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

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

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ABSTRACT

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

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In this study, the aim was to investigate the effects of exponential feeding strategy on benzaldehyde lyase (BAL) production by recombinant *Escherichia coli* BL21. For this purpose, the effects of medium components were investigated to optimize the initial medium composition of the fed-batch fermentations. For the batch bioreactor operations, the highest cell concentration and BAL activity were achieved in a media containing 30 g L^{-1} pretreated molasses, and 5 g L^{-1} $(NH_4)_2HPO_4$ as 5.07 g L^{-1}, and 1611 U ml^{-1} at t=8 h, respectively. Thereafter, in order to increase the cell growth and BAL production while avoiding acetate accumulation, fed-batch bioreactor operations were conducted with exponential feeding at different specific growth rates namely, 0.1 h^{-1} (μ-0.1), 0.15 h^{-1} (μ-0.15), and 0.2 h^{-1} (μ-0.2), and a combined exponential and constant feeding (μ-0.2+) strategy. In the experiments, 9 hours of batch-wise operation with the optimized

production medium was followed by a fed-batch operation phase using the pre-determined exponential feeding profiles and for μ-0.2+ operation after 10 hours of exponential feeding at $\mu=0.2\ h^{-1}$, where the feed rate was kept constant at 21.6 g h^{-1}. Additionally, the plasmid stability was investigated using the feeding method of μ-0.2+ operation with antibiotics in the feed solution, and it was observed that the plasmid was stable. Among the three exponential feeding conditions, the highest cell concentration and BAL activity were determined in μ-0.15 operation as 21.7 g L^{-1} and 8379 U ml^{-1}, respectively. In μ-0.2+ operation, the maximum cell concentration was achieved as 28.1 g L^{-1} which was higher than μ-0.15 experiment, though a lower maximum BAL activity was obtained as 6695 U ml^{-1}.

Keywords: Benzaldehyde Lyase, *Escherichia coli*, Molasses, Fed-Batch Operation, Exponential Feeding

ÖZ

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

Taşpınar, Hatice
Yüksek Lisans, Biyoteknoloji
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Bu çalışmada, rekombinant *Escherichia coli* BL21 ile benzaldehit liyaz (BAL) üretiminde eksponansiyel besleme stratejisinin etkilerinin araştırılması amaçlanmıştır. Bu amaç ile yarı-kesikli üretimlerle kullanılacak olan başlangıç üretim ortam bileşenlerinin derişimlerinin etkileri incelenmiştir. Kesikli biyoreaktör üretimleri arasında en yüksek hücre derişimi ve BAL aktivitesi, 30 g L^{-1} melas ve 5 g L^{-1} $(NH_4)_2HPO_4$ içeren ortamda t=8 st' te, sırasıyla 5.07 g L^{-1} ve 1611 U ml^{-1} olarak elde edilmiştir. Daha sonra, asetik asit oluşumu önlenerek hücre büyümesi ve BAL üretiminin arttırılması için yarı-kesikli üretimler, 0.1 st^{-1} (μ-0.1), 0.15 st^{-1} (μ-0.15) ve 0.2 st^{-1} (μ-0.2) spesifik büyüme hızlarında eksponansiyel besleme stratejisi ile birleştirilmiş eksponansiyel ve sabit besleme (μ-0.2+) stratejisi kullanılarak gerçekleştirilmiştir. Bu fermantasyon deneylerinde optimize edilmiş üretim ortamı ile 9 saatlik kesikli fazın ardından, önceden belirlenmiş eksponansiyel besleme profilleri kullanılarak yarı-kesikli faza

geçilmiştir. μ-0.2+ koşulunda 10 saatlik bir eksponansiyel besleme (μ=0.2 s^{-1}) fazından sonra besleme hızı 21.6 g st^{-1} hızında sabit tutulmuştur. Ayrıca, plasmitin stabil olup olmadığı ortama μ-0.2+ metodu ile beslenen melas çözeltisine antibiyotik eklenerek araştırılmış ve stabil olduğu sonucuna varılmıştır. Gerçekleştirilen üç eksponansiyel besleme deneyi içerinde en yüksek hücre derişimi 21.7 g L^{-1}, en yüksek BAL aktivitesi ise 8379 U ml^{-1} olarak μ-0.15 koşulunda elde edilmiştir. μ-0.2+ koşulu ile μ-0.15 koşulunda elde edilen maksimum hücre konsantrasyonu artmış (28.1 g L^{-1}); ancak maksimum BAL aktivitesi, 6695 U ml^{-1}, μ-0.15 koşulunun maksimum BAL aktivite değerinden daha düşük elde edilmiştir.

Anahtar Kelimeler: Benzaldehit Liyaz, *Escherichia coli*, Melas, Yarı-Kesikli İşletim, Eksponansiyel Besleme

To my family

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NOMENCLATURE

A	Benzaldehyde lyase activity, U ml^{-1}
A_X	Specific Activity of Benzaldehyde lyase, U mg^{-1} DCW
C_O	Dissolved oxygen concentration, mol m^{-3} or g L^{-1}
C_O*	Oxygen saturation concentration, mol m^{-3} or g L^{-1}
C	Concentration, g L^{-1}
C_X	Cell concentration, g dry cell L^{-1}
Da	Damköhler number; Maximum possible oxygen utilization rate per maximum mass transfer rate
F	Substrate feed rate, g h^{-1}
K_La_0	Physical overall liquid phase mass transfer coefficient, s^{-1}
K_La	Overall liquid phase mass transfer coefficient, s^{-1}
N	Agitation rate, min^{-1}
m	Maintenance coefficient, g g^{-1} DCW h^{-1}
Q	Volumetric substrate feed rate, L h^{-1}
q	Specific formation or consumption rate, g g^{-1} DCW h^{-1}
r	Reaction rate, g L^{-1} h^{-1}
T	Bioreaction medium temperature, °C
t	Bioreactor cultivation time, h
U	One unit of an enzyme
V	Volume, L
Y	Yield, g g^{-1}

Greek Letters

η	Effectiveness factor
μ	Specific cell growth rate, h^{-1}

μ_{max}	Maximum specific cell growth rate, h^{-1}
λ	Wavelength, nm

Subscripts

0	Initial condition
G	Glucose
F	Fructose
M	Molasses
O	Oxygen
P	Product
S	Substrate
X	Cell

Abbreviations

BAL	Benzaldehyde lyase
bal	Gene of benzaldehyde lyase
DCW	Dry cell weight
DO	Dissolved oxygen
EC	Enzyme Commission
HPLC	High pressure liquid chromatography
OD	Oxygen demand
OUR	Oxygen uptake rate
OTR	Oxygen transfer rate
OTR_{max}	Maximum possible mass transfer rate
TCA	Tricarboxylic acid

CHAPTER 1

INTRODUCTION

Use of microorganisms for production of industrial products especially for food and beverage has a great history. Growing cells in bioreactors for industrial productions were referred as fermentation processes, which have many applications since the ancient times (Nielsen et al., 2003; Najafpour, 2007). Today, combination of fermentation processes with recombinant DNA technology have enabled to produce various important biological molecules in suitable amounts for research, clinical and industrial purposes, which cannot be produced naturally (Lee, 1996; Nielsen et al., 2003; Choi et al., 2006). Fermentation processes have a broad range of products in pharmaceutical, food, agriculture, energy, and chemical industries; and enzymes are one of the most important products of microbial fermentations.

Enzymes which are proteins, except a small group of catalytic RNA molecules (Nelson and Cox, 2005), are biocatalysts catalyzing biological reactions produced by living cells (Underkofler et al., 1958). They increase the reaction rate by reducing the activation energy; they are highly specific to their substrates due to their unique three dimensional structures (Watson et al., 1987). Some enzymes are enantioselectivite that they can recognize the differences between the stereoisomer substrates (Scheve, 1984). Enantiopure compounds are important especially for pharmaceuticals, agrochemicals and flavors, and there is an increasing demand for marketing chiral products as a single enantiomer than a

racemic mixture (Adam et al., 1999; Rouhi, 2004). Since enzymes are chiral molecules, single enantiomers may be produced in high enantiomeric excess from racemic mixtures by catalytic asymmetric induction or kinetic resolution. Thus, enantioselectivity can be referred as the most important property of the enzymes (Adam et al., 1999).

Benzaldehyde lyase (BAL, EC 4.1.2.38) is an enantioselective enzyme which is used for the synthesis of various enantiopure α-hydroxy ketones (Sanchez-Gonzalez and Rosazza, 2003). It only converts (R)-benzoin into benzaldehyde, and does not catalyze the reaction with (S)-benzoin (Demir et al., 2001). BAL was first described by Gonzàlez and Vicuña (1989) from *Pseudomonas fluorescens* Biovar I, and thereafter the gene encoding benzaldehyde lyase was cloned and expressed in *Escherichia coli* strains. The characteristic properties of BAL have been investigated by various researchers, and they have been reported that it is a thiamin diphophate (ThDP) dependent enzyme, requires cofactors as Mg^{+2}, and cosolvents (DMSO or PEG) are needed for enzyme stability and activity. It is stable between pH 6 and 8, and inactive below pH 6 (Gonzàlez and Vicuña, 1989; Demir et al., 2001; Domínuguez de María et al. 2006; Janzen et al., 2006).

Escherichia coli is one of the most commonly used host organism for production of recombinant proteins in industrial biotechnology, since its whole genome is known and it has a well-characterized system (Yee and Blanch, 1993; Lee, 1996). In *E. coli* fermentations high cell densities and high volumetric productivities can be achieved with simple nutritional requirements, thus high cell density aerobic cultures of *E. coli* are frequently used (Lee, 1996). However, significant amounts of acetate formation occurs in *E. coli* cultivations both under anaerobic (Åkesson et al., 2001), and aerobic fermentations running at high growth rates (Åkesson et al., 1999). Accumulation of acetate in high concentrations inhibits the cell growth and recombinant protein production; therefore, to avoid acetate accumulation and to reach high cell densities with high product concentrations, fed-batch operations are commonly preferred (Lee, 1996). In the literature, various feeding strategies

were successfully used for *E. coli* cultivations with lowering acetate formation by supplying limiting amounts of carbon source (Paalme et al., 1990; Kleman et al., 1991; Kleman and Strohl, 1994; Yoon et al., 1994; Lin et al., 2001). Exponential feeding strategy, which ensures the control of the specific growth rate below the critical value, seems to be useful for *E. coli* fermentations (Lee, 1996).

The studies in the literature on BAL production were published by Çalık et al. (2004, 2006), Kaya-Çeliker et al. (2009), and Çalık and Levent (2009a, 2009b). Firstly the effects of bioprocess parameters, as oxygen transfer characteristics and pH, on BAL production were investigated by Çalık et al. (2004, 2006) by recombinant *E. coli* K12 carrying modified pUC18::bal_{HIS} gene. Furthermore, Kaya-Çeliker et al. (2009) reported the effects of oxygen transfer in more detail on BAL production by recombinant *E. coli* BL21 (DE3) pLySs carrying pRSETA::bal_{HIS}. Recently, Çalık and Levent (2009a, 2009b) used initially pretreated beet molasses as the carbon and energy source. They investigated the effects of molasses concentration on *E. coli* fermentation and found that pretreated-beet molasses can be a good carbon source for *E. coli* fermentation (Çalık and Levent, 2009a); besides, they reported the effects of pulse feeding strategies based on the air-flow detection, and glucose and fructose concentrations in the fermentation medium (Çalık and Levent, 2009b).

In this study, the aim was to develop a feeding strategy for BAL production by recombinant *E. coli* using pretreated molasses as the carbon and energy source, to increase the cell growth and product formation. Exponential feeding experiments were conducted with different feeding rates using the optimized medium. Additionally, a combined exponential and constant feeding strategy was developed, and also stability of the plasmid in long term fermentations was investigated.

CHAPTER 2

LITERATURE SURVEY

2.1. Benzaldehyde Lyase

Benzaldehyde lyase (BAL, EC 4.1.2.38) is a thiamin diphosphate (ThDP) dependent enzyme, which catalyzes the cleavage of acyloin linkage of benzoin to form benzaldehyde (Gonzàlez and Vicuña, 1989; Demir et al., 2001, 2002). It was first identified from *Pseudomonas fluorescens* Biovar I by Gonzàlez and Vicuña (1989). Due to the ability of BAL to convert benzoin into benzaldehyde, this strain can use benzoin as the sole carbon and energy source (Gonzàlez and Vicuña, 1989). Subsequent to the identification, the gene encoding benzaldehyde lyase was cloned and characterized (Hinrichsen et al, 1994); thereafter it was expressed in different *E. coli* strains (Demir et al., 2001; Pohl et al., 2002; Sanchez-Gonzalez and Rosazza, 2003; Janzen et al., 2006; Kaya-Çeliker et al., 2009).

Gonzàlez and Vicuña (1989) defined a purification procedure for benzaldehyde lyase, and determined the characteristic properties of BAL, such as molecular weight, cofactor requirements, substrate specificity, and pH range for activity. They reported that BAL requires ThDP to show enzymatic activity; the maximum activity was obtained at 0.01 mM ThDP, and ThDP became inhibitory above a concentration of 0.5 mM. In addition, a divalent cation was needed as a cofactor; it was found that BAL shows optimal activity with either 1 mM Mn^{2+}, Mg^{2+}, or

Ca^{2+} ion. The pH range for maximal activity was reported as pH 7.5 to 8.5, and below pH 6.0 the enzyme was inactive. They stated that BAL is highly specific, since only benzoin and anisoin (4,4′-dimethoxybenzoin) were found as substrates. Additionally, a catalytic mechanism was proposed in this study.

Benzoin is slightly soluble in aqueous buffer, therefore when racemic benzoin was reacted with BAL in potassium phosphate buffer, it was seen that a very small amount of benzaldehyde was formed. To overcome this problem 20% DMSO (v/v) or 15% polyethylene glycol (PEG 400) (v/v) was used as a cosolvent and increased benzaldehyde formation was observed (Demir et al., 2001).

In another study, Domínuguez de María et al. (2006) investigated the effects of cosolvents, cofactors and the pH of the reaction medium on the stability and activity of BAL, which are important parameters for the industrial applications. They reported that the presence of the cofactors ThDP and Mg^{2+} significantly increased the stability; also the optimum concentration of cosolvent DMSO for the stability and activity of BAL was found as 30% (v/v). Furthermore, the maximum catalytic activity was obtained at pH 9.5.

Additionally, the influence of pH, temperature, buffer salt, cofactors (Mg^{2+} and ThDP) and cosolvents (DMSO and PEG) on the stability of BAL was investigated by Janzen et al. (2006). According to this study, BAL is stable up to 37 ^{0}C, and between pH 6 and 8, where pH 8 is the optimum value for the reactions and the enzyme is inactive below pH 6. Cofactors are needed for stability and the optimal concentrations are 0.1-5 mM and 1-5 mM, for ThDP and $MgSO_4$, respectively. Optimal buffers were found as potassium phosphate and Tris; moreover, 20 % DMSO gave better results than PEG-400 in terms of enzyme stability, substrate and product solubility.

