COMPARATIVE ANALYSIS OF PRODUCT AND BY-PRODUCT DISTRIBUTIONS IN DEFINED AND COMPLEX MEDIA IN SERINE ALKALINE PROTEASE PRODUCTION BY RECOMBINANT *Bacillus subtilis*

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ABSTRACT

COMPARATIVE ANALYSIS OF PRODUCT AND BY-PRODUCT DISTRIBUTIONS IN DEFINED AND COMPLEX MEDIA IN SERINE ALKALINE PROTEASE PRODUCTION BY RECOMBINANT *Bacillus subtilis*

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In this study, firstly the effects of aspartic acid group amino acids -which were reported to be the potential bottleneck in serine alkaline protease (SAP) synthesis- on SAP production were investigated by substituting at a concentration range of 0-15 mM by using recombinant *Bacillus subtilis* carrying pHV1434::*subC* gene. All aspartic acid group amino acids except threonine inhibited SAP activity when $C_{AA} \geq$ 2.5 mM. The highest SAP activities with asparagine, aspartic acid, lysine, threonine, isoleucine and methionine were

found to be 1.89-, 1.87-, 1.61-, 1.48-, 1.4-, and 1.4-fold higher than the reference medium activity, respectively, when the concentration of each amino acid was C_{AA}=0.25 mM. The product and by-product distributions in defined and complex media in SAP production were also analyzed and compared in order to obtain a depth in-sight on functioning of the metabolic reaction network. The highest SAP activity in complex medium was found to be 3–fold higher than defined medium activity, while, specific SAP production rate was 1.2- fold higher. The highest cell concentration in complex medium (C_X= 14.3 g/dm^{-3}) was 8.1-fold higher than that obtained in defined medium (C_X= 1.75 g/dm^{-3}). In both media, oxaloacetic acid was observed extracellularly and intracellularly. In complex medium there was also succinic acid in the extracellular medium indicating that the operation of TCA cycle was insufficient. In both media serine, valine and glycine were observed neither in the extracellular nor in the intracellular media indicating that the synthesis of these amino acids can be a secondary rate limiting step. In defined medium asparagine was present neither in the cell nor in fermentation broth whereas, methionine was observed in the cell in high amounts, probably due to the lower flux values towards asparagine. Thus, in defined medium the synthesis of asparagine can also be a potential bottleneck in SAP production in defined medium.

Keywords: *Bacillus*, serine alkaline protease, defined medium, complex medium, by-product, amino acid, organic acid, extracellular, intracellular.

ÖZ

REKOMBINANT *Bacillus subtilis* İLE SERİN ALKALİ PROTEAZ ÜRETİMİ SIRASINDA TANIMLANMIŞ VE KOMPLEKS ORTAMLARDAKİ ÜRÜN VE YAN-ÜRÜN DAĞILIMLARININ KARŞILAŞTIRMALI ANALİZİ

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Bu çalışmada ilk olarak, serin alkali proteaz (SAP) üretiminde potansiyel darboğaz oluşturduğu söylenilen aspartik asit grubu amino asitlerin SAP üretimine etkisi pHV1434::*subC* geni taşıyan recombinant *Bacillus subtilis* kullanılarak 0-15 mM konsantrasyon aralığında araştırılmıştır. Triyonin dışındaki bütün aspartik asit grubu amino asitler $C_{AA} \geq$ 2.5 mM iken SAP aktivitesini inhibe etmiştir. Herbir amino asit derişimi, C_{AA}= 0.25 mM iken; asparajin, aspartik asit,

lözin, triyonin, izolözin ve metiyoninin için elde edilen en yüksek SAP aktiviteleri sırasıyla referans ortam aktivitesinin 1.89, 1.87, 1.61, 1.48, 1.4 ve 1.4 katı olarak bulunmuştur. Biyokimyasal tepkime sisteminin işleyişi ile ilgili derinlemesine bilgi elde etmek amacıyla, tanımlanmış ve kompleks ortamlardaki ürün ve yan-ürün dağılımları analiz edilmiş ve karşılaştırılmıştır. Kompleks ortamdaki en yüksek SAP aktivitesi tanımlanmış ortamdakinin 3-katı iken, özgül SAP üretim hızı 1.2-katı olarak bulunmuştur. Kompleks ortamdaki en yüksek hücre konsantrasyonu (C_X= 14.3 g dm^{-3}), tanımlanmış ortamdakinin (C_X= 1.75 g dm^{-3}) 8.1-katı olarak bulunmuştur. Her iki ortamda da okzalik asit hem hücre içinde hem de hücre dışında gözlenmiştir. Kompleks ortamda ayrıca süksinik asit de bulunmuştur. Bu bulgular, TCA döngüsünün yetersiz çalıştığını göstermektedir. Tanımlanmış ve kompleks ortamların ikisinde de serin, valin ve glisin ne hücredışında ne de hücreiçinde gözlenmiştir. Bu amino asitlerin sentezinin hız kısıtlayıcı bileşik olabilme potansiyeline sahiptir. Tanımlanmış ortamda asparajin hücreiçi ve hücredışında gözlenmezken, metiyonin hücre içinde fazla miktarda bulunmaktadır. Büyük olasılıkla asparajine giden akının düşük oluşundan kaynaklanmaktadır. Sonuç olarak aspartik asit grup amino asit asparajin sentezi SAP üretiminde tanımlanmış ortamda darboğaz oluşturma potansiyeline sahiptir.

Anahtar Kelimeler: *Bacillus*, serin alkali proteaz, tanımlanmış ortam, kompleks ortam, yan-ürün, amino asit, organik asit, hücredışı, hücreiçi.

To The Memory of My Grandfather and Grandmother,

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LIST OF SYMBOLS AND ABBREVIATIONS

A	SAP activity, U cm^{-3}
A'	Specific SAP activity, U g_X^{-1}
Ac	Acetate
AcCoA	Acetyl coenzyme A
ADP	Adenosine 5’-diphosphate
Ala	L-Alanine
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartate
ATP	Adenosine 5’-triphosphate
CaP	Carbamoyl-phosphate
C_{AA}	Amino acid concentration
C_{Asp}^0	Initial aspartic acid concentration, mM
C_{Asn}^0	Initial asparagine concentration, mM
C_{Ile}^0	Initial isoleucine concentration, mM
C_{Lys}^0	Initial lysine concentration, mM
C_{Met}^0	Initial methionine concentration, mM
C_{Thr}^0	Initial threonine concentration, mM
C_G	Glucose concentration, g dm^{-3}
C_X	Cell concentration, g dm^{-3}
Chor	Chorismate
CO_2	Carbondioxide

Cys	L-Cysteine
DNA	Deoxyribonucleic acid
Fum	Fumarate
Gln	L-Glutamine
Glu	L-Glutamate
Gly	L-Glycine
His	L-Histidine
Ile	L-Isoleucine
αKG	α-ketoglutarate
Kval	Ketovaline
Lac	Lactic acid
Leu	L-Leucine
Lys	L-Lysine
mDAP	meso-Diaminopimelate
Met	L-Methionine
MeTHF	N^5- N^{10}-methenyl-THF
NADH	Nicotinamide-adeninedinucleotide (reduced)
NADPH	Nicotinamide-adeninedinucleotide phosphate (reduced)
OA	Oxalacetate
Orn	Ornithine
PEP	Phosphoenolpyruvate
PG3	Glycerate 3-phosphate
Phe	L-Phenylalanine
Pi	Inorganic ortophosphate
Pro	L-Proline
Pyr	Pyruvate
RNA	Ribonucleic acid

r_P	Specific serine alkaline protease production rate, U $g_X^{-1}h^{-1}$
Ser	L-Serine
Suc	Succinic acid
t	Residence time, h
TE	Tris EDTA
THF	Tetrahydrofolate
Thr	L-Threonine
Trp	L-Tyrptophan
Tyr	L-Tyrosine
UDPNAG	UDP-N-Acetyl-glucosamine
Val	L-Valine

CHAPTER 1

INTRODUCTION

Enzymes are a major resource utilized by the food, chemical, and allied industries to produce a wide range of biotechnology products and have already been recognized as valuable catalysts for various organic transformations and production of fine chemicals and pharmaceuticals. Enzymes can either be produced by isolation from plant and animal tissues or by microorganisms, plant and animal cells. The latter is known as bioprocesses. In industrial production of enzymes, microorganisms are preferred because;

1. they are generally cheaper to produce,
2. bioprocesses in which microorganisms are used have short operation time,
3. enzyme production can be increased by modifying the medium composition or by genetic manipulation of the microorganism.

Table 1.1 summarizes 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). Table 1.2 summarizes the major producers of industrial enzymes. The majority of the industrial enzyme production belongs to Novo Industri A/S (Denmark), Gist-brocades (Netherlands), and Miles Laboratories (U.S.), controlling about 70% of the market between them (Kalizs, 1988).

Table 1.2 Major producers of industrial enzymes (Kalizs, 1998)

Company	Market Share (%)
Novo Industri A/S (Denmark)	40
Gist-brocades (Netherlands)	20
Miles Laboratories (USA)	10
Hansen (Denmark)	5
Sanofi (France)	5
Finnish Sugar (Finland)	5
Others	15

Most commercial enzymes are produced by microorganisms belonging to the *Bacillus* and *Aspergillus* genera. The majority of the *Bacillus* species are harmless and are well known for their ability to excrete enzymes such as amylases and proteases and are therefore, excellent candidates for large-scale production of these enzymes. (Moon and Parulekar, 1991)

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 account for approximately 35% of the microbial enzyme sales. 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, there are some important criteria that must be taken into account in order to have high product yield and selectivity. These are; (1) selection of microorganism, (2) medium design and (3) bioreactor operation parameters. 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. Serine alkaline proteases, like most of the commercial enzymes, are produced by organisms belonging to the *Bacillus* species since they are able to secrete large number of extracellular enzymes (Priest, 1977). In bioprocesses carbon and energy sources and their concentrations are indeed important as they are tools for bioprocess medium design (Çalık *et al.*, 2001). Culture medium supplies the microorganism with all the essential elements for growth. 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. There are three types of media; defined, semi-defined and complex. 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.

Bioreactor operation conditions such as oxygen transfer rate, pH and temperature 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). Product yield and selectivity are also strongly affected by pH. Some bioprocesses require controlled-pH conditions, and some require uncontrolled-pH operations.

Microorganisms act as microbioreactors in the bioreactor system and thousands of reactions take place in the microbioreactor that are coupled with the medium composition and bioreactor operation conditions. Cellular growth is 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 then the metabolic products are excreted back into the extracellular medium. SAP synthesis depends on good coupling of supply and demand of the amino acids in the cell, the syntesis of which are dependent on the intracellular concentrations of intermediatary metabolites and intracellular reaction network enzyme activities.

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. 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 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 whereupon the rate limiting step 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. 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. 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. In the literature there are no studies related with the influence of aspartic acid amino acids on SAP production, which were reported to be a potential bottleneck in SAP synthesis (Çalık *et al.*, 1999), and also there are no publications that compares the product and by-product distributions in serine alkaline protease production in defined and complex media.

Similar to the synthesis of other proteins, SAP synthesis depends on good coupling of supply and demand of amino acids in the cell, thus in a recombinant bacilli the intracellular concentrations of intermediary metabolites and intracellular reaction network enzyme activities and intracellular control mechanisms are indeed important. Each microorganism can tolerate different certain intracellular amino acid concentrations and depending on the intracellular regulations and control mechanisms different inhibitions take place. Consequently, insufficient synthesis of some of the amino acid(s) creates reaction rate limitations in the bioreaction network for SAP production as the controlling amino acids. In this case, supply of the controlling amino acids to the fermentation broth and supplied period are indeed important.

In this context, in this study, first of all the effects of aspartic acid group amino acids, which were reported to be the potential bottleneck in SAP synthesis (Çalık *et al.*, 1999), were investigated in defined medium by using recombinant *Bacillus subtilis*; thereafter, the product and by-product distributions of the bioprocess for SAP production were determined in defined and complex media. Lastly, the results were compared, in order to analyze the differences between defined and complex media and to find out the potential strategies in order to increase SAP production further by medium modification, since the attainable enzyme activity and cell yields are much higher in complex medium, than that of defined medium.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

Enzymes, which are proteins in nature, are catalysts of extraordinary efficiency and specificity. They are so effective as biological catalysts that most of the reactions they catalyze would not proceed in a reasonable time without extremes of temperature, pressure or pH (Horton, 1992). They increase the rates of the reactions by factors of at least one million compared to the uncatalyzed reactions. They lower the activation energy of the catalyzed reaction by binding the substrate and forming an enzyme-substrate complex (Kirk and Othmer, 1994). Enzymes have a number of distinct advantages over conventional chemical catalysts. Foremost amongst these are their specificity and selectivity not only for particular reactions but also in their discrimination between similar parts of molecules (regiospecificity) or optical isomers (stereospecificity). Enzymes work under generally mild processing conditions of temperature, pressure and pH. 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).

Enzymes, like other proteins, have molecular weights ranging from about 12,000 to over 1 million. Some enzymes require no chemical groups other than their amino acid residues for activity. 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).

Enzymes have been classified into six main types, depending on the nature of the reaction catalysed. 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.

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. Proteases are involved mainly in degradation of large polypeptide substrates into peptides and amino acids before cellular uptake. Proteases 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; and
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, whatever their amino acid composition is, 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. SAP are 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 (Çalık *et al*., 2001).

