# BIOPROCESS OPERATION PARAMETERS FOR RECOMBINANT BENZALDEHYDE LYASE PRODUCTION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMICAL ENGINEERING

AUGUST 2004

Approval of the Graduate School of Natural and Applied Sciences

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# ABSTRACT


BIOPROCESS OPERATION PARAMETERS FOR RECOMBINANT BENZALDEHYDE LYASE PRODUCTION

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August 2004, 85 pages

In this study, the effects of bioprocess operation parameters on benzaldehyde lyase production were systematically investigated. For this purpose, the research program was carried out in mainly four parts. In the first part of the study, *Escherichia coli* K12 (ATCC 10798), having the highest benzaldehyde lyase production capacity, was selected as the host microorganism. Next, using the selected microorganism, the production medium was designed in terms of its carbon and nitrogen sources. Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as 1.8 kg $m^{-3}$ and 745 U $cm^{-3}$, respectively, in the medium containing 8.0 kg $m^{-3}$ glucose, 5.0 kg $m^{-3}$ $(NH_4)_2HPO_4$ and the salt solution. Thereafter, by using the designed medium, the effects of bioreactor operation parameters, i.e., oxygen transfer and pH, were investigated in pilot scale bioreactor. Oxygen transfer effects on benzaldehyde lyase production were investigated at $Q_O/V_R$=0.5 vvm; N=250, 375, 500, 625, 750 $min^{-1}$ and at $Q_O/V_R$=0.7 vvm, N=750 $min^{-1}$ conditions. The highest cell concentration and benzaldehyde lyase activity were obtained at 0.5 vvm, 500 $min^{-1}$ condition as 2.3 kg $m^{-3}$ and 860 U $cm^{-3}$, respectively.

Finally, the effect of pH was investigated for benzaldehyde lyase production process at $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$ condition, at $pH_C$=5.0, 6.4, 6.7, 7.0, 7.2 and 7.8 values. Among the investigated pH values, the highest cell concentration and enzyme activity were obtained at $pH_C$=7.0 condition as 2.1 kg $m^{-3}$; 775 U $cm^{-3}$. However, the values obtained at this condition, were lower than the values obtained at $pH_{UC}$=7.2 uncontrolled pH operation. Hence, medium oxygen transfer condition and uncontrolled pH operation are found to be favorable for benzaldehyde lyase production.

**Keywords:** Benzaldehyde Lyase, Production, Recombinant *E. coli*, Oxygen Transfer, pH Effects

# ÖZ

## REKOMBİNANT BENZALDEHİT LİYAZ ÜRETİMİ İÇİN BİYOPROSES İŞLETİM PARAMETRELERİNİN ARAŞTIRILMASI

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Ağustos 2004, 85 sayfa

Bu çalışmada, biyoproses işletim parametrelerinin benzaldehit liyaz üretimine etkileri sistematik olarak incelenmiştir. Bu amaçla, araştırma programı başlıca dört bölümde yürütülmüştür. Çalışmanın ilk bölümünde, en yüksek benzaldehit liyaz üretim kapasitesine sahip olan *Escherichia coli* K12 (ATCC 10798), konak hücre olarak seçilmiştir. Daha sonra, seçilen bu mikroorganizma kullanılarak, karbon ve azot kaynaklarının etkisinin araştırılmasıyla ortam tasarımı yapılmıştır. İncelenen koşullarda, en yüksek hücre derişimi ve enzim aktivitesi sırasıyla 1.8 kg $m^{-3}$ ve 745 U $cm^{-3}$ olarak; 8.0 kg $m^{-3}$ glukoz, 5.0 kg $m^{-3}$ $(NH_4)_2HPO_4$ ve tuz çözeltisi içeren ortamda elde edilmiştir. Tasarlanan üretim ortamı kullanılarak, biyoreaktör işletim parametrelerinden oksijen aktarımı ve pH etkileri; fermantasyon ve oksijen aktarım karakteristikleri pilot ölçek biyoreaktörde incelenmiştir. Benzaldehit liyaz üretiminde oksijen aktarımının etkileri, hava giriş hızı $Q_0/V_R$=0.5 vvm; karıştırma hızları N=250, 375, 500, 625, 750 $dk^{-1}$ ve $Q_0/V_R$=0.7 vvm, N=750 $dk^{-1}$ koşullarında incelenmiştir. İncelenen koşullar arasında en yüksek hücre derişimi ve enzim aktivitesine sırasıyla 2.3 kg $m^{-3}$ ve 860 U $cm^{-3}$ olarak ulaşılmıştır. Son olarak benzaldehit liyaz üretiminde pH etkileri en uygun oksijen aktarım koşulu olarak belirlenmiş olan 0.5 vvm, 500

$dk^{-1}$ koşulunda incelenmiştir. Bu amaçla altı ayrı kontrollü pH deneyi $pH_C$=5.0, 6.4, 6.7, 7.0, 7.2, 7.8 koşullarında gerçekleştirilmiştir. En yüksek hücre derişimi ve enzim aktivitesine sırasıyla 2.1 kg $m^{-3}$ ve 775 U $cm^{-3}$ olarak, $pH_C$=7.0 koşulunda ulaşılmıştır; ancak, bu değerler $pH_{UC}$=7.2 pH kontrolsüz koşulda ulaşılan değerlerden düşüktür. Bu nedenlerle, benzaldehit liyaz üretiminde orta oksijen aktarım koşulu ve kontrolsüz pH koşulu uygulanmasına karar verilmiştir.

**Anahtar Kelimeler:** Benzaldehit Liyaz, Üretim, Rekombinant *E. coli*, Oksijen Aktarımı, pH Etkisi

To My Family

Lena, Esat and Çağlar Yılgör

# ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Pınar Çalık, for her continuous support, guidance and help, in all the possible way, throughout this study.

I am grateful to Prof. Dr. Ayhan Sıtkı Demir, for introducing me to the world of organic chemistry and for being an accessible source of knowledge throughout this study.

I wish to thank to Prof. Dr. H. Tunçer Özdamar and Prof. Dr. Güzide Çalık for their valuable comments and for giving me the opportunity to perform the HPCE analysis in Ankara University.

Beyond Ms. degree, what I really earned in this study is the friendship I was lucky to find among Müge Arifoğlu, Ayşegül Namaldı, İlkin Ece Telli, Peruze Ayhan and Pınar Kocabaş; without them, the endless time spent in the lab would never worth remembering. I am also very grateful to my seniors Eda Çelik and Ceren Oktar for sharing their time and knowledge, and for helping me whenever I needed.

Financial Support provided by TUBITAK through project MISAG-258 and METU-Research Fund are gratefully acknowledged.

Above all, I would like to thank to my family for being right beside me; loving, supporting and encouraging me all through my life.

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| $C_{OA}$ | Organic acid concentration, kg $m^{-3}$ |
| $C_O$ | Dissolved oxygen concentration, mol $m^{-3}$; kg $m^{-3}$ |
| $C_O^*$ | Oxygen saturation concentration, mol $m^{-3}$; kg $m^{-3}$ |
| $C_G$ | Glucose concentration, kg $m^{-3}$ |
| $C_G^0$ | Initial glucose concentration, kg $m^{-3}$ |
| $C_N$ | Nitrogen concentration, kg $m^{-3}$ |
| $C_P$ | Product concentration, kg $m^{-3}$ |
| $C_S$ | Concentration of the substrate, mM; kg $m^{-3}$ |
| $C_X$ | Cell concentration, kg dry cell $m^{-3}$ |
| Da | Damköhler number (=OD / $OTR_{max}$; Maximum possible oxygen utilization rate per maximum mass transfer rate) |
| E | Enhancement factor (=$K_La$ / $K_La_o$); mass transfer coefficient with chemical reaction per physical mass transfer coefficient |
| $K_La_0$ | Physical overall liquid phase mass transfer coefficient; $s^{-1}$ |
| $K_La$ | Overall liquid phase mass transfer coefficient; $s^{-1}$ |
| $K_m$ | Michealis constant, mM |
| N | Agitation rate, $min^{-1}$ |
| $m_0$ | Rate of oxygen consumption for maintenance, kg oxygen $kg^{-1}$ dry cell weight $h^{-1}$ |
| $m_S$ | Maintenance coefficients for substrate, kg substrate $kg^{-1}$ dry cell weight $h^{-1}$ |
| $pH_0$ | Initial pH |
| Qo | Volumetric air feed rate, $m^3$ $min^{-1}$ |
| $q_o$ | Specific oxygen uptake rate, kg kg $^{-1}$ DW $h^{-1}$ |
| $q_s$ | Specific substrate consumption rate, kg kg $^{-1}$ DW $h^{-1}$ |
| r | Volumetric rate of reaction, mol $m^{-3}$ $s^{-1}$ |
| $r_{max}$ | Maximum rate of reaction, mol $m^{-3}$ $s^{-1}$ |
| $r_0$ | Oxygen uptake rate, mol $m^{-3}$ $s^{-1}$; kg $m^{-3}$ $h^{-1}$ |
| $r_X$ | Rate of cell growth, kg $m^{-3}$ $h^{-1}$ |
| T | Bioreaction medium temperature, $^oC$ |

| | |
|---|---|
| t | Bioreactor cultivation time, h |
| U | One unit of an enzyme |
| $V_R$ | Volume of the bioreaction medium, $m^3$ |
| $Y_{X/S}$ | Yield of cell on substrate, kg $kg^{-1}$ |
| $Y_{X/O}$ | Yield of cell on oxygen, kg $kg^{-1}$ |
| $Y_{S/O}$ | Yield of substrate on oxygen, kg $kg^{-1}$ |
| $Y_{P/X}$ | Yield of product on cell, kg $kg^{-1}$ |
| $Y_{P/S}$ | Yield of product on substrate, kg $kg^{-1}$ |
| $Y_{P/O}$ | Yield of product on oxygen, kg $kg^{-1}$ |

*Greek Letters*

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

*Abbreviations*

| | |
|---|---|
| ATCC | American Type Culture Collection |
| BAL | Benzaldehyde lyase |
| DNS | Dinitrosalicylic acid |
| DO | Dissolved oxygen |
| EC | Enzyme Commission |
| OD | Oxygen demand (=$\mu_{max}$ $C_X$ / $Y_{X/O}$; mol $m^{-3}$ $s^{-1}$) |
| OUR | Oxygen uptake rate, mol $m^{-3}$ $s^{-1}$ |
| OTR | Oxygen transfer rate, mol $m^{-3}$ $s^{-1}$ |
| $OTR_{max}$ | Maximum possible mass transfer rate (=$K_La C_O$*; mol $m^{-3}$ $s^{-1}$) |
| TCA | Tricarboxylic acid |

# CHAPTER 1

# INTRODUCTION

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, often far superior to conventional chemical catalysts, for which reason they are being employed increasingly in today's high-technological society, as a highly significant part of the biotechnological expansion. Their utilization has created a billion dollar business, including a wide diversity of industrial processes, consumer products, and the field of biosensors (Chaplin Bucke,1990). Enzymes have a number of distinct advantages over chemical catalysts:

1. *Enzymes are very efficient catalysts.* Typically the rates of enzyme-mediated processes are accelerated, compared to those of the corresponding non-enzymatic reactions, by a factor of $10^8$-$10^{10}$, which is far above the values that chemical catalysts are capable of achieving.

2. *Enzymes are environmentally acceptable.* Unlike heavy metals, biocatalysts are environmentally benign reagents since they are completely degradable.

3. *Enzymes act under mild conditions.* Enzymes act in a pH range of about 5-8, typically around 7, and in a temperature range of 20–40$^{o}$C, preferably at around 30$^{o}$C. This minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology.

4. *Enzymes are compatible with each other.* Since enzymes generally function under the same or similar conditions, several biocatalytic reactions can be carried out in a reaction cascade in one flask. Thus sequential reactions are feasible by using multi-enzyme systems in order to simplify reaction processes (Faber, 2000).

In nature, there exists an enzyme-catalyzed process equivalent to almost every type of organic reaction. On the other hand, the reactions catalyzed by enzymes can often be achieved by classical chemical methods with a large expenditure of time and effort; moreover, some biocatalysts can accomplish reactions impossible to emulate in organic chemistry.

Enzymes display three major types of selectivities:

1. *Chemoselectivity*: Since the purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, do survive. As a result, reactions generally tend to be 'cleaner' and laborious purification of product(s) from impurities can largely be omitted.

2. *Regioselectivity and Diastereoselectivity*: Due to their complex three-dimensional structure, enzymes may distinguish between functional groups, which are chemically situated in different regions of the same substrate molecule.

3. *Enantioselectivity:* Almost all enzymes are made from L-amino acids and thus are chiral catalysts. As a consequence, any type of chirality present in the substrate molecule is 'recognized' upon the formation of the enzyme-substrate complex. Thus a prochiral substrate may be transformed into an optically active product through enzymes.

Enantioselectivity is the most important property that enzymes display. Today, enantiomerically pure compounds are becoming increasingly more important in the production of pharmaceuticals, agrochemicals, and flavors. The world market for single-isomer chiral drugs amounted to ca. 73 billion U.S. dollars in 1996, and in the year 2000 this will increase to 90 billion (Adam et al., 1999).

The importance of enantiomerically pure compounds is based on the fact that, all the major biochemical events taking place in an organism are governed by enzymes; and since the majority of enzymes are highly selective with respect to the chirality of a substrate, it is obvious that the enantiomers of a given bioactive compound, such as a pharmaceutical, cause different biological effects. For example, R-enantiomer of the penicillamine molecule has an antiarthritic effect and it is used in drugs with this property; where S-penicillamine is toxic (Faber, 2000).


**Figure 1.1** R- and S- enantiomers of penicillamine molecule.

Of the numerous methods which have been developed in the past decades for the preparation of optically active compounds, the biocatalytic processes are becoming more and more popular since substances can be produced in high enantiomeric excess using enzymes.

Benzaldehyde lyase (BAL, EC 4.1.2.38), is used for the synthesis of enantiomerically pure $\alpha$-hydroxy ketones, an important class of compounds in natural product and drug synthesis (Demir et al., 2001, 2002, 2003). Benzaldehyde lyase is a thiamine pyrophosphate (TPP) dependent enzyme, and it catalyzes the cleavage of the acyloin linkage of benzoin to form two molecules of benzaldehyde (Gonzales and Vicuna, 1989).

In the literature, Gonzales and Vicuna (1989) firstly reported the growth of *Pseudomonas fluorescens* Biovar I on benzoin as a sole carbon and energy source due to the benzaldehyde lyase production potential of the microorganism; thereafter, Hinrichsen et al. (1994) cloned the gene encoding benzaldehyde lyase to an *Escherichia coli* strain using vector pUC18.

Demir et al. (2001, 2002, 2003), described the mechanism of the reaction catalyzed by benzaldehyde lyase; and they found that only R-benzoin is the substrate of the reaction. Finally, Dünkelmann et al. (2002), presented the ability of the enzyme to asymmetrically synthesize mixed benzoins.

However, there is no work in the literature reporting the effects of bioprocess operation parameters on benzaldehyde lyase production. But related with other biomolecules using the host *E. coli*; Ryan et al. (1989) reported the effects of oxygen transfer on beta-lactamase production with Luria-Bertani (LB) medium by *E. coli* JM103; without stating the oxygen transfer rate and oxygen uptake rate, they reported that the decrease in air inlet rate decreased the growth rate. They also investigated the effect of pH on the growth of recombinant *E. coli* at three different controlled pH values, i.e., 7.0, 7.4, 8.0; and reported that cell concentration increases when the culture pH decreases, however, enzyme activity attain its highest value at pH values in the vicinity of pH=7.4. Bhattacharya and Dukey (1997) reported the effects of oxygen transfer with LB medium on DNA methyltransferase production by *E. coli* K12 and reported that an oxygen sufficient condition was necessary to obtain optimal expression and cell productivity. Castan et al. (2002) reported the effects of air supply enrichment either by oxygen (30 and 40%) or carbon dioxide (12 and 20%) on human growth hormone production using glucose based defined medium by *E. coli* K12. In their study, they kept the pH constant at 7.0 with aqueous $NH_3$. Lastly, Leon et al. (2003) investigated the effect of the dissolved oxygen on penicillin acylase production with glucose based defined medium by *E. coli* JM101, low oxygen transfer condition was studied in detail; kinetic and stoichiometric parameters were evaluated. In this study, pH was kept constant throughout the bioprocess at 7.4 with 10 N NaOH. Also, in the studies of Akesson et al. (2001) (pH=6.9 by 25% ammonia) and Yang et al. (2003) (pH=7.0 by 2 M NaOH) pH value was kept constant throughout the bioprocesses. Hence, there is no systematic investigation in the literature, on the effects of oxygen transfer and pH on any biomolecule production by the host *Escherichia coli*.

In this study, the effects of bioprocess operation parameters on benzaldehyde lyase production were systematically investigated. In this context, pUC18*::bal* gene was transformed to several *E. coli* strains and using the *E. coli* strain having the highest benzaldehyde lyase and biomass production capacity, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher benzaldehyde lyase production. Thereafter, by using the designed medium, the effects of bioreactor operation parameters, i.e., oxygen transfer conditions and pH, and the fermentation and oxygen transfer characteristics of the bioprocess, were investigated in the pilot scale bioreactor.

# CHAPTER 2

# LITERATURE SURVEY

## 2.1 Enzymes

### **2.1**.1 General Characteristics

Enzymes are usually proteins of high molecular weight that acts as catalysts in biological systems. They accelerate hundreds of reactions taking place simultaneously in the cell, without undergoing any permanent chemical change and without affecting the reaction equilibrium; by lowering the activation energy of the reaction via binding the substrate and forming an enzyme-substrate complex. Enzymes are specific, versatile, and very effective biological catalysts, resulting in much higher reaction rates as compared to chemically catalyzed reactions under ambient conditions (Shuler and Kargı, 2002). Enzyme specificity is thought to be a consequence of its elaborate three-dimensional conformation which allows formation of the active site responsible for the catalytic ability of the enzyme (Bailey, 1986).

Enzymes catalyze only the reactions of very narrow ranges of reactants, which may consist of a small number of closely related classes of compounds, a single class of compounds, or a single compound. This means that the chosen reaction can be catalyzed to the exclusion of side-reactions, eliminating undesirable by-products. Thus, higher productivities may be achieved, reducing material costs (Chaplin and Bucke, 1990).

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

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

Biologically active enzymes may be extracted from any living organism. A very wide range of sources is used for commercial enzyme production- from *Actinoplanes* to *Zymomonas*, from spinach to snake venome. Of the hundred or so enzymes being used industrially, over half are from fungi and yeast and over a third are from bacteria, with the remainder divided between animal (%8) and plant (%4) sources (Chaplin and Bucke, 1990). It is a remarkable fact that enzymes with the same name but obtained from different organisms often have different amino acid sequences and hence different properties and catalytic activities (Bailey, 1986).

## **2.1**.2 Classification of Enzymes

Enzymes are classified according to the report of the International Union of Biochemistry. The Enzyme Commission (EC) numbers divide enzymes into six main groups according to the type of reaction catalyzed. For identification purposes, every enzyme has a four-digit number EC A.B.C.D; the following properties are encoded (Faber, 2000):

A denotes the main type of reaction (Table 2.1).
B stands for the subtype, indicating the substrate type.
C indicates the nature of the co-substrate.
D is the individual enzyme number.

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

**Table 2.1** International classification of enzymes.

| No | Class | Type of reaction catalyzed |
|---|---|---|
| 1 | Oxidoreductases | Transfer of electrons |
| 2 | Transferases | Group-transfer reactions |
| 3 | Hydrolases | Transfer of functional groups to water |
| 4 | Lyases | Addition of groups to double bonds or the reverse |
| 5 | Isomerases | Transfer of groups within molecules to yield isomeric forms |
| 6 | Ligases | Formation of C-C, C-S, C-O, and C-N bonds by condensation reactions coupled to ATP cleavage |

**2.1**.3 Enzyme Activity

The amount of enzyme present or used in a process is difficult to determine in absolute terms (e.g. grams), as its purity is often low and a proportion may be in an inactive, or partially active, state. The activity is a measure of enzyme content that is clearly of major interest when the enzyme is to be used in a process. For this reason, enzymes are usually marketed in terms of activity rather than weight. Enzyme activity is usually determined from a rate assay and expressed in terms of the activity unit; which was defined by the Commission on Enzymes as (Chaplin and Bucke, 1990):

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

A change in temperature, pH, and/or substrate concentration affects the reaction rate; therefore, these parameters must be carefully controlled in order to achieve reproducible results (Kirk and Othmer, 1994). Also, a comparison of

the activity of different enzyme preparations is only possible if the assay procedure is performed exactly in the same way (Faber, 2000).

### 2.1.4 Enzyme Kinetics

It is established that enzymes form a bound complex with their substrates during the course of their catalysis and prior to the release of products (Chaplin and Bucke, 1990). This can be simply illustrated, using a mechanism based on that of Michaelis and Menten for a one-substrate reaction, by the reaction sequence:

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

where, enzyme E and substrate S combine to form a complex ES, which then dissociates into product P. The Michealis-Menten equation (Eq 2.1) is simply derived from the rate of the above reaction by substituting $K_m$ for $(k_{-1}+k_{+2})/k_{+1}$:

$$r = \frac{r_{max} C_S}{K_m + C_S} \qquad (2.1)$$

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

The Michaelis-Menten equation in its original form (Eq 2.1) is not well suited for estimation of the kinetic parameters $r_{max}$ and $K_m$. Rearrangement of equation (2.1) gives:

$$\frac{1}{r} = \frac{1}{r_{max}} + \frac{K_m}{r_{max}} \cdot \frac{1}{C_S} \qquad (2.2)$$

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

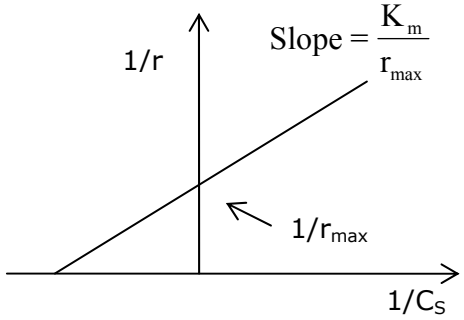



**Figure 2.1** Lineweaver-Burk plot.

### 2.2 Benzaldehyde Lyase

Benzaldehyde lyase (BAL, EC 4.1.2.38), is a thiamine pyrophosphate (TPP) dependent enzyme as a cofactor; and it reversibly catalyzes the cleavage and formation of the acyloin linkage of R-benzoin (Figure 2.2). The R- and S-enantiomers of benzoin molecule is given in Figure 2.3.

