INFLUENCE OF OXYGEN TRANSFER ON BENZALDEHYDE LYASE PRODUCTION BY RECOMBINANT *Escherichia coli* BL21(DE3) pLySs

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INFLUENCE OF OXYGEN TRANSFER ON BENZALDEHYDE LYASE PRODUCTION BY RECOMBINANT *Escherichia coli* BL21(DE3) pLySs

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ABSTRACT

INFLUENCE OF OXYGEN TRANSFER ON BENZALDEHYDE LYASE PRODUCTION BY RECOMBINANT *Escherichia coli* BL21(DE3) pLySs

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In this study, the effects of oxygen transfer conditions on the synthesis of the enzyme benzaldehyde lyase as intracellular in recombinant *E. coli BL21* (DE3) pLysS was investigated sistematically and a comprehensive model was developed to determine benzaldehyde lyase activity. For this purpose, the research program was carried out in mainly two parts. In the first part of study, the effects of oxygen transfer together with the mass transfer coefficient (K_La), enhancement factor E ($=K_La/K_La_o$), volumetric oxygen transfer rate, volumetric and specific oxygen uptake rates, mass transfer and biochemical reaction resistances; moreover, the variation in product and by-product distribution, specific substrate uptake rates, yield and maintenance coefficient were investigated in the pilot scale batch bioreactor at Q_O/V_R = 0.5 vvm and agitation rates of N= 250, 500, 625, and 750 min^{-1}, and dissolved oxygen levels DO= 20%, 40% conditions, while medium components were $C_{Glucose}$= 8.0 kg m^{-3}, $C_{(NH4)2HPO4}$= 5.0 kg m^{-3} and salt solution at controlled pH_c=7.2. The highest cell concentration and benzaldehyde lyase activity were obtained at DO=40% condition as 3.0 kg m^{-3} and A=1095 Ucm^{-3}, respectively.

Then a mathematical model was proposed to estimate benzaldehyde lyase activity as function of time, agitation rate, cell concentration, dissolved oxygen concentration, and by-product concentration with reasonable accuracy.

Keywords: Benzaldehyde Lyase, Production, Recombinant *E. coli*, Oxygen Transfer, Mathematical Model.

ÖZ

REKOMBİNANT *Escherichia coli* BL21(DE3) pLySs İLE OKSİJEN AKTARIMININ BENZALDEHİT LİYAZ ÜRETİMİNE ETKİSİ

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Bu çalışmada rekombinant *Eschericihia coli BL21* (DE3) pLysS ile oksijen aktarımının benzaldehit liyaz üretime etkisi araştırılmıştır ve modellenmiştir. Araştırmanın birinci alt-propgramında laboratuvar ölçekte bulunan en uygun koşullarda, V_R=1.65 dm^3 hacimli, mekanik karıştırmalı, pH, sıcaklık, çözünmüş oksijen ve karıştırma hızı kontrollü pilot-ölçek kesikli biyoreaktörlerde Q_O/V_R = 0.5 vvm hava giriş hızında ve N= 250, 500, 625, and 750 dk^{-1} karıştırma hızlarında ve çözünmüş oksijen DO= 20%, 40% koşullarında oksijen aktarım etkileri yan ürün dağılımları ile birlikte incelenmiş, oksijen aktarımı ve biyoproses karakteristikleri belirlenmiştir. En yüksek hücre derişimi ve benzaldehit liyaz üretimi C_{Glukoz}= 8.0 kg m^{-3}, $C_{(NH4)2HPO4}$= 5.0 kg m^{-3} ; pH_c=7.2 ve DO=40%'da sırasıyla 3.0 kg m^{-3} ve A=1095 Ucm^{-3} olarak bulunmuştur. İkinci alt-programda, benzaldehit liyaz aktivitesi zaman, karıştırma hızına, hücre derişimi çözünmüş oksijen derişimi ve yan ürün derişimlerine bağlı matematik modelle ifade edilmiştir.

Anahtar Kelimeler: Benzaldehit Liyaz, Üretim, Rekombinant *E. coli*, Oksijen Aktarımı, Matematik Model.

To My Family

And

My Husband

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NOMENCLATURE

A	Benzaldehyde lyase activity, U cm^{-3}
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_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

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

Abbreviations

ATCC	American Type Culture Collection
BAL	Benzaldehyde lyase
DO	Dissolved oxygen
EC	Enzyme Commission
OD	Oxygen demand (=μ_{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

Biocatalysts (enzymes) are usually proteins or RNA molecules that act as catalysts. 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 Kargi, 1992). They are used in a multitude of processes, in industry as well as in the daily household, and enzymatically based processes are replacing many classical processes due to their mild reaction conditions and environmentally friendly outcome. The market (approximately 1.5 billion USD in 2000) is expanding, and new applications of enzymes are constantly being explored (Nielsen, Villadsen, Liden, 2003).

The advantages of biocatalysts when compared with chemical catalysts can be summarized as:

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}.

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).

5. Enzymes can catalyze a broad spectrum of reactions. Like all catalysts, enzymes only accelerate a reaction, but they have no impact on the position of the thermodynamic equilibrium of the reaction. Thus, in principle, some enzyme-catalyzed reactions can be run in both directions.

The unique catalytic property of enzyme is based on its three-dimensional structure and on an active site whose chemical groups may be brought into close proximity from different regions of the polypeptide chain. The stereospecificity of an enzyme for substrate has been compared to a lock-and-key relationship. This analogy implies that enzyme has an active site that fits the exact attachment of substrate, the enzyme may undergo conformational changes that provide a more perfect fit between it and the substrate (Bhagavan, 2001).

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 certain extent under chemical catalysis, do survive. As result, reaction 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 region of the same substrate molecule.

3. Enantioselectivity: Almost all enzyme are made of form L-amino acids and thus are cherial 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 transformed into an optically active product through an asymmetrization process.

One of the important and unique properties of enzyme is enantioselsectivity and the use of enantionerically pure compounds are becoming increasingly more important in the production of pharmaceuticals, agrochemicals (e.g., pesticides, fungicides, herbicides), and flavors (Adam et al., 1999). 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 pharmaceutical or an agrochemical cause different biological effects. consequently, they must be regarded as two distinct species. The isomer with the highest activity is denoted as the 'eutomer', whereas its enantiomeric counterpart, possessing less or even undesired activities, is termed as the 'distomer'. The range of effects derived from the distomer can extend from lower activity, no response or toxic events. A representive example of different biological effects is given in scheme 1.1.

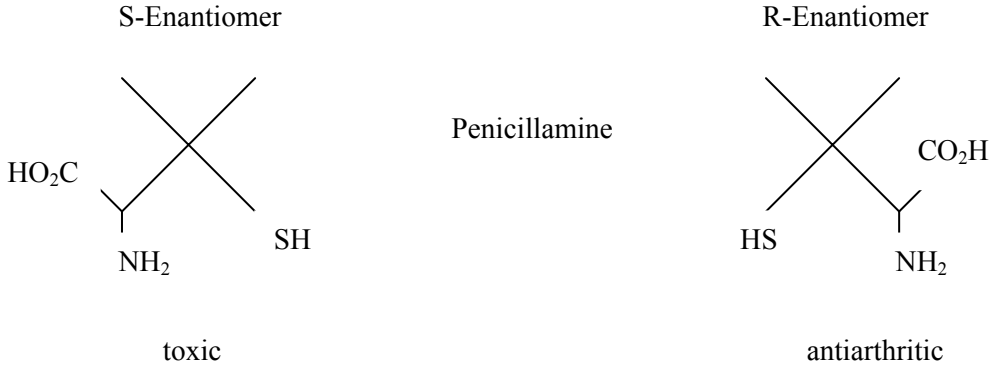

Figure 1.1 R- and S- enantiomers of penicillamine molecule.

Of the alternative ways of obtanining enantiomerically pure compounds, the enzymatic methods are becoming more and more popular for the asymmetic synthesis of fine chemicals (Faber, 2000).

Benzaldehyde lyase (BAL, EC 4.1.2.38), a thiamin diphosphate (ThDP) dependent enzyme, is a valuable tool for chemo-enzymatic syntheses beacuse it generates various enantiomerically pure 2-hydroxyketones through aldehyde ligation or by partial decomposition of racemic mixtures. The (R)- and (S)-enantiomers of benzoin are shown in Figure 1.2.

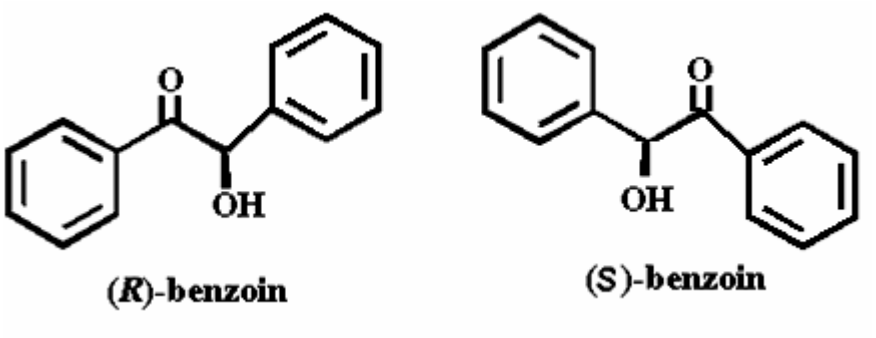

Figure 1.2 R- and S- enantiomers of benzoin molecule.

In the literature, BAL was firstly reported by Gonzales and Vicuna (1989) who isolated it from the strain *Pseudomonas Fluorescens Biovar I*, which was found in wood scraps in a cellulose factory. They showed that this strain can grow on lignin-like substrate, beacuse the endogenous BAL can cleave the acyloin linkage of R-benzoin and R-anision to use these compounds as a carbon and energy source. Thereafter, the gene encoding banzaldehyde lyase was cloned to *Escherichia coli* HB101 by Hinrichsen et al. (1994). Then Pohl et al. (2002) corrected and re-submitted the DNA sequence which was published by Hinrichsen; and for easier purification, fused hexa-histidine tag to the C-terminus of the enzyme and expressed in *E. coli* SG130009.

The mechanism of the reaction catalyzed by benzaldehyde lyase and substrate range of the enzyme was described by Demir et al., (2001, 2002,

2003, 2004). The ability of BAL to asymmetrically synthesize mixed benzoins was also reported by Dünkelmann et al. (2002).

Recently, modeling of benzaldehyde lyase structure and active site identification of the enzyme (Keen et al., 2005) together with obtaining information about homotetramer structure of BAL (Mosbacher et al., 2005) provided a useful knowledge for future studies. Finally, Maria et al. (2005) investigated the effects of parameters such as cofactors, cosolvents, and pH, on the stability and the activity of the BAL.

The reaction engineering of benzaldehyde lyase was presented by Stillger et al. (2006); based on kinetic studies a continuous process was developed and by the application of a continuous process in combination with membrane technology, high space time yields (1120 g L-1 d-1, ee > 99%) of the product as well as high total turnover numbers of the biocatalyst (mol of product/mol of biocatalyst 188.000) were obtained.

The stability of BAL with respect to pH, temperature, buffer salt, cofactors and organic cosolvents was studied by Janzen et al. (2006). In this study reported that the reaction temperature should not exceed 37 C^{o} and the enzyme was stable between pH 6 and 8, with pH 8 being the pH-optimum of both the lyase and the ligase reactions.

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

The effects of bioprocess operation parameters on benzaldehyde lyase production was firstly reported by Çalık et al. (2004, 2006); using the recombinant *E. coli* K12 carrying modified pUC18::*bal* plasmid on a defined medium with glucose and $(NH_4)_2HPO_4$ as the sole carbon and nitrogen sources, respectively. The highest BAL activity was obtained at 0.5 vvm, 500 min^{-1} as 860 Ucm^{-3} with a cell concentration of 2.3 kg m^{-3} at uncontrolled pH of 7.2.

In the study of Kaya, (2006), T7 regulated system was chosen in order to increase the productivity of BAL as an alternative to *trc* promoter, which was present in modified pUC18::*bal* plasmid. For this purpose, the gene encoding benzaldehyde lyase, *bal*, was cloned into pUC derived pRSETA (under the control of T7 promoter) and as the host cell *E. coli* BL21 (DE3) pLySs strain was utilized, which was specifically designed for T7 regulated genes. Thereafter, by using the developed recombinant *E. coli* BL21 (DE3) pLySs cells, the effect of glucose and $(NH_4)_2HPO_4$ and their concerted effects were investigated in the production of recombinant benzaldehyde lyase.

The only study about the effect of oxygen transfer on cell physiology of *E. coli* BL21 cells was performed by Kaya, (2006). However, the oxygen transfer conditions investigated as bioreactor operation parameters showed similar trends and at all conditions oxygen transfer was the rate limiting step. As a result lower BAL expression was observed.

Therefore, in this study, it was aimed to investigate the effects of oxygen transfer conditions on the synthesis of benzaldehyde lyase by the recombinant *E. coli* BL21 (DE3) pLySs in a systematic way and in detail to explain the variations in cell physiology in terms of product and by-product distributions and biomass formation. Moreover, the oxygen transfer characteristics like mass transfer coefficient (K_La), enhancement factor E ($=K_La/K_La_o$), volumetric oxygen transfer rate, volumetric and specific oxygen uptake rates, mass transfer; and biochemical reaction resistances like specific substrate uptake rates, yield and maintenance coefficients were reported . Also, in this study, by using multiple regression technique, mathematical model was proposed to estimate the benzaldehyde activity as fuction of time, agitation rate, cell concentration, dissolved oxygen concentration, and by- product concentration.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

2.1.1 General Characterisatics

Enzymes are catalysts and are functional units of cellular metabolism. They are usually proteins but may include RNA molecules as well (Bhagavan, 2001). The main function characterisatics of enzymes compared with chemical catalysts are their high efficiency, their specificity and their capacity for regulation (Matthews,1997).

An enzyme- catalyzed reaction is initiated when the enzyme binds to its substrate to form an enzyme-substrate complex. Substrate binding to the enzyme occurs at a specific and specialized region known as the active site, a cleft or pocket in the surface of the enzyme that constitues only a small portion of the enzyme molecule. Catalytic function is accomplished at this site beacuse various chemical groups important in substrate binding are brougth together in a spatial arrangment that confers specificity on the enzyme. Thus, the unique catalytic property of an enzyme is based on its three-dimentional structure and on an active site whose chemical groups may be brougth into close proximity from different regions of the polypeptide chain (Bhagavan, 2001).

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 °C, and pH optima range from acidic, i.e., 1.0, to alkaline, i.e., 10.5 (Krik and Othmer, 1994). The pH dependence of enzyme activity is the result of several effects. Ionizable groups in the active site of the enzyme (or elsewhere), in the

substrate, or in the enzyme- substrate complex can effect catalysis depending on whether the protons on the reactive groups are dissociated or undissociated (Bhagavan, 2001). Another distinguishing characteristic of enzymes is their frequent need for cofactors. A cofactor is a non-protein compound which combines with an other- wise inactive protein to give a catalytically active complex. The simplest cofactors are metal ions like Ca^{2+}, Zn^{2+}, Co^{2+}, etc (Bailey, 1986).

Most of the enzymes used on an industrial scale are derived from microbial sources. Enzymes isolated from microorganisims have impressive credentials as catalysts for the synthesis of industrial chemicals. Among the advantages derived from using microbial cells are: 1) potential for reduced catalyst cost; 2) increased enzyme stability; 3) ease of running multi-catalystic processes; and 4) decreased time for catalyst production (moses and Cape, 1991).

2.1.2 Classification of Enzymes

Enzymes are generally named for the substrate or chemical group on which they act, and the name takes the suffix –ase. Systematic nomenclature for the enzymes has been developed by the Enzyme Commission of the International Union of Biochemistry (Bhagavan, 2001). This enzyme commission assiged each enzyme a recommended name and four-part distinguishing number (Chaplin, 1990). In this system, enzymes are divided into six main groups (Table 2.1) according to the type of reaction catalyzed (Faber, 2000). Each of the major classes is futher divided into numerical subclasses and sub-subclasses according to the individual reactions involved. The forth number in the classification is the serial number of the enzyme within a subclass (Atkinson and Mavituna, 1991).

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

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

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

2.1.3 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. More relevant parameters are the activity of the enzyme preparation and the activities of any contaminating enzymes. 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 (Chaplin and Bucke, 1990), which was defined by the Commission on Enzymes:

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

A comparison of the activity of different enzyme preparations was only possible if the assay procedure is performed exactly in the same way (Faber, 2000). However, these so-termed optimal conditions vary even between laboratories and suppliers (Chaplina and Bucke, 1990). Therefore, the parameters such as temperature, pH, and/or substrate concentration that affects the reaction rate must be carefully controlled in order to achieve reproducible results (Kirk and Othmer, 1994).

2.1.4 Enzyme Kinetics

A model for enzyme kinetics that has found wide applicability was proposed by Michaelis and Menten in 1913 and later modified by Briggs and Haldane. The Michaelis- Menten equation relates the initial rate of an enzyme catalyzed reaction to the substrate concentration and to a ratio of rate constants. This equation is a rate equation, derived for a single substrate-enzyme-catalyzed reaction. In the reaction

$$E + S \xrightarrow{k1} ES \xrightarrow{k2} P$$

An enzyme E combines with substrate S to form an ES complex with a rate constant k_1. The ES complex formed can dissociate back to E and S with rate constant $k_{-1,}$ or it can rise to a product p with rate constant k_2 (Bhagavan, 2001). The Michaelis- Menten equation (Eq 2.1) is simply derived from the rate of the above reaction by substituting k_m for $(k_{-1}+k_2)/k_1$:

$$r = \frac{r_{\max} Cs}{k_m + Cs} \qquad (2.1)$$

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).

Rearrangement of equation (2.1) gives

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

A plot of 1/r versus $1/C_S$ (known as a Lineweaver-Burk plot, Figure 2.1) provides a means of determinig the parameters k_m and r_{max} from experimental data (Doran, 1995).

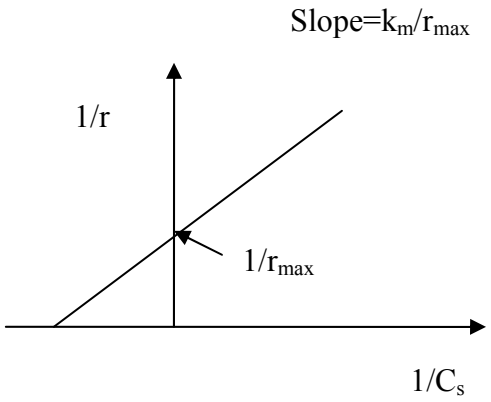

Figure 2.1 Lineweaver-Burk plot.

2.2 Benzaldehyde Lyase

The enzymic modification of raw materials has been important component of industrial processing for centuries (Moses and Cape, 1991). As cherial materials, enzymes can be used in the synthesis of optically active substances in high enantiomeric excess from prochiral or racemic substrates by catalytic asymetric induction or kinetic resolution. Therefore, the development of enantiometrically pure compounds used in the production of

pharmeceuticals, agrochemicals, and flavors are recently becoming more important (Adem et al., 1999).

Benzaldehyde lyase (BAL; E.C. 4.1.2.38), a thiamine pyrophosphate (TPP)_ and Mg^{2+}_ dependent enzyme is used for synthesis of enatio pure 2_hydroxyl ketones, an important type of compounds in natural product and drug synthesis, in very good yields and high enantioselectivities. This enzyme catalyzes cleavage of the carbon-carbon bond of benzoin to form two benzaldehydes. BAL also catalyzes the reverse acyloin condensation of benzaldehydes resulting in the synthesis of (R)-benzoins. Only one enantiomer of benzoin acts as substrate when racemic mixture of benzoin is reacted with BAL. In other words, only (R)-benzoin is converted into benzaldehyde through BAL catalysis, while (S)-benzoin gives no reaction at all. The catalytic mechanism of the enzyme is schematized in Figure 2.2 (Demir et. al., 2001).