2.3 Bioprocess Parameters for Serine Alkaline Protease Production

In enzyme production by bioprocesses, there are some important criteria that must be taken into account 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 fermentations and produce large cell mass per volume quickly on cheap media (Kirk and Othmer, 1994).

2.3.1.1 The Genus *Bacillus*

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 Gram positive and are 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 excreate 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. Its ability to secrete a wide variety of proteins far exceeds than of its prokaryotic competitor, *E.coli*,
6. The tendency of *B.subtilis* to produce several proteases is able to degrade foreign proteins either intracellularly or extracellularly. ,

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

Carbon and energy sources and their concentrations are indeed important as they are tools for bioprocess medium design (Çalık *et al.*, 2001). Culture medium supplies the microorganism with all the essential elements for growth.

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 the 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 The composition of a bacteria (*E.coli*)

Species	Content (g/g of 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 micronutrients 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 solubles
n-Alkanes	Cottonseed extract
Gas oil	Dried blood
Sulfite waste liquor	Corn steep liquor
Domestic sewage	Fish solubles 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. In this study, the components of the complex medium are glucose and defatted soybean which was reported to have the highest SAP activity than the other complex medium components (Özdemir, 2003).

Glucose

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 require economically inefficient processing (Cejka, 1985).

Soybean

Soybean is most frequently used in bioprocesses. It is commercially available in three different grades according to the fat content: full-fat meal with a minimum of 18 % fat, low fat meal with 4.5-9 % fat, and defeated meal with a maximum of 2 % fat. The amino acid content and inorganic content of soybean are summarized in Table 2.6 and Table 2.7.

Table 2.6 Amino acid content of soybean (Cejka, 1985)

Amino acid	Percentage
Arginine	7.5
Histidine	2.3
Lysine	6.7
Tyrptophan	1.6
Phenylalanine	5.2
Threonine	3.9
Valine	5.4
Leucine	8.1
Isoleucine	5.3
Methionine	1.4
Glutamic acid	18.4

Table 2.7 Inorganic content of soybean (Cejka, 1985)

Constituent	Percentage
Potassium	1.67
Sodium	0.34
Calcium	0.28
Magnesium	0.22
Phosphorus	0.66
Sulfur	0.41
Chloride	0.024
Iodine	0.000054
Iron	0.0097
Copper	0.0012
Manganese	0.0028
Zinc	0.0022
Aluminum	0.0007

Carbon and energy sources and their concentrations are indeed important as they are tools for bioprocess medium design (Çalık *et al.*,2001). *Bacillus* strains can utilize alanine (Ala), arginine (Arg), asparagines (Asn), aspartate (Asp), glutamate (Glu), glutamine (Gln), histidine (His), isoleucine (Ile), ornithine (Orn), proline (Pro), threaonine (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 the literature there are a lot of 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. Frakena *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 compex 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. 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.

In the literature there are no studies related with the influence of aspartic acid amino acids on SAP production, which were reported to be a potential bottleneck in SAP synthesis, and also there are no publications that compares the product and by-product distributions in serine alkaline protease production in defined and complex media.

2.3.3 Bioreactor Operation Parameters

Bioreactor operation conditions such as oxygen transfer rate, pH and temperature 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 parameter that is normally desired to keep both these variables constant and at their optimal values throughout the fermentation process. 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).

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 (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 a remarkable 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 8.2-8.5, whereas for netrophilic *Bacillus* species (e.g. *B.subtilis*, *B.licheniformis*) this value is 7.5 (Çalık *et al.*, 2001). 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 without any explanation or discussion. 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. 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 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 whereupon the rate limiting step of the bioprocess. In all the reported works in the literature, the temperature is between 35-40 °C. Frankena *et al.* (1985) studied protease production at pH=7, T=37°C; Kole *et al.*(1988) at pH=6.9-7.2, T=35°C; Wright *et al.* (1992) at pH=7.5, T= 34°C; Hübner *et al.*, (1993) and van Putten *et al.*(1996), at initial pH=6.8, T=39.5°C 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.

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 favourable 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 then the metabolic products are excreted 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.

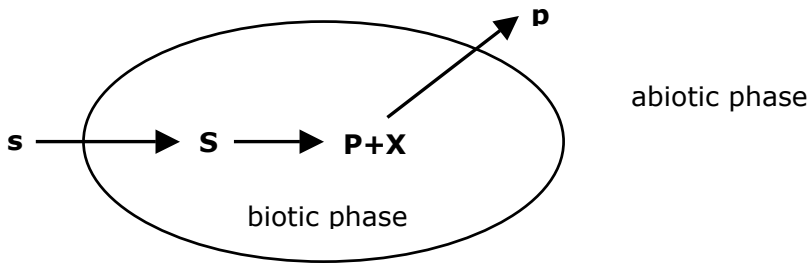

Figure 2.1 Reactions involved in cellular growth: Upper-case letters represent intracellular species (S, substrates; P, products; X biomass components) and lower-case letters represent extracellular species (s, substrates; p, products)

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

Tricaboxylic 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 completely oxidized to CO_2. TCA cycle functions not only as a process for energy generation but also a supply of precursors for biosynthesis of amino acids and nucleotides. In TCA cycle there are two key intermediates that are used in the synthesis of necessary amino acids for SAP production (Horton, 1992). From OA; aspartic acid group amino acids, from α-ketoglutarate glutamic acid group amino acids are synthesized.

Similar to the synthesis of other proteins, SAP synthesis depends on good coupling of supply and demand of amino acids in the cell, thus in a recombinant bacilli the intracellular concentrations of intermediary metabolites and intracellular reaction network enzyme activities and intracellular control mechanisms are indeed important. Each microorganism can tolerate different certain intracellular amino acid concentrations and depending on the intracellular regulations and control mechanisms different inhibitions take place. Consequently, insufficient synthesis of some of the amino acid(s) creates reaction rate limitations in the bioreaction network for SAP production as the controlling amino acids. In this case, supply of the controlling amino acids to the fermentation broth and supplied period are indeed important.

2.4.1 Synthesis of Aspartic Acid Group Amino Acids

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 via oxalo acetic 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 brances. From one branch lysine; from the other branch methionine, threonine and isoleucine are synthesized.

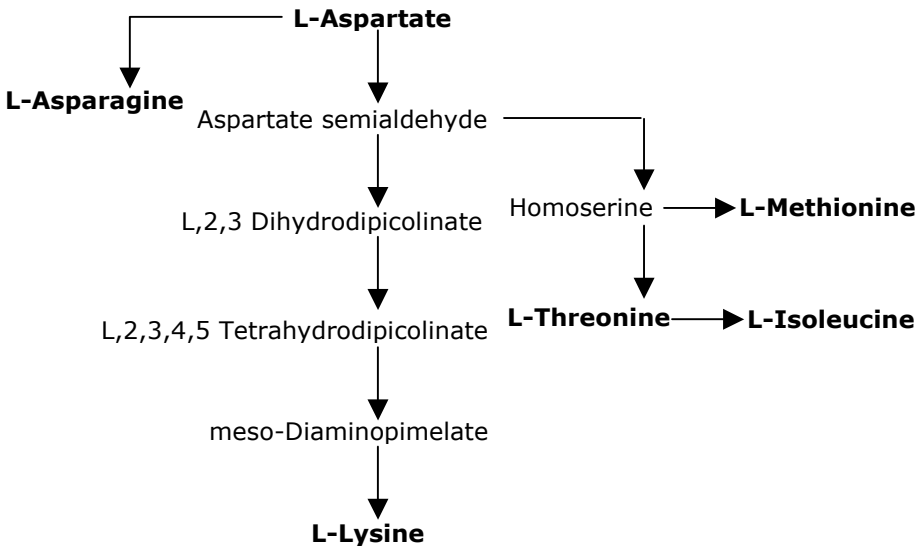

Figure 2.2 Aspartic acid pathway

CHAPTER 3

MATERIALS AND METHOD

3.1 Microorganism

Recombinant *Bacillus subtilis* (BGSC-1A751), carrying pHV1434::*subC* gene (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 micobank, 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 25 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 250 ml air-filtered Erlenmeyer flasks with a working volume of 44 ml. The composition of the reference defined and reference complex media are given in Table 3.3 and Table 3.4 respectively.

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

Table 3.4 The composition of reference complex medium for recombinant *Bacillus* sp.

Compound	Concentration, kg m^{-3}
Soybean	20
Glucose	8.0
NaH_2PO_4	2.815
$Na_2HPO_4.2H_2O$	0.028
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 and also DNA and RNA concentrations were determined. Figure 3.1 summarizes the analysis carried out for the samples.

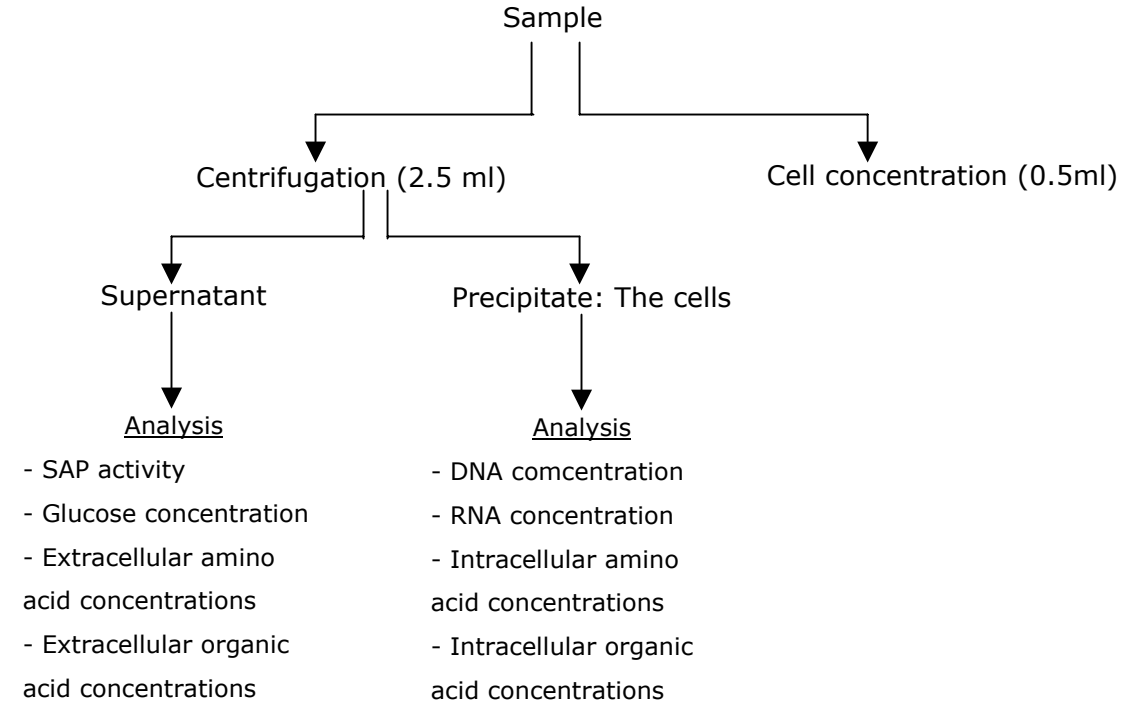

Figure 3.1 Analysis carried out for samples

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), the so-called 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 constructed. The preparation 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 constructing a calibration curve, which gives the exact relationship between A550 and glucose concentration.

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 Metabolite Concentrations

In order to determine the intracellular metabolites and their concentrations, biomass was harvested by centrifugation at 13500 g for 10 min (The supernatant was stored at -20°C for the determination of glucose, extracellular amino acid and organic acid concentrations.). After a washing step with distilled water (4 °C) and 0.009 M NaOH solution (4 °C) the biomass was hydrolyzed using 750 μL, 6 M HCl at 105°C for 18 h. After the hydrolysis, 525 μL isopropanol was added and the sample was kept at -20°C for 20 min in order to accelerate the precipitate of DNA and RNA. Thereafter, the sample was centrifuged at 12000 g and at T=0°C. The supernatant was stored at -20°C for the determination of intracellular amino acid and organic acid concentrations. The precipitate was used to determine DNA and RNA concentrations; for this aim DNA and RNA was dissolved in 0.1 M TE buffer.

3.5.6.1 Determination of DNA Concentration by Diphenylamine Reaction

The procedure involves chemical hydrolysis of DNA: when heated (e.g. 95°C) in acid (e.g. perchloric acid at a concentration exceeding 1 molar), the glycosidic bond linking the base to the sugar is hydrolyzed with subsequent hydrolysis of the phosphodiester bond linking that nucleotide to the next in a polynucleotide chain.(The reaction requires a deoxyribose sugar and therefore is specific for DNA). Under these conditions, the 2-deoxyribose is converted to w-hydroxylevulinyl aldehyde, which reacts with the compound, diphenylamine, to produce a blue-colored compound. The amount the blue-colored compound formed is determined by measuring the intensity of absorbance of the solution at 600nm. This is related to the DNA concentration by constructing a calibration curve, which gives the exact relationship between A_{600} and DNA concentration.

A. Supplies

* 1.6 M HClO4

* Diphenylamine reagent (freshly prepared): 1 g diphenylamine+ 98 ml glacial acetic acid+2 ml conc. sulfuric acid (use fume hood to make stock solution)

* TE buffer (Appendix E)

B. Procedure

A set of reaction tubes was marked (tape and Sharpie should be used; marking shouldn't be done directly on glassware), and samples (dissolved in TE buffer) were placed into the tubes and diluted to 1 ml with distilled water. Then 1 ml acid was added to each and heated for 15 min at 95oC; thereafter the tubes were cooled to room temperature (≈5 min). In the meantime the spectrophotometer was turned on and is zeroed in at 540 nm; water was used as a blank. After 5 min diphenylamine reagent was added and the tubes were

transferred to a boiling water boiling water bath for 10 min. The tubes were then cooled in an ice bath (≈5 min). The absorbance was read at 540 nm for each tube. Concentrations of the samples were determined by using the previously constructed calibration curve (Appendix F).