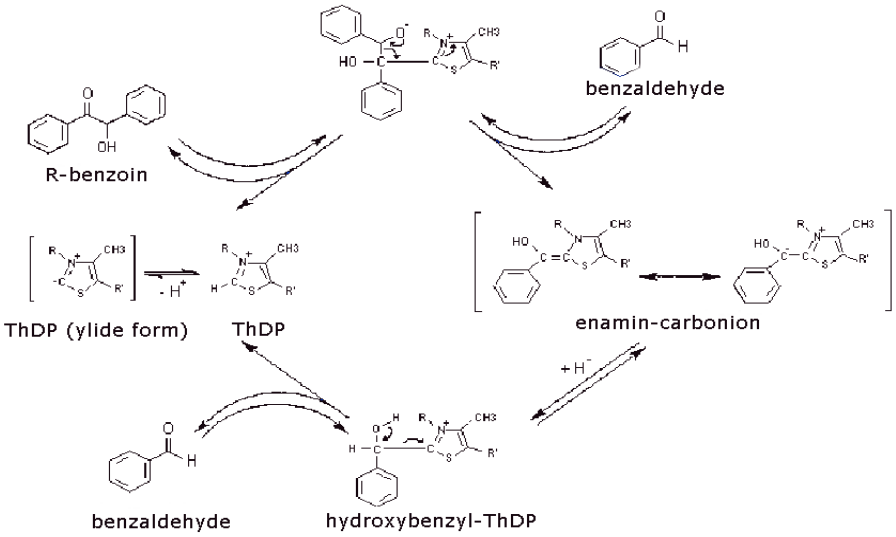
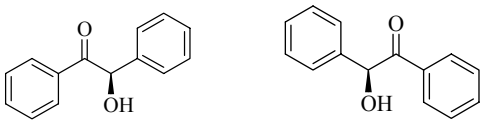



**Figure 2.2** Benzaldehyde lyase catalyzed cleavage and synthesis of benzoin.



R-benzoin S-benzoin

**Figure 2.3** R- and S- enantiomers of benzoin molecule.

The most important property of this reaction catalyzed by benzaldehyde lyase was recognized by Demir et. al. (2001, 2002, 2003); which indicates that only R-benzoin is converted into benzaldehyde through benzaldehyde lyase catalysis; where S-benzoin gave no reaction at all. With this property benzaldehyde lyase is used for the synthesis of enantiopure $\alpha$-hydroxy ketones, an important class of compounds in natural product and drug synthesis.

The synthesis of enantiomerically pure compounds are becoming increasingly more important in the production of pharmaceuticals, agrochemicals (e.g., pesticides, fungicides, herbicides), and flavors (Adam et. al., 1999). These compounds frequently act by interacting with receptors, enzymes, carrier molecules, and the like. All such interactions are highly stereospecific; which means, one stereoisomer of a drug may interact tightly with a particular receptor, whereas the other streoisomer may have a different target. For example; the R-isomer of the antitubercular drug ethambutol, has potent antitubercular activity, whereas the S-isomer causes degeneration of the optic nerve, leading to blindness (Glazer and Nikaido, 1998). Therefore, the development of efficient and environmentally acceptable processes for the preparation of enantiomerically pure compounds is essential, but still presents a major challenge in organic chemistry. However, enantiomerically pure substances can be produced in high enantiomeric excess by utilizing biocatalytic processes since enzymes are chiral materials. Considering the above mentioned problems, it is obvious that enzymatic methods represent a valuable addition to the existing toolbox available for the enantioselective synthesis of fine chemicals (Faber, 2000).

Benzaldehyde lyase, which is used for the synthesis of enantiopure $\alpha$-hydroxy ketones, was firstly reported by Gonzales and Vicuna (1989) from *Pseudomonas fluorescens* Biovar I. This strain can grow on benzoin as a sole carbon and energy source due to the benzaldehyde lyase production potential of the microorganism. In this first study, it was found that the molecular weight of the enzyme is 80,000 and and was suggested that the native enzyme may be either a dimer or a single polypeptide with a nonspherical shape. Maximum

activity of benzaldehyde lyase was reached at 0.01 mM TPP, while concentrations higher than 0.5 mM were inhibitory.

Benzaldehyde lyase shows maximal activity between pH 7.5 and 8.5, whereas it is inactive below pH 6.0. It is highly specific; of a variety of structurally related compounds, only benzoin and anisoin acts as substrates.

In the literature, after the detection of the enzyme by Gonzales and Vicuna (1989); Hinrichsen et al. (1994) cloned the gene encoding benzaldehyde lyase to an *Escherichia coli* strain using vector pUC18. Demir et al. (2001, 2002, 2003), described the mechanism of the reaction catalyzed by benzaldehyde lyase; and they found that only R-benzoin is the substrate of the reaction. Finally, Dünkelmann et al. (2002), presented the ability of the enzyme to asymmetrically synthesize mixed benzoins.

### 2.3 Bioprocess Parameters in Enzyme Production

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

Until about 1950, the predominant method of producing industrial enzymes was by extraction from animal or plant sources; by 1993, this accounts for less than 10% (Kirk and Othmer, 1994). Industrial enzymes are now produced by microorganisms grown in aqueous suspension in large vessels, i.e., by fermentation. Microorganisms are preferred to plants and animals as sources of enzymes because (Chaplin and Bucke, 1990):

1. They are generally cheaper to produce;
2. Their enzyme contents are more predictable and controllable;
3. Reliable supplies of raw material of constant composition are more easily arranged;
4. Plant and animal tissues contain more potentially harmful materials, including phenolic compounds (from plants) and endogenous enzyme inhibitors.

Enzymes are usually sensitive to harsh physical and chemical conditions, which demands careful selection of production processes and conditions for each individual enzyme. In aerobic bioprocesses, the important criteria that must be taken into account, in order to have high product yield, are:

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

### 2.3.1 Microorganism

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

Few microorganisms fulfill all the above criteria and are used for production of industrial enzymes. *Escherichia coli* is one of the most important host microorganisms.

Benzaldehyde lyase is naturally produced by wild-type *Pseudomonas fluerescens*. Hinrichsen et. al. (1994), cloned the gene encoding benzaldehyde lyase to an *Escherichia coli* strain; the studies until then has been performed using this host microorganism.

#### 2.3.1.1 *Escherichia coli*

If posttranslational modifications are unnecessary, *E. coli* is most often chosen as the initial host. The main reason for the popularity of *E. coli* is the broad knowledge base for it. *E. coli* physiology and its genetics are probably far

better understood than for any other living organism. This large knowledge base greatly facilitates sophisticated genetic manipulations. Also, an important engineering contribution was the development of strategies to grow cultures of *E. coli* to high cell densities (Shuler and Kargı, 2002).

The average *E. coli* cell is a rod shaped body with diameter and length of approximately 1 and 2 micrometers, respectively. It has a wet mass of $9.5*10^{-13}$ g, or $2.9*10^{-13}$ g dry mass. Elemental essay of the dry mass of *Escherichia coli* cells reveals a fairly typical composition of the protoplasm: 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% potassium, 2% sodium, 1% sulfur, 0.05% calcium, 0.05% magnesium, 0.05% chlorine, 0.2% iron and a total of 0.3% trace elements including manganese, cobalt, copper, zinc and molybdenium.

*E. coli* is a Gram-negative bacterium. The murein layer of the Gram-negative bacteria is much thinner than that of Gram-positive bacteria, and they make a completely different structure- an outer membrane; which has the ability to resist damaging chemicals. *E. coli* is a facultative aerobe bacterium which can grow in the presence or absence of oxygen. Under anaerobic conditions, *E. coli* can utilize $NO_3$ as a source of nitrogen, but when growing aerobically it requires a reduced nitrogen source.

The maximum temperature of growth for *E. coli* is approximately 48$^{o}$C, its optimal temperature is 37$^{o}$C, its minimum temperature is 8$^{o}$C, and its normal temperature range extends from about 21$^{o}$C to 39$^{o}$C. In the absence of methionine, growth stops at 45$^{o}$C. *E. coli* grows well near neutrality at pH values from 6.0 to 8.0. Regardless of the value of the external pH, within the cell, pH is maintained at a value quite close to 7.6 (Neidhardt et. al., 1990).

#### 2.3.1.2 Cell Growth, Kinetics and Yield Factors

For microorganisms, growth is their most essential response to their physiochemical environment. Growth is a result of both replication and change in cell size. In a suitable nutrient medium, organisms extract nutrients from the medium and convert them into biological compounds. Parts of these nutrients are used for energy production and parts are used for biosynthesis and product formation.

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

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

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

When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. A typical batch growth curve includes the following phases (Shuler and Kargı, 2002):

1. Lag phase; occurs immediately after inoculation and is a period of adaptation of cells to a new environment ($\mu \approx 0$).

2. Exponential growth phase; the cells have adjusted to their new environment. Growth achieves its maximum rate ($\mu \approx \mu_{max}$).

3. Deceleration phase; growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth ($\mu < \mu_{max}$).

4. Stationary phase; the net growth rate is zero (no cell division) or the growth rate is equal to the death rate ($\mu = 0$).

5. Death phase; cells lose viability and lyse ($\mu < 0$).

Despite the complexity occurring in cell growth, yield principles can be applied to cell metabolism to relate flow of substrate in metabolic pathways to formation of biomass and other products. Yield coefficients allow to quantify the nutrient requirements and production characteristics of an organism (Doran, 2000). Yield coefficients are defined based on the amount of consumption of another material.

For example, the growth yield in a fermentation is:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \tag{2.4}$$

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

$$Y_{X/S} = \frac{dX}{dS} = \frac{dX/dt}{dS/dt} = \frac{r_X}{r_S} \tag{2.5}$$

Yield coefficients based on other substrates or product formation may be defined; for example,

$$Y_{X/O} = \frac{\Delta X}{\Delta O} \tag{2.6}$$

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

For organisms growing aerobically on glucose, $Y_{X/S}$ is typically 0.4 to 0.6 g/g for most yeast and bacteria, while $Y_{X/O}$ is 0.9 to 1.4 g/g. Anaerobic growth is less efficient, and the yield coefficient reduced substantially (Shuler and Kargı, 2002).

A list of frequently used yield coefficients is given in Table 2.2.

**Table 2.2** Definition of yield coefficients.

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

In an aerobic process, oxygen is consumed for cell growth, product and by-product formations, and maintenance. The oxygen consumption rate for cell growth can be defined as:

$$-r_{O1} = \frac{dC_X/dt}{Y'_{X/O}} \tag{2.8}$$

where $Y'_{X/O}$ is the cell yield per oxygen consumed for cell. Oxygen consumption rate for product formation is defined as:

$$-r_{O2} = \frac{dC_P/dt}{Y'_{P/O}} \tag{2.9}$$

If oxygen consumption rate for maintenance is first order with respect to cell concentration:

$$-r_{O3} = m_O C_X \tag{2.10}$$

Then, total oxygen consumption rate becomes:

$$-r_O = (-r_{O1}) + (-r_{O2}) + (-r_{O3}) \tag{2.11}$$

Combining equations (2.8), (2.9), (2.10) and (2.11):

$$r_O = \frac{dC_X/dt}{Y_{X/O}} + \frac{dC_P/dt}{Y_{P/O}} + m_O C_X \quad (2.12)$$

By using the definitions;

$$q_p = \frac{1}{C_X}\ \frac{dC_P}{dt} \quad (2.13)$$

$$\mu = \frac{1}{C_X}\ \frac{dC_X}{dt} \quad (2.14)$$

If equation (2.12) is reorganized, by using equations (2.13) and (2.14), and divided by $C_X$:

$$\frac{r_O}{C_X} = \frac{\mu}{Y_{X/O}} + \frac{q_P}{Y_{P/O}} + m_O \quad (2.15)$$

In order to make use of this equation, $q_P$ is defined, based on the study of Leudeking and Piret, as:

$$q_P = \alpha.\mu + \beta \quad (2.16)$$

Combining equations (2.15) and (2.16) and rearranging gives:

$$\frac{1}{Y_{X/0}} = \left(\frac{\alpha}{Y'_{P/O}} + \frac{1}{Y'_{X/O}}\right) + \left(\frac{1}{\mu}\right)\left(mo + \frac{\beta}{Y'_{P/O}}\right) \quad (2.17)$$

Then, the slope of the plot 1/μ vs $1/Y_{X/O}$ gives the value of oxygen consumption rate for maintenance, $m_O$. The Leudeking and Piret constants can be evaluated as follows:

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

Similarly, from the slopes of 1/μ versus $1/Y_{X/S}$ plots, $m_S$, maintenance coefficient for substrate, values can be obtained.

### 2.3.2 Medium Composition

Bacteria grow only if their environment is suitable; if it is not optimal, growth may occur at a lower rate or not at all- or the bacteria may die, depending on species and conditions.

Essential requirements for growth include a supply of suitable nutrients, a source of energy, water, an appropriate temperature, an appropriate pH, appropriate levels (or absence) of oxygen (Singleton, 1995).

Cells need nutrients as raw materials for growth, maintenance and division. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation (Shuler and Kargı, 2002). Typical components of a fermentation medium are:

1. *Water;* is the major component of all fermentation media.
2. *Macronutrients;* are needed in concentrations larger than $10^{-4}$ M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, $Mg^{2+}$, and $K^{+}$ are major macronutrients, from which living matter is made. Carbon compounds are major sources of cellular carbon and energy. Nitrogen constitutes about 10-14% of cell dry weight. Oxygen is present in all organic cell components and cellular water and constitutes about 20% of the dry weight of cells. Also, molecular oxygen is required as a terminal electron acceptor in the aerobic metabolism.
3. *Micronutrients;* are needed in concentrations of less than $10^{-4}$ M. Trace elements such as $Mo^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Ca^{2+}$, $Na^{2+}$, vitamins, growth hormones, and metabolic precursors are micronutrients. Trace elements are essential to microbial nutrition. Lack of essential trace elements increases the lag phase, and may decrease the specific growth rate and the yield.
4. *Buffers;* may be necessary to control the pH of the fermentation medium.
5. *Inducers;* must be present in the medium if the product of interest is an induced enzyme which is synthesized only in response to the presence of an inducer in the environment.
6. *Antifoams;* are surface active agents, reducing the surface tension in the foams. They usually have no metabolic effect but may substantially reduce the oxygen transfer rate.

Two major types of growth media are defined and complex media. Defined media contain specific amounts of pure chemical compounds with known chemical compositions. Complex media contain natural compounds whose chemical composition is not exactly known. A complex medium usually can provide the necessary growth factors, vitamins, hormones and trace elements; often resulting in higher cell yields, compared to the defined medium. Often, complex media are less expensive than defined media. The primary advantage of defined media is that the results are more reproducible and the operator has better control of the fermentation. Further, recovery and purification of a product is often easier and cheaper in defined media (Shuler and Kargı, 2002).

In literature, there is no work on the production of benzaldehyde lyase; hence, there is no reported medium for benzaldehyde lyase production. But related with other biomolecules using the host *E. coli*, Ryan et al. (1989) and Bhattacharya and Dukey (1997) used Luria-Bertani (LB) medium; Castan et al. (2002), Leon et al. (2003), Akesson et. al. (2001) and Yang et. al. (2003) used glucose based defined medium.

### 2.3.3 Bioreactor Operation Parameters

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

#### 2.3.3.1 Temperature

Generally, for a given type of bacterium, growth proceeds most rapidly at a particular temperature: the optimum growth temperature; the rate of growth tails off as temperatures increase or decrease from the optimum (Neidhardt et. al., 1990).

Rates of all reactions, including those catalyzed by enzymes, rise with increase in temperature in accordance with the Arrhenius equation:

$$k = A\, e^{-\Delta G/RT} \qquad (2.19)$$

Typical standard free energies of activation give rise to increases in rate by factors between 1.2 and 2.5 for every 10°C rise in temperature. So, it would be preferable to use enzymes at high temperatures in order to make use of this increased rate of reaction plus the protection it affords against microbial contamination (Chaplin and Bucke, 1990). For enzymes, however, denaturation begins to occur at 45 to 50°C and is severe at 55°C. One physical mechanism for this phenomenon is obvious: as the temperature increases, the atoms in the enzyme molecule have greater energies and a greater tendency to move. Eventually, they acquire sufficient energy to overcome the weak interactions holding the globular protein structure together, and deactivation follows.

Temperature also affects product formation. However, the optimum temperature for growth and product formation may be different. When temperature is increased above the optimum temperature, the maintenance requirements of cells increase. The yield coefficient is also affected by temperature. Temperature also may affect the rate-limiting step in a fermentation process. At high temperatures, the rate of bioreaction might become higher than the diffusion rate, and diffusion would then become the rate-limiting step (Shuler and Kargı, 2002).

In literature, the production of *E. coli* is performed at 37°C, which is the bacteria's optimal temperature of growth.

2.3.3.2 pH

Enzymes are amphoteric molecules containing a large number of acidic and basic groups, situated mainly on the surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzyme and the distribution of charge on its exterior surface, in addition to the reactivity of the catalytically active groups. Thus, the catalytically active enzyme may be a large or small fraction of the total enzyme present, depending upon the pH (Bailey, 1986). In a similar manner, the charge and the charge distribution on the substrate(s), product(s) and coenzymes (where applicable) will also be affected by pH changes. These charge variations, plus any consequent structural alterations, may be reflected in changes in the binding of the substrate, the catalytic efficiency and the amount of active enzyme (Chaplin and Bucke, 1990).

For these reasons, enzymes have a characteristic pH at which their activity is maximal; above or below this pH the activity declines. However, the pH-activity profiles of enzymes are not always bell-shaped. The optimum pH of an enzyme is not necessarily identical with the pH of its normal intracellular surroundings, which may be on the ascending or descending slope of its pH-activity profile. This fact suggests that the pH-activity relationship of an enzyme may be a factor in intracellular control of enzymatic activity (Lehninger, 1970).

For many bacteria, pH optima ranges from 3 to 8; and many organisms have mechanisms to maintain intracellular pH at a relatively constant level in the presence of fluctuations in environmental pH. When pH differs from the optimal value, the maintenance-energy requirements increase. In most fermentations, pH can vary substantially. Often the nature of the nitrogen source can be important. Furthermore, pH can change due to the production of organic acids, utilization of acids (particularly amino acids), or the production of bases. Thus, pH control by means of a buffer or an active pH control system is important (Shuler and Kargı, 2002). Nevertheless, some bioprocesses require controlled pH conditions, while others might require uncontrolled pH operations, in order to increase the product yield and selectivity (Çalık et al., 2002).

The studies in the literature using *E. coli* as a host microorganism, differs in the pH value they utilize. Ryan et al. (1989) investigated the effect of pH on the growth of recombinant *E. coli* at three different controlled pH values, i.e., 7.0, 7.4, 8.0; and reported that cell concentration increases when the culture pH decreases, however, enzyme activity attain its highest value at pH values in the vicinity of pH=7.4. Castan et al. (2002) kept the pH constant at 7.0 with aqueous $NH_3$. In the study of Leon et al. (2003) pH was kept constant throughout the bioprocess at 7.4 with 10 N NaOH. Also, in the studies of Akesson et al. (2001) (pH=6.9 by 25% ammonia) and Yang et al. (2003) (pH=7.0 by 2 M NaOH) pH value was kept constant throughout the bioprocesses. Hence, there is no systematic investigation in the literature, on the effects of pH on any biomolecule production and by-product distributions by the host *E. coli*.

2.3.3.3 Oxygen Transfer

2.3.3.3.1 The Importance and Mechanism of Oxygen Transfer

As microorganisms require oxygen for their metabolic activities like respiration, growth and product formation in aerobic fermentations, the concentration of the dissolved oxygen in the medium and oxygen uptake rate are very important parameters (Çalık et. al., 1998). Oxygen is present in all organic cell components and cellular water and constitutes about 20% of the dry weight of the cells. Molecular oxygen is required as a terminal electron acceptor in the aerobic metabolism. Gaseous oxygen is introduced into growth media by sparging air; and oxygen transfer rate can be adjusted by either changing the air inlet rate or agitation rate.

Cells in aerobic culture take up oxygen from the liquid. The rate of oxygen transfer from gas to liquid is therefore of prime importance, especially at high cell densities when cell growth is likely to be limited by availability of oxygen in the medium. According to the two-film theory, the oxygen must pass through a series of transport resistances, the relative magnitudes of which depend on bubble hydrodynamics, temperature, cellular activity and density, solution composition, interfacial phenomena, and other factors. These arise from different combinations of the following resistances; all of these resistances appear in Figure 2.3 (Doran, 2000):

1. Transfer from the interior of the bubble to the gas-liquid interface,

2. Movement across the gas-liquid interface,

3. Diffusion through the relatively stagnant liquid film surrounding the bubble,

4. Transport through the bulk liquid,

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

6. Movement across the liquid-cell interface,

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

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

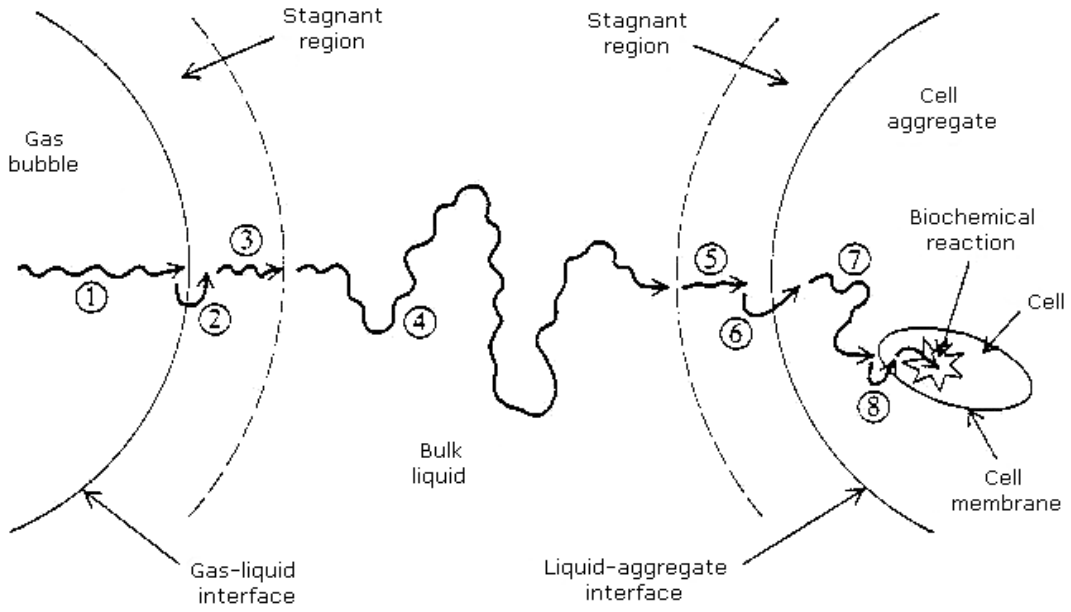



**Figure 2.4** Schematic diagram of steps involved in transport of oxygen from a gas bubble to inside a cell.

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

$$OTR = k_L a(C_O^* - C_O) \qquad (2.20)$$

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

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

2.3.3.3.2 Oxygen Transfer Characteristics

The transfer of oxygen into the microbial cell in aerobic fermentation processes strongly affects product formation by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999); therefore, to fine-tune bioreactor performance in relation with the physiology of the microorganism the extent of oxygen transfer requirement needs to be clarified (Çalık et. al., 2000).

