

FEEDING STRATEGY DEVELOPMENT FOR BENZALDEHYDE LYASE  
PRODUCTION BY RECOMBINANT *Escherichia coli* BL21

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**FEEDING STRATEGY DEVELOPMENT FOR BENZALDEHYDE LYASE  
PRODUCTION BY RECOMBINANT *Escherichia coli* BL21**

Submitted by **HANDE LEVENT** in partial fulfillment of the requirements for the degree of **Master of Science in Chemical Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen

Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Gürkan Karakaş

Head of Department, **Chemical Engineering, METU**

Prof. Dr. Pınar Çalık

Supervisor, **Chemical Engineering Dept., METU**

**Examining Committee Members:**

Prof. Dr. Tunçer H. Özdamar

Chemical Engineering Dept., Ankara University

Prof. Dr. Pınar Çalık

Chemical Engineering Dept., METU

Prof.Dr. Ufuk Bakır

Chemical Engineering Dept., METU

Prof. Dr. Tülin Kutsal

Chemical Engineering Dept., Hacettepe University

Assist.Prof.Dr. Nuray Oktar

Chemical Engineering Dept., Gazi University

**Date: 03.06.2008**

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Name, Last Name: Hande LEVENT

Signature :

## ABSTRACT

### FEEDING STRATEGY DEVELOPMENT FOR BENZALDEHYDE LYASE PRODUCTION BY RECOMBINANT *Escherichia coli* BL21

Levent, Hande

M.S., Department of Chemical Engineering

Supervisor: Prof. Dr. Pınar Çalık

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This study focuses on the molasses based complex medium design for benzaldehyde lyase production by recombinant *E. coli* BL21 and development of a feeding strategy based on the designed complex medium. For this purpose, firstly, the effects of molasses were investigated in laboratory scale bioreactors. As *E. coli* BL21 was not able to utilize sucrose, molasses was pretreated and hydrolyzed to fructose and glucose. Thereafter, effect of pretreated molasses concentration was investigated in the range of 16 to 56 kg m<sup>-3</sup> by batch-bioreactor experiments; and the highest cell concentration and benzaldehyde lyase activity were obtained as  $C_x=5.3$  kg m<sup>-3</sup> and  $A=1617$  U cm<sup>-3</sup>, respectively, in the medium containing 7.5 kg m<sup>-3</sup> glucose and 7.5 kg m<sup>-3</sup> fructose. Then, different feeding strategies were developed to produce efficient cells with high concentration and BAL activity. In the first strategy, after 10 hours of batch-cultivation with molasses based medium having 7.5 kg m<sup>-3</sup> glucose and 7.5 kg m<sup>-3</sup> fructose concentration, based on the airflow rate, pretreated molasses was fed to the system. When air flow rate decreased considerably, fed was given to the system that results in increase in glucose and fructose concentration in the medium to 2.5 kg m<sup>-3</sup>. At the end of the

process, the highest cell concentration obtained was  $C_x=7.4 \text{ kg m}^{-3}$ . The maximum activity was reached at 20<sup>th</sup> hour as  $A=2360 \text{ U cm}^{-3}$ . On the other hand, as air flow variation only demonstrated the absence of glucose not fructose, a second strategy, based on the detection of the fructose and glucose concentrations during the process, was applied. In this strategy when glucose and fructose were depleted, fed was given to the system that results in increase in glucose and fructose concentration in the medium to  $2.5 \text{ kg m}^{-3}$ ; and the highest BAL activity was obtained as  $2370 \text{ U cm}^{-3}$  at  $t= 26 \text{ h}$  where the cell concentration was  $7.5 \text{ kg m}^{-3}$ . At the last strategy, when glucose and fructose were depleted, fed was given to the system that results in increase in  $C_{\text{Glucose}}=1.5 \text{ kg m}^{-3}$  and  $C_{\text{Fructose}}=1.5 \text{ kg m}^{-3}$  in the production medium to decrease the accumulation of acetic acid. By this strategy highest cell concentration was obtained as  $8.04 \text{ kg m}^{-3}$  at  $t=24 \text{ h}$  and the highest BAL activity was  $2315 \text{ U cm}^{-3}$ . These strategies could be accepted having the same BAL activity with little distinctions. However, cell concentration of the last one was higher than others and also the lowest amount of carbon source was used. Thus, last one could be chosen as the most favorable strategy.

**Keywords:** Benzaldehyde Lyase, Production, Molasses, Fed Batch Operation, Recombinant *E. coli*.

## ÖZ

### REKOMBİNANT *Escherichia coli* BL21 İLE BENZALDEHİT LİYAZ ÜRETİMİ İÇİN BESLEME STRATEJİSİ GELİŞTİRİLMESİ

Levent, Hande

Yüksek Lisans, Kimya Mühendisliği Bölümü

Tez Yöneticisi: Prof. Dr. Pınar Çalık

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Bu çalışmada, rekombinant *E. coli* BL21 ile benzaldehide liyaz üretimi için melas temelli kompleks ortam tasarlanmış ve tasarlanan ortam temel alınarak besleme stratejisi geliştirilmiştir. Bu amaçla, ilk olarak, melas derişimi etkisi, laboratuvar ölçekli biyoreaktörlerde incelenmiştir. *E. coli* BL21 sukrozu tüketemediği için, kimyasal önışlem uygulanarak melas fruktoz ve glukozu indirgenmiş ve melas derişiminin etkisi  $16 \text{ kg m}^{-3}$  ile  $56 \text{ kg m}^{-3}$  aralığında incelenmiştir. En yüksek hücre derişimi ve benzaldehit liyaz aktivitesi  $C_{\text{Glukoz}}=7.5 \text{ kg m}^{-3}$  ve  $C_{\text{Fruktoz}}=7.5 \text{ kg m}^{-3}$  derişimlerinde sırasıyla  $5.3 \text{ kg m}^{-3}$  ve  $1617 \text{ U cm}^{-3}$  olarak bulunmuştur. Sonraki aşamada, yüksek hücre derişimi ve BAL üretimine ulaşabilmek için besleme stratejileri geliştirilmiştir. Optimum koşullarda, kesikli biyoreaktör işletiminde  $t=10 \text{ st}$ 'te hava giriş hızının minimuma ulaştığı gözlenmiştir. Bu durum ortamdaki karbon kaynağının bittiğini gösterdiği varsayıldığı için birinci stratejide besleme yapılarak glukoz ve fruktozun  $2.5 \text{ kg m}^{-3}$  e çıkması sağlanmıştır. Bu strateji proses süresince tekrarlanmıştır. En yüksek hücre derişimi  $t=24 \text{ st}$ 'te  $C_x=7.4 \text{ kg m}^{-3}$ , en yüksek BAL aktivitesi ise  $t=20 \text{ st}$  'te  $A=2360 \text{ U cm}^{-3}$  olarak elde edilmiştir. Ancak hava giriş hızının değışimine bağıli geliştirilen strateji yalnızca glukozun tüketildiğini

gösterdiği sonucuna varıldığı için, glukoz ve fruktoz derişimi proses süresince izlenerek yeni bir strateji geliştirilmiştir. Yeni stratejide glukoz ve fruktoz tüketildiğinde derişimleri üretim ortamında  $2.5 \text{ kg m}^{-3}$  e çıkarılmış; en yüksek BAL aktivitesi  $t=26 \text{ st'te } A=2370 \text{ U cm}^{-3}$  olarak bulunmuştur. Bu noktadaki hücre derişimi ise  $C_x=7.5 \text{ kg m}^{-3}$ ' tür. Asetik asit birikimini azaltmak için yapılan beslemelerin glukoz ve fruktoz derişimi  $1.5 \text{ kg m}^{-3}$  e düşürülmüştür. Bu son strateji ile  $t= 24 \text{ st'te}$  en yüksek BAL aktivitesi ve hücre derişimi sırasıyla  $A=2315 \text{ U cm}^{-3}$  ve  $C_x=8.04 \text{ kg m}^{-3}$  olarak bulunmuştur. İncelenen stratejilerin hepsinde aktiviteler yaklaşık aynı olmasına rağmen son koşulda en yüksek hücre derişimine en az girdi kullanılarak ulaşılmıştır.

**Anahtar Kelimeler:** Benzaldehit Liyaz, Üretim, Melas, Ön-işlem, Yarı-kesikli İşletim, Rekombinant *E. coli*.

To My Family

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## NOMENCLATURE

A	Benzaldehyde lyase activity, U cm <sup>-3</sup>
A <sub>X</sub>	Specific Activity of Benzaldehyde lyase, U mg <sup>-1</sup> DW
C <sub>Ac</sub>	Acetic acid concentration, kg m <sup>-3</sup>
C <sub>OA</sub>	Organic acid concentration, kg m <sup>-3</sup>
C <sub>O</sub>	Dissolved oxygen concentration, mol m <sup>-3</sup> ; kg m <sup>-3</sup>
C <sub>O</sub> <sup>*</sup>	Oxygen saturation concentration, mol m <sup>-3</sup> ; kg m <sup>-3</sup>
C <sub>G</sub>	Glucose concentration, kg m <sup>-3</sup>
C <sub>F</sub>	Fructose concentration, kg m <sup>-3</sup>
C <sub>G</sub>	Glucose concentration, kg m <sup>-3</sup>
C <sub>M</sub>	Molasses concentration, kg m <sup>-3</sup>
C <sub>P</sub>	Product concentration, kg m <sup>-3</sup>
C <sub>S</sub>	Concentration of the substrate, mM; kg m <sup>-3</sup>
C <sub>X</sub>	Cell concentration, kg dry cell m <sup>-3</sup>
Da	Damköhler number (=OD / OTR <sub>max</sub> ; Maximum possible oxygen utilization rate per maximum mass transfer rate)
E	Enhancement factor (=K <sub>La</sub> / K <sub>La0</sub> ); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
K <sub>La0</sub>	Physical overall liquid phase mass transfer coefficient; s <sup>-1</sup>
K <sub>La</sub>	Overall liquid phase mass transfer coefficient; s <sup>-1</sup>
K <sub>m</sub>	Michealis constant, mM
N	Agitation rate, min <sup>-1</sup>
m <sub>0</sub>	Rate of oxygen consumption for maintenance, kg oxygen kg <sup>-1</sup> dry cell weight h <sup>-1</sup>
m <sub>S</sub>	Maintenance coefficients for substrate, kg substrate kg <sup>-1</sup> dry cell weight h <sup>-1</sup>
pH <sub>c</sub>	Controlled pH
Q <sub>o</sub>	Volumetric air feed rate, m <sup>3</sup> min <sup>-1</sup>
q <sub>o</sub>	Specific oxygen uptake rate, kg kg <sup>-1</sup> DW h <sup>-1</sup>
q <sub>s</sub>	Specific substrate consumption rate, kg kg <sup>-1</sup> DW h <sup>-1</sup>
r	Volumetric rate of reaction, mol m <sup>-3</sup> s <sup>-1</sup>

$r_{\max}$	Maximum rate of reaction, $\text{mol m}^{-3} \text{s}^{-1}$
$r_0$	Oxygen uptake rate, $\text{mol m}^{-3} \text{s}^{-1}$ ; $\text{kg m}^{-3} \text{h}^{-1}$
$r_X$	Rate of cell growth, $\text{kg m}^{-3} \text{h}^{-1}$
T	Bioreaction medium temperature, $^{\circ}\text{C}$
t	Bioreactor cultivation time, h
U	One unit of an enzyme
$V_R$	Volume of the bioreaction medium, $\text{m}^3$
$Y_{X/S}$	Yield of cell on substrate, $\text{kg kg}^{-1}$
$Y_{X/O}$	Yield of cell on oxygen, $\text{kg kg}^{-1}$
$Y_{S/O}$	Yield of substrate on oxygen, $\text{kg kg}^{-1}$
$Y_{P/X}$	Yield of product on cell, $\text{kg kg}^{-1}$
$Y_{P/S}$	Yield of product on substrate, $\text{kg kg}^{-1}$
$Y_{P/O}$	Yield of product on oxygen, $\text{kg kg}^{-1}$

#### *Greek Letters*

$\eta$	Effectiveness factor (=OUR/OD; the oxygen uptake rate per maximum possible oxygen utilization rate)
$\mu$	Specific cell growth rate, $\text{h}^{-1}$
$\mu_{\max}$	Maximum specific cell growth rate, $\text{h}^{-1}$
$\lambda$	Wavelength, nm

#### *Abbreviations*

BAL	Benzaldehyde lyase
<i>bal</i>	Gene of Benzaldehyde Lyase
DO	Dissolved oxygen
EC	Enzyme Commission
OD	Oxygen demand ( $=\mu_{\max} C_X / Y_{X/O}$ ; $\text{mol m}^{-3} \text{s}^{-1}$ )
OUR	Oxygen uptake rate, $\text{mol m}^{-3} \text{s}^{-1}$
OTR	Oxygen transfer rate, $\text{mol m}^{-3} \text{s}^{-1}$
$\text{OTR}_{\max}$	Maximum possible mass transfer rate ( $=K_L a C_O^*$ ; $\text{mol m}^{-3} \text{s}^{-1}$ )
TCA	Tricarboxylic acid

## **CHAPTER 1**

### **INTRODUCTION**

The biotechnology industry is one of the fastest growing industrial sectors, and in recent years many new products has been launched. Thus, many new pharmaceuticals are currently produced based on growth of microbial and cell cultures. The exploitation of cell cultures for production of industrial products involves growth of the cells in so called bioreactors and this is often referred as fermentation process (Nielsen and Villadsen, 2003). The living cell is the site of many chemical reactions, most of which require catalysts if they are to proceed at a sufficient rate under physiological conditions for the requirements of the cell. One of the most important functions of proteins is as protein catalysts or enzymes.

Enzymes are proteins that catalyze the biological reactions. They increase the reaction rate, without any permanent change in their structure; by reducing the activation energy of the reaction (Watson et al., 1987).The molecule to which they bind and create a complex together is called substrate. Enzymes are specific to their substrates. Their specificity is due to their three dimensional conformation and it differs for each enzyme. Absolutely specific enzymes can react with only one substrate where group specific ones can work with similar molecules that have same functional group. Enzymes are very efficient catalysts. Enzymes do not require elevated temperatures to promote reaction nor do they require that the reactions they catalyze take place in acid, alkali or non-aqueous solution. Enzyme reactions take place at body temperature and, with a few exceptions, take place in aqueous solution near pH 7. No material is wasted in side reactions. They are biodegradable and environmentally acceptable. The yield from enzyme-catalyzed reactions is usually only limited by the equilibrium point of the reaction being catalyzed. Furthermore some enzymes require the presence of another molecule, called

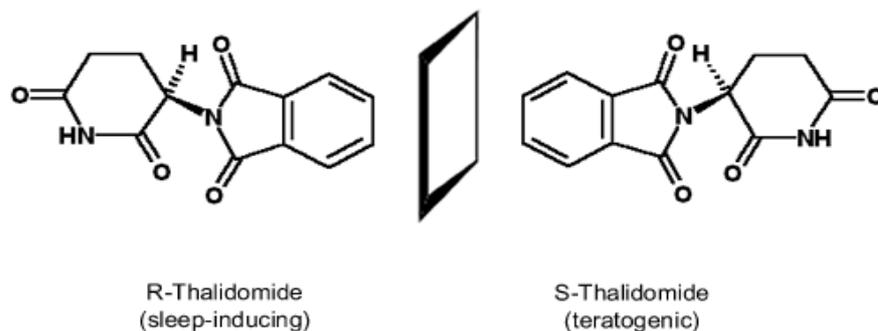
cofactor. Some metal ions such as  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Co}^{2+}$  can serve as cofactors. Moreover some vitamins can be converted to cofactors and help enzymes to function (Watson et al., 1987).

The polypeptide chain of enzyme is folded in such a way that the active site on the surface of the enzyme acts as a keyhole for a specific substrate which results in specificity property of the enzyme. Enzymes show three major types of selectivities:

1. Chemoselectivity: Enzyme exhibits specificity on a single type of a functional group.
2. Regioselectivity and Diastereoselectivity: Enzyme acts on a functional groups, which are situated in different regions of the same substrate.
3. Enantioselectivity: Certain enzymes can discriminate the differences between the stereoisomer compounds (Scheve, 1984).

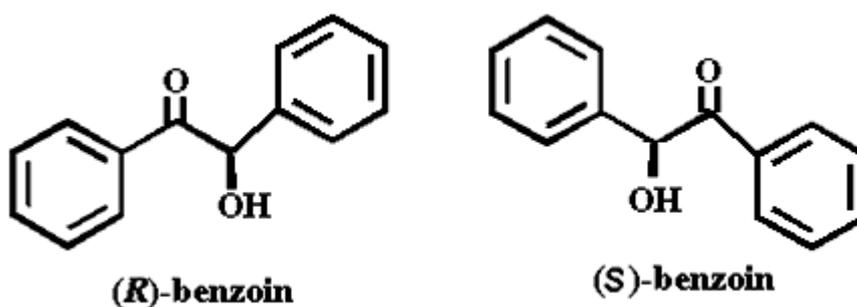
Molecular recognition, especially enantiomer recognition, is a key to the biological specificity of many current drugs. Enantiopure compounds for pharmaceutical intermediates and other uses is currently a rapidly growing multibillion business worldwide (Rouhi, 2004). Therefore it has become increasingly important for chiral products to be marketed as a single enantiomer rather than racemic mixture. This need gave rise to the development of new methods that provide only a single enantiomer.

The enzymes are the most convenient means of producing single enantiomers of a compound since the enzyme substrate binding mechanism and chirality both depends on the three dimensional conformation of the molecules. Enantioselective enzymes will process only one enantiomer while giving no product with the other. These enzymes can be used in the synthesis of enantiopure products such as drugs. For instance, R-Thalidomide is an important sleep inducing drug, while S- enantiomer of Thalidomide is teratogenic (Figure 1.1). Hence, the development of enantiomerically pure compounds are becoming increasingly more essential in the production of pharmaceuticals, agrochemicals (e.g., pesticides, fungicides, herbicides), and flavors. Hence, enantioselectivity is the most important property that enzymes display (Adam et al., 1999). To be able to obtain the target enantiomer can improve the economics of the process and lead to reduced quantities of side reactions and thus to reduced environmental impact (Faber, 2000).



**Figure 1.1** R- and S- enantiomers of Thalidomide molecule (Friedman and Kimmel, 1999).

Benzaldehyde lyase (BAL, EC 4.1.2.38), which is the central element of this study, is an enzyme which is used for synthesis of enantio pure 2-hydroxyl ketones, which are an important class of compounds in natural product and drug synthesis. The enzyme realizes the cleavage of the carbon-carbon  $\alpha$ -hydroxy ketone bond of benzoin to form two benzaldehydes and it works with R-benzoin while giving no product with S-benzoin (Figure 1.2), (Demir et al., 2002).



**Figure 1.2** R- and S- enantiomers of benzoin molecule (Demir et al., 2002).

BAL has been first described by Gonzales and Vicuna (1989). It was isolated from the strain *Pseudomonas Fluorescens Biovar I*, which was found in wood scraps in a cellulose factory. The authors established the requirement for thiamin diphosphate (ThDP) and a divalent cation such as  $Mg^{+2}$ . After that, Hinrichsen et al., (1994) first founded some information about the nucleotide sequence containing open reading frame encoding benzaldehyde lyase and cloned the gene to an *Escherichia coli* HB101 using vector pUC18. The sequence was later corrected and the substrate specificity was studied with modified pUC18::*bal* plasmid, where hexa histidine was inserted to the C-terminus of the mature gene fragment and expressed in *E. coli* SG130009, by Pohl et al., (2002).

Demir et al. (2001) has firstly described the reaction mechanism and reversible conversion of R- benzoin to benzaldehyde. In further studies, the substrate range of the enzyme was expanded (Demir et al., 2002, 2003, 2004; Dünkemann et al., 2002; Sanchez et al., 2003).

Recently, the homotetramer structure of BAL has been determined by X-ray diffraction analysis (Mosbacher et al., 2005). According to sequence alignments and homology models (Kneen et al., 2005) the residue similarities between benzaldehyde lyase and other ThDP dependent enzymes, like benzoylformate decarboxylase (BFD) and pyruvate decarboxylase (PDC), were declared. At the same year, Maria et al., (2005) investigated the effects of cofactors, cosolvents, and pH on the stability and the activity of the enzyme.

A deeper study has been done by Janzen et al., (2006) and the stability of BAL with respect to pH, temperature, buffer salt, cofactors and organic cosolvents was studied. The reaction temperature has been declared 37 C° as maximum and the enzyme was stable between pH 6 and 8, with pH 8 being optimum.

In the more recent study, Mikolajek et al., (2007) was investigated a carboligation for the first time as an enzymatic gas phase reaction, where benzaldehyde was converted to benzoin using thiamine diphosphate (ThDP)-dependent enzymes, namely benzaldehyde lyase (BAL) and benzoylformate decarboxylase (BFD).

The effects of bioprocess operation parameters were investigated by Çalık et al. (2004, 2006); using the defined medium with glucose and  $(NH_4)_2HPO_4$  as the sole carbon and nitrogen sources, respectively, the highest BAL activity was obtained at 0.5 vvm,  $500min^{-1}$  as  $860 U cm^{-3}$  with a cell

concentration of  $2.3 \text{ kg m}^{-3}$  at uncontrolled pH of 7.2, using the recombinant *E. coli* K12 carrying pUC18::*bal* plasmid.

In the master thesis study of Kaya (2006), the gene has been inserted into another, more effective plasmid, namely pRSETA, which has T7 promoter enabling more effective expression of the gene. The host cell *E. coli* BL21 (DE3) pLySs strain was utilized, which was specifically designed for T7 regulated genes. Hence, by using the developed recombinant *E. coli* BL21 (DE3) pLySs cells, the effect of glucose and  $(\text{NH}_4)_2\text{HPO}_4$  and their concerted effects were investigated in the production of recombinant benzaldehyde lyase. Thereafter, in the thesis study of Anghardi (2007), the effects of oxygen transfer conditions were examined on the synthesis of benzaldehyde lyase in order to increase the BAL activity by the recombinant *E. coli* BL21 (DE3) pLySs. Also, in this study, by using multiple regression technique, mathematical model was proposed to estimate the benzaldehyde activity as function of time, agitation rate, cell concentration, dissolved oxygen concentration, and by- product concentration.

Molasses, which is the by-product of sugar-beet or sugarcane extraction processes, is among the most important raw materials of the fermentation industry, especially for the production of baker's yeast, citric acid, feed yeasts, acetone/butanol, organic acids, amino acids, antibiotics, and enzymes. However, in order to use it in processes as carbon source, molasses might need to be pretreated with the removal of the inhibitors (Cejka, 1985). For this reason, Çalık et al. (2001, 2003) studied the use of molasses in glutamic acid and serine alkaline protease (SAP) production processes and explained the use of molasses with pretreatment conditions.

In spite of the industrial importance of BAL, most of the literature study examines the production of BAL using defined media. There is not any account available in the literature dealing with the complex media involving molasses for BAL production.

*Escherichia coli* remains the best established production organism in industrial biotechnology and aerobic high cell density cultures of *E. coli* are most frequently used to arrive at high mass yields and high protein concentrations. However, during aerobic fermentation runs at high growth rates, considerable amounts of acetate are accumulated as described by Akesson et al., (1999). Acetate production is a loss of carbon therefore an economic sink and also it is inhibitory to recombinant protein production and inhibits cell growth (Shiloach et al., 1995). The level of acetate produced

during aerobic fermentations is depending on the *E.coli* strain, the growth conditions, the actual glucose concentration in the medium and the overall composition of the fermentation medium. Fed batch fermentations are preferred as a mode of operation as in that strategy carbon source concentration could be limited. In the literature, several specific fermentation strategies to reduce acetate production levels are mentioned. These strategies include various glucose feeding approaches (Lee, 1996; Riesenber et al., 1999), limitations of growth by substrate limited fed batch schemes (Akesson et al., 2001; Lee et al., 1999), and utilization of alternative feeds such as glycerol (Pflug et al., 2007). All the studies in the literature about BAL were based on the batch operations (Çalık et al., 2004 & 2006) although fed batch operation gives a lot of advantages listed above when compared with others.

For this purpose, in this work, in order to increase BAL production further, molasses based complex medium was designed and thereafter a feeding strategy was developed which can be used to avoid overflow metabolism. This is the first study using molasses based medium for *E.coli* cultivation and also the first study applying fed batch strategy for BAL production.

## CHAPTER 2

### LITERATURE SURVEY

#### 2.1 Enzymes

##### 2.1.1 General Characteristics

Catalysts increase the rate of otherwise slow or imperceptible reactions, without undergoing any net change in their structure. The early development of the concept of catalysis in the nineteenth century went hand in hand with the discovery of powerful catalysts from biological sources. These were called enzymes and were later found to be proteins. They mediate all synthetic and degradative reactions carried out by living organisms. They are very efficient catalysts for that reason they are being employed increasingly in today's high technological society as a part of biotechnological expansion. Their utilization has created a billion dollar business, including a wide diversity of industrial processes (Chaplin and Bucke, 1992).

All enzymes are proteins. As catalysts, they are characterized by high substrate and functional specificities. The substrate specificity means that the enzyme catalyzes the reaction of only one chemical compound or a group of chemically very similar compounds. The functional specificity is expressed by the fact that an enzyme catalyzes only one definite reaction and the substrate undergoes a definite conversion with high stereo and regiospecificities.

With the exception of solid-phase enzyme synthesis, all enzymes are derived from living sources. Although all living cells produce enzymes, one of the three sources –plant, animal, or microbial- may be favored for a given enzyme or utilization (Bailey and Ollis, 1986).

### **2.1.2 Enzyme Activity**

When the enzyme is part of a crude preparation, its concentration is in terms of 'units'. A unit is the amount of enzyme that gives a predetermined amount of catalytic activity under specific conditions (Shuler and Kargi, 2002).

Enzyme activity, expressed as activity unit (U), is defined by the Commission on Enzymes as follows: One unit of enzyme activity is defined as the amount which will catalyze the transformation of one mole of substrate per minute under defined conditions (Chaplin and Bucke, 1990).

Activity of the enzymes can be altered by environmental factors, such as temperature and pH, and the presence and concentration of substrate, cofactors and products.

### **2.1.3 Enantioselectivity**

Despite being composed of exact same atoms, chiral molecules draw extremely different biological responses in living organisms because of their three dimensional arrangement. These two molecules are nonsuperimposable mirror images of each other and called enantiomers. As an example; S-naproxen is an important anti-inflammatory drug while its enantiomer R-naproxen, is a liver toxin (Hart et al., 1999).

Because of their identical structures, enantiomers have identical chemical properties except towards chiral reagents and receptors which are usually enantioselective i.e. they prefer to interact with one enantiomer over the other, in much the same way that a right-handed glove will not easily fit a left hand (Pang, 1989).

### **2.1.4 Intracellular and Extracellular Enzymes**

For industrial applications, enzymes are produced by the microorganisms. One of the most important characteristic is the way this happens. There are two possibilities. The microorganism can produce and use it in the cell (intracellular enzyme) or secrete it for using it outside the cell (extracellular enzyme). Extracellular enzymes have advantages in industrial applications over the intracellular enzymes. They are cheaper and easier to isolate and purify, more stable in the production medium. Intracellular enzymes are more fragile and sensitive to operation conditions, these make them commercially less attractive; however, they are much more common in nature than extracellular enzymes (Kirk and Othmer, 1994).

### 2.1.5 Nomenclature of Enzymes

Many enzymes have been named by adding the suffix “-ase” to the name of their substrate or to a word or phrase describing their activity. Sometimes the same enzyme has two or more names, or two different enzymes have the same name. Because of such ambiguities, and the ever increasing number of newly discovered enzymes, biochemists, by international agreement, have adopted a system for naming and classifying enzymes. This system divides enzymes into six classes, each with subclasses, based on the type of reaction catalyzed. Each enzyme is assigned a four-part classification number (enzyme commission, EC) and a systematic name, which identifies the reaction it catalyzes (Nelson et al., 2005). International Union of Biochemistry –The Enzyme Commission (EC) report defines how enzymes should be designated. Each enzyme has a four digit number in the form of:

EC A.B.C.D.

EC stands for the Enzyme Commission, while A, B, C and D denote specific properties of the enzyme.

A: Main type of reaction which the enzyme catalyzes

B: Substrate type

C: Nature of co-substrate

D: Individual enzyme number

Main reaction types are designated by the first number (A) showing the international classification of enzymes:

1) Oxidoreductases: Catalyze oxidation and reduction reactions by the transfer of hydrogen and/ or electrons.

2) Transferases: Catalyze the transfer of certain groups from a donor molecule to a suitable acceptor molecule.

3) Hydrolases: Catalyze hydrolytic reactions.

4) Lyases: Catalyze cleavage reactions nonhydrolytically, leaving a double bond, or catalyze the addition of groups to double bonds.

5) Isomerases: Catalyze reversible transformations of isomeric compounds.

6) Ligases : Catalyze the covalent linkage of the molecules with simultaneous cleavage of an energy- rich bond.

