DETERMINATION OF METABOLIC BOTTLENECKS USING REACTION ENGINEERING PRINCIPLES IN SERINE ALKALINE PROTEASE PRODUCTION BY RECOMBINANT *Bacillus* sp.

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ABSTRACT

DETERMINATION OF METABOLIC BOTTLENECKS USING REACTION ENGINEERING PRINCIPLES IN SERINE ALKALINE PROTEASE PRODUCTION BY *Bacillus* SPECIES

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In this study, firstly, bioprocess characteristics for Serine Alkaline Protease (SAP) production, using recombinant *Bacillus subtilis* carrying pHV1431::*subC*, were examined. The cell concentration, substrate concentration, SAP activity and SAP synthesis rate profiles demonstrated that the system reaches to a steady state in terms of cell growth and SAP synthesis between t=15-25 h, therefore, this time interval is appropriate to employ both metabolic flux analysis and metabolic control analysis, which apply strictly to steady state systems.

After that, three separate perturbations were introduced by addition of aspartate to the production medium at a certain time of the bioprocess. The response of the cells were observed and; by comparing the changes in intracellular reactions of aspartate pathway, Asn, Thr and Ile productions were determined to be the bottlenecks in aspartate pathway and the branchpoints splitting from Asp and AspSa were identified to be weakly rigid branchpoints.

Lastly, metabolic control analysis principles were applied to determine the elasticity and flux control coefficients of the simplified aspartate pathway. Aspartate formation reaction and Lys, Thr, Ile, Met producing group share the control of asparagine synthesis. The results revealed that lysine producing branch flux dominates the other branch fluxes, therefore to eliminate bottlenecks and increase SAP production, the activity of the branches leading to the formation of Asn, Thr and Ile should be increased while decreasing the activity of lysine synthesizing branch. This could be achieved either by genetic manipulation or by addition of specific inhibitors or activators to the system.

Keywords: *Bacillus*, serine alkaline protease, metabolic flux analysis, branchpoint identification, metabolic control analysis.

ÖZ

Bacillus TÜRLERİ İLE SERİN ALKALİ PROTEAZ ÜRETİMİNDEKİ DARBOĞAZLARIN REAKSİYON MÜHENDİSLİĞİ PRENSİPLERİYLE BELİRLENMESİ

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Bu çalışmada ilk olarak, pHV1431::*subC* geni taşıyan recombinant *Bacillus subtilis* kullanılarak serin alkali proteaz (SAP) üretimi biyoprosesinin genel karakteristikleri incelenmiştir. Hücre derişimi, glukoz derişimi, enzim aktivitesi ve enzim üretim hız profilleri, t=15-25 st arasında hücre büyümesi ve SAP üretimi açısından bir yatışkın hale ulaşıldığını göstermiştir. Bu sebeple, bu zaman aralığının metabolik akı analizi ve sadece yatışkın halde uygulanabilen metabolik kontrol analizi için uygun olduğuna karar verilmiştir. Belirlenen zaman aralığında, aspartik asit eklenerek sisteme üç ayrı pertürbasyon verilmiş ve sistemin tepkisi incelenmiştir.

Hücreiçi akı değerlerinin karşılaştırılmasıyla aspartik asit yolizinde asparajin, treonin ve izolözin üretimlerinin darboğaz oluşturduğu, aspartik asit ve aspartat semialdehitten ayrılan dallanma noktalarının da yarı esnek dallanma noktaları olduğu sonucuna varılmıştır.

Son olarak, metabolik kontrol analizi prensipleri uygulanarak aspartik asit yolizinin basitleştirilmiş bir formunun esneklik ve akı kontrol katsayıları hesaplanmıştır. Aspartik asit üretim tepkimesi ve lizin, treonin, izolözin ve metiyonin üreten grubun, asparajin üretiminin kontrolünü paylaştığı tespit edilmiştir. Tüm sonuçlar, lizin üretimine giden yoldaki akının baskın olduğunu göstermektedir. Dolayısıyla, yolizindeki darboğazları gidermek ve SAP üretimini artırmak için asparajin, treonin ve izolözin üreten dallardaki enzimlerin aktivitesinin artırılması ve eşzamanlı olarak lizin üreten dalın aktivitesinin düşürülmesi gerekir. Bunun için genetik mühendisliği yöntemleri kullanılabileceği gibi enzimler için spesifik inhibitör ya da aktivatörler kullanılabilir.

Anahtar Kelimeler: *Bacillus*, serin alkali proteaz, metabolik akı analizi, dallanma noktası tanımlanması, metabolik kontrol analizi.

To my Mom and Dad,

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A	SAP activity, U cm^{-3}
A'	Specific SAP activity, U g_X^{-1}
Asn	L-Asparagine
Asp	L-Aspartate
AspSa	Aspartate semialdehyde
C_G	Glucose concentration, g dm^{-3}
C_i^J	Flux control coefficient of enzyme i on J
C_i^x	Concentration control coefficient of enzyme i on X.
C_X	Cell concentration, g dm^{-3}
ε_x^J	Elasticity coefficient of metabolite X on flux J.
DC	L,2,3-dihydrodipicolinate
Gln	L-Glutamine
Glu	L-Glutamate
Gly	L-Glycine
His	L-Histidine
Ile	L-Isoleucine
αKG	α-ketoglutarate
Lac	Lactic acid
Leu	L-Leucine
Lys	L-Lysine
MDAP	meso-Diaminopimelate
Met	L-Methionine
r_P	Specific serine alkaline protease production rate, U $g_X^{-1}h^{-1}$
Ser	L-Serine
T	Cultivation time, h
Thr	L-Threonine
Trp	L-Tyrptophan
Tyr	L-Tyrosine
Val	L-Valine

CHAPTER 1

INTRODUCTION

Enzymes are extraordinarily efficient and selective biological catalysts and are regarded as one of the most important biological products. They find applications in many industries including detergent, food and pharmaceutical industries. Enzymes are valuable in manufacturing because of their rapid and efficient action at low concentrations under mild pH values and temperatures, their high degree of substrate specificity, low toxicity and the ease of stopping their action by mild treatments. They can be produced by isolation from plant and animal tissues or by microorganisms, plant and animal cells. 90 per cent of all enzymes produced is obtained from microorganisms. The reasons for using microorganisms are as follows;

1. enzyme fermentations are quite economical on large scale due to short fermentation times and inexpensive media,
2. screening procedures are simple and thousands of cultures can be examined in a reasonably short time,
3. different species can produce different enzymes catalyzing the same reaction, allowing flexibility in operating conditions of the reactor.

Table 1.1 summarises the sources and application areas of some industrially important enzymes. As it is seen from Table 1.1, proteases can be produced by bacteria, fungi, animal and plant cells in order to be used in detergent, food processing, brewing, leather, baking and cheese industries (Kalisz, 1988).

Table 1.1 Some examples of industrially important enzymes, their sources and application areas

Enzyme	Source	Application area
Amylases	Bacteria, fungi, plant and animal cells	Baking, brewing, detergents, starch, textile
Catalase	Bacteria, animal cell	Food, fruit juices, soft drinks
Cellulase	Bacteria, fungi	Food, soft drinks, feed, pharmaceuticals
Lipases	Bacteria, plant and animal cells	Food, diagnostics
Proteases	Bacteria, fungi, plant and animal cells	Detergent, food, leather, brewing, food, baking, cheese

In 1998, the world-wide enzyme sales amounted to over $1.5 billion (OEDC, 1998). There are approximately 470 companies producing industrial quantities of enzyme types. It is estimated that 60 per cent of the total world supply of industrial enzymes is prepared in Europe. Some 15 per cent is produced in North America and 12 to 15 per cent is from a considerable number of Japanese companies. The world market for enzymes is estimated to be between $1.7 and $2 billion in 2005 (Godfrey, 1996). The majority of the industrial enzyme production belongs to Novo Industri A/S (Denmark), Gist-brocades (Netherlands), and Miles Laboratories (U.S.A) (Kalizs, 1988).

Serine alkaline protease (SAP), which is the product of interest, is widely used in detergent, leather and meat industries. All detergent proteases that are found on the market today are serine alkaline proteases (Eriksen, 1996). They account for approximately 35% of the microbial enzyme sales, therefore, they can be regarded as one of the most important industrial enzymes. Serine alkaline proteases catalyze the hydrolysis of peptide bonds and have serine, histidine and aspartic acid residues in the active site. They are most active around pH 10 and have molecular weights in the range of 15-30 kDa.

In enzyme production by bioprocesses; selection of microorganism, medium design and bioreactor operation parameters are very important in order to have high product yield and selectivity. It is crucial to begin with the most active

strain available. Selecting the proper microorganism is important in order to obtain the desired product with adequate yields without producing toxins or any other undesired byproducts. Serine alkaline proteases, like most of the commercial enzymes, are produced by organisms belonging to the *Bacillus* species since they are harmless and are able to secrete large number of extracellular enzymes (Priest, 1977). Once a good strain is obtained, other fermentation parameters must be optimized to maximize enzyme production. The first to consider is the design of a suitable production medium that meets the microorganism's basic needs for sources of carbon, nitrogen, phosphorus, sulfur, potassium and trace elements. The culture conditions that promote production of enzymes like proteases are frequently significantly different from the culture conditions promoting cell growth (Moon and Parulekar, 1991). Therefore it is necessary to formulate a medium that is optimum for both cell growth and product formation. The designed media can be defined, semi-defined or complex. In industrial bioprocesses complex media is preferred because of its lower cost due to the high enzyme activity and cell yields compared to those of defined media since they include necessary growth factors, vitamins, hormones, and trace elements.

The performance of an industrial microorganism is affected strongly by the bioreactor operation conditions such as oxygen transfer rate, pH and temperature. Oxygen transfer conditions should be considered carefully due to the importance of oxygen in aerobic processes. Some bioprocesses require high oxygen transfer rate conditions while others require controlled oxygen transfer rate conditions (Çalık *et al.*, 1999). Product yield and selectivity are also strongly affected by pH. Some bioprocesses require controlled-pH conditions, and some require uncontrolled-pH operations while temperature is desired to keep constant during the process.

Microorganisms act as microbioreactors in the bioreactor system and thousands of reactions take place in the microbioreactor. Metabolism is the entire network of chemical reactions carried out by living cells. Metabolic reactions are catalyzed by enzymes, therefore, a complete description of metabolism includes not only the reactants, intermediates and products but also the characteristics of the relevant enzymes.

It is possible to improve commercial production of biochemicals by altering the bioprocess parameters or by using molecular biology and recombinant DNA technologies. However, regulatory controls that exist in the metabolic pathways

usually oppose the changes introduced. Regulatory mechanisms are the most important features of the microorganisms since they provide adaptation to different environments and optimization of growth, which is a consequence of evolution. However, microbial metabolism may strictly enhance growth while avoiding the formation of the desired product via regulatory mechanisms. Therefore, it is very important to investigate the control exerted on the metabolic pathways leading to the formation of the desired product.

SAP synthesis depends on good coupling of supply and demand of the amino acids in the cell, therefore, it is of great importance to examine the intracellular reactions and contributing enzymes, in other words metabolism, in order to gain knowledge about the regulatory mechanisms in the cell and understand the control exerted on the production of the amino acids needed to produce our product of interest, SAP.

Metabolism is regulated via enzymatic action since metabolic pathways consist of enzyme catalyzed reactions. The regulation could be by changing either the enzyme activity or the enzyme concentration. Regulation by altering enzyme activity can be accomplished through modulation of the activity or affinity of regulatory enzymes. This is mainly achieved by allosteric effects and covalent modification of enzymes. Regulation by altering enzyme concentration includes transcriptional and translational controls at the genetic level.

The overall response of the system to a certain change in its environment is the cumulative of all these control mechanisms. Branch points, or nodes, are the points in a metabolic network, where the reaction sequence splits to two or more different branches. Flux partitioning at some critical branch points determines the yield of the product. Therefore it is wise to examine the control of those nodes by calculating the fluxes. This is achieved by metabolic flux analysis (MFA), which is a powerful method to calculate the pathway fluxes, using a stoichiometric model for the major intracellular reactions and applying mass balances around intracellular metabolites.

Although MFA provides information about the pathway fluxes and interactions between different pathways, it fails to give quantitative results related with the control exerted in a metabolic network. However, if the controlling steps and their relative potential to control the pathway flux is to be understood, quantitative results are needed. Metabolic Control Analysis (MCA) is a sensitivity analysis technique that provides a quantitative basis for studying metabolic regulation. The basis of the theory is a set of sensitivity coefficients known as

control coefficients, mainly flux and concentration control coefficients and elasticity coefficients.

In the literature there have been various publications related with protease production. Hanlon *et al.* (1981) investigated bacitracin and protease production in relation with sporulation during exponential growth of *Bacillus licheniformis* on poorly utilized carbon and nitrogen sources; then in 1982 they studied the influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. Frankena *et al*. (1985) examined the bioenergetic aspects of growth and production of exocellular protease in *Bacillus licheniformis* in continuous culture; in their next publication in 1986, they investigated the effect of different limitations in chemostat cultures on growth and product formation of exocellular protease by *Bacillus licheniformis.* Kole *et al.* (1988) studied the production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. Moon and Parulekar (1991) were focused on formulation of a semi-defined medium that enhances synthesis and secretion of an alkaline protease in batch and fed-batch cultures of *Bacillus firmus.* Wright *et al.* (1992) investigated the enhancement and regulation of extracellular protein production through manipulation of cell culture conditions by using polypeptone, inorganic salts, glucose or fructose for *Bacillus brevis*. Hübner *et al.* (1993) used both semi-defined and complex media and developed on-line process monitoring techniques for the production of alkaline protease by *Bacillus licheniformis.* From the same research group van Putten *et al*. (1996) used the same medium with Hübner *et al.* (1993) and tested various control strategies on pH and oxygen in order to maximize the protease concentration in a stirred tank reactor using *Bacillus licheniformis*. Çalık *et al.* (1998) studied the effects of oxygen transfer in serine alkaline protease fermentation by *Bacillus licheniformis* in a defined medium where citric acid was the sole carbon source. Çalık *et al.* (1999), reported the effects of the bioreactor operation parameters on the production and product distributions in serine alkaline protease (SAP) fermentation by the wild type *Bacillus licheniformis* in relation to the physiology of the bacilli in a defined medium. The calculated intracellular flux distributions showed the importance of aspartate group amino acids in SAP synthesis. In the following article in 2000, Çalık *et al.* investigated the effects of oxygen transfer on product and by-product distributions in a wider range at nine different oxygen transfer

conditions and with the provided constant oxygen transfer conditions they designed an oxygen transfer strategy depending on the periods of the bioprocess. Although the activity obtained with citrate (Çalık et al., 2000) was higher than that of glucose (Çalık et al., 2002) the enzyme was not able to maintain its high activity; therefore, Çalık et al. (2002) used glucose as the carbon source. Christiansen *et al.* (2002) investigated the uptake of the amino acids in alkaline protease production in a semi-rich medium containing 15 of the 20 amino acids, normally present in proteins using fully labeled glucose in batch cultivation and they also analyzed the structure of the metabolic network of *B.clausii* and estimated the metabolic fluxes in batch and continuous culture on a minimal medium. Çalık *et al.* (2002a) used glucose as the sole carbon source in SAP production by *B.licheniformis* and investigated the influence of pH conditions on metabolic regulations in the production of this enzyme. In order to increase SAP production, Çalık et al. (2003a) cloned SAP encoding gene subC to a multi-copy plasmid (pHV1431) and expressed in *B.licheniformis* and studied the bioprocess characteristics of wild-type and recombinant *B.licheniformis* strains in a defined simple synthetic medium with a single carbon source glucose under well-defined bioreactor operation conditions. In 2003, on the basis of the findings of the previous study, the same research group investigated the regulatory effects of pH and pH control on recombinant *B.licheniformis* between pH=6.8 and 7.25 on product and by-product formations as well as the oxygen transfer characteristics, and the perturbation effect of initial pH and bioreactor operation conditions on the intracellular metabolic reaction network rates. Thereafter, Çalık *et al.*(2003b) used chemically and/or physically pre-treated molasses, having different glucose+fructose and/or sucrose concentrations, in small-scale bioreactors using four different recombinant *Bacillus* species, and investigated the effects of pre-treatments on resulting SAP activity. Among the recombinant species the highest SAP production was obtained with recombinant *B.subtilis* carrying pHV1431*::sub*C. In the same study they also investigated the effects of oxygen transfer in SAP production process in larger scale together with the oxygen transfer parameters. Lastly, from our reaserch group Çalık *et al.* (2003c) reported on the design of a complex medium based on simple and complex carbon sources using recombinant *B.subtilis* carrying pHV1431*::sub*C.

In literature, there are no studies related with the investigation and identification of bottlenecks in SAP production. Although Çalık et al. reported that asparagine production could be a potential bottleneck in SAP production, no detailed study is performed on the identification of branch-points and, therefore, bottlenecks. In addition, no application of MCA to enzyme or protein production is available.

In this study, response of *B. subtilis* to different perturbations are examined by changing the aspartate content of the extracellular medium. MFA results are utilized to identify the branch-points and determine the bottlenecks in aspartic acid pathway, leading to the formation of many important amino acids in SAP formation such as asparagine, isoleucine and threonine. Moreover, MCA is applied to a simplified form of aspartate pathway. Potential strategies to eliminate the bottlenecks and increase the serine alkaline protease production are discussed.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

Enzymes are proteins that are synthesized in a living cell, catalyzing a thermodynamically possible reaction so that the rate of the reaction is compatible with the biochemical processes essential for the maintenance of a cell (Conn, 1972). Most of the reactions they catalyze would not proceed in a reasonable time without extremes of temperature, pressure or pH (Horton, 1996). They increase the rates of the reactions by factors of at least one million compared to the un-catalyzed reactions without undergoing a permanent chemical change. They lower the activation energy of the catalyzed reaction by binding the substrate and forming an enzyme-substrate complex (Kirk and Othmer, 1994).

Enzymes offer several advantages over alternative chemical catalysts. The main advantage is their specificity avoiding undesirable side reactions. In addition, enzymes are present in low concentrations; therefore, they do not need to be removed after usage. They can effect the necessary reactions under mild processing conditions of temperature, pressure and pH (Kalisz, 1988). This decreases the energy requirements, reduces the capital costs due to corrosion-resistant process equipment and further reduces unwanted side-reactions (Chaplin and Bucke, 1990).

The high specificity of an enzyme is due to its highly complex structure and substrate molecules are bound in an active site. Because of enzyme specificity, thousands of enzymes are required with each enzyme catalyzing only one reaction or a closely related reaction (Horton, 1996).

Some enzymes require no chemical groups other than their amino acid residues for activity while others require an additional chemical group called a cofactor. The cofactor may be either one or more inorganic ions, such as Fe^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+}, or a complex organic or metallorganic molecule called a coenzyme (Lehninger,1993). Most enzymes have a characteristic pH and temperature at which their activity is maximum (Scragg, 1988).

Most enzymes are named by adding the suffix *–ase* to the name of the substrate they act on or to a descriptive term for the reactions they catalyze. A classification scheme, categorizing enzymes according to the general class of organic chemical reactions catalyzed, is maintained (Horton, 1996). Enzymes have been classified into six main types and these groups are further subdivided according to the nature of substrate involved. A numbering scheme for enzymes have been developed by Enzyme Commission based on this division and the prefix E.C. is generally employed with the numerical scheme (Blanch and Clark, 1997). For instance the E.C. number of serine alkaline protease is EC 3.4.21.14. Table 2.1 summarizes the classification of enzymes.

There are several thousand different enzymes identified and characterized. Many of these are coming into industrial application. Enzymes can be obtained from microbial, plant or animal sources while microbial enzymes represent approximately 90 per cent of all enzyme materials produced for industrial processing. The commercial usage of enzymes has progressed rapidly and estimations for the whole world market is in the range between $1.7 and $2 billion in 2005 (Godfrey, 1996).

Table 2.1 International classification of enzymes

No	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group-transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to H_2O)
4	Lyases	Addition of groups to double bonds or formation or double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C-C, C-S, C-O, and C-N bonds by condensation reactions coupled to ATP cleavage

2.2 Proteolytic Enzymes

Proteolytic enzymes constitute one of the most important groups of industrial enzymes, being extensively used in food, detergent, and other industries. Microbial proteases are classified into two major groups -peptidases and proteinases- on the basis of their nature of attack (Moon and Parulekar, 1991). The exopeptidases remove terminal amino acids or dipeptides and are of secondary importance since they cannot rapidly complete digestion. Proteinases are a highly complex group of enzymes which are produced intra- and extracellularly. They play an important role in the metabolic and regulatory processes of animal and plant cells, as well as in those of prokaryotic and eukaryotic microorganisms.

Extracellular proteinases are involved mainly in the hydrolysis of large polypeptide substrates, such as proteins, into peptides and amino acids before cellular uptake. Intracellular proteases play a key role in metabolic processes. Proteases have also been implicated in the regulation of gene expression, DNA repair and DNA synthesis. They are classified by their catalytic mechanism into four groups; these are (Kalisz, 1988);

1. Serine Proteases (3.4.21)

2. Cysteine Proteases (3.4.22)

3. Aspartic Proteases (3.4.23)

4. Metalloproteases (3.4.24)

2.2.1 Serine Proteases

The serine proteases are the most widely distributed group of proteolytic enzymes of both microbial and animal origin. The enzymes have a reactive serine residue in the active site and are generally inhibited by either diisopropyl fluorophosphates (DFP) or phenylmethylsulphonyl fluoride (PMSF). Serine proteases have broad substrate specificities and are generally active at neutral and alkaline pH, with an optimum between 7-11 (Moon and Parulekar, 1991). They have generally low molecular weight in between 18.5-35 kDa. Most have isoelectric points between pH 4.4 and 6.2. Serine proteases can be divided into four sub-groups, according to their side chain specificity against oxidized insulin B-chain (Kalizs, 1991).

