BIOPROCESS DESIGN PARAMETERS FOR BETA-LACTAMASE PRODUCTION BY *Bacillus* SPECIES

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ABSTRACT

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In this study, the effects of bioprocess design parameters on β-lactamase production were systematically investigated using wild type *Bacillus* species. For this purpose, the research programme was carried out in mainly four parts. Initially, potential β-lactamase producers were screened and *Bacillus licheniformis* ATCC 25972, a constitutive β-lactamase producer, was selected. Next, the effects of bioprocess medium components, i.e., carbon sources (glucose, fructose, sucrose, citric acid and glycerol), inorganic nitrogen sources ($(NH_4)_2HPO_4$ and NH_4Cl) and organic nitrogen sources (yeast extract, peptone and casamino acids), were investigated in agitation and heating rate controlled laboratory scale bioreactors. Thereafter, by using the designed medium, the effects of bioprocess operation parameters, i.e., pH and temperature, on β-lactamase activity were investigated in order to achieve a higher β-lactamase

production. Among the investigated bioprocess conditions, the highest β-lactamase activity was obtained as 275 U cm^{-3}, in the medium with 10.0 kg m^{-3} glucose, 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} yeast extract and the salt solution, at pH_0=6.0, T=32°C, N=200 min^{-1}, which was 7.9 fold higher than the activity obtained in the reference medium. Finally, using the optimum bioprocess parameters obtained in laboratory scale experiments, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in 3.0 dm^3 pilot scale bioreactor, having temperature, pH, foam and stirring rate controls, at Q_0/V=0.5 vvm and N=500 min^{-1} oxygen transfer conditions. The variations in β-lactamase activity, cell, glucose, amino acid and organic acid concentrations with the cultivation time; the oxygen uptake rate and the liquid phase mass transfer coefficient values were determined. Throughout the bioprocess, overall oxygen transfer coefficient (K_La) varied between 0.008-0.016 s^{-1}; oxygen uptake rate varied between 0.001-0.003 mol m^{-3} s^{-1}. Furthermore, rate limiting step analysis was performed; the yield and maintenance coefficients for the bioprocess as well as the kinetic parameters for β-lactamase were determined.

Keywords: β-lactamase, production, *Bacillus*, medium design, bioprocess operation parameters, oxygen transfer

ÖZ

Bacillus TÜRLERİ İLE BETA-LAKTAMAZ ÜRETİMİ İÇİN BİYOPROSES TASARIM PARAMETRELERİNİN BELİRLENMESİ

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Bu çalışmada, doğal *Bacillus* türleri kullanılarak, biyoproses tasarım parametrelerinin β-laktamaz üretimine etkileri sistematik olarak incelenmiştir. Bu amaçla, araştırma programı başlıca dört bölümde yürütülmüştür. Öncelikle, β-laktamaz üretim potansiyeline sahip mikroorganizmalar taranmış ve β-laktamaz üretimi için biyoproses tasarım parametrelerinin incelenmesi amacıyla *Bacillus licheniformis* ATCC 25972 seçilmiştir. Üretim ortamı, karbon kaynaklarının (glikoz, fruktoz, sukroz, sitrik asit ve gliserol), inorganik azot ($(NH_4)_2HPO_4$ ve NH_4Cl) ve organik azot kaynaklarının (maya özütü, pepton ve kazamino asitler) etkisi araştırılarak tasarlanmıştır. Tasarlanan üretim ortamı kullanılarak, biyoproses işletim parametrelerinden pH ve sıcaklığın β-laktamaz üretimi üzerine etkisi incelenmiştir. İncelenen biyoproses koşullarında, en yüksek aktivite, 10.0 kg m^{-3} glikoz, 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} maya özütü ve tuz çözeltisini

içeren ortamda, pH_0=6.0, T=32°C, N=200 min^{-1} koşullarında, 275 U cm^{-3} olarak elde edilmiştir. Bu aktivite, referans ortamda elde edilen aktivitenin 7.9 katıdır. Son olarak, laboratuvar ölçekte bulunan en uygun koşullarda, fermantasyon ve oksijen aktarımı karakteristikleri, 3.0 dm^3 hacimli, sıcaklık, pH, köpük ve karıştırma hızı kontrollu, pilot-ölçek biyoreaktörde, Q_0/V=0.5 vvm ve N=500 min^{-1} oksijen aktarımı koşullarında, araştırılmıştır. β-laktamaz aktivitesi, hücre, glikoz, amino asit ve organik asit konsantrasyonlarının zamanla değişimi; oksijen tüketim hızı ve sıvı faz kütle aktarım katsayısı bulunmuştur. Biyoproses süresince, kütle aktarım katsayısı (K_La), 0.008-0.016 s^{-1} arasında; oksijen tüketim hızı ise 0.001-0.003 mol $m^{-3}s^{-1}$ arasında değişmiştir. Ayrıca, hız kısıtlayıcı basamak analizi gerçekleştirilmiş; verim ve yaşam katsayıları ile β-laktamaz enzimi için kinetik parametreler belirlenmiştir.

Anahtar Kelimeler: β-laktamaz, üretim, *Bacillus*, ortam tasarımı, biyoproses işletim parametreleri, oksijen aktarımı

To My Family

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NOMENCLATURE

A	Beta-lactamase activity, U cm^{-3}
A_λ	Absorbance
C_{OA}	Organic acid concentration, kg m^{-3}
C_{AA}	Amino 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_G	Glucose concentration, kg m^{-3}
C_G^0	Initial glucose concentration, kg m^{-3}
C_N	Nitrogen concentration, kg m^{-3}
C_P	Product concentration, kg m^{-3}
C_S	Concentration of the substrate, mM; kg m^{-3}
C_X	Cell concentration, kg dry cell m^{-3}
C_{YE}^0	Initial yeast concentration, kg m^{-3}
Da	Damköhler number (=OD / OTR_{max} ; Maximum possible oxygen utilization rate per maximum mass transfer rate)
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
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, mM
N	Agitation rate, min^{-1}
m_0	Rate of oxygen consumption for maintenance, kg oxygen kg^{-1} dry cell weight h^{-1}
m_S	Maintenance coefficients for substrate, kg substrate kg^{-1} dry cell weight h^{-1}
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, mol m^{-3} s^{-1}
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_P	Product formation rate, kg m^{-3} h^{-1}
r_X	Rate of cell growth, kg m^{-3} h^{-1}
T	Bioreaction medium temperature, °C
t	Bioreactor cultivation time, h
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}
$Y_{P/X}$	Yield of product on cell, kg kg^{-1}
$Y_{P/S}$	Yield of product on substrate, kg kg^{-1}
$Y_{P/O}$	Yield of product 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
Arg	Arginine
Ala	Alanine
Asn	Asparagine
Asp	Aspartic acid

ATCC	American Type Culture Collection
BGSC	*Bacillus* Genetic Stock Center
But	Butyric acid
Cys	Cysteine
DNS	Dinitrosalicilyc acid
DO	Dissolved oxygen
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EC	Enzyme Commission
Gln	Glutamine
Glu	Glutamic acid
His	Histidine
Iso	Isoleucine
Lac	Lactic acid
Leu	Leucine
NRLL	Northern Regional Research Center
Phe	Phenylalanine
Pro	Proline
OA	Oxaloacetic acid
OD	Oxygen demand ($=\mu_{max} C_X / Y_{X/O}$; mol m^{-3} s^{-1})
OUR	Oxygen uptake rate, 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})
Ser	Serine
TCA	Tricarboxylic acid
Thr	Threonine
Tyr	Tyrosine
Val	Valine

CHAPTER 1

INTRODUCTION

Antibiotics are currently the most important products of microbial biotechnology, apart from such "traditional" products as alcoholic beverages and cheese. Beta-lactams, i.e., penicillins and cephalosporins, are probably the most significant and commonly used class of antibiotics, ever since the discovery of penicillin by A. Fleming in 1928 (Glazer and Nikaido, 1995). Beta-lactamase (EC 3.5.2.6) (also known as penicillinase) enzymes are highly efficient catalysts for a very specific reaction, i.e., the hydrolytic cleavage of the β-lactam ring in penicillins, cephalosporins and some closely related structures. This process renders such materials useless as antibiotics and is a primary defense mechanism of many bacteria (Smyth et al., 2000).

Beta-lactamases make-up 20 % of the $55 million biocatalyst market (out of a total world industrial enzyme market of over $700 million) (Godfrey and West, 1996). They are manufactured for the specific assay of penicillins, destruction of residual 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 β-lactamase dependent prodrugs with the applications in antibody-directed enzyme prodrug therapy (ADEPT) has been an area of

particular interest (Tang et al., 2003). The β-lactamase dependent prodrugs (Smyth et al., 2000) and ADEPT (Senter and Springer, 2001) are reviewed elsewhere.

Beta-lactamases are industrially produced mostly by *Bacillus* species due to their ability to secrete high amounts of enzyme into the extracellular medium. *Bacillus* species, having a transparent genome, being non-pathogenic and free of endotoxins, are widely used in industry.

Besides the presence of the most potential producer, bioprocesses require a closely controlled environment for optimal performance, and this necessity markedly influences their design. Special precaution is necessary because of the labile process-sensitive nature of the biocatalyst, thus avoiding protein denaturation, microorganism inactivation and eventual death.

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. On the other hand, little information is available on the effects of bioprocess design parameters for β-lactamase production.

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), temperature (Bernstein et al., 1967; Kuennen et al.,1980; Hemila et al., 1992) and dissolved oxygen (Sargantanis and Karim, 1996; 1998) on β-lactamase production are reported. However, the above mentioned studies were not intended to attain high enzyme yield and cell growth.

In this study, the effects of bioprocess design parameters on β-lactamase production were systematically investigated. In this context, *Bacillus* species were screened and using the *Bacillus* strain having the highest β-lactamase production capacity, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher β-lactamase production. Thereafter, by using the designed medium, the effects of bioprocess operation parameters, i.e., pH and temperature, on β-lactamase activity were investigated in laboratory scale bioreactors. Finally, using the optimum bioprocess parameters obtained in the previous steps, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in the pilot scale bioreactor.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

2.1.1 General Characteristics

Enzymes are protein and/or RNA molecules that catalyze chemical reactions in biological systems. The main functional characteristics of enzymes compared with chemical catalysts are their high efficiency, their specificity and their capacity for regulation (Matthews, 1997). It is a remarkable paradox that many enzymes display high specificities for a specific type of reaction while accepting a wide variety of substrate structures (Faber, 2000).

The ability to obtain very rapid reaction rates under very mild conditions facilitates the processing of relatively sensitive substrates where extremes of temperature, pH, or pressure would be undesirable (Moses and Cape, 1991). Enzymes do not affect the free-energy change or the equilibrium constant. They lower the activation energy of the reaction catalyzed by binding the substrate and forming an enzyme-substrate complex (Shuler and Kargi, 1992).

Another distinguishing characteristic of enzymes is their frequent need for cofactors. A cofactor is a nonprotein compound which combines with an otherwise inactive protein to give a catalytically active complex. The simplest cofactors are metal ions like Ca^{2+}, Zn^{2+}, Co^{2+}, etc (Bailey, 1986).

Any organism, whether 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).

Enzymes and other proteins produced by the cell tend to be classed into intracellular, membrane or extracellular. Conventionally, extracellular enzymes are defined as those that have crossed the cell membrane (Gacesa and Hubble, 1987).

2.1.2 Classification of Enzymes

At present, about 3000 enzymes have been recognized by the International Union of Biochemistry. As depicted in Table 2.1, enzymes have been classified into six categories according to the type of reaction they can catalyze (Faber, 2000). Each of the major classes is further divided into numerical subclasses and sub-subclasses according to the individual reactions and the nature of substrates involved (Atkinson and Mavituna, 1991). Each enzyme is then assigned an EC (enzyme commission) four-digit classification number and a systematic name, which identifies the reaction catalyzed. 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 cleavage9

2.1.3 Enzyme Activity

The qualitative description of the chemical reactions they catalyse 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:

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

2.1.4 Enzyme Kinetics

The kinetics of most enzyme reactions are reasonably well represented by the Michealis-Menten equation:

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

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).

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 $1/C_S$ (known as a Lineweaver-Burk plot, Figure 2.1) provides a means of determining the parameters K_m and r_{max} from experimental data (Doran, 1995; Atkinson and Mavituna, 1991).

The K_m values of enzymes range widely, but for most industrially used enzymes they lie in the range 10^{-1} to 10^{-5} M when acting on biotechnologically important substrates, under normal reaction conditions. 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).

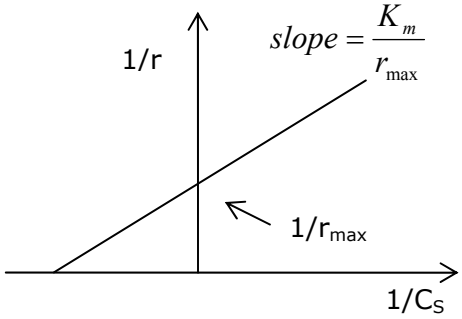
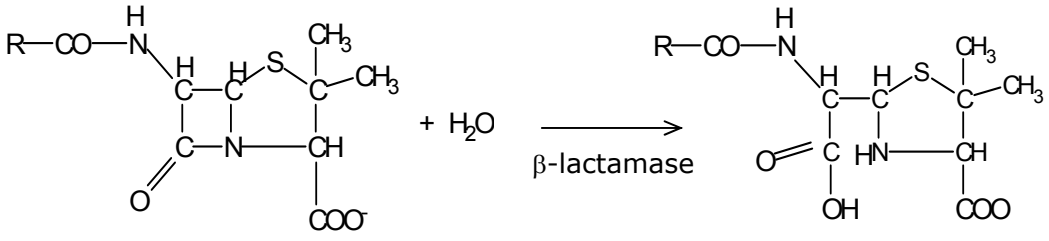


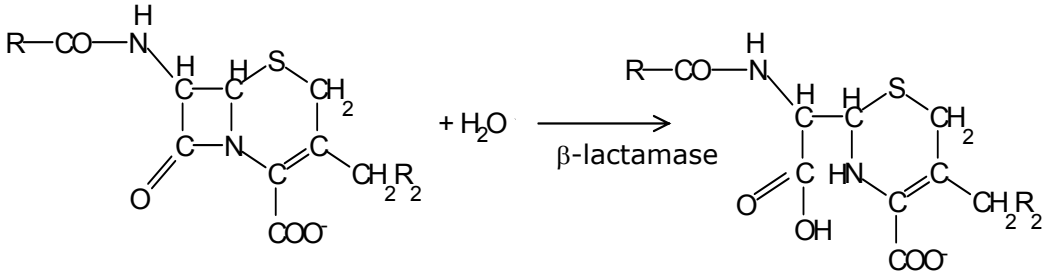
Figure 2.1 Lineweaver-Burk plot.

2.2 Beta-Lactamases

Beta-lactamases (EC 3.5.2.6) are enzymes which catalyse the hydrolysis of β-lactam ring in β-lactam antibiotics, i.e. penicillins and cephalosporins (Figure 2.2). The products of this hydrolysis reaction are antibiotically inactive biomolecules. Therefore, the synthesis of β-lactamases, being the major mechanism of bacterial resistance to β-lactam antibiotics, is observed in a wide range of bacteria in varying amounts. It has also been found in blue-green algae (Kushner and Breuil, 1977) and yeast (Mehta and Nash, 1978). The presence of this enzyme in non-bacterial systems suggests that it may have a more widespread role.



