined value for glucose transport by S. *lividans* (A21), 6.2 mM .

It is interesting to note that no one has reported the isolation of a carbohydrate transport mutant using toxic sugar analogues such as 2-deoxyglucose (Hodgson, 1982) and 2-deoxygalactose (Kendall *et al.,* 1987). Perhaps there are multiple transport systems present, as observed for glucose in S. *aureofaciens* (A 14) (Novotna *et al.,* 1985) and S. *lividans* (A21). The high-affinity glucose permease in S. *lividans* (A21) has a V_{max} of 66 nmol min^{-1} mg $(protein)^{-1}$. The equivalent value for the low-affinity system is 27 nmol min^{-1} mg $(protein)^{-1}$.

It appears that S. *clavuligerus* NRRL 3585 (J71) is a natural glucose transport mutant (Garcia-Dominguez *et al.,* 1989). This strain has the ability to grow on starch,

maltose and glycerol, but not on glucose or galactose. It was demonstrated that the strain had an ATP-dependent glucokinase. A mutant, *gut1,* was isolated that could grow with glucose as sole energy and carbon source. The mutant also grew on galactose. The kinetics of glucose transport was examined in both wild type and mutant. Two transport systems were identified in both strains. A high-affinity, (5-6 μM) low-capacity (140-190 nmol min^{-1} mg (dry weight)$^{-1}$ transport was present in both strains. A low-affinity (3.7 mM) high-capacity (1.0 mmol min^{-1} mg (dry weigh)$^{-1}$ transport system was present in the mutant that had higher affinity, but lower capacity, than the equivalent system in the wild type (K_m= 12.5 mM and V_{max} = 2.5 mmol min^{-1} mg (dry weight)$^{-1}$. It is presumed that the changes in the kinetic parameters of the low-affinity transport system are responsible for the ability of the *gutl* mutant to use glucose as a carbon source.

There is now c1ear evidence that disaccharide transport in streptomycetes is via five component ABC permeases: maltose in S. *coelicolor* A3(2) (A21) (van Wezel *et al.,* 1997a,b); cellobiose/cellotriose in S. *reticuli* (Schloesser and Schrempf, 1996; Schloesser *et al.,* 1997,1999); and cellobiose, xylobiose and maltose in *S lividans* (A21) (Hurtubise *et al.,* 1995; Schloesser *et al.,* 1997). A specific cellobiose-binding lipid-anchored protein has been identified in S. *reticu!i*. This protein is equivalent to the sugar-binding proteins found in the periplasm of Gram-negative bacteria. As streptomycetes are Gram-positive, they do not have a periplasm and so such 'periplasmic' proteins have to be anchored by a fatty acid adduct, in this case palmitate (Schloesser and Schrempf, 1996). A gene product, MsiK, has been identified in S. *lividans* (A21) and S. *reticuli* that is involved in the independent trans ort s stems for cellobiose, xylobiose and maltose in the former streptomycete, and for cellobiose in the latter streptomycete (Hurtubise *et al.,* 1995; Schloesser *et al.,* 1997). Loss of MsiK leads to loss of ability to transport all of the different disaccharides. The maltose-induced, glucose-repressed maltose transport genes, *malEFG,* of S. *coelicolor* A3(2) (A21) are subject to the control of a repressor, MaIR. This repressor is related to the LacI-GalR family of repressors. Loss of *malE* led to loss of ability to transport maltase. Loss of MaIR led to constitutive expression of *malEFG* and

expression was no longer subject to glucose repression (van Wezel *et al.,* 1997a, b). The homologue of MalR in S. *lividans* (A21), Reg1, was shown to be required for repression of amylase and chitinase genes. Loss of Reg1 led to inducer-independent expression of the extracellular enzymes and loss of glucose repression (Nguyen *et al.,* 1997a).

1.9.1.2.2. Inducible Intracellular Carbohydrate Catabolic Enzymes

Over the last few years a number of carbohydrate catabolism genes have received detailed attention. The glycerol, xylose and galactose catabolism genes have been cloned and subjected to molecular analysis.

1.9.1.2.2.1. Glycerol Catabolism.

Glycerol is dissimilated by glycerol kinase encoded by GUT1 (Sprague and Cronan, 1977; Pavlik et al., 1993) and a specific flavin adenine dinucleotide (FAD)-dependent and mitochondrion-located glycerol-3-phosphate dehydrogenase (Gut2p) encoded by GUT2 (Ronnow and Kielland-Brandt, 1993). Mutants defective in GUT1 or GUT2 are unable to utilize glycerol as carbon source (Pavlik et al., 1993; Ronnow and Kielland-Brandt, 1993). The product formed by glycerol kinase, glycerol-3-phosphate, can be used either as a precursor for lipid biosynthesis or for conversion to dihydroxyacetone phosphate. The latter intermediate can either be transformed to glyceraldehyde-3-phosphate by a triose phosphate isomerase into the center metabolic pathway or can serve as a substrate for the synthesis of other metabolites (Grauslund et al., 1999). Gpd1p, Gpd2p and Gut2p also contribute to the ‘glycerol-3-phosphate shuttle’ of yeast, which may play an important role during aerobic growth of S. cerevisiae (Larsson et al., 1998). This route of glycerol dissimilation has also been observed in a number of other yeasts such as D. hansenii (Adler et al., 1985), Z. Rouxii , C. and S. pombe glycerinogenes (Wang et al., 2000).

S. *coelicolor* A3(2) (A21) (Seno and Chater, 1983), S. *lividans* (A21) and S. *griseus* (A1B) (Biro and Chater. 1987) catabolise glycerol via a glycerol-inducible, glucose-repressible glycerol transport facilitator (the *gylC* product), glycerol kinase (the *gylA* product) and glycerol-3-phosphate dehydrogenase (the *gylB* product). The operon is arranged as *gylCABX,* where *gylX* is of unknown function (Hindle,1994). There is strong evidence that the true inducer of the operon is glycerol-3-phosphate, the product of glycerol kinase activity (Seno and Chater, 1983).

Upstream of the operon is *gylR* (formerly the 0.9-kb gene), which encodes a 27 600 Mr protein with a helix-turn-helix domain characteristic of DNA-binding proteins. Loss of this gene leads to constitutive expression of the operon; i.e. it is a repressor. Point mutations in the *gylR* gene that cause a Gyl- phenotype must, therefore, be superrepressor mutations. Interestingly, the constitutively expressed operon in the $gylR^{\Delta}$ mutant is no longer subject to glucose repression (Hindle, 1994). The *gylR* region of S. *griseus* (A1B) has also been sequenced (Bolotin and Biro, 1990). The *gylR* gene showed 81 % sequence identity to the S. *coelicolor* A3(2) (A2l) *gylR* gene. This value shows agreement with that obtained from analysing the levels of hybridization and restriction site polymorphism between the two strains; i.e. about 87% (Biro and Chater, 1987). They also reported that the genes of S. *lividans* (A21) were 99% similar, and those of S. *parvulus* (A12) were 96% similar, to the S. *coelicolor* A3(2) genes.

1.9.1.2.2.2. Galactose Catabolism.

The galactose operon of S. *lividans* (A21) consists of three cistrons: *galTEK,* where *galT* encodes galactose--phosphate uridylyltransferase; *galE* encodes the UDPgalactose 4-epimerase; and *galK* encodes galactose kinase (Fornwald *et al.,* 1987; Adams *et al.,* 1988). The restriction site map is very similar to that of S. *coelicolor* A3(2) (A21) (Kendal *et al.,* 1987). There is no regulatory protein gene associated with the operon.

As with the *gyl* operon, two promoters were found by S1 nuclease analysis. Promoter gal_{p1} is located 5' of *galT* and was responsible for the generation of the *galTEK* polycistronic message. The second promoter, gal_{P2} was situated between the *galT* and *galE* and produced a dicistronic *galEK* mRNA. The gal_{P1} promoter was galactose-inducible and glucose-repressible. The gal_{p2} promoter was expressed constitutively. This explains the observation that there is a constitutive low level of galactose kinase expression (Fornwald *et al.,* 1987). It has been suggested that the cell required a supply of galactose-l-phosphate and UDPgalactose for galactosyl lipid synthesis (Adams *et al.,* 1988) and perhaps cell wall synthesis. Hence *galE* and *galK* have an anabolic as well as a catabolic role. Adams *et al.* (1988) further reported that constitutive expression of *galK* is necessary for correct induction of gal_{P1} by galactose, implying that galactose-1-phosphate may be the true inducer of the operon, similar to glycerol-3-phosphate and the *gylCABX* operon. However, there has been a recent report that different forms of RNA polymerase with different sigma factors transcribe the two *gal* promoters (Bramwell *et al.,* 1998). This is similar to the situation reported by Servin-Gonzalez *et al.* (1994) for the agarase gene of S. *coelicolor* A3(2) (A21). In the *gal* operon case, the physiological significance of the use of alternative sigma factors may be more obvious than in the *dagA* case because gal_{p1} and gal_{P2} are regulated differently.

1.9.1.2.2.3. Fructose and Mannose Catabolism.

Sabater *et al.* (1972 a) identified a fructose-inducible, ATP-dependent- fructose kinase in S. *violaceoruber* (A21) that was unusual in its specificity for its substrate. This unusual specificity allowed it to be used in a specific assay for fructose (Sabater and Sebastiane, 1972). The enzyme showed unusually complex kinetics which implied positive cooperativity induced by nuc1eotides and negative cooperativity induced by fructose (Sabater and Delafuente, 1975). These findings are perhaps surprising considering the catabolic role of fructose kinase. It was suggested that high ATP or low ADP would inhibit the enzyme whilst low ATP and high ADP would stimulate it; however, the opposite was the case. Inbar and Lapidot (1992)

used ^{13}C NMR to study fructose metabolism in S. *parvulus* (A12). During rapid growth, fructose was rapidly transported into the cell and pools of mannitol, fructose and glucose-6-phosphate were formed rapidly. The accumulation of fructose and mannitol implied that fructose kinase was inactive in the strain; once glucose-6-phosphate decreased the enzyme appeared to be activated, implying the phosphosugar inhibited the enzyme. Examination of the growth conditions reveals an alternative explanation; i.e. fructose transport was constitutive but fructose catabolism has to be induced as in S. *coelicolor* A3(2) (A21) (Hodgson, 1982). It was not clear from the experiments that time had been allowed for fructose kinase induction.

Sabater *et al.* (1972a) identified a mannose-inducible, ATP-dependent, mannose kinase in S. *violaceoruber* (A21). This enzyme could also phosphorylate glucose, albeit with a lower affinity (4 mM) than for mannose (O.05mM). The inability of the enzyme to phosphorylate fructose was a unique feature.

1.9.1.3. Carbon Storage Compounds in Actinomyceteles

The glucose homopolymer glycogen, the α-1,1-linked glucose disaccharide trehalose, polyhydroxybutyrate and neutral lipids such as triacylglycerols have been implicated as carbon storage molecules in streptomycetes. Studies indicate that these molecules have different roles in the cell, and that glycogen and trehalose are closely associated with morphological development (i.e. sporulation).

1.9.1.3. 1. Glycogen

Hey-Ferguson *et al.* (1973) reported that the spores of S. *hygroscopicus* (A32) contained glycogen and that it was metabolized late in germination; essentially after the trehalose had been used up. However, no glycogen was reported in mature S. *antibioticus* (A31) spores (Brana *et al,* 1986a) or S. *venezuelae* (A6) (Ranade and Vining. 1993).

Brana *et al.* (1980) reported the accumulation and disappearance of polysaccharide granules during morphological development of S. *viridocll1v-l710genes* (A27). The first appearance of the granules was coincident with formation of sporulation septa in aerial hyphae. Once the sporulation septa were complete, the now enclosed immature spores filled with granules. About halfway through the spore maturation process the granules began to decrease in size and had disappeared by the time full maturation had been achieved. The kinetics of the process led the authors to suggest that the polysaccharide was a temporary carbon store which the organism used to hold the carbon and energy sources required later in the developmental process in an insoluble but readily mobilizable form. Later work (Brana *et al.,* 1982) revealed the polysaccharide to be glycogen. These authors also reported that a number of other streptomycetes contained glycogen as a carbon store.

Chemical and cytological analysis of the accumulation of glycogen in S. *antibioticus* (A31) confirmed the observations with S. *viridochromogenes* (A27). However, there is an additional glycogen accumulation step, phase I, in substrate mycelium at the onset of aerial mycelium formation. These reserves were catabolized as aerial mycelium development progressed. The second stage of glycogen accumulation, phase II, and degradation corresponded with the accumulation and disappearance of granules in immature spores. This led to the suggestion that phase-I accumulation, and the other products released during substrate mycelium lysis, were used as carbon and energy sources to support the growth of aerial hyphae (Mendez *et al.,* 1985; Brana *et al.,* 1986). Altering the concentration of the nitrogen source supplied to the cells could modulate glycogen accumulation, but only when they had reached the correct developmental stage (Miguelez *et al.,* 1997).

Detailed electron microscopy confirmed an identical situation in S. *coelicolor* A3(2) (A21) to that of S. *antibioticus* (A31) (Plaskitt and Chater, 1995). Analysis of cloned glycogen synthesis *(glg)* genes revealed two separate gene clusters that

encode isozymes for each step of the biosynthetic duster. Inactivation of one cluster led to modification of one phase of glycogen accumulation. This has been shown to be true for S. *coelicolor* A3(2) (A21) (Martin *et al.,* 1997) and S. *aureofaciens* (A14) (Homerova *et al.,* 1996). These observations are consistent with the idea that glycogen has a role as a carbon and energy store within the spore. On the other hand; research related with glycogen of *Actinomycetes* are so limited.

1.9.1.3.2. Trehalose

Every *Actinomycetes* so far examined contains trehalose in the vegetative mycelium (McBride and Ensign, 1987). The pioneering work of Elbein showed that contain the disaccharide and that it was synthesized in a way different from the mechanism seen in insects and fungi. Streptomycetes used GDP-D-glucose as the donor to glucose-6-phosphate in the formation of trehalose-6-phosphate. In insects and fungi the preferred donor is UDP-D-glucose. The *Km* of the streptomycete enzyme for both of its substrates was reported to be 0.7 mM.

lnbar and Lapidot (1992) investigated the activity of the enzymes of trehalose anabolism (trehalose phosphate synthase and trehalose phosphate phosphatase) and catabolism (trehalase) during spore germination in S. *hygroscopicus* (A32). Trehalase activity increased after germination before outgrowth in a nutrient medium. There was a concomitant in trehalose. The phosphatase from a high activity in spores and levelled off only after outgrowth had begun. The trehalose phosphate synthase was low in the spores and Increased with cell mass only when outgrowth had commenced. These observations are consistent with a role for trehalose as a carbon and energy store in streptomycete spores.

McBride and Ensign (1987) confirmed and expanded the foregoing work to a number of other streptomycetes and actinomycetes. They demonstrated that the final concentration of trehalose in the spores was dependent on the culture conditions of

the streptomycete. If the culture was grown on glucose-limited media, the spores contained less trehalose. In contrast, limitation of the nitrogen or phosphate source stimulated trehalose accumulation. Prolonged incubation of spores, in conditions where germination did not occur, led to a slow decrease in trehalose content, whilst germination led to a rapid metabolism of trehalose. Addition of glucose to trehalose-depleted spores stimulated trehalose accumulation, even in conditions where protein and RNA synthesis was inhibited. This latter observation would imply that if trehalose was an osmoprotectant *in* S. *griseus* (A1B), accumulation was not osmotically regulated. Killham and Firestone (1984) had reported that proline, glutamine and alanine were the osmoprotectants of S. *griseus* (A1B) and S. *californicus* (A9), and the accumulation of these amino acids was regulated osmotically.

1.9.1.3. 3 Triacylglycerols

Some bacteria do not accumulate neutral lipids, but Olukoshi and Packter (1994) demonstrated this assumption to be incorrect. It had of ten been noticed, during electron microscopy of thin sections, that streptomycete hyphae appeared to contain' vacuoles that may have been fat droplets (Plaskitt and Chater, 1995). Olukoshi and Packter (1994) demonstrated that S. *griseus (*A15*),* S. *albus (*A16*),* S. *coelicolor* A3(2) (A21) and S. *lividans* (A21) accumulated significant amounts of triacylglycerols, starting in the exponential phase and continuing into the late stationary phase. There was evidence of these lipids in the spores. Activity of the final enzyme of triacylglycerol synthesis, diacyglycerol acyltransferase, was found in S. *coelicolor A3(2) (A21)* and S. *lividans (A21).* The authors speculate that triacylglycerols provide the carbon units for antibiotic synthesis.

1.9.1.3.4. Polyhydroxybutyrate

There have been few reports of polyhydroxybutyrate formation in streptomycetes. This compound is formed by reduction and polymerization of acetoacetyl CoA and is

common storage compound in bacteria. Ranade and Vining (1993) reported small amounts (1μg mg /dry weight)$^{-1}$ in S. *aureofaciens* (A14) and S. *antibioticus* (A31), and ten-fold less in S. *venezuelae* (A6). The polymer was found only in the mycelium, not the spores, of S. *venezuelae (A6)* and was a minor storage compound. Glycogen and trehalose were the main storage compounds: trehalose in spores and glycogen in hyphae. Polyhydroxybutyrate could not be found in the hyphae of streptomycetes examined by Olukoshi and Packter (1994).

1.9.1.4. Catabolite Repression in Actinomyceteles

1.9.1.4. 1. Carbon Catabolite Repression

Catabolite repression of intracellular and extracellular catabolic enzymes and of carbohydrate transport systems have been reported in *Actinomycetes*. In numerous instances catabolite repression of induction of an enzyme may, to different degrees, be exhibited by a number of catabolites. An example is the Class I cellobiose of S. *venezuelae (A6)* The induction of this enzyme can be repressed by glucose and, to a lesser extent, by amino acids and acetate.

As an object lesson, it might be relevant to consider what is probably a special case of indirect glucose repression. Surowitz and Pfister (1985b) demonstrated that the accumulation of organic acids was the result of glucose-induced uncoupling of glycolysis and the TCA cycle. This uncoupling could be reversed by adenine. It appears special case that in this case 'glucose repression' is, in fact, glucose-induced metabolic poisoning. Glucose-induced organic acid accumulation is a common event in streptomycetes, depending on culture conditions, and a well buffered medium needs to be used to ensure 'true' catabolite repression is involved.

1.9.1.4. 2. Amino Acid Catabolic Repression

Roth *et al.* (1985, 1986) investigated the repressive effect of amino acids on maltase production by amino acids in S. *hygroscopicus* (A32). They used a simple pH indicator plate test which was based on the observation that cells growing on maltose were acidogenic; cells growing on amino acids were alkalinogenic; and cells growing in the presence of maltose plus amino acids were alkalinogenic because they used the latter substrate preferentially. They reasoned that cells that had lost the amino acid repression system would be able to use maltose in the presence of amino acids and hence reduce the level of alkali no genesis. Following mutagenesis and growth in a fermenter under a specifically designed selection regime, which involved switching the carbon and energy source from maltose to amino acids and back again over a number of cycles, they isolated a number of derepressed mutants. One of these mutants (M36) possessed a constitutive a-amylase and a constitutive maltase that were not subject to amino acid repression. A second mutant (M39) had lost amino acid repression of both enzymes but they remained inducible. The nature of the genetic regulatory network implied by these results - namely, a global activator of a-amylase and maltase and amino acid repression of the activator - has yet to be confirmed.

It should be noted that amino acid repression may have nothing to do with nitrogen regulation. Rather, the carbon skeletons left after deamination of the amino acids (i.e. organic acids) may be the 'true' repressor(s). Acetate and citrate were good repressors of the S. *venezuelae* (A6) maltase, and reported citrate 'repression' of glucose catabolism in S. *niveus* (A1B).

1.9.1.4. 3 Carbohydrate Repression in Actinomycetes

Diauxic lag is a phenomenon characteristic of glucose repression and inducer exc1usion in the enteric bacteria. When a cell is presented with growth-limiting glucose and excess glucose-repressible catabolite, the cell metabolizes the glucose alone first.

Once glucose is exhausted, the cell ceases growing until the enzymes necessary for the catabolism of the second catabolite have been induced. This period of growth cessation is referred to as the 'diauxic lag'.

A number of groups have sought to demonstrate diauxic lag in *Actinomycetes* cultures supplied with growth-limiting repressing catabolite and excess repressible catabolite. A diauxic lag when S. *venezuelae* (A6) was incubated in a medium initially containing 0.6% glucose and 1.4% lactose. Analysis of residual sugar in the medium revealed the complete catabolism of the glucose, the exhaustion of which correlated with the initiation of the lag in cell growth. The lag ended upon initiation of lactose catabolism. When the same strain was incubated in the presence of 0.6% glucose and 1.4% cellobiose, the glucose was not exhausted before cellobiose catabolism commenced, and no diauxic lag was seen.

Hodgson (1982) could not find a diauxic lag phase when S. *coelicolor* A3(2) (A2l) was grown in medium containing growth-limiting glucose (0.56 mM) and excess alternative (glucose repressible) carbon and energy source. Diauxic growth could be seen, however, as the cells grew with the characteristic doubling time (1.9 h) of glucose first and then slowed down to the characteristic doubling time seen for growth on the alternative carbon source. The point of changeover from the first phase to the second occurred at precisely the point where growth ceased in cultures initially supplied with 0.56 mM of glucose alone. Hanel *et al.* (1987) were also unable to find a diauxic lag phase when the growth of S. *chrysomallus* (A1B) on glucose and xylose was examined. Glucose and xylose were co-catabolized, although glucose was preferentially used.

Since glucose transport systems in streptomycetes have low affinity for their substrate - *Km* values of 0.12 mM to 6.1 mM - glucose limitation cou1d occur even though glucose was still present in the medium. Taking this into consideration with the observations that comparative growth rates of streptomycetes are slower than those of enteric bacteria, whilst the time taken to activate, transcribe and translate

genes are about the same, it is perhaps not surprising that diauxic lags are rarely seen in streptomycetes. It should also be noted that the PEP:PTS-dependent mechanism of inducer exc1usion makes a significant contribution to delay of induction of the catabolism genes, and thus to the creation of diauxic lag.

1.9.1.4.3.1 Inducer Exclusion

A number of constitutive carbohydrate transport systems of streptomycetes are inhibited by glucose. This raises the possibility that, where the carbohydrate catabolism enzymes are inducible, the repressing carbohydrate could exert its repressing effect by exc1uding the inducer. Whilst inducer exc1usion may have a role in catabolite repression, it cannot be the sole mechanism if the results of Hodgson (1982) in S. *coelicolor* A3(2) (A2l) have a more universal applicability. Mutants were isolated that had lost the mechanism of glucose repression; however, these mutants still retained the ability for glucose to inhibit transport of inducers (in this case fructose and galactose) into the cell. Thus, when these mutants were cultured in the presence of glucose and the inducer, enough inducer could get into the cell to fully induce the catabolic enzymes. Servin-Gonzalez *et al.* (1994) also conc1uded inducer exc1usion was not the basis of glucose repression of agarase induction in S. *coelicolor A3(2) (A21*).

1.9.1.4. 3.2 Cyclic 3',5'-AMP Involved in the Control of Glucose Repression

It has been shown to occur in a number of *Actinomycetes*. The concentration of the nucleotide varies, however, from 4 to 400 pmol (mg protein)$^{-1}$. Only a minority (about 10%) of the cAMP present in a culture is intracellular, the rest being present in the culture medium. Attempts to implicate cAMP as the regulatory molecule of carbon catabolite repression of both primary and secondary metabolism have failed to provide unambiguous results in all the species examine. In a number of cases (Gersch, 1980) it was found that in contrast to what is seen in *E. coli and* other. intracellular cAMP concentration was greatest in media

containing glucose where growth rate was highest and lowest in media containing either no carbon and energy source or one that could not be catabolized. However, they reported that cAMP synthesis was possible only in the presence of a carbon and energy source, and confirmed that adenyl cyclase was most active in cells with a utilizable carbon and energy source. In this case it appeared that cAMP had the kinetics of a secondary metabolite.

In summary, high growth rate correlates with high intracellular cAMP concentration; and low growth rate correlates with low intracellular cAMP level. This situation is reminiscent of that in the yeast *Saccharomyces cerevisiae* (Postma, 1986).