These sub-groups are;

1. Trypsin-Like Proteases

2. Alkaline Proteases

3. Myxobacter α-Lytic Proteases

4. Staphylococcal Proteases

2.2.1.1 Serine Alkaline Proteases (SAP)

Serine alkaline proteases (SAP) are one of the most important group of industrial enzymes that are widely used in detergent, leather and meat industries. They are produced by various bacteria, moulds and yeasts (Kalisz, 1988). They account for approximately 35% of the microbial enzyme sales. The common properties of all serine alkaline proteases are (Çalık *et al*., 2001):

1. They all involve a particular serine residue that is essential for their catalytic activity

2. They are most active at approximately pH=10

The amino acid sequence of these enzymes depends on the microorganism that they are produced by. However, regardless of their amino acid composition, they fold in such a way that histidine, aspartic acid and serine form a catalytic triad. Near the active site is a hydrophobic binding site, a slit-like pocket that preferably accommodates the non-polar side chains; thus serine alkaline proteases are specific for aromatic or hydrophobic residues such as tyrosine, phenylalanine, tryptophane and leucine (Çalık *et al*., 2001). SAP is sensitive to DFP and potato inhibitor. They are most active at around pH 10 and their molecular weights are in the 15-30 kDa range. The isoelectric point of SAP is normally around pH 9 (Kalisz, 1988).

2.3 Bioprocess Parameters for Serine Alkaline Protease Production

In developing a fermentation process, a variety of applied issues must be taken into consideration in order to have high product yield and selectivity. These are; (1) microorganism, (2) medium composition and (3) bioreactor operation parameters (pH, temperature and oxygen transfer).

2.3.1 Microorganism

In bioprocesses, selecting the proper microorganism is important in order to obtain the desired product. The microorganism that is to be used should give adequate yields, be able to secrete large amounts of protein and should not

produce toxins or any other undesired products. Potential hosts should be suitable for industrial fermentation and produce large cell mass per volume quickly in inexpensive media (Kirk and Othmer, 1994).

2.3.1.1 The Genus *Bacillus*

The genus *Bacillus* includes a variety of industrially important species that are commonly used as hosts in the fermentation industry (Sonenshein, 1993). Bacillus species are attractive microbioreactors due to their secretion ability of large amounts of enzyme (Çalık, 2003a).

The rod shaped bacteria that aerobically form refractile endospores are assigned to the genus *Bacillus.* The endospores of the bacilli are more resistant than the vegetative cells to heat, drying, disinfectants, and other destructive agents and thus may remain viable for centuries. Cell strain is Gram positive and is motile by petrichoous flagella. The genus *Bacillus* encompasses a great diversity of strains. Some species are strictly aerobic, others are facultatively anaerobic. Although the majority are mesophobic, there are also psycrophilic and thermophilic species. Some are acidophiles while others are alkalophiles. Strains of some species grow well in a solution of glucose, ammonium phosphate and a few mineral salts, others need additional growth factors or amino acids, and still others have increasingly complex nutritional requirements (Laskin and Lechevalier, 1973). *Bacilli* are well known for their ability to secrete enzymes such as amylases and proteases and are, therefore, excellent candidates for large-scale production of these enzymes (Moon and Parulekar, 1991).

Advantages of *B. subtilis* for production of foreign proteins can be stated as follows (Fogarty and Kelly, 1990):

1. It is non-pathogenic,

2. It can be manipulated by current genetic engineering techniques,

3. It lacks both endotoxins (a characteristic important in the production of proteins for medical or foodstuff application) and protein modification mechanisms which may create inactive enzyme forms,

4. It can be grown more easily and has greater rates of protein synthesis than many eucaryotic systems,

5. It has the ability to secrete a wide variety of proteins far exceeds than of its prokaryotic competitor, *E.coli*,

6. It has the tendency to produce several proteases, which is able to degrade foreign proteins either intracellularly or extracellularly.

Undesirable aspects of *Bacillus* metabolism for enzyme production, such as the formation of heat-resistant endospores, or production of antibiotics or unwanted enzyme activities, can be eliminated by development of mutants deficient in these properties.

In the literature, Hanlon *et al.* (1981, 1982), Frankena *et al*. (1985, 1986), van Putten *et al.* (1995, 1996) and Çalık *et al.* (1998, 2000) used *B.licheniformis*; Hageman *et al.* (1984), Kole *et al*. (1988) used *B.subtilis*, Levisohn and Aronson (1967, 1971) used *B.cereus*, Wright et al. (1992) used *B.brevis* and Moon and Parulekar (1991) used *B.firmus* for serine alkaline protease production.

2.3.2 Medium Design

It is important to develop a suitable production medium that meets the microorganism's basic needs for sources of carbon, nitrogen, sulfur, potassium, and trace elements in order to maximize growth and product yield (Sonenshein, 1993). Carbon and energy sources and their concentrations are indeed important as they are tools for bioprocess medium design (Çalık *et al.*, 2001).

Certain microorganisms are capable of synthesizing all of their cellular constituents from carbon and nitrogen sources. However, most microorganisms require some source of micronutrients (i.e., amino acids, trace elements, vitamins, etc.). The culture conditions that promote production of enzymes like proteases are frequently significantly different from the culture conditions promoting cell growth (Moon and Parulekar, 1991). Therefore, it is necessary to formulate a medium that is optimum for both cell growth and product formation.

2.3.2.1 Cell Composition

Cells are composed of high molecular weight polymeric compounds such as proteins, nucleic acids, polysaccharides, lipids, and other storage materials. Formation of macromolecules which constitute the major part of the cell mass requires production of the necessary building blocks followed by polymerization of the building blocks (Nielsen and Villadsen, 1994). Table 2.2 summarizes typical composition of a typical bacterial cell (*E.coli*). In addition to these biopolymers, cells contain other metabolites in the form of inorganic salts (e.g., NH_4^+, PO_4^{3-}, K^+, Ca^{2+}, Na^+, SO_4^{2-}), metabolic intermediates (e.g., pyruvate, acetate), and vitamins. A typical bacterial cell is composed of 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorus, and 1% sulfur, with small amounts of K^+, Ca^{2+}, Na^+, Mg^{2+}, Cl^- , and vitamins (Table 2.3) (Shuler and Kargi, 2002).

Most of the products formed by organisms are produced as a result of their response to environmental conditions, such as nutrients, growth hormones, and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation.

Table 2.2 Typical composition of a bacteria (*E.coli*) (Neidhardt, 1990).

Species	Content (g/g_{dw} cell)
Protein	0.55
RNA	0.2
rRNA	0.16
tRNA	0.03
mRNA	0.01
DNA	0.03
Lipid	0.09
Lipopolysaccharide	0.03
Peptidoglycan	0.03
Glycogen	0.03
Building blocks etc.	0.04
Total	**1.00**

Table 2.3 The elemental composition of a bacteria

Element	**% of dry weight**
Carbon	50
Oxygen	20
Nitrogen	14
Hydrogen	8
Phosphorus	3
Sulfur	1
Potassium	1
Sodium	1
Calcium	0.5
Magnesium	0.5
Chlorine	0.5
Iron	0.2
All others	≈ 0.3

Nutrients required by the cells can be classified into two categories (Shuler and Kargi, 2002):

1. Macronutrients are needed in concentrations larger than 10^{-4} M, such as carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+}. Table 2.4 lists the major macronutrients and their physiological functions

2. Micronutrients are needed in concentrations less than 10^{-4} M. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, vitamins, growth hormones and metabolic precursors are known as micronutrients.

Table 2.4 The major macronutrients and their physiological functions

Element	Physiological Function	Required Concentration (mol/L)
Carbon	Constituents of organic cellular material. Often the energy source.	$> 10^{-2}$
Nitrogen	Constituents of proteins, nucleic acids, and coenzymes.	10^{-3}
Hydrogen	Organic cellular material and water.	-
Oxygen	Organic cellular material and water. Required for aerobic respiration	-
Sulfur	Constituents of proteins and certain coenzymes.	10^{-4}
Phosphorus	Constituents of nucleic acids, phospholipids, nucleotides, and certain coenzymes.	10^{-4} to 10^{-3}
Potassium	Principle inorganic cation in the cell and cofactor for some enzymes.	10^{-4} to 10^{-3}
Magnesium	Cofactor for many enzymes and chlorophyls and present in cell walls and membranes.	10^{-4} to 10^{-3}

Alkaline protease is comprised of 53.8 % carbon and 15.6 % nitrogen. Production of protease depends heavily on the availability of both carbon and nitrogen sources in the medium. Either an excess or a deficiency of carbon and nitrogen may cause repression of the synthesis of protease by prokaryotes (Moon and Parulekar, 1991).

Some carbon and nitrogen sources utilized by fermentation industry are summarized in Table 2.5.

Table 2.5 Some carbon and nitrogen sources utilized by fermentation industry

Carbon Sources	Nitrogen Sources
Starch waste (maize and potato)	Soybean meal
Molasses (cane and beet)	Yeast extract
Whey	Distillers
n-Alkanes	Cottonseed extract
Gas oil	Dried blood
Sulfite waste liquor	Corn steep liquor
Domestic sewage	Fish and meal
Cellulose waste	Groundnut meal
Carbon bean	Casein

2.3.2.2 Types of Media

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a culture medium, and depending upon the special needs of particular bacteria a large variety and types of culture media have been developed with different purposes and uses (Todar, 2000). There are two major types of media depending on their composition or use. A chemically defined (synthetic) medium is one in which the exact chemical composition is known. A complex (undefined) medium is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemicals; a medium containing glucose, KH_2PO_4, $(NH_4)_2HPO_4$, and $MgCl_2$ is an example of a defined medium. Complex media usually contain complex materials of biological origin such as soybean, yeast extract, peptone, molasses or cornsteep liquor, the exact chemical composition of which is obviously undetermined. In industrial bioprocesses complex media is preferred since the attainable enzyme activity and cell yields are much higher than that of defined media due to the presence of necessary growth factors, vitamins, hormones, and trace elements.

Bacillus strains can utilize alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), glutamate (Glu), glutamine (Gln), histidine (His), isoleucine (Ile), ornithine (Orn), proline (Pro), threonine (Thr), and valine (Val) as the nitrogen source; and Ala, Arg, Glu, Gln, His and Pro as the carbon source (Sonenshein, 1993). Besides these amino acids, *Bacillus* strains can also use organic acids like citric, acetic, succinic, pyruvic and α-ketoglutaric acids; glucose and the other hexoses; saccarose and glycerol.

In this study, glucose is used as the sole carbon source for SAP production. Glucose ($C_6H_{12}O_6$) is frequently used in bioprocesses intended to yield highly purified products, especially where colored carbohydrate-containing substrate mixtures would lead to economically inefficient processing (Cejka, 1985).

In the literature there are many studies on protease production by using different liquid media. Hanlon and Hodges (1981) used a medium containing (kg m^{-3}) : 0.015, glucose; 0.04, $Na_2HPO_4.12H_2O$; 0.026, KH_2PO_4; 0.01 NH_4Cl; $0.5x10^{-4}$, $MgSO_4.7H_2O$; $1x10^{-4}$, $CaCl_2$; $1x10^{-4}$, $MnCl_2$; $1x10^{-4}$, $FeSO_4.7H_2O$ for *B.licheniformis* and investigated the effect of glucose on protease production. Frankena *et al*. (1985) used (kg m^{-3}): 1.8, glucose; 10.1, K_2HPO_4; 1.2, KH_2PO_4; 2.0, NH_4Cl; 0.2, $MgSO_4.7H_2O$; $2.2x10^{-3}$, $CaCl_2$; and citric acid, $MnCl_2$, $CoCl_2$, $ZnSO_4$ at trace levels for *B.licheniformis* and investigated the efficiency of growth and energy conservation in both glucose-limited and glucose/acetate-limited chemostat cultures. Frakena *et al*. (1986) also investigated the effects of citric acid as the carbon source at two concentrations that are 10 and 20 mM. Moon and Parulekar (1991) used the medium reported by Frakena *et al*. (1985) for their parametric study for *B.firmus* and investigated the effects of important culture parameters including pH, dissolved oxygen, and concentrations of nitrogen and phosphorous sources and yeast extract on cell growth, synthesis and secretion of protease. Write *et al.* (1992) used polypeptone, inorganic salts, glucose or fructose for *B.brevis* and studied the enhancement and regulation of extracellular protein production through manipulation of cell culture conditions. Hübner *et al.* (1993) used (kg m^{-3}): 12, glucose.H_2O; 10, casein peptone; 5, yeast extract; 1.6, $(NH_4)_2HPO_4$; 0.5, Na_2HPO_4; 0.3, K_2HPO_4; 0.2, $MnSO_4.4H_2O$; 0.05, $FeSO_4.7H_2O$; 0.05, $MgSO_4.7H_2O$ as the semi-synthetic medium; and 10, cornstarch; 0.4, amylase; 27, Na-caseinate; 23, soy-flour; 7, cornsteep liquor;

0.5, $(NH_4)_2HPO_4$; 0.3, K_2HPO_4; 0.2, $MnSO_4.4H_2O$; 0.05, $FeSO_4.7H_2O$; 0.05, $MgSO_4.7H_2O$ as the complex medium and developed on-line process monitoring techniques for the production of alkaline protease for *B.licheniformis*. Çalık *et al.* (1998) used a defined medium containing (kg m^{-3}): 9, citric acid; 4.7, $(NH_4)_2HPO_4$; 2.0, KH_2PO_4 for SAP production by *B.licheniformis* and investigated the effects of initial citric acid concentration (C_C) on growth and SAP activity and obtained a maximum biomass concentration at C_C= 6.0 kg m^{-3}. Optimum citric acid concentration for maximum SAP activity was reported as C_C= 9.0 kg m^{-3}. Although the activity obtained with citrate (Çalık et al., 2000) was higher than that of glucose (Çalık et al., 2002) the enzyme was not able to maintain its high activity; therefore, Çalık et al. (2002) used glucose as the carbon source. Following this study, in order to increase SAP production Çalık et al. (2003a) cloned SAP encoding gene subC to a multi-copy plasmid (pHV1431) and expressed in *B.licheniformis* and studied the bioprocess characteristics of wild-type and recombinant *B.licheniformis* strains in a defined simple synthetic medium with a single carbon source glucose under well-defined bioreactor operation conditions. Thereafter, Çalık *et al.* (2003b) used chemically and/or physically pre-treated molasses, having different glucose+fructose and/or sucrose concentrations, in small-scale bioreactors using four different recombinant *Bacillus* species, and investigated the effects of pre-treatments on resulting SAP activity. Among the recombinant species the highest SAP production was obtained with recombinant *B.subtilis* carrying pHV1431*::sub*C. Lastly, Çalık *et al.* (2003c) reported on the design of a complex medium based on simple and complex carbon sources using recombinant *B.subtilis* carrying pHV1431*::sub*C. Complex medium initially containing (kg m^{-3}): 20, defatted-soybean; 15, sucrose; 0.021, Na_2HPO_4; 2.81, NaH_2PO_4 gave the highest SAP production.

2.3.3 Bioreactor Operation Parameters

The performance of an industrial microorganism is affected strongly by the bioreactor operation conditions such as oxygen transfer rate, pH and temperature. These show diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 2001).

2.3.3.1 Temperature and pH

The reaction temperature and pH of the growth medium are important bioprocess parameters. The influence of temperature and pH on a bioprocess can be very different, and since the growth process is the result of many enzymatic processes, the influence of both culture parameters on the overall bioreaction is quite complex (Çalık et al., 2001).

The influence of temperature on the maximum specific growth rate of a microorganism is similar to that observed for the activity of an enzyme: An increase with increasing temperature up to a certain point where protein denaturation starts, and a rapid decrease beyond this temperature (Nielsen and Villadsen, 1994).

Temperature is normally desired to keep constant and at its optimal value throughout the fermentation process. In literature, the bioreactor operation temperature for SAP is in the range of 35 to 40ºC. Frankena et al. (1985) and Çalık et al. (1998,2000) achieved SAP production at 37ºC; Kole et al. (1988) at 35ºC; Wright et al. (1992) at 34ºC; Hübner et al. (1993) and van Putten et al. (1996) at 39.5ºC.

Culture pH strongly affects many enzymatic processes and transport of several species across the cell membrane. Variation in pH alters acid-base equilibria and fluxes of various nutrients, inducers and growth factors between the abiotic and biotic phase because cell membrane enzymes are influenced (Moon and Parulekar, 1991). The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in pH. Enzymes are normally active only within a certain pH interval and the total enzyme activity of the cell is therefore a complex function of the environmental pH.

Microbial cells have the ability to maintain the intracellular pH at a constant level even with large variations in the pH of the extracellular medium (Nielsen and Villadsen, 1994). The intracellular aqueous (cytoplasmic) pH of alkalaphilic *Bacillus* species (e.g. *B.firmus*) is reported to be 8.2-8.5, whereas for *Bacillus* species (e.g. *B.subtilis*, *B.licheniformis*) this value is 7.5 (Çalık *et al.*, 2001). However, in spite of this ability, microorganisms might fail to keep pH at a certain value throughout the bioprocess and the intracellular pH might change influencing the biochemical reactions.

Some bioprocesses require controlled pH conditions, while others might require uncontrolled pH operations in order to increase the product yield and selectivity (Çalık et al., 2002a). Frankena *et al.* (1986), Kole *et al.* (1988), Moon and Parulekar (1991), and Wright *et al.* (1992) studied protease production in controlled pH conditions; Hübner *et al.* (1993), van Putten *et al.* (1996) reported the results obtained with uncontrolled-pH operation. Çalık *et al.* (1998, 2000) reported the time course results of the uncontrolled-pH operation in relation with the oxygen transfer conditions in a wide range. Frankena *et al.* (1985) studied protease production at pH=7; Kole *et al.*(1988) at pH=6.9-7.2; Wright *et al.* (1992) at pH=7.5; Hübner *et al.*, (1993) and van Putten *et al.*(1996), at initial pH=6.8; and Çalık *et al.*(1998, 2000) at pH=7.25, T=37°C. Among these studies only Moon and Parulekar (1991) investigated the controlled pH effect and reported optimum pH as pH=7.7.

Çalık *et al.* (2002a) used glucose as the sole carbon source in SAP production by *B.licheniformis* and investigated the influence of pH conditions on metabolic regulations in the production of the enzyme. In 2003, on the basis of the findings of the previous study, the same research group investigated the regulatory effects of pH and pH control on recombinant *B.licheniformis* between pH=6.8 and 7.25 on product and by-product formations as well as the oxygen transfer characteristics, and the perturbation effect of initial pH and bioreactor operation conditions on the intracellular metabolic reaction network rates.

2.3.3.2 Oxygen Transfer

Oxygen transfer show diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes. According to cell growth conditions and metabolic pathway analysis some bioprocesses require high oxygen transfer rate conditions while others require controlled oxygen transfer rate conditions (Çalık *et al.*, 1999). It has been extensively investigated in defined (Çalık et al. 1998, 1999, 2000) and molasses based complex medium (Çalık et al. 2003) for serine alkaline protease production and medium oxygen transfer conditions were found to be favorable for SAP production.

2.4 Intracellular Biochemical Reactions

Cellular growth and product formation are the result of a very large number of chemical reactions that occur inside individual cells and involves transport of substrates into the cell, followed by conversion of the intracellular substrates into biomass and metabolic products, and lastly excretion of the metabolic products back into the extracellular medium. Cellular processes can therefore be divided into three categories (Fig. 2.1) (Nielsen and Villadsen, 1994):

1. transport of substrates into the cell
2. intracellular reactions by which the substrates are converted into cellular components and metabolic products
3. excretion of metabolic products to the abiotic phase.

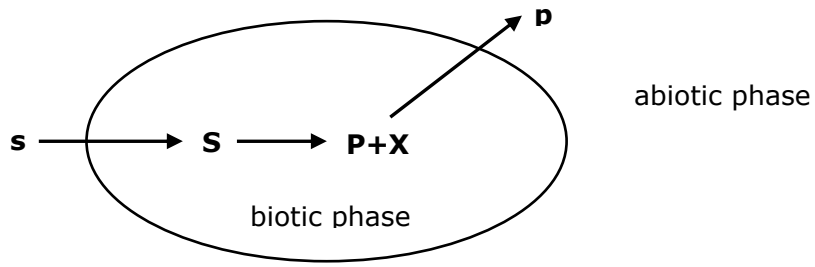

Figure 2.1 Reactions involved in cellular growth: Upper-case letters represent intracellular species (S, substrates; P, products; X biomass components) and lower-case letters represent extracellular species (s, substrates; p, products)

Metabolism is the entire network of chemical reactions carried out by living cells. Metabolites are the small molecules that are intermediates in the degradation or synthesis of biopolymers. The sequence of reactions in which the product of one enzyme-catalyzed reaction is the substrate of the next, is called a "metabolic pathway".

Although a variety of adaptations are observed when different organisms are examined, they demonstrate some common characteristics in their metabolism.

First of all, they extract energy from external sources to perform energy consuming reactions. They grow and reproduce according to their genetic material and they respond to environmental influences. The specific internal concentrations of inorganic ions, metabolites and enzymes are maintained at a certain level although many of them are continually synthesized and degraded (Horton et al., 1996).