Benzaldehyde lyase, which is used for the synthesis of enantiopure α-hydroxy ketones, was firstly reported by Gonzales et al. (1989) from *Pseudomonas fluorescens Biovar* I. Due to the benzaldehyde lyase activity of the microorganism, this strain can grow on benzoin as a sole carbon and energy source. In this study, the purification and the characterization of the enzyme were described and the ability of BAL to catalyze the cleavage of acyloin linkage of benzoin to form benzaldehyde was also described. It was also stated that benzaldehyde lyase requires ThDP for catalytic activity which was maximum at 0.01 mM ThDP, while concentrations higher than 0.5 mM were inhibitory. The activity loss was observed when treated with EDTA which can be restored by addition of 1.0 mM concentrations of $MgCl_2$, $MnSO_4$, or $CaSO_4$. The enzyme showed maximal activity between pH 7.5 and 8.5, whereas it was inactive below pH 6.0. After the identification of BAL by Gonzales (1989); Hinrichsen et al. (1994) cloned the gene encoding benzaldehyde lyase to an *Escherichia coli* strain using vector pUC18. In this study, the nucleotide sequence and the location of the gene were also determined.

Afterward, the DNA sequence published was corrected and re-submitted; and for easier purification, hexa-histidine tag was inserted to the C-terminus of the enzyme and expressed in *E. coli* SG130009 by Pohl et al. (2002).

In further studies, the catalytic activity and substrate range of benzaldehyde lyase like the enantioselective C-C bond cleavage and formation to generate (R)- and (S)-benzoins and (R)-2hydroxypropiophenone ((R)-2HPP) derivatives (Demir et al., 2001); C-C bond formation from aromatic aldehydes and acetaldehyde (Demir et al., 2002) and methoxy- and dimethoxy-acetaldehydes (Demir et al., 2003) on a preparative scale in buffer/DMSO solution; asymmetrical synthesis of mixed benzoins (Dünkelmann et al., 2002); mixed acyloin condensation between methoxy- substituted benzaldehydes and phenylacetaldehyde (Sanchez et al., 2003); and hydroxymethylation of aromatic aldehydes with formaldehyde (Demir et al., 2004) were investigated.

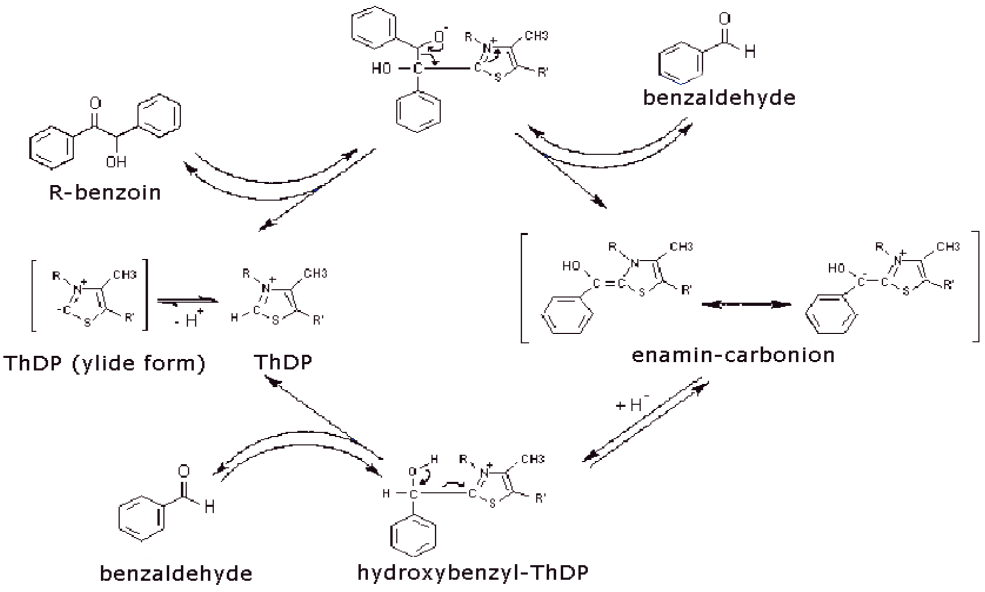

Figure 2.2 Benzaldehyde lyase catalyzed cleavage and synthesis of benzoin. The first step of the catalytic cycle is the attack of ylide form of ThDP on the carbonyl carbon of (R) - benzoin to produce an adduct. The enamine, intermediate product of ThDP dependent enzymes catalyzing the formation of 2-hyroxy ketones, and the first free aldehyde molecule is formed. Protonation of this intermediate then releases the second molecule of aldehyde and restores the cofactor. In the presence of an acceptor aldehyde and enamine intermediate is able to undergo a C-C bond formation

Recently, to determine the similarities and differences between THDP dependent enzymes like benzoylformate decarboxylase (BFD) and benzaldehyde lyase which result in the different catalytic activities, the X-ray structure of BFD and pyruvate decarboxylase (PDC) were used as template for modeling benzaldehyde lyase. The model shows that the glutamine residue, Gln113, present in the active site plays an important role where replacement with alanine or histidine resulted in 200 fold activity loss (Kneen et al., 2005). Thereafter, the structure of BAL was determined by X-ray diffraction and identified that benzaldehyde lyase is a homotetramer, which each subunit binds one THDP molecule using one Mg^{2+} ion. It was also founded that the molecular weight of the enzyme is 4×58919 Da corresponding to 4×563 amino acid residues. The information obtained from X-ray diffraction exposed the active center, reaction geometry, and substrate specificity of benzaldehyde lyase which can later be used to expand the substrate range of the enzyme (Mosbacher et al., 2005).

Maria et al. (2005) investigated the effects of cofactors, cosolvents, and pH, on the stability and the activity of the BAL to obtain higher chemical yields for the synthesis of benzoins by carboligation of benzaldehyde derivatives. Among the investigated parameters, it was shown that 30% DMSO content in the presence of potassium phosphate buffer with 0.5mM Mg^{+2}, 0.5 mM ThDP and 1mM DTT, a well known stabilizer of hyrolases, were the optimal for enzyme activity and stability. It was shown that the enzyme prepared in potassium phosphate buffer supplemented with ThDP, Mg^{2+}, DTT as a cofactor and 30% DMSO as a cosolvent leaded to 50% activity loss after 30 h, while the enzyme losses its activity almost completely within 3 h in water. It was also shown that best enzymatic activity appeared when the pH of the reaction medium was increased from 8.0 to 9.5.

In the other study, Hischer et al. (2005) performed benzoin condensation using recombinant benzaldehyde lyase. To enable the conversion of hydrophobic substrates, the enzyme was entrapped in polyvinyl alcohol and suspended in hexane. Compared to the reported application of the biocatalyst in an aqueous phase containing 20% DMSO, the productivity of the resulting gel stabilised two-phase system was 3-fold better. The entrapment process had an efficiency of >90%, no enzyme or cofactor was lost during reaction or

storage. The entrapped enzyme was stable in hexane for 1 week at 4 ^{o}C and more than 1 month at -20 ^{o}C.

Stillger et al. (2006), presented the reaction engineering of BAL and based on kinetic studies a continuous process was developed. By the application of a continuous process in combination with membrane technology, high space time yields (1120 g L-1 d-1, ee > 99%) of the product as well as high total turnover numbers of the biocatalyst (mol of product/mol of biocatalyst 188.000) were obtained. A kinetic model was developed to simulate the continuously operated reactor and to determine optimal production conditions.

Janzen et al. (2006) also studied the stability of BAL with respect to pH, temperature, buffer salt, cofactors and organic cosolvents. It was shown that the reaction temperature should not exceed 37 C^{o} and the enzyme was stable between pH 6 and 8, with pH 8 being the pH-optimum of both the lyase and the ligase reactions.

Thereafter Mikolajek et al. (2007) investigated a carboligation for the first time as an enzymatic gas phase reaction, where benzaldehyde was converted to benzoin using thiamine diphosphate (ThDP)-dependent enzymes, namely benzaldehyde lyase (BAL) and benzoylformate decarboxylase (BFD). The biocatalyst was immobilized per deposition on non-porous support. Some limitations of the gas/solid biocatalysis were discussed based on this carboligation and it was also demonstrated that the solid/gas system is an interesting tool for more volatile products.

The studies in the literature reporting the effects of bioprocess operation parameters on benzaldehyde lyase production from *E. coli* K12 were published by Çalık et al. (2004, 2006). The host microorganism having the highest benzaldehyde lyase productivity was determined as *E. coli* K12 (ATCC 10798) carrying modified pUC18::*bal* plasmid where inducible hybrid t*rc* promoter is present. 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, the effects of uncontrolled-pH and controlled pH operations and effects of oxygen transfer conditions on

benzaldehyde lyase productivity were systematically investigated using the recombinant *E. coli* K12 carrying pUC18::*bal* plasmid. Among the controlled-pH operations, the highest cell concentration and BAL activity were obtained as 2.1 Kg m^{-3} and 775 U cm^{-3}, respectively at pH_c 7.0; whereas they were 2.3 Kg m^{-3} and 860 U cm^{-3}, respectively at 0.5 vvm, 500 min^{-1} and uncontrolled pH 7.2 condition.

2.3 Bioprocess Parameters in Enzyme production

Bioprocess engineering is the application of engineering principles to design, develop and analyze processes using biocatalyst for the production of a desirable product or for the destruction of unwanted or hazardous substances.

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

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

In aerobic bioprocess, there are some important criteria that must be taken into account in order to have high product yield. These are:

1. Microorganism
2. Medium design
3. Bioreactor operation parameters

i.Oxygen transfer rate

- Air inlet rate (Q_o/V)
- Agitation rate (N)

ii. pH and temperature.

2.3.1 Microorganism

In bioprocesses the first critical step is to identify the most proper microbial strain for the production of the product, screening a wide range of candidate microorganisms. The micoorganism should be able to secrete large amounts of the desired product using cheaper substrates, be suitable for industrial fermentations, give sufficient yields, 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).

Benzaldehyde lyase is naturally produced by wild-type *Pseudomonas fluorescens (*Gonzales et al., 1984). The gene encoding benzaldehyde lyase was firstly cloned to an *Escherichia coli* by Hinrichsen et al. (1994), and further studies about the catalysis ability of the enzyme in stereospecific manner were performed by using *E. coli* SG130009 as the host microorganism. In the literature concerning benzaldehyde lyase production, Çalık et al. (2004, 2006) selected *E. coli* K12 strain as host microorganism among the investigated *E. coli* strains, namely *E. coli* JM109, *E. coli* XL-1 Blue, *E. coli* K12 and *E. coli* GBE 180 (Çalık, 2004, 2006; Kaya, 2006). As an alternative to these, to achieve extracellular production of BAL recombinant *Bacillus species* (Kaya, 2006) and *Pichia pastoris* (Büyüksungur, 2006) was studied as the host.

2.3.1.1 *Escherichia coli*

Escherichia coli is most often chosen as a host for the production of enzymes and recombinant proteins. Reasons for the popularity of *Escherichia coli* are the ease of genetic manipulation and wealth of the availability of genetic information coupled with fast growth rate, standardized cultivation techniques and cheap media (Çalık et al., 2002-b)

The typical *E. coli* cell is a rod shaped microorganism 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 (Neidhardt et al., 1990).

E.coli is a gram-negative bacterium. It has a peptidoglycan layer covered by an outer membrane. The outer membrane is an asymetric lipid bilayer membrane: A lipopolysaccharide forms the exterior layer, and phospholipid forms the inner layer. The presence of the outer membrane on gram negative bacteria confers a higher resistance to antibiotics, such as penicillin, and to degradative enzymes, such as lysozyme (Glazer and Nikaido, 1995). The cell envelope serves to retaine important cellular compounds and to preferentially exclude undesirable compounds in the environment. The cell envelope is critical to the transport of selected material in and out of the cell (Schuler and Kargı, 2002).

The maximum temperature of growth for *E.coli* is approximately 48°C, its optimal temperature is 37°C, its minimal temperature is 8°C, and its normal temperature range extends from about 21°C to 39°C. *E. coli* grows well near neutrality at pH value 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).

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

2.3.1.2 Cell Growth, Kinetics and Yield Factors

Cellular growth is the net result of the uptake and conversion of nutrients (substrates) into new cell marerial (biomass). Cellular growth can also be considered as an increase in the number of individuals in the population as a result of both replication and change in the cell size due to the chemical reactions occur inside the cell. Microbial growth is an autocatalytic process, as

one cell is responsible for the synthesis of more cells, and at conditions with excess nutrients growth is therefore exponential with a certain doubling time for the number of cells (Nielsen and Villadsen, 2003).

The rate of growth is directly related to cell concentration, and cellular reproduction is the normal out come of this reaction. The rate of cellular 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 microorganisms selectively take up dissolved nutrients from the medium and convert them into biomass.

A typical batch growth curve include the following phases (Shuler and Kargı, 2002):

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

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

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

4. Stationary phase; the net 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 ($\mu < 0$).

The typical growth curve for a bacterial population is schematized in Figure 2.3

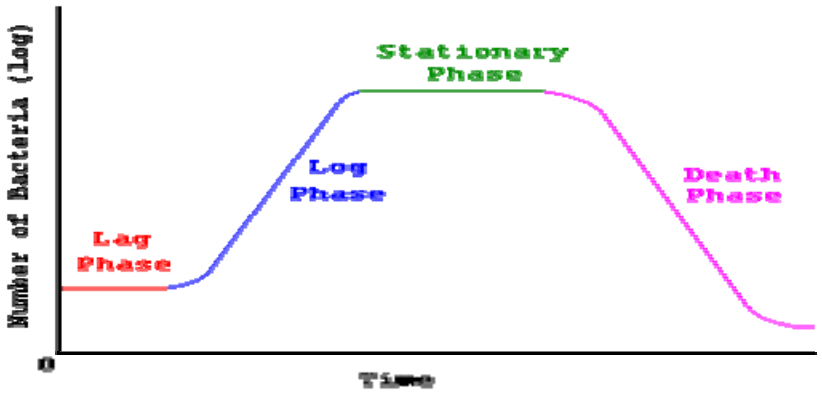

Figure 2.3 Typical growth curve for a bacterial population

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

$$r_x = \frac{dC_x}{dt} = \mu C_x \tag{2.4}$$

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

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

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

To better describe growth kinetics, some stoichiometrically related parameters are defined. Yield coefficients are defined based on the amount of consumption of another material (Shuler and Kargi, 2002). A list of frequently used yield

coefficients is given in Table 2.2. When yields for fermentation are reported, the time or time period to which they refer should be stated (Doran, 1995).

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 utilized 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

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

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

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

The oxygen consumption for oxygen denoted by m_0 is the oxygen consumption for maintenance. In an aerobic process oxygen is consumed for cell growth, product formation and by-product formations, and maintenance. The oxygen consumption rate for cell growth can be defined as (Calık et al., 2006):

$$-r_{O1} = \frac{dCx/dt}{Y_{X/O}} \tag{2.9}$$

Oxygen consumption for by-product formation is defined as:

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

Oxygen consumption for maintenance is defined as:

$$-r_{O3} = m_0 Cx \tag{2.11}$$

The total oxygen consumption rate is as follows:

$$-r_O = (-r_{O1}) + (-r_{O2}) + (-r_{O3}) \tag{2.12}$$

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

$$-\frac{dCo}{dt} = -r_O = \left(\frac{1}{Y_{X/O}}\right)\frac{dCx}{dt} + \left(\frac{1}{Y_{BP/O}}\right)\frac{dC_{BP}}{dt} + m_0 Cx \tag{2.13}$$

Also,

$$q_{BP} = \frac{1}{Cx}\frac{dC_{BP}}{dt} \tag{2.14}$$

And

$$\mu = \frac{1}{Cx}\frac{dCx}{dt} \tag{2.15}$$

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

$$-r_O = \left(\frac{1}{Y_{X/O}}\right)(\mu Cx) + \left(\frac{1}{Y_{BP/O}}\right)(q_{BP}Cx) + m_0 Cx \qquad (2.16)$$

Dividing (2.13) by Cx

$$\frac{-r_O}{Cx} = \frac{\mu}{Y_{X/O}} + \frac{q_{BP}}{Y_{BP/O}} + m_0 \qquad (2.17)$$

q_{BP} can be defined as follows, where α is the term for growth associated organic acid formation and β is the term for non-growth associated organic acid formation:

$$\frac{dC_{BP}}{dt} = \alpha \frac{dCx}{dt} + \beta Cx \qquad (2.18)$$

Dividing (2.18) to Cx,

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

Substituting (2.19) into (2.17),

$$\frac{-r_O}{Cx} = \frac{\mu}{Y_{X/O}} + \frac{\alpha\mu + \beta}{Y_{BP/O}} + m_0 \qquad (2.20)$$

Dividing (2.20) by μ,

$$\frac{-r_O}{\mu Cx} = \frac{1}{Y_{X/O}} + \frac{\alpha + (\beta/\mu)}{Y_{BP/O}} + \frac{m_0}{\mu} \qquad (2.21)$$

Rearranging (2.21),

$$\frac{-r_O}{\mu Cx} = \left(\frac{\alpha}{\bar{Y}_{BP/O}} + \frac{1}{\bar{Y}_{X/O}}\right) + \left(\frac{1}{\mu}\right)\left(m_0 + \frac{\beta}{\bar{Y}_{BP/O}}\right) \quad (2.22)$$

And,

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

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

weight h^{-1}) and from the intercept, $\left(\frac{\alpha}{\bar{Y}_{BP/O}} + \frac{1}{\bar{Y}_{X/O}}\right)$ could be determined,

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

When by-product formation is neglected, equation (2.13) becomes:

$$-\frac{dCo}{dt} = -r_O = \left(\frac{1}{\bar{Y}_{X/O}}\right)\frac{dCx}{dt} + m_0 Cx \quad (2.24)$$

If the above equation is reorganized:

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

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

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

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

2.3.2 Medium Composition

An important aspect in design of a fermentation process is the choice of fermentation medium, which represents the raw material for the process. The fermentation medium should fulfill the following criteria:

- It should contain a carbon, nitrogen and energy source
- It should contain all essential minerals required for growth
- It should contain all necessary growth factors to ensure rapid growth and high yield of the desired product
- It should be of a consistent quality and be readily available throughout the year
- It causes a minimum of problems in the downstream processing
- It causes a minimum of problems in other aspects related to the fermentation process, i.e., it has no negative effect on the gas-liquid mass transfer (Nielsen, Villadsen, Liden, 2003).

Nutrients required by cells can be classified in two categories (Shuler and Kargı, 2002):

1. Macronutrients: These compounds are needed in the concentrations of larger than 10^{-4} M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{+2}, and K^{+} are major macronutrients, from which living matter is made. These molecules are the bulk of the cellular components such as proteins, lipids, membrane structures, and nucleic acids. Microorganisms exhibit some diversity in utilizing the macronutrients as a source.

2. Micronutrients: These compounds are needed in the concentrations of less than 10^{-4} M. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Na^{+}, vitamins, growth hormones, and metabolic precursors are micronutrients. Microorganisms show diversity in the usage of micronutrients as a source.

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. Often, complex media is less expensive than defined media. However, the primary advantage of defined media is that the results are more reproducible and the operator has better control of the fermentation. Further, recovery and purification of a product is often easier and cheaper in defined media (Shuler and Kargi, 2002).

The advantages of applying complex media are that they often contain an organic nitrogen source, essential minerals and different growth factors. Thus, two frequently applied complex media, corn steep liquor and paramedical; both contain a very large variety of amino acids, many different minerals and many different vitamins (Nielsen, Villadsen, Liden, 2003). The disadvantages of complex media are that:

1) There may be a seasonal variation in the composition,

2) The composition changes with storage

3) There may be compounds present that are undesirable.

In the study of Choi et al. (1997), the effects of various culture conditions such as medium (LB or M9), temperature, and expression time to optimize the production of bovine growth hormone (bGH) in *E. Coli* BL21 were examined and LB medium was reported slightly better than M9 medium containing 2.0 kg m^{-3} casamino acid. In another study performed by Christensen et al. (2002), glucose and glycerol effects as carbon sources in batch cultures of *E. coli* BL21 and recombinant strain were investigated and lower acetate accumulation was observed in glycerol based medium.

Furthermore, Çalık et al. (2004, 2006) designed a glucose based defined medium containing 8.0 Kg m^{-3} glucose, 5.0 Kg m^{-3} $(NH_4)_2HPO_4$ and salt solution, and by using this medium they investigated the effects of bioreactor operation parameters in *E. Coli* K12 carrying *pUC18::BAL* plasmid.

