EFFECTS OF pH AND FEEDING STRATEGY ON METABOLITE PROFILING OF BETA-LACTAMASE PRODUCING *Bacillus licheniformis*

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis and for the degree of Master of Science.

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ABSTRACT

EFFECTS OF pH AND FEEDING STRATEGY ON METABOLITE PROFILING OF BETA-LACTAMASE PRODUCING *Bacillus licheniformis*

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In this study, the effects of pH and different feeding modes on β-lactamase production and the cell metabolism were investigated with *Bacillus licheniformis* (ATCC 25972). For this purpose, first, the effects of pH on β-lactamase activity, cell formation, substrate consumption, as well as intracellular and extracellular sodium, potassium, ammonium ion, amino acid and organic acid concentrations were investigated in V=3.0 dm^3 batch bioreactors consisting of temperature, pH, foam, stirring rate and dissolved oxygen controls. Among the investigated uncontrolled pH operation with pH_0=7.5 and controlled pH operations, pH_C=6.75 yielded in the highest cell concentration and β-lactamase activity as C_X=0.60 kg m^{-3} and A=54 U cm^{-3}, respectively. Next, the production medium was redesigned in terms of initial glucose and phosphate ion concentrations in order to increase the enzyme activity and cell growth rate, and to determine the feeding strategy in laboratory scale batch-bioreactors using shake bioreactors having V=33 ml working volumes. The medium containing (kg m^{-3}), glucose, 2.5 (7.0); Na_2HPO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2HPO_4$, 7.1 and salt solution at pH_C=6.75 was accepted as optimized medium for fed-batch (batch) processes. Using this optimized medium the feeding strategy was investigated for linear and exponential feeding

profiles and compared with batch operation. Throughout the fermentation, cell, substrate and intracellular and extracellular by-product, sodium, potassium, ammonium ion concentrations, β-lactamase activity, yield coefficients, specific rates, oxygen uptake rates and the liquid phase mass transfer coefficient values were determined. The highest β-lactamase activity was obtained at fed-batch operation with exponential feeding (FB1) condition as A=108 U cm^{-3}, which is ca. 1.7-fold higher than that of the batch operation with optimized medium. Finally, to investigate the physiological state of the culture media, viability of the cells was monitored throughout the cultivation time for repeated FB1, pH_C=6.75, and pH_{UC}=7.5 experiments. About 9% of the cells were found to be dead through the end of FB1 and pH_{UC}=7.5 operations.

Keywords: β-lactamase, *Bacillus*, pH, feeding strategy, viability.

ÖZ

pH VE BESLEME STRATEJİSİNİN BETA-LAKTAMAZ ÜRETEN *Bacillus licheniformis* MİKROORGANİZMASININ METABOLİT PROFİLLERİ ÜZERİNDEKİ ETKİLERİ

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Bu çalışmada, *Bacillus licheniformis* (ATCC 25972) ile pH ve besleme koşullarının β-laktamaz üretimine ve hücre metabolizmasına olan etkileri araştırılmıştır. Bu amaçla, önce, pH'in β-laktamaz aktivitesine, hücre oluşumuna, substrat tüketimine, hücreiçi ve hücredışı sodyum, potasyum, amonyum iyonları, amino asit ve organik asit derişimlerine olan etkileri V=3.0 dm^3 hacimli sıcaklık, pH, köpük, karıştırma ve çözünmüş oksijen derişimi kontrollü kesikli biyoreaktörde araştırılmıştır. İncelenen kontrollu- ve kontrolsuz-pH işletim koşullarında, pH_C=6.75'te en yüksek hücre derişimi ve β-laktamaz aktivitesi sırasıyla C_X=0.60 kg m^{-3} ve A=54 U cm^{-3} olarak belirlenmiştir. Daha sonraki aşamada, enzim aktivitesini, hücre çoğalma hızını arttırmak ve besleme stratejisini belirlemek için üretim ortamı başlangıç glukoz ve fosfat iyon derişimleri açısından laboratuar ölçekli, V=33 ml çalışma kapasitesine sahip kesikli biyoreaktör kullanılarak tekrar tasarlanmıştır. pH_C=6.75'te, (kg m^{-3}) glucose, 2.5 (7.0); Na_2HPO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2HPO_4$, 7.1 ve tuz çözeltisinden oluşan ortam yari-kesikli işletim için optimum ortam olarak kabul edilmiştir. Bu ortam kullanılarak, besleme stratejisi, yari-kesikli işletim koşularında, üstel glukoz besleme (FB1) ve lineer glukoz besleme (FB2) stratejileri açısından araştırılmış ve kesikli işletim ile

karşılaştırılmıştır. Proses süresince, hücre, substrat, hücreiçi ve hücredışı yan-ürün, sodyum, potasyum, amonyum iyon derişimleri, β-laktamaz aktivitesi, verim katsayıları, spesifik hızlar, oksijen tüketim hızları, ve sıvı faz kütle aktarım katsayıları belirlenmiştir. En yüksek β-laktamaz aktivitesi ve hücre derişimi sırasıyla FB1 ve FB2 koşullarında A=108 U cm^{-3} ve C_x=0.54 kg m^{-3} olarak bulunmuştur. Son aşamada, hücrenin fizyolojik durumunu belirlemek amacıyla, tekrarlanan FB1, pH_C=6.75, ve pH_{UC}=7.5 koşulları için hücre canlılıkları proses suresince izlenmiştir. FB1 ve pH_{UC}=7.5 proseslerinin sonunda hücrelerin yaklaşık %9'unun canlılığını yitirdiği saptanmıştır.

Anahtar Kelimeler: β-laktamaz, *Bacillus*, pH, besleme stratejisi, canlılık.

To My Mum and Dad

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

NOMENCLATURE

a	The gas liquid interfacial area per unit liquid volume, $m^2\ m^{-3}$
A	Beta-lactamase activity, U cm^{-3}
A_λ	Absorbance
C_{AA}	Amino acid concentration, kg m^{-3}
C_G	Glucose concentration, kg m^{-3}
C_G^0	Initial glucose concentration, kg m^{-3}
C_{OA}	Organic acid concentration, kg m^{-3}
C_O	Dissolved oxygen concentration, mol m^{-3}; kg m^{-3}
Co_0	Initial dissolved oxygen concentration, mol m^{-3}; kg m^{-3}
C_O^*	Oxygen saturation concentration, mol m^{-3}; kg m^{-3}
C_s	Concentration of the substrate, kg m^{-3}
C_X	Cell concentration, kg dry cell m^{-3}
Da	Damköhler number (=OD / OTR_{max}; Maximum possible oxygen utilization rate per maximum mass transfer rate)
D	Bioreactor diameter, m
D_I	Impeller diameter, m
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
En	Enzyme
K_La_0	Physical overall liquid phase mass transfer coefficient; s^{-1}
K_La	Overall liquid phase mass transfer coefficient; s^{-1}
K_m	Michealis constant for the substrate
N	Agitation or shaking rate, min^{-1}
P	Product
pH_0	Initial pH
Qo	Volumetric air feed rate, $m^3\ min^{-1}$
q_o	Specific oxygen uptake rate, kg kg^{-1} DW h^{-1}
q_s	Specific substrate consumption rate, kg kg^{-1} DW h^{-1}
r	Volumetric rate of reaction, mol $m^{-3}\ s^{-1}$
r_{max}	Maximum rate of reaction at infinite reactant concentration
r_0	Oxygen uptake rate, mol $m^{-3}\ s^{-1}$; kg $m^{-3}\ h^{-1}$
r_S	Substrate consumption rate, kg $m^{-3}\ h^{-1}$
r_X	Rate of cell growth, kg $m^{-3}\ h^{-1}$
S	Substrate

t	Bioreactor cultivation time, h
T	Bioreaction medium temperature, °C
T_{AA}	Total amino acid concentration, kg m^{-3}
T_{OA}	Total organic acid concentration, kg m^{-3}
U	One unit of an enzyme
V	Volume of the bioreactor, m^3
V_R	Volume of the bioreaction medium, m^3
$Y_{X/S}$	Yield of cell on substrate, kg kg^{-1}
$Y_{X/O}$	Yield of cell on oxygen, kg kg^{-1}
$Y_{S/O}$	Yield of substrate on oxygen, kg kg^{-1}

Greek Letters

η	Effectiveness factor (=OUR/OD; the oxygen uptake rate per maximum possible oxygen utilization rate)
μ	Specific cell growth rate, h^{-1}
μ_{max}	Maximum specific cell growth rate, h^{-1}
λ	Wavelength, nm

Abbreviations

Ac	Acetic acid
ADEPT	Antibody-Directed Enzyme Prodrug Therapy
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATCC	American Type Culture Collection
Cys	Cysteine
DO	Dissolved oxygen
EC	Enzyme Commission
Gln	Glutamine
Glc	Glucose
Gluc	Gluconate
Gly	Glycine
His	Histidine

Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Mal	Malate
Met	Methionine
NH_3	Ammonia
OA	Oxaloacetic acid
Orn	Ornithine
OD	Oxygen demand ($=\mu_{max}\ C_X\ /\ Y_{X/O}$; mol $m^{-3}\ s^{-1}$)
OTR	Oxygen transfer rate, mol $m^{-3}\ s^{-1}$
OTR_{max}	Maximum possible mass transfer rate ($=K_La C_O^*$; mol $m^{-3}\ s^{-1}$)
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Suc	Succinate
Val	Valine
Tyr	Tyrosine
Thr	Threonine
Trp	Tyrptophan

CHAPTER 1

INTRODUCTION

Biochemical engineering may be defined as the use of living organisms, or their products (for example enzymes), to produce chemical or biological materials or develop new processes. It integrates features of biochemistry, cell and molecular biology, bioorganic and bioinorganic chemistry, and the discipline of chemical engineering at its core. In this sense, the practice of biochemical engineering has a long history, beginning with the fermentation of grapes for wine, brewing of beer, cheese manufacture, leavening of bread, and effluent disposal. Recently, on the other hand, biochemical engineers have been engaged in a number of economic sectors such as antibiotic fermentations, production of industrial solvents, organic acids, vaccines, blood and tissue products, animal feedstuffs, commercial enzymes and in the treatment of effluents and wastes (Blanch and Clark, 1996).

Enzymes are one of the most important resources utilized in industry to produce a wide range of biotechnology products, and have significant applications in medicine. Enzyme production and utilization are a multibillion dollar business with a great potential expansion. The estimated value of world enzyme market was about US $ 1.3 billion in 2000 and it has been forecasted to grow to almost US $5.1 billion by 2009 (Leisola et al., 2001; www.freedoniagroup.com).

Enzymes are protein, glycoprotein, or RNA molecules that have a key role in catalyzing biologically important chemical transformations; and thus, the essential components of all living systems. Enzymes have extraordinary catalytic power, often far greater than that of synthetic or organic catalysts. They have a high degree of specificity for their substrates, they accelerate chemical reactions, and they function under very mild conditions of temperature and pH. Few nonbiological catalysts have all these properties (Lehninger, 2000). Enzymes can either be produced by isolation from plant or animal tissue or by microorganisms, plant and animal cells. However, for many reasons, microbial

enzymes have largely replaced the traditional plant and animal enzymes used in industry; and among them, hydrolytic enzymes are the most commonly used ones in biotechnology recently.

In enzyme production by bioprocesses, there are some important factors (such as selection of microorganism, medium design, and fermentation conditions; i.e., oxygen transfer rate, pH, temperature, operating mode) that must be taken into account in order to have high product yield and selectivity. Among these parameters, pH shows diverse effects on product formation by influencing metabolic pathways and changing metabolic fluxes. Whereupon, some bioprocesses require uncontrolled-pH conditions, while others favor a constant regulated pH of the culture media (Çalık et al., 2003).

On the basis of the interactions between the cell and the bioreactor through a process, carried out at either controlled- or uncontrolled pH conditions, intracellular pH can be widely different and variable during the course of the fermentation. While some bacteria maintain fairly constant pH_i values upon changes in extracellular pH, others maintain a constant pH gradient at least at limited pH intervals (Hornbæk et al., 2004). The intracellular pH is of utmost importance since it has a major influence on the metabolic activity of cells. It determines the in vivo activity of the enzyme and often modulates the transport kinetics of nutrients and metabolism. Moreover, pH gradient across cell membranes also controls ATP generation, and more generally the cellular energetics. Consequently, if one aims towards a quantitative understanding of the cell metabolism, one has to take into account the time variations of the intracellular pH and its effects on the *in-vivo* kinetics of the metabolic steps involved (Levyal et al., 1997).

The transport mechanism by which the cells can sense their extracellular environment is another factor that is to be considered. A cell must take nutrients from its extracellular environment if it is to grow or retain metabolic activity. Which nutrients enter the cell and at what rate can be important in regulating metabolic activity (Shuler and Kargi, 1992).

The bioreactor is the center of all biochemical processing (Blanch and Clark, 1996). One of the most important decisions for constructing any process

concerns the configuration the reactor system should take. The choice of reactor and operating strategy determines product concentrations, number and types of impurities, degree of substrate conversion, and yields and whether sustainable, reliable performance can be achieved. Bioreactors using suspended cells can be operated in many modes intermediate between a batch reactor and a single-stage chemostat. Although a chemostat has potential productivity advantages for primary products, considerations of genetic instability, process flexibility, and process reliability have greatly limited the use of chemostat units. The fed-batch system, on the other hand, combines the features of continuous culture and batch that allow the manufacturer to maintain flexibility and ease of intervention (Shuler and Kargi, 1992).

Throughout the course of any cultivation process whether batch or fed-batch, considered above, or another approach, namely continuous culture, it is also essential to monitor cell proliferation and viability. Accurate estimates of biomass concentration are important if informed decisions on process control are to be made. In this way, products can be harvested at optimum concentrations and inducible systems can be activated at the appropriate time so that high product yields are achieved. Information on cell physiological state is also important since a large number of dead or dormant cells present during any part of a cultivation will have a detrimental effect on the synthesis of any desired product (Hewitt et al., 1999).

Hence, in this study, beta-lactamase was the target enzyme to be investigated. Beta-lactamase enzymes (EC 3.5.2.6) are a group of highly efficient hydrolytic enzymes that catalyze the hydrolysis of the beta-lactam ring in penicillins, cephalosporins and their analogues. Beta-lactamases make-up about 20% of the world's biocatalyst market (Godfrey and West, 1996). They are manufactured for the specific assay of penicillins/ cephalosporins in body fluids and culture media, sterility tests of penicillins, treatment of penicillin sensitivity reactions, penicillin electrodes and for drug design (White and White, 1997). Recently, the development of beta-lactamase dependent prodrugs with the applications in antibody-directed enzyme prodrug therapy (ADEPT) has been an area of particular interest (Tang et al., 2003).

Because of the medical implications of this enzyme, it has been focus of intense research over the last half century, however, mostly from the point of view of enzyme induction, secretion, and purification. Related with medium design, the effect of glucose was investigated for improving β-lactamase production (Hemila et al., 1992). Among the bioreactor operation parameters, the effects of agitation rate (Wase and Patel, 1987), pH (Sargent et al., 1968; Hemila et al., 1992) and dissolved oxygen (Sargantanis and Karim, 1996; 1998) on β-lactamase production by *Bacillus* species were reported. In more recent studies, Celik and Çalık (2004) investigated the variations in product and by-product distributions, and the fermentation and oxygen transfer characteristics of the β-lactamase production process by *B.licheniformis* in a semi-designed medium at V= 3.0 dm^3 bioreactor system with a V_R=1.65 dm^3 working volume at Q_O/V_R= 0.5 vvm and N=500 min^{-1} and reported that the oxygen transfer resistances were effective in cell growth phase.; and Çalık et al (2005) investigated the effect of oxygen transfer conditions within a wide range, from limiting to high oxygen transfer thoroughly on glucose-based defined medium in β-lactamase production by *Bacillus licheniformis*.

Related with other enzymes produced by *B. licheniformis*, on the other hand, Çalık et al. (2002, 2003) investigated the effects of the controlled and uncontrolled pH conditions together with the initial pH on product and by-product distributions and oxygen transfer characteristics in SAP production on a defined medium for pH ranges between 7-7.5 and 6.80-7.25 in batch bioreactors. Moreover, Hornbæk and Jakobsen (2003, 2004) studied the effect of intracellular pH to describe different physiological states within viable populations encountered during cultivation in a laboratory-scale bioreactor; and identified the effect of pH upshifts by measuring subsequent growth, ΔpH and global gene transcription, after transferring *B.lichniformis* cells in the early-stationary growth phase to fresh media with different extracellular pH values. However, in the literature there is no study related with influence of pH as well as feeding strategy on transport mechanism and physiological state of culture media throughout the production of β-lactamase by *B.licheniformis*.

Hence, in this study, the effects pH on enzyme activity, transport mechanism, product and by-product distributions were investigated in previously defined batch bioreactor conditions first. Thereafter, to increase the product

yield, the medium was redesigned in terms of its phosphate source in laboratory scale bioreactors. Then, using the optimum bioprocess parameters, the effects of feeding profiles on cell yield, enzyme activity, transport mechanism, and product and by-product distributions were studied in fed-batch cultivation. Finally, by using the optimum process parameters on whole, the experiments were repeated to observe the effects of pH and feeding profile on cellular physiology of β-lactamase producing *B.licheniformis*.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

2.1.1 General Characteristics

Enzymes are one of the essential components of all living systems. These macromolecules have a key role in catalyzing the chemical transformations that occur in all cell metabolisms. The nature and specificity of their catalytic activity is primarily due to the three-dimensional structure of the folded protein, which is determined by the sequence of the amino acids that make up the enzyme (Blanch and Clark, 1996). Recently, it has been shown that some RNA molecules are also catalytic, but the vast majority of cellular reactions meditated by protein catalysts. Enzymes lower the activation energy of the reaction catalyzed by binding the substrate and forming an enzyme-substrate complex. They do not affect the free-energy change or the equilibrium constant. They are highly specific, versatile, and very effective macromolecules, resulting in much higher reaction rates as compared to chemically catalyzed reactions under ambient conditions (Shuler and Kargı, 2002).

Enzymes, like other proteins, have molecular weights ranging from about 12,000 to over 1 million. Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called a cofactor -either one or more inorganic ions, such as Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, etc., or a complex organic or metalloorganic molecule called a coenzyme (Lehninger, Nelson, and Cox, 2000).

Each enzyme has a specific temperature and pH range where it functions to its optimal capacity, the optima for these proteins usually lie between 37-47^{o}C, and pH optima range from acidic, i.e., 1.0, to alkaline, i.e., 10.5 (Kirk and Othmer, 1994).

Enzymes tend to be classed into intracellular, membrane, or extracellular. Any organism, i.e., any plant, animal, or microorganism, is a potential source of enzymes. Of those enzymes used on an industrial scale, the majority are derived from microbial sources (Moses and Cape, 1991). Enzymes with the same name but obtained from different organisms often have different amino acid sequences and hence different properties and catalytic activities (Bailey, 1986).

2.1.2 Classification of Enzymes

As depicted in Table 2.1, enzymes have been classified into six main types, depending on the nature of the reaction catalyzed. A numbering scheme for enzymes has been developed, in which the main classes are distinguished by the first of four digits. The second and third digits describe the type of reaction catalyzed, and the fourth digit is employed to distinguish between enzymes of the same function on the basis of the actual substrate in the reaction catalyzed. This scheme has proven useful in clearly delineating many enzymes that have similar activities. It was developed by the Enzyme Commission and the prefix E.C. is generally employed with the numerical scheme (Blanch and Clark, 1996). For example, the EC number of β-lactamase is EC 3.5.2.6, which catalyzes the hydrolysis of C-N bond in β=lactam ring.

Table 2.1 International classification of enzymes.

No	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons
2	Transferases	Group-transfer reactions
3	Hydrolases	Transfer of functional groups to water
4	Lyases	Addition of groups to double bonds or the reverse
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

2.1.3 Enzyme Activity

The qualitative description of the chemical reactions they catalyze forms the basis for their classification, while their catalytic activity is quantitatively expressed in terms of units of activity. The quantitative activity of enzymes give indication of how much enzyme should be used to achieve a required effect (product yield) and forms the basis for comparison of several similar enzyme products (Godfrey and West, 1996). However, a comparison of the activity of different enzyme preparations is only possible if the assay procedure is performed exactly in the same way (Faber, 2000). The Commission on Enzymes suggested that a standard unit definition of enzyme activity should be as (Celik, 2003):

> One unit (U) of enzyme activity is defined as the amount which will catalyze the transformation of one micromole of substrate per minute under defined conditions.

2.1.4 Enzyme Kinetics

Kinetics of simple enzyme-catalyzed reactions are often referred to as Michaelis-Menten kinetics or saturation kinetics. An enzyme solution has a fixed number of active sites to which substrates can bind. At high substrate concentrations, all these sites may be occupied by substrates or the enzyme is saturated. Saturation kinetics can be obtained from a simple reaction scheme that involves a reversible step for enzyme-substrate complex formation and a dissociation step of the ES complex (Shler and Kargi, 1992).

$$En + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} P$$

It is assumed that the ES complex is established rather rapidly and the rate of the reverse reaction of the second step is negligible. The assumption of an

irreversible second reaction often holds only when product accumulation is negligible at the beginning of the reaction (Shuler and Kargi, 1992). Thus, the Michealis-Menten equation (Eq 2.1) is simply derived from the rate of the above reaction by substituting K_m for $(k_{-1}+k_{+2})/k_{+1}$:

$$r = \frac{r_{max} C_S}{K_m + Cs} \tag{2.1}$$

where, r is the volumetric rate of reaction (mol m^{-3} s^{-1}), C_S is the concentration of the substrate (mM), r_{max} is the maximum rate of reaction at infinite reactant concentration, and K_m is the Michealis constant for the substrate (mM). K_m is a measure of the affinity of an enzyme for a particular substrate, a low K_m value representing a high affinity and a high K_m a low affinity (Godfrey and West, 1996).

Rearrangement of equation (2.1) gives:

$$\frac{1}{r} = \frac{1}{r_{max}} + \frac{K_m}{r_{max}} \cdot \frac{1}{C_S} \tag{2.2}$$

A plot of 1/r versus $1/C_S$, which is known as the Lineweaver-Burk plot (Figure 2.1), should give a straight line with slope K_m/r_{max} and intercept $1/r_{max}$.

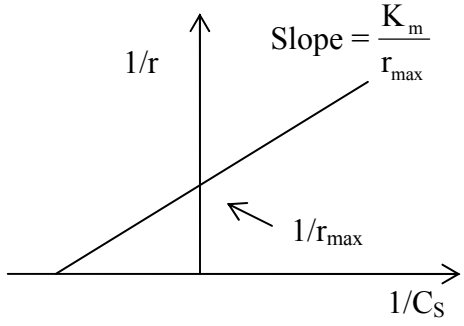

Figure 2.1 Lineweaver-Burk plot.

2.2 Beta-lactamases

Beta-lactamases (EC 3.5.2.6) are the second major group of enzymes which interact with penicillins (Figure 2.2). These were discovered almost as soon as the penicillin structure became known and they have been an ominous presence throughout the history of the development of penicillins and other β-lactams (Fogarty and Kelly, 1990). The products of the hydrolysis of penicillins by β-lactamases are antibiotically inactive biomolecules.

Beta-lactamases are produced by most bacteria, including blue-green algae but have also been reported from yeast and in human kidney (Fogarty and Kelly, 1990). The presence of this enzyme in non-bacterial systems suggests that it may have a more widespread role (Çelik, 2003).

In most cases the reported molecular weight for β-lactamases from gram-positive bacteria is within the range of 28000-30000 Da. With benzylpenicillin as the substrate, typical pH-activity and temperature-activity curves obtained with β-lactamases from gram-positive bacteria shows maxima in the range of pH 6.0-7.0 and 30°-40°C respectively. The enzyme from *Bacillus* species are reasonably stable between pH 3.0-10.0 and are quite thermostable (Çelik, 2003).

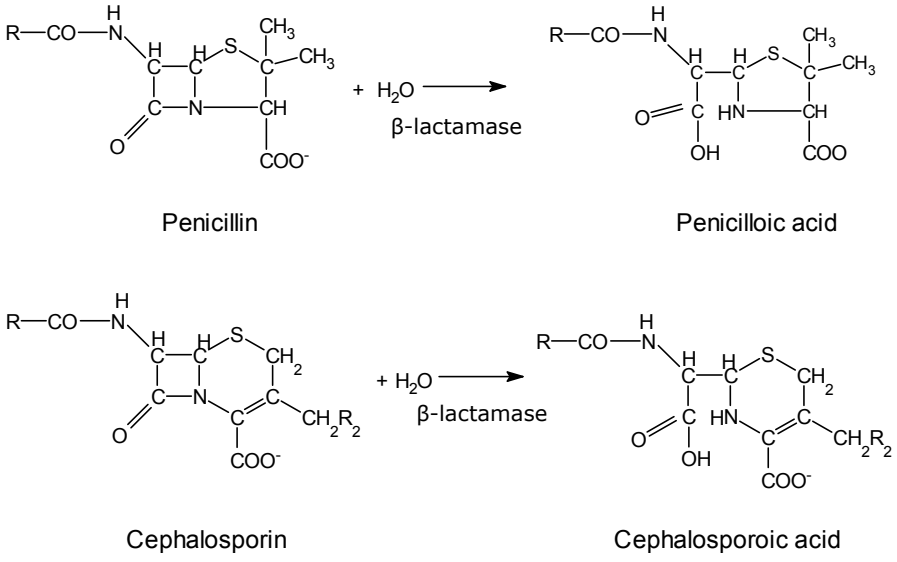

Figure 2.2 Hydrolysis of penicillins and cephalosporins by beta-lactamase.

Beta-lactamases consist of a single polypeptide chain with the complete absence of cysteine residue. Specifically β-lactamase from *B.licheniformis* consists of 165 amino acids (Çelik, 2003). They owe their success in proliferating continuously to the transposable nature of β-lactamase genes. These genes may be located chromosomally or extrachromosomally and can be mobilized within an organism by transportation and between organisms by mechanisms such as transduction, conjugation or transformation. β-lactamases can be classified on the basis of microbial origin, size, substrate and inhibition profiles, isoelectric point and, increasingly, by sequence. Numerous attempts to classify this group of enzymes have been made and as new data is gathered, especially gene sequence data, additional schemes will be offered (Fogarty and Kelly, 1990).

Beta-lactamases are manufactured for the specific assay of the penicillins, destruction of residual penicillin/cephalosporins in body fluid and culture media, sterility tests of penicillins, treatment of penicillin sensitivity reactions and for drug design (White and White, 1997).

Beta-lactamses have been the subject of numerous reviews, such as Medeiros & Jacoby (1986), Amyes (1987) and Sanders (1987) (Fogarty and Kelly, 1990). In more recent studies, on the other hand, for improving β-lactamase production the effect of glucose was investigated (Hemila et al., 1992). Among the bioreactor operation parameters, the effects of agitation rate (Wase and Patel, 1987), pH (Sargent et al., 1968; Hemila et al., 1992) and dissolved oxygen (Sargantanis and Karim, 1996; 1998) on β-lactamase production by *Bacillus* species were reported. Moreover, Celik and Çalık (2004) investigated the variations in product and by-product distributions, and the fermentation and oxygen transfer characteristics of the β-lactamase production process by *B.licheniformis* in a semi-designed medium at V= 3.0 dm^3 bioreactor system with a V_R=1.65 dm^3 working volume at Q_O/V_R=0.5 vvm and N=500 min^{-1} and reported that the oxygen transfer resistances were effective in cell growth phase; and Çalık et al. (2005) investigated the effect of oxygen transfer conditions within a wide range, from limiting to high oxygen transfer thoroughly on glucose-based defined medium in β-lactamase production by *Bacillus licheniformis*.

2.3 Bioprocess Parameters in Enzyme Production

Any operation involving the transformation of some raw material (biological or non-biological) into some product by means of microorganisms, animal or plant cell cultures, or by materials derived from them (e.g. enzymes, organelles), may be termed as a "bioprocess" (Moses and Cape, 1991).

Until about 1950, the predominant method of producing industrial enzymes was by extraction from animal or plant sources (Kirk and Othmer, 1994). However, today, microbial enzymes have largely replaced the traditional plant and animal enzymes used in industry. Enzymes are usually sensitive to harsh physical and chemical conditions, which demands careful selection of production processes and conditions for each individual enzyme (Yılgör, 2004). In aerobic bioprocesses, there are some important criteria that must be taken into account, in order to have high product yield. These are:

1. Microorganism
2. Medium composition
3. Bioreactor operation parameters
    i. Temperature
    ii. pH
    iii. Oxygen transfer rate
        * Air inlet rate (Q_0/V_R)
        * Agitation rate (N)

2.3.1 Microorganism

In bioprocesses, the selection of host microorganism for production of industrial enzymes is often critical for the commercial success of the product. Potential hosts should give sufficient yields, be able to secrete large amounts of protein, be suitable for industrial fermentations, produce a large cell mass per volume quickly and on cheap media, be considered safe based on historical experience or evaluation by regulatory authorities, and should not produce harmful substances or any other undesirable products (Kirk and Othmer, 1994).

Beta-lactamases are produced by most, if not all, bacterial species, blue green algae and yeasts. Among many species, *Bacillus* strains, which fulfill all the above criteria, are attractive as microbioreactors under well-designed bioreactor operation conditions due to their secretion ability of large amounts of enzyme into the bioreactor medium (Çalık et al., 2003b), and this makes the genus *Bacillus* more favorable than the others for β-lactamase production.

In the literature concerning β-lactamase production, Çalık et al. (2005), Celik & Çalık (2004), and Sargent et al. (1968), used *B.licheniformis*; Bernstein et al. (1967), Kuennen et al. (1980) and Wase and Patel (1987) used *B.cereus*; whereas, Hemila et al. (1992), and Sargantanis and Karim (1996, 1998) used *B.subtilis*.

2.3.1.1 The Genus *Bacillus*

The genus, *Bacillus*, encompasses about 35 currently recognized species, plus scores of proposed new species. The broad range of DNA content of recognized species is an indication of their genetic heterogeneity. Their morphology ranges from rod-shaped to spherical to mycelial. The major unifying morphological characteristic is endospore formation (Fogarty and Kelly, 1990). The endospores of the bacilli are resistant to heat, drying, disinfectants and other destructive agents, and thus may remain viable for centuries (Celik, 2003).

This genus exhibits a wide diversity of physiological abilities; although the majority is mesophilic, there are also psychrophilic and thermophilic species. Some species are strictly aerobic, others are facultatively aerobic. Some are acidophiles while others are alkalophiles. Some are haloduric. Some grow well on a single organic carbon/energy source in inorganic salts media while others require amino acids and vitamins (Fogarty and Kelly, 1990). The most widely studied *Bacillus*, *B.subtilis*, is known to have 2.6 μm-lengths, 0.65 μm-widths, $6x10^{-8}$ cm^2 cell surface area, $9x10^{-3}$ cm^3 cell volume, and $2.2x10^{-13}$ g dry cell weight.

Among these strains, *B.licheniformis* produces oval endospores that do not swell the mother cell. It is gram positive, is motile by peritrichous flagella, and produces acids from a range of sugars. It is listed by the American Food and

Drug Administration (FDA) as a GRAS organism. *B.licheniformis* is a facultative anaerobe, having pH and temperature tolerance in the range of 5.0-7.5 and 15°C-50°C respectively (Priest, 1993; and Laskin and Lechevalier, 1973).

2.3.1.2 Cell Growth, Kinetics and Yield Factors

For microorganisms, growth is their most essential response to their physiochemical environment (Yilgor, 2004).Growth can be considered as the orderly increase in all of the chemical constituents of an organism which, for unicellular organisms, leads to an increase in the number of individuals in the population (Scragg, 1988). In order to ensure orderly and energy-efficient growth, most of the reactions have to coupled, and the flux through the various pathways inside the cell is therefore carefully controlled (Nielsen and Villadsen, 1994). Unicellular microbial growth is autocatalytic such that the rate of growth is proportional to the cell concentration already present (Scragg, 1988), i.e. the specific growth rate of the total biomass, μ, is defined as,

$$\mu = \frac{1}{C_x} \cdot \frac{dC_x}{dt} \tag{2.3}$$

where C_X is the cell mass concentration (kg m^{-3}), t is time (h), and μ is the specific growth rate (h^{-1}). When a suitable growth supporting medium is inoculated with cells a characteristic sequence of events termed the growth cycle takes place (Scragg, 1998). Table 2.2 provides a summary of a typical batch growth cycle.