Approximately two thousand distinct metabolic reactions participate in the growth and product formation of an ordinary bacterial cell. On the basis of their primary functions, these metabolic reactions are categorized as catabolic reactions and anabolic reactions (Neidhardt, 1990). Catabolic reactions degrade molecules to produce smaller molecules as well as energy. Living organisms use the released energy to drive anabolic reactions, which synthesize the molecules needed for cell maintenance, growth and product formation. Organisms also require energy to perform transport, cell movement and regulation of certain metabolic processes (Horton et al., 1996).

Metabolic reactions are catalyzed by enzymes, therefore, a complete description of metabolism includes not only the reactants, intermediates and products but also the characteristics of the relevant enzymes. The complexity of the metabolism can be overcome by dividing into segments or branches.

Although metabolic pathways embrace thousands of different enzymatic reactions, the central metabolic pathways are few in number and are remarkably similar in all organisms (Lehninger, Sonenshein, 1993, 1993). There are three central metabolic pathways; glycolysis, pentose phosphate pathway and tricarboxylic acid (TCA) cycle.

Gylcolysis is a universal metabolic pathway for the catabolism of glucose to pyruvate accompanied by the formation of ATP. Glycolysis can be divided into two stages: a hexose stage, in which ATP is consumed, and a triose phase, a net gain of ATP is realized (Horton, 1992) . During glycolysis biomass components, serine, aromatic and alanine group amino acids are synthesized.

Pentose phosphate pathway is an alternative path for glucose degradation. It is also known as hexose monophosphate shunt. This pathway has both aerobic and

anaerobic parts. Pentose phosphate pathway provides nucleotide, RNA and DNA synthesis besides histidine synthesis (Horton, 1992).

Tricarboxylic acid cycle, whose driving force is oxygen, is the major pathway of carbohydrate oxidation in aerobic cells (Lehninger, 1993). This is the pathway by which pyruvate is oxidized to CO_2. The TCA cycle is of the greatest importance to biosynthesis, as it provides the carbon skeletons needed as starting materials and the energy for the reactions (Aiba et al., 1965).

The main pathways of metabolism serve three purposes: generation of energy in the form of ATP, production of reducing power mainly in the form of NADPH and formation of precursor metabolites required in the synthesis of building blocks (Stephanopoulos, 1998). Among 12 precursor metabolites, glucose-6-phosphate, fructose-6-phosphate, glyceraldehyde-3-phosphate, 3-phosphoglycerate, phosphoenolpyruvate and pyruvate are produced in the glycolysis pathway; ribose-5-phosphate and erythrose-4-phosphate are formed in the pentose phosphate pathway; and acetyl-CoA, α-ketoglutarate, succinyl-CoA and oxaloacetate are produced in the TCA cycle (Nielsen and Villadsen, 1994).

Majority of the precursor metabolites are utilized as intermediates in the synthesis of the amino acids for SAP production. Hence, the three main pathways together with the biosynthesis of necessary amino acids are of great importance for SAP production. Insufficient synthesis of some of the amino acids may create bottlenecks in the bioreaction network for SAP production. Therefore, it is necessary ,to examine the intracellular reactions and contributing enzymes in order to gain knowledge about the regulatory mechanisms in the cell and understand the control exerted on the production of the amino acid(s) creating bottleneck(s), if any.

2.4.1 Regulation of Metabolism

Bacteria have the ability to rapidly adapt to their environment. They respond to changes in the availability of nutrients, they can cope with frequent changes in the physical quality of their environment and they can protect themselves from harmful agents and conditions by coordinating their metabolic reactions (Neidhardt, 1990). The responses of organisms to changing conditions range from fine-tuning to drastically reorganizing the metabolic processes. Many pathways or only a few may be affected (Horton, 1996).

Metabolism is regulated via enzymatic action since metabolic pathways consist of enzyme catalyzed reactions. Metabolic intermediates within the cell is employed for the purpose of metabolic regulation; certain compounds function both as metabolic intermediates and as metabolic regulators of key enzymes. Although

not necessarily in physical contact, each enzyme is informed of the state of the remaining of the pathway by specific chemical signals, such as concentrations of substrate, product or specific regulators (Newsholme, 1974). The regulation could be by changing either the enzyme activity or the enzyme concentration.

2.4.1.1 Regulation of Enzyme Activity

In all organisms, rapid control can be accomplished through modulation of the activity of regulatory enzymes. These enzymes are usually located at the first step that is unique to a metabolic pathway and their activity changes in response to metabolic signals, allowing the enzyme to adjust the flux of entire pathway (Horton, 1996).

The activity could be altered by concentrations of substrates, products or other regulatory metabolites, also termed effectors, modifiers or modulators. The effectors usually modify the affinity of the enzyme for its substrate and/or other reaction components (Larner, 1971).

Feedback regulation of enzyme activity is the most flexible and biologically widespread mechanism of metabolic control (Newsholme, 1974). It is the ability of a building block to inhibit an early enzyme of its own biosynthetic pathway (Neidhardt, 1990). Several types of control system can be included under this general heading (Larner, 1971):

1. Isofunctional enzymes: Multiple enzymes, catalyzing the same reaction while inhibited by different effectors, insure the pathway is active even when one of the end products is in excess.
2. Concerted feedback inhibition: Two or more end products must be present simultaneously and in excess amounts to give inhibition.
3. Cooperative feedback inhibition: Each end product causes partial inhibition, however, presence of two or more end products result in a total inhibition which is greater than the simple sum of the individual effects.

4. Cumulative feedback inhibition: Inhibition by each product is separate and independent.
5. Sequential feedback inhibition: Each end product inhibits the first reaction of its own branch and the intermediate at the branch point inhibits the first reaction of the common branch (Neidhardt, 1990).

Similarly, feed-forward activation is possible when a metabolite produced early in a pathway activates an enzyme that catalyzes a reaction further down the pathway (Horton, 1996).

Generally, allosteric (other shape) phenomena are responsible for the reversible control of regulatory enzymes. Allosteric enzymes are proteins that have, in addition to their active site, another site with its own affinity for binding small molecules called regulatory site or allosteric site (Neidhardt, 1990). An allosteric effector binds to the regulatory site and causes a conformational change in the regulatory enzyme changing the affinity of that enzyme to its substrate (Horton, 1996).

Alternatively, activation or inactivation of regulatory enzymes by covalent modification may occur. Some examples are acetylation/deacetylation, phosphorylation/dephosphorylation, methylation/demethylation etc. (Neidhardt, 1990).

2.4.1.2 Regulation of Enzyme Concentration

An alternate method to modify the rate of the enzymatic reaction is to alter the total amount of enzyme. Bacteria display major changes in enzyme levels to regulate the metabolism. The reason is that it is cheaper to adjust an enzyme level by modulating enzyme synthesis than making an enzyme and not using it (Neidhardt, 1990).

It is achieved in two ways: control of DNA transcription and control of RNA translation. Control at transcription initiation is preferred in bacteria to prevent production of unnecessary mRNA (Stephanopoulos, 1998).

2.4.1.3 Regulation of Metabolic Networks

The regulatory mechanisms, explained in the preceding sections, do not function in isolation, but rather they are members of higher level regulatory networks.

The final response of a living cell to a change in its environment is the cumulative of these mechanisms.

The flow of material through a metabolic pathway, or flux, is determined by the kinetics and regulation of the individual enzymes in the pathway. In certain cases, the pathway flux is controlled by the total amount of an enzyme that is low enough to qualify as a rate controlling step. In other cases, flux control is distributed between the several enzymes of the pathway (Stephanopoulos, 1998).

In biological processes, productivity is determined by the overall activity of enzymes in the product branch. However, flux split ratios at branch points determines the yield of the product. Branch points, or nodes, are the points in a metabolic network where the reaction sequence splits to two or more different branches (Stephanopoulos, 1998).

The metabolic control exerted in each organism is different. However, it is possible to classify the nodes in three groups according to their rigidity (Stephanopoulos, 1998).

- Flexible nodes: Flux partitioning changes readily according to the requirements of the cell.
- Weakly rigid nodes: Flux partitioning can change but it is dominated by one branch.
- Strongly rigid nodes: Flux partitioning does not change due to strict control mechanisms.

Since control of branchpoints are important for the yield of product of concern, it is wise to examine the network fluxes and flux partitioning at the nodes in the biosynthetic pathways producing the building blocks that make up the final product.

2.5 Metabolic Flux Analysis

Determination of pathway fluxes (rates) is crucial to gain knowledge about the regulations performed on branchpoints. A powerful methodology for the determination of metabolic pathway fluxes is metabolic flux analysis (MFA), in which fluxes are calculated using a stoichiometric model for the major

intracellular reactions and applying mass balances around intracellular metabolites (Stephanopoulos, 1998).

The first step is to list the stoichiometric reactions taking place in the cell and determine the intermediate metabolites in the reaction sequence. The reaction network can be simplified by lumping some of the reactions without loosing accuracy. In addition, the cells are assumed to behave identically throughout the process.

Second step is to write the mass balance equations around intracellular metabolites such as:

A: $-r_1+r_2 = dC_A/dt$

B: $r_2-r_3+r_5 = dC_B/dt$

Since hundreds of reactions are included in the model it is better to use the matrix forms of the mass balance equations as:

$A*r(t)=c(t)$

A is the stoichiometric coefficients matrix of the metabolic network of size m x n where m is the number of metabolites and n is the number of reactions. r(t) is the flux vector and c(t) is the metabolite accumulation vector. c(t) is actually composed of two subvectors:

$c(t)=c_1(t)+c_2(t)$

where $c_1(t)$ and $c_2(t)$ are extracellular and intracellular metabolite accumulation vectors, respectively. As it is expressed in Section 2.4, the concentrations of intracellular metabolites are maintained at a certain level in the cell. Therefore, a pseudo-steady state approximation may be accepted for the intracellular metabolites and $c_2(t)$ can be assumed to be 0. $c_1(t)$ can be determined from the analysis of extracellular medium.

The solution of equation $A*r(t)=c_1(t)$ for r(t) gives the fluxes for the metabolic network of concern. If number of metabolites is equal to or greater than number of reactions the model gives an exact solution since the number of unknowns does not exceed the number of known variables. However, if the number of metabolites is less than the number of reactions, metabolic fluxes can be obtained by maximizing or minimizing an objective function. As a result, the best

metabolic pathway utilization that would fulfill the stated objective is obtained (Çalık et al., 2002b). The mathematical formulation for the objective function is:

$$Z=\sum \alpha_i r_i$$

where Z is a linear combination of the fluxes r_i and α_i is the coefficient of the component i in the stoichiometric equation of the corresponding reactions. The matrix is solved in least squares method and a unique solution is obtained.
Results of MFA enable to calculate the intracellular reaction rates and the theoretical metabolic capacities of the microorganism. The results, then, can be used to modify the medium composition, to improve the bioreactor operation parameters, to identify the different pathways and above all to identify the types of branchpoints (Çalık et al., 2002b).

MFA was originated from the work of Aiba et al. (1979) and the methodology of stoichiometrically based analysis was first introduced by Stephanopoulos et al. (1991). Since then, MFA has been applied to many processes including SAP production.

Çalık et al. (1999b) developed a mass balance-based stoichiometric model for *Bacillus licheniformis* in order to obtain the flux distributions for SAP production. The results demonstrated that amino acid fluxes are important for SAP production. Çalık et al. (1999) investigated the effects of oxygen transfer rate on intracellular fluxes in SAP synthesis. They concluded that design of an oxygen transfer strategy is needed to improve the production and verified their conclusion in a more recent article (Çalık et al., 2000). In addition, they stated that asparagine synthase or aspartate kinase which are included in aspartic acid pathway could be a potential metabolic engineering site since the flux value toward asparagine is rather low. Moreover, the same group (Çalık et al., 2000a) investigated SAP overproduction capacity of *B.licheniformis* to reveal the upper limits of the microorganism. Their results showed that SAP production can theoretically be increased. In addition, there may be a bottleneck due to the low transcription of SAP synthesizing gene *subC* and insufficient production of amino acids used in SAP production.

Çalık et al. (2002a), reported on the influence of pH conditions on metabolic fluxes in SAP production by *B.licheniformis*. Their results revealed that addition

of the TCA cycle organic acids and controlling amino acids to the fermentation broth at limiting levels during SAP production might increase SAP synthesis.
Based on the results of their previous studies, Çalık et al. (2003a) investigated the effect of overexpression of SAP gene in *B.licheniformis* in SAP production and intracellular fluxes. They concluded that higher SAP production obtained in the recombinant microorganism is due higher fluxes towards the amino acids.

Furthermore, they (Çalık et al., 2003) used the recombinant *B.licheniformis* they developed to investigate the effects of pH on flux distributions. Their results showed that supporting the medium with certain amino acids, such as threonine, leucine, valine etc., increases the SAP synthesis indicating insufficient synthesis of some amino acids.

2.6 Metabolic Control Analysis

As it was expressed previously, the control of flux through a pathway is controlled by the total amount of an enzyme that is low enough to qualify as a rate controlling step. In other cases, flux control is distributed between the several enzymes of the pathway. MFA is useful to determine the pathway fluxes and to study the interactions between pathways, however, it does not provide a quantitative measure of the control exerted.

Metabolic Control Analysis (MCA), initially developed by Kacser and Burns (1973) and Heinrich and Rapoport (1974), is a sensitivity analysis technique that provides a quantitative basis for studying metabolic regulation. The basis of the theory is a set of sensitivity coefficients known as control coefficients. These coefficients allow the biotechnologists to quantify the metabolic response of a microorganism to changes on individual steps or segments of a metabolic network. In addition, MCA provides results to understand the relative importance of different controlling enzymes when some of them operate simultaneously (Delgado, 1993).

MCA strictly applies to steady-state (or pseudo-steady-state) conditions, and one basic assumption is that a stable steady state is defined by the activities of the enzymes in a pathway. That is, enzyme activities are considered as system parameters in addition to the concentrations of substrate to the first reaction and product of the last reaction. System parameters can be changed and they

completely define the system while the properties determined by the system parameters are accepted as system variables. One objective of the MCA is to relate the variables of a system to its parameters, such as relating the flux to the activity of one enzyme in the pathway (Stephanopoulos, 1998).

2.6.1 Flux and Concentration Control Coefficients

The most important control coefficients are Flux Control Coefficients (FCC) defining the relative change in the steady state flux of a branch resulting from a relative infinitesimal change in the activity of an enzyme.

$$C_i^{J_k} = \frac{E_i}{J_k}\frac{dJ_k}{dE_i} = \frac{d\ln J_k}{d\ln E_i}$$

J_k is the steady state flux through the k^{th} reaction in the pathway and E_i is the activity of i^{th} enzyme. Note that since FCCs are defined in terms of relative fluxes and activities, they are dimensionless (Kacser and Burns, 1973).

One important point about the FCCs is that they all sum up to unity with respect to each flux value. This is called the summation theorem.

$$\sum_{i=1}^{L} C_i^{J_k} = 1$$

The summation theorem proves that control of a flux in a pathway is shared between the enzymes of the pathway. It is derived from the fact that if all the enzyme amounts could be simultaneously increased by the same ratio, then all the fluxes would increase by this fraction because the rates are proportional to enzyme amount (Fell, 1997).

Similarly, Concentration Control Coefficients (CCCs) are defined in terms of the effect of an infinitesimal change in enzyme activity to the concentration of a metabolite.

$$C_i^{X_j} = \frac{E_i}{c_j}\frac{dc_j}{dE_i} = \frac{d\ln c_j}{d\ln E_i}$$

c_j is the j^{th} metabolite in the pathway. CCCs have a summation theorem, as well.

$$\sum_{i=1}^{L} C_i^{X_j} = 0$$

This result is derived from the fact that when all enzyme activities are changed by the same ratio, level of the intermediates remain unchanged. It also demonstrates that there should be at least one enzyme exerting a negative control on a metabolite, that is, when the amount of an enzyme increases the level of a metabolite decreases or vice versa (Stephanopoulos, 1998).

Both FCCs and CCCs are systemic properties including both direct and indirect effects of the activity change.

2.6.2 Elasticity Coefficients

Elasticity coefficients are local properties of the individual enzymes unlike FCCs or CCCs, which are systemic properties. They are the sensitivities of reaction rates to a certain metabolite. As in the case of FCCs and CCCs, elasticity coefficients are normalized and dimensionless.

$$\varepsilon_{c_j}^{i} = \frac{c_j}{J_k}\frac{\partial J_k}{\partial c_j} = \frac{\partial \ln J_k}{\partial \ln c_j}$$

Since elasticities are local properties, partial derivatives are used to indicate that all other variables must be kept constant. In other words, elasticities include only the direct effect of metabolite concentration change (Stephanopoulos, 1998).

2.6.3 Connectivity of Elasticity Coefficients and Flux Control Coefficients

Enzymes that have high elasticity coefficients tend to have low flux control coefficients. In other words, perturbation of an enzyme having a high elasticity coefficient would be elastic enough to compensate for the changes and its effect on the entire network would not be significant, therefore, it will have a low FCC.

On the other hand, if the elasticity coefficient of an enzyme is low, perturbation of that enzyme would cause a significant flux change in the system because of its inability to compensate for the changes, resulting in a high FCC value. This fact is demonstrated by Kacser and Burns (1973):

$$\sum_{i=1}^{L} C_i^{j_k} \varepsilon_{X_j}^i = 0$$

Usage of connectivity theorem with elasticity coefficients and summation theorem makes the determination of FCCs possible without *in vivo* enzyme activity measurements, which is a quite difficult task due to the necessity for new and sensitive methods.

2.6.4 Determination of Flux Control Coefficients

Flux control coefficient of an enzyme demonstrates the role of that enzyme in control of the specified flux. It gives some idea about the increase in flux when activity of that enzyme is altered. Therefore, it is crucial to determine the FCC values in order to increase a desired flux in a pathway to increase the yield of an intermediate or the product itself. However, determination of FCCs are quite difficult and many direct and indirect methods are developed to achieve metabolic control analysis. Direct methods determine the FCCs directly from flux and enzyme activity measurements while indirect methods use elasticities, connectivity and summation theorems.

Direct methods include genetic alteration of expressed enzyme activity, titration with purified enzyme and titration with specific inhibitors. The drawback of these methods is their difficulty to apply.

Indirect methods include double modulation, single modulation, top-down approach and kinetic models. These methods are preferred over the direct methods because of their relative simplicity (Stephanopoulos, 1998). Another method utilizing transient metabolite measurements have been proposed by Delgado (1992), however, Ehlde et al. (1996) reported that this method is highly sensitive to experimental errors.

Among these methods usage of kinetic models is widely used, however, it necessitates detailed knowledge about the kinetics of the enzyme. Once the information is obtained, elasticity coefficients are simply the derivative of the rate expression with respect to the specified metabolite. Nevertheless, experimental information is not always available for all enzymes in a pathway of interest.

Top-down approach is one of the preferred methods because it simplifies the experimental procedure significantly. This method is based on lumping the reactions in blocks or groups properly, and obtaining group elasticities and group control coefficients. This gives an idea about the control exerted by blocks and enables the investigators to concentrate on the blocks having high group control coefficients by segmenting those blocks into smaller blocks (Fell, 1997).

Methodology of MCA is developed by Kacser and Burns (1973) and, Heinrich and Rapoport (1974). Since application of MCA is difficult due to the necessity for troublesome experimental procedure, it has been mainly applied in small and simple pathways using the kinetic data.

In the literature, there are many reports describing the theory of MCA and summarizing the methods used in MCA (Fell, 1992; Liao and Delgado, 1993; Ehlde et al., 1997; Visser et al., 2002).

Fell et al. (1988), used kinetic data to apply MCA to mammalian serine biosynthesis. They reported that the pathway product serine controls the pathway flux by feedback inhibition. Nielsen et al. (1995), used kinetic data and MCA to identify the rate controlling step in penicillin biosynthetic pathway in *Penicillium chrysogenum*. They have found that the control shifts between the two pathway enzymes during the process and concluded that it is better to amplify the activity of both enzymes in order to obtain higher penicillin production. In 1996, Brand introduced the top-down approach. Krauss et al. (1996), used top-down approach to apply MCA to fatty acid oxidation and ketogenesis and they reported that CPT I enzyme has a high control potential. Thomas et al. (1997), investigated controlling enzymes using the kinetic data of glycolysis in tuber tissue of potato (*Solanum tuberosum*). Their results revealed that control lies in the dephosphorylation of phosphoenolpyruvate and/or in the

steps beyond, while phosphofructokinase has little control over glycolytic flux.

Hua et al. (2000) applied MCA to lysine synthesis using *Corynebacterium glutamicum* based on kinetic models. The controlling steps were determined to be aspartokinase (ASK) and lysinepermease. They verified their results experimentally and obtained a 20% increase in lysine production by overexpressing ASK. Similarly, Chassagnole et al. (2001) investigated the control over threonine synthesis pathway in *E. Coli* and reported that aspartate kinase, aspartate semialdehyde dehydrogenase and homoserine dehydrogenase share the control of flux through the pathway. Cronwright et al. (2002), used the MCA methodology to investigate the control characteristics of glycerol synthesis in *S. cerevisiae*. They constructed a kinetic model to apply MCA and concluded that NAD^+ dependent glycerol 3-phosphate dehydrogenase catalyzed reaction exercises the majority of the control of the pathway flux.

In literature, there is no application of MCA on enzyme or protein production as well as on processes using *Bacillus* species.

2.7 Synthesis of Aspartic Acid Group Amino Acids

Results of previous studies revealed that there is a potential bottleneck for SAP production in aspartate pathway, probably asparagine synthesis (Çalık et al., 1999, 2000). Therefore understanding the regulations and identification of the branchpoints in aspartic acid pathway is crucial in order to increase the SAP production.