3.5.6.2 Determination of RNA Concentration by Orcinol Reagent

RNA concentrations in the cells were determined by Orcinol reagent method (Herbert *et al.*, 1971).

A. Supplies

Solution A: 1g orcinol is dissolved in 100 ml distilled water.

Solution B: 0.09 g $FeCl_3.6H_2O$ is dissolved in 100 ml HCl.

Orcinol reagent: 4-volume solution A is mixed with 1 volume solution B (This solution should be freshly prepared.).

B. Procedure

1. After the biomass was hydrolyzed and DNA and RNA were precipitated by the addition of isopropanol followed by centrifugation. The precipitate was dissolved in TE buffer.

2. 100 μL of the sample was mixed with 200 μL distilled water then 100 μL $HClO_4$ and 700 μL orcinol reagent was added.

3. This mixture was heated in a boiling water bath for 20 min then cooled in an ice bath for 5 min.

4. The spectrophotometer was zeroed in by using a solution which passed through the same steps and contain 100 μL distilled water instead of the sample.

5. 500 μL of this reaction mixture was added to 2000 μL n-butanol and the

absorbance was measured at 670 nm. The absorbance was related to the RNA concentration by constructing a calibration curve, which gives the exact relationship between A670 and RNA concentration. The calibration curve for RNA is given in Appendix G.

3.5.6.3 Determination of Intracellular Amino Acid and Organic Acids Concentrations

After precipitating the DNA and RNA, the supernatent was dried at 60 °C then dissolved in 100 μL acetonitryl and by using the methods given in part 3.5.4 and part 3.5.5, 5 μL and 20 μL of this sample were used to determine the intracellular amino acid and intracellular organic acid concentrations respectively.

CHAPTER 4

RESULTS AND DISCUSSION

Serine alkaline protease (SAP) synthesis depends on good coupling of supply and demand of amino acids in the cell, the synthesis of which are dependent on the intracellular concentrations of intermediary metabolites and intracellular reaction network enzyme activities. Each microorganism can tolerate different certain intracellular amino acid concentrations and depending on the intracellular regulations and control mechanisms different inhibitions take place. Consequently, insufficient synthesis of some of the amino acid(s) creates reaction rate limitations in the bioreaction network for SAP production as the controlling amino acids. In this case, supply of the controlling amino acids to the fermentation broth and supplied period are indeed important. In this study, at first the effects of supply of aspartic acid group amino acids, which were reported to be the potential bottleneck in SAP synthesis (Çalık *et al.*, 1999), were investigated in defined medium by using recombinant *Bacillus subtilis*; thereafter, the product and by-product distributions of the bioprocess for SAP production were determined in defined and complex media. Lastly, the results were compared, in order to analyze the differences between defined and complex media and to find out the potential strategies in order to increase SAP production further by medium modification, since the attainable enzyme activity

and cell yields are much higher in complex medium, than that of the defined medium.

4.1 Effect of Aspartate on Biomass Concentration and SAP Activity

The effects of initial aspartic acid concentration on growth and SAP activity were investigated using r-*B.subtilis* in laboratory scale bioreactors in the range of 0-15mM by using the reference defined production medium given in Table 3.3. Variation in biomass concentration and SAP activity is given in Figure 4.1. The cell growth and the cell concentrations obtained at the end of the fermentation were not affected with the supply of aspartic acid indicating that aspartic acid is synthesized sufficienty in *r-B.subtilis*. On the other hand, the highest SAP production was obtained at 0.25 mM aspartic acid concentration with a value of 1074 U cm^{-3} which was 1.87-fold higher than that obtained for the reference medium indicating that in SAP synthesising period the amount of the aspartic acid is important as it is the precursor of the aspartic acid group amino acids; and aspartic group amino acid content is 26 % of the total amino acids in the SAP molecule. With the further increase in aspartic acid concentration SAP production decreased slightly but for $C_{Asp}^{o} \geq 2.5$ mM inhibited drastically (Figure 4.1).

4.2 Effect of Asparagine on Biomass Concentration and SAP Activity

The effect of initial asparagine concentration on growth and SAP activity were investigated using r-*B.subtilis* in laboratory scale bioreactors in the range 0-15mM by using the reference defined production medium given in Table 3.3. Variation in biomass concentration and SAP activity with the addition of asparagine is given in Figure 4.2. Cell concentrations increased up to t=15h and then reached to stationary phase. Maximum cell concentration was obtained at an asparagine concentration of 15 mM. While supply of 2.5 and 5.0 mM

asparagine didn't change the biomass concentration considerably,higher concentrations increased biomass formation. For low asparagine concentrations (0.25, 0.5, 1.0 mM), SAP activity decreased with the increase in asparagine concentration. The highest SAP activity was obtained as 1082 U cm^{-3} for an asparagine concentration of 0.25 mM at t = 41h. This indicates that with the addition of asparagine to the medium at t=0 h, the cells regulate themselves in order to keep the asparagine concentration at a certain level. For this purpose, the intracellular enzyme levels should be regulated in such a way that asparagine can be synthesized in sufficient amounts when needed. The highest activity is 1.89-fold higher than the reference medium activity. On the other hand, asparagine concentrations higher than 2.5 mM inhibited SAP production. Addition of asparagine increased cell concentration however, the addition of aspartic acid didn't affect cell growth. In contrast to the aspartic acid results, asparagine synthesis should be insufficient and this can be a potential bottleneck in SAP production.

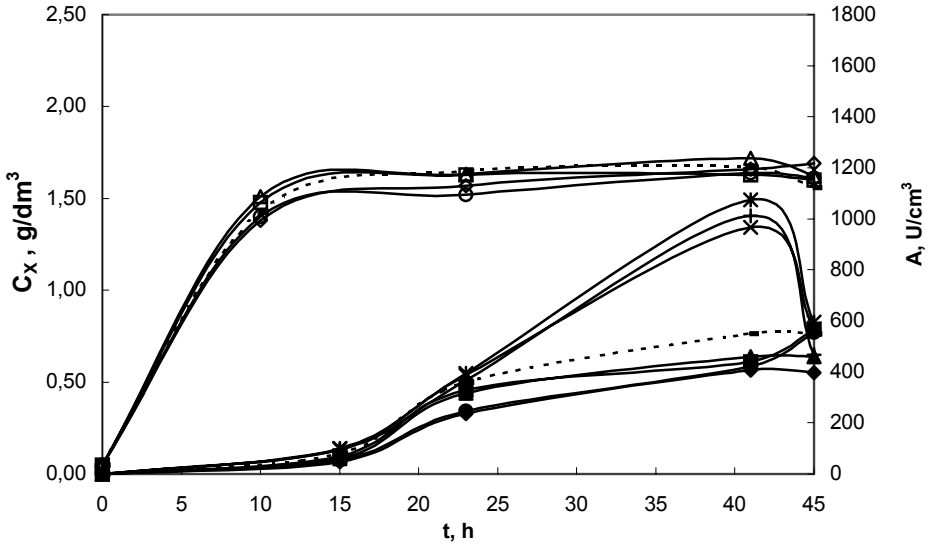

Figure 4.1. Effect of aspartate on biomass concentration and SAP activity. C_{Asp}^0 : (∗) 0.25 mM; (+) 0.5 mM; (×) 1.0 mM; (□ , ■) 5.0 mM; (△,▲) 7.5 mM; (○, ●) 12.5 mM; (◆,◇) 15.0 mM

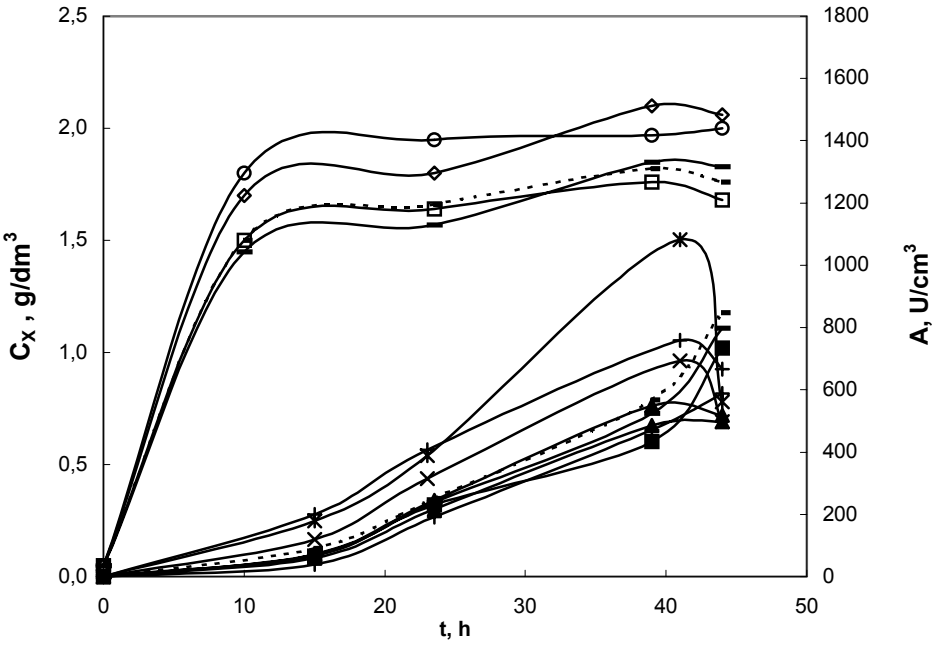

Figure 4.2. Effect of asparagine on biomass concentration and SAP activity. C_{Asn}^0 : (∗) 0.25 mM; (+) 0.5 mM; (×) 1.0 mM; (–,–) 2.5 mM; (□ , ■) 5.0 mM; (○, ●) 12.5 mM; (◆,◇) 15.0 mM

4.3 Effect of Isoleucine on Biomass Concentration and SAP Activity

The effects of initial isoleucine concentration on growth and SAP activity were investigated in the range of 0-15mM using r-*B.subtilis* in laboratory scale bioreactors. Variation of biomass concentration and SAP activity are given in Figure 4.3. Cell growth was inhibited with the addition of isoleucine at t = 0 h, probably as a result of feedback inhibition of isoleucine. Isoleucine supply to the fermentation medium at low concentrations (0.25, 0.5 and 1.0 mM) increased SAP production, SAP activity was almost the same and greater than that of the reference medium. The highest SAP activity was obtained as 813 U cm^{-3} for 0.25 mM concentration at t = 41 h This was 1.4-fold higher than the reference medium activity. At isoleucine concentrations higher than 2.5 mM, SAP activity decreased significantly below that of the reference medium probably due to the feedback inhibition of isoleucine.

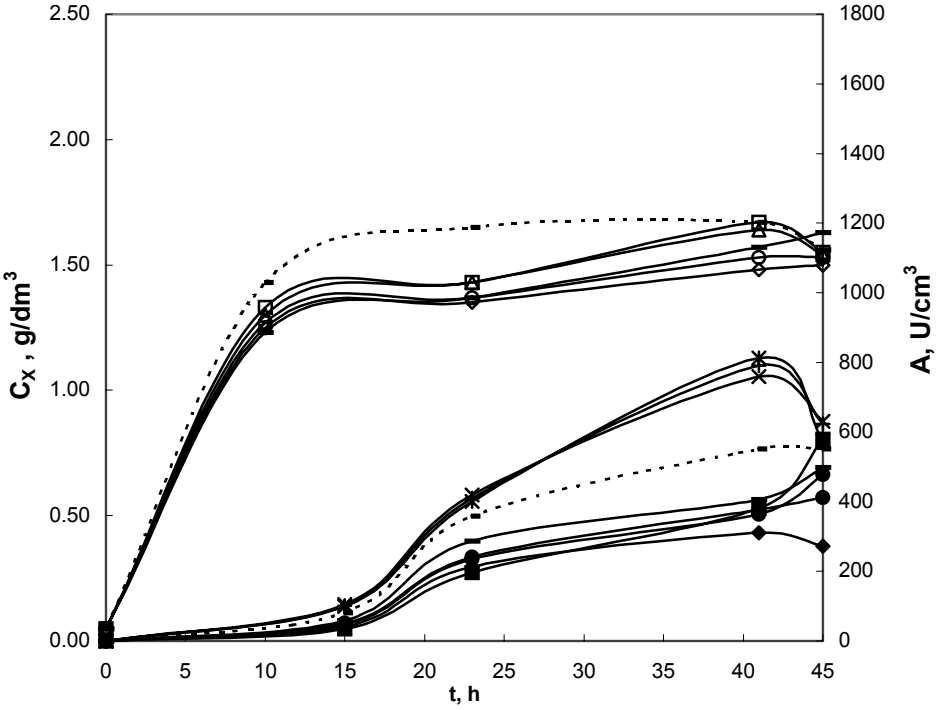

Figure 4.3. Effect of isoleucine on biomass concentration and SAP activity. C_{Ile}^0 : (∗) 0.25 mM; (+) 0.5 mM; (×) 1.0 mM; (–,–) 2.5 mM; (□ , ■) 5.0 mM; (△,▲) 7.5 mM; (○, ●) 12.5 mM; (◆,◇) 15.0 mM

4.4 Effect of Lysine on Biomass Concentration and SAP Activity

The effects of initial lysine concentration on growth and SAP activity were investigated in the range of 0-15mM using r-*B.subtilis* in laboratory scale bioreactors. Variation of biomass concentration and SAP activity are given in Figure 4.4. Cell growth was slightly inhibited with the addition of lysine. At lower lysine concentrations (0.25, 0.5 and 1.0 mM) SAP activity increased when initial lysine concentration was decreased and a maximum SAP activity of 926 U cm^{-3} was obtained at a lysine concentration of 0.25 mM which was 1.61–fold higher than the reference medium activity. On the other hand at $C_{Lys}^o \geq 2.5$ mM SAP activity was lower than that obtained for the reference medium and decreased with the increase in the initial lysine concentration. Similar to asparagine results, this indicates that with the addition of lysine alone to the medium at t=0 h, the

cells regulate themselves in order to keep the lysine concentration at a certain level. For this purpose, the cell enzyme levels should be regulated in such a way that lysine can be synthesized in sufficient amounts when needed.