The concentrations of exogenous cAMP (5-10 mM) required to inhibit germination and stimulate vegetative growth were the same. Susstrunk *et al.* (1998) reported that exogenous1y supplied cAMP could mimic autogenous stimulation of amino acid transport systems by their substrates. This stimulation occurred apparently at the level of transcription.

The importance of medium acidification in the cAMP mutant raised some interesting questions. A number of mutants of S. *coelicolor* A3(2) (A21), *bldA, bldC,* and *bldD,* were isolated that could not produce aerial mycelium in the presence of glucose but could in its absence when growing on 'non-repressing' carbohydrates such as mannitol. It was suggested that, in these mutants, development had become glucose-repressible. Susstrunk *et al.,* (1998) demonstrated that *bldA, bldB, bldC, bldD* and *bldG* mutants irreversibly acidified the medium when growing on glucose. Addition of cAMP or pH-buffering the medium did not suppress the Bld- phenotype. it would be interesting to see whether the mannitol-suppressible *bld* mutants produced acid on 'non-repressing' carbohydrate catabolites. Again the observation of acid formation and 'glucose repression' are seen to be linked in streptomycetes. It was concluded that cAMP has nothing to do with the mechanism of carbon catabolite repression in streptomycetes.

1.9.1.4. 3.3 A Role for Glucose Kinase in Glucose Repression

Hodgson (1982) studied glucose repression of primary metabolism in S. *coelicolor* A3(2) (A21). Having shown that a number of carbohydrate transport and catabolism systems were glucose-repressed, glucose-derepressed mutants were selected on media containing arabinose, a glucose-repressed catabolite, and excess of the non-catabolizable glucose-analogue, 2-deoxyglucose. The idea was that as the 2-deoxyglucose could not be catabolized, yet it repressed the use of arabinose, mutants would be selected that had lost the glucose repression system. Such mutants were isolated and appeared to have lost the ability to grow on glucose because of the loss of glucose kinase. Initially the evidence for loss of the enzyme was based on sugar catabolism patterns and glucose uptake studies. Biochemical studies confirmed this conclusion (Seno and Chater, 1983).

Angell *et al.* (1994) reported that glucose kinase had a regulatory role in addition to its glucose phosphorylation function. Isolation of suppressers of the glucose non-utilization phenotype of the *glkA* deletion mutants yielded unstable strains that could grow on glucose, had glucose kinase activity and yet failed to glucose-repress agar utilization. The authors speculated that there could have been activation of a silent glucose kinase gene in the suppresser strains. Recently the genome-sequencing project has yielded a gene with extensive similarity to the *glkA* gene (AL109950). The fact that a strain with wild-type levels of glucose kinase activity failed to exhibit glucose repression implies that glucose phosphorylation activity was not important in glucose repression. This was proven when the glucose kinase gene of *Zymomonas mobilis* was expressed in the *glkA* deletion mutant and shown to produce substantial glucose kinase activity, yet the strain failed to exhibit glucose repression of agarase. Clearly glucose flux through the glycolytic pathways or into the cell cannot be important in the control of glucose repression in streptomycetes. The regulatory role for glucose kinase in glucose repression in S. *coelicolor* A3(2) was confirmed by Kwakman and Postma (1994), and they also demonstrated that ten-fold over-expression of the *glkA* gene led to loss of glucose repression, implying that the repressive signal was titratable.

Hodgson (1982) was fortuitous in the choice of 2-deoxyglucose for selection of glucose-derepressed mutants. The product of the reaction of the analogue with the kinase, 2-deoxyglucose-6-phosphate, like all other phosphorylated sugars, is toxic to the cell because of its ability to react destructively with proteins and DNA (Lee and Cerami, 1987). Therefore the only cells that could survive either could not import it or could not phosphorylate it; i.e. be mutant in the glucose kinase. If S. *coelicolor* A3(2) (A21) has more than one glucose transport system, like its close relative S. *lividans* (A21), transport mutants would not be a class of analogue-resistant mutants. A further glucose-derepressed mutant of S. *coelicolor* A3(2) was isolated by screening for loss of glucose-repression of the extracellular enzyme agarase (Hodgson, 1982). Again this mutant proved to have lost glucose kinase activity.

The concomitant loss of a global glucose repression system and glucose kinase activity is very reminiscent of the situation in *Saccharomyces cerevisiae,* where the hexokinase pH isozyme has been strongly implicated in control of glucose repression. The nature of the role of this enzyme in glucose repression is not yet clear.

1.9.1.5. Nitrogen sources of Actinomyces

The production of secondary metabolites is linked to the metabolism of nitrogen and, therefore, this phenomenon has been investigated for several years. Nitrogen metabolism in *Streptomyces* species is best understood in *S.coelicolor*. Like in B.subtilis, no biochemical data on the uptake of ammonium are available. When ammonium has entered the cell, it is assimilated in S.coelicolor via GDH when present in high concentrations, while the GS/GOGAT pathway is employed when ammonium is limiting.

In all streptomycetes so far studied the main pathway of nitrogen assimilation is via glutamate synthetase (GS) and glutamate: 2-oxoglutarate transaminase (GOGAT) (Fisher, 1988). Surprisingly, all streptomycetes studied have two GSs; GSI resembles

those found in most prokaryotes and GSII is more similar to the eukaryotic type (Behrmann et al., 1990). An additional surprise is that GSI is regulated by adenylation, like the GSs of Gram-negative enteric bacteria, but unlike those of Gram-positive bacilli. Assimilation of nitrogen via GS is energy-expensive, but is possible at low ammonia concentrations. In many bacteria a 'cheaper" method of assimilation uses a glutamate dehydrogenase (GDH:GOGAT couple, but this mechanism works only at high ammonia concentrations. Many streptomycetes seem to have this couple but some, including *S. clavuligerus and S. aureofaciens*, do not (Vancurova et al., 1988). This led to speculation that alanine dehydrogenase and alanine:2-oxoglutarate transaminase could be used instead, but the latter enzyme could not be detected in the species lacking GDH. One is forced to conclude that the "expensive" GS: GOGAT couple is the sole means of nitrogen assimilation in some streptomycetes.

1.9.1.5.1. Catabolism of Amino acid

Most amino acid catabolism in streptomycetes seems to be via pathways previously described in other bacteria. An exception is arginine, which is catabolised via γ-guanidino butyramide and γ- guanidinobutyrate ; the enzymes involved were induced more than 15-fold by arginine. The histidine and proline catabolic pathways are induced by the cognate amino acid, but for histidine the true inducer is an intermediate of the pathway (Smith et al.,1995). About half the pathways studied seem to be constitutive, but the levels of the enzymes are very low and difficult to assay.

Global catabolite repression of amino acid catabolism has been reported in streptomycetes, but carbon catabolite repression of amino acid catabolism, common in enteric bacteria, does not seem to occur. (Hood et al., 1992). Ammonium repression of amino a catabolism has been reported, as has ammonium repression of secondary metabolism. However, the two do not seem to be directly linked because mutations that abolish global repression of primary metabolism do not abolish secondary metabolism (Bascaran et al., 1989). Much evidence has been presented

that “ammonium repression” of some secondary metabolites is due to ammonium inhibition of enzymes involved in amino acid catabolism that supply precursors to secondary metabolism, e.g. valine dehydrogenase (Omura and Tanaka, 1986).

1.9.1.5.2 Biosynthesis of Amino acid

The pathways of amino acid biosynthesis have been examined in numerous streptomycetes. The amino acids are apparently synthesised in the same way as they are in most bacteria, with a coup of minor differences. Tyrosine is synthesised via arogenate as in pseudomonads, rather than via hydroxyphenylpyruvate as in enteric bacteria (Keller et al., 1985). A more important difference is the presence of a trans-sulphurase pathway in streptomycetes, which is characteristic of fungi (Kern and Inamine, 1981); this means that sulphur can be transferred either way between methionine and cysteine via cystathionine γ-lyase and cystathionine β-synthase.

A major difference between amino acid biosynthesis in streptomycetes and the enteric bacteria bacilli is its apparent lack of regulation. In the unicellular bacteria, most regulation is at the transcriptional level, with the amino acid product negatively controlling expression of the genes encoding biosynthetic enzymes. The method of inhibition includes attenuation and amino acid binding to repressor proteins. There is very little evidence of such feedback inhibition of gene expression in streptomycetes. Most amino acid biosynthetic enzymes, like those for their catabolism, are expressed at very low constitutive levels (so low it is often difficult to believe that the assays are meaningful) (Hood et al., 1992). There are reports that biosynthesis of arginine and the common aromatic amino acids may be subject to feedback repression. Recently an attenuator was found upstream of the *trpEG* gene, which encodes the first enzyme of the tryptophan-specific pathway. Genes encoding the enzymes for the final part of the pathway appeared to be regulated by growth phase (on in exponential phase, off in stationary phase) and growth rate (the faster the growth the more enzyme) (Hu et al., 1999).

It was speculated that the tight biosynthetic regulation reflects the growth of the streptomycetes under typically nutrient-limited conditions, compared with the enteric bacteria. In the gut a plentiful supply of nutrients washes through at regular intervals, so the enteric bacteria have developed the ability to react quickly to feast and famine. For the streptomycetes, starvation is the rule and so they seem not to have evolved, or to have lost, the ability to regulate amino acid biosynthesis at the gene level. How streptomycetes manage to avoid the apparent futile cycle of constitutive synthesis of an amino acid followed by its degradation by a constitutive catabolic enzyme is a mystery.

1.9.1.5.3. Problems of Studying Primary Metabolism in Actinomycetes

The *Actinomycetes* colony consists of a number of different cell types. Within any one of these cell types new metabolic pathways may be activated, and it may be difficult to distinguish between metabolites that are involved in differentiation and secondary metabolites. It might be claimed that in liquid culture the cells are exclusively of the substrate mycelium type. However, there have been reports that some *Actinomycetes* can produce spores in liquid culture and that others can go through a microsporulation cycle (Hodgson, 1992).

The induction of metabolic differentiation is unlikely to be synchronous throughout even a liquid culture, which is a problem when studying *Actinomycetes* metabolism. It is exacerbated by the tendency of *Actinomycetes* to form pellets and for growth to occur on the walls of the container above the tidemark during liquid culture, especially in minimal media. The cells on the outside of the pellet will be in a different physiological state from those on the inside. Because liquid culture is the easiest way to obtain biomass for physiological studies, a number of methods have been used to minimize pellet growth. These include the use of baffles in the vessel, and the incorporation polymeric molecules (e.g. 1unlon and polyethylene glycol) in the medium to inhibit cell-cell adhesion (Hodgson, 1982; Hobbs *et al., 1*989). Such actions, however, merely reduce the problem rather than abolish it. Microscopic examination of the cultures reveals that, unless the strain is

one that fragments to an unusual degree, the cells form diffuse microcolonies, the cells at the centres of which are probably in a different physiological state from those on the outside.

Another approach is to filter out smaller microcolonies from larger ones in a culture in the hope that 'smaller' means younger. Unfortunately the filtering process may break up 'old' large colonies. To be sure of obtaining young cells, spores can be spread on cellophane discs on solid media and harvested before cellular differentiation has occurred. Riesenberg and Bergter (1984) used freshly germinated and outgrown *Actinomycetes* spores as a source of material. They demonstrated, by monitoring ATP, DNA, RNA and protein synthesis, and correlating these parameters with increase in mycelial length, that the cells underwent balanced growth for at least two cell doublings.

It is important to consider these problems because of the rapidly growing list of the number of *Actinomycetes* in which isozymes of particular enzymes have been discovered. These isozymes have distinct properties and are expressed during different physiological states of the cell. Isozymes of enzymes important in primary metabolism have been shown to fall into two types: those that result in antibiotic autoimmunty; and those that are involved in secondary metabolism. An example of the former which is of direct relevance to primary metabolism was the report of Maurer *et al.* (1983) in which they announced the discovery of pentalenolactone-resistant and –sensitive isozymes of glyceraldehydes-3-phosphate dehydrogenase in S. *arenae* (A 18), the producer of the antibiotic.

Two isozymes of kynurenine formamidase were found in S. *parvulus* (A12) (Brown *et al.,* 1986). One was involved in biosynthesis of actinomycin D, and was inducible; the other which was constitutive, was postulated to have a role either in NAD synthesis or tryptophan breakdown. A similar situation was found in candicidin production. The enzyme p-aminobenzoic acid synthesis is an important enzyme in the synthesis of folic acid and the synthesis of candicidin S. *griseus* (AlB). The presence of two isozymes has been implied: one of which is regulated by phosphate

and the aromatic amino acids and is involved in the synthesis of the antibiotic; an one that as not been detected but is presumably involved in primary metabolism.

Another problem of studies of primary metabolism in streptomycetes is the nature of strains used. If a comparison of primary metabolism and secondary metabolism is to be made it is essential that the production of the secondary metabolite be consistently inducible and be at such a level that it be easily assayable. This again may present problems. Many strains isolated directly from the environment may not produce much secondary metabolite. Therefore, there is the temptation to use strains have been improved for secondary metabolite production and compare them with still more highly producing strains. The potential problem with this approach is that the improvement of secondary metabolite yield may have come about by the specific deregulation of the metabolic events one is hoping to study. There are some secondary metabolites that are produced at high levels in natural isolates, in particular pigments. Each case will depend on the research to be attempted, but the caveat should be borne in mind when considering primary metabolism studies on improved strains.

1.9.2. Secondary Metabolism

Secondary metabolism results in the synthesis of metabolites that have no further apparent function in metabolism. Streptomycetes have been studied extensively in the area of secondary metabolism, because of the obvious biotechnological importance of the products.

The term *secondary metabolites* was introduced by Bu'Lock in the early 1960s (the term had been previously used by plant physiologists to indicate plant alkaloids) to indicate microbial metabolites found as the products of differentiation in restricted taxonomic groups, and not essential for cell metabolism.

These substances are thus clearly distinguished from primary metabolites-products, such as amino acids or nucleotides, participating in the basic cellular metabolism.

This expression has been questioned by several authors. The alternative term *specific metabolites,* as opposed to *general metabolites,* appears indeed more appropriate in that it underlines the fact that these products represent the individual diversity in the biochemistry of the microbial world. However, the term secondary metabolite is so widespread that it cannot practically be replaced.

Secondary metabolites are low-molecular-weight compounds with the following characteristics:

1. They are synthesized by only some microbial strains, and are the expression of the biochemical differentiation of the producing organism.
2. They have no obvious function in the growth of cultures or colonies. Strains able to make these molecules may lose, because of mutations, the capacity to synthesize them, without any apparent effect on their general metabolism.
3. They are often produced in connection with differentiation processes.
4. They are often made as families of similar products.

Not all of these characteristics are shared by all secondary metabolites. It is important to stress that "no obvious function in the growth" is different from "no function at all." Lack of essential metabolic function does not imply lack of selective function in evolutionary terms; for example, with respect to other microorganisms. The essential aspect of secondary metabolism is that it is a manifestation of the organism's individuality, i.e., of cellular differentiation, often expressed only under conditions of limited growth.

According to this definition, antibiotics are secondary metabolites. Studies aimed at uncovering a role as regulatory agents in the producer metabolism have been attempted for several antibiotics. In one case only, that of pamamycin, has a function been demonstrated-as an inducer of aerial mycelium formation in actinomycetes.

It is important to emphasize that many secondary metabolites, besides antibiotics, are produced by microorganisms. Examples are some pigments, numerous protease inhibitors, and toxins. However, in more recent times, the search for microbial metabolites endowed with different biological activities has revealed a high number of new products. Such inhibitors of physiological functions of higher organisms are termed bioactive microbial metabolites, and are discovered with a lower frequency, in comparison with antimicrobial substances.

1.9.2.1. Antimicrobial and Other Biological Activities of Secondary Metabolism

The general biological activity of secondary metabolites in relation to their antimicrobial activity, the following different situations are observed:

1. Several typical antibiotics demonstrate, in addition to their antimicrobial activity, an appreciable activity on other biological systems. This is the case, for instance, of the cholesterol-lowering activity of rifamycins; the induction of gastric motility by erythromycin; and the ability of ristocetin to trigger platelet aggregation. These activities have no relation to the mechanism of action of the antibiotic and may be enhanced in semisynthetic derivatives, without a corresponding increase, or even with the disappearance, of the antimicrobial activity. These types of activities as adventitious: in fact, the chances are that when thousands of molecules are tested on a high number of biological systems a few of them will show a positive or negative interaction. This is not surprising, in view of the experience of the pharmacological screening of synthetic compounds successfully performed for many years by the research laboratories of the pharmaceutical industry.

2. The same biological action can be effective both on the microbial cell and on cells or systems of higher organisms. This is the case for compounds such as

mevinolin or compactin that, by inhibiting the isoprenoid biosynthesis, are active as fungistatic and are cholesterol owering agents in man. Analogous cases may be those of the immunosuppressant agents cyclosporin A and FK 506, which inhibit two different enzymes having a peptidyl-prolyl isomerase activity. The binding of these metabolites to corresponding fungal enzymes results in the formation of complexes toxic for the microorganism.

3. Many bioactive metabolites do not show any microbiological activity. Among these are cytostatic compounds, insecticides and antiparasites, and herbicides, but the vast majority result from the search for substances potentially active as pharmacological agents. These are predominantly inhibitors of physiologically (or pathologically) relevant enzymes, antagonists of hormones or other regulatory molecules that act by competing with the natural ligand for cellular receptors. There are also immunomodulators, antagonists of cell growth factors, and free radical scavengers.

It is not known whether some of these substances have an activity related to the producer cell metabolism, or a function in cell-to-cell or species-to-species communication. The variety of their biological activities and chemical structures certainly preclude any general interpretation of their relation to the producer organisms.

1.9.2.2. Actinomycetes Secondary Metabolites

As well as the more renowned antibacterial antibiotics such as chloramphenicol, produced by S. *venezuelae* (A6), and streptomycin, produced by S. *griseus* (A15) and others, there are the herbicide bialaphos produced by S. *hygroscopicus* (A32), the immunosuppressive agent FK506 produced by S. *tsukabiensis*, and the β-lactamase inhibitor, clavulanic acid, produced by S. *clavuligerus* (J71) (Wagman & Weinstein, 1980). One of the more exotic streptomycete natural products is stroptozocin produced by S. *achromogenes* var. *streptozoticus;* this compound contains a dinitrogen bond that resembles that of the most potent alkylating agent N-methyl-N'-nitro-N-nitroso-guanidine. To assemble this dinitrogen bond, the streptomycete must

generate nitrous acid, another potent mutagen, within its cytoplasm. How the bacterium avoids mutating its own DNA during streptozocin biosynthesis is not yet clear.

1.9.2.3. Control of Secondary Metabolism

At the same time as *Actinomycetes* undergo morphological differentiation, they also undergo morphological differentiation and secondary metabolism is activated. This led to the proposal that one of the criteria for naming a compound as a secondary metabolite was that it was produced after the cell had stopped growing. This aspect of the definition has often been challenged. However, it should be noted that when secondary metabolism is seen associated with the growth phase, the cells are of ten growing very slowly.

It is becoming a truism that all the genes involved in the biosynthesis of, and resistance to, a particular secondary metabolite are clustered at a single locus of the *Actinomycetes* genome and that they are very tightly regulated. This gene localization makes the coordinated regulation of the gene cluster simpler.

Secondary metabolite regulators have usually been identified as gene mutations, which lead either to overproduction of metabolite (i.e. a repressor) or non-production of a metabolite (ie. an activator). The former class has been reported far less frequently than the latter. Different classes of non-production mutant have been identified: (I) mutations that are pathway-specific and map to the biosynthetic gene locus; (II) mutations that block production of more than one secondary metabolite; and (III) mutations that block morphological differentiation in addition to secondary metabolism. Other classes of 'regulatory' genes were identified as multicopy suppressors or activators of secondary metabo1ism.

There is the danger of over-interpretation of a metabolite loss overproduction phenotype. Loss of metabolite production could be due to loss of a gene necessary for expression that does not of itself control expression. A good example is the *bldA*

gene of S. c*oelicolor* A3(2) (A2l) which encodes a tRNA gene that is required for translation of a number of pathway-specific regulators of metabolite production; i.e. *act* II orf4 and *redZ* for actinorhodin and undecylprodigiosin, respectively (Guthrie *et al.,* 1998). The *bldA* gene is part of the 'wiring' of the switch, rather than the switch itself. The *bld* mutants are very pleiotropic; in addition to loss of secondary metabolism they also do not produce an aerial mycelium and are deregulated for glucose repression of carbohydrate catabolism. They fall into class III of secondary metabolite non-production mutations.

There is currently a great deal of work to understand how the class I, class II and class III gene s interact and how these genes interact with global regulatory networks such as stringent response and cAMP control (Bibb, 1996; Susstrunk *et al.*1998).
A great deal of effort has been concentrated on the pathway-specificregulatory genes; their loss leads to an inability to express, or over expression of gene products involved in the biosynthetic pathway. As might be expected, these genes of ten encode DNA-binding proteins. RedZ, a gene product required for undecylprodigiosin gene *(red)* expression, turned out to be of the very common response regulator type but lacks the acid pocket and phosphate receptor aspartate residue characteristic of response regu1ators (Guthrie *et al.,* 1998). A number of pathway-specific regulatory genes have been found to belong to a recently identified protein family with an unusual DNA binding domain with homology to the OmpR DNA-binding fold. This new class of protein, which includes RedD and ActII-orf4 of S. *coelicolor* (A3(2) (A21), have been called SARPs - standing for *Streptomyces* antibiotic regulatory proteins (Wietzorrek and Bibb, 1997).

1.9.2.4. Antibiotic Production as Secondary Metabolism

Antibiotics are an important group of pharmaceuticals in today’s medicine. In addition to the treatment of human infections, they are also used in veterinary medicine.

Recently, there has been a growing interest in the presence of pharmaceutical substances in the aquatic environment.

Specific production rates of antibiotics, besides other drugs, are not reported in the literature. However, in 1994 the overall production amount of antibiotics in Germany was 1831 tons with 624 tons for the penicillins alone Statistisches Bundesamt Wiesbaden, 1994 . The application of drugs in veterinary medicine and as growth promoters are presently not under federal regimentation. Hence, no estimations concerning this area can be made. However, the numbers of prescribed daily doses for human medication are available. For the year under investigation, the total amount of antibiotics used for human medication was calculated to be 37.7 tons. Also, they stated a production rate of antibiotics for veterinary medication of 49.7 tons while 94 tons of growth promoters were used for pig livestock production. These numbers are generally in the same range as the applied amounts of some pesticides in Germany which were subject to extensive studies of their behavior in the aquatic environment for many years.

1.9.2.5. Actinomycetes as Antibiotic Producers

It is well known that members of the *Actinomycetes* are increasingly important in industrial processes. Their ability to secrete large amounts of enzyme and secondary metabolites, particularly antibiotics, make them commercially very attractive.

1.9.2.5.1 Regulation of Antibiotic Biosynthesis

The principal mechanisms of regulation which apply to both primary and secondary metabolite biosynthesis are the repression of enzyme synthesis and the inhibition of enzyme activity. However, the fact that these operate in the control of secondary metabolite biosynthesis has only recently been well accepted. Some of these mechanisms are specific for antibiotic production but, remembering that secondary metabolism is a form of differentiation, it is not surprising that its

regulation often falls under more general systems of control governing other forms of differentiation, such as the sporulation process or the formation of aerial mycelium.

1.9.2.5.1.1 Feedback Regulation

The assumption that secondary metabolites may regulate their own biosynthesis by feedback inhibition has been experimentally supported in several cases (Malik, 1982). Most of the evidence is based on the cessation of antibiotic production observed when the fermentation product is added to cultures of the producing organism. Other indirect evidence includes the Increase in production yields achieved by continuously removing the produced antibiotic from the fermentation broth. This can be accomplished by adsorption onto a resin, or as in the case of cycloheximide production by *Streptomyces griseus,* by dialysis-extraction. This type of experiment cannot conclusively establish whether the underlying mechanism is repression of enzyme synthesis or inhibition of enzyme activity. However, at least in the case of aurodox production by *Streptomyces goldiniensis,* the short time elapsing between addition of antibiotic and block of the synthesis suggests that inhibition, rather than repression, is the likely mechanism.
Only in a few cases has the biosynthetic enzyme involved been identified. In *Streptomyces venezuelae,* the producer of chloramphendicol the addition to the fermentation medium of the final product, or of its p-methylthio analogue, inhibits the *de novo* formation of the antibiotic (Horinouchi & Beppu, 1992). This effect is caused by repression of arylamine synthet the enzyme converting chorismate to p-aminophenylpyruvate, which is the first specific metabolite in chloramphenicol biosynthesis.