The rate of oxygen transfer in fermentation broths is influenced by several physical and chemical factors that change either the value of $K_La$, or the driving force for mass transfer, ($C_O^*$- $C_O$). Therefore, the oxygen uptake rate of the cells, and the liquid phase mass transfer coefficient, $K_La$, are important characteristics of oxygen transfer.

The rate at which oxygen is consumed by cells in fermenters determines the rate at which it must be transferred from gas to liquid. Many factors influence oxygen demand; the most important of which are cell species, culture growth phase, and nature of the carbon source in the medium. In batch culture, rate of oxygen uptake varies with time. The reasons for this are twofold. First, the concentration of cells increases during the course of batch culture and the total rate of oxygen uptake is proportional to the number of cells present. In addition, the rate of oxygen consumption per cell, known as the specific oxygen uptake rate ($q_O$), also varies. Oxygen uptake rate (OUR), $-r_O$, per unit volume of broth is given by (Doran, 2000):

$$-r_O = q_O C_X \qquad (2.21)$$

The inherent demand of an organism for oxygen ($q_O$) depends primarily on the biochemical nature of the cell and its nutritional environment. Choice of substrate for fermentation can also significantly affect oxygen demand. Because

glucose is generally consumed more rapidly than other sugars or carbon-containing substrates, rates of oxygen demand are higher when glucose is used.

The liquid phase mass transfer coefficient, $K_La$, is an important parameter in bioreactors, which indicates the oxygen transfer rate from gas to the liquid phase. It is important to estimate whether the dissolved oxygen in the medium is successfully transferred to the cells, and it is possible by determining the liquid phase mass transfer coefficient, $K_La$. Dynamic method is widely used for the determination of the value of $K_La$ experimentally, and it can be applied during the fermentation process (Rainer, 1990)

The method is based on an unsteady state mass balance for oxygen given by the following equation:

$$\frac{dC_O}{dt} = K_L a(C_O^* - C_O) - q_0 C_X \qquad (2.22)$$

As shown in Figure 2.5, at some time $t_0$, the broth is de-oxygenated by stopping the air flow. During this period, dissolved oxygen concentration, $Co_0$, drops, and since there is no oxygen transfer, equation (2.22) becomes:

$$\frac{dC_O}{dt} = -r_0 \qquad (2.23)$$

Using equation (2.23) in region-II of Figure 2.5, oxygen uptake rate, $-r_0$, can be determined.

Air inlet is then turned back on, and the increase in $C_O$ is monitored as a function of time. In this period, region-III, equation (2.22) is valid. Combining equations (2.21) and (2.22) and rearranging,

$$C_O = -\frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_0\right) + C_O^* \qquad (2.24)$$

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

The Dynamic Method can also be applied to conditions under which there is no reaction, i.e., $r_0$=0 (Nielsen and Villadsen, 1994). In this case, the broth is de-oxygenated by sparging nitrogen into the vessel. Air inlet is turned back on and again the increase in Co is monitored as a function of time. Modifying equation (2.24)

$$C_O = \frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \qquad (2.25)$$

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

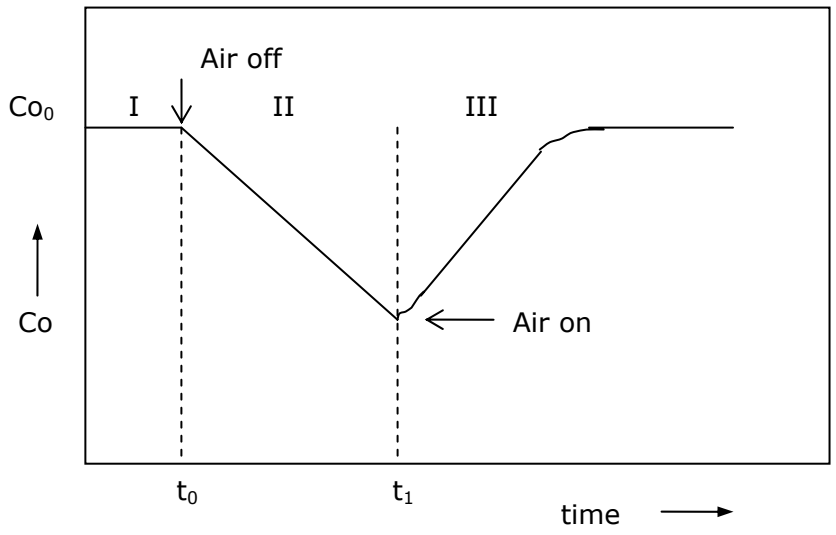



**Figure 2.5** Variation of dissolved oxygen concentration with time in dynamic measurement of $K_L a$.

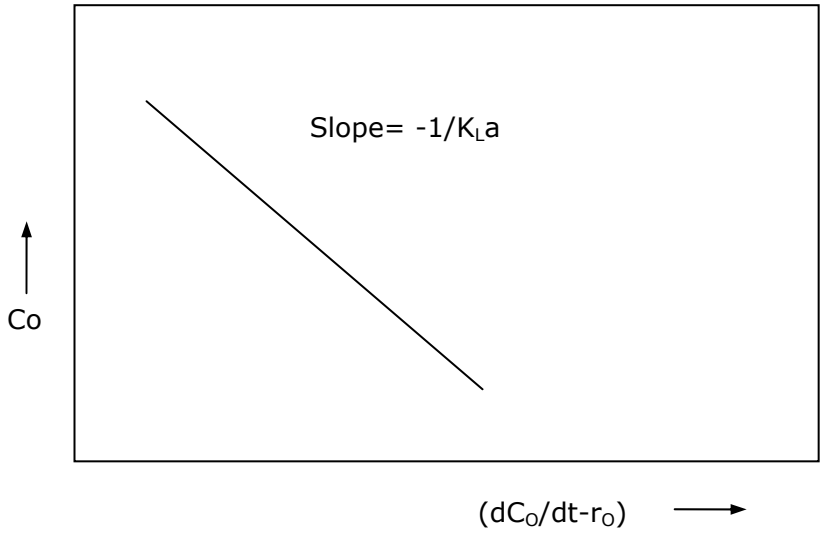



**Figure 2.6** Evaluting $K_L$a using the Dynamic Method.

There is no work in the literature reporting the effects of oxygen transfer on benzaldehyde lyase production. But related with other biomolecules using the host *E. coli*; Ryan et al. (1989) reported the effects of oxygen transfer on beta-lactamase production by *E. coli* JM103; without stating the oxygen transfer rate and oxygen uptake rate, they reported that the decrease in air inlet rate decreased the growth rate. Bhattacharya and Dukey (1997) reported the effects of oxygen transfer on DNA methyltransferase production by *E. coli* K12 and reported that an oxygen sufficient condition was necessary to obtain optimal expression and cell productivity. Castan et al. (2002) reported the effects of air supply enrichment either by oxygen (30 and 40%) or carbon dioxide (12 and 20%) on human growth hormone production by *E. coli* K12. Lastly, Leon et al. (2003) investigated the effect of the dissolved oxygen on penicillin acylase production by *E. coli* JM101, low oxygen transfer condition was studied in detail and kinetic and stoichiometric parameters were evaluated. In addition to these, studies have been performed in the fed batch mode, with particular emphasis on the control strategy for controlling dissolved oxygen levels, through agitation, air inlet rate and glucose feed rate (Akesson, 2001; Johnston et al., 2003). Hence, there is no systematic investigation in the literature, on the effects of oxygen transfer on any biomolecule production by the host *Escherichia coli*.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Chemicals

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

## 3.2 The Microorganism

*Escherichia coli* K12 (ATCC 10798), *Escherichia coli* JM109, *Escherichia coli* XL1-Blue, *Escherichia coli* GBE180 (CGSC 7569), *Escherichia coli* W3110 (CGSC 4474), carrying pUC18*::bal* gene, were used as the potential producers of benzaldehyde lyase (BAL, EC 4.1.2.38). pUC18*::bal* was transferred into *E. coli* strains by $CaCl_2$ Method (Appendix A). The microorganisms were received as a few drops of glycerolized culture placed on a filter disc and wrapped in plastic film. They were sub-cultured immediately, by placing each on an appropriate plate, adding sterile diluents and streaking. Afterwards, the microorganisms were re-inoculated onto a solid medium, and stored at 4ºC.

## 3.3 The Solid Medium

The microorganisms, stored on agar slants at 4ºC, were inoculated onto the freshly prepared agar slants under sterile conditions, and were incubated at 37ºC for 10 h 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 $m^{-3}$ |
|---|---|
| Soytryptone | 10.0 |
| Yeast Extract | 5.0 |
| NaCl | 10.0 |
| Agar | 15.0 |
| Ampicillin | 0.1 |

### 3.4 The Precultivation Medium

Microorganisms grown in the solid medium were inoculated into precultivation medium and incubated at 37ºC and N=200 $min^{-1}$ for 14 h. Experiments were conducted in agitation and heating rate controlled orbital shakers, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. LB medium was used as the precultivation medium for biomass production, and its constituents is given in Table 3.2.

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

| Compound | Concentration, kg $m^{-3}$ |
|---|---|
| Soytryptone | 10.0 |
| Yeast Extract | 5.0 |
| NaCl | 10.0 |
| Ampicillin | 0.1 |

### 3.5 The Production Medium

After incubation in the precultivation medium for 14 hours, microorganisms were inoculated to the production medium, contained either in the laboratory scale bioreactor (V=150 ml) or pilot scale bioreactor (V=3.0 $dm^3$), with 1/10 inoculation ratio.

For microorganism selection and medium design experiments, air-filtered Erlenmeyer flasks 150 ml in size, that had working volume capacities of 33 ml,

were used as bioreactors. Agitation and heating rate controlled orbital shakers, at a cultivation temperature of 37ºC and an agitation rate of 200 $min^{-1}$ were used as incubators. Details of the bioreactor system, which was used for oxygen transfer and pH effect experiments, are given in section 3.6.

After the selection of the proper microorganism, a defined medium design was performed; furthermore, effects of oxygen transfer and pH was investigated in order to increase the biomass and benzaldehyde lyase production. The parameters investigated for benzaldehyde lyase production are given in Table 3.3.

**Table 3.3** The investigated parameters for benzaldehyde lyase production.

| Medium Components | Bioreactor Operation Parameters |
|---|---|
| Glucose | Oxygen Transfer Rate |
| $(NH_4)_2HPO_4$ | pH |
| $NH_4Cl$ | |
| $(NH_4)_2SO_4$ | |

The reference defined medium for benzaldehyde lyase production and starting point of the medium design experiments was chosen to be the M9 glucose minimal salts broth; the composition is given in Table 3.4.

**Table 3.4** The composition of the reference BAL production medium.

M9 Glucose Minimal Salts Broth

| Component | Concentration, kg $m^{-3}$ |
|---|---|
| Glucose | 4.0 |
| $Na_2HPO_4$ | 6.7 |
| $KH_2PO_4$ | 3.1 |
| NaCl | 0.5 |
| $MgSO_4.7H_2O$ | 0.5 |
| $NH_4Cl$ | 4.0 |
| Ampicillin | 0.1 |

Before being used in the experiments, all the medium components were steam sterilized at 121°C for 20 min, glucose being sterilized separately.

### 3.6 The Pilot Scale Bioreactor System

The pilot scale 3.0 $dm^3$ batch bioreactor (Braun CT2-2), having a working volume of 0.5-2.0 $dm^3$, a diameter of 121 mm, and consisting of temperature, pH, foam and stirring rate controls, was used. The bioreactor utilized an external cooler, steam generator and a jacket around the bioreactor for sterilization and temperature control. The bioreactor was stirred with two Rushton turbines and consisted of four baffles, two six-bladed impellers, each of which has a diameter of 53 mm, and a sparger.

### 3.7 Analysis

Throughout the bioprocesses, samples were taken at characteristic cultivation times. After determining the cell concentration, the medium was centrifuged at 13500 $min^{-1}$ for 10 min at 4°C to precipitate the cells. Precipitate was used to determine benzaldehyde lyase activity after the lysis of the cell wall; supernatant was used for the determination of glucose concentration. In bioreactor system experiments, besides enzyme activity, cell and glucose concentrations, organic acid concentrations, oxygen uptake rate and liquid phase mass transfer coefficient values were determined.

#### 3.7.1 Cell Concentration

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

#### 3.7.2 Benzaldehyde Lyase Activity

Benzaldehyde lyase activity was determined by measuring the conversion of benzoin into benzaldehyde. Samples from the culture broth was harvested by centrifugation (Sigma 1-15) at 13500 $min^{-1}$ for 10 min at 4°C. To determine the activity of the intracellular benzaldehyde lyase enzyme, the cell walls was lyzed

at $f$=10 $s^{-1}$ for 10 minutes at the agitator bead mill (Retsch, MM 200) by using 30% suspension of glass beads in tris-HCl buffer. Fresh substrate solutions were prepared daily containing 0.035 mM benzoin, 20 mM tris-HCl (pH=8.0), 0.01 mM TPP, 0.1 mM $MgCl_2$ and incubated at 37°C. Reaction was started by the addition of 20 µl of diluted crude extract to 3 $cm^3$ 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):

$$\text{Nanomoles of product formed} = [2(A_f - A_i)/(\varepsilon_s - 2\varepsilon_p)]*10^6$$

With $\varepsilon_s$ and $\varepsilon_p$ 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.

### 3.7.3 Reduced Sugar Concentration

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

1. 3 $cm^3$ of DNS solution was added into test tubes containing 1 $cm^3$ of sample at different glucose concentrations.
2. The test tubes were put into boiling water for 5 min and then into ice for another 5 min.
3. The sample passing through the same steps but do not contain any reducing sugar is used as blank and the absorbance values of the samples were measured by a UV spectrophotometer at 550 nm.

### 3.7.4 Organic Acids Concentrations

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

### 3.7.5 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 2.3.3.3.2, was used.

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

# CHAPTER 4

# RESULTS AND DISCUSSION

The research program for the investigation of the effects of bioprocess operation parameters on benzaldehyde lyase production was carried out in mainly four parts. The selection of the host *Escherichia coli* strain having the highest benzaldehyde lyase production capacity had the primary importance. Next, using the selected microorganism, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher benzaldehyde lyase production. Thereafter, by using the designed medium, the effects of bioreactor operation parameters, i.e., oxygen transfer conditions and pH, and the fermentation and oxygen transfer characteristics of the bioprocess, were investigated in the pilot scale bioreactor.

## 4.1 Microorganism Selection

Plasmid pUC18 carrying benzaldehyde lyase gene was transformed via $CaCl_2$ method (Appendix A) into several *Escherichia coli* strains, in order to select the host *E. coli* strain having the highest benzaldehyde lyase production capacity.

pUC18*::bal* was transformed into *E. coli* JM 109, *E. coli* K12, *E. coli* XL-1 Blue, *E. coli* W3110 and *E. coli* GBE 180. However, plasmid transformation into *E. coli* W3110 was not successful; growth on solid medium can not be achieved. Remaining four *E. coli* strains were grown in LB medium (Table 3.2); and the strain having the highest cell production capacity was chosen to be the most potential benzaldehyde lyase producer, since benzaldehyde lyase is an intracellular enzyme in *E. coli*.

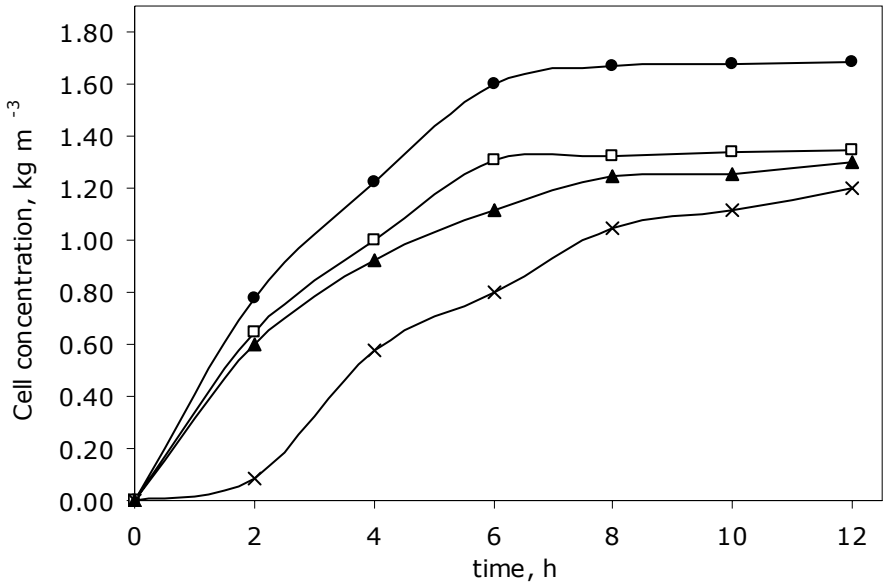



**Figure 4.1** The variations in cell concentration with the cultivation time for various strains of *E. coli* carrying pUC18*::bal* gene in LB medium, V=33 $cm^3$, T=37°C, N=200 $min^{-1}$. *E. coli* strains: K12, (●); JM 109, (□); XL1-Blue, (▲); GBE 180, (x).

As it is seen from Figure 4.1, the highest cell concentration was obtained with *Escherichia coli* K12 in LB medium as 1.7 kg $m^{-3}$. Therefore, in the first part of the study, *E. coli* K12 (ATCC 10798) was selected as the benzaldehyde lyase producer.

### 4.2 Medium Design

After the selection of the producer microorganism, a defined medium was designed to optimize benzaldehyde lyase production and cell growth. In Table 3.4, the composition of the reference production medium in which the cell concentration and benzaldehyde lyase activity were obtained respectively as 1.3 kg $m^{-3}$ and 450 U $cm^{-3}$, is given. This medium was considered as the starting point for medium design experiments, and named as RPM medium. In this context, the effects of the concentrations of carbon source, i.e., glucose; and nitrogen sources, i.e., $(NH_4)_2HPO_4$, $NH_4Cl$, $(NH_4)_2SO_4$; were investigated in agitation (N=200 $min^{-1}$) and heating rate (T=37°C) controlled laboratory scale bioreactors.

4.2.1 The Effect of Glucose Concentration

Glucose, being easily metabolized by microorganisms, is widely used in fermentation media. The effects of glucose on benzaldehyde lyase production were investigated in the RPM medium, at the initial concentrations of $C_G^o$=4.0, 6.0, 8.0, 10.0, 12.0, 15.0 and 20.0 kg $m^{-3}$. Cell concentration and benzaldehyde lyase activity profiles had a similar trend in response to initial glucose concentration. With the increase in initial glucose concentration, cell concentration and benzaldehyde lyase activity first increased and they both give a maximum when the initial glucose concentration was 8.0 kg $m^{-3}$. On the other hand, cell concentration and benzaldehyde lyase activity decreased at higher initial glucose concentrations, due to the substrate inhibition. So, it was concluded that the initial glucose concentration for benzaldehyde lyase production should be 8.0 kg $m^{-3}$, having values of cell concentration $C_x$=1.5 kg $m^{-3}$ and benzaldehyde lyase activity of A=600 U $cm^{-3}$ at t=12 h of the bioprocess (Figure 4.2).

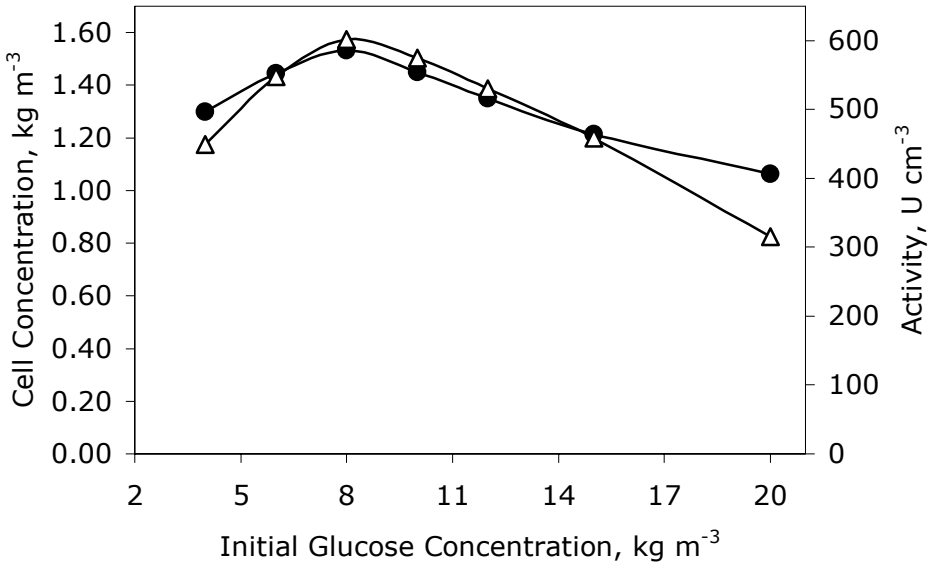



**Figure 4.2** The variation in cell concentration and benzaldehyde lyase activity with the initial glucose concentration, at t=12 h, V=33 $cm^3$, T=37°C, N=200 $min^{-1}$. Cell concentration:(●); Benzaldehyde lyase activity:(Δ).

4.2.2 The Effects of Nitrogen Sources

To investigate the effect of nitrogen sources, $NH_4Cl$ was omitted from the RPM medium. Since $C_G^o$ = 8.0 kg $m^{-3}$ was found to be the optimum for benzaldehyde lyase production, glucose at $C_G^o$ = 8.0 kg $m^{-3}$ was used as the carbon source and the effects of $(NH_4)_2HPO_4$, $NH_4Cl$, $(NH_4)_2SO_4$ were investigated with the initial concentrations of $(NH_4)_2HPO_4$, (kg $m^{-3}$): 4.0, 5.0, 6.0, 7.0, 8.0; $NH_4Cl$, (kg $m^{-3}$): 3.0, 4.0, 5.0, 6.0, 7.0, 8.0; and $(NH_4)_2SO_4$, (kg $m^{-3}$): 4.0, 5.0, 6.0. It was observed that, in the presence of $(NH_4)_2SO_4$, cell concentration and activity values decreased. This was due to the potential inhibition effect of $SO_4^=$ on the metabolism. Furthermore, as can be seen from Table 4.1, with the increase in the initial $NH_4Cl$ and $(NH_4)_2HPO_4$ concentrations; cell concentration and benzaldehyde lyase activity values first increased; but higher nitrogen content had an inhibitory effect on both cell concentration and benzaldehyde lyase activity values.