Table 2.2 Batch Cell Growth Cycle

Phase	Type of growth	Specific growth rate
Lag	Adaptation of the cells to he new env.	$\mu \approx 0$
Acceleration	Growth starts to occur	$\mu < \mu_{max}$
Growth	Growth achieves its maximum rate	$\mu \approx \mu_{max}$
Deceleration	Growth slows due to nutrient exhaustion or build-up of inhibitory products	$\mu < \mu_{max}$
Stationary	Growth ceases	$\mu = 0$
Death	Cells lose viability and lyse	$\mu < 0$

During the exponential growth period including lag, acceleration and growth phases, in batch cultivation, rate of cell growth, r_X, is described by the following equation (Shuler and Kargı, 1992):

$$r_X = \frac{dC_X}{dt} = \mu C_X \tag{2.4}$$

Similarly the substrate consumption rate, r_S, and product formation rate, r_P, are described as follows:

$$r_P = \frac{dC_P}{dt} \tag{2.5}$$

$$r_S = \frac{dC_S}{dt} \tag{2.6}$$

The specific, or relative, rates of synthesis and consumption are distinguished from the absolute rates of consumption or formation as follows:

$$q_P = \frac{1}{C_X}\frac{dC_P}{dt} \tag{2.7}$$

$$q_S = \frac{1}{C_X}\frac{dC_S}{dt} \tag{2.8}$$

Despite the complexity occurring in cell growth, yield principles can be applied to cell metabolism to relate flow of substrate in metabolic pathways to formation of biomass and other products. Yield coefficients allow quantifying the nutrient requirements and production characteristics of an organism (Doran, 2000). They are defined as the mass of biomass or product formed per unit mass of another material consumed. Thus,

$$Y_{X/S} = \frac{dX}{dS} = \frac{dX/dt}{dS/dt} = \frac{r_X}{r_S} \tag{2.9}$$

$$Y_{P/S} = \frac{-dP}{dS} = \frac{-dP/dt}{dS/dt} = \frac{r_p}{r_S} \quad (2.10)$$

where, $Y_{X/S}$ and $Y_{P/S}$ are the yield coefficients, X, P and S are mass of the cell, product and substrate, respectively, involved in metabolism. However, the usual method of measuring yields is the measure the amounts of biomass and or product formed and substrate consumed over some time period. Then, the overall biomass and product yields can be defined as:

$$\overline{Y}_{X/S} = \frac{\Delta X}{\Delta S} \quad (2.11)$$

$$\overline{Y}_{P/S} = \frac{\Delta P}{\Delta S} \quad (2.12)$$

A list of frequently used yield coefficients is given in Table 2.3. When yield for fermentation are reported, the time or time period to which they refer should be stated (Doran, 1995).

Table 2.3 Definition of yield coefficients.

Symbol	Definition	Unit
$Y_{X/S}$	Mass of cells produced per unit mass of substrate consumed	kg cell kg^{-1} substrate
$Y_{X/O}$	Mass of cells produced per unit mass of oxygen consumed	kg cell kg^{-1} oxygen
$Y_{S/O}$	Mass of substrate produced per unit mass of oxygen consumed	kg substrate kg^{-1} oxygen
$Y_{P/X}$	Mass of product formed per unit mass of substrate consumed	kg product kg^{-1} cell
$Y_{P/S}$	Mass of product formed per unit mass of substrate consumed	kg product kg^{-1} substrate
$Y_{P/O}$	Mass of product formed per unit mass of oxygen consumed	kg product kg^{-1} oxygen

2.3.1.3 Transport processes

A fundamental requirement for all organisms is the efficient transport of solutes and solvent. Such transport supplies chemicals for cellular metabolism, and energy generation as well as signals to change cellular functions. In order to regulate the movement of molecules into and through cells, specialized mechanisms have developed in cells to transport molecules efficiently. Thus, ion gradients are maintained across cell membranes; and cells use the energy of the ions to drive chemical reactions and to transmit information (Truskey, Yuan, and Katz, 2003).

Species may be transported across the plasma membrane by three different mechanisms: (1) free diffusion, (2) facilitated transport, and (3) active transport. In free and facilitated diffusion, species are transported down a concentration gradient. The difference between the two mechanisms is that facilitated diffusion is carrier-mediated, i.e. a specific carrier or transmembrane protein is involved in the transport, driven by a differential of chemical potentials (Stephanopoulos, Aristidou, Nielsen, 1998).

The third mechanism, active transport resembles facilitated diffusion because specific membrane localized proteins-called permeases-mediate the transport process. In contrast to facilitated diffusion, active transport can occur against a concentration gradient and therefore is a free energy-consuming process. The free energy required for the transport process may be provided by high-energy phosphate bonds in, for example, ATP (primary active transport). Alternatively, the transport process may be coupled to another transport process with a downhill concentration gradient (secondary active transport). In secondary active transport, the transport of the compound is coupled with the transport of another compound along a favorable concentration gradient. If the compounds are transported in the same physical direction, the transport is called symport, and if the compounds are transported in opposite physical directions, the transport is called antiport. Even though proton symport is the most common secondary transport mechanism, symport and antiport may also occur with Na^+, K^+, and Mg^{2+} (Stephanopoulos, Aristidou, Nielsen, 1998).

Active transport can only occur at intact, closed membranes. Such membranes can envelop very different compartments, like the whole cell, vesicles, the mitochondrial matrix, etc. As a result of active transport can ions and metabolites be concentrated within the respective compartment or the cell and the steady state of the metabolism can be kept constant despite of large fluctuations in the external medium's composition. Ions, especially sodium, potassium, calcium, magnesium and phosphate have an important part in the regulation of the metabolism. The best-known and best-studied ion pumps are the sodium-potassium- and the calcium pump. However, almost all the active transport in cells is carried out by only the sodium pump and proton pump.

The sodium pump occurs in a cycle of conformational changes apparently triggered by phosphorylation of the pump. As currently understood, the sequence of events can be summarized as follows (See Figure 2.3):

1. The pump, with bound ATP, binds three intracellular Na^+ ions.

2. ATP is hydrolyzed, leading to phosphorylation of a cytoplasmic loop of the pump and release of ADP.

3. A conformational change in the pump exposes the Na^+ ions outside to the cell, where they are released.

4. The pump binds two extracellular K^+ ions, leading somehow to dephosphorylation of the alpha subunit.

5. ATP binds and the pump reorients to release K^+ ions inside the cell.

6. The pump is ready to go again.

The sodium-potassium pump is a system that enables the cell to admit ions needed for important biological processes, such as the conduction of nerve impulses through the body; import of glucose, amino acids, nutrients into the cell; absorption of water, etc. In just one second, each channel can move more than 300 sodium ions out of the cell. When there are very few sodium ions in the

cell, facilitated infusion channels enable sodium ions to rush back into the cell. However, the channels must be opened. Some are opened by electrical currents, and others are opened only when the sodium ions are paired to partner molecules such as sugar or amino acids. Because so many ions rush back in through these channels, large numbers of partner molecules are pulled through as well, even if they are already present in high concentrations within the cell.

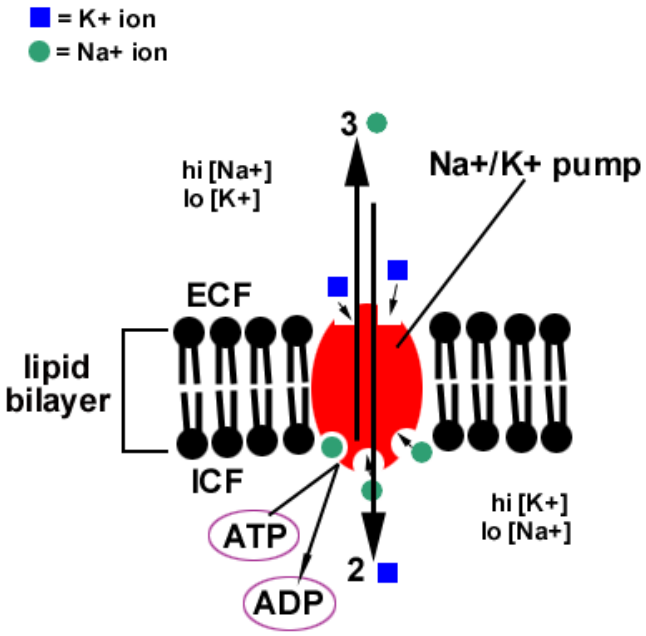

Figure 2.3 Schematic model of sodium potassium pump in operation (Bamberg and Schoner, 1994).

Although cells relay on the sodium-potassium pump to take in ions, they use a proton pump to pump protons across membranes. To function, this active transport system depends upon chemicals. The proton pump expels protons until large numbers of protons build up outside a membrane. The result is a proton gradient, in which outside face of the membrane has a lower pH and a more positive charge. This energized state of the membrane represents potential energy that can perform useful work. The controlled passage of protons back across the membrane through specific membrane proteins is used to drive ion transport, or ATP synthesis.

In the literature, Trchounian et al. (1987) studied the energy-dependent K^+ uptake and H^+-K^+ exchange in *S.typhimurium* and concluded that exchange of 2 H^+ from a cell for one K^+ of the medium in anaerobically grown *S.typhimurium* was carried out by the H^+-ATPase complex $F_0.F_1$ and the Trk-like system strongly united together, and this supercomplex functioned as the H^+-K^+ pump. Bagramyan and Martirosov (1989) proposed a model of a multienzyme transport supercomplex which consists of three genetically independent mechanisms: F_0F_1 H^+-ATPase to provide energy, the K^+-transporting Trk system as energy sink and formate-hydrogen lyase as donor of reducing equivalents, and concluded that oxidative phosphorylation or photophosphorylation could not proceed through direct transfer of energy but only with mediation of $\Delta\mu H^+$. Trchounian et al. (2001) studied the multiple uptake systems of *Escherichia coli* and found that in cells grown under fermentative conditions at pH 5.5, K^+ influx by a wild type strain upon hyper-osmotic stress at pH 5.5 was accompanied by a marked decease in H^+ efflux. Similar results with a mutant defective in Kdp and TrkA uptake systems but with a functional Kup system but not in a mutant defective in *Kdp* and *Kup* but having an active TrkA system suggested that Kup operates as a H^+-K^+ symporter. Finally, in a more recent study of Christiansen and Nielsen (2003) the extracellular potassium ion concentration was measured during production of Savinase by *B.clausii* in fed-batch cultivation and it was concluded that the specific glucose uptake rate increased with time for constant specific growth rate indicating that the maintenance requirements increased with time, possibly due to a deceasing K^+ concentration.

2.3.2 Medium Design

All living cells require certain nutrients for their growth and development. These nutrients must contain the chemical elements which constitute the cellular materials and structures, as well as those elements which are required for membrane transport, enzyme activity, and for the generation of the energy required for biosynthetic processes (Scragg, 1988). Hence, the qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation (Shuler and Kargı, 2002). Nutrients required by cells can be classified in two categories:

1. *Macronutrients;* are needed in concentrations larger than 10^{-4} M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} are major macronutrients.
2. *Micronutrients;* are needed in concentrations of less than 10^{-4} M. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Na^{2+}, vitamins, growth hormones, and metabolic precursors are micronutrients.

Nutrient media can be simple mixtures of a few pure chemical compounds, or contain a large number of preformed monomers as growth factors. As long as well-characterized chemicals are used, the medium is defined. However, it is simpler to add amino acids, vitamins and other natural compounds as crude digests of animal or plant substances. Thus, this type of media is called complex, because its precise chemical composition is not known. Often, complex media is less expensive than defined media. However, the primary advantage of defined media is that the results are more reproducible and the operator has better control of the fermentation. Further, recovery and purification of a product is often easier and cheaper in defined media (Shuler and Kargı, 2002).

In literature, Çalık et al. (2005) designed a glucose based defined medium containing 7.1 kg m^{-3} $(NH_4)_2HPO_4$, 7 kg m^{-3} glucose and salt solution, and using this media they investigated the effects of bioreactor operation parameters on beta-lactamase production. In the study of Celik and Çalık (2004), the effects of medium components for beta-lactamase by *Bacillus* species were investigated and the medium containing 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} yeast extract, 10 kg m^{-3} glucose and salt solution gave maximum beta-lactamase production. In addition, in the study of Hemila et al. (1992), the effect of glucose was investigated for improving beta-lactamase production in a strain of *B.subtilis*, and the addition of 60 kg m^{-3} glucose and 100 mM potassium phosphate was defined as the favorable condition for higher yields and stability, but with the disadvantage of retardation of growth in the exponential phase.

2.3.3 Bioreactor Operation Parameters

Bioreactor operation parameters, i.e. oxygen transfer, pH, and temperature, influences growth and product formation by affecting metabolic pathways and changing metabolic fluxes.

2.3.3.1 Temperature

Like all chemical reactions microbial growth is affected by temperature. As the temperature is increased toward optimal growth temperature, the growth rate approximately doubles for every 10°C increase in the temperature. Above the optimal temperature range, the growth rate decreases and thermal death may occur. The increased death rate at higher temperatures is mainly due to thermal denaturation of proteins resulting in an increase in cell maintenance energy requirement for repair mechanisms. At low temperatures, on the other hand, the regulatory mechanisms of the cell are affected in addition to diffusional limitations such as transport of substrates into and within the cell. As a result the biomass yield falls at extremes of temperature (Scragg, 1988; Shuler and Kargı, 2002).

In literature concerning β-lactamase production, Wase and Patel (1987) and Sargent et al. (1968) conducted β-lactamase production at T=30°C and Sargantanis and Karim (1996, 1998) at T=37°C, without investigating the effect of temperature. Bernstein et al. (1967) investigated the effect of temperature on β-lactamase production in the range T=18°C-46°C, and reported that the basal level of β-lactamase production in an inducible strain of *B.cereus* reaches a maximum at T=42°C. They have also proposed that culturing at 42°C and lowering the temperature to 37°C leads to an increase in β-lactamase production. Kuennen et al. (1980) also investigated the effect of temperature, and reported that β-lactamase activity was relatively constant when a constitutive strain of *B.cereus* was grown at a temperature range of 33°C to 42°C, above which a rapid decrease in activity was observed. In the study with *B.subtilis* β-lactamase by Hemila et al. (1992), it was concluded that in the range 27°C to 40°C, 30°C was the optimum cultivation temperature for β-lactamase production. In a more recent study, Celik and Çalık (2004) investigated the effects of temperature in the range T=29°C-37°C, and 37°C was found to be the most favorable condition for β-lactamase production by *B.licheniformis*.

2.3.3.2 pH

Microorganisms tend to grow over a limited pH range, and even within this range they frequently shift their metabolism as result of even a 1-1.5 pH unit change (Scragg, 1988). For many bacteria, pH optima range from 3 to 8.

In most fermentations, pH can vary substantially. Often the nature of the nitrogen source can be important. Furthermore, pH can change due to the production of organic acids, utilization of acids (particularly amino acids), or the production of bases. Thus, pH control by means of a buffer or an active pH control system is important (Shuler and Kargı, 2002). Nevertheless, some bioprocesses require controlled pH conditions, while others might require uncontrolled pH operations, in order to increase the product yield and selectivity (Çalık et al., 2002).

Even though fermentation processes are generally carried out at a constant regulated pH of the culture media, internal pH can be expected to be widely different and variable during the course of the fermentation. While some bacteria maintain a fairly constant pH_i values, only at the expense of a significant increase in the maintenance demands, upon changes in extracellular pH, i.e. pH homeostasis, others maintain a constant pH gradient. The intracellular pH has a major influence on the metabolic activity of cells. It determines the in-vivo activity of the enzymes and often modulates the transport kinetics of nutrients and metabolites. Moreover pH gradient across cell membranes also controls ATP generation, and more generally the cellular energetics. Thus, under stressful pH conditions, regulation of intracellular pH in bacteria involves proton-driven transporters and more passive mechanisms, such as changes of the lipid composition of the membrane and the buffering capacity of the cytoplasm. Consequently, if one aims towards a quantitative understanding of the cell metabolism, one has to take into account the time variations of the intracellular pH and its effects on the in-vivo kinetics of the metabolic steps involved (Levyal et al., 1997; Hornbæk et al, 2004).

In the study by Sargent et al. (1968), specific β-lactamase activity of cells was shown to be constant irrespective of pH over the range of pH 5.5 to 7.5. However, the fraction of the total enzyme secreted into the extracellular medium

was favorable around pH 7.5. In the study with *B.subtilis* β-lactamase by Hemila et al. (1992), it was concluded that in the pH range 5.8 to 7.4, pH 6.0 was the optimum cultivation pH for β-lactamase production. Sargantanis and Karim (1996) stated that pH control was not beneficial for β-lactamase productivity, and conducted their experiments at an initial pH of 7.0, without investigating its effect. Celik and Çalık (2004) investigated the effects of pH in the range of 5.8-7.2 in media with NaH_2PO_4-Na_2HPO_4 buffer having a buffering capacity of 0.02 M, and in media without buffer, where the initial pH was set by the addition of either NaH_2PO_4 or Na_2HPO_4. The uncontrolled pH operation with an initial pH of 6.25 was found to be the most favorable condition for β-lactamase production. In a more recent study, Çalık et al (2005), investigated the effect of pH control in an initial pH range of 5.8-8.0, in the media with NaH_2PO_4-Na_2HPO_4 buffer, and in media without buffer, and concluded that pH_0=7.5 uncontrolled-pH operation gave the highest β-lactamase activity.

Related with the production of other biomolecules using *Bacillus licheniformis*, Çalık et al. (2002) investigated the effects of the controlled and uncontrolled pH conditions, as well as of the value of initial pH in the range of 7-7.5, on serine alkaline protease (SAP) production on a defined medium with single carbon source glucose in batch bioreactors, and concluded that for SAP production, uncontrolled pH operation was more favorable than controlled pH operations; and pH_0=7.25 was optimum for SAP production, while, the highest cell concentration was obtained at pH_0=7 in uncontrolled operation. In a more recent study of Çalık et al. (2003), the influence of controlled- and uncontrolled-pH conditions together with the initial pH on the product and by-product distributions and oxygen transfer characteristics, whereupon the process rate limitations in relation to the intracellular reaction rates were investigated in SAP fermentation process by recombinant *B.licheniformis* carrying pHV1431*::subC* on a defined medium with sole carbon source glucose in the pH range of 6.80-7.25 in batch bioreactors. It was found that although the same amount of cell was produced at all conditions in each type of operation, with increase of the initial pH the cell formation rates increased; moreover, uncontrolled-pH operation was favorable for the cell formation. Similarly, the SAP synthesis rates and concentrations were higher at uncontrolled-pH operations.

Related with the effects of intracellular pH, Hornbæk et al. (2002) proved that the existence of a pH gradient over the cytoplasmic membrane influences the duration of the lag phase in populations of *B.licheniformis*. In the most recent study of Hornbæk et al. (2004), the effect of transferring *B.licheniformis* cells at the early stationary growth phase (pH 5.3) to fresh growth medium at pH 5.0-8.0 was investigated. The optimum extracellular pH value for growth of *B.licheniformis* was found to be pH 7.0, resulting in the shortest lag phase, highest maximum specific growth rate and maximum biomass formation. Moreover, an average pH gradient of approximately 1.0 was found 15 min. after transfer to pH_{ex} 5.0-8.0. Mechanisms involved in pH_i regulation appeared to include changes in fatty acid synthesis to yield a more rigid cell membrane structure at low pH_{ex} values and conversion of pyruvate to acetion instead of acetate for neutralization of low pH_{ex} values.

2.3.3.3 Oxygen Transfer

2.3.3.3.1 The Importance and Mechanism of Oxygen Transfer

Many important fermentations have been found to depend critically on the availability of dissolved oxygen in the aqueous phase in contact with the organism. Molecular oxygen is required as a terminal electron acceptor in the aerobic metabolism. Since oxygen is only sparingly soluble in aqueous solution it must be continuously supplied. The transfer of oxygen from the gas to the microorganism takes place in several steps (Scragg, 1988). These steps are:

1. Diffusion of oxygen from the bulk gas to the gas-liquid interface,

2. Movement across the gas-liquid interface,

3. Diffusion of oxygen through a relatively stagnant liquid region adjacent to the gas bubble, i.e., from the gas-liquid interface to the well-mixed bulk liquid,

4. Transport through the bulk liquid,

5. Diffusion through the relatively stagnant liquid film surrounding the cells,

6. Movement across the liquid-cell interface,

7. If the cells are in a floc, clump or solid particle, diffusion through the solid to the individual cell,

8. Transport through the cytoplasm to the site of reaction.

In general, according to cell growth conditions and metabolic pathway analysis, some bioprocesses require high oxygen transfer rate conditions while others require controlled oxygen transfer rates in order to regulate oxygen uptake rate (Çalık et al., 1998). Gaseous oxygen is introduced into growth media by sparging air; and oxygen transfer rate can be adjusted by either changing the air inlet rate or agitation rate.

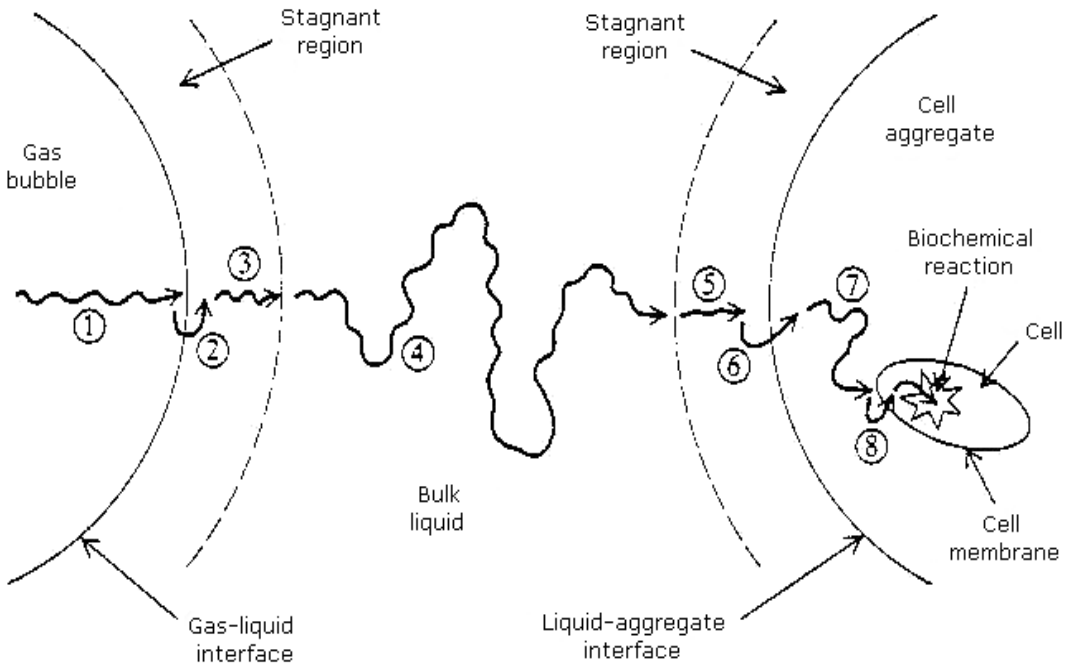

Figure 2.4 Schematic diagram of steps involved in transport of oxygen from a gas bubble to inside a cell.

When cells are dispersed in the liquid, and the bulk fermentation broth is well mixed, the major resistance to oxygen transfer is the liquid film surrounding the gas bubbles; therefore the rate of oxygen transfer from gas to liquid is of prime importance (Nielsen and Villadsen, 1994). An expression for oxygen transfer rate (OTR) from gas to liquid is given by the following equation:

$$OTR = k_L a(C_O^* - C_O) \tag{2.13}$$

where, k_L is the oxygen transfer coefficient, a is the gas-liquid interfacial area, k_La is the volumetric oxygen transfer coefficient, C_O^* is saturated dissolved oxygen concentration, C_O is the actual dissolved oxygen concentration in the broth.

Since solubility of oxygen in aqueous solutions is very low, the liquid phase mass transfer resistance dominates, and the overall liquid phase mass transfer coefficient, K_La, is approximately equal to liquid phase mass transfer coefficient, k_La.

2.3.3.3.2 Oxygen Transfer Characteristics

A common engineering challenge in large-scale aerobic fermentations is ensuring an adequate level of dissolved oxygen to achieve the desired cell growth and productivity (Stephanopoulos, Aristidou, Nielsen, 1998), as oxygen influences metabolic pathways and changes metabolic fluxes (Çalık et al., 1999). The rate of oxygen transfer in fermentation broths is influenced by several physical and chemical factors that change either the value of K_La, or the driving force for mass transfer, (C_O^*- C_O). Therefore, the oxygen uptake rate of the cells, and the liquid phase mass transfer coefficient, K_La, are important characteristics of oxygen transfer.

The rate at which oxygen is consumed by cells in bioreactors determines the rate at which it must be transferred from gas to liquid. Many factors influence oxygen demand. The more important of these are cell species, culture growth phase, carbon nutrients, pH, and nature of the desired microbial process. In batch culture, rate of oxygen uptake varies with time. In addition, the rate of

oxygen consumption per cell, known as the specific oxygen uptake rate (q_O), also varies. Thus, oxygen uptake rate (OUR), $-r_O$, per unit volume of broth is given by (Doran, 2000):

$$-r_O = q_O C_X \tag{2.14}$$

For the design of aerobic biological reactors correlations of data more or less approximating the situation of interest are frequently used to establish whether the slowest process step is the oxygen transfer rate or the rate of cellular utilization of oxygen (or other limiting substrate). The maximum possible mass-transfer rate is simply that found by setting $C_0=0$ in equation (2.13): all oxygen entering the bulk solution is assumed to be rapidly consumed. The maximum possible oxygen utilization rate can be found by the following equation:

$$-r_{O\,max} = C_x \mu_{max}/Y_{x/o} \tag{2.15}$$

Evidently, if maximum possible oxygen transfer rate is much larger than maximum possible oxygen utilization rate, the main resistance to increased oxygen consumption is microbial metabolism and the reaction appears to be biochemically limited. Conversely, the reverse inequality apparently leads to C_0 near zero, and the reactor seems to be in the mass-transfer-limited mode. The situation is actually slightly more complicated. In general above some critical bulk oxygen concentration the cell metabolic machinery is saturated with oxygen. In this case, sufficient oxygen is available to accept immediately all electron pairs which pass through the respiratory chain, so that some other biochemical process within the cell is rate limiting (Bailey and Ollis, 1986).

The liquid phase mass transfer coefficient, K_La, is an important parameter in bioreactors, which indicates the oxygen transfer rate from gas to the liquid phase. It is dependent on the physico-chemical properties of the bioreactor media, and on the physical properties and operating conditions of the vessel. The value of K_La volumetric mass transfer coefficient can be controlled by the agitation conditions and the air flow rate. Oxygen is a substrate which limits

growth, however, above a certain concentration, growth will become independent of oxygen concentration. Knowledge of K_La behavior allows the operation of bioreactors at conditions where oxygen is not a limiting factor for growth (Scragg, 1988).

Numerous methods have been developed for the experimental determination of K_La values. Among these methods, dynamic method is a widely used simple method that can be applied during a fermentation process. This method is based on a material balance on the oxygen in the liquid phase,

$$\frac{dC_O}{dt} = K_L a(C_O^* - C_O) - q_0 C_X \qquad (2.16)$$

As shown in Figure 2.5, at some time t_0, the broth is de-oxygenated by stopping the air flow. During this period, dissolved oxygen concentration, Co_0, drops, and since there is no oxygen transfer, equation (2.16) becomes:

$$\frac{dC_O}{dt} = r_0 \qquad (2.17)$$

Using equation (2.17) in region-II of Figure 2.6, oxygen uptake rate, $-r_0$, can be determined.

Air inlet is then turned back on, and the increase in C_O is monitored as a function of time. In this period, region-III, equation (2.16) is valid. Combining equations (2.14) and (2.16) and rearranging,

$$C_O = -\frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_0\right) + C_O^* \qquad (2.18)$$

From the slope of a plot of C_O versus ($dC_O/dt - r_0$), K_La can be determined (Figure 2.6).

The Dynamic Method can also be applied to conditions under which there is no reaction, i.e., $r_0=0$ (Nielsen and Villadsen, 1994). In this case, the broth is

de-oxygenated by sparging nitrogen into the vessel. Air inlet is turned back on and again the increase in Co is monitored as a function of time. Modifying equation (2.18)

$$C_O = \frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \qquad (2.19)$$

From the slope of a plot of C_O versus dC_O/dt, the physical mass transfer coefficient, $K_L a_0$, can be determined.

A large number of different empirical correlations for the volumetric mass transfer coefficient $K_L a$ have also been presented in the literature. Most of these correlations can be written in the form:

$$K_L a = k\left(\frac{P_G}{V_R}\right)^{\beta} V_S^{\alpha} \qquad (2.20)$$

where the parameters are specific for the considered system, i.e. for the bioreactor design. Thus for different stirrers and different tank geometry the parameter values may change significantly, and a certain set of parameters can be reasonably used only when studying a system which the parameters were originally derived. Some of the parameter values reported in the literature for stirred tanks are listed in Table 2.4.

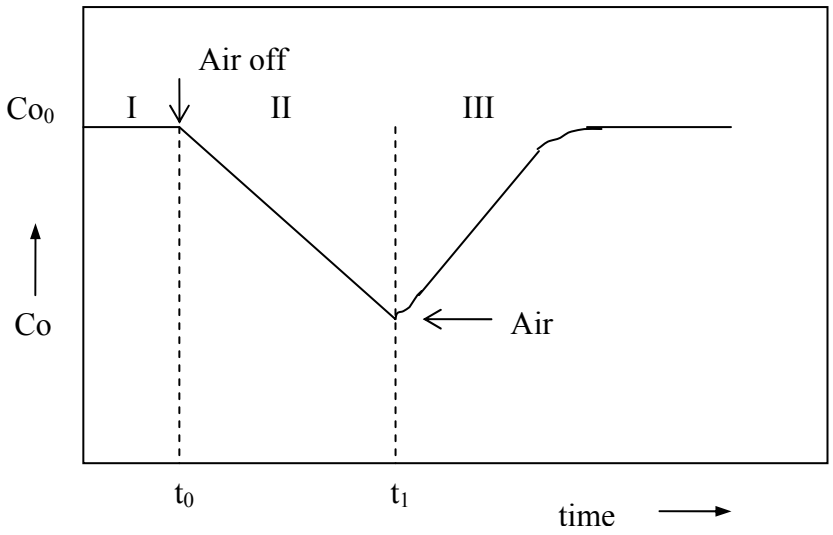

Figure 2.5 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La.

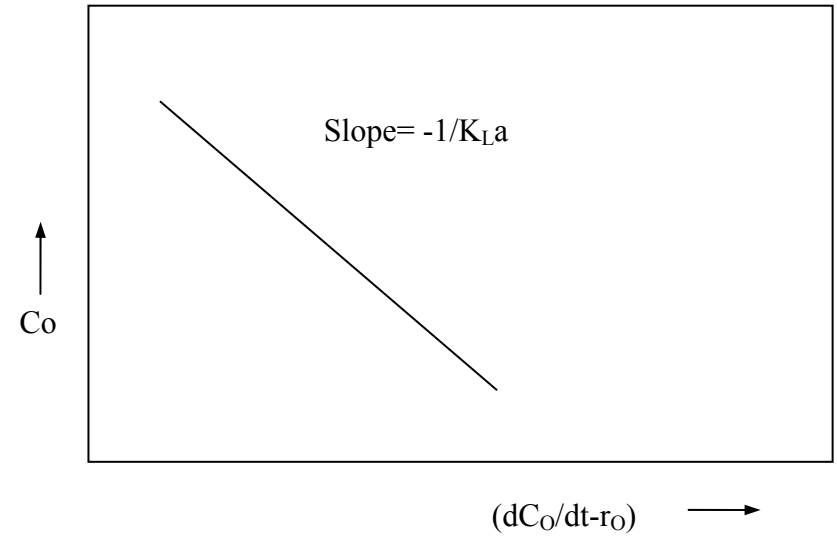

Figure 2.6 Evaluating K_La using the Dynamic Method.

Table 2.4 Parameter values for the empirical correlation of K_La.

Medium	Eq #	k	α	β	Agitator	Reference
Coalescing	1	0.025	0.5	0.4	Six-bladed Rushton turbines	Moo-Young and Blanch (1981)
	2	0.00495	0.4	0.593	Six-bladed Rushton turbines	Linek et al. (1987)
	3	0.01	0.4	0.475	Various agitators	Moo-Young and Blanch (1981)
	4	0.026	0.5	0.4	Not specified	van't Riet (1979)
	5	0.01	0.4	0.475	Four-bladed Rushton turbines	Çalık et al. (2005)
Noncoalescing	6	0.0018	0.3	0.7	Six-bladed Rushton turbines	Moo-Young and Blanch (1981)
	7	0.02	0.4	0.475	Various agitators	Moo-Young and Blanch (1981)
	8	0.002	0.2	0.7	Not specified	van't Riet (1979)
	9	0.00525	0.2	0.7	Not specified	Çalık et al. (1998)

The effect of oxygen transfer conditions on β-lactamase production by *Bacillus* species was investigated by Sargantanis and Karim (1996, 1998). They mainly focused on the performance of dissolved oxygen (DO) control strategy, and compared β-lactamase activities observed at different dissolved oxygen levels, which were kept constant during the bioprocess using an adaptive pole placement control algorithm. They performed the experiments at 3%, 5%, 8% and 15% constant dissolved oxygen levels and reported the highest productivity of β-lactamase at the highest DO level (15%), but that the rate of β-lactamase degradation by proteases was the highest at this level also, without giving the protease activities. Therefore they concluded that β-lactamase production was much higher at low DO levels (3%), although it occurred at a later stage of the fermentation, and that limited growth conditions favored long-term β-lactamase production.