In the puromycin producer *Streptomyces alboniger,* the activity of the last enzyme of the biosynthetic pathway, an O-methyltransfese, is inhibited by the presence of the end product. Similarly, in *Streptomyces fradiae* tylosin inhibits the O-methyltransferase involved in its own biosynthesis and in the mycophenolic acid

producer, *Penicillium stoloniferum,* the last enzyme of the biosynthetic pathway, again an 0methyltransferase, is also inhibited by the end product.

Antibiotic production can be indirectly regulated by feedback inhibition of products of primary metabolism involved in their biosynthesis. For example, in *Penicillium chrysogenum,* lysine interferes with penicillin production by inhibiting the first enzyme involved in its own biosynthesis (homocitrate synthase) the product of which (L-α-aminoadipate) is also a precursor of the antibiotic. The decreased availability of the precursor molecule results in a corresponding decrease of the final product yields. Another example is, in *Streptomyces griseus* fermentation, the inhibition of candicidin biosynthesis by aromatic amino acids tryptophan in particular. p-Aminobenzoic acid, a precursor of the aromatic moiety of candicidin, is synthesized through the shikimate pathway, subject to the feedback inhibition of the aromatic amino acids.

1.9.2.5.1.2. Regulation by Nutrient Concentration

When producing strains are cultured in nutritionally rich media, high levels of secondary metabolites are usually produced only after cellular growth is completed. On this basis the presence of a growth phase, called trophophase, distinct from a production phase, called idiophase has been considered a general characteristic of secondary metabolite fermentations (Demain, 1992). This is certainly an oversimplification, antibiotics, such as chloramphenicol, etamycin, and the rifamycins, are produced during the exponential growth phase also. Moreover in defined media supporting only a slow growth rate, the two phases often overlap, since production is observed while growth is occurring.

It is clear that nutrient limitation can result in a slow growth rate and the expression of biosynthetic genes simultaneously. The link between the two phenomena is complex, and it is very difficult in most cases to distinguish between cause and effect.

However, the direct interference of a particular nutrient level on antibiotic biosynthesis has been clearly demonstrated in several cases.

1.9.2.5.1.2.1. Carbon Source Repression

Glucose, usually an excellent carbon source, is known to interfere with the biosynthesis of many antibiotics, produced either by actinomycetes or by fungi and belonging to different biosynthetic families, such as aminoglycosides, polyketides, peptides, and β-lactams. In most cases, the experimental support is limited to the difference of production observed when slowly metabolized carbon sources are substituted for glucose in the fermentation media. However, in some cases specific repression of the synthesis of enzymes of the antibiotic biosynthetic pathways has been demonstrated (Demain, 1989).

For example, in *Streptomyces antibioticus,* producer of actinomycin, phenoxazinone synthase is repressed by glucose. These enzymes normally synthesized after cessation of cell growth and its presence increases following glucose depletion. Since the levels of the specific mRNA follow a parallel course, it appears that regulation is exerted at the level of transcription. All of the other enzymes of the actinomycin biosynthetic pathway so far studied also appear to be affected by glucose repression. Similarly, in *Nocardia lactamdurans* cephamycin biosynthesis is under glucose regulation, exerted through repression of at least two enzymes of the biosynthetic pathway, ACV synthase and expandase.

Other antibiotics produced by actinomycetes in which the effect of glucose on the biosynthesis has been studied are tetracycline, puromycin, and the aminoglycosides kanamycin, neomycin, and streptomycin. In all of these cases, the repression of one of the biosynthetic enzymes has been observed. Since in some cases this represents the only enzyme studied, it is quite possible that the repression also affects other, or all, enzymes of the pathway (Martin & Demain, 1980).

β-Lactam production in fungi is also regulated by rapidly utilized carbon sources. Penicillin production in *Penicillium chrysogenum* fermentations is depressed by glucose, fructose, and sucrose, as is depressed the production of cephalosporin in *Cephalosporium acremonium* by glucose or glycerol.

Glucose does not inhibit the activity of the penicillin-forming enzymes in *P. chrysogenum.* but represses the synthesis of the cyclase (isopenicillin N synthase) and to some extent that of the ACV synthase. In C. *acremonium* the cyclase is also moderately repressed by glucose or glycerol, whereas expandase is markedly depressed. This, and the fact that expandase is a labile enzyme, explains why intermediate concentrations of glucose result in a low cephalosporin production and in accumulation of isopenicillin N. Surprisingly, it has been found that in *Cephalosporium* the formation of ACV synthase is not depressed by carbon sources but that the activity of this enzyme is inhibited by glucose or by intermediates of the glycolytic pathway.

Since studies on the carbon source effect are normally performed on whole cells, it is not known whether glucose or one of its metabolites is the intracellular repressor. Carbon source regulation of antibiotic biosynthesis is often referred to as carbon catabolite repression, in analogy with the well-known repression of inducible enzymes in *E. coli* and other eubacteria. However, the mechanism by which the regulation is exerted appears to be different. The intracellular effector in *E. coli* is cyclic AMP. Both in actinomycetes and in fungi most of the accumulated evidence excludes participation of this molecule in the repressive mechanism.

1.9.2.5.1.2.2 Nitrogen Source Regulation

Depression of production by high concentrations of ammonium ions is a common feature of antibiotic fermentation (Shapiro, 1989). In fact, it has often been observed that antibiotic production starts only when most of the ammonium in the medium has

been depleted. Evidence of this is also the increase of yields obtained when slowly metabolized nitrogen sources are substituted for ammonia in some antibiotic fermentation. The classical example is streptomycin production by *Streptomyces griseus* that was enhanced threefold by substituting proline for ammonia as the sole nitrogen source in a chemically defined medium. Moreover, addition of magnesium phosphate, an ammonium ion trapping agent, to fermentations of macrolides or streptomycin also results in substantial increases in the yields of these antibiotics.

Kinetic studies on cephalosporin production by *Streptomyces clavuligerus* have suggested that the action involves repression of enzyme synthesis, rather than inhibition of enzyme activity, and this is consistent with observations made on other fermentations. However, there is no generally accepted hypothesis on the mechanism by which the repression is exerted. Contrasting results have been reported on the involvement of glutamine, whose synthesis is normally repressed by high concentrations of ammonium ions. In fungi, ammonia represses the enzymes involved in the utilization of a number of nitrogen sources. Specifically, in aspergilli it inhibits the expression of a gene, *areA,* coding for a protein which is a positive regulator of transcription. However, a link between this repression and inhibition of antibiotic biosynthesis has not been established.

In a few cases the biosynthetic enzymes affected by ammonium ions have been identified. In C. *acremonium* two enzymes of the cephalosporin biosynthetic pathway, ACV synthase and expandase, are repressed.

An interesting interpretation has been proposed for the negative effect of ammonia on macrolide production. The building blocks constituting the polyketide chain of these antibiotics, particularly propionate, derive from the degradation of branched amino acids. The first step of the degradation is the conversion of the amino acids to the keto acids, catalyzed by either valine dehydrogenase or valine transaminase. It has been demonstrated that ammonium ions both repress and inhibit

valine dehydrogenase, suggesting that the lack of suitable precursors is the cause of antibiotic production depression.

1.9.2.5.1.3. Phosphate Control

Phosphate control of the formation of secondary metabolites is a commonly observed phenomenon among bacteria, fungi, and also plants. The suppression of practically all of the secondary metabolites of a culture is most often observed at high concentrations of phosphate. However, the sensitivity to phosphate inhibition may differ from product to product, even when produced by the same cell, because of the differential expression of certain genes as a function of phosphate concentration. In this respect the effect of different phosphate concentration on *S. clavuligerus* fermentation is noteworthy. This microorganism produces both clavulanic acid and a cephamycin. Whereas the biosynthesis of the first is inhibited by phosphate, the production of the second is practically unaffected, so that it is possible to dissociate cephamycin biosynthesis from that of clavulanic acid by adjusting the phosphate level in the culture medium. Phosphate control has been described for several groups of antibiotics, including aminoglycosides, tetracyclines, macrolides, polyenes, and polyether ionophores. Generally speaking, the biosynthesis of antibiotics directly assembled from amino acids tends to be less sensitive to phosphate regulation than that, for example, of polyketides and aminoglycosides.

As illustrated by the following examples, repression of enzyme synthesis appears to be the mechanism by which antibiotic production is normally depressed by phosphate.

1.10. Antibiotics

1.10.1. Antibiotic Modes of Action

By the early 1960's it was possible to distinguish known antibiotics into five groups. Figure 1.1, illustrates the sites of action in bacterial cell for various

antibiotics (Neu, 1992; Walsh, 2000). These categories were defined by using simple systems of non-growing cell suspensions incubated with media of varying complexity that each process and its inhibition could be studied.

1. Energy metabolism
2. Bacterial membrane function
3. Protein synthesis
4. Nucleic acid metabolism
5. Peptidoglycan synthesis

Much of the experimentation during this period demonstrated that different antibiotics within any group could have different modes of action. So in order to understand the molecular basis of action for a particular drug the total elucidation of the biochemistry of the 'group site' was required (Gale et al 1972) This process is still on-going, even in 2004. Today, we have a better understanding of many of the biosynthetic processes in bacteria and many of the steps in these biosynthetic pathways are now known however, though total elucidation of the mode of action is still being worked out for many antibiotics.

1.10.2.Antibiotic Resistance

From the early studies of penicillin, two important facts stand out. Pencillin G kills gram-positive bacteria far more readily than gram-negative bacteria, and it kills growing cells but not resting ones (Strominger et al, 1965; Keller and Zengler, 2004). The first fact highlights the structural differences between bacteria but the second fact highlights bacteria's survival mechanisms through selective pressure (Walsh, 2000). Indeed, bacteria have lived on Earth for more than 3 billion years and during that time, they have encountered a wide range of naturally occurring antibiotics from other microbes(Coates et al., 2002). In order to survive, bacteria have developed an inexhaustible range of resistance mechanisms. The following resistance mechanisms are the most common.

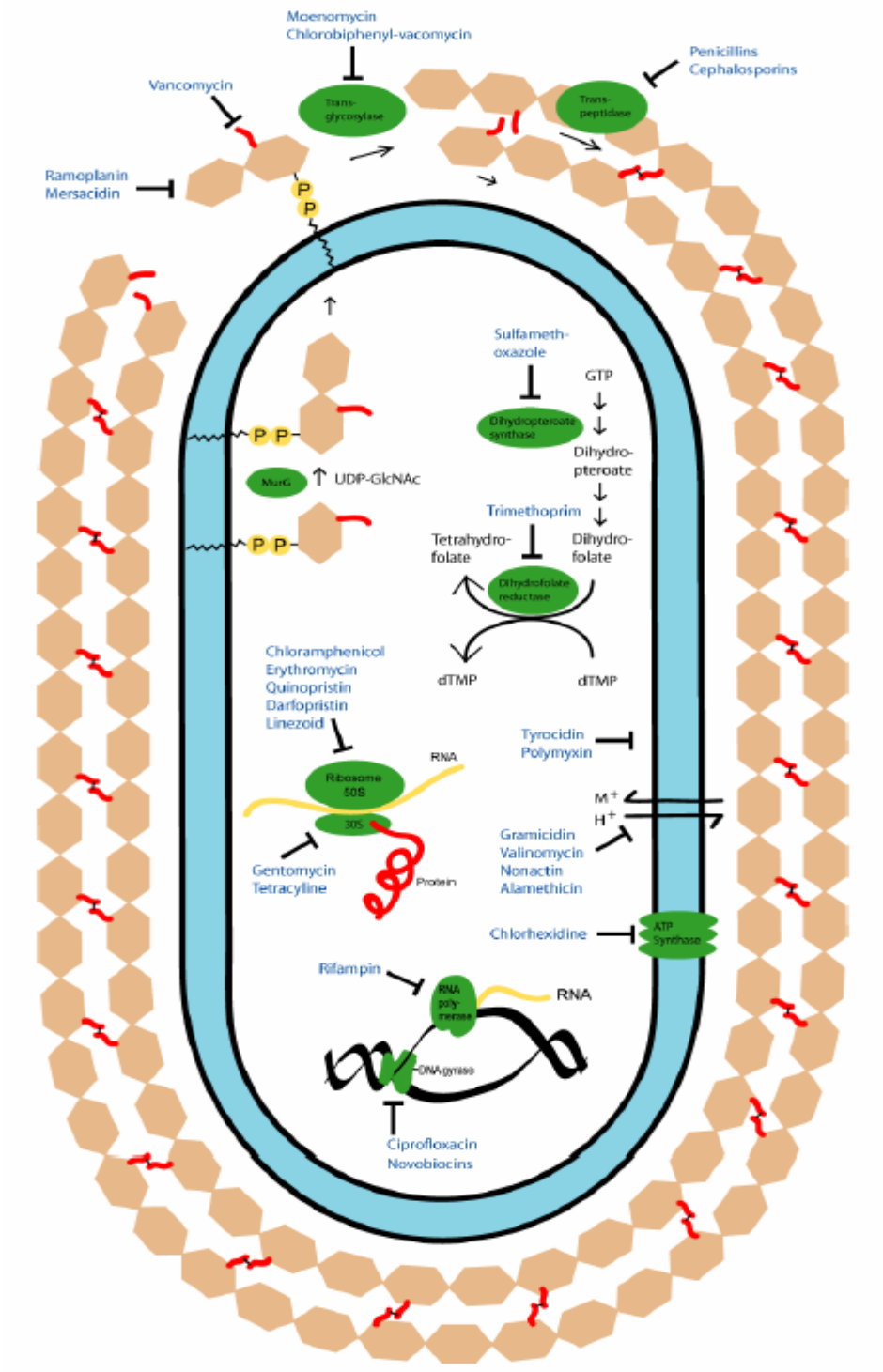

Figure 1.1 Bacterial Cell Metabolic Process and Antibiotics That Target Them

Bacteria can resist antibiotics as a result of chromosomal mutation, for example a change in a single amino acid in the enzyme's target site. Resistance can also involve the inductive expression of a latent chromosomal gene where the resulting protein causes enzymatic hydrolysis or modification of the antibiotic, or modification in the cell's permeability (Neu, 1992). These early methods are often caused by the use of suboptimal levels of antibiotic which has the effect of killing susceptible bacteria and selecting for resistance. (Walsh,2000). Finally, bacteria can exchange of genetic material through transformation (exchange of DNA), transduction (bacteriophage), conjugation of plasmids (extra chromosomal DNA), or by transposon mutagenesis(Neu, 1992). Antibiotics most susceptible to the first resistance mechanism are those that interact directly with the enzymatic target and some examples include sulfonamides, β-lactams, and the macrolides. Examples of inductive expression of a latent gene due to the presence of the antibiotic include β lactamases and aminoglycoside-inactivating enzymes.

These proteins inactivate, in the first case, by hydrolyzing the β lactam at the active site thereby destroying the antibiotic or as in the second case, by acetylation, adenylation, or phosphorylation so that the aminoglycoside binding affinity to ribosome severely reduced. The alteration of permeability or efflux of an agent is also a problem for β lactams, aminoglycoside and tetracyclines (Neu, 1992). Those antibiotics that bind substrate take longer to induce resistance because it takes reprogramming of a biosynthetic process that utilizes the substrate to avoid the antibiotic and thus be resistant. Substrate binders include the glycopeptides vancomycin and teicoplanin, the glycodepsipeptide ramoplanin, and bacitracin. Indeed, for these antibiotics, it took about 29 years for clinically relevant resistance to develop (Walsh, 2000). It follows then that the exchange of genetic material is a serious issue. Bacterial strains that produce antibiotics as a defense mechanism have a set of genes located on the chromosome or on a plasmid that allows the strain to survive in the presence of the antibiotic. These resistance genes are typically transferred by conjugation via plasmids or by transposons that enter transmissible plasmids or chromosomes. This type of resistance can also be spread horizontally by

plasmids or conjugative transposons to other species. For example, it has been postulated that *E.coli* transferred the ability to produce β-lactamase enzymes to *H.parainfluenzae.* Intergenus spread of resistance can occur between Gram-positive species such as staphylococci and enterococci, and between *Enterobacteriaceae* and *Pseudomonas* and anaerobes such as *Bacteroides.* Gram-positive can transfer genetic material to Gram-negative but the reverse is uncommon (Neu, 1992).

1.10.2.1. Enterococci

In the past decade, enterococcal infections have emerged as the major source of hospital acquired infections. There are 12 species of enterococci of which *E faecalis* accounts for 90% and *E.faecium* for 5% of all cases. They are an important cause of infection in organ transplant recipients, and become a common intestinal colonizer among hospitalized patients. Traditionally, resistance to β lactams due to the presence of β lactamases and altered penicillin binding proteins was overcome by combination treatment with aminoglycosides to generate a synergistic bactericidal effect. However, in the past 15 years, serious high-level resistance to the aminoglycosides has emerged and has led to the failure of this combination as a permanent approach. As a result, vancomycin either alone or in combination therapy, replaced the β lactam-aminoglycoside treatment regimen in the hospital setting. However, plasmid mediated resistance to vancomycin has become widespread in the past decade (0.3% in 1989 to 18% in 1996) and thus, has severely limited the use of this drug. In addition, these vancomycin resistant strains are often resistant to β-lactams and aminoglycosides. Thus, the management of enterococci, in particular for *E.faecium* and *E.faecalis*, in the hospital setting is a tremendous problem. Alternative treatments for these resistant strains are limited. Tetracyclines and fluoroquinolones show good activity in vitro but there are some isolates that are resistant to the serum achievable levels, and fluoroquinolones are not indicated for children or adolescents. Of particular concern is the ability of enterococci, staphylococci, and streptococci to share genetic material by conjugation.

Thus, this vancomycin resistance may be transferred to these other pathogens, specifically MRSA.

1.10.2.2.Staphylococcus aureus

In 1941, virtually all strains of *S.aureus* worldwide were susceptible to penicillin G but by 1944, isolates of *S.aureus* emerged that were capable of destroying penicillin with β lactamase. Today, the majority of *S.aureus* strains produce β lactamases and hence are resistant to penicillin and many other types of β lactams such as methicillin. Methicillin resistant *S.aureus* (MRSA) is a major nosocomial pathogen causing infections in hospitals and long-term care facilities throughout the world. MRSA accounts for 12% of all bacteraemias, and the pathogen has been implicated in surgical wound infections (28%), and skin infections (21%). *Staphylococcus aureus* resistant to methicillin are often resistant to other bacterial agents such as aminoglycosides, macrolides, lincosamides, tetracyclines, β-lactamase inhibitor combinations, trimethoprim and sulphonamides. Initially, *S.aureus* was susceptible to the quinolones however, resistance has rapidly developed and in 1997 more than 80% of MRSA isolates are resistant to quinolones. Currently, vancomycin is used to treat MRSA infections.

1.10.3.Glycopeptides

1.10.3.1. Glycopeptide Classification

Glycopeptides including vancomycin belong to a group of antibiotics that inhibit PG biosynthesis in bacteria by binding the D-Ala-D-Ala carboxyl termini of PG precursor, presumably lipid II. Eli Lilly scientists discovered vancomycin in 1956 in soil samples collected in the jungles of Borneo. The soil microbe *Amycolaptasis orientalis* (formerly classified as Norcardia and Streptomyces) was identified to produce vancomycin.

Vancomycin is highly effective against a wide spectrum of pathogens: almost all aerobic Gram-positive cocci (staphylococci, streptococci, and enterococci), bacilli (actinomycetes, listeria, corynebacteria), some anaerobic Gram-positive (clostridium) and some aerobic Gram-negatives (brucella spp., and Bordetella pertussis) (Giesbrecht et al., 1998). The exceptional potency combined with efficacy against a broad spectrum of pathogens led to the pursuit of discovering structural diversity in glycopeptides. Currently, there are about 50 organisms known to produce over one hundred different types of natural glycopeptides (Lu et al., 2004). Even greater numbers of chemically modified glycopeptides have been reported in an attempt to further improve efficacy

The natural glycopeptides can be classified into four (Loll and Axelsen, 2000), based on their chemical composition. All glycopeptides have a characteristic heptapeptide core (aglycon) structure constructed by seven aminoacids through a series of phenolic oxidative couplings, and various sugars attached to the aglycon. Vancomycin belongs to the Type I glycopeptides characterized by the aliphatic aminoacids asparagine and methylated-leucine at the first and third positions of the a glycon from the N-terminus. In Type II, the first and the third residues are replaced by aromatic amnoacids. Avoparcin is an example of a Type II glycopeptide. Type III and IV glycopeptides also have aromatic aminoacids at the first and third position like type II, but they are cross-linked by a phenolic-ether linkage. Ristocetin is an example of Type III, in which its aglycon is further modified with six sugar adducts. Type IV have identical core structures as type III but slight variations in sugar adducts occur, with the main distinction of a long acyl chain(C_{11}) attached to one of the sugars. Teicoplanin is classified as type IV and is clinically available only Europe. Off all the types classified, the glycopeptides of type I are the most important drugs in clinical usage today.

1.10.3.1. 1. Structure of Vancomycin

The first aminoacid of the vancomycin heptapeptide core is methylated leucine. The state of methylation does not seem to affect the antimicrobial activity of

vancomycin, but the removal of leucine (desleucyl-vancomycin) by Edman degradation results in the complete loss of antimicrobial activity. The second residue of vancomycin is chlorinated hydroxyphenylglycine. The chlorination of the 2nd residue is importantfor antimictobial activity, and renders the compound 2 to 10 fold more potent than its non-chlorinated counter part.

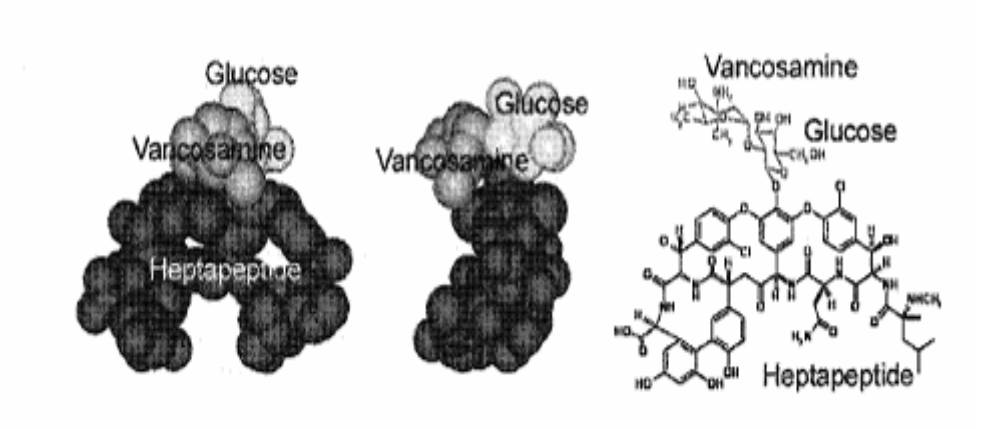

Figure 1.2. Crystal structure of vancomycin

Vancomycin consist of heptapeptide core (aglycon) with glucose and vancosamine attached at 4th aminoacid position. Vancomycin is shown in spaced filled representation with a view facing the D-Ala-D-Ala binding site (Left), and in 90 degrees rotation (Middle). The chemical structure of vancomycin (right) shows heptapeptide core structure is highly crosslinked.

The third residue is asparagine, which is not directly involved in bond formation with D-Ala-D-Ala. Lengthening the side chain with glutamine analogs results in reduced antimicrobial activity, likely due to the steric hindrance. Modifications of the charge characteristics of the heptapeptide core are made by the introduction of negatively charged residues. The aspartic acid in place of asparagine at the 3rd amino acid position reduced activity without changing conformation, presumably due to the

repulsion of the negatively charged side chain of aspartic acid in the binding pocket with the carboxyl terminus of D-Ala-D-Ala (Kaplan *et al.*, 2001).