In a more recent study, Schmidt et al. (2009) systematically investigated the influence and also the interactive effect of ionic strength, pH, and concentration of

DMSO on benzaldehyde lyase activity for the first time. It was found that a correlation is present between pH and DMSO concentration, which was explained by a direct interaction of DMSO with an amino acid in the catalytic site of the enzyme. The outcomes of this study are important in order to understand the catalytic mechanism of BAL and for the determination of optimum conditions for BAL-catalyzed reactions.

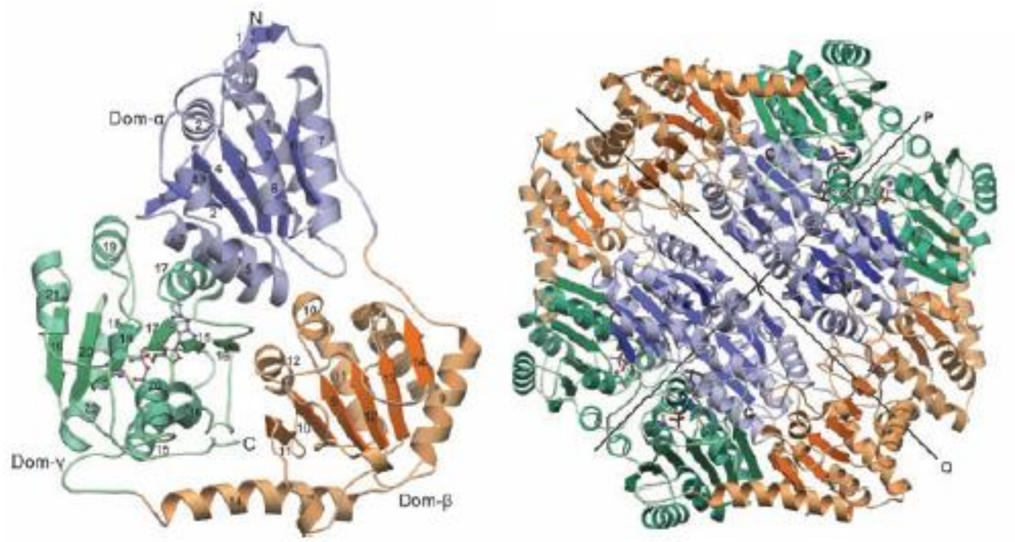

Figure 2.1. Stereo ribbon plot of a BAL subunit and BAL tetramer (Mosbacher et al., 2005).

In the literature there are some studies on the determination of the structure of benzaldehyde lyase. Mosbacher et al. (2005) reported the cyrstal structure of BAL with bound ThDP, and the substrate specifity on structural basis. BAL, having a molecular weight of 4x58.9 kDa, is a homotetramer with a chain of 4x563 amino acids, and each subunit binds to one ThDP molecule using one Mg^{2+} ion. The structure of a BAL subunit and BAL homotetramer is shown in Figure 2.1.

Overall size of the BAL homotetramer was found approximately as 95x95x75 $Å^3$ (Mosbacher et al., 2005). Subsequently, Maraite et al. (2007) crystallized BAL to investigate the molecular structure of catalysis and enantioselectivity of a BAL catalyzed reaction with a higher resolution.

The reaction engineering of BAL catalyzing the carboligation of benzaldehyde and acetaldehyde producing (R)-hydroxypropiophenone [(R)-HPP] was reported by Stillger et al. (2006). They were developed a kinetic model for the continuous process of BAL-catalyzed reaction. In terms of reaction system characterization they reported that benzoin formation as an intermediate was occurred, which then converted into HPP. Kinetic characterization of the benzoin formation from benzaldehyde, HPP formation from benzaldehyde and benzoin was conducted. It was also indicated that the maximum enzyme stability and the highest activity was seen at pH 7 and 9, respectively. As a conclusion they suggested that backmixed flow reactor will be appropriate for BAL catalyzed HPP synthesis. Besides, Hildebrand et al. (2007) investigated a reactor system for selective synthesis of (R)-HPP derivatives, and a kinetic model was derived which is used to define optimal parameters for a continuously operating enzyme membrane reactor.

As benzaldehyde lyase is an enantioselective enzyme, it is used for enantioselective synthesis of various enantiopure α-hydroxy ketones (Sanchez-Gonzalez and Rosazza, 2003; Demir et al., 2004; Pohl et al., 2004). Demir et al. (2001) investigated if two enantiomers of benzoin converted into benzaldehyde through BAL catalysis; they found that only (R)-benzoin acts as substrate, and no reaction was obtained with (S)-benzoin. It was also found that equilibrium is present between the synthesis and cleavage of (R)-benzoin. Moreover, it was demonstrated that BAL has a potential for both carbon-carbon (C-C) bond cleavage and C-C bond formation (Demir et al., 2001). The catalytic mechanism and the reactions catalyzed by BAL are given in Figure 2.2.

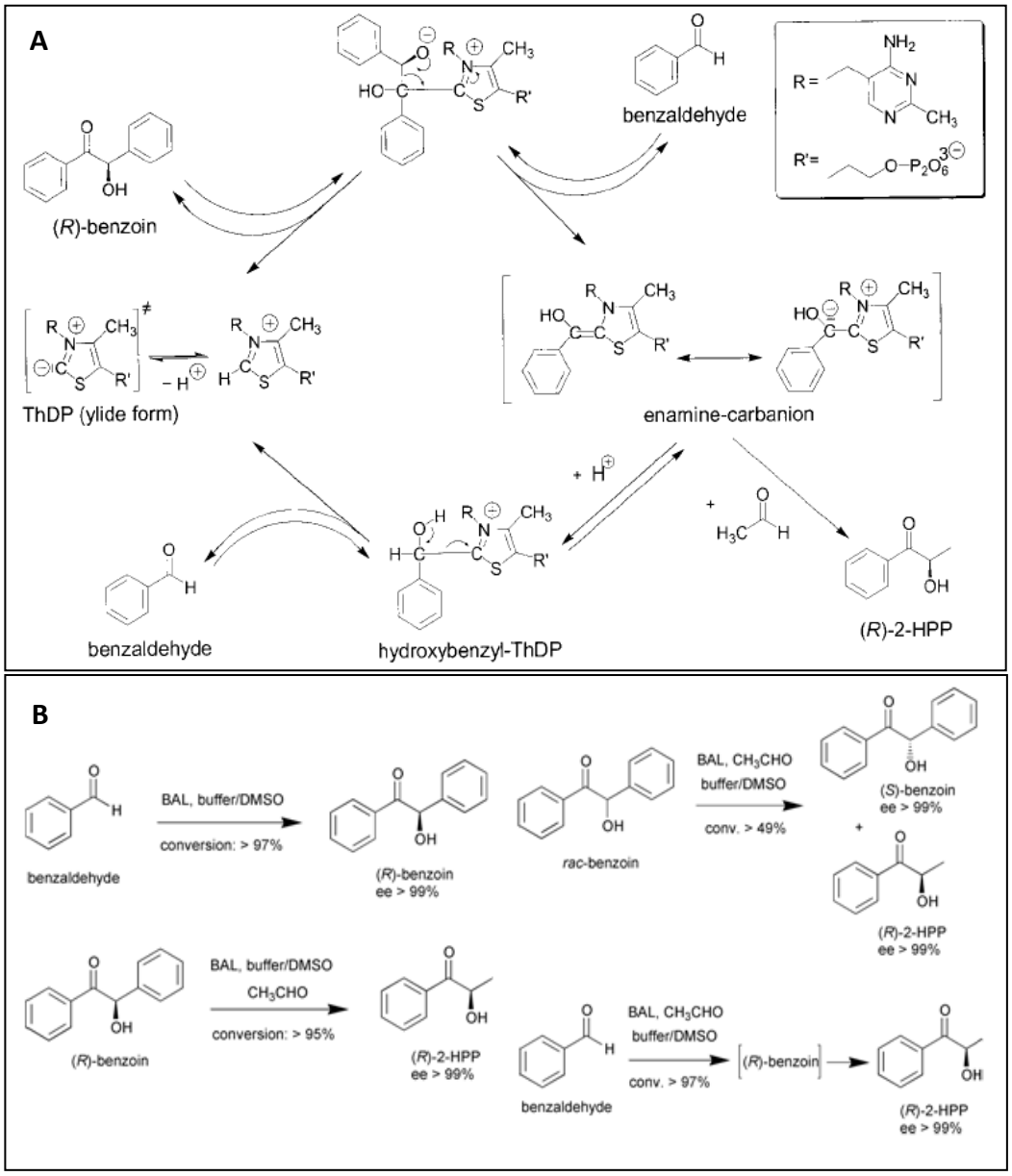

Figure 2.2. (A) Mechanism for the benzaldehyde lyase catalyzed formation and cleavage of benzoin; (B) a variety of reactions catalyzed by BAL (Demir et al., 2001).

Knoll et al. (2006) investigated the binding site of BAL, and a detailed illustration was given for the shape of the binding site (Figure 2.3). For a better understanding of the enantioselective property of BAL, (R)- and (S)-benzoins were placed into

the binding site of the enzyme, and it was observed that it is not possible to fit the S enantiomer while R enantiomer can be fitted. With structural studies, substrate specifity, stereo- and enantioselectivity of the enzyme was given in detail; thus further investigations to increase the substrate range of benzaldehyde lyase can be expected.

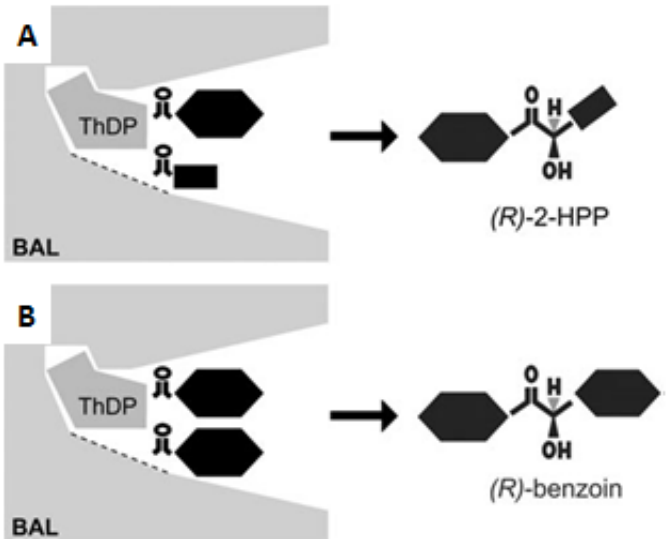

Figure 2.3. Schematic illustrations for the shape of the binding site of BAL. (A) Formation of (R)-2-HPP with acceptor acetaldehyde, donor benzaldehyde; (B) Formation of (R)-benzoin with acceptor, and donor benzaldehyde (Knoll et al., 2006).

The substrate range of benzaldehyde lyase was investigated by Demir et al. (2002) which resulted in highly selective synthesis of (R)-benzoins and (R)-2-hydroxypropiophenone [(R)-2-HPP] derivatives by BAL catalyzed C-C bond formation from aromatic aldehydes and acetaldehyde. On the basis of this study with further investigation methoxy and dimethoxy acetaldehydes were also found

as good choices (Demir et al., 2003). Additionally, it was shown that enantioselective synthesis of mixed benzoins can be achieved with BAL catalysis (Dünkelmann et al., 2002). In another study it was reported that BAL catalyzed mixed acyloin reactions with phenylacetaldehyde and methoxybenzaldehydes (Sanchez-Gonzalez and Rosazza, 2003). Moreover, the effects of formaldehyde on the synthesis of hydroxyacetophenones with BAL catalysis were investigated (Demir et al., 2004).

Moreover, for the resolution of racemic benzoin, a new method named as enzyme enhanced ultrafiltration was developed by Ölçeroğlu et al. (2008). In this chiral seperation method, the apo form of benzaldehyde lyase was used as the ligand, since it only binds to R-benzoin, and the reason for using the apo-form of the enzyme was to prevent the reaction. For the separation of the two enantiomers, the addition of ligand to the benzoin solution was followed by ultrafiltration. It was concluded that, enzyme enhanced ultrafiltration method is suitable for the chiral separation of racemic molecules.

2.2. Bioprocess Parameters

Microorganisms have been used to produce fermented foods from biological materials since the ancient times. Later on, bioprocesses have been developed for a variety of commercial products from relatively cheap to expensive materials; i.e. industrial alcohol, antibiotics, therapeutic proteins, vaccines, enzymes and living cells such as bakers' and brewers' yeast (Doran, 1995). To produce microbial enzymes, it is typical to use an aerobic submerged culture in a stirred reactor with a microorganism which is mostly genetically engineered (Aehle, 2007).

For enzyme production firstly the identification of the microbial strain producing the enzyme with desired physical properties and catalytic specifity is required. Generally, genetic modification of the microorganism is necessary to increase the enzyme production. Subsequent to the microorganism selection, determination of

the composition of the medium and the fermentation conditions such as pH, temperature, dissolved oxygen concentration and mode of operation, are needed for a high yield and low cost bioprocess (Glazer and Nikaido, 1995).

2.2.1. Microorganism Selection

Different microorganisms from eukaryotic systems such as yeasts and fungi to prokaryotic systems from both the gram-negative and gram-positive families have been used so far for the production of enzymes (Aehle, 2007). The selection and thereafter the improvement of the appropriate host play a crucial role in the production of the interested product (Nielsen et al., 2003). Strain selection and improvement is necessary to enhance the productivity of the culture resulting in the reduced costs of the processes, and feasible industrial applications. This can be achieved by selection of a strain which can synthesize the highest product with the same amount of the raw materials. Mutation, selection, and genetic recombination are the general methods used for strain improvement with increasing their metabolic capacities (Parekh et al., 2000).

There are many advantages of using *E. coli* as the host microorganism. It is possible to achieve high yields and good protein secretion with *E. coli* cultivations. Additionally, gene expression control is easy and a wide choice of cloning vectors is present. Since having an expression system with a high productivity is required for the low cost productions, *E. coli* becomes a suitable host microorganism. However, it has disadvantages as lack of post-translational modifications, high endotoxin content and protein aggregation (Nielsen et al., 2003).

In this study, the host microorganism used for the enzyme benzaldehyde lyase production was *E. coli*. *Pseudomonas fluorescens* was found as the natural producer of benzaldehyde lyase (Gonzàlez and Vicuña, 1989). In the literature, cloning and expression studies of the gene encoding benzaldehyde lyase to

different *E. coli* strains were reported (Hinrichsen et al, 1994; Demir et al., 2001; Pohl et al., 2002; Sanchez-Gonzalez and Rosazza, 2003; Janzen et al., 2006). Moreover, bioprocess development studies for benzaldehyde lyase production were conducted by Çalık et al. (2004, 2006) and Çalık and Levent (2009a, 2000b) with various *E. coli* strains. Additionally the potential of some *Bacillus* species (Kaya, 2006) and *Pichia pastoris* (Büyüksungur, 2006) were investigated for extracellular production of BAL; however due to the ineffective production with these hosts, *E. coli* remains its importance for benzaldehyde lyase production.