In the study of Çelik and Çalık (2004), oxygen transfer characteristics ($-r_0$, K_La) of the bioprocess for β-lactamase production by *B.licheniformis* were investigated in a semi-defined medium containing glucose, $(NH_4)_2HPO_4$, yeast extract and the salt solution, at pH_0=6.0, T=32°C at Q_0/V=0.5 vvm and N=500 min^{-1} oxygen transfer conditions and found that throughout the bioprocess, overall oxygen transfer coefficient varied between 0.006-0.016 s^{-1}; oxygen uptake rate varied between 0.001-0.003 mol m^{-3} s^{-1}.

In the recent study (Çalık et al, 2005) the effects of oxygen transfer on β-lactamase production and on intracellular reaction rates were investigated at three different air inlet (Q_0/V_B=0.2, 0.5 and 1vvm) and at three agitation rates (N=250, 500, 750 min^{-1}) in V=3.0 dm^3 batch bioreactors with *B.licheniformis* using a glucose-based defined medium. The highest β-lactamase activity was obtained at 0.5 vvm 500 min^{-1} and at 0.2 vvm 500 min^{-1} conditions while the highest cell concentration was obtained at 0.5 vvm 750 min^{-1} and at 0.2 vvm 750 min^{-1} conditions. K_La, increased with increase in the agitation and aeration rates and its value varied between 0.007-0.044 s^{-1} and oxygen uptake rate varied between 0.4-1.6 mol m^{-3} s^{-1}.

Related with the production of other biomolecules using *Bacillus licheniformis*, effects of oxygen transfer were investigated in the production of serine alkaline protease by Çalık et al. (1999) at nine different oxygen transfer

conditions by forming a 3x3 matrix using three agitation rates N=150, 500, 750 min^{-1} and three air inlet rates Q_0/V=0.2, 0.5, 1.0 vvm with the initial substrate concentration C_0=9.0 kg m^{-3} by using a laboratory-scale 3.5 dm^3 batch bioreactor consisted of a system of working volume 2.0 dm^5 equipped with two four-blade Rushton turbines, and dissolved oxygen, temperature, pH, foam, air-inlet and stirring rate measurements and control. In the bioreactor the oxygen uptake rate values as well as the oxygen transfer rate increased with the increasing oxygen transfer, i.e. Q_0/V_R and N, up to the medium oxygen transfer condition, Q_0/V_R=0.5 vvm and N=750 min^{-1}. Nevertheless, at higher oxygen transfer conditions the cell growth, and consequently the OUR and OTR decreased. The impact of the further increase in Q_0/V_R and/or N after Q_0/V_R=0.5 vvm and N=750 min^{-1} condition increases the shear fields in the bioreactor; and the exposure of the bacilli to higher shear fields at high oxygen transfer conditions causes considerable dynamic influences that decease OUR as well as the OTR and consequently perturbs the metabolism of *B.licheniformis*. With the carbon source citrate, the consumption rate of the substrate increases with increasing agitation rate and air inlet rate, and cell concentration was the highest at low oxygen transfer condition.

2.4 Operation Modes

Reactor operation includes discontinuous and continuous operations, steady-state and transient-state modes, and a group of intermediate modes that can be referred to collectively as semicontinuous operation (Moser, 1988).

2.4.1 Batch Operation

The batch operation is the most frequently used type of operation in biotechnological productions. Virtually all food processing, pharmaceutical and agricultural bioprocesses are carried out in batch reactors (Dam-Mieras, Jeu, Vries, Currell, James, Lach, Patmore, 1992).

Batch operation requires a relatively simple setup. It has the advantage of being easy to perform and can produce large volumes of data experimental data in a short period of time. The disadvantage is that the experimental data are difficult to interpret as there are dynamic variations throughout the operation,

i.e. the environmental conditions experienced by the cells vary with time. By using well-instrumented bioreactors at least some variables, e.g., pH and dissolved oxygen tension, may, however, be controlled at a constant level (Stephanopoulos, Aristidou, Nielsen, 1998).

In the literature, Hemilä et al. (1992) studied the effect of glucose, pH and temperature on the production level of *E.coli* β-lactamase; Sagantanis and Karim (1996, 1997) studied the effect of oxygen limitation and adaptive pole placement control for DO-control during β-lactamase production; and Çalık et al. (2002, 2003) studied the effect of pH on SAP by *B.licheniformis*, in batch cultivations.

2.4.2 Continuous Operation

A typical operation of the continuous bioreactor is the so-called bioreactor is the so-called *chemostat*, where the added medium is designed such that there is a single rate-limiting substrate. This allows for controlled variation in the specific growth rate of the biomass. The advantage of the continuous bioreactor is that a steady-state can be obtained, which allows for precise experimental determination of specific rates under well-defined environmental conditions. These conditions can be further varied by changing the feed flow rate to the bioreactor. This allows valuable information concerning the influence of the environmental conditions on cellular physiology to be obtained. The disadvantage of the continuous bioreactor is that it is laborious to operate as large amounts of fresh, sterile medium have to be prepared and requires long periods of time for a steady state to be achieved. Despite the advantages of continuous operation, it is rarely used in industrial processes because it is sensitive to contaminations, e.g., via the feed stream, and to genetic instability that may lead to the formation of fast-growing mutants that out-compete the production strain. Other examples of continuous operation are the *pH-stat*, where the feed flow is adjusted to maintain constant pH in the bioreactor, and the *turbidostat*, where the feed flow is adjusted to maintain the biomass concentration at a constant level (Stephanopoulos, Aristidou, Nielsen, 1998).

In the literature, concerning continuous operation mode, Wase and Patel (1987) investigated the effects of changes in agitation rate on steady-state penicillinase titers in continuously-cultivated *bacillus cereus*; and Christiansen

and Nielsen (2002) studied the production of extracellular protease and glucose uptake in *Bacillus claussi* in steady-state and transient continuous cultures.

2.4.3 Fed-batch Operation

This is probably the most common operation in industrial practice, because it allows for control of the environmental conditions, e.g., maintaining the glucose concentration or specific growth rate at a certain level, and it enables formation of much higher titers (up to several hundreds grams per liter of some metabolites), which is of importance in the subsequent downstream processing. The basic characteristic of fed-batch microbial processes is that the concentrations of nutrients fed into the culture liquid of the bioreactor can be controlled voluntarily by changing the feed rate. In ideal batch bioreactor, nutrient concentrations are not controlled and are progressively used up; whereas in an ideal continuous bioreactor at steady state, the concentrations of all nutrients, including the growth limiting substrate, are kept constant. Hence, fed-batch is superior to conventional batch operation especially when changing concentrations of a nutrient affect the yield or productivity of the desired metabolite (Yamané, and Shimizu, 1984). At the same time, the fed-batch operation is a convenient experimental system for maintaining steady environmental conditions to facilitate physiological studies. The physiological state of the culture during any part of a cultivation is important since a high number of dead or dormant cells have a detrimental effect on the synthesis of any desired product, and this is especially relevant in fed-batch cultivations wherein the cells may be undergoing prolonged and severe nutrient limitation (Stephanopoulos, Aristidou, Nielsen, 1998).

In the literature, Yamané, and Shimizu (1984) reviewed the history and characteristics of fed-batch technique in microbial reactions and classified various fed-batch techniques according to the mode of nutrient feeding. Strandberg et al. (1994) studied the use of fed batch cultivation for achieving high cell densities in the production of a recombinant protein in *E.coli* and concluded that fed-batch cultivation made it possible to increase the final cell concentration compared to batch cultivation. Hewitt et al. (1999) investigated the use of multi-parameter flow cytometry to compare the physiological response of *E.coli* W3110 to glucose limitation during batch, fed-batch and

continuous culture cultivations, and stated that during the latter stages of fed-batch cultivations there was a considerable drop in cell viability, as characterized by cytoplasmic membrane depolarization and permeability. However, this trend was not observed in either batch or continuous culture cultivations. In a more recent study, Christiansen and Nielsen (2003) studied the production of Savinase and population viability of *B.clausii* during high-cell-density fed-batch cultivations with linear and exponential feeding profiles and concluded that in the latter parts of the fed-batch cultures with linear feed profile, a large portion of the cell population was found to have a permeable membrane, indicating a large percentage of dead cells. Moreover, the highest overall yield was obtained with an exponential feed profile, however, the yields in fed-batch cultures with linear feed profiles were only marginally smaller, and the highest total amount of Savinase was obtained with linear feed profiles.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Benzylpenicillin (penicillin G) was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. 5-(and 6-)-carboxy SNARF-1, acetoxymethyl ester, acetate, SNARF-4F 5-(and-6-)-carboxylic acid, acetoxymethyl ester, acetate *cell permeant*, and LIVE/DEAD *Bac*Light Bacterial Viability Kit *for microscopy and quantitative assays* were purchased from Moleclar Probes Inc., Eugene, OR. All other chemicals were analytical grade, and obtained either from Sigma Ltd., Difco Laboratories, or Merck Ltd.

3.2 The Microorganism

Bacillus licheniformis 749/C (ATCC 25972) was used as the microbial source of β-lactamase (EC 3.5.2.6). The microorganisms, which were freeze dried when received, were kept at -20ºC, and brought to an active state by incubating for 30 min, at 30ºC, in a liquid medium, V=0.3ml, that contained (kg m^{-3}): soytryptone, 5; peptone, 5; $MnSO_4.2H_2O$, 0.010. Afterwards, the microorganisms were inoculated onto a solid medium, and stored at 4ºC.

3.3 The Solid Medium

The microorganisms, stored on agar slants at 4ºC, were inoculated onto the newly prepared agar slants under sterile conditions, and they were incubated at 30ºC for 24h. The composition of the solid medium for β-lactamase production by *Bacillus* sp. is given in Table3.1.

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

Compound	Concentration, kg m^{-3}
Soytryptone	5.00
Peptone	5.00
$MnSO_4.2H_2O$	0.01
Agar	15.00

3.4 The Precultivation Medium

Microorganisms grown in the solid medium for 24 h, were inoculated into precultivation medium, and incubated at 37ºC and N=200 min^{-1}, for 3.5 h in laboratory scale bioreactor experiments, and for 12 h in pilot scale bioreactor experiments. Microorganism growth was conducted in orbital shakers under agitation and heating rate control, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. The constituents of the precultivation medium for cell growth and β-lactamase production is given in Table 3.2.

Table 3.2 The composition of the precultivation medium.

Compound	Concentration, kg m^{-3}
Soytryptone	15.0
Peptone	5.00
Na_2HPO_4	0.25
$CaCl_2$	0.10
$MnSO_4.2H_2O$	0.01

3.5 The Production Medium

When the microorganism concentration in the precultivation medium reached to 0.30 kg m^{-3}, the microorganisms were inoculated to the production medium in the laboratory scale bioreactor (V=150 ml) with an inoculation ratio of 1/10. For pilot scale bioreactor experiments (V=3.0 dm^3), on the other hand,

7.5-fold diluted microorganisms, incubated in the precultivation medium for 12 hours, were inoculated to the production medium with 1/10 inoculation ratio.

Laboratory scale batch fermentations were conducted in agitation and heating rate controlled orbital shakers at an agitation rate of 200 min^{-1} and a cultivation temperature of 37ºC, using air filtered 150 cm^3 shake bioreactor that contained V_R = 33 cm^3 glucose based defined medium whose composition is given in Table 3.3 (Arifoglu, 2004), unless otherwise stated. The laboratory scale experiment results enabled the design of the defined medium for the β-lactamase production in terms of the initial phosphate source concentrations. Pilot scale β-lactamase fermentation, on the other hand, were accomplished in 3.0 dm^3 batch bioreactor (Braun CT2-2), having a working volume of 0.5-2.0 dm^3 and a diameter of 12.1 cm, and consisting of temperature, pH, foam, stirring rate and dissolved oxygen controls. The bioreactor utilized an external cooler, steam generator and a jacket around the bioreactor for sterilization and temperature control. The bioreactor was stirred with two four-blade Rushton turbines with a diameter of 5.4 cm, and consisted of four baffles and a sparger.

Table 3.3 The composition of the glucose based defined medium.

Macronutrients	Concentration, kg m^{-3}
Glucose	7.0
$(NH_4)_2HPO_4$	7.1
Micronutrients (salt solution)	Concentration, kg m^{-3}
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	1.0×10^{-3}
$ZnSO_4.7H_2O$	1.0×10^{-3}
$MnSO_4.H_2O$	7.5×10^{-5}
$CuSO_4.5H_2O$	1.0×10^{-5}

All the medium components except the salt solution were steam sterilized at 121°C for 20 min, glucose being sterilized separately. The micronutrients all together, referred to as the salt solution, was filter sterilized with a sterile filter of 0.2 μm pore size.

3.6 Analysis

Throughout the bioprocesses, samples were taken at characteristic cultivation times. After determining the cell concentration, the medium was centrifuged at 13500 min^{-1} for 10 min at 4°C to precipitate the cells. Supernatant was used for the determination of β-lactamase activity. In pilot scale experiments, besides cell concentration, β-lactamase activity, reduced sugar, sodium, and potassium ion concentrations, and phosphate ion concentration for exponential feeding with fed-batch cultivation; amino acid and organic acid concentrations; and viability were determined. In addition, in order to determine the intracellular sodium, potassium, and ammonium ions, as well as amino acid, and organic acid concentrations, samples were taken at regular time intervals. Moreover, besides the mentioned parameters oxygen uptake rate and liquid phase mass transfer coefficient values were determined in bioreactor system experiments.

3.6.1 Cell Concentration

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

3.6.2 Beta-Lactamase Activity

Beta-lactamase activity was determined by measuring the hydrolysis of benzylpenicillin. Samples from the culture broth was harvested by centrifugation (Sigma 1-15) at 13,500 g for 10 min. Fresh substrate solutions were prepared daily and maintained at 30°C, by dissolving 0.25 kg m^{-3} benzylpenicillin in 0.1 M phosphate buffer, pH 7.0. 0.1 cm^3 sample of centrifuged culture supernatant, diluted properly, was added to 3 cm^3 of substrate solution and immediately analyzed, by following the change in absorbance in one minute at 232 nm with a UV spectrophotometer (Thermo Spectronic, Heλios α) (Wase and Patel, 1987). One unit of β-lactamase activity was defined as the amount of enzyme that could hydrolyze 1μmol of benzylpenicillin at 30°C and pH 7.0 in one minute, (Appendix B).

3.6.3 Glucose Concentration

Reduced sugar, glucose, concentration was determined by the glucose oxidation method at 505 nm with a UV spectrophotometer (Boyaci et. al., 2005). D-glucose is oxidized in the presence of glucose oxidase enzyme (eqn. 3.1) and peroxide formed due to oxidation reaction is further reacted with 4-aminoantipyrine and phenol in the catalysis of peroxidase to form iminoquinone (eqn. 3.2) which gives spectro-photometrically observable red color in proportion with glucose concentration.

$$\text{D - glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} Gluconate + H_2O_2 \quad (3.1)$$

$$H_2O_2 + 4\text{ - Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Im}inoquinone + H_2O \quad (3.2)$$

The preparation method of analysis solution and standard glucose solutions were given in Appendix C. The calibration curve was obtained from the slope of absorbance versus known standard glucose concentration for each experiment separately. The method used in analysis of samples is given below:

1. The samples containing more than 1 g/L glucose were diluted to a final concentration less than or equal to 1g/L.
2. 2 ml analysis solution was added to standard glucose solutions and 0.05 ml samples, respectively. Due to analysis procedure the test tubes and analysis solution should be kept at room temperature.
3. Treated samples were incubated at either room temperature for 20 minutes or at 37°C for 10 minutes. The sample passing through the same steps but do not contain any reducing sugar is used as blank and the absorbance values of the samples were measured by a UV spectrophotometer at 505 nm.

3.6.4 Determination of Sodium and Potassium Ion Concentrations

Extracellular sodium and potassium ion concentrations were determined using flame-photometer (JENWAY, PFP7). Samples taken from the broth were centrifuged at 13500 min^{-1} for 10 min. The supernatant was extracted and 0.25-

0.5 µl of extract was diluted with de-ionized water to a final volume 30-40 ml to be analyzed.

3.6.5 Determination of Phosphate ion Concentration

Phosphate ions were determined by molybdenum blue reaction method (Murphy and Riley, 1962) at 690 nm with UV-spectrophotometer. The preparation of ammonium molybdate and stannous chloride solutions were given in Appendix D. To detect the presence of phosphate ion, each sample was treated with two color reagents which convert the phosphate ion to a blue molybdenum complex. The intensity of the blue color is directly proportional to the concentration of phosphate. The calibration curve was drawn (Appendix E) for known concentrations of standards (Appendix F) versus absorbance.

The method used in analysis of samples is given below:

1. One drop of phenolphthalein was added to samples and the acidty was adjusted with strong acid solution until no color change was observed.
2. Samples were diluted to the final volume of 100 ml; thereafter, 4 ml of ammonium molybdate, $(NH_4)_6Mo_7O_{24}$ solution and 0.5 ml stannous chloride, $SnCl_2.2H_2O$ solution were added.
3. Samples were incubated for 10 min. at room temperatures. Treated samples were incubated at either room temperature for 20 minutes or at 37°C for 10 minutes. The sample passing through the same steps but do not contain any reducing sugar is used as blank and the absorbance values of the samples were measured by a UV spectrophotometer at 505 nm.

3.6.6 Amino Acid 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

3.6.7 Determination of the Viability

The viability of the cells was determined by using LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Inc.) and flow cytometry (FACSCalibur with Cellquent Software). Each ml of $1x10^6$ cells/ml of bacterial suspension was dyed with 5 µl of equivolumes of SYTO 9 green-fluorescent nucleic acid strain and red-fluorescent nucleic acid strain, propidium iodide, provided in the LIVE/DEAD *Bac*Light Bacterial Viability Kit. These strains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 strain generally labels all bacteria in the population, those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 strain fluorescence when both dyes are present. The suspension prepared was incubated for 15 min in the dark. At the end of the incubation, suspension was diluted with PBS and loaded into flow cytometry to determine the live cell percent in terms of intensities of fluorescent signals.

3.6.8 Organic Acids Concentrations

Organic acid concentrations were measured with an organic acid analysis system (Waters, HPLC, alliance 2695). The method is based on reversed phase HPLC, in which organic acid concentrations were calculated from the

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

Column	Capital Optimal ODS, 5µm
Column dimensions	4.6 x250 mm
System	:Reversed phase chromatography
Mobile phase flow rate	:0.8 ml/min
Column temperature	:30 °C
Detector and wavelength	:Waters 2487 Dual absorbance detector, 254 nm nm
Injection volume	:5 µl
Analysis period	:15 min

3.6.9 Determination of the Intracellular Metabolite Concentrations

The method proposed by Teusink et al. (1998) was modified in order to determine the intracellular metabolite concentrations. 2 ml samples were quenched into 4 ml of methanol at -60°C at regular time intervals. The cells were washed three times with 4 ml of 60% (v/v) methanol by centrifugation at 13500 min^{-1} for 5 minutes at -10°C, and thereafter, cells were extracted by the addition of 0.5 mL of 5% perchloric acid and 0.15 ml of 60% methanol solution at -40°C to reach a final volume of approximately 0.65 ml. These steps were performed rapidly to prevent the possible leakage of intracellular metabolites out of the cells, as reported by Jensen et al. (1999), as much as possible.

In order to determine the intracellular organic and amino acid concentrations, the extract was centrifuged and the supernatant is neutralized by addition of 5M KOH and 5M H_3PO_4. The salt is removed by further centrifugation and the supernatant is loaded onto a high performance liquid chromatography (HPLC) column.

Intracellular sodium and potassium ion concentrations, on the other hand, were determined by using flame-photometer (JENWAY, PFP7) after diluting the samples with deionized-water to a final volume of 10 ml.

For intracellular ammonium ion concentration, ion-meter (Sartorius Professional Meter, PP-50) was used. After diluting the samples to a final volume of 50 ml, 1 ml of 10M NaOH solution was added and the samples were analyzed.

3.6.10 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

In order to determine the liquid phase mass transfer coefficient and oxygen uptake rate in the β-lactamase production process, the Dynamic Method (Rainer 1990), as explained in section 2.3.3.3.2, was used.

Prior to inoculation of the microorganism to the production medium in the bioreactor, the physical mass transfer coefficient (K_La_0) was determined. After inoculation of the microorganism to the bioreactor, the dynamic oxygen transfer experiments were carried out at certain cultivation times for a short period of time, so that the biological activities of the microorganisms are unaffected. During this period, while the air inlet was totally ceased, the agitation rate was lowered to N=100 min^{-1} in order to lower the effect of surface aeration (Çelik, 2003).

CHAPTER 4

RESULTS AND DISCUSSION

This study focuses on the effects of pH and feeding strategy on metabolite profiling in terms of sodium, potassium, ammonium, and phosphate ion concentrations; as well as organic acid, and amino acid concentrations of β-lactamase producing *Bacillus licheniformis* on a defined medium. For this purpose, the study was carried out in three parts. First, the effect of pH was investigated in pilot scale bioreactor using the glucose based defined medium containing glucose at a concentration of C_G^o= 7 kg m^{-3} (Table 3.3). Thereafter, glucose based defined medium was modified in terms of phosphate concentration to increase the cell yield and productivity. Finally, the effect of batch and fed-batch fermentation on β-lactamase production and cell viability was investigated.

4.1 Effects of pH

The effect of pH, which is known to have a great influence on enzyme activity and microbial growth rate, was investigated for β-lactamase production at the optimum oxygen transfer condition, Q_o/V_R=0.5 vvm, N=500 min^{-1}, in pilot scale batch bioreactor. For this purpose, five different controlled-pH conditions which were pHc: 6.5, 6.75, 7, 7.25, and 7.5 and one uncontrolled-pH condition which was pHuc: 7.5, which was reported as the optimum pH condition by Çalık et al. (2005), were investigated to explain the effect of hydrogen ion concentration on β-lactamase activity, specific cell growth rate, and yield; glucose consumption, oxygen consumption and uptake rates, overall mass transfer coefficients; as well as the effect of pH on sodium, potassium, intracellular ammonium, organic and amino acid concentrations.

4.1.1 Cell growth profile

The variations in the cell concentration with cultivation time and the pH conditions applied are illustrated in Figure 4.1. Cell concentrations increased between t= 1-6 h and the maximum values were obtained at t=5-6 h, in general, then reached to their stationary phase at t=6-8. The highest cell

concentration was obtained at pH_C = 6.75 as C_x = 0.60 kg/m^3, and the lowest cell concentration was obtained at pH_C = 7.25 as C_x = 0.51 kg/m^3. In addition the cell concentration at pH_C=7.5 was close to that of pH_{UC}=7.5. In the literature, Çalık et al. (2005) reported the variations in cell concentration profile throughout the β-lactamase production process at 37^0C, pH_0 = 7.5, N=500 min^{-1}, and Q_0/V_R = 0.5 vvm in the semi-defined medium. The cell concentration at pH_{UC}=7.5 was almost the same as that of reported in Çalık et al. (2005).

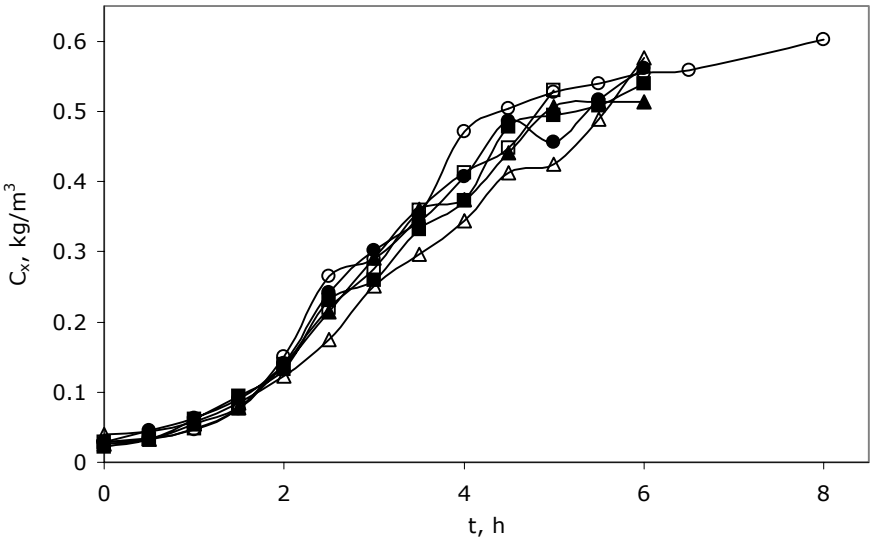

Figure 4.1 Variations in cell concentration with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□).

4.1.2 Glucose Concentration Profiles

The effect of pH on glucose concentration throughout the process is given in Figure 4.2. For all pH operations, glucose concentration, at an initial concentration of C_G^0=7.0 kg m^{-3}, declined throughout the process as expected. The highest consumption rate was obtained at pH_C=6.75, where the cell concentration was the highest; whereas, the lowest glucose consumption rate was attained at pH_{UC}=7.5 operation where the cells require a lesser amount of

maintenance energy; as in controlled operations there exists a permanent regulatory force on the system, whereas in uncontrolled operation no such force is present, hence the system tend to maintain the optimum living environment with the lowest energy requirement. When controlled- and uncontrolled-pH 7.5 operations are compared, it is observed that the glucose consumption rates are significantly higher for controlled pH operations, due to mainly maintenance requirement.

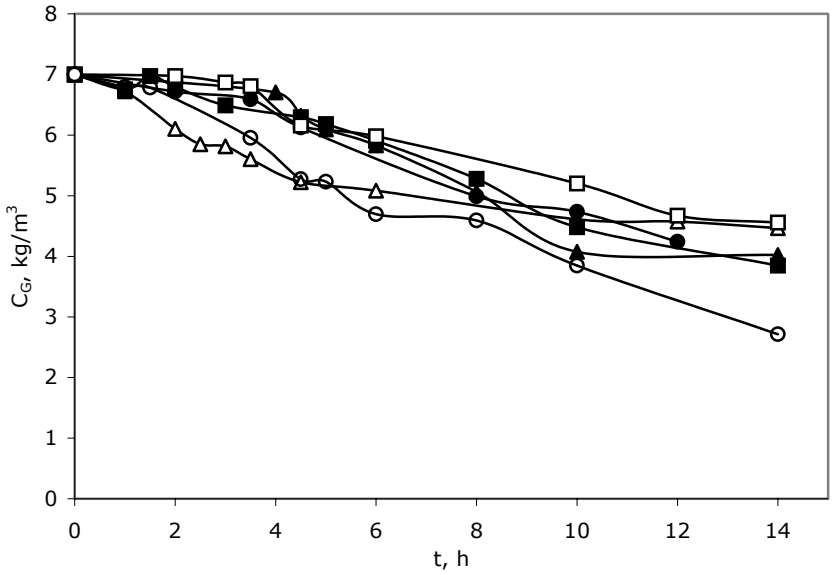

Figure 4.2 Variations in glucose concentration with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□).

4.1.3 β-lactamase Activity Profiles

The variations of β-lactamase activity with pH and cultivation time are given in Figure 4.3. The highest β-lactamase activity was obtained at pH_{UC}=7.5 (ca. A=57 U/cm^3), where the β-lactamase activity was also higher than all the other conditions throughout the process; and the lowest β-lactamase activity was obtained at pH_C=7.5 as A=37 U/cm^3. The low activity values at controlled

pH operations should be because of the high maintenance requirements of the cells and hence the low enzyme concentrations. However, among controlled pH operations the highest enzyme activity was obtained at pH_C=6.75 as ca. A=54 U/cm^3, which is only slightly less than that of pH_{UC}=7.5. Thus, since controlled operations are more preferable in industrial applications, where complex media are extensively used that results in extreme pH changes and hence negative effect on production, pH_C=6.75 was selected as the optimum pH operation in terms of enzyme activity for further studies.

In the literature, Çalık et al. (2005) reported the variations in beta-lactamase activity profile with cultivation time at 37^{0}C, pH_0 = 7.5, N=500 min^{-1}, and Q_0/V_R = 0.5 vvm in the semi-defined medium. In this study, the enzyme activity at pH_{UC}=7.5 was about 0.85-fold lower than that of reported in Çalık et al. (2005), because of lower cell concentration obtained.

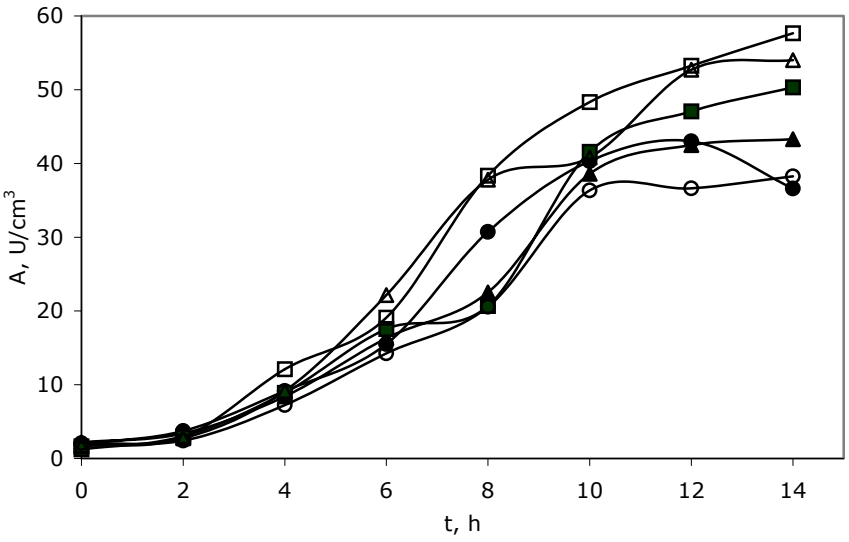

Figure 4.3 Variations in β-lactamase activity with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□).

4.1.4 Dissolved Oxygen Profiles

The variations of the dissolved oxygen concentration with pH and cultivation time are given in Figure 4.4. At all pH conditions due to the high oxygen demand of the cells at the beginning of the bioprocesses, a decrease was observed in the dissolved oxygen profiles; after reaching a minimum around 50-65% of the saturation (C_{DO}=0.1-0.13 mol m^{-3}) profiles gradually increased in general until the end of the bioprocesses. The loci of the profiles are similar; pH_C=7.5 and pH_{UC}=7.5 operations showed the same trend till the end of t=6h; and in general controlled pH operations have lower dissolved oxygen values and higher decrease rates. As it can be seen from Figure 4.4., the oxygen transferred to media was most effectively used at pH_C=6.75 operation where the highest cell concentration was obtained. On the other hand, for the first 6 h of the operation the oxygen in media was least effectively utilized at pH_C=6.5 where the cell concentration is the lowest for that time course of operation, and for t=6-14h of the operation it was least effectively utilized at pH_C=7.5.

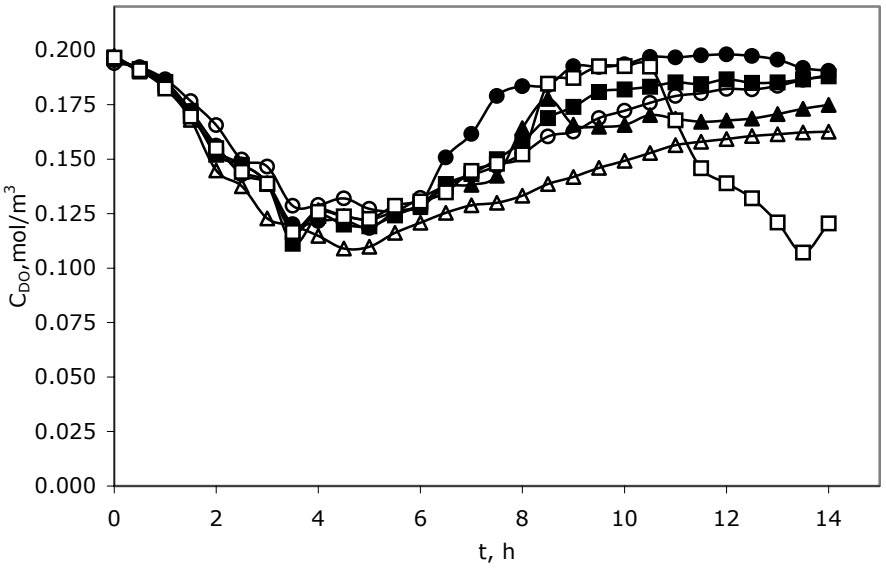

Figure 4.4 The variations in dissolved oxygen concentration with the cultivation time and pH, C_{DO}*= 0.20 mol m^{-3}. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□).

4.1.5 Amino Acid concentration profiles

For the production of proteins, regulation of the metabolic reaction network should cause good coupling of supply and demand for the amino acids (Çalık et al., 2000), as they may be the rate limiting step in metabolic reaction network. The variations in intracellular and extracellular amino acid concentrations with cultivation time for controlled and uncontrolled pH operations are given in Tables 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, and Appendix H. At all pH conditions applied, generally aspartic acid and the asparagine were the amino acids excreted extracellularly; whereas methionine, valine, cysteine, and tryptophan are the major amino acids expelled intracellularly. In general the amino acid concentrations in the fermentation broth were low.