The aspartic acid group amino acids constitute 26 % of the total amino acids in serine alkaline protease and they play a critical role not only in growth and serine alkaline protease production, but also in sporulation that precedes protease synthesis (Çalık *et al.*, 1999). Figure 2.2 presents the aspartic acid pathway via which aspartic acid group amino acids are synthesized. Aspartic acid group amino acids are produced from oxaloacetic acid. After aspartate is produced the pathway splits into two branches, from one branch asparagine and from the other branch aspartate semialdehyde are produced. After aspartate semialdehyde the pathway again splits into two branches. From one branch

lysine; from the other branch methionine, threonine and isoleucine are synthesized.

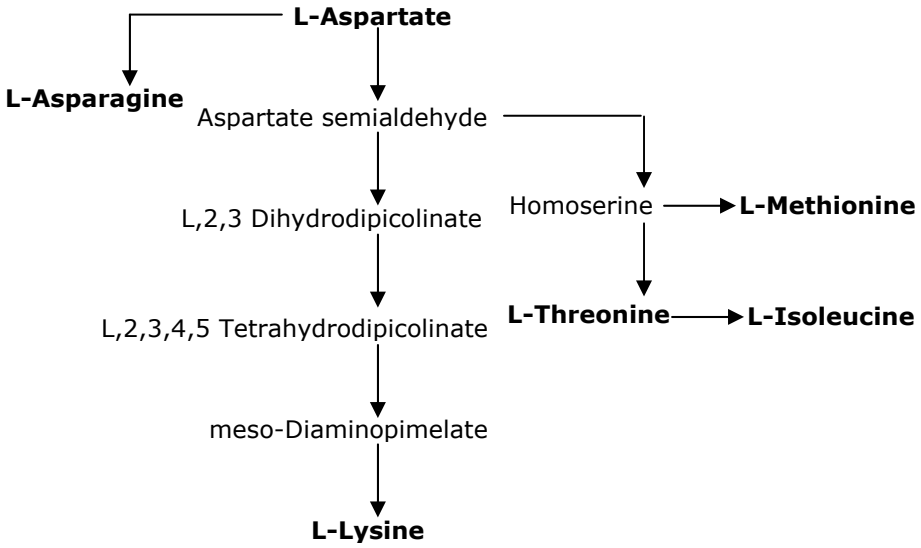

Figure 2.2 Aspartic acid pathway

As expressed before, there is no study in the literature, reporting on the identification of bottlenecks and branchpoints, and on application of MCA in SAP production.

CHAPTER 3

MATERIALS AND METHOD

3.1 Microorganism

Recombinant *Bacillus subtilis* (BGSC-1A751), carrying pHV1431::*subC* (Kalender, 2000) was used as a producer of serine alkaline protease (SAP, EC:3.4.21.14) enzyme in the defined and complex media.

3.1.1 Microorganism Storage

Microorganisms were stored in Microbanks (PRO-LAB, Microbank^{TS}) and kept at T=-20°C.

3.2 Solid Medium

The microorganisms, stored in microbank, were inoculated to the newly prepared agar slants under sterile conditions and they were incubated at 30°C for 36h. Table 3.1 gives the composition of solid medium for serine alkaline protease production by *Bacillus* sp. (Çalık, 1998).

3.3 Precultivation Medium

The microorganisms, grown in solid medium for 36 h, were inoculated into precultivation medium and they were grown at a temperature of 37°C and an agitation rate of N=200 rpm for 5.5 to 6 hours (until an absorbance of 0.28-0.35 at 600 nm was reached). Microorganism growth was conducted in orbital shakers under agitation and heating rate control, using 150 ml air-filtered Erlenmeyer flasks with a working volume of 33 ml. The composition of the precultivation

medium for cell growth and enzyme production is given in Table 3.2 (Çalık, 1998).

Table 3.1 The composition of solid medium for recombinant *Bacillus* sp.

Compound	Concentration, kg m^{-3}
$MnSO_4.2H_2O$	0.01
Agar	15.0
Peptone	2.5
Azocasein	2.0
Chloramphenicol	0.007

Table 3.2 The composition of precultivation medium for recombinant *Bacillus* sp.

Compound	Concentration, kg m^{-3}
$MnSO_4.2H_2O$	0.01
Peptone	5.0
Soytryptone	15.0
Na_2HPO_4	0.25
$CaCl_2$	0.1
Chloramphenicol	0.007

3.4 Production Medium

After incubation to a sufficient growth in the precultivation medium, microorganisms were transferred to the laboratory scale bioreactors with an inoculation ratio of 1/10. Laboratory scale SAP production experiments were conducted in orbital shakers under agitation (N=200 rpm) and heating rate

(37°C) control, using 150 ml air-filtered Erlenmeyer flasks with a working volume of 33 ml. Pilot scale batch bioreactor experiments were conducted at N= 750 min^{-1} , Q_0/V_R=0.5 vvm and pH_0=7.1 in bioreactor systems (BBraun, Germany) with V_R=1.1 dm^3 working volume, and equipped with temperature, pH, foam, air inlet and stirring rate controls. The composition of the reference defined medium is given in Table 3.3.

Table 3.3 The composition of reference defined medium for recombinant *Bacillus* sp.

Compound	**Concentration, kg m^{-3}**
Glucose	8.0
$(NH_4)_2HPO_4$	4.71
KH_2PO_4	2.0
NaH_2PO_4	5.63
$Na_2HPO_4.2H_2O$	0.055
$Mg(CH_3COO)_2$	0.87
$CaCl_2$	0.2
Chloramphenicol	0.007

3.5 Analysis

During the bioprocess, samples were taken at characteristic cultivation times and cell concentration, SAP activity, glucose, amino acid, organic acid concentrations were determined. Figure 3.1 summarizes the analysis carried out for the samples.

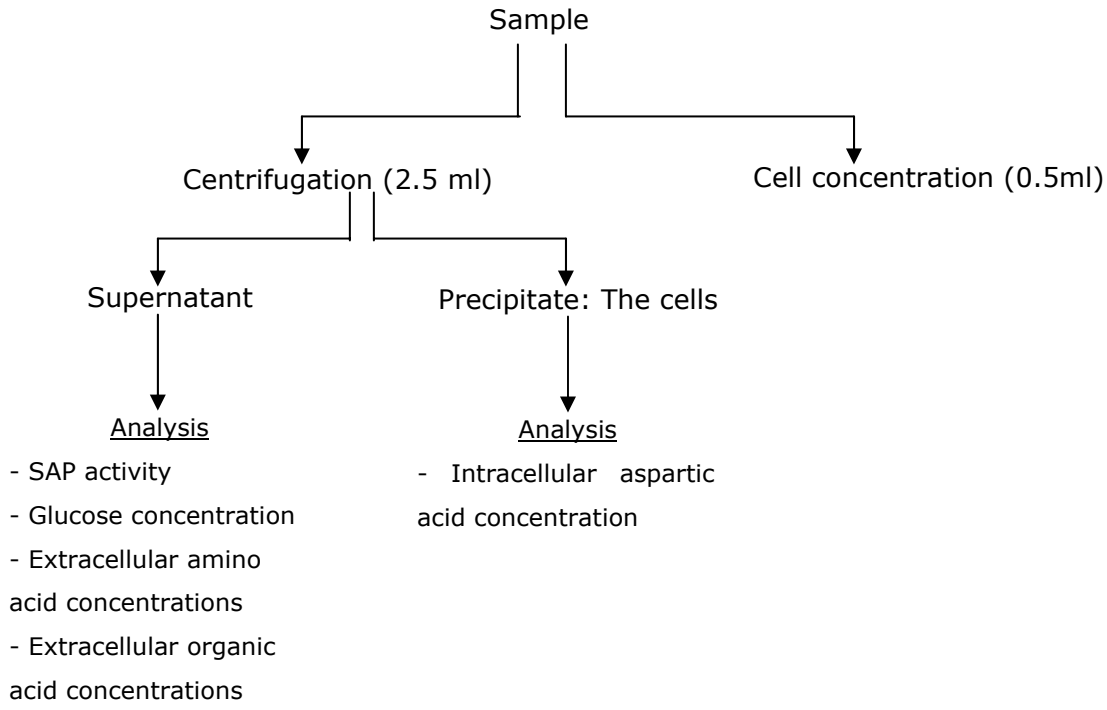

Figure 3.1 Analysis carried out for samples

3.5.1 Cell Concentration

Cell concentrations based on dry weights were measured with a UV-Vis spectrophotometer using a calibration curve obtained at 600 nm (Appendix A) (Çalık, 1998).

3.5.2 Serine Alkaline Protease (SAP) Activity

Proteolytic activity was measured by hydrolysis of casein. The culture broth was harvested by centrifugation at 13500 g for 10 min. Hammersten casein (2 ml of 0.5% w/v) in borate buffer was mixed with 1 ml of diluted bacterial broth and

hydrolyzed under T= 37°C, pH=10 for 20 min. The reaction was stopped by adding 10 % (w/v) trichloroacetic acid (TCA) and the reaction mixture was centrifuged at 10500 g for 10 min at +4°C, then the absorbance of the supernatant was measured at 275 nm with a UV-Vis spectrophotometer using a calibration curve (Appendix B). One unit protease activity was defined as the activity that liberates 4 nmole tyrosine min^{-1} (Çalık, 1998).

3.5.3 Reduced Sugar Concentration by DNS Method

DNS method (Miller, 1959) tests for the presence of free carbonyl group (C=O), known also as reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions. Reduced sugar (glucose) concentrations were determined at a wavelength of 550 nm by using a UV-Vis spectrophotometer and a pre-constructed calibration curve (Appendix C). For every newly prepared DNS solution, calibration curve should be reconstructed. The preparation details of DNS solution is given in Appendix D. The procedure for DNS method is as follows:

1. DNS solution was added to 1 cm^3 of diluted sample.
2. This mixture was placed into a boiling water bath and heated for 5 min.
3. After 5 min the mixture was placed into an ice bath and cooled for 5 min.
4. The spectrophotometer was zeroed in by using a solution which passed through the same steps but do not contain any reduced sugar.
5. The absorbance value of the sample was measured by a UV-Vis spectrophotometer at 550 nm. This was related to the glucose concentration by the calibration curve.

3.5.4 Determination of Amino Acids Concentrations

Amino acid concentrations were measured with an amino acid analysis system (Waters, HPLC), using the Pico Tag method (Cohen, 1983). The method is based on reversed phase HPLC, using a precolumn derivation technique with a gradient program developed for amino acids. The amino acid concentrations were

calculated from the chromatogram, based on the chromatogram of the standard amino acids solution. The analysis was performed under the conditions specified below:

Column	: Amino acid analysis column (Nova-Pak C18, Millipore)
Column dimensions	: 3.9 mm x 30 cm
System	: Reversed phase chromatography
Mobile phase flow rate	: 1 ml/min
Column temperature	: 38 °C
Detector and wavelength	: UV/VIS, 254 nm
Injection volume	: 4 µl
Analysis period	: 20 min

3.5.5 Determination of Organic Acids Concentrations

Organic acid concentrations were determined with a high performance capillary electrophoresis at 254 nm (Waters HPCE, Quanta 4000E). The samples were analyzed at 20kV and 15°C with a negative power supply by hydrostatic pressure injection, using an electrolyte containing 5mM potassium hydrogen phtalate and 0.5mM OFM Anion Bt (Waters) as the flow modifier at pH=5.6 (for α-ketoglutaric acid, acetic acid, malic acid, fumaric acid, succinic acid, lactic acid, oxalacetate and gluconic acid) and at pH=7.0 (for, pyruvic acid, citric acid, lactic acid, gluconic acid) (Çalık et al., 1998).

3.5.6 Determination of Intracellular Aspartate Concentrations

In order to determine the intracellular aspartate concentrations, a method very similar to Teusink et al. (1998) is used. Samples of 2 ml were quenched at regular time intervals into 4 ml of methanol at -60°C. The cells were washed three times in 4 ml of 60% (vol/vol) methanol by centrifugation at 13500 g for 5 minutes at the lowest possible temperature (-10°C) and after centrifugation the cells were extracted at - 40°C by the addition of 0.5 ml of 5% perchloric acid and 60% methanol solution. The final concentration of the samples were

measured to be approximately 0.65 ml. These steps are performed rapidly to prevent the possible leakage of intracellular aspartate out of the cells, as reported by Jensen et al. (1999), as much as possible. The extract was centrifuged and the supernatant is neutralized by addition of 10 M KOH. The salt is removed by further centrifugation and the supernatant is loaded onto a high performance liquid chromatography (HPLC) column.

3.6 Metabolic Flux Analysis

The metabolic reactions for *Bacillus subtilis* are given in Appendix E. The reaction network of *B.subtilis* includes 105 metabolites and 144 reactions. MFA methodology explained in section 2.5 were used to determine the pathway fluxes. A pseudo steady state approximation for the intracellular metabolites is accepted and the accumulation rates of extracellular metabolites were determined throughout the process. The linear optimization program GAMS 2.25 (General Algebraic Modeling System, GAMS Development Corp., Washington, DC) was used to solve the mass-flux balance equation defined in section 2.5.

Since SAP is an extracellular enzyme, the objective function, Z, was defined as the difference between SAP synthesis rate and SAP secretion rate, and the flux distributions were determined by minimizing Z.

3.7 Metabolic Control Analysis

Top-down approach is used to apply MCA to aspartate pathway of *B.subtilis*. The detailed pathway of Figure 2.2 is simplified as seen in Figure 3.2.

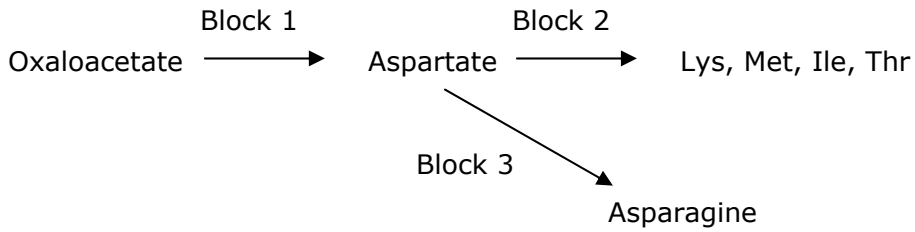

Figure 3.2 Simplified form of the aspartate pathway.

Perturbations were introduced by addition of aspartate and elasticity coefficients defined in section 2.6.2 were determined from ln J vs. ln C_{Asp} plots. Summation and connectivity theorems defined in sections 2.6.1 and 2.6.3, respectively, are used together with a branch point equation derived by Fell (1997).

$$C_1^{J_3} + C_2^{J_3} + C_3^{J_3} = 1$$

$$C_1^{J_3}\varepsilon_{Asp}^1 + C_2^{J_3}\varepsilon_{Asp}^2 + C_3^{J_3}\varepsilon_{Asp}^3 = 0$$

$$\frac{C_1^{J_3}}{J_1} + \frac{C_2^{J_3}}{J_2} = 0$$

Simultaneous solution of those three equations gives the FCC values for distribution of control in asparagine producing branch.

CHAPTER 4

RESULTS AND DISCUSSION

Microorganisms act as microbioreactors in the bioreactor system and thousands of reactions take place in the microbioreactor. These reactions are influenced by the production medium and bioreactor operation conditions, therefore, it is possible to improve production of biochemicals by altering the bioprocess parameters. However, regulatory controls that exist in the metabolic pathways usually oppose the changes introduced. Regulatory mechanisms provide adaptation to different environments and optimization of growth. However, microbial metabolism may strictly enhance growth while avoiding the formation of the desired product via regulatory mechanisms. Therefore, it is very important to investigate the control exerted on the metabolic pathways leading to the formation of the desired product.

Medium design and optimization of bioreactor operation conditions have been achieved for SAP production, and considerable increase in production using complex medium was achieved. However, in order to obtain higher yield, it is of great importance to examine the intracellular reactions and contributing enzymes, in other words metabolism, to gain knowledge about the regulatory mechanisms in the cell and understand the control exerted on the production of the amino acids needed to produce our product of interest, SAP.

Results of previous studies revealed a potential bottleneck for SAP production in aspartate pathway, probably asparagine synthesis (Çalık et al.,

1999, 2000). Understanding the regulations and identification of the bottlenecks in aspartic acid pathway is crucial since the information could enable the elimination of existing bottlenecks and, consequently, the increase in SAP production.

In this study, firstly, bioprocess characteristics for SAP production by *B.subtilis* using the production medium demonstrated in Table 3.3 and the bioreactor operation parameters given in section 3.4 were examined. After that, three separate perturbations were introduced by the supply of aspartate to the production medium at a certain time of the bioprocess, which correspond to a steady state in terms of SAP production, glucose consumption and cell growth. The perturbations were introduced such that the aspartate concentration in the production medium increased to 0.1, 0.2 and 0.3 mM. The response of the cells were observed and; by comparing the changes in intracellular reactions of aspartate pathway, bottlenecks were determined and branchpoint classification was achieved. Lastly, metabolic control analysis principles were applied to the simplified form of aspartate pathway, shown in Figure 3.2, to determine the elasticity coefficients. Since the objective of the MCA was to investigate the control exerted on production of asparagine, the aspartate pathway was simplified in such a form that there are three blocks, mainly aspartate production from oxaloacetate, asparagine production from aspartate and the rest of the reactions including Lys, Thr, Met and Ile production.

4.1 Bioprocess Characteristics

The pilot scale batch bioreactor experiments were conducted using the agitation rate N=750 min^{-1}, air inlet rate Q_0/V_R=0.5 vvm at T= 37 ºC with initial pH of pH_0=7.1.

4.1.1 Variation in Glucose and Cell Concentrations

Variations of glucose and cell concentrations are given in Figure 4.1. Cell concentration increased at a high rate and went through an exponential growth phase until t=6 h and then growth ceased, a stationary phase was reached.

Glucose concentration decreased at a high rate until t= 6 h due to the exponential growth phase that the cells were experiencing. Consumption rate of glucose decreased between t= 6 h and t= 12 h due to the stationary phase (Figure 4.2). After t= 12 h substrate utilization rate was very low and glucose concentration was almost constant at a very low level.

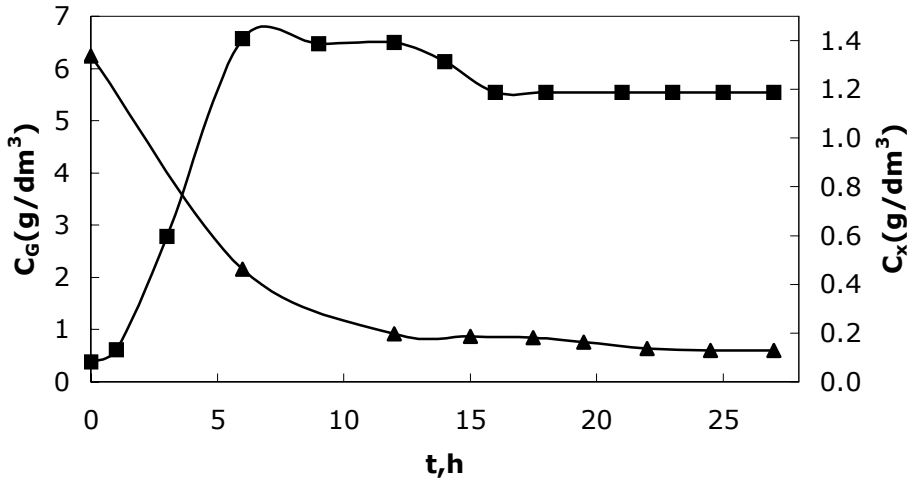

Figure 4.1. Variations in biomass and substrate concentrations with the cultivation time. (■), C_x; (▲), C_G.

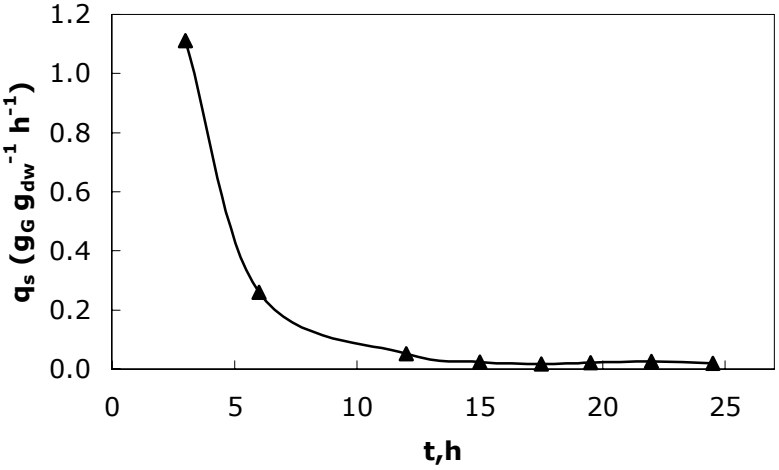

Figure 4.2. Variation in specific glucose consumption rate with cultivation time.

4.1.2 Variation in Serine Alkaline Protease Activity

The variations of serine alkaline protease activity (A) and specific serine alkaline protease activity (A') and specific serine alkaline protease production rate (r_p) with the cultivation time are shown in Figure 4.3 and 4.4, respectively. During the cell formation period (t= 0-6 h), the rate of increase in serine alkaline protease activity was low. After t=6 h serine alkaline protease activity started to increase at a high rate up to t=15 h. High serine alkaline protease production rates correspond to the stationary phase of the growth since serine alkaline protease synthesis is non-growth associated. Both serine alkaline protease activity and specific serine alkaline protease production increased up to t=25 h then gave maximums at t=25 h. The highest serine alkaline protease activity was obtained at t=25 h and its value was 460 U cm^{-3}.