2.2.1.1. *Escherichia coli*

Escherichia coli is a rod shaped gram-negative bacterium which was identified in 1885 by Theodor Escherich. It is one of the best studied, and accordingly one of the most important model organisms in biotechnology, since it grows rapidly on a simple media, and its genetics, biochemistry, and physiology is well known (Glazer and Nikaido, 1995; Choi et al., 2006). Additionally, its whole genome is known and it has a well characterized system; having these advantages, *E. coli* is one of the most frequently used host microorganism for recombinant protein production (Yee and Blanch, 1993; Lee, 1996).

In *E. coli* cultivations with high growth rates high cell densities can be achieved, and together with the high expression levels, extremely high volumetric productivities can be obtained (Shuler and Kargı, 1992; Choi et al., 2006). The studies on the high cell density cultivation of *E. coli* (Lee, 1996) enabled the production of various recombinant proteins and non-protein products with high productivities (Lee, 1996; Jeong and Lee, 1999; Gerigk et al., 2002; Choi et al., 2006). At the present time, it is possible to easily obtain *E. coli* cultures having cell concentrations above 100 g DCW L^{-1} using fed-batch techniques (Lee, 1996; Shiloach and Fass, 2005).

The accumulation of acetate, a metabolic by product, is the major problem in *E. coli* fermentations; as high amounts of acetate inhibit cell growth, and thereby reduce recombinant protein production (Luli and Strohl, 1990; Han et al., 1992; Åkesson et al., 2001). Acetate formation in *E. coli* cultures occurs both under anaerobic conditions, which the mechanism is generally stated as mixed-acid fermentation (Åkesson et al., 2001), and under fully aerobic conditions, where acetate formation is thought to be an overflow metabolism, which takes place at high specific growth rates and when excess carbon source present in the fermentation medium (Åkesson et al., 1999). In order to reduce acetate formation, strain selection, and determination of the medium composition, carbon source supply strategy and cultivation conditions are important (Van de Walle and Shiloach, 1998; Åkesson et al., 2001).

Acetate formation is directly related with the specific growth rate and the threshold specific growth rate value changes depending on the medium and the strain used (Han et al., 1992; Lee, 1996). As an example, with a continuous culture of *E. coli* K12 D1 it was found that acetate formation in complex medium occurs at a lower specific growth rate than in a chemically defined medium; acetate was formed above 0.2 h^{-1} and 0.35 h^{-1} for complex and defined medium, respectively (Meyer et al., 1984). Additionally, initial medium composition also affects acetate formation (Lee, 1996); acetate is not produced when glycerol is used as a carbon source (Holms, 1986), and when initial glucose concentration in the medium is below 0.748 g/L (Marison and von Stocker, 1986).

Since high concentrations of the carbon source in the medium causes acetate formation, different fed-batch strategies by supplying limited carbon source, which is most often glucose, were investigated to reduce or avoid acetate production (Paalme et al., 1990; Kleman et al., 1991; Kleman and Strohl, 1994; Yoon et al., 1994; Lin et al., 2001). Besides, fed-batch mode of operation is commonly preferred to reach high cell densities with high product yields while minimizing acetate production (Yee and Blanch, 1993; Lee, 1996). Moreover, in

aerobic fermentations oxygen limitations can be avoided with a feeding strategy which is developed considering the dissolved oxygen concentration (Yee and Blanch, 1993).

Due to the higher cell concentration and protein production results in fed-batch fermentations when compared with the other modes of operation (Wong et al., 1998); different feeding strategies have been carried out to achieve high cell density cultures of *E. coli* (Yee and Blanch, 1993; Yoon et al., 1994; Lee, 1996; Choi et al., 2006). Feeding methods such as pH-stat (Robbins and Taylor, 1989; Wong et al., 1998), DO-stat (Mori et al., 1979; Johnston et al., 2002-2003), according to the glucose (Luli and Strohl, 1990; Gao et al., 2002) and acetate (Shimizu et al., 1998) concentrations in the medium, and controlled specific growth rate (Paalme et al., 1990; Riesenberg et al., 1991) have been used in *E. coli* fermentations.

The effects of feeding methods on cell growth, recombinant protein production, and acetate accumulation for different *E. coli* strains were investigated by researchers thus far (Table 2.1). Among the feeding methods in fed-batch cultures, the exponential feeding strategy seems to be useful for *E. coli* fermentations, since the specific growth rate can be controlled below the critical value with avoiding the inhibitory levels of acetate accumulation (above 5 g L^{-1}) (Lee, 1996).

In the literature, *E. coli* BL21 strain has been reported as a low acetate producer (Van de Walle and Shiloach, 1998; Åkesson et al., 1999; Choi et al., 2006); even at high concentrations of glucose *E. coli* B strain accumulate lower amounts of acetate than *E. coli* K strain (Shiloach et al., 1996; Choi et al., 2006). In the present study *E. coli* BL21 (DE3) pLySs carrying pRSETA::bal_{HIS} (Kaya, 2006), a high benzaldehyde lyase and low acetate producer strain, was used as the host microorganism for the production of recombinant benzaldehyde lyase.

Table 2.1. Examples of *E. coli* fermentations with fed-batch operations.

Strain	Product	Feeding Method	Outcomes	Reference
E. coli B *E. coli* MC1060 *E. coli* HB101 *E. coli* JM105	-	Glucose feed-back controlled feeding	Comparison of acetate production by different strains for the first time and a model was derived for growth inhibition by acetate	Luli and Strohl, 1990
E. coli K12 W3350	-	Computer controlled fixed growth rate resulting in glucose limited fermentation	By choosing low specific growth rates, the concentration of the acid by-products can be reduced	Paalme et al., 1990
E. coli MC1061	Human growth hormone	Fixed and stepwise increased feeding	With glucose limited fed-batch fermentations significantly higher specific product yields and lower acetate concentrations were obtained	Jensen and Carlsen, 1990
E. coli X90	Trypsin	Exponential feeding at $\mu = 0.2\ h^{-1}$	Increase in volumetric yield of the product	Yee and Blanch, 1993

Table 2.1. Examples of *E. coli* fermentations with fed-batch operations *(continued)*.

Strain	Product	Feeding Method	Outcomes	Reference
E. coli BL21 (λDE3)	Exotoxin A	Dissolved oxygen concentration controlled feeding (DO at 30%)	Low and high glucose concentrations were investigated; for BL21 strain similar growth at both two cases, for JM109 strain higher cell concentrations at lower glucose case was obtained, acetate accumulation was reduced with low glucose case	Shiloach et al., 1996
E. coli JM109	Maltose binding protein			
E. coli BL21	Bioadhesive protein	pH-stat	Post-induction feeding was investigated; protein production was significantly affected by post-induction feeding strategy where the highest productivity was achieved by linear feeding	Wong et al., 1998
		Exponential feeding		
		Constant feeding		
		Linear feeding		
E. coli BL21 (DE3)	Protein L	Short pulses to an exponential feeding	With dissolved oxygen responses to the short pulses in the feed rate, on-line detection of acetate formation was obtained	Åkesson et al., 1999
E. coli TOPP1	Xylanase			

Table 2.1. Examples of *E. coli* fermentations with fed-batch operations *(continued)*.

Strain	Product	Feeding Method	Outcomes	Reference
E. coli BL21 (DE3) *E. coli* K12 UL635 *E. coli* K12 UL634	Proprietary protein	Automated feeding which avoids overflow metabolism and anaerobic conditions	Very low concentrations of acetate and glucose was obtained while having a high glucose feed rate, the method was independent of the strain and the operating conditions	Åkesson et al., 2001
E. coli K12 W3110 *E. coli* RB791	-	Combined exponential and afterwards constant feeding Constant feeding	A dynamic model was derived which considers changing capacities for glucose and oxygen uptake and results in a better fit of experimental data for glucose in fed-batch cultures	Lin et al., 2001
E. coli DH5α (pSI026)	Nuclease	Pseudo-exponential feeding (constant volumetric feed rate with changing glucose concentration in the feed)	The maximum product concentration was achieved at the specific growth rate of 0.05 h^{-1}; however, the specific production rate was increased with increasing specific growth rate values	Cheng et al., 2003

Table 2.1. Examples of *E. coli* fermentations with fed-batch operations *(continued)*.

Strain	Product	Feeding Method	Outcomes	Reference
E. coli BL21 (DE3)	Human interferon-gamma	Exponential feeding at constant (μ=0.12 h^{-1}) and variable (μ=0.12 - 0.52 h^{-1}) growth rates	With variable growth rate fed-batch fermentation; plasmid stability and specific yield was increased, and acetate production was lowered	Khalilzadeh et al., 2003
E. coli AF1000	β-galactosidase	Combined exponential and afterwards constant feeding	Protein production rate was higher at higher μ, and acetate accumulation was higher for higher production; also it was proposed that production at high μ is precursor-limited, while production at low μ is carbon and/or energy limited	Sandén et al., 2003
E. coli K12	Poly(3-hydroxybutyrate)	Exponential feeding (at μ=0.1 and 0.3 h^{-1}) combined with pH-stat	The proposed method overcomes the limitation of simple exponential feeding and pH-stat, and with the low specific growth rate case, higher cell densities were achieved by lowering the acetate production	Kim et al., 2004

Table 2.1. Examples of *E. coli* fermentations with fed-batch operations *(continued)*.

Strain	Product	Feeding Method	Outcomes	Reference
E. coli BL21 (DE3) pLySs	Benzaldehyde lyase	Pulse feeding of molasses based on air-flow rate and glucose-fructose detection	Cell concentrations and enzyme activities were significantly increased with fed-batch cultivations; and the best strategy was reported as the case in which fructose and glucose concentrations were increased to 1.5 g L^{-1} when they were depleted in the medium	Çalık and Levent, 2009b

2.2.2. Medium Design

Determination of the fermentation medium composition is an important step for the design of a fermentation process (Nielsen et al., 2003). First of all, the medium have to supply all the essential elements needed for cell growth. Microorganisms require nutrients for biosynthesis of cellular substances, products of cell operation and maintenance, as well as for a source of energy (Kampen, 1997).

Fermentation media have to contain the suitable sources of carbon, nitrogen and sulfur, additionally essential minerals, vitamins and other growth factors (Kampen, 1997; Nielsen et al., 2003). The carbon source can be referred as the most important substance in the fermentation medium since the 50% of the biomass consist of carbon, and it also serves as an energy source for the cells. Subsequent to the carbon source, nitrogen source is the other most plentiful element in the medium. Nitrogen and sulfur occur in reduced forms in the biomass (Kampen, 1997). In addition, essential minerals are required in trace amounts for the cell growth and the growth factors can be needed to achieve rapid growth with high yields of the product (Nielsen et al., 2003). Furthermore, the concentrations of the necessary elements are important since they have to ensure the required amounts (Kampen, 1997), and also above a concentration some nutrients can inhibit cell growth (Lee, 1996).

The growth media can be divided into two main groups as defined and complex media. Defined or synthetic medium is prepared with pure compounds in defined and exact amounts. Defined media can usually be preferred for investigating the effects of the specific nutrients on cell growth and product formation (Kampen, 1997). The important advantages of defined media are as follows; the results are easily reproducible, the media have low foaming tendency, product recovery and purification is easier.

Complex media do not have an exactly known chemical composition. They contain natural compounds, and they are usually preferred in industrial fermentations (Shuler and Kargı, 1992; Kampen, 1997). However, in some industrial applications, due to the difficulties in downstream processing and less reproducible results, defined media can be preferred (Nielsen et al., 2003). Molasses, meat and yeast extracts, whey, barley malt, corn steep liquor, and corn starch are examples of complex media (Kampen, 1997; Nielsen et al., 2003). Complex media were relatively cheap and they can be reached in large quantities. They often contain an organic nitrogen source, many different essential minerals and vitamins, also various growth factors (Nielsen et al., 2003). Rich nutrient content of complex media result in higher cell and product yields when compared to the defined media (Shuler and Kargı, 1992). Various complex carbon sources and their composition are given in Table 2.2. However, there are also some disadvantages of complex media as the composition can be variable, in storage the composition can be changed, and in addition undesired compounds can be present (Nielsen et al., 2003). Furthermore, addition of inorganic nutrients, particular to the microorganism, is usually necessary for the complex media (Kampen, 1997).

Initial addition of the required nutrients to the growth medium is usually enough for *E. coli* cultivations. Growing *E. coli* up to a cell density of 1 g L^{-1} DCW can be achieved by using the commercial complex Luria Bertani (LB) broth with appropriate environmental conditions. For higher densities, the concentrations of the nutrients must be increased in order to compensate the requirements of denser cultures, and also addition of phosphorus, sulfur and trace elements are necessary (Schiloach and Fass, 2005). However, high concentrations of the required nutrients for growth can be inhibitory for *E.coli*. The following substances are reported as inhibiting *E. coli* growth above the given concentrations; glucose, 50 g L^{-1}; ammonia, 3 g L^{-1}; iron, 1.15 g L^{-1}; magnesium, 8.7 g L^{-1}; phosphorus, 10 g L^{-1}; zinc, 0.038 g L^{-1} (Riesenberg, 1991; Lee, 1996). In *E. coli* cultivations with a defined medium in which the nutrients are having maximum non-inhibitory concentrations, cell densities of approximately 15 g L^{-1} DCW can be achieved

(Schiloach and Fass, 2005). Besides, with fed-batch cultivations it is possible to obtain higher cell densities, either with defined or complex media (Lee, 1996).

Table 2.2. Composition of various complex carbon sources (Atkinson and Mavituna, 1991).

Carbon source	Amino acid (%)	Protein (%)	Carbohydrate (%)
Molasses	-	3	54
Corn steep liquor	4.9	24.0	68.9
Corn gluten meal	8.8	62.0	20.0
Soybean meal	23.2	51-52	-
Yeast hydrolysate	34.1	40-65	-
Wheat flour	6.0	13.2	69.0
Whey powder	7.4	12.0	68.0
Rice flour	4.4	8.0	65.0

In the literature there are many studies investigating the effects of the medium components in *E. coli* cultivations. Neidhardt et al. (1974) investigated various defined minimal media for *E. coli*, and developed a media containing only simple inorganic salts and a carbon source. Yee and Blanch (1993) used various amino acids, salts, vitamins, and trace elements to optimize the growth medium. Choi and Lee (1997) investigated the effects of culture conditions, including medium effects with LB medium and M9 medium containing casamino acid (M9CA), on different strains of *E. coli* carrying plasmid for bovine growth hormone production; LB medium was found slightly better than M9CA medium. In more recent studies Çalık et al. (2004, 2006) reported the media containing, 8.0 g L^{-1}

glucose, 5.0 g L^{-1} $(NH_4)_2HPO_4$ and salt solution as the optimal media for BAL production by *E. coli* K12.

It is obvious that to achieve high cell density growth of *E. coli*, a well-investigated and designed medium and appropriate feeding strategies are needed (Lee, 1996; Schiloach and Fass, 2005). The development of the fermentation media requires experimental and trial-and-error-based processes, thus the optimization of the medium is an experiment-intensive process (Lee, 1996).