2.3.3 Bioreactor Operation Parameters

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

2.3.3.1 Temperature

Temperature is an important factor affecting the performance of cells. According to their temperature optima, organism can be classified in three groups:

1) Psychrophiles ($T_{opt} < 20^oC$)
2) Mesophiles ($20^oC \leq T_{opt} < 50^oC$)
3) Thermophiles ($T_{opt} \geq 50^oC$).

As the temperature is increased toward optimal growth temperature, the growth rate approximately doubles for every 10^oC increase in temperature. Above the optimal temperature ranges, the growth rare decreases and thermal death may occure.

Temperature also effects product formation. However the temperature optimum for growth and product formation may be different. When temperature is increased above the optimum value, the maintenance requirements of cells increase so that yield coefficient is also affected by temperature (Shuler and Kargi, 1992).

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

2.3.3.2 pH

Hydrogen ion concentation (pH) affects the activity of enzymes and therefore the microbial growth rate (Shuler and Kargi, 1992). The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in the pH. Enzymes are normally active only within a certain pH interval, and the total enzyme activity of the cells is therefore a complex function of the environmental pH (Nielsen, Villadsen, Liden, 2003). The optimal pH for growth may be different from that for product formation. Different organisms have different pH ranges optima: the pH optima for many bacteria ranges from pH=3 to 8 (Shuler and Kargi, 1992).

Although hydrogen ion concentration of the extracellular broth (fermentation medium) changes according to the metabolic activities of the cell, the intracellular pH of the cell remains constant. The proton gradient between extracellular and interacellular medium is necessary for the energy production through the transport of the materials and also for the interacellular reaction netwerk (Nielsen and Villadsen, 1994).

In most fermentation, pH can vary substantially. Often the nature of the nitrogen source can be important. If the ammonia is the sole nitrogen source, hydrogen ions are released into the medium as a result of the microbial utilization of ammonia, resulting in a decrease in pH. Also, the pH can change because of the production of organic acids, the utilization of acids (particularly amino acids), or the production of bases. The evolution or supply of CO_2 can alter pH greatly in some systems. Thus, pH control by means of a buffer or an active pH control system is important (Shuler and Kargi, 2002).

In the study by Çalık et al. (2006), the effect of value of pH in the range of 5.0-7.8 on benzaldehyde lyase production on a defined medium with glucose as a sole carbon source in batch bioreactors was examined. The pH value of 7.2 was reported as optimum pH for production of benzaldehyde lyase by *E. coli*. Therefore, in the current study pH of the fermentation medium was arranged as 7.2 with 5M KOH and 5M H_3PO_4.

2.3.3.3 Oxygen Transfer

The transfer of oxygen into microbial cell in aerobic fermentation processes affects product formation by influencing metabolic pathways and changing metabolic fluxes. Metabolic pathway analysis and cell growth conditions show that the oxygen requirement of bioprocesses can be different so that dissolved oxygen concentration has been accepted as an important bioreactor operational parameter. Futhermore, in order to design, scale-up, and operate the bioreactor with adequate mass transfer , the oxygen consumption rates and oxygen transfer coefficient which are the indicators of the mass transfer characteristics of a fermentation process are required; nevertheless, due to the complex composition of the fermentation liquid, it can be difficult to predict these parameters with reasonable accuracy (Çalık et al., 1999; Çalık et al., 1998).

To establish optimum conditions for microbial growth, sufficient levels of oxygen must be supplied to the liquid broth. Often, the oxygen treansfer rate is the limiting factor of a bioreactor system. The transfer of oxygen from gas to microorganism takes place in several steps (Figure 2.4), which are 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 (Scragg, 1988 and Bailey, 1986).

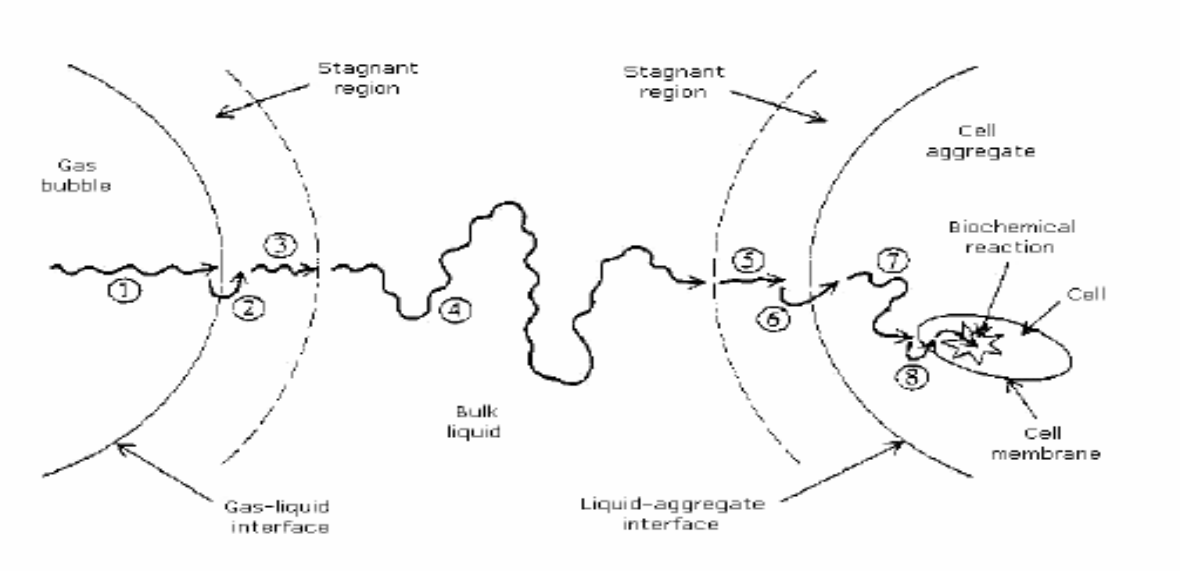

Figure 2.4 Schematic diagram of steps involved in transport of oxygen from a gas bubble to inside a cell (Bailey and Ollis, 1986)

The rate of oxygen transfer from the gas to liquid phase is given by

$$\text{OTR} = NO_2 = k_L a(C_O^* - C_O) \qquad (2.26)$$

where k_L is the oxygen transfer coefficient (cm/h), a is the gas-liquid interfacial area (cm^2/cm^3), k_La is the volumetric oxygen transfer coefficient (h^{-1}), C_o* is saturated concentration (mg/L), C_o is the actual DO concentration in the broth (mg/L) and the N_{O2} is the rate of oxygen transfer (mg O_2/l.h) (Shuler and Kargi, 2002).

The rate of oxygen uptake is denoted as OUR (oxygen uptake rate) where q_{O2} is the specific rate of oxygen consumption (mgO_2/g dw cells .h) (Shuler and Kargi, 2002

$$OUR = q_0 C_X \qquad (2.27)$$

2.3.3.3.1 Measurement of k_La: Dynamic Method

Dynamic method is a widely used method for the determination of the value of k_La experimentally, and it can be applied during the fermentation

process. This method based on the unsteady-state mass balance for oxygen given by the following question (Scragg, 1988 and Rainer, 1990):

$$\frac{dCo}{dt} = K_L a(Co^* - Co) - q_0 C_X \qquad (2.28)$$

A typical response curve of the dynamic method is given in Figure 2.5. 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 (region-II), equation reduces to:

$$\frac{dC_O}{dt} = r_0 \qquad (2.29)$$

Using equation 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 is valid. Combining equations and rearranging,

$$C_O = -\frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_0\right) + C_O^* \qquad (2.30)$$

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 (Figure 2.7). Modifying equation (2.30):

$$C_O = \frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \qquad (2.31)$$

From the slope of a plot of C_O versus dC_O/dt, the physical mass transfer coefficient, K_La_0, can be determined.

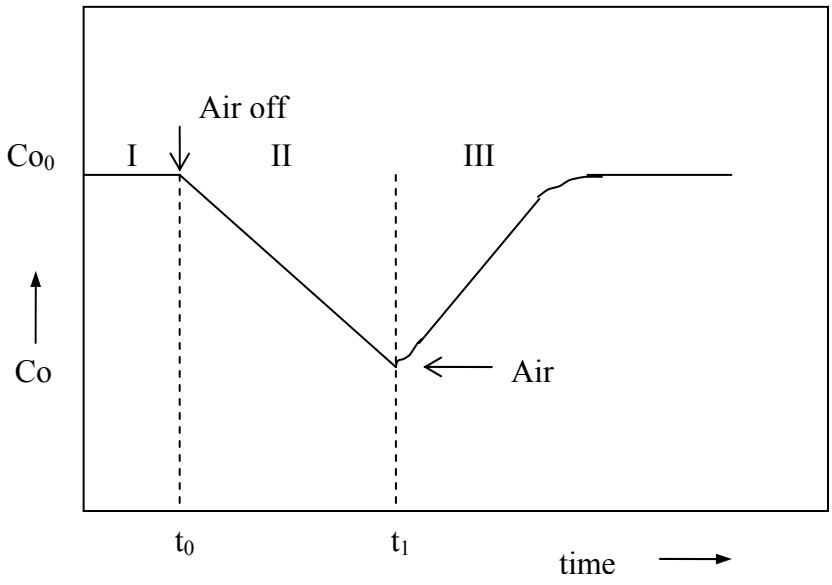

Figure 2.5 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La.

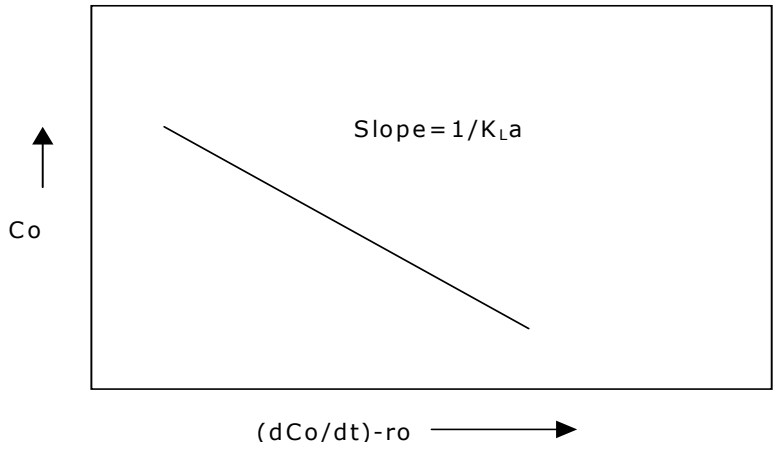

Figure 2.6 Evaluating K_La using the Dynamic Method

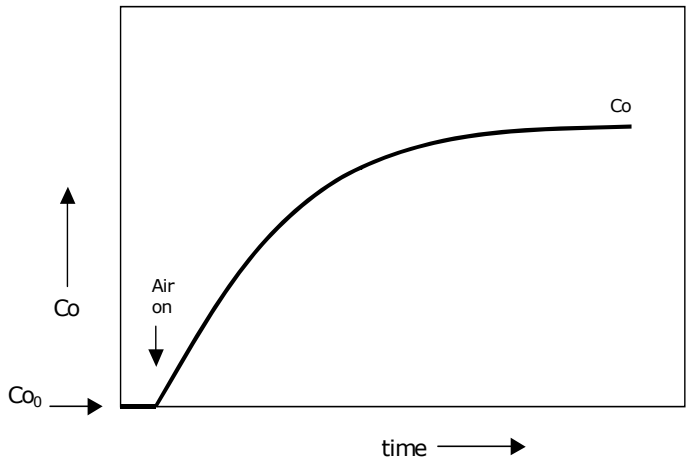

Figure 2.7 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La_0 using the Dynamic Method

2.3.3.3.2 Oxygen Transfer Characteristics

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

$$OD = \frac{\mu_{max} Cx}{Y_{X/O}} \tag{2.32}$$

And the maximum possible mass transfer rate is defined as,

$$OTR_{max} = k_L a C_{DO}^{*} \tag{2.33}$$

Should be determined throughout the bioprocess.

In order to express the oxygen limitation in an aerobic process, the effectiveness factor, η (oxygen uptake rate per maximum possible oxygen utilization rate) and modified Damköhler number, Da (maximum possible

oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2004)) are defined according to the equations given below:

$$\eta = \frac{OUR}{OD} \tag{2.34}$$

$$Da = \frac{OD}{OTR_{\max}} \tag{2.35}$$

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

$$E = \frac{K_L a}{K_L a_o} \tag{2.36}$$

The studies in the literature reporting the effect of oxygen transfer on recombinant benzaldehyde lyase production were published by Çalık et al. (2004, 2006). The study was done in six different conditions with parameters, air inlet rate of Q_o/V_R= 0.5 vvm, and agitation rate of N=250, 375, 500, 750 min^{-1} and at Q_o/V_R= 0.7 vvm, N=750 min^{-1}, in a pilot scale bioreactor with 1.65 dm^3 working volume. The highest BAL activity and cell concentration were obtained at 0.5 vvm, 500 min^{-1} condition as 860 U cm^{-3} and 2.3 Kg m^{-3}, respectively. Oxygen transfer characteristics of OUR and K_La values together with the yield and maintenance coefficients and by-product distribution were also determined. K_La increased with cultivation time and agitation rate, with values changing between 0.008- 0.046 s^{-1}.

Related with other biomolecules production by using *E. coli* as host, Ryan et al. (1989) investigated the effect of oxygen trasfer on β-lactamase production by *E.coli JM103* and reported that the decrease in air inlet rate deceased the cell growth rate. Bhattacharya and Dukey (1997) investigated the over experssion of target gene (MspI methylase) in recombinant *E. coli* K12 in response to dissolved oxygen and oxygen mass transfer and stated that an oxygen-sufficient condition was necessary to obtain optimal experssion and cell productivity. Thereafter, in the research of Castan et al. (2002), the effects of elevated concentration of oxygen and carbon dioxide on human growth hormone production by *E. coli* K12 were investigated. In most of

studies performed with *E. Coli* dissolved oxygen level was controlled at constant value (10-30%) by changing the air flow rate (Luli et al., 1990) or agitation rate in fed batch mode with an automated controller (Akesson et al., 2001; Johnston et al., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

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

3.2 The Microorganism

Escherichia coli BL21 (DE3) pLySs strain carrying the pRSETA::*bal* plasmid (Kaya, 2006) was used as the potential producer of benzaldehyde lyase (BAL, EC 4.1.2.38). The recombinant microorganism is stored in the microbanks (PRO-LAB), by inoculating young colonial growth into cyropreservative fluid present in the vial. After providing the adsorption of microorganism into the porous beads, excess cryopreservative was aspirated and inoculated cyrovial stored at -70^{o}C.

3.3 The Solid Medium

The recombinant *E. coli* strain, stored on agar slants at 4ºC, was inoculated onto the freshly prepared agar slants under sterile conditions, and was incubated at 37ºC overnight on Luria-Bertani (LB) medium (Table 3.1); thereafter inoculated into the precultivation medium.

Table 3.1 The composition of the solid medium.

Compound	Concentration,Kg m^{-3}
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Agar	15.0
Ampicillin	0.1
Chloramphenicol	0.035

3.4 The Precultivation Medium

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

Table 3.2 The composition of the precultivation medium.

Compound	Concentration, kg m^{-3}
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Ampicillin	0.1
Chloramphenicol	0.035

3.5 The Production Medium

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

Table 3.3 The composition of BAL production medium.

Component	Concentration, kg m^{-3}
Glucose	8.0
Na_2HPO_4	6.7
KH_2PO_4	3.1
$(NH_4)_2HPO_4$	5.0
NaCl	0.5
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	1.0×10^{-3}
$ZnSO_4.7H_2O$	0.1×10^{-3}
$MnSO_4.7H_2O$	7.5×10^{-5}
$CuSO_4.5H_2O$	1.0×10^{-5}
Ampicillin	0.1
Chloramphenicol	0.035

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

The pilot scale batch bioreactor with a 3.0 dm^3 volume (B.Braun CT2-2), having a working volume of 1.65 dm^3, and consisting of temperature, pH, foam and stirring rate controls, was used for the investigation of oxygen

transfer effects. 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.

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

3.6 Analysis

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

3.6.1 Cell Concentration

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

3.6.2 Benzaldehyde Lyase Activity

Benzaldehyde lyase activity was determined by measuring the conversion of benzoin into benzaldehyde. Samples from the culture broth were harvested by centrifugation (Sigma 1-15) at 13500 min^{-1} for 10 min at 4°C. In order to determine activity of the intracellular benzaldehyde lyase in r-*E. coli* strains, the cell walls were 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 activity buffer of 40 mM Tris-HCl (pH=8.0), 0.02 mM TPP, 0.2 mM $MgCl_2$ buffer. Fresh substrate solutions were prepared daily by mixing 0.5 volume activity buffer

with 0.35 volume stock benzoin (0.1mM) and 0.15 volume 15%PEG solution with a final concentrations of 0.035 mM benzoin, 20 mM Tris-HCl (pH=8.0), 0.01 mM TPP, 0.1 mM $MgCl_2$, 7.5% PEG and incubated at 37°C. Stock benzoin solution (0.1mM) was prepared in 15% PEG solution and used after overnight incubation at room temperature. The conversion reaction was carried out at 37°C.

Reaction was started by the addition of 20 µl of samples 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):

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

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

3.6.3 Reduced Sugar Concentration

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

$$\text{D - glucose} + O_2 + H_2O \xrightarrow{\text{Glucoseoxydase}} \text{Gluconate} + H_2O_2 \qquad (3.1)$$

$$H_2O_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Iminoquinone} + H_2O \qquad (3.2)$$

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

The method used in analysis of samples is given below:

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

3.6.4 Organic Acids Concentrations

Organic acid concentrations were measured with an organic acid analysis system (Waters, HPLC, Alliance 2695). The method is based on reversed phase HPLC, in which organic acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard organic acids solution. Samples were filtered with 45 μm filters (ACRODISC CR PTFE) and loaded to the analysis system with a mobile phase of 3.12% (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4. Needle and seal wash were conducted with 20% (v/v) acenotrile. The analysis was performed under the conditions specified below:

Column	Capital Optimal ODS, 5μm
Column dimensions	4.6 x250 mm
System	:Reversed phase chromatography
Mobile phase flow rate	:0.8 ml/min

Column temperature	:30 °C
Detector and wavelength	:Waters 2487 Dual absorbance detector, 210 nm
Injection volume	:5 µl
Analysis period	:15 min

3.6.5 Amino Acids Concentrations

Amino acid concentrations were measured with an amino acid analysis system (Waters, HPLC), using the Pico Tag method (Cohen, 1983). The method is based on reversed phase HPLC, using a pre-column derivation technique with a gradient program developed for amino acids. The amino acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard amino acids solution. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to the analysis system with a mobile phase of 6.0% (v/v) acenotrile, 1.79% (w/v) NaAc from solvent A and 66.6% (v/v) acenotrile from solvent B.

The analysis was performed under the conditions specified below:

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

3.6.6 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

In order to determine the liquid phase mass transfer coefficient and oxygen uptake rate in the benzaldehyde lyase production process, the Dynamic Method (Rainer, 1990), as explained in section 2.3.3.3, 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=50 min^{-1} in order to lower the effect of surface aeration.

3.6.7 Saturation oxygen concentration determination

In order to calculate the dissolved oxygen concentration in the fementation during the bioprocesses, saturation oxygen concentrations, C_{O2}^*, were determined. For the saturation oxygen concentration measurements, approximately 1000 ml of fermentation broth was prepared according to the medium composition used at the beginning of the bioprocess and 1000 ml of fermentation broth was collected at the end of each experiment performed in pilot scale bioreactor. Fermentation broth sample was set to 37°C and stirred with a magnetic stirrer. Air was compressed to the fermentation broth and dissolved oxygen concentration was measured by an oxygen probe. After a short period, dissolved oxygen concentration attained a constant value, namely the saturation oxygen concentration, C_{O2}^*.

CHAPTER 4

RESULTS AND DISCUSSION

This study focuses on the effects of oxygen transfer conditions on the intracellular synthesis of the enzyme benzaldehyde lyase in recombinant *E. coli* BL21 (DE3) pLysS. For this purpose, the effects of oxygen transfer together with the mass transfer coefficient (K_La), enhancement factor E ($=K_La/K_La_o$), volumetric oxygen transfer rate, volumetric and specific oxygen uptake rates, mass transfer and biochemical reaction resistances; moreover, the variation in product and by-product distribution, specific substrate uptake rates, yield and maintenance coefficient were investigated on a defined medium with glucose and $(NH_4)_2HPO_4$ as the carbon and nitrogen sources (Table 3.3) in 3.0 dm^3 pilot scale batch bioreactor (Braun CT2-2) equipped with temperature, pH, foam, air inlet and stirring rate controls with V_R=1.65 dm^3 working volume at six different conditions.