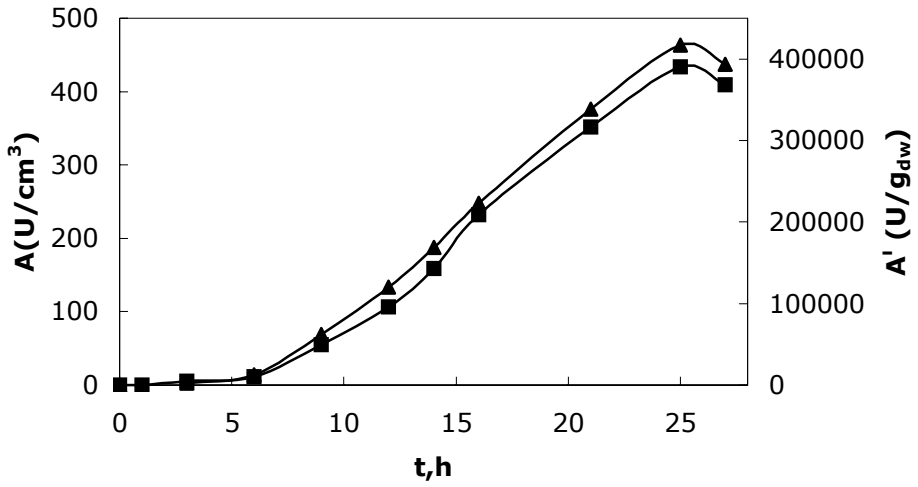

Figure 4.3. Variations in SAP activity and specific SAP activity in defined medium with cultivation time; (■), specific SAP activity; (▲), SAP activity.

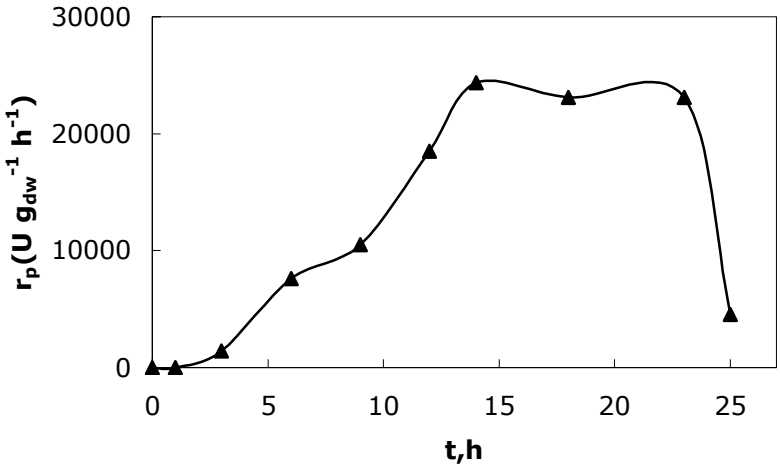

Figure 4.4. Variation in specific SAP production rate in defined medium with cultivation time.

It is observed from Figures 4.1, 4.2, 4.3 and 4.4 that, the system reached to a steady state between t=15-25 h in terms of SAP production, glucose consumption and cell growth. Therefore, it was decided to give the perturbations, and perform the MFA and MCA, between t=15-25 h since MCA strictly applies to systems at steady state. Perturbation time was chosen to be t=15 h.

4.1.3 Dissolved Oxygen and pH Profiles

Variations in dissolved oxygen and pH with the cultivation time are given in Figure 4.5. pH decreased until t=8 h down to 6.18 due to the consumption of medium contents by cells during growth. After t=8 h, pH values increased along with the SAP production in stationary phase until t=15 h and after that time pH values remained nearly constant.

Dissolved oxygen tended to decrease until t= 8 h during exponential growth period since oxygen uptake rate by the cells was higher than the oxygen transfer rate to the medium. As the cells went through the stationary phase, dissolved oxygen content increased due to the decrease in oxygen uptake rate until t=15 h and after that time it remained constant reaching to nearly 100% of the saturation concentration probably because the cell growth ceased and more

oxygen is supplied than needed by the cells for maintenance and SAP production.

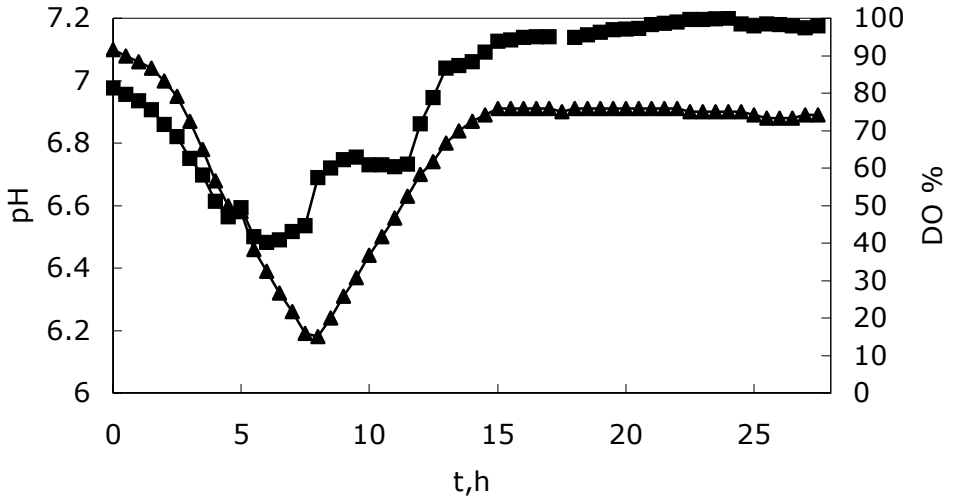

Figure 4.5. Variation of medium pH and dissolved oxygen % with cultivation time. (▲), pH; (■), % dissolved oxygen (C_{DO}^{*}= 0.21 mol/m^3)

4.1.4 Oxygen Transfer Characteristics

Oxygen transfer characteristics were investigated by determining the mass transfer coefficient, k_La, oxygen uptake rate, OUR, and specific oxygen uptake rate, q_0. They were obtained using the method outlined in Appendix G. Variations in oxygen transfer parameters with the cultivation time is demonstrated in Table 4.1. Mass transfer coefficient decreased at t= 2.5-15 h of the process and then increased at t=25 h. Oxygen uptake rate by the cells increased from t=2.5 h to t=5 h, and after that time it decreased throughout the process. In order to determine the oxygen need of the cells, specific oxygen uptake rates were calculated and it was observed that oxygen need by the cells decreased with the cultivation time. High specific oxygen uptake rate values correspond to the exponential growth phase of the cells. The need for oxygen decreased as the cells experience a stationary phase, as expected.

Table 4.1 Variations in oxygen transfer parameters with cultivation time.

t,h	k_La, s^{-1}	OUR,mol/m^3s	C_x, kg/m^3	q_0, mol/kg s
2.5	0.0407	0.00167	0.597	0.00279
5	0.0216	0.00204	1.41	0.00145
10	0.0257	0.00148	1.39	0.00107
15	0.0187	0.000826	1.19	0.000695
25	0.0320	0.000717	1.19	0.000604

4.1.5 Extracellular Amino Acid and Organic Acid Concentrations

Extracellular amino acid concentrations for the reference medium and for the cases of aspartate perturbations are presented in Table 4.2 and 4.4. Generally, Asp, Pro, Tyr, Leu and Lys were observed in the medium at the time of perturbations and after the perturbations. The other amino acids could not be detected in the extracellular medium. Existence of Asp, Pro, Tyr, Leu and Lys in the medium demonstrates the fact that the cells synthesize these amino acids in excess amount and transport the remaining outside the cell after usage for the metabolism. Existence of Asp is quite interesting since the pathway leading from Asp is believed to include a bottleneck in *Bacillus licheniformis*. No other amino acids produced from aspartic acid pathway, except for Lys, could be observed in the extracellular medium and, this could be a sign of insufficient production of these amino acids. Although Asp is present in excess amount, it might not be further converted to Asn, Thr, Ile or Met because of a bottleneck in one the production steps. These results might prove the existence of a bottleneck in aspartate pathway for *Bacillus subtilis*, as well.

The amino acid content of extracellular medium changed with the amount of perturbation. No correlation between the amino acid content and Asp perturbation amount could be made, however, it is obvious that addition of Asp to the medium enhanced the Asp utilization by the cells. It is also observed that t= 1 h after perturbation, the cells tended to reach to approximately same extracellular Asp concentration for all cases.

Table 4.3 and 4.5 shows the organic acid concentrations for the reference medium and for the cases of aspartate perturbations. Generally; acetic, butyric, glutamic and gluconic acid is present in the extracellular medium. The results mean that those organic acids are produced in excess and transported to extracellular medium by the cells.

Glutamate is used for the biosynthesis of serine, alanine, valine, leucine, aspartate, meso-Diaminopimelate, isoleucine, phenylalanine, tyrosine, proline and ornithine, it is also used in the catabolism of alanine, proline, arginine, valine, glutamine, histidine and by the biosynthesis reactions of asparagine, GMP, CaP, UDPNAG. Consequently, glutamate was more than the demand of the cells and excreted to the fermentation medium. Acetate was observed to be present in rather high amounts. This may be due to the cells' necessity to activate a new pathway probably because of a bottleneck in the metabolism or the need for more ATP.

Table 4.2 Variation in extracellular amino acid concentrations with no perturbation and after 0.1 mM Asp perturbation.

	Sampling time after perturbation, min									
	No perturbation					0.1 mM Asp				
g/l	0	10	20	30	60	0	10	20	30	60
Asn	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Asp	0.0052	0.0073	0.0054	0.0052	0.0054	0.0179	0.0041	0.0069	0.0047	0.0047
Gln	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ser	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gly	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
His	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Arg	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ala	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pro	0.0010	0.0011	0.0015	0.0011	0.0013	0.00	0.0008	0.00	0.00	0.00
Tyr	0.0074	0.0000	0.0080	0.0095	0.00	0.0166	0.0104	0.00	0.0062	0.00
Val	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cys	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
i-leu	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Leu	0.0017	0.0029	0.0009	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phe	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Orn	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Trp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	0.0407	0.0409	0.0344	0.0432	0.0673	0.0450	0.0446	0.0469	0.0099	0.00

Table 4.3 Variation in extracellular organic acid concentrations with no perturbation and after 0.1 mM Asp perturbation.

	Sampling time after perturbation, min									
	No perturbation					0.1 mM Asp				
g/l	0	10	20	30	60	0	10	20	30	60
Cit	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
a-KG	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Suc	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pyr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ac	0.5621	0.5505	0.5331	0.5223	0.5299	0.9675	0.9200	0.9034	0.8278	0.8384
Lac	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
But	0.1115	0.1205	0.1238	0.1197	0.1315	0.1458	0.1271	0.1221	0.1092	0.1249
Glu	0.0713	0.0722	0.0742	0.0780	0.0836	0.0767	0.0815	0.0827	0.0911	0.0789
Gluc	0.0960	0.1081	0.1038	0.1053	0.1187	0.0994	0.0908	0.0819	0.0818	0.0856

Table 4.4 Variation in extracellular amino acid concentrations after 0.2 mM and 0.3 mM Asp perturbation.

	Sampling time after perturbation, min									
	0.2 mM Asp					0.3 mM Asp				
g/l	0	10	20	30	60	0	10	20	30	60
Asn	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Asp	0.0321	0.0053	0.0051	0.0059	0.0056	0.0448	0.0065	0.0236	0.0054	0.0046
Gln	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ser	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gly	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
His	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Arg	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ala	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pro	0.0008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tyr	0.00	0.00	0.00	0.00	0.00	0.0099	0.00	0.00	0.00	0.00
Val	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cys	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
i-leu	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Leu	0.00	0.00	0.00	0.00	0.0010	0.0009	0.00	0.00	0.00	0.00
Phe	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Orn	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Trp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	0.00	0.00	0.00	0.00	0.00	0.0045	0.0060	0.0632	0.0430	0.0386

Table 4.5 Variation in extracellular organic acid concentrations after 0.2 mM and 0.3 mM Asp perturbation.

	Sampling time after perturbation, min									
	0.2 mM Asp					0.3 mM Asp				
g/l	0	10	20	30	60	0	10	20	30	60
Cit	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
a-KG	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Suc	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pyr	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ac	0.7127	0.7159	0.6276	0.6314	0.5770	0.9713	0.9644	0.9420	0.9213	0.8542
Lac	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
But	0.1040	0.1110	0.0983	0.1219	0.1090	0.1182	0.1348	0.1154	0.1162	0.1156
Glu	0.0757	0.0875	0.0779	0.0739	0.0564	0.0635	0.0708	0.0664	0.0671	0.0688
Gluc	0.0773	0.0854	0.1119	0.1074	0.1011	0.0889	0.0895	0.1003	0.1086	0.0908

4.1.6 Intracellular Aspartic Acid Concentrations

Intracellular aspartic acid concentrations for the reference medium and for the cases of aspartate perturbations are presented in Table 4.6. As observed from the table, intracellular Asp concentrations change rapidly according to the needs of the cell. The initial concentrations are almost the same, as expected, however, changes are observed after perturbations. In case of no perturbation, intracellular aspartate concentration first increases and than decreases with time due to the usage of Asp in the cell. In case of three separate perturbations, aspartate concentration increased after 10 min due to the transport of added Asp into the cell. After 10 min, transported Asp is utilized by the cell as observed from the concentration decrease. 60 min after perturbation, it is interesting to observe that the cells managed to regulate the metabolism such that intracellular Asp is almost at the same levels before the perturbation. This demonstrates the fact that cells try to keep the concentrations of intracellular metabolites at approximately same levels even in the presence of perturbations via regulatory mechanisms.

Table 4.6 Variation in intracellular aspartic acid concentrations with no perturbations and after 0.1, 0.2, 0.3 mM Asp perturbation.

	Sampling time after perturbation, min				
g/g_{dw}	0	10	20	30	60
No Asp	0.000541	0.00158	0.000397	0.000290	0.000584
0.1 mM Asp	0.000493	0.000735	0.000602	0.000371	0.000576
0.2 mM Asp	0.000573	0.000580	0.000470	0.000697	0.000522
0.3 mM Asp	0.000557	0.000769	0.000544	0.000539	0.000509

4.2 Metabolic Flux Analysis

Analysis of the extracellular medium for amino and organic acids were combined with the MFA methodology explained in Section 2.5, and intracellular reaction rates were determined for four conditions including three separate perturbations. The effect of aspartate perturbation was examined in main pathways.

The glycolysis pathway was active since glucose was the soul carbon source. Glycolysis pathway is naturally influenced by the perturbations, however, the effect could be accepted as insignificant although it was expected to be affected by aspartate perturbations because of the reaction connecting oxaloacetate to pyruvate. There is no correlation between glycolysis pathway fluxes and amount of aspartate perturbation. Flux values did not change significantly with the change in aspartate perturbation concentration as well as with time.

Pentose phosphate pathway experienced more significant changes due to the introduction of aspartate to extracellular medium. The pathway was active after and before the perturbations, however, a significant decrease in pathway fluxes was noticed especially 10 minutes after aspartate addition. After 20 minutes, the flux values for perturbed and un-perturbed systems were low but close to each other. The non-oxidative part of the pentose phosphate pathway was active for all conditions. The oxidative part was active 10 min after introducing 0.3 mM, and 20 min after introducing 0.1 and 0.3 mM aspartate.

The effect of aspartate addition was most obviously observed in TCA cycle. This should be expected since aspartate is synthesized from oxaloacetate, which functions in the TCA cycle. Generally, fluxes increased with increasing perturbation amount. For 10 and 20 minutes after perturbation, flux values increased with aspartate amount for 0.1 and 0.2 mM Asp, then decreased for 0.3 mM Asp but the values were still higher than the un-perturbed system. After 30 minutes, the values demonstrated an increasing trend with increasing aspartate perturbation amount. When time course results are inspected, the system with no aspartate added increased until 20 minutes and then the fluxes decreased. For 0.1 and 0.2 mM aspartate added, the flux values tend to decrease. Finally for 0.3 mM, the trend is an increase in fluxes with time.

4.2.1 Bottleneck Determination and Branchpoint Identification

In order to determine the bottlenecks and identify the branchpoints, three separate perturbations were introduced, as expressed before. Analysis of the extracellular medium for amino and organic acids were combined with the MFA methodology explained in Section 2.5, and flux distributions for the aspartic acid pathway for four different conditions were determined. Figure 4.6 represents the aspartic acid pathway and the numbers represent the reaction numbers assigned to the reactions for the sake of simplicity. Tables 4.7, 4.8 and 4.9 show specific flux values with no perturbation and with addition of aspartate to the production medium at after 10, 20 and 30 minutes of perturbation, respectively. Specific fluxes were determined by division of the real fluxes with substrate uptake rate and used for comparison.

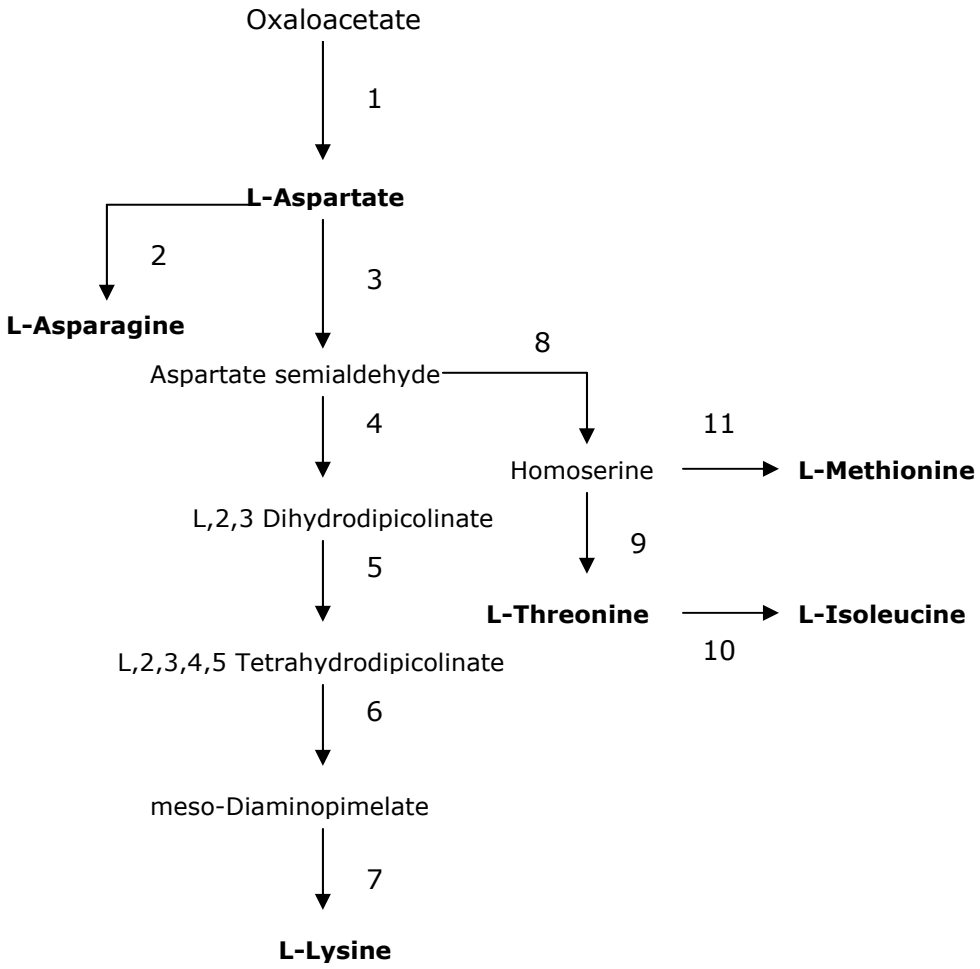

Figure 4.6. Aspartic acid pathway including the reaction numbers assigned.

Table 4.7 Specific flux values determined 10 min after perturbation.

	Aspartate perturbation concentration, mM			
Reaction number	0	0.1	0.2	0.3
1	1.6207	2.8426	2.6717	0.9946
2	0.0077	0.0035	0.0075	0.0054
3	1.5996	3.1834	3.5264	1.6116
4	1.5843	3.1661	3.4264	1.5880
5	1.5843	3.1661	3.4264	1.5880
6	1.5843	3.1661	3.4264	1.5880
7	1.5843	3.1661	3.4264	1.5880
8	0.0134	0.0173	0.0981	0.0236
9	0.0115	0.0156	0.0962	0.0218
10	0.0038	0.0017	0.0038	0.0036
11	0.0019	0.0010	0.0019	0.0015

Table 4.8 Specific flux values determined 20 min after perturbation.

	Aspartate perturbation concentration, mM			
Reaction number	0	0.1	0.2	0.3
1	1.1801	2.8270	1.4075	0.9909
2	0.0077	0.0035	0.0075	0.0054
3	1.2318	2.8010	1.3698	1.0127
4	1.2165	2.2699	1.3547	1.0018
5	1.2165	2.2699	1.3547	1.0018
6	1.2165	2.2699	1.3547	1.0018
7	1.2165	2.2699	1.3547	1.0018
8	0.0134	0.5294	0.0151	0.0109
9	0.0115	0.5294	0.0132	0.0091
10	0.0038	0.0017	0.0038	0.0036
11	0.0019	0.0010	0.0019	0.0015

Table 4.9 Specific flux values determined 30 min after perturbation.