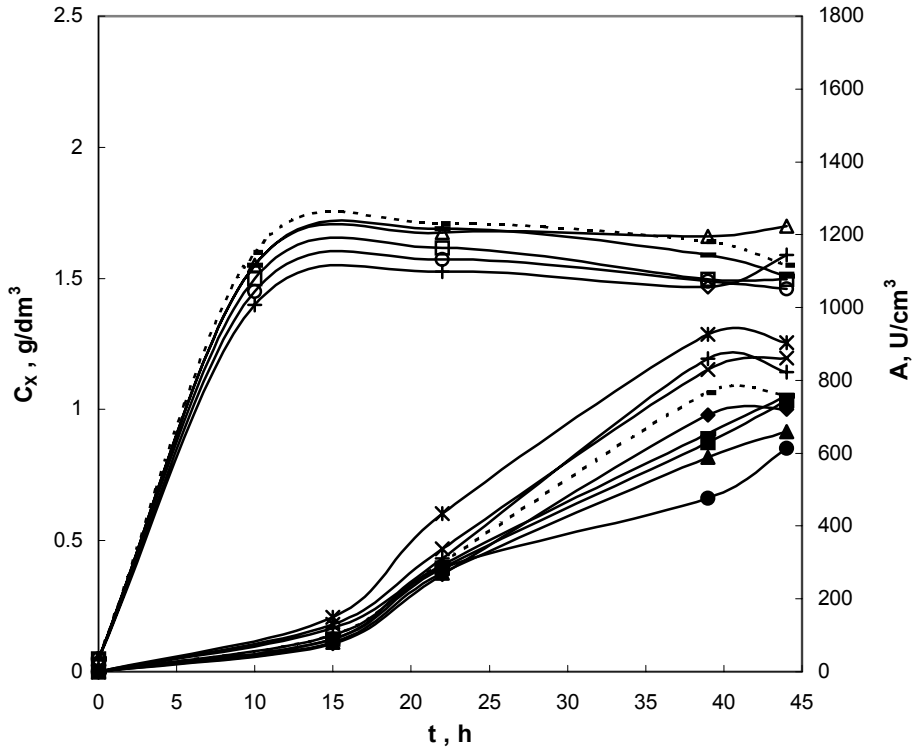

Figure 4.4. Effect of lysine on biomass concentration and SAP activity. C_{Lys}^0 : (∗) 0.25 mM; (+) 0.5 mM; (×) 1.0 mM; (–,–) 2.5 mM; (□ , ■) 5.0 mM; (△,▲) 7.5 mM; (○, ●) 12.5 mM; (◆,◇) 15.0 mM

4.5 Effect of Threonine on Biomass Concentration and SAP Activity

The effects of initial threonine concentration on growth and SAP activity were investigated using r-*B.subtilis* in the range of 0-15mM. Variation of biomass concentration and SAP activity are given in Figure 4.5. Cell growth and cell concentrations at the end of the fermentation were higher than that in the reference medium. The media with initial threonine concentrations of 0.25 and 0.5 mM had similar trends in SAP activity profiles. Moreover, at the

concentration range of 1.0-15mM SAP activities were almost the same with the reference medium. The highest SAP activity was obtained as 900 U cm^{-3} with an initial threonine concentration of 0.25 mM. This activity was 1.48-fold higher than the reference medium activity.

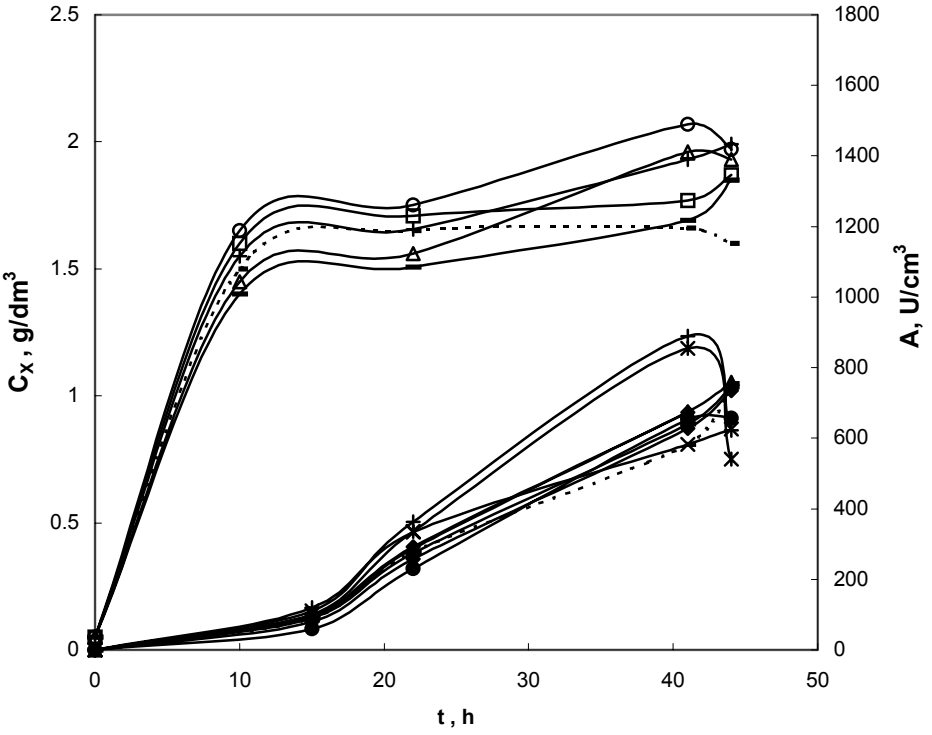

Figure 4.5. Effect of threonine on biomass concentration and SAP activity. C_{Thr}^0 : (∗) 0.25 mM; (+) 0.5 mM; (×) 1.0 mM; (–,–) 2.5 mM; (□ , ■) 5.0 mM; (△,▲) 7.5 mM; (○, ●) 12.5 mM; (◆,◇) 15.0 mM

4.6 Effect of Methionine on Biomass Concentration and SAP Activity

The effects of initial methionine concentration on growth and SAP activity were investigated using r-*B.subtilis* in the range of 0-15mM. Variation of biomass concentration and SAP activity are given in Figure 4.6. Cell concentrations were higher than that in the reference medium before t = 23 h but then started to increase up to t = 41 h and remained constant thereafter. For the concentration range of 0.25-1.0 mM.

SAP activity was greater than that obtained for the reference medium and the highest protease activity was 792 U cm^{-3} at a methionine concentration of 0.25 mM. This activity was 1.4-fold higher than that obtained in the reference medium. At concentrations higher than 2.5 mM SAP activity were inhibited.

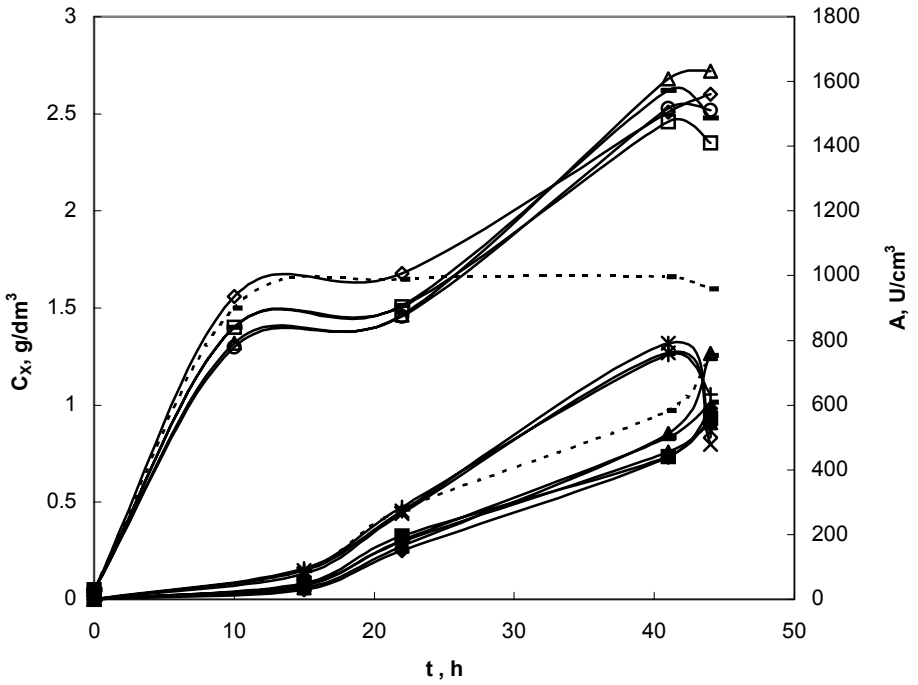

Figure 4.6. Effect of methionine on biomass concentration and SAP activity. C_{Met}^0 : (∗) 0.25 mM; (+) 0.5 mM; (×) 1.0 mM; (–,–) 2.5 mM; (□ , ■) 5.0 mM; (△,▲) 7.5 mM; (○, ●) 12.5 mM; (◆,◇) 15.0 mM

4.7 Product and By-Product Distributions in SAP Production in Defined and Complex Media

The product and by-product distributions of the bioprocess for SAP production were analyzed in both defined and complex media in order to compare, and obtain depth in-sight on intracellular metabolite concentrations, consequently their effects on metabolism and SAP production.

4.7.1 Variation in Glucose and Cell Concentrations in Defined Medium

The variations in glucose -that enters into the carbon metabolism from the glycolysis pathway- and cell concentrations in defined medium with the cultivation time are presented in Figure 4.7. It can be seen from the figure that glucose was consumed at a high rate in the first 5 h of the bioprocess, then up to 40 h the consumption rate was almost the same and after 40 h the consumption rate of glucose decreased. The cell concentration increased at a high rate between t= 0-5 h, and reached to its maximum value 1.75 g/dm^3 at t= 25 h. Variation of specific glucose consumption and growth rates are presented in Figure 4.8. Both specific glucose consumption and growth rates decrease with the cultivation time. After t=10 h specific glucose consumption rate remained constant whereas specific growth rate decreased up to t=25 h then after t=30 h as the cell concentration did not change the specific growth rate became zero.

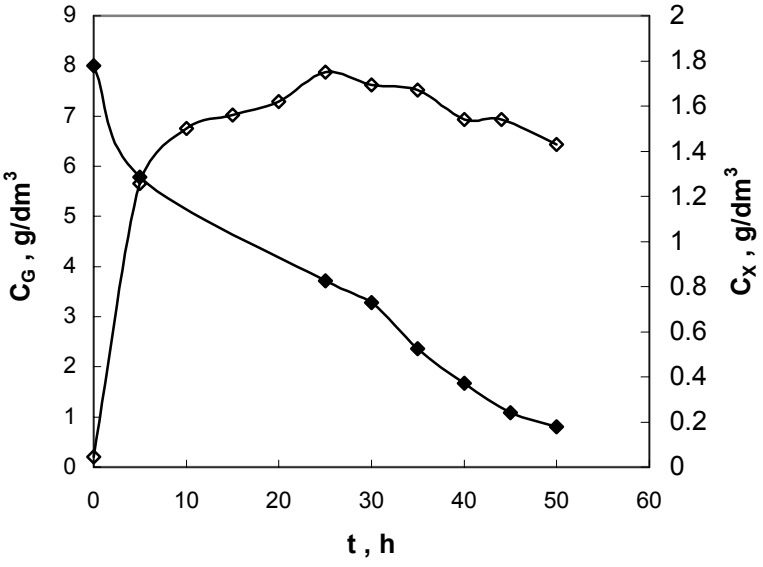

Figure 4.7 Variations of glucose (◆) and cell (◇) concentrations in defined medium with the cultivation time

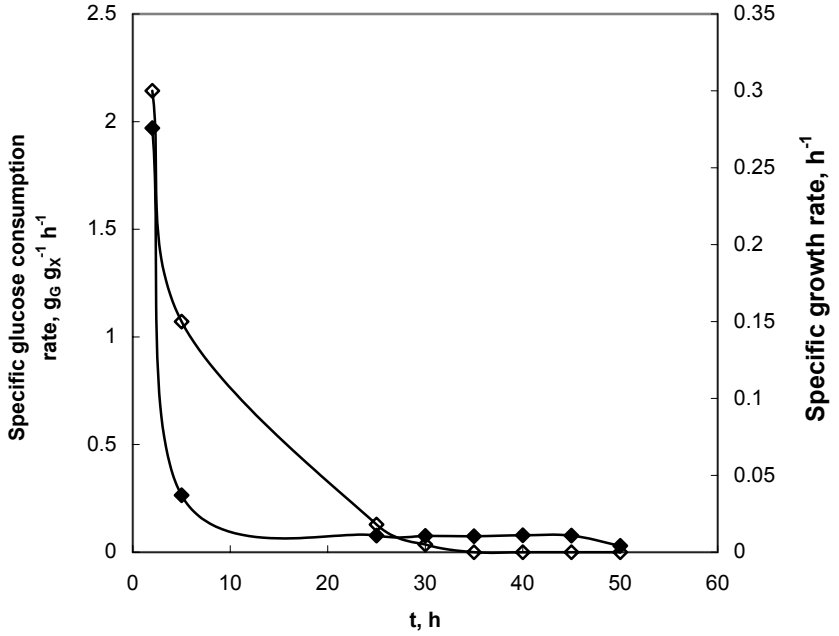

Figure 4.8 Variations in specific glucose consumption (◆) and specific growth (◇) rates in defined medium with the cultivation time

4.7.2 Variation in Serine Alkaline Protease Activity in Defined Medium

The variations of serine alkaline protease activity (A) and specific serine alkaline activity (A') and specific serine alkaline protease production rate (r_p) with the cultivation time are shown in Figure 4.9 and 4.10, respectively. During the cell formation period (t= 0-8 h), the rate of increase in serine alkaline protease activity was low. After t= 8 h serine alkaline protease activity started to increase at a high rate up to t=25 h. High serine alkaline protease production rates corresponds to the stationary phase of the growth since serine alkaline protease synthesis is a non-growth associated. Both serine alkaline protease activity and specific serine alkaline protease production increased up to t=50 h then gave maximums at t=50 h. The highest serine alkaline protease activity was obtained at t=50 h and its value was 700 U cm^{-3}.