For example, the EC number of benzaldehyde lyase is EC 4.1.2.38, which catalyzes the cleavage and synthesis of benzoin:

EC 4. Lyases  
EC 4.1. Carbon-Carbon Lyases  
EC 4.1.2. Aldehyde-Lyases  
EC 4.1.2.38. Benzaldehyde lyase

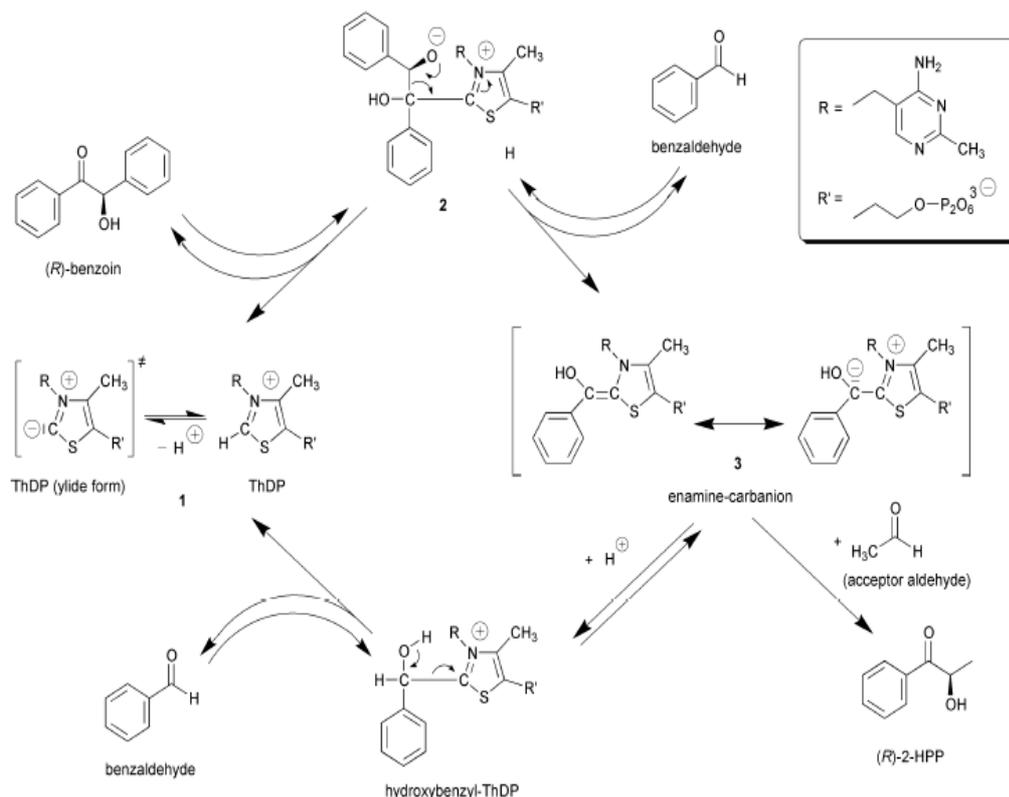
## **2.2 Benzaldehyde Lyase**

### **2.2.1 Characterization**

Benzaldehyde lyase (BAL, EC 4.1.2.38) is a thiamin diphosphate (ThDP) dependent enzyme. It was first detected in *Pseudomonas fluorescens* biovar I. It is shown that *Pseudomonas fluorescens* biovar I can grow on benzoin as a sole carbon and energy source, due to the ability of BAL to catalyze the cleavage of the acyloin linkage of benzoin yielding benzaldehyde (Gonzalez and Vicuna, 1989). In this study, the purification and the characterization of the enzyme were described and the ability of BAL to catalyze the cleavage of acyloin linkage of benzoin to form benzaldehyde was irreversible described. It was also stated that benzaldehyde lyase is highly specific requiring ThDP for catalytic activity maximum at 0.01 mM, while ThDP concentrations higher than 0.5 mM were inhibitory. The enzyme showed maximal activity between pH 7.5 and 8.5, whereas it was inactive below pH 6.0. Hinrichsen et al., (1994) cloned the BAL gene into pUC18 plasmid and expressed in *E. coli* HB101 in 1994. In this study, the nucleotide sequence contains open reading frame encoding benzaldehyde lyase and the location of the gene was determined. However, there was some conflicting points in DNA sequence hence it was corrected and submitted; and for easier purification, hexa-histidine tag was fused to the C-terminus of the enzyme and expressed in *E. coli* SG130009 by Pohl et al. (2002).

### **2.2.2 Enantioselective Reactions catalyzed by BAL**

Benzaldehyde lyase is an enantioselective enzyme that only converts R-benzoin into benzaldehyde lyase (Demir et al., 2001). With this property of the enzyme is used for the synthesis of enantiopure  $\alpha$ -hydroxy ketones, an important class of compounds in natural product and drug synthesis. The catalytic mechanism of the enzyme is shown in Figure 2.1.



**Figure 2.1** Benzaldehyde lyase catalyzed cleavage and synthesis of benzoin. The first step of the catalytic cycle is the attack of ylide form of ThDP on the carbonyl carbon of (R) - benzoin to produce an adduct. The enamine, intermediate product of ThDP dependent enzymes catalyzing the formation of 2-hydroxy ketones, and the first free aldehyde molecule is formed. Protonation of this intermediate then releases the second molecule of aldehyde and restores the cofactor. In the presence of an acceptor aldehyde and enamine intermediate is able to undergo a C-C bond formation reaction. Since BAL catalysis this reaction reversibly, cleavage and formation of (R) - benzoin reactions are in equilibrium (Demir et al., 2001).

Following studies investigated the catalytic activity and substrate range of benzaldehyde lyase like the enantioselective C-C bond cleavage and formation to generate (R)- and (S)-benzoin and (R)-2hydroxypropiophenone ((R)-2HPP) derivatives (Demir et al., 2001); C-C bond formation from aromatic aldehydes and acetaldehyde (Demir et al., 2002) and methoxy- and dimethoxy-acetaldehydes (Demir et al., 2003) on a preparative scale in buffer/DMSO solution; asymmetrical synthesis of mixed benzoin

(Dünkemann et al., 2002); mixed acyloin condensation between methoxy-substituted benzaldehydes and phenylacetaldehyde (Sanchez et al., 2003); and hydroxymethylation of aromatic aldehydes with formaldehyde (Demir et al., 2004).

In order to evaluate the industrial applicability of BAL-catalyzed carboligations, Maria et al., (2005) investigated the influence of cofactors, co solvents, and especially the pH-influence on the stability and activity of BAL. It was shown that 30% DMSO content in the presence of potassium phosphate buffer with 0.5mM  $Mg^{+2}$ , 0.5 mM ThDP and 1mM DTT, a well known stabilizer of hydrolyses, were the optimal for enzyme activity and stability. It was shown that the enzyme prepared in potassium phosphate buffer supplemented with ThDP,  $Mg^{2+}$ , DTT as a cofactor and 30% DMSO as a co solvent leaded to 50% activity loss after 30 h, while the enzyme losses its activity almost completely within 3 h in water. It was also shown that best enzymatic activity appeared when the pH of the reaction medium was increased from 8.0 to 9.5.

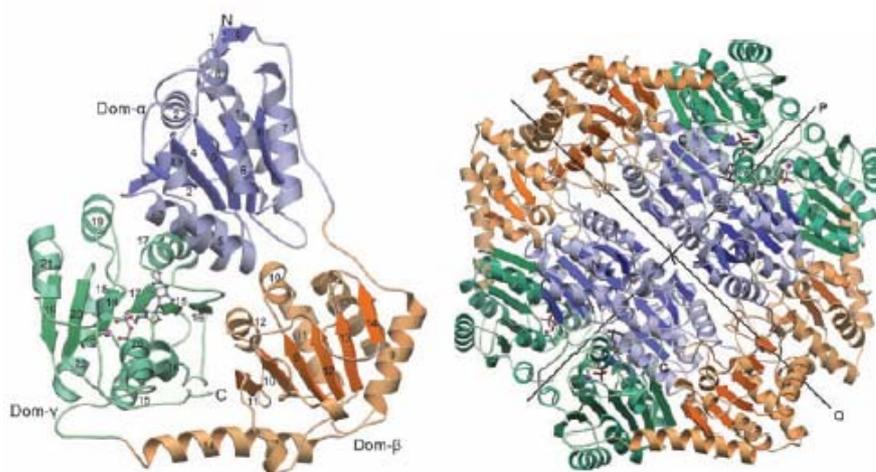
Janzen et al., (2006) studied the stability of BAL with respect to pH, temperature, buffer salt, cofactors and organic cosolvents. It was shown that the reaction temperature should not exceed 37 C° and the enzyme was stable between pH 6 and 8, with pH 8 being the pH-optimum of both the lyase and the ligase reactions. Potassium phosphate and Tris were identified as optimal reaction buffers and the addition of 20 vol% DMSO is useful to enhance both the solubility of aromatic substrates and products and the stability of BAL.

### 2.2.3 Structure

Kneen et al., (2005) have used a homology model of benzaldehyde lyase to identify several residues with the potential to be involved in substrate binding and catalysis. The X-ray structures of BFD and pyruvate decarboxylase (PDC) were used as templates for modeling benzaldehyde lyase.

Mosbacher et al., (2005) reported the crystal structure of BAL with bound cofactor ThDP at 2.6 Å resolution and determined the geometry of the reaction and explain the substrate specificity in structural terms. The results of X-ray diffraction firstly exposed that BAL is a homotetramer, where each subunit binds to one ThDP molecule using one  $Mg^{2+}$  ion. The enzyme is composed of 4 x 563 amino acid residues and has a molecular weight of 4 x 58919 Da. The BAL homotetramer has an overall size of approximately 95x95x75 Å<sup>3</sup>. No significant structural differences were found between the four crystallographically independent subunits of the tetramer (Figure 2.2). In the

study of Maraitte et al. (2007), BAL was crystallized in an alternative space group and its structure refined to a resolution of 1.65 Å, allowing detailed observation of the water structure. In addition to revealing the details of active-site interactions with the well defined cofactor, shows electron density for the co-crystallization agent 2-methyl-2,4-pentandiol at hydrophobic regions of the enzyme surface.



**Figure 2.2** Stereo ribbon plot of homotetramer structure of BAL. The cofactor ThDP is shown as a ball-and-stick model and  $Mg^{2+}$  as a sphere (Mosbacher et al., 2005).

#### 2.2.4 Kinetics

In the same year, BAL-catalyzed benzoin condensations were performed using an alternative reaction system with two immiscible phases of water and hexane by Hischer et al., (2005). The enzyme was entrapped in the aqueous phase, which was solidified by polyvinyl alcohol to ease handling and recovery of BAL. The entrapment process had an efficiency of 90%, no enzyme or cofactor was lost during reaction or storage. The entrapped enzyme was stable in hexane for 1 week at 4 °C and more than 1 month at -20 °C.

The reaction engineering of BAL was studied by Stillger et al. (2006) and based on kinetic studies a continuous process was developed. The coupling of

benzaldehyde and acetaldehyde has been successfully carried out in a continuous process in combination with membrane technology with high space time yields. A kinetic model was developed to simulate the continuously operated reactor and to determine optimal production conditions. In 2007, Hildebrand et al., (2007) reported the development of a reactor system that allows the selective production of substituted (R)-2-hydroxy-1-phenylpropan-1-one ((R)-HPP-) derivatives like BAL. This model was used to describe the HPP production in a continuously operated enzyme membrane reactor. The reactor type used combined the advantages of high conversion and excellent selectivity with high space-time yields and total turnover numbers.

The first kinetic study for carbonylation reaction by using ThDP-dependent enzymes was done Mikolajek et al. (2007) in a solid/gas bioreactor. By using the carbonylation of two propanal molecules as a test reaction. Benzaldehyde lyase (BAL) and benzoylformate decarboxylase (BFD) were compared in terms of enzymatic activity, enantioselectivity, biocatalyst stability and kinetics. The biocatalyst was immobilized per deposition on non-porous support. Some limitations of the gas/solid biocatalysis were discussed. For both enzymes, a kinetic model was developed, which correlated well with the experimental data.

### 2.2.5 Production

The studies in the literature on BAL production were published by Çalık et al. (2004, 2006). The host microorganism having the highest benzaldehyde lyase productivity was determined as *E. coli* K12 (ATCC 10798) carrying modified pUC18::*bal* plasmid where inducible hybrid *trc* promoter is present. Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as 1.8 kg m<sup>-3</sup> and 745 U cm<sup>-3</sup>, respectively, in the medium containing 8.0 kg m<sup>-3</sup> glucose, 5.0 kg m<sup>-3</sup> (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and the salt solution. Thereafter, the effects of uncontrolled-pH and controlled pH operations and effects of oxygen transfer conditions on benzaldehyde lyase productivity were systematically investigated using the recombinant *E. coli* K12 carrying pUC18::*bal* plasmid. Among the controlled-pH operations, the highest cell concentration and BAL activity were obtained as 2.1 kg m<sup>-3</sup> and 775 U cm<sup>-3</sup>, respectively at pH<sub>C</sub> 7.0; whereas they were 2.3 kg m<sup>-3</sup> and 860 U cm<sup>-3</sup>, respectively at 0.5 vvm, 500 min<sup>-1</sup> and uncontrolled pH 7.2 condition.

In the master thesis study of Kaya (2006), the benzaldehyde lyase production in *E. coli* BL21 (DE3) pLySs as intracellular and in *Bacillus* species as extracellular were investigated, and comparison of the production capacity of the enzyme in the developed recombinant microorganisms were compared. The gene has been inserted into another, more effective plasmid, namely pRSETA, which has T7 promoter enabling more effective expression of the gene. The host cell *E. coli* BL21 (DE3) pLySs strain was utilized, which was specifically designed for T7 regulated genes. Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as  $2.0 \text{ kg m}^{-3}$  and  $1060 \text{ U cm}^{-3}$ , respectively, in the medium containing  $20.0 \text{ kg m}^{-3}$  glucose,  $11.8 \text{ kg m}^{-3} (\text{NH}_4)_2\text{HPO}_4$  and the salt solution. Thereafter, oxygen transfer effects on benzaldehyde lyase production were investigated at air inlet rate of  $Q_{O_2}/V_R = 0.5 \text{ vvm}$ , and agitation rates of  $N=500$  and  $750 \text{ min}^{-1}$  and at  $Q_{O_2}/V_R = 0.7 \text{ vvm}$ ,  $N=750 \text{ min}^{-1}$  in pilot scale bioreactor and the highest cell concentration and volumetric BAL activity were found as  $1.7 \text{ kg m}^{-3}$  and  $990 \text{ U cm}^{-3}$ , respectively, at  $0.5 \text{ vvm}$ ,  $750 \text{ min}^{-1}$  condition. Furthermore, no extracellular production of benzaldehyde lyase was observed in the developed recombinant *Bacillus* species, probably because of ineffective secretion system, inefficient folding of heterologous protein, and degradation of enzyme due to proteolytic activity or high inactivation rate of the enzyme.

The same year, in the master thesis study of Büyüksungur (2006), benzaldehyde lyase gene from *Pseudomonas fluorescens* Biovar was cloned into *Pichia pastoris*, with the aim of the extra cellular production of the enzyme. It was showed that concentration of the tetrameric form of benzaldehyde lyase enzyme, active form, was much less than the monomeric form of the enzyme indicating that the enzyme produced by recombinant *P.pastoris* mostly could not fold into multimeric form in the fermentation medium.

Recently, in the thesis study of Anghardi (2007), the effects of oxygen transfer conditions were examined on the synthesis of benzaldehyde lyase in order to increase the BAL activity by the recombinant *E. coli* BL21 (DE3) pLySs. Also, in this study, by using multiple regression technique, mathematical model was proposed to estimate the benzaldehyde activity as function of time, agitation rate, cell concentration, dissolved oxygen concentration, and by- product concentration.

## 2.3 Bioprocess Parameters in Enzyme Production

In the production of proteins by recombinant organisms, process engineering principles are combined with molecular biology knowledge to design successful operations to grow genetically engineered organisms. Microorganisms are preferred when compared to animal or plant cells because they are easy and cheaper to produce, much more easily controlled. (Prave, 1987). Any operation involving the transformation of some raw material (biological or non-biological) into some product by means of microorganisms, animal or plant cell cultures, or by materials derived from them (e.g. enzymes, organelles), may be termed as a "bioprocess" (Moses and Cape, 1991). From the economical point of view, the aim is to optimize the system, i.e. to maximize the yield and productivity of industrial products.

Microorganism selection, culture medium design and bioprocess operation parameters are the most important factors affecting the product yield.

### 2.3.1 Microorganism

Selection of the host microorganism is the first point to determine the success of bioprocess. The host organism should be carefully examined to have the following characteristics (Kirk and Othmer, 1994):

It should,

- ❖ give sufficient yields,
- ❖ be able to produce large amounts of target protein,
- ❖ be suitable for industrial fermentation,
- ❖ produce a large cell mass/volume quickly and on cheap media,
- ❖ be considered safe based on historical experience or evolution by regulatory authorities,
- ❖ not produce harmful substances or any other undesirable products.

Benzaldehyde lyase is naturally produced by wild-type *Pseudomonas fluorescens* (Gonzales et al., 1984). Then, the gene encoding benzaldehyde lyase was firstly cloned to an *Escherichia coli* by Hinrichsen et al. (1994), and further studies were performed by using *E. coli* SG130009 as the host microorganism. For the benzaldehyde lyase production, Çalık et al. (2004, 2006) selected *E. coli* K12 strain as host microorganism among the investigated *E. coli* strains, namely *E. coli* JM109, *E. coli* XL-1 Blue, *E. coli* K12 and *E. coli* GBE 180 (Çalık et al., 2004, 2006; Kaya, 2006). As an alternative

to these, to achieve extracellular production of BAL recombinant *Bacillus species* (Kaya, 2006) and *Pichia pastoris* (Büyüksungur, 2006) was studied as the host.

#### 2.3.1.1 *Escherichia coli*

It is the most widely used microorganism in genetic studies owing to the extended studies and collected knowledge about its physiology and genetics. Ever since the first cloning of benzaldehyde lyase, *Escherichia coli* is used as a host organism for the scientific studies. Reasons for the popularity of *Escherichia coli* are the ease of genetic manipulation and wealth of the availability of genetic information coupled with fast growth rate, standardized cultivation techniques and cheap media (Çalık et al., 2002). Its scientific classification is as follows:

Kingdom: Bacteria  
Phylum: Proteobacteria  
Class: Gamma Proteobacteria  
Order: Enterobacteriales  
Family: Enterobacteriaceae  
Genus: *Escherichia*  
Species: *Escherichia coli*

It is a Gram-negative bacterium that has an outer membrane in contrast to the Gram-positive bacteria. This outer membrane also serves as a protective layer to harmful chemicals. It has the ability to grow in both aerobic and anaerobic conditions. Its optimum growth temperature and pH is 37°C and 7.6, respectively (Kirk and Othmer, 1994).

In the literature, *E. coli* BL21 strains were used as the most popular host microorganism in both batch (Choi and Lee, 1997; Christensen et al., 2002) and fed batch (Shiloach et al., 1996; Akesson et al., 2001; Johnston et al., 2003) cultivations. In this study recombinant *E. coli* BL21 (DE3) pLySs *E.coli* (Kaya, 2006) was selected as a potential producer of benzaldehyde lyase because this strain was reported to be a high BAL and low acetate producer.

#### 2.3.2 Medium Design

Optimization of the fermentation conditions for the productivity and the cost per unit of recombinant enzyme is becoming one of the major goals in biotechnology today and the productivity of any cultivation is affected by process parameters and media composition. (Park and Reardon, 1996) A

cultivation medium is designed to reflect the elemental composition and the biosynthetic capacity of a given microbial cell (Prescott et al., 2002).

Cells need several materials in the media in which they grow. Water is the major element in a cultivation medium. Carbon compounds are needed as energy sources. Oxygen, nitrogen, hydrogen, sulfur, phosphorus, magnesium and potassium are other macronutrients needed. Furthermore, some metal ions, vitamins, hormones must be present in trace amounts in the medium to increase the growth rate and yield. Some special inducers which repress the translation of the enzyme may be required in special cases (Atkinson and Mavituna, 1991). Media should be of a consistent quality and be readily available throughout the year. The downstream processing should cause minimum problem (Nielsen and Villadsen, 2003).

There are two major types of growth media: defined and complex. Defined media is prepared with exactly known composition of molecules in it. Control over the process is high and reproducibility is possible. Use of defined media requires the knowledge of the exact nutritional requirements of the organism. Hence, the results are more reproducible and the operator has better control of the fermentation. In addition, recovery and purification of a product is often easier and cheaper in defined media (Shuler and Kargi, 2002). Complex media, however, contains natural compounds, necessary for cell growth, of unknown compositions (Chaplin and Bucke, 1990). They usually provide the full range of growth factors that may be required by an organism hence, may be more usefully used to cultivate bacteria whose nutritional requirements are complex. Traditionally complex media from the agricultural sector are used, as these are present in large quantities and are relatively cheap.

Generally, microorganisms grow more vigorously in complex media than in defined media, because complex media provides carbon, nitrogen, vitamins and trace elements necessary for cell growth and metabolite production. Furthermore, economic constraints make the large-scale production of low-cost products reliant on cheap sources of carbon and nitrogen, such as molasses from the sugar industry, corn steep liquor from the starch industry, spent sulphite liquor from the forest products industry and cheese whey from the dairy industry (Miller et al., 1986, Dahod et al., 1999). The disadvantages of applying complex media in the fermentation are that there may be a variation in the composition due to season or storage; there may be

compounds present that are undesirable (Nielsen and Villadsen, 2003). Hence decision should be taken due to these pros and cons.

When growing *E. coli* to low density, all the required nutrients can be added initially into the basal broth. The popular complex Luria Bertani (LB) broth allows the growth of *E. coli* in a temperature-, pH-, and oxygen-controlled environment up to a cell density of 1 g/l dcw. To accommodate the nutritional requirements of denser cultures, concentrations of media components must be increased and phosphorus, sulfur and trace elements must be added (Lee, 1996; Reisenberg et al., 1991). However, there are some drawbacks when adding all of the needed supplements from the beginning of the cultivation (Yamane and Shimizu, 1984).

Examples of these are:

- ❖ The supplement can be used for other purposes than intended, i.e. by-product formation.
- ❖ The composition of the complex medium differs, i.e. different amounts should be added to each cultivation in order to allow for reproducible operation.
- ❖ If the process knowledge is low, it is difficult to know in advance exactly how much to add of the needed supplement.
- ❖ A high concentration of the needed supplement might be inhibitory.

Furthermore, the need of the supplement might change during the course of the cultivation, e.g. in the transition from growth to production.

The need for adequate nutrient supply served as the obvious motivation to develop a sophisticated feeding technique. The strongest motivation to develop the fed batch methods to achieve higher density of *E. coli* culture was to decrease acetate accumulation, since high acetate concentration can inhibit growth and recombinant protein production (Majewski et al., 1990; Han et al., 1992; Luli and Strohl, 1990; Bentley et al., 1990; Shimizu et al., 1991).

In the study of Choi and Lee (1997), various mediums were examined for *E. coli* BL21 in the production of bovine growth hormone (bGH). LB medium was reported slightly better than M9 medium containing 2.0 kg m<sup>-3</sup> casamino acid. Christensen et al. (2002) investigated glucose and glycerol effects as carbon sources in batch cultures of *E. coli* BL21 and lower acetate accumulation was observed in glycerol based medium. Recently, Çalık et al. (2004, 2006) designed a glucose based defined medium containing 8.0 Kg m<sup>-3</sup> glucose, 5.0 Kg m<sup>-3</sup> (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and salt solution, and by using this medium

they investigated the effects of bioreactor operation parameters in *E. coli* K12 carrying *pUC18::bal* plasmid.

From the above data, it is clear that a well-designed medium and feeding strategies are needed for high-dense growth of *E. coli*. The objective of this study was to investigate the effect of molasses based complex medium on the expression of recombinant gene *E. coli* BL21 and to optimize and improve the medium for increased production of BAL.

#### 2.3.2.1 Molasses

Molasses is a by product of the sugar industry; it is the mother liquor remaining after crystallization and removal of sucrose from the juices of sugar cane or sugar beet and is used in a variety of food and non food applications . The final molasses product from sugarcane factories is molasses, containing 35–55% sucrose and 15–25% each glucose and fructose. Because of the high mineral (primarily KCl) and browning polymer content, blackstrap is too bitter for human consumption; most is used for animal feed, alone or as an ingredient, and it is traded in international commerce for this purpose. Refinery molasses, and blends of both factory and refinery with various lighter syrups, are the sources of a wide range of food-grade molasses, known as treacle in Europe. Molasses is fermented to ethanol at sugarcane factories in almost all cane-growing areas outside the United States, for industrial alcohol. Molasses is the basis for almost all rum production (some rum is produced directly from sugarcane juice in the French-speaking Caribbean), and for other beverage alcohol, in Asian countries. Molasses has been used as a carbon source in a multitude of chemical and microbial reactions; it is usually the sugars in molasses that serve as the carbon source; hence, these products are included herein (Kirk and Othmer, 1994).

The principal reasons for widespread use of molasses as substrate are its low price compared to other sources of sugar, and the presence of several other compounds and vitamins which are valuable for the fermentation (Makkar and Cameotra, 2002). Molasses is comparatively rich in various nutrients besides sucrose compared to other sources of sugar (Table 2.1).

In the studies of Çalık et al., (2001, 2003) chemically and physically pretreated molasses having different glucose, fructose and/or sucrose concentrations was employed in small-scale bioreactors using *Bacillus* species, and the effects of the pretreatments on resulting SAP activity was investigated.

Beet molasses, the by-product of sugar refinery process, containing 45–50% sugars, is the most economical source of carbohydrate for various industrial fermentations (Agarwal et al., 2007). The beet molasses was taken from Sugar Factory in Ankara and the composition is listed in Table 2.2.

**Table 2.1** Various Sources of Carbon (Atkinson and Mavituna, 1991).

	Amino acid, %	Protein, %	Carbohydrate, %
Molasses	-	3	54
Corn steep liquor	4.9	24.0	68.9
Corn gluten meal	8.8	62.0	20.0
Soybean meal	23.2	51-52	-
Starch	-	-	80-90
Yeast hydrolysate	34.1	40-65	-
Oat flour	4.3	12.0	54.0
Wheat flour	6.0	13.2	69.0
Whey powder	7.4	12.0	68.0
Peanut meal	22.7	45.0	23.0
Rice flour	4.4	8.0	65.0

In the present investigation, a study was made to optimize the cultural conditions for maximizing the production of BAL from *Escherichia coli* BL21 in an inexpensive beet molasses based medium using statistical approaches. Furthermore, a feasibility of large-scale production was attempted in a laboratory bioreactor. This is the first report on use of molasses based medium in an *E.coli* fermentation process.

**Table 2.2** Composition of molasses (Sugar Research Institute, Ankara).

Dry Solids, (%)	82
Sucrose	51
Raffinose	1
Invert Sugar	1
Organic Nonsugar Substances	21
Inorganics	6
Nitrogen	2
Water, (%)	18
pH	7.5

### 2.3.3 Bioreactor Operation Parameters

In aerobic fermentation processes; oxygen transfer, pH, and temperature are the major bioreactor operation parameters, having essential effects on product formation by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999). Therefore these parameters should be taken into consideration and examine profoundly because they affect the yields and protein production.

- ❖ Temperature is the first important physiological parameter for cell growth that must be monitored and controlled continuously. If the cells are grown at a temperature below the optimum, rate of cellular production is reduced. On the other hand, if the growth occurs in too high a temperature, not only will cell death occur, but in situations where the target protein may be under the control of temperature sensitive promoter, it may be expressed prematurely, lowering product yield (Nielsen and Villadsen, 2003). In the literature, the production of *E. coli* is performed at 37°C, which is the bacteria's optimal temperature of growth.

- ❖ Another parameter that must be controlled continuously is the medium pH. As metabolic products are released into the medium, pH may change. Different organisms have different pH optima, however, for many bacteria, pH optima ranges from 3.0 to 8.0. Thus, pH control by means of a buffer or an active pH control system is important (Shuler and Kargı, 2002). Nevertheless, some bioprocesses require controlled pH conditions, while others might require uncontrolled pH operations, in order to increase the product yield and selectivity (Çalık et al., 2002). In the study by Çalık et al. (2006), the effect of controlled and un-controlled pH conditions, as well as of the value of pH in the range of 5.0-7.8, on benzaldehyde lyase production on a defined medium with glucose as a sole carbon source in batch bioreactors was investigated. pH of 7.2 was reported to be most favorable in terms of enzyme activity and cell concentration. Therefore, in the current study, initial pH of the fermentation medium was arranged as 7.2 with 5M NaOH and 5M H<sub>3</sub>PO<sub>4</sub>.
- ❖ Optimal growth of host microorganisms of recombinant genes require large amounts of dissolved oxygen. Since the oxygen is sparingly soluble in water it must be supplied continuously into a growing medium generally in the form of sterilized air (Prave, 1987). Oxygen transfer rate can be adjusted by changing the rate of supplied air or by changing the rate of agitation.
- ❖ Last point to consider in a bioprocess is the agitation rate. Adequate mixing is critical for ensuring adequate supply of nutrients and prevention of the accumulation of metabolic wastes. It also affects the oxygen and heat transfer rates. Insufficient agitation will cause death of cells due to insufficient oxygen supply and metabolic waste accumulation, while excessive agitation again cause death of cells due to mechanical damage. Hence, optimum rate of agitation must be adjusted according to operation needs.