Penicillin Penicilloic acid



Cephalosporin Cephalosporoic acid

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

Gram-positive bacteria generally produce a highly soluble extracellular β-lactamase and a hydrophobic membrane-bound form while containing only a single structural gene for β-lactamases. In the case of gram-negative bacteria, the enzyme synthesized within the cell is secreted into the periplasm region. Furthermore, according to the species, β-lactamase synthesis can be either inducible or constitutive.

Beta-lactamases consist of a single polypeptide chain with the complete absence of cysteine residue. Specifically β-lactamase from *Bacillus licheniformis* consists of 265 amino acids.

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.

Beta-lactamase displays typical Michealis-Menten kinetics. The K_m value for benzylpenicillin varies slightly according to the source of the enzyme, but all reported values lie between 2×10^{-4} M and 4.8×10^{-5} M (Pollock, 1960).

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

2.2.1 Determination of Beta-Lactamase Activity

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).

In the literature, there are five different basic assay techniques used for quantization of β-lactamase activity:

1. Manometric or acidimetric method
2. Iodometric assay
3. Hydroxylamine assay
4. Microbiological assay
5. Spectrophotometric assay

Spectrophotometric assay is a rapid and widely used technique, ideal for following enzyme kinetics and determination of the enzyme activity at various substrate concentrations without losing accuracy and sensitivity (Samuni, 1975).

Among studies using the spectrophotometric assay, a variety of substrates (benzylpenicillin, which is most widely used, nitrocefin or cephalotin), substrate concentrations, buffers (potassium or sodium phosphate), wavelengths (232 nm, which is most widely used, 240 nm or 255 nm) and temperatures (25 or 30°C) were used in β-lactamase assays. Furthermore, there are numerous definitions for one unit of enzyme activity. Therefore, it was difficult to compare the activities obtained in this study with the literature.

In this study, the spectrophotometric assay described by Wase and Patel (1987), was modified and used.

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 (e.g. enzymes, organelles) derived from them, may be termed as a "bioprocess" (Moses and Cape, 1991).

In industrial production of enzymes, microorganisms are mainly utilized for the following basic reasons:

1. Enzyme levels can be increased by environmental and genetic manipulations;
2. Enzyme fermentations are quite economical on a large scale;
3. Screening procedures are simple (Fogarty and Kelly, 1990).

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 design
3. Bioreactor operation parameters
    i. Oxygen transfer rate
        * Air inlet rate (Q_0/ V)
        * Agitation rate (N)
    ii. pH and temperature.

2.3.1 Microorganism

In bioprocesses, the selection of host microorganism for production of industrial enzymes is 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). Traditionally, identification of the most suitable enzyme source involves screening a wide range of candidate microorganisms.

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, 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 rod-shaped bacteria that aerobically form endospores are assigned to the genus *Bacillus*. The endospores of the bacilli are resistant to heat, drying, disinfectants and other destructive agents, and thus may remain viable for centuries.

The genus *Bacillus* encompasses a great diversity of strains. Specifically, *B.subtilis* and *B.licheniformis* assigned to Group 2, produce oval endospores that do not swell the mother cell. They are gram positive, are motile by peritrichous flagella, and produce acids from a range of sugars. They are listed by the

American Food and Drug Administration (FDA) as a GRAS (Generally recognized as Safe) organism. While *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; *B.subtilis*, generally regarded as an aerobe, has pH and temperature tolerance in the range of 5.4-8.0 and 20°C-55°C respectively (Priest, 1993; and Laskin and Lechevalier, 1973).

2.3.1.2 Cell Growth, Kinetics and Yield Factors

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

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

Microbial growth is a good example of an autocatalytic reaction. The rate of growth is directly related to cell concentration, and cellular reproduction is the normal outcome of this reaction. The rate of microbial growth is characterized by the specific growth rate, μ, which is defined as,

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

where C_X is the cell mass concentration (kg m^{-3}), t is time (h), and μ is the specific growth rate (h^{-1}) (Shuler and Kargi, 1992).

Several phases of cell growth are observed in batch culture. Table 2.2 provides a summary of batch cell growth and metabolic activity during the phases of batch culture (Doran, 1995).

Table 2.2 Summary of batch cell growth.

Phase	Description	Specific growth rate
Lag	Cells adapt to the new environment	$\mu \approx 0$
Acceleration	Growth starts	$\mu < \mu_{max}$
Growth	Growth achieves its maximum rate	$\mu \approx \mu_{max}$
Decline	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 growth and decline phases in batch growth, rate of cell growth, r_X, is described by the following equation:

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

Similarly, substrate consumption rate, $-r_S$ and product formation rate, r_P, is described by the following equations respectively:

$$-r_S = \frac{dC_S}{dt} \tag{2.5}$$

$$r_P = \frac{dC_P}{dt} \tag{2.6}$$

To better describe growth kinetics, some stoichiometrically related parameters, namely yield coefficients, are defined. As a general definition,

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \tag{2.7}$$

where, $Y_{P/S}$ is the yield coefficient, P and S are product and substrate, respectively, involved in metabolism. ΔP is the mass or moles of P produced, and ΔS is the mass or moles of S consumed. This definition gives an overall yield representing some sort of average value for the entire culture period. However, in batch processes, the yield coefficients may show variations throughout the process for a given microorganism in a given medium, due to the growth rate and metabolic functions of the microorganism. Therefore, it is sometimes necessary to evaluate the instantaneous yield at a particular point in time. Instantaneous yield can be calculated as follows:

$$Y_{P/S} = -\frac{dP}{dS} = -\frac{dP/dt}{dS/dt} = \frac{r_P}{r_S} \tag{2.8}$$

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

To understand the above mentioned variations in yield coefficients, it is useful to breakdown substrate consumption into three parts: assimilation into cell mass, provision of energy for cell synthesis, and provision of energy for maintenance. Maintenance here refers to the collection of cell energetic requirements for survival, or for preservation of a certain cell state, which are not directly related to or coupled with the synthesis of more cells. Such activities include active transport of ions and other species across cell membranes, and replacement synthesis of decayed cell constituents (Bailey and Ollis, 1986).

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

The maintenance coefficients for oxygen denoted by m_0, is the rate of oxygen consumption for maintenance. Omitting oxygen uptake for product formation, oxygen consumption rate for cell growth, $-r_{01}$, is defined as

$$-r_{01} = \frac{dC_X / dt}{\bar{Y}_{X/0}} \tag{2.9}$$

and oxygen consumption rate for cell maintenance, $-r_{02}$, is defined as

$$-r_{02} = m_0 C_X \tag{2.10}$$

Total oxygen consumption rate, $-r_0$, being defined as,

$$-r_0 = (-r_{01}) + (-r_{02}) \tag{2.11}$$

equations (2.9) and (2.10) are substituted into (2.11) to obtain,

$$-r_0 = (1/\bar{Y}_{X/0})\frac{dC_X}{dt} + m_0 C_X \quad (2.12)$$

Dividing equation (2.12) by C_X,

$$\frac{-r_0}{C_X} = \frac{\mu}{\bar{Y}_{X/0}} + m_0 \quad (2.13)$$

Dividing equation (2.13) by μ,

$$\frac{-r_0}{\mu C_X} = \frac{1}{\bar{Y}_{X/0}} + m_0 \frac{1}{\mu} \quad (2.14)$$

$$\frac{-r_0}{dC_X/dt} = \frac{1}{Y_{X/0}} = \frac{1}{\bar{Y}_{X/0}} + m_0 \frac{1}{\mu} \quad (2.15)$$

From the slope of the plot of $1/Y_{X/0}$ versus $1/\mu$, m_0 (kg oxygen kg^{-1} dry cell weight h^{-1}); and from the intercept, cell yield on oxygen, in the case where oxygen uptake for product formation is omitted, $\bar{Y}_{X/0}$, could be determined.

Similarly, the maintenance coefficients for substrate denoted by m_S could be determined. m_0 and m_S may differ with the change in bioprocess parameters such as, type of microorganism, type of substrate, pH and temperature.

2.3.2 Medium Design

Conceptually, the cells function as semi-batch microbioreactors with volume V wherein the biochemical reactions take place. A number of compounds of the intracellular biochemical reaction network (e.g substrate(s), oxygen, H^+, H_2O, CO_2, amino acids, organic acids of the glycolysis or gluconeogenesis pathways and the tricarboxylic acid (TCA) cycle, and extracellular β-lactamase

enzyme) is exchanged or transferred with facilitated and active transport mechanisms between the metabolic system and the bioreactor medium which is defined as the environment. Furthermore, most of the products formed by organisms are produced as a result of their response to environmental conditions, such as nutrients, growth hormones, and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation (Shuler and Kargi, 1992). Typical components of a fermentation medium are explained below:

1. *Water* is the major component of all fermentation media.
2. *Macronutrients* are needed in concentrations larger than 10^{-4}M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} are major macronutrients.
3. *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.
4. *Buffers* may be necessary to control the pH of the fermentation medium.
5. *Vitamins and growth factors* are required in some fermentation processes.
6. *Inducers* must be present in the medium if the product of interest is an induced enzyme which is synthesized only in response to the presence of an inducer (typically the substrate for the enzyme or a structurally related compound) in the environment.
7. *Antifoams* are surface active agents, reducing the surface tension in the foams. They usually have no metabolic effect but may substantially reduce the oxygen transfer rate.

While Table 2.4 lists the eight major macronutrients, their physiological functions, typical amount required in fermentation broth, and common sources; in Table 2.5 the compositions of commonly utilized proteins in fermentation medium as nitrogen sources are given.

In literature, there is no work on systematic investigation of the effects of medium components for β-lactamase production by *Bacillus* species. Nevertheless, in the study of Hemila et al. (1992), the effect of glucose was investigated for improving β-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

Oxygen transfer, pH, and temperature, which are the major bioreactor operation parameters, show diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999).

2.3.3.1 Temperature

Temperature is one of the most important bioprocess parameters which is normally desired to be kept constant at its optimum value throughout the bioprocess (Nielsen and Villadsen, 1994). It may affect both the growth rate and the product formation; however the optimum temperature for growth and product formation may be different. When temperature is increased above the optimum temperature, the maintenance requirements of cells increase. The yield coefficient is also affected by temperature (Shuler and Kargi, 1992).

Table 2.4 The major macronutrient elements, their physiological functions, growth requirements and common sources.

Element	Physiological Function	Required Concentration (mol l^{-1})	Common Source
Carbon	Constituent of organic cellular material. Often the energy source.	> 10^{-2}	Glucose, sucrose fructose (for defined medium)
Nitrogen	Constituent of proteins, nucleic acids, and coenzymes.	10^{-3}	Ammonia, ammonia salts, proteins, amino acids
Hydrogen	Constituent of organic cellular material and water.		Carbon compounds such as carbohydrates
Oxygen	Constituent of organic cellular material and water. Required for aerobic respiration.		Sparging air or surface aeration
Sulfur	Constituent of proteins and certain coenzymes.	10^{-4}	Sulphate salts
Phosphorus	Constituent of nucleic acids, phospholipids, nucleotides and certain coenzymes.	10^{-4} to 10^{-3}	Inorganic phosphate salts (KH_2PO_4, K_2HPO_4)
Potassium	Principal inorganic cation in the cell and cofactor for some enzymes.	10^{-4} to 10^{-3}	Inorganic potassium salts (KH_2PO_4, K_2HPO_4, K_3PO_4)
Magnesium	Cofactor for many enzymes and chlorophylls and present in the cell walls and membranes.	10^{-4} to 10^{-3}	$MgSO_47H_2O$, $MgCl_2$

Table 2.5 Analysis of microbiological media (Difco Manual, Ninth Edition).

Constituent	Yeast Extract	Peptone	Casamino Acids
Ash %	10.1	3.53	3.64
Total nitrogen %	9.18	16.16	11.15
Ammonia Nitrogen %		0.04	
Free Ammonia Nitrogen%		3.20	
Arginine %	0.78	8.0	3.8
Aspartic acid %	5.1	5.9	0.49
Cysteine %		0.22	
Glutamic acid %	6.5	11.0	5.1
Glycine %	2.4	23.0	1.1
Histidine %	0.94	0.96	2.3
Isoleucine %	2.9	2.0	4.6
Leucine %	3.6	3.5	9.9
Lysine %	4.0	4.3	6.7
Methionine %	0.79	0.83	2.2
Phenylalanine %	2.2	2.3	4.0
Threonine %	3.4	1.6	3.9
Tryptophane %	0.88	0.42	0.8
Tyrosine %	0.60	2.3	1.9
Valine %	3.4	3.2	7.2
Organic sulfur %		0.33	
Inorganic sulfur %		0.29	
Phosphorous %	0.29	0.07	0.35
Potassium %	0.04	0.22	0.88
Sodium %	0.32	1.08	0.77
Magnesium %	0.030	0.056	0.0032
Calcium %	0.040	0.058	0.0025
Chloride %	0.190	0.27	11.2
Manganese (mg/L)	7.8	8.6	7.6
Copper (mg/L)	19.00	17.00	10.00
Zinc (mg/L)	88.00	18.00	8.00
Biotin (µg/gm)	1.4	0.32	0.102
Thiamine (µg/gm)	3.2	0.50	0.12
Riboflavin (µg/gm)	19.00	4.00	0.03

In the 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 a more recent 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.

2.3.3.2 pH

As the phases of a bioprocess are dynamic, and are the consequences of directed functioning of the bioreaction network interacting strongly with the micro-environment of the cell, the influence of the bioreactor operation parameter pH on the bioreaction network is indeed important and needs clarification in order to develop a bioreactor operation strategy. Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level even with large variations in the pH of the extracellular medium, but only at the expense of a significant increase in the maintenance demands, since Gibbs free energy has to be used for maintaining the proton gradient across the cell membrane. Under the influence of the operational conditions pH and oxygen transfer including the designed medium, the cell pumps H^+ ions in or out through the membrane (Çalık et al., 2003a).

In most fermentation processes, 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 Kargi, 1992). 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).

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.

2.3.3.3 Oxygen Transfer

The transfer of oxygen into the microbial cell in aerobic fermentation processes affects product formation by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999). Oxygen transfer rate can be adjusted by either changing the air inlet rate or agitation rate.

Agitation of the fermentation broth also affects other factors such as the assurance of an adequate supply of nutrients to the cells, efficient heat transfer, accurate measurement of specific metabolites in the culture fluid and efficient dispersion of added solutions such as antifoaming agents (Glick and Pasternak,

1998). However, excessive agitation of a fermentation broth can cause shear damaging the cells, increase in temperature and foam formation.

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. An expression for oxygen transfer rate (OTR) from gas to liquid is given by the following equation:

$$OTR = K_L a(C_O^* - C_O) \tag{2.16}$$

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.

The rate at which oxygen is consumed by cells in fermenters determines the rate at which it must be transferred from gas to liquid. Many factors influence oxygen demand; the most important of which are cell species, culture growth phase, and nature of the carbon source in the medium. Oxygen uptake rate (OUR), $-r_0$, per unit volume of broth is given by

$$-r_0 = q_0 C_X \tag{2.17}$$

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). These are bubble characteristics, rheological properties of the medium, air inlet rate, agitation rate, antifoam agents, temperature, and the presence of cells and solutes.