2.2.2.1. Molasses

Sugar beet molasses is the main by-product of the sugar industry; where blackstrap molasses, the common type of cane molasses, is the residual by-product of raw sugar production from sugar cane. In the fermentation industry, both blackstrap and beet molasses are commonly used (Kampen, 1997), and they are the most important raw materials, particularly for the production of baker's yeast, citric acid, feed yeasts, acetone/butanol, enzymes, organic and amino acids (Çalık et al., 2003). Molasses has important advantages as having low prices, being readily available (Park and Baratti, 1991; Makkar and Cameotra, 2002), having high sugar and rich nutrient content when compared to other sugar sources (Makkar and Cameotra, 2002). In Table 2.3, the average composition of European beet molasses from French and Dutch molasses (1990) is given as an example (Kampen, 1997). The sugar content of molasses is usually serves as the carbon source which is used in various chemical and microbial reactions. It has been used for industrial ethanol production at sugarcane factories; for rum production; and for other beverage alcohol. (Kirk and Othmer, 1994).

Table 2.3. An example for average composition of beet molasses from European samples (Kampen, 1997).

Component	Average composition (%)
Water	16.5
Sucrose	51.0
Glucose and Fructose	1.0
Raffinose	1.0
Organic non-sugars	19.0
Ash components:	11.5
SiO_2	0.1
K_2O	3.9
CaO	0.26
MgO	0.16
P_2O_5	0.06
Na_2O	1.3
Fe_2O_3	0.02
Al_2O_3	0.07
CO_3	3.5
Sulfates as SO_3	0.55
Cl	1.6
Vitamins (mg/100 g)	
Thiamine (B1)	1.3
Riboflavin (B2)	0.4
Nicotinic acid	51.0
Ca-pantothenate (B3)	1.3
Folic acid	2.1
Pyridoxine-HCl (B2)	5.4
Biotin	0.05

In the literature, Agarwal et al. (2006) used cane molasses for succinic acid production by *E. coli*. They were reported that with complex media, which was inexpensive than the defined media, succinic acid production was increased. Furthermore, statistical optimization of this process was conducted to obtain high yields of succinic acid (Agarwal et al., 2007). Çalık and Levent (2009a, 2009b) were used pretreated-beet molasses for benzaldehyde lyase production by *E. coli,* the enzyme production was increased with using molasses as a complex carbon source in batch and pulse feeding fermentations. Besides, the pretreatment process of molasses, to achieve glucose and fructose from sucrose, was reported by Çalık et al. (2003), and the effects of the pretreated molasses on serine alkaline protease production with *Bacillus* species were investigated. In a more recent study, Ye et al. (2010) investigated the effects of untreated molasses on heterologous protein production by *E. coli,* to develop an economical medium that can be used in industrial applications.

In the present study, molasses was used as the whole carbon and energy source for benzaldehyde lyase production by *E. coli*. The effects of molasses concentration on the cell and product concentrations were investigated and thereafter fed-batch cultivations with molasses feeding were conducted. Molasses was taken from Ankara Sugar Factory, and the composition is given in Table 2.4.

Table 2.4. Composition of molasses used in the present study (Ankara Sugar Factory).

Dry solids (%)	83.04
Sucrose	51.07
Raffinose	0.67
Invert sugar	0.40
Total nitrogen	2.05
Organic non-sugars	16.16
Ash	12.69
Water (%)	16.69

2.2.3. Bioreactor Operation Parameters

In bioprocesses, the cell growth and product formation are effected by environmental conditions such as temperature, pH, and dissolved oxygen concentration for aerobic fermentations (Shuler and Kargı, 1992). These parameters should be taken into account since they directly affect the production and product yields.

2.2.3.1. Temperature

Temperature is one of the most important bioreactor operation parameters which affects the performance of cells and should be kept at the optimum value during fermentation. Cellular growth rate, product formation, and also the yield coefficients are affected by temperature; however the optimum temperature for cellular production and product formation can be different. When temperature is below the optimum value, cell growth will be reduced; furthermore when temperature is higher than optimum, cell death may occur and product yield will be lowered (Shuler and Kargı, 1992, Nielsen et al., 2003).

In the literature *E. coli* fermentations have been conducted at 37 ^{0}C, which is the optimum growth temperature for *E. coli*.

2.2.3.2. pH

pH is the other parameter which is needed to be monitored and controlled throughout the bioprocess. Microbial growth rate is affected by pH, since the activity of enzyme is directly affected by pH. During the fermentation, pH can change substantially due to the production of by-products or the utilization of the nitrogen; therefore, it is necessary to control the pH of the fermentation medium. The optimum pH range differs from organism to organism; however, for many bacteria, it is between pH 3 and 8 (Shuler and Kargı, 1992).

In the literature, *E. coli* fermentations producing various proteins have been conducted at different pH values (Åkesson et al., 2001; Castan et al., 2002; Leon et al., 2003); also the effects of medium pH on *E. coli* growth with different products were investigated (Ryan et al., 1989; Çalık et al., 2006). Çalık et al. (2006) investigated the effects of un-controlled and controlled pH operations from pH 5.0 to 7.8 on recombinant *E. coli* producing benzaldehyde lyase. They reported that un-controlled pH 7.2 was the best case in terms of cell growth and BAL activity; additionally among controlled pH operations pH 7.0 was reported as the most favorable operation. Also in the studies of Çalık and Levent (2009a, 2009b), pH controlled 7.2 operations were used with molasses based medium.

2.2.3.3. Dissolved Oxygen

Dissolved oxygen (DO) can be a limiting component in aerobic fermentations due to the low solubility of oxygen in water; therefore the control of the dissolved oxygen concentration in the medium is important. Oxygen limitations can occur at high cell growth rates when oxygen consumption rate exceed the oxygen transfer rate (Shuler and Kargı, 1992). Dissolved oxygen concentration can be controlled by manipulating the rate of air supplied to the system or by changing the agitation rate; thus controlling the oxygen transfer rate.

In the literature, the effects of oxygen transfer on *E. coli* fermentations were investigated for various products and different fermentation conditions (Ryan et al., 1989; Castan et al., 2002; Leon et al., 2003). For benzaldehyde lyase production by *E. coli*, Çalık et al. (2004) systematically investigated the effects of oxygen transfer conditions. Afterwards, in the master thesis study of Angardi (2007), the optimum oxygen transfer condition for BAL production by *E. coli* was reported at 40% dissolved oxygen concentration in the medium.

2.2.4. Modes of Operation

In microbial processes, culture techniques can be classified as batch, fed-batch and continuous operations.

2.2.4.1. Batch Fermentations

In batch mode of operation, all nutrients required for cell growth and product formation are added to the medium before cultivation. During cultivations, the only supplements to the bioreactor are oxygen for aerobic fermentations, and acid and/or base for pH adjustment (Yamané and Shimizu, 1984; Harada et al., 1997). Cell growth is restricted to the total consumption of the limiting substrate in the medium (Harada et al., 1997). Additionally, the final products are only removed at the end of the process (Yamané and Shimizu, 1984). As advantages, with batch fermentations the risk of contamination and mutation is low and complete conversion of substrate is possible (Winkler, 1990; Nielsen et al., 2003). Most commercial bioprocesses are batch systems; due to the high productivity for secondary metabolites, genetic and contamination considerations, flexibility of using the system for different products. However, with batch operations variations in product quality and concentration may occur, which is undesirable (Shuler and Kargı, 1992).

In the literature, BAL production is mostly studied with batch mode of operation (Çalık et al., 2004, 2006; Çalık and Levent, 2009a). They used both defined (Çalık et al., 2004, 2006) and complex media (Çalık and Levent, 2009a); the highest BAL activities were reported as 860 U ml^{-1} and 1617 U ml^{-1}, and the maximum cell concentrations as 2.3 g L^{-1} and 5.3 g L^{-1} were obtained for defined and complex media, respectively.

2.2.4.2. Continuous Fermentations

In the continuous mode of operation, all nutrients are continuously added to the bioreactor and the cultured broth is simultaneously removed from the bioreactor at the same flow rate, thus constant culture volume is maintained (Yamané and Shimizu, 1984; Harada et al., 1997). This mentioned continuous process operates without feedback control; besides, continuous fermentations with feedback control of pH, cell and nutrient concentration are present. Continuous fermentations can be useful for the optimization of media formulation and for the investigation of the physiological state of the microorganism (Harada et al., 1997).

There are some important advantages of continuous fermentations, such as good utilization of the bioreactor, high productivity due to the autocatalytic nature of microbial reactions, steady-state operation and constant product quality. Also, continuous systems can be the preferred mode of operation due to its advantage of automation. In addition, for the productions in which catabolite repression is present, it is necessary to use continuous or fed-batch mode of operation. However, infection and mutation are the major problems of continuous processes, and these processes are inflexible, thus it is generally necessary to make adaptations to use a system with different productions (Nielsen et al., 2003).

2.2.4.3. Fed-Batch Fermentations

In fed-batch operations, one or more nutrients are fed continuously or intermittently to the bioreactor during cultivation (Yamané and Shimizu, 1984; Harada et al., 1997). Feeding can be start in the beginning of the cultivation or after a batch process (Harada et al., 1997), additionally the fermentation medium held in the bioreactor during the cultivation (Yamané and Shimizu, 1984). As a basic characteristic of fed-batch fermentation, the concentrations of the key nutrients fed into the bioreactor can be controlled by adjusting the feed rate (Yamané and Shimizu, 1984; Winkler, 1990).

Fed-batch mode of operation shows a behavior between the ideal batch and continuous fermentations (Yamané and Shimizu, 1984), and the advantages of both two fermentation systems are combined in fed-batch operations (Nielsen et al., 2003). Fed-batch processes can be used to avoid substrate inhibition, glucose effect, and catabolite repression, as well as for auxotrophic mutants (Harada et al., 1997). As an example, if substrate has an inhibitory effect, intermittent addition of the substrate improves the productivity by maintaining the concentration below the inhibitory level (Shuler and Kargı, 1992). Fed-batch fermentations are excellent for the control and the optimization of the given production criterion (Nielsen et al., 2003).

The selection of the feeding method is important, since it effects the maximum cell concentration, cell productivity, and also product formation (Lee, 1996). Fed-batch processes can be classified according to the feeding mode. In Table 2.5, the classification of fed-batch cultivations and essential explanations of each group are given. The feeding methods can be classified broadly into; without and with feedback control. In fed-batch operations with direct feedback control, the feeding rate is directly controlled by the concentration of the substrate (the carbon source) in the medium; whereas with indirect feedback control, nutrient feeding is controlled according to the measurements of physical parameters such as pH, DO, CO_2 evolution rate (CER) or cell concentration changes. In both cases, substrate concentration in the medium is kept at a constant or an optimal level. Additionally, recent technical developments in on-line sensors and the control strategies made various and sophisticated feeding modes possible (Yamané and Shimizu, 1984; Lee, 1996).

The changes in the controlled parameters in indirect feedback control strategies are related with the substrate consumption profiles of the microorganisms. For example, the DO-stat method is based on the sharp increase in DO when substrate is depleted. In this case with feeding substrate at a predetermined amount, when DO increases above the pre-set value, will provide the control of the substrate in a

desired range. Similarly, the pH-stat method is based on the changes in pH when substrate is depleted (Lee, 1996).

Table 2.5. Feeding methods in fed-batch fermentations (Yamané and Shimizu, 1984; Lee, 1996).

Without feedback control	
Constant feeding	feeding nutrient at a predetermined constant rate
Increased feeding	feeding nutrient at an increasing rate: gradual, stepwise or linear rate
Exponential feeding	feeding nutrient at an exponential rate with a predetermined constant μ
With feedback control	
Indirect feedback control	Feeding nutrient according to a controlled parameter: DO-stat, pH-stat, CER, cell concentration
Direct feedback control	Substrate concentration control

In fed-batch operations without feedback control, the feed rate is changing during fermentation with a predetermined profile. In constant feeding strategy, nutrients are fed to the system at a predetermined constant rate, and this strategy is regarded as the simplest fed-batch method (Yamané and Shimizu, 1984). With constant feeding, the specific growth rate continuously decreases due to the increase in medium volume and cell concentration in the bioreactor. Also, the increase in cell concentration slows down over time.

The increased feeding strategy with an increasing rate as stepwise, gradual, or linear feeding can increase cell growth by supplying more substrate at higher cell concentrations. Exponential growth can be achieved throughout the fermentation, if the growth limiting substrate is fed increasingly in proportion to cell growth (Lee, 1996).

In exponential feeding strategy, nutrients are fed to the system exponentially with a pre-determined constant specific growth rate. In ideal conditions it is possible to achieve an exponential cell growth and constant specific growth rate (Yamané and Shimizu, 1984; Lee, 1996). Additionally, with exponentially increased feeding of the growth limiting substrate, a high cell growth for a long period of time can be achieved, and the substrate concentration in the medium can be kept constant (Yamané and Shimizu, 1984). However, the specific growth rate may deviate in some cases due to the unexpected conditions during fermentation, in such cases feeding methods with additional control mechanisms may be necessary (Lee, 1996).

The exponential fed-batch fermentations are generally the most suitable operations to obtain maximum amounts of cell concentrations in the shortest time (Yamané and Shimizu, 1984). Exponential feeding methods have been successfully used for *E. coli* fermentations; where the specific growth rate is usually selected between 0.1 and 0.3 h^{-1} to avoid acetate accumulation (Yee and Blanch, 1993; Lee, 1996; Khalilzadeh et al., 2003; Kim et al., 2004).

Fed-batch fermentations of *E. coli* are already pointed out in Section 2.2.1.1. Since *E. coli* is the most commonly used host microorganism, various feeding strategies have been investigated with different types of products. Recently, as the only fed-batch study for BAL production, pulse feeding strategies according to the air flow rate and glucose-fructose concentrations were developed with a pretreated molasses based medium (Çalık and Levent, 2009b). In the DO-stat pulse feeding strategy, when the air flow was reached its minimum, pulse feed was given to the

system resulting in a 2.5 g L^{-1} glucose and 2.5 g L^{-1} fructose concentration in the medium. In the substrate concentration controlled pulse feeding strategies, glucose and fructose concentrations in the medium were measured on-line and when they were depleted pulse feed was given to the system with two independent operations, resulting in an increase of 2.5 or 1.5 g L^{-1} concentrations of glucose and fructose each. The highest BAL activity and cell concentration results were reported as 8.04 g L^{-1} and 2315 U ml^{-1}, respectively.

In the current study, exponential feeding at pre-determined constant specific growth rates and combined exponential-constant feeding strategies were developed for BAL production by *E. coli.*

2.2.5. Bioprocess Characteristics

2.2.5.1. Cell Growth Kinetics, Yield Coefficients and Specific Rates

When microorganisms start to grow in a liquid media, typically a curve including several phases, as lag phase, exponential (logarithmic) growth phase, stationary phase and death phase, are observed. Lag phase is the adaptation phase of cells to the environment. It is followed by an exponential growth phase in which cell concentration increase exponentially. Thereafter, along with the depletion of the nutrients and/or accumulation of the toxic by-products, a stationary phase occurs where the growth rate is equal to the death rate. Finally, a death phase follows the stationary phase (Bailey and Ollis, 1986; Shuler and Kargı, 1992; Doran, 1995).