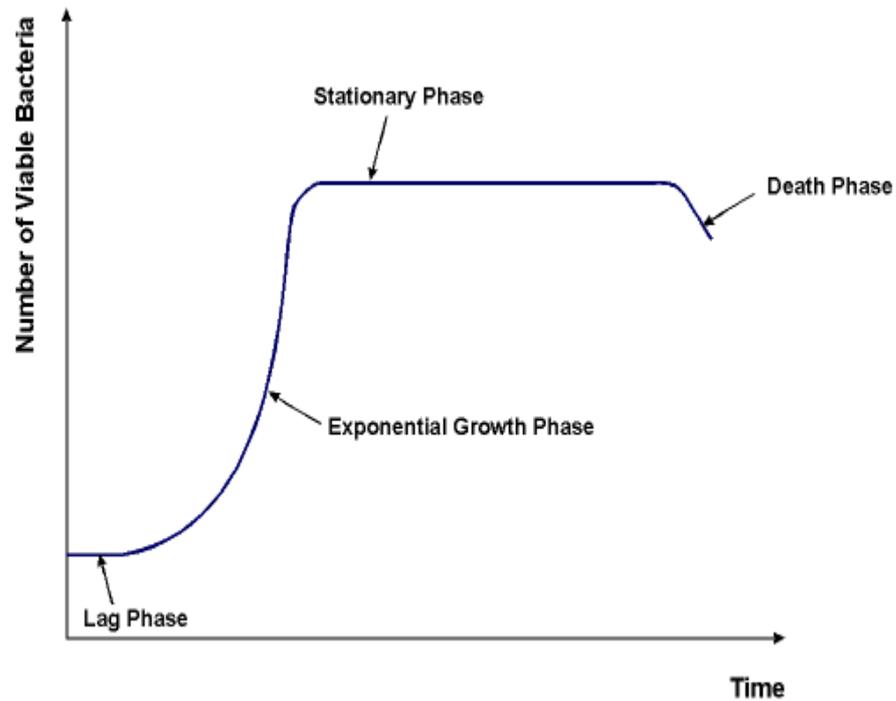
## 2.3.4 Computation of Bioprocess Characteristics

### 2.3.4.1 Cell Growth, Kinetics and Yield Factors

Cell growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. As a result of both replication and change in cell size due to the chemical reactions occur inside the cell, microbial growth can be considered as an increase in the number of individuals in the population (Nielsen and Villadsen, 1994; Scragg, 1988).

The schematic growth curve (Figure 2.3) shown below is associated with simplistic conditions known as a batch culture. It refers to a single bacterial culture, introduced into and growing in a fixed volume with a fixed (limited) amount of nutrient. Industrial situations tend to be much more complex in nature than such a simplified model.

During **lag phase**, microorganisms adapt themselves to growth conditions. It is the period where the individual microorganism are maturing and not yet able to divide. During the **exponential phase** (sometimes called the log phase), the number of new microorganisms appearing per unit time is proportional to the present population. This gives rise to the classic exponential growth curve, in which the logarithm of the population density rises linearly with time. The actual rate of this growth (i.e. the slope of the line in the Figure 2.3) depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes. During **stationary phase**, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the microorganisms begin to exhaust the resources that are available to them. At **death phase** microorganism run out of nutrients and die. (Atkinson and Mavituna, 1991)



**Figure 2.3** Typical growth curve for a bacterial population

The rate of growth is directly related to cell concentration, and cellular reproduction and characterized by the specific growth rate,  $\mu$ , which is defined as,

$$\mu = \frac{1}{C_x} \cdot \frac{dC_x}{dt} \quad (2.1)$$

Where  $C_x$  is the cell mass concentration ( $\text{kg m}^{-3}$ ),  $t$  is time (h), and  $\mu$  is the specific growth rate ( $\text{h}^{-1}$ ).

Within the different phases of microbial growth, specific growth rate is not constant and varies with respect to phases. Batch cell growth and the metabolic activities are summarized in Table 2.3 (Atkinson and Mavituna, 1991; Shuler and Kargi, 2002).

**Table 2.3** Batch Cell Growth Cycle Depending on Specific growth rate

Phase	Specific growth rate
Lag	$\mu \approx 0$
Exponential	$\mu \approx \mu_{\max}$
Deceleration	$\mu < \mu_{\max}$
Stationary	$\mu \leq 0$
Death	$\mu < 0$

Rate of cell growth,  $r_x$ , is described by the following equation (Shuler and Kargi, 1992):

$$r_x = \frac{dC_x}{dt} = \mu C_x \quad (2.2)$$

Similarly, substrate consumption rate,  $r_s$ , and product formation rate,  $r_p$ , are described by the following equations respectively where  $C_s$  is the substrate concentration and  $C_p$  is the product concentration:

$$r_p = \frac{dC_p}{dt} \quad (2.3)$$

$$r_s = \frac{dC_s}{dt} \quad (2.4)$$

The product and biomass yields are other important parameters which demonstrate the efficiency of conversion of the substrate into product and biomass. A general method can be defined as shown on equations 2.5 and 2.6:

$$\bar{Y}_{X/S} = \frac{\Delta C_X}{\Delta C_S} \quad (2.5)$$

$$\bar{Y}_{P/S} = \frac{\Delta C_P}{\Delta C_S} \quad (2.6)$$

Where  $C_x$ ,  $C_p$  and  $C_s$  are mass of cell, product and substrate, respectively, involved in metabolism. A list of frequently used yield coefficients is given in Table 2.4 When yields for fermentation are reported, the time or time period to which they refer should be stated (Doran, 1995).

Yield coefficients are not constant throughout the growth phase since they change with growth rate due to the maintenance energy ( $m$ ) requirement (Scragg, 1988). Energy is needed to repair damaged cellular components, to transfer some nutrients and products in and out of the cell, for motility, and to adjust the osmolarity of the cells' interior volume (Shuler and Kargi, 2002). Microbial growth, product formation and substrate utilization rates are usually expressed in the form of specific rates:

$$q_p = \frac{1}{C_x} \frac{dC_p}{dt} \quad (2.7)$$

$$q_s = \frac{1}{C_x} \frac{dC_s}{dt} \quad (2.8)$$

In an aerobic process oxygen is consumed for cell growth, product formation and by-product formations, and maintenance. The oxygen consumption rate for cell growth can be defined as (Çalık et al., 2006):

$$-r_{O1} = \frac{dC_x / dt}{Y_{X/O}} \quad (2.9)$$

Oxygen consumption for by-product (BP) formation is defined as:

$$-r_{O2} = \frac{dC_{BP} / dt}{Y_{BP/O}} \quad (2.10)$$

The oxygen consumption for oxygen denoted by  $m_0$  is the oxygen consumption for maintenance and can be defined as:

$$-r_{O3} = m_0 C_x \quad (2.11)$$

The total oxygen consumption rate is as follows:

$$-r_o = (-r_{O1}) + (-r_{O2}) + (-r_{O3}) \quad (2.12)$$

**Table 2.4** Definition of yield coefficients

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

Equations (2.9)-(2.11) are substituted into (2.12) to obtain,

$$-\frac{dC_o}{dt} = -r_o = \left(\frac{1}{Y_{X/O}}\right) \frac{dC_X}{dt} + \left(\frac{1}{Y_{BP/O}}\right) \frac{dC_{BP}}{dt} + m_o C_X \quad (2.13)$$

Also,

$$q_{BP} = \frac{1}{C_X} \frac{dC_{BP}}{dt} \quad (2.14)$$

and

$$\mu = \frac{1}{C_X} \frac{dC_X}{dt} \quad (2.15)$$

Substituting (2.14) and (2.15) into (2.13),

$$-r_o = \left(\frac{1}{Y_{X/O}}\right)(\mu C_X) + \left(\frac{1}{Y_{BP/O}}\right)(q_{BP} C_X) + m_0 C_X \quad (2.16)$$

Dividing (2.13) by  $C_X$ ,

$$\frac{-r_o}{C_X} = \frac{\mu}{Y_{X/O}} + \frac{q_{BP}}{Y_{BP/O}} + m_0 \quad (2.17)$$

Where  $\alpha$  is the term for growth associated organic acid formation and  $\beta$  is the term for non-growth associated organic acid formation,  $q_{BP}$  can be defined as follows,:

$$\frac{dC_{BP}}{dt} = \alpha \frac{dC_X}{dt} + \beta C_X \quad (2.18)$$

Dividing (2.18) to  $C_X$ ,

$$q_{BP} = \frac{1}{C_X} \frac{dC_{BP}}{dt} = \alpha \mu + \beta \quad (2.19)$$

Substituting (2.19) into (2.17),

$$\frac{-r_o}{C_X} = \frac{\mu}{Y_{X/O}} + \frac{\alpha \mu + \beta}{Y_{BP/O}} + m_0 \quad (2.20)$$

Dividing (2.20) by  $\mu$ ,

$$\frac{-r_o}{\mu C_X} = \frac{1}{Y_{X/O}} + \frac{\alpha + (\beta / \mu)}{Y_{BP/O}} + \frac{m_0}{\mu} \quad (2.21)$$

Rearranging (2.21),

$$\frac{-r_o}{\mu C_X} = \left(\frac{\alpha}{Y_{BP/O}} + \frac{1}{Y_{X/O}}\right) + \left(\frac{1}{\mu}\right)\left(m_0 + \frac{\beta}{Y_{BP/O}}\right) \quad (2.22)$$

And,

$$\frac{1}{Y_{X/O}} = \left( \frac{\alpha}{Y_{BP/O}} + \frac{1}{Y_{X/O}} \right) + \left( \frac{1}{\mu} \right) \left( m_0 + \frac{\beta}{Y_{BP/O}} \right) \quad (2.23)$$

From the slope of the plot of  $\frac{1}{Y_{X/O}}$  versus  $\frac{1}{\mu}$ ,  $\left( m_0 + \frac{\beta}{Y_{BP/O}} \right)$  (g oxygen g<sup>-1</sup> dry cell)

weight h<sup>-1</sup>) and from the intercept,  $\left( \frac{\alpha}{Y_{BP/O}} + \frac{1}{Y_{X/O}} \right)$

where Y represents the apparent and  $\bar{Y}_{X/O}$  represents the true yield (Çalık et al., 2006).

When by-product formation is neglected, equation (2.23) becomes:

$$-\frac{dC_o}{dt} = -r_o = \left( \frac{1}{\bar{Y}_{X/O}} \right) \frac{dC_X}{dt} + m_0 C_X \quad (2.24)$$

If the above equation is reorganized:

$$\frac{-r_o}{dC_X/dt} = \frac{1}{\bar{Y}_{X/O}} = \frac{1}{\bar{Y}_{X/O}} + \frac{m_0}{\mu} \quad (2.25)$$

is obtained (Çalık et al., 2004). From the slope of  $\frac{1}{\bar{Y}_{X/O}}$  versus  $\frac{1}{\mu}$ , oxygen consumption for maintenance is obtained.

From the slope of the plot of  $1/\bar{Y}_{X/O}$  versus  $1/\mu$ ,  $m_0$  (kg oxygen kg<sup>-1</sup> dry cell weight h<sup>-1</sup>), and the intercept, cell yield on oxygen, in the case where oxygen uptake for product formation is omitted,  $\bar{Y}_{X/O}$ , 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.

#### 2.3.4.2 Oxygen Transfer

In an aerobic process, oxygen is a key substrate and because of its low solubility in aqueous solutions, a continuous transfer of oxygen from the gas phase to the liquid phase is decisive for maintaining the oxidative metabolism of the cells (Nielsen and Villadsen, 2003).

The oxygen requirement of bioprocesses can be different according to the process depending on cell growth conditions and metabolic pathway analysis. Hence, dissolved oxygen concentration has been accepted as an important bioreactor operation parameter. The effects of oxygen transfer on the activity and bioprocess residence time profiles of *in vitro* metabolic intermediates and catabolic end products should be investigated in order to understand the details of metabolism and increase the product selectivity. Furthermore, in order to design, scale-up, and operate the bioreactor with adequate mass transfer, the oxygen consumption rates and oxygen transfer coefficients which are the indicators of the mass transfer characteristics of a fermentation process are required; nevertheless, due to the complex composition of the fermentation liquid, it can be difficult to predict these parameters with reasonable accuracy (Çalık et al., 1998).

The expression for the rate of oxygen transfer from the gas to liquid phase is given by the following equation:

$$\text{OTR} = k_L a (C_o^* - C_o) \quad (2.26)$$

where  $k_L$  is the oxygen transfer coefficient (cm/h),  $a$  is the gas-liquid interfacial area ( $\text{cm}^2/\text{cm}^3$ ),  $k_L a$  is the volumetric oxygen transfer coefficient ( $\text{h}^{-1}$ ),  $C_o^*$  is saturated concentration (mg/L),  $C_o$  is the actual DO concentration in the broth (mg/L) and the OTR is the rate of oxygen transfer ( $\text{mg O}_2/\text{l.h}$ ) (Shuler and Kargi, 2002).

The rate of oxygen uptake, OUR, can be denoted as:

$$\text{OUR} = -r_o = q_o C_x \quad (2.27)$$

where  $q_o$  is the specific rate of oxygen consumption and  $C_x$  is the cell concentration (Shuler and Kargi, 2002).

A large number of different empirical correlations have been developed for the experimental determination of  $K_L a$  values. The most common method used during fermentation processes is dynamic method for the determination of the value of  $K_L a$  experimentally. This method is based on measurement of the dissolved oxygen concentration in the medium. However, it does not require measurement of the gas composition and therefore cheaper to establish (Nielsen and Villadsen, 2003).

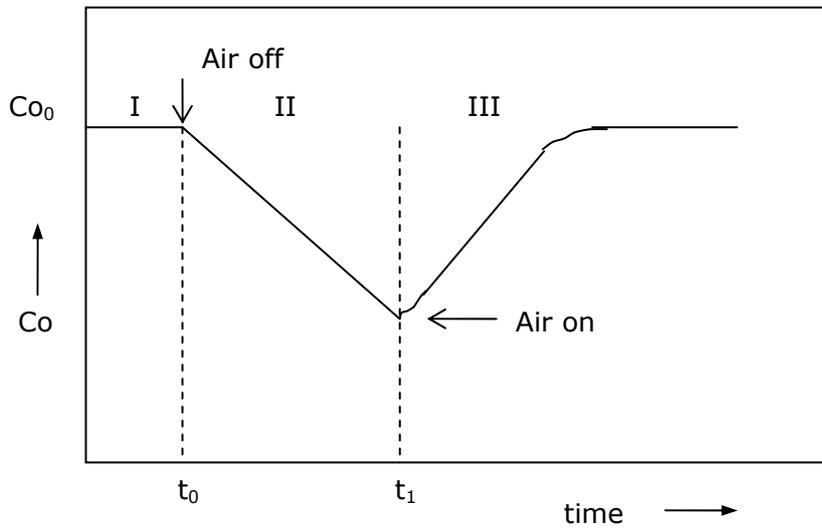
The dynamic mass balance for the dissolved oxygen concentration is:

$$\frac{dC_o}{dt} = K_L a(C_o^* - C_o) - q_o C_x \quad (2.28)$$

If the gas supply to the bioreactor is turned off, the first term on the right hand side of the equation immediately drops to zero and equation reduces to:

$$\frac{dC_o}{dt} = -r_o \quad (2.29)$$

A typical response curve of the dynamic method is given in Figure 2.4. When the reactor is de-oxygenated, dissolved oxygen concentration,  $C_o$ , drops since there is no oxygen transfer (region-II).



**Figure 2.4** Variation of dissolved oxygen concentration with time in dynamic measurement of  $K_La$ .

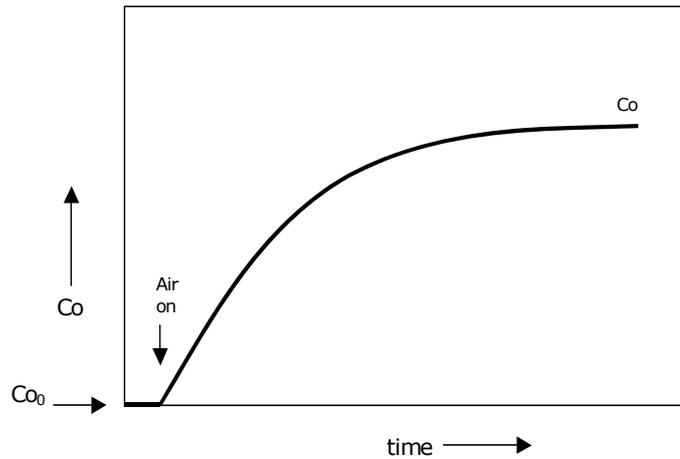
Gas supply is then given to the system, and the increase in  $C_o$  is monitored as a function of time (Region III). In this period, the equation (2.28) is valid. Combining equations (2.28) and (2.29) and rearranging,

$$C_o = -\frac{1}{K_La} \left( \frac{dC_o}{dt} - r_o \right) + C_o^* \quad (2.30)$$

From the slope of a plot of  $C_o$  versus  $(dC_o/dt - r_o)$ ,  $K_La$  can be determined.

When there is no reaction, the Dynamic Method can also be applied, i.e.,  $r_o=0$  (Nielsen and Villadsen, 2003). 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  $C_o$  is monitored as a function of time in order to determine the physical mass transfer coefficient,  $K_La_0$  from the slope of a plot of  $C_o$  versus  $dC_o/dt$  (Figure 2.5).

$$C_o = -\frac{1}{K_La} \left( \frac{dC_o}{dt} \right) + C_o^* \quad (2.31)$$



**Figure 2.5** Variation of dissolved oxygen concentration with time in dynamic measurement of  $K_La_0$  using the Dynamic Method.

In order to compare the relative rates of maximum oxygen transfer and biochemical reactions and find the rate limiting step of the bioprocess, the maximum possible oxygen utilization rate (OD=oxygen demand) which is defined as (Çalık et al., 2004),

$$OD = \frac{\mu_{\max} CX}{Y_{X/O}} \quad (2.32)$$

And the maximum possible mass transfer rate is defined as,

$$OTR_{\max} = k_L a C_{DO}^* \quad (2.33)$$

Oxygen limitations is very essential in an aerobic process hence in order to understand the concept deeply, the effectiveness factor,  $\eta$  (oxygen uptake rate per maximum possible oxygen utilization rate) and modified Damköhler number,  $Da$  (maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2004)) are defined according to the equations given below:

$$\eta = \frac{OUR}{OD} \quad (2.34)$$

$$Da = \frac{OD}{OTR_{\max}} \quad (2.35)$$

And the enhancement factor,  $E$  (oxygen transfer coefficient per physical oxygen transfer coefficient) can be also defined as;

$$E = \frac{K_L a}{K_L a_o} \quad (2.36)$$

In the literature, there are studies reporting the effect of oxygen transfer on recombinant benzaldehyde lyase production (Çalık et al. 2004 & 2006, Anghardi, 2007). In the study of Anghardi, (2007), six different conditions were investigated with parameters, air inlet rate of  $Q_o/V_R = 0.5$  vvm, and agitation rate of  $N=250, 375, 500, 750 \text{ min}^{-1}$  and at  $Q_o/V_R = 0.7$  vvm,  $N=750 \text{ min}^{-1}$ , in a pilot scale bioreactor with  $1.65 \text{ dm}^3$  working volume. The highest BAL activity and cell concentration were obtained at  $0.5$  vvm,  $500 \text{ min}^{-1}$  condition as  $860 \text{ U cm}^{-3}$  and  $2.3 \text{ kg m}^{-3}$ , respectively. Oxygen transfer characteristics of OUR and  $K_L a$  values together with the yield and maintenance coefficients and by-product distribution were also determined.  $K_L a$  increased with cultivation time and agitation rate, with values changing between  $0.008$ - $0.046 \text{ s}^{-1}$ . Then in the thesis study of Anghardi, (2007) the effects of oxygen transfer on benzaldehyde lyase production were investigated at six different conditions with the parameters, air inlet rate of  $Q_o/V_R = 0.5$  vvm and agitation rates of  $N= 250, 500, 625,$  and  $750 \text{ min}^{-1}$ , and dissolved oxygen levels  $DO= 20\%, 40\%$  ( $C_{DO}=0.04, 0.08 \text{ mol m}^{-3}$ ) of the saturation. Volumetric mass transfer coefficients,  $K_L a$ , values and subsequently the enhancement factor,  $E$ , found were increased with the increase in the oxygen transfer. Among the investigated oxygen transfer conditions, the highest cell concentration and benzaldehyde lyase activity was observed at the condition of  $DO= 40\%, N=625 \text{ rpm}$ . These values were approximately  $1.27$  and  $1.30$  fold higher than the values obtained in the study of of Çalık et al. (2004).

Other than BAL, there are different studies investigating the effect of oxygen in *E. coli* fermentation. Ryan et al. (1989) investigated the effect of

oxygen transfer on  $\beta$ -lactamase production by *E. coli JM103* and reported that the decrease in air inlet rate decreased the cell growth rate. Bhattacharya and Dukey, (1997) investigated the over expression of target gene (MspI methylase) in recombinant *E. coli* K12 in response to dissolved oxygen and oxygen mass transfer and stated that an oxygen-sufficient condition was necessary to obtain optimal expression and cell productivity. Thereafter, in the research of Castan et al. (2002), the effects of elevated concentration of oxygen and carbon dioxide on human growth hormone production by *E. coli* K12 were investigated. In most of studies performed with *E. coli* dissolved oxygen level was controlled at constant value (10-30%) by changing the air flow rate (Luli and Strohl, 1990) or agitation rate in fed batch mode with an automated controller (Akesson et al., 2001; Johnston et al., 2003).

### **2.3.5 Modes of Operation**

#### **2.3.5.1 Batch Operation**

Batch processing gives fermentation process a number of advantages. Batch processes have a lower risk of contamination, infection by an undesirable organism (Winkler, 1990). Hence it is the most frequently used type of operation in biotechnological productions. Virtually all food processing, pharmaceutical and agricultural bioprocesses are carried out in batch reactors. Batch operation offers great flexibility of production planning, control of the system and simple setup. It has the advantage of producing large volumes of experimental data in a short period of time. The disadvantage is that the experimental data are difficult to interpret as there are dynamic variations throughout the operation, i.e. the environmental conditions experienced by the cells vary with time. By using well-instrumented bioreactors at least some variables, e.g., pH and dissolved oxygen tension, may, however, be controlled at a constant level (Stephanopoulos et al., 1998).

Most of the literature about BAL production studied the batch fermentation (Çalik et al., 2004, 2006). They can only achieve a BAL activity of 860 U cm<sup>-3</sup> and a cell concentration of 2.3 kg m<sup>-3</sup>.

#### **2.3.5.2 Continuous Operation**

The advantage of the continuous bioreactor is that a steady-state can be obtained, which allows for precise experimental determination of specific rates under well-defined environmental conditions. These conditions can be further

varied by changing the feed flow rate to the bioreactor. This allows valuable information concerning the influence of the environmental conditions on cellular physiology to be obtained. The disadvantage of the continuous bioreactor is that it is laborious to operate as large amounts of fresh, sterile medium have to be prepared and requires long periods of time for a steady state to be achieved.

Continuous fermentation is suited to a process where there is low risk of contamination, or contamination is unimportant, as in waste water treatment, and there are no problems with the genetic stability of the organisms, where the product is growth associated, so that the process can be stabilized in a positive growth phase and for which demand or storage characteristics justify long production runs (Winkler, 1990). A continuous bioreactor can take over 20 h to reach a new steady state after each step changes in manipulated variables. Examples of continuous operation are *chemostat*, (Christiansen et al., 2002) where the added medium is designed such that there is a single rate-limiting substrate, *pH-stat*, where the feed flow is adjusted to maintain constant pH in the bioreactor, and the *turbidostat*, where the feed flow is adjusted to maintain the biomass concentration at a constant level (Stephanopoulos et al., 1998).

There is no study in the literature studying continuous mode fermentation for BAL production.

#### 2.3.5.3 Fed-Batch Operation

In practice, fed batch fermentation is the most common operation. This gives some control over the concentration of a key nutrient, which would otherwise vary in an uncontrolled fashion throughout the course of the batch, e.g., maintaining the glucose concentration at a certain level, and it enables formation of much higher titers (up to several hundred grams per liter of some metabolites), which is of importance in the subsequent downstream processing.

The basic characteristic of fed-batch microbial processes is that the concentrations of nutrients fed into the culture liquid of the bioreactor can be controlled voluntarily by changing the feed rate. In ideal batch bioreactor, nutrient concentrations are not controlled and are progressively used up; whereas in an ideal continuous bioreactor at steady state, the concentrations of all nutrients, including the growth limiting substrate, are kept constant. Hence, fed-batch is superior to conventional batch operation especially when

changing concentrations of a nutrient affect the yield or productivity of the desired metabolite (Yamane and Shimizu, 1984). At the same time, the fed-batch operation is a convenient experimental system for maintaining steady environmental conditions to facilitate physiological studies. The physiological state of the culture during any part of cultivation is important since a high number of dead or dormant cells have a detrimental effect on the synthesis of any desired product, and this is especially relevant in fed-batch cultivations wherein the cells may be undergoing prolonged and severe nutrient limitation (Stephanopoulos et al., 1998).

In the literature, Yamane and Shimizu (1984) reviewed the history and characteristics of fed-batch technique in microbial reactions and classified various fed-batch techniques according to the mode of nutrient feeding. The author classified fed batch operations broadly into those processes without feedback control and those with feedback control. Gupta et al. (1999) studied the use of fed batch cultivation for achieving high cell densities in the production of a recombinant protein in *E.coli* and concluded that fed-batch cultivation made it possible to increase the final cell concentration compared to batch cultivation. Hewitt et al. (1999) investigated the use of multi-parameter flow cytometry to compare the physiological response of *E.coli* W3110 to glucose limitation during batch, fed-batch and continuous culture cultivations, and stated that during the latter stages of fed-batch cultivations there was a considerable drop in cell viability, as characterized by cytoplasmic membrane depolarization and permeability. However, this trend was not observed in either batch or continuous culture cultivations. In a more recent study, Christiansen and Nielsen (2003) studied the production of savinase and population viability of *B.clausii* during high-cell-density fed-batch cultivations with linear and exponential feeding profiles and concluded that in the latter parts of the fed-batch cultures with linear feed profile, a large portion of the cell population was found to have a permeable membrane, indicating a large percentage of dead cells. Moreover, the highest overall yield was obtained with an exponential feed profile, however, the yields in fed-batch cultures with linear feed profiles were only marginally smaller, and the highest total amount of savinase was obtained with linear feed profiles.

To achieve a good productivity, high cell concentration and high cell productivity are desired and this is usually obtained from fed-batch cultivations. Much work is done on how to determine the addition of the growth-limiting carbon (Reisenberg et al., 1999; Lee, 1996). Using such

models, an optimal concentration of all components and feed rates can be calculated, and the carbon feed becomes limiting and determines the growth rate. One of the obstacles in attaining high product yields and high productivity is the accumulation of the metabolic byproduct acetate which inhibits the growth as well as the production of recombinant protein. In aerobic *E. coli* cultures, formation of acetate occurs when the specific glucose uptake rate exceeds a critical value (Han et al., 1992). The acetate production is thought of as an overflow phenomenon where flux of AcetylCoA is directed to acetate, instead of entering the TCA-cycle (Majewski and Domach, 1990). A characteristic feature is that the specific oxygen uptake rate reaches an apparent maximum at the onset of acetate formation (Akesson et al., 2001). In Akesson et al. (1999), it was demonstrated how this phenomenon can be exploited for on-line detection of acetate formation. Under glucose-limited conditions, superimposed pulses in the glucose feed rate give rise to changes in the glucose uptake. These changes imply variations in the oxygen uptake that can be seen in the dissolved oxygen measurement.

Formation of acetate in *E. coli* cultures occurs under anaerobic conditions but also under fully aerobic conditions in situations with excess carbon source. These two mechanisms are often referred to as mixed-acid fermentation and overflow metabolism, respectively. The accumulation of acetate can be reduced by manipulation of strains, media, and cultivation conditions, and much research has been devoted to this topic (Lee, 1996; Riesenberget al., 1999). As the cells grow, the feed rate can be increased gradually without causing production of acetate. However, this implies increased oxygen consumption and eventually the maximum oxygen transfer capacity of the reactor may be reached. This will impose another limitation on the feed rate, since it will lead to anaerobic conditions if the resulting oxygen consumption exceeds the maximum oxygen transfer capacity. Consequently, by keeping the feed rate sufficiently low it is possible to avoid accumulation of acetate. On the other hand, choosing a feed rate that is unnecessarily low will give a low growth rate and hence a long cultivation time and low productivity. The challenge is thus to keep a high feed rate while avoiding overflow metabolism and anaerobic conditions.

The temperature limited fed-batch technique is described in Silfversparre et al. (2002). The basic idea is to decrease the cultivation temperature to control O<sub>2</sub> consumption rate and thereby avoiding O<sub>2</sub> limitation. Also, the substrate has to be fed in excess in order to prevent substrate limitation. Use

of this technique has, in previous trials, led to minimization of released endotoxins (Silfversparre et al. 2002), as well as to lowered proteolysis rate, when compared to substrate-limited fed-batch (Gao et al., 2002).

The probing feeding strategy described in Akesson et al. (2001) is a good strategy used to avoid acetate accumulation. By superimposing short pulses in the substrate feed-rate, on-line detection of acetate formation can be made using the dissolved oxygen sensor (Akesson et al., 1999). As a result *E.coli* was studied a lot in the literature as a fed batch operation and high cell concentrations was achieved. The applications and outcomes are summarized in the Table 2.5.

For BAL production, fed batch was not used up to now. Hence this would be the first study developing a fed batch strategy for BAL production.