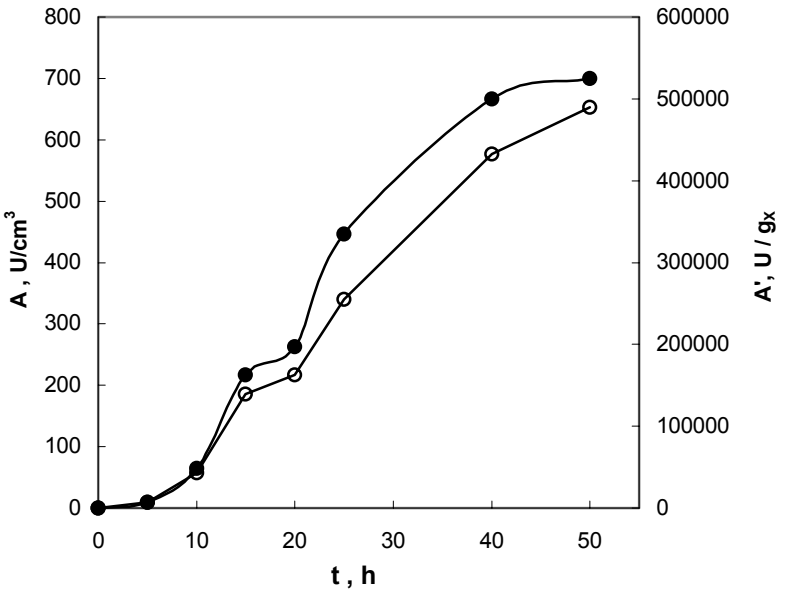

Figure 4.9 Variations in SAP activity (●) and specific SAP activity (○) in defined medium with cultivation time

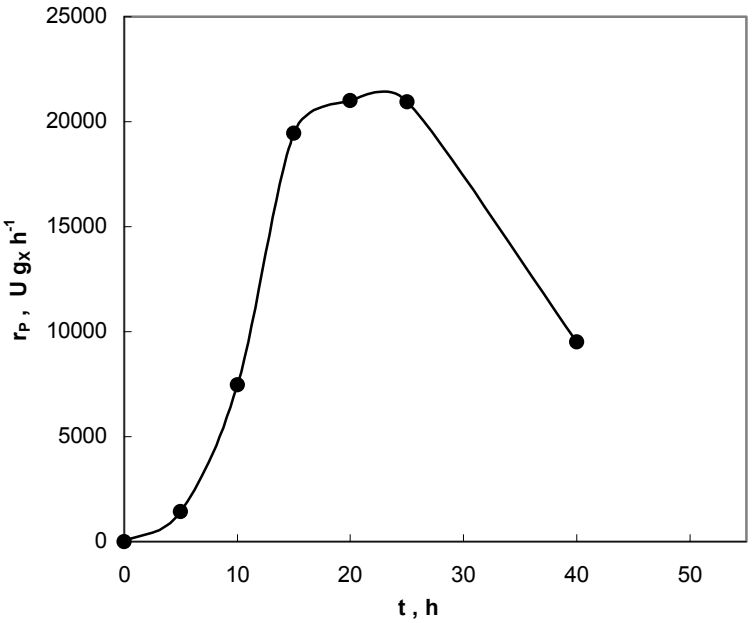

Figure 4.10 Variation in specific SAP production rate in defined medium with cultivation time

4.7.3 Variations in the Extracellular Amino Acid and Organic Acid Concentrations in Defined Medium

Extracellular amino acid concentrations are presented in Table 4.1. In the beginning of bioprocess (t= 0 h) there were cysteine, methionine, ornithine, phenyalanine, proline and tyrosine in the fermentation broth. Since there were no amino acids in the reference defined medium, these amino acids should be excreted to the broth during the growth in precultivation medium and supplied by the inoculation. In the first 5 h of the bioprocess, glutamate was excreted to the fermentation broth while cysteine and tyrosine initially present were utilized. Throughout the bioprocess initially present (t= 0 h) extracellular ornithine concentration decreased with the cultivation time therefore, one can conclude that ornithine was supplied by the cells from the fermentation broth and utilized for cellular growth. Extracellular phenyalanine and proline concentrations were in the range between 0.082-0.134 g dm^{-3} and 0.3-0.6 g dm^{-3}, respectively. Within the time interval between t=10-20h and at t=50 h cysteine was detected in the medium at low concentrations. Proline, which belongs to glutamic acid family amino acids was present in the extracellular medium throughout the whole bioprocess. This indicates that proline was being sufficiently synthesized by the cell that the excess of this amino acid was excreted to the medium and since the total glutamic acid content is only 8 % of the total amino acids in SAP molecule, glutamic acid group amino acids may not be a potential bottleneck in SAP production.

Table 4.2 shows the variations in the extracellular organic acid concentrations. As it is seen from the table the only organic acid excreted to the fermentation broth was oxalo acetic acid – the branch point for aspartic acid group amino acids- in a concentration range between $0.207x10^{-3}$ – $0.261x10^{-3}$ g dm^{-3}.

Oxaloacetic acid is synthesized either from the glycolyisis pathway metabolites, i.e., pyruvate and phosphoenolpyruvate by the anaplerotic reactions, or from the TCA cycle metabolite malic acid; and used for the energy generation in the TCA cycle and in the synthesis of aspartic acid. The existence of oxalo acetic acid in the fermentation broth denotes the insufficient operation of the TCA cycle probably due to the oxygen limitation.

Table 4.1 Variations in the extracellular amino acid concentrations in defined medium

	cultivation time, h										
g/L	0	5	10	15	20	25	30	35	40	45	50
Ala	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Arg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cys	0.023	0.0	0.054	0.012	0.018	0.0	0.0	0.0	0.0	0.0	0.028
Glu	0.0	0.055	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gly	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
His	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ile	0.0	0.0	0.0	0.0	0.0	0.005	0.0	0.0	0.0	0.0	0.0
Leu	0.0	0.0	0.0	0.0	0.0	0.005	0.0	0.0	0.0	0.0	0.0
Met	0.032	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Orn	0.24	0.213	0.15	0.176	0.14	0.092	0.088	0.058	0.090	0.06	0.0
Phe	0.134	0.088	0.087	0.087	0.085	0.084	0.125	0.106	0.130	0.126	0.082
Pro	0.320	0.344	0.313	0.396	0.490	0.4	0.4	0.39	0.52	0.560	0.662
Ser	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.005	0.0	0.0	0.0	0.0	0.0	0.0	0.002	0.0	0.0	0.0
Val	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.2 Variation in the extracellular organic acid concentrations in defined medium

	cultivation time, h						
g/L*10^6	5	15	20	25	30	35	40
OA	261	253	230	0	0	0	207
Suc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mal	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lac	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KG	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gluc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glu	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cit	0.0	0.0	0.0	0.0	0.0	0.0	0.0
But	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asp	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ac	0.0	0.0	0.0	0.0	0.0	0.0	0.0

4.7.4 Variations in the Intracellular Amino Acid and Organic Acid Concentrations in Defined Medium

In the bioprocess for serine alkaline protease production, the intracellular amino acid concentrations are indeed important since insufficient synthesis of some of the amino acid(s) creates reaction rate limitations in the bioreaction network as the controlling amino acids. Table 4.3 shows the variations of intracellular amino acid concentrations with the cultivation time in defined medium. At t=0 h there were isoleucine, leucine, methionine and phenylalanine in the cell. In the first 5h of the process; intracellular isoleucine and leucine were consumed; on the other hand, in addition to methionine, and phenylalanine initially present alanine, proline and tyrosine were present in the cell at low concentrations; but phenylalanine had a higher concentration than those of four amino acids. Moreover, phenyalanine was detected in high amounts in the cell throughout the bioprocess. Methionine concentration increased up to t=15 h, gave a maximum and decreased thereafter. Furthermore it was only observed in the extracellular medium at t=0 h. At the onset of SAP synthesis (t=10 h) all of the amino acids

were present in the cell except histidine, isoleucine, leucine, tryptophan and valine. Furthermore, glutamate, ornithine, methionine and phenylalanine were appeared at higher concentration in the cell than the others at t=10 h. Between t=15 h and t=35 h the amino acids concentrations were low, due to their consumption for serine alkaline protease production. In this time interval phenylalanine and proline concentrations were nearly constant. Moreover, phenylalanine and proline were present both in the extracellular and intracellular media throughout the whole bioprocess. Serine and valine, which are 11.6 and 11.3 % of the amino acid content of SAP, were neither observed in the cell nor in the extracellular medium during the bioprocess. Isoleucine and leucine were present at t=0 h and t=40 h in the intracellular medium, and only at t=25 h in the fermentation broth indicating that these amino acids were synthesized and then consumed by the cell for growth and product formation while with the decrease in cell functions the consumption rate of these amino acids should be lower than the production rate; consequently produced more than the demand of the cells. Alanine (14.5 % of the total amino acid content of SAP), was observed in low amounts in the intracellular medium only at t=5, 10 and 40 h; while, was not detected in the extracellular medium. At t=40 h, where the serine alkaline protease activity was about to reach its maximum value, all of the amino acids were present in the cell except alanine, cysteine, glysine, histidine, serine, tryptophan and valine. As with the decrease in cell functions still these amino acids were not observed in the fermentation broth, the synthesis of these amino acids can be rate-limiting step in SAP synthesis.

In this study variations in the intracellular organic acid concentrations with the cultivation time were also determined. As it is seen from Table 4.4 lactic acid was present in the cell throughout the whole bioprocess and oxalic acid was detected in the cell in the exponential phase of SAP production and t=40 h. The

existence of these organic acids in the cell indicates the insufficient operation of the TCA cycle probably due to the oxygen limitation.

Table 4.3 Variation in the intracellular amino acid concentrations in defined medium

	cultivation time, h										
g/g*10^6	0	5	10	15	20	25	30	35	40	45	50
Ala	0.0	0.11	1.66	0.0	0.0	0.0	0.0	0.0	0.0	0.23	0.0
Asp	0.0	0.0	7.63	0.0	0.0	0.0	0.0	0.0	0.49	0.0	0.0
Asn	0.0	0.0	7.57	0.0	0.0	0.0	0.0	0.0	0.49	0.0	0.0
Arg	0.0	0.0	1.77	0.0	0.0	0.0	0.0	0.0	0.13	0.0	0.0
Cys	0.0	0.0	5.32	0.0	0.0	0.0	0.0	0.0	0.00	3.23	0.0
Glu	0.0	0.0	30.66	0.0	0.0	0.0	0.0	0.0	1.40	0.67	0.0
Gly	0.0	0.0	4.14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
His	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ile	1.46	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.94	0.0	0.0
Leu	1.46	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.94	0.0	0.0
Met	1.08	0.99	21.50	91.13	74.02	0.77	0.0	0.0	4.32	3.92	2.02
Orn	0.0	0.0	31.56	16.73	34.32	33.39	18.57	0.0	35.56	37.49	35.83
Phe	10.46	11.84	13.88	11.36	11.36	11.42	10.80	9.56	12.60	12.97	15.08
Pro	0.0	3.66	6.41	4.16	4.84	4.90	4.93	4.54	4.57	4.79	4.36
Ser	0.0	0.0	1.41	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	0.0	0.0	1.21	0.0	0.0	0.0	0.0	0.0	0.09	0.0	0.0
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.0	0.29	8.01	0.25	0.0	0.0	0.0	0.0	0.73	0.62	0.25
Val	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.4 Variation in the intracellular organic acid concentrations in defined medium

	cultivation time, h						
g/g*10^6	5	15	20	25	30	35	40
OA	0.0	0.012	0.011	0.0	0.0	0.0	0.009
Suc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mal	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lac	0.0	0.007	0.005	0.003	0.004	0.003	0.001
KG	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gluc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gluc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cit	0.0	0.0	0.0	0.0	0.0	0.0	0.0
But	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asp	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ac	0.0	0.0	0.0	0.0	0.0	0.0	0.0

4.7.5 Variations in Glucose and Cell Concentrations in Complex Medium

The variations in glucose and cell concentrations in complex medium with the cultivation time are presented in Figure 4.11. Besides glucose soybean was also utilized as the carbon source.