As illustrated in Tables 4.1, 4.2, 4.3, 4.4, 4.5, 4.6 the total amino acid concentration excreted extracellularly and intracellularly were highest at pH_{UC}=7.5 t=0h and pH_C=7.25 t=12h as $T_{AA,\ ext}$= 1.433 kg m^{-3} and $T_{AA,int}$= 0.327 g g_{cell}^{-1} (7.987 kg m^{-3}), respectively. In contrast, the minimum total amounts were obtained at pH_C= 7.0 t=10 h and pH_C=7.5 t=8 h as $T_{AA,\ ext}$= 0.0117 kg m^{-3} and $T_{AA,int}$= 0.0058 g g_{cell}^{-1} (0.141 kg m^{-3}), respectively. For all pH conditions applied, tyrosine concentration was the highest extracellular and intracellular amino acid concentration obtained at pH_C=6.75 t=8h as 0.968 kg m^{-3}, and at pH_C=7.25 t=12 h as 0.250 g g_{cell}^{-1} (6.121 kg m^{-3}). Generally speaking, a relation was observed between asparagine and aspartic acid as well as between cysteine and methionine in extracellular and intracellular samples, respectively; with lower amounts of cysteine and higher and/or lower amounts of aspartic acid. This is an expected trend, since methionine is produced from cysteine, whereas asparagine is produced from and degraded to aspartic acid. On the whole, intracellular amino acid concentrations in terms of kg m^{-3} were, at maximum, 683-folds higher than extracellular amino acid concentrations.

4.1.6 Organic Acid concentration profiles

The variations in intracellular and extracellular organic acid concentrations with cultivation time for controlled and uncontrolled pH operations are given in Tables 4.7, 4.8, 4.9, 4.10, 4.11, 4.12, and Appendix H. At all pH conditions applied, all organic acids, except oxaloacetic acid, which is known to be produced

in cell regeneration, were excreted. However, acetic acid, fumaric acid, succinic acid could not be presented among intracellular components of the cells as they were excreted in trace amounts ($C_{OA,ext}$ < 0.0003 kg m^{-3}, $C_{OA,int}$ < 0.000001 g g_{cell}^{-1}). In general the organic acid concentrations in the fermentation broth were low and intracellular amounts were, at maximum, 19.8-folds higher than extracellular amounts (in terms of kg m^{-3}).

As illustrated in Tables 4.7, 4.8, 4.9, 4.10, 4.11, 4.12 the total organic acid concentration excreted extracellularly and intracellularly were highest at pH_C=6.75 t=14h and pH_C=7.5 t=2h as $T_{OA, ext}$= 1.897 kg m^{-3} and $T_{OA,int}$= 0.054 g g_{cell}^{-1} (1.304 kg m^{-3}), respectively. In contrast, the minimum total amounts were obtained at pH_C= 7.25 t=2 h and pH_C=7.0 t=12 h as $T_{OA, ext}$= 0.066 kg m^{-3} and $T_{OA,int}$= 0.005 g g_{cell}^{-1} (0.105 kg m^{-3}), respectively. For all pH conditions applied, lactic acid concentration was the highest extracellular organic acid concentration obtained at pH_C =6.75 t=10h as 0.687 kg m^{-3}, while oxalic acid concentration was the highest intracellular amino acid concentration, obtained at pH_C=7.0 t=2 h as 0.028 g g_{cell}^{-1} (0.695 kg m^{-3}). On the whole, it could be seen that lactic acid- pyruvic acid and α-ketoglutaric acid- pyruvic acid were converted to each other continuously throughout the course of fermentation. Moreover, from malic acid, succinic acid, fumaric acid, and α-ketoglutaric acid; pyruvic acid, fumaric acid, malic acid, and fumaric and succinic acids were produced, respectively.

Table 4.1 Variations in extracellular and intracellular amino acid concentrations with cultivation time at pH_C=6.5

C 6.5 – Extracellular, C_{AA} ($kg.m^{-3}$)												
Time (h)	Asn	Asp	His	Thr	Ala	Pro	Met	Cys	Lys	Phe	Orn	Trp
0	0.085	-	-	0.001	0.001	0.001	-	-	0.007	0.006	0.008	0.006
2	0.082	-	0.047	0.002	0.003	0.002	-	0.015	0.028	0.019	0.025	0.019
4	-	0.142	-	-	-	-	0.128	-	0.011	0.033	0.013	0.033
6	-	0.093	-	-	-	-	-	-	-	-	-	-
8	-	0.104	-	-	-	-	-	-	-	-	-	-
10	-	0.044	-	-	-	-	-	0.026	-	-	-	-
12	-	0.055	-	-	-	-	-	0.028	-	-	-	-
14	-	0.082	-	-	-	-	-	0.018	-	-	-	-
C 6.5 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)												
0	-	-	-	-	-	0.003	0.036	0.008	-	-	0.007	-
2	-	-	-	-	-	0.048	-	0.026	-	-	-	-
4	-	-	-	-	-	0.008	0.149	0.026	-	-	0.085	-
6	-	-	-	-	-	-	0.099	0.002	-	-	0.003	-
8	-	-	-	-	-	-	0.102	0.020	-	-	0.031	-
10	-	-	-	-	-	-	0.093	0.018	-	-	0.147	-
12	-	-	-	-	-	-	0.094	0.019	-	0.025	-	0.133
14	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2 Variations in extracellular and intracellular amino acid concentrations with cultivation time at pH_C=6.75

C 6.75 – Extracellular, C_{AA} ($kg.m^{-3}$)															
Time (h)	Asn	Asp	His	Arg	Ala	Pro	Tyr	Val	Met	Cys	Leu	Lys	Phe	Orn	Trp
0	0.030	-	-	0.110	0.010	0.007	-	-	0.373	-	-	0.008	0.013	0.017	0.013
2	-	0.109	-	0.084	-	-	-	-	0.304	-	-	0.010	0.009	0.012	0.009
4	0.141	-	-	-	-	-	-	-	-	-	-	0.015	0.007	0.009	0.007
6	0.020	-	-	-	-	-	-	-	-	-	-	-	0.005	0.004	0.005
8	0.098	-	-	-	-	-	0.968	-	-	-	-	-	0.004	0.005	0.004
10	0.061	-	-	-	-	-	-	-	-	-	-	-	0.004	0.003	0.004
12	0.027	-	0.027	-	-	-	-	0.020	-	-	-	-	0.005	0.006	0.005
14	0.023	-	0.022	-	-	-	-	0.015	-	-	-	-	0.006	0.008	0.006
C 6.75 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)															
0	-	-	-	-	-	-	-	-	0.088	0.021	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	0.086	0.120	-	-	-	-
4	-	-	-	-	-	-	-	-	0.128	0.021	0.033	-	-	-	-
6	-	-	-	-	-	-	0.023	-	0.126	0.023	0.026	-	-	-	-
8	-	-	-	-	-	-	-	-	0.072	0.034	0.020	0.004	-	-	-
10	-	-	-	-	-	-	-	-	0.132	0.033	-	-	-	-	-
12	-	-	-	-	-	-	-	-	0.082	0.026	-	-	-	-	-
14	-	-	-	-	-	-	-	-	0.119	0.035	-	-	-	-	-

Table 4.3 Variations in extracellular and intracellular amino acid concentrations with cultivation time at pH_C=7.0

C 7 – Extracellular, C_{AA} ($kg.m^{-3}$)											
Time (h)	Asn	Gln	Thr	Val	Met	Cys	Leu	Lys	Phe	Orn	Trp
0	-	-	-	0.017	-	-	-	-	0.006	0.007	0.006
2	-	-	-	-	-	-	-	-	-	-	-
4	0.119	-	-	0.052	-	-	-	0.006	0.005	0.007	0.005
6	-	-	-	-	-	-	-	-	0.004	0.005	0.004
8	-	-	-	-	-	-	-	-	0.004	0.004	0.004
10	-	-	-	-	-	-	-	-	0.004	0.005	0.004
12	0.093	-	-	0.026	-	-	-	-	0.003	0.004	0.003
14	0.075	-	-	0.035	-	-	-	-	0.004	0.006	0.004
C 7 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)											
0	-	0.006	0.006	-	0.028	0.018	-	-	-	0.001	0.009
2	-	-	-	-	-	0.137	-	-	-	0.022	0.081
4	-	-	-	-	0.085	0.114	0.026	-	-	0.004	0.029
6	-	0.210	-	-	0.025	0.034	-	-	-	-	0.014
8	-	-	-	-	0.059	0.034	-	-	-	-	0.017
10	-	-	-	-	0.055	0.034	-	-	-	-	0.017
12	-	-	-	-	0.027	0.044	-	-	-	0.005	0.017
14	-	-	-	-	0.125	0.058	-	-	-	0.009	-

Table 4.4 Variations in extracellular and intracellular amino acid concentrations with cultivation time at pH_C=7.25

C 7.25 – Extracellular, C_{AA} ($kg.m^{-3}$)												
Time (h)	Asn	Gln	Ala	Tyr	Val	Met	Cys	Ile	Leu	Phe	Orn	Trp
0	0.143	-	-	-	0.114	0.103	-	-	-	-	-	-
2	0.129	-	-	-	0.091	0.058	-	-	-	0.010	0.013	0.010
4	0.241	-	-	-	0.108	0.043	0.026	-	-	0.010	0.013	0.010
6	0.059	-	-	-	0.097	0.023	0.030	-	-	0.006	0.008	0.006
8	0.122	-	0.010	0.003	0.122	0.032	-	-	-	0.008	0.010	0.008
10	0.147	-	-	0.003	0.131	0.029	0.030	-	-	0.006	0.009	0.006
12	0.111	-	-	0.002	0.127	0.030	0.030	-	-	0.006	0.008	0.006
14	0.225	-	-	0.003	0.106	0.048	0.033	-	-	0.007	0.010	0.007
C 7.25 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)												
0	-	-	-	0.015	-	0.001	-	-	-	-	-	0.036
2	-	-	-	0.156	-	0.038	-	-	-	-	0.053	-
4	-	0.161	-	0.052	-	0.006	-	0.025	-	-	0.016	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	-	0.071	-	0.042	-	0.006	-	0.027	-	-	0.012	0.092
10	-	0.090	-	0.047	-	0.007	-	0.030	-	-	0.011	0.092
12	-	-	-	0.250	-	-	-	-	0.067	-	0.010	
14	-	-	-	0.090	-	-	-	0.036	0.043	-	-	0.095

Table 4.5 Variations in extracellular and intracellular amino acid concentrations with cultivation time at pH_C=7.5

C 7.5 – Extracellular, C_{AA} ($kg.m^{-3}$)												
Time (h)	Asn	Asp	Ser	Thr	Tyr	Val	Met	Cys	Ile	Lys	Phe	Trp
0	-	-	-	-	-	-	-	-	-	-	0.053	-
2	-	-	-	-	-	-	-	-	-	0.008	0.048	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	0.041	-	-	-	-
8	-	0.029	0.037	-	0.687	-	-	0.017	-	0.019	-	0.051
10	0.031	-	-	-	-	-	-	0.009	-	-	-	0.042
12	-	-	-	-	-	-	-	0.093	-	-	-	-
14	0.080	-	-	-	-	-	-	0.035	-	-	-	0.067
C 7.5 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)												
0	-	-	-	-	-	-	-	0.007	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	0.006	-	0.032	-	-	-	-
6	-	-	-	-	-	-	0.004	0.031	-	-	-	-
8	-	-	-	-	-	0.006	-	-	-	-	-	-
10	-	-	-	-	-	-	-	0.039	0.011	-	-	-
12	-	-	-	-	-	-	-	0.024	-	-	-	-
14	-	-	-	0.001	-	0.007	0.004	0.022	-	-	-	-

Table 4.6 Variations in extracellular and intracellular amino acid concentrations with cultivation time at pH_{UC}=7.5

UC 7.5 – Extracellular, C_{AA} ($kg.m^{-3}$)															
Time (h)	Asn	Asp	Gla	His	Thr	Ala	Tyr	Val	Met	Cys	Ile	Lys	Phe	Orn	Trp
0	-	0.059	-	-	-	-	1.339	-	-	0.035	-	-	-	-	-
2	0.024	-	-	-	-	-	-	-	-	-	-	-	0.060	-	-
4	0.028	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	0.122	-	-	-	-	-	-	-	-	0.015	-	-	-	-	0.109
8	0.108	-	-	-	-	-	0.024	0.018	0.020	0.043	-	-	-	-	0.231
10	0.181	-	-	-	-	-	-	-	-	0.023	-	-	-	-	-
12	0.500	-	0.028	0.051	0.183	-	0.007	-	0.025	0.084	-	-	-	-	0.165
14	0.098	-	-	-	-	-	-	-	0.012	-	-	-	-	-	-
UC 7.5 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)															
0	-	-	-	-	-	0.002	-	0.004	0.003	0.002	-	0.001	-	-	-
2	-	-	-	-	-	-	-	0.037	0.033	0.018	-	-	-	-	-
4	-	-	-	-	-	0.004	-	0.003	0.013	0.009	0.017	-	-	-	-
6	-	-	-	-	-	-	-	0.010	0.014	-	-	-	-	-	-
8	-	-	-	-	-	-	0.006	0.012	0.012	0.008	0.007	-	-	-	-
10	-	-	-	-	-	-	-	-	0.007	0.005	-	-	-	-	-
12	-	-	-	-	-	-	-	0.012	0.008	0.003	-	-	-	-	-
14	-	-	-	-	-	-	-	0.009	0.013	-	-	-	-	-	-

Table 4.7 Variations in extracellular and intracellular organic acid concentrations with cultivation time at pH_C=6.5

C 6.5 – Extracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.033	0.054	-	-	0.002	0.011	-	0.001	0.005	-	0.001	-
2	0.083	0.042	-	0.001	0.003	0.016	-	0.001	-	-	0.002	0.165
4	0.094	-	-	0.002	0.008	0.049	-	0.002	-	-	0.002	0.305
6	0.092	-	-	0.002	0.014	0.087	-	0.001	-	-	0.001	0.248
8	0.128	-	0.006	0.002	0.057	0.344	0.001	0.050	0.004	-	0.001	-
10	0.053	-	0.006	0.002	0.040	0.240	-	0.001	0.004	-	0.001	0.338
12	0.104	0.016	0.014	0.004	0.105	0.614	0.001	0.008	0.004	-	0.001	-
14	0.056	0.013	0.012	0.003	0.073	0.432	0.001	0.006	0.004	-	0.001	0.427
C 6.5 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	0.001	-	-	0.001	-	-	0.003	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	0.005	-	-	-	-	0.002	0.008	-	-	-
6	-	-	-	-	-	-	-	-	0.005	-	-	-
8	-	-	-	-	-	-	-	-	0.007	-	-	-
10	-	-	-	-	-	-	-	0.001	0.005	-	-	-
12	-	-	-	-	-	-	-	-	0.004	-	-	-
14	-	-	-	-	0.001	0.003	-	-	0.009	-	-	-

Table 4.8 Variations in extracellular and intracellular organic acid concentrations with cultivation time at pH_C=6.75

C 6.75 – Extracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.058	0.091	-	-	0.002	0.012	-	0.002	0.005	-	0.002	-
2	0.096	0.020	-	-	0.002	0.012	-	0.002	-	-	0.002	-
4	0.094	-	-	0.001	0.006	0.037	-	0.001	0.002	-	0.001	0.092
6	0.147	-	-	0.002	0.046	0.281	0.001	0.001	-	-	0.001	-
8	0.148	-	0.007	0.003	0.104	0.596	0.001	0.006	0.004	-	0.001	-
10	0.117	-	0.012	0.006	0.127	0.740	0.001	0.007	0.004	-	0.001	-
12	0.063	0.013	0.006	0.005	0.093	0.543	0.001	0.005	0.004	-	0.001	0.901
14	0.061	0.014	0.011	0.006	0.117	0.687	0.001	0.007	0.005	-	0.001	0.987
C 6.75 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	0.001	-	-	-	-	-	0.002	-	-	-
2	-	-	0.010	-	-	-	-	0.006	0.021	-	0.004	-
4	-	-	0.010	-	-	-	-	0.001	0.003	-	0.001	-
6	-	-	0.003	-	-	-	-	0.001	0.002	-	-	-
8	-	-	0.004	-	-	-	-	0.008	0.006	-	-	-
10	-	-	0.003	-	-	-	-	0.001	0.003	-	0.001	-
12	-	-	0.004	-	-	-	-	-	0.007	-	-	-
14	-	-	0.004	-	-	-	-	-	0.003	-	-	-

Table 4.9 Variations in extracellular and intracellular organic acid concentrations with cultivation time at pH_C=7.0

C 7.0 – Extracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.034	0.059	-	-	0.001	0.006	-	0.001	0.008	-	0.002	-
2	0.137	0.027	-	-	0.003	0.007	-	0.003	-	-	0.003	-
4	0.183	0.088	-	-	0.007	0.044	-	0.002	0.004	-	0.003	-
6	0.180	0.201	-	-	0.043	0.203	0.001	0.008	-	-	0.004	-
8	0.149	-	0.004	0.002	0.061	0.345	0.001	0.006	0.006	-	0.001	-
10	0.090	-	0.006	0.001	0.046	0.264	0.001	0.004	0.005	-	0.001	-
12	0.037	-	0.012	0.001	0.156	0.601	0.001	0.006	0.028	-	0.002	0.011
14	0.184	0.157	0.016	0.006	0.123	0.682	-	0.007	0.013	-	0.001	-
C 7.0 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	-	-	-	-	-	-	0.002	-	-	-
2	-	-	-	-	-	-	-	-	0.028	-	-	-
4	-	-	-	-	-	-	-	0.001	0.010	-	-	-
6	-	-	-	-	-	-	-	-	0.007	-	-	-
8	-	-	-	-	-	-	-	0.001	0.007	-	-	-
10	-	-	-	-	-	-	-	0.002	0.004	-	-	-
12	-	-	-	-	-	-	-	0.001	0.004	-	-	-
14	-	-	-	-	0.001	-	-	-	0.010	-	-	-

Table 4.10 Variations in extracellular and intracellular organic acid concentrations with cultivation time at pH_C=7.25

C 7.25 – Extracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.016	0.040	-	-	-	-	-	0.003	0.001	-	0.001	-
2	0.040	0.018	-	-	-	-	-	0.001	0.005	-	0.002	-
4	0.092	-	-	0.001	0.004	0.023	-	0.001	0.002	-	0.002	-
6	0.094	-	-	0.001	0.016	0.093	0.001	0.003	0.004	-	0.002	-
8	0.153	-	-	0.001	0.047	0.263	0.001	0.007	0.016	-	0.002	-
10	0.138	-	0.008	0.001	0.053	0.289	0.001	0.006	0.014	-	0.001	0.038
12	0.155	-	0.009	0.002	0.065	0.361	0.001	0.006	0.019	-	0.001	0.061
14	0.227	0.140	0.012	-	0.089	0.484	0.001	0.007	0.029	-	0.001	0.104
C 7.25 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	0.002	-	-	0.001	-	-	0.004	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	0.005	-	-	0.003	-	-	0.008	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	0.005	-	-	-	-	-	0.004	-	-	-
14	-	-	0.005	-	-	0.002	-	-	0.005	-	-	-

Table 4.11 Variations in extracellular and intracellular organic acid concentrations with cultivation time at pH_C=7.5

C 7.5 – Extracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.072	0.066	-	-	0.004	0.025	0.001	0.003	-	-	0.001	-
2	0.086	-	-	-	0.003	0.016	0.001	0.003	0.005	-	0.001	-
4	0.156	0.017	-	0.001	0.005	0.031	-	0.003	0.002	-	0.002	-
6	0.096	-	-	-	0.012	0.069	0.001	0.004	0.002	-	0.001	-
8	0.187	0.070	-	-	0.043	0.233	0.002	0.011	0.005	-	0.001	-
10	0.253	0.087	-	-	0.061	0.309	0.003	0.017	0.024	-	0.001	0.323
12	0.254	0.096	0.015	-	0.048	0.250	0.002	0.017	0.030	-	0.001	0.443
14	0.074	-	0.019	0.001	0.013	0.069	0.001	0.006	0.006	-	-	-
C 7.5 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	0.002	0.010	-	0.002	0.038	-	0.002	-
4	-	-	-	-	-	-	-	-	0.018	-	-	-
6	-	-	-	-	-	0.002	-	-	0.009	-	-	-
8	-	-	0.005	-	-	-	-	-	0.005	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	0.004	-	0.001	0.004	-	-	0.008	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.12 Variations in extracellular and intracellular organic acid concentrations with cultivation time at pH_{UC}=7.5

UC 7.5 – Extracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.033	0.060	-	-	-	-	-	0.001	0.004	-	0.001	-
2	0.066	-	0.015	-	0.002	0.011	-	0.010	0.002	-	0.001	-
4	0.115	-	-	0.001	0.007	0.044	-	0.003	0.007	-	0.001	-
6	0.102	-	0.003	-	0.013	0.079	0.001	0.003	0.004	-	0.001	-
8	0.270	0.075	-	-	0.065	0.357	0.002	0.010	0.003	-	0.001	-
10	0.154	-	0.006	0.001	0.042	0.241	0.001	0.008	0.009	-	0.001	0.151
12	0.254	-	0.006	0.002	0.052	0.289	0.001	0.015	0.015	-	0.001	-
14	0.143	-	-	0.002	-	-	0.001	0.005	0.036	-	0.001	-
UC 7.5 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	0.026	-	-	-	-	-	0.009	-	-	-
4	-	-	0.004	-	-	-	-	0.001	0.005	-	-	-
6	-	-	0.003	-	-	-	-	-	0.005	-	-	-
8	-	-	0.003	-	-	-	-	-	0.010	-	-	-
10	-	-	0.004	-	-	-	-	0.001	0.005	-	-	-
12	-	-	0.005	-	-	-	-	-	0.002	-	-	-
14	-	-	0.005	-	-	-	-	0.001	0.005	-	-	-

4.1.7 Sodium and Potassium Ions Profiles

The variations of the intracellular and extracellular sodium and potassium ion concentrations with pH and cultivation time are given in Figure 4.5 and Figure 4.6, respectively. For pH_C=6.75, 7.5 and pH_{UC}=7.5 the extracellular sodium ion concentrations were ranging between 80-120 ppm throughout the processes; while for pH_C=6.5, 7.0, and 7.25 the extracellular sodium ion concentrations were ranging between 50-90 ppm showing an increasing trend with increasing pH. On the other hand, the extracellular potassium ion concentrations increased significantly with increasing pH, reaching a maximum of 840 ppm at pH_C=7.5, for controlled pH operations due to the addition of 5M KOH to the system to maintain the pH of the media constant; whereas it was about 120 ppm for pH_{UC}=7.5.

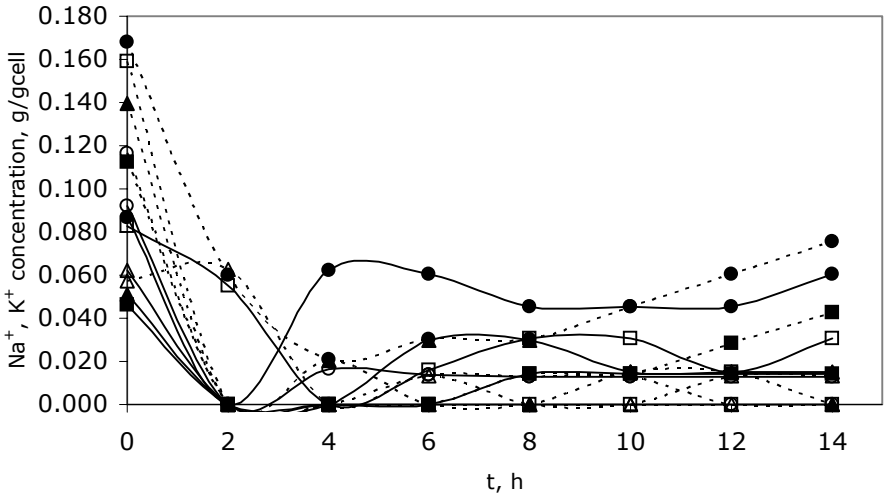

Figure 4.5 Variations in intracellular sodium, potassium ion concentration with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□); rigid lines show potassium concentration, dashed lines show sodium concentration.

The intracellular sodium and potassium amounts per gram cell, however, were increasing with increasing pH; whereas the sodium amount was almost 0 for pH_{Uc}=7.5, and the potassium amount was almost 0 for pH_C=6.5. Similarly, at t=0 h, the intracellular amounts of these ions were the highest at pH=7.5 for both controlled- and uncontrolled-pH operations, and the lowest for pH_C=6.5. From these intra- and extracellular ion concentration profiles, the total amount of sodium and potassium transferred into and out of the cell was calculated, the result of which is shown in Figure 4.7. Figure 4.7 clearly illustrates that there exists a sodium/potassium ion pump in the system to maintain the electropotential gradient across the cell membrane.

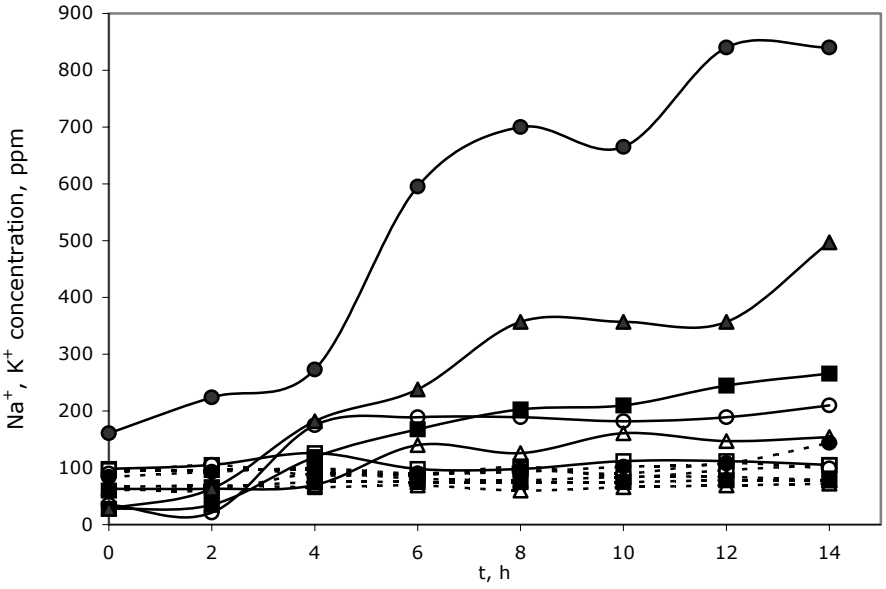

Figure 4.6 Variations in extracellular sodium, potassium ion concentration with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□); rigid lines show potassium concentration, dashed lines show sodium concentration.

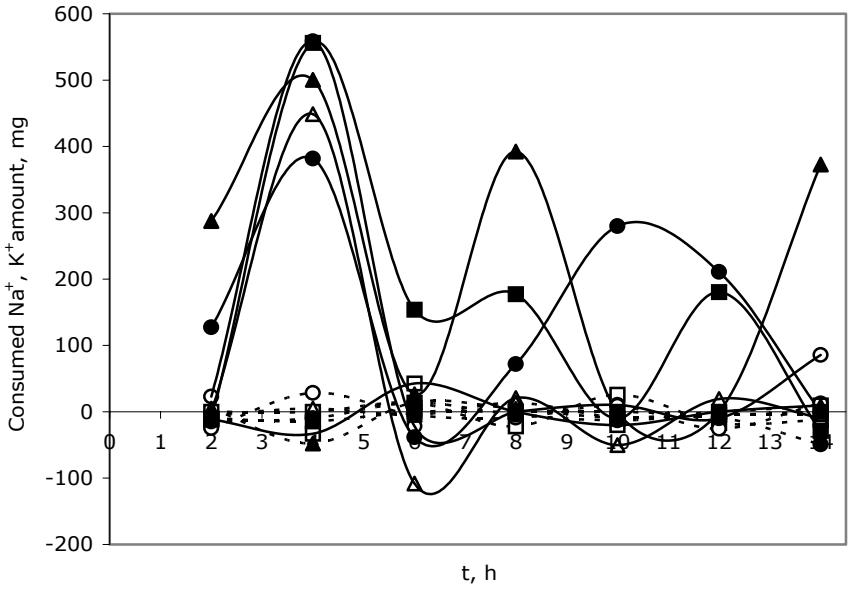

Figure 4.7 Variations in consumed amount of sodium, potassium ions with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□); rigid lines show amount of consumed potassium ions, dashed lines show amount of consumed sodium ions.

4.1.8 Intracellular Ammonium Ion

Ammonium is the primary source of nitrogen. The variation of the intracellular ammonium ion amounts per gram cell with pH and cultivation time is given in Figure 4.8. As it is illustrated in Figure 4.8 the ammonium ion amounts had a maxima at the t=2h of the processes due to high specific growth rate of the cells at the first hours of the cultivations; whilst, it was relatively constant at t=4-14h for all pH operations. At pH_C=6.5 the ammonium ion amount had its maximum; whereas it was the lowest at pH_{UC}=7.5 and pH_C=7.5.

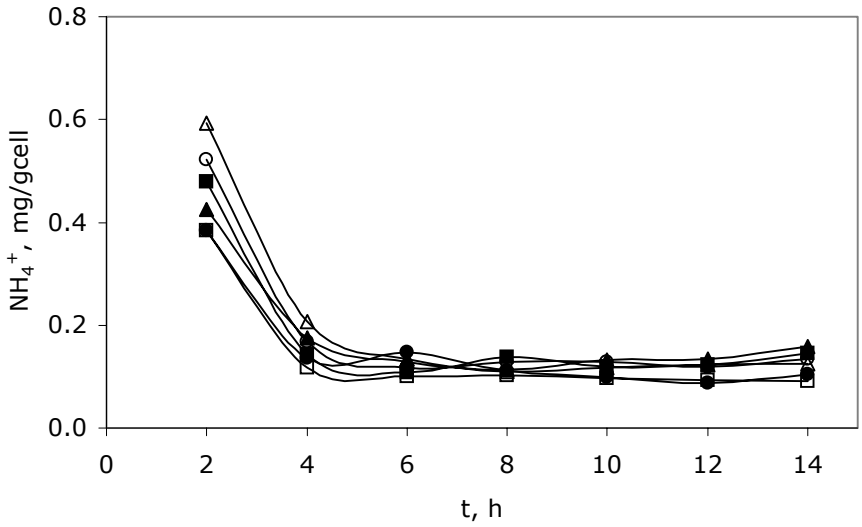

Figure 4.8 Variations in intracellular ammonium ion concentration with the cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□).

4.1.9 Oxygen Transfer Characteristics

Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r_0, and oxygen transfer coefficient, K_La. At t<0 h, the physical oxygen transfer coefficient K_La_o was measured in the medium in the absence of the microorganism. The variations in K_La, oxygen uptake rate, oxygen transfer rate and the enhancement factor E (=K_La/K_La_o) with cell growth (GP) and enzyme production (PP) phases are given in Table 4.13.

K_La and consequently the enhancement factor E are higher in β-lactamase production phase than in growth phase of the bioprocesses, varing between 0.0100-0.0311 and 0.98-2.22, respectively; having the highest values at the enzyme production phase of pH_C=7.5 condition. Furthermore, it is seen that K_La and E values were approximately 2-fold higher in pH_C=7.5 compared to pH_{UC}=7.5; and K_La and E tend to increase with increasing pH in general. The reason for low E values in GP should be because of low actual C_{DO}^* values

compared to C_{DO}^{*}= 0.20 mol m^{-3} assumption. In addition, low enhancement factor E values in the bioprocess indicate that the mass transfer rate is much higher than the reaction rate. Moreover, K_La depends on agitation rate, temperature, rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. The observed increase in K_La, could be the result of the decrease in viscosity of the medium due to secreted metabolites, as temperature and agitation rate were kept constant throughout the bioprocesses.

The oxygen transfer rate, which is proportional to the difference between the equilibrium concentration and the dynamic dissolved oxygen concentration in the medium, was decreased to an extent depending on the oxygen uptake rate. In general, oxygen uptake rates were higher at GP of the processes, where the cell formation and the substrate consumption rates, i.e. metabolic activity of the cells, were high; and it increased with increasing pH. Comparing controlled- and uncontrolled pH 7.5 operations, it could be seen that oxygen uptake rate was higher at pH_C=7.5 for GP, and lower at pH_{UC}=7.5 for PP of the bioprocess.