4.1 Oxygen Transfer Effects

In aerobic processes, oxygen is a key substrate for cell growth and, therefore, continuous transfer of oxygen from the gas phase to liquid phase is crucial. The effect of oxygen transfer was studied systematically for benzaldehyde lyase production in recombinant *E. coli* BL21 (DE3) pLySs strain by altering the parameters of air inlet rate, Q_O/V_R, agitation rate, N, and dissolved oxygen level, DO, at the stated conditions in Table 4.1.

This study was carried out at six different conditions with the parameters, air inlet rate of Q_O/V_R = 0.5 vvm and agitation rates of N= 250, 500, 625, and 750 min^{-1}, and dissolved oxygen levels DO= 20%, 40% (C_{DO}= 0.04, 0.08 mol m^{-3}) of the saturation, while medium components were C_G= 8.0 kg m^{-3}, C_N= 5.0 kg m^{-3} and salt solution at controlled pH_c=7.2.

The operation conditions applied at Q_o/V_R= 0.5 vvm and N= 250, 500, 625, and 750 min^{-1}, were named as OT_1, OT_2, OT_3, and OT_4 conditions; and the last two operations at DO=20%, 40% were named as OT_5 and OT_6 conditions.

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, dissolved oxygen level, air inlet and stirring rate controls with V_R =1.65x10^{-3} m^3 working volume.

Table 4.1 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	OT_1	N=750, Q_O/V_R= 0.5	OT_4
N=500, Q_O/V_R = 0.5	OT_2	DO=20%	OT_5
N=625, Q_O/V_R = 0.5	OT_3	DO=40%	OT_6

4.1.1 Dissolved oxygen

The variations in dissolved oxygen concentration with cultivation time, air inlet rate and agitation rate are given in Figures 4.1 and 4.2. Solubility of oxygen, C_{O2}^* in cultivation medium at 37°C was found as 0.21 mol m^{-3}.

Dissolved oxygen (DO) level in the fermentation broth depends on the extent of the oxygen transfer rate to the medium and to the oxygen uptake rate of the cells. At all the oxygen transfer conditions, except OT_5 and OT_6 operations, illustrated on Figure 4.1, a generous decrease due to the rapid uptake of dissolved oxygen by the microorganism was observed in the dissolved oxygen concentration profiles but at different rates depending on the amount of oxygen transferred to the medium and amount of oxygen demanded by the microorganism. At OT_1 condition, there was no oxygen accumulation throughout the bioprocesses after t=0.5 h of bioprocess. At OT_2 condition, due to the high oxygen demand of the cells between t=3-11 h, transferred oxygen was totally consumed but oxygen profile suddenly increased at the end of the bioprocesses which indicates that there is a decrease in oxygen uptake of cells.

However at OT_3 and OT_4 conditions dissolved oxygen level decreased in the first 5 hours; after t=5h , due to the decrease in oxygen uptake rate dissolved oxygen level increased.

When the dissolved oxygen concentration profiles of the current study were compared with that of obtained by Çalık et al. (2004), similar trends were observed in the dissolved oxygen concentration profiles at the same conditions.

At the OT_5 and OT_6 conditions, dissolved oxygen levels were controlled at the values of DO= 20, 40% respectively, by keeping agitation rate at constant value of N=625 min^{-1} and by considering air inlet rate as variable parameter. As seen in the figure 4.2 at OT_5 and OT_6 conditions a sharp increase was observed in the air flow rate profiles between t=3-5 h of bioprocess but at different rate depending on oxygen demand of the cells during the growth phase; before starting to decrease at the remaining hours of bioprocess.

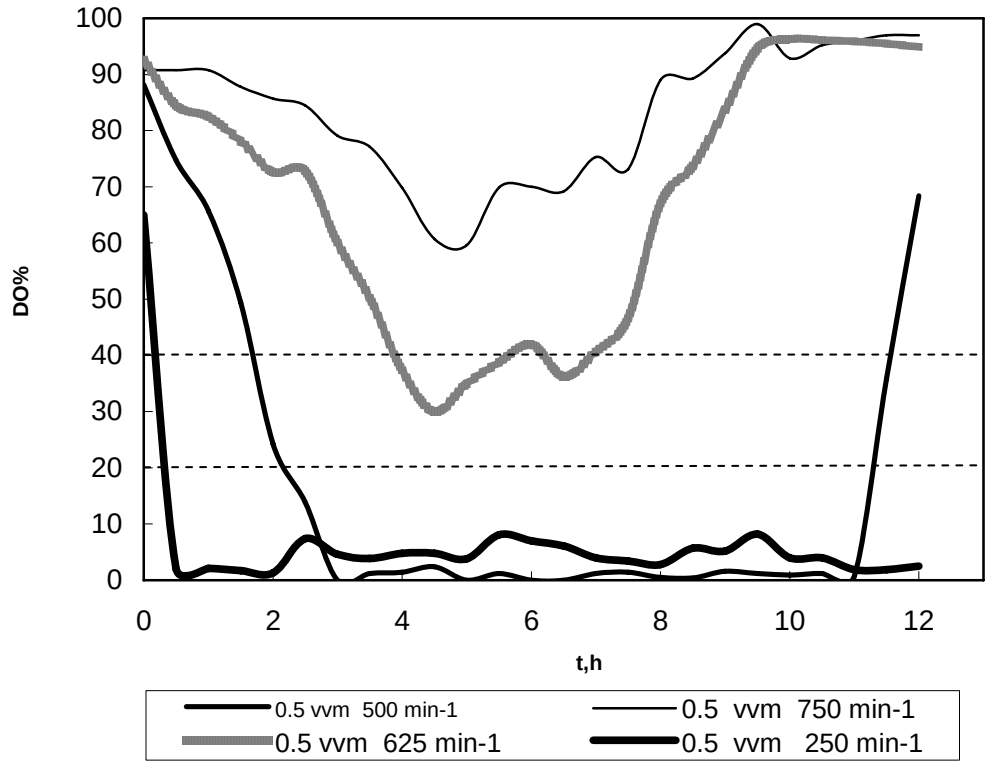

Figure 4.1 The variations in the dissolved oxygen concentration with the cultivation time, agitation and air inlet rates applied. C_G=8.0 Kg m^{-3}, T=37°C, V_R=1.65×10^{-3} m^3, pH_c=7.2.

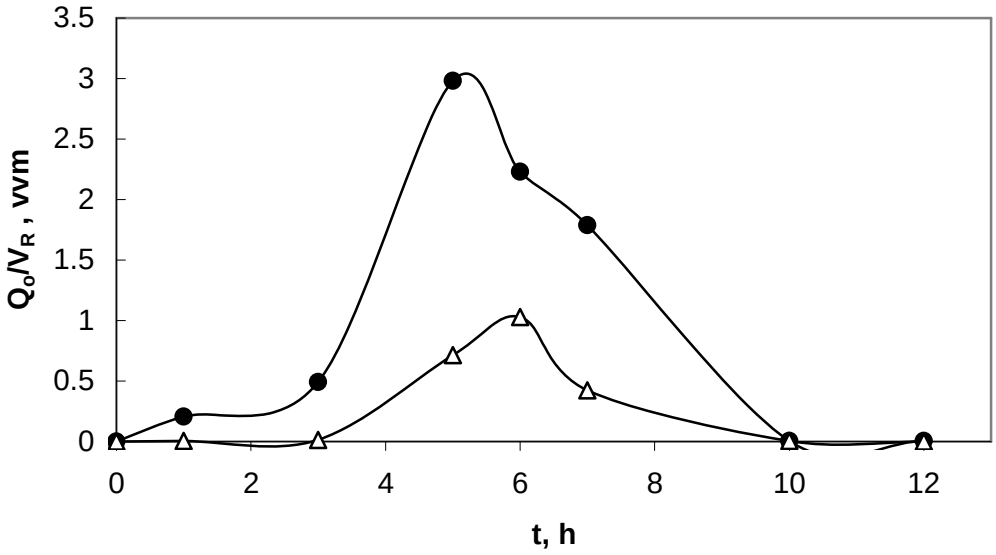

Figure 4.2 The variations in air flow rate with the cultivation time: OT_5 (Δ); OT_6 (●). C_G=8.0 Kg m^{-3}, T=37°C, V_R=1.65×10^{-3} m^3, pH_c=7.2.

4.2 Cell Growth Profiles

The variations in the cell concentration with cultivation time agitation and air inlet rates applied are given in Figure 4.3.

At t=0-4 h of bioprocess, in all conditions except in OT_1 condition, cell formation did not change significantly with respect to the oxygen transfer conditions applied. On the other hand, after t=4 h cell profiles altered considerably throughout the bioprocess. In general, cell growth reached to stationary phase after t=8 h when cell formation rate decreased at all the conditions. The highest cell concentration was obtained at OT_6 condition with the value of C_X=3.0 kg m^{-3} and the lowest cell concentration was observed at OT_1 condition as C_X=1.19 kg m^{-3} where oxygen supply seems to be insufficient for maintenance of oxidative metabolism among the investigated oxygen transfer conditions.

The variation of cell concentration with the cultivation time should be related to the dissolved oxygen concentration of the fermentation broth. In a typical batch culture, the dissolved oxygen concentration decreases in the media along with the growth time, until the microorganism reaches the stationary phase and then the dissolved oxygen concentration increases. This decreasing and increasing pattern in dissolved oxygen concentration with respect to cell growth can be seen well on Figures 4.1 and 4.3.

In the study of Çalık et al. (2004), among the investigated oxygen transfer conditions, the condition with agitation rate of N=500 min^{-1}, air inlet rate of Q_o/V_R=0.5 vvm, and medium composed of 8.0 kg m^{-3} glucose and 5.0 kg m^{-3} $(NH_4)_2HPO_4$ were reported to be the optimum condition in terms of the cell growth with a value of 2.3 kg m^{-3} in recombinant *E. coli K12* carrying pUC18::*bal* plasmid at pilot scale bioreactor with a working volume of 1.65 dm^3. When the cell concentration of OT_2 condition (C_X=2.1 kg m^{-3}) of the current study is compared with that of the same condition (C_X=2.3 kg m^{-3}) of Çalık et al. (2004), it can be concluded that the oxygen requirement of the *E. coli* BL21 (DE3) pLySs strain is higher than that of *E. coli* K12 strain which result in the lower cell concentration in the same condition.

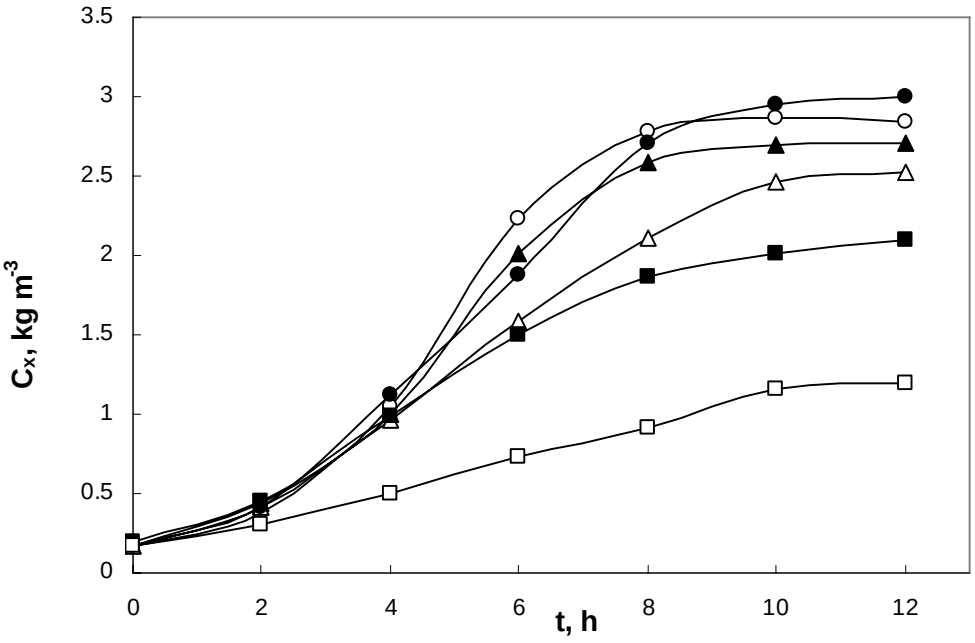

Figure 4.3 The variations in cell concentration with the cultivation time, agitation rates and air inlet rates applied and medium contents: OT_1 (□); OT_2 (■); OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G=8.0 Kg m^{-3}, T=37°C, V_R=1.65×10^{-3} m^3, pH_c=7.2.

4.3 Glucose Concentration Profiles

The effect of oxygen transfer conditions on the consumption of glucose which enters to the carbon metabolism from the beginning of the glycolysis pathway, throughout the process is given in Figure 4.4.

In the first two hours of the bioprocess, the oxygen transfer conditions applied did not affect the glucose consumption rate considerably; because during this period the cell growth profiles did not also change considerably with respect to the different oxygen transfer conditions. On the other hand, between t=2-8 h with the increase in the cell formation rate, glucose consumption rate increased.

At OT_3, OT_4, OT_5, and OT_6 conditions glucose depleted at t=8 h while at OT_1 and OT_2 conditions it was totally consumed at t=10 h of the bioprocess.

Among the investigated oxygen transfer parameters, the highest consumption rate was observed at OT_6 condition, where the cell concentration was the highest; whereas, the lowest glucose consumption rate was attained at OT_1 condition, where the cell concentration was low due to the oxygen limitation. So it can be concluded that glucose concentration profiles coincide with the cell growth at all operation conditions. Where the higher glucose utilization rate was observed, the higher cell growth rate was obtained.

When the glucose concentration profiles of the present study were compared with the findings of Çalık et al. (2004) at the same oxygen transfer conditions, where the glucose consumption rates were the lower, it can be concluded that, *E. coli* BL21 (DE3) pLySs strain requires more glucose as carbon source to maintain the optimum state of cell growth than *E. coli* K12 dose.

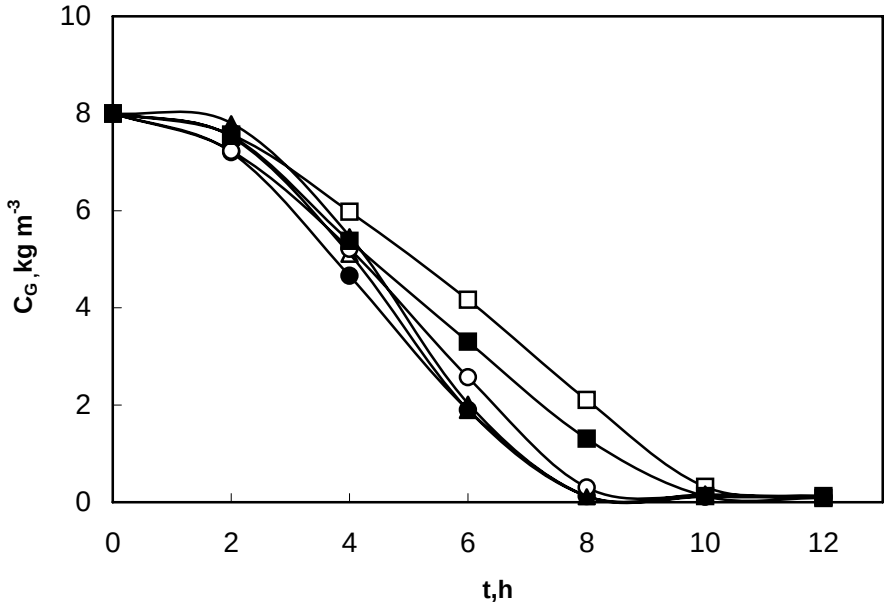

Figure 4.4 The variations in glucose concentration with the cultivation time, agitation rates and air inlet rates applied and medium contents: OT_1 (□); OT_2 (■); OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G=8.0 Kg m^{-3}, T=37°C, V_R=1.65×10^{-3} m^3, pH_c=7.2.

4.4 Benzaldehyde Lyase Activity Profiles

The variations of benzaldehyde lyase volumetric activity with the cultivation time at the oxygen transfer conditions applied are given in Figure 4.5.

The highest benzaldehyde lyase volumetric activity was obtained at OT_6 condition (A=1095 U/cm^3), where the highest cell concentration and the highest glucose consumption rate was obtained. On the other hand, at t=12 h the lowest benzaldehyde lyase volumetric activity (A=459 U cm^{-3}) was obtained at OT_1 condition among the investigated oxygen transfer conditions.

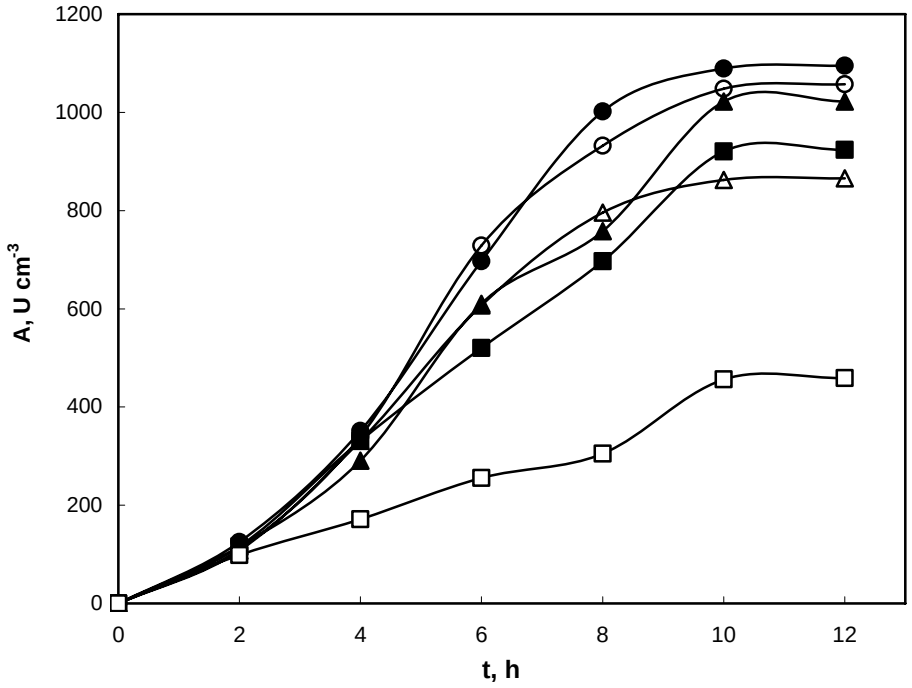

Figure 4.5 The variations in benzaldehyde lyase volumetric activity with the cultivation time, agitation and air inlet rates applied: OT_1 (□); OT_2 (■); OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G=8.0 Kg m^{-3}, T=37°C, V_R=1.65×10^{-3} m^3, pH_c=7.2.

When benzaldehyde lyase volumetric activity (A=923 U cm^{-3}) at OT_2 condition of the current study is compared with the optimum benzaldehyde lyase volumetric activity (A=860 U cm^{-3}) of Çalık et al. (2004), it is concluded that, the capability of *E. coli* BL21 (DE3) pLySs strain in the benzaldehyde lyase production is better than *E. coli* K12 strain. Since in production, the aim is to obtain the maximum volumetric benzaldehyde lyase activity and cell concentration; it is essential to increase the concentration of the benzaldehyde lyase producing cells. Therefore, it is concluded that to increase benzaldehyde lyase production by *E. coli* BL21 (DE3) pLySs strain , the bioreactor should be operated at OT_6 (DO=40%) condition, where the highest amount of the cell was formed.

In most of the studies performed with *E. coli*, dissolved oxygen level was kept constant ranging between 10-30% by either changing the air flow rate (Luli et al., 1990) or agitation rate in fed batch processes with automated control systems (Akesson et al., 2001; Johnston et al., 2003). Similarly, in this study the optimum condition in terms of cell concentration and benzaldehyde lyase activity was achieved at constant dissolved oxygen level determined by changing the air flow rate, and the optimum constant dissolved oxygen value was obtained at OT_6 condition as DO=40%. When the air inlet flow rate profile of OT_5 operation was compared with that of OT_6, it can be concluded that the insufficient oxygen supply was the reason of low cell concentration and enzyme activity at this condition.