The fourth residue of all glycopeptides has a conserved phenolic moiety that is cross-linked to the aromatic residues of the second and fifth residues by an ether linkage. Similarly, the benzyl moieties of the fifth and seventh residues that are cross-linked are also conserved in all glycopeptides. The lack of structural analogues in such conserved structures suggest that these residues are critical for shaping the binding cleft. In general, the modifications of the binding cleft strongly influence the antimicrobial properties of the glycopeptides. However, it is interesting to note that within a reasonable range, the binding affinities of glycopeptides to their substrate do not correlate with antimicrobial properties.

Lastly, all the aglycons of the glycopeptides are decorated with sugar adducts (glocose, mannose, vancosamine, or 4-epi-vancosamine). Vancomycin has D-glucose and L- vancosamine attached to the phenolic moiety of the fourth residue. The selective removal of the sugars of vancomycin results in the reduction of activities by 2 to 5 fold while adding L-epi-vancosamine to the sixth residue of the aglycon enhances the activity by 2 to 5 fold.

The structure of vancomycin bound to the PG precursor analogue Acyl-L-Lys-D-Ala-D-Ala (dipeptide) has been determined using solution-state NMR by D.H.Williams and his coworkers in the early 1980's. The substrate-binding site in vancomycin is defined by heptapeptide core structure by cross-linking the residues(2, 4 and 6) and the residues (5 and7) forming a rigid cage like structure, to which the D-Ala-D-Ala portion of the PG precursor analogue is bound (Nitanai et al., 2002) (Figure 1.3).

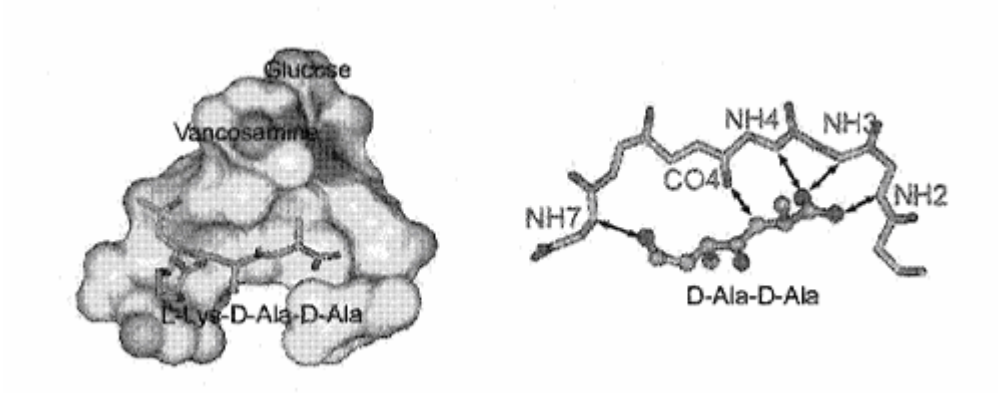

Figure 1.3. Vancomycin bound to dipeptide.

Crystal structure of vancomycinbound to L-Lys-D-Ala-D-Ala (Nitanai et al., 2002). The disaccharide does not participate in the binding. Five hydrogen bounds formed between the heptapeptide core structure (labeled by the residue numbers) and the dipeptide D-Ala-D-Ala are shown with arrows.

1.10.3.1. 2. Vancomycin Mode of Action

Among the first clues in the investigation of the MOA of vancomycin was the inhibition of PG synthesis in cell extracts of S.aureus. The effects of vancomycin on the radioactively labelled aminoacids (Lys, Glu and Ala), and the sugar nucleotide (Glc-Nac) incorporation into the mature PG were monitored using the cell extracts. The results suggested that vancomycin inhibited the incorporation of glycine into the lipid II as well as the formation of the PG .This suggested that vancomycin must inhibit several enzymatic steps in both the first and the second stages of PG assembly.

This confusion was cleared when radioactive iodine-labeled vancomycin was found localized only to the exterior side of the cytoplasmic membrane (Kaplan *et al.*, 2001), mostly at the PG, without permeating into the cytoplasm (Figure 1.4). The pulse chase experiment using radioactively labeled aminoacids in whole bacteria showed that vancomycin results in the accumulation of cytoplasmic precursors

(Park's Nucleotide) and membrane bound PG precursors. Therefore, vancomycin inhibits the PG biosynthesis steps proceeding the formation of lipid II at the exterior of the cytoplasmic membrane.

The vancomycin target site was determined when vancomycin failed to inhibit in an in vitro PG polymerization assay using UDP-MurNac-tetrapeptide precursors purified from the membrane fractions of *Gaffkya homari*. Since the UDP-MurNac-tetrapeptide precursors lack the terminal D-Ala of the pentapeptide stem, it was inferred that the vancomycin MOA must involve binding to the terminal D-Ala-D-Ala portion of lipid II. When the D-Ala-D-Ala peptide was added to the growth media, it had an antagonistic effect on vancomycin potency suggesting that the D-Ala-D-Ala peptide is a competitive substrate for vancomycin. Finally, solution-state NMR structure of vancomycin bound to the PG precursor mimic peptide Acyl-L-Lys-D-Ala-D-Ala confirmed the MOA.

Vancomycin dissociation constant to Acyl-L-Lys-D-Ala-D-Ala is 4.3 μM, as determined by capillary electrophoresis. Vancomycin is thought to bind to D-Ala-D-Ala of lipid II, thereby affecting the incorporation of the PG precursors into the growing nascent PG (Smith, et al., 2000). And interfering with the transglycosylase step ((Reipert et al., 2003). There is no known structure of vancomycin bound to lipid II, and the nature of vancomycin interaction to lipid II is unknown.

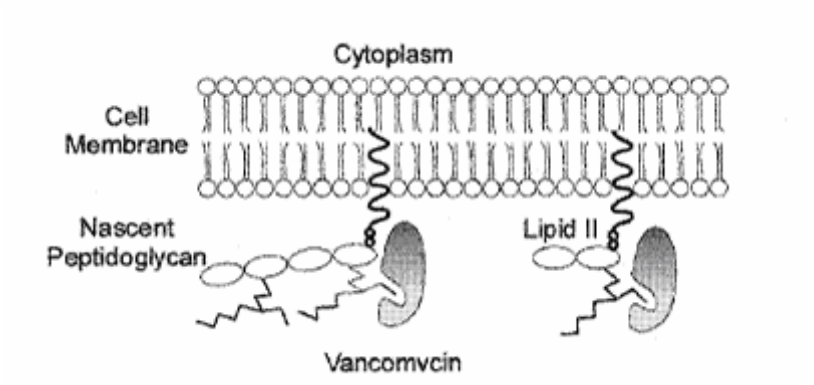

Figure1.4. Vancomycin and mode of action

Vancomycin binding to lipid II is an effective means of sequestering the lipid transporter C_{35}. The C_{35} transporter is the limiting factor in PG biosynthesis. It is present only in a small number of copies per bacterium, and is only regenerated from lipid II during the transglycosylation step (Goffin & Ghuysen 2002). This effective sequestering of C_{35} by vancomycin explains its high potency. The depletion of available C_{35} by vancomycin disables the bacteria's ability to transport the PG precursors from the cytoplasm to the exterior of the cytoplasmic membrane. The cascading effect is the dramatic reduction or lipid associated PG precursors despite the accumulation of Park's nucleotide in the cytoplasm.

Vancomycin perturbs the intricate balance between the cell wall synthesis and degradation essential for the normal bacterial growth and cell division by inhibiting only the biosynthesis. As the bacteria proceed with the cell wall turnover through selective cell wall digestion, the entire PG biosynthesis is inhibited by vancomycin and thereby induce. Cell death through lysis. This effect is evident from the EM images of bacteria grown in the presence of vancomycin (Figure 1.5), which show the characteristic thinning of the cell wall and septa, eventually leading to lysis.

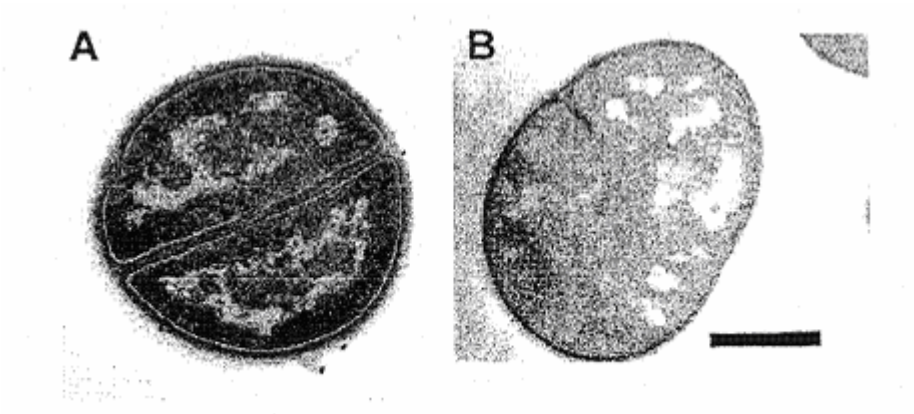

Figure 1.5 Effects of vancomycin on S. aureus.

(A) Electron micrographsof healthy dividing S.aureus. (B) After 90 mins of exposure to vancomycin, the cells show thinning of septa and cell wall, which eventually leads to lysis (Chen et al., 1998).

CHAPTER TWO
MATERIAL AND METHOD

2.1 Media and Growth Conditions

Amycolatopsis orientalis subsp. orientalis 40040 was obtained from "DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (German Collection of Microorganisms and Cell Cultures). Spore cultures of *Amycolatopsis orientalis* were prepared by inoculating solid medium M65. This medium contains 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$, 12 g Agar, 20 g starch in 1 litre of ultra-pure water (Lechevalier et al, 1986). The basal chemically-defined fermentation medium contained 0.6 g $MgSO_4$ $7H_2O$, 3.5 g KH_2PO_4, 2.0 g asparagine, 10 g glycerol, 1 g Bacto Yeast, 1 g NH_4Cl, 21.0 g 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, and 1 ml trace salts solution (containing 1.0 g$FeSO_4$ $7H_2O$, 1g $MnCl_2$ $4H_2O$, 1.0 g $ZnSO_4$ H_2O, 1.0 g $CaCl_2$) in 1 litre of ultra-pure water. The pH was adjusted to 7.0 before autoclaving. The cultures were inoculated with spore suspensions (OD_{600} = 0.15) and incubated with agitation at 150 rpm at 28°C in 500 ml shaking flasks containing 50 ml of culture for 96 hours. After the cultivation process, the cells were collected by centrifugation followed by washing twice with distilled water and kept at -20°C.

2.2 Dry Weight Determinations

Ten-milliliter aliquots of culture were centrifuged and the pellets were washed and centrifuged twice with deionized water and the placed at 105 ^{0}C for approximately 24 h, until the weight remained constant. Optical density was measured at 620 nm with a spectrophotometer.

2.3 Preparation of Cell-Free Extracts

For preparation of cell extracts, wet *A. orientalis* cells were harvested by centrifugation, washed twice with 10 mM potassium-phosphate buffer, pH 7.5,

containing 2 mM EDTA and stored at -20°C. Before assaying, the samples were thawed, washed, and resuspented in 100mM potassium-phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol in a volume equal to 1.5 times its weight. A 600 µl cell suspension was ground in 1.5 ml plastic vials with 0.6 g of glass beads (0.25 mm ϕ) for 10 min at optimized conditions. Cell debris was removed by centrifugation at 15000 rpm for 15 min.

2.4 Enzyme Activity Assay in Crude Extract

2.4.1 Glucose Kinase Activity Assay

Glucose kinase was assayed by measuring the initial rate of increase in $NADP^+$ absorbancy at 365 nm. The reaction mixture contained 100 mM Tris-HCl, pH 7.5, 5.0 mM $MgCl_2$, 0.5 mM $NADP^+$, 10 mM glucose, 1 mM ATP, 0.7 U glucose-6-phosphate dehydrogenase (Postma E et al., 1988).

2.4.2 Pyruvate Kinase Activity Assay

Pyruvate kinase was assayed by measuring the initial rate of decrease in NADH absorbancy at 340 nm. The reaction mixture contained 100 mM triethanolamine, pH 7.6, 0.75 mg/ml phosphoenolpyruvate, (in 0.05M $MgSO_4$, 0.2 M KCl), 4.7 mM, ADP, 0.2 mM NADH, 0.2 mM lactate dehydrogenase **(**de Jong-Gubbels P et al 1995).

2.4.3 α- Ketoglutarate Dehydrogenase Activity Assay

α- ketoglutarate dehydrogenase activity was assayed by measuring the initial rate of increase in NAD^+ absorbancy at 340 nm. The standard reaction mixture as optimized during this study contained 100 mM Tris-hydrochloride buffer (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 2 mM thiamine pyridoxal 5'-phosphate, 1 mM sodium α -ketoglutarate, 1 mM NAD, 0.2 mM coenzyme A, (CoA), and enzyme sufficient to produce an increase in absorbancy (Meixner-Monori et al., 1985).

2.4.4 Isocitrate lyase Activity Assay

Isocitrate lyase was assayed mixture for isocitrate lyase contained, in 2 ml, 15 μmol of $MgCl_2$ $6H_2O$, 10 μmol of phenylhydrazine-HCl, and 6 μmol cysteine-HCl. The reaction for the assay was started by addition of crude cell-free extract to be assayed and 0.2 ml or 5 μmol of trisodium DL-isocitrate. The formation of glyoxylic acid phenylhydrazone was followed by monitoring absorbance at 324 nm. The enzyme activity was expressed as U/mg protein, with 1 unit enzyme activity equal to 1 nmol glyoxalate formation (Dixon et al., 1959).

2.5 Intra-Extracellular Metabolite Assay

2.5.1 Intra-Extracellular Metabolite Extraction

Intracellular metabolites were obtained from resting cells prepared essentially as described by Babul et al. by using a boiling water extraction (Babul et al., 1993). The extracted sample was cooled on ice, quickly frozen, and then lyophilized. The lyophilyzate was brought up in 1 ml of water and centrifuged twice to eliminate cell debris and supernatant was filtered through a 0.45 μm filter before injecting into the HPLC.

Extracellular metabolites, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.45 μm syringe filter for HPLC analysis.

2.5.2 Organic Acid Analysis

2.5.2.1. Organic Acid Analysis with HPLC

The HPLC system used was equipped with Alltech IOA-1000 column, a UV detector, and a differential refractive index detector. 0.4 ml/min mobile phase using

9.0 mM H_2SO_4 solution was applied to the column. The column was operated at 42 ^{0}C. Standards were prepared for glucose, α-ketoglutarate, citrate, malate, succinate, fumarate for the both the RI detector and UV detector (210 nm), and calibration curves were created.

2.5.2.2. Pyruvate Assay with Spectrofotometric

Pyruvate concentrations were determined with 2,4-dinitrophenylhydrazine (Aras & Erşen, 1975). The coloured complex that has a maximum absorbance at 520nm formed. Samples and pyruvate solutions containing 0-2.6 10^{-2} mg/ml pyruvate volume up to 3 ml were pipetted in to test tubes. One millilitre of 1 mg/ml 2,4-dinitrophenylhydrazine reagent (dissolved in 2 N HCl) was added to the test tube and the contents were mixed. Absorbance at 520 nm was measured after ten minutes. The amounts of pyruvate were plotted against the corresponding absorbance resulting in a standard curve used to determine the pyruvate in unknown samples.

2.5.3. Glycogen Assay

Glycogen was determined as glucose after treatment with amyloglucosidase (Devos et al., 1983).

2.6. Vancomycin Isolation and Determination

Extracellular vancomycin in culture filtrates was recovered by solid phase extractions using Varian Bond Elut LRC C18 EWP cartridges (Bauchet et al., 1987). The antibiotic concentration was measured by high-performance liquid

chromatography (HPLC) gradient and singles multiple wavelength detection at 210 nm (Backes et al., 1998) by using Varian Cromsep C8 column.

2.7. Protein Determination

The protein content was determined by the method of Bradford et al. using bovine serum albumin as standard (Bradford, 1976).

2.8. Statistical analysis

Tukey test, one of the multiple comparisons, was used for statistical significance analyses. The values are the mean of three separate experiments. Also comparison was made with ***Pearson correlation*** for each substrate and/or enzyme depending glucose concentration with respect to incubation time.

CHAPTER THREE
RESULTS

3. Results

In the first step of the research; growth curve, the variations of pH, intra-extracellular glucose levels and vancomycin levels of *Amycolatopsis orientalis* growth in various glucose (5-20 g/L) and glycerol (2.5-20 g/L) concentrations were investigated during the incubation period as the fundamental parameters.

In the second step; some important glycolytic enzymes such as glucose kinase, pyruvate kinase, pyruvate decarboxylase as well as pyruvate as an end product of *Amycolatopsis orientalis* growth in various glucose(5-20 g/L) and glycerol (2.5-20 g/L) concentrations were investigated during the incubation period. In this step, glycogen level as a glucose storage metabolite was also investigated.

In the third step; the variations of tricarboxylic acid (TCA) cycle metabolites such as citrate, α-ketoglutarate, succinate, fumarate, malate and α-ketoglutarate dehydrogenase, and glyoxalate cycle enzyme isocitrate lyase of *Amycolatopsis orientalis* growth in various glucose (5-20 g/L) and glycerol (2.5-20 g/L) concentrations were investigated depending on the incubation period.

3.1. Glucose as a Carbon Source in *A. oriantalis* Growth Medium

3.1.1.Growth Curve Variations of A. orientalis Depending on Glucose Concentrations and Incubation Periods

As shown in Figure 3.1, *A. orientalis* displays logarithmic growth in growth medium during the first 24-36 hour for 5 g/L glucose and 24-48 hour in the range of 10-20 g/L glucose, after which it enters to stationary phase. *A. orientalis* growth rate increased when the concentration of glucose was increased up to 15 g/L. From this concentration and above, growth rate did not increase significantly indicating that glucose was no longer a growth limiting factor to research the greater iomass-concentrations.

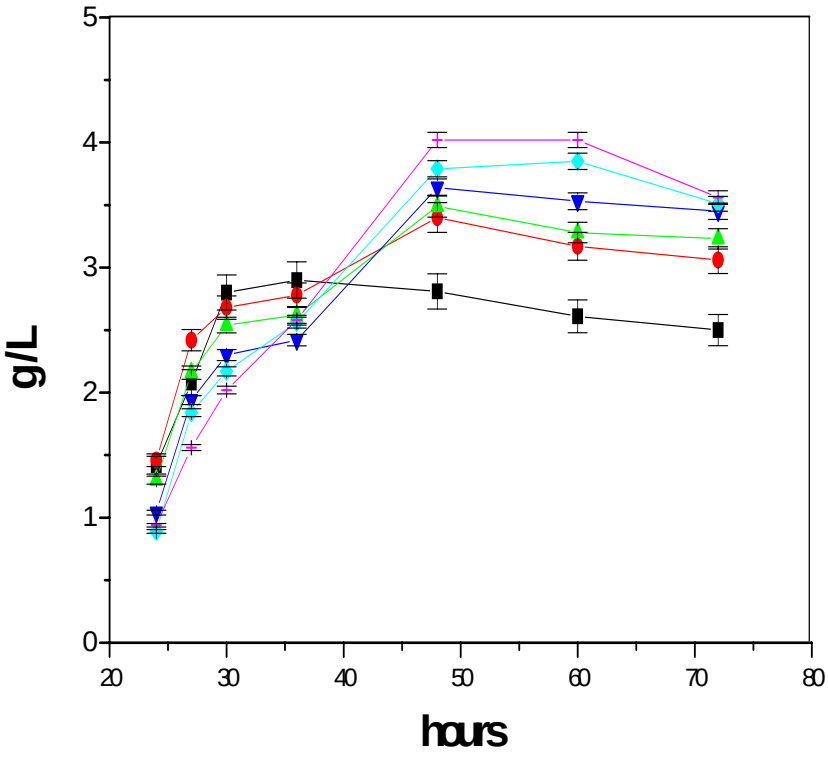

Figure 3.1 Biomass concentration variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.2. pH Level Variations of A. orientalis Growth Medium Depending on Glucose Concentrations and Incubation Periods

According to our results, pH level of *A. orientalis* showed a rise up to 36th hour for all used glucose concentration and then decreased continuingly until 48th for 5-12.5 g/L, 60th for 15.0 g/L and 72nd hour for 17.5 and 20.0 g/L glucose (Figure 3.2). These decreases in pH values were increased with increases in glucose concentrations in the culture medium. The highest pH decrease was determined at 20 g/L glucose as approximately 1 pH units. On the other hand, in the following incubation periods, pH variations were slightly increased.

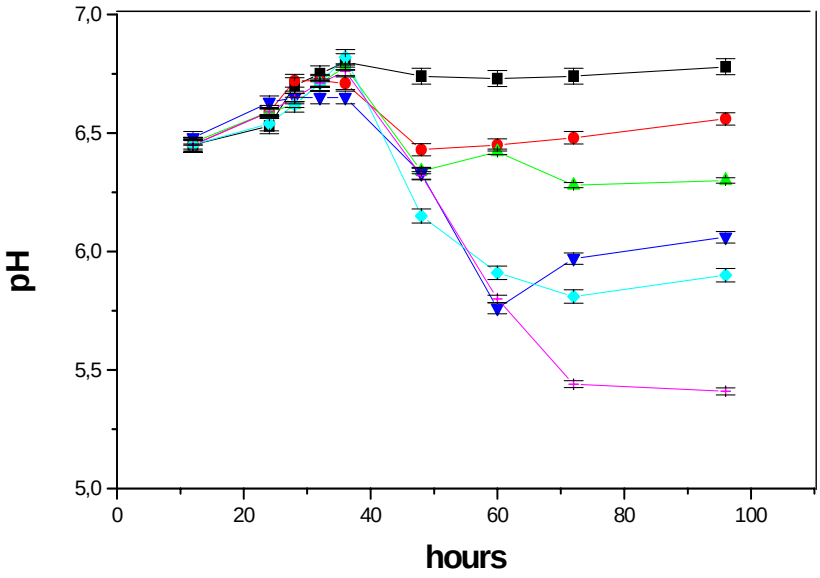

Figure 3.2 Variations of pH values in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.3. Intra- and Extracellular Glucose Level Variations of A. orientalis Depending on Glucose Concentrations and Incubation Periods

Figure 3.3a shows that intracellular glucose levels increased with respect to increasing initial glucose concentrations of the growth medium and reached their maximum levels on the 24th hour ($r = 0.568$, $p<0.01$). On the other hand, a positive correlation was determined between consumption time of extracellular glucose levels and glucose concentration in the culture medium ($r = 0.618$, $p<0.01$) (Figure 3.3b). According to the results, glucose in the culture medium was consumed rapidly during the same time period of incubation time as stationary phase was observed.