In Table 4.13, the maximum possible oxygen utilization rate (OD), the maximum possible mass transfer rate (OTR_{max}), Damköhler number (Da), effectiveness factor (η), and k/K_La values were given to compare mass transfer and biochemical reaction rates. For all operations mass transfer resistances were more effective (Da>>1). Da was slightly higher at pH_{UC}=7.5 than at pH_C=7.5. As it is seen in Table 4.13, the effectiveness factor, η, was low indicating that the cells are consuming lower oxygen than the dissolved oxygen. The effectiveness factor showed varying trend with pH and it was slightly higher at pH_{UC}=7.5 than that at pH_C=7.5. Finally, k and k/K_La values were calculated assuming pseudo-steady state first order reaction. For all conditions applied, k/K_La values revealed that operations were limited by mass transfer resistances; and the values were higher at PP than at GP, as well as at pH_C=7.5 than at pH_{UC}=7.5. Comparing Da and k/K_La, it is seen that Da values were higher than k/K_La, as Da is defined for maximum utilization and transfer rates, whereas k/K_La was calculated for specific time interval that includes the average but not the maximum values. Moreover, the calculated k values may be different from the actual k, as the biochemical reaction rates should not be the first order in ideal

cases; i.e., if the reaction is in the second order or higher, k and hence k/K_La values will increase. Indeed, calculated k and k/K_La were the minimum values that could be obtained for the operations assuming homogeneous irreversible reactions. Therefore, higher k and hence k/K_La values should be obtained for the ideal case.

4.1.10 Specific Growth Rate and Yield Coefficients

The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_o, the specific substrate consumption rate, q_s, and the yield coefficients with cultivation time and pH are given in Table 4.14. At all pH conditions applied, the specific growth rate (μ) decreased with cultivation time, as expected; and $\mu=0\ h^{-1}$ at t=6-8h in general. As it is seen in Figure 4.9, the maximum value of specific growth rate was attained at pH=6.75, t=0.5 h as $\mu=4.18\ h^{-1}$, where the highest cell concentration was obtained, as expected. Specific substrate utilization rates (q_S), on the other hand, decreased at PP for all conditions except $pH_C=6.5$, where qs is higher at PP; and increased with decreasing pH in general. At $pH_{UC}=7.5$ GP, q_S was higher and at PP it was lower than that of $pH_C=7.5$. The highest value of q_S was obtained at $pH_C=6.5$ (PP) as 2.97 kg kg^{-1} h^{-1}; where the lowest value was obtained at $pH_{UC}=7.5$ (PP) as 0.33 kg kg^{-1} h^{-1}. Similarly, specific oxygen uptake rates (q_0), were higher at GP of bioprocesses. The highest value of q_0 was obtained at $pH_C=7.5$ (GP) as 0.454 kg kg^{-1} h^{-1}; where the lowest value was obtained in the same process at PP as 0.082 kg kg^{-1} h^{-1}. Contrary to q_s, at $pH_C=7.5$, q_0 was higher at GP and lower at PP than $pH_{UC}=7.5$.

The yield of cell on substrate, the yield of cell on oxygen and the yield of substrate on oxygen were also given in Table 4.14. The highest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at $pH_{UC}=7.5$ and $pH_C=6.5$ (PP) as 0.613; 1.189; 11.88 kg kg^{-1}, respectively. In contrast, the lowest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at $pH_C=6.75$ and $pH_C=7.0$ (GP) 0.223; 0.638; 1.863 kg kg^{-1}; respectively. At $pH_{UC}=7.5$, $Y_{X/S}$ and $Y_{X/O}$ were higher and $Y_{S/O}$ values were lower than those of $pH_C=7.5$. The reason for low $Y_{X/O}$ and $Y_{S/O}$ values is the inefficient use of the oxygen through biochemical reaction network, increased energy requirement or decrease in the efficiency of energy metabolism with the cultivation time.

Table 4.13 The variations in oxygen transfer parameters with cultivation time and pH (GP: Growth phase, PP: Enzyme production phase).

Condition	t	k_La (s^{-1})	E k_La/k_La_0	OTRx10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}x10^3$ ($molm^{-3}s^{-1}$)	OURx10^3 ($molm^{-3}s^{-1}$)	ODx10^3 ($molm^{-3}s^{-1}$)	Da OD/OTR_{mx}	η OUR/OD	k/ K_La
pH_C:6.5	GP	0.0108	0.9941	0.6437	2.2767	0.9177	14.141	6.2112	0.0649	1.4380
	PP	0.0132	1.2204	0.4336	2.7760	0.7605	-	-	-	2.0820
pH_C:6.75	GP	0.0103	0.9868	0.7638	2.1660	1.0043	15.473	7.1436	0.0649	1.3200
	PP	0.0127	1.0181	0.6443	2.6660	0.9107	-	-	-	1.4000
pH_C:7.0	GP	0.0112	0.9870	0.7590	2.3600	1.0333	16.067	6.8081	0.0643	1.3858
	PP	0.0137	1.0485	0.4118	2.8760	0.9000	-	-	-	2.5795
pH_C:7.25	GP	0.0111	0.9830	0.7353	2.3267	1.0650	15.692	6.7443	0.0679	1.5038
	PP	0.0141	1.1712	0.5570	2.9580	0.5964	-	-	-	4.2562
pH_C:7.5	GP	0.0119	0.9940	0.7090	2.5050	1.0000	15.316	6.1147	0.0653	1.4121
	PP	0.0311	2.2247	0.4373	6.5440	0.5600	-	-	-	4.3530
pH_{UC}:7.5	GP	0.0100	0.9852	0.6540	2.0953	0.9000	13.316	6.3552	0.0676	1.4062
	PP	0.0122	1.0629	0.8205	2.5620	0.9000	-	-	-	3.3350

Table 4.14 Variations in specific rates and yield coefficients with cultivation time and pH.

Condition	t	r_o (kg O_2/m^3h)	q_0 (kg/kg h)	q_s (kg/kg h)	$Y_{x/o}$ (kg/kg)	$Y_{x/s}$ (kg/kg)	$Y_{s/o}$ (kg/kg)
pH_C:6.5	GP	0.104	0.389	1.669	1.015	0.527	3.288
	PP	0.092	0.136	2.971	-	-	11.880
pH_C:6.75	GP	0.115	0.340	1.107	0.638	0.223	2.736
	PP	0.104	0.161	0.795	-	-	5.108
pH_C:7.0	GP	0.115	0.453	0.868	0.848	0.455	1.863
	PP	0.104	0.176	0.505	-	-	2.867
pH_C:7.25	GP	0.127	0.454	1.026	0.867	0.374	1.972
	PP	0.069	0.101	0.492	-	-	3.584
pH_C:7.5	GP	0.115	0.455	0.857	0.885	0.422	2.080
	PP	0.069	0.082	0.486	-	-	6.884
pH_{UC}:7.5	GP	0.104	0.439	0.921	1.189	0.613	1.991
	PP	0.104	0.195	0.328	-	-	2.041

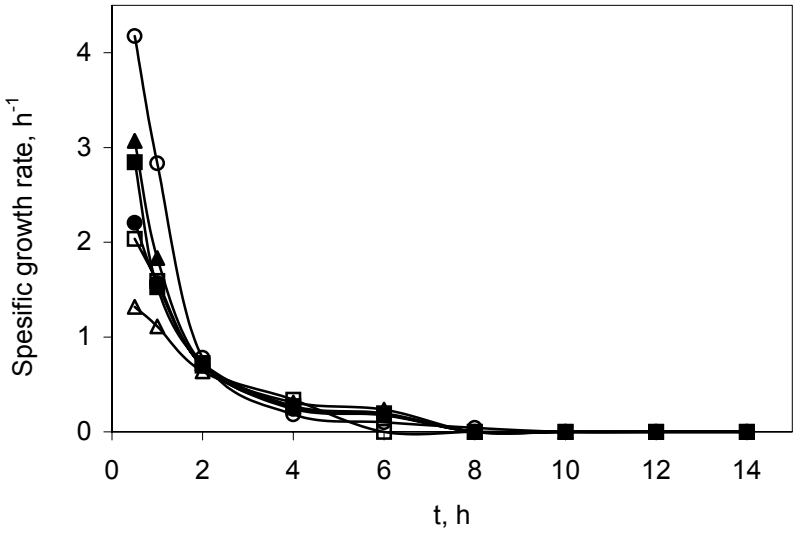

Figure 4.9 Variations in the specific growth rate with cultivation time and pH. C_G^o = 7.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}: pH_C=7.5, (●); pH_C=7.25, (▲); pH_C=7.0, (■); pH_C=6.75, (○); pH_C=6.5, (Δ), pH_{UC}=7.5, (□).

4.2 Medium Design

The medium described in Çalık et al. (2005) containing 7.0 kg m^{-3} glucose, 7.1 kg m^{-3} $(NH_4)_2HPO_4$, and the salt solution in which the β-lactamase activity was reported as 115U/cm^3 at t=27 h, respectively, was used as a reference medium in this study.

In order to increase the enzyme activity and cell growth rate, and to determine the feeding strategy for fed-batch processes the phosphate ion effect and initial glucose concentration effect with time were investigated in agitation (N=200 min^{-1}) and heating rate (T=37°C) controlled laboratory scale bioreactors.

4.2.1 Effect of Phosphate Concentration

Phosphate is known to have an influence on the metabolism of the microorganisms in terms of altering operation time while increasing the cell concentration. In this context, the effect of phosphate source with the initial

concentrations of phosphate compounds (kg/m^3): KH_2PO_4, 2 ; NaH_2PO_4, 5.63; $Na_2HPO_4.2H_2O$, 0.055 (Oktar, 2003), and N (kg/m^3):Na_2HPO_4, 5.0, 4.2, 2.1, 1.0 ; K_2HPO_4, 5.0, 4.2, 2.1, 1.0 (Christiansen et al.,2003) in addition to that of reference media were investigated. The cell concentration values at t=5h and t=6 h, and β-lactamase activity at t=24 values were measured and results were listed in Table 4.15. The highest cell concentration at t=5h together with the activity value was obtained in the case of initial phosphate concentration of N (kg/m^3):Na_2HPO_4, 1.0; K_2HPO_4, 1.0, as 0.49 kg/m^3 and 110.4 U/cm^3, respectively; whereas, the highest cell concentration at t=6h and the minimum activity were achieved for the medium containing (kg/m^3): KH_2PO_4, 2 ; NaH_2PO_4, 5.63; $Na_2HPO_4.2H_2O$, 0.055, as 0.50 kg/m^3 and 41.4 U/cm^3, respectively. It was observed that higher Na_2HPO_4 and K_2HPO_4 concentrations resulted in decreased cell concentration and activity values, because of the inhibitory effects of $HPO_4^=$ on the metabolism. Since the highest cell concentration and activity values were obtained for lower amounts of phosphate source a second set of experiment was performed to find the optimum amount of phosphate, the results of which is given in Table 4.15. Although the medium containing phosphate source of N (kg/m^3):Na_2HPO_4, 1.5; K_2HPO_4, 1.5 in addition to that of reference media components, gave highest β-lactamase activity, 120.7 U/cm^3, the medium containing N (kg/m^3):Na_2HPO_4, 1.0; K_2HPO_4, 1.05, in addition to that of reference media components, yielded a slightly less activity, 119.9 U/cm^3, hence was selected as the optimal concentration for phosphate source.

4.2.2 Effect of Glucose Concentration

The initial concentration of most preferable carbon source, glucose, that is to be used in fed-batch experiments, was investigated by using different glucose concentrations (C_G^o=2.0, 4.0, 6.0 kg/m^3) compared to the reference medium. Moreover, to observe the effect of excess and/or limiting glucose amount on the system, 2kg/m^3 glucose was added at t=2 h and t=4 h to the fermentation broth; and results were given in Table 4.16. At all conditions the cell concentration values decreased as the amount of the glucose was increased even as the activity show an increasing trend for 2kg/m^3 of initial glucose concentration and for 4 kg/m^3 of initial glucose concentration for the first addition contrary to the case for 6 kg/m^3 of initial glucose concentration which was declined relatively by the addition of glucose. Since cell concentration and β-lactamase activity were in an increasing trend for 2kg/m^3 initial glucose

concentration and for 4 kg/m^3 initial glucose concentration with the first addition, the initial concentration that should be adapted in fed-batch cultivation was determined to be in the range of 2-4 kg/m^3. Depending on previous pH_C=6.75 batch operation results this value was determined to be 2.5 kg/m^3, as the amount of glucose utilized at the feeding time (t=7h) was approximately 2.5 kg/m^3.

Table 4.15 Effects of phosphate concentration on cell concentration and beta-lactamase activity

Set No	Exp. No	Phosphate Source	C_x kg/m^3 t= 5h	C_x kg/m^3 t= 6h	Activity U/cm^3 t=24h
1	1	P (kg/m^3): KH_2PO_4,2 ; NaH_2PO_4, 5.63; $Na_2HPO_4.2H_2O$, 0.055	0.459	0.501	41.37
	2	N (kg/m^3):Na_2HPO_4, 5.0; K_2HPO_4, 5.0	0.275	0.337	54.44
	3	N (kg/m^3):Na_2HPO_4, 4.2; K_2HPO_4, 4.2	0.307	0.325	45.46
	4	N (kg/m^3):Na_2HPO_4, 2.1; K_2HPO_4, 2.1	0.429	0.458	61.03
	5	**N (kg/m^3):Na_2HPO_4, 1.0; K_2HPO_4, 1.0**	**0.486**	**0.475**	**110.44**
2	1	N (kg/m^3):Na_2HPO_4, 0.0; K_2HPO_4, 0.0	0.651	0.708	70.07
	2	N (kg/m^3):Na_2HPO_4, 0.5; K_2HPO_4, 0.5	0.564	0.699	98.11
	3	**N (kg/m^3):Na_2HPO_4, 1.0; K_2HPO_4, 1.0**	**0.583**	**0.617**	**119.90**
	4	N (kg/m^3):Na_2HPO_4, 1.25; K_2HPO_4, 1.25	0.596	0.684	103.96
	5	N (kg/m^3):Na_2HPO_4, 1.5; K_2HPO_4, 1.5	0.533	0.600	120.71

Table 4.16 Effects of glucose concentration and its feeding time on cell concentration and beta-lactamase activity

Exp. No	Glucose amount added to the medium kg/m^3 and time of addition	C_x kg/m^3 @ t= 6h	Activity U/cm^3 @ t=22h
1	2, t=0 h	0.367	37.08
2	2, t=0 h; 2, t=2 h	0.341	39.25
3	2, t=0 h; 2, t=2 h; 2, t=4 h	0.328	48.79
4	4, t=0 h	0.366	56.92
5	4, t=0 h; 2, t=2 h	0.333	60.35
6	4, t=0 h; 2, t=2 h; 2, t=4 h	0.286	56.17
7	6, t=0 h	0.369	69.26
8	6, t=0 h; 2, t=2 h	0.339	66.61
9	6, t=0 h; 2, t=2 h; 2, t=4 h	0.310	58.19

4.2.3 Effects of Phosphate and Glucose Concentrations (LSB)

In order to investigate the effect of phosphate concentration together with initial glucose concentration, mediums containing glucose (kg/m^3), 2.5, 7; Na_2HPO_4 (kg/m^3), 0.0, 1.0; K_2HPO_4 (kg/m^3), 0.0, 1.0 in addition to $(NH_4)_2HPO_4$ (kg/m^3), 7.1 and salt solution were prepared and results were compared with reference medium (Table 4.17). It can obviously be seen that the phosphate had a positive effect on enzyme activity in the range of 5.1-5.7 kg m^{-3} initial $PO_4^{=}$ concentration. Moreover, it is concluded that due to the inhibitory effect of glucose, smaller amounts should be used in fed-batch. Therefore, the medium containing, glucose, 2.5 kg/m^3; Na_2HPO_4, 1.0 kg/m^3; K_2HPO_4, 1.0 kg/m^3 $(NH_4)_2HPO_4$ (kg/m^3), 7.1 and salt solution was accepted as optimized medium for fed-batch processes.

Table 4.17 Comparison of the cell concentration and beta-lactamase activity of reference medium with the medium enhanced with phosphate

Exp. No	Phosphate and Glucose Amount added to the medium kg/m^3	C_x kg/m^3 t= 5h	C_x kg/m^3 t= 6h	Activity U/cm^3 t= 24h
1	Ref. Medium	0.649	0.651	71.02
2	Glucose, 2.5	0.580	0.624	53.20
3	Glucose, 7; Na_2HPO_4, 1.0; K_2HPO_4, 1.0	0.505	0.513	103.15
4	Glucose, 2.5; Na_2HPO_4, 1.0; K_2HPO_4, 1.0	0.548	0.554	75.97

4.3 Effects of feeding strategy

Through controlled- and uncontrolled-pH batch operations, intracellular and extracellular metabolite concentrations were investigated. The highest cell concentration and β-lactamase activity were found as 0.60 kg/m^3 and 54 U/cm^3 at pH_C=6.75 condition. Next, phosphate and initial glucose concentrations were studied in laboratory scale bioreactor; and, the optimum medium and initial conditions for fed-batch processes were determined to be (kg m^{-3}) : glucose, 2.5; Na_2HPO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2HPO_4$, 7.1 and salt solution; and controlled pH, pH_C: 6.75 with optimum oxygen transfer condition of Q_o/V_R=0.5 vvm, N=500 min^{-1}, respectively.

In the third part of the study, to increase the yield and productivity, the feeding strategy was investigated for linear and exponential feeding profiles and compared with batch operation, in order to increase the β-lactamase activity, specific cell growth rate, and yield; and to observe the change in glucose consumption rate, oxygen consumption and uptake rate, overall mass transfer coefficients; as well as the effects on sodium, potassium, intracellular ammonium, organic and amino acid concentrations.

For both fed-batch processes, at an initial concentration of C_G^0= 2.5 kg m^{-3}, feeding started at t=7.5 h by adding fresh glucose of 320 kg/m^3 with a constant rate of 0.031 ml/min for linear feeding and rate of $1.017e^{0.11t(h)}$ ml/h for exponential feeding, to the fermentation broth of 1.65 cm^3 initial volume.

4.3.1 Cell growth profile

The variations in the cell concentration with cultivation time for controlled pH, pH_C: 6.75 (N-pH_C=6.75) batch process with optimized medium; fed-batch process with exponential feeding (FB1); and fed-batch process with linear feeding (FB2) were presented in Figure 4.10. For all process types cell concentrations increased between 2-6 to their optima, then reached to stationary phase. The highest cell concentration was obtained at FB2 as C_x = 0.54 kg/m^3, whereas the lowest cell concentration was gained at N-pH_C=6.75 as C_x = 0.51kg/m^3. Comparing N-pH_C=6.75 with pH_C=6.75, it could be seen that phosphate had a negative on cell concentration, resulting in 0.85-fold lower cell concentration at N-pH_C=6.75.

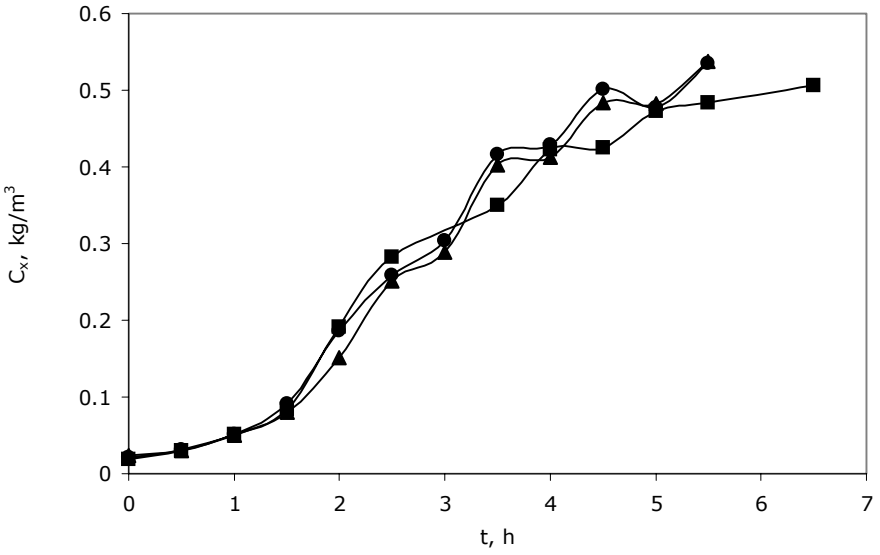

Figure 4.10 Variations in cell concentration with the cultivation time. C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1, (●); FB2, (▲); N-pH_C=6.75, (■).

For fed-batch processes the feeding started at t=7.5 h that corresponds to the beta-lactamase production phase, whereas the cell growth was stopped. Therefore, cell concentration profiles were only considered for the initial batch phase of the fed-batch processes.

4.3.2 Glucose Concentration Profiles

The change in glucose concentration throughout the investigated processes was illustrated in Figure 4.11. For both fed-batch processes glucose concentration, at an initial concentration of C_G^0= 2.5 kg m^{-3}, declined till the end of the initial batch phase at t=7.5 h, thereafter a sharp increase was occurred due to the addition of fresh glucose of 320 kg/m^3 with a constant rate of 0.031ml/min for linear feeding and rate of $1.017e^{0.11t(h)}$ ml/h for exponential feeding, to the fermentation broth of 1.65 cm^3 initial volume. The glucose concentration was increased almost linearly up to 4.8 kg/m^3 in fed-batch fermentation with linear feeding while the glucose concentration increased quite sharply with a maximum of 8.4 kg/m^3 in the case of fed-batch fermentation with exponential feeding.

However, when glucose consumption rate of batch process, with initial glucose concentration of 7kg/m^3 and at optimum pH and oxygen transfer conditions, was compared with that of both fed-batch processes, an almost parallel trend was observed as in the initial batch phase of fed-batch cultivations, where the glucose was consumed more rapidly at the beginning of the process till t=4h while it was almost remained stationary for the rest of the process. Furthermore, unlike at pH_C=6.75, glucose consumption rate was slightly lower at N-pH_C=6.75 because of the lower cell concentration.

In Table 4.18, the glucose amount consumed with cultivation time for FB1, FB2, N-pH_C=6.75 is given. In general, glucose consumption showed similar profile for FB1 and FB2 until the end of t=12h, where the feeding profiles were the same, then glucose consumption increased for FB2. Comparing batch and fed-batch processes, it is observed that the glucose consumption was higher for batch process than for the initial batch phase of fed-batch processes. Afterwards, the amount of glucose consumption decreased compared to fed-batch processes.

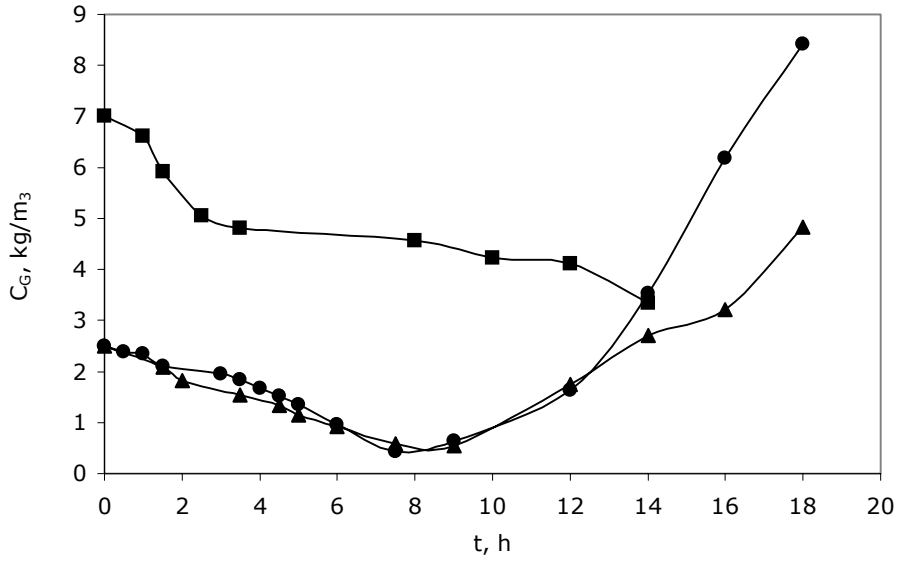

Figure 4.11 Variations in glucose concentration with the cultivation time. C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1, (●); FB2, (▲); N-pH_C=6.75, (■).

Table 4.18 Glucose consumption with cultivation time and feeding strategy.

Time (h)	Consumed Glucose, g		Time (h)	Consumed Glucose, g
	FB1	FB2		N-pH_C= 6.75
2	1.018	1.054	2	2.559
4	0.849	0.898	4	1.972
6	0.735	0.779	6	0.736
7.5	0.904	0.513	8	0.399
9	1.604	1.982	10	0.989
12	2.775	2.516	12	0.299
14	1.273	2.519	14	0.402
16	1.336	4.140	-	-
18	3.149	3.907	-	-

4.3.3 β-lactamase Activity Profiles

The variation of β-lactamase activity with cultivation time is given in Figure 4.12. The highest β-lactamase activity was obtained as A=108 U/cm^3 for FB1 while the lowest value belongs to the batch system with N-pH_C= 6.75, as expected. The enzyme activity at N-PH_C=6.75 increased about 1.21-fold compared to that of at pH_C=6.75 operation. The low activity values at batch operations should be the result of inhibitory effect of glucose on cell metabolism. Since the cell concentrations of both fed-batch operations were only slightly different from each other, insufficient amount of glucose, due to different feeding strategy and hence high glucose consumption rates of cells, at FB2 after t=9h was probably resulted in lower activity values for fed-batch operation with linear feeding. Therefore, the fed-batch process with exponential feeding was selected as optimal operation type in terms of β-lactamase activity with a value of 108 U/cm^3.

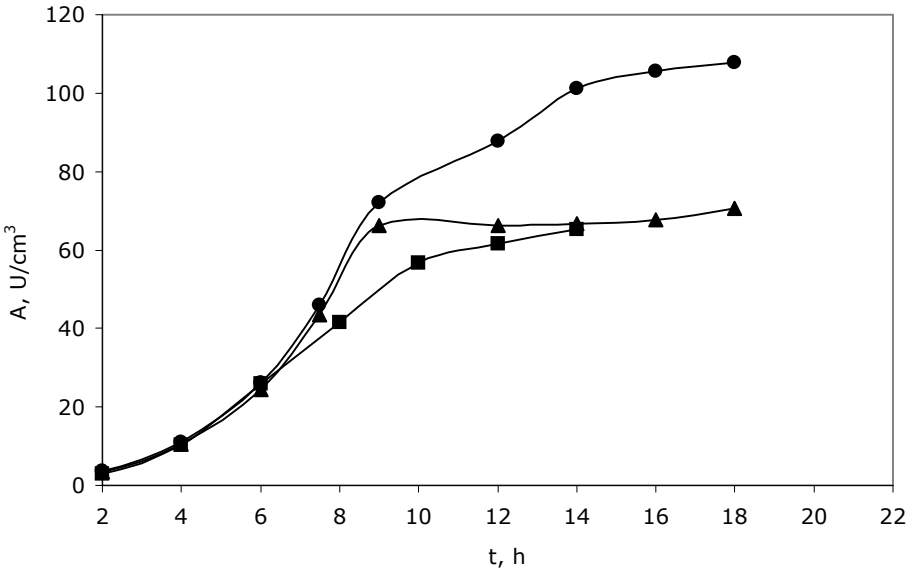

Figure 4.12 Variations β-lactamase activity with the cultivation time. C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1, (●); FB2, (▲); N-pH_C=6.75, (■).

4.3.4 Dissolved Oxygen Profiles

The variation of the dissolved oxygen concentration of different operation types with cultivation time is given in Figure 4.13. For all operation types, dissolved oxygen declined reaching to 55-65% of saturation value (C_{DO}=0.11-0.13 mol m^{-3}) due to the high demand of oxygen at the beginning of the processes till t=5 h. Thereafter, dissolved oxygen profiles gradually increased and remained almost stationary until the end of the processes. Comparing N-pH_C=6.75 and pH_C=6.75 operations, it is observed that locus of dissolved oxygen concentration profiles were similar with lower values at pH_C=6.75 due to higher cell concentration.

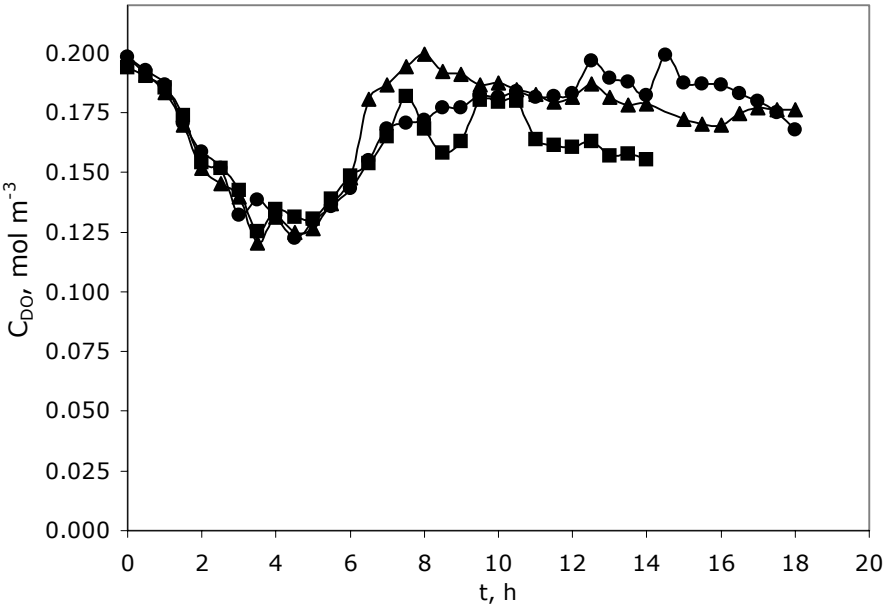

Figure 4.13 Variations in dissolved oxygen concentration with the cultivation time. C_{DO}^{*}=.20 mol m^{-3}, C_G^{o} = 2.5 kg m^{-3} for FB1 and FB2, C_G^{o} = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1, (●); FB2, (▲); N-pH_C=6.75, (■).

4.3.5 Amino Acid concentration profiles

The variations in intracellular and extracellular amino acid concentrations with cultivation time for batch and fed-batch operations are given in Tables 4.19, 4.20, 4.21, and Appendix H. At all conditions applied, asparagine, methionine, valine, phenylalanine, and tyrosine were the amino acids excreted extracellularly, in common; whereas prolin, phenylalanine, and asparagine were the major amino acids expelled intracellularly. Similar to previous cases, the amino acid concentrations in the fermentation broth were low.

The total amino acid concentration excreted extracellularly and intracellularly were highest at N-pH_C=6.75 t=14h and FB2 t=2h as $T_{AA,\ ext}$= 0.1604 kg m^{-3} and $T_{AA,int}$= 0.247 g g_{cell}^{-1} (6.029 kg m^{-3}), respectively. On the contrary, the minimum total amounts were obtained at FB1 t=9 h and FB2 t=14 h as $T_{AA,\ ext}$= 0.0626 kg m^{-3} and $T_{AA,int}$= 0.0006 g g_{cell}^{-1} (0.015 kg m^{-3}), respectively. Among all conditions, asparagine concentration was the highest extracellular and intracellular amino acid concentration obtained at N-pH_C=6.75 t=4h as 0.0791 kg m^{-3}, and at FB2 t=2 h as 0.1623 g g_{cell}^{-1} (3.968 kg m^{-3}). As it is seen in Table 4.19, 4.20, and Table 4.21, there was relation between methionine-cystein and phenylalanine-tyrosine with higher amounts of methionine and phenylalanine. This is an expected result, as methionine is produced from cystein; phenylalanine is degraded to tyrosine; and both phenylalanine and tyrosine are produced from chorismate and glutamate with higher production rate of phenylalanine.

4.3.6 Organic Acid concentration profiles

The variations in intracellular and extracellular organic acid concentrations with cultivation time for batch and fed-batch operations are given in Tables 4.22, 4.23, 4.24, and Appendix H. At all conditions applied, except oxaloacetic acid, all organic acids were excreted; whereas acetic acid, fumaric acid, lactic acid, α-ketoglutaric acid and succinic acid were not presented among intracellular components because of their minute amounts. Similar to previous cases, the organic acid concentrations in the fermentation broth were low.

As illustrated in Tables 4.21, 4.22, 4.23 the total organic acid concentration excreted extracellularly and intracellularly were highest at FB2 t=18h and N-

pH_C=6.75 t=2h as $T_{OA,\ ext}$= 1.698 kg m^{-3} and $T_{OA,int}$= 0.076 gg_{cell}^{-1} (1.835 kgm^{-3}), respectively. In contrast, the minimum total amounts were obtained at N-pH_C= 6.75 t=2 h and FB1 t=6 h as $T_{OA,\ ext}$= 0.125 kg m^{-3} and $T_{OA,int}$= 0.004 g g_{cell}^{-1} (0.108 kg m^{-3}), respectively. For all pH conditions applied, lactic acid concentration was the highest extracellular organic acid concentration obtained at FB2 t=18h as 0.945 kg m^{-3}, while citric acid concentration was the highest intracellular amino acid concentration, obtained at FB2 t=2 h as 0.044 g g_{cell}^{-1} (1.087 kg m^{-3}). As in controlled and uncontrolled pH batch fermentations, lactic acid-pyruvic acid and α-ketoglutaric acid-pyruvic acid were converted to each other continuously throughout the operations. In addition, from malic acid, succinic acid, fumaric acid, and α-ketoglutaric acid; pyruvic acid, fumaric acid, malic acid, and fumaric and succinic acids were produced, respectively.