4.5 Organic Acid Concentration Profiles

The variations in the organic acids detected in the fermentation broth with the cultivation time and oxygen transfer conditions were demonstrated in Table 4.2.

Apparently, for all oxygen transfer conditions, acetic acid is the major by-products in the fermentation medium. One of the main challenges in total productivity in *E. coli* cultivation is onset of acetic acid production which is known to inhibit the cell growth and recombinant protein production (Shiloach et al., 1996; Johnston et al., 2003; Akesson et al., 2001; Luli et al., 1990; Çalık et al., 2004, 2006). The reason is the accumulation of undissociated

(protonated- CH_3COOH) form of acetic acid in the medium. Since undissociated state of acetate is lipophilic, it can freely permeate the cell membrane and accumulate in the medium. When a fraction of undissociated acid present extracellularly re-enters the cell, it dissociates (ionized- $CH3COO^-$) at the relatively higher intracellular pH. When this process continues, the intracellular pH decreases and hence the ΔpH component of the promotive force collapses. Thus, apart from energy consumption for rapid growth, *E. coli* requires energy for maintenance of optimum intracellular pH which leads to inhibition on cellular efficiency (Stephanopoulos, 1998; Luli et al., 1990).

E. coli BL21 (DE3) pLySs strain was derived from *E. coli* B which was reported to be a low acetate producer (Luli et al., 1990; Shiloach et al., 1996). In general, formation of acetate in *E. coli* occurs when oxygen limitation and excess carbon source, especially glucose, are present. The latter cause of aerobic acidogenesis under glucose mediated fermentations is known as Crabtree effect or overflow metabolism (Doelle et al., 1982).

Table 4.2 Organic acids detected in the fermentation broth with cultivation time at the oxygen transfer conditions applied.

OT_1, C_{OA} ($kg.m^{-3}$)						
Time(h)	2	4	6	8	10	12
Ac	0.1148	0.5902	0.8430	1.3836	3.1608	2.6332
Form	0.0060	0.0969	0.0713	0.1037	0.0777	0.0557
Cit	0.0747	0.1185	0.1977	0.3535	0.7992	0.9495
Mal	-	-	-	-	-	-
Suc	-	-	-	-	-	-
T_{OA}	**0.1955**	**0.8056**	**1.1120**	**1.8408**	**4.0377**	**3.6384**
OT_2, C_{OA} ($kg.m^{-3}$)						
Time(h)	2	4	6	8	10	12
Ac	0.0778	0.2518	0.6958	1.7248	1.0952	1.4076
Form	0.0044	0.0086	0.0013	0.0288	0.0183	0.0181
Cit	0.0898	0.1138	0.2409	0.3006	0.0875	0.0770
Mal	-	-	-	-	-	-
Suc	-	-	-	0.0232	0.0707	0.0614
T_{OA}	**0.1720**	**0.3742**	**0.9380**	**2.0774**	**1.2717**	**1.5641**

OT_3, C_{OA} ($kg.m^{-3}$)

Time (h)	2	4	6	8	10	12
Ac	0.0504	0.1722	0.2688	0.3512	0.5940	0.4680
Form	-	0.0482	0.2838	0.8282	0.0213	0.0090
Cit	0.0950	0.0173	0.1767	0.1823	0.1023	0.0883
Mal	-	-	-	0.1590	0.1886	0.1433
Suc	-	0.0189	0.0408	-	0.1460	0.1075
T_{OA}	**0.1454**	**0.2566**	**0.7701**	**1.5207**	**1.0522**	**0.8161**

OT_4, C_{OA} ($kg.m^{-3}$)

Time (h)	2	4	6	8	10	12
Ac	0.0596	0.1640	0.2596	0.3448	0.3824	0.3596
Form	0.0044	0.0093	0.3000	0.4460	0.0068	0.0130
Cit	0.0878	0.0986	0.1645	0.1828	0.0784	0.0735
Mal	-	-	0.0582	0.1340	0.1322	0.1261
Suc	-	0.0215	-	-	0.0780	0.0740
T_{OA}	**0.1518**	**0.2934**	**0.7823**	**1.1076**	**0.6778**	**0.6462**

OT_5, C_{OA} ($kg.m^{-3}$)

Time (h)	2	4	6	8	10	12
Ac	0.0596	0.2892	0.4016	0.5108	0.6436	0.4976
Form	-	0.0620	0.1530	0.2605	0.0198	0.0075
Cit	0.0864	0.1161	0.1357	0.1807	0.1527	0.0392
Mal	-	-	-	0.0907	0.1387	0.1087
Suc	-	0.0114	0.0235	0.0169	0.0819	0.0603
T_{OA}	**0.1460**	**0.4787**	**0.7138**	**1.0596**	**1.0367**	**0.7133**

OT_6, C_{OA} ($kg.m^{-3}$)

Time (h)	2	4	6	8	10	12
Ac	0.0498	0.2322	0.3118	0.4064	0.3468	0.3388
Form	0.0387	0.0390	0.2079	0.2079	0.0333	0.0880
Cit	0.0954	0.0982	0.1451	0.2424	0.0494	0.0491
Mal	-	0.0076	-	0.1474	0.1035	0.1233
Suc	0.0155	0.0288	0.0684	0.0654	0.0920	0.1127
T_{OA}	**0.1994**	**0.4058**	**0.7332**	**1.0695**	**0.6250**	**0.7119**

Almost for all the oxygen transfer conditions applied, acetic, formic, citric, malic and succinic acids were detected in the fermentation broth. Presence of

the organic acids of the TCA cycle, i.e., citric, succinic and malic acid may be a consequence of inadequate oxygen supply which leads to repression on the TCA cycle enzymes or their improper activity. Apart from the effect of limiting oxygen, due to glucose depletion at t=8h, the lower amount of organic acid at t=10 was obtained in almost all oxygen transfer conditions. Similarly, in the study of Çalık et al. (2004), under oxygen limiting conditions the TCA cycle organic acids of α-ketoglutaric acid and succinic acid were found in the medium showing insufficient operation of the TCA cycle under low oxygen transfer conditions and high acetate accumulation, up to 2.5 kg m^{-3} was observed, where recombinant *E. coli* K12 was the host microorganism.

As seen in the figure 4.6 the highest acetate accumulation occurred in OT_1 condition, where the lowest cell concentration and the lowest glucose consumption rate were observed, as C_{AC}=3.16 kg m^{-3} at t=10 h; and the lowest amount of the acetate was detected at OT_6 condition, where the highest cell concentration was obtained, as C_{AC}=0.34 kg m^{-3} at t=12h. All the oxygen transfer conditions except OT_2 and OT_6, show similar trend in terms of acetate accumulation in the fermentation broth. Acetate concentration increases and reaches a peak value at t=10h (except OT_2), then decreases (Figure 4.6). However in OT_2 condition acetate concentration gives a maximum at t=8 h. The change in the metabolic activity can be explained by activation of the acetate utilization pathways in response to an acetate accumulation in the fermentation broth, or a decrease in the glucose uptake rate, or with their concerted effect (Shiloach et al., 1996).

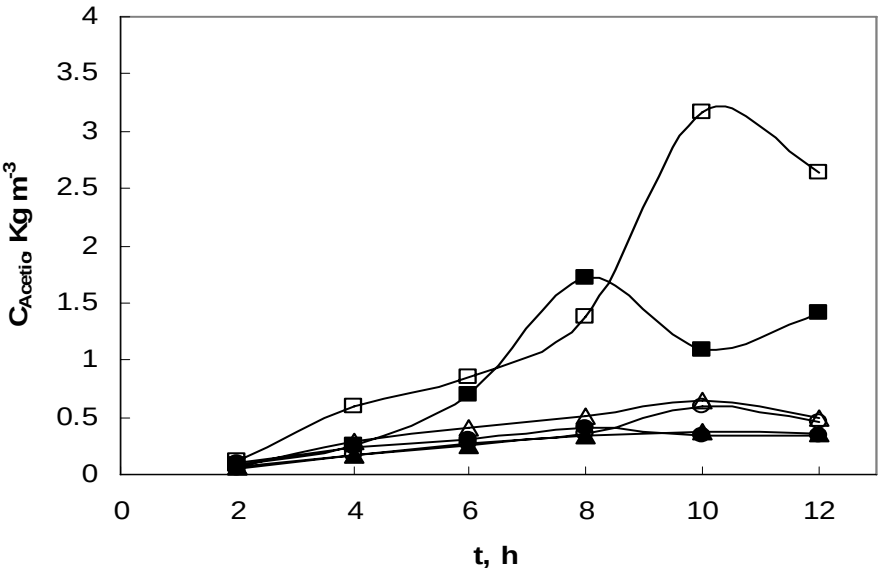

Figure 4.6 The variations in acetic acid concentration with the cultivation time, agitation and air inlet rates: OT_1 (□); OT_2 (■); OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o =8.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, pH_c=7.2.

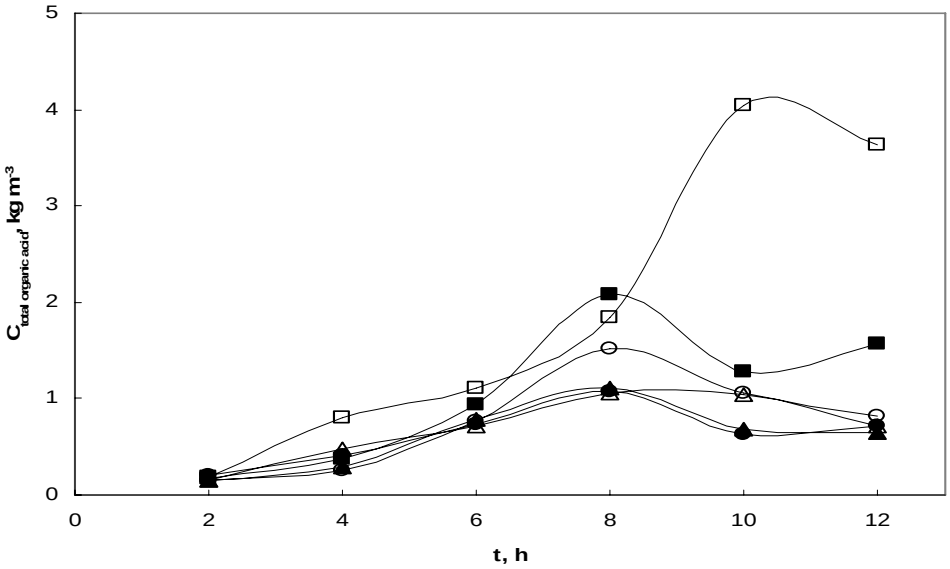

Figure 4.7 The variations in total organic acid concentration with the cultivation time, agitation and air inlet rates: OT_1 (□); OT_2 (■); OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o =8.0 kg m^{-3}, T=37°C, V_R=1.65x10^{-3} m^3, pH_c=7.2.

4.6 Amino Acid Concentration Profiles

The variations in amino acid concentrations with the cultivation time with respect to oxygen transfer conditions used were listed in Table 4.3.

Most microorganisms have the metabolic machinery to synthesize all essential amino acids from carbon and nitrogen sources for the production of proteins. Aspartic acid, asparagine, cysteine, glutamic acid, isoleucine, leucine, lysine, serine, threonine, tyrosine, valine, and ornithine are the amino acids that were detected in the medium, but in very low amounts. The highest total amino acids produced was at OT_6 condition at t=10 h as T_{AA}=0.1737 kg m^{-3}, followed by OT_1 condition as a T_{AA}=0.0734 kg m^{-3}. For all conditions studied, aspartic acid was the major amino acid excreted, which indicates that the demand for aspartic acid should be lower than the produced amount, thus it was excreted to the fermentation broth.

Table 4.3 The variations in amino acid concentrations with the cultivation time at the oxygen transfer conditions applied.

	OT_1, C_{AA} (kg.m^{-3})						
Time (h)	0	2	4	6	8	10	12
Asp	0.0128	-	0.012	0.038	-	0.015	-
Asn	0.0	-	0.0	0.019	-	0.011	-
Cys	0.0056	-	0.0016	0.0	-	0.0	-
Glu	0.0	-	0.0	0.0	-	0.0	-
Iso	0.0	-	0.0	0.0	-	0.0	-
Leu	0.0	-	0.01	0.087	-	0.038	-
Lys	0.0095	-	0.0	0.0	-	0.0	-
Ser	0.0044	-	0.0	0.0028	-	0.0014	-
Orn	0.0	-	0.0	0.0	-	0.0	-
Thr	0.073		0.0	0.0	-	0.0	-
Tyr	0.0	-	0.018	0.017	-	0.008	-
Val	0.039	-	0.006	0.0	-	0.0	-
T_{AA}	**0.1443**	**-**	**0.0476**	**0.1638**	**-**	**0.0734**	**-**

OT_2, C_{AA} ($kg.m^{-3}$)							
Time (h)	0	2	4	6	8	10	12
Asp	0.0128	-	0.018	0.011	-	0.0	-
Asn	0.0	-	0.0	0.0	-	0.0	-
Cys	0.0056	-	0.01	0.008	-	0.0	-
Glu	0.0	-	0.0	0.0	-	0.0	-
Iso	0.0	-	0.0	0.0	-	0.0	-
Leu	0.0	-	0.0	0.0	-	0.0	-
Lys	0.0095	-	0.0	0.0	-	0.0	-
Ser	0.0044	-	0.0	0.0	-	0.0	-
Orn	0.0	-	0.0	0.0	-	0.0	-
Thr	0.073		0.0	0.0	-	0.0	-
Tyr	0.0	-	0.0	0.0	-	0.0	-
Val	0.039	-	0.0	0.0	-	0.0	-
T_{AA}	**0.1443**	**-**	**0.028**	**0.019**	-	**0.0**	**-**

OT_3, C_{AA} ($kg.m^{-3}$)							
Time (h)	0	2	4	6	8	10	12
Asp	0.0128	-	0.0	0.016	-	0.03	-
Asn	0.0	-	0.01	0.0	-	0.0	-
Cys	0.0056	-	0.0	0.0	-	0.0	-
Glu	0.0	-	0.0	0.0	-	0.0	-
Iso	0.0	-	0.0004	0.002	-	0.0	-
Leu	0.0	-	0.008	0.0	-	0.0	-
Lys	0.0095	-	0.0	0.0	-	0.0	-
Ser	0.0044	-	0.01	0.009	-	0.0045	-
Orn	0.0	-	0.0	0.0	-	0.002	-
Thr	0.073	-	0.0	0.0	-	0.0	-
Tyr	0.0	-	0.0	0.006	-	0.006	-
Val	0.039	-	0.0	0.0	-	0.0	-
T_{AA}	**0.1443**	**-**	**0.0284**	**0.033**	-	**0.0425**	**-**

OT_4, C_{AA} ($kg.m^{-3}$)							
Time (h)	0	2	4	6	8	10	12
Asp	0.0128	-	0.207	0.016	-	0.014	-
Asn	0.0	-	0.0	0.0	-	0.0	-
Cys	0.0056	-	0.0	0.0	-	0.0	-
Glu	0.0	-	0.0	0.0	-	0.0	-
Iso	0.0	-	0.0	0.0	-	0.0	-
Leu	0.0	-	0.0057	0.0	-	0.0	-
Lys	0.0095	-	0.006	0.0	-	0.0046	-
Ser	0.0044	-	0.003	0.005	-	0.0035	-
Orn	0.0	-	0.0	0.0	-	0.0	-
Thr	0.073	-	0.0	0.0	-	0.0	-
Tyr	0.0	-	0.01	0.0	-	0.0061	-
Val	0.039	-	0.0	0.0	-	0.0	-
T_{AA}	**0.1443**	**-**	**0.2287**	**0.021**	-	**0.0282**	**-**

OT_5, C_{AA} ($kg.m^{-3}$)							
Time (h)	0	2	4	6	8	10	12
Asp	0.0128	0.0267	0.011	0.015	0.012	0.012	0.011
Asn	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cys	0.0056	0.003	0.0	0.0	0.0	0.003	0.0
Glu	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Iso	0.0	0.0	0.0	0.0025	0.0	0.0	0.0017
Leu	0.0	0.0	0.0	0.013	0.0	0.0	0.0
Lys	0.0095	0.0	0.0	0.0	0.0043	0.012	0.0
Ser	0.0044	0.0031	0.003	0.008	0.0079	0.007	0.0055
Orn	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	0.073	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.0	0.008	0.0	0.0	0.0034	0.004	0.0
Val	0.039	0.0	0.0	0.0	0.0	0.0	0.0
T_{AA}	**0.1443**	**0.0408**	**0.014**	**0.0385**	**0.0276**	**0.038**	**0.0182**

OT_6, C_{AA} ($kg.m^{-3}$)							
Time (h)	0	2	4	6	8	10	12
Asp	0.0128	0.0	0.0	0.0	0.196	0.0	0.0
Asn	0.0	0.0	0.0	0.0	0.01	0.017	0.0
Cys	0.0056	0.0043	0.0	0.014	0.0055	0.0	0.0
Glu	0.0	0.0	0.0	0.0	0.0	0.009	0.0
Iso	0.0	0.0	0.0032	0.0	0.0	0.0	0.0
Leu	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lys	0.0095	0.0079	0.011	0.0096	0.016	0.145	0.001
Ser	0.0044	0.0049	0.0044	0.017	0.0	0.0027	0.0099
Orn	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	0.073	0.0	0.0	0.0	0.054	0.0	0.0
Tyr	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Val	0.039	0.0	0.0	0.0	0.016	0.0	0.0
T_{AA}	**0.1443**	**0.0171**	**0.0186**	**0.0406**	**0.2975**	**0.1737**	**0.0109**

4.7 Oxygen Transfer Characteristics

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r_0, and oxygen transfer coefficient, K_La. 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, enhancement factor E (=KLa/KLao), oxygen uptake rate (OUR), oxygen transfer rate (OTR),

maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor (η) calculated throughout the bioprocess are given in Table 4.4.

Volumetric mass transfer coefficients (K_La) and the enhancement factor (E) found were increased with the increase in the oxygen transfer, i.e. Q_O/V_R and N, at all the operation conditions except OT_5 and OT_6. The low values of K_La could be explained as result of the high values of driving force at OT_5 and OT_6 conditions where dissolved oxygen levels were controlled at the values of DO= 20, 40%, respectively.

At all the operation conditions K_La and E values were higher in the growth phase of the microorganism than in the benzaldehyde lyase production phase. K_La depends on agitation rate, temperature, and rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. Temperature and agitation rate were kept constant throughout the bioprocess, therefore the reason for the observed decrease at some conditions in K_La, could be due to the increase in viscosity of the medium due to the 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 by increasing particle diameter; therefore, along with the increase in the concentration of the rod-shaped micro-bioreactors, the mass transfer coefficient may increase or decrease depending on the diameter and concentration of the biomass (Çalık et al., 1998). The highest K_La and consequently E were obtained in OT_4 operation at t=5 h of the bioprocess as 0.039 s^{-1} and 1.86 respectively. The lowest K_La value as 0.005 s^{-1} at t=10 h of the bioprocess was obtained in OT_6 condition where the highest cell concentration was observed.