**Table 2.5** Fed Batch operations about *E. coli* in the literature.

Microorganism	Product	Fed Batch Method	Carbon Source	Operation mode of Carbon source	Advantage	Source	
<i>E. coli</i> BL21	Recombinant exotoxin	Controlling dissolved oxygen at 30 %	Glucose	1. 2 g/l batch+ reduced to 0.5 g/l fed batch	Low values of acetate accumulation	Shiloach et al, 1995	
				2. 40 g/l batch+ reduced to 5 g/l for fed batch			
<i>E. coli</i> DH5a	Beta-galactosidase	Controlling the synthesis of T7-RNA polymerase	Glucose	Heat Shock	The product concentration increased 18-fold.	Gupta et al., 1998	
<i>E. coli</i> B	Recombinant Protein	Evolutionary Programming & Dynamic programming	Glucose & Lactose	Programmed to add lactose efficiently as both inducer and carbon source	High accuracy with the experiments	Levisauskas et al., 1998	
							pH stat
							Exponential feeding
<i>E. coli</i> BL21	Bioadhesive protein	Controlling dissolved oxygen at 30 %	Glucose	Post induction of 4 different strategy when glucose depleted	Linear feeding was found to be best having highest productivity.	Wong et al., 1998	
							Constant feeding
							Linear feeding
<i>E. coli</i> BL21 & <i>E. coli</i> K-12	Proprietary protein	Controlling dissolved oxygen at 30 %	Glucose	Stirrer speed control indicating that glucose has been depleted.	Aerobic conditions with very tiny amount of acetate. Only sensor required is oxygen probe.	Akeson et al., 2001	
							Linear feeding
<i>E. coli</i> DH5a	Extracellular hydroxybutyric acid	Controlling glucose concentration	Glucose	Continuous addition	High productivity.	Gao et al., 2002	

**Table 2.5** Fed Batch operations about *E.coli* in the literature (continued).

Microorganism	Product	Fed Batch Method	Carbon Source	Operation mode of Carbon source	Results	Source
<i>E.coli BL21</i>	Recombinant insoluble protein	1. pH stat 2. Starvation based DO control 3. Feed up DO transient control	Glucose	No glucose was in the base medium until maximum biomass levels are reached. Then glucose feed was given.	Optimal protein productivity.	Johnston et al., 2002
<i>E.coli BL21</i>	Recombinant insoluble protein	DO transient control to signal acetate threshold	Super Luria complex media	Precise addition of feed by controller	Complex media provides a difficult environment by decreasing productivity for the application of acetate threshold.	Johnston et al., 2003
<i>E.coli BL21</i>	Human interleukin-6	pH stat	Semi complex medium based on a medium with glucose and yeast extract	When glucose was depleted, a concentrated feed medium began to be fed based on a pH-stat feeding strategy with high limit	Simple to apply and effective in protein production.	Kim et al., 2005
<i>E.coli BL21</i> & <i>E. coli</i> W3110	Xylanase	Mid-ranging controller to manipulate temperature and stirrer speed to control the dissolved O <sub>2</sub> tension	Glucose	The temperature is decreased to lower the O <sub>2</sub> demand and the growth rate. Feed was started when the dissolved O <sub>2</sub> tension increased abruptly	Limited the growth rate without the risk of acetate accumulation. 20% increase in cell mass was achieved.	De Mare et al., 2005

**Table 2.5** Fed Batch operations about *E. coli* in the literature (continued).

Microorganism	Product	Fed Batch Method	Carbon Source	Operation mode of Carbon source	Results	Source
<i>E. coli</i> DSM1099 & DSM6968	-	Dissolved oxygen control at 20 % for <i>E. coli</i> DSM1099 and 30% for <i>E. coli</i> DSM6968	Glucose +Lysine for DSM1099 and Glucose + Yeast extract for DSM6968	Feed was started when the dissolved oxygen increased abruptly, indicating the depletion of the initial glucose.	Shorten the process development phase considerably.	De Mare et al., 2006
<i>E. coli</i> BL21	Recombinant cytochrome P450 monooxygenase CYP102A1	DO-spike controlled feeding	1. Glycerol 2. Glucose	When initial carbon source was depleted feed pulses were given maintaining the concentration at a specific value.	Glycerol feeding proved to be superior over glucose as carbon source since the formation of larger amounts of acetate was prevented.	Pflug et al., 2007
<i>E. coli</i> MG1655	-	Chemostat	Glucose	Monitoring the influent vessel and the effluent vessel, allowing precise measurement of the feed rate during chemostat operation.	A combination of reducing the carbon flow to acetate and anticipating on the underlying metabolic and regulatory mechanism that leads to acetate is the most promising approach to overcome acetate formation.	De Mey et al., 2007

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Chemicals

All chemicals were analytical grade, and obtained from Sigma Ltd., Difco Laboratories, Fluka Ltd. and Merck Ltd.

#### 3.2 The Microorganism

*Escherichia coli* BL21 (DE3) pLYSs strain carrying the pRSETA::*bal* plasmid (Kaya, 2006) was used as the potential producer of benzaldehyde lyase (BAL, EC 4.1.2.38). The recombinant microorganism has been stored in the microbanks (PRO-LAB), by inoculating young colonial growth into cryopreservative fluid present in the vial. After providing the adsorption of microorganism into the porous beads, excess cryopreservative was aspirated and inoculated cyrovial stored at -55°C.

#### 3.3 Procedure for Benzaldehyde Lyase Production in Recombinant *Escherichia coli* strain

Benzaldehyde lyase (BAL, EC 4.1.2.38) was produced from *E. coli* BL21 (DE3) pLYS carrying pRSETA::*bal* gene (Kaya, 2006). It was first inoculated into solid medium, then transferred into precultivation medium and finally inoculated into the production medium. The details were given in the following sections.

##### 3.3.1 The Solid Medium

The recombinant *E. coli* strain, stored on agar slants at 4°C or stored on microbanks at -55°C, was inoculated onto the freshly prepared agar slants under sterile conditions. According to the antibiotic resistance ability of the microorganisms, antibiotic was added to the agars after steam sterilization. The recombinant *E. coli* strain was incubated at 37°C overnight on Luria-

Bertani (LB) medium (Table 3.1); thereafter inoculated into the precultivation medium.

**Table 3.1** The composition of the solid medium.

Compound	Concentration, kg m <sup>-3</sup>
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Agar	15.0
Ampicilin	0.100
Chloramphenicol	0.035

### 3.3.2 The Precultivation Medium

The recombinant *E. coli* strains, grown in the solid medium, were inoculated into precultivation medium and incubated at 37 °C and  $N=200 \text{ min}^{-1}$  for 12 h in agitation and heating rate controlled orbital shakers (B.Braun, Certomat BS-T) using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. The compositions of precultivation mediums were listed in Table 3.2. The selective antibiotics were added to the precultivation medium to increase the stability of the plasmid in amounts stated in Table 3.2 after sterilization.

**Table 3.2** The composition of the precultivation medium.

Compound	Concentration, kg m <sup>-3</sup>
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Ampicilin	0.100
Chloramphenicol	0.035

### 3.3.3 The Production Medium

The microorganisms inoculated in precultivation medium, were further inoculated in production medium. The reference production medium (RPM) for benzaldehyde lyase production in *E. coli* was chosen to be medium described in Çalık et al. (2004, 2006); after omitting the glucose the composition is given in Table 3.3.

Hydrolyzed molasses were used as the alternative carbon source to glucose in the fermentation process. The effect of the concentration of molasses has been investigated in the range of 8 to 40 kg m<sup>-3</sup> in order to increase the biomass and benzaldehyde lyase production in recombinant organisms.

The laboratory scale experiments for microbial growth and medium design were executed in Erlenmeyer flasks, 150 ml in size with working volume capacities of 33 ml. Microorganisms were inoculated at a cultivation temperature of 37 °C and an agitation rate of 200 min<sup>-1</sup> in agitation and heating rate controlled orbital shakers.

The pilot scale batch bioreactor with a 3.0 dm<sup>3</sup> volume (B.Braun CT2-2), having a working volume of 1.65 dm<sup>3</sup>, and consisting of temperature, pH, air flow, foam and stirring rate controls, was used for the bioreactor experiments. It was utilized by an external cooler, steam generator and a jacket around the bioreactor for sterilization and temperature control. The bioreactor was stirred with two Rushton turbines and consisted of four baffles, two six-bladed impellers, each of which has a diameter of 53 mm, and a sparger.

**Table 3.3** The composition of BAL production medium.

Component	Concentration, kg m <sup>-3</sup>
<b>Molasses</b>	<b>8.0 - 40.0</b>
Na <sub>2</sub> HPO <sub>4</sub>	6.7
KH <sub>2</sub> PO <sub>4</sub>	3.1
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	5.0
NaCl	0.5
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.2 x 10 <sup>-5</sup>
MnSO <sub>4</sub> .7H <sub>2</sub> O	0.1
CoCl <sub>2</sub> .6H <sub>2</sub> O	2.0 x 10 <sup>-5</sup>
Ampicillin	0.1
Chloramphenicol	0.035

All of the medium components were steam sterilized at 121°C for 20 min, molasses being sterilized separately. Antibiotics were added to the medium when the culture temperature was about 50°C; the final concentrations were arranged as given in Table 3.3.

### 3.4 Pretreatment of Molasses

Beet molasses, the composition and properties of which was defined elsewhere (in section 2.3.2.1) was chemically modified with acid hydrolysis following dilution and centrifugation. The details of the physical and chemical pretreatments are as follows: (Çalık et al., 2003)

- i. Dilution: 100 g molasses was diluted with water to obtain a 200 cm<sup>3</sup> solution.
- ii. Centrifugation: Diluted molasses was centrifuged for 20 min at 6000 g. Hence the impurities not soluble in water and the foam formed during sugar process removed from the medium.
- iii. HCl hydrolysis: 37% HCl was used to adjust pH of the molasses solution to 1.8 at room temperature. Hydrolysis reaction was carried out at 90°C;

temperature for 3 h. pH of the solution then rearranged by 5M KOH to 7.2. Therefore, molasses was ready to use in the fermentation process after sterilization.

### 3.5 Analysis

Throughout the bioprocesses, samples were taken at characteristic cultivation times. After determining the cell concentration, the medium was centrifuged at  $13500 \text{ min}^{-1}$  for 10 min at  $4^{\circ}\text{C}$  to precipitate the cells. In recombinant *E. coli* strains, precipitate was used to determine benzaldehyde lyase activity after the lysis of the cell wall; supernatant was used for the determination of glucose and fructose concentrations, organic acid concentrations, oxygen uptake rate and liquid phase mass transfer coefficient values.

#### 3.5.1 Cell Concentration

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

#### 3.5.2 Benzaldehyde Lyase Activity

Benzaldehyde lyase activity was determined by measuring the conversion of benzoin into benzaldehyde. Samples from the culture broth were harvested by centrifugation (Sigma 1-15) at  $13500 \text{ min}^{-1}$  for 10 min at  $4^{\circ}\text{C}$ . In order to determine the intracellular benzaldehyde lyase activity in r-*E. coli* strains, the cell walls were lysed at  $f=10 \text{ s}^{-1}$  for 10 minutes at the agitator bead mill (Retsch, MM 200) by using 30% suspension of glass beads in activity buffer of 40 mM Tris-HCl (pH=8.0), 0.02 mM TPP, 0.2 mM  $\text{MgCl}_2$  buffer. Fresh substrate solutions were prepared daily by mixing 0.5 volume activity buffer with 0.35 volume stock benzoin (0.1mM) and 0.15 volume 15%PEG solution with a final concentrations of 0.035 mM benzoin, 20 mM Tris-HCl (pH=8.0), 0.01 mM TPP, 0.1 mM  $\text{MgCl}_2$ , 7.5% PEG and incubated at  $37^{\circ}\text{C}$ . Stock benzoin solution (0.1mM) was prepared in 15% PEG solution and used after overnight incubation at room temperature. The conversion reaction was carried out at  $37^{\circ}\text{C}$ .

Reaction was started by the addition of 20  $\mu\text{l}$  of samples to 3  $\text{cm}^3$  of substrate solution and further incubated for 10 seconds. Enzymatic activity was monitored spectrophotometrically at 250 nm by following the change in

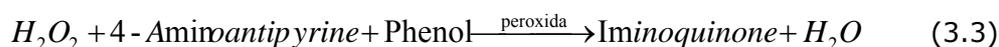
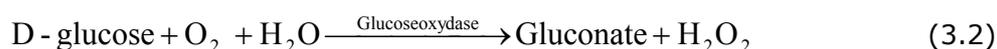
absorbance in ten seconds. The substrate benzoin and the product benzaldehyde have similar absorbance maxima. However, since two molecules of benzaldehyde are produced per each molecule of benzoin cleaved, the following formula was used to relate absorbance change to product formed (Gonzalez and Vicuna, 1989):

$$\text{Nanomoles of product formed} = [2(A_f - A_i)/(\epsilon_s - 2\epsilon_p)] * 10^6 \quad (3.1)$$

With  $\epsilon_s$  and  $\epsilon_p$  being molar extinction coefficients of the substrate and the product, respectively.  $A_f - A_i$  is the change in optical density during the reaction time, measured at 250 nm. One unit of enzymatic activity was defined as the amount of enzyme that catalyzes the cleavage of benzoin into one nanomoles of benzaldehyde at 37°C and pH 8.0 in one second (Çalık et al., 2004).

### 3.5.3 Reduced Sugar Concentration

Reduced sugar, glucose, concentration was determined by the glucose oxidation method at 505 nm with a UV spectrophotometer (Boyaci et. al., 2005). D-glucose is oxidized in the presence of glucose oxidase enzyme (equation 3.2) and peroxide formed due to oxidation reaction is further reacted with 4-aminoantipyrine and phenol in the catalysis of peroxidase to form *Iminoquinone* (equation 3.3) which gives spectro-photometrically observable red color in proportion with glucose concentration.



The calibration curve was obtained from the slope of absorbance versus known standard glucose concentration (Appendix B).

The method used in analysis of samples is given below:

1. The samples containing more than 1 g L<sup>-1</sup> glucose were diluted to a final concentration less than or equal to 1 g L<sup>-1</sup>.

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

#### **3.5.4 Fructose Concentration**

The concentration of D-fructose was measured using the colorimetric assay of Dische and Borenfreund (1951), known as cysteine-carbazole-sulfuric acid method. 60 µl of freshly prepared 1.5%(w/v) cysteine-HCl, 60 µl of 0.12%(w/v) carbazole in 95% ethanol, and 1.8 ml of 70% sulfuric acid were added to 600 µl of the sample mixture and the absorbance at 560 nm was measured after a 30 minute incubation at room temperature. The absorbance was compared to a linear standard curve prepared by using D-fructose in the reaction mix (Appendix C).

#### **3.5.5 Organic Acids Concentrations**

Organic acid concentrations were measured with an organic acid analysis system (Waters, HPLC, Alliance 2695). The method is based on reversed phase HPLC, in which organic acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard organic acids solution. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to the analysis system with a mobile phase of 3.12% (w/v) NaH<sub>2</sub>PO<sub>4</sub> and 0.62x10<sup>-3</sup>% (v/v) H<sub>3</sub>PO<sub>4</sub>. Needle and seal wash were conducted with 20% (v/v) acetonitrile. The analysis was performed under the conditions specified as:

**Table 3.4** Conditions for HPLC system for organic acid analysis.

Column	Capital Optimal ODS, 5 $\mu$ m
Column dimensions	4.6 x250 mm
System	:Reversed phase chromatography
Mobile phase flow rate	:0.8 ml min <sup>-1</sup>
Column temperature	:30 °C
Detector and wavelength	:Waters 2487 Dual absorbance detector At 210 nm
Injection volume	:5 $\mu$ l
Analysis period	:15 min

### 3.5.6 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 benzaldehyde lyase production process, the Dynamic Method (Rainer, 1990), as explained in section 2.3.4.2, was used.

Prior to inoculation of the microorganism to the production medium in the bioreactor, the physical mass transfer coefficient ( $K_{La0}$ ) 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  $N=50 \text{ min}^{-1}$  in order to lower the effect of surface aeration.

### 3.5.7 Saturation oxygen concentration determination

In order to calculate the dissolved oxygen concentration in the fermentation during the bioprocesses, saturation oxygen concentrations,  $C_{O_2}^*$ , were determined. For the saturation oxygen concentration measurements, approximately 1000 ml of fermentation broth was prepared according to the medium composition used at the beginning of the bioprocess and 1000 ml of

fermentation broth was collected at the end of each experiment performed in pilot scale bioreactor. Fermentation broth sample was set to 37°C and stirred with a magnetic stirrer. Air was compressed to the fermentation broth and dissolved oxygen concentration was measured by an oxygen probe. After a short period, dissolved oxygen concentration attained a constant value, namely the saturation oxygen concentration,  $C_{O_2}^*$ .

## CHAPTER 4

### RESULTS AND DISCUSSION

This study focuses on the development of a feeding strategy based on molasses based complex medium for benzaldehyde lyase production by recombinant *E. coli* BL21. For this purpose, the study was carried out in two parts. Firstly, the effects of different molasses concentrations were investigated in a bioreactor system as batch mode. Thereafter, a feeding strategy was developed in order to increase BAL activity and cell concentration.

#### 4.1 Molasses Based Complex Medium Design

A molasses based complex medium was designed to increase benzaldehyde lyase production. For this purpose, the medium given by Çalık et al. (2006) was taken as the reference production medium (Table 3.3). After omitting the glucose from this medium, the medium was considered as the starting point of the medium design experiments. In this context, the effects of molasses was investigated in agitation ( $N=200 \text{ min}^{-1}$ ) and heating rate ( $T=37^\circ\text{C}$ ) controlled laboratory scale bioreactors. However it has been seen that, molasses, which is a sucrose source, could not be used as a carbon source for *E. coli* cultivation. Hence molasses was pretreated. As a result of hydrolysis, fructose and glucose in 50 % were obtained.

Effect of molasses was investigated at  $C_M=16, 24, 30$  and  $56 \text{ kg m}^{-3}$  as batch mode in the bioreactor system consisted of temperature, pH, foam, dissolved oxygen level, air inlet and stirring rate controls with  $V_R = 1.65 \times 10^{-3} \text{ m}^3$  working volume. The experiments were performed at the optimum oxygen transfer conditions (Anghardi, 2007),  $\text{DO}=40\%$  cascade to airflow,  $N=625 \text{ min}^{-1}$ , at  $T=37^\circ\text{C}$  and at the controlled pH of 7.2 in pilot scale batch bioreactor. Batch bioreactor conditions and their abbreviations are given in Table 4.1.

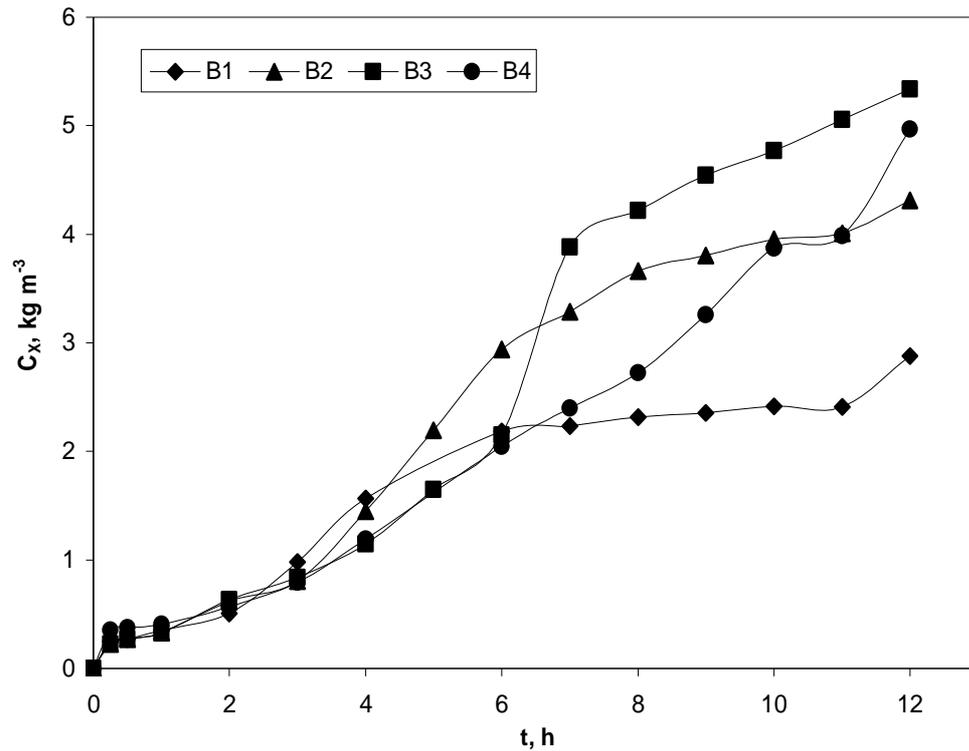
**Table 4.1** Batch bioreactor conditions and their abbreviations.

Molasses Concentration (kgm <sup>-3</sup> )	Sucrose Concentration (kgm <sup>-3</sup> )	Fructose - Glucose Concentration (kgm <sup>-3</sup> )	Abbreviation
16	8	4-4	B1
24	12	6-6	B2
30	15	7.5-7.5	B3
56	28	14-14	B4

#### 4.1.1 Cell Growth Profile

The variations in the cell concentration with the cultivation time and the molasses concentration applied are illustrated in Figure 4.1. For the first four hours of the bioprocess, cell formation did not change significantly with respect to molasses concentration. At  $t > 4$ h, cell profiles showed alteration considerably throughout the bioprocess. In B1 condition which has the lowest molasses concentration, cell concentration reached to stationary phase after  $t=6$  h where maximum cell concentration was  $C_X=2.85$  kg m<sup>-3</sup> at  $t=12$  h. On the other hand, in the other conditions, cell concentration continued to increase throughout the processes. The highest cell concentration was obtained at B3 as  $C_X=5.34$  kg m<sup>-3</sup> whereas with the further increase in molasses concentration from  $C_M=30$  kg m<sup>-3</sup> to 56 kg m<sup>-3</sup>, cell concentration decreased to  $C_X=5.14$  kg m<sup>-3</sup>. Thus, higher concentration of molasses appeared to be inhibitory for the cell growth.

It is preferable to increase the concentration of the benzaldehyde lyase producing cells with the minimum input. Therefore, B3 condition where the highest amount of cell was generated would be the best for the operation of the bioreactor.



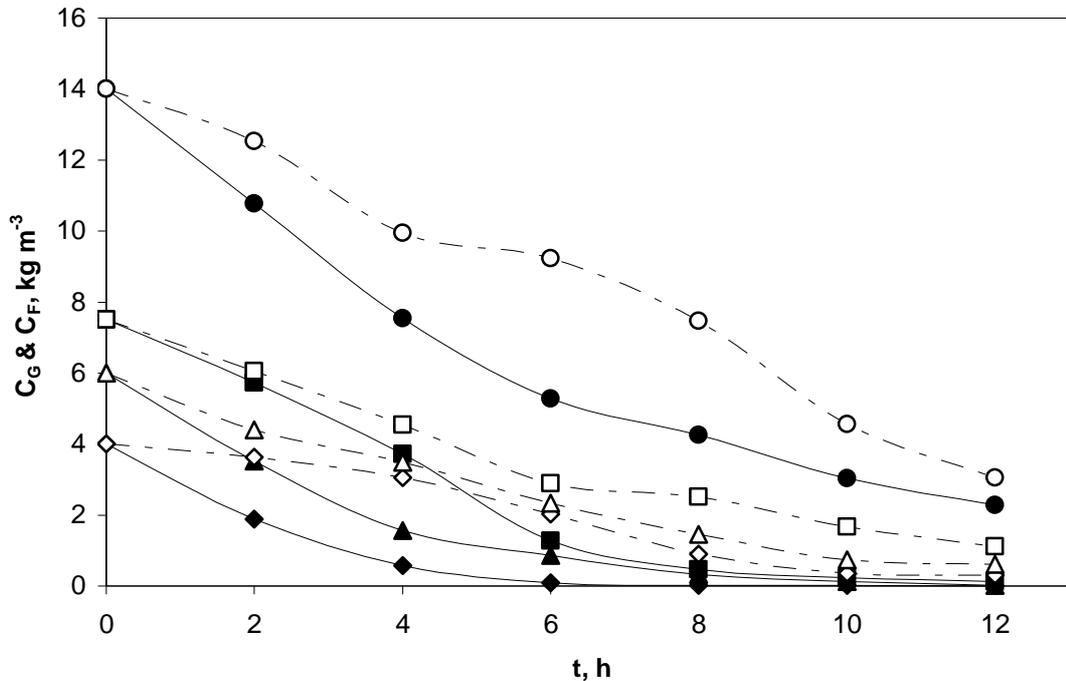
**Figure 4.1** The variations in cell concentration with the cultivation time and molasses concentration: B1, (◆); B2, (▲); B3, (■); B4, (●); T=37°C,  $V_R=1.65 \times 10^{-3} \text{ m}^3$ , DO=40%,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

Çalık et al., (2006) reported the optimum bioreactor operation conditions for BAL production as  $N=500 \text{ min}^{-1}$ ,  $Q_0/V_R=0.5 \text{ vvm}$ ,  $\text{pH}_C=7.2$ , and the medium containing  $8.0 \text{ kg m}^{-3}$  glucose and  $5.0 \text{ kg m}^{-3}$   $(\text{NH}_4)_2\text{HPO}_4$  in which the highest cell growth was obtained as  $C_X=2.3 \text{ kg m}^{-3}$  by recombinant *E. coli* K12 carrying pUC18::bal plasmid in a bioreactor with a working volume of  $1.65 \text{ dm}^3$ . In the thesis study of Anghardi (2007), the highest cell concentration was obtained as  $3.0 \text{ kg m}^{-3}$  in the same medium of Çalık et al. (2006); however, at the optimum oxygen transfer condition of DO=40% cascade to airflow,  $N=625 \text{ min}^{-1}$  and at  $\text{pH}_C=7.2$  by recombinant *E. coli* BL21. When the maximum cell concentration of the current study is compared with those, it was seen that using molasses based medium with the concentration of  $C_{\text{Glucose}}=7.5 \text{ kg m}^{-3}$  and  $C_{\text{Fructose}}=7.5 \text{ kg m}^{-3}$  was resulted in 1.8 fold higher cell concentration than the highest one found in literature.

#### 4.1.2 Glucose and Fructose Concentration Profiles

From the fructose, glucose and sucrose analyses, it was found that, molasses contains 50 % of sucrose and 25 % each of glucose and fructose. In order to decide whether molasses is a good carbon source for *E. coli* fermentation, the variations of glucose and fructose with cultivation time should be analyzed. The variations of glucose and fructose concentrations throughout the process are given in Figure 4.2.

From the figure 4.2, it is clear that at all the conditions; glucose was preferred by the cells to fructose throughout the process. At B1, B2 and B3 conditions, glucose was depleted at the end of the process. However, at B4 condition, glucose built up after 4<sup>th</sup> hour explaining the reason of the lower cell concentration of B4 condition.



**Figure 4.2** Variations in glucose (solid lines) and fructose (dashed lines) concentrations with the cultivation time and molasses concentration: B1, ( $\blacklozenge, \blacklozenge$ ); B2, ( $\blacktriangle, \blacktriangle$ ); B3, ( $\blacksquare, \square$ ); B4, ( $\bullet, \circ$ );  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pHC}=7.2$ .

The most frequently applied energy source for cellular growth is glucose since it can be metabolized easily by microorganisms. This result could be

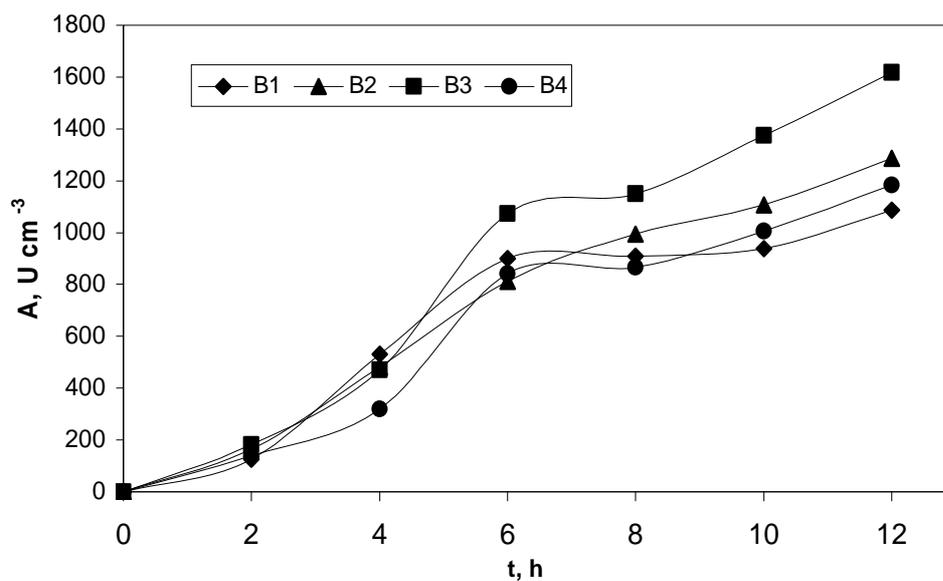
proved in the Figure 4.2, since fructose was not preferred by the cells when there was glucose in the medium. After 4<sup>th</sup> hour, for B1, B2 and B3 conditions, fructose started to be consumed since glucose concentration reduced to half of its initial concentration. After 12 hours of cultivation, nearly all of the fructose was used up except the accumulation of it in B4 case.

When figure 4.1 was compared with figure 4.2, glucose and fructose concentration profiles coincide with the cell growth at all operation conditions. When the higher glucose utilization rate was observed, the higher cell growth rate was obtained.