**Table 4.1** The variations in cell concentration and benzaldehyde lyase activity with the initial nitrogen source concentration, at t=12 h, V=33 $cm^3$, T=37°C, N=200 $min^{-1}$.

| Component | kg $m^{-3}$ | Cx, kg $m^{-3}$ | A, U $cm^{-3}$ |
|---|---|---|---|
| $NH_4Cl$ | 3.0 | 1.4 | 545 |
| | 4.0 | 1.5 | 600 |
| | 5.0 | 1.5 | 660 |
| | 6.0 | 1.6 | 695 |
| | 7.0 | 1.6 | 585 |
| | 8.0 | 1.5 | 510 |
| $(NH_4)_2HPO_4$ | 4.0 | 1.6 | 690 |
| | **5.0** | **1.8** | **745** |
| | 6.0 | 1.7 | 640 |
| | 7.0 | 1.6 | 625 |
| | 8.0 | 1.4 | 560 |
| $(NH_4)_2SO_4$ | 4.0 | 1.1 | 420 |
| | 5.0 | 1.4 | 530 |
| | 6.0 | 1.4 | 500 |

Among the investigated nitrogen sources, the highest cell concentration (1.8 kg $m^{-3}$) and benzaldehyde lyase activity (745 U $cm^{-3}$) were obtained when $(NH_4)_2HPO_4$ was used as the nitrogen source with the initial concentration of 5.0 kg $m^{-3}$.

4.2.3 The Optimized Medium

As a result, among the investigated media, the highest cell concentration and benzaldehyde lyase activity was obtained as 1.8 kg $m^{-3}$ and 745 U $cm^{-3}$, respectively, in the medium containing 8.0 kg $m^{-3}$ glucose, 5.0 kg $m^{-3}$ $(NH_4)_2HPO_4$ and the salt solution. The activity obtained in the optimized medium was 1.6-fold higher than the activity obtained in the reference medium.

## 4.3 Bioreactor Operation Parameters

By using *E. coli* K12 (ATCC 10798) and the designed medium, the bioreactor operation parameters, given in Table 3.3, i.e., the effect of oxygen transfer and pH, were investigated together with the fermentation and oxygen transfer characteristics of the bioprocess, in the pilot scale bioreactor.

## 4.4 Oxygen Transfer Effects

The effect of oxygen transfer, which is one of the most important parameters of the bioprocesses, was investigated in detail for benzaldehyde lyase production. This investigation was done at six different conditions with the parameters, air inlet rate of $Q_O/V_R$ = 0.5 vvm, and agitation rates of N=250, 375, 500, 625, 750 $min^{-1}$ and at $Q_O/V_R$ = 0.7 vvm, N=750 $min^{-1}$. The first two operations were named as "low oxygen transfer" conditions; following two operations are "medium oxygen transfer" conditions; and the last two operations were named as "high oxygen transfer" conditions. Dissolved oxygen, pH, cell concentration, benzaldehyde lyase volumetric and specific activities, glucose concentration, organic acid concentrations, yield values, maintenance coefficients, overall mass transfer coefficients, specific growth rates and oxygen uptake rates were determined throughout the bioprocesses.

The investigation of the effects of oxygen transfer on benzaldehyde lyase production was performed by batch-bioreactor experiments, in the bioreactor

system consisted of temperature, pH, foam, air inlet and stirring rate controls with $V_R$ =1.65 $dm^3$ working volume. The oxygen transfer conditions applied were abbreviated as given in Table 4.2.

**Table 4.2** Oxygen transfer conditions and their abbreviations.

| Oxygen Transfer Condition Applied N, $min^{-1}$; $Q_O/V_R$, vvm | Abbreviation | Oxygen Transfer Condition Applied N, $min^{-1}$; $Q_O/V_R$, vvm | Abbreviation |
|---|---|---|---|
| N=250, $Q_O/V_R$ = 0.5 | $LOT_1$ | N=625, $Q_O/V_R$ = 0.5 | $MOT_2$ |
| N=375, $Q_O/V_R$ = 0.5 | $LOT_2$ | N=750, $Q_O/V_R$ = 0.5 | $HOT_1$ |
| N=500, $Q_O/V_R$ = 0.5 | $MOT_1$ | N=750, $Q_O/V_R$ = 0.7 | $HOT_2$ |

4.4.1 Dissolved Oxygen and pH Profiles

The variations in the dissolved oxygen and medium pH with the cultivation time, agitation rate and air inlet rates are respectively shown in Figs. 4.3 and 4.4. Dissolved oxygen in the medium depends on the extent of the oxygen transfer rate to the medium and to the oxygen uptake rate of the cells. At $LOT_1$ and $LOT_2$ conditions, throughout the bioprocesses, there was no oxygen accumulation; thus, oxygen transfer rate (OTR) was equal to the oxygen uptake rate (OUR). At $MOT_1$, due to the high oxygen demand of the cells between t=3-7 h of the bioprocess, transferred oxygen was totally consumed. However, at $MOT_2$, $HOT_1$ and $HOT_2$ conditions, dissolved oxygen concentration in the medium did not change considerably throughout the bioprocess as the oxygen transfer rate was high enough (Fig. 4.3).

The initial pH value was $pH_0$=7.2 at all conditions. pH has a tendency to decrease along with the bioprocesses with the cultivation time; and the decrease rate of pH was the highest at $LOT_1$ and $LOT_2$ conditions (Fig. 4.4). Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level, even with large variations in the pH of the extracellular medium, but only at the expense of a significant increase in the maintenance demands, since Gibbs free energy has to be used for maintaining the proton gradient across the cell membrane (Nielsen and Villadsen, 1994).

For instance, for *E. coli*, regardless of the value of the external pH, within the cell, pH is maintained at a value quite close to 7.6. To maintain the intracellular pH at a constant level, protons are pumped in or out through the cell membrane. The inward flow of protons is enzymatically mediated by ATPase, which participates in the formation of ATP. The proton transport is reversible; i.e., the ATPase may also pump protons out of the cells with the expenditure of ATP. Whereupon, the pH of the environment has a natural tendency to change along with the bioprocess despite the fact that intracellular pH is kept constant. At all the oxygen transfer conditions, as the pH decreased with the cultivation time considerably (Fig. 4.4) the proton electro-chemical gradient to generate energy was high enough. The increase in $H^+$ ion concentration in the fermentation broth was the highest at $LOT_1$ and $LOT_2$ conditions, indicating the high energy generation potential, the lowest amount of cell was formed under these two conditions, in spite of the energy generation potential, due to the oxygen limitation.

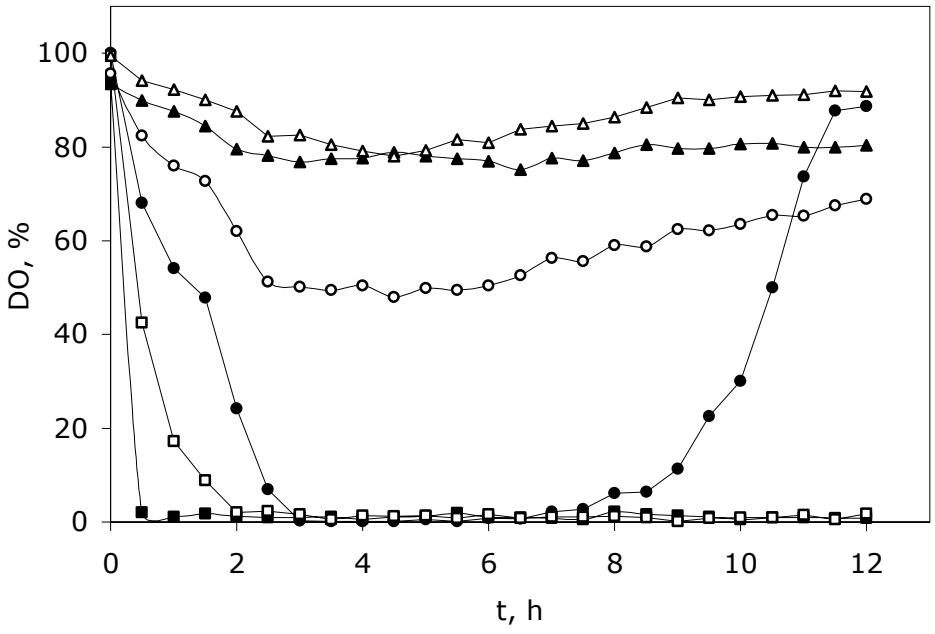



**Figure 4.3** The variations in the dissolved oxygen concentration with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ). $C_{DO}^*$=0.21 mol $m^{-3}$.

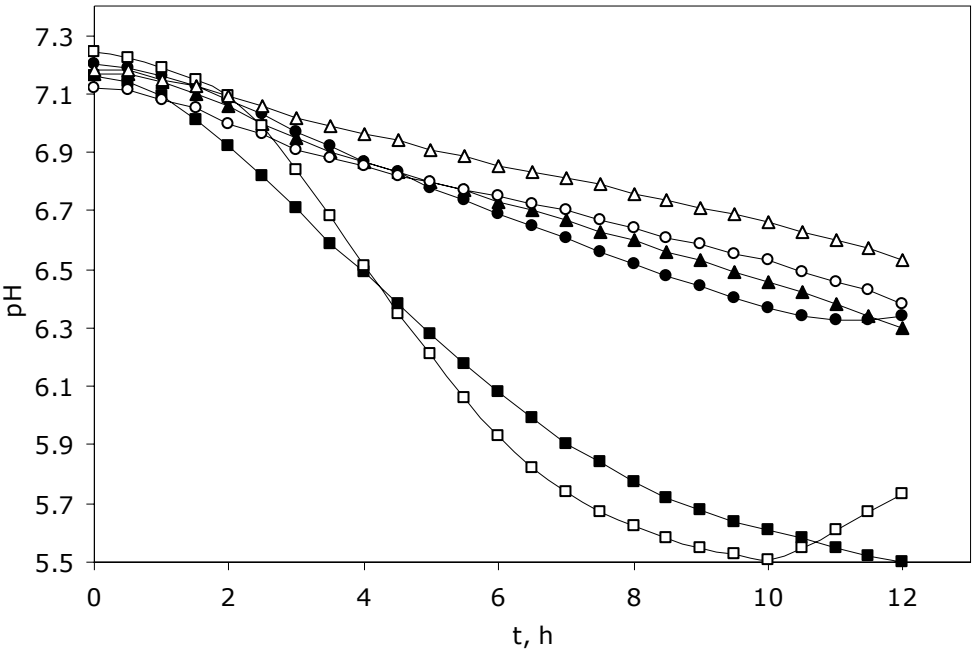



**Figure 4.4** The variations in the medium pH with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

In literature, there is no work reporting the variations in pH and the dissolved oxygen profiles throughout the benzaldehyde lyase production process.

### 4.4.2 Glucose and Cell Concentration Profiles

The variations of the glucose -that enters to the carbon metabolism from the beginning of the glycolysis pathway- and cell concentrations with the cultivation time, agitation rate and air inlet rates are given in Figs. 4.5 and 4.6, respectively. In the first 4 h of the bioprocesses, the oxygen transfer condition applied did not affect the glucose consumption rate considerably; however, at t>4 h glucose consumption rate was the highest at 375-500 $min^{-1}$ and it decreased at higher agitation and/or air inlet rates.

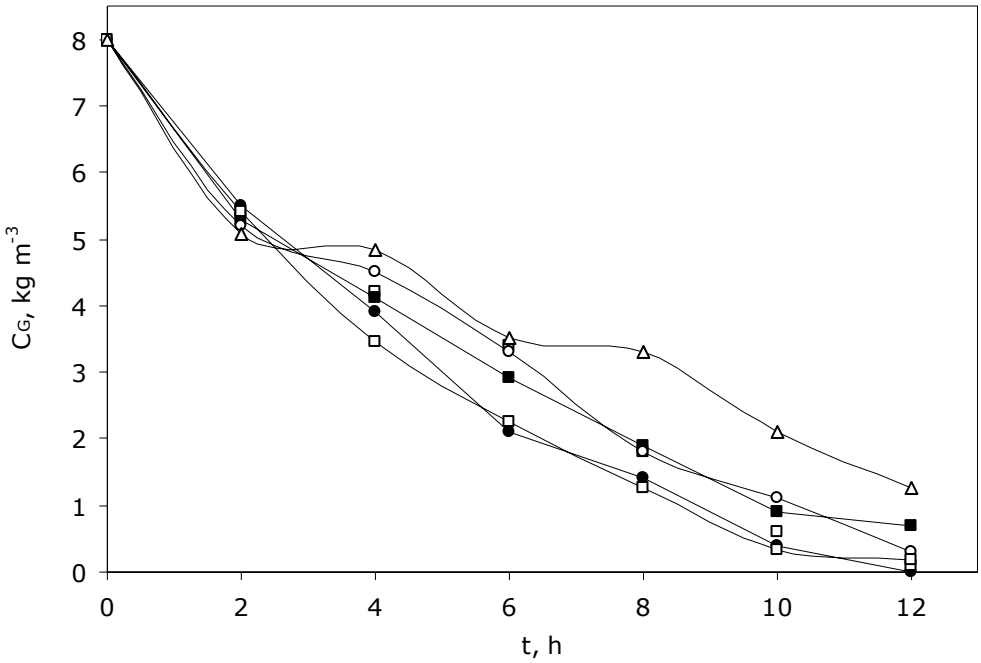



**Figure 4.5** The variations in the glucose concentration with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

At t=0-2 h cell formation did not change considerably with the oxygen transfer condition applied, however, at t=2-4 h with the increase in agitation rate, cell formation increased; and the highest cell formation rate was obtained at $HOT_1$ condition. Cell formation rate decreased with the cultivation time at all the oxygen transfer conditions, as expected; but the decrease rate was higher at high oxygen transfer conditions. Consequently, at the end of the bioprocesses, the highest cell concentration value was obtained at $MOT_1$ condition as 2.3 kg $m^{-3}$, while the lowest cell concentration value was obtained at $LOT_1$ condition as 1.0 kg $m^{-3}$ (Fig. 4.6).

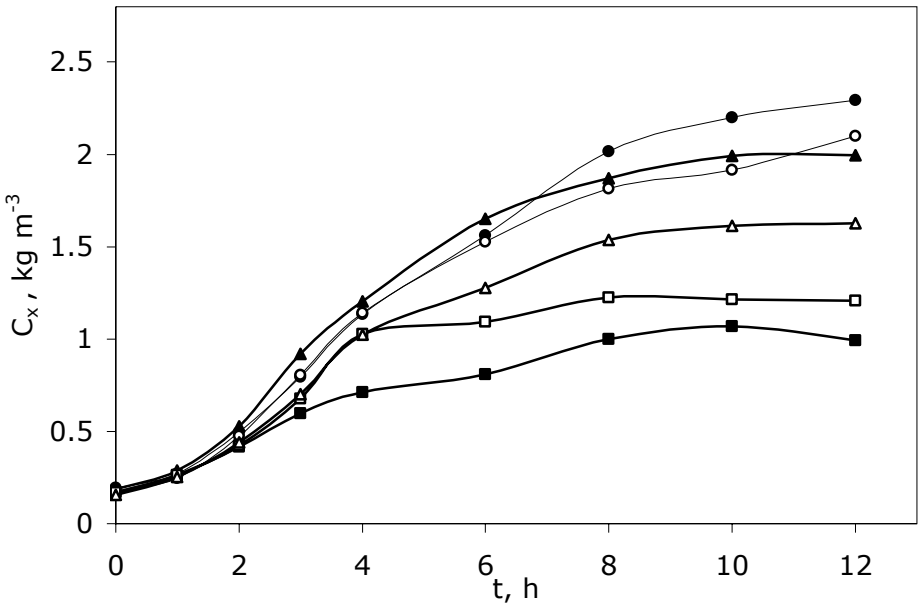



**Figure 4.6** The variations in the cell concentration with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

4.4.3 Benzaldehyde Lyase Activity Profiles

The variations of benzaldehyde lyase volumetric activity (A) and specific activity ($A_x$) with the cultivation time at the oxygen transfer conditions applied are respectively shown in Figs. 4.7 and 4.8. The highest benzaldehyde lyase volumetric activity was obtained at $MOT_1$ condition, as 860 U $cm^{-3}$, where the highest cell concentration was obtained. On the other hand, at t=12 h the lowest benzaldehyde lyase volumetric activity (400 U $cm^{-3}$) and the highest specific activity (400 U $mg^{-1}$DW), which was also close to the other oxygen transfer conditions, was obtained at $LOT_1$ condition. Since in production, the aim is to obtain the maximum volumetric benzaldehyde lyase activity and concentration; it is essential to increase the concentration of the benzaldehyde lyase producing cells. Therefore, it was concluded that for benzaldehyde lyase production, the bioreactor should be operated at medium oxygen transfer conditions, where the highest amount of cell was generated.

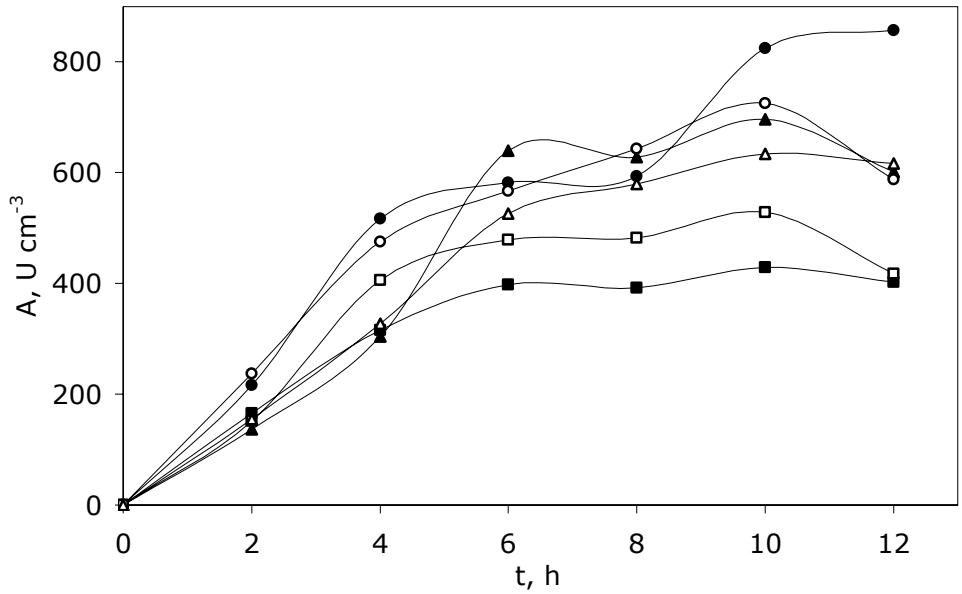



**Figure 4.7** The variations in the benzaldehyde lyase volumetric activity with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

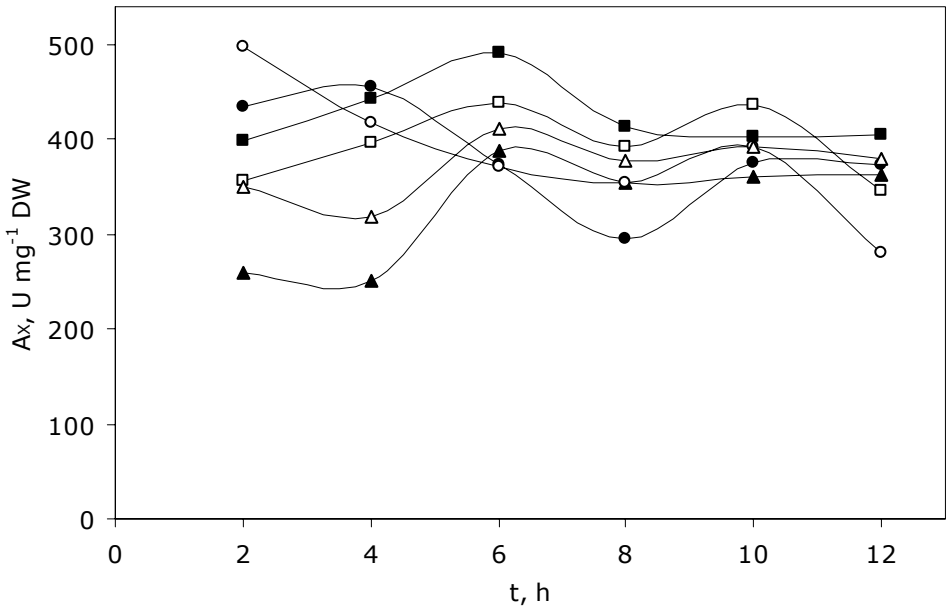



**Figure 4.8** The variations in benzaldehyde lyase specific activity with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

4.4.4 Organic Acid Concentration Profiles

The organic acids detected in the fermentation broth with the cultivation time at the oxygen transfer conditions applied are given in Table 4.3. As it is seen from the table, at $LOT_1$ and $LOT_2$ conditions, especially by the end of the bioprocesses; citric, α-ketoglutaric, lactic, butyric and succinic acids were detected in the fermentation broth, most of which did not appear at higher oxygen transfer conditions. Especially, detection of TCA cycle organic acids, i.e., citric, α-ketoglutaric and succinic acids in the fermentation broth is important as it signals the insufficient operation of the TCA cycle in benzaldehyde lyase production process, at low oxygen transfer conditions. This was concluded; since the TCA cycle organic acids were accumulated in the broth instead of being directed in the TCA cycle.

**Table 4.3** Organic acids detected in the fermentation broth with the cultivation time at the oxygen transfer conditions applied.

| t, h | $LOT_1$ | $LOT_2$ | $MOT_1$ | $MOT_2$ | $HOT_1$ | $HOT_2$ |
|---|---|---|---|---|---|---|
| 4 | Citric<br>Acetic<br>Lactic | Citric<br>Acetic | Acetic | Acetic | Acetic | Acetic |
| 8 | Citric<br>α-KG<br>Acetic<br>Lactic | α-KG<br>Succinic<br>Acetic<br>Lactic | Succinic<br>Acetic | Succinic<br>Acetic | Pruvic<br>Succinic<br>Acetic | Succinic<br>Acetic |
| 12 | Citric<br>α-KG<br>Acetic<br>Lactic<br>Butric | α-KG<br>Succinic<br>Acetic<br>Lactic | Succinic<br>Acetic | Succinic<br>Acetic | Succinic<br>Acetic<br>Glutamic | Succinic<br>Acetic<br>Gluconic |

Also, it is clear in Figure 4.9 that, the total organic acid concentration in the fermentation broth was higher at low oxygen transfer conditions $LOT_1$ and $LOT_2$, explaining the reason of the highest rate of decrease in the medium pH at these conditions (Fig. 4.4). At the end of the bioprocesses, the lowest amount of organic acids was observed at $MOT_1$ condition, where the highest cell concentration was reached (Figure 4.6). Hence, it is concluded that the accumulation of organic acids in the fermentation broth inhibits cell formation.