In fermentation processes calculation of the yield coefficients, and the specific rates of cell growth, product formation and substrate consumption is important for an accurate evaluation of the bioprocess. The rates of cell and product formation and substrate consumption can be determined simply with measuring the concentrations of each one throughout the bioprocess. The specific rates are the normalized rates according to the cell concentrations, and it is better to compare

fermentations with specific rates since cell concentrations can differ between different experiments.

The rate of the cell growth, r_x, which is related with the cell concentration and specific growth rate, is described as (Bailey and Ollis, 1986),

$$r_X = \mu\, C_X \tag{2.1}$$

and combining equation (2.1) with the general mass balance for biomass for fed-batch processes,

$$r_X V = \frac{d(C_X V)}{dt} = \mu\, C_X V \tag{2.2}$$

where C_x is the cell mass concentration (g L^{-1}), t is time (h), V is volume (L) and μ is the specific growth rate (h^{-1}).

Volume change in the system is due to the molasses feeding (Q, L h^{-1}),

$$\frac{dV}{dt} = Q \tag{2.3}$$

Substituting equation (2.3) into (2.2),

$$\frac{dC_X}{dt} = (\mu - \frac{Q}{V})C_X \tag{2.4}$$

and with rearranging equation (2.4), specific growth rate can easily be calculated easily from equation (2.5);

$$\mu = \frac{dC_X}{dt}\frac{1}{C_x} + \frac{Q}{V} \tag{2.5}$$

Similar to the equation (2.1), substrate consumption rate, r_s, can be expressed as,

$$r_S = \frac{dC_s}{dt} = q_S C_X \tag{2.6}$$

where q_s is the specific substrate consumption rate (h^{-1}), and C_s is the substrate concentration (g L^{-1}).

For fed-batch experiments where substrate is fed to the system continuously, in the present study molasses was fed to the bioreactor with exponential or constant rate, mass balance for substrate can be written as,

$$QC_{So} + r_S V = \frac{d(C_S V)}{dt} \tag{2.7}$$

Substituting equation (2.6) into (2.7);

$$QC_{So} + q_S C_X V = V\frac{dC_S}{dt} + C_S\frac{dV}{dt} \tag{2.8}$$

With quasi-steady-state assumption where the substrate feed rate is nearly equal to consumption rate and no significant accumulation of substrate occurs, which is typical for fed-batch fermentations, the terms on the right hand side are generally negligible,

$$QC_{So} + q_S C_X V \approx 0 \tag{2.9}$$

Rearranging equation (2.9) and dividing by volume (V),

$$-q_S = \frac{Q}{V}\frac{C_{So}}{C_X} \tag{2.10}$$

q_s can be calculated from equation (2.10) in the case of quasi-steady-state growth condition.

Similar to equation (2.1), product formation can be described by the following equations for batch and fed-batch fermentations, respectively,

$$r_P = q_P\, C_X \tag{2.11}$$

$$r_P V = \frac{d(C_P V)}{dt} = q_P C_X V \tag{2.12}$$

where q_p is the specific product formation rate (h^{-1}), and C_P is the product concentration (g L^{-1}).

Inserting equation (2.11) into (2.12),

$$\frac{dC_P}{dt} = q_P C_X - \frac{Q}{V} C_P \tag{2.13}$$

and rearranging equation (2.13) for calculation of specific product formation rate q_s,

$$q_P = \left(\frac{dC_P}{dt} + \frac{Q}{V} C_P\right) \frac{1}{C_x} \tag{2.14}$$

Additionally, the term Q/V is the dilution rate which exists in fed-batch operations, and for batch cases it is equal to zero.

Yield coefficients (Table 2.6) of cell and product are important parameters, since they demonstrate the efficiency of utilization of substrate or oxygen for the

formation of product and cell. As a general definition for cell and product yield on substrate is given in the following equations respectively,

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \tag{2.15}$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \tag{2.16}$$

where ΔX, and ΔP are the mass or moles of cell and product produced, respectively; and ΔS is the mass or moles of substrate consumed. $Y_{X/S}$ and $Y_{P/S}$ are the overall yield coefficients.

Table 2.6. Definitions of frequently used yield coefficients.

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

The overall coefficients represent an average value for the whole bioprocess; however the yield coefficients may change throughout the fermentations. Thus, calculation of an instantaneous yield for a certain time can be necessary (Doran, 1995). Calculation of the instantaneous yield of product on substrate concentration is as follows,

$$Y'_{P/S} = \frac{dP}{dS} = \frac{dP/dt}{dS/dt} \tag{2.17}$$

In aerobic fermentations, total oxygen consumption rate, r_o, including oxygen used cell growth and maintenance and neglecting by-product formation, can be expressed as (Çalık et al., 2006),

$$-\frac{dC_O}{dt} = -r_O = \left(\frac{1}{Y_{X/O}}\right)\frac{dC_X}{dt} + m_O C_X \tag{2.18}$$

where C_O is the dissolved oxygen concentration (g L^{-1}), $Y_{X/O}$ is the overall yield coefficient for cell growth on oxygen, and m_o is the maintenance coefficient for oxygen (g oxygen g^{-1} DCW h^{-1}).

Reorganizing equation (2.18),

$$-\frac{r_O}{dC_X/dt} = \frac{1}{Y'_{X/O}} = \frac{1}{Y_{X/O}} + \frac{m_O}{\mu} \tag{2.19}$$

is obtained which can be used for the calculation of the maintenance coefficient for oxygen (Çalık et al., 2004). From the slope of the plot of $1/Y'_{X/O}$ versus $1/\mu$, m_O can be determined. Also, the maintenance coefficient for substrate, m_S, can be determined with a similar approach.

2.2.5.2. Oxygen Transfer Characteristics

Oxygen transfer between microorganism and fermentation medium occurs in several steps; mass transfer resistances are present at the interfaces of gas-liquid, liquid-solid, and also within the cell (Bailey and Ollis, 1986; Nielsen et al., 2003). Oxygen is usually supplied to the system by sparging air through the medium. Oxygen transfer to the microorganism from gas bubbles is usually limited by the transfer through liquid film surrounding the bubble (Shuler and Kargı, 1992). The oxygen transfer rate (OTR, mg O_2 l^{-1} h^{-1}) from gas to liquid phase is given by,

$$OTR = K_L a(C_O^* - C_O) \tag{2.20}$$

where, C_O^* is saturated oxygen concentration (mg/L), C_O is the dissolved oxygen concentration in the medium (mg/L), K_L is the overall oxygen transfer coefficient (cm/h), a is the gas-liquid interfacial area (cm^2/cm^3), and K_La is the overall volumetric oxygen transfer coefficient (h^{-1}) (Shuler and Kargi, 1992). Due to the fact that the liquid phase mass transfer resistance is dominate, the overall liquid phase mass transfer coefficient, K_La, can be assumed as equal to liquid phase mass transfer coefficient, k_La. Furthermore, the maximum oxygen transfer rate is the transfer rate when the dissolved oxygen concentration present in the medium is equal to zero,

$$OTR_{max} = K_L a C_O^* \tag{2.21}$$

The rate of oxygen uptake (OUR) of microorganisms is given by the following equation,

$$OUR = -r_O = q_O C_X \tag{2.22}$$

where q_O is the specific oxygen consumption rate (Shuler and Kargı, 1992).

The Dynamic Method (Bandyopadhyay and Humprey, 1967), which is applied during fermentation simply with measuring the dissolved oxygen concentration, is the most common empirical method used for the determination of $K_L a$ values. The method is based on the following mass balance for dissolved oxygen concentration;

$$\frac{dC_O}{dt} = OTR - OUR = K_L a(C_O^* - C_O) + r_O \qquad (2.23)$$

Application of the Dynamic Method is shown in Figure 2.4; first air flow is turned off (region-II), and then again air is supplied to the system (region-III). In region II, the medium is de-oxygenated by turning off the air flow and lowering the agitation rate. During the de-oxygenation period, there is no oxygen transfer and microorganisms consume the oxygen already present in the medium, therefore dissolved oxygen concentration, C_O, drops. When the air supply given to the bioreactor is turned off, equation (2.23) becomes;

$$\frac{dC_O}{dt} = r_O \qquad (2.24)$$

At region-II, oxygen uptake rate, $-r_o$, can be determined with using equation (2.24).

Thereafter, in region-III, air flow is turned on again and the increase in dissolved oxygen concentration is monitored. Equation (2.23) is valid for this period, with rearranging this equation,

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

From the slope of the plot of C_O versus (dC_o/dt-r_o), K_La can be determined. In region-III the r_o value is assumed to be the same as the value obtained in period-II for the same analyses set.

The Dynamic Method can also be applied when there is no microorganism in the medium, thus r_O=0, and with this experiment the physical mass transfer coefficient K_La_0 can be determined (Nielsen et al., 2003). For the application of the Dynamic Method in this condition, the medium is de-oxygenated by sparging nitrogen. Afterwards, air flow is turned on and the increase in dissolved oxygen is monitored as a function of time. In this case equation (2.25) is reduces to,

$$C_O = -\frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \tag{2.26}$$

When C_O versus dC_O/dt is plotted, the slope is the physical mass transfer coefficient, K_La_0.

The maximum possible oxygen utilization rate (OD = oxygen demand) which is defined as (Çalık et al., 2000),

$$OD = \frac{\mu_{max} C_X}{Y_{X/O}} \tag{2.27}$$

This term enables the comparison of the relative rates of maximum oxygen transfer and biochemical reactions, thus the rate limiting step of the bioprocess can be determined.

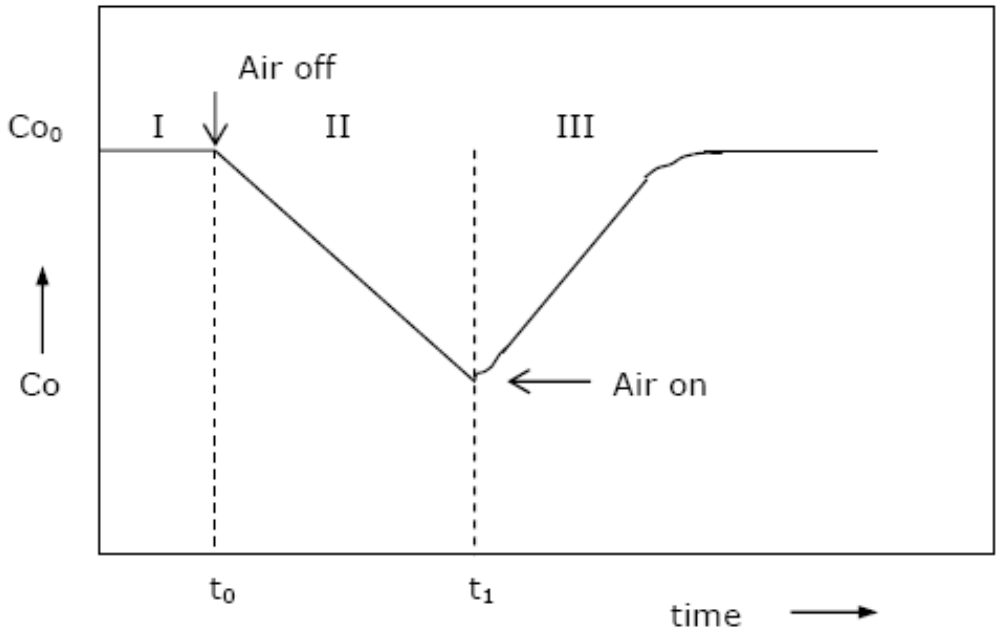

Figure 2.4. Variations in dissolved oxygen concentration with time while applying dynamic method for measurement of K_La.

The oxygen limitations in an aerobic process can be interpreted from the calculations of effectiveness factor, η, which is the oxygen uptake rate per maximum possible oxygen utilization rate, and Damköhler number, Da, which is the maximum possible utilization rate per maximum transfer rate. The related equations for η and Da are as follows, respectively;

$$\eta = \frac{OUR}{OD} \tag{2.28}$$

$$Da = \frac{OD}{OTR_{max}} \tag{2.29}$$

2.3. Benzaldehyde Lyase Production

In the literature, the effects of oxygen transfer conditions (Çalık et al., 2004), and subsequently the effects of controlled and uncontrolled pH operations (Çalık et al., 2006) were investigated on benzaldehyde lyase production by recombinant *Escherichia coli*. Prior to these studies in the master thesis of Yılgör (2004), different *E. coli* strains were investigated and *E. coli* K12 (ATCC 10798) carrying modified pUC18::*bal* plasmid was selected as the host microorganism since it has the highest BAL activity. The optimal production media was also investigated; the media containing, 8.0 g L^{-1} glucose, 5.0 g L^{-1} $(NH_4)_2HPO_4$ and the salt solution was selected where the highest cell concentration and benzaldehyde lyase activity were obtained. Among the investigated oxygen transfer conditions with uncontrolled pH operations of pH=7.2, the maximum BAL activity and cell concentration were achieved at 0.5 vvm and 500 min^{-1} condition as 860 U ml^{-1}, and 2.3 g L^{-1}, respectively (Çalık et al., 2004). Besides, with controlled pH operations the highest cell concentration and BAL activity were reported at pH 7.0 operation as 2.1 g L^{-1} and 775 U ml^{-1}, respectively (Çalık et al., 2006).

Subsequently, intracellular benzaldehyde lyase production by *E. coli* BL21 (DE3) pLySs and extracellular production by *Bacillus* species were investigated in the master thesis study of Kaya (2006). In this study, *bal* gene was cloned into pRSETA expression plasmid carrying T7 promoter using NdeI- *bal* Forward and *bal* Reverse primers to combine the gene encoding benzaldehyde lyase in pRSETA and thereafter the recombinant plasmid was transformed into *E. coli* BL21 (DE3) pLySS strain. Furthermore, the medium composition was investigated where the maximum cell concentration and BAL activity were achieved in the media containing 20.0 g L^{-1} glucose, 11.8 g L^{-1} $(NH_4)_2HPO_4$ and the salt solution, as 2.0 g L^{-1} and 1060 U ml^{-1}, respectively. The oxygen transfer effects were also investigated in pilot scale bioreactor and the highest cell concentration and BAL activity were found at air inlet rate of Q_0/V_R=0.5 vvm and agitation rate of N=750 min^{-1} operation as 1.7 g L^{-1} and 990 U ml^{-1}, respectively.

However, extracellular production of benzaldehyde lyase could not be achieved with the developed recombinant *Bacillus* species, probably due to the ineffective secretion system, inefficient folding of heterologous protein, and degradation of enzyme due to proteolytic activity or high inactivation rate of the enzyme.

Simultaneously, in the master thesis of Büyüksungur (2006), extracellular production of BAL by recombinant *Pichia pastoris* was investigated, though it was reported that the concentration of the active (tetrameric) form of BAL was much lower than the monomeric form showing that the enzyme could not fold into multimeric form in the fermentation medium.