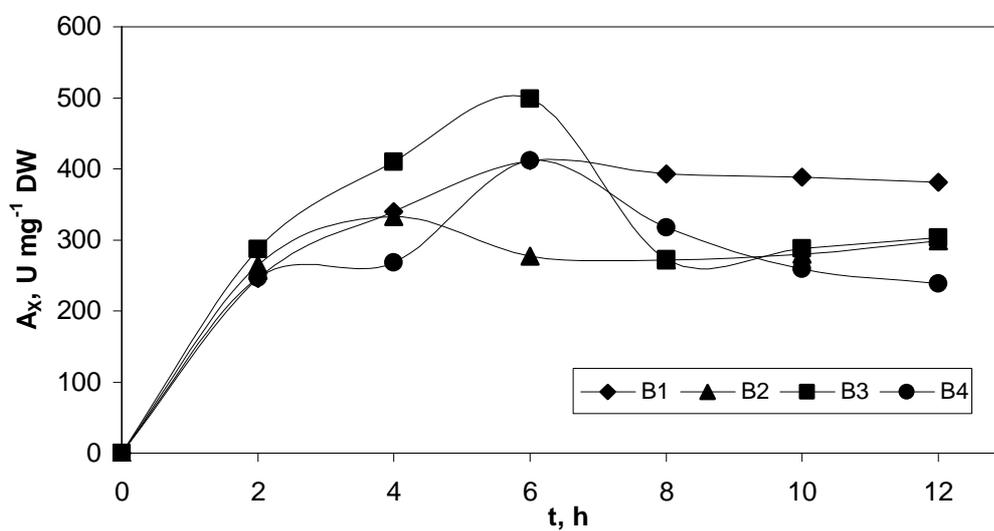
When this founding was compared with the findings of Çalık et al. (2004), where glucose consumption rates were lower, it can be concluded that, *E. coli* BL21 (DE3) pLySs strain requires more glucose as a carbon source to maintain the optimum state of cell growth than *E. coli* K12 does. In the studies of Kaya (2006), Anghardi (2007) and in most of the batch processes, where *E. coli* BL21 was used, the same trend was observed in the glucose consumption even if at higher initial glucose concentrations of 20.0 kg m<sup>-3</sup> (Luli and Strohl, 1990), or 40.0 kg m<sup>-3</sup> (Shiloach et al., 1996) were preferred in production mediums.

#### **4.1.3 Benzaldehyde Lyase Activity Profiles**

The variations of the benzaldehyde lyase activity and specific activity with the molasses concentration and the cultivation time are given in Figure 4.3 and Figure 4.4, respectively. The highest BAL activity was obtained at B3 as 1617 U cm<sup>-3</sup>, and the lowest BAL activity was obtained at B1 as 1088 U cm<sup>-3</sup>. The difference between the conditions might be the result of glucose depletion at the end of the exponential phase of growth and increased proteolytic activity. *E. coli* BL21 (DE3) pLySs is protease deficient strain, so glucose depletion can lead to increase in protease production which may result in lower productivity. In the study of Choi and Lee (1997), a decrease in expression level of bovine growth hormone was observed due to the starvation for a required nutrient in *E. coli* BL21 strain. Thus, it can be concluded that the protease degradation caused by nutrient depletion can account for the decrease in BAL volumetric activity.



**Figure 4.3** The variations in benzaldehyde lyase activity with the cultivation time and molasses concentration: B1, (◆); B2, (▲); B3, (■); B4, (●); T=37°C,  $V_R = 1.65 \times 10^{-3} \text{ m}^3$ , DO=40%, N=625  $\text{min}^{-1}$ ,  $\text{pH}_C = 7.2$ .



**Figure 4.4** The variations in benzaldehyde lyase specific activity with the cultivation time and molasses concentration: B1, (◆); B2, (▲); B3, (■); B4, (●); T=37°C,  $V_R = 1.65 \times 10^{-3} \text{ m}^3$ , DO=40%, N=625  $\text{min}^{-1}$ ,  $\text{pH}_C = 7.2$ .

The same trend like in the cell growth profile was observed in activity profile. As the molasses concentration increased the benzaldehyde lyase activity increased and gave a maximum when both glucose and fructose concentration are  $7.5 \text{ kg m}^{-3}$ . A lower activity value of  $1184 \text{ U cm}^{-3}$  was obtained in B4 where glucose and fructose concentrations were higher than B3 condition. The cell concentration and the benzaldehyde lyase activity profile reveal that higher glucose and fructose concentrations as in B4 condition show inhibitory effect and the optimum condition was found as B3, giving the highest cell concentration and benzaldehyde lyase activity.

In the literature, the highest benzaldehyde lyase volumetric activity was obtained as  $1095 \text{ U cm}^{-3}$  by Anghardi (2007) with  $8 \text{ kg m}^{-3}$  initial glucose concentration. When it is compared with the activities found in the current study, it can be seen that, molasses based medium overcome all the media used (Çalık et al. 2004 & 2006, Kaya, 2006). It seems that, initial glucose and fructose concentration of  $7.5 \text{ kg m}^{-3}$  is ideal for the optimum cell growth and BAL yield in recombinant *E. coli* strains.

Specific activity of BAL did not change for the first two hours of the process. At B1 and B2 conditions, specific activity increased and reached a stationary phase after 6<sup>th</sup> hour. On the other hand, at B3 and B4 condition, specific activity increased during  $t=0-6 \text{ h}$  and then decreased. Interestingly, in B3 condition specific activity showed a sharp decrease after  $t=6 \text{ h}$ . This was due to the rapid increase of cell concentration at B3 condition whereas activity of that did not increased with the same rate.

#### **4.1.4 The Optimized Medium**

As a consequence, among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as  $5.3 \text{ kg m}^{-3}$  and  $1617 \text{ U cm}^{-3}$ , respectively, in the medium containing  $7.5 \text{ kg m}^{-3}$  glucose and  $7.5 \text{ kg m}^{-3}$  fructose. The activity obtained in the optimized medium was 1.8-fold higher than the activity obtained in the reference production medium (Çalık et al., 2006) and 1.5-fold higher than the highest activity found in the literature with glucose based medium having a concentration of  $8 \text{ kg m}^{-3}$  (Anghardi, 2007).

Table 4.2 summaries the results in point of cell concentration and the benzaldehyde lyase activity.

**Table 4.2** The variations in cell concentration and benzaldehyde lyase activity with glucose and fructose concentration in molasses based medium at  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ ,  $t=12 \text{ h}$ .

Glucose Concentration $\text{kg m}^{-3}$	Fructose Concentration $\text{kg m}^{-3}$	$C_x, \text{ kg m}^{-3}$	$A, \text{ U cm}^{-3}$
4	4	2.9	1088
6	6	4.3	1286
<b>7.5</b>	<b>7.5</b>	<b>5.3</b>	<b>1617</b>
14	14	5.2	1184

#### 4.1.5 Inducer Effect

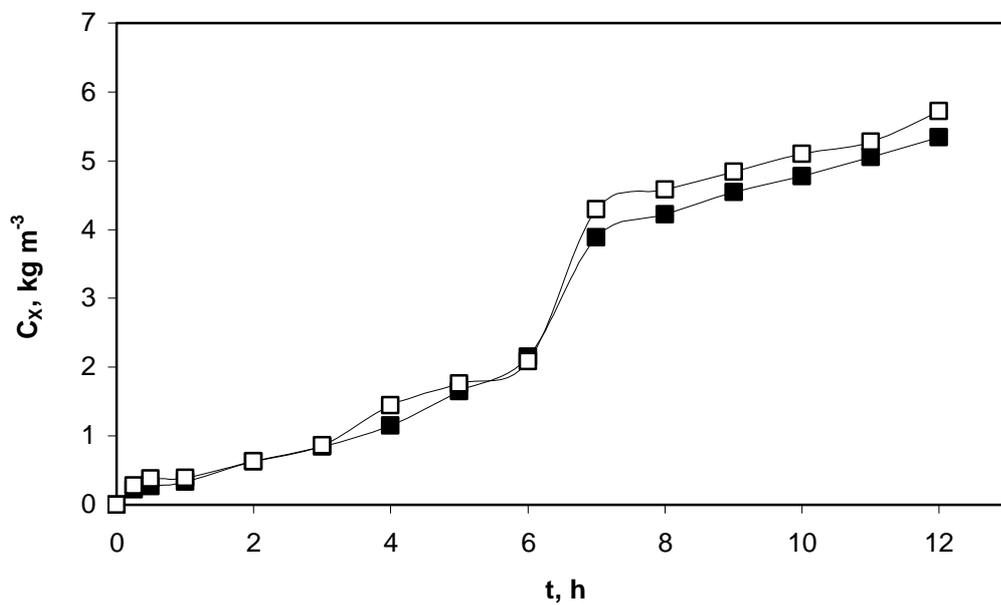
In intracellular BAL synthesis by *E.coli* BL21, the T7 RNA polymerase gene is under the control of the *lacUV5* promoter and repressed until IPTG is added to the growth medium. However, IPTG was reported as a source of the decrease in cellular growth. Moreover, its use in large-scale production is scarce because of its high cost and its toxicity towards humans (Menzella et al. 2003). As an alternative to IPTG, lactose is also used as an inducer. Lactose can activate the same promoters as IPTG while serving as a source of carbon for the bacteria (Gombert et al., 1998).

As the alternative to IPTG, lactose is used as an alternative inducer. Hence it was reasonable to make an experiment using the optimized medium but without IPTG inducer as molasses contains lactose in a few amounts. The variations in benzaldehyde lyase activity and cell concentration with the cultivation time and IPTG effect at B3 condition are shown in figures 4.5 and 4.6.

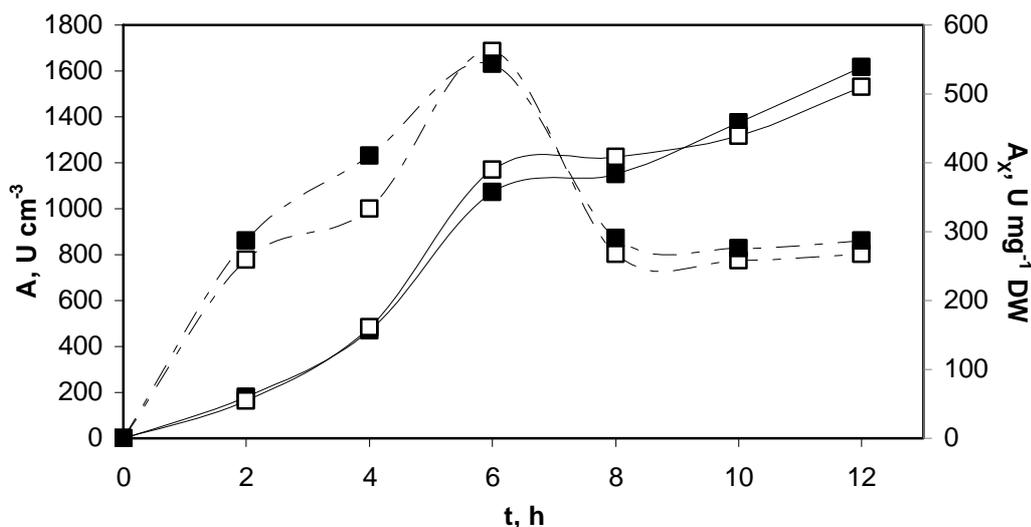
IPTG was added to medium at  $t= 4\text{h}$ . Until  $t=6 \text{ h}$ , cell concentration did not change significantly with respect to inducer. Though, after  $t=6 \text{ h}$  cell profiles varied. However, this difference was in the boundaries of the experimental errors. Hence it could be ignored.

The variation of BAL activity and specific activity were given in Figure 4.6 and it has not changed for the first four hours. After 6<sup>th</sup> hour, BAL activity profile was seemed to be varied; nevertheless this might be neglected again

due to the little distinction. The matter of interest here was to prove the existence of lactose in the medium. Hence these tiny differences did not cause a big problem concerning the disadvantages of IPTG. As a result, it was found to be reasonable to cultivate BAL in a molasses based medium in the absence of IPTG. Specific activities of both cases showed the similar trend during the process increasing up to 6<sup>th</sup> hour then decreased and reached a stationary phase after 8<sup>th</sup> hour.



**Figure 4.5** The variations in cell concentration with the cultivation time and IPTG at B3 condition: without IPTG, (□); with IPTG, (■); T=37°C, V<sub>R</sub>= 1.65x10<sup>-3</sup> m<sup>3</sup>, DO=40%, N=625 min<sup>-1</sup>, pH<sub>C</sub>=7.2.



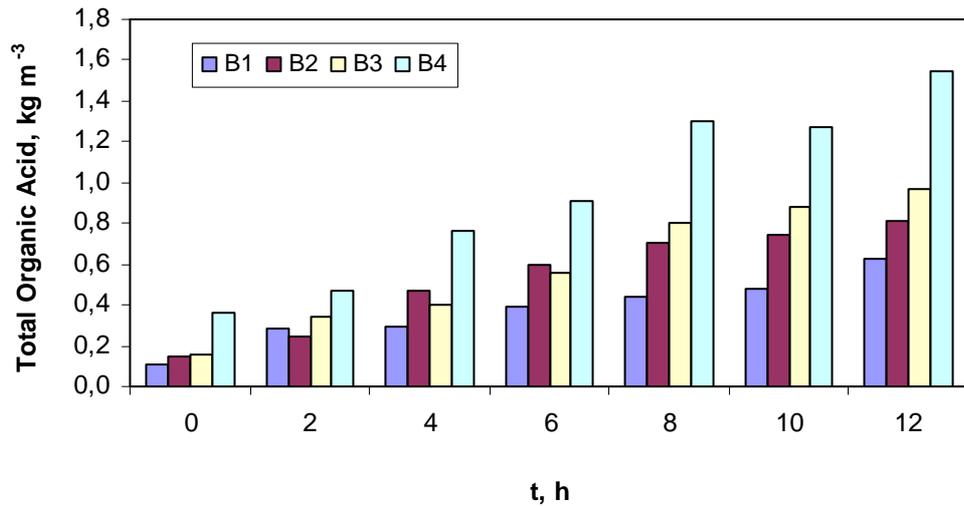
**Figure 4.6** The variations in benzaldehyde lyase activity (solid lines) and specific activity (dashed lines) with the cultivation time and IPTG at B3 condition: without IPTG, (□); with IPTG, (■); T=37°C,  $V_R = 1.65 \times 10^{-3} \text{ m}^3$ , DO=40%,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

#### 4.1.6 Organic Acid Concentration Profiles

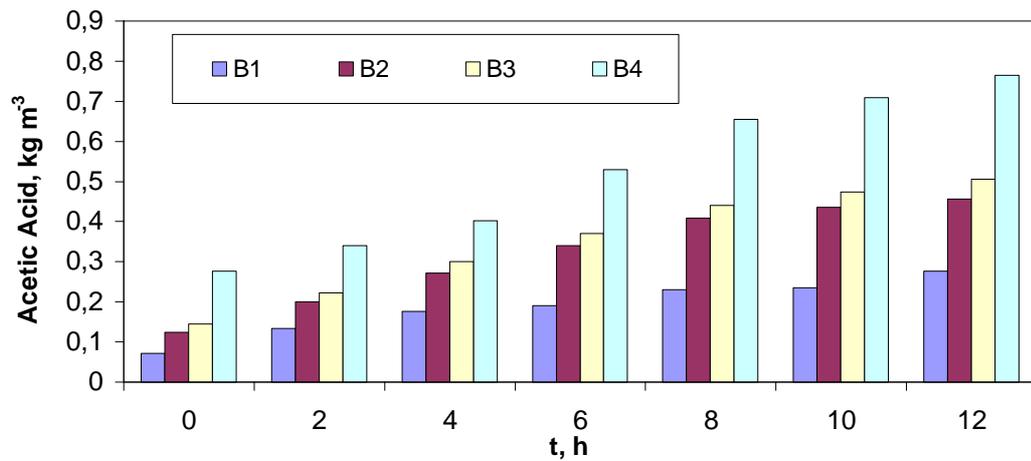
The variations in the organic acids detected in the fermentation broth with the cultivation time and batch conditions were demonstrated in Table 4.3. For all of the conditions applied, acetic, formic, succinic, citric fumaric and malic acids were found in the fermentation broth in increasing amounts.

Almost all of these organic acids were detected in the molasses based medium at  $t=0 \text{ h}$  and throughout the fermentation. In figure 4.7, variation in total organic acid concentration with cultivation time was given. It is clear from the figure that with the increase in cultivation time, organic acid concentration increased at all the conditions. However, at B4 condition the highest total concentration was obtained ( $C_{\text{OA}}=1.55 \text{ kg m}^{-3}$ ) at the end of the process. On the other hand, in the optimum medium, B3, total organic acid concentration was  $C_{\text{OA}}=0.97 \text{ kg m}^{-3}$  at  $t=12 \text{ h}$ .

As expected, for all of the conditions, acetic acid is the major by-product in the fermentation medium. The variations with acetic acid concentration with cultivation time and batch conditions were shown in the Figure 4.8.



**Figure 4.7** The variations in total organic acid concentrations with cultivation time in batch mode bioreactor conditions;  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .



**Figure 4.8** The variations in acetic acid concentrations with cultivation time in batch mode bioreactor conditions;  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

In all of the batch mode conditions, acetic acid formation increased with increasing cultivation time. The highest acetate accumulation occurred in B4 condition, where the highest glucose and fructose accumulation were observed, as  $C_{Ac}=0.764 \text{ kg m}^{-3}$  at  $t=12 \text{ h}$ ; followed by B3 condition as

$C_{Ac}=0.506 \text{ kg m}^{-3}$ , B2 condition as  $C_{Ac}=0.456 \text{ kg m}^{-3}$  and B1 as  $C_{Ac}=0.277 \text{ kg m}^{-3}$ . The change in the metabolism can be explained by utilization pathway of the acetate with respect to glucose and fructose uptake rate (Shiloach et al., 1996). In the literature, by the same oxygen transfer conditions, the highest acetic acid concentration was obtained as  $0.34 \text{ kg m}^{-3}$  at  $t=12\text{h}$  in glucose based medium (Anghardi, 2007). This value is lower than the one obtained ( $C_{Ac}=0.506 \text{ kg m}^{-3}$ ) in the optimized medium, B3, of the current study. However, molasses based medium contains acetic acid before the cultivation ( $C_{Ac}=0.144 \text{ kg m}^{-3}$  at  $t=0 \text{ h}$ ). In general, formation of acetate in *E. coli* occurs under anaerobic conditions but also under fully aerobic conditions in situations with excess carbon source. As glucose enters the glycolysis pathway for the synthesis of the monomers of cell and BAL, the glycolysis pathway; for the synthesis of the nucleotides, pentose phosphate pathway; and for the synthesis of glutamic acid and aspartic acid group, TCA cycle should be active and acetic acid, which is produced from AcCoA that is substrate of the TCA cycle was detected in highest amount in the fermentation broth (Çalık et al., 2006). The formation of acetate has been suggested to be caused by an imbalance between glucose metabolism and respiration, a condition which influx of carbon into the cell exceeds demands for biosynthesis, the presence of excess NADH, the repression of tricarboxylic acid cycle enzymes or uncoupled metabolism. It is likely that all of these interrelated causes are involved (Luli and Strohl, 1990). In this study in order to decrease acetate accumulation, *E. coli* BL21 (DE3) pLySs strain, which was derived from *E. coli* B a low acetate producer (Luli and Strohl, 1990; Shiloach et al., 1996), was used as the host microorganism.

Presence of the organic acids of the TCA cycle, i.e., citric, succinic, fumaric and malic acid may be a consequence of inadequate oxygen supply which leads to repression on the TCA cycle enzymes or their improper activity. Apart from the effect of limiting oxygen, due to glucose depletion at  $t=8\text{h}$ , the lower amount of organic acid at  $t=10$  was obtained in almost all oxygen transfer conditions. Similarly, in the study of Anghardi, (2007) under oxygen limiting conditions the TCA cycle organic acids of  $\alpha$ -ketoglutaric acid and succinic acid were found in the medium showing insufficient operation of the TCA cycle under low oxygen transfer conditions and high acetate accumulation, up to  $2.5 \text{ kg m}^{-3}$  was observed, where recombinant *E. coli* K12 was the host microorganism.

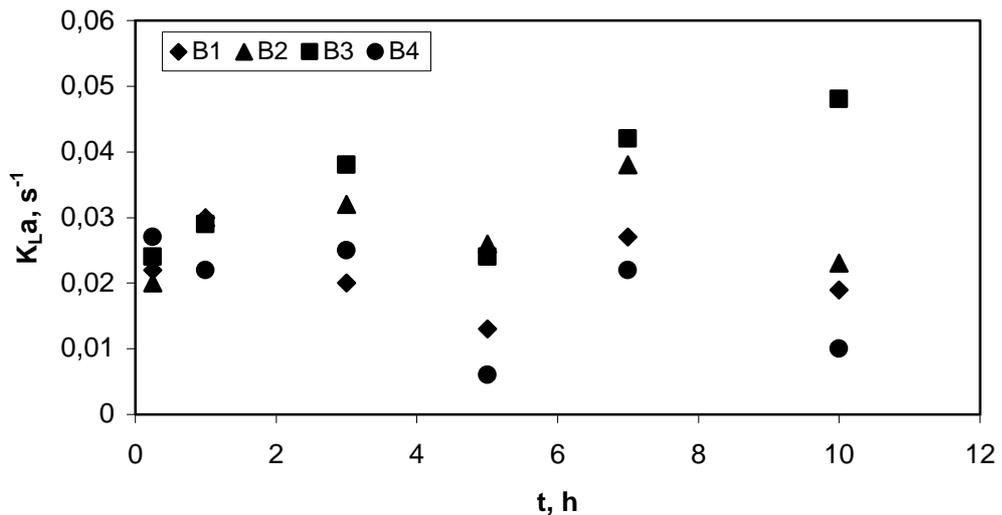
**Table 4.3** Organic acids detected in the fermentation broth with cultivation time at the batch mode bioreactor conditions.

	t, h	Acet. (kgm <sup>-3</sup> )	Form. (kgm <sup>-3</sup> )	Succ. (kgm <sup>-3</sup> )	Citr. (kgm <sup>-3</sup> )	Fuma. (kgm <sup>-3</sup> )	Mali. (kgm <sup>-3</sup> )	Total (kgm <sup>-3</sup> )
B1	0	0.071	0.025	-	0.001	0.009	6.78E-05	0.11
	2	0.134	0.107	-	0.031	0.016	-	0.29
	4	0.176	0.056	0.042	-	0.021	-	0.30
	6	0.190	0.116	0.081	-	-	-	0.39
	8	0.231	0.119	0.091	-	-	-	0.44
	10	0.235	0.133	0.110	-	-	-	0.48
	12	0.277	0.147	0.124	0.080	-	-	0.63
B2	0	0.124	0.013	-	-	-	0.011836	0.15
	2	0.200	0.049	-	-	-	-	0.25
	4	0.272	0.079	0.053	0.020	0.031	0.012803	0.47
	6	0.340	0.131	-	0.080	0.041	-	0.59
	8	0.408	0.137	-	0.096	0.047	0.01689	0.70
	10	0.436	0.151	-	0.110	0.050	-	0.75
	12	0.456	0.210	0.126	-	-	0.021578	0.81
B3	0	0.144	0.015	-	-	-	-	0.16
	2	0.223	0.052	-	0.014	0.031	0.018564	0.34
	4	0.301	0.040	-	-	0.043	0.019902	0.40
	6	0.371	0.107	-	0.015	0.048	0.020436	0.56
	8	0.441	0.199	0.066	0.015	0.055	0.022751	0.80
	10	0.473	0.213	0.119	0.017	0.057	-	0.88
	12	0.506	0.226	0.131	0.023	0.057	0.028765	0.97
B4	0	0.277	0.040	-	-	0.047	-	0.36
	2	0.340	0.125	-	-	-	-	0.46
	4	0.403	0.210	0.045	0.049327	0.051	-	0.76
	6	0.529	0.296	0.084	-	-	-	0.91
	8	0.656	0.381	0.123	0.0769	0.061	-	1.30
	10	0.710	0.417	0.140	-	-	-	1.27
	12	0.764	0.453	0.158	0.08904	0.082	-	1.55

#### 4.1.7 Oxygen Transfer Characteristics

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR),  $r_0$ , and oxygen transfer coefficient,  $K_{La}$ . The variations in  $K_{La}$ , oxygen uptake rate, and oxygen transfer rate, maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor ( $\eta$ ) calculated throughout the bioprocess are given in Table 4.4.

The variations in  $K_{La}$  with the cultivation time and molasses concentration  $K_{La}$  values varied between 0.006 and 0.048. As molasses concentration increased,  $K_{La}$  values did not show a stable trend. This could be due to many reasons.  $K_{La}$  depends on agitation rate, temperature, rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone.

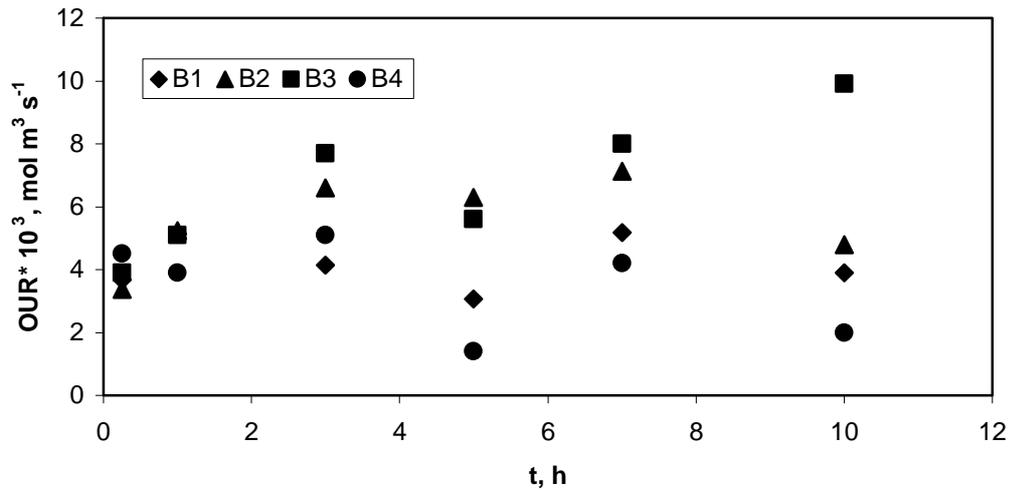


**Figure 4.9** The variations in  $K_{La}$  with the cultivation time and molasses concentration in batch mode bioreactor conditions: B1, ( $\diamond$ ); B2, ( $\blacktriangle$ ); B3, ( $\blacksquare$ ); B4, ( $\bullet$ );  $T=37^{\circ}C$ ,  $V_R= 1.65 \times 10^{-3} m^3$ ,  $DO=40\%$ ,  $N=625 min^{-1}$ ,  $pH_C=7.2$ .

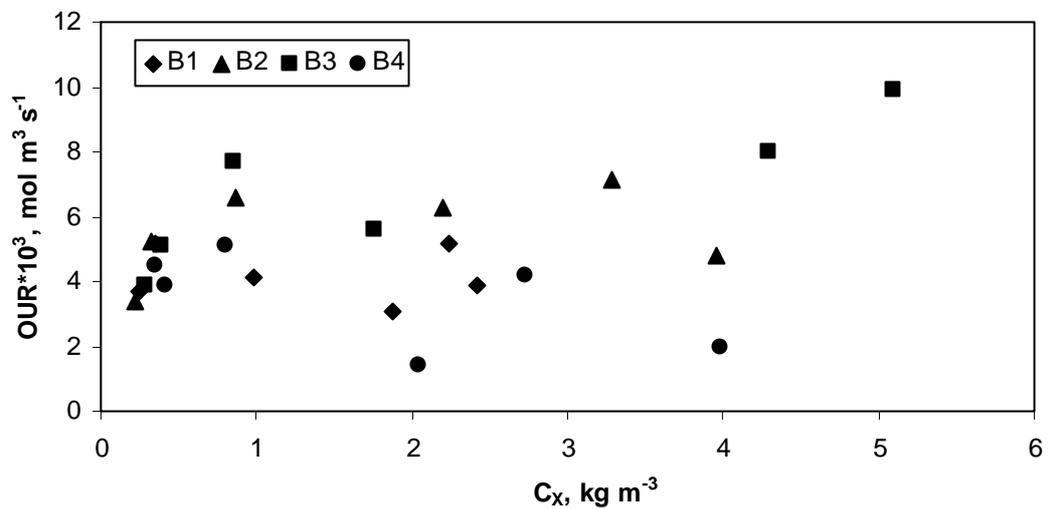
Temperature and agitation rate were kept constant throughout the bioprocess. Hence, the presence of complex medium components from the molasses and by-products formed can significantly alter the bubble size and liquid film resistance around the gas bubble. Therefore, particles with a

diameter somewhat greater than the thickness of the mass transfer layer enhance the gas absorption and the enhancement decreases by increasing particle diameter; therefore, along with the increase in the concentration of the rod-shaped micro-bioreactors, the mass transfer coefficient may increase or decrease depending on the diameter and concentration of the biomass (Çalık et al., 1998). Taking concerted effect of the dynamic shear stress caused by eddies and viscous stress into account, it can be said that observed increase in  $K_{La}$ , could be the result of the decrease in viscosity of the medium, which, also, explains higher  $K_{La}$  values obtained for B3 condition when compared with other conditions as it can be seen from the table 4.4. In the optimum condition, B3,  $K_{La}$  values increased with the cultivation time and reached a maximum at  $t=10$  h as  $0.048\text{ s}^{-1}$ . Lowest  $K_{La}$  values were obtained in B4 condition having the minimum as  $0.006\text{ s}^{-1}$  at  $t=5$ h. This decrease in  $K_{La}$  values could be explained as result of the high values of driving force at conditions where dissolved oxygen levels were controlled at the values of  $DO=40\%$ .