2.3.3.1.1 Measurement of K_La: Dynamic Method

This widely used, simple method can be applied during the fermentation process to determine the value of K_La experimentally. The method is based on an unsteady state mass balance for oxygen given by the following equation:

$$\frac{dC_O}{dt} = K_L a(C_O^* - C_O) - q_0 C_X \tag{2.18}$$

As shown in Figure 2.3, 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 (2.18) becomes:

$$\frac{dC_O}{dt} = r_0 \tag{2.19}$$

Using equation (2.19) in region-II of Figure 2.3, 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 (2.18) is valid. Combining equations (2.17) and (2.18) and rearranging,

$$C_O = -\frac{1}{K_L a}(\frac{dC_O}{dt} - r_0) + C_O^* \tag{2.20}$$

From the slope of a plot of C_O versus ($dC_O/dt - r_0$), K_La can be determined (Figure 2.4).

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 (2.20)

$$C_O = -\frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \qquad (2.21)$$

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.

The only study on the effect of oxygen transfer conditions for β-lactamase production by *Bacillus* species was conducted 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 reported that the highest productivity of β-lactamase corresponds with 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 literature, there is no work reporting the oxygen transfer characteristics ($-r_0$, $K_L a$) of the process for β-lactamase production by *Bacillus* species.

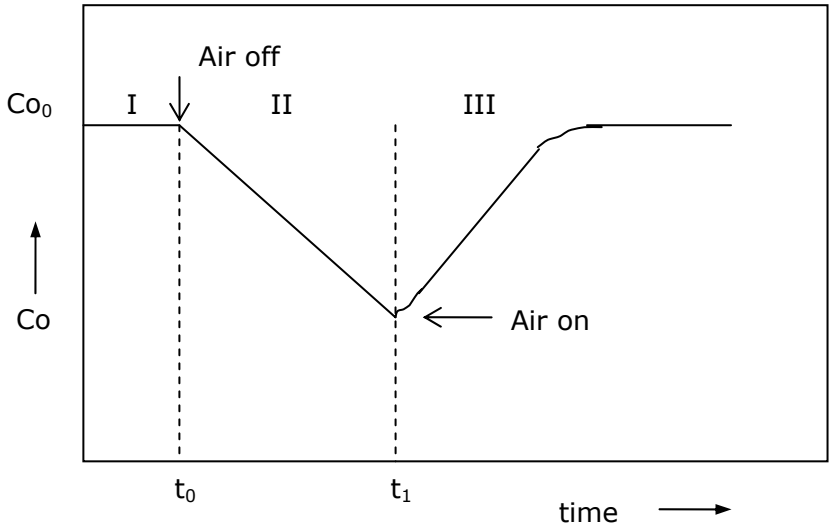


Figure 2.3 Variation of dissolved oxygen concentration with time in dynamic measurement of $K_L a$.

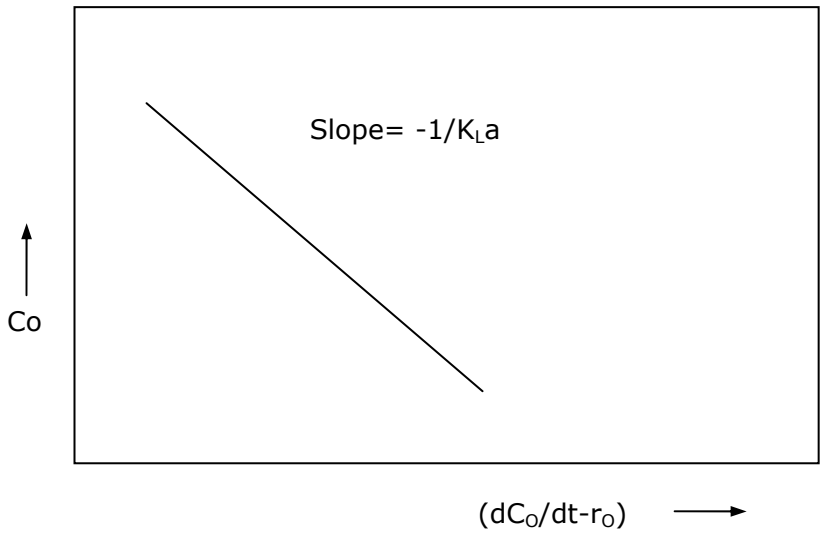


Figure 2.4 Evaluting $K_L a$ using the Dynamic Method.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Benzylpenicillin (penicillin G) was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. 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), *Bacillus licheniformis* (NRRL 978), *Bacillus licheniformis* (NRRL 243), *Bacillus licheniformis* (DSM 1969), *Bacillus subtilis* (NRRL 1125), *Bacillus subtilis* (BGSC 168) were used as the potential producers 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 freshly prepared agar slants under sterile conditions, and were incubated at 30ºC for 24h; thereafter inoculated into the pre-cultivation medium. The composition of the solid medium for β-lactamase production is given in Table 3.1.

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

Compound	Concentration, kg m^{-3}
Soytryptone	5.0
Peptone	5.0
$MnSO_4.2H_2O$	0.01
Agar	15.0

3.4 The Precultivation Medium

Microorganisms grown in the solid medium were inoculated into precultivation medium, and incubated at 37ºC and N=200 min^{-1} for 3h. Experiments were conducted in agitation and heating rate controlled orbital shakers, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. The composition 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 for *Bacillus* sp.

Compound	Concentration, kg m^{-3}
Soytryptone	15.0
Peptone	5.0
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, contained either in the laboratory scale bioreactor (V=150 ml) or pilot scale bioreactor (V=3.0 dm^3), with 1/10 inoculation ratio.

For laboratory scale experiments, air-filtered Erlenmeyer flasks 150 ml in size, that had working volume capacities of 33 ml, were used as bioreactors. Agitation and heating rate controlled orbital shakers, at a cultivation temperature of 37ºC and an agitation rate of 200 min^{-1}, unless otherwise stated, were used as incubators. Details of the pilot scale experiments are given in section 3.6.

The parameters investigated in the production medium for β-lactamase production are given in Table 3.3. The activity values, obtained in the designed medium using the designed bioreactor operation parameters, were compared with the activity of the reference medium in order to determine the enhancement factor. The composition of the reference medium is given in Table 3.4 (Pollock, 1965).

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.

Table 3.3 The investigated parameters for β-lactamase production.

Medium Components	Bioreactor Design Parameters
Glucose	pH
Fructose	Temperature
Sucrose	Oxygen Transfer Rate
Citric acid	
Glycerol	
$(NH_4)_2HPO_4$	
NH_4Cl	
Yeast extract	
Casamino acids	
Peptone	

Table 3.4 The composition of the reference production medium.

Macronutrients	Concentration, kg m^{-3}
Glucose	10.0
Casamino acids	10.0
Na_2HPO_4	2.1
$NaH_2PO_4.2H_2O$	0.8
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}

3.6 The Pilot Scale Bioreactor System

The pilot scale 3.0 dm^3 batch bioreactor (Braun CT2-2), having a working volume of 0.5-2.0 dm^3, and consisting of temperature, pH, foam and stirring rate controls, was used. 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, and consisted of four baffles and a sparger.

3.7 Analysis

Throughout the bioprocess, samples were taken at different cultivation times. After determining the cell concentration, the medium was centrifuged at 13500 min^{-1} for 10 min to separate the cells. Supernatant was used for the determination of β-lactamase activity and glucose concentration. In bioreactor system experiment, besides β-lactamase activity, cell and glucose concentrations; amino acid and organic acid concentrations, oxygen uptake rate and liquid phase mass transfer coefficient values were determined.

3.7.1 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.

3.7.2 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).

3.7.3 Reduced Sugar Concentration

Reduced sugar, glucose, concentration was determined by the DNS (dinitrosalisylic acid) method (Miller, 1959) at 550 nm with a UV spectrophotometer. The calibration curve and the preparation method of the DNS solution are given in Appendix C and D, respectively. The method used in analysis of samples and preparation of the calibration curve is given below:

1. 3 cm^3 of DNS solution was added into test tubes containing 1 cm^3 of sample at different glucose concentrations.
2. The test tubes were put into boiling water for 5 min and then into ice for another 5 min.
3. 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 550 nm.

3.7.4 Amino Acids Concentrations

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

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

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

3.7.5 Organic Acids Concentrations

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

3.7.6 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 2.3.3.1.1, 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.

CHAPTER 4

RESULTS AND DISCUSSION

The research programme for the investigation of the effects of bioprocess design parameters on β-lactamase production was carried out in mainly four parts. The selection of a *Bacillus* strain having the highest β-lactamase production capacity had the primary importance. Next, using the selected microorganism, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher β-lactamase production. Thereafter, by using the designed medium, the effects of bioprocess operation parameters, i.e., pH and temperature, on β-lactamase activity were investigated in laboratory scale bioreactors. Finally, using the optimum bioprocess parameters obtained in the previous steps, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in the pilot scale bioreactor.

4.1 Microorganism Selection

To select the *Bacillus* strain having the highest β-lactamase production capacity, a two stage screening programme was followed.

4.1.1 Microorganism Selection in Solid Medium

Several strains of *Bacillus licheniformis* and *Bacillus subtilis* potentially producing β-lactamase were screened in the solid medium. *B. licheniformis* 749/C and *B. subtilis* NRRL 1125 were selected as potential producers based on susceptibility to increasing amounts of benzylpenicillin added into the solid medium. The selected strains were maintained on agar slants, and stored at 4°C.

4.1.2 Microorganism Selection in Production Medium

After screening potential β-lactamase producers in the solid medium containing benzylpenicillin, an inducible (*B. subtilis* NRRL 1125) and a constitutive (*B. licheniformis* 749/C) β-lactamase producer was selected for further investigation of productivity in liquid medium. However the induction strategy, i.e., the amount of inducer added, had to be studied first, since β-lactamase is synthesized only in response to the presence of an inducer if its coded by an inducible gene.

4.1.2.1 Induction Strategy

Bacillus subtilis NRRL 1125, an inducible microorganism for β-lactamase production, was grown in the reference β-lactamase production medium (Table 3.4) and induced with benzylpenicillin, at the concentrations given in Table 4.1, when the cell concentration was 0.2 kg dry cell m^{-3} (Pollock, 1957). As it is seen in Table 4.1, the highest β-lactamase activity was obtained when the production medium was induced with 1.0 U cm^{-3} of benzylpenicillin.

Table 4.1 Comparison of relative maximum β-lactamase activities with respect to the amount of inducer added to the reference medium.

Amount of inducer, U cm^{-3}	Relative Maximum β-Lactamase Activity
0.5	0.34
1.0	**1.00**
2.0	0.77

4.1.2.2 Selection of the Potential Beta-lactamase Producer

The constitutive β-lactamase producer grown in the reference β-lactamase production medium, had an 1.8 fold higher activity, than the inducible producer grown in the same medium but induced with 1.0 U cm^{-3} of benzylpenicillin. However, when the constitutive β-lactamase producer grown in the reference β-lactamase production medium, was induced with 1.0 U cm^{-3} of benzylpenicillin, no progress in productivity was obtained, as expected. Therefore, the constitutive strain *B. licheniformis* 749/C, was selected as the β-lactamase producer, having 35 U cm^{-3} β-lactamase activity.

4.2 Medium Design

After the selection of the microorganism, a semi-defined medium was designed to optimize β-lactamase production and cell growth. In Table 3.4 the composition of the reference β-lactamase production medium, in which the β-lactamase activity was obtained as 35 U cm^{-3}, is given. However, it was found that, the medium containing 2.0 kg m^{-3} yeast extract and 5.0 kg m^{-3} NH_4Cl, instead of the 10.0 kg m^{-3} casamino acids in the reference medium, had a β-lactamase activity of 55 U cm^{-3}.

Therefore, the medium that contained (kg m^{-3}): glucose, 10.0; NH_4Cl, 5.0; yeast extract, 2.0 and the salt solution, given in Table 3.4, was considered as the starting point for medium design experiments. This medium was named as the GNY medium.

In this context, the effects of the concentrations of carbon sources, i.e., glucose, fructose, sucrose, citric acid and glycerol; inorganic nitrogen sources, i.e., $(NH_4)_2HPO_4$ and NH_4Cl; the organic nitrogen sources, i.e., yeast extract, peptone and casamino acids and their concerted effects, were investigated in agitation (N=200 min^{-1}) and heating rate (T=37°C) controlled laboratory scale bioreactors.

4.2.1 The Effect of Glucose Concentration

Glucose, being easily metabolized by microorganisms, is widely used in fermentation media. The effects of glucose on β-lactamase production were investigated in the GNY medium, at the initial concentrations of $C_G°$=0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 20.0 kg m^{-3}. With the increase in initial glucose concentration, cell concentration increased. On the other hand, β-lactamase activity was the highest at $C_G°$=8.0 kg m^{-3} having a value of A=65 U cm^{-3} at t=35 h (Figure 4.1).

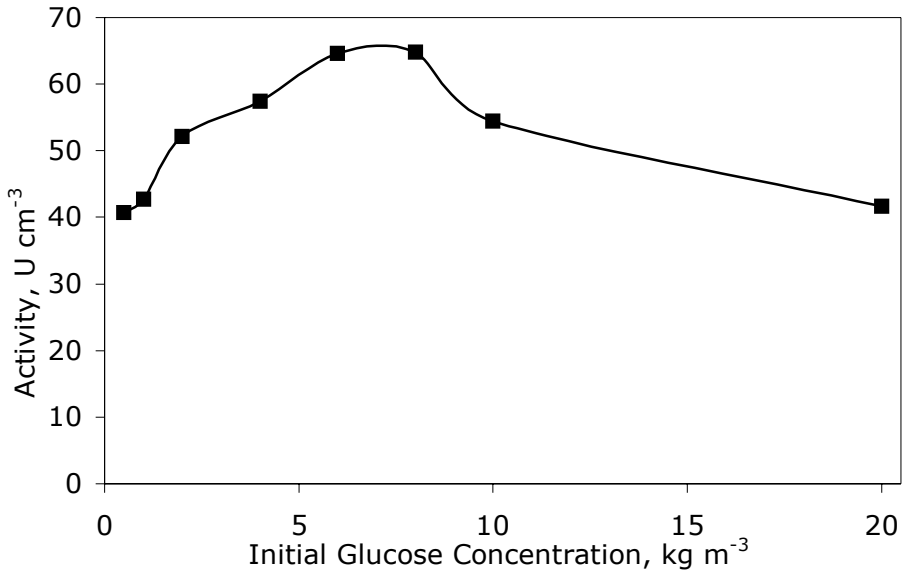


Figure 4.1 The variation in β-lactamase activity with the initial glucose concentration, at t=35 h, V=33 cm^3, T=37°C, pH =7.2, N=200 min^{-1}.

4.2.2 The Effects of Inorganic Nitrogen Sources

To investigate the effect of inorganic nitrogen sources, yeast extract was omitted from the GNY medium. Since $C_G°$ = 8.0 kg m^{-3} was found to be the optimum for β-lactamase activity, glucose at $C_G°$ = 8.0 kg m^{-3} was used as the carbon source and the effects of $(NH_4)_2HPO_4$ and NH_4Cl were investigated within the range corresponding to nitrogen concentration of 0.25 – 4.0 kg m^{-3}. It was observed that, lower nitrogen content was preferable for higher β-lactamase activity. In the presence of $(NH_4)_2HPO_4$, twice the activity was obtained compared to the medium containing NH_4Cl, as shown in Figure 4.2. This was either due to the reported induction effect of PO_4 (Collins, 1979), or the inhibition effect of Cl^-. Furthermore, as can be seen from Figure 4.2, higher nitrogen content had an inhibitory effect on both cell concentration and β-lactamase activity.