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
	0.25	0.008	1.05	1.08	1.68	0.70	1.71	1.02	0.41
	1	-	-	-	-	-	-	-	-
OT_1	3	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-
	0.25	0.017	1.42	1.03	3.57	1.2	1.27	0.36	0.94
	1	0.019	1.58	1.47	3.99	1.5	1.66	0.42	0.90
OT_2	3	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-
	0.25	0.022	1.57	0.63	4.62	0.90	0.99	0.22	0.90
	1	0.023	1.65	0.74	4.83	1.3	2.08	0.43	0.62
OT_3	3	0.025	1.78	2.14	5.25	2.2	3.62	0.69	0.60
	5	0.021	1.5	2.63	4.41	2.2	7.07	1.60	0.31
	10	0.020	1.43	0.2	4.20	0.40	81.69	19.45	0.005
	0.25	0.034	1.62	0.81	7.56	1.10	1.18	0.16	0.93
	1	0.036	1.71	0.85	6.93	1.30	1.66	0.22	0.78
OT_4	3	0.038	1.81	1.92	8.19	2.40	4.13	0.52	0.58
	5	0.039	1.86	2.48	8.61	2.50	7.74	0.94	0.32
	10	0.037	1.76	0.55	5.88	0.60	138.81	17.86	0.04
	0.25	0.007	-	0.90	1.41	0.90	1.18	0.83	0.76
	1	0.008	-	1.20	1.76	1.20	1.69	0.96	0.71
OT_5	3	0.011	-	1.50	2.28	1.50	3.37	1.48	0.44
	5	0.008	-	1.40	1.75	1.40	5.11	2.93	0.27
	10	0.007	-	0.80	1.44	0.80	56.21	38.99	0.01
	0.25	0.007	-	0.90	1.37	0.90	1.29	0.94	0.69
	1	0.01	-	1.20	2.16	1.20	1.98	0.92	0.60
OT_6	3	0.017	-	2.00	3.57	2.00	3.29	0.92	0.60
	5	0.014	-	1.90	2.91	1.90	8.26	2.84	0.23
	10	0.005	-	0.60	1.07	0.60	60.66	56.42	0.01

The dynamic method was applied to the conditions where there is no reaction, i.e., r_o=0 and physical K_La, K_La_o, values were determined to investigate the influence of operating parameters, e.g, the stirring speed and the gas flow rate, on the volumetric mass transfer condition in model media. K_La_o values increased with the increase in the oxygen transfer condition, as expected, and E (=K_La/K_La_o) values varied between 1.05 and 1.86. In the study of Çalık et al. (2004), it was also shown that physical K_La_o values were increasing with oxygen amount supplied to the reactors ranging between 0.007-0.020.

Enhancement factor is a measure of relative rates of the mass transfer and biochemical reactions and presence of the cells in the mass transfer zone. The low E values obtained show that slow reaction is accompanied with mass transfer, as expected in most of the fermentation processes accomplished in stirred bioreactors (Çalık et al., 2004).

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). The oxygen uptake rate, OUR, which depends on the cultivation time and cell concentration is one of the important factors of the bioprocess. The change in the OUR values with the cell concentration is shown in Figure 4.8. Firstly, the OUR increased during the lag and exponential phases of the microbial growth and reach to their maximum values between t=3-5 h of the bioprocess. The highest OUR value was obtained at t=5h of OT_4 condition as 0.0025 $molm^{-3}s^{-1}$, and the lowest one was attained at t=10 h of OT_3 operation as 0.0004 $molm^{-3}s^{-1}$. The oxygen transfer rate first increased, and then decreased with the cultivation time (Table 4.4). Although higher oxygen transfer rates were obtained at OT_3 and OT_4 conditions when compared to OT_6 condition, the cell formation and hence benzaldehyde lyase activity was lower than that of OT_6 as a result of the inhibition of oxygen on the metabolism of *E. coli* BL21 (DE3) pLySs. Due to the rapid decrease of the dissolved oxygen in the first hours of the bioprocess at the OT_1 and OT_2 oıperation conditions, k_La and the other oxygen transfer parameters can not to be calculated at the remaining hours of the bioprocess.

In order to analyze the limiting step of the growth process, the maximum possible OUR (OD) and the maximum oxygen transfer rate were calculated in the course of the fermentation. Relation between both maximum rates is the modified Damköhler number, Da (Çalık et al., 2000). The evolution of Da with cell concentration was shown in Figure 4.9. As seen in the Figure and Table 4.4, Da highly depends on the stirrer speed. The highest and lowest profiles were achieved OT_5 and OT_4 respectively. It can be concluded that OT_5 operation was a mass-transfer-limited condition, which resulted a higher profile of Da. Da increased, when the maximum oxygen transfer rate decreased by the increase in mass transfer resistance. As OT_4 condition attained the highest maximum oxygen transfer rate profile and hence the lowest mass transfer resistance, it had the lowest profile for Da.

The effectiveness factor, η, defined as the ratio between the observed biochemical reaction rate and the formal reaction rate, without mass transfer (Calık et al., 2000). As seen in Table 4.4, η took value close to 1.0 at OT_2, OT_3 and OT_4 conditions at t=0-0.25 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 OT_1, OT_5 and OT_6 conditions, starting from the beginning of the processes, cells consumed oxygen lower than the oxygen demand (OD) due to the low oxygen transfer rate.

Film effectiveness factor, η, is inversely proportional with Da and cell concentration and this proportionally can be observed in Figures 4.10 and 4.11. As the maximum oxygen utilization rate increased with increase in the cell concentration, Da increased and consequently, η decreased which is inversely proportional to the maximum oxygen utilization rate.

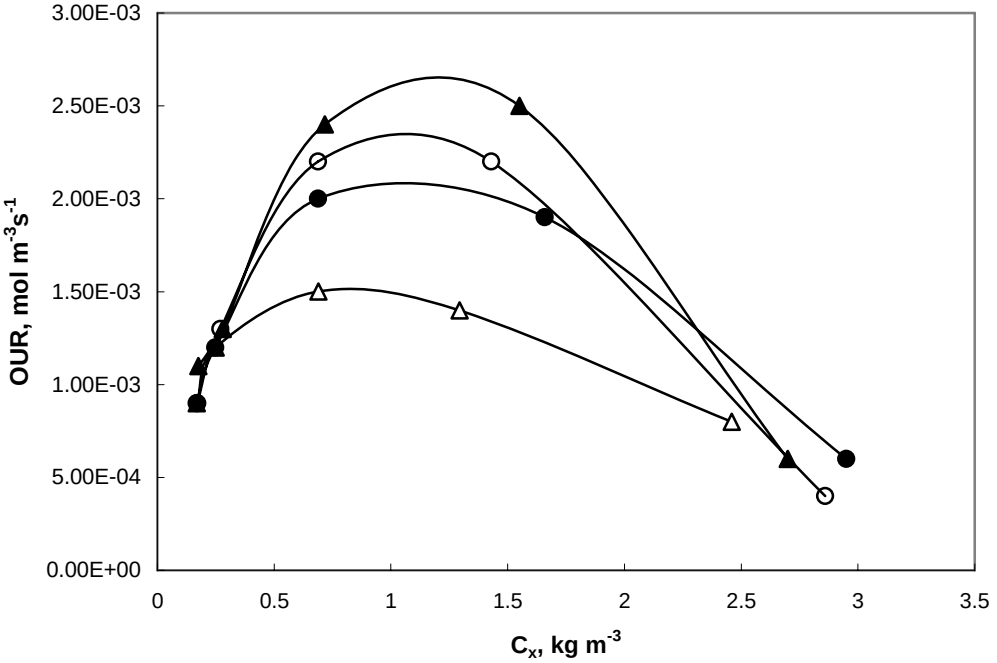

Figure 4.8 The variation of OUR with cell concentration, agitation and air inlet rates: OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o = 8.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, pH_c=7.2.

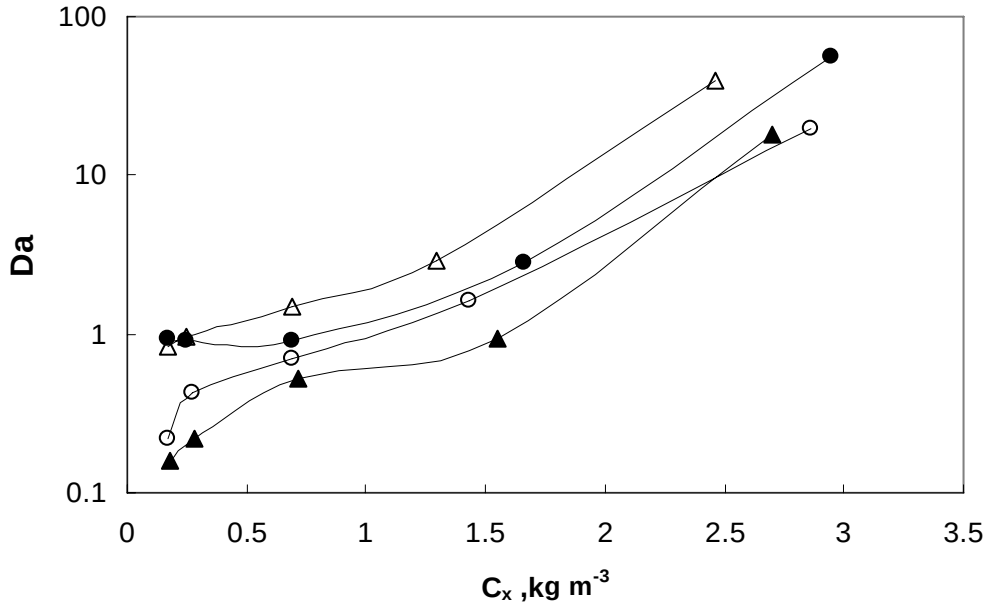

Figure 4.9 The variation of Da (OD/OTR_{max}) with cell concentration, agitation and air inlet rates: OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o = 8.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, pH_c=7.2.

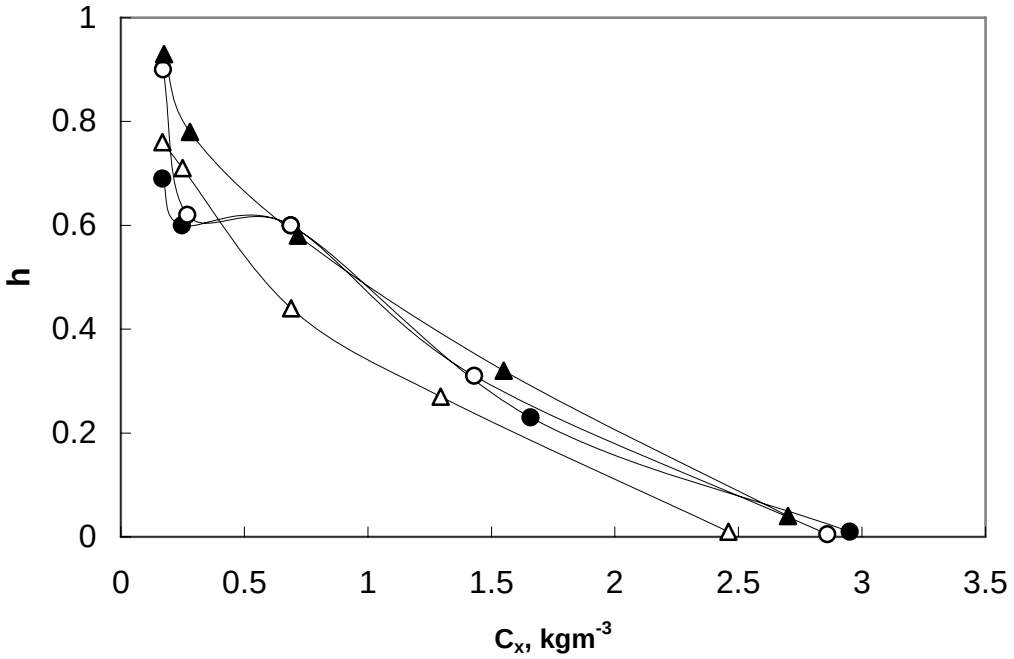

Figure 4.10 The variation of η (OUR/OD) with cell concentration , agitation and air inlet rates:OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o = 8.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, pH_c=7.2.

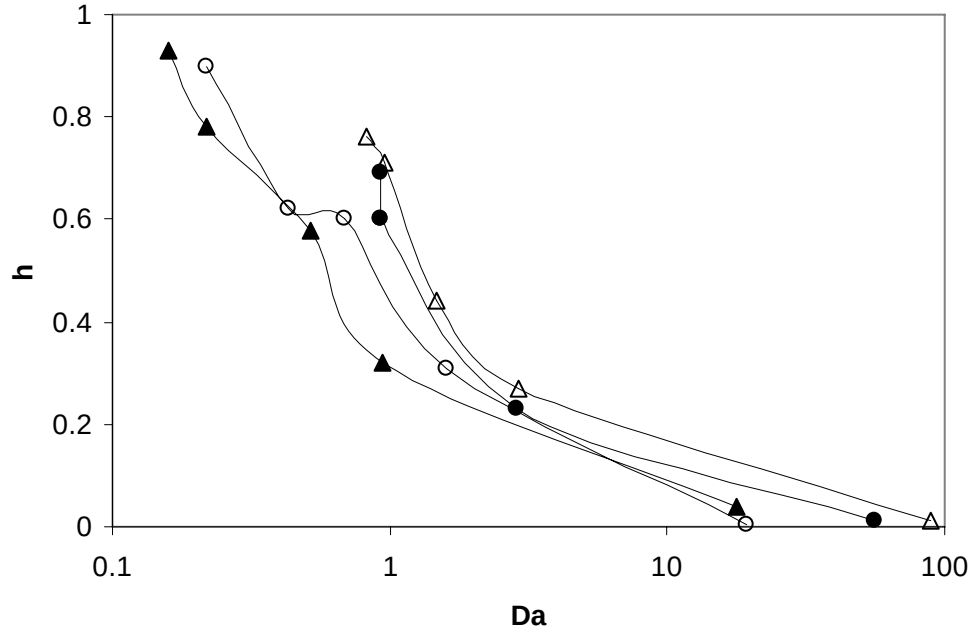

Figure 4.11 The relationship between Da and η at different oxygen transfer conditions: OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o = 8.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, pH_c=7.2.

4.8 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_0, maintenance coefficients for oxygen, m_0 and m_0', and maintenance coefficients for substrate, m_s and m_s' and the yield coefficients with the cultivation time are given in Table 4.6.

As it is seen in the Figure 4.12, specific growth rates decreased with the cultivation time and approaches to 0 as the cells get closer to the stationary phase at all the oxygen transfer conditions, as expected; but the decrease in specific growth rate was higher at high oxygen transfer conditions. The highest specific growth rate (μ) was obtained as μ_{max}=0.81 h^{-1} at t=0.25 h at OT_2 condition and the highest cell concentration was achieved among the operation conditions at OT_6 as C_x=3.0 Kgm^{-3} at t=12h. The maximum specific growth rate in the study of Çalık et al. (2004) was reported as 1.19 h^{-1} at t=1 h at HOT_1 (N=750 min^{-1}, Q_o/V_R=0.5 vvm) condition and the same trend in the specific growth rate values was observed.

Specific oxygen uptake rates (q_O) and specific substrate utilization rates (q_S) decreased with the cultivation time between t=1-10 h of bioprocess at all the oxygen transfer conditions. The highest value of q_O was obtained at t=1 h of OT_4 and OT_2 conditions as 0.72 kg kg^{-1} h^{-1}; where the lowest value was obtained at t=10 h of OT_3 condition as 0.016 kg kg^{-1} h^{-1}. The highest value of q_S was obtained at t=1 h of OT_6 condition as 1.97 kg kg^{-1} h^{-1}.

The yield coefficients, which represent the overall distribution and the efficiency of conversion reactions, were given in Table 4.6. The highest overall specific cell yield on substrate ($Y_{X/S}$) value was obtained at OT_6 as 0.354, due to the increased efficiency of the utilization of the carbon source for product formation; while the lowest value was 0.13 obtained at OT_1 condition.

Cell yield on oxygen values increased between t=1-5 h of bioprocess with cultivation time and took the maximum value between t=3-5 h before the reduction at the remaining time of the process, where the cell growth reached to stationary phase. The highest $Y_{X/O}$ value was obtained at OT_5 condition at

t=5 h as 1.88 kg kg^{-1} followed by OT_6 condition with a value of 1.55 kg kg^{-1}at t=3 h. The lowest $Y_{X/O}$ value was obtained at OT_4 at t=10 h as 0.14 kg kg^{-1} indicating the inefficient use of the oxygen through biochemical reaction network, probably due to the inhibition effect of oxygen on metabolism.

Similarly, the amount of substrate metabolized per amount of oxygen used ($Y_{S/O}$) increased between t=1-5 h of bioprocess with cultivation time and took the maximum value at t=5 h before the reduction at the remaining time of the process signaling either the increased energy requirement or decrease in the efficiency of energy metabolism with the cultivation time (çalık et al., 2006). The highest $Y_{S/O}$ was obtained as 6.54 kg kg^{-1} at OT_6 condition at t= 5 h; while the lowest $Y_{S/O}$ was obtained as 0.68 kg kg^{-1} at OT_5 condition at t= 10 h (Table 4.6). The amount of substrate consumed per amount of oxygen utilized, $Y_{S/O}$, values were higher in *E. coli* BL21 (DE3) pLySs cells relative to *E. coli* K12 cells (Çalık et al., 2004) at the exponential phase of the processes. Therefore, there can be suggested that *E. coli* BL21 (DE3) pLySs cells require much more energy for effective glucose utilization to maintain the optimum state of cell growth than *E. coli* K12 does.

From the slope of the plot of $1/Y_{X/O}$ versus $1/\mu$, the rate of oxygen consumption for maintenance were obtained, and the variation of m_O and m_O' with the oxygen transfer conditions applied is given in Table 4.6. The m_O values varied between 0.02-0.43 kg kg^{-1} h^{-1}, and m_O' varied between 0.08-0.42 kg kg^{-1} h^{-1}. The highest value for m_O and m_O' were obtained at OT_3 and OT_6 conditions as 0.43 and 0.42 kg kg^{-1} h^{-1} respectively. And similarly, the rate of substrate consumption for maintenance m_S and m_S' values were obtained for each oxygen transfer condition. The m_S values varied between 0.01-0.35 kg kg^{-1} h^{-1}, and m_s' varied between 0.04-0.11 kg kg^{-1} h^{-1}. The highest value for m_s and m_s' were obtained at OT_3 and OT_6 conditions as 0.35 and 0.11 kg kg^{-1} h^{-1} respectively. The calculation procedure of maintenance coefficients was explained in section 2.3.1.2; the Leudeking-Piret constants calculated for the bioprocesses are given in Table 4.5.

Table 4.5 The variations in Leudeking-Piret constants

	α	β
OT_1	2.4521	0.1221
OT_2	0.3975	0.1034
OT_3	0.3069	0.1248
OT_4	0.1607	0.0763
OT_5	0.5179	0.0206
OT_6	0.3124	0.0889

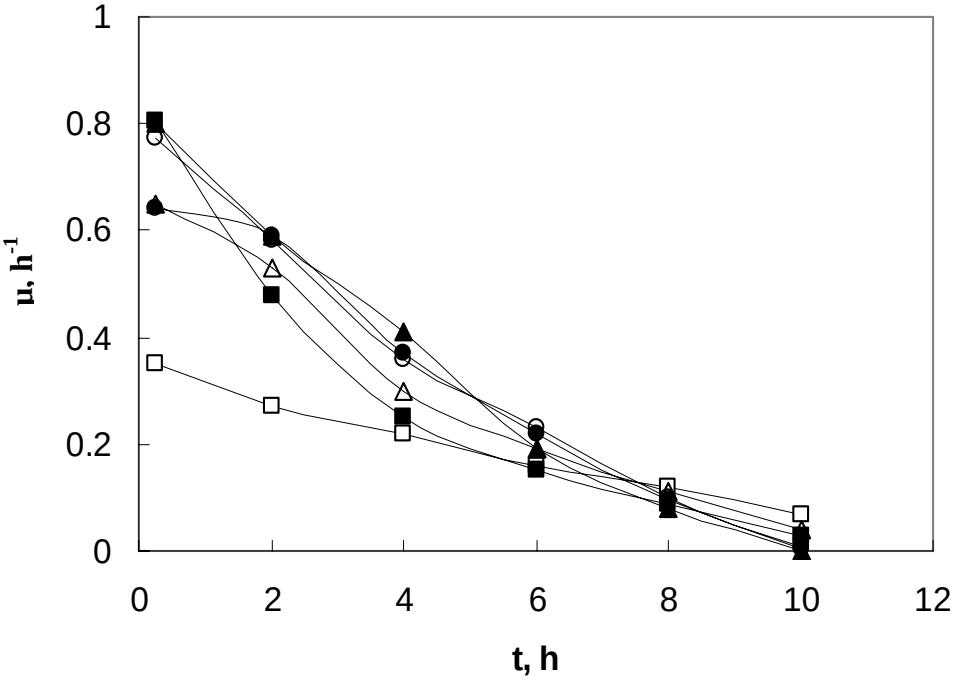

Figure 4.12 The variations in specific growth rate with the cultivation time, agitation and air inlet rates: OT_1 (□); OT_2 (■); OT_3 (○); OT_4 (▲) OT_5 (Δ); OT_6 (●). C_G^o = 8.0 kg m^{-3}, T=37°C, V_R= 1.65x10^{-3} m^3, pH_c=7.2.