As it is seen from the figure, glucose was consumed at a high rate in the first 5 h of the bioprocess, then at t=5-30 h the consumption rate was almost constant and after 30 h the glucose concentration remained constant till the end of the bioprocess. The cell concentration increased until t=35 h then remained constant. The highest cell concentration was reached at t=35 h and its value was 14.3 g dm^{-3}. Variations of specific glucose consumption and growth rates are presented in Figure 4.12. Both specific glucose consumption and growth rates decreased with the cultivation time.

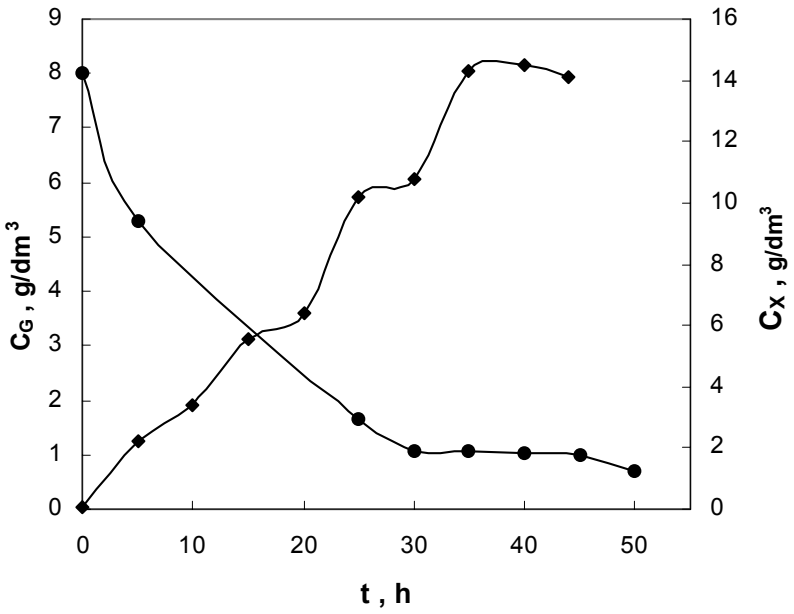

Figure 4.11 Variations in glucose (◆) and cell (◇) concentrations in complex medium with the cultivation time

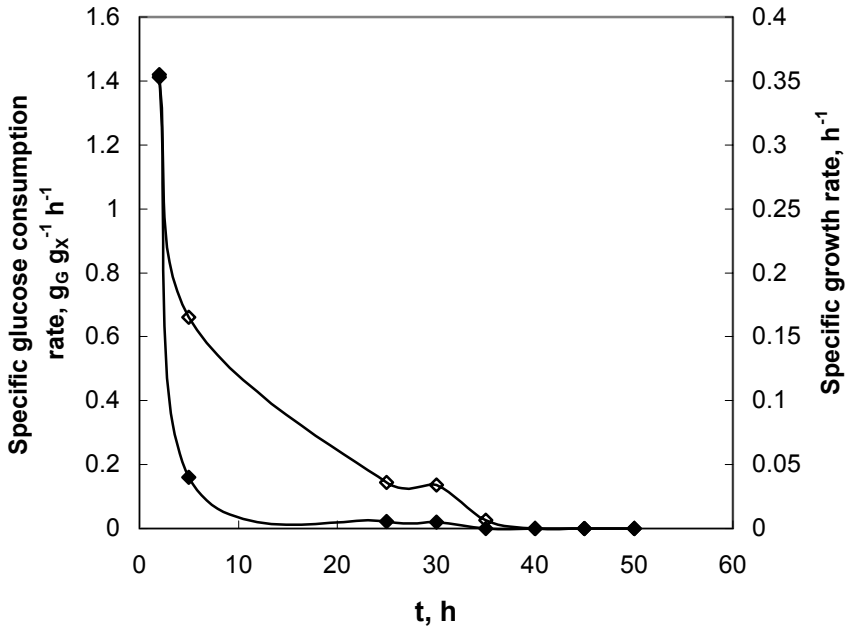

Figure 4.12 Variation in specific glucose consumption (◆) and specific growth (◇) rates in complex medium with the cultivation time

4.7.6 Variation in Serine Alkaline Protease Activity in Complex Medium

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 in complex medium are given in Figure 4.13 and 4.14, respectively. Serine alkaline protease activity started to increase at a high rate after t=15h up to t= 25 h, then the rate of increase in SAP activity decreased and the highest serine alkaline protease activity was observed at t=50 h having a value of 2015 U cm^{-3}. Same trend was observed in the variation of specific SAP production. Specific SAP production rate increased after t=10 h and a maximum was observed at t=20 h, where glucose reached its limiting value indicating that SAP is produced when glucose is limited.

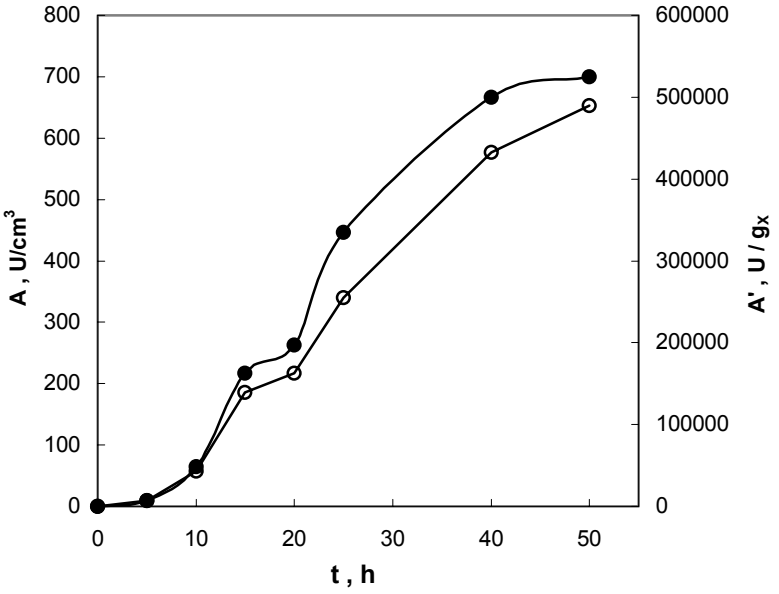

Figure 4.13 Variations in SAP activity (●) and specific SAP activity (○) in complex medium with cultivation time

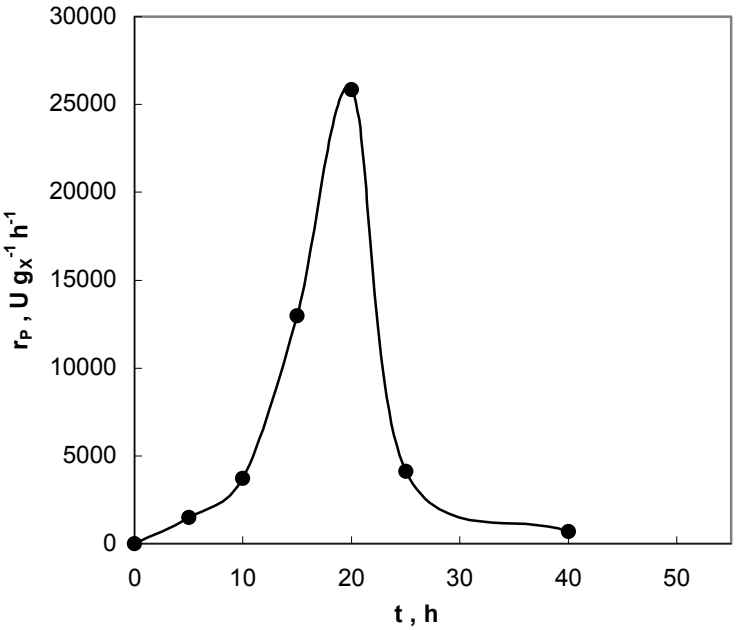

Figure 4.14 Variations in specific SAP activity in complex medium with cultivation time

4.7.7 Variations in the Extracellular Amino Acid and Organic Acid Concentrations in Complex Medium

In this study, soybean was used as a carbon source for complex medium which contains arginine (7.5%), histidine (2.3%), lysine (6.7%), tryptophan (1.6%), phenylalanine (5.2%), threonine (3.9%), valine (5.4%), leucine (8.1%), isoleucine (5.3%), methionine (1.4%) and glutamine (18.4%). Variations in the extracellular amino acid concentrations are given in Table 4.5. In SAP production with complex medium as the precultivation medium used for defined medium experiments was used; on the basis of the amino acid composition at t= 0 h of the defined medium, cysteine, methionine, ornithine, phenyalanine, proline and tyrosine have to be supplied to the medium from precultivation medium. In complex medium at t= 0 h, all the amino acids were present in the extracellular medium except alanine, glycine, and serine, that are not present in soybean structure, and tryptophan, and valine. At t=5 h of the bioprocess, all the amino acids present in the broth at the beginning of the fermentation were consumed except cysteine, glutamate, ornithine, phenylalanine and proline. As the soybean amino acids were not present in the fermentation broth at t= 5 h one can conclude that the consumption rate of the soybean protein amino acids should be either equal or higher than their liberation rates. At the beginning of SAP synthesis that corresponds to t=10 h alanine, glutamate, ornithine, phenylalanine and tyrosine were present in the fermentation broth, and the other amino acids were not observed, similar to t= 5h results, at t= 10 h the consumption rate of the soybean protein amino acids should be either equal or higher than their liberation rates. Glutamate and glutamine serve as the donor of virtually all amino and amide groups in cellular components, either by the direct participation of glutamine in biosynthetic reaction or through the action of glutamate as a substrate for transamination reaction. 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, produced by the below reactions, was more than the demand of the cells and excreted to the fermentation medium.

Glutamate consuming reactions:

* PG3 + Glu $\rightarrow$ Ser + αKG + NADH + Pi
* Pyr + Glu $\rightarrow$ α KG + Ala
* KVal + Glu $\rightarrow$ αKG + Val
* KVal + AcCoA + Glu $\rightarrow$ NADH + CO_2 + α KG + Leu
* OA + Glu $\rightarrow$ Asp + αKG
* Tet + AcCoA +Glu $\rightarrow$ Ac + αKG + mDAP
* Thr + Pyr + NADPH + Glu $\rightarrow$ Ile + αKG + NH3 +CO_2
* Chor + Glu $\rightarrow$ αKG + Phe + CO_2
* Chor + Glu $\rightarrow$Tyr + αKG + NADH + CO_2
* Glu + ATP + NH3 $\rightarrow$ Gln + ADP + Pi
* Glu + ATP + 2 NADPH $\rightarrow$ Pro + ADP + Pi
* 2Glu + AcCoA + ATP + NADPH $\rightarrow$ Orn + Ac + ADP + Pi + αKG

Glutamate synthesizing reactions:

* αKG + Ala $\rightarrow$ Pyr + Glu
* Pro $\rightarrow$ Glu + NADPH
* Arg+ αKG $\rightarrow$ 2Glu + NH_3+ NADPH + CO2
* Val + αKG $\rightarrow$ KVal + Glu
* Gln + αKG + NADPH $\rightarrow$ 2Glu
* His +THF $\rightarrow$ Glu + MeTHF

At t=20-30 glutamate, cysteine, ornthine, tyrosine and phenylalanine were present in the fermentation broth. The initial phenylalanine concentration was high and phenylalanine was further supplied from the soybean, and as tyrosine that was present in the fermentation both although it was not present in the stucture of soybean, phenylalanine should be converted to tyrosine (Phe $\rightarrow$ Tyr +NADPH). As a result of this conversion, tyrosine concentration should be more than the demand of the cells since it was excreted to the fermentation broth. Extracellular ornithine concentration did not change considerably throughout the fermentation and was between 0.11-0.33 g dm^{-3} At the time where SAP activity was maximum, cysteine, ornithine and phenyalanine were present in the extracellular medium. Throughout the whole bioprocess for SAP production; glycine (12.7% of SAP), serine (11.6% of SAP), valine (11.3% of SAP), and alanine (14.5 % of SAP) (except t=10 h and t= 25 h) were not observed in the fermentation broth similar to the defined medium.

Table 4.6 shows the variations in the extracellular organic acid concentrations in complex medium. In the bioprocess for SAP production in complex medium oxalic acid, succinic acid and lactic acid were excreted to the fermentation broth. The presence of these organic acids indicates the oxygen limitation. Compared to the defined medium oxygen requirement in complex medium should be higher.