Table 4.19 Variations in extracellular and intracellular amino acid concentrations with cultivation time at N-pH_C=6.75

N 6.75 – Extracellular, C_{AA} (kg.m^{-3})													
Time (h)	Asn	His	Thr	Ala	Pro	Tyr	Val	Met	Cys	Lys	Phe	Orn	Trp
0	0.066	-	0.004	-	0.004	0.009	0.010	0.008	-	0.012	0.015	-	-
2	0.052	-	0.002	-	0.003	0.009	0.005	0.007	-	0.010	0.016	-	-
4	0.079	-	-	-	-	0.018	-	-	0.002	0.008	-	-	0.017
6	0.063	0.003	-	-	-	0.009	0.006	0.003	0.001	0.006	-	0.010	-
8	0.034	-	-	-	-	0.011	0.007	-	0.003	0.010	0.016	-	-
10	0.047	-	-	-	-	-	0.009	0.005	0.002	0.004	0.062	-	-
12	0.071	0.004	-	0.006	-	0.011	0.009	0.002	0.001	0.004	0.012	-	-
14	0.054	0.002	-	0.007	-	0.017	0.010	-	0.003	0.004	0.065	-	-
N 6.75 – Intracellular, C_{AA} (g.g$_{cell}^{-1}$)													
0	0.002	-	-	-	-	-	-	-	-	-	-	0.002	-
2	0.006	-	-	-	0.003	0.011	-	-	0.002	-	0.004	0.006	-
4	0.003	-	-	-	0.001	-	-	-	-	0.001	0.001	0.003	-
6	0.004	-	-	-	0.001	-	-	-	-	0.001	-	0.004	-
8	-	-	-	-	0.001	-	-	-	-	0.001	0.001	-	-
10	-	-	-	-	0.001	-	-	-	-	0.001	0.001	-	-
12	0.006	-	-	-	0.001	0.004	-	0.006	0.001	-	0.001	0.006	-
14	0.007	-	-	-	0.001	0.004	-	0.008	0.001	0.001	0.001	0.007	-

Table 4.20 Variations in extracellular and intracellular amino acid concentrations with cultivation time at FB1

FB1 – Extracellular, C_{AA} ($kg.m^{-3}$)															
t, h	Asn	Gla	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys	Ile	Lys	Phe	Trp
0	0.046	0.008	-	-	0.003	0.003	0.002	0.020	0.021	0.006	0.002	-	0.008	0.018	-
2	0.046	0.004	-	-	0.002	-	0.003	0.019	0.031	0.004	0.003	0.024	0.007	0.016	-
4	0.012	-	0.005	-	0.002	-	-	0.008	0.020	-	-	-	0.006	0.011	-
6	0.009	-	-	-	0.001	-	-	0.013	0.026	0.003	-	-	-	0.078	-
7.5	0.009	0.005	-	-	-	-	0.004	0.009	0.021	0.002	0.001	-	-	0.082	-
9	0.014	0.005	-	-	-	-	0.002	0.012	0.018	0.002	0.002	-	-	-	0.008
12	0.014	0.004	0.006	-	-	-	0.003	0.011	0.019	-	0.001	-	-	0.006	-
14	0.014	0.004	0.003	-	-	-	0.002	0.016	0.017	-	0.002	-	-	0.009	-
16	0.021	0.003	0.010	-	0.001	-	0.003	0.014	0.019	0.003	0.004	-	-	0.008	-
18	0.023	-	0.006	-	-	-	0.003	0.017	0.019	0.002	0.006	-	-	0.008	-
FB1 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)															
0	0.018	-	-	-	0.008	-	-	0.037	-	0.002	-	-	0.001	0.001	-
2	0.153	-	-	0.003	-	-	0.002	0.014	-	0.010	-	-	0.006	0.002	-
4	0.064	-	-	-	0.011	-	-	-	-	0.012	-	-	0.002	0.016	-
6	0.058	-	-	-	-	-	-	0.011	-	0.004	-	-	0.001	0.011	-
7.5	0.036	-	-	-	-	-	0.001	0.009	-	0.004	-	-	0.001	0.011	-
12	0.052	-	-	-	-	-	0.001	0.011	-	0.003	-	-	0.002	0.015	-
14	0.045	-	-	-	-	-	0.001	0.007	-	0.005	-	-	0.002	0.017	-
16	0.057	-	-	-	-	-	0.001	0.013	-	0.007	-	-	0.001	0.013	-
18	0.011	-	-	-	-	-	0.001	0.002	-	0.002	-	-	0.002	0.009	-

Table 4.21 Variations in extracellular and intracellular amino acid concentrations with cultivation time at FB2

FB2 – Extracellular, C_{AA} ($kg.m^{-3}$)																
t, h	Asn	Gla	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys	Ile	Lys	Phe	Trp
0	0.056	0.006	-	0.004	-	0.004	0.004	-	-	0.018	0.004	-	-	0.012	0.021	-
2	0.044	-	0.006	0.006	-	0.003	0.007	-	0.010	0.013	0.003	-	-	0.012	0.020	-
4	0.054	-	-	0.002	-	0.023	-	-	-	-	0.007	-	0.030	0.007	-	0.011
6	0.047	-	-	-	-	-	-	-	0.015	0.014	0.002	-	-	0.002	0.009	-
7.5	0.048	-	-	-	-	0.019	-	-	-	0.012	-	-	-	-	0.012	-
9	0.053	-	-	-	-	-	-	-	0.020	0.019	-	-	-	-	0.015	-
12	0.046	-	-	-	-	-	-	0.003	0.017	0.015	0.002	0.002	-	-	0.011	-
14	0.055	-	-	-	-	-	-	0.003	0.017	0.020	-	0.005	-	-	0.012	-
16	0.065	-	-	-	-	-	-	0.003	0.025	0.016	-	0.004	-	-	0.015	-
18	0.074	-	-	-	-	-	-	0.004	0.028	0.020	0.002	0.006	-	-	0.012	-
FB2 – Intracellular, C_{AA} ($g.g_{cell}^{-1}$)																
0	0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	0.001	-
2	0.162	-	-	-	0.004	-	-	0.003	0.051	-	0.021	-	-	0.005	-	-
4	0.012	-	-	-	-	-	-	-	-	-	0.006	-	-	-	0.012	-
7.5	0.006	-	-	-	-	-	-	-	-	-	-	0.002	-	-	0.006	-
9	0.003	-	-	-	-	-	-	-	-	-	-	-	-	-	0.003	-
12	-	-	-	-	-	-	-	0.001	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	0.001	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	0.001	-	-	-	0.002	-	-	-	-
18	-	-	-	-	-	-	-	0.001	-	-	-	-	-	-	-	-

Table 4.22 Variations in extracellular and intracellular organic acid concentrations with cultivation time at N-pH_C=6.75

N 6.75 – Extracellular, C_{OA} (kg.m^{-3})												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.048	0.113	-	-	0.002	0.010	-	0.003	0.001	-	0.002	-
2	0.099	-	-	0.001	0.003	0.017	-	0.002	0.001	-	0.002	-
4	0.119	0.019	-	-	0.010	0.062	0.001	0.003	0.002	-	0.002	-
6	0.191	0.043	-	0.002	0.056	0.339	0.002	0.007	-	-	0.002	-
8	0.205	-	0.009	0.002	0.080	0.462	0.002	0.012	0.003	-	0.004	0.026
10	0.152	-	0.024	0.001	0.061	0.355	0.002	0.010	0.003	-	0.003	0.140
12	0.312	-	0.012	0.002	0.115	0.642	0.002	0.018	0.004	-	0.005	-
14	0.265	-	0.041	0.002	0.085	0.491	0.001	0.028	0.004	-	0.004	-
N 6.75 – Intracellular, C_{OA} (g.g_{cell}^{-1})												
0	-	0.025	0.002	-	-	-	0.001	0.004	-	-	-	-
2	-	-	0.020	-	-	-	0.001	0.025	0.026	-	0.004	-
4	-	0.037	0.007	-	-	-	0.001	0.007	0.011	-	-	-
6	-	0.026	0.007	-	-	-	0.001	0.006	0.008	-	-	-
8	-	0.026	0.007	-	-	-	0.001	0.005	0.008	-	-	-
10	-	0.013	0.006	-	-	-	0.001	0.004	0.008	-	-	-
12	-	0.015	0.007	-	-	-	0.001	0.005	0.008	-	0.001	-
14	-	-	0.008	-	-	-	0.001	0.003	0.009	-	-	-

Table 4.23 Variations in extracellular and intracellular organic acid concentrations with cultivation time at FB1

FB1 – Extracellular, C_{OA} ($kg.m^{-3}$)												
t, h	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.047	0.098	-	-	0.001	0.009	-	0.002	-	-	0.002	-
2	0.077	-	-	0.001	0.003	0.017	-	0.008	-	-	0.001	0.123
4	0.129	-	-	0.001	0.012	0.071	-	0.001	-	-	0.001	-
6	0.066	-	0.003	0.001	0.022	0.133	0.001	0.003	-	-	0.001	0.137
7.5	0.175	-	0.006	0.001	0.081	0.466	0.003	0.012	0.007	-	0.001	-
9	0.209	-	0.009	0.003	0.102	0.591	0.002	0.016	0.003	-	0.001	-
12	0.288	-	0.039	0.002	0.119	0.682	0.002	0.021	0.005	-	0.001	0.022
14	0.152	-	0.024	0.001	0.059	0.342	0.001	0.014	0.005	-	0.002	0.188
16	0.406	0.097	0.013	0.002	0.150	0.843	0.003	0.052	0.006	-	0.013	-
18	0.327	-	0.009	0.002	0.124	0.733	0.002	0.097	0.006	-	0.001	-
FB1 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	-	-	-	-	-	-	-	0.002	-	-	-
2	-	-	0.034	-	-	-	-	-	0.022	-	-	-
4	-	-	0.015	-	-	-	-	-	0.005	-	-	-
6	-	-	-	-	-	-	-	-	0.004	-	-	-
7.5	-	-	0.013	-	-	-	-	-	0.003	-	-	-
12	-	-	0.006	-	-	-	-	-	0.007	-	-	-
14	-	-	0.006	-	-	-	-	-	0.009	-	-	-
16	-	-	0.006	-	-	-	-	-	0.008	-	-	-
18	-	-	0.006	-	-	-	-	-	0.008	-	-	-

Table 4.24 Variations in extracellular and intracellular organic acid concentrations with cultivation time at FB2

FB2 – Extracellular, C_{OA} ($kg.m^{-3}$)												
t, h	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	0.067	0.132	-	-	0.002	0.011	0.001	0.001	-	-	0.002	-
2	0.116	-	-	0.001	0.003	0.020	-	0.002	0.002	-	0.002	-
4	0.140	-	-	0.002	0.014	0.086	-	0.001	0.003	-	0.001	-
6	0.148	-	-	0.001	0.051	0.307	0.001	0.006	0.003	-	0.001	-
7.5	0.182	-	0.007	0.001	0.084	0.488	0.003	0.011	0.004	-	0.001	-
9	0.266	-	0.009	0.002	0.139	0.777	0.002	0.017	-	-	0.001	0.027
12	0.319	-	0.048	0.003	0.143	0.799	0.002	0.021	0.005	-	0.001	-
14	0.339	-	0.040	0.002	0.134	0.765	0.002	0.037	0.005	-	0.004	-
16	0.395	-	0.020	0.002	0.155	0.883	0.002	0.037	0.006	-	0.007	-
18	0.439	0.077	0.019	0.002	0.165	0.945	0.001	0.041	0.006	-	0.003	-
FB2 – Intracellular, C_{OA} ($g.g_{cell}^{-1}$)												
0	-	0.017	0.002	-	-	-	0.001	0.002	0.001	-	-	-
2	-	0.044	0.020	-	-	-	0.001	0.014	0.014	-	-	-
4	-	0.037	0.007	-	-	-	0.001	0.006	0.005	-	-	-
6	-	0.026	0.007	-	-	-	0.001	0.006	0.003	-	-	-
7.5	-	0.016	0.008	-	-	-	0.001	0.004	0.004	-	-	-
9	-	0.011	0.008	-	-	-	0.001	0.003	0.003	-	-	-
12	-	0.009	0.006	-	-	-	0.001	0.002	0.003	-	-	-
14	-	-	0.007	-	-	-	0.001	0.002	0.003	-	-	-
16	-	-	0.007	-	-	-	0.001	0.002	0.003	-	-	-
18	-	0.009	0.005	-	-	-	0.001	0.002	0.003	-	-	-

4.3.7 Sodium and Potassium Ions Profiles

The variations of the intracellular and extracellular sodium and potassium ion concentrations of different feeding profiles with cultivation time are given in Figure 4.14 and Figure 4.15, respectively. For batch operation and fed-batch operation with linear feeding the extracellular sodium ion concentrations were ranging between 270-340 ppm throughout the processes; while for fed-batch cultivation with exponential feeding the extracellular sodium ion concentration was ranging significantly between 660-780 ppm. On the other hand, the extracellular potassium ion concentrations increased approximately 1.6-fold through the end of the cultivation for all operation types. Furthermore, at N-pH_C=6.75, extracellular sodium and potassium ion concentrations were approximately 3-fold higher than those of at pH_C=6.75, due to the additional Na^+, K^+ present in the optimized production media.

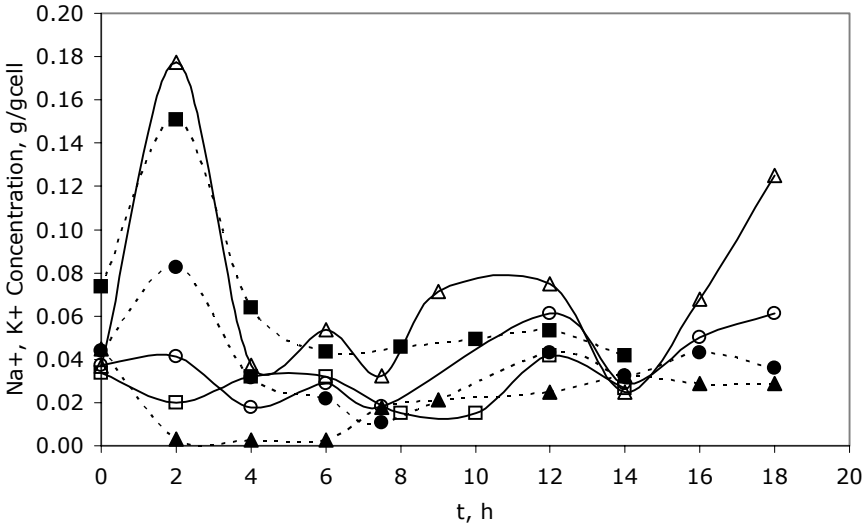

Figure 4.14 Variations in intracellular sodium, potassium ion concentration with the cultivation time C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1-Na^+ ion conc., (●); FB2-Na^+ ion conc, (▲); N-pH_C=6.75-Na^+ ion conc, (■); FB1-K^+ ion conc., (○); FB2-K^+ ion conc, (Δ); N-pH_C=6.75-K^+ ion conc, (□); rigid lines show potassium concentration, dashed lines show sodium concentration.

The intracellular sodium amounts per gram cell, however, were decreasing with increasing potassium amounts for fed-batch processes; whereas the higher sodium amounts were conversely ranging with potassium amounts in batch operation type. Similar to extracellular sodium and potassium ion concentrations, intracellular amounts of the ions were higher at N-pH_C=6.75 than at pH_C=6.75, due to the presence of additional sodium and potassium ions in production media. The total amount of sodium and potassium transferred into and out of the cell calculated using these profiles, Figure 4.16. Figure 4.16 illustrates that there exists a sodium/potassium ion pump in the system to maintain the electro-potential gradient across the cell membrane as in the previous cases.

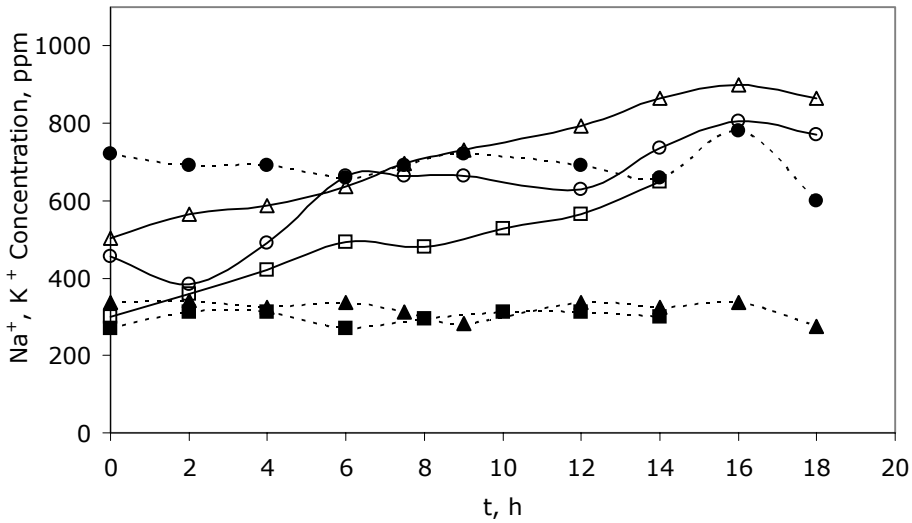

Figure 4.15 Variations in extracellular sodium, potassium ion concentration with the cultivation time C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1-Na^+ ion conc., (●); FB2-Na^+ ion conc, (▲); N-pH_C=6.75-Na^+ ion conc, (■); FB1-K^+ ion conc., (○); FB2-K^+ ion conc, (Δ); N-pH_C=6.75-K^+ ion conc, (□); rigid lines show potassium concentration, dashed lines show sodium concentration.

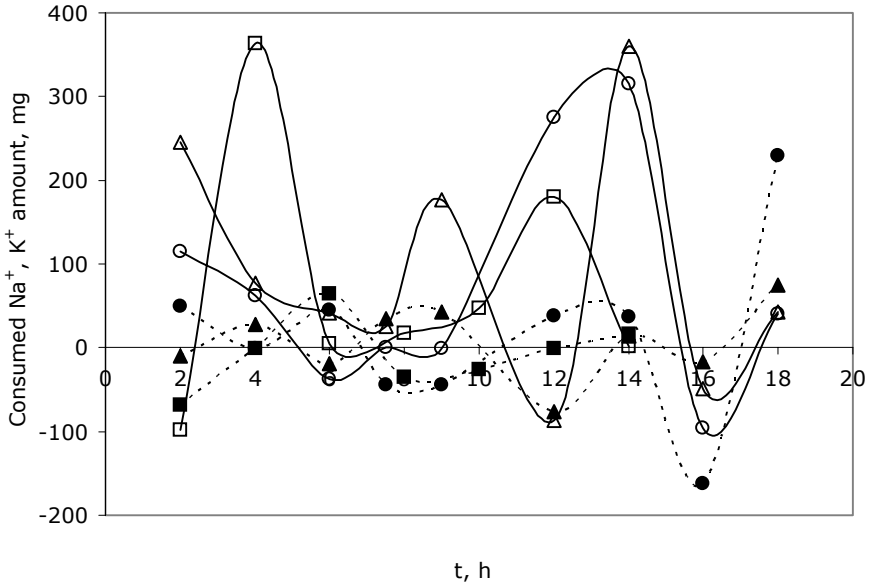

Figure 4.16 Variations in consumed amount of sodium, potassium ions with the cultivation time C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1-Na^+ ion conc., (●); FB2-Na^+ ion conc, (▲); N-pH_C=6.75-Na^+ ion conc, (■); FB1-K^+ ion conc., (○); FB2-K^+ ion conc, (Δ); N-pH_C=6.75-K^+ ion conc, (□); rigid lines show potassium concentration, dashed lines show sodium concentration.

4.3.8 Intracellular Ammonium Ion

The variation of the intracellular ammonium ion amounts per gram cell with pH and cultivation time is given in Figure 4.17. As in previous controlled- and uncontrolled-pH batch operations the ammonium ion amounts had a maxima at the t=2h of the processes due to high specific growth rate of the cells at the first two hours of the cultivations; whilst, it was relatively constant till the end of processes. When N-pH_C=6.75 and pH_C=6.75 operations are compared, it could be seen that intracellular ammonium amount at pH_C=6.75 was about 3-fold higher than that at N-pH_C=6.75. This should be due to the buffering capacity of phosphate ion, which diminished the need for ammonium ion.

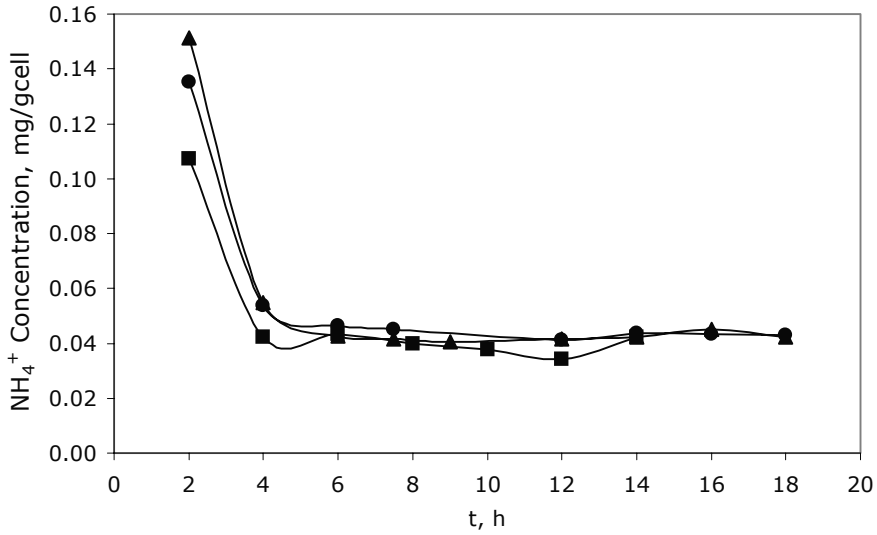

Figure 4.17 Variations in intracellular ammonium ion concentration with cultivation time. C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1, (●); FB2, (▲); N-pH_C=6.75, (■).

4.3.9 Phosphate Concentration Profiles

The variations of the intracellular and extracellular phosphate concentrations with cultivation time at FB1 condition are given in Figure 4.18 and Figure 4.19, respectively. Extracellular phosphate had maxima at t= 7.5, and 14 h of the bioprocess; and was relatively constant at 15000 ppm except these points. Since the phosphate amount at the beginning and end of the process were almost the same, the cells should not be suffering from phosphate deficiency. On the other hand, the intracellular phosphate amount per gram cell was increasing and decreasing throughout the process with lower fluctuations through the end of process. Using these profiles, the total amount of phosphate transferred into and out of the cell was calculated, and illustrated in Figure 4.20. Figure 4.20 illustrates that potassium is transported through the cell in the course of fermentation to maintain the electro-potential gradient across the cell membrane.

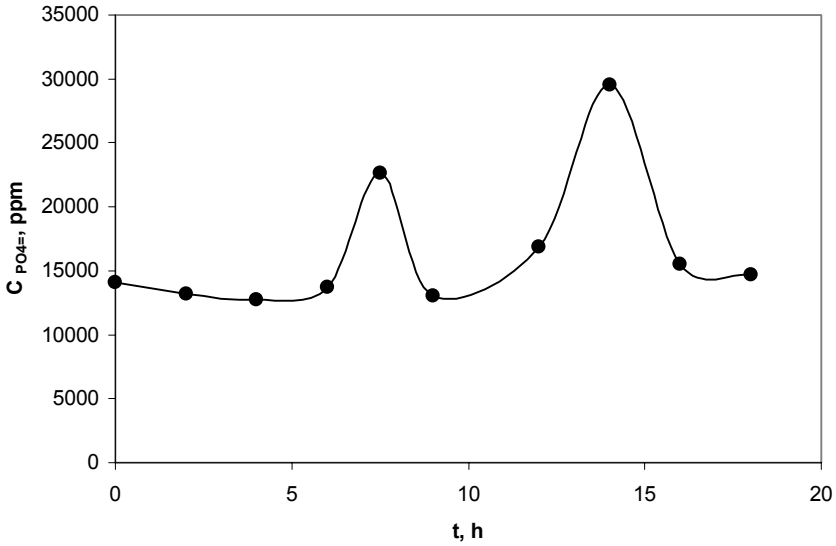

Figure 4.18 Variations in extracellular phosphate concentration with the cultivation time at FB1 condition. C_G^o = 2.5 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1-$PO_4^=$ ion conc., (●).

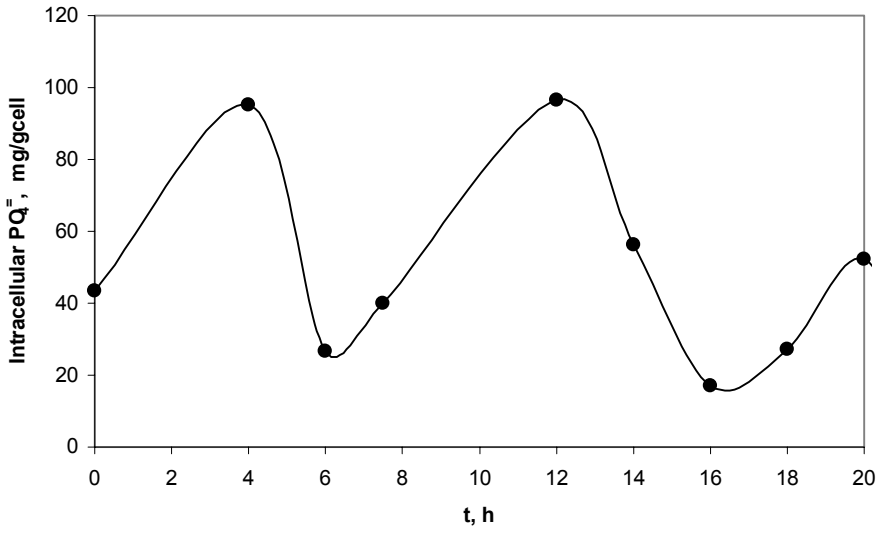

Figure 4.19 Variations in intracellular phosphate amounts with the cultivation time at FB1 condition. C_G^o = 2.5 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1-$PO_4^=$ ion conc., (●).

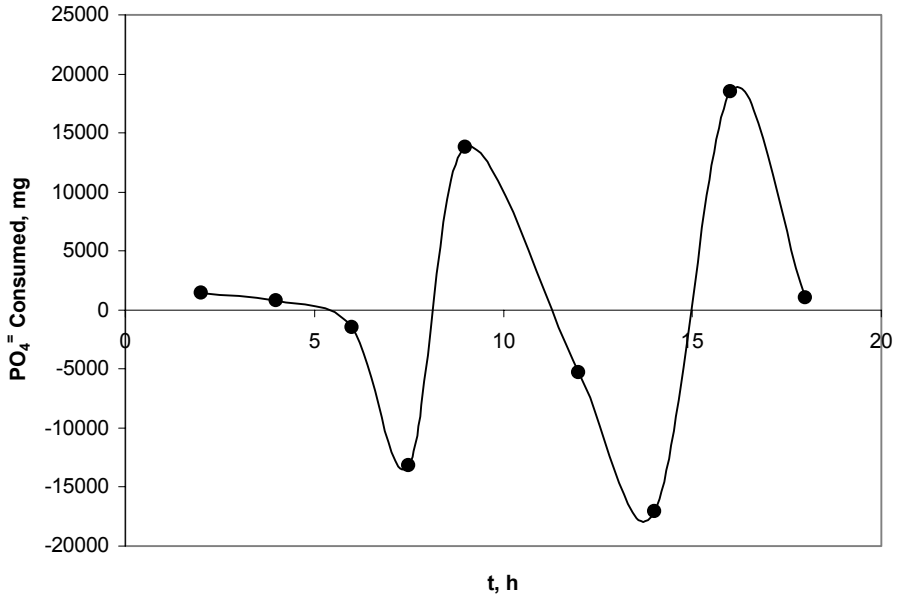

Figure 4.20 Variations in consumed amount of phosphate with the cultivation time for FB1 condition. C_G^o = 2.5 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1-$PO_4^=$ iamount, (●).

4.3.10 Oxygen Transfer Characteristics

The variations in K_La, oxygen uptake rate, oxygen transfer rate and the enhancement factor E (=K_La/K_La_o) throughout the bioprocesses are given in Table 4.25. As in previous cases K_La and E were higher at PP of the processes, varying between 0.0102-0.0186 and 0.99-1.55, respectively. The oxygen uptake rate, on the other hand, decreased in PP of the bioprocesses with decreasing metabolic activity of the cells. K_La, oxygen uptake rate, and E values did not show a similar trend in the initial batch phase of fed-batch processes and batch process, since the initial glucose concentrations were different. In the same manner, K_La and E values were slightly higher whereas oxygen uptake rate was lower at N-pH_C=6.75 than those at pH_C=6.75 operation.

To compare mass transfer and biochemical reaction rates, the maximum possible oxygen utilization rate (OD), the maximum possible mass transfer rate (OTR_{max}), Damköhler number (Da), effectiveness factor (η), and k/K_La values

were given in Table 4.25. For all conditions applied, Da and k/K_La values were higher than 1, except N-pH_C=6.75 PP condition (k/K_La=0.56) limited by chemical reactions, indicating that mass transfer resistance limited the operations. As in previous cases Da values were slightly higher than k/K_La, due to the reasons mentioned formerly. As it is seen in Table 4.24 the effectiveness factor, η, was low indicating that the cells are consuming lower oxygen than the dissolved oxygen. In general, the highest OD, Da, η, and k/K_La values were obtained at FB1 condition, and the lowest ones were attained at FB2 condition; whereas N-pH_C=6.75 batch process results were in between of the two fed-batch processes and lower than those at pH_C=6.75 batch process.

4.3.11 Specific Growth Rate and Yield Coefficients

For different feeding profiles, the variations in the specific growth rate, μ, the specific oxygen uptake rate, q_o, the specific substrate consumption rate, q_s, and the yield coefficients with cultivation time are given in Table 4.26 and Figure 4.21. The specific growth rate (μ) decreased with cultivation time for all conditions and as it is seen in Figure 4.21, the maximum value of specific growth rate was achieved at N-pH_C=6.75, t=0.5 h as μ=4.23 h^{-1}, which is slightly higher than pH_C=6.75 operation. In addition, the specific growth rate of FB1 and FB2 conditions showed a similar profile, as expected. Specific substrate utilization rates (q_S), however, generally increased at PP except FB2 condition where it was almost the same at both phases. The highest value of q_S was attained at FB1 PP condition as 5.76 kg kg^{-1} h^{-1}; where the lowest value was obtained at the same operation at GP as 0.85 kg kg^{-1} h^{-1}. Specific oxygen uptake rates (q_0), on the other hand, were higher at GP for all conditions applied. The highest value of q_0 was obtained at FB1 (GP) as 0.470 kg kg^{-1} h^{-1}; where the lowest value was obtained at N-pH_C=6.75 (PP) as 0.080 kg kg^{-1} h^{-1}.

The yield coefficients are also given in Table 4.26. The highest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at N-pH_C=6.75 and FB1; FB2; and FB1 (PP) as 0.434; 1.178; 57.19 kg kg^{-1}, respectively. In contrast, the lowest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at FB2; N-pH_C=6.75; and FB1 (GP) as 0.414; 0.708; 1.845 kg kg^{-1}; respectively. The variation in specific growth rate, μ, the specific oxygen uptake rate, q_o, the specific substrate consumption rate, q_s, and the yield coefficients in batch and initial batch phase of fed-batch processes is because of the difference in the initial glucose concentrations.

Table 4.25 Variations in oxygen transfer parameters with cultivation time.

Condition	t	k_La (s^{-1})	E k_La/k_La_0	OTRx10^3 ($molm^{-3}s^{-1}$)	$OTR_{max}x10^3$ ($molm^{-3}s^{-1}$)	OURx10^3 ($molm^{-3}s^{-1}$)	ODx10^3 ($molm^{-3}s^{-1}$)	Da OD/OTR_{mx}	η OUR/OD	k/ K_La
N-pH_C:6.75	GP	0.0112	0.9977	0.6373	2.3567	0.9333	14.382	6.1026	0.0649	1.4784
	PP	0.0186	1.5450	0.7110	3.8980	0.4600	-	-	-	0.5605
FB1	GP	0.0115	0.9961	0.6683	2.4200	1.1000	16.950	7.0041	0.0649	1.6934
	PP	0.0171	1.3360	0.4566	3.5814	0.6286	-	-	-	1.3221
FB2	GP	0.0102	0.9910	0.7740	2.1383	0.8333	12.327	5.7649	0.0676	1.0872
	PP	0.0117	1.1273	0.4321	2.4627	0.3857	-	-	-	1.1376

Table 4.26 The variations in specific rates and yield coefficients with cultivation time and feeding condition.