Oxygen uptake rate (OUR) is another important factor, which depends on the cultivation time, cell concentration and on the growth phases of the microorganisms. The variations in OUR with the cultivation time and cell concentration were shown in Figure 4.10 and 4.11, respectively. Since dissolved oxygen in the process was kept constant throughout the process, the OTR took the same values with the OUR and gave the same profile. Firstly increased during the lag phase and exponential phases of the microbial growth and reached a maximum at the exponential phase as it is the case in this analysis as it can be seen in Figure 4.10. Generally, the OUR and the OTR were maximum at  $t=5$  or  $7$  hours of the batch bioprocess and had a tendency to decrease towards the end of the process at the all conditions except B3; where the cell formation and the substrate consumption rates, and BAL activity of the cells, were highest (Figure 4.11). The OUR and the OTR took its maximum at  $t=10$  h in B3 condition as  $9.90\text{ mol m}^3\text{ s}^{-1}$  where the highest cell concentration was achieved also.



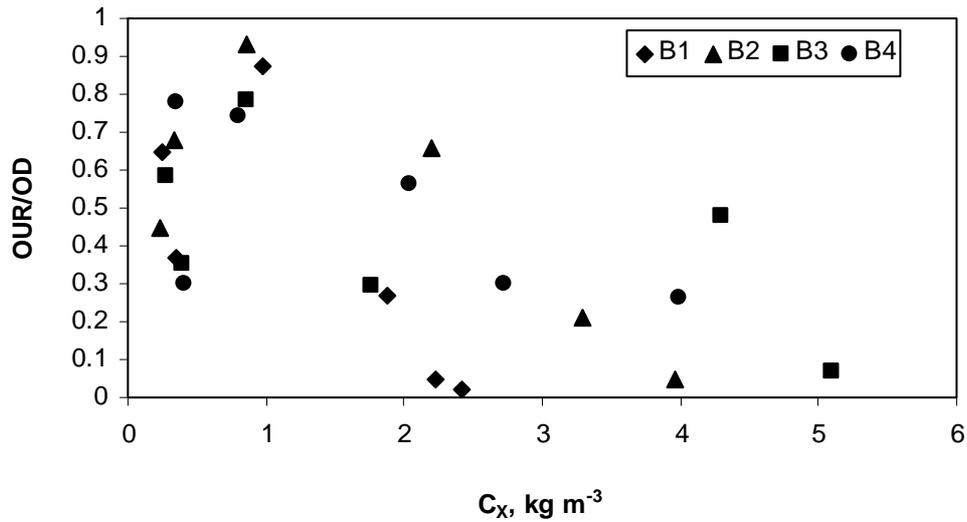
**Figure 4.10** The variations in the OUR with cultivation time and molasses concentration in batch mode bioreactor conditions: B1, ( $\blacklozenge$ ); B2, ( $\blacktriangle$ ); B3, ( $\blacksquare$ ); B4, ( $\bullet$ );  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .



**Figure 4.11** The variations in the OUR with the cell concentration and molasses concentration in batch mode bioreactor conditions: B1, ( $\blacklozenge$ ); B2, ( $\blacktriangle$ ); B3, ( $\blacksquare$ ); B4, ( $\bullet$ );  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

In order to determine the limiting step of the growth process, the maximum possible OUR (OD) and the maximum oxygen transfer rate were calculated in the course of the fermentation. Relation between both maximum rates is the modified Damköhler number,  $Da$  (Çalik et al., 2004). It is clear in Table 4.21 that, at all operation conditions, mass-transfer resistances, Damköhler number ( $Da$ ), which is the ratio between the maximum rate of oxygen consumption in the bioprocess and its maximum mass transfer rate ( $=OD/OTR_{max}$ ) were effective throughout the process ( $Da \gg 1$ ) indicating that mass transfer is the rate limiting step and the maximum possible oxygen utilization rate is significantly lower than at bulk phase condition. However, at some times of the process ( $t=0.25, 1$  and  $3$  h),  $Da$  value is smaller than 1 designating that the reaction resistance was superior than the mass transfer resistance. This showed that at those points, there is oxygen limitation because of high rates of cell formation. Correspondingly, 2.3 fold higher  $Da$  value was obtained at B3 condition at the end of the process with respect to that of Anghardi, (2007) although the only variable was carbon source (glucose).

Another important dimensionless parameter, effectiveness factor,  $\eta$ , is described as the ratio between the observed biochemical reaction rate and the formal reaction rate, without mass transfer (Çalik et al., 2004). As seen in Figure 4.12,  $\eta$  took its maximum  $t=3$  h for all of the batch cases. This indicates that cells are consuming oxygen with a high rate that maximum possible oxygen uptake rate (OD) value was attained. Then  $\eta$  decreased gradually meaning that the microorganisms are utilizing oxygen under the maximum oxygen demand.



**Figure 4.12** The variations in  $\eta$  (OUR/OD) with the cultivation time and molasses concentration in batch mode bioreactor conditions: B1, (◆); B2, (▲); B3, (■); B4, (●);  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

In the study of Anghardi (2007), at the same oxygen conditions, the lowest  $K_La$  value was as  $0.005 \text{ s}^{-1}$  at  $t=10 \text{ h}$  of the bioprocess and maximum was at  $t=3 \text{ h}$  as  $0.017 \text{ s}^{-1}$ . In the current study  $K_La$  values were higher than these ones. OUR and OTR values were alternating in that study lying between 0.6 and 1.9 whereas in the current study these values were much more higher showing oxygen consumption increase where the cell formation and the substrate consumption rates were also higher. From the  $Da$  profiles of two studies, it can be concluded that the study of Anghardi (2007) was a mass-transfer-limited condition, which resulted a higher profile of  $Da$ .  $Da$  increased, when the maximum oxygen transfer rate decreased by the increase in mass transfer resistance. As the current study attained higher oxygen transfer rate profile and hence the lowest mass transfer resistance, it had the lower profile for  $Da$ .

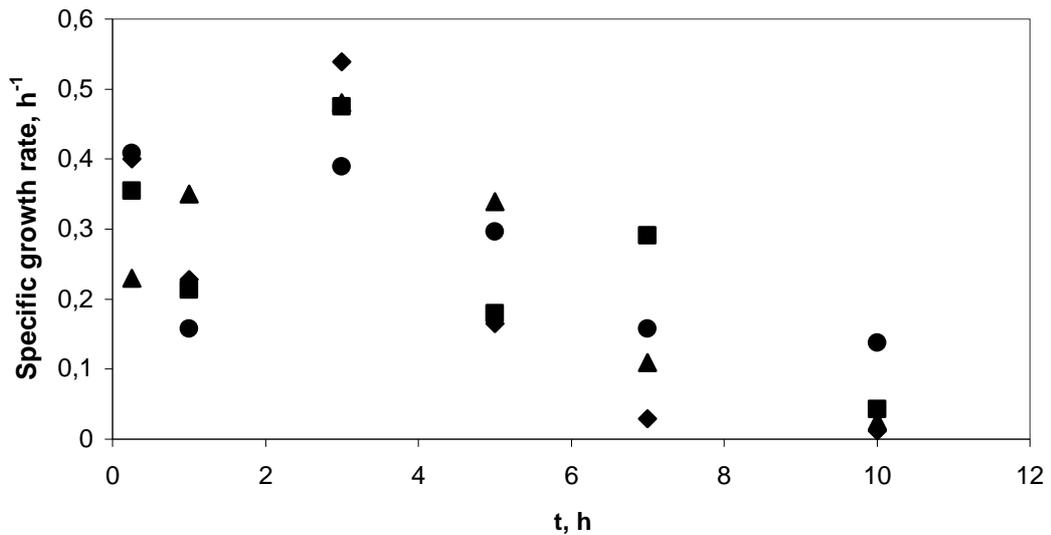
**Table 4.4** The variations in the oxygen transfer parameters in batch mode bioreactor conditions.

	t (h)	$K_L a$ ( $s^{-1}$ )	$OTR \cdot 10^3$ ( $mol m^{-3} s^{-1}$ )	$OTR_{max} \cdot 10^3$ ( $mol m^{-3} s^{-1}$ )	$OUR \cdot 10^3$ ( $mol m^{-3} s^{-1}$ )	$OD \cdot 10^3$ ( $mol m^{-3} s^{-1}$ )	Da (OD / $OTR_{max}$ )	$\eta$ (OUR /OD)
B1	0.25	0.022	3.68	7.15	3.68	0.65	0.80	0.65
	1	0.030	5.14	9.47	5.14	1.60	1.47	0.37
	3	0.020	4.14	6.50	4.14	0.55	0.73	0.87
	5	0.013	3.06	4.11	3.06	1.32	2.79	0.27
	7	0.027	5.18	8.78	5.18	12.71	12.56	0.05
	10	0.019	3.90	6.05	3.90	24.14	34.65	0.02
B2	0.25	0.020	3.37	6.54	3.37	7.58	1.16	0.44
	1	0.030	5.23	9.63	5.23	7.71	0.80	0.68
	3	0.032	6.59	10.35	6.59	6.59	0.64	0.93
	5	0.026	6.29	8.45	6.29	9.56	1.13	0.66
	7	0.038	7.13	12.08	7.13	33.51	2.77	0.21
	10	0.023	4.79	7.43	4.79	97.77	13.16	0.05
B3	0.25	0.024	3.90	7.57	3.90	6.68	0.88	0.58
	1	0.029	5.10	9.39	5.10	14.54	1.55	0.35
	3	0.038	7.70	12.09	7.70	9.85	0.81	0.78
	5	0.024	5.60	7.53	5.60	18.98	2.52	0.30
	7	0.042	8.00	13.56	8.00	16.74	1.23	0.48
	10	0.048	9.90	15.35	9.90	140.38	9.15	0.07
B4	0.25	0.027	4.50	8.74	4.50	5.78	0.66	0.78
	1	0.022	3.90	7.18	3.90	12.99	1.81	0.30
	3	0.025	5.10	8.01	5.10	6.87	0.86	0.74
	5	0.006	1.40	1.88	1.40	2.48	1.32	0.56
	7	0.022	4.20	7.12	4.20	13.97	1.96	0.30
	10	0.010	2.00	3.10	2.00	7.63	2.46	0.26

#### 4.1.8 Specific Growth Rate and Yield Coefficients

The variations in the specific growth rate,  $\mu$ , specific oxygen uptake rate,  $q_o$ , specific substrate utilization rate,  $q_s$ , and yield coefficients in batch conditions with cultivation time are given in Table 4.5.

Figure 4.13 shows the change of specific growth rate,  $\mu$ , with cultivation time. At all operation conditions applied,  $\mu$  decreases with cultivation time and approaches to 0 as the cells get closer to the stationary phase. The highest specific growth rate ( $\mu$ ) was obtained as  $\mu_{\max}=0.54 \text{ h}^{-1}$  at  $t=3 \text{ h}$  at B1 condition and the highest cell concentration was achieved among the operation conditions at B3 as  $C_x=5.3 \text{ kg m}^{-3}$  at  $t=12 \text{ h}$ . The maximum specific growth rate achieved in the same oxygen transfer condition in the study of Anghardi, (2007) was reported as  $\mu_{\max}=0.63 \text{ h}^{-1}$  at  $t=0.25 \text{ h}$  and the highest cell concentration was achieved as  $C_x=3.0 \text{ kg m}^{-3}$  at  $t=12 \text{ h}$ .



**Figure 4.13** The variations in specific growth rate with the cultivation time and molasses concentration in batch mode bioreactor conditions: B1, (♦); B2, (▲); B3, (■); B4, (●);  $T=37^\circ\text{C}$ ,  $V_R=1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

Specific oxygen uptake rates ( $q_o$ ) and specific substrate utilization rates ( $q_s$ ) decreased with the cultivation time at all the conditions having the highest values of 1.83 and 3.47  $\text{kg kg}^{-1} \text{h}^{-1}$  at  $t=1$  h at B2 operation, respectively, whereas the lowest values were found as 0.06 and 0.35 at  $t=10$  for B4 case when there occurred fructose accumulation after 6<sup>th</sup> hour. When they are compared with Anghardi (2007), although glucose consumption rates were higher, specific substrate utilization rates were obtained lower due to the low consumption values of fructose.

The yield coefficients, which represent the overall distribution and the efficiency of conversion reactions, were given also in Table 4.5. Cell yield on oxygen values altered throughout the process and at batch operations, the highest  $Y_{x/o}$  value was obtained at B4 condition at  $t=5$  h as 3.75  $\text{kg kg}^{-1}$ . The lowest  $Y_{x/o}$  value was obtained at B1 at  $t=7$  h as 0.11  $\text{kg kg}^{-1}$  indicating the inefficient use of the oxygen through biochemical reaction network.

Specific cell yield on substrate ( $Y_{x/s}$ ), in batch cases, increased between 1<sup>st</sup> and 5<sup>th</sup> hours and decreased gradually through the end of the process when cell growth rate decreased. The highest overall cell yield on substrate was obtained in B3 condition as 0.37  $\text{kg kg}^{-1} \text{h}^{-1}$  as it has the highest cell concentration and the lowest overall cell yield on substrate was determined as 0.18  $\text{kg kg}^{-1} \text{h}^{-1}$  since glucose and fructose was not consumed by cells much and cell concentration remained the same.

The amount of substrate metabolized per amount of oxygen used ( $Y_{s/o}$ ) increased between  $t=1-5$  h of bioprocess with cultivation time and then reduced at the remaining time of the process at B1 and B2 conditions however at B3 and B4 conditions the values altered most probably due to the oxygen limitations. The amount of substrate consumed per amount of oxygen utilized,  $Y_{s/o}$ , values were much more higher than the previous studies (Anghardi, 2007; Çalık et al., 2004). Therefore, there can be suggested that when molasses was used as medium, cells require much more energy for effective substrate utilization to maintain the optimum cell growth.

**Table 4.5** The variations in specific growth rate and yield coefficients in batch mode bioreactor conditions.

	t (h)	$\mu$ (h <sup>-1</sup> )	$q_o$ (kgkg <sup>-1</sup> h <sup>-1</sup> )	$q_s$ (kgkg <sup>-1</sup> h <sup>-1</sup> )	$Y_{x/s}$ (kgkg <sup>-1</sup> )	$Y_{x/o}$ (kgkg <sup>-1</sup> )	$Y_{s/o}$ (kgkg <sup>-1</sup> )	$m_o$	$m_s$
B1	0.25	0.40	1.70	2.80	0.14	0.24	1.65	0.18	0.12
	1	0.23	1.69	3.16	0.07	0.14	1.87		
	3	0.54	0.49	0.40	1.35	1.11	0.82		
	5	0.16	0.19	0.23	0.71	0.88	1.24		
	7	0.03	0.27	0.09	0.34	0.11	0.32		
	10	0.01	0.19	0.13	0.09	0.06	0.68		
	Overall				0.29				
B2	0.25	0.23	1.72	3.38	0.07	0.13	1.97	0.11	0.08
	1	0.35	1.83	3.47	0.10	0.19	1.90		
	3	0.48	0.88	1.07	0.45	0.55	1.22		
	5	0.34	0.33	0.27	1.26	1.03	0.82		
	7	0.11	0.25	0.11	1.03	0.44	0.43		
	10	0.03	0.14	0.09	0.29	0.18	0.62		
	Overall				0.33				
B3	0.25	0.35	1.61	1.54	0.23	0.22	0.96	0.1	0.05
	1	0.21	1.50	1.66	0.13	0.14	1.10		
	3	0.48	1.03	0.95	0.50	0.46	0.92		
	5	0.18	0.37	0.56	0.32	0.49	1.53		
	7	0.29	0.21	0.15	1.93	1.35	0.70		
	10	0.04	0.22	0.10	0.44	0.19	0.44		
	Overall				0.37				
B4	0.25	0.41	1.47	2.23	0.18	0.28	1.51	0.07	0.06
	1	0.16	1.10	2.88	0.05	0.14	2.62		
	3	0.39	0.74	1.65	0.24	0.53	2.23		
	5	0.30	0.08	0.54	0.55	3.75	6.81		
	7	0.16	0.18	0.26	0.60	0.89	1.49		
	10	0.14	0.06	0.35	0.40	2.38	6.01		

## 4.2 Development of a Feeding Strategy

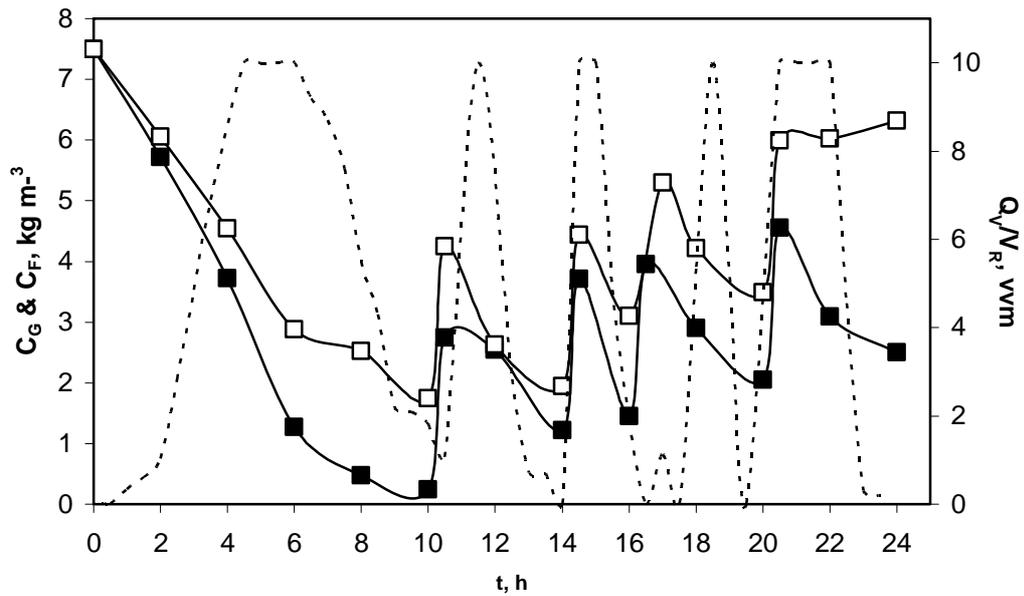
A molasses based complex medium was optimized for BAL production by recombinant *E.coli*. The fed batch cultivation was applied to produce efficient cells with higher cell concentration and BAL activity. The idea was based on the addition of fresh carbon source, after glucose and fructose was utilized. Nevertheless, there occurs acetate accumulation if it was added too much. Thus two kinds of feeding strategies that are complementary should be handled by combining their respective advantages. The study of Akesson et al. (2001) combined these two cases hence it was taken as a basis and a feeding strategy was developed.

### 4.2.1 Air Flow Variation Strategy- FB1

It is not known beforehand if or when overflow metabolism becomes limiting, and it is therefore necessary to monitor this continuously. 10 hours of cultivation with molasses based medium having glucose and fructose concentration of  $7.5 \text{ kg m}^{-3}$  was performed. While airflow controlled dissolved oxygen by oxygen probe, when the airflow was at its maximum this showed that the oxygen demand would exceed the oxygen transfer capacity (Akesson et al., 2001). Hence no feed was allowed until airflow was at or close to its minimum. At that moment when the airflow reached its minimum, this showed decrease in oxygen demand meaning that carbon source was about to finish. Thus, feed pulses with  $2.5 \text{ kg m}^{-3}$  glucose and fructose concentration were given to the system. In this way, aerobic conditions can be assured and at the same time overflow metabolism can be detected and avoided.

The variations in airflow rate and glucose and fructose concentrations with the cultivation time and feed pulses were given in Figure 4.12. Apparently, at the end of 10 hour batch process, air flow reached its minimum hence a feed pulse was given. Since  $2.5 \text{ kg m}^{-3}$  was a low glucose and fructose concentration, it was consumed in 4 hours time and a second pulse was given in 14<sup>th</sup> hour. This trend has been repeated for 16<sup>th</sup> and 20<sup>th</sup> hours. As air flow saturated after 20<sup>th</sup> hour, it was decided to finish the cultivation period at the end of 24 hour.

In order to verify the strategy, it is best to analyze the variations of fructose and glucose concentrations with feed pulses and cultivation time in Figure 4.14.



**Figure 4.14** The variations in airflow rate (dashed lines) and glucose (■) and fructose (□) concentrations (solid lines) with respect to cultivation time and feed pulses in FB1,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

From the figure 4.14, it is clear to see the pulse points. At  $t=10, 14, 16$  and  $20 \text{ h}$ , glucose concentration achieved nearly to minimum. An increased glucose concentration correlated well with the pulse response shortly after. Hence, it was logical to add fresh glucose.

As explained in 4.1.2, fructose is not a preferred source for *E.coli* consumption when there is glucose in the medium. From the figure 4.14, it could be understood that only at the times when there was no more glucose for consumption; fructose was preferred by the cells. Hence, for the 14 hours of the cultivation, fructose was used up. After 16<sup>th</sup> hour, fructose was built up. These results showed that, although there was fructose in the medium, air flow variation with the cultivation time illustrated that there was no more carbon source. This is due to the shift in metabolism from glucose to fructose.

A major advantage of the presented method is the general applicability. While many interesting approaches have been suggested to the problem, they often involve specialized instrumentation. In contrast, the only sensor required

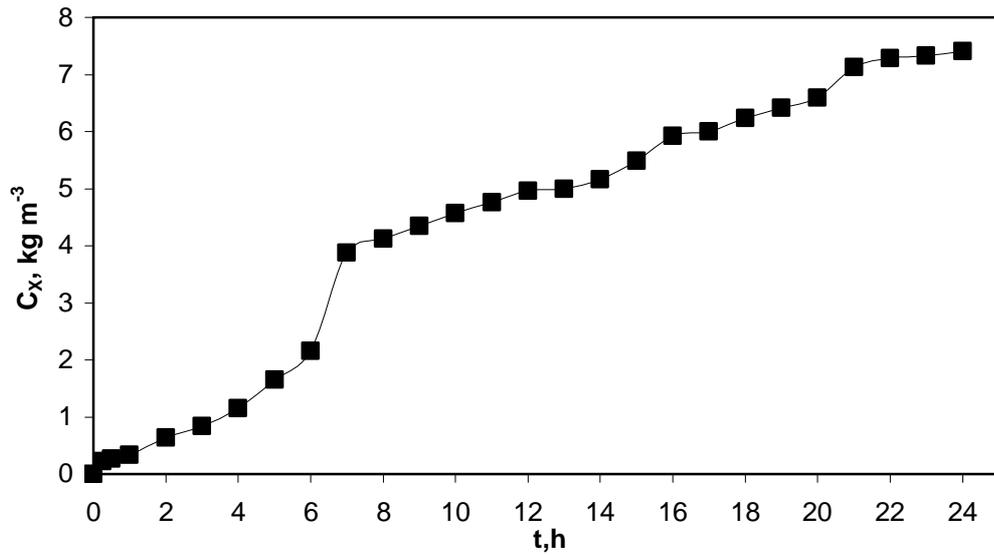
in the presented method is a dissolved oxygen probe, a standard measurement.

In fed-batch processes, glucose feeding strategies were arranged in order to keep cell growth at critical level where acetate accumulation starts with automated feeding systems (Johnston et al., 2003; Akesson et al., 2000) where lower initial glucose concentration up to  $2 \text{ kg m}^{-3}$  were preferred (Luli and Strohl, 1990; Shiloach et al., 1996). This concentration correlated well with the one used in the current study. In this way, aerobic conditions can be assured and at the same time overflow metabolism can be detected and avoided.

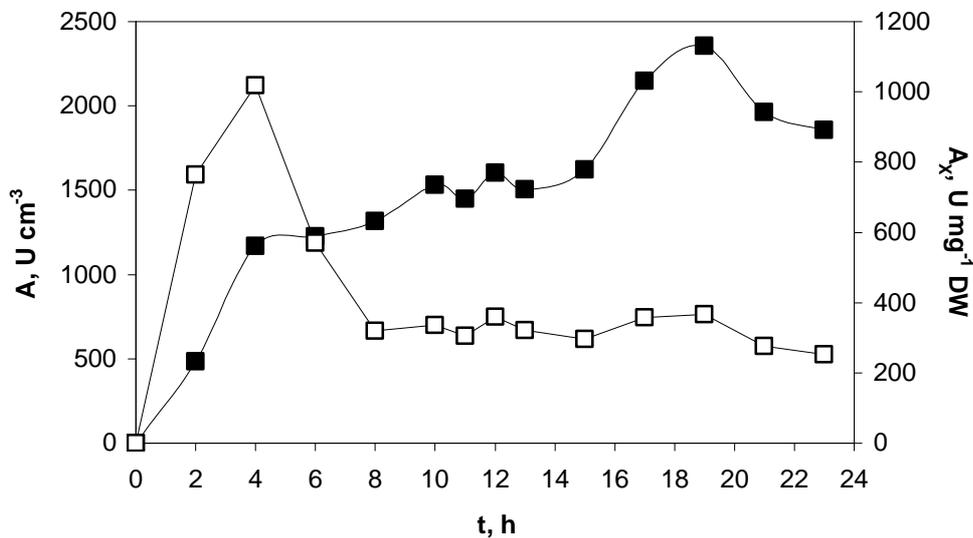
In the study of Akesson (2000), the strategy based on the fact that the maximum oxygen transfer capacity of the reactor was reached when the maximum stirrer speed attained its maximum. Hence the feed rate was decreased. However, this strategy had a weakness since the stirrer speed could only climb up to 1000-1200 rpm. Due to this limitation, the strategy was extended and air flow was preferred as the control parameter for the dissolved oxygen in the current study.

The outcomes of the strategy can be understood best from the variation of cell concentration with respect to cultivation time and feed pulses (Figure 4.15).

There is an increasing cell growth profile throughout the process. After the times when the pulses were introduced to the system, cell growth rate apparently increased. After 20<sup>th</sup> hour, the cells seemed to reach stationary phase. They achieved a concentration of  $7.4 \text{ kg m}^{-3}$  at the end of the process. This is the maximum cell growth for BAL production with *E. coli* cultivation in the literature.



**Figure 4.15** The variations of cell concentration with respect to cultivation time and feed pulses in FB1,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .



**Figure 4.16** The variations of Benzaldehyde Lyase activity(■) and specific activity(□) with respect to cultivation time and feed pulses in FB1,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

The variations of BAL activity and specific activity with the cultivation time are given in Figure 4.16. The activity profile was same as the batch case until the pulse was given. When the feed pulses were presented, activity increased considerably. The maximum activity was reached at 20<sup>th</sup> hour as 2360 U cm<sup>-3</sup>. Afterwards, cells lost the ability for BAL productivity probably because of the inhibition of glucose and fructose.

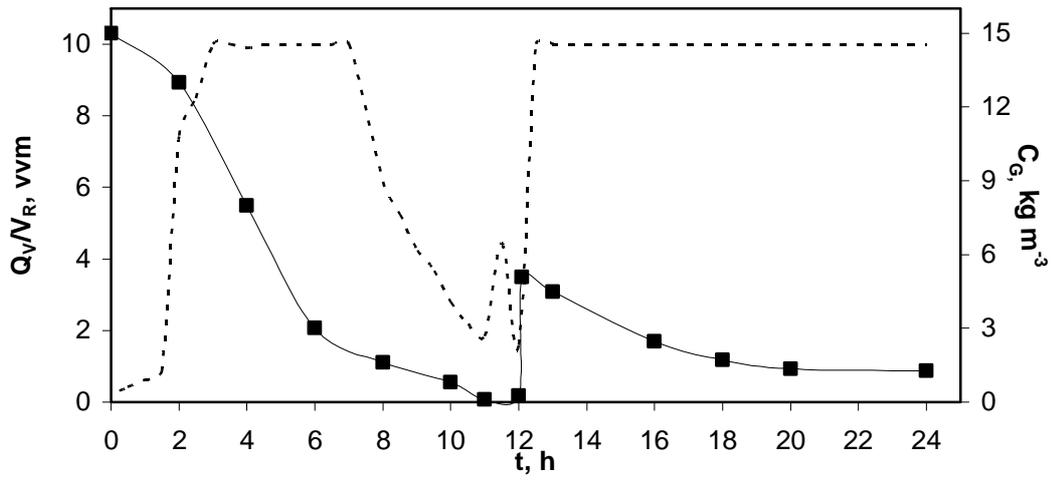
In the case of specific activity, the highest increase was seemed in the first four hours of the cultivation and reached a value of 1018 U mg<sup>-1</sup> DW then the specific activity decreased and reached a stationary phase at t= 8 h. After that time, specific activity did not changed at all until it began to decrease after t= 20 h. This showed that the pulses given increased the cell concentration and activity at the same rate.

The results of FB1 showed that presenting the last two feed pulses was not the right choice since there was no further increase in BAL activity after 20<sup>th</sup> hour. This strategy should be ended at t=20<sup>th</sup> hour. Hence the cell concentration at that time should be considered as the maximum cell concentration which was 6.6 kg m<sup>-3</sup> since the cells growing after that time was not carrying BAL producing cells.