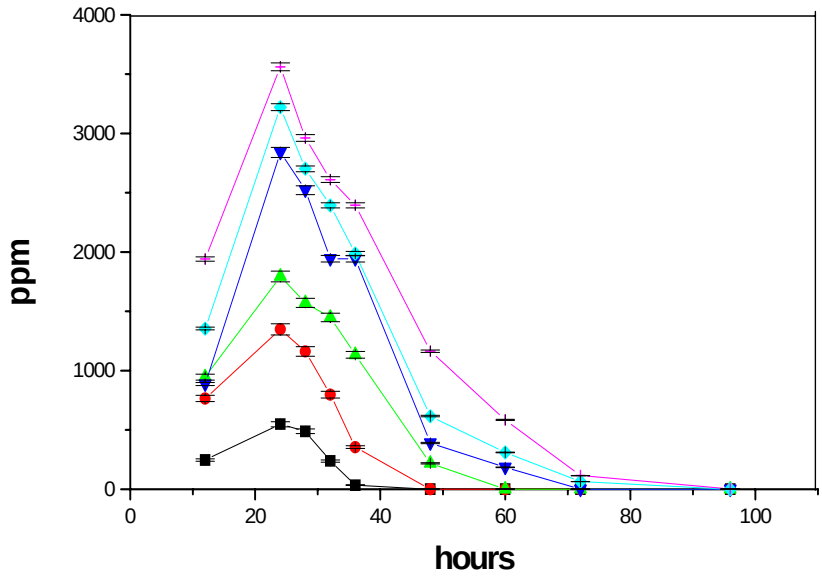

Figure 3.3a. Variations of intracellular glucose level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

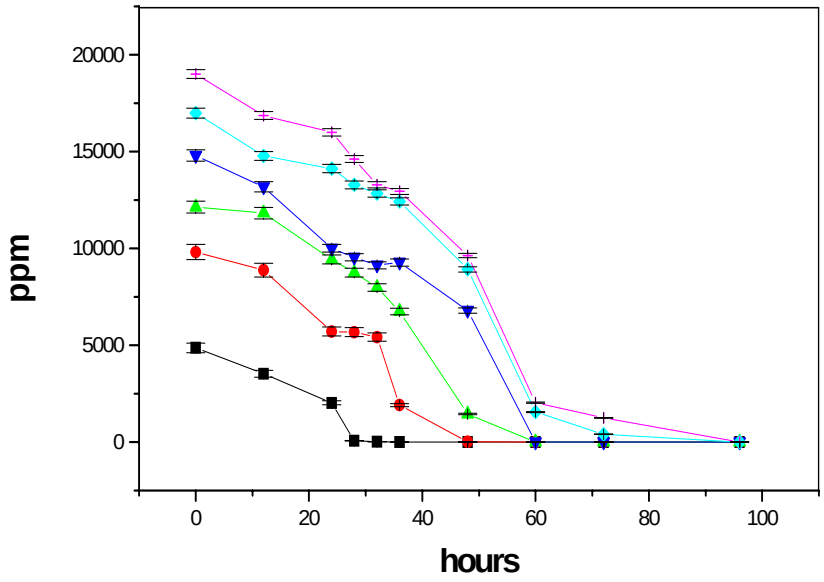

Figure 3.3b Variations of extracellular glucose level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.4. Variations of Vancomycin Production by A. orientalis Depending on Initial Glucose Concentrations and Incubation Periods

According to our results, vancomycin productions were reached their maximum values on the 48th hour for all used glucose concentrations except for 5 g/L glucose and afterward, all of them decreased slightly ($p>0.01$) (Figure 3.4). Vancomycin production increased with the increases in glucose concentrations up to 15 g/L and determined as 7.8 ± 0.086 ppm .

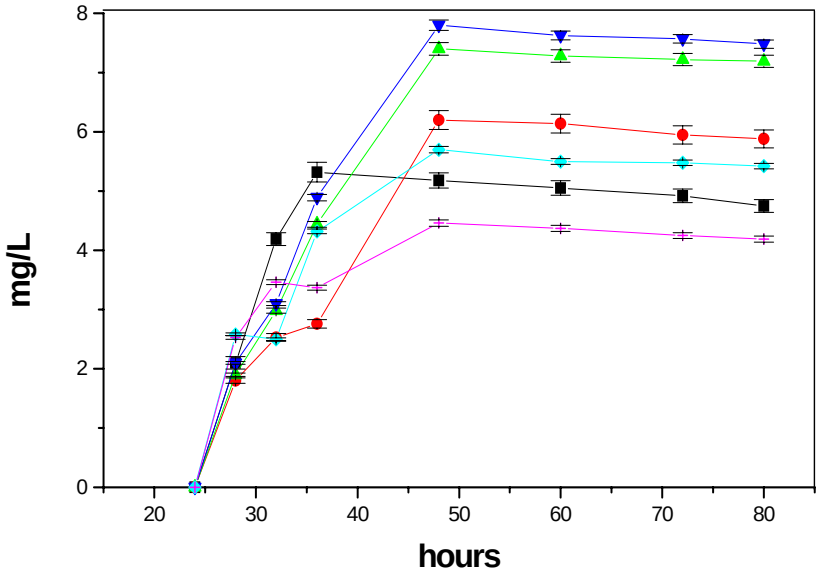

Figure 3.4 Vancomycin production variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.5. The Variations of Intra- and Extracellular Glycolysis Metabolite and Enzymes Levels of A. orientalis Depending on Glucose Concentrations and Incubation Periods

Glucose kinase, pyruvate kinase, pyruvate decarboxlase as well as pyruvate *Amycolatopsis orientalis* growth in various glucose (5-20 g/L) and glycerol (2.5-20 g/L) concentrations were investigated during the incubation period. In this step, glycogen level as a glucose storage metabolite was also investigated.

3.1.5.1. Variations of Pyruvate Level by A. orientalis Depending on Glucose Concentrations and Incubation Periods

As shown in Figure 3.5a, the pyruvate reached their maximum levels on 36th and 48th hours at initial glucose concentrations of 5,10 g/L and 12.5-20 g/L, respectively. Pyruvate levels were increased with the increases in glucose concentration.

The highest level was determined as 528.2 ± 10.32 ppm. Extracellular pyruvate levels were also increased significantly with increases in glucose concentration ($p<0.01$). Maximum levels were determined on the 48th and 60th hours (Figure 3.5b).

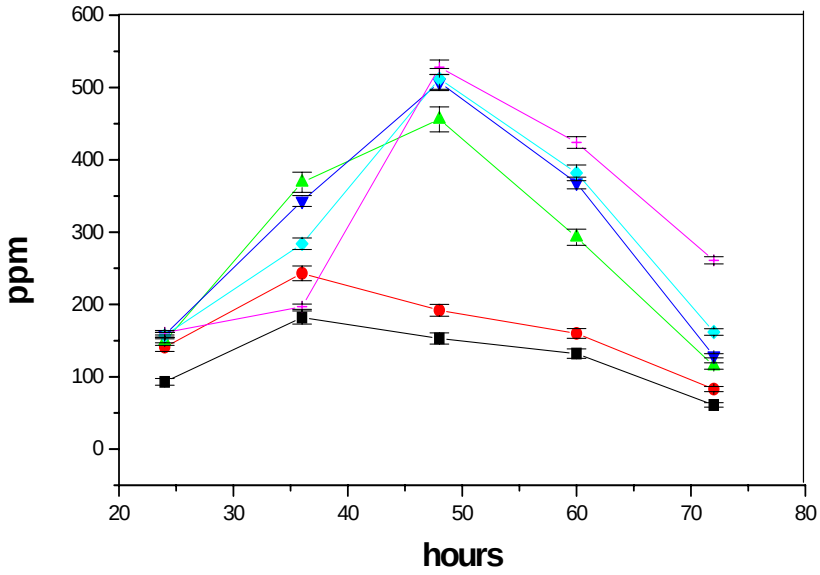

Figure 3.5a. Intracellular pyruvate level variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

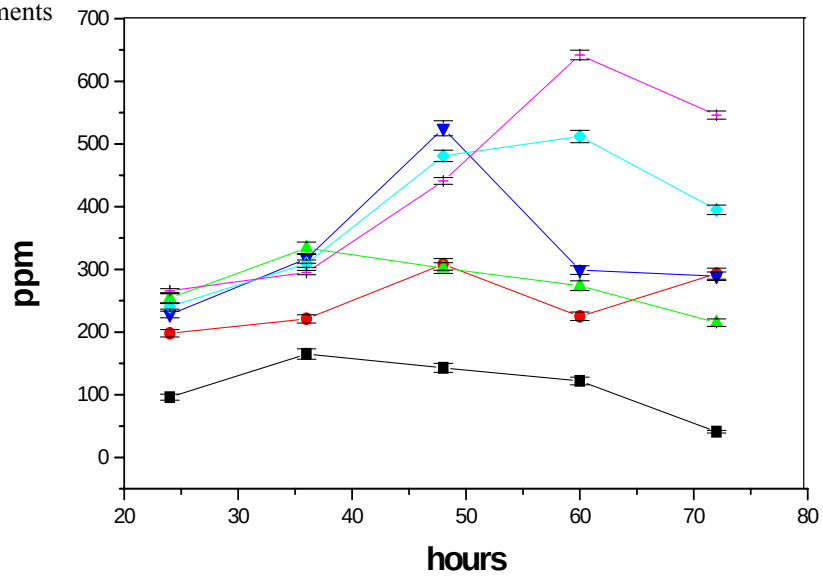

Figure 3.5b. Extracellular pyruvate level variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.5.2. Variations of Glycolytic Enzyme Activities by A. orientalis Depending on Glucose Concentrations and Incubation Periods

As an important enzyme of glycolysis, glucose kinase activities were showed positive correlation with the glucose concentration of the *A. orientalis* growth medium and their maximum levels were determined on the 48th hour ($p<0.01$) (Figure 3.6). The highest glucose kinase activity was determined as 1089 ± 14.17 U/mg at 20 g/L on the 48th hour ($p<0.01$).

As shown in Figure 3.7, pyruvate kinase activity was increased up to 15 g/L. In addition, pyruvate kinase activities were reached the maximum on the 36th hour at 5 g/L while it shifted 48th hour in the range of 10-20 g/L glucose containing medium ($p<0.01$). The highest pyruvate kinase activity was determined as 506 ± 10.6 at 15 g/L on the 48th hour.

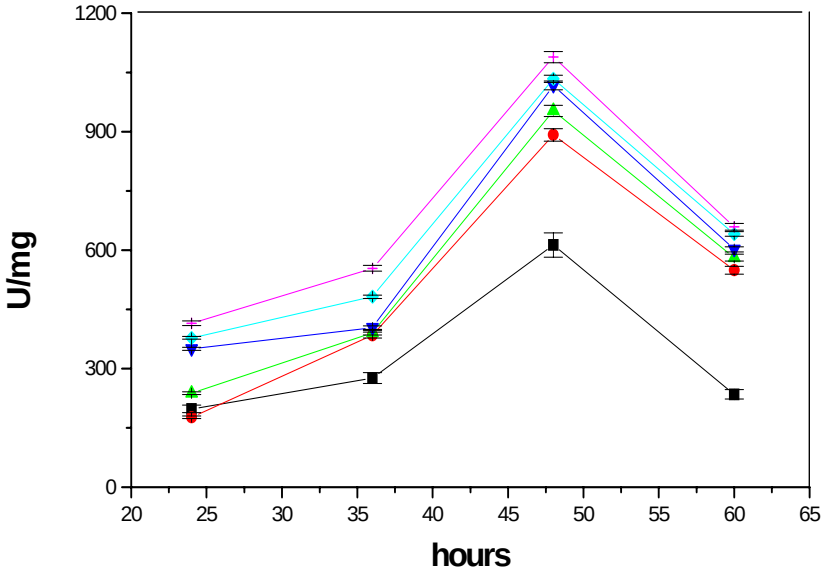

Figure 3.6. Variations of glucose kinase activity in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments.

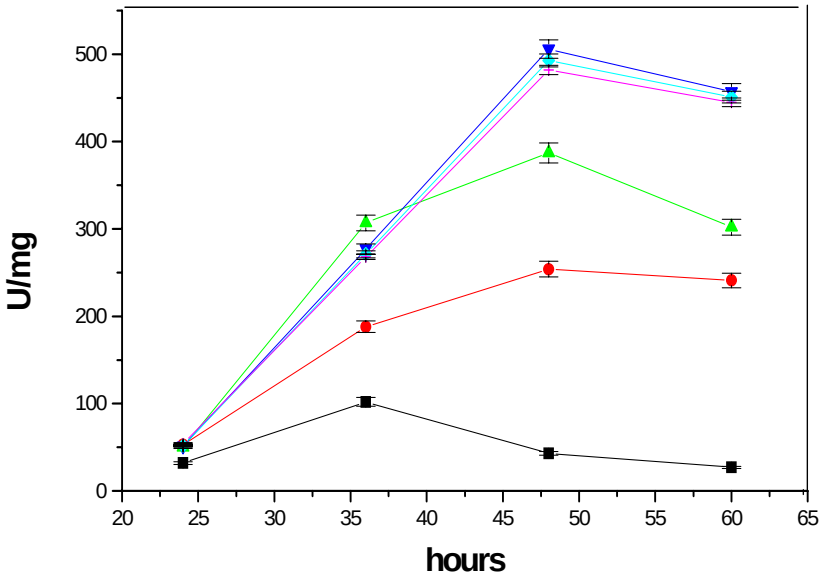

Figure 3.7. Variations of pyruvate kinase activity in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.6. Variations of Glycogen Level by A. orientalis Depending on Glucose Concentrations and Incubation Periods

As shown in Figure 3.8, the glycogen levels of *A. orientalis* reached their maximum levels on 48th and 60th hours at in the range of 5-12.5 and 15-20 g/L glucose, respectively ($p<0.01$). Glycogen levels were also showed positive correlation with the glucose concentration of the growth medium ($r =0.782$ $p<0.01$).

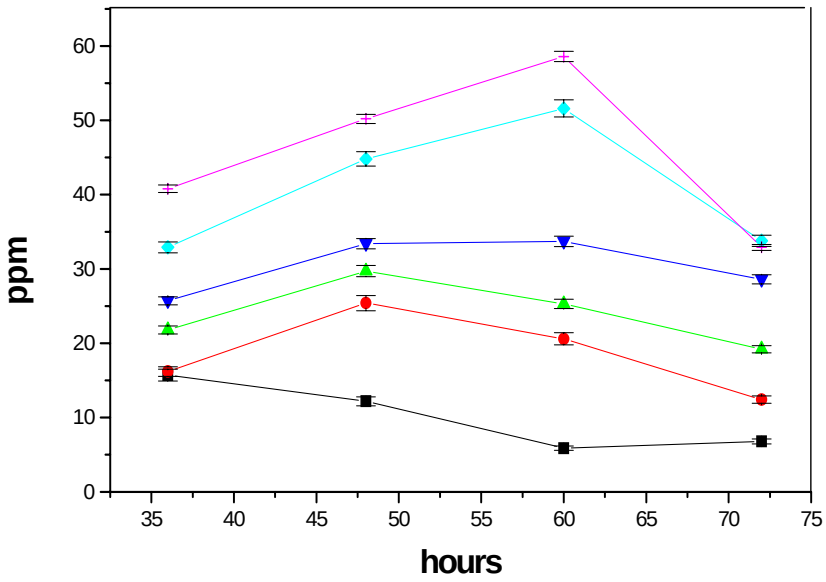

Figure 3.8 Glycogen level variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.7. The Variations of Intra- and Extracellular TCA Cycle Metabolite Levels of A. orientalis Depending on Glucose Concentrations and Incubation Periods

As shown in Figure 3.9 a, the maximum intracellular citrate levels of *A. orientalis* were determined on the 48th hour in the range of 10-20 g/L glucose in the culture medium, although it was 36th hour for 5 g/L glucose.

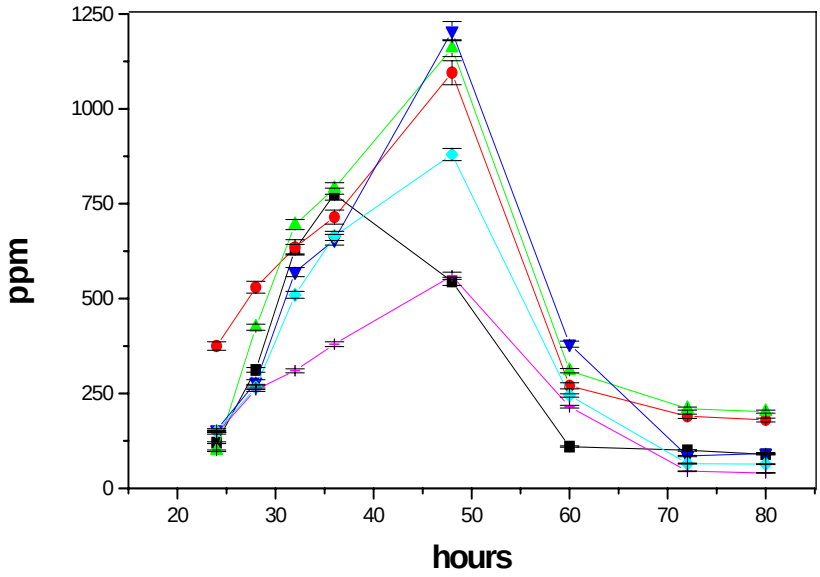

Figure 3.9a Variations of intracellular citrate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

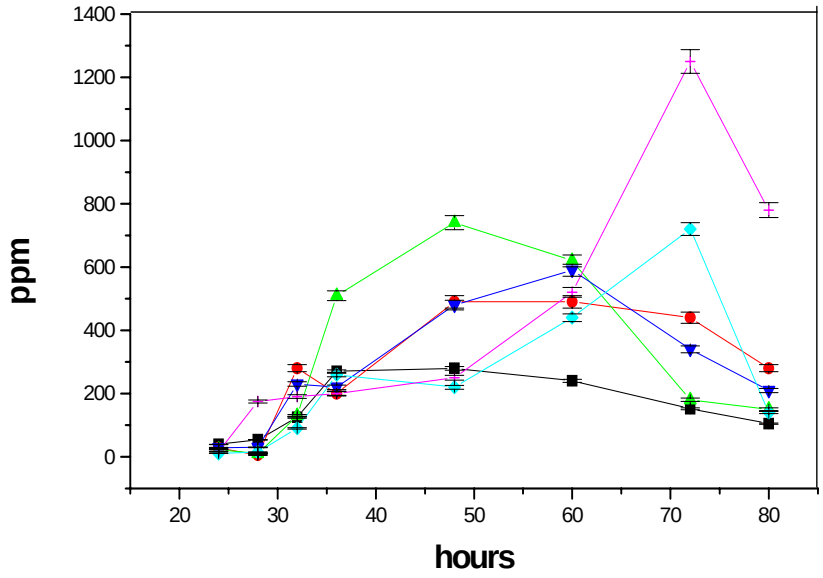

Figure 3.9b. Variations of extracellular (b) citrate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

Intracellular citrate levels indicated positive correlation with the rise of glucose concentration up to 15 g/L while further increases had an opposite effect and the highest level was determined as 1205 ± 13.76 ppm**.** Citrate level determined in 12.5 g/L was not lower significantly compared to 15 g/L glucose **(**$p>0.01$**).** On the other hand, extracellular citrate levels were reached the maximum on the 48th hour in the range of 5-12.5 g/L glucose containing medium while it shifted to 60th and 72nd hours for 15 g/L and 20 g/L glucose, respectively ($p<0.01$) (Figure 3.9 b).

As can be seen from Figure 3.10a, the levels of α-ketoglutarate of *A. orientalis* increased in the range of 10-20 g/L glucose in the initial hours of cultivation ($p<0.01$), reaching their maximum levels on the 48th hour, after which the levels began to decline ($p<0.01$). Intracellular α-ketoglutarate levels increased up to 12.5 g/L glucose on the 48th hour ($p<0.01$) and determined as 702 ± 10.24 ppm. The increases in extracellular α-ketoglutarate levels depend on the glucose concentration were showed similar trends with its intracellular levels ($p<0.01$) (Figure 3.10b). Nevertheless, maximum extracellular α-ketoglutarate levels were determined on the 60th and 72nd hours in the range of 5-17.5 g/L and 20 g/L glucose, respectively.

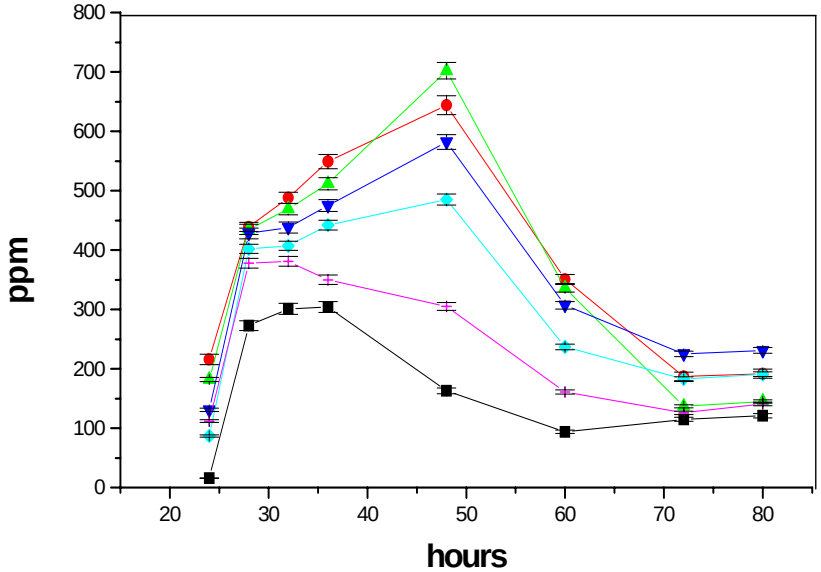

Figure 3.10a . Variations of intracellular α-ketoglutarate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—),20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments

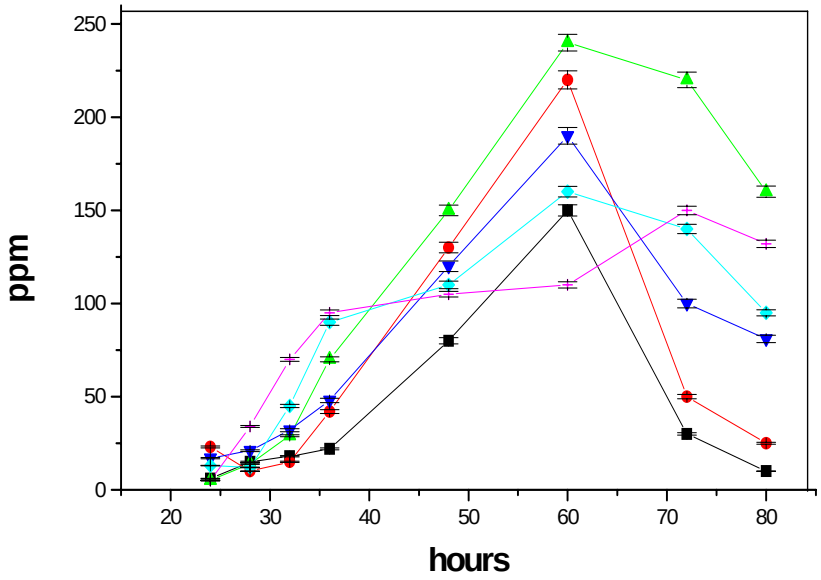

Figure 3. 10b . Variations of extracellular α-ketoglutarate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—),20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

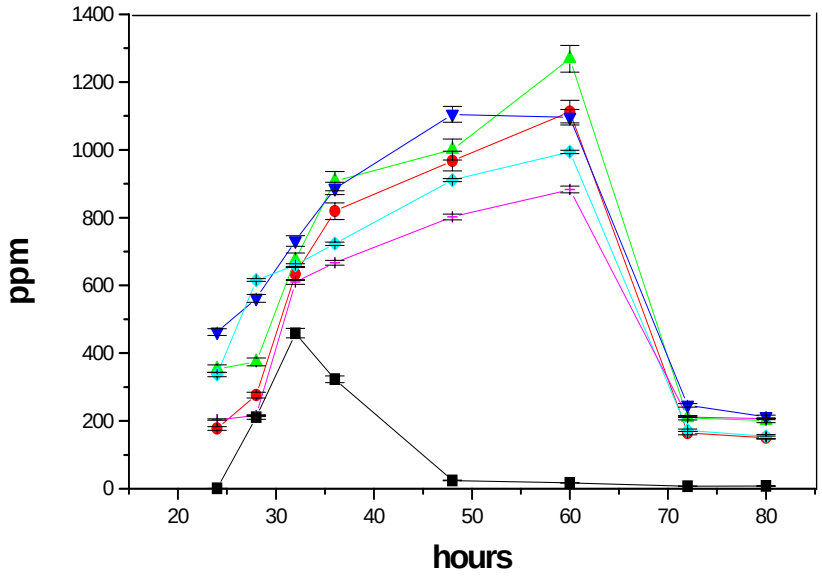

Figure 3.11a. Variations of intracellular succinate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

Figure 3.11a shows that intracellular succinate levels of *A. orientalis* showed apparent increases in the 24th - 36th hour incubation period range ($p<0.01$), and they reached maximum levels in all glucose concentrations used on the 60th hour except for 5 g/L on the 32nd hour. The incubation periods of maximum extracellular succinate levels were differed by shifting to right with the increases in glucose concentration of the culture medium ($p<0.01$) (Figure 3.11b).