Condition	t	r_o (kg O_2/m^3h)	q_0 (kg/kg h)	q_s (kg/kg h)	$Y_{x/o}$ (kg/kg)	$Y_{x/s}$ (kg/kg)	$Y_{s/o}$ (kg/kg)
N-pH_C:6.75	GP	0.104	0.343	1.52	0.708	0.434	3.382
	PP	0.058	0.080	2.08	-	-	35.03
FB1	GP	0.127	0.470	0.85	0.792	0.434	1.845
	PP	0.069	0.135	5.76	-	-	57.19
FB2	GP	0.092	0.391	1.19	1.178	0.414	2.870
	PP	0.046	0.089	1.18	-	-	24.06

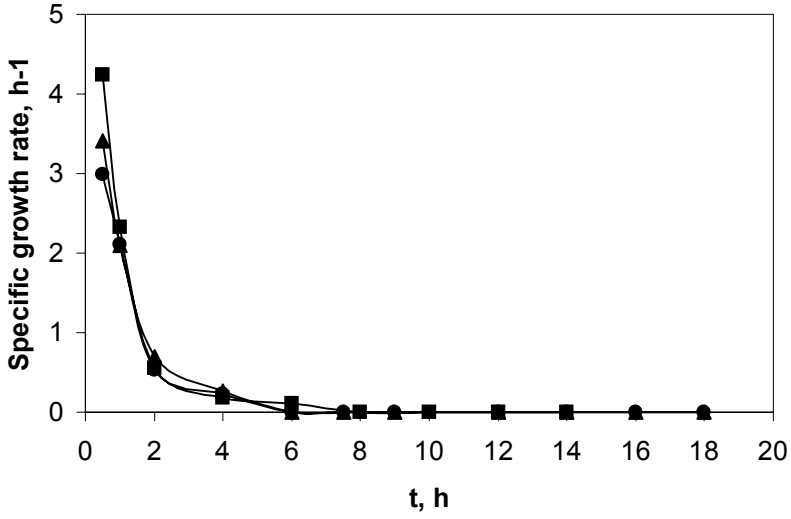

Figure 4.21 Variations in the specific growth rate with cultivation time. C_G^o = 2.5 kg m^{-3} for FB1 and FB2, C_G^o = 7.0 kg m^{-3} for N-pH_C=6.75, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_C=6.75, FB1, (●); FB2, (▲); N-pH_C=6.75, (■).

4.4 Viability of the Cells

To investigate the physiological state of the culture media, viability of the cells was monitored throughout the cultivation time for repeated FB1, pH_C=6.75, and pH_{UC}=7.5 experiments. The variation of viability with cultivation time is given in Table 4.27. At pH_C=6.75, the increase in the number of dead cells was not significant. However, through the end of FB1 and pH_{UC}=7.5 operations, about 9% of the cells were dead. Indeed, in fed-batch cultivation, this is an expected trend, as the cells are exposed to the prolonged nutrient limitation. Cell concentration, enzyme activity, and pH profiles of these operations were also measured at specific time intervals and the results are given in Appendix G. As it is seen in Appendix G, the results were reproducible in terms of cell concentration and enzyme activity. Similarly for pH_{UC}=7.5, the pH variation with time showed similar trend.

In the literature concerning *B.licheniformis*, Hornbæk et al. (2002) studied cell vitality in laboratory scale bioreactor, and proved that pH gradient correlates

with the lag phase of bacteria. However, there was no information about cell viability in pilot scale bioreactor. Concerning other *Bacillus* strains, Christiansen et al. (2003) investigated the physiological state of *B.clausii*, and found that at the end of 24h of fed-batch cultivation with exponential feeding the fraction of dead cells was about 47%. This value was significantly higher than that found at FB1. The reason for this may be explained by the high number of dead cells being carried over from the inoculum in the study of Christiansen et al. (2003) and/or inaccurate estimates made in this study and fluctuations in the feed.

Table 4.27 The variation of cell viability with cultivation time and operation mode.

Conditions	t (h)	Viability (%)
pH_{UC}	8	99.9
	14	90.3
pH_C	4	99.7
	8	99.4
	14	98.7
FB1	4	99.9
	8	99.9
	12	99.5
	18	91.5

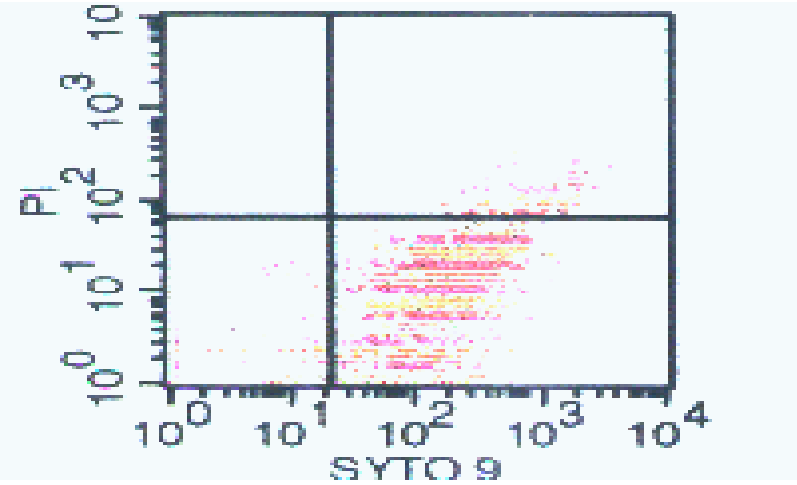

Figure 4.22 Analysis of relative viability of *B.licheniformis* cell suspensions by flow cytometry. Cell samples were taken at 18 h during FB1 cultivation.

4.5 Transport Mechanism

In sections 4.1.7 and 4.3.7, it was illustrated that a sodium/potassium imbalance existed in the system. It is known that, this imbalance is established and maintained by a primary active transport involving Na^+K^+ATPase; and for each molecule of ATP, this transporter moves two K^+ ions inward and three Na^+ ions outward making the inside of the cell membrane negatively charged compared to the outside. However, for all pH and feeding conditions applied, it was observed that the intracellular sodium ion concentration was only slightly less, if not even higher, than intracellular potassium ion concentration. This reveals that, ions other than Na^+ and K^+, such as H^+, should be involved in the transport mechanism of *B.licheniformis*, enabling the pumping of sugar, amino acids, or other related molecules. Hence, intracellular pH change throughout the cultivation should be known, in order to have a better perspective of transport within the system. In addition, higher variation in Na^+ and K^+ concentrations at N-pH_C=6.75 compared to pH_C=6.75 condition, showed that phosphate has activated the transport mechanism of the system.

Considering intracellular and extracellular organic acid content of the system, it was observed that intracellular amounts were usually high compared to extracellular contents. Thus, organic acids should be transferred through the cellular membrane by simple diffusion. Indeed, organic acids are among the most important compounds that are transported by free diffusion.

Transport of a substance across a lipid membrane by simple diffusion involves three steps: (1) Transfer of a substance from the extracellular medium to the membrane phase, (2) molecular diffusion through the lipid membrane, and (3) transfer from the lipid phase to the cytoplasm. Normally, the cytoplasm has physical and chemical properties similar to the properties of the extracellular medium, and steps 1 and 3 are therefore similar. Furthermore, the interphase processes can be assumed to be in equilibrium; i.e., the characteristic time of these processes to equilibrate is much smaller than the characteristic time for the molecular diffusion through the lipid layer. The concentration in the lipid layer at the interphase can therefore be described as the product of the concentration in the water phase and the so-called partitioning coefficient K_{par}, the ratio of the solubility of the compound in the liquid layer to its solubility in

water. Mass flux due to molecular diffusion follows Fick's first law, and the rate of mass transport of a compound into the cell (J, unit: mass per membrane area per time) through a lipid membrane of thickness d_{mem} can therefore be described by

$$J = \frac{D_{mem}}{d_{mem}} K_{par} \left(c_a - c_b \right)$$

D_{mem} is the diffusion coefficient for the compound under consideration in the liquid membrane, c_a and c_b are the concentrations of the compounds in, receptively, the abiotic phase (extracellular medium) and the biotic phase (the cytoplasm). The ratio $D_{mem}K_{par}/d_{mem}$ is called is the permeability coefficient P, and it is frequently used for calculation of the mass transport (Nielsen and Villadsen, 1997).

In Table 4.28, the calculated average permeability coefficients of *Bacillus licheniformis* for all conditions applied was given. In addition, the variation of permeability coefficients with pH and operation mode was illustrated in Appendix I. Permeability coefficients were calculated for the growth phase and production phase of the bioprocess. In the growth phase permeability coefficients were ranging between 2.73 x 10^{-9} – 4.35 x 10^{-6} cm s^{-1}, while in the production phase they were ranging between 0.14 x 10^{-7} – 1.62 x 10^{-6} cm s^{-1}. For both growth phase and production phase, the highest values were obtained for citric acid and succinic acid, and the lowest values were obtained for oxalic and citric acids, respectively. These results revealed that these compounds diffuse quite rapidly. The factors affecting permeability coefficient are known to be the size and ionization of the acid that is to be transported. In their undissociated form, organic acids are practically insoluble in the lipid membrane, whereas the undissociated form of many organic acids is quite soluble. Because it is mainly the undissociated form that diffuses freely, the total transport of these compounds is very sensitive to the degree of dissociation and, hence, the pH of both the cytosol and extracellular medium (Stephanopoulos, Aristidou, Nielsen, 1998). As seen in Table 4.28, the permeability coefficients were affected from the structure of the cell membrane. Hence, the transport of organic acids is rather complex and the structure of the cell membrane was predominant compared to other factors.

Table 4.28 Permeability coefficients for compounds in membranes of *Bacillus licheniformis*.

Compound		Permeability Coefficient (cm s^{-1})	Compound		Permeability Coefficient (cm s^{-1})
Acetic acid	GP	(1.29±1.22) x 10^{-6}	Maleic acid	GP	(6.23±0.00) x 10^{-8}
	PP	(6.92±4.34) x 10^{-7}		PP	(5.07±4.38) x 10^{-7}
Citric acid	GP	(2.46±1.86) x 10^{-6}	Malic acid	GP	(2.36±1.29) x 10^{-6}
	PP	(1.43±1.29) x 10^{-7}		PP	(7.75±4.81) x 10^{-7}
Formic acid	GP	-	Oxalic acid	GP	(4.63±1.90) x 10^{-9}
	PP	(2.30±1.95) x 10^{-7}		PP	(2.43±1.71) x 10^{-7}
Fumaric acid	GP	(7.75±2.10) x 10^{-7}	Pyruvic acid	GP	(1.40±1.06) x 10^{-6}
	PP	(6.00±3.89) x 10^{-7}		PP	(7.81±6.83) x 10^{-7}
Lactic acid	GP	(13.1±2.93) x 10^{-7}	Succsinic acid	GP	(5.31±0.00) x 10^{-7}
	PP	(4.40±2.51) x 10^{-7}		PP	(8.47±7.70) x 10^{-7}
α-ketoglutaric acid	GP	(13.3±7.65) x 10^{-7}			
	PP	(4.45±2.42) x 10^{-7}			

CHAPTER 5

CONCLUSION

In this study, the effects of pH and feeding strategy on β-lactamase production and metabolite profiling of *Bacillus licheniformis* were investigated in a defined medium. For this purpose, first, the effect of pH on cell concentration, enzyme activity, by-product distributions, and sodium, potassium, and ammonium ion concentrations was studied in pilot-scale bioreactor. Next, the reference medium was redesigned in terms of initial glucose and phosphate ion concentrations to increase cell yield and productivity. Finally, using the optimized medium the effects of different feeding profiles on cell generation, substrate consumption, by-product formation, ion concentration, and viability were investigated in the pilot scale bioreactor. In this context the following conclusions were drawn:

1. In order to investigate the effect of pH five different controlled pH, 6.5, 6.75, 7, 7.25, and 7.5, and one uncontrolled pH, 7.5, operations were conducted at oxygen transfer conditions of Q_o/V_R=0.5 vvm, N=500 min^{-1} in pilot scale batch bioreactor. The medium described by Çalık et al. (2005), containing (kg m^{-3}): glucose, 7; $(NH_4)_2HPO_4$, 7.1 was used.

2. For all conditions applied, the cell concentration reached the stationary phase at the end of t=6h, except pH_C=6.75 and pH_{UC}=7.5, where the cells were grown until the end of t=8 h and t=5h respectively. The highest cell concentration was obtained at pH_C = 6.75 as C_x = 0.60 kg/m^3, and the lowest cell concentration was obtained at pH_C = 7.25 as C_x = 0.51 kg/m^3.

3. Glucose with an initial concentration of C_G^0=7.0 kg m^{-3}, decreased throughout the process as expected. At pH_C=6.75, where the cell concentration was the highest, the decrease rate was also highest; whereas, the lowest glucose consumption rate was attained at pH_{UC}=7.5 operation.

4. The highest β-lactamase activity was obtained at pH_{UC}=7.5 (ca. A=57 U/cm^3) condition due to low maintenance requirements. However, among controlled pH operations the highest enzyme activity was obtained at pH_C=6.75 as ca. A=54 U/cm^3, which is only slightly less than that of pH_{UC}=7.5. Therefore, pH_C=6.75 was selected as the optimum pH for β-lactamase production, since controlled operations are usually preferred in industry.

5. At all pH conditions applied, a decrease was observed in dissolved oxygen profiles in the 6h of the processes due to the high metabolic activity of cells; then, C_{DO} increased, in general, until the end of the cultivation time. Controlled pH operations have lower dissolved oxygen values and higher decrease rates.

6. In general the amino acid concentrations in the fermentation broth were low and intracellular amino acid concentrations were higher than extracellular amino acid concentrations. On the whole, aspartic acid and asparagine; methionine, valine, cystein, and tryptophan were the major amino acids excreted extracellularly and intracellularly, respectively. Among all pH conditions, tyrosine concentration was the highest extracellular and intracellular amino acid concentration at pH_C=6.75 t=8h as 0.968 kg m^{-3}, and at pH_C=7.25 t=12 h as 0.250 g g_{cell}^{-1} (6.121 kg m^{-3}), respectively. In addition, the highest total amino acid concentrations excreted extracellularly and intracellularly were obtained at pH_{UC}=7.5 t=0h and pH_C=7.25 t=12h as $T_{AA,\ ext}$= 1.433 kg m^{-3} and $T_{AA,int}$= 0.327 g g_{cell}^{-1} (7.987 kg m^{-3}), respectively.

7. In general the organic acid concentrations in the fermentation broth were low and intracellular amounts were higher than extracellular amounts. All organic acids, except oxaloacetic acid, were present in the fermentation broth. The total organic acid concentration excreted extracellularly and intracellularly were highest at pH_C=6.75 t=14h and pH_C=7.5 t=2h as $T_{OA,\ ext}$= 1.897 kg m^{-3} and $T_{OA,int}$= 0.054 g g_{cell}^{-1} (1.304 kg m^{-3}), respectively. Similarly, for all pH conditions applied, lactic acid concentration was the highest extracellular

organic acid concentration obtained at pH_C =6.75 t=10h as 0.687 kg m^{-3}, while oxalic acid concentration was the highest intracellular amino acid concentration, obtained at pH_C=7.0 t=2 h as 0.028 g g_{cell}^{-1} (0.695 kg m^{-3}).

8. For pH_C=6.75, 7.5 and pH_{UC}=7.5 the extracellular sodium ion concentrations were higher than those of pH_C=6.5, 7.0, and 7, showing an increasing trend with increasing pH in general. On the other hand, for controlled pH operations the extracellular potassium ion concentrations increased significantly with increasing pH, reaching a maximum of 840 ppm at pH_C=7.5, due to the addition of 5M KOH to the system to maintain the pH of the media constant; whereas it was relatively constant at pH_{UC}=7.5.

9. In general, the intracellular sodium and potassium amounts per gram cell were increasing with increasing pH; whereas the sodium amount was almost 0 for pH_{Uc}=7.5, and the potassium amount was almost 0 for pH_C=6.5.

10. Relaying on intracellular and extracellular ion profiles, it was concluded that a sodium/potassium pump exists in the system to maintain the ion gradient across the cell membrane.

11. The profiles of intracellular ammonium ion amounts per gram cell *vs* time were similar for all pH conditions. The amounts per gram cell had maxima in the second hour of the bioprocesses where the metabolic activity of the cell was the highest. Then, it was relatively constant throughout the process. At pH_C=6.5 the ammonium ion amount had its maximum; whereas it was the lowest at pH_{UC}=7.5 and pH_C=7.5

12. K_La and consequently the enhancement factor E varied between 0.0100-0.0311 and 0.98-2.22, respectively; having the highest values at PP of pH_C=7.5 condition. In general, K_La and E are higher at PP than in GP of bioprocceses. The observed increase in K_La, could be the result of the decrease in viscosity of the medium due to

secreted metabolites; and, low enhancement factor E values indicate that the mass transfer rate is much higher than the reaction rate.

13. In general, for all pH conditions applied, oxygen uptake rates were higher at GP of the processes, where the cell formation and the substrate consumption rates, i.e. metabolic activity of the cells, were high.

14. For all operations mass transfer resistances were more effective (Da>>1). Moreover, the effectiveness factor, η, was low indicating that the cells are consuming lower oxygen than the dissolved oxygen. k and k/K_La values were calculated assuming pseudo-steady state first order reaction, as mass transfer resistances are much higher than the reaction rates (Da>>1). According to k/K_La values, operations were found to be limited by mass transfer resistances; and the values were higher at PP than at GP. Comparing Da and k/K_La, it is seen Da values were higher than k/K_La, as Da is defined for maximum utilization and transfer rates, whereas k/K_La was calculated for specific time interval that includes the average but not the maximum values.

15. The specific growth rate, μ, is zero at t=8h in general. At pH=6.75, t=0.5 h, the maximum value of specific growth rate was achieved as $\mu=4.18\ h^{-1}$.

16. Except $pH_C=6.5$, specific substrate utilization rates (q_S), decreased at PP. The highest value of q_S was obtained at $pH_C=6.5$ (PP) as 2.97 kg kg^{-1} h^{-1}; where the lowest value was obtained at $pH_{UC}=7.5$ (PP) as 0.33 kg kg^{-1} h^{-1}.

17. Similarly, specific oxygen uptake rates (q_0) were higher at GP of bioprocesses; reaching the highest value at $pH_C=7.5$ (GP) as 0.454 kg kg^{-1} h^{-1}; and the lowest value at $pH_C=7.5$ (PP) as 0.082 kg kg^{-1} h^{-1}.

18. The highest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at pH_{UC}=7.5 and pH_C=6.5 (PP) as 0.613; 1.189; 11.88 kg kg^{-1}, respectively. In contrast, the lowest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at pH_C=6.75 and pH_C=7.0 (GP) 0.223; 0.638; 1.863 kg kg^{-1}; respectively. Low $Y_{X/O}$ and $Y_{S/O}$ values are due to the inefficient use of the oxygen through biochemical reaction network, increased energy requirement or decrease in the efficiency of energy metabolism with the cultivation time.

19. In the second part, the phosphate ion effect and initial glucose concentration effect with time were investigated in laboratory scale bioreactors in order to increase the enzyme activity and cell growth rate and to determine the feeding strategy for fed-batch processes.

20. The medium containing N (kg/m^3):Na_2HPO_4, 1.0; K_2HPO_4, 1.0, in addition to that of reference media components, yielded in the highest activity value, 119.90 U/cm^3, and therefore was selected as the optimal concentration in terms of phosphate source.

21. Cell concentration and β-lactamase activity were in an increasing trend for minimal amounts of initial glucose with continuous glucose addition. Hence, the initial concentration to be adapted in fed-batch cultivation was determined to be in the range of 2-4 kg/m^3, and from pH_C=6.75 batch operation results this value was selected as 2.5 kg/m^3, since the glucose amount would decrease to its minimal amount before feeding would start. Thus, the medium containing, glucose, 2.5 kg/m^3; Na_2HPO_4, 1.0 kg/m^3; K_2HPO_4, 1.0 kg/m^3 $(NH_4)_2HPO_4$ (kg/m^3), 7.1 and salt solution was accepted as the optimized minimal medium for fed-batch processes.

22. In third part, the fed-batch cultivation with linear and exponential feeding profiles were studied and compared with the pH_C=6.75 batch operation with the optimized medium.

23. For all conditions applied, cell concentrations increased to their optima between 2-6, then reached to their stationary phase. The

highest cell concentration was obtained for FB2 as C_x = 0.54 kg/m^3; whereas the lowest cell concentration was gained at N-pH_C=6.75 as C_x = 0.51kg/m^3.

24. For both fed-batch processes, glucose concentration decreased till the end of the initial batch phase, thereafter a sharp increase was occurred due to the addition of fresh glucose of 320 kg/m^3 to the fermentation broth. Compared with the fed-batch processes, on the other hand, the glucose consumption rate of batch process with optimized medium showed an almost parallel trend with the initial batch phase of fed-batch cultivations. The glucose was consumed more rapidly till t=4h of the process, then was almost stationary for the rest of the process.

25. The highest β-lactamase activity was obtained as A=108 U/cm^3 for FB1 which is 1.6-fold higher than N-pH_C=6.75. Hence, the fed-batch process with exponential feeding was selected as optimal operation type in terms of β-lactamase activity.

26. For all operation types, dissolved oxygen declined for the first five hours of the operation reaching to 50-70% of saturation value due to the high demand of oxygen at the beginning of the processes. Thereafter, dissolved oxygen profiles gradually increased and remained almost stationary until the end of bioprocesses.

27. Similar to previous cases, the amino acid concentrations in the fermentation broth were low. Phenylalanine and asparagine were the major amino acids excreted in common. At all feeding conditions applied, asparagine concentration was the highest extracellular and intracellular amino acid concentration obtained at N-pH_C = 6.75 t=4h as 0.0791 kg m^{-3}, and at FB2 t=14 h as 0.1623 g g_{cell}^{-1} (3.968 kg m^{-3}). Further more, the highest total amino acid concentration excreted extracellularly and intracellularly were found at N-pH_C=6.75 t=14h and FB2 t=2h as $T_{AA,\ ext}$= 0.1604 kg m^{-3} and $T_{AA,int}$= 0.247 g g_{cell}^{-1} (6.029 kg m^{-3}), respectively.

28. At all conditions applied, except oxaloacetic acid, all organic acids were excreted extracellularly and intracellularly; whereas acetic acid, fumaric acid, lactic acid, α-ketoglutaric acid and succinic acid were expelled in trace amounts and hence not presented among intracellular components. For all pH conditions applied, lactic acid concentration was the highest extracellular organic acid concentration obtained at FB2 t=18h as 0.945 kg m^{-3}, while citric acid concentration was the highest intracellular amino acid concentration, obtained at FB2 t=2 h as 0.044 g g_{cell}^{-1} (1.087 kg m^{-3}). The total organic acid concentration excreted extracellularly and intracellularly were highest at FB2 t=18h and N-pH_C=6.75 t=2h as $T_{OA,\ ext}$= 1.698 kg m^{-3} and $T_{OA,int}$= 0.076 gg_{cell}^{-1} (1.835 kgm^{-3}), respectively. In contrast, the minimum total amounts were obtained at N-pH_C= 6.75 t=2 h and FB1 t=6 h as $T_{OA,\ ext}$= 0.125 kg m^{-3} and $T_{OA,int}$= 0.004 g g_{cell}^{-1} (0.108 kg m^{-3}), respectively.

29. For FB1 the extracellular sodium ion concentration was ranging between 660-780 ppm; whereas, for N-pH_C=6.75 and FB2 it was ranging between 270-340 ppm. On the other hand, for all operation types, the extracellular potassium ion concentrations increased approximately 1.6-fold through the end of the cultivation reaching about 600 ppm in N-pH_C=6.75 and 800 ppm in fed-batch processes. At N-pH_C=6.75, extracellular sodium and potassium ion concentrations were approximately 3-fold higher than those of at pH_C=6.75.

30. The intracellular sodium amounts per gram cell, however, were decreasing with increasing potassium amounts for fed-batch processes; whereas the higher sodium amounts were conversely ranging with potassium amounts in batch operation type.

31. The ammonium ion amounts had a maxima at the t=2h of the processes due to high specific growth rate of the cells at the first two hours of the cultivations as in previous controlled- and uncontrolled-pH batch operations; and, it was relatively constant till the end of the operations. The highest ammonium amount per gram

cell was obtained in FB2. On the other hand, ammonium amount at pH_C=6.75 was about 3-fold higher than that at N-pH_C=6.75, which should be due to the buffering capacity of phosphate ion that diminished the need for ammonium ion at N-pH_C=6.75.

32. At FB1 condition, the variations of the intracellular and extracellular phosphate concentrations with cultivation time were investigated. Since the phosphate amount at the beginning and end of the process were almost the same, the cells should not be suffering from phosphate deficiency. Moreover, from intracellular and extracellular phosphate amounts, the total amount of phosphate transferred into and out of the cell was calculated, and the result revealed that potassium is transported through the cell in the course of fermentation to maintain the electro-potential gradient across the cell membrane.

33. K_La and E were higher at PP of the processes, changing between 0.0102-0.0186 and 0.99-1.55, respectively. The oxygen transfer rate, on the other hand, decreased in PP of the bioprocesses with decreasing metabolic activity of the cells. K_La, oxygen uptake rate, and E values did not show a similar trend in the initial batch phase of fed-batch processes and batch process, since the initial glucose concentrations were different.

34. For all conditions applied, Da and k/K_La values were higher than 1, except N-pH_C=6.75 PP condition (k/K_La=0.56) limited by chemical reactions, indicating that mass transfer resistance limited the operations. As in previous cases Da values were slightly higher than k/K_La, On the other hand, the effectiveness factor, η, was low throughout the bioprocesses indicating low oxygen consumption.

35. The maximum specific growth rate was achieved at N-pH_C=6.75, t=0.5 h as μ=4.23 h^{-1}, which is slightly higher than pH_C=6.75 operation. Specific substrate utilization rates (q_S), on the other hand, increased at PP except FB2 condition where it was almost the same at both phases. The highest value of q_S was attained at FB1 PP

condition as 5.76 kg kg^{-1} h^{-1}; where the lowest value was obtained at the same operation at GP as 0.85 kg kg^{-1} h^{-1}. Specific oxygen uptake rates (q_0) were higher at GP for all conditions applied. The highest value of q_0 was obtained at FB1 (GP) as 0.470 kg kg^{-1} h^{-1}; where the lowest value was obtained at N-pH_C=6.75 (PP) as 0.080 kg kg^{-1} h^{-1}.

36. The highest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at N-pH_C=6.75 and FB1; FB2; and FB1 (PP) as 0.434; 1.178; 57.19 kg kg^{-1}; and the lowest $Y_{X/S}$, $Y_{X/O}$, and $Y_{S/O}$ values were obtained at FB2; N-pH_C=6.75; and FB1 (GP) as 0.414; 0.708; 1.845 kg kg^{-1}; respectively. The variation in specific growth rate, μ, the specific oxygen uptake rate, q_o, the specific substrate consumption rate, q_s, and the yield coefficients in batch and initial batch phase of fed-batch processes is because of the difference in the initial glucose concentrations.

37. For all pH and feeding conditions applied, ions other than Na^+ and K^+, such as H^+, should be involved in the transport mechanism of *B.licheniformis*, enabling the pumping of sugar, amino acids, or other related molecules, as the intracellular sodium ion concentration was slightly less than intracellular potassium ion concentration. In addition, higher variations in Na^+ and K^+ concentrations at N-pH_C=6.75 compared to pH_C=6.75 condition, showed that phosphate has activated the transport mechanism of the system.

38. Organic acids should be carried across the cytoplasmic membrane by simple diffusion, as the intracellular amounts were higher than extracellular amounts. Moreover, the permeability coefficients of *Bacillus licheniformis* for all conditions were calculated for the growth phase and production phase of the bioprocesses, and found to range between 2.73 x 10^{-9} – 4.35 x 10^{-6} cm s^{-1} in growth phase, and between 0.14 x 10^{-7} – 1.62 x 10^{-6} cm s^{-1} in the production phase. For both growth and production phases, the highest values were obtained for citric acid and succinic acid, and the lowest values

were obtained for oxalic and citric acids, respectively. These results revealed that these compounds diffuse quite rapidly. In addition, the transport of organic acids was rather complex and the structure of the cell membrane was predominant compared to other factors.

39. Finally to investigate the physiological response of culture media, viability of cells was investigated for controlled and uncontrolled pH operations with reference media, and for fed-batch process with exponential feeding. At pH_C=6.75, most of the cells were viable. On the other hand, for FB1 and pH_{UC}=7.5 operations, about 9% of the cells were dead.

REFERENCES

1. Arifoglu, M., 2004. Effects of Bioreactor Operation Parameters on Inracellular reaction Rate Distibution in Beta-Lactamase Production by *Bacillus* Species, MS Thesis, METU, Ankara.

2. Blanch, W. Harvey and Clark S. Douglas, 1996. Biochemical Engineering, M. Dekker, New York.

3. Bailey, J.E. and Ollis D.F., 1986. Biochemical Engineering Fundamentals, 2nd ed., McGraw-Hill Inc., New York.

4. Bamberg, E., Schoner, W., "The Sodium Pump: Structure Mechanism, Hormonal Control and Its Role in Disease" Steinkopff Verlag GmbH & Co., Darmstadt, 1994.

5. Boyaci I. H., 2005. A new approach for determination of enzyme kinetic constants using response surface methodology, Biochemical Engineering Journal, 25 (1), 55-62.

6. Çalık, P., Çalık, G., Özdamar, T.H., 1998. Oxygen Transfer Effects in Serine Alkaline Protease Fermentation by *Bacillus licheniformis*: Use of Citric Acid as the Carbon Source, Enzyme and Microbial Technology, 23(7-8), 451-461.

7. Çalık, P., Çalık, G., Takaç, S., Özdamar, T.H., 1999. Metabolic Flux Analysis for Serine Alkaline Protease Fermentation by *Bacillus licheniformis* in a Defined Medium: Effects of the Oxygen Transfer Rate, Biotechnology and Bioengineering, 64, 151-167.

8. Çalık, P., Çalık, G., Özdamar, T.H., 2000. Oxygen Transfer Strategy and its Regulation Effects in Serine Alkaline Protease Production by *Bacillus licheniformis*, Biotechnology and Bioengineering, 69, 301-311.

9. Çalık, P., Özdamar, T.H., 2001. Effects of Carbon Sources on Metabolic Capacity of *Bacillus Licheniformis* for Serine Alkaline Protease, Neutral Protease and α-Amylase Production, Biochemical Engineering Journal., 8(1), 61-8.

10. Çalık, P., Özdamar, T.H., 2002. Bioreaction Network Flux Analysis for Industrial Microorganisms: A Review, Reviews in Chemical Engineering, 18(6), 553-596.

11. Çalık, P., Bilir, E., Çalık, G., Özdamar, T. H., 2002. Influence of pH Conditions on Metabolic Regulations in Serine Alkaline Protease Production, Enzyme and Microbial Technology, 31(5), 685–697.

12. Çalık, P., Bilir, E., Çalık, G., Özdamar, T.H., 2003a. Bioreactor Operation Parameters as Tools for Metabolic Regulations in Fermentation Processes: Influence of pH Conditions, Chemical Engineering Science, 58, 759-766.

13. Çalık, P., Tomlin, G., Oliver, S.G., Özdamar, T.H., 2003b. Overexpression of Serine Alkaline Protease in Bacillus licheniformis and its Impact on the Metabolic Reaction Network, Enzyme and Microbial Technology, 32(6), 706-720.

14. Çalık, P., Arifoglu, M., Çalık, G., 2005. Oxygen transfer effects in beta-lactamase fermentation, by *Bacillus licheniformis* in a glucose-based defined medium, Journal of Chemical Technology and Biotechnology, in press.

15. Çelik, E., 2003. Bioprocess Design Parameters for Beta-Lactamase Production by *Bacillus* Species, MS Thesis, METU, Ankara.

16. Çelik, E., Çalık, P., 2004. Bioprocess Parameters and Oxygen Transfer Characteristics in β-Lactamase Production by *Bacillus* Species, Biotechnology Progress, 20(2), 491-499.

17. Christiansen, T., Michaelsen, S., Wumpelmann, M., Nielsen, J., 2003. Production of savinase and population viability of *Bacillus clausii* during high-cell-density fed-batch cultivations, Biotechnology and Bioengineering, 30, 344-352.

18. Doran, P., M., 2000. Bioprocess Engineering Principles, Academic Press Ltd., San Diego.

19. Faber, K., 2000. Biotransformations in Organic Chemistry, 4th ed., Springer, Germany.

20. Fogarty, M. William and Kelly, T. Catherine, 1990. Microbial enzymes and biotechnology, 2nd ed., Elsevier Applied Science, London.

21. Godfrey, T. and West, S., 1996. Industrial Enzymology, 2nd ed., Macmillan Press Ltd., London.

22. Goergen, J. L., Engasser, J.M., Debay, F., Levyal, D., 1997. Flow cytometry for the intracellular pH measurement of glutamate producing *Corynebacterium glutamicum*, Journal of Microbiological Methods, 29, 121-127.

23. Hemila, H., Pokkinen, M., Palva, I., 1992. Improving the Production of *E. coli* Beta-Lactamase in *Bacillus subtilis* - The Effect of Glucose, Ph and Temperature on the Production Level, Journal of Biotechnology, 26, 245-256.

24. Hornbæk, T., Dynesen, J., Jakobsen, M., 2002. Use of fluorescence ratio imaging microscopy and flow cytometry for estimation of cell vitality for *Bacillus licheniformis*, FEMS Microbiology Letters, 215, 261-265.