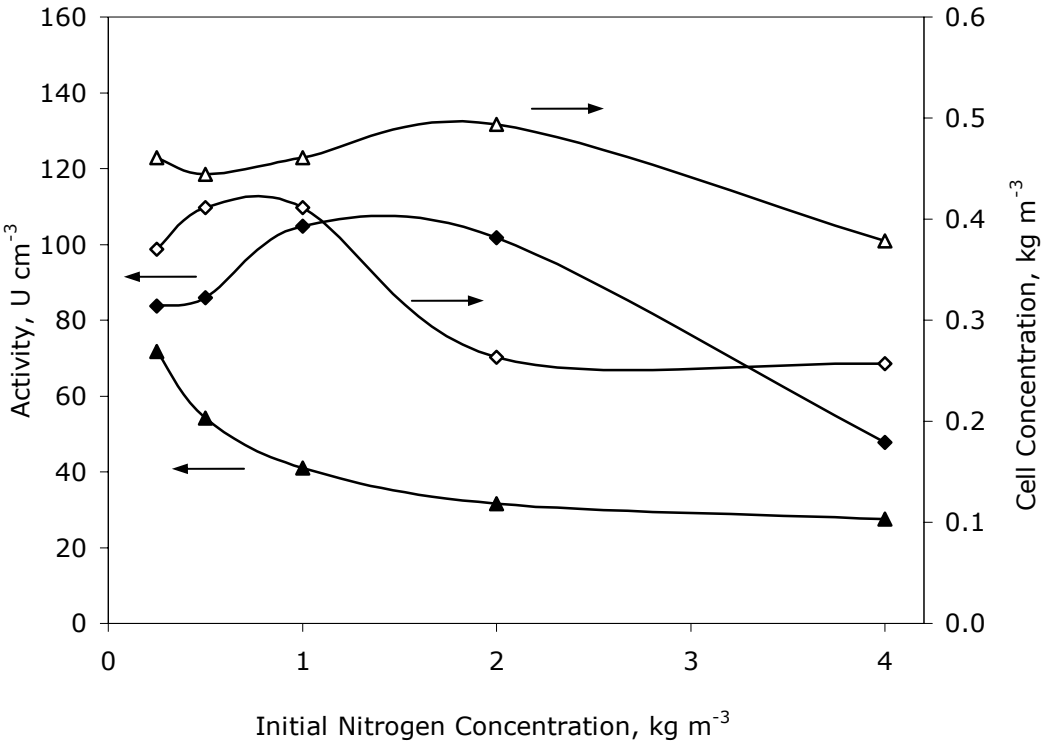


Figure 4.2 The variations in β-lactamase activity and cell concentration with the initial nitrogen concentration, at t=35 h, V=33 cm^3, T=37°C, pH=7.2, N=200 min^{-1}. $(NH_4)_2HPO_4$: (♦, ◇); NH_4Cl: (▲, Δ).

4.2.3 The Concerted Effects of $(NH_4)_2HPO_4$ and Yeast Extract

As lower concentrations of the inorganic nitrogen sources increased β-lactamase productivity, $(NH_4)_2HPO_4$ in the range corresponding to a nitrogen concentration of 0.125–0.5 kg m^{-3} was further investigated together with yeast extract at the initial concentrations of C_{YE}^{o}=0.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 kg m^{-3}. The highest β-lactamase activities obtained during the bioprocess, that corresponds to t=40 h, are tabulated in Table 4.2.

With the increase in yeast extract concentration, the activity increased giving its optimum value at 8.0 kg m^{-3} of yeast extract and 1.179 kg m^{-3} of $(NH_4)_2HPO_4$ (C_N=0.25 kg m^{-3}), as A=200 U cm^{-3} (Table 4.2). At lower yeast extract concentrations, i.e., C_{YE}^{o}=0.0 and 2.0 kg m^{-3}, with the increase in

$(NH_4)_2HPO_4$ concentration β-lactamase production increased, however at higher yeast extract concentrations, i.e., C_{YE}^{o}=4.0 and 8.0 kg m^{-3}, C_N=0.50 kg m^{-3} had an inhibitory effect on β-lactamase production. Furthermore, an initial yeast extract concentration above 10.0 kg m^{-3} had an inhibitory effect on β-lactamase production. (Table 4.2)

The increase in β-lactamase production with the increase in yeast extract concentration is due to the complex nature of yeast extract, which contains a mix of peptides, free amino acids, purine and pyrimidine bases and hydrosoluble vitamins of B group, as seen in Table 2.5 in detail. The inhibitory effect of $(NH_4)_2HPO_4$ at higher yeast extract concentrations indicates the consumption of the organic nitrogen source primarily.

Table 4.2 The variations in β-lactamase activity with the initial yeast extract and nitrogen source ($(NH_4)_2HPO_4$) concentrations.

C_{YE} (kg m^{-3})	C_N (kg m^{-3})	A (U cm^{-3})
0.0	0.125	65
0.0	0.250	93
0.0	0.500	114
2.0	0.125	78
2.0	0.250	90
2.0	0.500	101
4.0	0.125	96
4.0	0.250	114
4.0	0.500	102
6.0	0.250	186
8.0	0.125	187
8.0	**0.250**	**202**
8.0	0.500	164
10.0	0.250	201
12.0	0.250	180

4.2.4 The Effects of Organic Nitrogen Sources

While the highest β-lactamase activity obtained in the presence of inorganic nitrogen sources was 100 U cm^{-3} as seen in Figure 4.2, by the addition of an organic nitrogen source, yeast extract, the maximum activity obtained increased to 200 U cm^{-3} as seen in Table 4.2. Therefore, the effect of other commonly utilized organic nitrogen sources were investigated at the initial concentrations of 6.0, 8.0, 10.0, 12.0 kg m^{-3} in a medium containing 1.179 kg m^{-3} of $(NH_4)_2HPO_4$, 8.0 kg m^{-3} of glucose and the salt solution. As seen in Figure 4.3a and b, the highest activity was obtained again in the presence of 8.0 kg m^{-3} yeast extract as 200 U cm^{-3}.

In the presence of peptone and casamino acids, there was no improvement in β-lactamase production; moreover, increasing amounts of these compounds also had an inhibitory effect on β-lactamase production (Figure 4.3b). When the constituents of these complex nitrogen sources are compared (Table 2.5), the reasons for this inhibitory effect can be deduced. To start with, as can be seen from Table 2.5, a high concentration of chloride in casamino acids could be a reason, as the possibility of the inhibition effect of Cl^- was discussed previously. Moreover, the high potassium content of casamino acids could have an inhibitory effect on β-lactamase production.

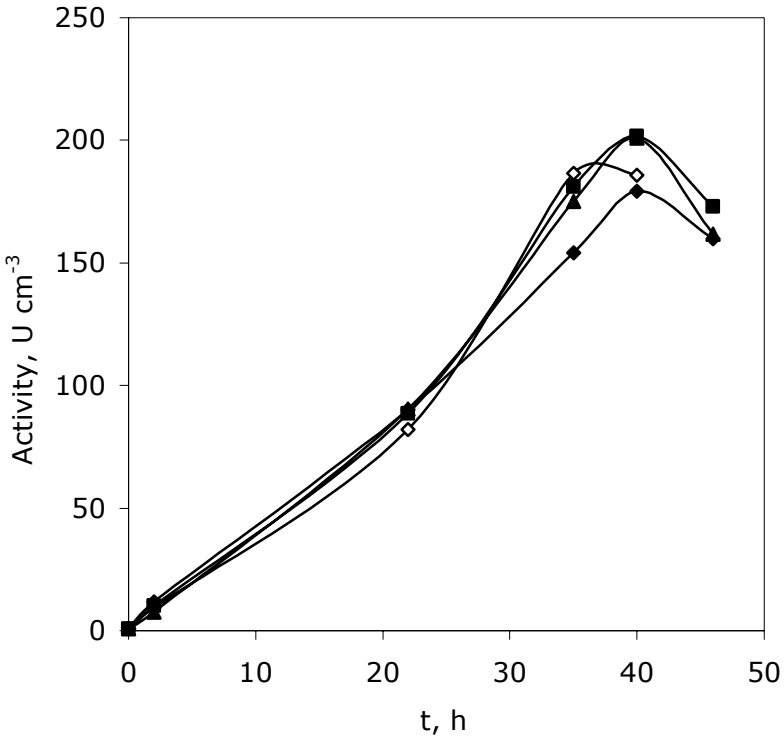


Figure 4.3.a The variations in β-lactamase activity with the cultivation time and the initial yeast extract concentration. V=33 cm^3, T=37°C, pH =7.2, N=200 min^{-1}. Yeast extract (kg/m^3): 6, (◇); 8, (■); 10, (▲); 12, (♦).

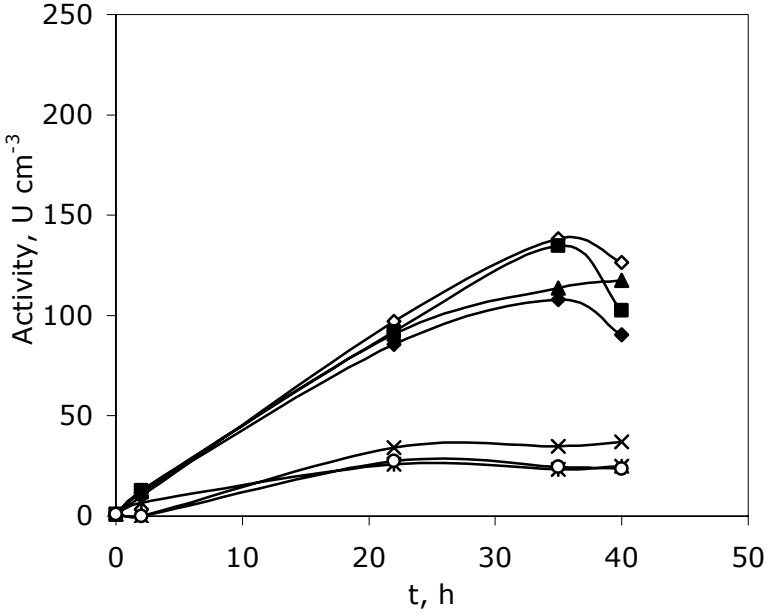


Figure 4.3.b The variations in β-lactamase activity with the cultivation time and the initial peptone and casamino acids concentrations. V=33 cm^3, T=37°C, pH =7.2, N=200 min^{-1}. Peptone (kg/m^3): 6, (◇); 8, (■); 10, (▲); 12, (♦); Casamino acids (kg/m^3): 6, (x); 8, (o);12, (∗).

4.2.5 The Effects of Alternative Carbon Sources

The variations in β-lactamase activity with the cultivation time utilizing different carbon sources were investigated next, in media containing 8.0 kg m^{-3} of yeast extract, 1.179 kg m^{-3} of $(NH_4)_2HPO_4$ and the salt solution (Figure 4.4). The initial concentrations were (kg m^{-3}): glucose, 8.0; fructose, 8.0; sucrose, 6.6; glycerol, 8.1; citric acid, 9.2; adjusted such that in each case, the amount of carbon in the medium was equivalent to the amount of carbon in the medium containing 8.0 kg m^{-3} of glucose.

Considering the entrance point of glucose and fructose to the glycolysis pathway, fructose, being more easily metabolized, resulted in a minor improvement in β-lactamase activity; however, glucose was preferred over fructose, from economics point of view. On the other hand, citric acid entering the carbon metabolism from TCA cycle did not enhance β-lactamase production; hence, the glycolysis pathway was probably preferred over the gluconogenesis pathway for energy generation.

The cells can utilize sucrose as a carbon source if invertase is secreted to the extracellular medium which degrades sucrose into glucose and fructose, which are then transported into the cell via group translocation and enter the glycolysis pathway. On the other hand, sucrose may be transferred to the cell by group translocation and then hydrolysed in the presence of invertase into glucose and fructose. As seen in Figure 4.4, the cells were able to utilize sucrose; however, it was not preferred over glucose in β-lactamase production.

Glycerol, unlike other carbon sources, passes the cell membrane by facilitated diffusion. A high concentration of glycerol (50 kg m^{-3}) as the sole carbon source was reported to increase the specific productivity of recombinant *E. coli* β-lactamase 5-fold in the flask culture, when compared with glucose

(Kwon et al., 1996). However, β-lactamase productivity in the presence of glycerol by *B. licheniformis* was 2.8-fold lower than the productivity obtained with glucose.

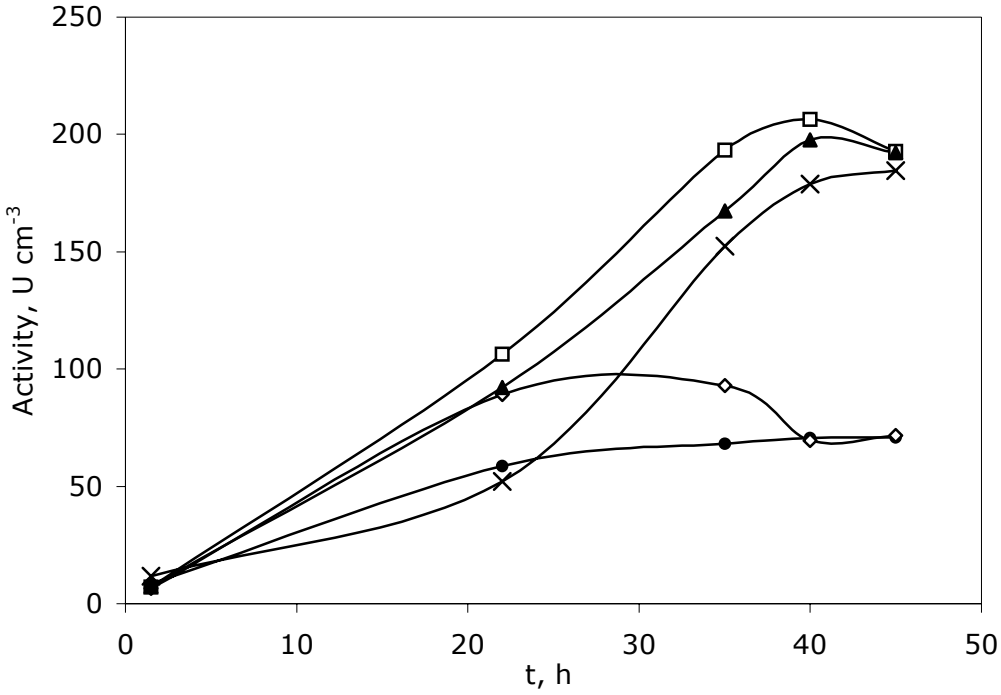


Figure 4.4 The variations in β-lactamase activity with the cultivation time and different carbon sources, containing equivalent amount of carbon to 8.0 kg m^{-3} glucose. V=33 cm^3, T=37°C, pH =7.2, N=200 min^{-1}. Carbon Source: Fructose, (□); Glucose, (▲); Sucrose, (x); Citric acid, (◇); Glycerol, (●).