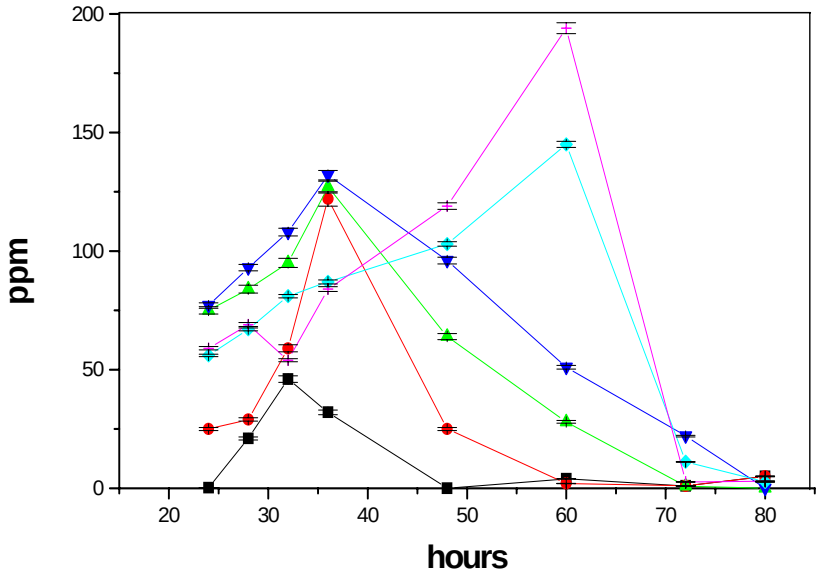

Figure 3. 11b. Variations of extracellular succinate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

Intracellular fumarate levels of *A. orientalis* grown in all glucose concentration used reached the maximum on the 48th hour and then decreased significantly with prolongation of the incubation period ($p<0.01$) (Figure 3.12a). As determined in citrate, succinate and α-ketoglutarate, intracellular fumaric acid increased with the increase of initial glucose concentration up to 12.5 g/L, but then decreased above this

value ($p<0.01$). Extracellular fumaric acid levels also increased with respect to raised glucose concentrations up to 15 g/L ($r = 0.405$, $p<0.05$), and reached the maximum values after 36th and 48th hours ($p<0.01$) (Figure 3.12b). The highest intra- and extra-cellular fumaric acid were determined as 865.0 ± 10.56 and 83.6 ± 2.26 ppm .

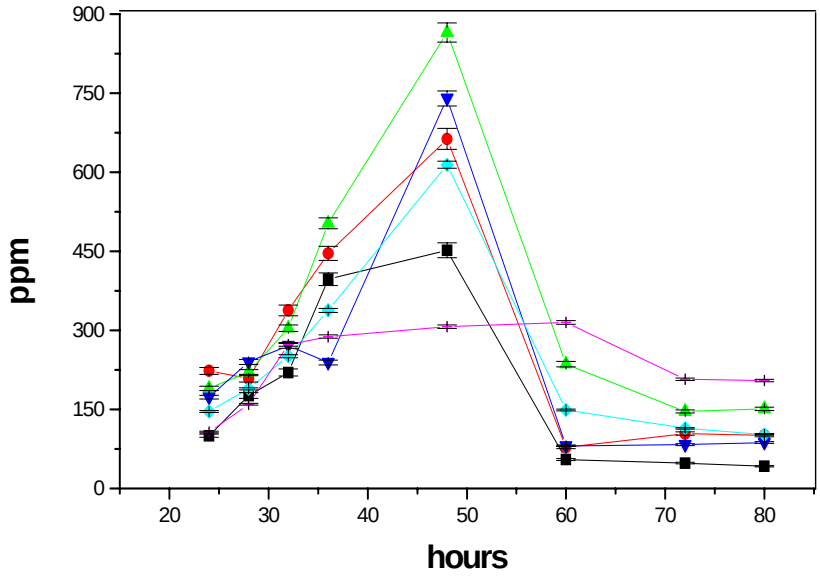

Figure 3.12a. Variations of intracellular fumarate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

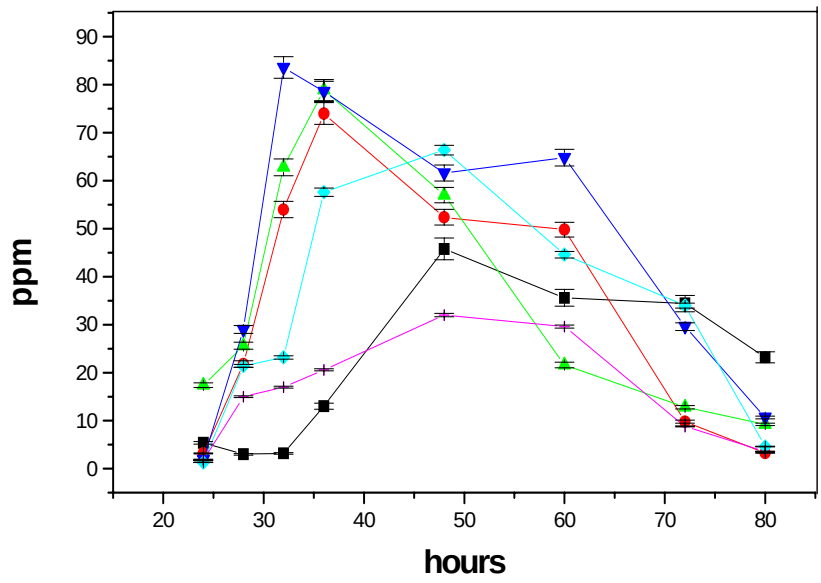

Figure 3.12b. Variations of extracellular fumarate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

As can be seen from Figure 3.13 a and 3.13b, intracellular malate levels increased with increases in glucose concentration from 5 to 15 g/L while extracellular levels increased up to 12.5 g/L glucose ($p<0.01$). When the initial glucose concentration exceeded these concentrations, malate levels decreased significantly ($p<0.01$). The highest levels were 2272.0 ± 26.8 and 398.0 ± 6.75 ppm, respectively.

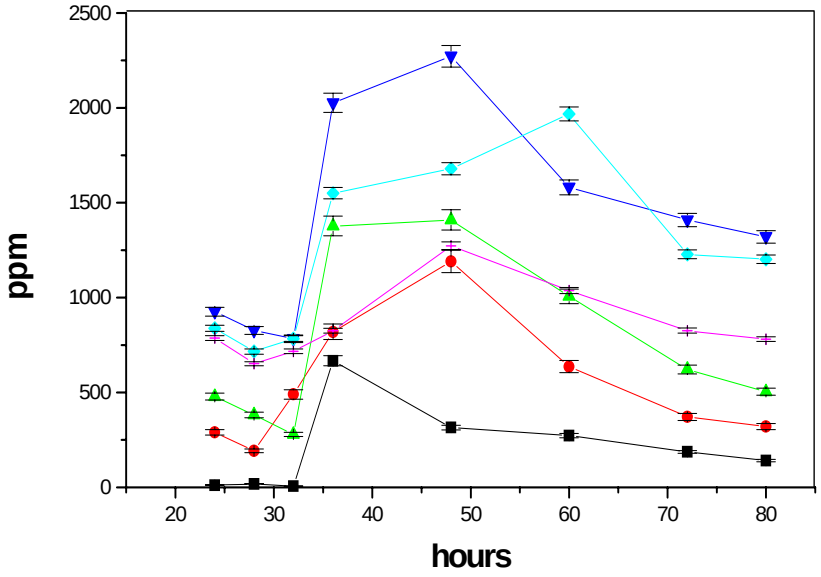

Figure 13a. Variations of intracellular malate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

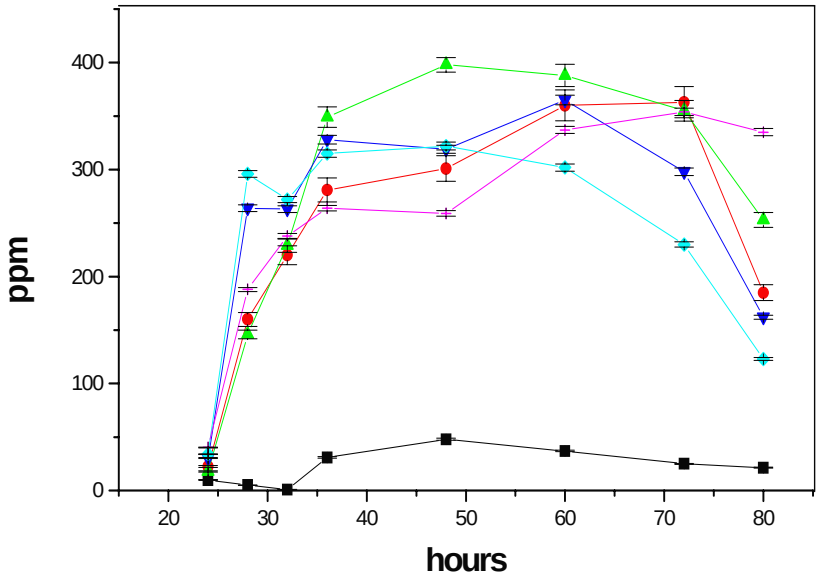

Figure 3.13b. Variations of intracellular malate level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments

3.1.8. Variations of TCA Enzyme Activities by A. orientalis Depending on Glucose Concentrations and Incubation Periods

One of TCA cycle key enzyme, α-ketoglutarate dehydrogenase activities were increased slightly but not significantly different depend on the glucose concentration up to 36th hour ($p>0.01$) (Figure 3.14). The activities were increased markedly up to 12.5 g/L glucose on the 48th hour ($p<0.01$) and decreased slowly in the following incubation period. The highest value was determined as 174.0 ± 3.39 IU/mg.

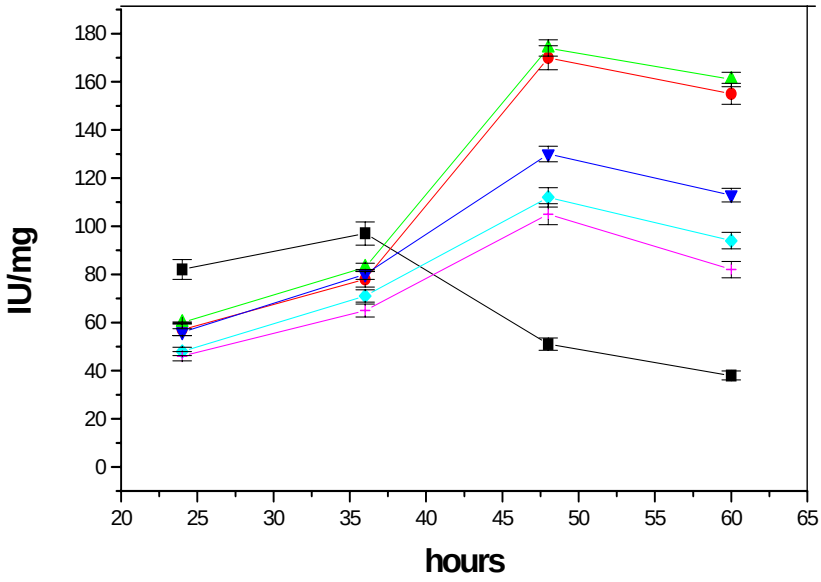

Figure 3.14. Variations of α-ketoglutarate dehydrogenase in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (— ┼ —) glucose. The values are the mean ±SEM for experiments of three separate experiments.

As a considered key enzyme of the glyoxalate shunt, isocitrate lyase showed positive correlation with glucose concentrations as well as incubation period ($r = 0.666, 0.663$; $p<0.01$) (Figure 3.15). The highest value was 1019.2 ± 25.48 IU/mg.

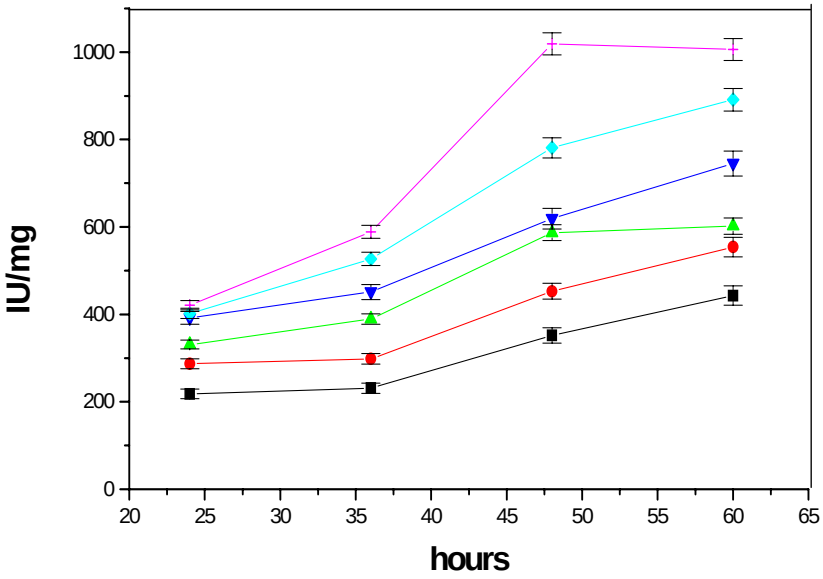

Figure 3.15. Variations of isocitrate lyase activity in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments.

Intracellular glycerol levels of *A. orientalis* grown in all glucose concentrations we tested reached their maximum levels on the 48th hour and then decreased significantly with prolongation of the incubation period ($p<0.01$) (Figure 3.16). Extracellular glycerol levels also increased with rising glucose concentrations.

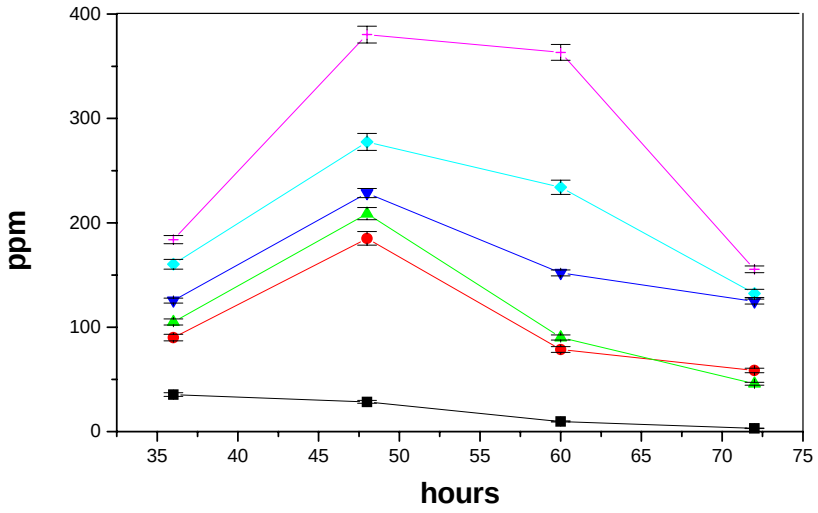

Figure 3.16. Variations of glycerol level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments.

3.2. Glycerol As a Carbon Source in *A. oriantalis* Growth Medium

3.2.1. Growth Curve Variations of A. orientalis Depending on Glycerol Concentrations and Incubation Periods

Figure 3.17 shows that logarithmic growth of *A. orientalis* were continued up to 36th hour in the range of 2.5 -10 g/L glycerol and 48th hours at 15, 20 g/L glycerol. In addition, *A. orientalis* growth rate increased when the concentration of glycerol was increased.

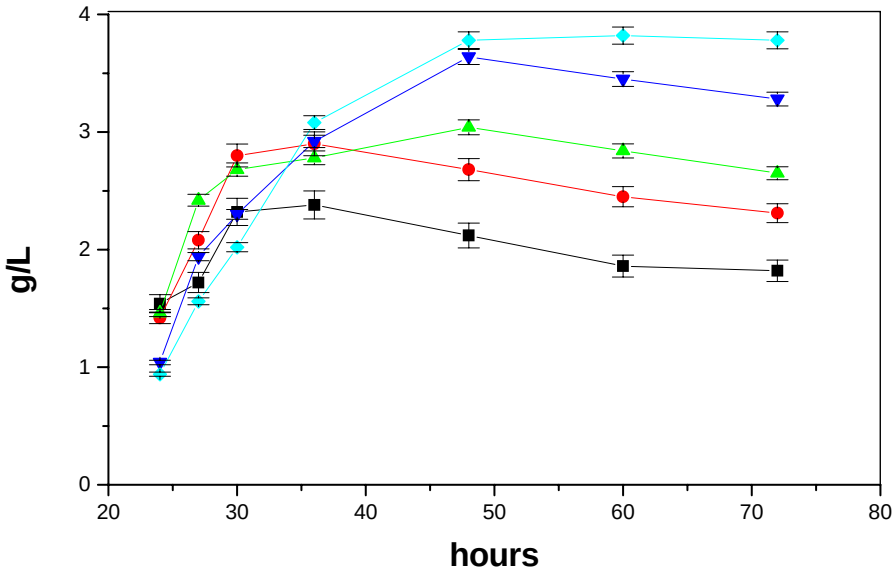

Figure 3.17. Biomass concentration variations in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.2. pH Level Variations of A. orientalis Growth Medium Depending on Glycerol Concentrations and Incubation Periods

As shown in Figure 3.18, pH level of *A. orientalis* showed a rise up to 28 for 5 and 10 g /L glycerol and up to 32th hour and then decreased continuingly until 36th for 5-12.5 g/L, 60th for 15.0 g/L and 72nd hour for 17.5 and 20.0 g/L glycerol. These decreases in pH values were increased with increases in glycerol concentrations in the culture medium. The highest pH decrease was determined at 20 g/L glycerol as 1.38 units. On the other hand, in the following incubation periods, pH variations were slightly increased.

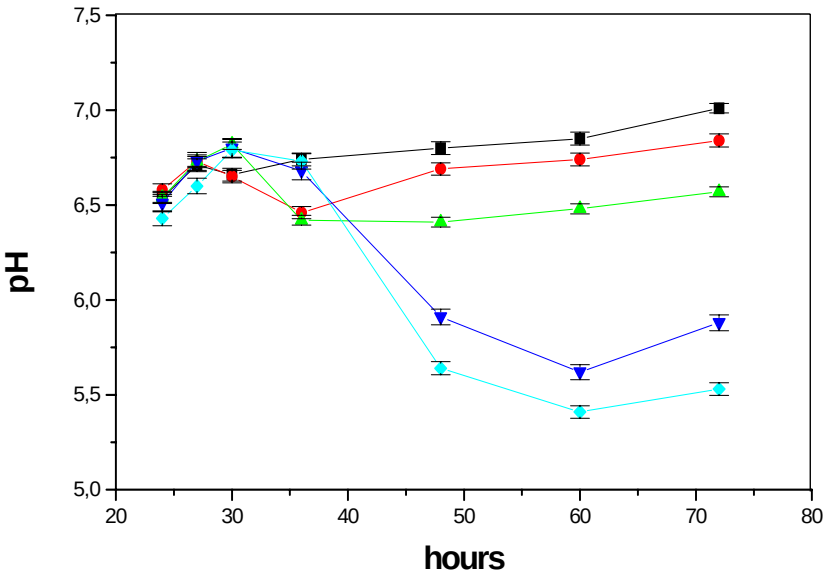

Figure 3.18. pH variations in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.3. Intra- and Extracellular Glycerol Level Variations of A. orientalis Depending on Glycerol Concentrations and Incubation Periods

As shown in Figure 3.19a, intracellular glycerol levels increased with respect to increases in glycerol concentrations of the growth medium and reached their maximum levels on the 36th hour ($r = 0.885$, $p < 0.01$). Glycerol levels were decreased approximately 4000 ppm for all used glycerol concentration during the 24-36th hours of incubation. In addition, the decreases of glycerol levels were increased to 7000 ppm at 15 and 20 g/L glycerol. The results showed that the rate of glycerol transport was increased with respect to incubation time, but it didn't change dependent on the glycerol concentration of the *A. orientalis* growth medium.

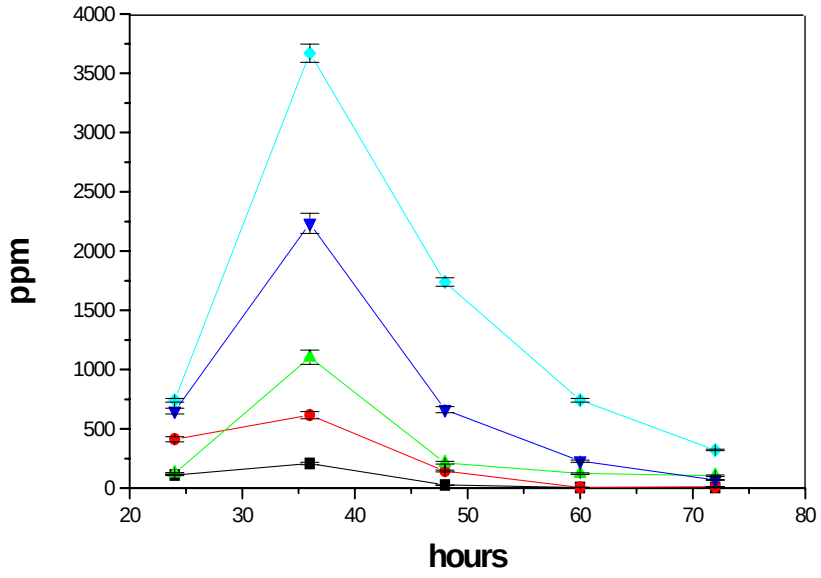

Figure 3.19a. Variations of intracellular glycerol level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

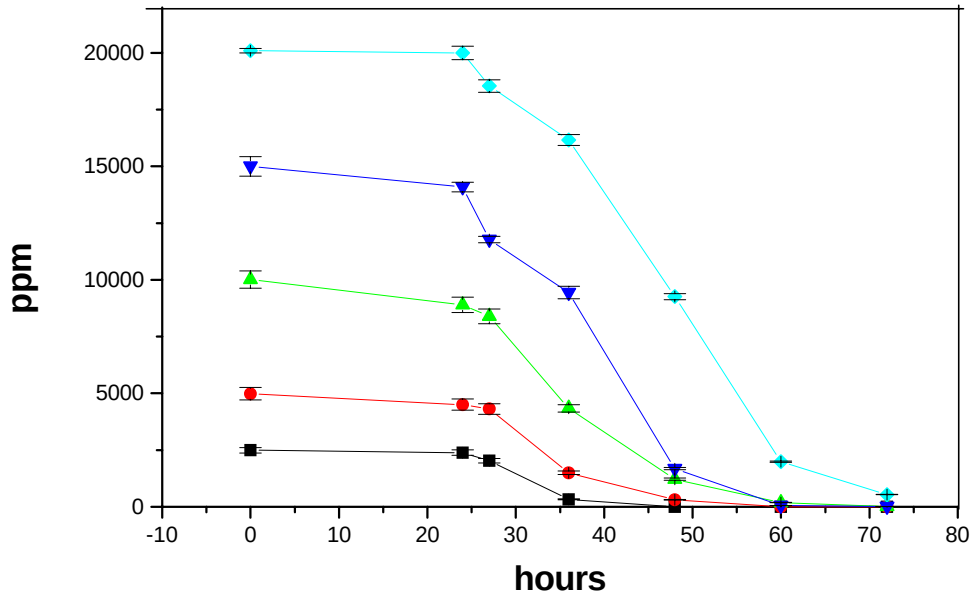

Figure 19b. Variations of extracellular glycerol level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.4. Variations of Vancomycin Production by A. orientalis Depend on Glycerol Concentrations and Incubation Periods

As shown in Figure 3.20, vancomycin productions were reached their maximum values at the 48th hour for all used glycerol concentrations. Vancomycin production increased with the increases in glycerol concentration from 5 to 10 g/L and it decreased significantly at the higher concentration of the glycerol. The maximum vancomycin level was determined as 4.01 ± 0.06 ppm at the 10g/L glycerol.