	Aspartate perturbation concentration, mM			
Reaction number	0	0.1	0.2	0.3
1	0.4693	2.2647	2.4245	1.7132
2	0.0077	0.0035	0.0075	0.0054
3	0.2816	2.3945	2.6943	2.0998
4	0.2682	2.3824	2.5170	2.0907
5	0.2682	2.3824	2.5170	2.0907
6	0.2682	2.3824	2.5170	2.0907
7	0.2682	2.3824	2.5170	2.0907
8	0.0134	0.0138	0.1774	0.0109
9	0.0115	0.0121	0.1736	0.0091
10	0.0038	0.0017	0.0038	0.0036
11	0.0019	0.0010	0.0019	0.0015

Examination of specific flux values reveals that addition of aspartate to the medium, consequently, increase in the intracellular aspartate concentration increases the aspartate pathway fluxes. It is observed that the flux of branch leading to lysine production, including the reactions 3, 4, 5, 6 and 7, is dominant over the other branches. However, although all the fluxes increased due to the addition of aspartate, branches for the production of Asn, Thr, Ile and Met have low flux values for all four cases compared to the lysine producing branch. That means the aspartic acid pathway is regulated such that production of lysine, and may be one or more other metabolites of that branch, is enhanced. Dipicolinate, which is produced from L,2,3 dihydrodipicolinate, is an

important intermediate for spore formation. In addition, meso-diaminopimelate is also utilized in cell wall and spore-cortex peptidoglycan (Sonenshein, 2002). The formation of those two metabolites are crucial especially in the SAP synthesis period of the bioprocess. The metabolism is regulated to have a high flux through those metabolites and when these are not used by the cell, they are further catalyzed to form lysine. That also explains the presence of lysine in extracellular medium.

From Tables 4.7 and 4.9, 10 and 30 minutes after perturbation, it is obviously seen that as aspartate perturbation amount increased from 0 to 0.2 mM, flux values increase, as well. However, when aspartate perturbation is increased from 0.2 to 0.3 mM, all fluxes decrease probably due to an inhibition effect. For Table 4.8, a different situation is present. 20 minutes after perturbation, fluxes increase for 0.1 mM aspartic acid perturbation and when the concentration is increased to 0.2 and 0.3 mM, a decrease in fluxes is noticed.

When the time course results of the perturbations are inspected, from Tables 4.7, 4.8 and 4.9; for no perturbation and 0.1 mM aspartate perturbation, the fluxes decrease as the time passes. However, for 0.2 and 0.3 mM perturbations, fluxes are higher after 10 minutes while they decrease after 20 minutes of perturbation. Flux values increase again after 30 minutes. These results show that fluxes of the pathway can fluctuate during the regulation process according to the needs of the metabolism.

Fluxes through asparagine, threonine, isoleucine and methionine producing branches are lower than those of the other branch. Although the values are low, perturbation with aspartic acid increased the fluxes through reactions 8 and 9, producing threonine from aspartate semialdehyde, considerably. However, reactions 10 and 11 still have low values. That means even if the flux to threonine production increases, the regulations avoid the rise in the fluxes of those two reactions, thus production of methionine and isoleucine. When these results are combined with the results of a previous study (Oktar, 2003), it is possible to say that Asn, Thr and, consequently, Ile productions create bottlenecks. In the study of Oktar (2003), it was observed that supplying the medium with Ile, Thr and Asn increased SAP production 1.4, 1.48 and 1.87 fold, respectively. Supplying the medium with Met did not have a significant effect on SAP production but increased the cell growth 1.43 fold. That means that the cells

do not necessitate Met for SAP production but Met affects cell growth probably because it is required for translation initiation in the cell and is important for most of methyltransferase reactions (Sonenshein, 1993). In SAP production period there is no shortage of Met, probably because Met can also be produced by other means.

Having determined the bottlenecks, the next step should be the identification of branchpoints in order to understand the regulations performed by the cells. As it was expressed before, flux split ratios at branch points determines the yield of the product. If production of Ile, Asn and Thr is to be increased; flux split ratios should be investigated. From Tables 4.10, 4.11 and 4.12, it is easily noticed that flux split ratios for both branchpoints can readily change while the fluxes to aspartate semialdehyde (AspSa) and L,2,3 dihydrodipicolinate (DC) is quite dominant in most of the cases. Since flux value of one branch is dominant, the branchpoints splitting from Asp and AspSa were both identified to be weakly rigid branchpoints. The reason for dominance of the lysine producing branch could be higher affinity of the enzymes in lysine branch to their substrates than those of the enzymes in branches leading to Asn or Thr and Ile.

Table 4.10 Specific flux values and flux split ratios for the branchpoints in Asp pathway 10 minutes after perturbation.

		Specific Flux to Product Formation					
Aspartate concentration, mM		Asn	AspSa	Ratio	HSer	DC	Ratio
	0	0.00766	1.60	209	0.0134	1.58	118
	0.1	0.00346	3.18	920	0.0173	3.17	183
	0.2	0.00755	3.53	467	0.0981	3.43	34.9
	0.3	0.00544	1.61	296	0.0236	1.59	67.3

Table 4.11 Specific flux values and flux split ratios for the branchpoints in Asp pathway 20 minutes after perturbation.

		Specific Flux to Product Formation					
Aspartate concentration, mM		Asn	AspSa	Ratio	HSer	DC	Ratio
	0	0.00766	1.23	161	0.0134	1.22	90.7
	0.1	0.00346	2.80	810	0.529	2.27	4.29
	0.2	0.00755	1.37	182	0.0151	1.35	89.8
	0.3	0.00544	1.01	186	0.0109	1.00	92.0

Table 4.12 Specific flux values and flux split ratios for the branchpoints in Asp pathway 30 minutes after perturbation.

		Specific Flux to Product Formation					
Aspartate concentration, mM		Asn	AspSa	Ratio	HSer	DC	Ratio
	0	0.00766	0.28	36.8	0.0134	0.27	20.0
	0.1	0.00346	2.39	692	0.0138	2.38	197
	0.2	0.00755	2.69	357	0.177	2.52	14.2
	0.3	0.00544	2.10	386	0.0109	2.09	192

It is not easy to prevent the flux from going through lysine synthesizing branch. However, efforts could be made to decrease the activity of lysine branch while increasing the activity of the other branches simultaneously. That is, levels of enzymes catalyzing the reactions producing Asn, Thr and Ile could be increased by using genetic engineering principles. Another way to improve the production could be increasing those enzymes' affinities towards their substrates by activators or inhibitors.

4.3 Metabolic Control Analysis

Application of MCA was achieved on the previously explained simple form of aspartic acid pathway. In the first step, elasticity coefficients were determined from the ln J vs ln C_{Asp} plots. Thereafter, flux control coefficients were calculated using the procedure given in section 3.7.

4.3.1 Determination of Elasticity Coefficients

Metabolic control analysis principles were applied on a simplified form of aspartic acid pathway in order to find the elasticity coefficients corresponding to three blocks. The simplified pathway and used equations were shown in Section 3.7 and is presented again in Figure 4.7.

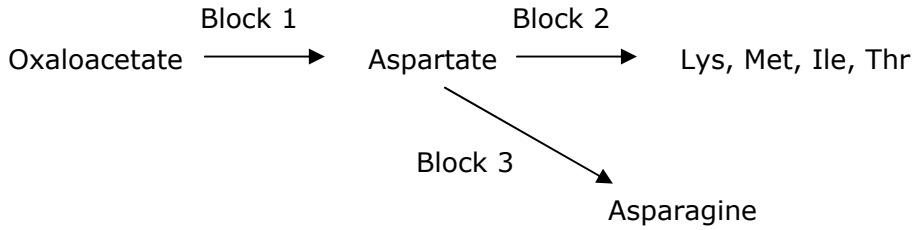

Figure 4.7 Simplified form of the aspartate pathway.

Intracellular aspartate concentrations measured was combined with the flux values for the aspartic acid pathway and elasticity coefficients were determined by plotting lnJ vs ln C_{Asp} and taking the slope of the graph. The graphs for each block is given in Appendix F. Table 4.13 demonstrates the elasticity coefficients for Blocks 1, 2 and 3 after 10, 20 and 30 minutes of perturbation.

Table 4.13 Elasticity coefficients for blocks 1, 2 and 3; 10, 20 and 30 minutes after perturbation.

	Sampling time after perturbation, min		
	10	20	30
Block 1	-0.56	2.15	1.91
Block 2	-0.82	2.04	2.70
Block 3	-1.86	-3.08	1.24

Elasticity coefficient values give important information about regulation of metabolism. It shows the effect of a relative change in an intracellular metabolite on flux of a branch. A positive elasticity value means the flux value of a certain branch is increased with the increase in the concentration of a certain metabolite. Keeping that in mind when the elasticities presented in Table 4.12 are inspected, it is possible to make explanations about the control exerted. From Figure 4.7, it is expected that increase in Asp concentration should decrease the flux of Block 1 due to a possible feedback inhibition effect; and

increase the fluxes of Blocks 2 and 3 since availability of more substrate to the pathway should increase the fluxes of the branches unless the enzymes are saturated with the substrate. That means elasticities for Block 1 should be negative while elasticities for blocks 2 and 3 should be positive. Elasticity coefficients determined after 10 minutes of perturbation are all negative indicating that the relative increase in Asp concentration tends to decrease the fluxes or relative decrease in Asp level tends to increase the flux values, 10 minutes after perturbation. Increase in Asp level causes an attempt to decrease the flux going through Block 1, as expected. Tendency to decrease in Block 2 and 3 fluxes could be because the cells have not regulate the pathway to access high fluxes in aspartate pathway yet, and intracellular Asp is directed to the other metabolic reactions utilizing Asp.

20 minutes after perturbation, positive values of elasticity coefficients for Blocks 1 and 2, a negative value for Block 3 are observed. Positive elasticity is expected for Block 2 due to the higher availability of substrate. For Block 1, it is an unexpected situation, however, the reason could be the microorganism's efforts to keep intracellular concentration of Asp at high levels since addition of aspartate increased the Asp levels compared to normal conditions and the reactions are regulated according to these high levels. Elasticity for block 3 is expected to be positive, the negative result is probably due to the direction of flux through block 2 not block 3. Block 2 is more active than block 3. Generally, aspartate is directed to be used in aspartate pathway rather than the other reactions unlike the situation 10 min after perturbation.

After 30 minutes, all elasticities posses a positive value. The reason for positive elasticities for blocks 1 and 2 should be the same as the previous case, efforts to keep high Asp levels and direction of flux mainly to block 2. As time passed, the regulations started to direct the flux through block 3, as well. However, from the numerical values, block 2 is more favorable than block 3 for the microorganism.

4.3.2 Determination of Flux Control Coefficients

Using the elasticity coefficients, flux control coefficients were calculated for asparagine synthesis at two different times after perturbation, 10 and 30 minutes. Sample calculations for determination of FCCs is given in Appendix H. Flux control coefficient values, to understand the control exerted on asparagine production, are given in Table 4.14.

Table 4.14 Flux control coefficients for asparagine production 10 and 30 min after perturbation.

	Time after perturbation, min	
FCC	10	30
C_1^3	6.8	6.1
C_2^3	-6.7	-3.7
C_3^3	0.9	-1.5

C_i^j stands for the control exerted on block j by block i. From Table 4.14, it is seen that flux control coefficient of block 1, that is control of Asp formation reaction on asparagine formation, is positive after both 10 min and 30 min. That means an increase in the activity or amount of the enzyme catalyzing the Asp formation reaction from oxaloacetate, would cause an increase in the flux of asparagine production reaction or vice versa.

The FCC for block 2, control of Lys, Thr, Ile and Met formation reactions on asparagine formation, is negative meaning that an overall increase in the activity of the block might increase the flux of the metabolites down their own branch while decreasing the amount available to flow down the other branch. These results are in good agreement with the results of MFA where a dominance of lysine producing branch in flux partition is observed.

Control of asparagine synthesis on itself changes with the time. 10 minutes after perturbation an increase in enzyme activity or amount leads to an increase in the flux as expected. However, after 30 min, it is noticed that there exists a negative control. The activity increase results in a flux decrease. That could be due to the high activity of the other branch, block 2, directing most of the flux to itself. Therefore, even if the activity of the enzyme producing asparagine increases,

flux through the other branch is so favored that the final result is a negative control.

Comparing the values of the FCC's, it is deduced that block 1 and 2 share the control of asparagine production almost equally since the absolute values are very close to each other 10 minutes after perturbation. After 30 minutes, they still share the control, nevertheless, aspartate formation controls more than the other branch. Still, FCC value of that branch is high, in this respect, it should not be ignored.

Investigation of metabolic fluxes, elasticity and flux control coefficients revealed that branch producing lysine is far more active than the other two branches synthesizing threonine, isoleucine and asparagine. In order to increase the production of the latter amino acids in the cell, it is compulsory to eliminate the bottlenecks by deregulation of the pathway. The activity of the lysine branch should be decreased without avoiding the synthesis of sufficient amount of especially L,2,3 dihydrodipicolinate, meso-diaminopimelate and lysine, and the enzymes of other branches should be activated simultaneously.

The reason for the lysine producing branch to be very active should be due to the existence of multienzyme complexes and lack of feedback inhibition mechanisms in order to produce especially L,2,3 dihydrodipicolinate and meso-diaminopimelate even in the presence of high levels of amino acids.

For improvements in asparagine production both aspartate formation reaction and the branch leading to Lys, Thr, Ile, Met should be taken into account. From metabolic control analysis, it is inspected that these two steps share the control and if asparagine flux is to be increased, activity of the enzyme catalyzing aspartate formation reaction, aspartate aminotransferase, should be increased while decreasing the activity of the other branch. Despite its central importance in aspartic acid pathway, aspartate aminotransferase of *B. subtilis* have not been investigated in detail. A detailed study of this enzyme would surely give information about the methods to increase the reaction flux. Moreover, asparagine synthesizing enzyme could be studied. There are three different glutamine dependent asparagine synthesizing enzymes, asparagine synthetases, in *B. Subtilis* (Sonenshein, 2002). Although they are active enzymes when cloned in *E.coli*, two of them contribute relatively little to Asn synthesis. The

reason for this could be the low activity or low levels of these enzymes. Asn synthesis could be increased if expression of the genes encoding these enzymes is increased using genetic manipulation. In addition, the gene responsible for encoding an NH_3 dependent asparagine synthase, which is not normally expressed in *B.subtilis*, in *E.coli* could be cloned in *B.subtilis* to investigate the effect of the existence of that enzyme in asparagine synthesis (Sonenshein, 2002). Inactivation of the enzymes producing aspartate semialdehyde from aspartate is not wise since it would affect the threonine production, as well.

In *C. glutamicum*, overproduction of threonine by mutants with reduced dihydrodipicolinate synthase, enzyme catalyzing the formation of dihydrodipicolinate, activity is achieved. The enzyme of *C. glutamicum* resembles that of *B. subtilis*, therefore, the competition between dihydrodipicolinate synthase and homoserine dehydrogenase, enzyme producing homoserine, possibly regulates the flow of intermediates into two main branches. Increasing the activity or level of homoserine dehydrogenase could significantly direct the flux through Thr formation. One problem with this enzyme is that it is inhibited by threonine and in the absence of feedback inhibition considerably more aspartate semialdehyde would be utilized in the synthesis of Thr, Met and Ile (Sonenshein, 1993). Efforts to prevent the inhibition as much as possible could result in elevated flux values for threonine branch.

Increasing Thr and Asn production does not eliminate all the bottlenecks in SAP production. MFA results revealed that although threonine producing fluxes increase considerably, Ile flux does not change much. Threonine is used for other purposes in the cell instead of Ile production. Isoleucine producing enzyme, threonine dehydratase, could be expressed genetically to improve the synthesis. However, since this enzyme is inhibited by Ile, efforts might not be adequate. It is known that threonine synthesizing enzyme, threonine synthase, also functions in formation of isoleucine without a feedback inhibition of Ile (Sonenshein, 1993). This enzyme could be an interesting metabolic engineering site if ways to direct the enzyme activity to Ile production could be made possible.

CHAPTER 5

CONCLUSIONS

Microorganisms act as microbioreactors in the bioreactor system and thousands of reactions take place in the microbioreactor. Regulatory mechanisms provide adaptation of microorganisms to different environments and optimization of growth. However, microbial metabolism may strictly enhance growth while avoiding the formation of the desired product via regulatory mechanisms. Therefore, it is very important to investigate the control exerted on the metabolic pathways leading to the formation of the desired product.

In order to obtain higher yield for SAP production, it is of great importance to examine the intracellular reactions and contributing enzymes and gain knowledge about the regulatory mechanisms in the cell and understand the control exerted on the production of the amino acids needed to produce our product of interest, SAP.

Results of previous studies revealed that there is a potential bottleneck for SAP production in aspartate pathway, probably asparagine synthesis (Çalık et al., 1999, 2000). Therefore, understanding the regulations and identification of the branchpoints in aspartic acid pathway is crucial since the information could enable the elimination of existing bottlenecks and, consequently, the increase in SAP production.

In this study, firstly, bioprocess characteristics for SAP production by *B.subtilis* using the production medium demonstrated in Table 3.3 and the bioreactor operation parameters given in section 3.4 were examined. After that, three separate perturbations were introduced by addition of aspartate to the

production medium at a certain time of the bioprocess. The response of the cells were observed and; by comparing the changes in intracellular reactions of aspartate pathway, bottlenecks were determined and branchpoint classification was achieved. Lastly, metabolic control analysis principles were applied to the simplified form of aspartate pathway to determine the elasticity and flux control coefficients.

Bioprocess characteristics were examined using the agitation rate N=750 min^{-1}, air inlet rate Q_0/V_R=0.5 vvm at T= 37 ºC with initial pH of pH_0=7.1. Cell concentration increased at a high rate and went through an exponential growth phase until t=6 h and then growth ceased, a stationary phase is reached. Glucose concentration decreased at a high rate in the exponential growth phase that the cells were experiencing. After that, specific consumption rate of glucose decreased due to the stationary phase. During the cell formation period, the rate of increase in serine alkaline protease activity was low. High serine alkaline protease production rates correspond to the stationary phase of the growth since serine alkaline protease synthesis is a non-growth associated product. The system reached to a steady state between t=15-25 h in terms of SAP production, specific glucose consumption and cell growth. Therefore, it was decided to give the perturbations, and perform the MFA and MCA, between t=15-25 h since MCA strictly applies to systems at steady state. Perturbation time was chosen to be t=15 h. Perturbations are given such that the aspartate concentration of the extracellular medium increased 0.1, 0.2 and 0.3 mM in separate experiments.

Existence of Asp, His, Pro, Tyr, Leu and Lys in the extracellular medium demonstrates the fact that the cells synthesize these amino acids in excess amount and transport the remaining outside the cell after usage for the metabolism. No other amino acids produced from aspartic acid pathway, except for Lys, could be observed in the extracellular medium and, this could be a sign of insufficient production of these amino acids. The amino acid content of extracellular medium changed with the amount of perturbation. No correlation between the amino acid content and Asp perturbation amount could be made, however, it is obvious that addition of Asp to the medium enhanced the Asp utilization by the cells. It is also observed that 1 h after perturbation, the cells tended to reach to approximately same Asp concentration for all cases.

Generally; acetic, butyric, glutamic and gluconic acid is present in the extracellular medium. The results mean that those organic acids are produced in excess and transported to extracellular medium by the cells.

Analysis for intracellular Asp concentrations revealed that Asp change rapidly according to the needs of the cell. The initial concentrations are almost the same, as expected, however, changes are observed after different perturbations. It is interesting to observe that the cells managed to regulate the metabolism such that intracellular Asp is almost at the same levels before and after 1 h of the perturbation. This demonstrates the fact that cells try to keep the concentrations of intracellular metabolites at approximately same levels even in the presence of perturbations via regulatory mechanisms.

Fluxes were determined using MFA methodology and analysis of extracellular medium. The glycolysis pathway was active for all the conditions and there was no significant influence of the perturbations. There is no correlation between glycolysis pathway fluxes and amount of aspartate perturbation. Pentose phosphate pathway was active after and before the perturbations. A significant decrease in pathway fluxes was noticed especially after aspartate addition. The effect of aspartate addition was most obviously observed in TCA cycle. This should be expected since aspartate is synthesized from oxaloacetate, which functions in the TCA cycle. Generally, fluxes increased with increasing perturbation amount.

Examination of MFA results revealed that the flux of branch leading to lysine production is dominant over the other branches. Branches for the production of Asn, Thr, Ile and Met have very low flux values for all four cases. That means the aspartic acid pathway is regulated such that production of lysine and may be one or more other metabolites of that branch is enhanced. When these results are combined with the results of another study (Oktar, 2003), it is possible to say that Asn, Thr and, consequently, Ile productions create bottlenecks.

Since flux value of one branch is dominant, the branchpoints splitting from Asp and AspSa were both identified to be weakly rigid branchpoints. The reason for dominance of the lysine producing branch could be higher affinity of the enzymes

in lysine branch to their substrates than those of the enzymes in branches leading to Asn or Thr and Ile.

It is not easy to prevent the flux from going through lysine synthesizing branch. However, efforts could be made to decrease the activity of lysine branch while increasing the activity of the other branches simultaneously. That is, levels of enzymes catalyzing the reactions producing Asn, Thr and Ile could be increased by using genetic engineering principles. Another way to improve the production could be increasing those enzymes' affinities towards their substrates by activators.

Determination and examination of elasticity coefficients revealed that 10 minutes after perturbation, increase in Asp level decreases the flux going through the reaction producing Asp from oxaloacetate. There is a flux decrease in asparagine producing block and Lys, Met, Thr, Ile producing block with an increase in intracellular Asp since the cells have not regulate the pathway to access high fluxes in aspartate pathway yet, and intracellular Asp is directed to the other metabolic reactions utilizing Asp. 20 minutes after perturbation, fluxes for Lys, Met, Thr, Ile production increased with increasing Asp level due to the higher availability of substrate. Flux of Asp producing branch also increased with increasing Asp unexpectedly, because of the microorganism's efforts to keep intracellular concentration of Asp at high levels since addition of aspartate increased the Asp levels compared to normal conditions and the reactions are regulated according to these high levels. Asparagine production flux decreased with increasing aspartate, probably due to the direction of flux through Lys, Met, Thr, Ile production not asparagine. This time, aspartate is directed to be used in aspartate pathway rather than the other reactions. After 30 minutes, the situation did not change for Asp producing and Lys, Met, Thr, Ile producing branches. However, as time passed, the regulations started to direct the flux through asparagine, as well. From the numerical values, Lys, Met, Thr, Ile production is more favorable than asparagine production.