4.2.6 The Optimized Medium

As a result, among the investigated media, the highest β-lactamase activity was obtained as 200 U cm^{-3}, in the medium containing 8.0 kg m^{-3} glucose, 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} yeast extract and the salt solution, which was 5.7-fold higher than the activity obtained in the reference medium. The variations in β-lactamase activity, glucose and cell concentrations with the cultivation time, in the optimized medium can be seen in Figure 4.5. Beta-lactamase production was growth associated as expected, however showed a diauxic growth profile probably due to consumption of the organic nitrogen source primarily.

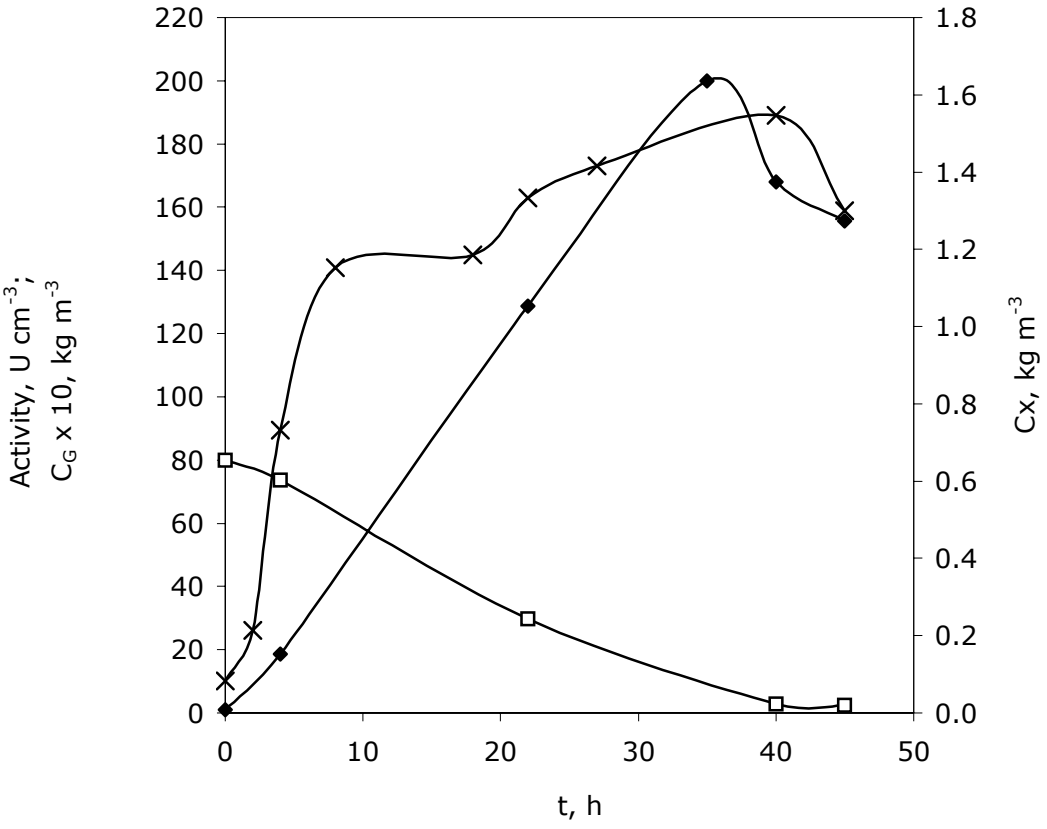


Figure 4.5 The variations in β-lactamase activity, glucose concentration and cell concentration with the cultivation time, in the optimized medium. V=33 cm^3, T=37°C, pH =7.2, N=200 min^{-1}. Beta-lactamase activity, (♦); C_G, (□); C_X, (x).

4.3 Bioreactor Operation Parameters

By using *B.licheniformis* 749/C and the designed medium, the bioreactor operation parameters, given in Table 3.3 were investigated. The effect of pH, temperature and the microorganism concentration of the precultivation medium at which the cells should be inoculated to the production medium were investigated in laboratory scale bioreactors. Thereafter, the fermentation and oxygen transfer characteristics of the bioprocess were determined in the pilot scale bioreactor.

4.3.1 The Effect of pH and Temperature

During microorganism selection and medium design experiments, the pH of the production medium was set to pH 7.2 with 0.02M sodium phosphate buffer. Under the scope of determining the most favorable bioreactor operation conditions, the effect of pH control was investigated first, since some bioprocesses require controlled pH conditions, while others might require uncontrolled pH operations.

The effect of pH control was investigated in an initial pH 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. As seen in Figure 4.6, the uncontrolled pH operation was favorable for β-lactamase production, which is consistent with the observations of Sargantanis and Karim (1996). Furthermore, the highest relative activity, i.e., activity obtained relative to the activity obtained in the optimized medium (0.02 M buffer, pH 7.2), was observed at pH_0=6.25, as 1.2. In other words, the highest β-lactamase activity was A=240 U cm^{-3}.

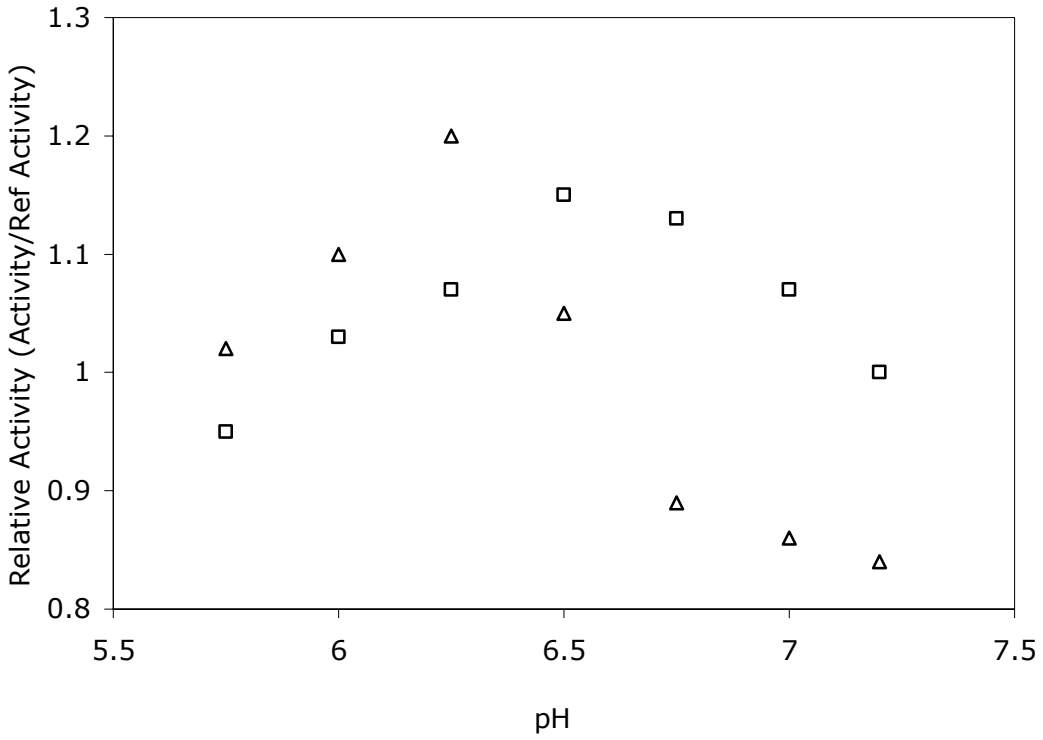


Figure 4.6 The variations in relative β-lactamase activity with pH of the buffer. V=33 cm^3, T=37°C, N=200 min^{-1}, t=40h, Buffer capacity: w/o buffer, (Δ); 0.02 M buffer, (□).

Therefore, the effects of pH and temperature were investigated in media without buffer. As seen in Figure 4.7, at a cultivation temperature of 37°C, pH=6.3 was the most favorable condition. However, at T=32°C, β-lactamase activity was about 1.3 times higher, and at this temperature the activity was not dependent on pH in the range pH_0=6.0 – 7.0. Decrease in the temperature further to T=29°C did not enhance the β-lactamase productivity. Moreover, from the variation in product yield on cell, $Y_{P/X}$, with pH_0 at T=32°C (t=40 h) given in Table 4.3, it is seen that the highest product yield on cell was obtained at pH_0=6.0.

As a result, T=32°C and pH_0=6.0 were found as the most favorable conditions for β-lactamase production, having a β-lactamase activity of 260 U cm^{-3}. These results are consistent with pH and temperature range used in literature for β-lactamase production by *Bacillus* species.

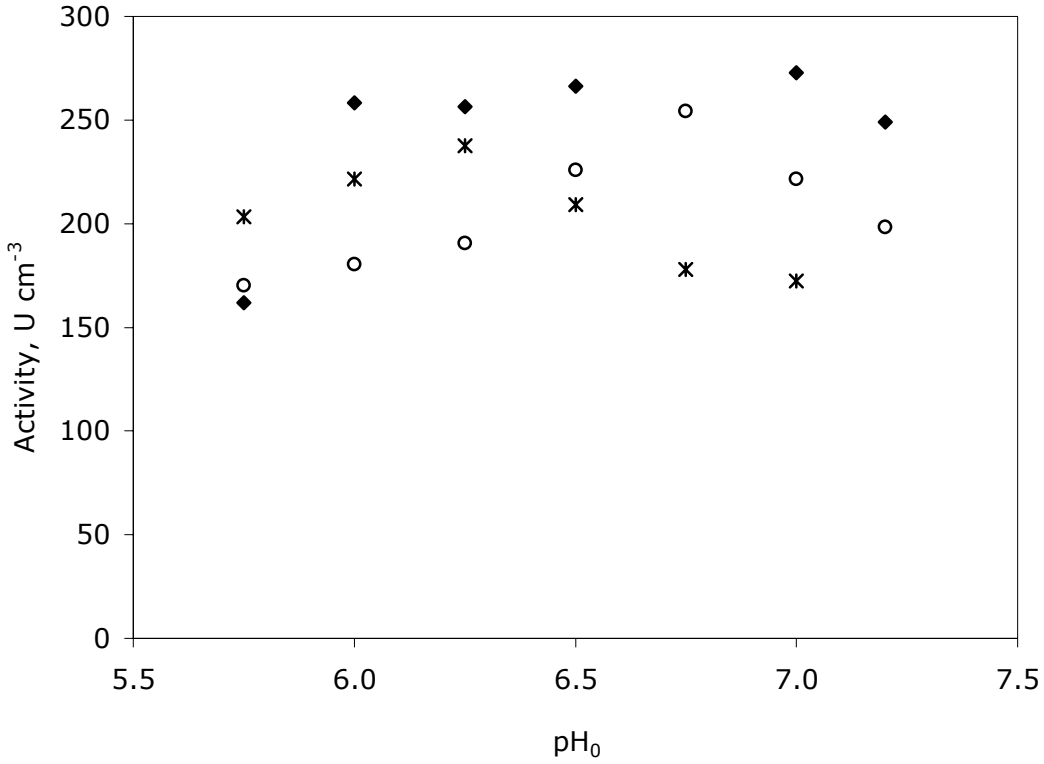


Figure 4.7 The variations in β-lactamase activity with initial pH and temperature of the cultivation medium. V=33 cm^3, N=200 min^{-1}, t=40h, T (°C) : 29 ,(o); 32, (♦); 37, (*).

Table 4.3 The variation in product yield on cell with pH_0, at t=40 h, V=33 cm^3, T=32°C, N=200 min^{-1}.

pH_0	$Y_{P/X}$, U mg^{-1}
6.0	309
6.3	273
6.5	250
7.0	282

4.3.2 The Effect of Cell Concentration of the Precultivation Medium

As mentioned before, when the microorganism concentration in the precultivation medium reached to 0.30 kg m^{-3}, the microorganisms were transferred to the production medium, with an 1/10 inoculation ratio. This amount of cell concentration, detected spectrophotometrically, is usually reached after 3 h of cultivation period in the precultivation medium. As 3.0 cm^3 of cells from the precultivation medium are transferred to the production medium having 33 cm^3 working volume capacity, the cell concentration of the precultivation medium is important.

Therefore, the effect of cell concentration of the precultivation medium was investigated in a cell concentration range of 0.23 – 0.42 kg m^{-3}. As seen in Figure 4.8, the proper cell concentration in the precultivation medium to obtain the highest β-lactamase productivity in production medium was found to be between 0.28 – 0.32 kg m^{-3}, above which the activity decreased sharply. Thus the metabolic state of the microorganism in the precultivation medium is indeed important for enzyme production.

4.3.3 Determination of Metabolic Shifts in Carbon Utilization

Prior to the pilot scale experiment, the effect of the initial glucose concentration was reinvestigated, in order to determine possible shifts in the metabolism, at the initial concentrations of $C_G°$= 6.0, 8.0, 10.0, 15.0 and 20.0 kg m^{-3}. In the medium containing 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} yeast extract and the salt solution, at T=32°C, pH_0=6.0, N=200 min^{-1}, β-lactamase activity was the highest at $C_G°$=10.0 kg m^{-3} having a value of A=275 U cm^{-3} at t=40 h (Figure 4.9). Previously, the effects of glucose on β-lactamase production were investigated in the medium containing (kg m^{-3}): glucose, 10.0; NH_4Cl, 5.0; yeast extract, 2.0 and the salt solution at T=37°C, pH=7.2, N=200 min^{-1} and the β-lactamase activity was found to be highest at $C_G°$=8.0 kg m^{-3} having a value of A=65 U cm^{-3} (section 4.2.1). The increase in the carbon source requirements was expected, since due to improved medium and bioprocess conditions, cell requirements changes.

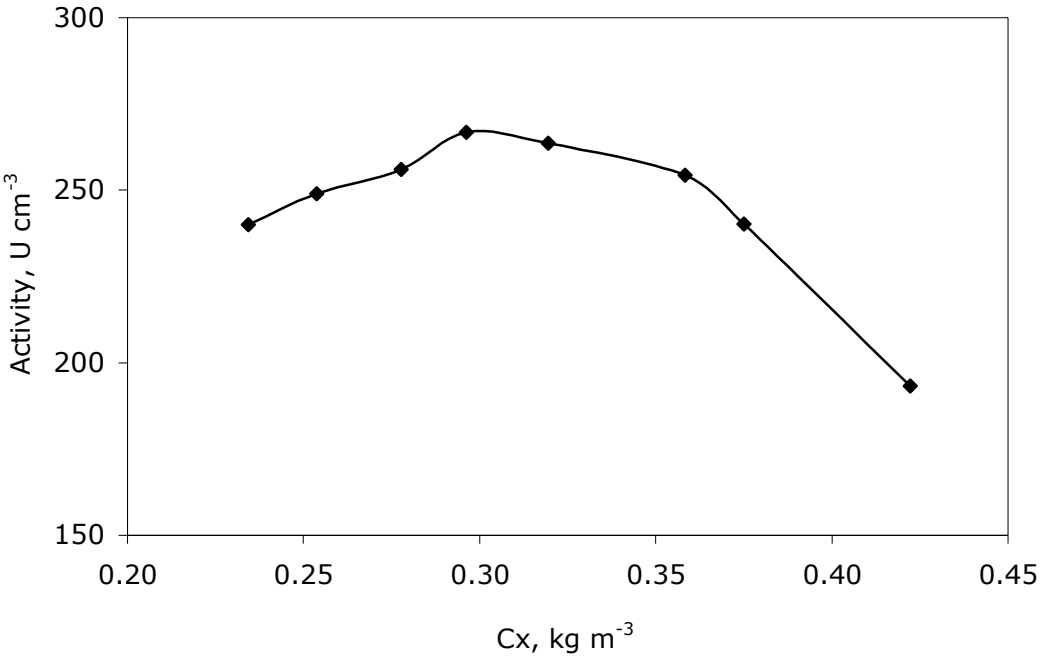


Figure 4.8 The variations in β-lactamase activity with cell concentration of the precultivation medium. V=33 cm^3, T=32°C, pH=6.0, N=200 min^{-1}, t=40 h.