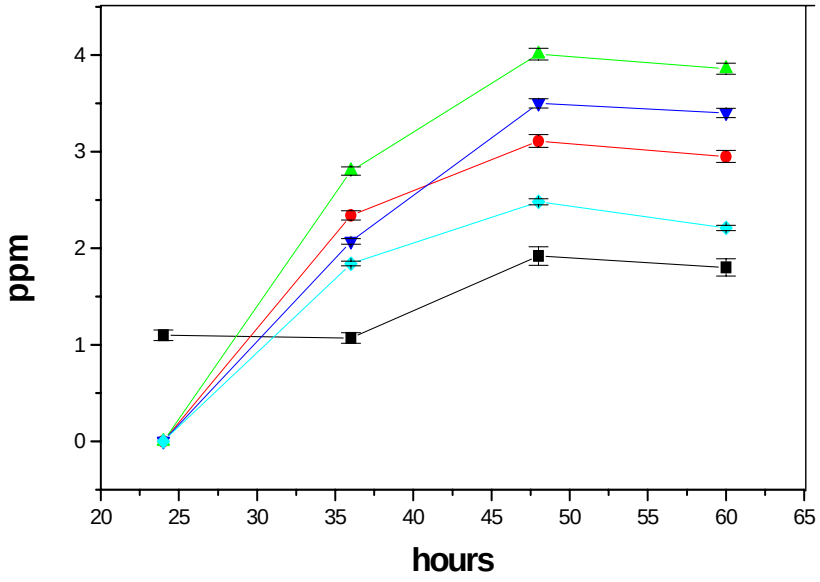

Figure 3.20. Variations of vancomycin production in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.5. The Variations of Intra- and Extracellular Glycolysis Metabolite and Enzymes Levels of A. orientalis Depend on Glycerol Concentrations and Incubation Periods

3.2.5.1. Variations of Pyruvate Level by A. orientalis Depend on Glycerol Concentrations and Incubation Periods

Maximum pyruvate levels of *A. orientalis* were determined on the 48th hour for all used glycerol concentrations (Figure 3.21a). In addition, pyruvate levels showed a positive correlation with the increases in glycerol concentration of the growth medium. The highest level was determined as 469 ± 8.9 ppm. Extracellular pyruvate levels were also increased significantly with increases in glycerol concentration ($p<0.01$) (Figure 3.21b). Maximum levels were determined on the 48th and 60th hours.

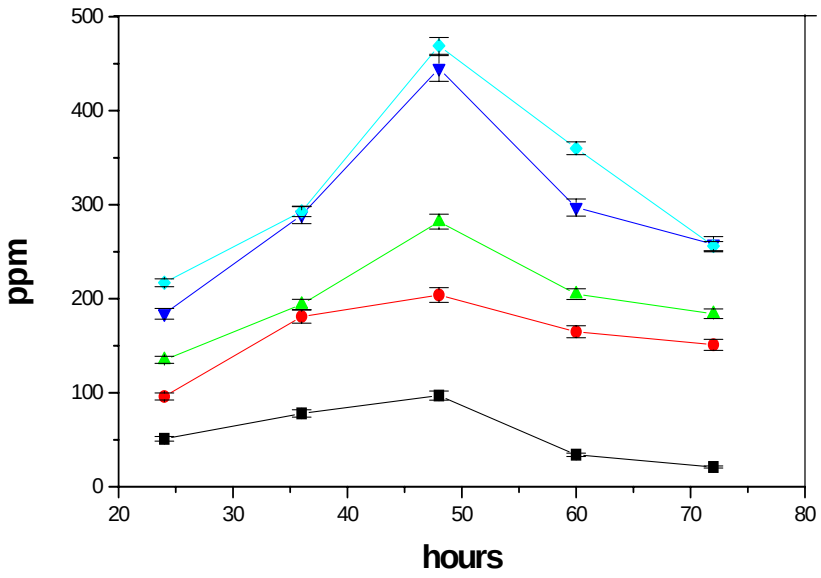

Figure 3.21a. Variations of intracellular pyruvate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

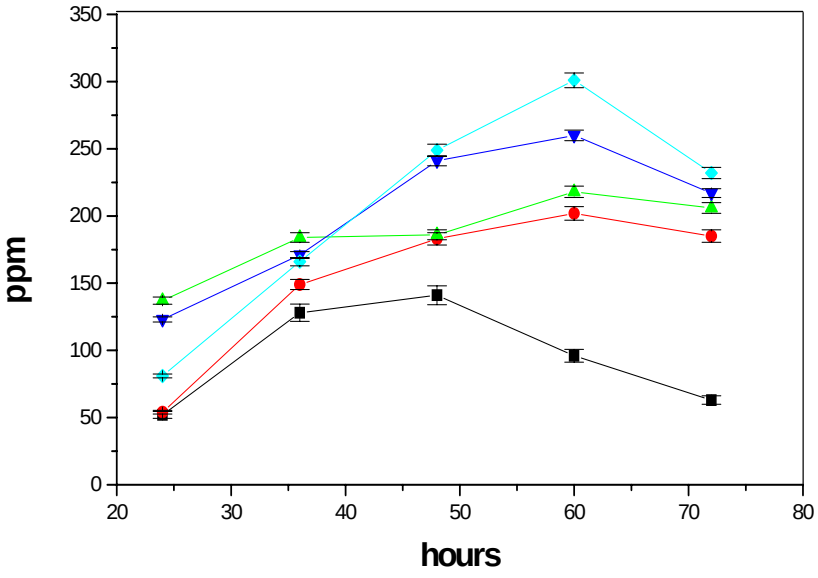

Figure 3.21b. Variations of extracellular pyruvate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

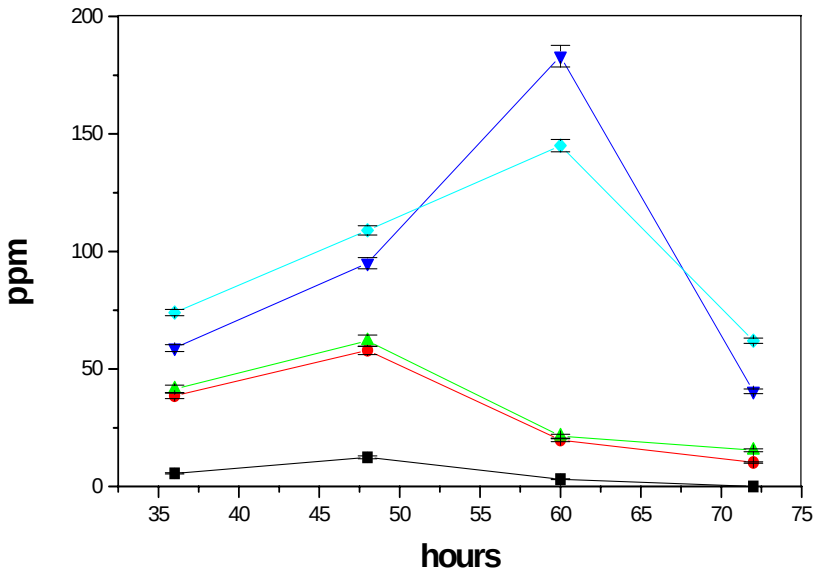

Figure 3.22. Variations of glucose level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

As shown in Figure 3.22, intracellular glucose levels reached their respective maxima at the 48th hour in the range of 2.5-10 g/L of glycerol while the incubation period corresponding to maximum values shifted to 60th for 15 g/L and 20 g/L of glycerol ($p<0.01$). In addition, intracellular glucose level showed positive correlation with glycerol concentration up to 15 g/L while further increases had an opposite effect.

3.2.5.2. Variations of Glycolytic Enzyme Activities by A. orientalis Depend on Glycerol Concentrations and Incubation Periods

Glucose kinase activities of *A. orientalis* reached their maximum levels on the 48th hour and the activities did not change significantly during the 48-60th hour (Figure 3.23). After 60th hour, the activities were decreased markedly ($p<0.01$). A positive correlation between glucose kinase activities and glycerol concentration of the growth medium was determined ($p<0.01$).

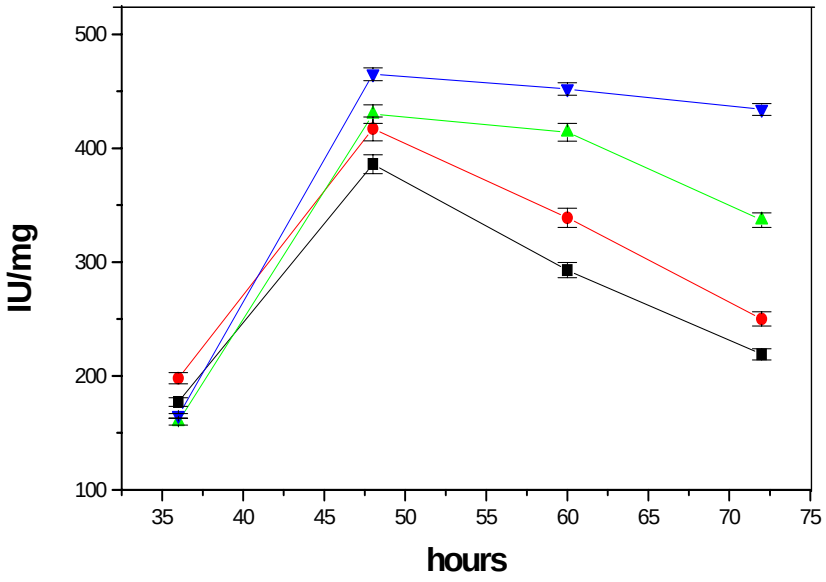

Figure 3.23. Variations of glucose kinase activity in *A. orientalis* depending on the incubation period in medium containing 5.0 (—■—), 10 g/l (—●—), 15g/l (—▲—), 20 g/l (—▼—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

As an another glycolysis enzyme, maximum pyruvate kinase activities determined on the 48th and 60th hours, for 2.5, 5.0 and 10-20 g/L of glycerol, respectively (Figure 3.24). As well as determined in glucose kinase, pyruvate kinase activities didn't change significantly during the 48-60th hour. In addition, pyruvate kinase activity increased with the increases in glycerol concentration of the medium ($p<0.01$).

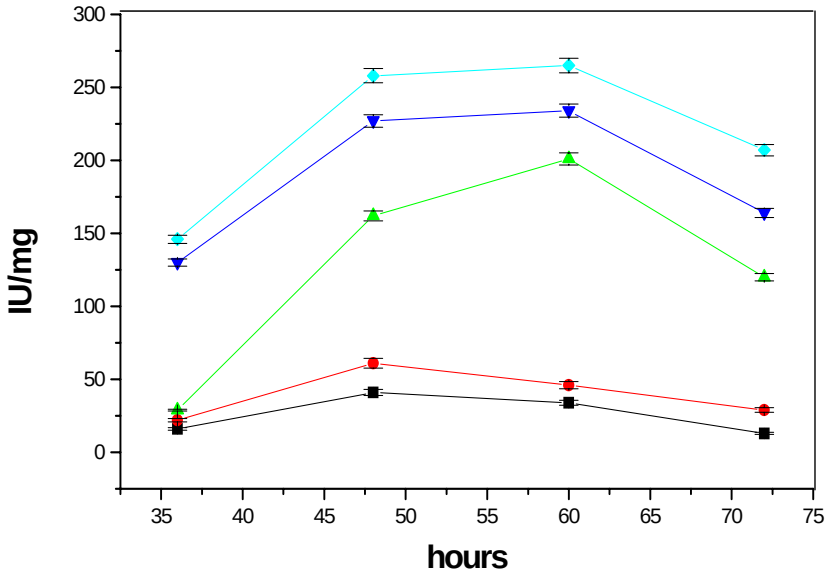

Figure 3.24. Variations of pyruvate kinase activity in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.6. Variations of Glycogen Level by A. orientalis Depending on Glycerol Concentrations and Incubation Periods

As shown in Figure 3.25, glycogen levels of *A. orientalis* reached their maximum levels on the 48th hour for all used glycerol concentration. Glycogen levels increased significantly when the glycerol concentration of the growth medium was increased from 2.5 to 20 g/L ($p<0.01$).

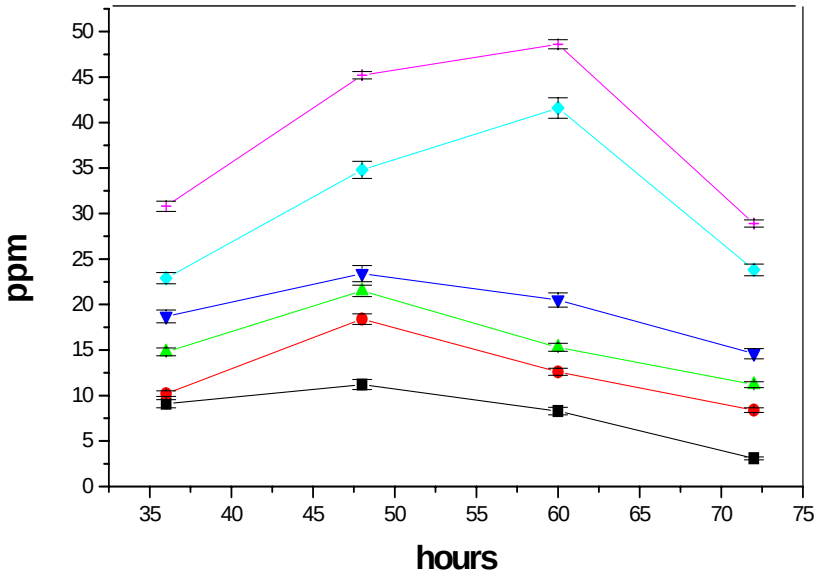

Figure 3.25 Variations of glycogen level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.7. The Variations of Intra- and Extracellular TCA Cycle Metabolite Levels of A. orientalis Depending on Glycerol Concentrations and Incubation Periods

Figure 3.26a shows that the maximum intracellular citrate levels of *A. orientalis* were determined on the 48th and 60th hour at 2.5 g/L glycerol and in the range of 5-20 g/L glycerol in the growth medium, respectively. In addition, intracellular citrate levels increased up to 10 g/L glycerol concentration while further increases had an opposite effect and the highest level was determined as 701 ± 5.6 ppm**.** On the other hand, extracellular citrate levels were reached the maximum on the 60th hour for all used glycerol concentration and they increased.

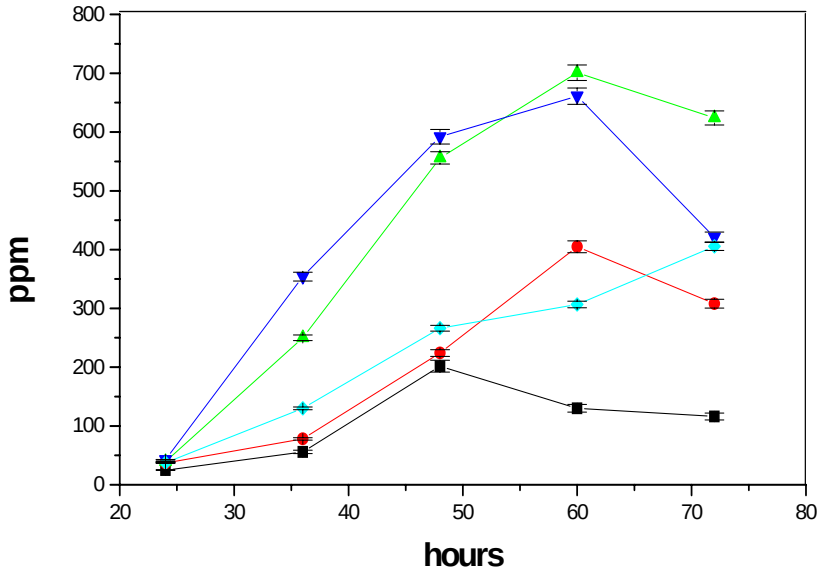

Figure 3.26a Variations of intracellular citrate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

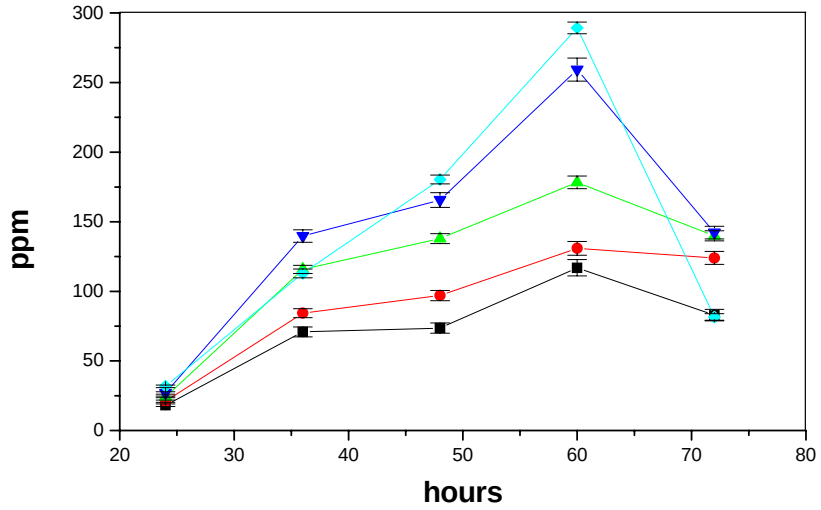

Figure 3.26b.Variations of extracellular citrate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

significantly with respect to increases in glycerol concentration of the growth medium ($p<0.01$) (Figure 3.26b).

As can be seen from Figure 3.27a, the levels of α-ketoglutarate of *A. orientalis* increased up to 36th hour for 2.5 g/L glycerol and 48th hour in the range of 5-20 g/L glycerol ($p<0.01$), after which the levels began to decline ($p<0.01$). Intracellular α-ketoglutarate levels increased with increases in glycerol concentration from 2.5 to 10 g/L on the 48th hour ($p<0.01$). The increases in extracellular α-ketoglutarate levels were reached their maximum levels on the 36th, 48th and 60th hours depend on the glycerol concentration (Figure 3.27b). However, they were increased significantly with respect to increase in glycerol concentration ($p<0.01$).

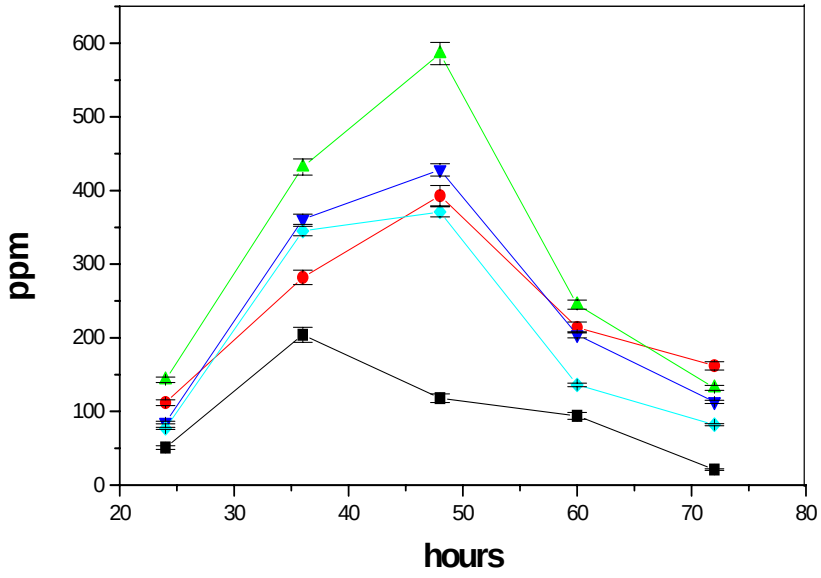

Figure 3.27a. Variations of intracellular α-ketoglutarate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

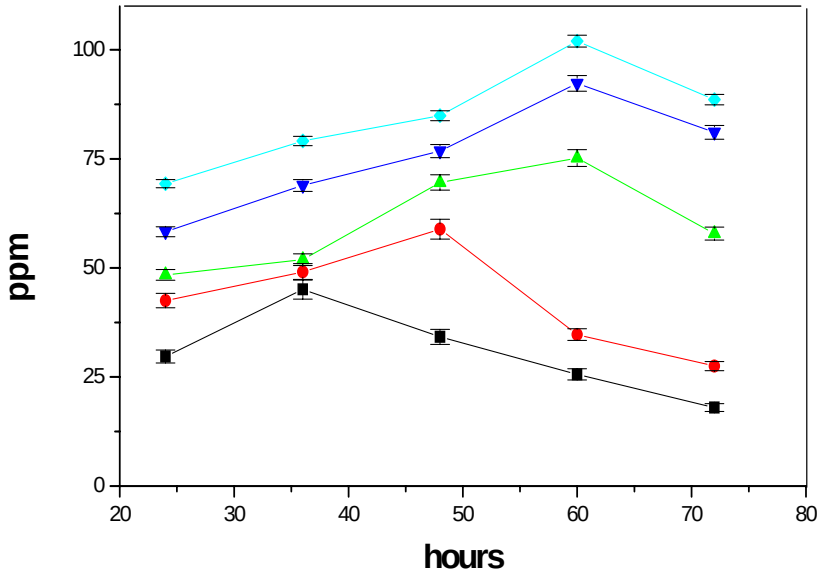

Figure 3.27b. Variations of extracellular α-ketoglutarate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

As shown in Figure 3.28a, the maximum intracellular succinate levels of *A. orientalis* were determined on the 60th hour in the range of 5-20 g/L glycerol, although it was 48th hour for 2.5 g/L glycerol. Intracellular succinate levels indicated positive correlation with the rise of glucose concentration up to 15 g/L while further increases had an opposite effect and the highest level was determined as 912 ± 14.13 ppm**.** Succinate level determined in 20 g/L was not lower significantly compared to 15 g/L glucose **(**$p>0.01$**).** On the other hand, extracellular succinate levels were reached the maximum on the 36th hour at 2.5 and 5 g/L glycerol while it shifted to 60th and 72nd hours at 10 g/L and 15, 20 g/L glycerol, respectively ($p<0.01$) (Figure 3.28 b).

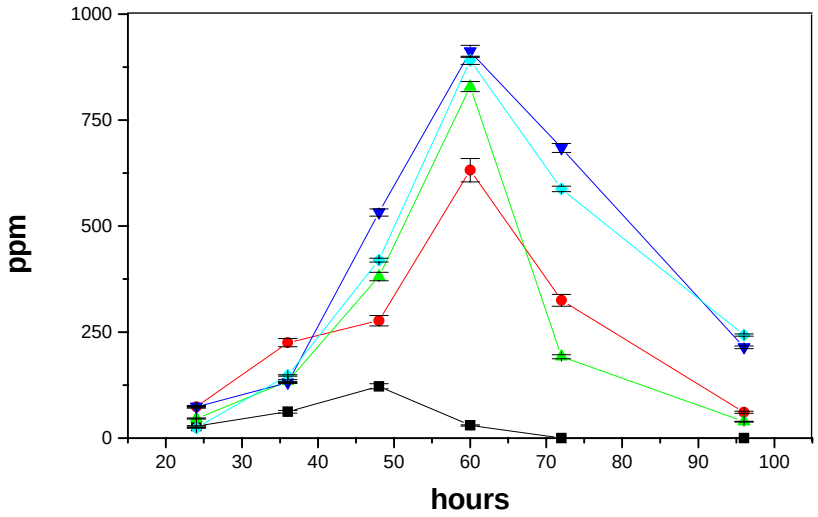

Figure 3.28a Variations of intracellular succinate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

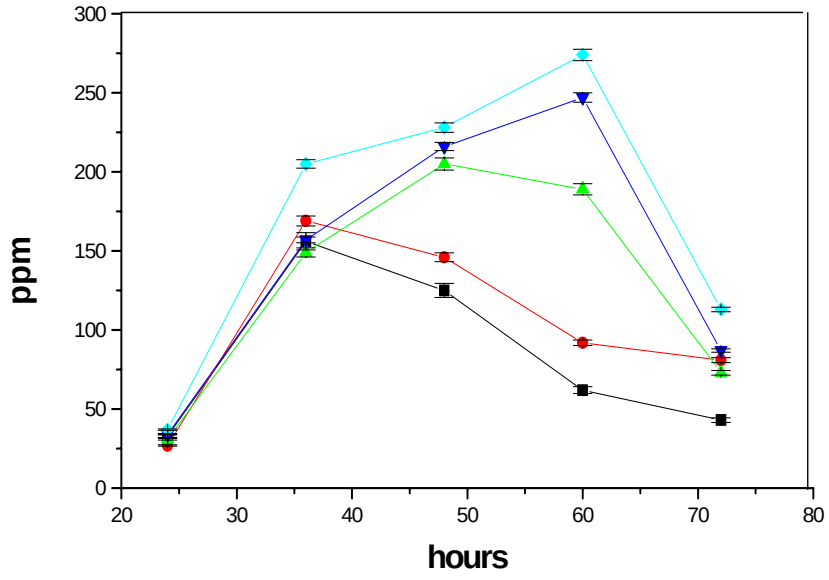

Figure 3.28b. Variations of extracellular succinate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

Intracellular fumarate levels of *A. orientalis* grown reached their maximum on the 48th and 60th hours as well as determined in succinate levels ($p<0.01$) (Figure 3.29a). Intracellular fumarate increased with the increase of glycerol concentration up to 10.0 g/L as determined in citrate, and α-ketoglutarate ($p<0.01$). Extracellular fumaric acid levels were increased up to different incubation times with respect to glycerol concentration of the growth medium and they raised with increases in glycerol concentration up to 15 g/L ($p<0.01$) (Figure 3.29b).