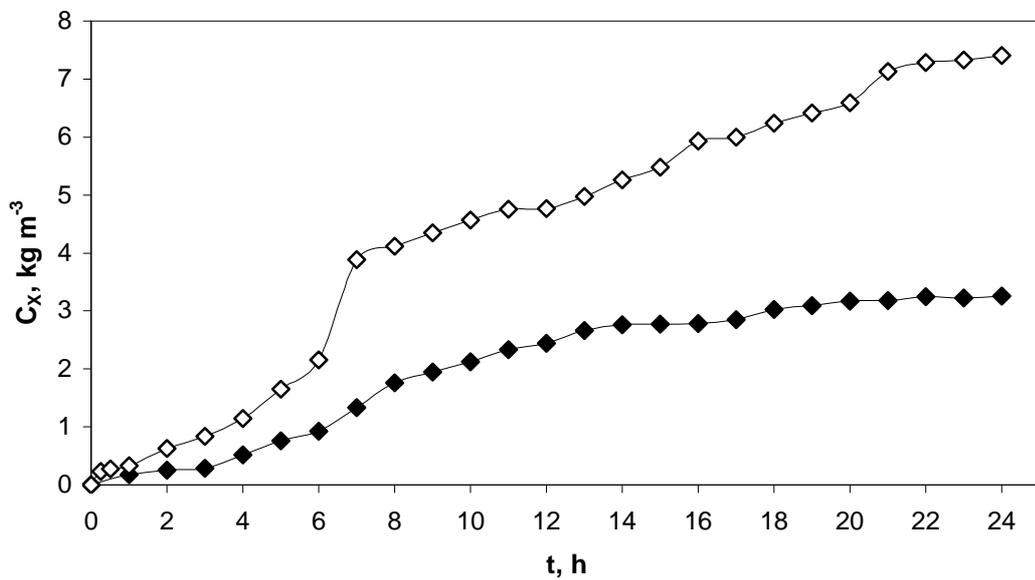
#### **4.2.2 Glucose Based Medium-FB2**

The feeding strategy was developed for molasses based medium. In order to see if it is valid for defined medium, the same strategy was applied to glucose based medium having a concentration of 15 kg m<sup>-3</sup>. The variations in airflow and glucose concentration with the cultivation time in glucose based medium (FB2) was shown in Figure 4.17. It is clear that, airflow reached its minimum only at t=12 h. Hence, only pulse with 5 kg m<sup>-3</sup> glucose concentration was given at that time. After 14<sup>th</sup> hour, air flow did not change at all indicating high oxygen demand during the process. As glucose did not depleted completely, air flow did not fall during the process. This reality is verified with glucose variation with time.

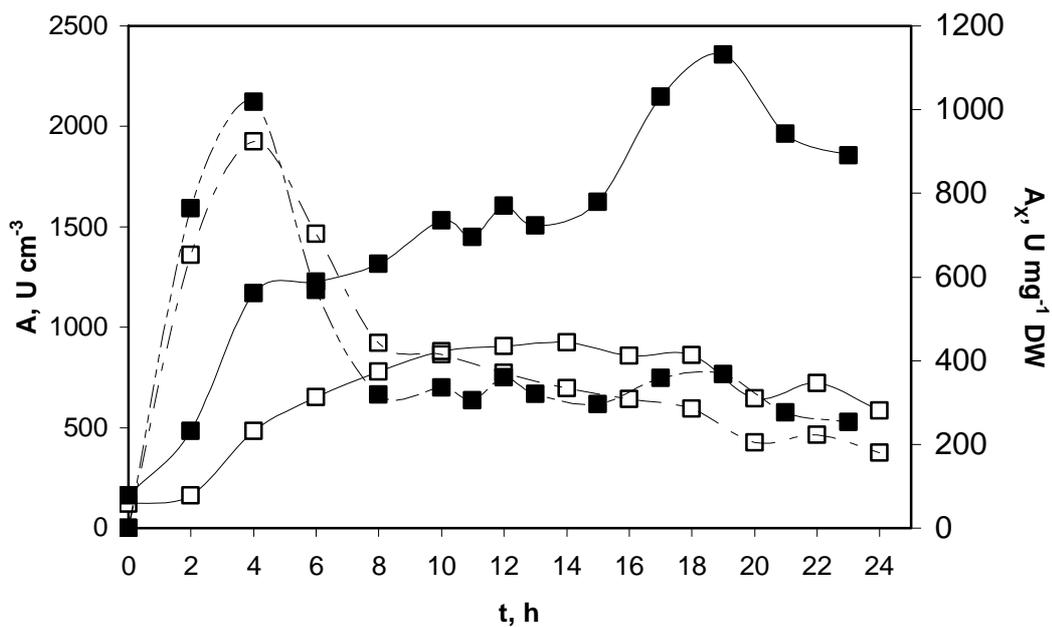
The cell concentration and BAL activity variation were given in figures 4.18 and 4.19 respectively. Apparently, the feeding influenced much neither the cell concentration nor the BAL activity. The maximum cell concentration and BAL activity achieved were 3.26 kg m<sup>-3</sup> and 924 U cm<sup>-3</sup>, respectively. Besides, activity decreased after 20<sup>th</sup> hour. This might be the result of inhibitory effect of glucose on cell metabolism.



**Figure 4.17** The variations in airflow (dashed lines) and glucose concentration (■) with the cultivation time in glucose based medium (FB2);  $T=37^{\circ}\text{C}$ ,  $V_R=1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .



**Figure 4.18** The variations in cell concentration with the cultivation time in FB1 (□) and FB2 (■);  $T=37^{\circ}\text{C}$ ,  $V_R=1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .



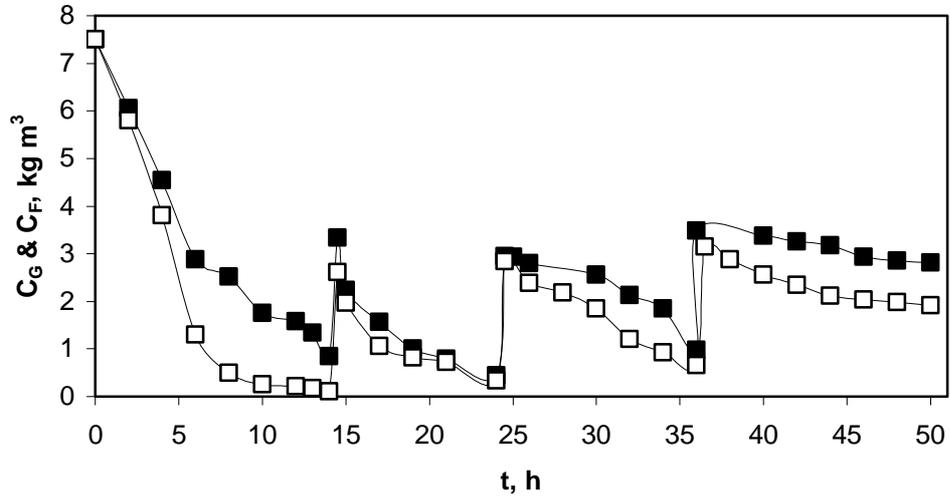
**Figure 4.19** The variations in BAL activity (solid lines) and specific activity (dashed lines) with the cultivation time in FB1 (■) and FB2 (□);  $T=37^{\circ}\text{C}$ ,  $V_R=1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

In the thesis study of Anghardi (2007), the highest cell concentration was obtained as  $3.0 \text{ kg m}^{-3}$  and the highest benzaldehyde lyase volumetric activity was obtained as  $1095 \text{ U cm}^{-3}$  in the same medium with  $8 \text{ kg m}^{-3}$  initial glucose concentration indicating this strategy is not reasonable for glucose based medium costing extended cultivation time and need for higher glucose concentration. As a result, although the same strategy was applied to both defined and complex medium, complex media was preferred by the cells because complex media provides more carbon, nitrogen, vitamins and trace elements desired for cell growth and metabolite production.

#### 4.2.3 Glucose and Fructose Detection- FB3

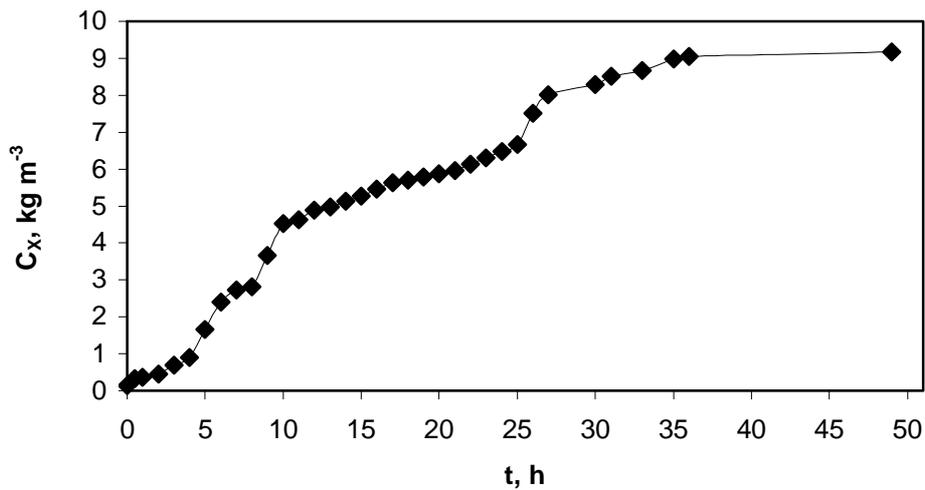
According to feeding strategy based on the air flow variation (FB1), the results showed that the decrease in the airflow only demonstrated the consumption of glucose. This lack in the strategy was able to be solved by detection of the fructose and glucose concentrations during the process and giving pulses to the system when glucose and fructose were about to finish. According to this strategy, pulses were given at  $t=14 \text{ h}$ ,  $t=24 \text{ h}$ , and  $t=36 \text{ h}$

worked well using less oxygen and giving since both glucose and fructose reached to the minimum values at those times.



**Figure 4.20** The variations in glucose (■) and fructose (□) concentration with respect to cultivation time and feed pulses in FB3;  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^\circ\text{C}$ ,  $V_R=1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

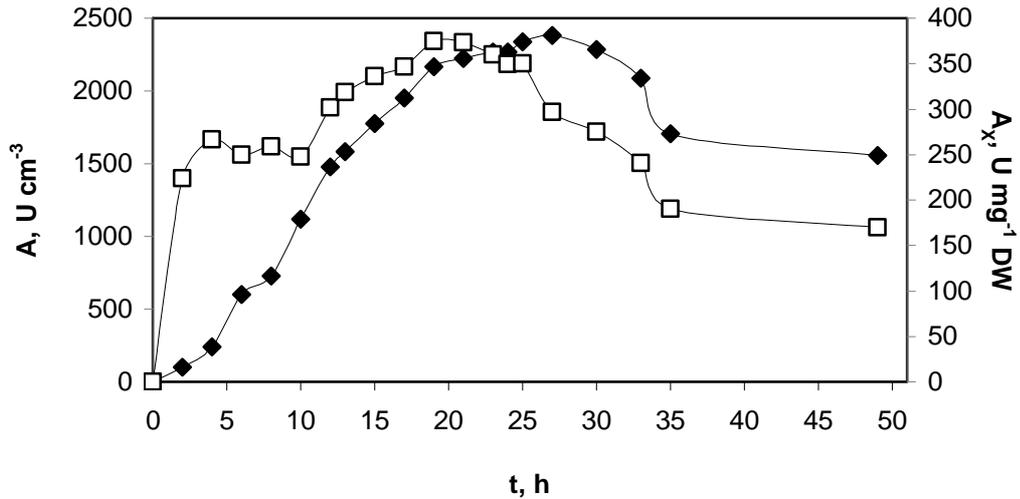
The variations of cell concentration and BAL activity were investigated with the extended strategy at B3 condition and were given in Figures 4.21 and 4.22, respectively. It was concluded from the Figure 4.21 that cell concentration increased between  $t=0-36 \text{ h}$  with the increase in cultivation time. Afterwards, the stationary phase was reached. The highest cell concentration was achieved at  $t=36 \text{ h}$  as  $9.1 \text{ kg m}^{-3}$ . This result is 1.2 fold higher than the one obtained at FB1 case at  $t=24 \text{ h}$ ; nevertheless, this time there is a cost of higher cultivation times.



**Figure 4.21** The variations in cell concentration with respect to cultivation time and feed pulses in FB3,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

The variation of BAL activity and specific activity with cultivation time is given in Figure 4.22. The highest BAL activity was obtained as  $2370 \text{ U cm}^{-3}$  at  $t= 26 \text{ h}$  then started to decrease. This showed that, although cell formation continued for further 10 hours, BAL producing cells were not produced at all. As a result this process should be stopped at  $t= 26\text{h}$  since there was no increase in BAL activity after that time. Hence the cell concentration of  $26^{\text{th}}$  hour should be taken into account as the maximum cell concentration which was  $7.5 \text{ kg m}^{-3}$ .

This study was based on the study of Akesson et al. (2000). However since the airflow variation did not worked well in the medium with both glucose and fructose, strategy was updated by detection of glucose and fructose.

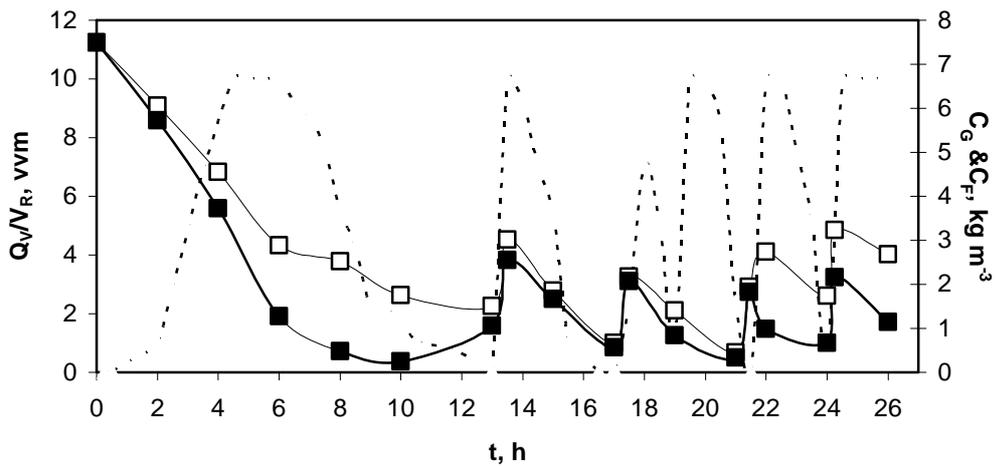


**Figure 4.22** The variations of Benzaldehyde Lyase activity (□) and specific activity(■) with respect to cultivation time and feed pulses in FB3,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

#### 4.2.4 Air Flow Variation Strategy (Extended) - FB4

When the results of FB1 were analyzed, two problems stood in the strategy. First one was about the excess amount of feed pulses that  $2.5 \text{ kg m}^{-3}$  concentration of each glucose and fructose might be plenty for such a strategy and decreasing the concentration of glucose and fructose could decrease the accumulation of fructose and glucose. Hence  $1.5 \text{ kg m}^{-3}$  was chosen at this time as the glucose and fructose concentration of the feed pulses. The second problem in FB1 condition was due to the shift in metabolism from glucose and fructose. In order to work out this problem, the idea was allowing time lag to watch the shift and add feed pulses according to that. (FB4)

In this case, four pulses were given to the system at  $t=13 \text{ h}$ ,  $17 \text{ h}$ ,  $21 \text{ h}$ ,  $24 \text{ h}$ . An increased glucose and fructose concentration correlated well with the pulse response shortly after. From the Figure 4.23 it could be clearly understood that, this time glucose and fructose accumulation was fewer than FB1 case so first problem was handled.

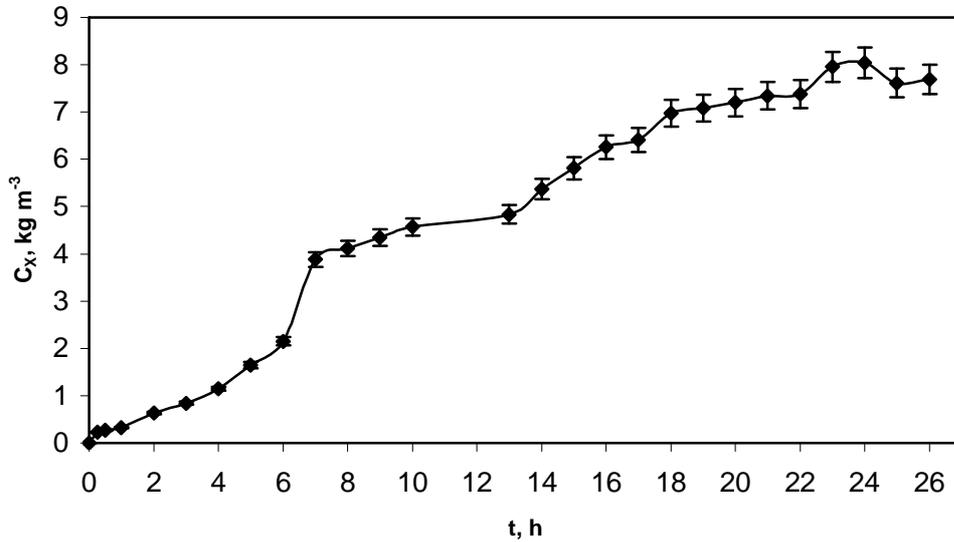


**Figure 4.23** The variations in airflow rate (dashed lines) and glucose (■) and fructose (□) concentrations (solid lines) with respect to cultivation time and feed pulses in FB4,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^\circ\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

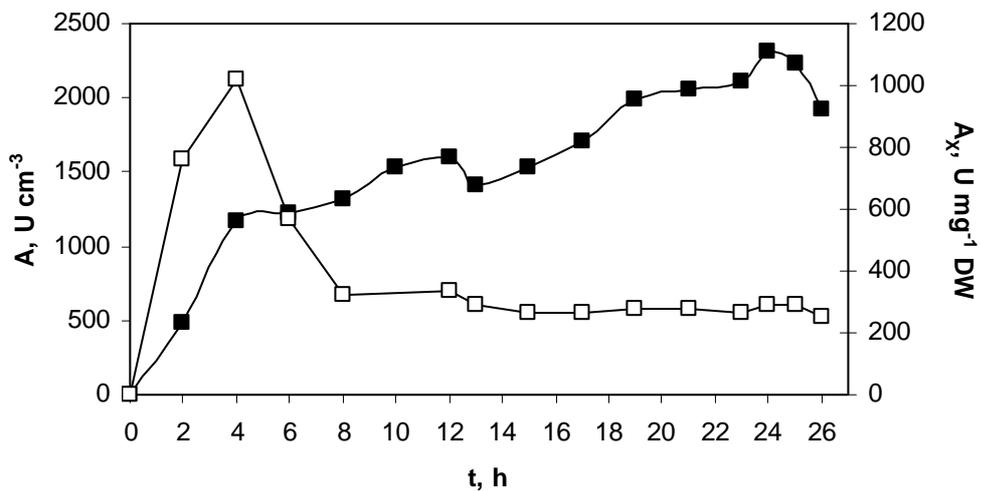
In the biochemical processes which contain both glucose and fructose in their growing media, there occurs a shift in metabolism from glucose to fructose. In FB1 and FB3 this was not taken into account while presenting pulses. However in FB4, visualizing this shift was aimed. From Figure 4.23, at  $t=19 \text{ h}$ , there was a decrease in airflow which was a sign of depletion of glucose and fructose. However when some time was allowed, it was clearly observed that there occurred again an increase in airflow. This showed that, at  $t=19 \text{ h}$  glucose was depleted and the decrease in airflow was due to that. After a while, there occurred an increase in airflow again demonstrating the start up of the fructose consumption. This behavior could not be observed in  $t=13 \text{ h}$ ,  $21 \text{ h}$  and  $t=24 \text{ h}$  since at  $t=13 \text{ h}$  shift was so fast that it could not be caught. On the contrary, at  $t=24 \text{ h}$ , shift time was too long to be observed.

The variations in cell concentration and BAL activity were given in Figure 4.24 and Figure 4.25. By this strategy a cell concentration of  $8.04 \text{ kg m}^{-3}$  was achieved at  $t=24 \text{ h}$  and a maximum activity of  $2315 \text{ U cm}^{-3}$  was obtained at the same time. According to these results, it did not need to present the last feed pulse since there is no increase in BAL activity due to that feed. Specific

activity did not changed a lot after 12<sup>th</sup> hour showing a balance between activity and cell concentration throughout the process.



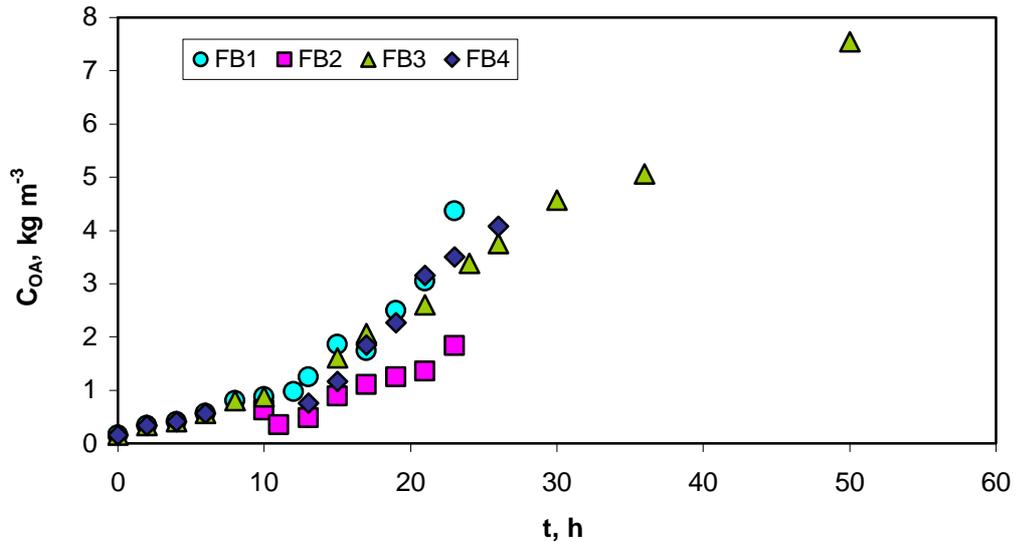
**Figure 4.24** The variations in cell concentration with respect to cultivation time and feed pulses in FB4,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .



**Figure 4.25** The variations of Benzaldehyde Lyase activity (□) and specific activity(■) with respect to cultivation time and feed pulses in FB4,  $C_{G\&F}=7.5 \text{ kg m}^{-3}$ ,  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

#### 4.2.5 Organic Acid Concentration Profiles

The variations in the organic acids detected in the fermentation broth with the cultivation time and fed batch conditions were demonstrated in Table 4.6. For the entire fed batch conditions applied, acetic, formic, succinic, citric fumaric and malic, acids were found in the fermentation broth in general increasing amounts.

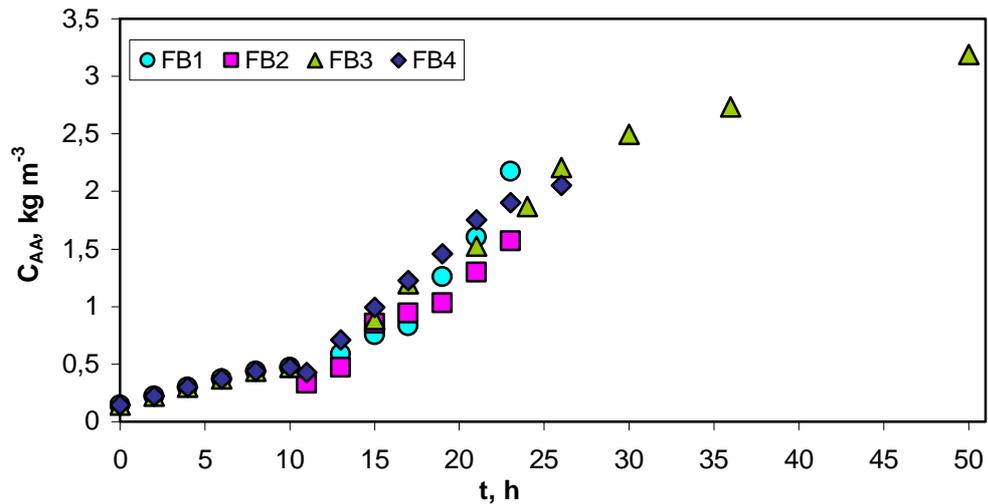


**Figure 4.26** The variations in organic acid concentrations with cultivation time and fed batch mode bioreactor conditions;  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65\times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

In figure 4.26, variation in total organic acid concentration with cultivation time was given. It is clear from the figure that with the increase in cultivation time, organic acid concentration increased at all the conditions. At the end of 23 hour total organic acid concentrations of FB1, FB2, FB3 and FB4 were  $4.36 \text{ kg m}^{-3}$ ,  $1.83 \text{ kg m}^{-3}$ ,  $3.38 \text{ kg m}^{-3}$  and  $3.50 \text{ kg m}^{-3}$ , respectively.

The strongest motivation to develop the fed batch methods to achieve higher density of *E.coli* culture was to decrease acetate accumulation, since high acetate concentration can inhibit growth and recombinant protein production (Luli and Strohl, 1990; Shimizu et al, 1991). Hence, the feeding strategy developed in this study based on the production of acetate. By controlling the carbon source with airflow, the goal was to assure aerobic

conditions and at the same time to detect the overflow metabolism in order not to produce acetate. Hence, acetic acid profile is essential. The variations with acetic acid concentration with cultivation time and fed batch conditions were shown in the Figure 4.27.



**Figure 4.27** The variations in acetic acid concentrations with cultivation time in fed batch mode bioreactor conditions;  $T=37^{\circ}\text{C}$ ,  $V_R= 1.65 \times 10^{-3} \text{ m}^3$ ,  $\text{DO}=40\%$ ,  $N=625 \text{ min}^{-1}$ ,  $\text{pH}_C=7.2$ .

In the current study, in FB1 case, acetate has accumulated after the batch phase and it had a concentration of  $0.439 \text{ kg m}^{-3}$  at  $t=11 \text{ h}$  at the beginning of fed batch phase (Table 4.6). Acetic acid concentration continued to increase with cultivation time and attained a concentration of  $2.17 \text{ kg m}^{-3}$  at  $t=23 \text{ hour}$ . This might due to fructose and glucose accumulation so acetate production and also addition of pulses having acetic acid as their content. Shiloach et al., (1996) had an acetate accumulation of  $2 \text{ kg m}^{-3}$  in batch mode and  $1 \text{ kg m}^{-3}$  in fed batch mode by *E. coli* BL21 and Akesson et al., (2001) reported much lower. However, these fermentations were done in a glucose based medium. It became a general consensus that acetate accumulation above  $2 \text{ kg m}^{-3}$  in the growth media slows down *E.coli* growth, may stop biomass build up and may inhibit recombinant protein biosynthesis. As a result, propagation techniques that keep acetate concentration below  $2 \text{ kg m}^{-3}$  are

aimed (Shiloach et al., 2005). Thereafter, the current study, a complex medium leading acetate accumulation of  $2.17 \text{ kg m}^{-3}$  could be thought as reasonable. Furthermore the acetate at  $t=21 \text{ h}$  should be considered as the acetate accumulation which was  $1.60 \text{ kg m}^{-3}$  since  $t=21 \text{ h}$  is the time when the process should be ended. In glucose based fed batch case, (FB2), an acetate accumulation of  $1.57 \text{ kg m}^{-3}$  was reached at the end of the process which is a good result although there was glucose accumulation after 16<sup>th</sup> hour. When glucose and fructose detection strategy was applied (FB3), the acetic acid accumulation rose to  $3.19 \text{ kg m}^{-3}$  at  $t=50 \text{ h}$  which was inhibitory for the medium and explains the lower activity of BAL. However this might be due to higher cultivation time. If the acetate concentration at  $t=26 \text{ h}$ , which was the time with the highest BAL activity, was taken into account it can be concluded that a value of  $2.21 \text{ kg m}^{-3}$  was in the interval of the accepted concentration of acetate. In the extended strategy (FB4), acetate accumulation was  $2.05 \text{ kg m}^{-3}$  at the end of 26 hour and  $1.90 \text{ kg m}^{-3}$  at  $t=23 \text{ h}$ .