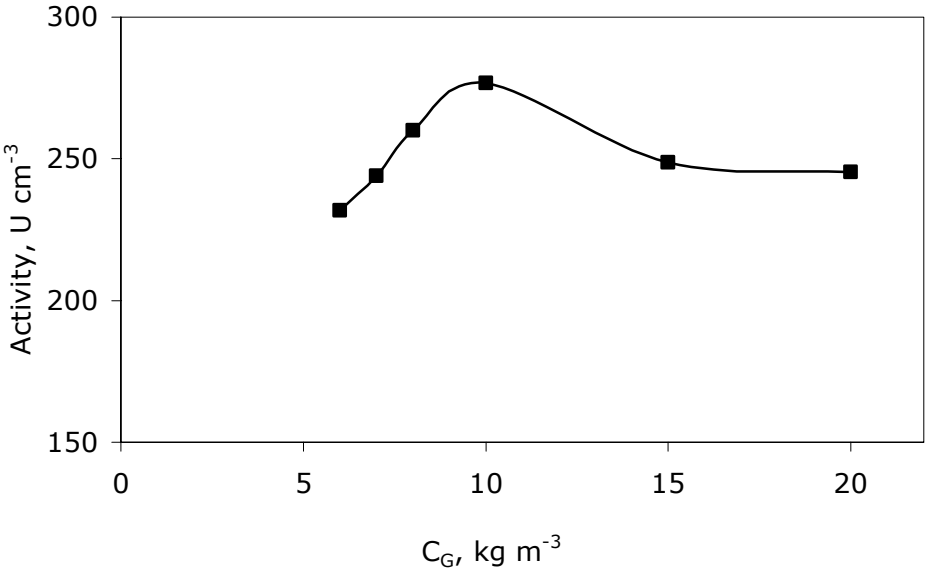


Figure 4.9 The variation in β-lactamase activity with the initial glucose concentration. V=33 cm^3, T=32°C, pH=6.0, N=200 min^{-1}, t=40 h.

4.4 The Pilot Scale Bioreactor Experiment

Using *B. licheniformis* 749/C in the previously optimized medium at the optimum bioprocess conditions obtained in the laboratory scale experiments, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in the pilot scale bioreactor system explained in section 3.6, where N=500 min^{-1}, Q_0/V_R=0.5 vvm, V_R=1.65 dm^3. Throughout the bioprocess, β-lactamase activity, cell concentration, glucose concentration, amino acid and organic acid concentrations, dissolved oxygen, pH, oxygen uptake rate, liquid phase mass transfer coefficient, specific growth rate, yield and maintenance coefficients were determined.

4.4.1 pH and Dissolved Oxygen Profiles

The variations in pH and dissolved oxygen with cultivation time are given in Figure 4.10. The initial pH value was pH_0=6.1. Throughout the bioprocess, pH decreased until t=4.5 h to pH=5.8, thereafter with the initiation of β-lactamase production, the pH of the fermentation broth increased due to H^+ transport into the cells and finally reached a constant value of pH=7.6 at t=27 h.

Whereupon, the pH of the fermentation medium has a natural tendency to change along with the bioprocess, every microorganism has a particular intracellular pH that is kept constant. For instance, the intracellular aqueous (cytoplasmic) pH of *B. licheniformis* is 7.5. To maintain the intracellular pH at a constant level, protons are pumped in or out through the cell membrane. The inward flow of protons is enzymatically mediated by ATPase, which participates in the formation of ATP. The proton transport is reversible; i.e., the ATPase may also pump protons out of the cells with the expenditure of ATP. Therefore, the extracellular pH being lower than the intracellular pH is a favorable situation for microorganisms. In this respect, the pH of the fermentation medium reaching a

constant value at t=27 h, signaled the end of β-lactamase production process and the start of β-lactamase degradation.

The DO exhibited a sudden drop at the early hours of the fermentation and at t=4.5-7 h the transferred oxygen was totally consumed. During t=7-15 h, oscillations were observed in the DO profile and thereafter the DO concentration gradually increased.

In literature, there is no work reporting the variations in pH and the uncontrolled-dissolved oxygen profiles throughout the β-lactamase production process.

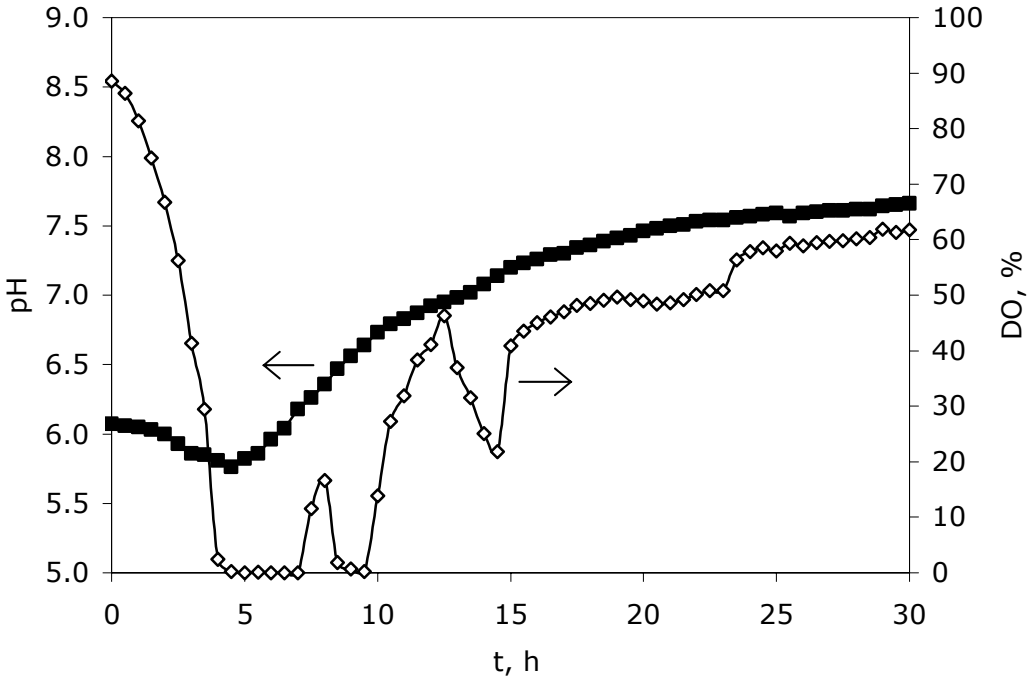


Figure 4.10 The variations in pH and dissolved oxygen with cultivation time T=32°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm, C_O^*=0.20 mol m^{-3}.

4.4.2 Glucose and Cell Concentration Profiles

The variations in glucose and cell concentrations with cultivation time are given in Figure 4.11. A diauxic growth profile was observed as in the laboratory scale experiments; the first logarithmic phase being completed at t=6 h and the increase in microorganism concentration after t=15 h indicating the consumption of the secondary nitrogen source. The maximum microorganism concentration observed during the bioprocess was C_X=1.85 kg m^{-3} at t=28h, which is lower than the maximum microorganism concentration observed during the laboratory scale experiments. Glucose concentration in the fermentation medium, initially present in the medium at an initial concentration of C_G^0=10 kg m^{-3}, decreased with a high rate until t=6 h and then with a lower rate after t=23 h. As seen in Figure 4.11, the carbon source was not totally consumed, probably either because of limitations in oxygen or another nutrient, or inhibition caused by a by-product.

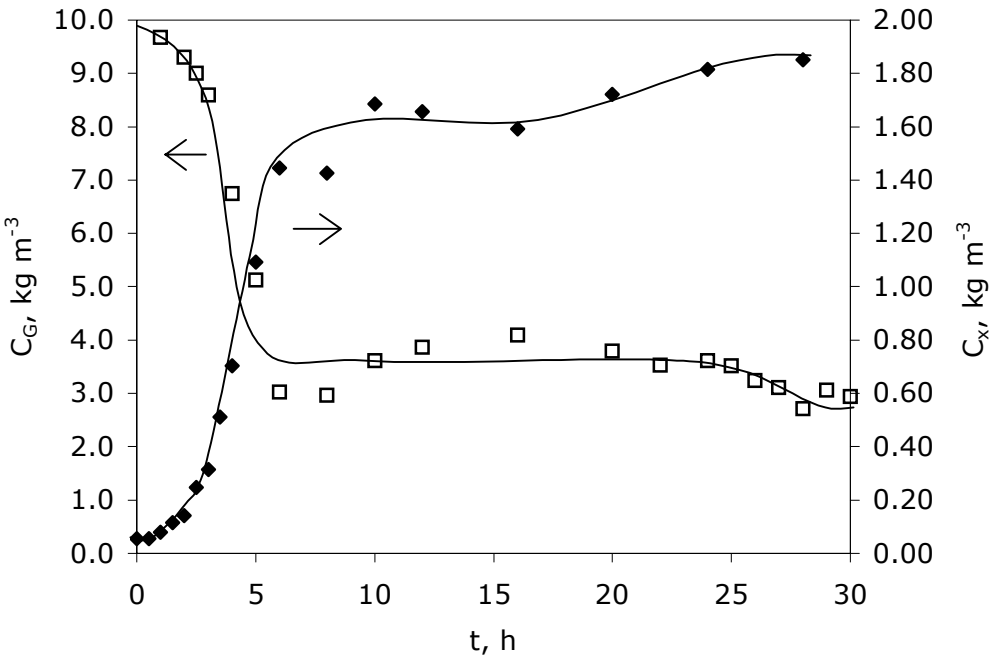


Figure 4.11 The variations in glucose and cell concentrations with cultivation time T=32°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm.

4.4.3 Beta-Lactamase Activity Profile

The variation in β-lactamase activity with cultivation time is given in Figure 4.12. Beta-lactamase synthesis started after t=4 h. The cultivation time in which the highest activity obtained shifted to the earlier stages of the fermentation (t=27h) when compared to the laboratory scale experiments with a cost of lower activity (210 U cm^{-3}). This is probably because of inappropriate oxygen transfer conditions, which in turn indicates that oxygen transfer conditions should be investigated in detail for β-lactamase production in pilot scale bioreactor system.

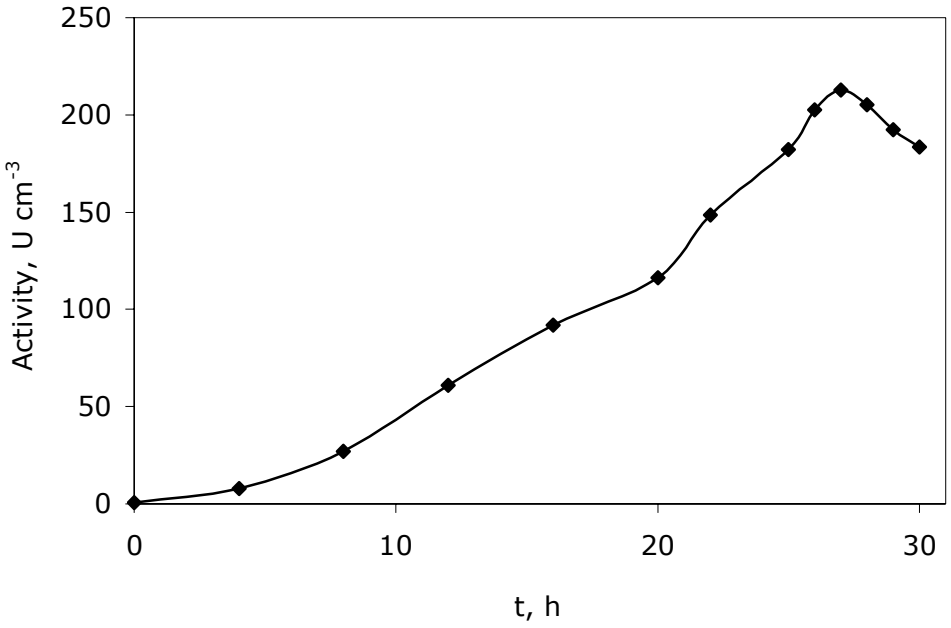


Figure 4.12 The variation in β-lactamase activity with cultivation time. T=32°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm.

4.4.4 Amino Acid and Organic Acid Concentrations

The variations in amino acid and organic acid concentrations in the fermentation broth with cultivation time are given in Table 4.4 and 4.5 respectively. The amino acid profiles could give an idea about the supply and demand for the amino acids, which is regulated by the metabolic reaction network.

Starting from the key intermediates or the products of the glycolytic pathway, pentose-phosphate pathway and the TCA cycle, the amino acid pathways should produce all the amino acids in the primary sequence of β-lactamase; however, methionine, tryptophane, glycine and lysine were not secreted to the fermentation broth. Moreover, cysteine, which is not present in primary sequence of β-lactamase, was transported into the cell in the early hours of the fermentation process, thereafter its concentration was constant at C_{Cys}=0.03 kg m^{-3}. At the end of the bioprocess, Phenylalanine concentration in the broth was the highest, C_{Phe}=0.100 kg m^{-3}. Glutamic acid, glutamine, serine, tyrosine, valine, isoleucine and leucine present in the medium at the early hours of fermentation, originating from either the yeast extract or the precultivation medium, are all consumed by t=10h.

Throughout the bioprocess acetic acid, butyric acid, lactic acid and oxaloacetic acid were detected in the fermentation broth (Table 4.5). The presence of oxaloacetic acid at the beginning of the fermentation medium and its concentration being constant afterwards indicates that oxaloacetic acid originates from the precultivation medium. Therefore, the TCA cycle organic acids not being excreted into the medium indicate the successful operation of the TCA cycle to produce key intermediates for growth and production. The presence of lactic acid at high concentrations during t=4-10 h is due to insufficient oxygen, which can

also be seen in Figure 4.10. Butyric acid, also originating from the precultivation medium, was consumed by the end of the bioprocess.

4.4.5 Oxygen Transfer Characteristics and Rate-Limiting-Step Analysis

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r_0, and oxygen transfer coefficient, K_La, during the cultivation times corresponding to the characteristic regions of the batch bioprocess. At t<0 h, the physical oxygen transfer coefficient K_La_o was measured in the medium in the absence of the microorganism. As C_O value was very low between t=5-10 h of the fermentation, the Dynamic Method could not be applied but the K_La values during this period were obtained from K_La versus t plot. The variations in K_La, oxygen uptake rate, oxygen transfer rate and the enhancement factor E ($=K_La/K_La_o$) throughout the growth phase of the bioprocess are given in Table 4.6.

K_La decreased with cultivation time, and the enhancement factor was always higher than 1.0. K_La depends on agitation rate, temperature, rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. Temperature and agitation rate were kept constant throughout the bioprocess, therefore the reason for the decrease in K_La could be the addition of antifoam by the foam control system of the bioreactor, which was no more than 10 ml; or the increase in viscosity of the medium due to secreted metabolites. Furthermore, particles with a diameter somewhat greater than the thickness of the mass transfer layer enhance the gas absorption, but the enhancement decreases with increasing particle diameter; therefore, along with the increase in the concentration of the rod-shaped biocatalyst *B. licheniformis* cells that have dimensions 0.6–0.8 μm x 1.5–3.0 μm, the mass transfer coefficient may increase or decrease depending on the diameter and concentration of the biomass (Çalık et al., 1998).