Furthermore, in the master thesis study of Angardi (2007), the effects of oxygen transfer conditions on BAL production by recombinant *E. coli* BL21 (DE3) pLySs were investigated and a model was developed to determine the BAL activity. The highest cell concentration and benzaldehyde lyase activity were obtained at 40% dissolved oxygen concentration with a medium containing 8 g L^{-1} glucose, 5 g L^{-1} $(NH_4)_2HPO_4$ and salt solution with controlled pH 7.2 operation, as 3.0 g L^{-1} and A=1095 U ml^{-1}, respectively.

Recently, the effects of initial pretreated beet molasses, as the carbon and energy source, for BAL production by *E. coli* BL21 (DE3) pLySs carrying pRSETA::bal_{HIS} were investigated (Çalık and Levent, 2009a) and a pulse feeding strategy with molasses was developed (Çalık and Levent, 2009b). Molasses concentration was investigated at 16, 24, 30 and 56 g L^{-1} with batch operations at 40% dissolved oxygen concentration, N=625 min^{-1} and controlled pH=7.2; where the optimum molasses concentration was found as 30 g L^{-1} having the highest cell concentration and benzaldehyde lyase activity as 5.3 g L^{-1} and 1671 U ml^{-1}, respectively (Çalık and Levent, 2009a). Afterwards, in order to investigate the effects of fed-batch operation, three pulse feeding strategies were conducted (Çalık and Levent, 2009b) using the previously reported conditions by Çalık and Levent (2009a). Feeding strategies based on air-flow rate detection, and two

different strategies based on glucose and fructose detection were investigated. The maximum cell concentration and BAL activity was obtained with the pulse feeding strategy in which the fructose and glucose concentrations were increased to 1.5 g L^{-1} when they were depleted in the medium, as 8.04 g L^{-1} and 2315 U ml^{-1}, respectively (Çalık and Levent, 2009b). Additionally it was stated that IPTG addition to induce recombinant protein production was eliminated with molasses, since galactose, one of the monomers of trisaccharide raffinose present in the beet molasses, induced the *lac* promoter (Çalık and Levent, 2009a). According to these findings it can be concluded that pretreated beet molasses is a good carbon source for BAL production by *E. coli* since higher cell concentration and BAL activities were obtained when compared to the glucose based medium; and with fed-batch cultivations it is possible to achieve higher cell and BAL production results.

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 pRSETA::*bal* plasmid (Kaya, 2006) was used for benzaldehyde lyase production. The microorganism has been stored in the microbanks (PRO-LAB) at -55 ^{0}C. Microbanks were prepared by inoculating young colonies into cyropreservative fluid present in a vial which also contains porous beads. After inoculation, microorganisms were adsorbed into the porous beads, and excess cyropreservative was aspirated.

3.3. Pretreatment of Molasses

As mentioned in Section 2.2.2.1, approximately 50 % of molasses is sucrose, but *E. coli* cannot utilize sucrose. Therefore, molasses is needed to be pretreated in order to obtain fructose and glucose from sucrose. Beet molasses (Ankara Sugar Factory, Turkey) was pretreated with acid hydrolysis followed by dilution and centrifugation as described by Çalık et al. (2003).

The steps of pretreatment procedure are as follows:

- ***Dilution:*** 100 g beet molasses was diluted to a final volume of 200 ml with water.
- ***Centrifugation:*** diluted molasses was centrifuged for 20 min at 6000 g and +4 ^{0}C. The impurities not soluble in water and foam formed during sugar process were removed from the samples.
- ***HCl hydrolysis:*** the pH of molasses solution was adjusted to 1.8 at room temperature with 37 % HCl. Hydrolysis reaction was carried out at 90 ^{0}C for 3 h.

Finally, pH of the hydrolyzed molasses solution was adjusted to pH 7.2 with 5M KOH. After sterilization this pretreated molasses was ready to use in fermentation experiments.

3.4. Benzaldehyde Lyase Production by *Escherichia coli*

In this study benzaldehyde lyase was produced from *E. coli* BL21 (DE3) pLysS carrying the recombinant plasmid pRSETA::*bal*. *E. coli* was first incubated on solid medium, then transferred into the precultivation medium, and lastly inoculated into the production medium.

3.4.1. Solid Medium and Growth Conditions

The recombinant *E. coli* strain, stored in microbanks at – 55 ^{0}C, was inoculated to the freshly prepared agar plates containing antibiotics added Luria-Bertani (LB) Agar medium under sterile conditions, and the agar plates were incubated at 37 ^{0}C. The composition of the solid medium is given in Table 3.1; antibiotics were added to the medium after medium was sterilized by autoclave at 121 ^{0}C for 20 min.

Table 3.1. The composition of the solid medium.

Compound	Concentration, g L^{-1}
Soytryptone	10.0
Yeast extract	5.0
NaCl	10.0
Agar	15.0
Ampicilin	0.100
Chloramphenicol	0.007

3.4.2. Precultivation Medium and Growth Conditions

The cells grown in the solid medium were then inoculated into the precultivation medium. The composition of the precultivation medium is given in Table 3.2. Cells were incubated using air-filtered Erlenmeyer flasks with a size of 150 ml and having a working volume of 33 ml, at 37 ^{0}C with an agitation rate of N=200 min^{-1} for 12 h in an agitation rate and heating controlled orbital shaker (B. Braun, Certomat BS-T). According to the antibiotic resistance property of the recombinant microorganism, the selective antibiotics (Table 3.2) were added to the medium after sterilization.

Table 3.2. The composition of the precultivation medium.

Compound	Concentration, g L^{-1}
Soytryptone	10.0
Yeast extract	5.0
NaCl	10.0
Ampicilin	0.100
Chloramphenicol	0.007

3.4.3. Production of BAL in Pilot Scale Bioreactor

After 12 h of precultivation, microorganisms were inoculated to the production medium, having an inoculation ratio of 1:10. Experiments were conducted in a pilot scale bioreactor having a volume of 3.0 L (B. Braun CT2-2) and consisting of temperature, pH, air flow, foam, and stirring rate controllers. The bioreactor system was supplemented by an external cooler, steam generator and a jacket around the vessel for sterilization and temperature control (Figure 3.1). The bioreactor was stirred with two four bladed Rushton turbines, and consisted of four baffles, and a sparger.

Cultivations were carried out at T=37 ^{0}C, and DO=40% cascade to air flow with a stirrer rate of 625 min^{-1}. Additional pure oxygen was mixed with air when it is necessary. Also, pH control was carried out with 5 mol l^{-1} KOH and 5 mol l^{-1} H_3PO_4.

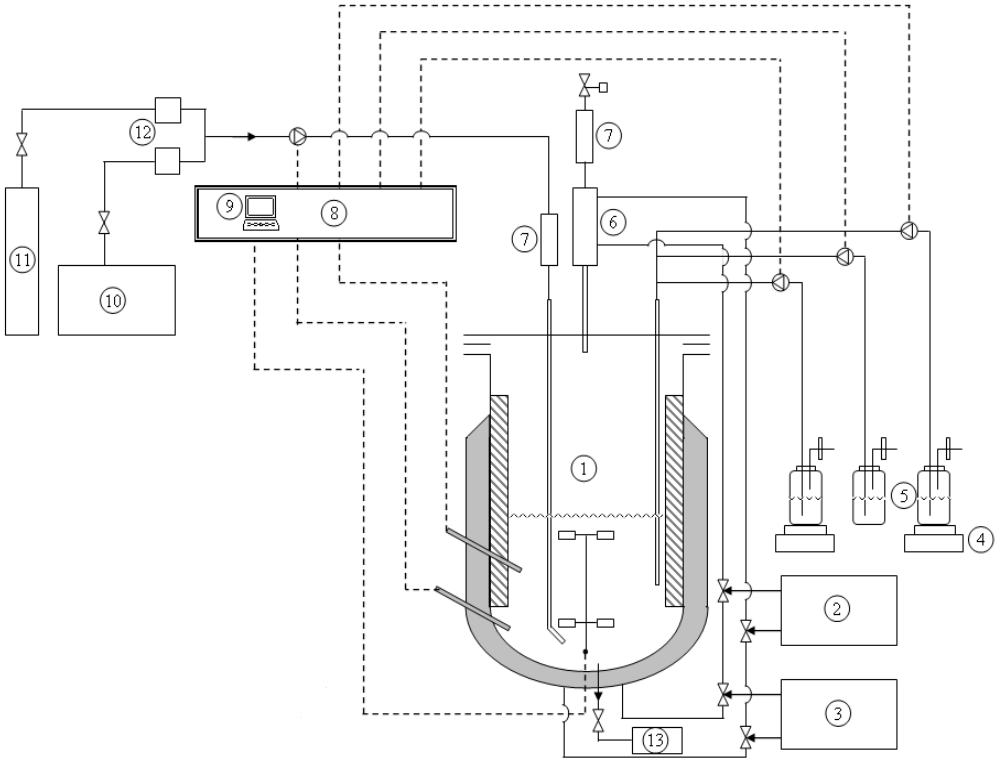

Figure 3.1. Schematic presentation of the pilot scale bioreactor system: (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2 tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008).

3.4.3.1. Batch Experiments

In order to investigate the effects of medium components on BAL production, four batch fermentations were conducted. Cells were inoculated to the bioreactor, as described in Section 3.4.3, having a working volume of 1.65 L. The production media used for batch experiments are given in Table 3.3.

Table 3.3. Production media used in batch pilot scale bioreactor experiments.

	BR1	**BR2**	**BR3**	**BR4**
Component	**Concentration, g L^{-1}**			
Molasses	30	30	24	24
Na_2HPO_4	6.7	6.7	6.7	6.7
KH_2PO_4	3.1	3.1	3.1	3.1
$(NH_4)_2HPO_4$	1.0	5.0	5.0	5.0
NaCl	0.5	0.5	0.5	0.5
$MgSO_4.7H_2O$	0.5	0.5	0.5	0.5
$ZnSO_4.7H_2O$	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}
$MnSO_4.7H_2O$	0.1	0.1	0.1	0.1
$CoCl_2.6H_2O$	2.0×10^{-5}	2.0×10^{-5}	2.0×10^{-5}	--
Ampicillin	0.1	0.1	0.1	0.1
Chloramphenicol	0.007	0.007	0.007	0.007

3.4.3.2. Fed-Batch Experiments

Fed-batch fermentations were conducted with the molasses based medium of BR2 having an initial volume of 1 L, and feeding was started at t=9 h of batch process. Throughout the processes pH was kept constant at pH 7.2. In order to develop an exponential feeding strategy, the effects of specific growth rate values of 0.1, 0.15, and 0.2 h^{-1} were investigated. Molasses was fed to the system according to the following equation (Weigand et al., 1979).

$$F(t) = \frac{\mu_0 V_0 C_{Xo}}{C_{So} Y_{X/S}} \exp(\mu_0 t) \quad (3.1)$$

where μ_0 is the desired specific growth rate, V_0 is the initial volume, C_{X0} is the initial cell concentration, C_{S0} is feed substrate concentration, and $Y_{X/S}$ is cell yield on substrate. For all the feeding experiments feed substrate concentration (total glucose and fructose concentration) was 208 g L^{-1}, and $Y_{X/S}$ value was selected according to Çalık and Levent (2009b) as 0.32 g g^{-1}.

Subsequently, a combined exponential and constant feeding strategy was developed, in which after 10 hours of exponential feeding at μ =0.2 h^{-1}, feed rate was kept constant at the rate of the 10^{th} hour of exponential feeding. Additionally, an experiment was conducted in order to investigate the stability of the plasmid with the exponential and constant feeding strategy. For this purpose antibiotics added to the feed stock of molasses in the concentrations of ampicillin, 0.11 g L^{-1}, and chloramphenicol, 0.0077 g L^{-1}.

3.5. Analysis

During fermentations, samples were taken with certain time intervals to measure cell concentrations, benzaldehyde lyase activities, substrate and organic acid concentrations. The medium was centrifuged at 13500 min^{-1} for 10 min at 4 ^{0}C to precipitate the cells and collect the supernatant. Cell precipitation was used for the determination of the enzyme activity, and supernatant was stored at -55 ^{0}C for further analysis. Analyses were performed in duplicates.

3.5.1. Cell Concentration

Cell concentrations were measured using a UV-Vis Spectrophotometer (Thermo Spectronic, Heλiosα) at 600 nm. Samples taken from the fermentation medium were diluted with distilled water. Cell concentrations, C_X, were calculated using

the equation obtained from the calibration curve given in Appendix A which converts the absorbance to cell concentration on dry cell weight basis.

3.5.2. Benzaldehyde Lyase Activity

Benzaldehyde lyase activity was determined by measuring the conversion of benzoin into benzaldehyde. Since BAL is produced as an intracellular enzyme in recombinant *E. coli*, separation of the biomass from the medium and cell lysis is necessary. Samples were collected from the fermentation medium by centrifugation (Sigma 1-15) at 13500 min^{-1} for 10 min at 4 ^{0}C. The cell walls were lyzed using an agitator bead mill (Retsch, MM 200) at f=10 s^{-1} for 10 minutes 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 containing 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 were prepared daily and incubated at 37 ^{0}C. Stock benzoin solution (0.1mM) was prepared in 15% PEG solution and used after stirred overnight with a low rate using magnetic stirrer at room temperature.

The conversion reaction was carried out with the addition of 20 µl of samples to 3 ml of substrate solution and further incubation for 10 seconds at 37 ^{0}C. Enzymatic activity was measured by UV-Vis Spectrophotometer 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 equation (3.2) was used to relate absorbance change to product formed (Gonzàlez and Vicuña, 1989).

$$\text{Nanomoles of product formed} = \left(2\frac{A_f - A_i}{\varepsilon_s - 2\varepsilon_p}\right) x\ 10^6 \qquad (3.2)$$

where 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 at 250 nm during the reaction time.

One unit of enzymatic activity was defined as one nanomole of benzaldehyde formed by the cleavage of benzoin via BAL catalysis at 37 ^{0}C and pH 8.0 in one second (Çalık et al., 2004).

3.5.3. Glucose Concentration

Glucose concentrations, C_G, were determined by the glucose oxidation method with a UV-Vis Spectrophotometer at 505 nm (Boyaci et. al., 2005) using a glucose analysis kit (Biasis, Ankara). According to this method, D-glucose is oxidized in the presence of glucose oxidase enzyme (equation 3.3) and thereafter hydrogen peroxide formed with this reaction is reacted with 4-aminoantipyrine and phenol in the catalysis of peroxidase to form iminoquinone (equation 3.4) which gives spectrophotometrically observable red color in proportion with glucose concentration.

$$D - glucose + O_2 + H_2O \xrightarrow{Glucose\ oxidase} Gluconate + H_2O_2 \qquad (3.3)$$

$$H_2O_2 + 4 - Aminoantiprine + Phenol \xrightarrow{Peroxidase} Iminoquinone + H_2O \qquad (3.4)$$

The analysis kit includes a glucose analysis reactive containing glucose oxydase, peroxidase and 4-aminoantipirin, and a glucose analysis buffer containing potassium dihydrogen phosphate and phenol. Samples were diluted with water if they contain more than 2 g L^{-1} glucose. 0.05 ml sample solutions were mixed with 0.05 ml analysis reactive, 0.40 ml analysis buffer and 2.05 ml distilled water;

and incubated at 37 ^{0}C for 20 min. After incubation absorbance values were measured by UV Spectrophotometer at 505 nm. In order to determine the concentrations, standard glucose solutions were analyzed according to the procedure and standard calibration curves were achieved as shown in Appendix B.