Using the elasticity coefficients, flux control coefficients were calculated for asparagine synthesis at two different times after perturbation, 10 and 30 minutes. The control of Asp formation reaction on asparagine formation is positive after both 10 min and 30 min. The control of Lys, Thr, Ile and Met formation reactions on asparagine formation is negative meaning that an overall increase in the activity of the block might increase the flux of the metabolites

down their own branch while decreasing the amount available to flow down the other branch. Control of asparagine synthesis on itself changes with the time. 10 minutes after perturbation an increase in enzyme activity or amount leads to an increase in the flux as expected. However, after 30 min, it is noticed that there exists a negative control. The activity increase results in a flux decrease.

Comparing the values of the flux control coefficients, it is deduced that Asp synthesis reaction and Lys, Thr, Ile, Met producing group share the control of asparagine production almost equally since the absolute values are very close to each other.

The reason for the high activity of lysine producing branch is probably the high activity and lack of inhibition of the branch enzymes. In order to improve SAP production, Asn, Thr, Ile synthesis should be increased. For asparagine production, both aspartate formation reaction and the branch leading to Lys, Thr, Ile, Met should be taken into account. Activity of the enzyme catalyzing aspartate formation reaction, aspartate aminotransferase, should be increased while decreasing the activity of the other branch. Moreover, asparagine synthesizing enzyme could be studied. Overexpression of glutamine dependent asparagine synthetases found in *B. subtilis* or expression of NH_3 dependent asparagine synthetase from *E. coli* could be beneficial. For threonine synthesis, increasing the activity or level of homoserine dehydrogenase could significantly direct the flux through Thr formation. Increasing Thr and Asn production is not enough to improve SAP production, isoleucine fluxes should be increased, as well. Isoleucine producing enzyme, threonine dehydratase, could be overexpressed to improve the synthesis. In addition, threonine synthesizing enzyme, threonine synthase, also functions in formation of isoleucine and if ways to direct the enzyme activity to Ile production could be made possible, it could eliminate the bottleneck related with the Ile production.

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APPENDIX A

CALIBRATION OF CELL CONCENTRATION

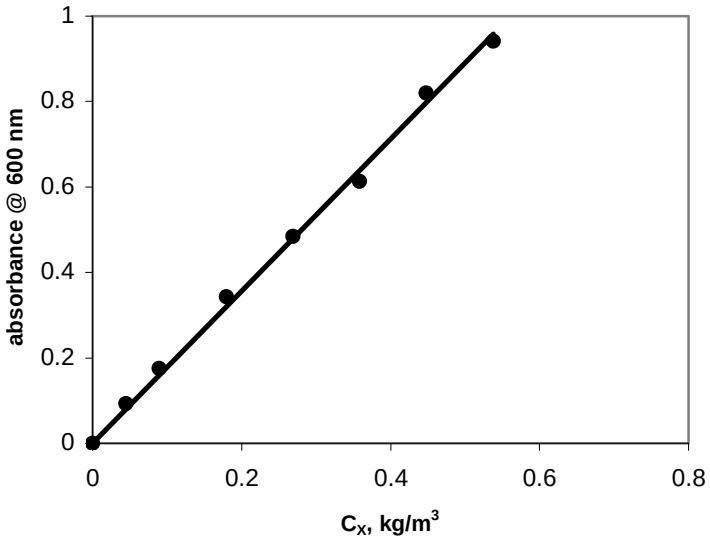

Figure A1 Calibration curve of microorganism concentration

Slope of the calibration curve, m=2.50 1/kg m^{-3} (λ = 600 nm)

$$C_X = \frac{\text{Absorbance}}{2.50} \times \text{Dilution Ratio}$$

APPENDIX B

CALIBRATION OF SERINE ALKALINE PROTEASE ACTIVITY

The slope of the calibration curve is m=0.8 1/(μmole cm^{-3}). One unit protease activity is defined as the activity that liberates 4 nmole of tyrosine per minute.

The activity;

$$A = \frac{\text{Absorbance}}{m} \times \text{Dilution Ratio} \times \frac{1U}{4 \times 10^{-3} \mu\text{mole}} \times \frac{1}{20\text{min}}$$

APPENDIX C

CALIBRATION OF REDUCED SUGAR CONCENTRATION

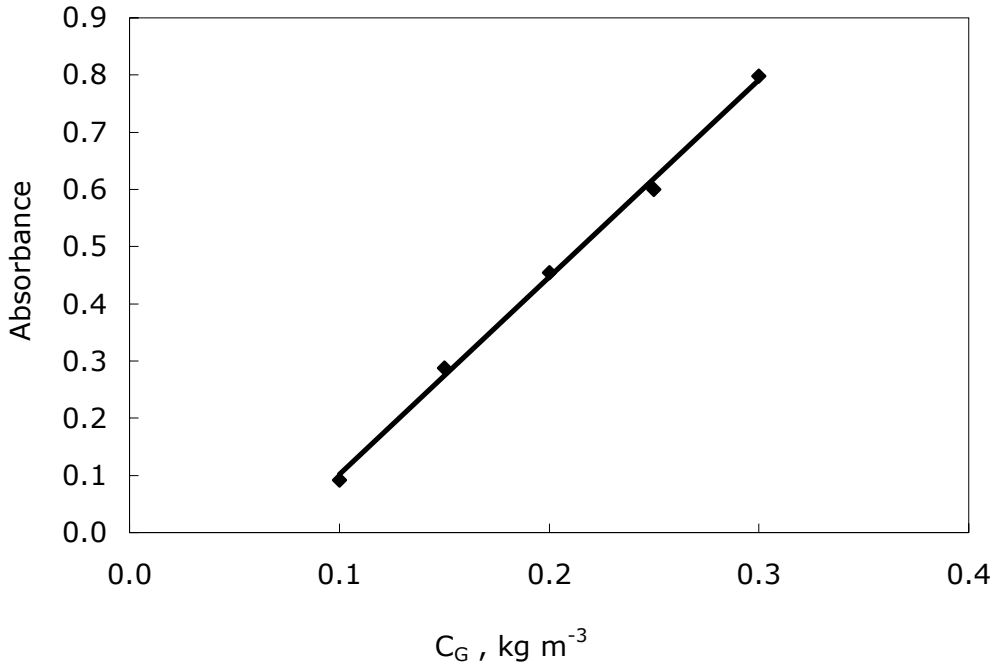

Figure A.2 Calibration Curve of the DNS solution

$$C_G = \left(\frac{\text{Absorbance} + 0.2432}{3.448} \right) \times \text{Dilution Ratio}$$

APPENDIX D

PREPARATION OF DNS SOLUTION

1. a) 880 cm^3 of 1 % (m/v) DNS solution is prepared by dissolving 8.8 g dinitrosalicylic acid in 880 cm^3 distilled water.

   b) After addition of 225 g ROCHELLE salt (sodium potassium tartarate), the solution is mixed.

   c) 300 cm^3 of 4.5 % NaOH, prepared by dissolving 13.5 g NaOH in 300 cm^3 distilled water, is added to this solution.

2. a) 22 cm^3 10 % NaOH, is prepared by dissolving 2.2 g NaOH in 22 cm^3 distilled water.

   b) 10 g christalyzed phenol and 100 cm^3 distilled water are added to the solution.

   c) 60 cm^3 is taken from this alkali-phenol mixture, 6.9 g $NaHCO_3$ is added and mixed.

The solution obtained from the first step is mixed with that from the second step and then they are stirred until ROCHELLE salt is dissolved. The prepared solution is kept in dark-colored bottle at 4°C for at least 48 h before use.

APPENDIX E

METABOLIC REACTIONS FOR *Bacillus subtilis*

Glycolysis and Gluconeogenesis Pathway

1. Glc + PEP → G6P + Pyr
2. G6P → Glc + Pi
3. F6P → G6P
4. G6P → F6P
5. Frc + PEP → F6P + Pyr
6. Man + PEP → Man6P + Pyr
7. Man6P → F6P
8. F6P + ATP → 2T3P + ADP
9. 2T3P → F6P + Pi
10. T3P + ADP + Pi → PG3 + ATP + NADH
11. PG3 + ATP + NADH→ T3P + ADP + Pi
12. PG3 → PEP
13. PEP → PG3
14. PEP +ADP → Pyr + ATP
15. Pyr + 2 ATP → PEP + 2ADP
16. Pyr → AcCoA + CO_2 + NADH

Pentose Phosphate Pathway

17. G6P → Gluc6P + NADPH
18. Glc → Gluc + NADH
19. Gluc + ATP → Gluc6P + ADP
20. Gluc6P → R5P + CO_2 + NADPH
21. R5P → Xyl5P
22. Xyl5P → R5P
23. R5P → Rib5P

24. Rib5P → R5P
25. Xyl5P + Rib5P → S7P + T3P
26. S7P + T3P → Xyl5P + Rib5P
27. Xyl5P + E4P → F6P + T3P
28. F6P + T3P → Xyl5P + E4P
29. T3P +S7P → F6P + E4P
30. F6P + E4P → T3P +S7P

Branches from Glycolysis

31. Pyr + NADH → Lac
32. Lac → Pyr + NADH
33. AcCoA + ADP + Pi → Ac + ATP
34. Ac + ATP → AcCoA + ADP + Pi

Anapleoric Reactions

35. Mal → Pyr + CO_2 + NADPH
36. OA → Pyr + CO_2

TCA Cycle

37. AcCoA + OA → Cit
38. Cit → ICit
39. ICit → αKG +CO_2+ NADPH
40. αKG → SucCoA + CO_2 + NADH
41. SucCoA + Pi + ADP → Suc + ATP + CoA
42. Suc + ATP → SucCoA + ADP + Pi
43. Suc → Fum + $FADH_2$
44. Fum → Mal
45. Mal → OA + NADH
46. ICit → Glx + Suc
47. Glx + AcCoA → Mal

Biosynthesis of Serine Family Amino Acids

48. PG3 + Glu → Ser + αKG + NADH + Pi
49. Ser + THF → Gly + MetTHF
50. Ser + AcCoA + H_2S → Cys + Ac

Biosynthesis of Alanine Family Amino Acids

51. Pyr + Glu → Ala + αKG

52. 2 Pyr + NADPH → KVal
53. KVal + Glu → Val + αKG
54. KVal + AcCoA + Glu → Leu + αKG + NADH + CO_2

Biosynthesis of Histidine

55. R5P + ATP → PRPP + AMP
56. PRPP + ATP + Gln → His + PRAIC + αKG + 2PPi + 2NADH + Pi

Biosynthesis of Aspartic Acid Family Amino Acids

57. OA + Glu → Asp + αKG
58. Asp + Gln + ATP → Asn + Glu + AMP + PPi
59. Asp + ATP + NADPH → AspSa + ADP + Pi
60. AspSa +Pyr → DC
61. DC + NADPH → Tet
62. Tet + AcCoA +Glu → Ac + αKG + mDAP
63. mDAP → Lys + CO_2
64. AspSa + NADPH → HSer
65. HSer + ATP → Thr + ADP + Pi
66. Thr + Pyr + NADPH + Glu → Ile + αKG + NH3 +CO_2
67. AcCoA + Cys + HSer + H_2S + MTHF → Met + Pyr + 2Ac + NH_3 + THF

Biosynthesis of Aromatic Amino Acids

68. 2 PEP + E4P + ATP + NADPH → Chor + ADP + 4 Pi
69. Chor + Glu → Phe + αKG + CO_2
70. Chor + Glu → Tyr + αKG + NADH + CO_2
71. Chor + NH3 + PRPP → Pyr + IGP + CO_2 + PPi
72. IGP + Ser → Trp + T3P

Biosynthesis of Glutamic Acid Family Amino Acids

73. αKG + NH3 + NADPH → Glu
74. Glu + ATP + NH3 → Gln + ADP + Pi
75. Glu + ATP + 2 NADPH → Pro + ADP + Pi
76. 2Glu + AcCoA + ATP + NADPH → Orn + αKG + Ac + ADP + Pi
77. Orn + CaP → Citr + Pi
78. Citr + Asp + ATP → Arg + Fum + AMP + PPi

Catabolism of the amino acids

79. αKG + Ala → Pyr + Glu
80. Arg+ αKG → 2Glu + NH_3+ NADPH + CO2
81. Asn → Asp + NH_3
82. Asp → Fum + NH_3
83. Cys → Pyr + NH_3+ H_2S
84. Gln + αKG + NADPH → 2Glu
85. Gly + MetTHF → Ser + THF
86. His +THF → Glu + MeTHF
87. Ile + αKG → Glu + $FADH_2$ + 2NADH + CO_2 + SucCoA + AcCoA
88. Leu + αKG + ATP → Glu + $FADH_2$ + NADH + 2AcCoA + ADP + Pi
89. Phe → Tyr+ NADPH
90. Pro → Glu + NADPH
91. Ser → Pyr + NH_3
92. Thr → Gly + NADH + AcCoA
93. Trp + NADPH → AcCoA + Ala
94. Tyr + αKG + SucCoA → Glu + Fum + AcCoA + Succ + CO_2
95. Val + αKG → Glu + $FADH_2$ + 3NADH + $2CO_2$ + SucCoA

Biosynthesis of Nucleotides

96. PRPP + 2Gln + Asp + 2H2O + CO2 + Gly + 4ATP + F10THF →
    2Glu + PPi + 4ADP + + 4Pi + THF + PRAIC + Fum
97. PRAIC + F10THF → IMP +THF
98. IMP + Gln + ATP → NADH + GMP + Glu + AMP + PPi
99. GMP + ATP → GDP + ADP
100. ATP + GDP → ADP + GTP
101. GTP + ADP → ATP + GDP
102. NADPH + ATP → dATP
103. NADPH + GDP + ATP → ADP + dGTP
104. IMP + GTP + Asp → GDP + Pi + Fum + AMP
105. AMP + ATP → 2ADP
106. PRPP + Asp + CaP → UMP + NADH + PPi + Pi + CO2
107. UMP + ATP → UDP + ADP
108. UDP + ATP → ADP + UTP
109. UTP + NH3 + ATP → CTP + ADP + Pi
110. ATP+ NADPH + CDP → dCTP + ADP
111. CDP + ATP → CTP + ADP

112. CTP + ADP → CDP + ATP

113. UDP + MetTHF + 2ATP + NADPH → dTTP + DHF + 2ADP + PPi

Biosynthesis of and interconversion of one-carbon units

114. DHF + NADPH → THF

115. MetTHF + CO_2 + NH_3 + NADH → Gly + THF

116. MetTHF + NADPH → MTHF

117. MetTHF → MeTHF + NADPH

118. MeTHF → F10THF

119. Gly + THF → MetTHF + NH_3+ NADH + CO_2

Transhydrogenation reaction

120. NADH → NADPH

121. NADPH → NADH

Electron Transport System (P/O=2)

122. NADH + 2ADP + 2Pi → 2ATP

123. FADH2 + ADP + Pi → ATP

Transport Reactions

124. CO_2 → exp

125. imp → CO_2

126. imp → NH_3

127. NH_3 → exp

128. 2ATP + 4 NADPH → AMP + ADP + H_2S + PPi + Pi

129. PPi → 2Pi

130. imp → Pi

131. Pi → exp

Biosynthesis of Fatty Acids

132. T3P + NADPH → GL3P

133. 7AcCoA + 6ATP + 12 NADPH → C14:0 + 6ADP + 6Pi

134. 7AcCoA + 6ATP + 11 NADPH → C14:1 + 6ADP + 6Pi

135. 8.2AcCoA + 7.2ATP + 14 NADPH → 7.2PI + 7.2ADP + PA

136. 2ATP + CO_2 + Gln → CaP + Glu + 2ADP + Pi

Other Biomass Components

137. F6P + Gln + AcCoA + UTP → UDPNAG + Glu + Ppi
138. PEP + NADPH + UGPNAG → UDPNAM + Pi
139. R5P + PEP + CTP → CMPKDO + PPi + 2 Pi
140. Ser + CTP + ATP → CDPEtN + ADP + PPi + CO_2
141. S7P + ATP → ADPHep + PPi
142. G6P → G1P
143. G1P → G6P
144. UTP + G1P → UDPGlc + PPi

Biomass Synthesis

145. 0.5352 Ala + 0.28 Arg + 0.22 Asn + 0.22 Asp + 0.09 Cys + 0.09 His + 0.25 Gln + 0.25 Glu + 0.58 Gly + 0.27 Ile + 0.42 Leu + 0.32 Lys + 0.14 Met + 0.0593 Orn + 0.17 Phe + 0.2 Pro + 0.377 Ser + 0.05 Trp + 0.13 Tyr + 0.24 Thr + 0.4 Val + 0.2 GTP + 0.13 UTP + 0.12 CTP +0.22 DATP + 0.02 DCTP + 0.02 DGTP + 0.02 DTTP + 0.129 GL3P + 0.0235 C140 + 0.0235 C141 + 0.259 PA + 0.0433 UDPNAG + 0.0276 UDPNAM + 0.0235 CMPKDO + 0.0235 CDPETN + 0.0157 UDPGLC + 0.02354ADPHEP + 0.154 G1P + 41.139 ATP → Biomass + 41.139 ADP + 41.139 Pi

Serine Alkaline Protease Synthesis

146. (0.145 Ala + 0.0146 Arg + 0.0657 Asn + 0.0328 Asp + 0.0255 Gln + 0.0182 Glu + 0.127 Gly + 0.0182 His + 0.0365 Ile + 0.0584 Leu + 0.0328 Lys + 0.0182 Met + 0.0146 Phe + 0.0365 Pro + 0.116 Ser + 0.0729 Thr + 0.0036Trp + 0.0474 Tyr + 0.113 Val) x 274 + 5.5 ATP → SAP + 5.5 ADP + 5.5 Pi

Maintenance

147. ATP → ADP + Pi
148. Pyr + CO_2 → OA

Abbreviations used in the metabolic reactions

Ac	Acetate
AcCoA	Acetyl coenzyme A
ADP	Adenosine 5′-diphosphate
ADPHep	ADP-D-glycerol-D-mannoheptose
Ala	L-Alanine
AMP	Adenosine 5′-monophosphate
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartate
AspSa	Aspartate semialdehyde
ATP	Adenosine 5′-triphosphate
C14:0	Myristic acid
C14:1	Hydroxymyristic acid
CaP	Carbamoyl-phosphate
CDP	Cytidine 5′-diphosphate
CDPEtN	CDP-ethanolamine
Cit	Citrate
Citr	Citruline
Chor	Chorismate
CMP	Cytidine 5′-monophosphate
CMPKDO	CMP-3-deoxy-D-manno-octulosonic acid
CO_2	Carbondioxide
CTP	Cytidine 5′-triphosphate
Cys	L-Cysteine
dATP	2′-Deoxy-ATP
dCTP	2′-Deoxy-CTP
dGTP	2′-Deoxy-GTP
dTTP	2′-Deoxy-TTP
DC	L-2,3 dihyrodipicolinate
DHF	7,8-Dihydrofolate
E4P	Erythrose 4-phosphate
F10THF	N^{10}-Formyl-THF
F6P	Fructose 6-phosphate
FA	Fatty acids
FADH	Flavine adeninedinucleotide (reduced)
Frc	Fructose

Fum	Fumarate
G1P	Glucose 1-phosphate
G6P	Glucose 6-phosphate
GDP	Guanosine 5′-diphosphate
GL3P	Glycerol 3-phosphate
Glc	Glucose
Gln	L-Glutamine
Glu	L-Glutamate
Gluc	Gluconate
Gluc6P	Gluconate 6-phosphate
Glx	Glyoxylate
Gly	L-Glycine
GMP	Guanosine 5′-monophosphate
GTP	Guanosine 5′-triphosphate
H_2S	Hydrogen sulfide
His	L-Histidine
HSer	Homoserine
ICit	Isocitrate
IGP	Indoleglycerolphosphate
Ile	L-Isoleucine
IMP	Inosinemonophosphate
αKG	α-ketoglutarate
Kval	Ketovaline
Lac	Lactate
Leu	L-Leucine
Lys	L-Lysine
Mal	Malate
Man	Mannose
Man6P	Mannose 6-phosphate
mDAP	meso-Diaminopimelate
Met	L-Methionine
MeTHF	N^5- N^{10}-methenyl-THF
MetTHF	N^5- N^{10}-methylene-THF
MTHF	N^5- methyl-THF
NADH	Nicotinamide-adeninedinucleotide (reduced)
NADPH	Nicotinamide-adeninedinucleotide phosphate (reduced)
NH_3	Ammonia
OA	Oxalacetate

Orn	Ornithine
PEP	Phosphoenolpyruvate
PG3	Glycerate 3-phosphate
Phe	L-Phenylalanine
Pi	Inorganic ortophosphate
PPi	Inorganic pyrophosphate
PRAIC	5’-Phosphoribosyl-4-carboxamide-5-aminoimidazole
Pro	L-Proline
PRPP	5-Phospho-D-ribosylpyrophosphate
Pyr	Pyruvate
R5P	Ribulose 5-phosphate
Rib5P	Ribose 5-phosphate
S7P	Sedoheptulose-7-phosphate
Ser	L-Serine
Suc	Succinate
SucCoA	Succinate coenzyme A
Xyl5P	Xylulose 5-phosphate
Tet	L-2,3,4,5 Tetrahydrodipicolinate
T3P	Triose 3-phosphate
THF	Tetrahydrofolate
Thr	L-Threonine
Trp	L-Tyrptophan
Tyr	L-Tyrosine
UDP	Uridine 5’-diphosphate
UDPGlc	UDP-glucose
UDPNAG	UDP-N-Acetyl-glucosamine
UDPNAM	UDP-N-Acetyl-muramic acid
UMP	Uridine 5’-monophosphate
UTP	Uridine 5’-triphosphate
Val	L-Valine

APPENDIX F

GRAPHS USED IN ELASTICITY COEFFICIENT DETERMINATION

In graphs, x-axis corresponds to intracellular aspartate concentration and y-axis corresponds to J_3, the flux of block 3.