Table 4.6 The variations in specific growth rate and yield coefficients.

Condition	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_o' ($kgkg^{-1}h^{-1}$)	m_s ($kgkg^{-1}h^{-1}$)	m_s' ($kgkg^{-1}h^{-1}$)
OT_1	0.25	0.35	0.44	1.19	0.29	0.79	2.69	-	-	-	-
	Overall				0.13						
OT_2	0.25	0.81	0.72	1.21	0.45	0.76	1.68	-	-	-	-
	1	0.77	0.72	1.46	0.53	1.07	2.03	-	-	-	-
	Overall				0.24						
OT_3	0.25	0.77	0.61	1.65	0.46	1.27	2.75	0.43	0.412	0.35	0.09
	1	0.53	0.55	1.39	0.38	0.96	2.53				
	3	0.51	0.37	1.63	0.34	1.41	4.15				
	5	0.31	0.18	0.92	0.29	1.50	5.18				
	10	0.004	0.02	0.02	0.19	0.26	1.37				
	Overall				0.34						

Condition	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_o' ($kgkg^{-1}h^{-1}$)	m_s ($kgkg^{-1}h^{-1}$)	m_s' ($kgkg^{-1}h^{-1}$)
OT_4	0.25	0.8	0.72	1.65	0.48	1.10	2.27				
	1	0.55	0.65	1.25	0.53	1.03	1.92				
	3	0.49	0.38	1.71	0.29	1.29	4.44				
	5	0.28	0.18	0.98	0.28	1.49	5.29	0.118	0.08	0.11	0.04
	10	0.002	0.02	0.02	0.16	0.14	0.91				
	Overall				0.32						
OT_5	0.25	0.65	0.62	1.39	0.46	1.06	2.31				
	1	0.61	0.55	1.17	0.53	1.10	2.09				
	3	0.38	0.25	0.79	0.48	1.52	3.16	0.117	0.19	0.1	0.1
	5	0.23	0.12	0.61	0.38	1.88	4.87				
	10	0.04	0.04	0.02	0.47	0.32	0.68				
	Overall				0.29						
OT_6	0.25	0.63	0.62	1.17	0.50	0.96	1.93				
	1	0.52	0.55	1.97	0.29	0.93	3.17				
	3	0.52	0.34	1.86	0.28	1.55	5.54	0.02	0.42	0.01	0.11
	5	0.26	0.13	0.86	0.23	1.49	6.54				
	10	0.008	0.02	0.02	0.39	0.36	0.91				
	Overall				0.35						

4.9 Mathematical Modeling to Estimate Benzaldehyde Activity

The mathematical model selected for predicting the effect of the different operation parameters on the benzaldehyde lyase activity, is given by

$$Activity = (f_1)(f_2)(f_3)(f_4)(f_5)(f_6) \quad (4.1)$$

Where f_1, f_2, f_3, f_4, f_5, and f_6 represent the functional relations between benzaldehyde lyase activity and variables such as cultivation time, agitation rate, dissolved oxygen concentration, cell concentration, and by-product concentration. Each of these functions contains constants which are shown as a_1, a_2, a_3, a_4, a_5, and a_6. Determination of these constants is accomplished by using a multiple regression analysis of data which were collected during this study at OT_1, OT_2, OT_3, and OT_4 operation conditions. In this study, Bourgoyne & Young's model (Bourgoyne et al., 1974) was modified in order to estimate benzaldehyde lyase activity.

Function (f_1) represents the effects of parameters that have not been mathematically modeled; for example the effects of temperature, pH, and inlet air flow rate.

$$f_1 = e^{a_1} = K \quad (4.2)$$

Effect of cultivation time (f_2) is defined by

$$f_2 = \left[\frac{t}{t_{ave}}\right]^{a2} \quad (4.3)$$

And it is assumed that benzaldehyde lyase activity is directly proportional to cultivation time as observed during the experiments. The average cultivation time (t_{ave}) is used in order to make function dimensionless and to normalize the variation of parameters. In this study, t_{ave} value is assumed to be t=7 h.

Effect of agitation rate (f_3) is defined by

$$f_3 = \left[\frac{N}{N_{ave}} \right]^{a3} \tag{4.4}$$

Where N_{ave}=531 min^{-1}.

Effect of cell concentration (f_4) is defined by

$$f_4 = \left[\frac{C_x}{C_{x\,ave}} \right]^{a4} \tag{4.5}$$

And it is assumed an increase in benzaldehyde lyase activity with the cell concentration. Where $C_{x\,ave}$=1.54 kg m^{-3}.

Effect of dissolved oxygen concentration (f_5) is defined by

$$f_5 = \left[\frac{C_{o2}}{C_{o2\,ave}} \right]^{-a5} \tag{4.6}$$

And it is assumed that benzaldehyde lyase activity is inversely proportional to dissolved oxygen concentration as observed during the experiments. Where $C_{o2\,ave}$= 0.05 mol m^{-3}.

Effect of by- product concentration (f_6) is defined by

$$f_6 = \left[\frac{C_{Bp}}{C_{Bp\,ave}} \right]^{-a6} \tag{4.7}$$

And it is assumed an decrease in benzaldehyde lyase activity with increase in by-product formation rate. Where C_{BPave}= 1.06 kg m^{-3}.

By substituting equations 4.2-7 into the equation 4.1, the benzaldehyde lyase activity model is obtained as

$$\frac{Activity}{(Activity)_{ave}} = \left[e^{a1}\right]\left[\frac{t}{t_{ave}}\right]^{a2}\left[\frac{N}{N_{ave}}\right]^{a3}\left[\frac{C_x}{C_{xave}}\right]^{a4}\left[\frac{C_{o2}}{C_{o2ave}}\right]^{-a5}\left[\frac{C_{BP}}{C_{BPave}}\right]^{-a6} \tag{4.8}$$

Where the average activity value is 553 U cm^{-1}.

4.9.1 The Dimensionless Analysis to calculate C_x, C_{o2}, and C_{BP}

Since the cell concentration, dissolved oxygen concentration, and by-product concentration also depended on agitation rate and cultivation time, the dimensionless correlations are developed to estimate them as function of agitation rate and cultivation time. The following dimensionless equations for C_x, C_{o2}, and C_{BP} are defined by:

$$\left[\frac{C_x}{C_{x_{ave}}}\right] = \left[e^{b1}\right]\left[\frac{t}{t_{ave}}\right]^{b2}\left[\frac{N}{N_{ave}}\right]^{b3} \tag{4.9}$$

$$\left[\frac{C_{o2}}{C_{o2_{ave}}}\right] = \left[e^{d1}\right]\left[\frac{t}{t_{ave}}\right]^{d2}\left[\frac{N}{N_{ave}}\right]^{d3} \tag{4.10}$$

$$\left[\frac{C_{BP}}{C_{BP_{ave}}}\right] = \left[e^{g1}\right]\left[\frac{t}{t_{ave}}\right]^{g2}\left[\frac{N}{N_{ave}}\right]^{g3} \tag{4.11}$$

Where b_1, b_2, b_3, d_1, d_2, d_3, g_1, g_2 and g_3 are equation's constants. The constants can be determined from a multiple regression analysis of data which were collected during this study at OT_1, OT_2, OT_3, and OT_4 operation conditions.

4.9.2 Development of the Proposed Model

The benzaldehyde lyase activity model is developed by considering the effects of different operation parameters. The model can be used for prediction of benzaldehyde lyase activity at the condition in which medium contains 8.0 kg m^{-3} glucose, 5.0 kg m^{-3} $(NH_4)_2HPO_4$ and the salt solution at T=37°C and pH=7.2. By using the data obtained from experiments, model constants can be determined. The procedure is presented as follows.

4.9.2.1 Selecting and sorting relevant and proper data

An Excel program has been provided to identify and store the experimental data. In this program firstly, data which were obtained from different bioreactor operation conditions (OT_1, OT_2, OT_3, and OT_4) were rearranged. Then these data normalized and sorted by dividing them to their average value. After taking the logarithm from the normalized values, a new data file created which can be used in multiple regression analysis.

4.9.2.2 Multiple Regression Analysis

Multiple regression technique was used to determine the coefficients of the proposed benzaldehyde lyase activity model and equations which calculate C_x, C_{o2}, and C_{BP}. A derivation of the multiple regression-analysis procedure is presented in detail as follows.

As mentioned before, the equation of the proposed model is:

$$\frac{Activity}{(Activity)_{ave}} = \left[e^{a1}\right]\left[\frac{t}{t_{ave}}\right]^{a2}\left[\frac{N}{N_{ave}}\right]^{a3}\left[\frac{C_x}{C_{xave}}\right]^{a4}\left[\frac{C_{o2}}{C_{o2ave}}\right]^{-a5}\left[\frac{C_{BP}}{C_{BPave}}\right]^{-a6} \qquad (4.12)$$

Taking the logarithm of both sides of the above equation yields equation (4.13):

$$\ln\left(\frac{Activity}{(Activity)_{ave}}\right)=a_1+(a_2)\ln\left[\frac{t}{t_{ave}}\right]+(a_3)\ln\left[\frac{N}{N_{ave}}\right]+(a_4)\ln\left[\frac{C_x}{C_{xave}}\right]-(a_5)\ln\left[\frac{C_{o2}}{C_{o2ave}}\right]$$
$$-(a_6)\ln\left[\frac{C_{BP}}{C_{BPave}}\right]$$

By considering

$$x_2=\ln\left[\frac{t}{t_{ave}}\right] \tag{4.14}$$

$$x_3=\ln\left[\frac{N}{N_{ave}}\right] \tag{4.15}$$

$$x_4=\ln\left[\frac{C_x}{C_{x\,ave}}\right] \tag{4.16}$$

$$x_5=-\ln\left[\frac{C_{o2}}{C_{o2\,ave}}\right] \tag{4.17}$$

$$x_6=-\ln\left[\frac{C_{Bp}}{C_{Bp\,ave}}\right] \tag{4.18}$$

The equation (4.13) can be rewritten as:

$$\ln\frac{Activity}{Activity_{ave}}=\left(a_1+\sum_{j=2}^{6}a_j x_j\right) \tag{4.19}$$

If the residual error of the *i*th data point, r_i, is defined by

$$r_i=\left(a_1+\sum_{j=2}^{6}a_j x_j\right)-\ln\frac{Activity}{Activity_{ave}} \tag{4.20}$$

In order to minimize the square of the residuals $\sum_{i=1}^{n} r_i^2$, the constants from a_1 to a_6 should be determined properly by taking derivative from the square of the residuals $\sum_{i=1}^{n} r_i^2$.

$$\frac{\partial\left(\sum_{i=1}^{n} r_i^{\,2}\right)}{\partial a_j} = \sum_{i=1}^{n} 2r_i \ \frac{\partial r_i}{\partial a_j} = \sum_{i=1}^{n} 2r_i x_j \qquad (4.21)$$

For j = 1, 2, 3, 4, 5, 6.

So that by simultaneously solving the system of equations obtained by expanding $\sum_{i=1}^{n} r_i x_j$, the constants a_1 through a_6 can be obtained.

The expansion of $\sum_{i=1}^{n} r_i x_j$ yields a serie of equations (4.22):

$$a_1 n + a_2 \sum x_2 + a_3 \sum x_3 + .a_4 \sum x_4 + a_5 \sum x_5 + a_6 \sum x_6 = \sum \ln \frac{Activity}{(Activity)_{ave}}$$

$$a_1 \sum x_2 + a_2 \sum x_2^2 + a_3 \sum x_2 x_3 + a_4 \sum x_2 x_4 + a_5 \sum x_2 x_5 + a_6 \sum x_2 x_6 = \sum x_2 \ln \frac{Activity}{Activity_{ave}}$$

$$a_1 \sum x_3 + a_2 \sum x_3 x_2 + a_3 \sum x_3^2 + a_4 \sum x_3 x_4 + a_5 \sum x_3 x_5 + a_6 \sum x_3 x_6 = \sum x_3 \ln \frac{Activity}{Activity_{ave}}$$

$$a_1 \sum x_4 + a_2 \sum x_4 x_2 + a_3 \sum x_4 x_3 + a_4 \sum x_4^2 + a_5 \sum x_4 x_5 + a_6 \sum x_4 x_6 = \sum x_4 \ln \frac{Activity}{Activity_{ave}}$$

$$a_1 \sum x_5 + a_2 \sum x_5 x_2 + a_3 \sum x_5 x_3 + a_4 \sum x_5 x_4 + a_5 \sum x_5^2 + a_6 \sum x_5 x_6 = \sum x_4 \ln \frac{Activity}{Activity_{ave}}$$

$$a_1 \sum x_6 + a_2 \sum x_6 x_2 + a_3 \sum x_6 x_3 + a_4 \sum x_6 x_4 + a_5 \sum x_6 x_5 + a_6 \sum x_6^2 = \sum x_6 \ln \frac{Activity}{Activity_{ave}}$$

In order to calculate the constants from a_1 to a_6, the following linear equation system should be solved:

$$\begin{bmatrix} n & \sum_{i=1}^{n} x_{2i} & \sum_{i=1}^{n} x_{3i} & \sum_{i=1}^{n} x_{4i} & \sum_{i=1}^{n} x_{5i} & \sum_{i=1}^{n} x_{6i} \\ \sum_{i=1}^{n} x_{2i} & \sum_{i=1}^{n} x_{2i}^2 & \sum_{i=1}^{n} x_{2i}x_{3i} & \sum_{i=1}^{n} x_{2i}x_{4i} & \sum_{i!=1}^{n} x_{2i}x_{5i} & \sum_{i=1}^{n} x_{2i}x_{6i} \\ \sum_{i=1}^{n} x_{3i} & \sum_{i=1}^{n} x_{3i}x_{2i} & \sum_{i=1}^{n} x_{3i}^2 & \sum_{i=1}^{n} x_{3i}x_{4i} & \sum_{i=1}^{n} x_{3i}x_{5i} & \sum_{i=1}^{n} x_{3i}x_{6i} \\ \sum_{i=1}^{n} x_{4i} & \sum_{i=1}^{n} x_{4i}x_{2i} & \sum_{i=1}^{n} x_{4i}x_{3i} & \sum_{i=1}^{n} x_{4i}^2 & \sum_{i=1}^{n} x_{4i}x_{5i} & \sum_{i=1}^{n} x_{4i}x_{6i} \\ \sum_{i=1}^{n} x_{5i} & \sum_{i=1}^{n} x_{5i}x_{2i} & \sum_{i=1}^{n} x_{5i}x_{3i} & \sum_{i=1}^{n} x_{5i}x_{4i} & \sum_{i=1}^{n} x_{5i}^2 & \sum_{i=1}^{n} x_{5i}x_{6i} \\ \sum_{i=1}^{n} x_{6i} & \sum_{i=1}^{n} x_{6i}x_{2i} & \sum_{i=1}^{n} x_{6i}x_{3i} & \sum_{i=1}^{n} x_{6i}x_{4i} & \sum_{i=1}^{n} x_{6i}x_{5i} & \sum_{i=1}^{n} x_{6i}^2 \end{bmatrix} \times \begin{bmatrix} a_1 \\ a_2 \\ a_3 \\ a_4 \\ a_5 \\ a_6 \end{bmatrix} = \begin{bmatrix} \sum_{i=1}^{n} y_i \\ \sum_{i=1}^{n} x_{2i}y_i \\ \sum_{i=1}^{n} x_{3i}y_i \\ \sum_{i=1}^{n} x_{4i}y_i \\ \sum_{i=1}^{n} x_{5i}y_i \\ \sum_{i=1}^{n} x_{6i}y_i \end{bmatrix}$$

Where n is the number of data points which are obtained from experiments and $y = \ln\left(\frac{Activity}{Activity_{ave}}\right)$. Also, the same procedure can be applied to calculate the coefficients of the equations from (4.9) to (4.11).

4.9.3 Model Application

After appling the procedure which were mentioned in the earlier section, the coefficient of the equations from (4.9) to (4.11) are calculated and presented in equations (4.23), (4.24), and (4.25). It is observed that the correlations can estimate the cell concentration, C_x, dissolved oxygen concentration, C_{o2}, and by-product concentration, C_{BP}, with a reasonable accuracy.

$$\left[\frac{C_x}{C_{x_{ave}}}\right] = \left[e^{0.003}\right]\left[\frac{t}{t_{ave}}\right]^{0.991}\left[\frac{N}{N_{ave}}\right]^{0.778} \tag{4.23}$$

$$\left[\frac{C_{o2}}{C_{o2_{ave}}}\right] = \left[e^{-1.626}\right]\left[\frac{t}{t_{ave}}\right]^{0.066}\left[\frac{N}{N_{ave}}\right]^{2.995} \tag{4.24}$$

$$\left[\frac{C_{BP}}{C_{BP_{ave}}}\right] = \left[e^{-0.238}\right]\left[\frac{t}{t_{ave}}\right]^{1.254}\left[\frac{N}{N_{ave}}\right]^{0.801} \qquad (4.25)$$

The data which obtained from these equations were used to predict the benzaldehyde lyase activity. Figures (4.13), (4.14), and (4.15) show the calculated data which obtained by using the Multiple Regression Analysis versus observed data from the experiments. As seen in Figures (4.13) and (4.15), C_{BP} and C_x can be predicted by using equations (4.23) and (4.25), with reasonable accuracy. Although the Figure (4.14) looks scattered, still the predictions from equation (4.24) are acceptable to be used in the benzaldehyde lyase activity model.