3.5.4. Fructose Concentration

The concentration of D-fructose, C_F, was determined by the colorimetric assay of Dische and Borenfreund (1951), known as cysteine-carbazole-sulfuric acid method. 60 µl of freshly prepared 1.5% (w/v) cysteine-HCl, 60 µl of 0.12% (w/v) carbazole in 95% ethanol, and 1.8 ml of 70% sulfuric acid were added to 600 µl of the sample and incubated for 30 minutes at room temperature. After incubation the absorbance was measured by UV-Vis Spectrofotometer at 560 nm. The standard calibration curves, for each sample set, were prepared by using D-fructose as a sample with the reaction mix given above. The fructose concentrations were calculated from the measured absorbance values using the standard calibration curve, which is give in Appendix C.

3.5.5. Organic Acid Concentrations

Organic acid concentrations were determined by a HPLC system (Waters HPLC, Alliance 2695, Milford, MA) having Capital Optimal ODS-5Xm column (Capital HPLC, West Lothian, UK). The method is based on reversed phase HPLC, and a mobile phase with 3.12% (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4 was used. The analysis was performed under the conditions given in Table 3.4. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and then loaded the HPLS system.

The concentrations of the organic acids were calculated using the area of the related peak in the chromatogram. The areas were converted into concentrations using the standard calibration curves obtained with the analysis of standard

organic acids solution. The standard calibration curves for the detected organic acids were given in Appendix D.

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

Column	**:**	Capital Optimal ODS, 5 μm
Column dimensions	**:**	4.6 x 250 mm
System	**:**	Reversed phase chromatography
Mobile phase flow rate	**:**	0.8 ml min^{-1}
Column temperature	**:**	30 ^{0}C
Detector and wavelength	**:**	Waters 2487 Dual absorbance detector, 210 nm
Injection volume	**:**	5 μl
Analysis period	**:**	15 min

3.5.6. Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

For the investigation of the oxygen transfer characteristics of the bioprocesses, the liquid phase mass transfer coefficients (K_La) and oxygen uptake rates in the BAL production processes were determined by the Dynamic Method (Bandyopadhyay and Humprey, 1967), as explained in Section 2.2.5.2, and also all the other calculations were done using the given equations in this section.

The physical mass transfer coefficient (K_La_0) was determined before the inoculation of microorganisms to the production medium. The Dynamic Method was applied during cultivations at certain time intervals; the experiments take a short period of time to prevent the possible effects to the biological activities of

microorganisms. During this period, the air inlet was turned off and to lower the effect of the surface aeration the agitation rate was lowered to N=50 min^{-1}.

The saturation oxygen concentration, C_{O2}*, which is necessary for the calculations, was determined with the production medium and at the same conditions as the experiments. The system was aerated, after a while a constant dissolved oxygen concentration was achieved and it was the C_{O2}* value.

CHAPTER 4

RESULTS AND DISCUSSION

In this study, it was aimed to investigate the effects of feeding strategy on benzaldehyde lyase production by recombinant *Escherichia coli* BL21 carrying pRSETA::*bal* plasmid, and an exponential feeding strategy was developed with molasses based medium. In the first part of the study, the effects of medium components on cell growth, product and by-product formation, and oxygen transfer were evaluated with batch bioreactor operations. In the second part, in order to increase BAL production and cell concentration, fed-batch operations were investigated using exponential feeding strategy at three different specific growth rates, and a combined exponential and constant feeding strategy. The effects of molasses feeding rate on cell growth, BAL production, oxygen transfer characteristics and by-product formation were determined. Additionally, plasmid stability in a long term fed-batch operation was examined by antibiotic addition to the feed solution.

4.1. Evaluation of the Medium Composition with Batch Operations

Effects of concentration of the nitrogen source $(NH_4)_2HPO_4$, molasses, and salt solutions were investigated both with laboratory-scale and pilot scale bioreactor operations. Laboratory-scale bioreactor experiments were conducted in an agitation and heating controlled orbital shaker at 37 ^{0}C and N=200 min^{-1} using air filtered Erlenmeyer flasks having a working volume of 33 ml. The results of the

laboratory-scale bioreactor experiments were used to design the production media for pilot scale bioreactor operations. Pilot scale batch bioreactor operations were conducted in the bioreactor system, having a working volume of V_R =1.65x10^{-3} m^3, and consisted of temperature, pH, foam, dissolved oxygen level, air inlet and stirring rate controls. The operation parameters were selected according to the previously reported optimum conditions as DO=40%, N=625 min^{-1}, at T=37°C and at the controlled pH of 7.2 (Çalık et al., 2006; Angardi, 2007; Çalık and Levent, 2009a, 2009b). The batch bioreactor medium compositions and their abbreviations were given in Table 3.3.

The effect of $(NH_4)_2HPO_4$ concentration was investigated with laboratory-scale bioreactor experiments at the concentrations of 2, 3, 4 and 5 g L^{-1}, where the other medium components were the same as the BR1. It was seen that, with increasing concentrations of $(NH_4)_2HPO_4$, cell concentrations were increased, indicating that a nitrogen limitation occurs in the lower concentrations. Furthermore, a pilot scale bioreactor operation (BR1) having 1 g L^{-1} $(NH_4)_2HPO_4$ was performed to investigate the effects of a nitrogen limited medium in detail. In this experiment pH was adjusted with 25% ammonia solution which also provided a nitrogen source in limiting amounts during the cultivation.

Though Çalık and Levent (2009a) reported the effects of molasses concentration; in the current study, it was reexamined with laboratory-scale bioreactor experiments at the concentrations of 24 and 30 g L^{-1}, which are selected according to the results of Çalık and Levent (2009a). In these two experiments, cell concentrations and maximum BAL activities were close to each other. Therefore, the effects of both two molasses concentrations were investigated with pilot scale bioreactor operations (BR2 and BR3).

Additionally, the effects of salt compositions were investigated with lowering and/or ejecting them one by one. The results were similar to each other for the experiments with different salt compositions; except the medium of BR4 in which

$CoCl_2$ was ejected from the medium. Since similar cell growth and product formation results were obtained by laboratory-scale bioreactor experiments for the media of BR4 and BR3 operations, it was decided to perform a pilot scale bioreactor operation without $CoCl_2$ salt in the medium.

4.1.1. Cell Growth Profiles

Cell growth results of batch fermentations are given in Figure 4.1. As can be seen from the figure, BR3 experiment which has low molasses concentration (24 g L^{-1}), reached the lowest maximum cell concentration. The cell concentration results of BR1 and BR4 operations were close to the results of BR3 as they presented a quite similar cell growth profile. The cell concentrations in BR2 operation were significantly higher than the other three cases, especially after t=6 h. Among the batch bioreactor operations, the highest cell concentration was achieved in BR2 experiment as 5.07 g L^{-1} at t=8 h where the cell growth was reached to the stationary phase. In the BR3 and BR4 operations with lower molasses concentrations, stationary phase occurred earlier (t=6 h) which resulted in lower cell concentrations. The maximum cell concentrations of BR3 and BR4 cases were obtained as 3.06 g L^{-1}, and 3.45 g L^{-1}, respectively; indicating that ejection of $CoCl_2$ from the medium increased the cell concentration. On the other hand, although BR1 and BR2 cases include same molasses concentration, in BR1 condition the maximum cell concentration could not reach that obtained in BR2 experiment, due to the nitrogen limitation. Additionally, the highest cell concentration of BR2 was approximately 1.44-, 1.65-, and 1.47-fold higher than the highest cell concentrations of BR1, BR3, and BR4 operations, respectively.

Moreover, in the study of Çalık and Levent (2009a), where the effects of molasses concentration were investigated for BAL production by *E. coli*, the medium of BR2 experiment has also been reported as the optimum medium.

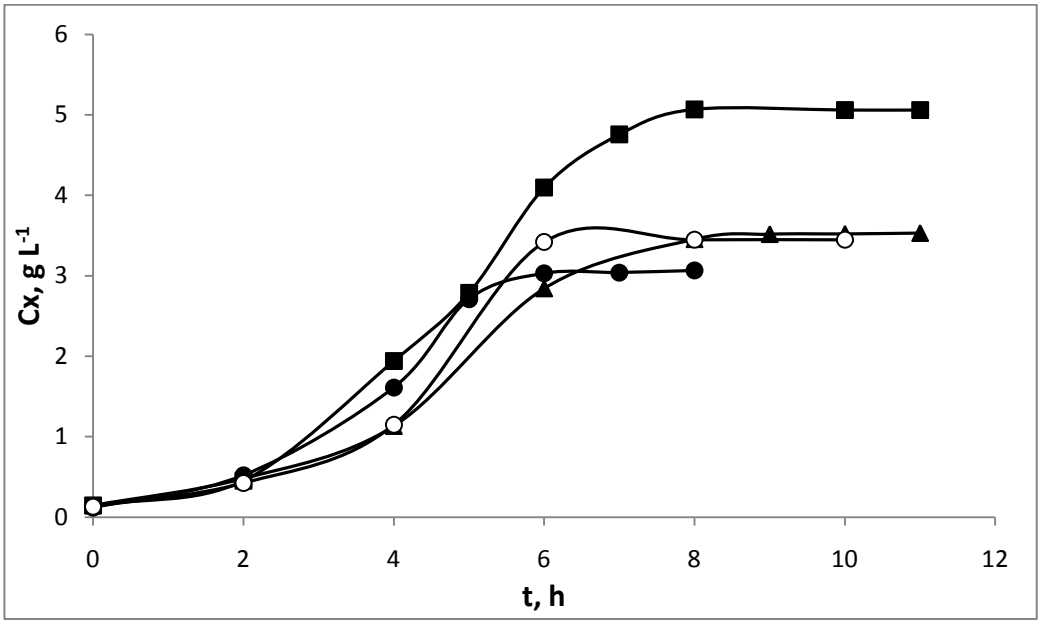

Figure 4.1. Variations in the cell concentration with the cultivation time for different batch bioreactor experiments; BR1 (▲), BR2 (■), BR3 (●), BR4 (○).

4.1.2. Glucose and Fructose Concentration Profiles

The consumption profiles of glucose and fructose are given in Figure 4.2 and Figure 4.3, respectively. 50 % of molasses is sucrose and after pretreatment process molasses contains 25 % glucose and 25 % fructose, therefore the media of BR1 and BR2 contain 7.5 g L^{-1} both glucose and fructose, where BR3 and BR4 contain 6 g L^{-1} each.

Glucose was totally consumed after t=6 h in all the batch bioreactor operations. The glucose concentration profiles for the operations having same initial molasses concentrations were very close to each other throughout the fermentations.

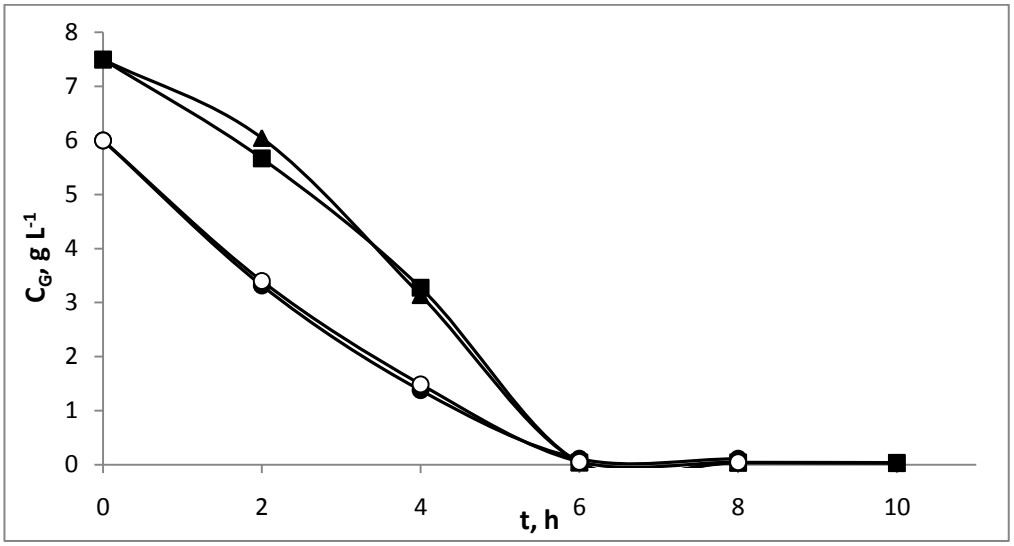

Figure 4.2. Variations in the glucose concentration with the cultivation time for different batch bioreactor experiments; BR1 (▲), BR2 (■), BR3 (●), BR4 (○).

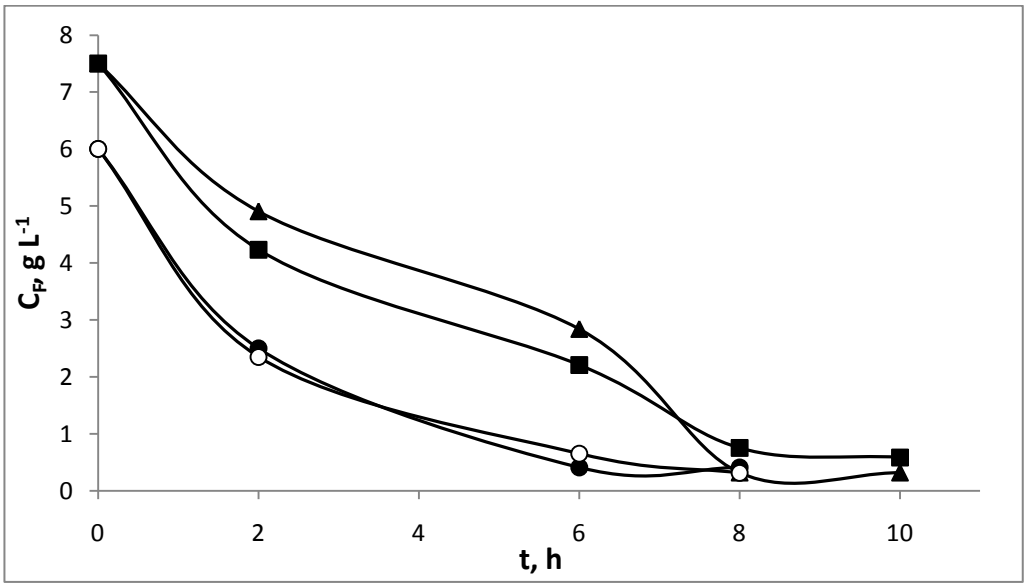

Figure 4.3. Variations in the fructose concentration with the cultivation time for different batch bioreactor experiments; BR1 (▲), BR2 (■), BR3 (●), BR4 (○).

Fructose was nearly depleted at t=6 in BR3 and BR4 cases, while in BR1 operation the depletion occurred at t=8 h; and in BR2 operation small amounts of fructose still remained in the medium at t=10 h. It can be concluded that earlier depletion of fructose in BR3 and BR4 operations resulted in an earlier stationary phase with lower cell concentrations.