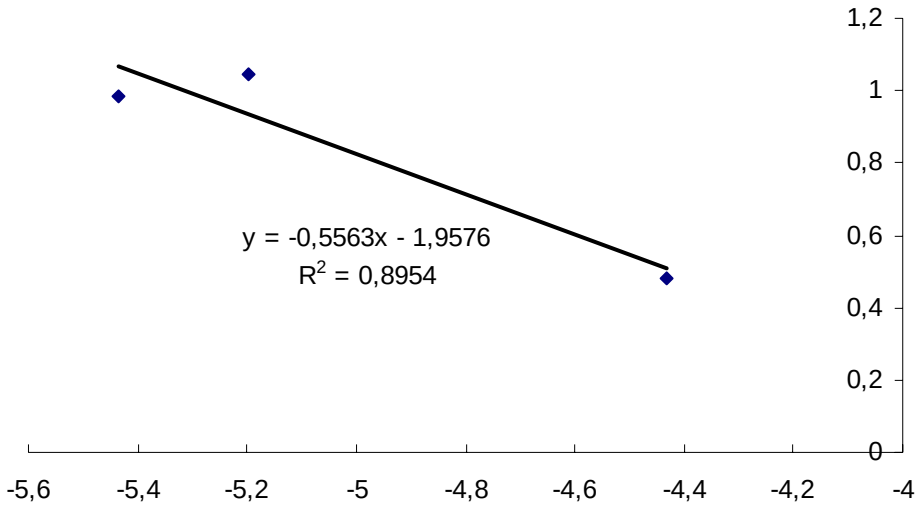

Figure A.3 Elasticity of Block 1 to intracellular Asp level 10 min after perturbation.

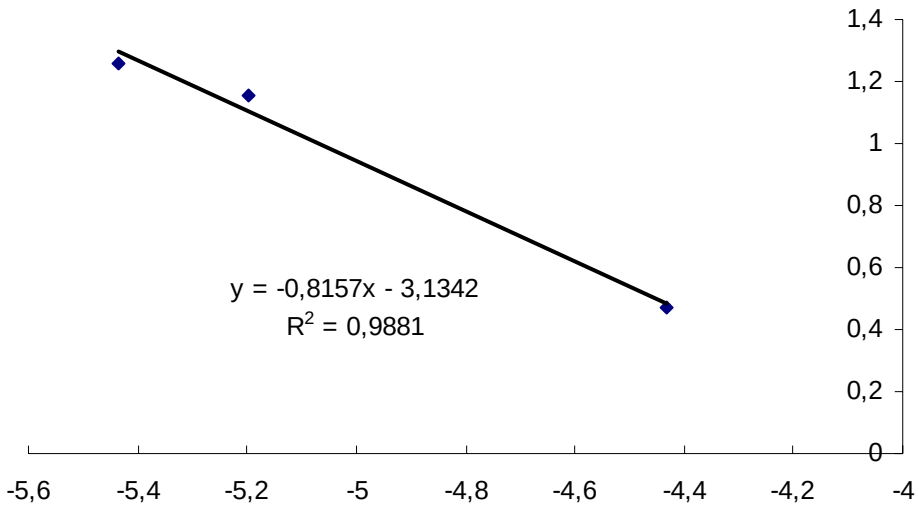

Figure A.4 Elasticity of Block 2 to intracellular Asp level 10 min after perturbation.

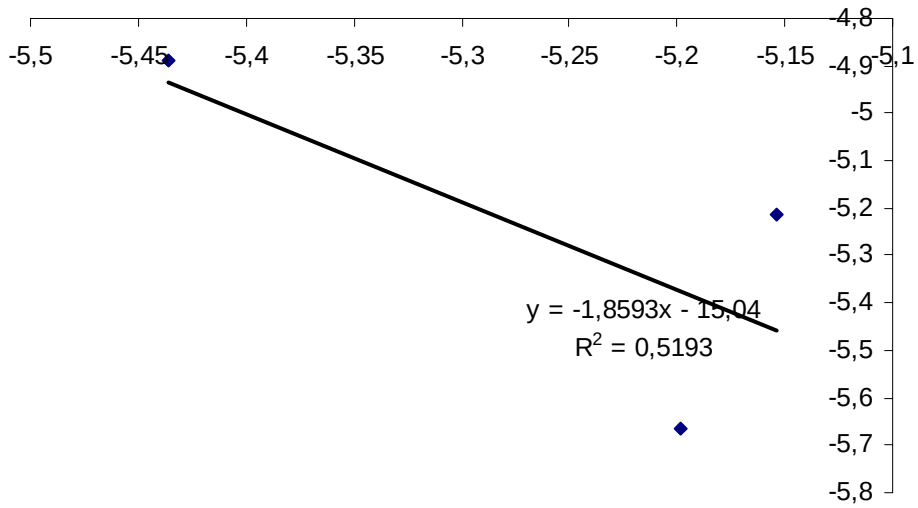

Figure A.5 Elasticity of Block 3 to intracellular Asp level 10 min after perturbation.

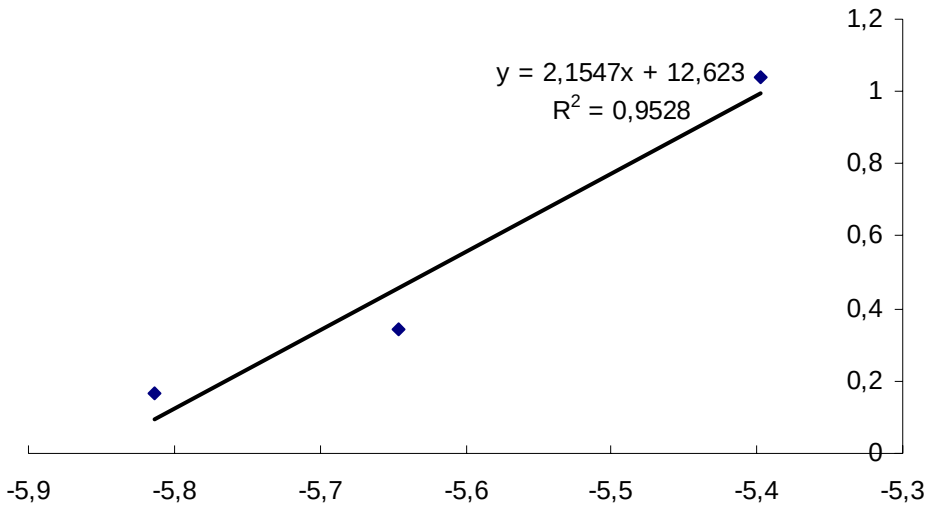

Figure A.6 Elasticity of Block 1 to intracellular Asp level 20 min after perturbation.

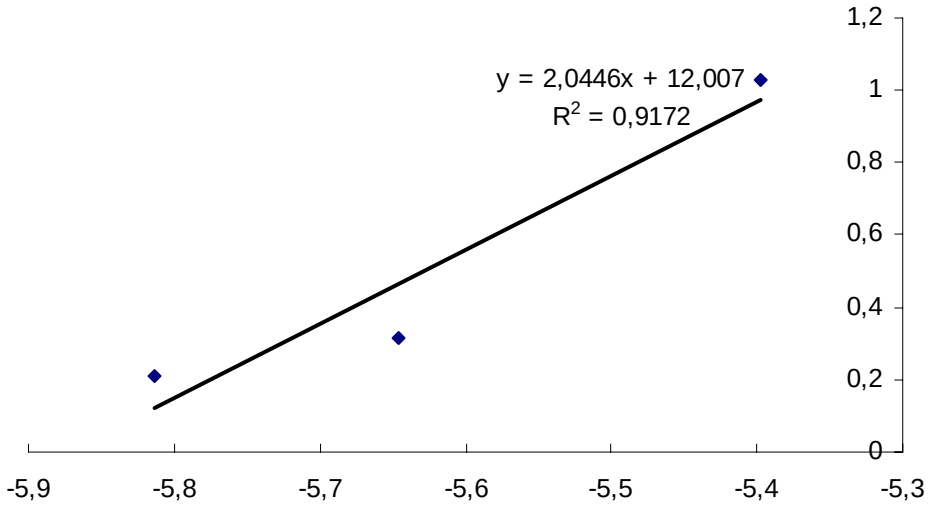

Figure A.7 Elasticity of Block 2 to intracellular Asp level 20 min after perturbation.

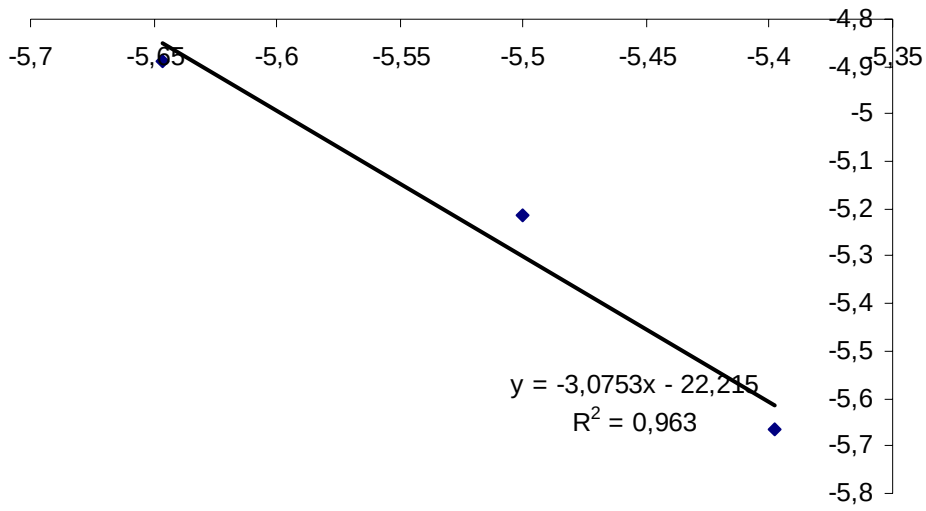

Figure A.8 Elasticity of Block 3 to intracellular Asp level 20 min after perturbation.

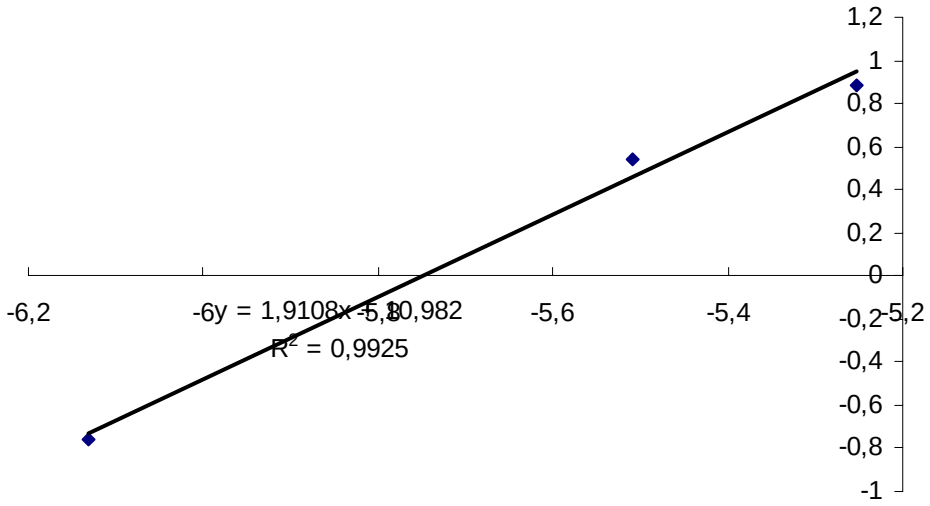

Figure A.9 Elasticity of Block 1 to intracellular Asp level 30 min after perturbation.

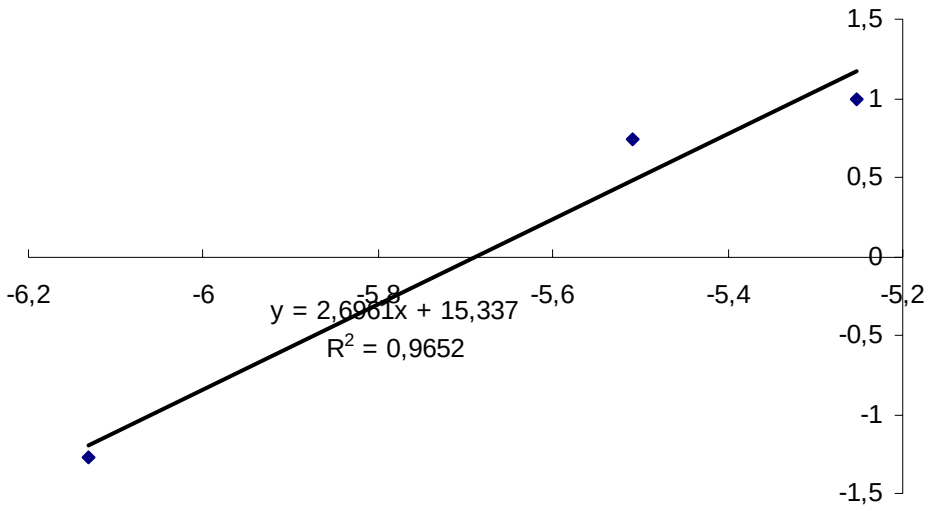

Figure A.10 Elasticity of Block 2 to intracellular Asp level 30 min after perturbation.

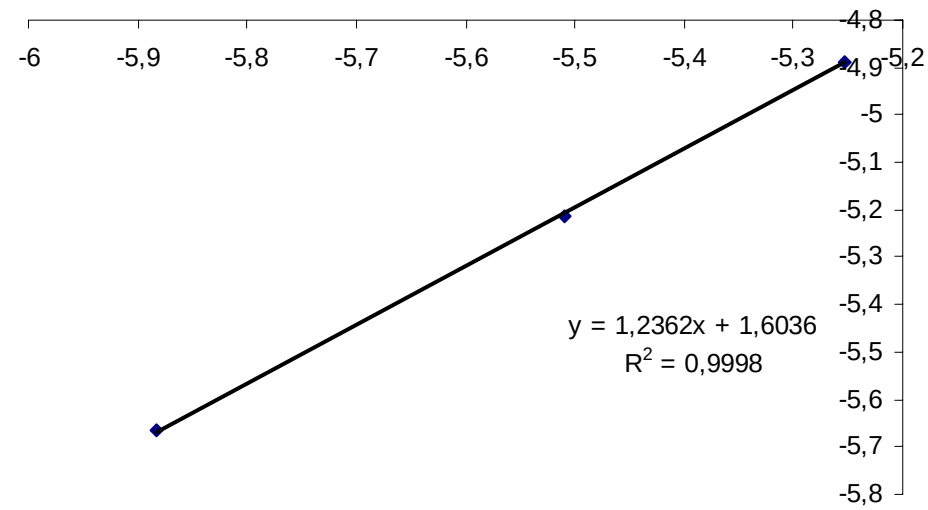

Figure A.11 Elasticity of Block 3 to intracellular Asp level 30 min after perturbation.

APPENDIX G

DETERMINATION OF LIQUID PHASE MASS TRANSFER COEFFICIENT AND OXYGEN UPTAKE RATE OF THE MICROORGANISM

Oxygen uptake rate (r_0) is determined by calculating the slope of t vs C_o diagram when airflow is switched off and the graph demonstrates a linear behavior.

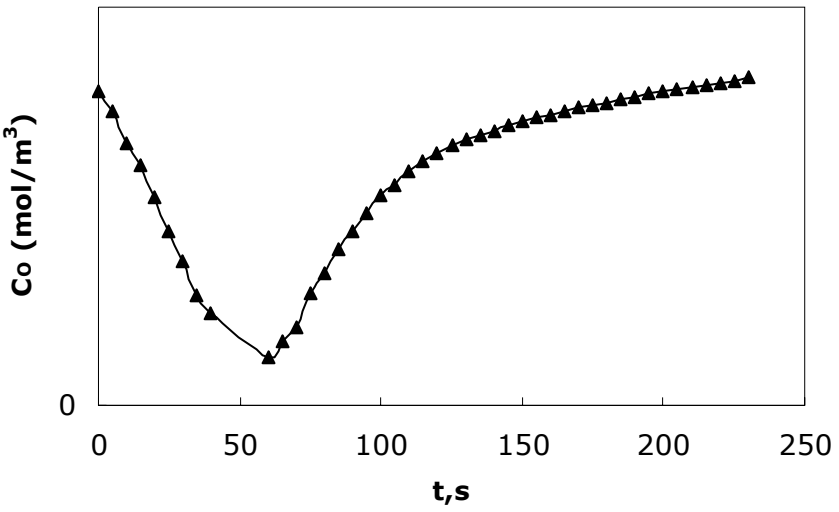

Figure A.12 Variation of oxygen concentration with time.

Slope= $-r_0$

$-r_0$= 0.002 mol m^{-3} s^{-1}

Liquid phase mass transfer coefficient, k_La, is determined by calculating the slope of ($dC_O/dt-r_0$) vs C_O diagram when the air flow is switched on.

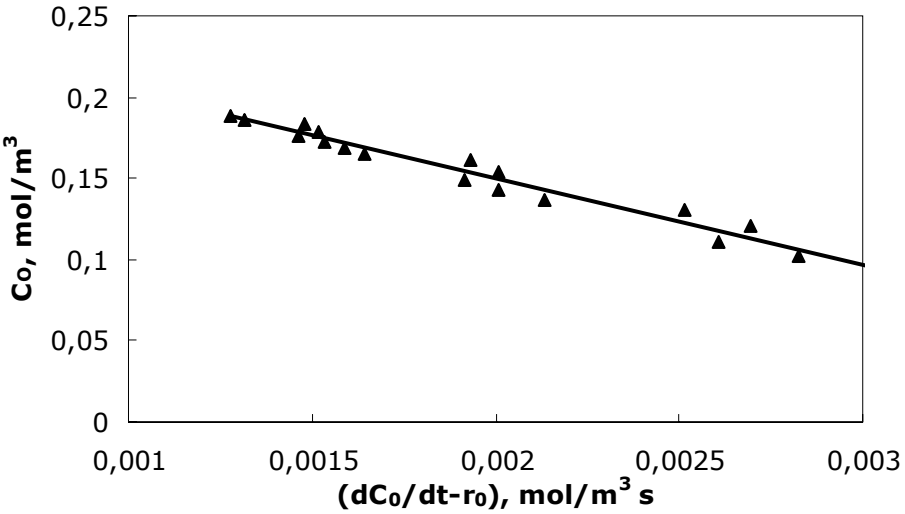

Figure A.13 Determination of k_La.

Slope= -1/ k_La

Slope= -38,921

k_La= 0.02569 s^{-1}

APPENDIX H

SAMPLE CALCULATIONS FOR DETERMINATION OF FLUX CONTROL COEFFFICIENTS

The equations to determine the FCCs were presented in section 3.7. Having determined the elasticity coefficients, one could determine the values for FCCs by simultaneous solution of the following three equations:

$$C_1^{J_3} + C_2^{J_3} + C_3^{J_3} = 1$$

$$C_1^{J_3}\varepsilon_{Asp}^1 + C_2^{J_3}\varepsilon_{Asp}^2 + C_3^{J_3}\varepsilon_{Asp}^3 = 0$$

$$\frac{C_1^{J_3}}{J_1} + \frac{C_2^{J_3}}{J_2} = 0$$

10 minutes after perturbation, the elasticity values are determined to be

ε^1_{Asp} = -0.56, ε^2_{Asp}=-0.82 and ε^1_{Asp}= -1.86.

Substituting the elasticity values to the constructed equations gives;

$$C_1^{J_3} + C_2^{J_3} + C_3^{J_3} = 1 \qquad (1)$$

$$-0.56C_1^{J_3} - 0.82C_2^{J_3} - 1.86C_3^{J_3} = 0 \qquad (2)$$

$$\frac{C_1^{J_3}}{1.62} + \frac{C_2^{J_3}}{1.60} = 0 \qquad (3)$$

Rearranging equation 3 and eliminating $C_1^{J_3}$ gives;

$$C_1^{J_3} = -1.013C_2^{J_3}$$

Substituting in equations 1 and 2;

$$-0.013C_2^{J_3} + C_3^{J_3} = 1 \qquad (4)$$

$$-0.25C_2^{J_3} - 1.86C_3^{J_3} = 0 \qquad (5)$$

Rearranging equation 5 and solving for $C_2^{J_3}$;

$$C_2^{J_3} = -7.38C_3^{J_3} \qquad (6)$$

Substituting this value in equation 4 to obtain $C_3^{J_3}$, consequently $C_1^{J_3}$ and $C_2^{J_3}$ gives the solution of the system as;

$C_1^{J_3} = 6.8$, $C_2^{J_3} = -6.7$ and $C_3^{J_3} = 0.9$