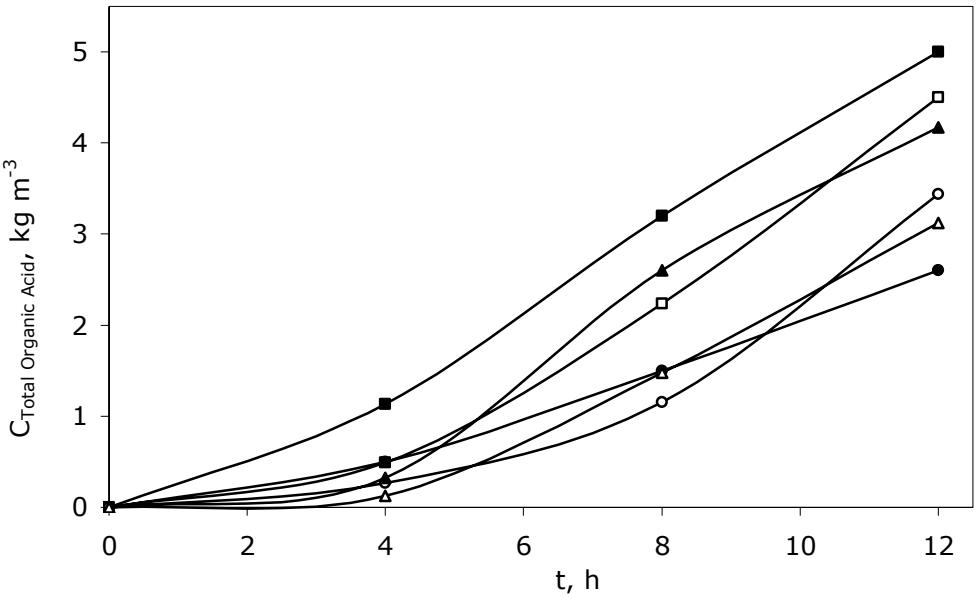



**Figure 4.9** The variations in total organic acid concentration with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg m$^{-3}$, T=37°C, $V_R$= 1.65x10$^{-3}$ m$^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

Apparently, the major organic acid that *E. coli* produces is acetic acid (Table 4.3). The accumulation of the metabolic by-product acetic acid was reported in many publications as one of the obstacles in attaining high product yields and high productivity with *E. coli*, which inhibits growth (Luli and Strohl, 1990) as well as production of recombinant proteins (Shimizu et al., 1988; Bauer et al., 1990; Bech Jensen and Carlsen, 1990; Turner et al., 1994). In benzaldehyde lyase production process, because of the oxygen limitation at low oxygen transfer conditions, AcCoA was converted into acetic acid instead of being directed to the TCA cycle due to the insufficient operation of the TCA cycle.

Hence, the accumulation of acetic acid in the fermentation broth was higher at low oxygen transfer conditions. Acetic acid concentration was the highest at $LOT_1$ condition at the $12^{th}$ hour of the bioprocess as 2.5 kg $m^{-3}$ (Figure 4.10). This situation is probably the reason of low cell formation and low enzyme production at low oxygen transfer conditions, due to the inhibition effect of acetic acid, as reported in the literature.

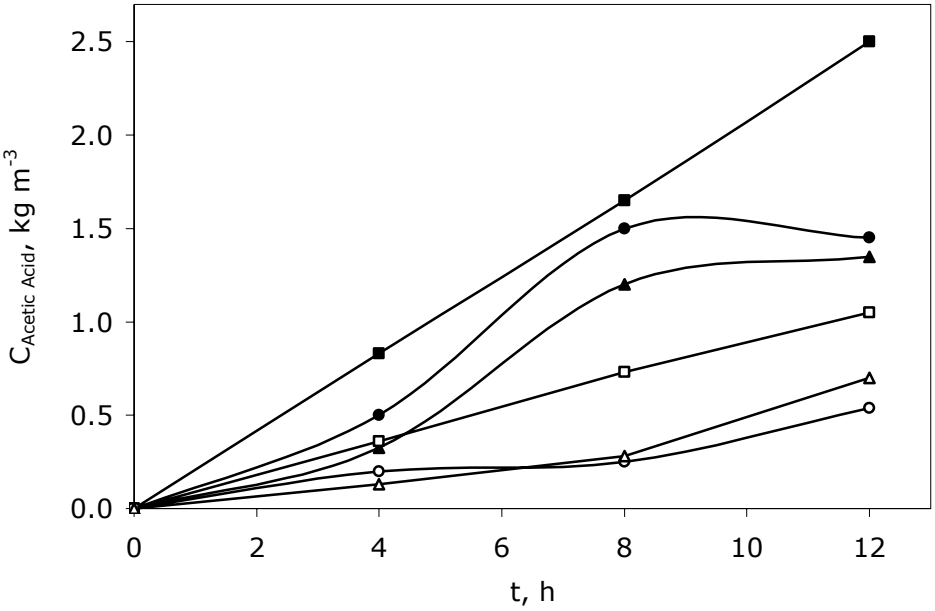



**Figure 4.10** The variations in acetic acid concentration with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

4.4.5 Mass Transfer Characteristics:

Oxygen Uptake Rate and Oxygen Transfer Coefficient

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), $r_0$, and oxygen transfer coefficient, $K_La$. At t<0 h, the physical oxygen transfer coefficient $K_La_o$ was measured in the medium in the absence of the microorganism. The variations in $K_La$, oxygen uptake rate, oxygen transfer rate and the enhancement factor E (=$K_La/K_La_o$) throughout the bioprocesses are given in Table 4.4.

**Table 4.4** The variations in oxygen transfer parameters.

| | t (h) | $k_La$ ($s^{-1}$) | E $k_La/k_La_0$ | $OTR^x10^3$ ($molm^{-3}s^{-1}$) | $OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$) | $OUR^x10^3$ ($molm^{-3}s^{-1}$) | $OD^x10^3$ ($molm^{-3}s^{-1}$) | Da $OD/OTR_{mx}$ | η OUR/OD |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 0.008 | 1.20 | 1.66 | 1.68 | 1.66 | 3.36 | 2.00 | 0.49 |
| | 3 | 0.008 | 1.20 | 1.66 | 1.68 | 1.66 | 9.77 | 5.82 | 0.17 |
| $LOT_1$ | 6 | 0.008 | 1.20 | 1.66 | 1.68 | 1.66 | 21.92 | 13.05 | 0.07 |
| | 8 | 0.008 | 1.20 | 1.64 | 1.68 | 1.64 | 47.67 | 28.37 | 0.03 |
| | 11 | 0.008 | 1.20 | 1.67 | 1.68 | 1.67 | 296.54 | 176.51 | 0.01 |
| | 1 | 0.009 | 1.20 | 1.56 | 1.89 | 1.56 | 2.25 | 1.19 | 0.69 |
| | 3 | 0.011 | 1.47 | 2.20 | 2.31 | 2.20 | 10.55 | 4.57 | 0.21 |
| $LOT_2$ | 6 | 0.012 | 1.60 | 2.20 | 2.52 | 2.20 | 31.53 | 12.51 | 0.07 |
| | 8 | 0.008 | 1.07 | 1.70 | 1.68 | 1.70 | 62.29 | 37.08 | 0.03 |
| | 11 | 0.007 | 1.00 | 1.00 | 1.05 | 1.00 | 38.95 | 37.09 | 0.02 |
| | 1 | 0.008 | 1.06 | 0.82 | 1.78 | 1.20 | 1.33 | 0.74 | 0.90 |
| | 3 | 0.013 | 1.62 | 2.72 | 2.73 | 2.72 | 10.24 | 3.75 | 0.27 |
| $MOT_1$ | 6 | 0.016 | 2.00 | 3.33 | 3.36 | 3.33 | 32.04 | 9.53 | 0.10 |
| | 8 | 0.018 | 2.25 | 3.55 | 3.78 | 3.55 | 55.16 | 14.59 | 0.06 |
| | 11 | 0.019 | 2.37 | 3.99 | 3.99 | 0.60 | 16.56 | 4.15 | 0.04 |
| | 1 | 0.026 | 1.13 | 1.31 | 5.46 | 1.30 | 1.33 | 0.24 | 0.98 |
| | 3 | 0.023 | 1.00 | 2.41 | 4.83 | 1.80 | 7.18 | 1.49 | 0.25 |
| $MOT_2$ | 6 | 0.029 | 1.26 | 3.02 | 6.09 | 2.00 | 20.95 | 3.44 | 0.09 |
| | 8 | 0.032 | 1.39 | 2.75 | 6.72 | 2.00 | 33.51 | 4.99 | 0.06 |
| | 11 | 0.027 | 1.17 | 1.97 | 5.67 | 1.60 | 59.76 | 10.54 | 0.03 |
| | 1 | 0.030 | 2.00 | 0.78 | 6.30 | 1.20 | 1.19 | 0.19 | 1.00 |
| | 3 | 0.037 | 2.47 | 1.80 | 7.77 | 2.20 | 9.09 | 1.17 | 0.24 |
| $HOT_1$ | 6 | 0.042 | 2.80 | 1.42 | 8.82 | 2.40 | 32.59 | 3.69 | 0.07 |
| | 8 | 0.043 | 2.87 | 1.92 | 9.03 | 1.70 | 57.54 | 6.37 | 0.03 |
| | 11 | 0.046 | 3.07 | 1.93 | 9.66 | 1.60 | 64.59 | 6.69 | 0.02 |
| | 1 | 0.046 | 2.30 | 0.74 | 9.66 | 1.20 | 1.46 | 0.15 | 0.82 |
| | 3 | 0.046 | 2.30 | 1.69 | 9.66 | 2.00 | 8.23 | 0.85 | 0.24 |
| $HOT_2$ | 6 | 0.044 | 2.20 | 1.76 | 9.24 | 2.00 | 22.92 | 2.48 | 0.09 |
| | 8 | 0.043 | 2.15 | 1.23 | 9.03 | 1.80 | 37.93 | 4.20 | 0.05 |
| | 11 | 0.042 | 2.10 | 0.78 | 8.82 | 1.80 | 204.76 | 23.22 | 0.01 |

The oxygen transfer rate ($K_La(C_{DO}^*-C_{DO})$) is proportional to the difference between the equilibrium concentration and the dynamic dissolved oxygen concentration in the medium, and it is increased to an extent depending on the oxygen uptake rate by increasing the concerted effect of the parameters air inlet rate ($Q_O/V_R$) and agitation rate (N). In the bioprocesses, oxygen uptake rate and oxygen transfer rate values increase with the increase in the oxygen transfer, i.e. $Q_O/V_R$ and N, up to $MOT_1$ condition. However, at t> 6 h at $MOT_2$, $HOT_1$ and $HOT_2$ conditions, the cell growth and consequently the oxygen uptake rate and oxygen transfer rate values decreased -in spite of the higher oxygen transfer- as a result of the inhibition of oxygen on metabolism. Similar trend was reported with *Bacillus licheniformis* for serine alkaline protease production (Çalık et al., 2000).

Oxygen transfer characteristics of the bioprocess; oxygen uptake rate, OUR, and liquid side overall oxygen transfer coefficient, $K_La$, that are interrelated with the applied oxygen transfer conditions, were determined at the cultivation times corresponding to the characteristic regions of the batch bioprocess (Table 4.4). $K_La_o$ values increased with the increase in the oxygen transfer condition, as expected. In general, $K_La$ and consequently the enhancement factor E ($=K_La/K_La_o$) have tendency to increase with the cultivation time. $K_La$ depends on agitation rate, temperature, rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. Temperature and agitation rate were kept constant throughout the bioprocesses, therefore the reason for the observed decrease at some conditions in $K_La$, could be the increase in viscosity of the medium due to secreted metabolites. Furthermore, particles with a diameter somewhat greater than the thickness of the mass transfer layer enhance the gas absorption, but the enhancement decreases with increasing particle diameter; therefore, along with the increase in the concentration of the rod-shaped biocatalyst, the mass transfer coefficient may increase or decrease depending on the diameter and concentration of the biomass (Çalık et al., 1998). E values varied between 1.00 and 3.07.

When mass transfer is accompanied by a chemical reaction, it can be enhanced several-folds depending on the relative rates of mass transfer and chemical reaction and presence of particles in the mass transfer zone. The low E values obtained, shows that slow reaction is accompanied with mass transfer, as

expected in most of the fermentation processes accomplished in stirred bioreactors. The oxygen uptake rate first increased, and then decreased with the cultivation time (Table 4.4); giving a maximum between t=3-6 h depending on the applied oxygen transfer condition. Its highest value was observed at $MOT_1$ condition at t=8 h as $3.55*10^{-3}$ mol $m^{-3}$ $s^{-1}$.

### 4.4.6 Relative Rates of Mass Transfer and Biochemical Reaction

In order to find the relative effects of mass transfer and biochemical reaction on the benzaldehyde lyase production process by recombinant *E.coli*, the maximum possible oxygen utilization rate ($OD=\mu_{max}C_X/Y_{X/O}$) and the maximum possible mass transfer rate ($OTR_{max}=K_La C_{DO}^*$) were also calculated throughout the bioprocesses. A kind of Damköhler number, Da, defined as maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2000, 2003); and effectiveness factor, η, defined as the oxygen uptake rate per maximum possible oxygen utilization rate values (Çalık et al., 2000, 2003) can be seen in Table 4.4.

It is clear in Table 4.4 that, at $LOT_1$ and $LOT_2$ conditions, throughout the bioprocesses, mass-transfer resistances were effective ($Da>>1$). At $MOT_1$, with the increase in cultivation time due to the increase in cell concentration and high oxygen consumption, at t>1 h mass-transfer resistances increased. At t=1 h of $MOT_1$ condition; and at $MOT_2$, $HOT_1$ and $HOT_2$ conditions at t=1-3 h, μ values were close to $\mu_{max}$; therefore, biochemical reaction resistances were effective; however, at higher cultivation times as μ values were much lower than $\mu_{max}$ values, these resistances were not effective.

The effectiveness factor (η) was close to 1.0 at $MOT_1$, $MOT_2$, $HOT_1$ and $HOT_2$ conditions at t=0-1 h indicating that the cells are consuming oxygen with such a high rate that maximum possible oxygen utilization (OD) value was approached; thereafter the decrease in η indicates that the cells are consuming lower oxygen than the OD. On the other hand, at $LOT_1$ and $LOT_2$ conditions, starting from the beginning of the processes, cells consumed oxygen lower than the oxygen demand (OD) due to the low oxygen transfer rate.

### 4.4.7 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in specific growth rate, μ, specific oxygen uptake rate, $q_o$, specific substrate utilization rate, $q_s$, yield and maintenance coefficients are given in Table 4.5. As it is seen in the table, specific growth rates decreased with the cultivation time at all the oxygen transfer conditions, as expected; but the decrease rate was higher at high oxygen transfer conditions. The highest specific growth rate (μ) was obtained as $\mu_{max}$=1.19 $h^{-1}$ at t=1 h at $HOT_1$ condition. At t>6 h the highest cell formation rate and μ was obtained at $MOT_1$; consequently at the end of the bioprocesses, the highest $C_X$ value was obtained at $MOT_1$ condition (Fig. 4.6).

Specific oxygen uptake rates ($q_O$) and specific substrate utilization rates ($q_S$) decreased with the cultivation time at all the oxygen transfer conditions. The highest value of $q_O$ was obtained at t=1 h of $LOT_1$ condition as 0.73 kg $kg^{-1}$ $h^{-1}$; where the lowest value was obtained at t=11 h of $MOT_1$ condition as 0.03 kg $kg^{-1}$ $h^{-1}$. The highest value of $q_S$ was obtained at t=1 h of $LOT_2$ condition as 4.42 kg $kg^{-1}$ $h^{-1}$; where the lowest value was obtained at t=11 h of the same condition as 0.09 kg $kg^{-1}$ $h^{-1}$. The variations in specific growth rate (μ), specific oxygen uptake rate ($q_o$) and specific substrate utilization rates ($q_s$) with the cultivation time at the oxygen transfer conditions applied are also given in Figures 4.11, 4.12 and 4.13, respectively. μ, $q_o$ and $q_s$ values decreased with the cultivation time at the oxygen transfer conditions applied. The decrease rate of specific growth rate (μ) was higher at high oxygen transfer conditions; whereas, the decrease rate of $q_o$ and $q_s$ was higher at low oxygen transfer conditions.

**Table 4.5** The variations in specific growth rate and yield coefficients.

| | t (h) | μ ($h^{-1}$) | $q_o$ (kg $kg^{-1}$ $h^{-1}$) | $q_s$ (kg $kg^{-1}$ $h^{-1}$) | $Y_{x/s}$ (kg $kg^{-1}$) | $Y_{x/o}$ (kg $kg^{-1}$) | $Y_{s/o}$ (kg $kg^{-1}$) | $m_o$ (kg$kg^{-1}h^{-1}$) | $m_s$ (kg$kg^{-1}h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| $LOT_1$ | 1 | 0.59 | 0.73 | 3.85 | 0.15 | 0.80 | 5.24 | 0.09 | 0.13 |
| | 3 | 0.20 | 0.36 | 1.39 | 0.14 | 0.56 | 3.88 | | |
| | 6 | 0.09 | 0.32 | 0.73 | 0.12 | 0.28 | 2.23 | | |
| | 8 | 0.04 | 0.17 | 0.42 | 0.09 | 0.25 | 2.54 | | |
| | 11 | 0.01 | 0.11 | 0.17 | 0.04 | 0.06 | 1.45 | | |
| | Overall | | | | 0.11 | | | | |
| $LOT_2$ | 1 | 0.83 | 0.68 | 4.42 | 0.19 | 1.22 | 6.48 | 0.08 | 0.12 |
| | 3 | 0.25 | 0.37 | 1.41 | 0.18 | 0.66 | 3.78 | | |
| | 6 | 0.08 | 0.23 | 0.59 | 0.14 | 0.36 | 2.53 | | |
| | 8 | 0.03 | 0.16 | 0.35 | 0.09 | 0.20 | 2.19 | | |
| | 11 | 0.03 | 0.09 | 0.09 | 0.32 | 0.32 | 0.99 | | |
| | Overall | | | | 0.13 | | | | |
| $MOT_1$ | 1 | 1.08 | 0.51 | 4.29 | 0.25 | 2.11 | 8.39 | 0.18 | 0.11 |
| | 3 | 0.32 | 0.39 | 1.22 | 0.26 | 0.80 | 3.10 | | |
| | 6 | 0.12 | 0.25 | 0.44 | 0.28 | 0.50 | 1.79 | | |
| | 8 | 0.08 | 0.20 | 0.25 | 0.31 | 0.38 | 1.23 | | |
| | 11 | 0.04 | 0.03 | 0.09 | 0.43 | 1.41 | 3.25 | | |
| | Overall | | | | 0.26 | | | | |
| $MOT_2$ | 1 | 1.16 | 0.61 | 3.62 | 0.32 | 1.91 | 5.95 | 0.04 | 0.53 |
| | 3 | 0.29 | 0.26 | 0.96 | 0.31 | 1.16 | 3.74 | | |
| | 6 | 0.11 | 0.15 | 0.39 | 0.29 | 0.76 | 2.64 | | |
| | 8 | 0.07 | 0.13 | 0.27 | 0.26 | 0.56 | 2.15 | | |
| | 11 | 0.03 | 0.09 | 0.17 | 0.19 | 0.34 | 1.78 | | |
| | Overall | | | | 0.25 | | | | |
| $HOT_1$ | 1 | 1.19 | 0.48 | 3.14 | 0.38 | 2.49 | 6.55 | 0.08 | 0.18 |
| | 3 | 0.29 | 0.27 | 0.87 | 0.33 | 1.05 | 3.15 | | |
| | 6 | 0.09 | 0.17 | 0.39 | 0.23 | 0.52 | 2.31 | | |
| | 8 | 0.03 | 0.10 | 0.28 | 0.12 | 0.34 | 2.72 | | |
| | 11 | 0.03 | 0.10 | 0.21 | 0.14 | 0.29 | 2.03 | | |
| | Overall | | | | 0.19 | | | | |
| $HOT_2$ | 1 | 0.98 | 0.54 | 2.90 | 0.34 | 1.80 | 5.36 | 0.06 | 0.10 |
| | 3 | 0.29 | 0.33 | 0.91 | 0.32 | 0.88 | 2.78 | | |
| | 6 | 0.10 | 0.18 | 0.38 | 0.27 | 0.58 | 2.13 | | |
| | 8 | 0.06 | 0.13 | 0.25 | 0.22 | 0.42 | 1.88 | | |
| | 11 | 0.01 | 0.13 | 0.15 | 0.07 | 0.08 | 1.16 | | |
| | Overall | | | | 0.22 | | | | |

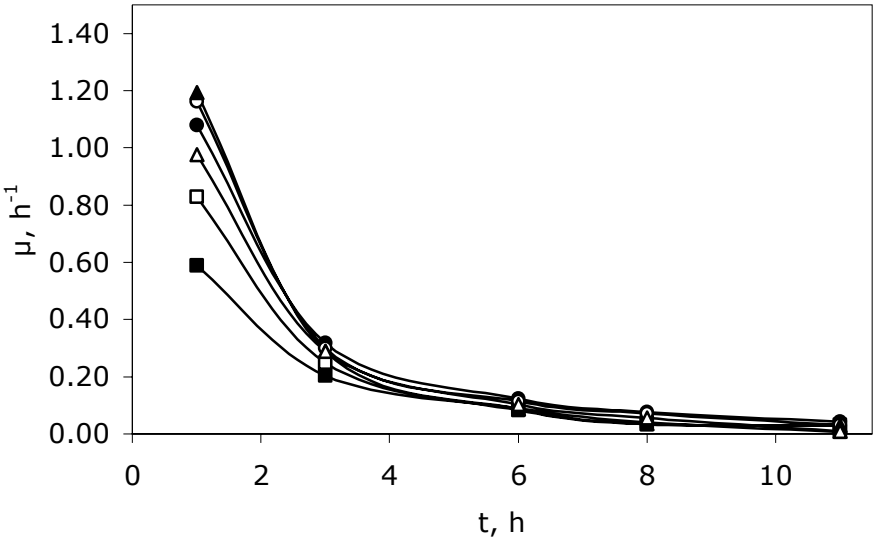



**Figure 4.11** The variations in specific growth rate with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

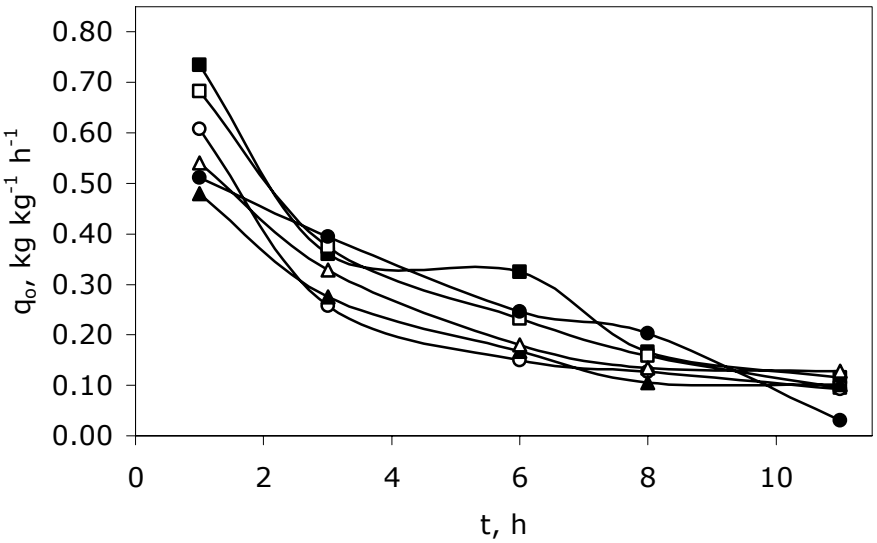



**Figure 4.12** The variations in specific oxygen uptake rate with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

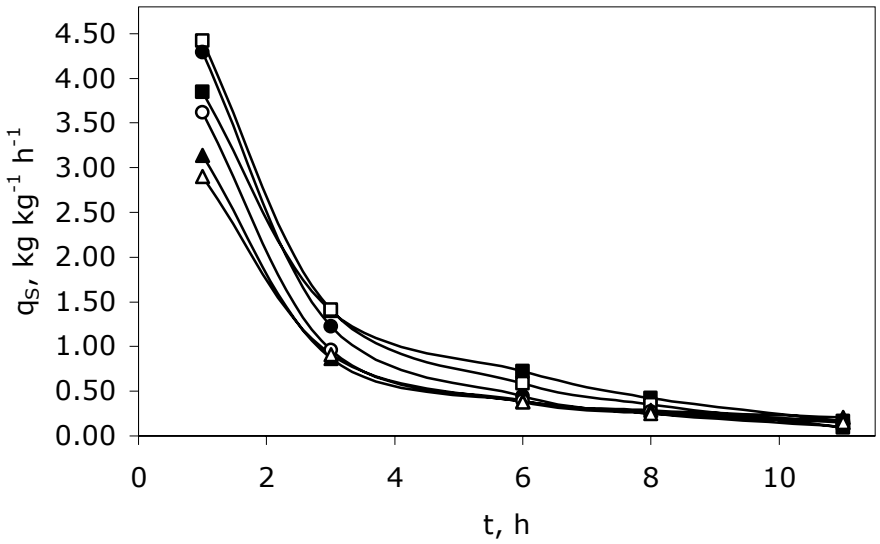



**Figure 4.13** The variations in specific substrate utilization rate with the cultivation time, agitation and air inlet rates. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$: $LOT_1$ (■); $LOT_2$ (□); $MOT_1$ (●); $MOT_2$ (○); $HOT_1$ (▲); $HOT_2$ (Δ).