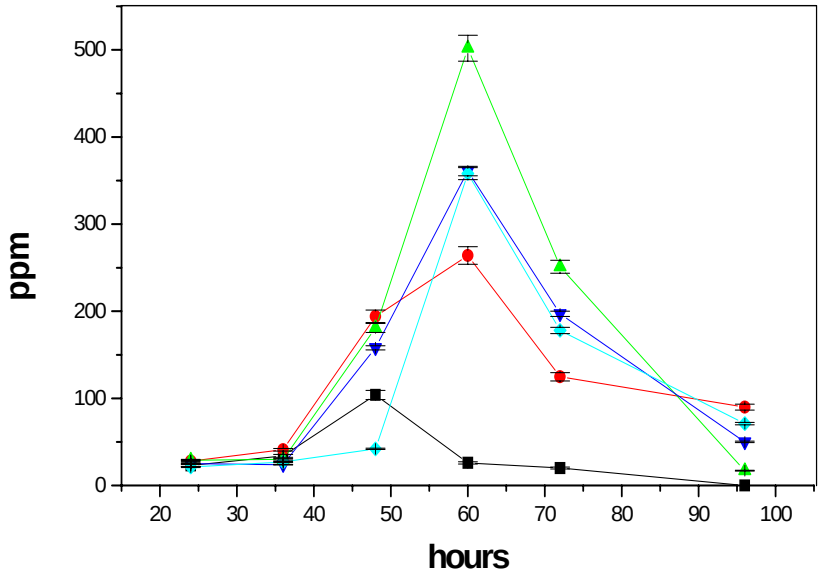

Figure 3.29a. Variations of intracellular fumarate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

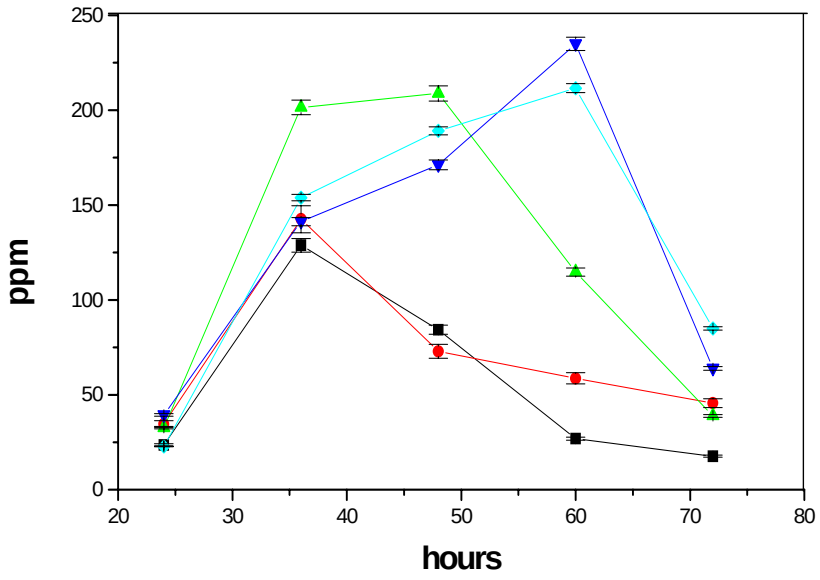

Figure 3.29b. Variations extracellular fumarate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

As can be seen from Figure 3.30a and 3.30b, maximum intracellular malate levels were determined on the 48th and 60th hours while they increased with increases in glycerol concentration from 5 to 15 g/L and the highest malate level was determined as 859 ± 13.05ppm. Similarly with citrate, α-ketoglutarate, succinate, extracellular levels showed positive correlation with the glycerol concentration ($p<0.01$). The highest extracellular malate level was 442 ± 6.15 ppm.

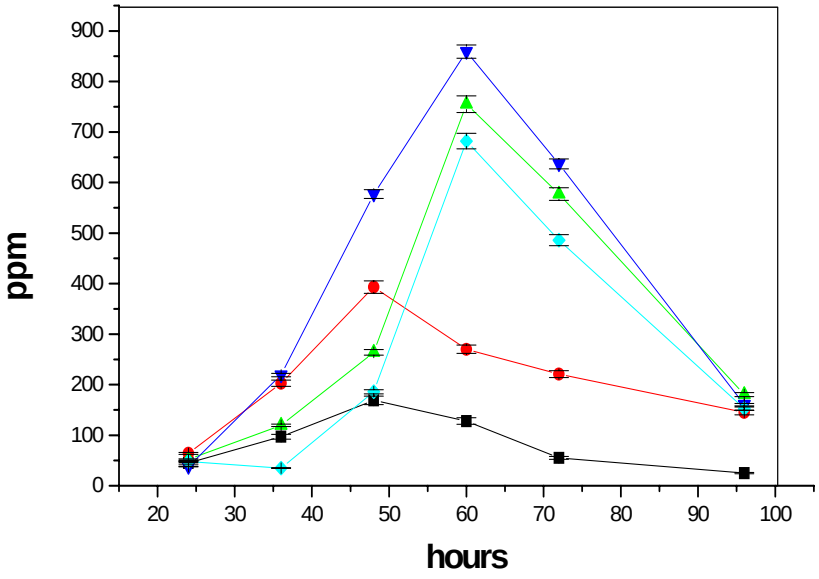

Figure 3.30a. Variations of intracellular malate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

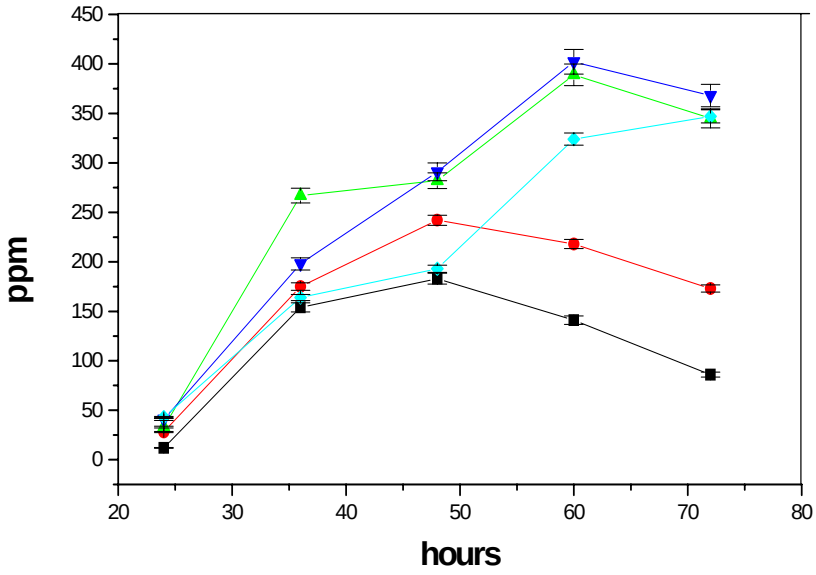

Figure 3.30b. Variations of extracellular malate level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

3.2.8. Variations of TCA and Glyoxalate Enzyme Activities by A. orientalis Depend on Glycerol Concentrations and Incubation Periods

As shown in Figure 3.31, maximum α-ketoglutarate dehydrogenase activities of *A. orientalis* were determined on the 48th hour in the range of 2.5-10 g/L glycerol while it shift to 60th hour at the 15 and 20 g/L glycerol. The maximum α-ketoglutarate dehydrogenase activity was determined at 15 g/L glycerol as 97 ± 1.6 IU/mg .

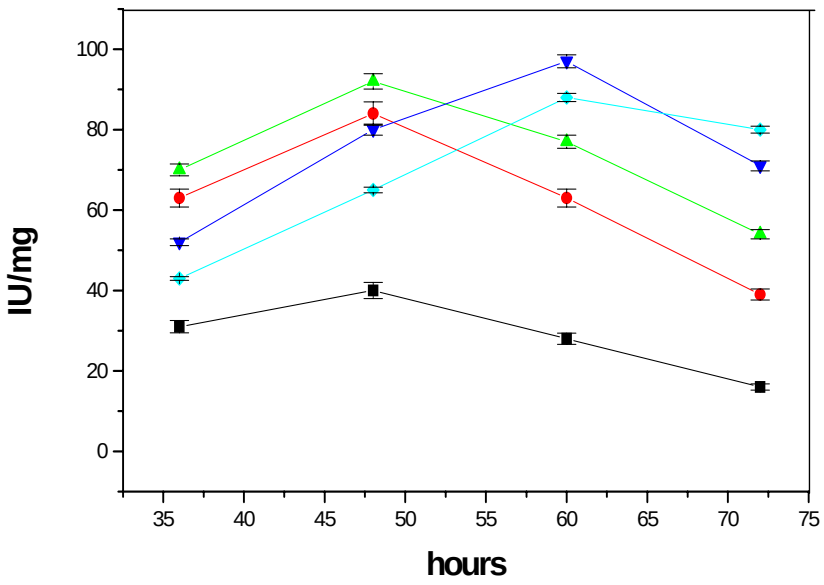

Figure 3.31. Variations of α- ketoglutarate dehydrogenase activity in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

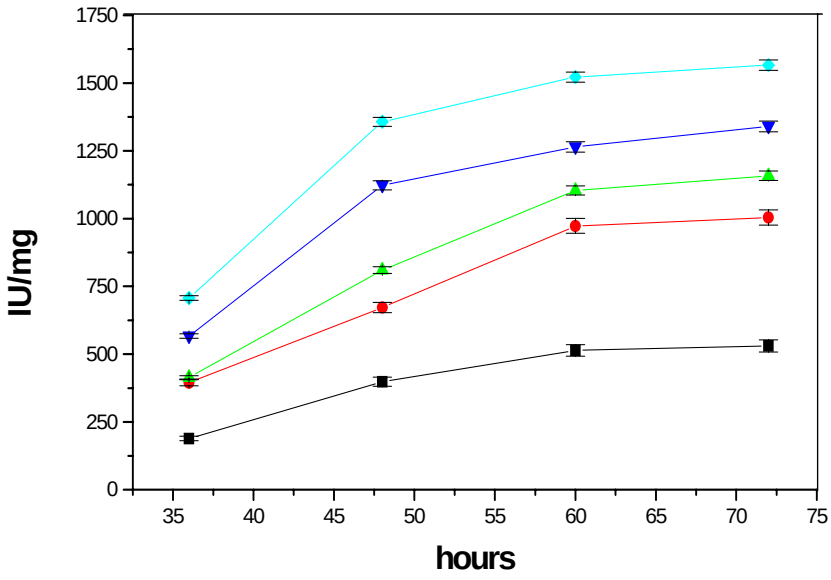

Figure 3.32. Variations of isocitrate lyase activity in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

Isocitrate lyase activity showed a positive correlation with the increases in glycerol concentration of the growth medium ($p<0.01$) as well as incubation period ($p<0.01$) (Figure 3.31). The highest isocitrate lyase activity was determined on the 20 g/L glycerol as 1566 ± 18.77 U/mg ($p<0.01$).

CHAPTER FOUR
DISCUSSION

A broad variety of secondary metabolites of commercial importance, including antibiotics, is produced by filamentous microorganisms, such as fungi and Actinomycetes in submerged cultures (Chater, 1993). Growth and production of secondary metabolites from filamentous microorganisms involves complex, and yet poorly understood processes, even for the most studied and commercially employed cases, such as penicillin production. Filamentous microorganisms present a multicompartment structure of morphologically heterogeneous hyphae. Cellular differentiation changes during growth and plays an important role on the production of secondary metabolites (Brana et al., 1986). Modeling the growth and product formation characteristics of several microorganisms is a very challenging task. The actinomycete *Amycolatopsis orientalis* produces the glycopeptide antibiotics vancomycin. The glycopeptide family of antibiotics have a highly specific mode of action against Gram-positive bacteria binding to the terminal carboxyl group of the developing peptidoglycan polymer (Nitanai et al., 2002). Vancomycin and related glycopeptides continue to be the antibiotics of choice against β-lactam resistant enterobacteria, although in recent years an increase in resistance to them has been reported (Kaplan *et al.*, 2001). Little information is available regarding vancomycin biosynthesis. Despite the excellent progress on the genetic and molecular biology of antibiotic biosynthesis, there still remains poor understanding of the physiological regulation and control of their production. The classical view that antibiotic production in batch culture occurs once the growth rate has declined as a consequence of nutrient limitation, is not always the case. Several examples of antibiotics that are produced during exponential growth, especially when chemically defined media are employed, have been reported.

Therefore, optimization of antibiotic production in actinomycetes strains is the main objective of genetically based investigations (Chater, 1993; Vining, 1995) and of investigations on the influence of cultivation conditions (Jonsbu et al., 2002; Poulsen, 1996). Whereas the genetic aspects of antibiotic production in

actinomycetes have been very well studied, less attention has focused upon primary metabolism (carbohydrate metabolism) and its role in the contribution of metabolites for antibiotic production (Hodgson, 1982). However, the knowledge of basic intracellular fluxes can be used for carefully directed optimization of antibiotic formation since the biosynthesis of most bioactive compounds starts from metabolites of the energy metabolism like TCA intermediates, pyruvate or acetyl-CoA (Pronk et al., 1996; Owen et al., 2002). Thus, a higher production yield may be achieved by increasing the availability of precursor metabolites for antibiotic formation (Dunn, 1998). Regarding the intracellular fluxes or biosynthetic pathways, the nodal points at the branches of the pathways are of special interest. It has been shown that changes in environmental conditions influence the secondary metabolite formation. Variation of temperature, pH-value or dissolved oxygen tension leads to drastic changes in specific growth rate and product concentrations. The variation of the growth rate itself in chemostate culture under carbon limitation leads to a different product pattern, as well (Brown et al., 1998; Vats et al., 2003). In this thesis, we give an insight into the relationship between vancomycin antibiotic production and intra- and extracellular metabolic variation dependence on carbon sources in *Amycolatopsis orientalis,* as a model organism for the investigation of antibiotic production.

In this thesis, *A. orientalis* growth rate increased when the concentration of glucose and glycerol was increased up to 15 g/L. From this concentration and above, growth rate did not increase significantly indicating that carbon sources were no longer a growth limiting factor for final biomass as it was reported also in the research of Jonsbu (Jonsbu E et al., 2002). In addition, biomass of *A. orientalis* growth in both glycerol and glucose supplemented medium was increased rapidly up to 36^{th} hour and decreases with increasing rate were determined in the range of 36^{th}-48^{th} hour incubation period. Decreases in rate observed more in glycerol medium compared to that of glucose. The determined highest biomass did not change significantly in glucose and glycerol supplemented medium.

According to the results, rapid consumption of glucose in the growth medium showed coherence with the significant increases of the intracellular glucose level on the 24th hour. After 24th hour of incubation, intracellular glucose levels decreased significantly in spite of the observed decreases in extracellular glucose concentrations. This result would have been resulted in an increase of the intracellular glucose consumption rate in order to maintain energy metabolism (Lin et al., 2005, Zamboni 2004).

In addition, uptake of glycerol showed also coherence with the decreases in extracellular glycerol level. The decreases in glycerol levels were so slow for the first 24 h incubation period and they increased with respect to glycerol concentration for the 28-48 hours. Intracellular glycerol levels were reached their maximum levels on the 36th hour. And, their levels were increased with respect to glycerol concentration of the medium. However, intracellular glucose levels were reached their maximum levels on the 24th hours. According to the results, maximum intracellular glycerol level and consuming time of extracellular glycerol level was determined at 12 hour late time compared to glucose results. In addition, intracellular maximum glycerol levels with respect to glycerol concentration of the growth medium were in the range of 100-750 ppm while the variation of intracellular maximum glucose levels was 500-3500 ppm. This rapid uptake of glucose compared to glycerol may be because of facilitated and rapid diffusion process of glucose as well as carbon catabolic repression.

pH levels of *A. orientalis* growth in both glycerol and glucose supplemented medium were similar up to 36th hour then decreased continuingly for following incubation period. These decreases in pH values were increased with increases in glucose and glycerol concentrations in the culture medium.

Pyruvate levels, which are end product of glycolysis, were reached their maximum levels on the 48th in the both glycerol and glucose supplemented medium and they decreased for the following incubation period. TCA intermediate levels

were also reached maximum levels on the glucose as well as pyruvate although maximum TCA intermediate levels in glycerol supplemented medium were determined on the 60th hour where the pyruvate levels showed decreasing trend. This situation caused to decreased TCA intermediate levels in glycerol medium compared to glucose. In addition, pyruvate levels in glucose medium were decreased slightly on the 48th-60th hour although it decreased sharply in glycerol medium. The results may have caused by decreased level of extracellular pyruvate level.

As glycolysis enzymes, glucose kinase activities were showed a positive correlation with the glucose concentration of the *A. orientalis* growth medium and their maximum levels were determined on the 48th hour. In addition, pyruvate kinase activities were reached the maximum on the 36th hour at 5 g/L while it shifted 48th hour in the range of 10-20 g/L glucose containing medium ($p<0.01$). Glucose kinase and pyruvate kinase activities with respect to glycerol concentration of the medium didn't change significantly during the 48th-60 th incubation range ($p<0.001$) in spite of determination of maximum pyruvate levels on the 48th hour and decreasing of the levels afterwards showed coherence TCA intermediates which reached their maximum levels on the 60th hour. This situation may suggest that in glycerol supplemented medium, glycolysis was slowed down during TCA cycle, which plays two major roles in the cell bioenergetics and biosynthetic, was faster after 48th hour (Imriskova et al., 2005).

It was suggested that TCA cycle is complete and active in a number of actinomyceteles. In the present thesis, almost all measured intracellular TCA cycle intermediates of *A. orientalis* grown in all glucose concentrations used were found to increase significantly within the incubation period of 12-48 hours. The only two exceptions were; succinate levels increased up to 60th hour, and maximum TCA cycle intermediates at 5 g/L glucose were reached their maximum levels generally on the 36th hour. In these cases, the production of organic acids were growth related. The results showed that in the glycerol supplemented medium; organic acids such as citrate, malate, succinate and fumarate levels were lower for the initiation

incubation time compared to glucose medium and their maximum levels were also lower in comparison to glucose have. In addition, α-ketoglutarate levels of glycerol medium were lower on the 24th and 36th hours but they were so similar in glycerol and glucose medium on the 48th hour. This situation may suggest that nitrogen sources, which are same in the both carbon source, are important parameter on the α-ketoglutarate production.

As key enzyme of glyoxalate pathway, isocitrate lyase activity which is known to catalyze the reversible cleavage of isocitrate into succinate and glyoxalate, showed positive correlation with increases in glucose concentration of the medium and it increased especially on the 36-48th hours incubation period and also after 48th hour in contrast to TCA cycle intermediates and enzymes. The results presented the point that excess glucose concentration and prolongation of incubation after 48th hour evoked over expression of isocitrate lyase in *A. orientalis*. Thus, it can be suggested that *A. orientalis* achieved adaptation depend on the conditions by induction of glyoxalate cycle as a possible alternative for normal anaplerotic reaction in order to use carbon metabolism economically (Huettner *et al.,* 1997). In glycerol medium, the isocitrate activity levels were higher compare to glucose and they increased at the same rate during the 60th hour and continued for all incubation period. This situation showed that glyoxalate pathway of *A. orientalis* growth in glycerol medium was active from the earlier time compared to glucose because of using carbon metabolism more economically.

In addition, the variations of TCA intermediate levels in glucose/glycerol were 1.36-2.0 while decrease of the rate to 0.56-1.0 because of the significant increases in isocitrate lyase activity, which caused to increases in succinate level by glyoxalate pathway, in glycerol medium compare to glucose. Succinate levels in glucose medium were increased significantly up to 48th hour and the increases were decreased on the 60th hour. However, succinate levels in glycerol supplemented medium were increased significantly at the same level on the both 36th-48th and 48th-60th hour incubation periods. This might be due to the decreases in increased rate of isocitrate lyase activities of *A. orientalis* growth in glucose supplemented medium on

the 60th hour compared to 36-48th incubation range while in glycerol medium, isocitrate lyase activities increased on the 48 th-60 th hour as well as other increases in TCA intermediates on the 60 th hour.

In this study, metabolite excretion of *A. orientalis* growth in glucose and glycerol supplemented medium was also observed. Nevertheless, extracellular α-ketoglutarate, fumarate, malate, succinate levels were lower values compared to intracellular levels. These results showed coherence some researches that *Actinomyceteles sp.* excrete organic acid such as pyruvate, α-ketoglutarate, lactate (Albrecht et al., 1997; Shoun et al., 1998). This can be explained that increases in intracellular intermediates levels lead to the excretion of organic metabolites because excess carbon could be excreted as organic acids if not oxidized to CO_2 for energy production. Results in the study also showed that pH decreased with the incubation period and these decreases in pH increased with the increases in glucose concentration of the growth medium because of excretion of organic acids by *A. orientalis*. A possible explanation is that intracellular pH was maintained at constant level by leading to the excretion of some organic acid which is profoundly dependent on the nature of the growth limitation and physiology of species. In general, extracellular organic acids reached their maximum levels on the 60th and 72th hours.

Glycogen levels of *A. orientalis* growth in both glucose and glycerol didn't change significantly and they were low levels. In addition glycogen levels in both carbon sources reached their maximum levels on the 48th hour where stationary phase was started and they decreased significantly for the following incubation period. On the other hand, intracellular glycerol levels in the glucose supplemented medium were significantly higher than glycogen levels which were determined in the both carbon sources. This situation may suggest that *A. orientalis*, was not used glycogen for the storage material possibly used trehalose via glycerol-3-P. These results showed coherence with the many authors that accumulation and disappearance of polysaccharide granules which is revealed to be glycogen during morphological development (Maitra et al., 2001) . The first appearance of the

glycogen was coincident formation of sporulation septa in aerial hyphae. Once the sporulation septa were complete, the now enclosed immature spores filled with granules. About halfway through the spore maturation process the granules began to decrease in size and had disappeared by the time full maturation had been achieved.

After 60^{th} hour of incubation, decreasing intracellular glucose and glycerol concentrations caused that all TCA cycle intermediates levels decreased significantly. This means that a decreasing intracellular substrate concentration must be compensated by the decreasing concentration of the produced these intermediates. Some authors also suggest that many microorganisms are able to use these organic acids as electron donors and carbon sources.

This result shows that the feeding of *A. orientalis* with 15 g/L of glucose and 10 g/L of glycerol could maintain cell metabolism, and thereby enhance effectively the production of the precursors of the amino acids leucine, tyrosine, aspartate, dihydroxyphenylglycine, and hydroxyphenylglycine which are in turn precursors of vancomycin . In addition, lower level of α-ketoglutarate compared to other TCA cycle intermediates may show that α-ketoglutarate allows more tyrosine production for the biosynthesis of vancomycin.

This is the first time that intracellular concentrations of a large number of primary metabolites have been measured during the cultivation period of *A. orientalis* and the results reported here provide new and significant information on the possible mechanisms of cellular adaptation. Results from this study highlighted several important physiological factors regulating vancomycin biosynthesis by *A. orientalis*.

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