4.7.8 Variations in the Intracellular Amino Acid and Organic Acid Concentrations in Complex Medium

Table 4.7 presents the variations in the intracellular amino acid concentrations in complex medium. As seen in the Table 4.7, at t=0 h there were isoleucine, leucine, methionine and phenylalanine in the cell similar to the defined medium since the same precultivation medium was used for complex medium experiments. At t=5 h all of the amino acids were present in the intracellular

medium except cysteine, ornithine, serine, threonine, tryptophan and valine. Furthermore, aspartate, asparagine, glutamate and glycine were only observed at t=5 h. Throughout the exponential phase of SAP production (t=15-25 h), methionine and ornithine were detected in the cell. Methionine concentration decreased with the cultivation time until the end of the exponential SAP synthesis phase (t=25h) then there was no methionine in the intracellular medium. Moreover methionine was not observed in the fermentation broth after the beginning of the bioprocess. This indicates that methionine that was liberated from soybean was supplied from the fermentation broth and not synthesized by the cells. Alanine, isoleucine and leucine were also present in the intracellular medium until t=25 h. Throughout the whole bioprocess ornithine was present both in the cell and in the extracellular medium; whereas glycine (except t=10 h), serine, tryptophan and valine were neither observed in the intracellular medium nor in the fermentation broth. This denotes that, glycine, serine, tryptophan and valine barely supply the requirement of the cell for growth and product formation. At the time where serine alkaline protease production was maximum (t=50h), arginine, methionine, proline and threonine were present at low amounts, whereas, ornithine and phenylalanine were observed in higher amounts in the cell.

Table 4.8 presents the variations in the intracellular organic acid concentrations in complex medium. In the bioprocess for SAP production in complex medium oxalic acid and butyric acid were observed in the cell. During the bioprocess oxalic acid was also present in the fermentation broth whereas, butyric acid was present at t=15 h and t=20 h only in the cell.

Table 4.5 Variations in the extracellular amino acid concentrations in complex medium

	cultivation time, h										
g / L	0	5	10	15	20	25	30	35	40	45	50
Ala	0.0	0.0	0.0004	0.0	0.0	0.0007	0.0	0.0	0.0	0.0	0.0
Asp	0.004	0.0	0.0	0.0	0.0036	0.0	0.0005	0.009	0.0	0.0012	0.0
Asn	0.004	0.0	0.0	0.0	0.0035	0.0	0.0004	0.009	0.0	0.0012	0.0
Arg	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cys	0.02	0.009	0.0	0.0	0.045	0.039	0.0474	0.0	0.0975	0.017	0.046
Glu	0.01	0.008	0.058	0.017	0.047	0.007	0.006	0.003	0.0067	0.0016	0.0
Gly	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
His	0.0	0.0	0.0	0.0	0.0170	0.0	0.0	0.0008	0.0	0.0	0.0
Ile	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Leu	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Met	0.02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Orn	0.33	0.12	0.113	0.12	0.26	0.112	0.12	0.0	0.15	0.27	0.13
Phe	0.11	0.19	0.098	0.13	0.12	0.122	0.14	0.0	0.16	0.27	0.14
Pro	0.0	0.039	0.0	0.0	0.0	0.0	0.0	0.04	0.0	0.0	0.0
Ser	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	0.0068	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.0053	0.0	0.0063	0.0	0.0021	0.0023	0.0017	0.0	0.0023	0.0041	0.0
Val	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.6 Variations in the extracellular organic acid concentrations in complex medium

	cultivation time, h						
g/L*10^6	5	15	20	25	30	35	40
OA	245	213	409	32	66.2	33.2	352
Suc	42	26	0.0	0.0	52.3	25.3	28.4
Pyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mal	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lac	0.0	0.0	20.5	196	0.0	0.0	0.0
KG	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gluc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glu	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cit	0.0	0.0	0.0	0.0	0.0	0.0	0.0
But	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asp	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ac	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.7 Variations in the intracellular amino acid concentrations in complex medium

	cultivation time, h										
g/g*10^6	0	5	10	15	20	25	30	35	40	45	50
Ala	0.0	1.42	0.11	0.18	0.04	0.0	0.0	0.0	0.0	0.0	0.0
Asp	0.0	0.55	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Asn	0.0	0.55	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Arg	0.0	0.48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.19
Cys	0.0	0.00	3.93	0.0	0.0	0.44	0.0	0.0	0.0	0.0	0.0
Glu	0.0	1.53	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gly	0.0	0.27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
His	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ile	1.46	4.75	0.0	3.38	0.41	0.0	0.0	0.0	0.0	0.0	0.0
Leu	1.46	4.75	0.0	3.38	0.41	0.0	0.0	0.0	0.0	0.0	0.0
Met	1.08	5.34	2.89	1.88	0.37	0.17	0.0	0.0	0.0	0.0	0.51
Orn	0.0	0.0	47.28	48.88	44.98	42.45	35.49	39.51	16.23	40.37	29.02
Phe	10.46	10.61	7.15	10.05	0.0	0.0	0.0	0.0	9.57	0.0	13.68
Pro	0.0	1.83	0.14	0.00	0.05	0.0	0.0	0.0	3.90	0.0	4.24
Ser	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00
Thr	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.13
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.0	2.51	1.56	0.75	0.18	0.19	0.0	0.24	0.0	0.27	0.0
Val	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4.8 Variations in the intracellular organic acid concentrations in complex medium

	cultivation time, h						
G/g*10^6	5	15	20	25	30	35	40
OA	0.093	0.009	0.014	0.002	0.003	0.009	0.054
Suc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mal	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lac	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KG	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gluc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gluc	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cit	0.0	0.0	0.0	0.0	0.0	0.0	0.0
But	0.0	0.0016	0.0023	0.0	0.0	0.0	0.0
Asp	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ac	0.0	0.0	0.0	0.0	0.0	0.0	0.0

4.7.9 Variations in the DNA and RNA Concentrations in Defined and Complex Media

Variations of DNA and RNA concentrations with the cultivation time were investigated in both defined and complex media. In defined medium DNA concentrations vary between 0.07-0.20 $g_{DNA}\ g_X^{-1}$ and RNA concentrations were in the range of 0.1-0.29 $g_{RNA}\ g_X^{-1}$. In complex medium DNA concentrations were between 0.07-0.20 $g_{DNA}\ g_X^{-1}$ and RNA concentrations vary between 0.06-0.21 $g_{RNA}\ g_X^{-1}$.

4.7.10 Comparison of Product and By-Product Distributions in Defined and Complex Media

The product and by-product distributions of the bioprocess for SAP production were analyzed in both defined and complex media in order to compare and obtain depth in-sight on intracellular metabolite concentrations, consequently their effects on SAP production.

In complex medium glucose consumption rate was higher than that of defined medium and glucose reached to the limiting value earlier than defined medium. The specific glucose consumption rate decreased with the cultivation time both in defined and complex media. However, the decrease in the glucose consumption rate was faster in complex medium than the defined medium. Maximum specific glucose consumption rates in defined medium and complex medium were 1.97 and 1.42 $g_G\ g_X^{-1}\ h^{-1}$, respectively. The maximum specific glucose consumption rate was lower in complex medium since there was an alternative carbon source, soybean, in the medium. In defined medium cell concentration increased up to t=5 h and thereafter remained constant. On the other hand in complex medium, cell concentration increased up to t=35 h then remained constant. The highest cell concentration in defined medium was 1.75 g dm^{-3} whereas, it was

14.3 g dm^{-3} in complex medium. The reason for such an increase in cell concentration in complex medium can be the utilization of hydrolyzed soybean as carbon source. Both in defined and complex media the specific growth rate decreased with the cultivation time. The maximum specific growth rates in defined medium and complex medium were 0.3 and 0.36 h^{-1}, respectively. The increase in the specific SAP production rate was also higher in complex medium than that in defined medium. The highest SAP activity obtained in complex medium was 3–fold higher than the SAP activity in defined medium as expected since in order to utilize soybean to use it as the nitrogen source microorganism should secrete protease. In both defined and complex media, glycine (12.7 % of the total amino acids in SAP), serine (11.6 % of the total amino acids in SAP) and valine (11.3 % of the total amino acids in SAP) were neither observed in the extracellular nor in the intracellular media indicating that these amino acids were synthesized and consumed by the cells or synthesized insufficiently. One can also conclude that the synthesis of these amino acids can be a rate-limiting step. Asparagine, which was reported to be the potential bottleneck in SAP synthesis (Çalık *et al.*, 1999) was not present in the fermentation broth and was observed only at t=10 h and t=40 h in the cell in defined medium. On the other hand, in complex medium asparagine was observed in the beginning of the fermentation and between t=20-45 h in the fermentation broth and t=5 h in the cell. Methionine -that initiates protein synthesis- was observed both in defined and complex media extracellularly and intracellularly. In complex medium the intracellular concentration of methionine decreased with the cultivation time until t=25 h, then no methionine was observed in the cell up to t=50 h. Moreover, methionine was present in complex medium extracellularly only in the beginning of the bioprocess. This indicates that methionine was supplied from the fermentation broth and did not synthesized by the cells. On the other hand in defined medium intracellular methionine concentration first increased until

t=15 h, gave a maximum and decreased thereafter. Furthermore, methionine was present in defined medium extracellularly only in the beginning of the bioprocess indicating that methionine was synthesized by the cells and did not supplied from the fermentation broth in defined medium. Both methionine and asparagine are in the aspartic acid group amino acids. In the aspartic acid pathway (Figure 2.2) from one branch asparagine and from another branch methionine is synthesized. The amino acid analysis results showed that in defined medium asparagine was neither present in the cell (except t=10 h and t=40 h) nor in the fermentation broth however, methionine was observed in the cell in high amounts in defined medium. This may indicate that the flux towards asparagine might be low compared to the flux values of the other branches in the aspartic acid pathway therefore asparagine synthesis might be insufficient and it might also be a potential bottleneck in SAP production. In defined medium proline was observed throughout the bioprocess in defined medium both extracellularly and intracellularly, whereas it was rarely observed in complex medium. In both defined and complex media, oxaloacetic acid was observed extracellularly and intracellularly. In complex medium there was also succinic acid in the extracellular medium indicating that the operation of TCA cycle was insufficient. In defined medium lactic acid was present in the cell throughout the whole bioprocess whereas in complex medium lactic acid was observed only at t=20-25 h in the fermentation broth. Excretion of lactic acid to the broth also indicates the insufficient operation of TCA cycle.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Serine alkaline protease (SAP) synthesis depends on good coupling of supply and demand of amino acids in the cell, thus in a recombinant bacilli the intracellular concentrations of intermediary metabolites and intracellular reaction network enzyme activities and intracellular control mechanisms are indeed important. Insufficient synthesis of some of the amino acid(s) creates reaction rate limitations in the bioreaction network for SAP production as the controlling amino acids. In this case, supply of the controlling amino acids to the fermentation broth and supplied period are indeed important. In this context, in this study, first of all the effects of aspartic acid group amino acids, which were reported to be the potential bottleneck in SAP synthesis (Çalık *et al.*, 1999), were investigated in defined medium by using recombinant *Bacillus subtilis*; thereafter, the product and by-product distributions of the bioprocess for SAP production were determined in defined and complex media. Lastly, the results were compared, in order to analyze the differences between defined and complex media and to find out the potential strategies in order to increase SAP production further by medium modification, since the attainable enzyme activity and cell yields are much higher in complex medium, than that of defined medium.

The effects of initial aspartic acid concentration on growth and SAP activity were investigated using r-*B.subtilis* in laboratory scale bioreactors in the range 0-15mM by using the reference defined production medium. The cell growth and the cell concentrations obtained at the end of the fermentation were not affected with the supply of aspartic acid. The highest SAP production was obtained at 0.25 mM aspartic acid concentration with a value of 1074 U cm^{-3}, which was 1.87 fold higher than that obtained for the reference medium. With the further increase in aspartic acid concentration SAP production decreased slightly but for $C_{Asp}^{o} \geq 2.5$ mM inhibited drastically.

The effect of initial asparagine concentration on growth and SAP activity were investigated using r-*B.subtilis* in laboratory scale bioreactors in the range 0-15mM by using the reference defined production medium. Maximum cell concentration was obtained at an asparagine concentration of 15 mM. At $C_{Asn}^{0} \geq 5$ mM increased biomass formation. The highest SAP activity was obtained as 1082 U cm^{-3} for an asparagine concentration of 0.25 mM, which was 1.89-fold higher than the reference medium activity. On the other hand, at $C_{Asn}^{0} \geq 2.5$ mM SAP production was inhibited. Addition of asparagine increased cell concentration however; the addition of aspartic acid didn't affect cell growth. In contrast to the aspartic acid results, asparagine synthesis should be insufficient and this can be the potential bottleneck in SAP production.

The effects of initial isoleucine concentration on growth and SAP activity were investigated in the range of 0-15mM using r-*B.subtilis* in laboratory scale bioreactors. Cell growth was inhibited with the addition of isoleucine at t = 0 h for all isoleucine concentrations. The highest SAP activity was obtained as 813 U cm^{-3} for 0.25 mM concentration, which was 1.4-fold higher than the reference medium activity. At $C_{Ile}^{0} \geq 2.5$ mM, SAP activity decreased significantly below that of the reference medium.

The effects of initial lysine concentration on growth and SAP activity were investigated in the range of 0-15mM using r-*B.subtilis* in laboratory scale bioreactors. Cell growth was slightly inhibited with the addition of lysine. The highest SAP activity of 926 U cm^{-3} was obtained at a lysine concentration of 0.25 mM, which was 1.61–fold higher than the reference medium activity. On the other hand at $C_{Lys}^{o} \geq 2.5$ mM SAP activity was lower than that obtained for the reference medium.

The effects of initial threonine concentration on growth and SAP activity were investigated using r-*B.subtilis* in the range of 0-15mM. Cell growth and cell concentrations at the end of the fermentation were higher than that in the reference medium. At $C_{Thr} \geq 1.0$ mM SAP activities were almost the same as the reference medium. The highest SAP activity was obtained as 900 U cm^{-3} with an initial threonine concentration of 0.25 mM which was 1.48-fold higher than the reference medium activity.