The yield coefficients are given in Table 4.5. Overall specific cell yield on substrate ($Y_{X/S}$) increased with the increase in oxygen transfer rate up to $MOT_1$ condition and it reached its highest value at $MOT_1$ as 0.26, due to the increased efficiency of the utilization of the carbon source for product formation; while the lowest value was 0.11 obtained at $LOT_1$ condition.

Cell yield on oxygen values decreased with the cultivation at all the oxygen transfer conditions applied; among the highest $Y_{X/O}$ value was obtained at $HOT_1$ condition at t=1 h as 2.49 kg $kg^{-1}$. The lowest $Y_{X/O}$ value was obtained at $LOT_1$ at t=11 h as 0.06 kg $kg^{-1}$ indicating the inefficient use of the oxygen through biochemical reaction network, probably because of the oxygen limitation.

Similarly, the amount of substrate metabolized per amount of oxygen used ($Y_{S/O}$) decreased with the cultivation time under all the oxygen transfer conditions signaling either the increased energy requirement or decrease in the efficiency of energy metabolism with the cultivation time. The highest $Y_{S/O}$ was

obtained as 8.39 kg $kg^{-1}$ at $MOT_1$ condition at t= 1 h; while the lowest $Y_{S/O}$ was obtained as 0.99 kg $kg^{-1}$ at $LOT_2$ condition at t= 11 h (Table 4.5).

There is no work in the literature on benzaldehyde lyase production reporting the yield coefficients. But for most bacteria growing aerobically on glucose, $Y_{X/S}$ is typically 0.4 to 0.6 kg $kg^{-1}$, while $Y_{X/0}$ is 0.9 to 1.4 kg $kg^{-1}$ (Shuler and Kargı, 2002). The decrease in $Y_{X/O}$ values at low oxygen transfer conditions is due to lack of oxygen. $Y_{X/S}$ values were very low, indicating the inefficient use of the carbon source through biochemical reaction network. The relatively high $Y_{S/O}$ values indicate that the carbon source is not efficiently utilized in the energy metabolism, and the decrease in $Y_{S/O}$ values with the cultivation time, shows the decrease in the efficiency of energy metabolism.

From the slope of the plot of $1/Y_{X/O}$ versus $1/\mu$, the rate of oxygen consumption for maintenance was obtained, and the variation of $m_O$ with the oxygen transfer conditions applied is given in Table 4.5. The $m_O$ value varied between 0.04-0.18 kg $kg^{-1}$ $h^{-1}$, and the highest value was obtained at $MOT_1$ condition. And similarly from the slope of the plot of $1/Y_{S/O}$ versus $1/\mu$, the rate of substrate consumption for maintenance ($m_S$) values were obtained for each oxygen transfer condition. The $m_S$ values varied between 0.10-0.53 kg $kg^{-1}$ $h^{-1}$, and the lowest value was obtained at $HOT_2$ condition. The calculation procedure of maintenance coefficients were explained in section 2.3.1.2; the Leudeking-Piret constants calculated for the bioprocesses are given in Table 4.6.

**Table 4.6** The variations in Leudeking-Piret constants.

| | $\alpha$ | $\beta$ |
|---|---|---|
| $LOT_1$ | 3.062 | 0.269 |
| $LOT_2$ | 0.539 | 0.214 |
| $MOT_1$ | 0.789 | 0.012 |
| $MOT_2$ | 0.446 | 0.269 |
| $HOT_1$ | 1.994 | 0.034 |
| $HOT_2$ | 0.881 | 0.327 |

Among the investigated oxygen transfer conditions, the highest cell concentration and benzaldehyde lyase activity was observed at $MOT_1$ condition. These values were approximately 1.2 and 1.9 fold higher than the values obtained in the designed medium and in the reference production medium, respectively. So it was concluded that the operation of the bioreactor for benzaldehyde lyase production cannot be achieved successfully at low oxygen transfer conditions but at higher rates; and the increase in the oxygen transfer rate should be in harmony with the oxygen uptake rate of the cells, without lifting the oxygen stress on the microorganism.

## 4.5 pH Effects

The effect of pH, which is another important parameter of the bioprocesses, was investigated for benzaldehyde lyase production process at $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$ condition, which was found as optimum. For this investigation, six different controlled-pH conditions were applied; which were $pH_C$= 5.0, 6.4, 6.7, 7.0, 7.2 and 7.8. The pH of the fermentation medium was kept constant at given values throughout the bioprocesses, and; dissolved oxygen concentrations, cell concentrations, benzaldehyde lyase volumetric activities, glucose concentrations, organic acid concentrations, yield values, maintenance coefficients, overall mass transfer coefficients, specific growth rates and oxygen uptake rates were determined throughout the bioprocesses.

Further, these results were compared with the uncontrolled $pH_{UC}$=7.2 operation; which was also conducted at $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$ condition; and abbreviated as $MOT_1$ at the previous part of the study.

The investigation of the effects of pH on benzaldehyde lyase production was performed by batch-bioreactor experiments, in the bioreactor system explained in section 3.6.

### 4.5.1 Dissolved Oxygen Profiles

The variations in the dissolved oxygen concentration with the cultivation time and pH are shown in Figure 4.14. As it is seen from the figure, at the first 3 h of the bioprocesses, the pH condition applied did not affect the dissolved

oxygen profiles considerably; only at the highest controlled-pH operation ($pH_C$=7.8), dissolved oxygen concentration in the medium was slightly higher than that of $pH_C$= 6.4-7.2 conditions. However, at the lowest controlled-pH operation ($pH_C$=5.0), until the sixth hour of the bioprocess; the cell concentration, and the oxygen uptake rate of these cells were so low that the observed decrease in the dissolved oxygen profiles at other pH conditions, can not be observed. Considering this delay, at all the pH conditions due to the high oxygen demand of the cells at the beginning of the bioprocesses, a decrease was observed in the dissolved oxygen profiles; after reaching a minimum around 0-15% of the saturation, profiles gradually increased until the end of the bioprocesses.

At t=0-3 h, the dissolved oxygen profiles of $pH_{UC}$=7.2 and $pH_C$=7.2 operations did not differ from each other; however, at t=3 h, the transferred oxygen was totally consumed at $pH_{UC}$=7.2. At t>8h at $pH_{UC}$=7.2 operation, $C_{DO}$ increased with a higher rate as the cells reached the stationary phase; whereas at $pH_C$=7.2 operation, the increase in $C_{DO}$ was lower, as the cell concentration continue to increase slightly.

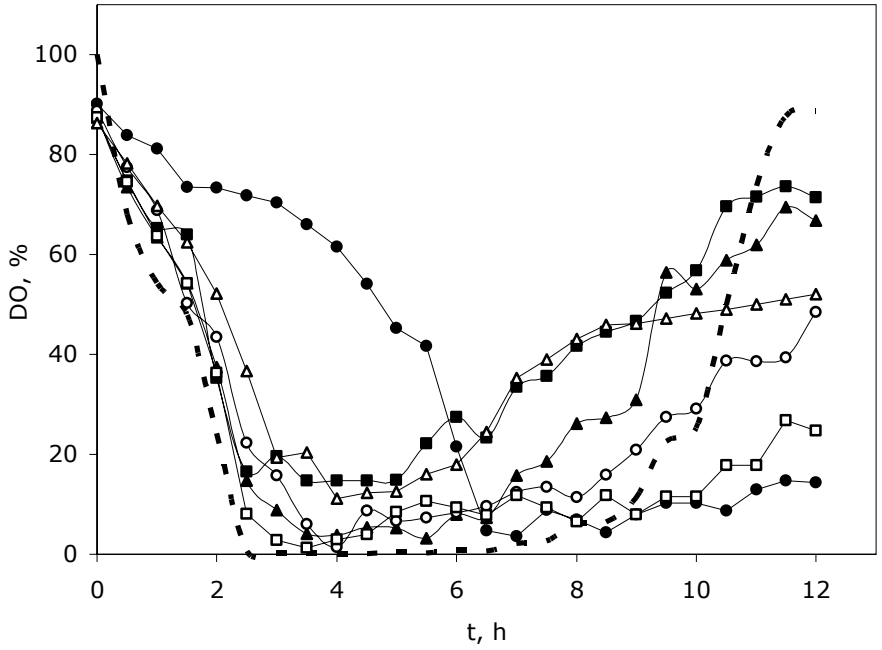



**Figure 4.14** The variations in dissolved oxygen concentration with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ); $pH_{UC}$=7.2, (-). $C_{DO}^*$=0.21 mol $m^{-3}$.

4.5.2 Glucose and Cell Concentration Profiles

The variations of glucose and cell concentrations with the cultivation time and pH are given in Figs. 4.15 and 4.16, respectively. Glucose concentration, at an initial concentration of $C_G^0$=8 kg $m^{-3}$, decreased until the 12$^{th}$ h of the bioprocesses. At $pH_C$=5.0 condition, the cell formation rate, and consequently the oxygen and glucose consumption rates were low; on the other hand, the decrease in glucose concentration was not considerably affected from the other pH conditions applied. Comparing the controlled- and uncontrolled- pH=7.2 operations; it can be observed that at the first four hours of the bioprocesses, although they have similar cell formation rates, the glucose consumption rate was higher at $pH_C$=7.2 operation. Between t=4-8 h, glucose consumption rate was higher at $pH_{UC}$=7.2 condition; where, after t=8 h, the profiles were similar. Probably, glucose was consumed at a higher rate at the beginning of the $pH_C$=7.2 operation, because of the cells' attempt to decrease the external pH to increase to proton electrochemical gradient ($\Delta$pH=0.4) for energy generation. During the first four hours, the pH of the medium decreased to pH=6.9 value at $pH_{UC}$=7.2 condition; causing the gradient to be larger ($\Delta$pH=0.7) that in turn caused lower glucose consumption rate.

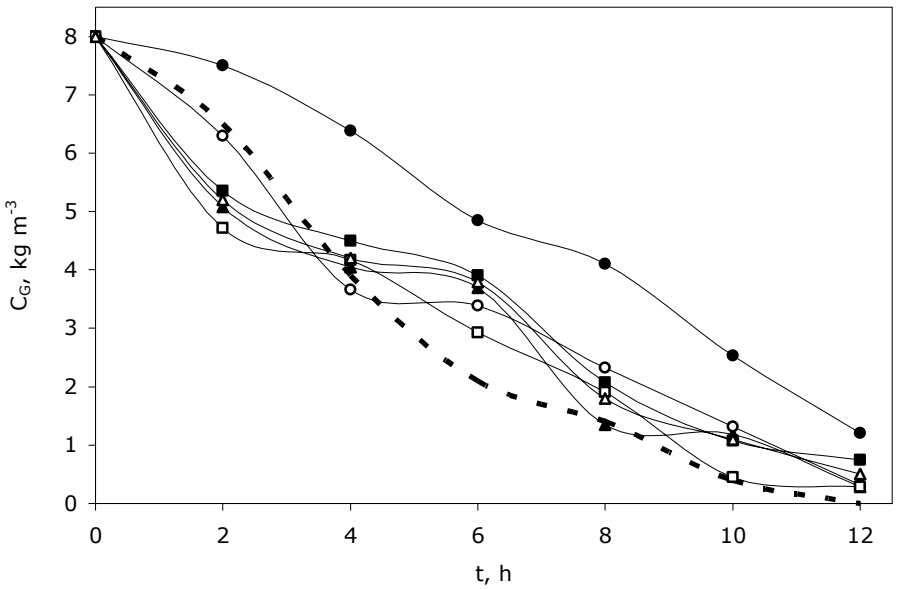



**Figure 4.15** The variations in glucose concentration with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ); $pH_{UC}$=7.2, (-).

At t=0-2 h, cell formation did not change considerably with the pH condition applied. However, after the second hour of the bioprocesses, cell formation rates were higher at $pH_C$=6.4, 6.7, 7.0 and 7.2 conditions, not being affected by the pH condition much. At the end of the bioprocesses, the highest cell concentration value was attained at $pH_C$=7.0 condition as 2.05 kg $m^{-3}$, while the lowest cell concentration value was obtained at $pH_C$=5.0 condition ($C_X$=1.41 kg $m^{-3}$) which was 1.45-fold lower than the highest cell concentration obtained among the controlled-pH operations. Cell formation was not dependent on whether the pH was controlled or uncontrolled at pH=7.2 value at t=0-6 h, but towards the end of the fermentations, the cell formation rate was higher at $pH_{UC}$=7.2 operation; giving a cell concentration of 2.29 kg $m^{-3}$ at the end of the process. The reason of lower cell concentration at $pH_C$=7.2 operation should be related with the higher acetic acid concentration that caused shifts in the intracellular bioreaction network.

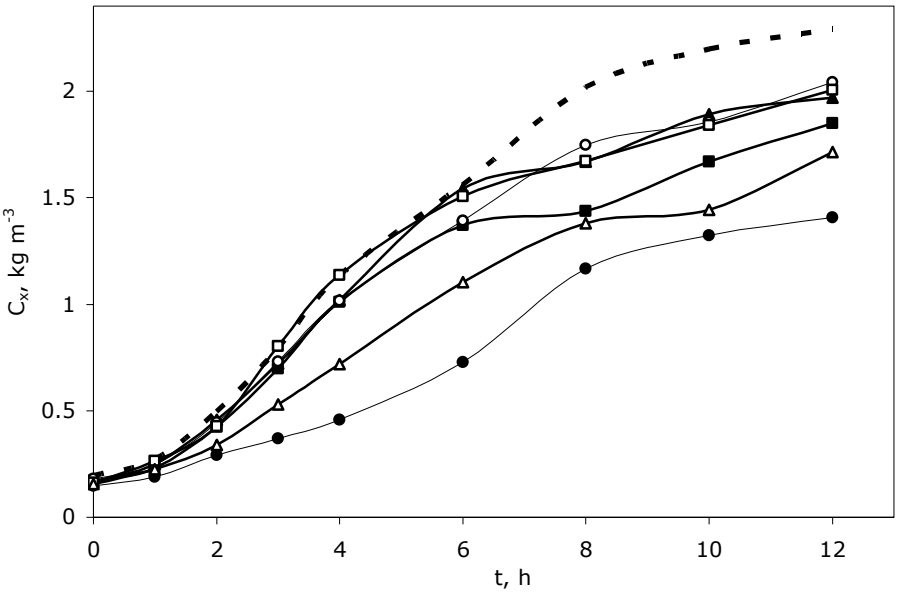



**Figure 4.16** The variations in the cell concentration with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ); $pH_{UC}$=7.2, (-).

4.5.3 Benzaldehyde Lyase Activity Profiles

The variations of benzaldehyde lyase volumetric activity (A) with the cultivation time at the pH conditions applied are shown in Figure 4.17. The highest benzaldehyde lyase volumetric activity was obtained at $pH_C$=7.0 condition, as 775 U $cm^{-3}$, where the highest cell concentration was obtained. On the other hand, at the end of the bioprocesses the lowest benzaldehyde lyase volumetric activity was obtained at $pH_C$=5.0 condition, as 380 U $cm^{-3}$, where the least amount of cell was generated. This result in turn indicates that, benzaldehyde lyase production could be enhanced by increasing the concentration of benzaldehyde lyase producing cells, probably either by making use of complex production medium, or developing a substrate feeding strategy; or a combination of both. The uncontrolled pH=7.2 operation gave higher activities of the enzyme; this result is interesting especially at t=0-6 h, where the cell concentration to produce enzyme was approximately the same at controlled- and uncontrolled- operations. This was observed, probably, because the fluxes were shifted towards acetic acid production at $pH_C$=7.2 operation.

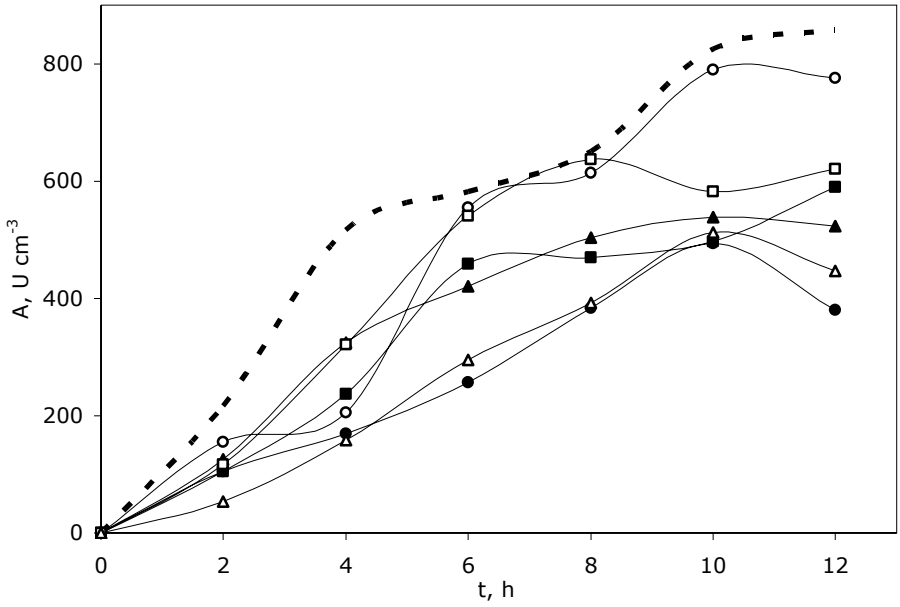



**Figure 4.17** The variations in BAL activity with the cultivation time and pH. $C_G^o$=8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ); $pH_{UC}$=7.2, (-).

When the variation of cell concentration and benzaldehyde lyase activity with the pH conditions is considered (Figure 4.18), it can be concluded that the optimum pH value for benzaldehyde lyase production is $pH_C$=7.0; as the highest amount of cell and enzyme was obtained at this condition, among the investigated controlled-pH operations. However, the values obtained at $pH_C$=7.0 condition ($C_X$=2.043 kg $m^{-3}$; A=775 U $cm^{-3}$), were lower than the values obtained at $pH_{UC}$=7.2 uncontrolled-pH operation ($C_X$=2.293 kg $m^{-3}$; A=857 U $cm^{-3}$); hence, uncontrolled-pH operation can be concluded to be favorable for benzaldehyde lyase production.

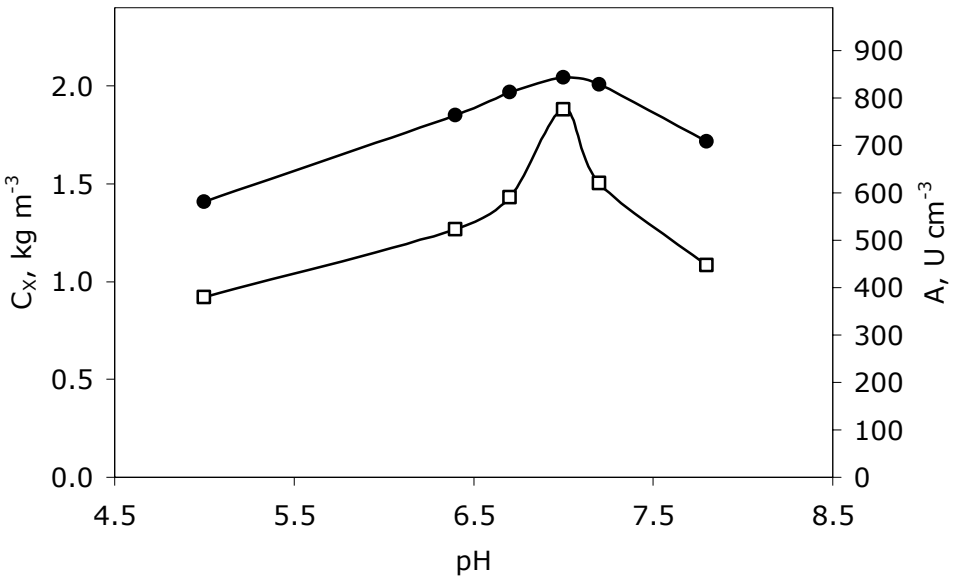



**Figure 4.18** The variations in cell concentration and benzaldehyde lyase volumetric activity with pH. t=12 h, $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$. Cell concentration: (●); BAL activity: (□).