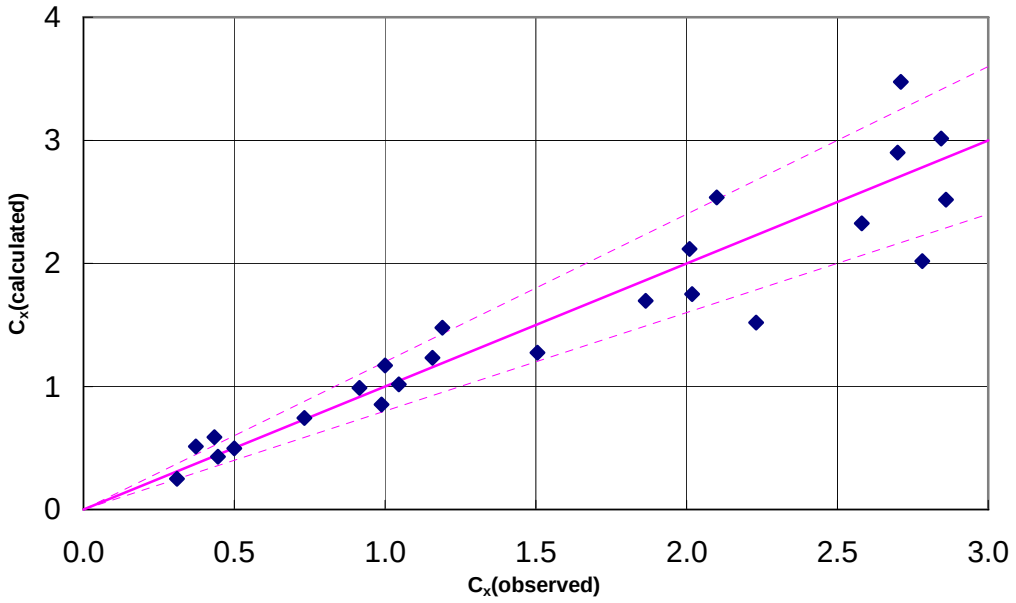

Figure4.13 Calculated C_x versus Observed C_x

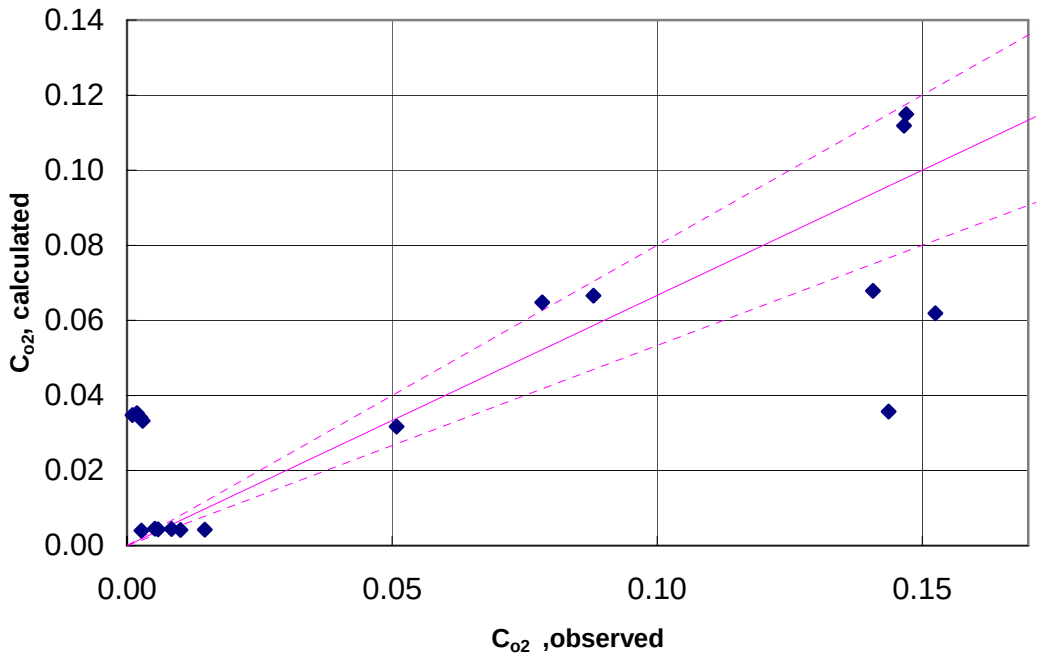

Figure 4.14 Calculated C_{o2} versus Observed C_{o2}

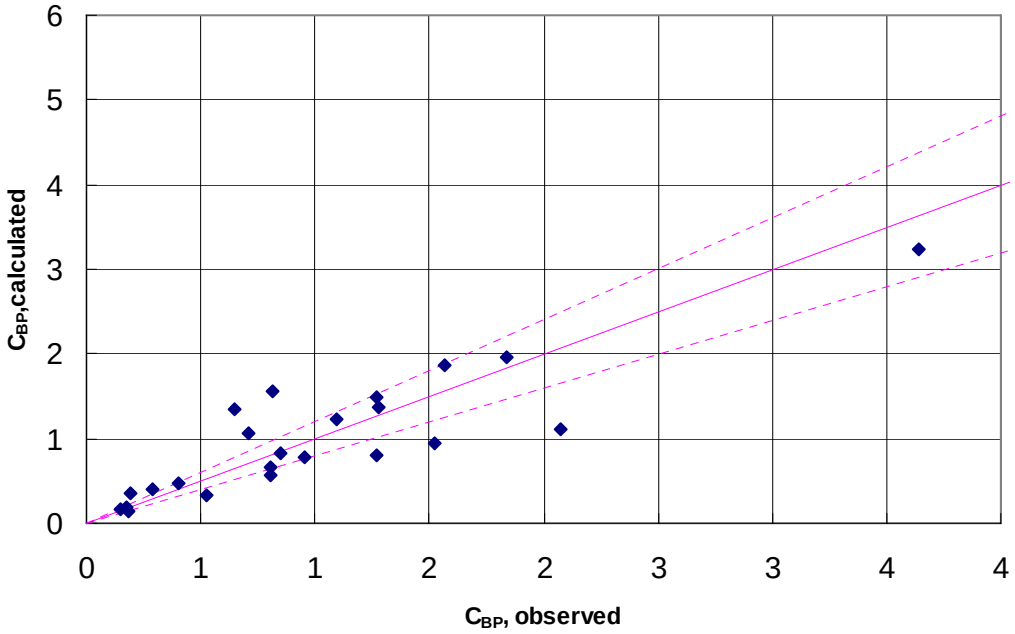

Figure 4.15 Calculated C_{BP} versus Observed C_{BP}

After determining cell concentration, dissolved oxygen concentration, and by-product concentration, Multiple Regression analysis is applied to calculate the benzaldehyde lyase activity model's constants. Equation (4.26) represents benzaldehyde lyase activity model constants.

$$\frac{Activity}{(Activity)_{ave}} = \left[e^{-1.38}\right]\left[\frac{t}{t_{ave}}\right]^{1.43}\left[\frac{N}{N_{ave}}\right]^{3.02}\left[\frac{C_x}{C_{xave}}\right]^{4.08}\left[\frac{C_{o2}}{C_{o2ave}}\right]^{-2.72}\left[\frac{C_{BP}}{C_{BPave}}\right]^{-3.30} \qquad (4.26)$$

By using equation (4.26), benzaldehyde lyase activity can be calculated at experiment conditions. In order to test the performance of the proposed model, benzaldehyde lyase activities are estimated for each data point. The results are posted in Table 4.7. The regression index of correlation G is used to check the persistence of Multiple Regression Analysis. The regression index of correlation G is defined by

$$G = \sqrt{1.0 - \frac{\sum\left[\left(\ln(Activity)\right)_{observed} - \left(\ln(Activity)\right)_{calculated}\right]^2}{\sum\left[\left(\ln(Activity)\right)_{observed} - \overline{\left(\ln(Activity)\right)}\right]^2}} \qquad (4.27)$$

By calculating (G) for experimental data, it is observed that the model can estimate enzyme activity with an error of ±10 % when compared with the data obtained from laboratory experiments.

Table 4.7 The result of Analysis for Experiment's Data

Activity(cal)	Activity(ob)	Activity(cal)	Activity(ob)
120	116	141	110
271	331	316	340
435	520	508	729
609	697	710	932
790	921	922	1048
977	924	1141	1057
160	116	74	99
359	290	167	171
576	610	269	256
806	758	376	305
1046	1022	488	456
1295	1022	604	459

The graphical representation of Table 4.7 is presented in Figure 4.16 As seen from figure, it can be concluded that the proposed model can estimate benzaldehyde lyase activity with a reasonable accuracy.

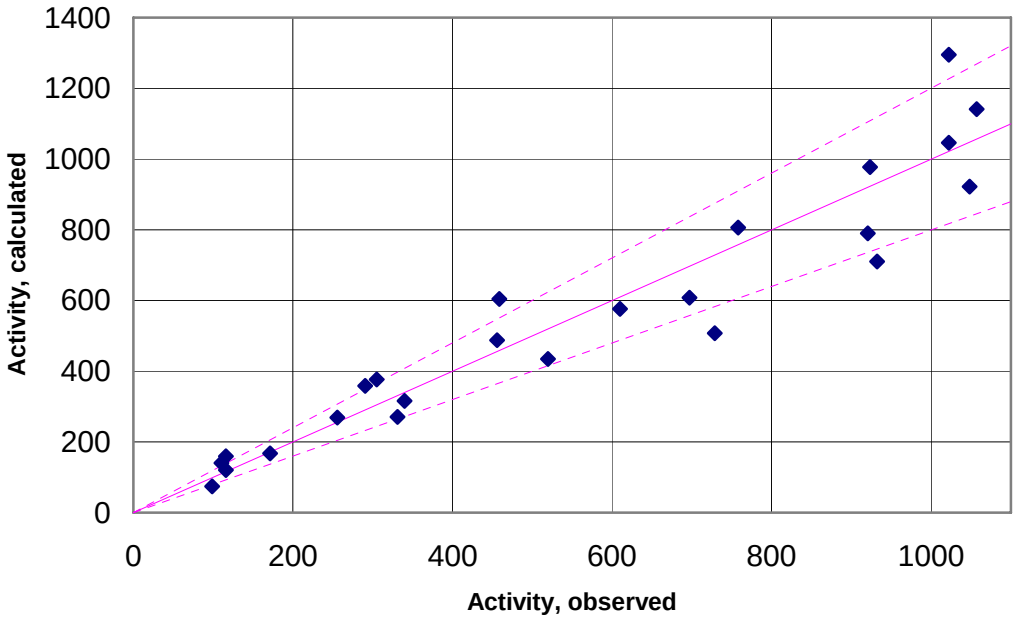

Figure 4.16 Calculated Activity versus Measured Activity

CHAPTER 5

CONCLUSION

In this study, the effects of oxygen transfer conditions on the synthesis of the enzyme benzaldehyde lyase as intracellular in recombinant *E. coli BL21* (DE3) pLysS was investigated and a comprehensive model was developed to determine benzaldehyde lyase activity. For this purpose, the effects of oxygen transfer together with the mass transfer coefficient (K_La), enhancement factor E ($=K_La/K_La_o$), volumetric oxygen transfer rate, volumetric and specific oxygen uptake rates, mass transfer and biochemical reaction resistances; moreover, the variation in product and by-product distribution, specific substrate uptake rates, yield and maintenance coefficient were investigated in the pilot scale batch bioreactor at six different conditions then the data obtained during the experiments were used to develop a model to estimate benzaldehyde lyase activity. In this context the following conclusions were drawn:

1. *Escherichia coli* BL21 (DE3) pLySs carrying recombinant pRSETA::*bal* plasmid (Kaya, 2006) was selected as the benzaldehyde lyase producer, due to the highest BAL production capacity of this strain in RPM medium.

2. The medium containing 8.0 kg m^{-3} glucose, 5.0 kg m^{-3} $(NH_4)_2HPO_4$ and the salt solution was selected as a production medium, due to the highest cell concentration and benzaldehyde lyase activity which were obtained in the study of Çalık et. al. (2004).

3. The effects of oxygen transfer on benzaldehyde lyase production were investigated at six different conditions with the parameters, air inlet rate of Q_O/V_R = 0.5 vvm and agitation rates

of N= 250, 500, 625, and 750 min^{-1}, and dissolved oxygen levels DO= 20%, 40% (C_{DO}=0.04, 0.08 mol m^{-3}) of the saturation.

4. At OT_1 condition, there was no oxygen accumulation throughout the bioprocesses after t=0.5 h of bioprocess. At OT_2 condition, due to the high oxygen demand of the cells between t=3-11 h, transferred oxygen was totally consumed however oxygen profile suddenly increased at the end of the bioprocesses which indicates that there is a decrease in oxygen uptake of cells. At the OT_3 and OT_4 conditions dissolved oxygen level decreased in the first 5 hours; after t=5h , due to the decrease in oxygen uptake rate dissolved oxygen level increased. However at OT_5 and OT_6 conditions, dissolved oxygen levels were controlled at the values of DO= 20, 40% respectively, by keeping agitation rate at constant value of N=625 min^{-1} and by considering air inlet rate as variable parameter.

5. The highest cell concentration with a value of 3.0 kg m^{-3} was obtained at OT_6 condition and the lowest cell concentration of 1.19 kg m^{-3} was observed at OT_1 condition where oxygen supply seems to be insufficient for maintenance of oxidative metabolism.

6. Among the investigated oxygen transfer parameters, the highest glucose consumption rate was observed at OT_6 condition, where the cell concentration was the highest; whereas, the lowest glucose consumption rate was attained at OT_1 condition, where the cell concentration was low due to the oxygen limitation.

7. Almost for all oxygen transfer conditions applied, acetic, formic, citric, malic and succinic acids were detected in the fermentation broth. Presence of the organic acids of TCA cycle, i.e., citric, succinic and malic acid may be a consequence of inadequate oxygen supply which leads to repression on TCA cycle enzymes.

8. For the all oxygen transfer conditions, acetic acid was the major by-products in the fermentation medium. the highest acetate accumulation occurred in OT_1 condition, where the lowest cell concentration and the lowest glucose consumption were observed, as 3.16 kg m^{-3} at t=10 h; and the lowest amount of the acetate was detected at OT_6 condition, where the highest cell concentration was obtained, as 0.34 Kg m^{-3} at t=12h.

9. Aspartic acid, asparagine, cysteine, glutamic acid, isoleucine, leucine, lysine, serine, threonine, tyrosine, valine, and ornithine are the amino acids that were detected in the medium, but in very low amounts. The highest total amino acids produced was at OT_6 condition at t=10 h as T_{AA}=0.1737 kg m^{-3}, followed by OT_1 condition as a T_{AA}=0.0734 Kg m^{-3}.

10. Volumetric mass transfer coefficients, K_La, values and subsequently the enhancement factor, E, found were increased with the increase in the oxygen transfer, i.e. Q_O/V_R and N, at all operation conditions except OT_5 and OT_6. The low values of K_La could be explained as result of the high values of driving force at OT_5 and OT_6 conditions where dissolved oxygen levels were controlled at the values of DO= 20, 40% respectively.

11. The highest K_La and consequently E were obtained in OT_4 operation at t=5 h of the bioprocess as 0.039 s^{-1} and 1.86 respectively. The lowest K_La value as 0.005 s^{-1} at t=10 h of the bioprocess was obtained in OT_6 condition where the highest cell concentration was observed.

12. Damköhler number (Da), effectiveness factor (η) was determined to compare the mass transfer and biochemical reaction rates. At the all operations mass transfer resistances were effective (Da>>1) through the end of the process; while they were smaller than 1 at the beginning of the processes, signaling the low mass transfer resistance. Effectiveness factor, η, were close 1 at OT_2, OT_3 and OT_4 conditions indicating that the cells are consuming oxygen with such a high rate that

maximum possible oxygen utilization (OD) value was approached. The effectiveness factor dropped at the end of process, where mass transfer resistances were the greatest.

13. The maximum value of μ was obtained as 0.81 h^{-1} at t=0.25 h of OT_2 condition and the highest cell concentration was achieved among the operation conditions at OT_6 as C_x=3.0 Kgm^{-3} at t=12h.

14. The yield coefficients of overall specific cell yield on substrate ($Y_{X/S}$), overall specific cell yield on oxygen ($Y_{X/O}$), and overall specific substrate yield on oxygen ($Y_{S/O}$) values were calculated according to empirical data. The highest overall specific cell yield on substrate ($Y_{X/S}$) value was obtained at OT_6 as 0.354, due to the increased efficiency of the utilization of the carbon source for product formation; and the lowest value was 0.13 obtained at OT_1 condition; while $Y_{X/O}$ and $Y_{S/O}$ increased between t=1-5 h of bioprocess with cultivation time and took the maximum value at t=5 h before the reduction at the remaining time of the process.

15. Specific oxygen uptake rates (q_O) and specific substrate utilization rates (q_S) decreased with the cultivation time between t=1-10 h of bioprocess at all the oxygen transfer conditions. The highest value of q_O was obtained at t=1 h of OT_4 condition as 0.72 kg kg^{-1} h^{-1}; where the lowest value was obtained at t=10 h of OT_3 condition as 0.016 kg kg^{-1} h^{-1}. The highest value of q_S was obtained at t=1 h of OT_6 condition as 1.96 kg kg^{-1} h^{-1}; where the lowest value was obtained at t=10 h of the same condition as 0.021 kg kg^{-1} h^{-1}.

16. The rate of oxygen consumption for maintenance, m_0, varied between 0.02-0.43 kg kg^{-1} h^{-1}, and the highest value was obtained as 0.43 kg kg^{-1} h^{-1} at OT_3, when the oxygen consumption for by-product production was neglected. However when oxygen consumption for by-product production is considered, the rate of oxygen consumption for maintenance

attained as 0.41 kg kg^{-1} h^{-1} at OT_3. As a result, when the by-product formation was taken into account, m_0 took lower values.

17. The rate of substrate consumption for maintenance m_S and m_S' values were obtained for each oxygen transfer condition. The m_S values varied between 0.01-0.35 kg kg^{-1} h^{-1}, and m_s' varied between 0.04-0.11 kg kg^{-1} h^{-1}. The highest value for m_s and m_s' were obtained at OT_3 and OT_6 conditions as 0.35 and 0.11 kg kg^{-1} h^{-1} respectively.

18. Among the investigated oxygen transfer conditions, the highest cell concentration and benzaldehyde lyase activity was observed at OT6 condition. These values were approximately 1.27 and 1.30 fold higher than the values obtained in the study of of Çalık et al. (2004).

19. Proposed mathematical model can estimate benzaldehyde lyase activity as function of time, agitation rate, cell concentration, dissolved oxygen concentration, and by-product concentration with reasonable accuracy.

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APPENDIX A

Calibration of *Escherichia coli* Concentration

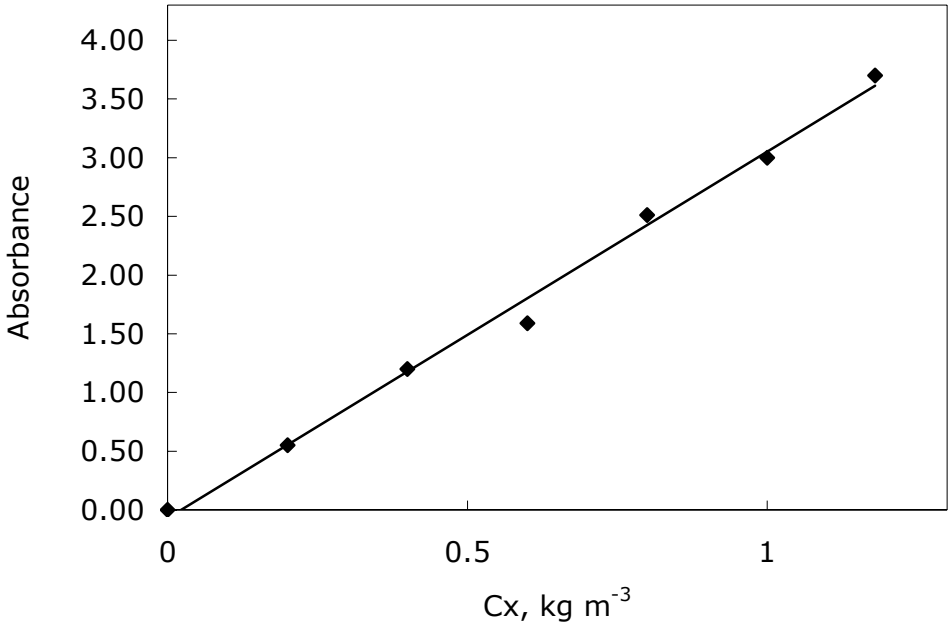

Figure A.1 Calibration curve for *Escherichia coli* concentration

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

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

APPENDIX B

Calibration of Reduced Sugar Concentration

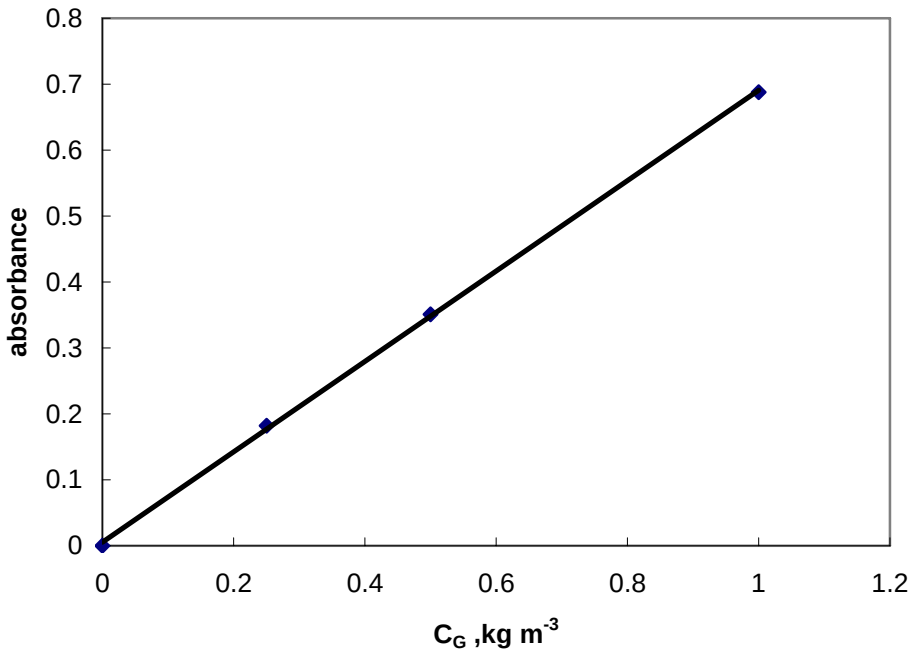

Figure B.1 Calibration curve of the glucose analysis solution

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

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