**Table 4.6** Organic acids detected in the fermentation broth with cultivation time at the fed batch operations.

	Time, h	Acet. (kgm <sup>-3</sup> )	Form. (kgm <sup>-3</sup> )	Succ. (kgm <sup>-3</sup> )	Citr. (kgm <sup>-3</sup> )	Fuma. (kgm <sup>-3</sup> )	Mali. (kgm <sup>-3</sup> )	Total (kgm <sup>-3</sup> )
FB1*	11	0.44	0.22	0.13	-	0.05	-	0.85
	13	0.59	0.43	0.14	-	0.07	0.02	1.24
	15	0.76	0.55	0.14	0.12	0.08	0.21	1.86
	17	0.83	0.73	0.15	-	0.03	-	1.74
	19	1.26	0.80	0.16	-	0.09	0.20	2.50
	21	1.60	0.92	0.18	-	0.11	0.23	3.05
	23	2.17	1.31	0.23	0.26	0.12	0.27	4.36
FB2	11	0.34	0.010	-	-	-	-	0.34
	13	0.47	0.010	-	-	-	-	0.48
	15	0.85	0.015	0.015	-	-	-	0.88
	17	0.94	0.020	0.020	0.118	-	-	1.10
	19	1.03	0.035	0.035	0.144	-	-	1.24
	21	1.30	0.005	0.056	-	-	-	1.36
	23	1.57	0.008	0.100	0.156	-	-	1.83
FB3*	2	0.25	0.05	0.10	-	-	0.15	0.55
	10	0.50	0.20	-	-	-	0.21	0.91
	15	0.89	0.60	-	0.12	-	-	1.61
	17	0.94	0.81	-	-	-	0.31	2.07
	21	1.53	0.94	0.14	-	-	-	2.60
	23	1.87	1.22	-	0.29	-	-	3.38
	26	2.21	1.35	0.19	-	-	-	3.75
	30	2.50	1.50	0.23	0.35	-	-	4.58
	36	2.73	1.80	0.53	-	-	-	5.07
	50	3.19	2.40	1.04	0.47	-	0.44	7.55
FB4*	13	0.71	-	-	-	0.05	-	0.76
	15	0.99	-	-	0.11	0.07	-	1.16
	17	1.22	0.31	0.15	-	0.08	0.07	1.84
	19	1.46	0.41	0.21	-	0.10	0.09	2.26
	21	1.76	0.65	0.25	0.25	0.10	0.15	3.15
	23	1.90	0.98	0.27	-	0.11	0.25	3.50
	26	2.05	1.04	0.29	0.32	0.12	0.25	4.08

\*The organic acids detected in the first 10 hours of FB1, FB3 and FB4 operations were given in Table 4.3 as B3 condition.

#### 4.2.6 Oxygen Transfer Characteristics

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR),  $r_0$ , and oxygen transfer coefficient,  $K_La$ . The variations in  $K_La$ , oxygen uptake rate, and oxygen transfer rate, maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor ( $\eta$ ) calculated throughout the bioprocess are given in Table 4.7.

In fed batch cases,  $K_La$  values altered due to the pulses given to the system. The highest  $K_La$  was determined at  $t=20$  h as  $0.026\text{ s}^{-1}$  in FB1 case where the glucose and fructose accumulation increased a lot. Decreases might be explained by the climbing up the cell concentrations a lot. In FB2 case,  $K_La$  values showed a decreasing profile starting from  $0.05\text{ s}^{-1}$  and was stationary at  $K_La=0.04\text{ s}^{-1}$  after  $t=14$  h and minimum at  $t=23$  h. This decrease was due to no more cell formation after 14<sup>th</sup> hour. In FB3 and FB4 cases,  $K_La$  again altered and increases occurred after pulses were given.

In fed batch cases, dissolved oxygen in the process was kept constant throughout the process hence OTR took the same values with OUR and gave the same profile. Comparing the batch and fed batch operations, fed batch operation worked well using less oxygen but giving high cell concentrations as it could be understood from the low values of OUR and OTR. The highest OUR value was obtained at the beginning of the process in all cases but having maximum at  $t=20$  h as  $3.9\text{ mol m}^3\text{ s}^{-1}$  in FB1 where the maximum activity was achieved. The lowest values of OTR were obtained at the end of the processes in all cases having minimum as  $0.60\text{ mol m}^3\text{ s}^{-1}$  in FB4 at  $t=15$  h. In fed batch processes, OUR increased when pulses given and decreased until the next pulse. This indicates that the cell growth and consequently the OUR decreased as a result of carbon source depletion rate.

In order to know the limiting step of the growth process, the maximum possible OUR (OD) and the maximum oxygen transfer rate have been calculated in the course of the fermentation and Damköhler number (Da), which is the ratio between the maximum rate of oxygen consumption in the bioprocess and its maximum mass transfer rate ( $=OD/OTR_{\max}$ ) was determined. At the beginning of FB1 and during FB4, Da value is smaller than 1 showing that reaction is the limiting resistance in the process when compared with mass transfer resistance. This showed that at those points, there is oxygen limitation because of high rates of cell formation. Nevertheless, at the points except these, mass transfer resistances were effective through the end

of the process ( $Da \gg 1$ ) indicating that mass transfer is the rate limiting step and maximum possible oxygen utilization rate is significantly lower than at bulk phase condition.

Film effectiveness factor,  $\eta$ , is inversely proportional with  $Da$  and cell concentration and this proportionally can be observed in Table 4.7. As the maximum oxygen utilization rate increased with increase in the cell concentration,  $Da$  increased and consequently,  $\eta$  decreased which is inversely proportional to the maximum oxygen utilization rate. As seen in Table 4.7,  $\eta$  took its maximum at  $t=12$  and  $t=13$  h for FB1, FB2 and FB4 cases having maximum at  $t=12$  h as 1.86 in FB1 operation. This indicates that at the beginning of the fed batch, cells are consuming oxygen with a high rate that maximum possible oxygen uptake rate (OD) value was attained. Then  $\eta$  decreased gradually meaning that the microorganisms are utilizing oxygen under the maximum oxygen demand.  $\eta$  took its minimum as 0.04 for FB2 operation where there was nearly no oxygen consumption.

**Table 4.7** The variations in oxygen transfer parameters in fed batch operations.

	t (h)	$K_La$ ( $s^{-1}$ )	$OTR \cdot 10^3$ ( $molm^3s^{-1}$ )	$OTR_{max} \cdot 10^3$ ( $molm^3s^{-1}$ )	$OUR \cdot 10^3$ ( $molm^3s^{-1}$ )	$OD \cdot 10^3$ ( $molm^3s^{-1}$ )	Da	$\eta$
FB1*	12	0.018	3.50	5.85	3.50	1.88	0.32	1.86
	14	0.007	1.80	2.39	1.80	1.85	0.77	0.97
	16	0.015	2.90	4.79	2.90	5.37	1.12	0.54
	18	0.012	2.50	3.68	2.50	6.04	1.64	0.41
	20	0.026	3.90	8.44	3.90	5.81	0.69	0.67
	23	0.003	0.80	0.93	0.80	7.96	8.54	0.10
FB2	12	0.005	1.03	1.71	1.03	0.79	1.35	0.76
	14	0.004	0.85	1.20	0.85	2.17	2.50	0.33
	16	0.004	0.89	1.34	0.89	2.99	2.86	0.22
	18	0.004	0.87	1.36	0.87	0.93	1.08	0.69
	20	0.004	0.77	1.14	0.77	2.88	2.51	0.23
	23	0.003	0.73	1.01	0.73	18.85	16.79	0.04
FB3*	13	0.013	2.05	4.08	2.05	6.18	1.51	0.33
	14	0.009	1.43	2.78	1.43	3.70	1.33	0.39
	16	0.008	1.64	2.72	1.64	10.83	3.98	0.15
	18	0.008	1.67	2.63	1.67	9.23	3.51	0.18
	22	0.009	1.92	2.79	1.92	6.33	2.27	0.30
	25	0.005	1.30	1.74	1.30	1.51	0.87	0.86
FB4*	13	0.006	1.10	1.82	1.10	5.41	2.97	0.20
	14	0.009	1.87	2.77	1.87	1.87	0.67	1.00
	15	0.003	0.60	0.93	0.60	1.13	1.22	0.53
	17	0.007	1.72	2.36	1.72	3.21	1.36	0.54
	19	0.007	1.62	2.16	1.62	8.28	3.83	0.20
	21	0.009	1.74	2.94	1.74	3.77	1.28	0.46
	25	0.009	1.85	2.92	1.85	7.84	2.68	0.24

\* The variations in oxygen transfer parameters in the first 10 hours of FB1, FB3 and FB4 operations were given in Table 4.4 as B3 condition.

#### 4.2.7 Specific Growth Rate and Yield Coefficients

The variations in specific growth rate,  $\mu$ , specific oxygen uptake rate,  $q_o$ , specific substrate utilization rate,  $q_s$ , and yield coefficients with cultivation time are given in Table 4.8.

In fed batch cases, specific growth rates showed an alternating profile since when pulses were given,  $\mu$  increased and the highest specific growth rate was attained in FB1 condition at  $t=12$  h as 0.15 which is the very beginning of the process and so growth rate was maximum. Whereas lowest specific growth rate was obtained at  $t=23$  h as 0.002 in FB2.

Specific oxygen uptake rates ( $q_o$ ) and specific substrate utilization rates ( $q_s$ ) showed a decreasing profile with the cultivation time at all the conditions. However when pulses were presented to the system throughout the process; there occurred some alterations. The highest  $q_o$  and  $q_s$  values were obtained as  $0.09 \text{ kg kg}^{-1} \text{ h}^{-1}$  at  $t=12$  h in FB1 and  $0.45 \text{ kg kg}^{-1} \text{ h}^{-1}$  at  $t=12$  h in FB2, respectively.

The yield coefficients, which represent the overall distribution and the efficiency of conversion reactions, were given also in Table 4.8. At fed batch operations,  $Y_{x/o}$  values reached its maximum at  $t=14$  h in FB1 as 1.90, at  $t=18$  in FB2 as 1.24, at  $t=25$  h in FB3 as 3.44 and at  $t=15$  in FB4 as 4.34, respectively. The maximum value was achieved at FB4 condition showing that it is the optimum condition for cell yield on oxygen. The lowest value was obtained as 0.09 at  $t=23$  h at FB2 case. Falls at the specific cell yield on oxygen signaled that less energy was used for cell formation at that time.

Although, almost the same trend of  $Y_{x/s}$  values were observed at batch and fed batch operations throughout the processes, the highest value of  $2.57 \text{ kg kg}^{-1} \text{ h}^{-1}$  at  $t=14$  h was found at FB1 condition due to the increased efficiency of the utilization of the carbon source for product formation. The lowest value was obtained at  $t=23$  h as  $0.02 \text{ kg kg}^{-1} \text{ h}^{-1}$  in FB2 since glucose and fructose was not consumed much and cell concentration remained the same. The overall values of  $Y_{x/s}$  are very essential for a process since it shows the overall yield of substrate on cell. These were found as  $0.20 \text{ kg kg}^{-1} \text{ h}^{-1}$  in FB1,  $0.15 \text{ kg kg}^{-1} \text{ h}^{-1}$  in FB2,  $0.32 \text{ kg kg}^{-1} \text{ h}^{-1}$  in FB3 and  $0.29 \text{ kg kg}^{-1} \text{ h}^{-1}$  in FB4.

The amount of substrate metabolized per amount of oxygen used ( $Y_{s/o}$ ) was also analyzed throughout the process. In fed batch cases, before the pulses given,  $Y_{s/o}$  obtained the highest values indicating the absence of the carbon source. The highest value was achieved in FB1 as  $22.48 \text{ kg kg}^{-1} \text{ h}^{-1}$  at  $t=23$  h

signaling decrease in the efficiency of energy metabolism with the cultivation time (Çalık et al., 2006). On the other hand, in FB2, FB3 and FB4 conditions, the highest  $Y_{S/O}$  was obtained as  $9.21 \text{ kg kg}^{-1}$  at  $t=12 \text{ h}$ ,  $6.46 \text{ kg kg}^{-1}$  at  $t=14 \text{ h}$  and as  $9.17 \text{ kg kg}^{-1}$  at  $t=15 \text{ h}$ , respectively when the first pulse was given. The lowest  $Y_{S/O}$  was obtained as  $0.74 \text{ kg kg}^{-1}$  at FB1 condition at  $t= 14 \text{ h}$  when there was no more substrate in the growing medium at that time.

**Table 4.8** The variations in specific growth rate and yield coefficients in fed operations.

	t (h)	$\mu$ (h <sup>-1</sup> )	$q_o$ (kgkg <sup>-1</sup> h <sup>-1</sup> )	$q_s$ (kgkg <sup>-1</sup> h <sup>-1</sup> )	Y <sub>x/s</sub> (kgkg <sup>-1</sup> )	Y <sub>x/o</sub> (kgkg <sup>-1</sup> )	Y <sub>s/o</sub> (kgkg <sup>-1</sup> )	$m_o$	$m_s$
FB1*	12	0.15	0.09	0.35	0.43	1.65	3.82	0.06	0.29
	14	0.08	0.04	0.03	2.57	1.90	0.74		
	16	0.04	0.06	0.38	0.11	0.77	6.71		
	18	0.03	0.05	0.04	0.84	0.72	0.86		
	20	0.05	0.07	0.26	0.20	0.79	3.88		
	23	0.01	0.01	0.28	0.03	0.64	22.48		
	Overall				0.20				
FB2	12	0.045	0.049	0.45	0.10	0.94	9.21	0.03	0.11
	14	0.019	0.035	0.24	0.08	0.55	6.65		
	16	0.013	0.037	0.25	0.05	0.36	6.74		
	18	0.041	0.033	0.09	0.45	1.24	2.78		
	20	0.014	0.028	0.03	0.41	0.50	1.22		
	23	0.002	0.026	0.10	0.02	0.09	4.03		
	Overall				0.15				
FB3*	13	0.03	0.05	0.37	0.29	0.63	1.46	0.03	0.16
	14	0.03	0.03	0.31	0.12	1.08	6.46		
	16	0.01	0.03	0.29	0.34	0.39	1.33		
	18	0.02	0.03	0.05	0.33	0.48	1.48		
	22	0.03	0.04	0.04	0.69	0.75	1.26		
	25	0.03	0.02	0.08	1.03	3.44	2.27		
	Overall				0.32				
FB4*	13	0.02	0.03	0.10	0.18	0.58	3.31	0.01	0.04
	14	0.09	0.04	0.08	1.14	2.26	1.99		
	15	0.05	0.01	0.10	0.47	4.34	9.17		
	17	0.05	0.03	0.07	0.68	1.71	2.52		
	19	0.02	0.03	0.07	0.24	0.68	2.89		
	21	0.01	0.03	0.10	0.43	1.66	3.86		
	25	0.04	0.03	0.07	0.29	0.81	2.81		
	Overall				0.29				

\* The variations in specific growth rate and yield coefficients in the first 10 hours of FB1, FB3 and FB4 operations were given in Table 4.5 as B3 condition.

## CHAPTER 5

### CONCLUSIONS

In this study, a feeding strategy of molasses based complex medium was developed in order to increase BAL production and cell growth by recombinant *E. coli* BL21 (DE3) pLysS. For this purpose, firstly, the effect of molasses concentration on cell concentration and BAL activity was investigated. Finally, using the optimized molasses based complex medium a feeding strategy was developed. In this context; the following conclusions were drawn:

#### 1. Molasses Based Complex Medium Design

*Escherichia coli* BL21 (DE3) pLySs carrying recombinant pRSETA::*bal* plasmid was selected as the benzaldehyde lyase producer, since this strain was reported to be a high BAL and low acetate producer. Molasses was chosen as the alternative carbon source to glucose because of its high sugar content and low cost. Molasses which is a sucrose source could not be used by *E. coli* BL21 hence it is hydrolyzed and glucose and fructose were obtained in 50 %.

The effect of molasses concentration was investigated at four different concentrations of molasses; 16 kg m<sup>-3</sup> (B1), 24 kg m<sup>-3</sup> (B2), 30 kg m<sup>-3</sup> (B3), 56 kg m<sup>-3</sup> (B4). Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as 5.3 kg m<sup>-3</sup> and 1617 U cm<sup>-3</sup>, respectively, in the medium containing 7.5 kg m<sup>-3</sup> glucose and fructose. The activity obtained in the optimized medium was 1.8-fold higher than the activity obtained in the reference production medium (Çalık et. al., 2006) and 1.5-fold higher than the highest activity found in the literature (Anghardi, 2007). The difference between the cases might be the result of glucose depletion at the end of exponential phase of growth and increased proteolytic activity.

An experiment using the optimized medium but without IPTG inducer was made as molasses contains lactose. According to variations of cell concentration and BAL activity, it has been concluded that, molasses did a good job without IPTG.

## 2. Development of a Feeding Strategy

The fed batch cultivation was applied to produce efficient cells with higher cell concentration and BAL activity. The idea was based on the addition of fresh carbon source, after glucose and fructose was utilized. Nevertheless, there occurs acetate accumulation if it was added too much. Thus two kinds of feeding strategies that are complementary should be handled by combining their respective advantages. It became a general consensus that acetate accumulation above  $2 \text{ kg m}^{-3}$  in the growth media slows down *E.coli* growth, may stop biomass build up and may inhibit recombinant protein biosynthesis. As a result, propagation techniques that keep acetate concentration below  $2 \text{ kg m}^{-3}$  were aimed (Shiloach et al., 2005). For that reason 4 strategies were applied in the current strategy.

First strategy was based on the airflow control of dissolved oxygen in the medium by oxygen probe. 10 hours of batch cultivation was performed with molasses based medium, having glucose and fructose concentration of  $7.5 \text{ kg m}^{-3}$ . When airflow rate was at or close to its minimum, feed pulses that lead to increase in glucose and fructose concentration in the medium to  $2.5 \text{ kg m}^{-3}$  were introduced to the system (FB1). In FB1, the cultivation time enlarged to 24 hours giving 4 pulses at  $t=10, 14, 16, 20$  hours. The feeding strategy worked well giving a maximum cell concentration of  $7.4 \text{ kg m}^{-3}$  at  $t=24 \text{ h}$  and maximum BAL activity of  $2360 \text{ U cm}^{-3}$  at  $t=20 \text{ h}$ . Since there was no further increase in BAL activity after 20<sup>th</sup> hour this strategy should be ended at  $t=20^{\text{th}}$  hour. Hence the cell concentration at that time should be considered as the maximum cell concentration which was  $6.6 \text{ kg m}^{-3}$  since the cells growing after that time was not carrying BAL producing cells. Furthermore, acetate at  $t=21 \text{ h}$  should be considered as the acetate accumulation which was  $1.60 \text{ kg m}^{-3}$ . A major advantage of the presented method is the general applicability. The only sensor required is an oxygen probe. However, although there was fructose in the medium, air flow variation with the cultivation time illustrated that there was no more carbon source. This is due to the shift in metabolism from glucose to fructose.

When the same strategy applied to the glucose defined medium (FB2), BAL activity and cell concentration did not increase at all since glucose accumulated after  $t = 20$  h. In glucose based fed batch case, (FB2), an acetate accumulation of  $1.57 \text{ kg m}^{-3}$  was reached at the end of the process which is a good result although there was glucose accumulation after  $t = 16$  h.

FB1 based on the air flow variation and the results showed that the decrease in the airflow only demonstrated the absence of glucose not fructose. This lack in the strategy was able to be solved by detection of the fructose and glucose concentrations during the process (FB3). The pulses were given at  $t = 14$  h,  $t = 24$  h, and  $t = 36$  h since both glucose and fructose reached to the minimum values at those times. The highest cell concentration was achieved at  $t = 36$  h as  $9.1 \text{ kg m}^{-3}$ . This result is 1.2 fold higher than the one obtained at FB1 case at  $t = 24$  h showing the extended strategy worked well with a cost of higher cultivation times. The highest BAL activity was obtained as  $2370 \text{ U cm}^{-3}$  at  $t = 26$  h then started to decrease. This showed that, although cell formation continued for further 10 hours, BAL producing cells were not produced at all. Hence the cell concentration of 26<sup>th</sup> hour should be taken into account as the maximum cell concentration which was  $7.5 \text{ kg m}^{-3}$ . If the acetate concentration at  $t = 26$  h, time where corresponding to the highest BAL activity, was taken into account, it can be concluded that a value of  $2.21 \text{ kg m}^{-3}$  was out of the interval of the accepted concentration of acetate.

Decreasing the concentration of glucose and fructose could decrease the accumulation of acetate. Hence at the last strategy  $1.5 \text{ kg m}^{-3}$  was chosen as the glucose and fructose concentration of the feed pulses. In order to work out the second problem which was the shift in metabolism from glucose and fructose, the idea was allowing time lag to watch the shift and add feed pulses according to that. (FB4) In this case, four pulses were given to the system at  $t = 13$  h, 17 h, 21 h, 24 h. By this strategy a cell concentration of  $8.04 \text{ kg m}^{-3}$  was achieved at  $t = 24$  h and a maximum activity of  $2315 \text{ U cm}^{-3}$  was obtained at the same time. According to these results, it did not need to present the last feed pulse since there is no increase in BAL activity due to that feed. An increased glucose and fructose concentration correlated well with the pulse response shortly after. This time glucose and fructose accumulation was fewer than FB1 case so first problem was handled and the metabolism shift could be visualized after the second pulse. The results of this strategy showed that, decreasing the molasses concentration of the pulses made a distinct increase in both cell concentration and BAL activity. An extra advantage of the

extended strategy was that acetate accumulation was  $2.05 \text{ kg m}^{-3}$  at the end of 26 hour and  $1.90 \text{ kg m}^{-3}$  at  $t=23\text{h}$ .

In general, at all the conditions applied, acetic, formic, succinic, citric fumaric and malic acids were found in the fermentation broth at increasing amounts. Presence of the organic acids of the TCA cycle, i.e., citric, succinic, fumaric and malic acids may be a consequence of inadequate oxygen supply which leads to repression on the TCA cycle enzymes or their improper activity.

In fed batch cases,  $K_La$  values altered due to the pulses given to the system. The highest  $K_La$  was determined at  $t= 20 \text{ h}$  as  $0.026 \text{ s}^{-1}$  in FB1 case where the glucose and fructose accumulation increased a lot. Decreases might be explained by the climbing up the cell concentrations a lot.

Comparing batch and fed batch operations, in fed batch operation higher cell concentrations were obtained; in addition to this, less oxygen was used. The highest OUR value was obtained at the beginning of process in all cases. In fed batch processes, OUR increased when pulses were given and decreased until the next pulse.

**Table 5.1** Outcomes of the fed batch strategies

	Process time, h	Input Carbon Source Concentration, $\text{kg m}^{-3}$	A, $\text{U cm}^{-3}$	$C_x$ , $\text{kg m}^{-3}$	$C_{AA}$ , $\text{kg m}^{-3}$
FB1	20	30	2360	6.60	1.60
FB2	20	20	924	3.17	1.30
FB3	26	25	2370	7.51	2.21
FB4	24	24	2315	8.04	1.90

When all the results of the strategies were considered, it was concluded that every strategy had both advantages and disadvantages. FB1, FB3 and FB4 could be accepted having almost the same activity; however, cell concentration of FB4 was higher than those of FB1 and FB3 and also in FB4 lower amount of carbon source was used. As a result, FB4 could be chosen as the most favorable strategy. On the other hand, FB4 strategy was slightly longer and had higher acetic acid concentration. However, these cost are within toleration limits. The future plan would be applying a different feeding strategy like exponential or linear feeding since these includes adding carbon sources continuously and so acetic acid formation and carbon source addition could be decreased and activity and cell concentration could increase.

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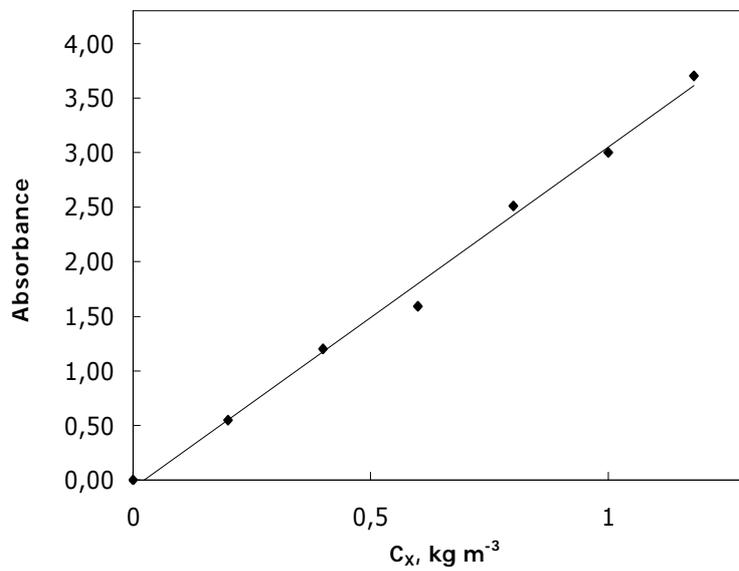
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## APPENDIX A

### CALIBRATION OF *Escherichia coli* CONCENTRATION



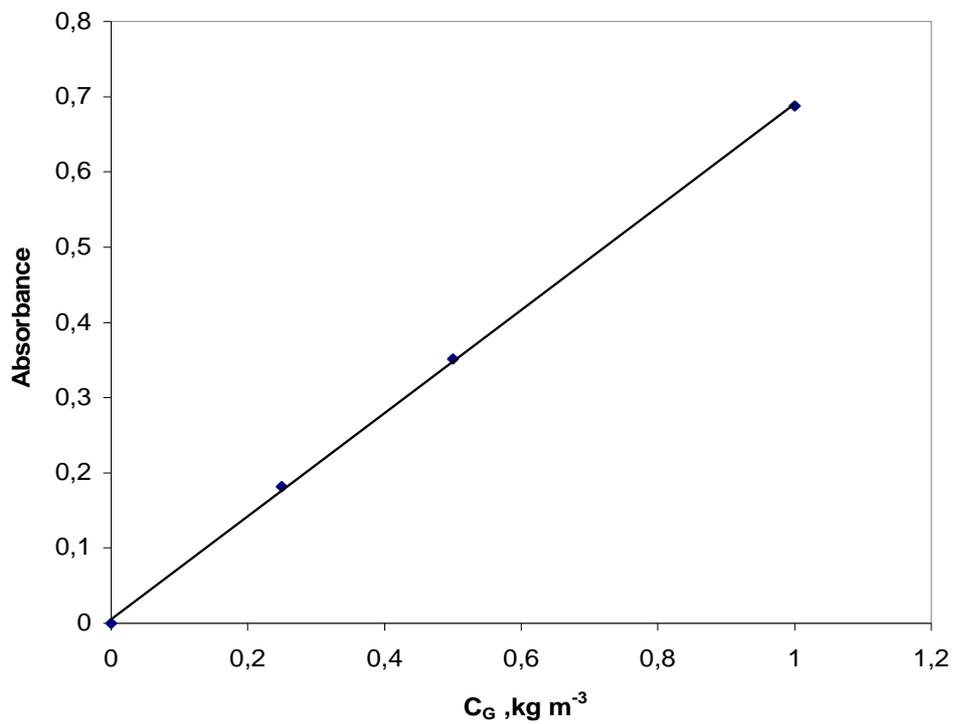
**Figure A.1** Calibration curve for *Escherichia coli* concentration

Slope of the calibration curve,  $m=2.8782 \text{ 1/kg m}^{-3}$  ( $\lambda=600 \text{ nm}$ )

$$C_x = \frac{\text{Absorbance}}{2.8782} \times \text{Dilution Rate} \quad (\text{A.1})$$

## APPENDIX B

### CALIBRATION OF GLUCOSE CONCENTRATION



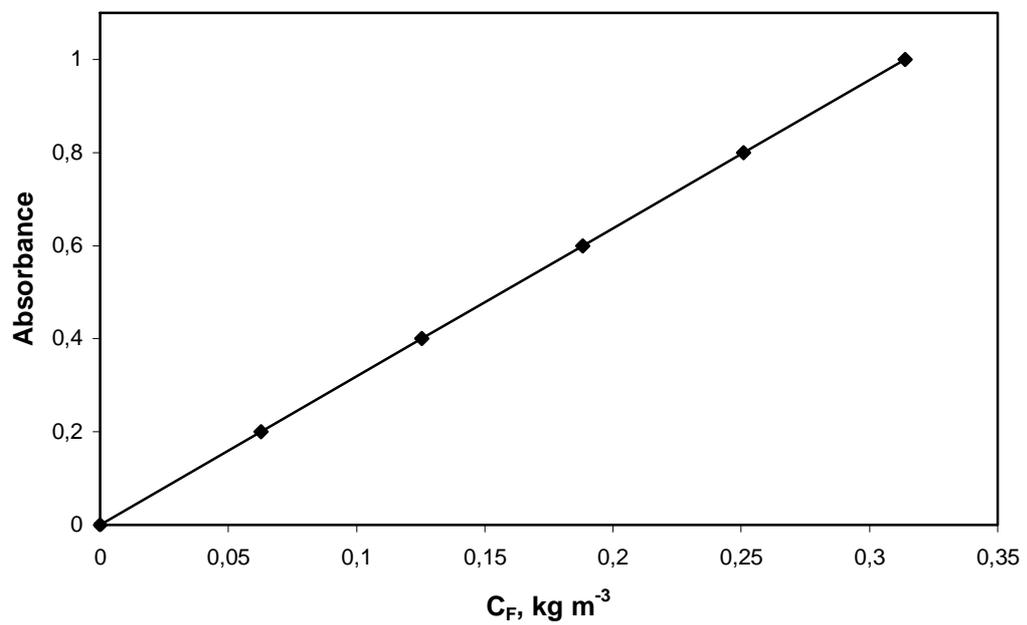
**Figure B.1** Calibration curve for glucose concentration.

Slope of the calibration curve,  $m=0.6854 \text{ 1/kg m}^{-3}$ , ( $\lambda=505 \text{ nm}$ )

$$C_G = \frac{(\text{Absorbance} - 0.0054)}{0.6854} \times \text{Dilution Rate} \quad (\text{B.1})$$

## APPENDIX C

### CALIBRATION OF FRUCTOSE CONCENTRATION



**Figure C.1** Calibration curve for Fructose concentration,  $\lambda=560\text{nm}$

Slope of the calibration curve,  $m=3.187 \text{ 1/kg m}^{-3}$  ( $\lambda=560 \text{ nm}$ )

$$C_F = \frac{\text{Absorbance}}{3.187} \times \text{Dilution Rate} \quad (\text{C.1})$$