In the first 12 h of the fermentation, OUR was almost equal to OTR, due to very low accumulation of oxygen in the fermentation medium. In the first 5 h of the bioprocess OUR, which depends on the metabolic functions of the biomass, tends to increase, due to the increase in biomass production rate, the biomass concentration and the substrate consumption rate. After t=5 h, because of the decrease in the substrate consumption rate, biomass production rate and OUR decreased.

In order to find the rate limiting step and the degree of the limitation in the β-lactamase production process by *B. licheniformis*, the maximum possible oxygen utilization rate ($OD=\mu_{max}C_X/Y_{X/O}$) and the maximum possible mass transfer rate ($OTR_{max}=K_La{C_O}^*$) were also calculated throughout the growth phase of the bioprocess. A kind of Damköhler number, Da, defined as maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2000 and 2003); and effectiveness factor, η, defined as the oxygen uptake rate per maximum possible oxygen utilization rate values (Çalık et al., 2000 and 2003) can be seen in Table 4.6.

It is clear in Table 4.6 that, in the first 2 h of the bioprocess, the process is at biochemical reaction limited condition (Da<1); at t=3.5 h both mass-transfer and biochemical reaction resistances are effective; and after t= 5 h mass-transfer resistances are effective (Da>>1), thus the process shifts to the mass-transfer limited condition. As it is apparent from Table 4.6, the high effectiveness factor, η, values until t=3.5 h indicate that the cells are consuming oxygen with such a high rate that maximum possible oxygen utilization (OD) value is approached and thereafter the decrease in η indicates that the cells are consuming lower oxygen than the oxygen demand (OD).

4.4.6 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in specific growth rate, μ, specific oxygen uptake rate, q_o, and yield coefficients are given in Table 4.7. As it is seen in the table, the specific growth rate first increases then decreases in the logarithmic growth phase, reaching maximum specific growth rate of μ_{max}=0.743 h^{-1} at t=2 h. The specific oxygen uptake and the specific substrate consumption rates were also at their maximum at t=2 h, and then decreased with the cultivation time due to the increased cell concentration.

The yield of cell on substrate reached a maximum value, $Y_{X/S}$=0.34, at t=5 h, the yield of cell on oxygen reached a maximum, $Y_{X/O}$=1.40, at t=3.5 h, the yield of substrate on oxygen reached a maximum, $Y_{S/O}$=6.94, at t=3.5 h, and thereafter they all decreased till the end of the bioprocess (Table 4.7). There is no work on β-lactamase production by *Bacillus* species reporting the yield coefficients. But for most bacteria growing aerobically on glucose, $Y_{X/S}$ is typically 0.4 to 0.6 kg kg^{-1}, while $Y_{X/0}$ is 0.9 to 1.4 kg kg^{-1} (Shuler and Kargi, 1992). The $Y_{X/O}$ values until t=5 h are in a consistent range with literature, whereas at t=6 h the decrease in $Y_{X/O}$ value is due to lack of oxygen. $Y_{X/S}$ values were very low, indicating the inefficient use of the carbon source through biochemical reaction network probably because of the oxygen limitation. The relatively high $Y_{S/O}$ values indicate that the carbon source is not efficiently utilized in the energy metabolism, and the decrease in $Y_{S/O}$ values with the cultivation time, shows the decrease in the efficiency of energy metabolism.

In the period where oxygen uptake for product formation is omitted, i.e., t=2-6 h, from the slope of the plot of $1/Y_{X/O}$ versus 1/μ, the rate of oxygen consumption for maintenance was obtained as m_0=0.13 kg $kg^{-1}h^{-1}$ (equation 2.15) and similarly from the slope of the plot of $1/Y_{S/O}$ versus 1/μ, the rate of substrate consumption for maintenance was obtained as m_s=3.02 kg $kg^{-1}h^{-1}$.

Table 4.4 The variations in amino acid concentrations in the fermentation broth with cultivation time, T=32°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm.

C_{AA}, kg m^{-3}	Cultivation time, h													
	0.5	2	4	6	10	12	16	20	22	26	27	28	29	30
Asn+Asp	0.100	0.123	0.108	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glu	0.185	0.199	0.117	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Gln+Ser	0.295	0.269	0.123	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
His	0.049	0.054	0.084	0.086	0.047	0.000	0.000	0.000	0.000	0.000	0.013	0.015	0.014	0.013
Arg	0.184	0.207	0.174	0.136	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Thr	0.027	0.028	0.029	0.028	0.012	0.006	0.008	0.007	0.007	0.000	0.001	0.008	0.007	0.007
Ala+Pro	0.000	0.000	0.024	0.017	0.000	0.000	0.000	0.025	0.025	0.005	0.000	0.023	0.027	0.023
Tyr	0.115	0.131	0.115	0.092	0.035	0.006	0.000	0.002	0.001	0.000	0.004	0.002	0.003	0.003
Val	0.010	0.010	0.007	0.004	0.003	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Cys	0.045	0.034	0.031	0.029	0.033	0.028	0.017	0.031	0.030	0.027	0.027	0.030	0.030	0.030
Iso+Leu	0.060	0.067	0.057	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Phe	0.165	0.122	0.080	0.075	0.088	0.079	0.075	0.097	0.096	0.077	0.096	0.100	0.097	0.098

Table 4.5 The variations in organic acid concentrations in the fermentation broth with cultivation time, T=32°C, N=500 min^{-1}, Q_0/V_R=0.5 vvm.

C_{OA} kg m^{-3} x10^3	Cultivation time, h													
	0.5	2	4	6	8	10	12	16	20	22	26	27	28	29
OA	0.309	0.363	0.311	0.361	0.304	0.295	0.306	0.300	0.337	0.302	0.341	0.242	0.289	0.457
Ac	0.000	0.030	0.024	0.050	0.000	0.021	0.000	0.022	0.037	0.021	0.027	0.054	0.050	0.023
Lac	0.287	0.536	0.952	1.483	1.090	0.987	0.000	0.166	0.067	0.000	0.065	0.033	0.045	0.000
But	0.359	0.369	0.346	0.296	0.102	0.127	0.014	0.010	0.025	0.022	0.017	0.012	0.010	0.011

Table 4.6 The variations in oxygen transfer parameters with cultivation time.

t (h)	K_La (s^{-1})	E K_La/K_La_o	OTR*10^3 (mol $m^{-3}s^{-1}$)	OTR_{max}*10^3 (mol $m^{-3}s^{-1}$)	OUR*10^3 (mol $m^{-3}s^{-1}$)	OD*10^3 (mol $m^{-3}s^{-1}$)	Da OD/OTR_{max}	η OUR/OD
0	0.007	1.00	-	-	-	-	-	-
2	0.016	2.29	1.1	3.2	1.1	1.1	0.3	1.00
3.5	0.016	2.29	2.3	3.2	2.3	2.7	0.8	0.85
5	0.014	2.00	2.8	2.8	2.8	5.0	1.8	0.56
6	0.013	1.86	2.6	2.6	2.6	19.3	7.4	0.13
8	0.010	1.43	2.0	2.0	2.0	-	-	-
10	0.008	1.14	1.6	1.6	1.6	-	-	-
12	0.009	1.29	1.1	1.8	1.1	-	-	-

Table 4.7 The variations in specific growth rate and yield coefficients.

t (st)	C_x (kg m^{-3})	dC_x/dt (kg m^{-3} h^{-1})	μ (h^{-1})	r_o (kg O_2 m^{-3} h^{-1})	dC_s/dt (kg m^{-3} h^{-1})	q_0 (kg kg^{-1} h^{-1})	q_s (kg kg^{-1} h^{-1})	$Y_{x/o}$ (kg kg^{-1})	$Y_{x/s}$ (kg kg^{-1})	$Y_{s/o}$ (kg kg^{-1})
0.5	0.06	0.029	0.483	-	0.21	-	3.50	-	0.14	-
1	0.08	0.050	0.625	-	0.26	-	3.25	-	0.19	-
2	0.14	0.104	0.743	0.127	0.51	0.91	3.64	0.82	0.20	4.02
3.5	0.51	0.320	0.627	0.265	1.84	0.52	3.61	1.21	0.17	6.94
5	1.09	0.453	0.416	0.323	1.33	0.30	1.22	1.40	0.34	4.12
6	1.44	0.143	0.099	0.300	0.43	0.21	0.30	0.48	0.33	1.43
23	1.80	0.017	0.009	-	0.09	-	0.05	-	0.19	-
25.5	1.83	0.010	0.005	-	0.16	-	0.09	-	0.06	-

4.4.7 Enzyme Kinetics

The K_m and r_{max} values for β-lactamase were assessed using the unpurified enzyme produced in the pilot scale bioreactor experiment. As can be seen in Figure 4.15, β-lactamase displays typical Michealis-Menten kinetics. From the Lineweaver-Burk plot and equation 2.2, r_{max} and K_m values were determined, at T=30°C, as 3.6 mol m^{-3} s^{-1} and 0.17 mM, respectively. The K_m value for benzylpenicillin varies slightly according to the source of the enzyme, but all reported values lie between 0.05 and 0.20 mM (Pollock, 1960). The K_m value obtained in this study for β-lactamase by *B. licheniformis* was therefore consistent with literature.

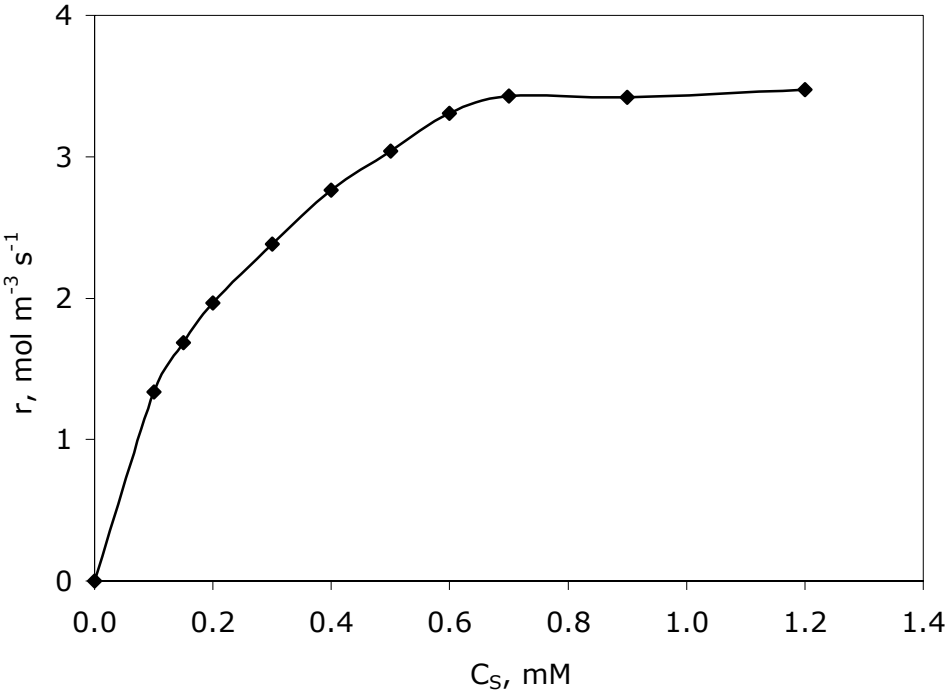


Figure 4.13 Michealis-Menten plot of β-lactamase.

CHAPTER 5

CONCLUSION

In this study, the effects of bioprocess design parameters on β-lactamase production were investigated. For this purpose, *Bacillus* species were screened and using the *Bacillus* strain having the highest β-lactamase production capacity, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher β-lactamase production. Thereafter, by using the designed medium, the effects of bioprocess operation parameters, i.e., pH and temperature, on β-lactamase activity were investigated in laboratory scale bioreactors. Finally, using the optimum bioprocess parameters obtained in the previous steps, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in the pilot scale bioreactor. In this context, the following conclusions were drawn:

1. The constitutive β-lactamase producer *B. licheniformis* 749/C was selected for β-lactamase production, having 35 U cm^{-3} activity in the reference β-lactamase production medium.
2. Prior to a systematic investigation of the effects of medium components, it was observed that while the β-lactamase activity in the reference medium was 35 U cm^{-3}, the medium containing 2.0 kg m^{-3} yeast extract and 5.0 kg m^{-3} NH_4Cl instead of the 10.0 kg m^{-3} casamino acids in the reference medium, had a β-lactamase activity

of 55 U cm^{-3}. Therefore, the medium that contained (kg m^{-3}): glucose, 10.0; NH_4Cl, 5.0; yeast extract, 2.0 and the salt solution, given in Table 3.4, was considered as the starting point for medium design experiments, and this medium was named as the GNY medium.

3. The effects of glucose on β-lactamase production were investigated in the GNY medium, at the initial concentrations of $C_G°$=0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 20.0 kg m^{-3}. β-lactamase activity was the highest at $C_G°$=8.0 kg m^{-3} having a value of A=65 U cm^{-3}.

4. The effect of inorganic nitrogen sources ($(NH_4)_2HPO_4$ and NH_4Cl) were investigated in the GNY medium, from which the yeast extract was omitted and $C_G°$ = 8.0 kg m^{-3}, within the range corresponding to nitrogen concentration of 0.25 – 4.0 kg m^{-3}. It was observed that, higher nitrogen content had an inhibitory effect on cell concentration and β-lactamase activity. In the presence of $(NH_4)_2HPO_4$, twice the activity was obtained compared to the medium containing NH_4Cl.

5. The concerted effects of $(NH_4)_2HPO_4$ and yeast extract were investigated at different combinations of C_{YE}^{o}=0.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 kg m^{-3} and C_N^{o}=0.125-0.5 kg m^{-3}, in the medium containing $C_G°$= 8.0 kg m^{-3} and the salt solution. With the increase in yeast extract concentration, the activity increased giving its optimum value at 8.0 kg m^{-3} of yeast extract and 1.179 kg m^{-3} of $(NH_4)_2HPO_4$ (C_N=0.25 kg m^{-3}), as A=200 U cm^{-3}; and β-lactamase production was inhibited above C_{YE}^{o}=10.0 kg m^{-3}. Furthermore, at C_{YE}^{o}=4.0 and 8.0 kg m^{-3}, C_N^{o}=0.50 kg m^{-3} had an inhibitory effect on β-lactamase production, which indicates the consumption of the organic nitrogen source primarily.