4.5.4 Organic Acid Concentration Profiles

The organic acids detected in the fermentation broth and the variations of their concentrations with the cultivation time at the pH conditions applied are respectively given in Table 4.7 and Figure 4.19. As it is seen from the Table 4.7, various organic acids were detected in the fermentation broth at lower pH

conditions, i.e., $pH_C$=5.0, 6.4, 6.7; however, their total concentrations were lower than that of $pH_C$=7.2 and 7.8 conditions (Figure 4.19). At $pH_C$=7.2 and 7.8, the glucose consumption profiles did not differ from each other significantly, however, while the higher cell concentration was obtained at $pH_C$=7.2; higher total organic acid concentration was detected at $pH_C$=7.8. This results indicates that the *E.coli* cells utilized glucose to decrease extracellular pH by producing organic acids in order to increase the energy generation potential, via proton electrochemical gradient. The proton electrochemical gradient is related to the proton concentrations in the bulk extracellular and intracellular aqueous phases and the membrane potential (adjusted to compensate for changes in the extracellular-intracellular pH difference) produced by *E.coli* whose intracellular aqueous (cytoplasmic) pH is 7.6.

**Table 4.7** Organic acids detected in the fermentation broth with the cultivation time at the pH conditions applied.

| t,h | pH=5.0 | pH=6.4 | pH=6.7 | pH=7.0 | pH=7.2 | pH=7.8 |
|---|---|---|---|---|---|---|
| 4 | Pruvic<br>Acetic<br>Gluconic | Acetic | Acetic | Acetic | Acetic | Acetic |
| 8 | Acetic | Pruvic<br>Acetic<br>Butyric<br>Gluconic | Succinic<br>Acetic<br>Butyric | Acetic | Acetic | Pruvic<br>Acetic |
| 12 | Acetic<br>Lactic | Pruvic<br>Acetic<br>Gluconic | Succinic<br>Acetic<br>Gluconic | Succinic<br>Acetic<br>Gluconic | Acetic | Pruvic<br>Acetic |

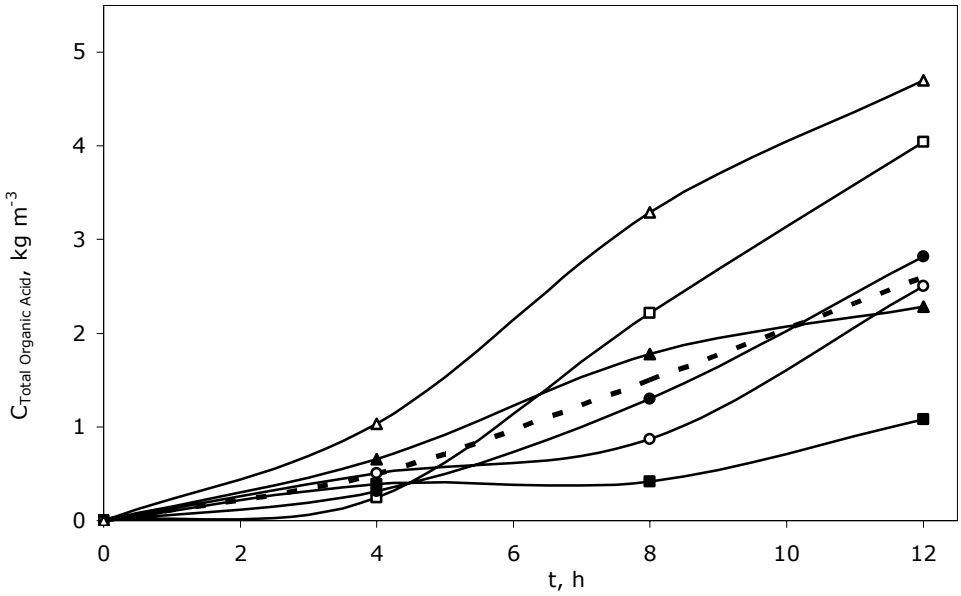



**Figure 4.19** The variations in total organic acid concentration with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ); $pH_{UC}$=7.2, (-).

Acetic acid again appears to be the major organic acid at this set of bioprocesses. The variation of the concentration of acetic acid with the cultivation time at the pH conditions applied is given in Figure 4.20. A similar trend at the profiles, and also, at the end of the bioprocesses, a close value at the concentration of acetic acid was observed at $pH_C$=5.0, 6.4 and 6.7 conditions. However, it should be noticed that, at $pH_C$=5.0 condition, the amount of cells producing acetic acid was approximately the half of the cells at $pH_C$=6.4 and 6.7 conditions. The accumulation of the metabolic by-product acetic acid was the highest at $pH_C$=7.2 and 7.8 conditions. The reason for the production of lower amount of cell at $pH_C$=7.8 condition is probably because of the extracellular pHs' being higher than intracellular pH; an unfavorable proton electrochemical gradient; and the inhibition effect of acetic acid which is highly accumulated in the fermentation broth. Furthermore, it is obvious that, decreasing the concentration of accumulating acetic acid in the medium by keeping the glucose concentration at low levels (Akesson et al., 2000), could be a way to enhance benzaldehyde lyase production at $pH_C$=7.2 condition.

The accumulation of acetic acid in the fermentation broth was higher at $pH_C$=7.2 condition when compared to $pH_{UC}$=7.2 condition; which explains the lower amount of cell generated at controlled-pH operation.

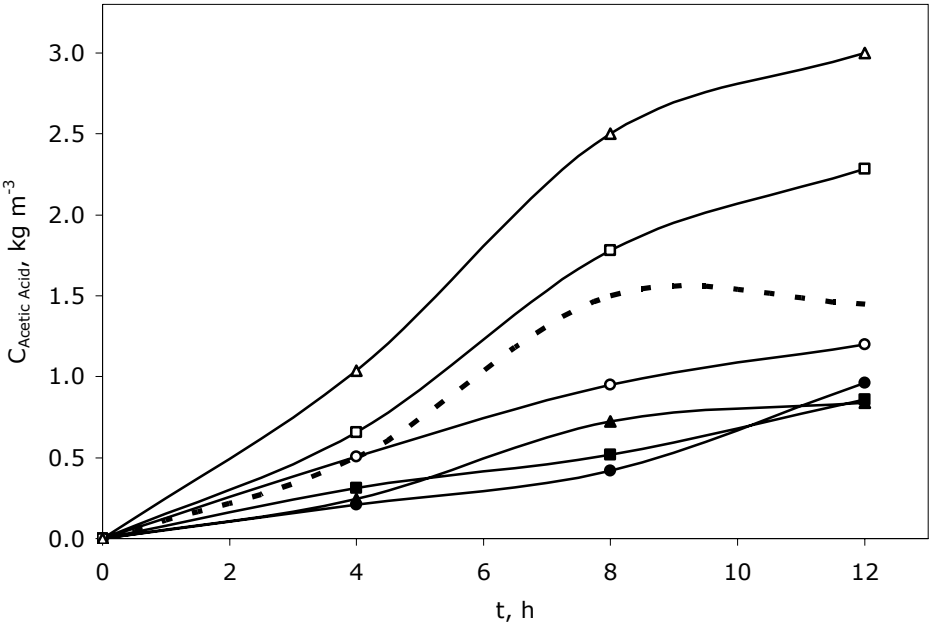



**Figure 4.20** The variations in acetic acid concentration with the cultivation time and pH. $C_G^o$ = 8.0 kg m$^{-3}$, T=37°C, $V_R$= 1.65x10$^{-3}$ m$^3$, $Q_o/V_R$=0.5 vvm, N=500 min$^{-1}$: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ); $pH_{UC}$=7.2, (-).

4.5.5 Oxygen Transfer Characteristics and Rate-Limiting-Step Analysis

The variations in $K_La$, oxygen uptake rate, oxygen transfer rate and the enhancement factor E (=$K_La/K_La_o$) throughout the bioprocesses are given in Table 4.8.

Liquid phase overall mass transfer coefficient, $K_La$, and E($K_La/K_La_0$), values varied between 0.010-0.033 and 1.00-3.30, respectively; having the highest values at $pH_C$=6.7 condition at t=6 h. In general, oxygen uptake rate increased at t=3-6 h that correspond to the high glucose consumption period and then decreased because of the decrease in metabolic activity of the cells.

Its highest value was observed at $pH_C$=6.7 condition at t=3 h as $3.88*10^{-3}$ mol $m^{-3}$ $s^{-1}$.

Comparing the controlled- and uncontrolled- pH=7.2 operations; it can be observed that $K_La$, and E values were 1.6-fold higher at $pH_{UC}$=7.2 operations, similarly, OUR was 1.5-fold higher at $pH_{UC}$=7.2.

In order to find the rate limiting step and the degree of the limitation, the maximum possible oxygen utilization rate (OD), the maximum possible mass transfer rate ($OTR_{max}$), Damköhler number (Da), and effectiveness factor (η), were also calculated and given in Table 4.8. At the first hours of the bioprocesses, biochemical reaction limitations were effective (Da<1); however, after the sixth hour of the bioprocesses, mass-transfer resistances became more effective (Da>>1). η was close to 1.0 at $pH_C$=6.4, 6.7, 7.0, 7.2 conditions at t=0-1 h indicating that the cells are consuming oxygen with such a high rate that maximum possible oxygen utilization (OD) value was approached; thereafter the decrease in η indicates that the cells are consuming lower oxygen than the OD. On the other hand, at $pH_C$=5.0 and 7.8 conditions, starting from the beginning of the processes, cells consumed oxygen lower than the oxygen demand (OD) due to the low cell growth rate.

**Table 4.8** The variations in oxygen transfer parameters with pH.

| | t (h) | $k_La$ ($s^{-1}$) | E $k_La/k_La_0$ | $OTR^x10^3$ ($molm^{-3}s^{-1}$) | $OTR_{max}{}^x10^3$ ($molm^{-3}s^{-1}$) | $OUR^x10^3$ ($molm^{-3}s^{-1}$) | $OD^x10^3$ ($molm^{-3}s^{-1}$) | Da $OD/OTR_{mx}$ | η OUR/OD |
|---|---|---|---|---|---|---|---|---|---|
| $pH_C$= 5.0 | 1 | 0.018 | 1.12 | 0.71 | 3.78 | 0.70 | 1.46 | 0.39 | 0.48 |
| | 3 | 0.016 | 1.00 | 0.99 | 3.36 | 1.00 | 3.86 | 1.15 | 0.26 |
| | 6 | 0.016 | 1.00 | 2.64 | 3.36 | 2.64 | 18.93 | 5.63 | 0.14 |
| | 8 | 0.016 | 1.00 | 3.13 | 3.36 | 3.13 | 34.56 | 10.28 | 0.09 |
| | 11 | 0.016 | 1.00 | 2.92 | 3.36 | 2.92 | 35.82 | 10.66 | 0.08 |
| $pH_C$= 6.4 | 1 | 0.014 | 1.40 | 1.08 | 2.94 | 1.10 | 1.23 | 0.42 | 0.89 |
| | 3 | 0.013 | 1.30 | 2.49 | 2.73 | 2.49 | 9.56 | 3.50 | 0.26 |
| | 6 | 0.012 | 1.20 | 2.32 | 2.52 | 2.32 | 26.35 | 10.45 | 0.09 |
| | 8 | 0.011 | 1.10 | 1.71 | 2.31 | 1.10 | 18.45 | 7.99 | 0.06 |
| | 11 | 0.010 | 1.00 | 0.08 | 2.10 | 1.60 | 65.80 | 31.33 | 0.02 |
| $pH_C$= 6.7 | 1 | 0.014 | 1.40 | 1.02 | 2.94 | 1.10 | 1.34 | 0.45 | 0.82 |
| | 3 | 0.023 | 2.30 | 3.88 | 4.83 | 3.88 | 16.72 | 3.46 | 0.23 |
| | 6 | 0.033 | 3.30 | 5.03 | 6.93 | 1.10 | 12.63 | 1.82 | 0.09 |
| | 8 | 0.019 | 1.90 | 2.33 | 3.99 | 1.50 | 23.66 | 5.93 | 0.06 |
| | 11 | 0.014 | 1.40 | 0.83 | 2.94 | 1.70 | 60.57 | 20.60 | 0.03 |
| $pH_C$= 7.0 | 1 | 0.012 | 1.20 | 0.78 | 2.52 | 1.00 | 1.18 | 0.47 | 0.84 |
| | 3 | 0.013 | 1.30 | 2.29 | 2.73 | 2.29 | 9.34 | 3.42 | 0.25 |
| | 6 | 0.015 | 1.50 | 2.89 | 3.15 | 2.89 | 28.97 | 9.19 | 0.09 |
| | 8 | 0.016 | 1.60 | 2.98 | 3.36 | 2.98 | 46.64 | 13.88 | 0.06 |
| | 11 | 0.018 | 1.80 | 2.32 | 3.78 | 1.30 | 35.82 | 9.47 | 0.04 |
| $pH_C$= 7.2 | 1 | 0.012 | 1.20 | 0.91 | 2.52 | 1.00 | 1.19 | 0.47 | 0.84 |
| | 3 | 0.010 | 1.00 | 2.04 | 2.10 | 1.60 | 6.87 | 3.27 | 0.23 |
| | 6 | 0.011 | 1.10 | 2.09 | 2.31 | 2.00 | 22.29 | 9.65 | 0.09 |
| | 8 | 0.012 | 1.20 | 2.35 | 2.52 | 2.30 | 38.62 | 15.32 | 0.06 |
| | 11 | 0.012 | 1.20 | 2.07 | 2.52 | 2.10 | 86.40 | 34.29 | 0.02 |
| $pH_C$= 7.8 | 1 | 0.015 | 1.50 | 0.95 | 3.15 | 0.90 | 1.37 | 0.43 | 0.66 |
| | 3 | 0.023 | 2.30 | 3.89 | 4.83 | 1.00 | 3.92 | 0.81 | 0.25 |
| | 6 | 0.018 | 1.80 | 3.10 | 3.78 | 0.90 | 8.74 | 2.31 | 0.10 |
| | 8 | 0.011 | 1.10 | 1.31 | 2.31 | 1.50 | 20.99 | 9.08 | 0.07 |
| | 11 | 0.010 | 1.00 | 1.05 | 2.10 | 1.05 | 21.31 | 10.15 | 0.05 |

### 4.5.6 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in specific growth rate, μ, specific oxygen uptake rate, $q_o$, specific substrate utilization rate, $q_s$, yield and maintenance coefficients are given in Table 4.9. As it is seen in the table, specific growth rate (μ) decreased with the cultivation time at all the pH conditions; the decrease rate was lowest at $pH_C$=5.0 and 7.8 conditions; it attained its highest value at $pH_C$=6.4 condition as 1.07 $h^{-1}$ at t=1 h of the bioprocess. Specific oxygen uptake rate ($q_O$) and specific substrate utilization rates ($q_S$) also decreased with the cultivation time at the pH conditions applied. The highest value of $q_O$ was obtained at t=1 h of $pH_C$=6.7 condition as 0.55 kg $kg^{-1}$ $h^{-1}$; where the lowest value was obtained as 0.08 kg $kg^{-1}$ $h^{-1}$ at t=8 h of $pH_C$=6.4 and t=11 h of $pH_C$=7.0 conditions. The highest value of $q_S$ was obtained at t=1 h of $pH_C$=7.8 condition as 3.84 kg $kg^{-1}$ $h^{-1}$; where the lowest value was obtained at t=11 h of the $pH_C$=7.2 condition as 0.14 kg $kg^{-1}$ $h^{-1}$; the decrease rate was lowest at $pH_C$=5.0 condition. The variations in specific growth rate (μ), specific oxygen uptake rate ($q_o$) and specific substrate utilization rate ($q_s$) with the cultivation time at the pH conditions applied are also given in Figures 4.21, 4.22 and 4.23, respectively.

The yield coefficients are also given in Table 4.8. Overall specific cell yield on substrate ($Y_{X/S}$) reached its highest value at $pH_C$=7.0 and 7.2 conditions as 0.23 kg $kg^{-1}$, due to the increased efficiency of the utilization of the carbon source for product formation; while the lowest value was 0.16 obtained at $pH_C$=5.0 condition. Cell yield on oxygen ($Y_{X/O}$) values decreased with the cultivation at all the pH conditions applied; among the highest $Y_{X/O}$ value was obtained at $pH_C$=7.2 condition at t=1 h as 2.30 kg $kg^{-1}$. The lowest $Y_{X/O}$ value was obtained at the same condition at t=11 h as 0.23 kg $kg^{-1}$ indicating the inefficient use of the oxygen through biochemical reaction network. Similarly, the amount of substrate metabolized per amount of oxygen used ($Y_{S/O}$) decreased with the cultivation time under all the pH conditions signaling either the increased energy requirement or decrease in the efficiency of energy metabolism with the cultivation time. The highest $Y_{S/O}$ was obtained as 8.35 kg $kg^{-1}$ at $pH_C$=7.8 condition at t=1 h; while the lowest $Y_{S/O}$ was obtained as 1.10 kg $kg^{-1}$ at $pH_C$=7.2 condition at t=11 h.

**Table 4.9** The variations in specific growth rate and yield coefficients with pH.

| | t (h) | μ ($h^{-1}$) | $q_o$ ($kgkg^{-1}h^{-1}$) | $q_s$ ($kgkg^{-1}h^{-1}$) | Yx/s ($kgkg^{-1}$) | Yx/o ($kgkg^{-1}$) | Ys/o ($kgkg^{-1}$) | $m_o$ ($kgkg^{-1}h^{-1}$) | $m_s$ ($kgkg^{-1}h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| $pH_C$ = 5.0 | 1 | 0.57 | 0.43 | 2.14 | 0.27 | 1.35 | 5.02 | 0.23 | 0.08 |
| | 3 | 0.31 | 0.31 | 1.29 | 0.24 | 0.99 | 4.16 | | |
| | 6 | 0.17 | 0.42 | 0.81 | 0.21 | 0.39 | 1.93 | | |
| | 8 | 0.11 | 0.31 | 0.56 | 0.19 | 0.35 | 1.83 | | |
| | 11 | 0.09 | 0.25 | 0.56 | 0.17 | 0.39 | 2.28 | | |
| | Overall | | | | 0.16 | | | | |
| $pH_C$ = 6.4 | 1 | 1.07 | 0.51 | 3.64 | 0.29 | 2.11 | 7.17 | 0.02 | 0.34 |
| | 3 | 0.31 | 0.39 | 1.09 | 0.29 | 0.78 | 2.74 | | |
| | 6 | 0.10 | 0.17 | 0.39 | 0.27 | 0.61 | 2.25 | | |
| | 8 | 0.07 | 0.08 | 0.28 | 0.25 | 0.94 | 3.75 | | |
| | 11 | 0.03 | 0.09 | 0.15 | 0.19 | 0.30 | 1.57 | | |
| | Overall | | | | 0.22 | | | | |
| $pH_C$ = 6.7 | 1 | 0.98 | 0.55 | 3.61 | 0.27 | 1.79 | 6.57 | 0.06 | 0.41 |
| | 3 | 0.28 | 0.34 | 1.05 | 0.26 | 0.43 | 1.64 | | |
| | 6 | 0.10 | 0.09 | 0.43 | 0.24 | 1.13 | 4.59 | | |
| | 8 | 0.08 | 0.12 | 0.34 | 0.22 | 0.63 | 2.79 | | |
| | 11 | 0.03 | 0.11 | 0.19 | 0.18 | 0.30 | 1.69 | | |
| | Overall | | | | 0.21 | | | | |
| $pH_C$ = 7.0 | 1 | 1.01 | 0.47 | 3.71 | 0.27 | 2.16 | 7.95 | 0.06 | 0.35 |
| | 3 | 0.29 | 0.36 | 1.08 | 0.27 | 0.81 | 2.92 | | |
| | 6 | 0.12 | 0.24 | 0.44 | 0.27 | 0.49 | 1.84 | | |
| | 8 | 0.08 | 0.19 | 0.28 | 0.27 | 0.39 | 1.43 | | |
| | 11 | 0.04 | 0.08 | 0.16 | 0.27 | 0.56 | 2.09 | | |
| | Overall | | | | 0.23 | | | | |
| $pH_C$ = 7.2 | 1 | 1.00 | 0.44 | 3.59 | 0.28 | 2.30 | 8.23 | 0.09 | 0.09 |
| | 3 | 0.28 | 0.23 | 1.01 | 0.27 | 1.21 | 4.41 | | |
| | 6 | 0.11 | 0.15 | 0.40 | 0.26 | 0.70 | 2.64 | | |
| | 8 | 0.07 | 0.16 | 0.28 | 0.25 | 0.45 | 1.78 | | |
| | 11 | 0.03 | 0.12 | 0.14 | 0.21 | 0.23 | 1.10 | | |
| | Overall | | | | 0.23 | | | | |
| $pH_C$ = 7.8 | 1 | 0.79 | 0.46 | 3.84 | 0.20 | 1.71 | 8.35 | 0.01 | 0.22 |
| | 3 | 0.30 | 0.22 | 1.43 | 0.21 | 1.40 | 6.58 | | |
| | 6 | 0.12 | 0.09 | 0.54 | 0.23 | 1.31 | 5.68 | | |
| | 8 | 0.08 | 0.12 | 0.35 | 0.25 | 0.68 | 2.77 | | |
| | 11 | 0.06 | 0.08 | 0.19 | 0.29 | 0.77 | 2.59 | | |
| | Overall | | | | 0.19 | | | | |

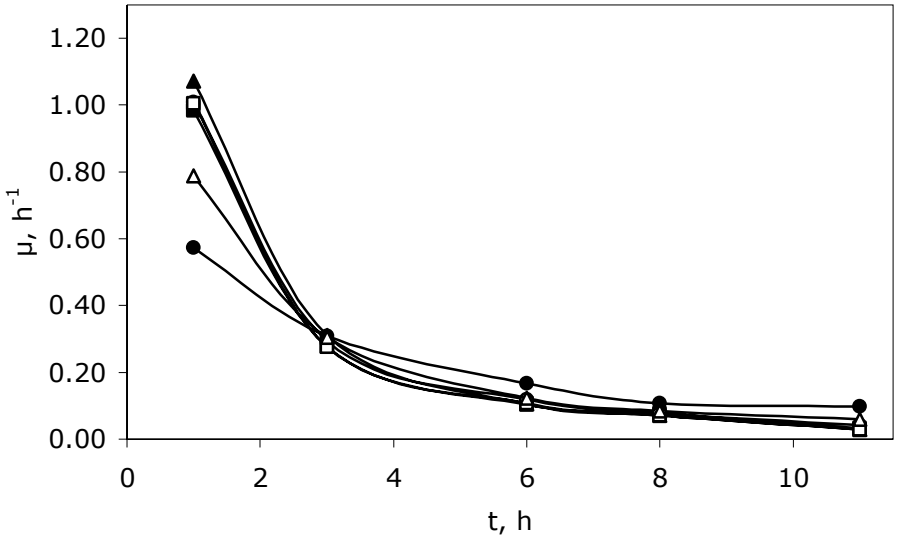



**Figure 4.21** The variations in specific growth rate with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 rpm: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ).