The effects of initial methionine concentration on growth and SAP activity were investigated using r-*B.subtilis* in the range of 0-15mM. For the concentration range of 0.25-1.0 mM SAP activity was greater than that obtained for the reference medium and the highest protease activity was 792 U cm^{-3} at a methionine concentration of 0.25 mM which was 1.4-fold higher than that obtained in the reference medium. At $C_{Met} \geq 2.5$ mM SAP activity were inhibited.

In the study, the product and by-product distributions of the bioprocess for SAP production were also analyzed in both defined and complex media in order to compare, and obtain depth in-sight on intracellular metabolite concentrations, consequently their effects on metabolism and SAP production. In complex medium the glucose consumption rate was higher than in defined medium and glucose reached to the limiting value earlier than defined medium.

The specific glucose consumption rate decreased with the cultivation time both in defined and complex media. The maximum specific glucose consumption rates in defined medium and complex medium were 1.97 and 1.42 $g_G\ g_X^{-1}\ h^{-1}$, respectively. The highest cell concentration in defined medium was 1.75 g dm^{-3} whereas; it was 14.3 g dm^{-3} in complex medium. Maximum specific growth rates in defined medium and complex medium were 0.3 and 0.36 h^{-1}, respectively. The increase in SAP production rate was also higher in complex medium than that in defined medium. The highest SAP activity obtained in complex medium was 3–fold higher than defined medium SAP activity as expected, since in order to utilize soybean to use it as the nitrogen source microorganism should secrete protease.

In both defined and complex media glycine (12.7 % of the total amino acids in SAP), serine (11.6 % of the total amino acids in SAP) and valine (11.3 % of the total amino acids in SAP) were neither observed in the extracellular nor in the intracellular media indicating that these amino acids were synthesized and consumed by the cells or synthesized insufficiently. One can also conclude that the synthesis of these amino acids can be rate-limiting step. Alanine which is 14.5 % of the total amino acids in SAP was not present in the fermentation broth and was observed only at t=5-10 h in the cell in defined medium. On the other hand, in complex medium alanine was observed between t=5 h and t=20 h in the cell and decreased with the cultivation time indicating that alanine present in the cell at t=5 h was consumed by the cells in the following hours for growth and product formation. Asparagine, which was reported to be the potential bottleneck in SAP synthesis (Çalık *et al.*, 1999) was not present in the fermentation broth and was observed only at t=10 h and t=40 h in the cell in defined medium. On the other hand, in complex medium asparagine was observed in the beginning of the fermentation and between t=20-45 h in the fermentation broth and t=5 h in

the cell. Methionine was observed both in defined and complex media extracellularly and intracellularly. In complex medium the intracellular concentration of methionine decreased with the cultivation time up to t=25 h then no methionine was observed in the cell up to t=50 h. Moreover, methionine was present in complex medium extracellularly only in the beginning of the bioprocess. One can conclude that methionine was supplied from the fermentation broth and did not synthesized by the cells. On the other hand in defined medium intracellular methionine concentration first increased up to t=15 h, gave a maximum and decreased thereafter. Furthermore, methionine was present in defined medium extracellularly only in the beginning of the bioprocess indicating that methionine was synthesized by the cells and did not supplied from the fermentation broth. Both methionine and asparagine are in the aspartic acid group amino acids. In the aspartic acid pathway (Figure 2.2) from one branch asparagine and from another branch methionine are synthesized. The amino acid analysis results showed that in defined medium asparagine was neither present in the cell (except t=10 h and t=40 h) nor in the fermentation broth however, methionine was observed in the cell in high amounts in defined medium. This is probably due to the lower flux values towards asparagine. The synthesis of asparagine might also be a potential bottleneck in SAP production. In both defined and complex media, oxaloacetic acid was observed extracellularly and intracellularly. In complex medium there was also succinic acid in the extracellular medium indicating that the operation of the TCA cycle was insufficient. In defined medium lactic acid was observed in the cell throughout the whole bioprocess whereas in complex medium lactic acid was observed only at t=20-25 h in the fermentation broth. Excretion of lactic acid to the broth also indicates the insufficient operation of the TCA cycle.

In SAP production it is known that insufficient synthesis of some of the amino acid(s) creates reaction rate limitations in the bioreaction network as the

controlling amino acids. In the study, the effect of supply of aspartic acid group amino acids on SAP synthesis were investigated at a concentration range between 0.25 and 15 mM. The effect of these amino acids on SAP synthesis should also be investigated at concentrations higher than that in the intracellular medium. The amino acid analysis results showed that serine, valine and glysine were neither present in the cell nor in the fermentation broth in both defined and complex media. This may indicate the insufficient synthesis of these amino acids therefore; the supply of these amino acids to the medium may increase SAP production so it will be useful to investigate the effects of these amino acids on SAP production. In order to obtain more information about the intracellular control mechanisms and regulations in recombinant *Bacillus subtilis* during SAP synthesis, metabolic flux analysis can be done by using the obtained product and by-product distributions.

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APPENDIX A

CALIBRATION OF CELL CONCENTRATION

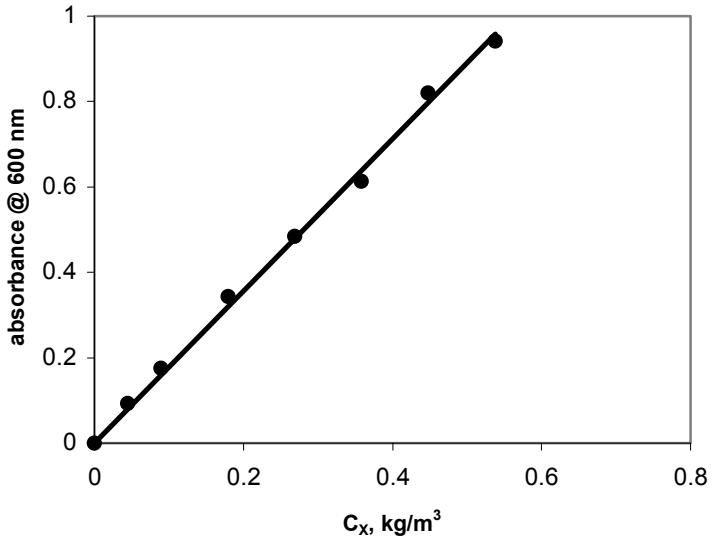

Figure A1 Calibration curve of microorganism concentration

Slope of the calibration curve, m=1.782 1/kg m^{-3} (λ = 600 nm)

$$C_X = \frac{\text{Absorbance}}{1.782} \times \text{DilutionRate}$$

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;

$$A = \frac{\text{Absorbance}}{m} \times \text{DilutionRate} \times \frac{1U}{4\text{nmole}^{-1}} \times \frac{1}{20\text{min}}$$

APPENDIX C

CALIBRATION OF REDUCED SUGAR CONCENTRATION

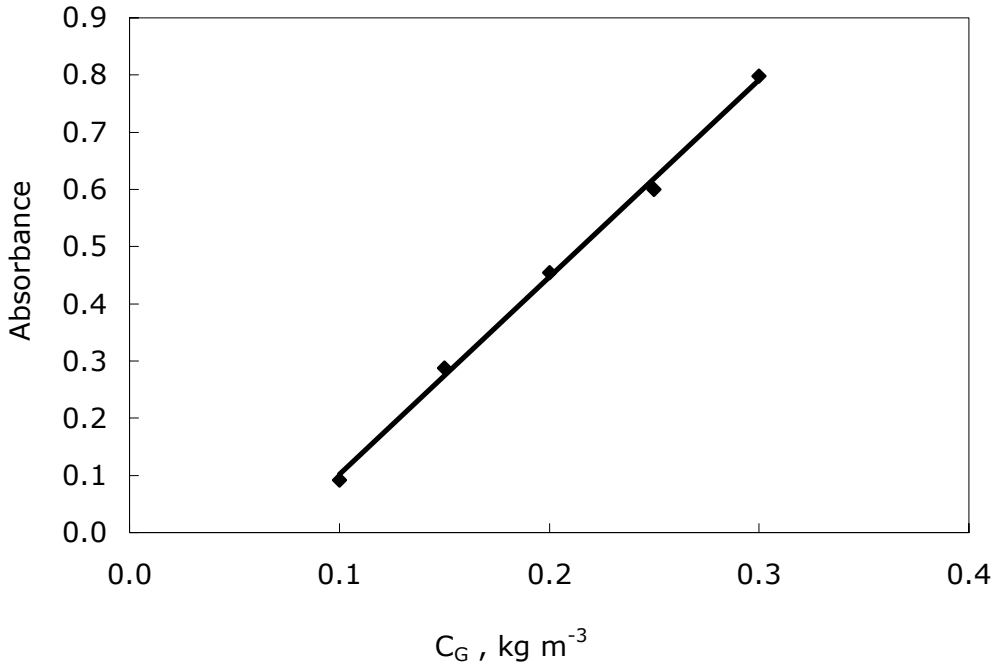

Figure A.2 Calibration Curve of the DNS solution

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

APPENDIX D

PREPARATION OF DNS SOLUTION

1. a) 880 cm^3 of 1 % (m/v) DNS solution is prepared by dissolving 8.8 g dinitrosalisilyc 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 christalized 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 and it should be used after 48 h.

APPENDIX E

TRIS AND EDTA SOLUTIONS AND TE BUFFER

Preparation of 0.5 M EDTA solution, pH 8.0-500 ml

- EDTA 93.05 g (disodium ethylenediaminetetraacetate)

- Distilled water 350 ml

Place on a magnetic stirrer and stir vigorously. Adjust the pH to 8.0 by adding approximately 10 g NaOH pellets. The disodium salt of EDTA will not go into solution until the solution is adjusted approximately pH 8.0. Bring to 500 ml total volume with dH_2O.

Preparation of 1.0 M Tris solution, pH 8.0-500 ml

- Tris (Tris(Hydroxymethyl) Aminomethane) 60.57 g

- Distilled water 350 ml

Dissolve 60.57 g Tris in dH_2O and adjust the pH to 8.0 with concentrated HCl (approximately 21 ml), cool to room temperature and make the final adjustments to the pH. Bring to 500 ml with dH_2O.

APPENDIX F

CALIBRATION OF DNA CONCENTRATION

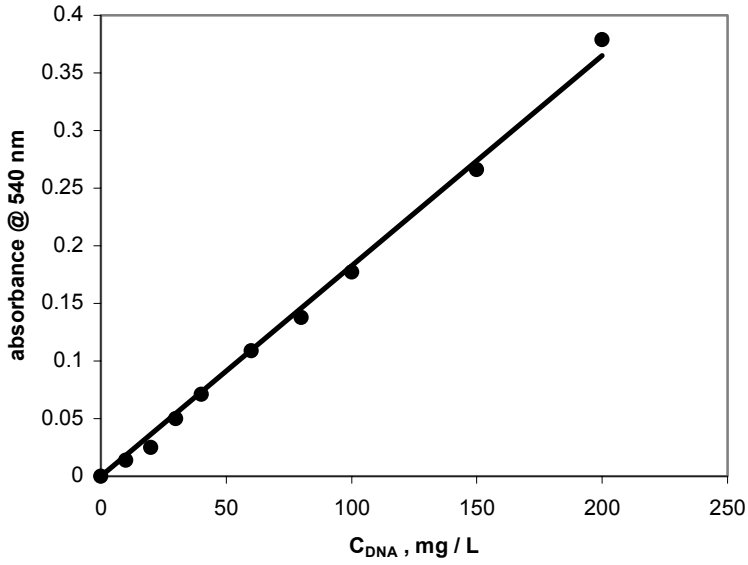

Figure A.3 Calibration curve for DNA

Slope of the calibration curve, m=0.0018 1/mg L^{-1} (λ = 540 nm)

$$C_{DNA} = \frac{\text{Absorbance}}{0.0018} \times \text{DilutionRate}$$

APPENDIX G

CALIBRATION OF RNA CONCENTRATION

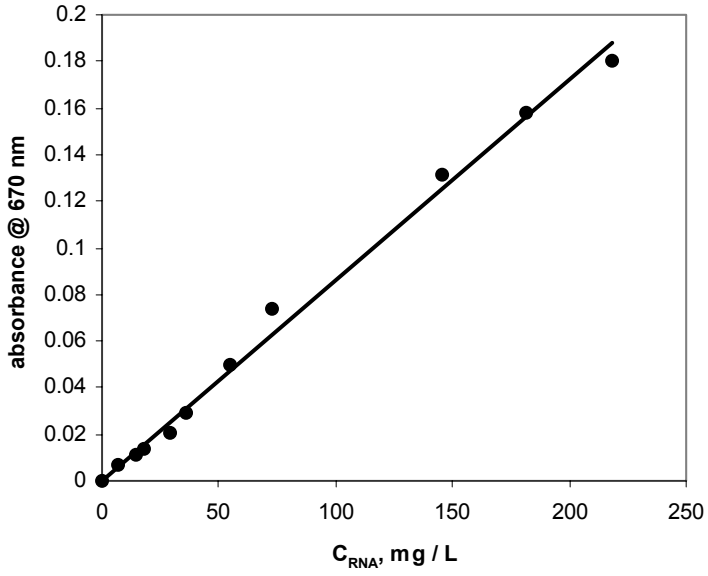

Figure A.4 Calibration curve for DNA

Slope of the calibration curve, m=0.0009 1/mg L^{-1} (λ = 670 nm)

$$C_{RNA} = \frac{\text{Absorbance}}{0.0009} \times \text{Dilution Rate}$$