6. While in the presence of an organic nitrogen source (yeast extract) twice the activity could be obtained compared to the medium containing an inorganic nitrogen source, the other commonly utilized organic nitrogen sources, i.e., peptone and casamino acids, had an inhibitory effect on β-lactamase production.
7. Among the carbon sources investigated, alternative to glucose, i.e., fructose, sucrose, citric acid and glycerol, only fructose resulted in a minor improvement in β-lactamase activity; however, glucose was preferred over fructose, from economics point of view.
8. As a result of the medium design experiments, the highest β-lactamase activity was obtained as 200 U cm^{-3}, in the medium containing 8.0 kg m^{-3} glucose, 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} yeast extract and the salt solution, at pH 7.2 (0.02 M phosphate buffer), T=37°C, N=200 min^{-1}, V=33 cm^3, which was 5.7-fold higher than the activity obtained in the reference medium.
9. The uncontrolled pH operation was found to be more favorable for β-lactamase production, than the presence of 0.02 M phosphate buffer. Furthermore, at T=37°C, the highest β-lactamase activity was obtained at pH_0=6.25 as A=240 U cm^{-3} in the optimized medium.
10. The effects of pH and temperature were investigated in media without buffer. At T=32°C, the highest β-lactamase activity was obtained and it was not dependent on pH in the range pH_0=6.0 – 7.0. Moreover, the highest product yield on cell, $Y_{P/X}$, at T=32°C was obtained at pH_0=6.0. As a result, T=32°C and pH_0=6.0 were found as the most favorable conditions for β-lactamase production, having a β-lactamase activity of 260 U cm^{-3}.
11. The effect of cell concentration of the precultivation medium was investigated in a cell concentration range of 0.23 – 0.42 kg m^{-3}. The

proper cell concentration in the precultivation medium to obtain the highest β-lactamase productivity in production medium was found to be between 0.28 – 0.32 kg m^{-3}, above which the activity decreased sharply. Thus, the importance of the metabolic state of the microorganism in the precultivation medium for enzyme production was verified.

12. When the effect of the initial glucose concentration was reinvestigated in order to determine possible shifts in the metabolism due to improved medium and bioprocess conditions, β-lactamase activity was the highest at $C_G°$=10.0 kg m^{-3}. That is, in the medium containing 10.0 kg m^{-3} glucose, 1.2 kg m^{-3} $(NH_4)_2HPO_4$, 8.0 kg m^{-3} yeast extract and the salt solution, at T=32°C, pH_0=6.0, N=200 min^{-1}, V=33 cm^3, a β-lactamase activity of 275 U cm^{-3} was obtained, which was 7.9-fold higher than the activity obtained in the reference medium.
13. To investigate the fermentation and oxygen transfer characteristics, the pilot scale bioreactor was operated at N=500 min^{-1}, Q_0/V_R=0.5 vvm, T=32°C, pH_0=6.1, V_R=1.65 dm^3 and *B. licheniformis* 749/C was inoculated to the medium designed (t=0h).
    a. By the end of the first 2 h the bioprocess, specific growth rate, specific oxygen uptake and specific substrate consumption rates reached their maxima; the process was at biochemical reaction limited condition and the cells were consuming oxygen with such a high rate that maximum possible oxygen utilization (OD) value was approached.
    b. During t=3.5-5 h, pH and DO reached their minima; β-lactamase synthesis started; K_La, OTR, OUR, and the yield coefficients reached their maxima.

c. By the end of 6 h of the bioprocess, the first logarithmic phase was completed, the substrate consumption rate reached to very low levels, the transferred oxygen was totally consumed and lactic acid secreted to the fermentation medium reached its maxima signaling anaerobic respiration. Furthermore, the process shifted to the mass-transfer limited conditions and the cells were consuming lower oxygen than the OD.

d. At t=27 h, the highest β-lactamase activity obtained was 210 U cm^{-3}, which was also signaled by pH of the fermentation medium reaching a constant value. When compared to the laboratory scale experiments, the cultivation time at which the highest β-lactamase activity obtained shifted from 40 h to 27 h, but with a cost of 1.3-fold lower β-lactamase activity. This was due to the lack of oxygen, which in turn indicates that oxygen transfer conditions should be investigated in detail for β-lactamase production in pilot scale bioreactor system.

e. At t=28 h, the maximum microorganism concentration was observed as C_X=1.85 kg m^{-3}.

f. The rate of oxygen consumption for maintenance was obtained as m_0=0.13 kg $kg^{-1}h^{-1}$ and the rate of substrate consumption for maintenance was obtained as m_s=3.02 kg $kg^{-1}h^{-1}$.

14. The K_m and r_{max} values for β-lactamase were determined using the unpurified enzyme produced in the pilot scale bioreactor experiment as 0.17 mM and 3.6 mol m^{-3} s^{-1}, respectively.

REFERENCES

Atkinson, B. and Mavituna, F., 1991. Biochemical Engineering and Biotechnology Handbook, 2nd ed., Macmillan Publishers Ltd., England.

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

Bernstein, A., Nickerson, K.W., Day, R.A, 1967. Thermal Penicillinase Expression and Temperature Dependence of Penicillinase Production Inducible and Constitutive Strains of *Bacillus cereus*, Archives of Biochemistry and Biophysics, 119, 50-54.

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.

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.

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.

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.

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.

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.

Cohen, A., 1983. The PICO TAG System: a New Method to Analyze Primary and Secondary Amino Acids with One Picomole Sensitivity, Biotechniques, Sept/Oct, 273-275.

Collins, J.R., 1979. The *Bacillus licheniformis* β-Lactamase System, In: Hamilton-Miller M.T. and Smith J.T. (ed.), Beta-Lactamases, Academic Press, Inc., (London), Ltd., London.

Doran, P.M., 1995. Bioprocess Engineering Principles, Academic Press Ltd., San Diego.

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

Fogarty, W.M. and Kelly, C.T., 1990. Microbial Enzymes and Biotechnology, 2nd ed., Elsevier Applied Sciences, London and New York.

Gacesa P. and Hubble J., 1987. Enzyme Technology, Open University Press, Oxford.

Glazer, A.N. and Nikaido, H., 1995. Microbial Biotechnology, W.H. Freeman and Company, New York.

Glick, B.R. and Pasternak J.J., 1998. Molecular Biotechnology, 2nd ed., ASM Press, Washington, D.C.

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

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.

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

Kuennen, R.W., Simone, M.J., Kreishman, G.P., 1980. The Temperature Dependence of the Production of Penicillinase in *Bacillus cereus* 569/H., Bioelectrochemistry and Bioenergetics, 7, 659-670.

Kushner, D.J. and Breuil, C., 1977. Archives of Microbiology, 112, 219-223.

Kwon, S., Kim, S., Kim, E., 1996. Effects of Glycerol on β-lactamase Production during High Cell Density Cultivation of Recombinant *Escherichia coli*, Biotechnology Progress, 12, 205-208.

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

Matthews, H.R., Freedland, R., Miesfeld, R.L., 1997. Biochemistry: A Short Course, Wiley-Liss Inc., New York.

Mehta, R.J. and Nash, C.H., 1978. Journal of Antibiotics, 31, 239-240.

Miller, G.L., 1959. Use of Dinitrosalicyclic Acid Reagent for Determination of Reduced Sugar, Analytical Chemistry, 31, 426-428.

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

Nielsen, J. and Villadsen, J., 1994. Bioreaction Engineering Principles, Plenum Press, New York.

Pollock, M.R., 1957. Activity and Specificity of Inducers of Penicillinase Production in *Bacillus cereus*, Strain NRRL-569, Biochemical Journal, 66(3), 419-428.

Pollock, M.R., 1960. Penicillinase. In: Boyer P.D., Lardy H., Myrback K., The Enzymes, 2nd ed., Academic Press, New York.

Pollock M.R., 1965. Purification and Properties of Penicillinases from Two Strains of *Bacillus licheniformis*: a Chemical, Physicochemical and Physiological Comparison, Biochemical Journal, 94, 666-675.

Priest F.G., 1993. Sytematics and Ecology of *Bacillus*. In: Sonenshein, A.L., Hoch, J.A. and Losick, R. (eds.), *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Phsiology, and Molecular Genetics, American Society for Microbiology, Washington D.C.

Rainer, B.W., 1990. Determination Methods of the Volumetric Oxygen Transfer KLa in Bioreactors, Chem. Biochem. Eng. Q., 4(4), 185-186.

Samuni, A., 1975. A Direct Spectrophotometric Assay and Determination of Michealis Constants for the β-Lactamase Reaction, Analytical Biochemistry, 63, 17-26.

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

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.

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.

Senter, P.D. and Springer C.J., 2001. Selective Activation of Anticancer Prodrugs by Monoclonal Antibody-Enzyme Conjugates, Advanced Drug Delivery Reviews, 53, 247-264.

Shuler, M.L. and Kargi, F., 1992. Bioprocess Engineering: Basic Concepts, Prentice Hall Inc., New Jersey.

Smyth, T.P., O'Donnell, M.E., O'Connor, M.J., St. Ledger, J.O., 2000. β-Lactamase Dependent Prodrugs-Recent Developments, Tetrahedron, 56, 5699-507.

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

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.

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

APPENDIX A

Calibration of *Bacillus licheniformis* Concentration

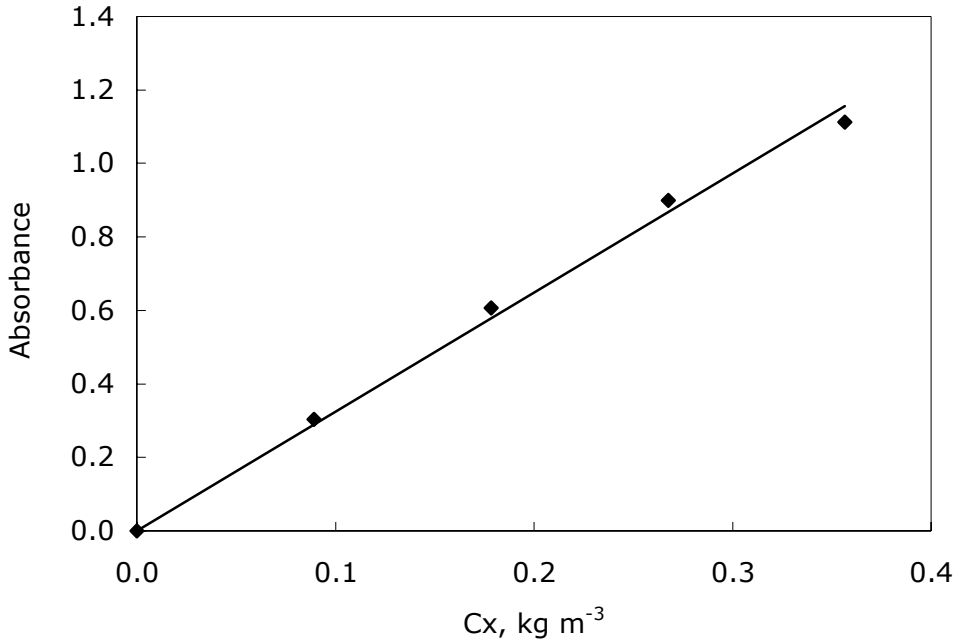


Figure A.1 Calibration curve for *Bacillus licheniformis* concentration

Slope of the calibration curve, m=3.240 1/kg m^{-3} (λ=600 nm)

$$Cx = \frac{Absorbance}{3.240} \times DilutionRate$$

APPENDIX B

Calibration of Beta-Lactamase Activity

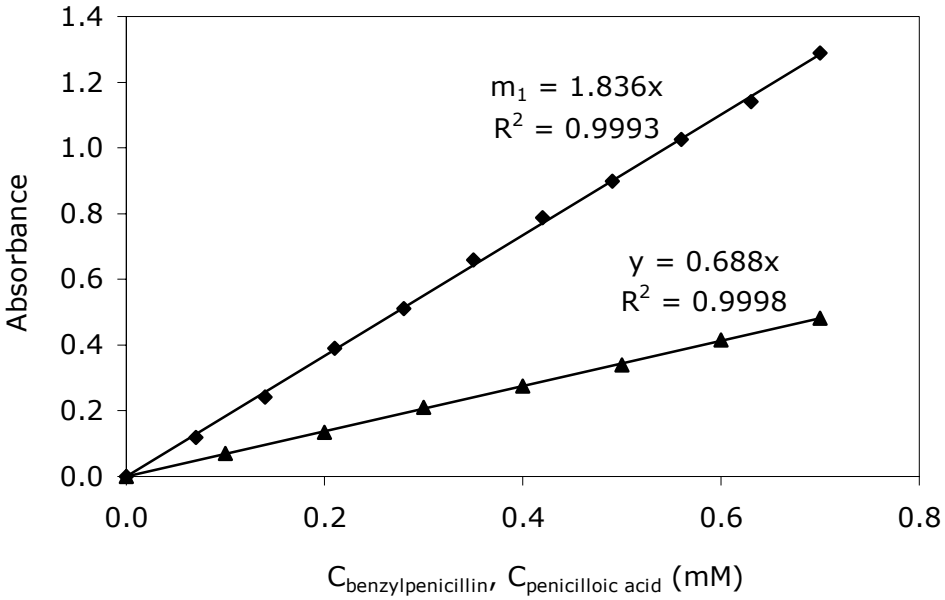


Figure A.2 Calibration Curve of the benzylpenicillin and penicilloic acid in 0.1 M phosphate buffer, pH=7.0, T=30°C, λ=232nm. Benzylpenicillin, (♦); Penicilloic acid, (▲).

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 A_λ mM^{-1}. The activity, U cm^{-3} is given by,

$$A = \frac{A_\lambda}{1.148 A_\lambda mM^{-1}} x \frac{1U}{10^{-3} mmol} x \frac{1l}{10^3 cm^3} xDilutionRatio$$

APPENDIX C

Calibration of Reduced Sugar Concentration

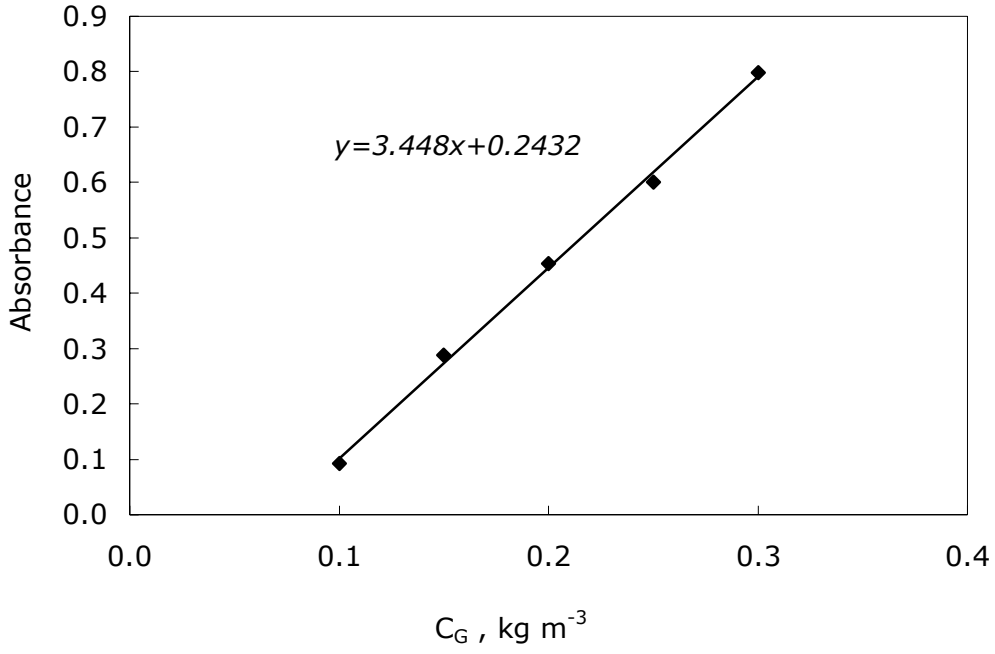


Figure A.3 Calibration Curve of the DNS solution

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

APPENDIX D

Preparation of DNS Solution

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

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

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

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

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

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

The solution obtained from the first step is mixed with that from the second step and then they are stirred until ROCHELLE salt is dissolved. The prepared solution is kept in dark-colored bottle at 4°C and it should be used after 48 h.