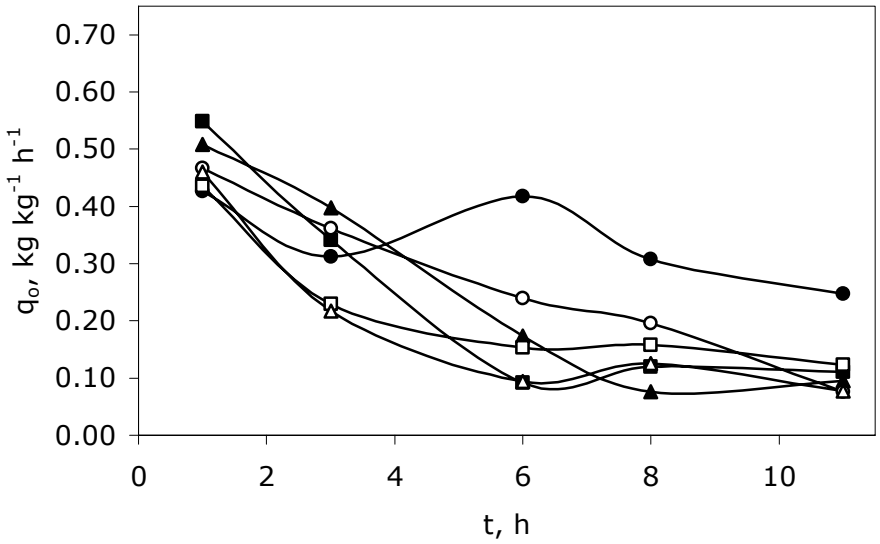



**Figure 4.22** The variations in specific oxygen uptake rate with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 rpm: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ).

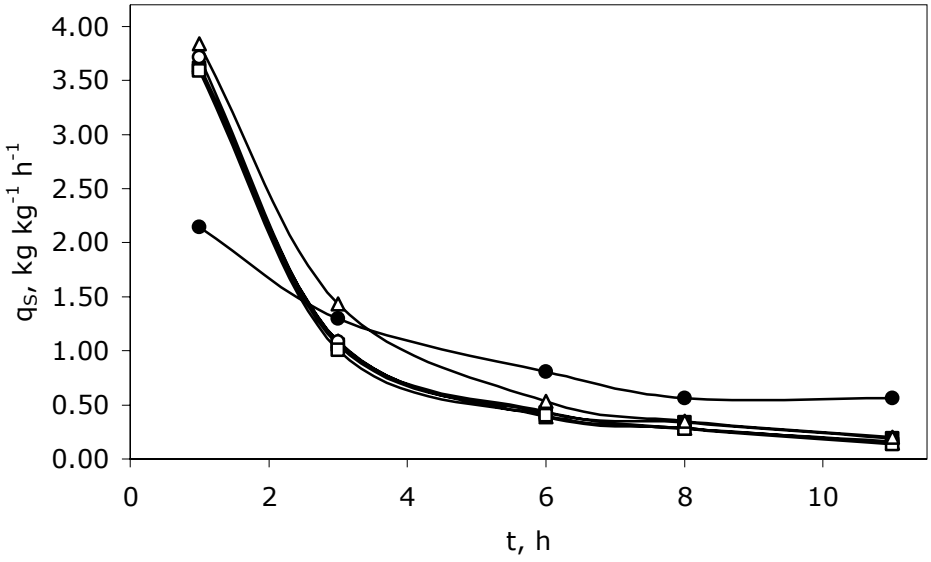



**Figure 4.23** The variations in specific substrate utilization rate with the cultivation time and pH. $C_G^o$ = 8.0 kg $m^{-3}$, T=37°C, $V_R$= 1.65x$10^{-3}$ $m^3$, $Q_o/V_R$=0.5 vvm, N=500 rpm: $pH_C$=5.0, (●); $pH_C$=6.4, (▲); $pH_C$=6.7, (■); $pH_C$=7.0, (○); $pH_C$=7.2, (□); $pH_C$=7.8, (Δ).

The variation of oxygen consumption for maintenance, $m_O$, with the pH conditions applied is given in Table 4.9. The $m_O$ value varied between 0.01-0.23 kg $kg^{-1}$ $h^{-1}$, and the highest value was obtained at $pH_C$=5.0 condition. The $m_S$ values varied between 0.08-0.41 kg $kg^{-1}$ $h^{-1}$, and the lowest value was obtained at $pH_C$=5.0 condition. The Leudeking-Piret constants calculated for the pH conditions applied, is given in Table 4.10.

**Table 4.10** The variations in Leudeking-Piret constants for the pH conditions applied.

| | α | β |
|---|---|---|
| $pH_C$=5.0 | 0.468 | 0.053 |
| $pH_C$=6.4 | 0.108 | 0.267 |
| $pH_C$=6.7 | 0.089 | 0.215 |
| $pH_C$=7.0 | 0.169 | 0.145 |
| $pH_C$=7.2 | 0.664 | 0.069 |
| $pH_{UC}$=7.2 | 0.789 | 0.012 |
| $pH_C$=7.8 | 1.236 | 0.289 |

# CHAPTER 5

# CONCLUSION

In this study, the effects of bioprocess operation parameters on benzaldehyde lyase production were investigated. For this purpose, pUC18*::bal* gene was transformed to several *Escherichia coli* strains and using the host *E. coli* strain having the highest benzaldehyde lyase production capacity, the production medium was designed in terms of its carbon and nitrogen sources, in order to achieve a higher benzaldehyde lyase production. Thereafter, by using the designed medium, the effects of bioreactor operation parameters, i.e., oxygen transfer conditions and pH, and the fermentation and oxygen transfer characteristics of the bioprocess, were investigated in the pilot scale bioreactor. In this context, the following conclusions were drawn:

1. *Escherichia coli* K12 (ATCC 10798) was selected as the benzaldehyde lyase producer, due to the highest cell production capacity of this strain in LB medium as 1.7 kg $m^{-3}$.
2. Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as 1.8 kg $m^{-3}$ and 745 U $cm^{-3}$, respectively, in the medium containing 8.0 kg $m^{-3}$ glucose, 5.0 kg $m^{-3}$ $(NH_4)_2HPO_4$ and the salt solution; T=37°C, N=200 $min^{-1}$, V=33 $cm^3$.
3. The effects of oxygen transfer on benzaldehyde lyase production were investigated within low-, medium-, and high-oxygen transfer rates at six different conditions with the parameters, air inlet rate of $Q_O/V_R$ = 0.5 vvm, and agitation rates of N= 250, 375, 500, 625, 750 $min^{-1}$ and at $Q_O/V_R$ = 0.7 vvm, N=750 $min^{-1}$.

4. At $LOT_1$ and $LOT_2$ conditions, no oxygen accumulation was observed throughout the bioprocesses, due to the low oxygen transfer to the medium than the oxygen uptake rate of the cells. At $MOT_1$ condition, the dissolved oxygen in the medium was totally consumed at the beginning of the bioprocess due to the high oxygen demand of the cells; after t=7 h of the bioprocess, dissolved oxygen in the medium increased as the microorganisms reached the stationary phase. At $MOT_2$, $HOT_1$ and $HOT_2$ conditions, the dissolved oxygen in the medium did not change considerably, as the oxygen transfer rate was high enough.
5. pH of the medium has a tendency to decrease along with the bioprocesses with the cultivation time. The decrease rate was the highest at $LOT_1$ and $LOT_2$ conditions; indicating the high energy generation potential; the lowest amount of cell was formed under these two conditions, due to the oxygen limitation.
6. Increase in the oxygen transfer rate increased cell formation rates up to $MOT_1$ condition; however, higher oxygen transfer conditions inhibited cell formation after t>6 h due to the inhibition effect of oxygen. The highest cell concentration was obtained at $MOT_1$ condition as 2.3 kg $m^{-3}$, while the lowest cell concentration value was obtained at $LOT_1$ condition as 1.0 kg $m^{-3}$.
7. Detection of TCA cycle organic acids, i.e., citric, $\alpha$-ketoglutaric and succinic acids in the fermentation broth at $LOT_1$ and $LOT_2$ conditions; signals the insufficient operation of the TCA cycle at low oxygen transfer conditions. Also, the total organic acid concentration in the medium was the highest; explaining the reason of the highest rate of decrease in the medium pH at this conditions. Furthermore, the accumulation of acetic acid in the medium was the highest at $LOT_1$ condition; which is probably the reason of low cell formation and low enzyme production at this condition.
8. With the increase in oxygen transfer, Damköhler number (Da), that is the oxygen transfer limitation, decreased; and at $MOT_2$, $HOT_1$ and $HOT_2$ conditions at t=1-3 h, μ values were close to $\mu_{max}$; therefore, biochemical reaction resistances were effective. The effectiveness factor (η) values were close to 1.0 at medium and high oxygen transfer conditions at the beginning of the processes as cells

consumed oxygen with such a high rate that maximum possible oxygen utilization rate value was approached; however, at low oxygen transfer conditions, η values were around 0.6, as cells consumed much less oxygen than maximum possible oxygen utilization rate because of the slow functioning of the bioreaction network due to oxygen limitation.

9. The rate of oxygen consumption for maintenance, $m_O$, varied between 0.04-0.18 kg $kg^{-1}$ $h^{-1}$, and the highest value was obtained at $MOT_1$ condition, indicating that higher amounts of oxygen was used for the maintenance of the gradients, and electrical potential, futile cycles and turnover of the macromolecules.
10. Among the investigated oxygen transfer conditions, the highest cell concentration and benzaldehyde lyase activity was observed at $MOT_1$ condition. These values were approximately 1.2 and 1.9 fold higher than the values obtained in the designed medium and in the reference production medium, respectively.
11. The effect of pH was investigated at $Q_o/V_R$=0.5 vvm, N=500 $min^{-1}$ condition, at six different pH conditions which were, $pH_C$=5.0, 6.4, 6.7, 7.0, 7.2 and 7.8.
12. At all the pH conditions, due to the high oxygen demand of the cells at the beginning of the bioprocesses, a decrease was observed in the dissolved oxygen profiles; after reaching a minimum around 0-15% of the saturation, profiles gradually increased until the end of the bioprocesses; except for the $pH_C$=5.0 condition in which a delay in the profile was observed, because of the low cell formation and low oxygen uptake rate at the first six hours of the bioprocess.
13. Glucose consumption rate was not considerably affected from the pH conditions applied; except for the $pH_C$=5.0 condition, in which the cell formation rate, and consequently the glucose consumption rates were low. At t=0-4 h, glucose consumption rate was higher at $pH_C$=7.2 operation in comparison with the $pH_{UC}$=7.2 operation; probably, because of the cells' attempt to decrease the external pH to increase to proton electrochemical gradient for energy generation.
14. Cell formation rates were higher at $pH_C$=6.4, 6.7, 7.0 and 7.2 conditions. The cell concentration was higher at $pH_{UC}$=7.2 compared to the $pH_C$=7.2, at the end of the bioprocesses.

The reason of lower cell concentration at $pH_C$=7.2 operation is thought to be related with the higher acetic acid accumulation in the medium that caused shifts in the intracellular bioreaction network.

15. The highest benzaldehyde lyase volumetric activity was obtained where the highest cell concentration was obtained; the lowest benzaldehyde lyase volumetric activity was obtained at where the least amount of cell was generated. This result in turn indicates that, benzaldehyde lyase production could be enhanced by increasing the concentration of benzaldehyde lyase producing cells, probably either by making use of complex production medium, or developing a substrate feeding strategy; or a combination of both.
16. On the bases of the organic acid profiles; it was concluded that the *E.coli* cells utilized glucose to decrease extracellular pH by producing organic acids in order to increase the energy generation potential, via proton electrochemical gradient.
17. The accumulation of the metabolic by-product acetic acid was the highest at $pH_C$=7.2 and 7.8 conditions in the fermentation medium; explaining the reason for the production of lower amount of cell at $pH_C$=7.8 condition: one probable reason is that the extracellular pHs' being higher than intracellular pH; an unfavorable proton electrochemical gradient; and also the inhibition effect of acetic acid which is highly accumulated in the fermentation broth could be another reason.
18. At the beginning of the bioprocesses, biochemical reaction limitations were effective (Da<1); however, after the sixth hour of the bioprocesses, mass-transfer resistances became more effective (Da>>1). η was close to 1.0 at $pH_C$=6.4, 6.7, 7.0, 7.2 conditions at t=0-1 h indicating that the cells are consuming oxygen with such a high rate that maximum possible oxygen utilization value was approached; thereafter the decrease in η indicates that the cells are consuming lower oxygen than the OD.
19. Specific growth rates, specific oxygen uptake rate ($q_O$), and specific substrate utilization rates ($q_S$), decreased with the cultivation time at all the pH conditions. Overall specific cell yield on substrate ($Y_{X/S}$) reached its highest value at $pH_C$=7.0 and 7.2 conditions, due to the increased efficiency of the utilization of the carbon source for product formation.

Cell yield on oxygen ($Y_{X/O}$) values decreased with the cultivation at all the pH conditions applied; similarly, the amount of substrate metabolized per amount of oxygen used ($Y_{S/O}$) decreased with the cultivation time under all the pH conditions signaling either the increased energy requirement or decrease in the efficiency of energy metabolism with the cultivation time.

20. Among the investigated controlled-pH conditions; the highest amount of cell and enzyme was obtained at $pH_C$=7.0 condition. However, the values obtained at $pH_C$=7.0 condition ($C_X$=2.043 kg $m^{-3}$; A=775 U $cm^{-3}$), were lower than the values obtained at $pH_{UC}$=7.2 uncontrolled-pH operation ($C_X$=2.293 kg $m^{-3}$; A=857 U $cm^{-3}$); hence, uncontrolled-pH operation can be concluded to be favorable for benzaldehyde lyase production.
21. On the bases of the results of the cell, glucose, organic acid and benzaldehyde lyase activity profiles; for recombinant protein production by *E.coli*, the following pH control strategy can be proposed:

The pH of the fermentation medium should be set to pH=7.2 value, and the pH of the fermentation medium can be let to decrease until pH=6.3 value. After that; pH of the medium should be controlled between pH=6.3-7.0, in order to have high biomass and protein production; and also low acetic acid formation. To achieve the control between pH=6.3-7.0 values:

a. If the pH is in the increasing period; it should be set to pH=6.3 and until it reaches to pH=7.0, no control is applied to the medium; and it should be repeated until process shifts to the pH decrease phase.

b. If the pH is in the decreasing period; it should be set to pH=7.0 until it reaches to pH=6.3, no control is applied to the medium; it is also repeated until process shifts to the pH increase phase.

By this mechanism, the pH of the fermentation medium is kept between the desired values; without increasing the stress on the microorganism.

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# APPENDIX A

**$CaCl_2$ Method for Plasmid Transformation** (Sambrook et. al., 1989)

1. Incubate *Escherichia coli* overnight in LB-solid medium at 37 $^oC$,
2. Transfer the microorganism to the 5ml-LB medium and further incubate overnight,
3. Transfer 1ml sample from the precultivation medium to an 100 ml- LB medium and incubate at 37 $^oC$ and 200 $min^{-1}$ for 3.5 hours,
4. Transfer microorganisms into sterile tubes; and place on ice for 10 minutes,
5. Separate the microorganisms by centrifugation at 4000 $min^{-1}$, 4$^oC$ for 10 minutes,
6. Separate the supernatant; let the cells dry on a paper tissue for 1 minute,
7. Add 10 ml of 0.1 M $CaCl_2$ solution onto the cells and make a complete solution by vortex, set on ice for 10 minutes,
8. Precipitate the microorganisms by centrifugation at 4000 $min^{-1}$, 4$^oC$ for 10 minutes,
9. Separate the supernatant; let the cells dry on a paper tissue for 1 minute,
10. Add 2 ml of 0.1M $CaCl_2$ solution onto the cells and make a complete solution by vortex, set on ice for 10 minutes,
11. Transfer 200 µl of solution to an eppendorf tube, and add 3 µl of plasmid DNA to this solution. Incubate on ice for 30 minutes,
12. Apply heat-shock to the solution at 42 $^oC$ for 90 seconds and quickly place the tube on ice for 1 minute,
13. Transfer the cell suspension to sterile culture tubes containing 800 µl of LB medium without antibiotics and incubate at 37 $^oC$ for 45 minutes with shaking at 140 $min^{-1}$ to recover cells,
14. Transfer 250 µl of the cultured cells onto the center of LB plate containing the desired antibiotic. Immediately spread the cells over the entire surface of the LB plate using a sterile, bent glass rod.
15. Invert the plates and incubate at 37 $^oC$ overnight. Selected colonies should be visible in 14 hours.

# APPENDIX B

**Calibration of *Escherichia coli* Concentration**

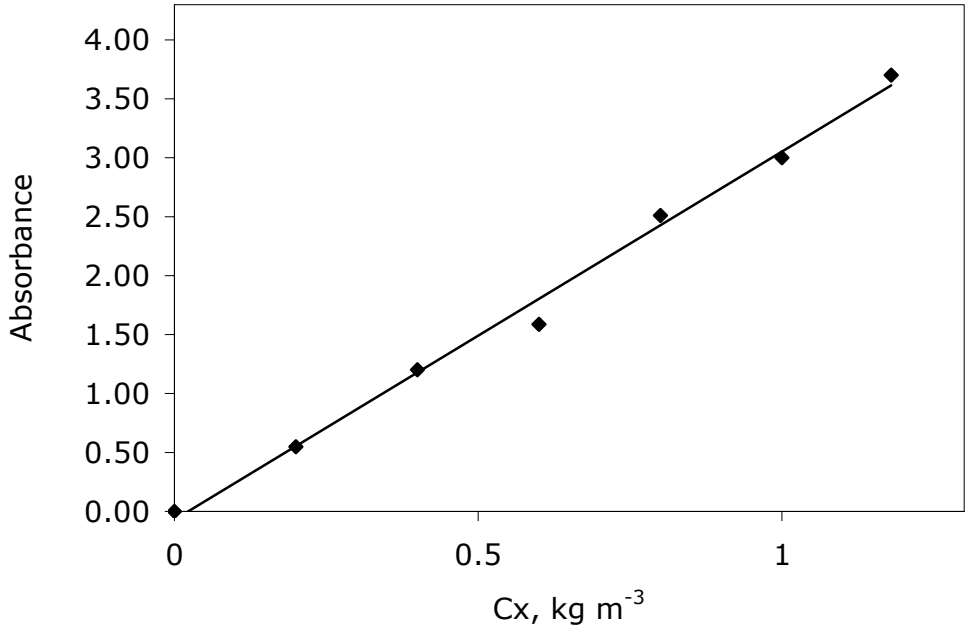


**Figure A.1** Calibration curve for *Escherichia coli* concentration

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

$$Cx = \frac{\text{Absorbance}}{2.8782} \times \text{Dilution Rate}$$

# APPENDIX C

**Calibration of Reduced Sugar Concentration**

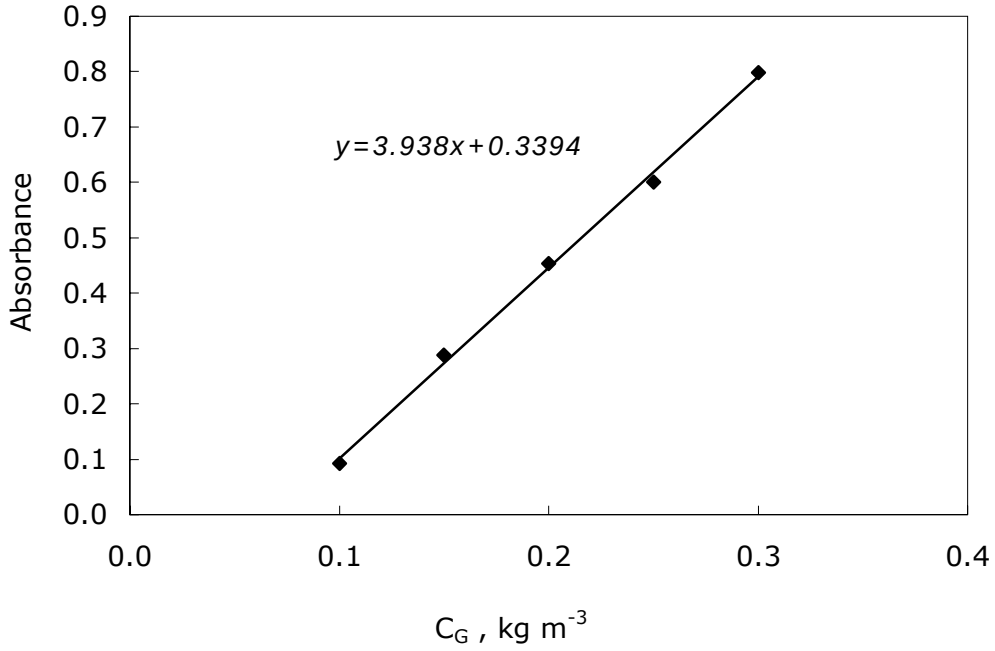


**Figure A.2** Calibration curve of the DNS solution

$$C_G = \frac{\text{Absorbance} + 0.3394}{3.938} \times \text{Dilution Rate}$$

# APPENDIX D

**Preparation of DNS Solution**

1. a) 880 $cm^3$ of 1 % (m/v) DNS solution is prepared by dissolving 8.8 g dinitrosalicilyc acid in 880 $cm^3$ distilled water.
   b) After addition of 225 g ROCHELLE salt (sodium potassium tartarate), the solution is mixed.
   c) 300 $cm^3$ of 4.5 % NaOH, prepared by dissolving 13.5 g NaOH in 300 $cm^3$ distilled water, is added to this solution.
2. a) 22 $cm^3$ 10 % NaOH, is prepared by dissolving 2.2 g NaOH in 22 $cm^3$ distilled water.
   b) 10 g christalized phenol and 100 $cm^3$ distilled water are added to the solution.
   c) 60 $cm^3$ is taken from this alkali-phenol mixture, 6.9 g $NaHCO_3$ is added and mixed.

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