25. Hornbæk, T., Dynesen, J., Jakobsen, M., Nielsen A.K., 2004. Global transcription profiles and intacellular pH regulation measured in *Bacillus licheniformis* upon external pH upshifts, Arch. Microbiology, 182, 467-474.

26. Laskin, A.I. and Lechevalier, H.A., 1973. Handbok of Microbiology, volume 1, CRC Press, USA.

27. Lehninger, A.L., Nelson, D.L., Cox, M.M., 2000. Principles of Biochemistry, 2nd edition, Worth publishers Inc., New York.

28. Kirk, R., E., Othmer, D., F., 1994. Encyclopedia of Chemical Technology, 4th ed., The Interscience Encyclopedia Inc., New York

29. Moser, A., 1988. Bioprocess Technology, Springer-Verlag, New York.

30. Moses, V. and Cape, R.E., 1991. Biotechnology: The Science and The Business, Harwood Academic Publishers, Switzerland.

31. Sargantanis, I.G. and Karim, M.N., 1996. Effect of Oxygen Limitation on Beta-lactamase Production, Biotechnology Progress, 12(6), 786-792.

32. Sargantanis, I.G. and Karim, M.N., 1998. Adaptive Pole Placement Control Algorithm for DO-Control in Beta-lactamase Production, Biotechnology and Bioengineering, 60(1), 1-9.

33. Sargent, M.G., Ghosh, B.K., Lampen, J.O., 1968. Characteristics of Penicillinase Release by Washed Cells of *Bacillus licheniformis*, Journal of Bacteriology, 96(4), 1231-1239.

34. Shuler, M.L. and Kargi, F., 2002. Bioprocess Engineering: Basic Concepts, Prentice Hall Inc., New Jersey

35. Stephanopoulos, G., Aristidou, A., and Nielsen, J., 1998. Metabolic Engineering: Principles and Applications, Academic Press.

36. Tang, X., Cai, T., Wang, P.G., 2003. Synthsis of Beta-Lactamses Activated Nitric Oxide Donors, Bioorganic and Medicinal Chemistry Letters, 13, 1687-1690

37. Truskey, GA, Yuan, F., and Katz, DF, 2003. Transport Phenomena in Biological Systems, Prentice-Hall.

38. Trchounian, A., Zakharyan, E., 2001. K^+ influx by K_{up} in *Escherichia coli* is accompanied by a decrease in H^+ efflux, FEMS Microbiology Letters, 204, 61-64.

39. Wase, D.A.J., Patel, Y.R., 1987. Effects of Changes in Agitation Rate on Steady-State Penicillinase Titers in Continuously-Cultivated *Bacillus cereus*, Journal of Chemical Technology and Biotechnology, 38, 277-282.

40. White J.S. and White D.C., 1997. Source Book of Enzymes, CRC Press, New York.

41. Yılgör, P., 2004. Bioprocess Operation Parameters for Recombinant Benzaldehyde Lyase Production Production, MS Thesis, METU, Ankara.

42. Yamané, T., Shimizu, S., 1984. Fed-batch techniques in microbial processes, Biochemical Eng., Biotech., 30.

APPENDIX A

Calibration of Bacillus *licheniformis* Concentration

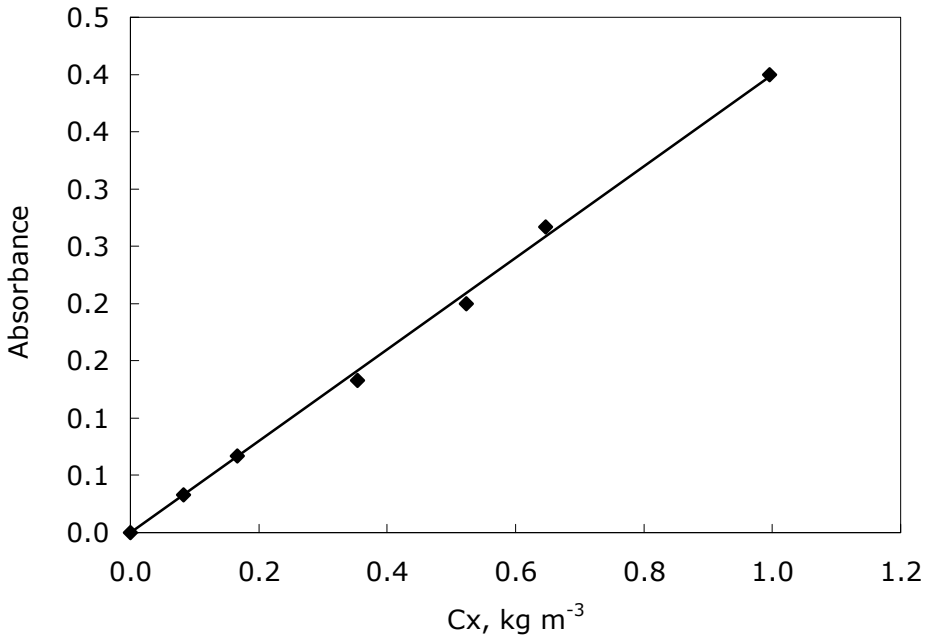

Figure A.1 Calibration curve for *Bacillus licheniformis* concentration

Slope of the calibration curve, m=2.49 1/kg m^{-3} (λ=600 nm)

$$Cx = \frac{Absorbance}{2.49} \times DilutionRatio$$

APPENDIX B

Calibration of Beta-Lactamase Activity

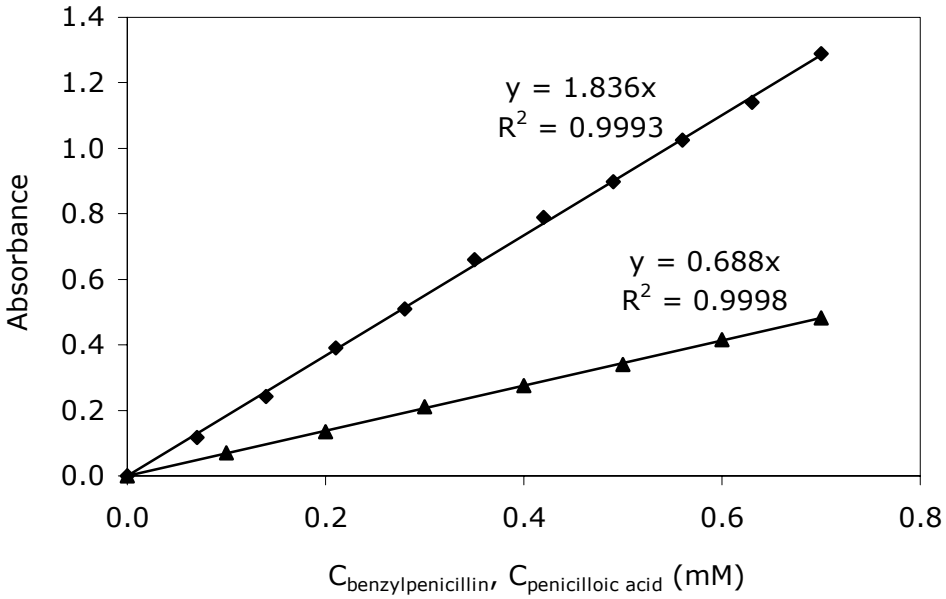

Figure A.2 Calibration Curve of the benzylpenicillin and penicilloic acid in 0.1 M phosphate buffer, pH=7.0, T=30°C, λ=232nm. Benzylpenicillin, (♦); Penicilloic acid, (▲).

One unit of β-lactamase activity was defined as the amount of enzyme that could hydrolyze 1μmol of benzylpenicillin at 30°C and pH 7.0 in one minute. The product of the hydrolysis reaction, penicilloic acid, also gives an absorbance at 232nm, therefore, the difference of the slopes is taken, m_1-m_2=1.148 mM^{-1}. The activity, U cm^{-3} is given by (Çelik, 2003),

$$A = \frac{C_{A0} m_1 - A_\lambda}{(m_1 - m_2) mM^{-1}} x \frac{1U}{10^{-3} mmol} x \frac{1l}{10^3 cm^3} x DilutionRatio$$

APPENDIX C

Preparation of Analysis Solution

1. Dilute the mixture containing glucose oxidase (900 U), peroxidase (500 U), and 4-aminoantiphrine (10mM) to final volume of 100 ml by 150 mM phosphate buffer at pH=7.5 consisting of phenol (4mM) and additives.

2. Mix thoroughly for 1 minute.

Preparation of Standard Glucose Solution

1. Prepare 10 ml 5g/L standard glucose solution using glucose standard and 150 mM phosphate buffer (pH=7.5) consisting of phenol (4mM) and additives.
2. Using this solution, prepare 0.25, 0.50, 0.75, and 1.0 g/L standard solutions.

APPENDIX D

Preparation of Molybdate Solution

1. Dissolve 25.0 g molybdate in 175 ml water.

2. Add 280 ml H_2SO_4 in 400 ml distilled water and cool the mixture.

3. Mix H_2SO_4 solution with molybdate solution to a final concentration of 1 lt.

Preparation of Stannous Chloride Solution

1. Dissolve 2.5 gr $SnCl_2.2H_2O$ in 100 ml water.

APPENDIX E

Calibration of Phosphate Concentration

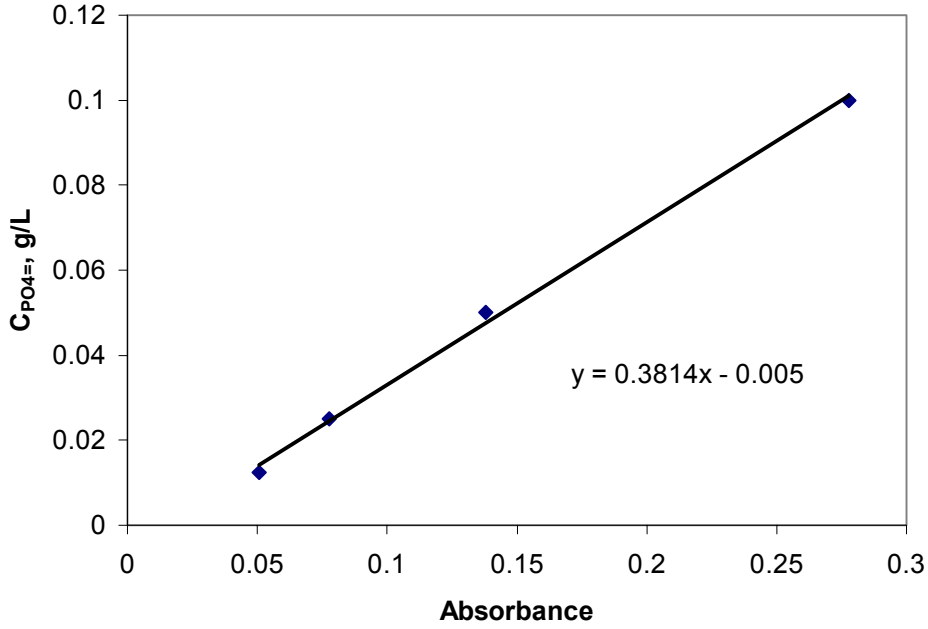

Figure E.3 Calibration Curve of the standard phosphate solution

$$C_{PO_4^{=}} = \left(Absorbance \times 3.938 - 0.3394\right) \times DilutionRatio$$

APPENDIX F

Preparation of Phosphate Standard Solution

1. Dissolve 0.7165 g KH_2PO_4 in 1 L distilled water. The concentration of this standard solution is 0.5 M.

2. Take 100 ml of 5M standard phosphate solution and dilute to the final volume and concentration of 1 L and 0.05M, respectively.

APPENDIX G

Reproducibility of repeated FB1, pH_C=6.75, and pH_{UC}=7.5 experiments

Table G.1 Variation of cell concentration with cultivation time for repeated experiments FB1, pH_C 6.75, pH_{UC} 7.5.

Time (h)	C_X, kg m^{-3}					
	FB1-1	**FB1-2**	**pH_C 6.75-1**	**pH_C 6.75-2**	**pH_{UC} 7.5-1**	**pH_{UC} 7.5-2**
1	0.0518	0.0530	0.0470	0.0494	0.0482	0.0928
2	0.1867	0.1554	0.1506	0.1663	0.1398	0.1795
3	0.3036	0.3530	0.2892	0.3167	0.2759	0.3289
4	0.4289	0.4663	0.4711	0.4940	0.4120	0.4217
5	0.4771	0.5518	-	-	0.5301	0.4795
6	0.5349	0.5518	-	-	0.5301	0.5398

Table G.2 Variation of enzyme activity with cultivation time for repeated experiments FB1, pH_C 6.75, pH_{UC} 7.5.

Time (h)	Activity U cm^{-3}		Time (h)	Activity U cm^{-3}			
	FB1-1	**FB1-2**		**pH_C 6.75 1**	**pH_C 6.75 2**	**pH_{UC} 7.5 1**	**pH_{UC} 7.5 2**
6	26.2	28.7	4	9.2	11.7	12.1	10.4
7.5	45.9	46.9	8	37.8	42.9	38.3	41.5
12	87.7	85.4	14	54.0	55.9	57.7	59.4
18	107.7	104.4	-	-	-	-	-

APPENDIX G (Continued)

Reproducibility of repeated FB1, pH_C=6.75, and pH_{UC}=7.5 experiments

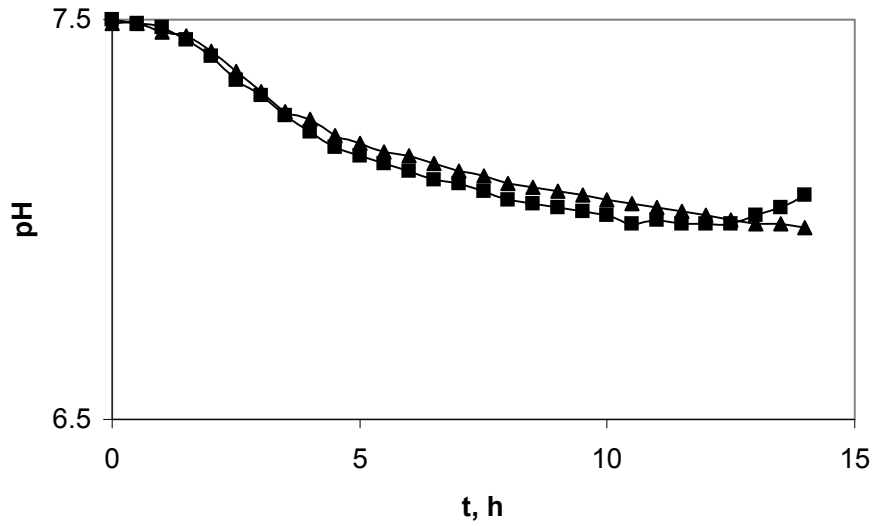

Figure G.1 The variation in pH with cultivation time. C_G^o = 7.5 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}, pH_{UC}=7.5-2, (▲); pH_{UC}=7.5-1, (■).

Appendix H

Table H.1 Variations in intracellular amino acid concentrations with cultivation time at pH_C=6.5 condition.

C 6.5 – Intracellular, C_{AA} ($kg.m^{-3}$)												
Time (h)	Asn	Asp	His	Thr	Ala	Pro	Met	Cys	Lys	Phe	Orn	Trp
0	-	-	-	-	-	0.083	0.872	0.197	-	-	0.165	-
2	-	-	-	-	-	1.167	-	0.622	-	-	-	-
4	-	-	-	-	-	0.198	3.641	0.640	-	-	2.087	-
6	-	-	-	-	-	-	2.409	0.044	-	-	0.084	-
8	-	-	-	-	-	-	2.484	0.489	-	-	0.747	-
10	-	-	-	-	-	-	2.282	0.442	-	-	3.596	-
12	-	-	-	-	-	-	2.306	0.457	-	0.600	-	3.253
14	-	-	-	-	-	-	-	-	-	-	-	-

Table H.2 The variations in intracellular amino acid concentrations with cultivation time at pH_C=6.75 condition.

C 6.75 – Intracellular, C_{AA} ($kg.m^{-3}$)															
Time (h)	Asn	Asp	His	Arg	Ala	Pro	Tyr	Val	Met	Cys	Leu	Lys	Phe	Orn	Trp
0	-	-	-	-	-	-	-	-	2.159	0.515	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	2.089	2.942	-	-	-	-
4	-	-	-	-	-	-	-	-	3.119	0.502	0.803	-	-	-	-
6	-	-	-	-	-	-	0.563	-	3.085	0.565	0.630	-	-	-	-
8	-	-	-	-	-	-	-	-	1.765	0.836	0.495	0.108	-	-	-
10	-	-	-	-	-	-	-	-	3.214	0.810	-	-	-	-	-
12	-	-	-	-	-	-	-	-	2.011	0.644	-	-	-	-	-
14	-	-	-	-	-	-	-	-	2.914	0.865	-	-	-	-	-

Table H.3 The variations in intracellular amino acid concentrations with cultivation time for pH_C=7.0 condition.

C 7 – Intracellular, C_{AA} ($kg.m^{-3}$)											
Time (h)	Asn	Gln	Thr	Val	Met	Cys	Leu	Lys	Phe	Orn	Trp
0	-	0.139	0.142	-	0.675	0.448	-	-	-	0.032	0.219
2	-	-	-	-	-	3.338	-	-	-	0.548	1.968
4	-	-	-	-	2.065	2.790	0.641	-	-	0.108	0.701
6	-	5.138	-	-	0.600	0.831	-	-	-	-	0.348
8	-	-	-	-	1.445	0.819	-	-	-	-	0.423
10	-	-	-	-	1.351	0.842	-	-	-	-	0.421
12	-	-	-	-	0.666	1.086	-	-	-	0.113	0.417
14	-	-	-	-	3.043	1.410	-	-	-	0.221	-

Table H.4 The variations in intracellular amino acid concentrations with cultivation time at pH_C=7.25 condition.

C 7.25 – Intracellular, C_{AA} (kg.m^{-3})												
Time (h)	Asn	Gln	Ala	Tyr	Val	Met	Cys	Ile	Leu	Phe	Orn	Trp
0	-	-	-	0.366	-	0.028	-	-	-	-	-	0.870
2	-	-	-	3.807	-	0.924	-	-	-	-	1.287	-
4	-	3.930	-	1.277	-	0.134	-	0.615	-	-	0.386	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	-	1.731	-	1.016	-	0.148	-	0.648	-	-	0.295	2.253
10	-	2.197	-	1.158	-	0.196	-	0.726	-	-	0.266	2.249
12	-	-	-	6.122	-	-	-	-	1.634	-	0.232	-
14	-	-	-	2.192	-	-	-	0.875	1.052	-	-	2.315

Table H.5 The variations in intracellular amino acid concentrations with cultivation time for pH_C=7.5 condition.

C 7.5 – Intracellular, C_{AA} ($kg.m^{-3}$)												
Time (h)	Asn	Asp	Ser	Thr	Tyr	Val	Met	Cys	Ile	Lys	Phe	Trp
0	-	-	-	-	-	-	-	0.168	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	0.151	-	0.783	-	-	-	-
6	-	-	-	-	-	-	0.108	0.751	-	-	-	-
8	-	-	-	-	-	0.141	-	-	-	-	-	-
10	-	-	-	-	-	-	-	0.942	0.261	-	-	-
12	-	-	-	-	-	-	-	0.585	-	-	-	-
14	-	-	-	0.026	-	0.179	0.098	0.538	-	-	-	-

Table H.6 The variations in intracellular amino acid concentrations with cultivation time at pH_{UC}=7.5 condition.

UC 7.5 – Intracellular, C_{AA} ($kg.m^{-3}$)															
Time (h)	Asn	Asp	Gla	His	Thr	Ala	Tyr	Val	Met	Cys	Ile	Lys	Phe	Orn	Trp
0	-	-	-	-	-	0.045	-	0.093	0.080	0.051	-	0.022	-	-	-
2	-	-	-	-	-	-	-	0.892	0.795	0.448	-	-	-	-	-
4	-	-	-	-	-	0.095	-	0.062	0.309	0.227	0.408	-	-	-	-
6	-	-	-	-	-	-	-	0.249	0.334	-	-	-	-	-	-
8	-	-	-	-	-	-	0.137	0.302	0.297	0.192	0.176	-	-	-	-
10	-	-	-	-	-	-	-	-	0.166	0.117	-	-	-	-	-
12	-	-	-	-	-	-	-	0.284	0.189	0.073	-	-	-	-	-
14	-	-	-	-	-	-	-	0.216	0.313	-	-	-	-	-	-

Table H.7 The variations in intracellular organic acid concentrations with cultivation time at pH_C=6.5 condition.

C 6.5 – Intracellular, C_{OA} (kg.m^{-3})												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	0.036	-	0.004	0.022	0.004	-	0.071	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	0.132	-	-	-	-	0.038	0.207	-	-	-
6	-	-	-	-	-	-	-	-	0.117	-	-	-
8	-	-	-	-	-	-	0.012	-	0.175	-	-	-
10	-	-	-	-	-	-	0.012	0.024	0.118	-	-	-
12	-	-	-	-	-	-	-	-	0.106	-	-	-
14	-	-	-	-	0.013	0.065	-	-	0.222	-	-	-

Table H.8 The variations in intracellular organic acid concentrations with cultivation time at pH_C=6.75 condition.

C 6.75 – Intracellular, C_{OA} (kg.m^{-3})												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	0.033	-	-	-	-	0.011	0.055	-	0.011	-
2	-	-	0.252	-	-	-	-	0.151	0.505	-	0.101	-
4	-	-	0.251	-	-	-	-	0.028	0.084	-	0.014	-
6	-	-	0.079	-	-	-	-	0.013	0.053	-	-	-
8	-	-	0.108	-	-	-	-	0.202	0.135	-	-	-
10	-	-	0.068	-	-	-	-	0.027	0.068	-	0.027	-
12	-	-	0.103	-	-	-	-	-	0.171	-	-	-
14	-	-	0.109	-	-	-	-	-	0.065	-	-	-

Table H.9 The variations in intracellular organic acid concentrations with cultivation time at pH_C=7.0 condition.

C 7.0 – Intracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	-	-	-	-	-	-	0.060	-	-	-
2	-	-	-	-	-	-	-	-	0.695	-	-	-
4	-	-	-	-	-	-	-	0.031	0.252	-	-	-
6	-	-	-	-	-	-	-	-	0.166	-	-	-
8	-	-	-	-	-	-	-	0.017	0.182	-	-	-
10	-	-	-	-	-	-	-	0.046	0.094	-	-	-
12	-	-	-	-	-	-	-	0.017	0.088	-	-	-
14	-	-	-	-	0.014	-	-	-	0.244	-	-	-

Table H.10 The variations in intracellular organic acid concentrations with cultivation time at pH_C=7.25 condition.

C 7.25 – Intracellular, C_{OA} (kg.m^{-3})												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	0.050	-	-	0.031	-	-	0.086	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	0.132	-	-	0.076	-	-	0.188	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	0.125	-	-	-	-	-	0.091	-	-	-
14	-	-	-	-	-	0.052	-	-	0.114	-	-	-

Table H.11 The variations in intracellular organic acid concentrations with cultivation time at pH_C=7.5 condition.

C 7.5 – Intracellular, C_{OA} (kg.m^{-3})												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	0.039	0.251	-	0.058	0.918	-	0.039	-
4	-	-	-	-	-	-	-	-	0.442	-	-	-
6	-	-	-	-	-	0.051	-	-	0.224	-	-	-
8	-	-	0.119	-	-	-	-	-	0.131	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	0.100	-	0.019	0.108	-	-	0.199	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-

Table H.12 The variations in intracellular organic acid concentrations with cultivation time for pH_{UC}=7.5 condition.

UC 7.5 – Intracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	0.642	-	-	-	-	-	0.226	-	-	-
4	-	-	0.104	-	-	-	-	0.016	0.110	-	-	-
6	-	-	0.083	-	-	-	-	-	0.114	-	-	-
8	-	-	0.081	-	-	-	-	-	0.255	-	-	-
10	-	-	0.089	-	-	-	-	0.024	0.129	-	-	-
12	-	-	0.114	-	-	-	-	-	0.061	-	-	-
14	-	-	0.117	-	-	-	-	0.032	0.110	-	-	-

Table H.13 The variations in intracellular amino acid concentrations with cultivation time for N-pH_C=6.75

N 6.75 – Intracellular, C_{AA} (kg.m^{-3})													
Time (h)	Asn	His	Thr	Ala	Pro	Tyr	Val	Met	Cys	Lys	Phe	Orn	Trp
0	0.039	-	-	-	-	-	-	-	0.004	-	0.007	0.039	-
2	0.150	-	-	-	0.079	0.277	-	-	0.069	-	0.088	0.150	-
4	0.062	-	-	-	0.021	-	-	-	-	0.035	0.021	0.062	-
6	0.099	-	-	-	0.022	-	-	-	-	0.029	-	0.099	-
8	-	-	-	-	0.016	-	-	-	-	0.030	0.018	-	-
10	-	-	-	-	0.017	-	-	-	-	0.023	0.014	-	-
12	0.152	-	-	-	0.025	0.102	-	0.138	0.019	-	0.033	0.152	-
14	0.165	-	-	-	0.020	0.107	-	0.206	0.016	0.018	0.278	0.165	-

Table H.14 The variations in intracellular amino acid concentrations with cultivation time at FB1 condition

FB1 – Intracellular, C_{AA} (kg.m^{-3})															
t, h	Asn	Gla	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys	Ile	Lys	Phe	Trp
0	0.450	-	-	-	0.205	-	0.005	0.897	-	0.041	-	-	0.016	0.019	-
2	3.739	-	-	0.069	-	-	0.045	0.342	-	0.235	-	-	0.146	0.040	-
4	1.573	-	-	-	0.257	-	-	-	-	0.281	-	-	0.037	0.396	-
6	1.416	-	-	-	-	-	-	0.270	-	0.093	-	-	0.033	0.257	-
7.5	0.881	-	-	-	-	-	0.017	0.210	-	0.102	-	-	0.033	0.264	-
12	1.264	-	-	-	-	-	0.017	0.277	-	0.070	-	-	0.047	0.364	-
14	1.100	-	-	-	-	-	0.023	0.167	-	0.119	-	-	0.055	0.419	-
16	1.400	-	-	-	-	-	0.013	0.323	-	0.159	-	-	0.031	0.312	-
18	0.267	-	-	-	-	-	0.019	0.058	-	0.041	-	-	0.036	0.231	-

Table H.15 The variations in intracellular amino acid concentrations with cultivation time at FB2 operation.

FB2 – Intracellular, C_{AA} (kg.m^{-3})																
t, h	Asn	Gla	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys	Ile	Lys	Phe	Trp
0	0.029	-	-	-	-	-	-	-	-	-	-	-	-	-	0.029	-
2	3.968	-	-	-	0.092	-	-	0.073	1.251	-	0.523	-	-	0.122	-	-
4	0.285	-	-	-	-	-	-	-	-	-	0.141	-	-	-	0.285	-
7.5	0.144	-	-	-	-	-	-	-	-	-	-	0.036	-	-	0.144	-
9	0.067	-	-	-	-	-	-	-	-	-	-	-	-	-	0.067	-
12	-	-	-	-	-	-	-	0.019	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	0.015	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	0.022	-	-	-	0.040	-	-	-	-
18	-	-	-	-	-	-	-	0.012	-	-	-	-	-	-	-	-

Table H.16 The variations in intracellular organic acid concentrations with cultivation time for N-pH_C=6.75

N 6.75 – Intracellular, C_{OA} ($kg.m^{-3}$)												
Time (h)	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	0.602	0.055	-	-	-	0.018	0.087	-	-	-	-
2	-	-	0.484	-	-	-	0.014	0.616	0.625	-	0.096	-
4	-	0.903	0.174	-	-	-	0.018	0.170	0.261	-	-	-
6	-	0.644	0.179	-	-	-	0.018	0.138	0.207	-	-	-
8	-	0.628	0.173	-	-	-	0.014	0.132	0.200	-	-	-
10	-	0.321	0.156	-	-	-	0.018	0.092	0.206	-	-	-
12	-	0.371	0.160	-	-	-	0.014	0.110	0.197	-	0.018	-
14	-	-	0.189	-	-	-	0.018	0.065	0.208	-	-	-

Table H.17 The variations in intracellular organic acid concentrations with cultivation time at FB1 operation.

FB1 – Intracellular, C_{OA} ($kg.m^{-3}$)												
t, h	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	-	-	-	-	-	-	-	0.053	-	-	-
2	-	-	0.829	-	-	-	-	-	0.532	-	-	-
4	-	-	0.364	-	-	-	-	-	0.121	-	-	-
6	-	-	-	-	-	-	-	-	0.108	-	-	-
7.5	-	-	0.311	-	-	-	-	-	0.083	-	-	-
12	-	-	0.135	-	-	-	-	-	0.177	-	-	-
14	-	-	0.152	-	-	-	-	-	0.213	-	-	-
16	-	-	0.144	-	-	-	-	-	0.199	-	-	-
18	-	-	0.139	-	-	-	-	-	0.199	-	-	-

Table H.18 The variations in intracellular organic acid concentrations with cultivation time at FB2 operation.

FB2 – Intracellular, C_{OA} (kg.m^{-3})												
t, h	Ac	Cit	Form	Fum	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc
0	-	0.407	0.060	-	-	-	0.019	0.060	0.032	-	-	-
2	-	1.087	0.483	-	-	-	0.019	0.334	0.334	-	-	-
4	-	0.901	0.176	-	-	-	0.028	0.153	0.111	-	-	-
6	-	0.634	0.178	-	-	-	0.023	0.137	0.068	-	-	-
7.5	-	0.386	0.184	-	-	-	0.019	0.094	0.104	-	-	-
9	-	0.265	0.187	-	-	-	0.018	0.069	0.069	-	-	-
12	-	0.219	0.150	-	-	-	0.014	0.041	0.068	-	-	-
14	-	-	0.164	-	-	-	0.014	0.041	0.068	-	-	-
16	-	-	0.160	-	-	-	0.014	0.037	0.073	-	-	-
18	-	0.230	0.133	-	-	-	0.014	0.060	0.069	-	-	-

Table I.1 Variations in permeability coefficients for compounds in membranes of *Bacillus licheniformis* with pH and operation mode.

Organic Acids	Conditions								
	pH_C: 6.5	pH_C: 6.75	pH_c: 7.0	pH_C: 7.25	pH_C: 7.5	pH_{UC}: 7.5	N-pH_C: 6.75	FB1	FB2
Acetic acid	8.43×10^{-7}	4.05×10^{-7}	1.10×10^{-6}	7.63×10^{-7}	5.21×10^{-7}	8.02×10^{-7}	5.40×10^{-7}	6.14×10^{-7}	3.45×10^{-7}
Citric acid	6.09×10^{-7}	2.19×10^{-6}	1.68×10^{-6}	2.81×10^{-6}	1.43×10^{-7}	-	3.40×10^{-8}	-	-
Formic acid	4.22×10^{-7}	5.47×10^{-8}	4.25×10^{-7}	8.58×10^{-8}	2.13×10^{-7}	-	1.28×10^{-7}	4.07×10^{-7}	1.07×10^{-7}
Fumaric acid	4.21×10^{-7}	3.64×10^{-7}	9.88×10^{-7}	6.61×10^{-7}	-	6.40×10^{-7}	6.71×10^{-7}	1.61×10^{-6}	5.69×10^{-7}
α-ketoglutaric acid	8.54×10^{-7}	6.29×10^{-7}	1.16×10^{-6}	5.59×10^{-7}	7.14×10^{-7}	7.70×10^{-7}	7.67×10^{-7}	8.69×10^{-7}	5.82×10^{-7}
Lactic acid	8.54×10^{-7}	6.27×10^{-7}	7.69×10^{-7}	5.50×10^{-7}	7.91×10^{-7}	7.60×10^{-7}	8.03×10^{-7}	7.45×10^{-7}	6.32×10^{-7}
Maleic acid	9.24×10^{-7}	-	-	-	4.84×10^{-7}	6.40×10^{-7}	6.56×10^{-8}	6.60×10^{-7}	2.64×10^{-7}
Malic acid	1.10×10^{-6}	1.25×10^{-7}	8.68×10^{-7}	1.16×10^{-6}	5.91×10^{-7}	1.68×10^{-6}	4.97×10^{-8}	1.09×10^{-6}	2.06×10^{-7}
Oxalic acid	-	7.43×10^{-9}	1.32×10^{-7}	1.22×10^{-6}	5.24×10^{-7}	4.19×10^{-7}	3.90×10^{-9}	4.14×10^{-7}	1.05×10^{-8}
Pyruvic acid	1.68×10^{-6}	1.93×10^{-8}	8.39×10^{-7}	1.57×10^{-6}	7.19×10^{-7}	-	4.21×10^{-7}	1.37×10^{-6}	8.09×10^{-7}
Succsinic acid	5.31×10^{-7}	7.70×10^{-8}	-	9.88×10^{-7}	2.84×10^{-7}	-	1.38×10^{-6}	1.51×10^{-6}	-