4.1.3. Benzaldehyde Lyase Activity Profiles

The variations in benzaldehyde lyase activities with the cultivation time for batch bioreactor operations are given in Figure 4.4. The maximum BAL activities of low molasses cases, BR3 and BR4, were obtained at t=6 h and they were significantly lower than that obtained in high molasses experiments. As it can be predicted from the cell growth profiles, the maximum BAL activity of BR4 experiment was higher than that obtained in BR3 case. In the experiments of high initial molasses concentrations, BR1 and BR2, maximum BAL activities were attained at t=8 h. The highest BAL activity among the batch bioreactor experiments was achieved in BR2 operation as 1611 U ml^{-1}, which was 1.24-, 1.93-, and 1.64-fold higher than that of BR1 (1298 U ml^{-1}), BR3 (836 U ml^{-1}), and BR4 (984 U ml^{-1}) experiments, respectively.

It can be concluded that when the cell growth reached to the stationary phase, BAL activities were decreased after giving a maximum. Additionally, substrate depletion was observed at the end of the exponential phases of all cases, which may affect the activities as well. It is known that nutrient depletion can lead to an increase in protease concentrations in *E. coli* fermentations. In the literature, Choi and Lee (1997) reported a decrease in expression level of bovine growth hormone due to the protease degradation caused by nutrient depletion. Therefore, protease concentrations may be increased due to the depletion of glucose and fructose, which can cause a decrease in BAL activities at the end of the fermentations.

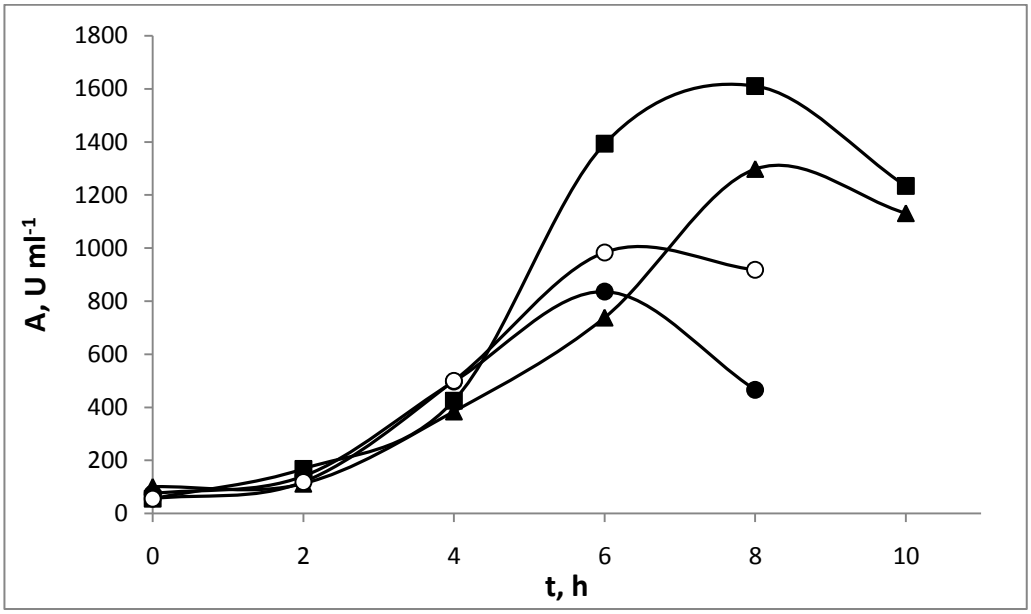

Figure 4.4. Variations in benzaldehyde lyase activities with the cultivation time for different batch bioreactor experiments; BR1 (▲), BR2 (■), BR3 (●), BR4 (○).

4.1.4. Organic Acid Concentration Profiles

Throughout the cultivations, acetic, formic, succinic, citric, fumaric, and oxalic acids were detected in the fermentation medium. The concentrations of the organic acids detected in the extracellular medium are given in Table 4.1, and the variations in the total organic acid concentrations with the cultivation time are shown in Figure 4.5 for batch bioreactor operations. Since acetic acid is the major by-product for *E. coli* cultivations, acetic acid concentration profiles are presented separately in Figure 4.6.

The highest total organic acid concentration was detected as 4.28 g L^{-1} due to the sharp increase in acetic acid after t=6 h in BR2 case, which was found as the optimum medium for the cell growth and product formation. The maximum acetic acid concentration among the batch bioreactor operations was achieved in BR2

experiment as 2.86 g L^{-1} at t=8 h. The lowest total organic and acetic acid concentrations were generally obtained in BR3 operation.

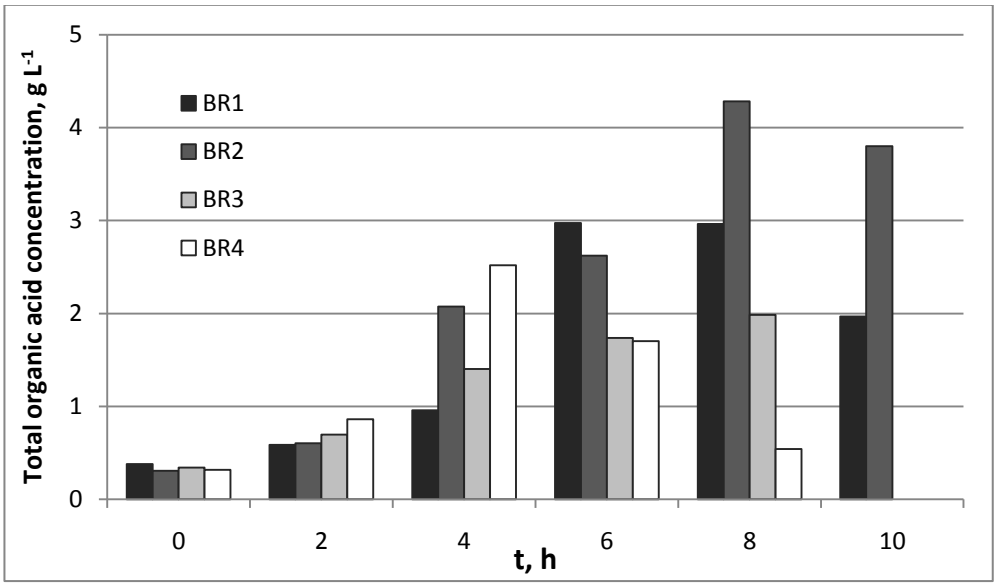

Figure 4.5. Variations in the total organic acid concentrations with the cultivation time for batch bioreactor experiments.

During the cultivations acetic acid concentrations were below the inhibitory level which is 5 g L^{-1} (Lee, 1996). Acetic acid formation occurs due to a limited TCA cycle capacity (Han et al., 1992) or an overflow phenomenon where flux of AcetylCoA is directed to acetic acid production instead of entering the TCA cycle (Åkesson et al., 2001). Acetic acid accumulation in *E. coli* cultivations can be observed both under anaerobic conditions, and under fully aerobic conditions with excess carbon source in the medium (Åkesson et al., 1999, 2001). Acetic acid concentrations were close to each other, for the operations having same amounts of initial molasses. Acetate concentrations detected in BR3 and BR4 operations

were significantly lower than BR1 and BR2 cases. Since glucose and fructose concentrations were detected in low amounts, higher concentrations of acetic acid may be caused by oxygen limitation. Furthermore, higher acetic acid concentrations were observed for higher cell concentrations, in which oxygen demands were higher, therefore oxygen supply may become inadequate.

Additionally, the presence of the TCA cycle organic acids as citric, succinic, and fumaric acid may be caused by an inadequate oxygen supply which results in repression on the TCA cycle enzymes or their improper activity. In the master thesis study of Angardi (2007), under oxygen limiting conditions, the TCA cycle organic acids were detected and also high acetate accumulation (2.5 g L^{-1}) was observed. In the present study the concentrations of TCA cycle organic acids were generally higher in BR2 operation, which is followed by BR1. These results also support the idea of insufficient oxygen supply in the cases having higher cell concentrations.

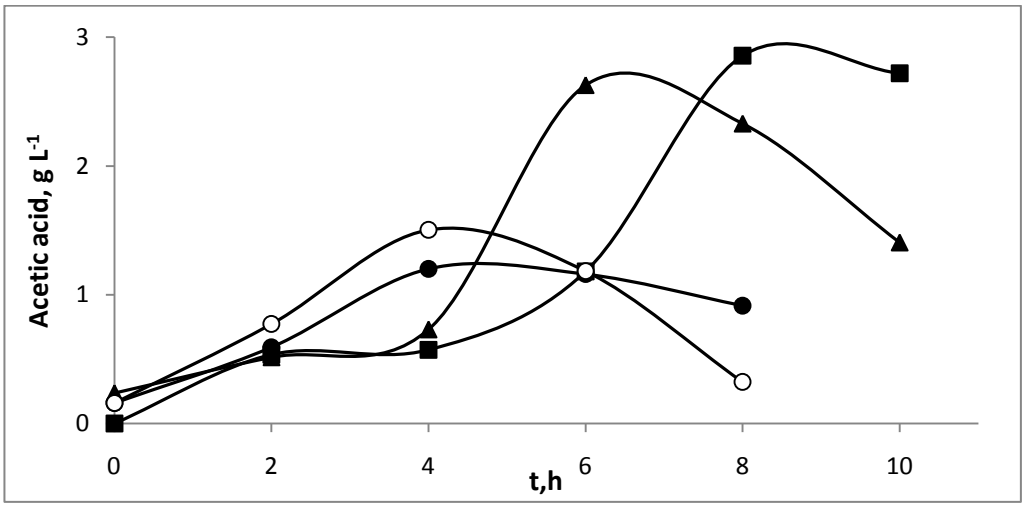

Figure 4.6. Variations in the acetic acid concentrations with the cultivation time for batch bioreactor experiments; BR1 (▲), BR2 (■), BR3 (●), BR4 (○).

Table 4.1. Variations in the organic acid concentrations with the cultivation time for batch bioreactor operations.

t, h	Concentrations, g L^{-1}						
	Acetic acid	**Formic acid**	**Succinic acid**	**Citric acid**	**Fumaric acid**	**Oxalic acid**	**Total**
				BR1			
0	0.2363	0.0617	0	0	0.0122	0.0696	0.3798
2	0.5140	0.0230	0	0	0.0010	0.0501	0.5880
4	0.7304	0.0677	0.1367	0	0.0249	0	0.9596
6	2.6272	0.0883	0.2591	0	0	0	2.9746
8	2.3284	0.0895	0.4930	0	0.0272	0.0236	2.9616
10	1.4053	0.0912	0.4158	0	0.0244	0.0318	1.9684
				BR2			
0	0	0.0163	0.2081	0	0.0177	0.0646	0.3066
2	0.5353	0.0272	0	0	0.0111	0.0299	0.6035
4	0.5725	0	0.8423	0.5777	0.0024	0.0806	2.0755
6	1.1805	0	0.8302	0.5102	0.0055	0.0961	2.6225
8	2.8563	0	0.8797	0.2418	0.0090	0.2960	4.2827
10	2.7191	0.0533	0.8535	0.1435	0.0041	0.0267	3.8001
				BR3			
0	0.1605	0.0291	0	0.0513	0.0367	0.0640	0.3415
2	0.5936	0.0596	0	0	0	0.0420	0.6952
4	1.2019	0.0528	0.1342	0	0	0.0147	1.4036
6	1.1609	0.0636	0.4959	0	0	0.0163	1.7366
8	0.9164	0.0635	0.9819	0	0	0.0231	1.9848
				BR4			
0	0.1606	0.0547	0	0	0.0373	0.0643	0.3170
2	0.7734	0.0451	0	0	0	0.0443	0.8628
4	1.5033	0.0702	0.8916	0	0.0360	0.0170	2.5181
6	1.1836	0.0605	0.4073	0	0.0345	0.0154	1.7013
8	0.3232	0.0311	0.1495	0	0.0251	0.0131	0.5419

4.1.5. Oxygen Transfer Characteristics

The oxygen transfer parameters ie., oxygen uptake rate (OUR), oxygen transfer coefficient (K_La), and oxygen transfer rate (OTR) were determined by the Dynamic Method throughout the fermentations. Moreover the oxygen utilization rate (OD), Damköhler number (Da), and effectiveness factor (η) were calculated. The variations in the oxygen transfer characteristics for batch experiments are given in Table 4.2.

During the batch cultivations K_La values were oscillated between 0.010 and 0.023 h^{-1}. However, K_La did not change significantly with the cultivation time in each operation; the values were close to each other especially in BR3 experiment. Since the temperature and the agitation rate were kept constant throughout the fermentations, K_La depends mostly on the rheological properties of the fermentation broth. Throughout fermentations besides the increase in the biomass concentration, the metabolites and by-products secreted in the medium are also create a resistance zone between the gas bubbles and the cells. Furthermore, the complex composition of molasses can significantly affect the bubble size and the liquid film resistance around the gas bubble. The maximum K_La values were achieved as 0.022, 0.018, 0.020, and 0.023 s^{-1} in BR1, BR2, BR3 and BR4 operations, respectively. Although they were close to each other, BR2, having the maximum cell and by-product concentration, showed the lowest maximum K_La value.

Since dissolved oxygen in the process was kept constant throughout the process, the oxygen uptake rates, OUR, were the same as the oxygen transfer rates, OTR. The higher OUR values were achieved in the exponential growth phase as expected, and decreased at the end of the fermentation as the cells stop growing. The highest OTR values were obtained as 4.91, 3.95, 4.44, and 7.91 mol m^{-3} s^{-1} at the times where the highest K_La values were achieved for BR1, BR2, BR3, and BR4, respectively.

In order to find the rate limiting step of the bioprocesses, the maximum possible oxygen utilization rate (OD) and the maximum oxygen transfer rate (OTR_{max}) were calculated throughout the fermentations (Table 4.2). As the ratio of the OD to OTR_{max} is defined as the modified Damköhler number (Da), when Da is higher than 1, mass transfer resistances are effective that means oxygen demand cannot be satisfied with maximum oxygen transfer rate. Da values lower than 1 shows that the reaction rate is dominating over the mass transfer resistance, which is the result of the oxygen limitation because of the high specific growth rates. Generally, lower Da values were obtained at the beginning of the fermentations which are lower than 1. However, at the end of the fermentations, Da values were considerably higher than 1. The maximum Damköhler numbers were attained as 18.64, 17.72, 6.26, and 4.46 for BR1, BR2, BR3, and BR4, respectively.

The effectiveness factor, η, is the ratio of the observed biochemical reaction rate to the reaction rate without mass transfer resistances. η values were higher at the beginning of the fermentations and then they were decreased gradually as η decreases oxygen transport becomes limited. η values lower than 1 indicates that oxygen was consumed below the maximum oxygen demand.

Table 4.2. Variations in the oxygen transfer parameters in batch bioreactor operations.

t h	K_La s^{-1}	OTR x 10^3 mol m^{-3} s^{-1}	OTR_{max} x 10^3 mol m^{-3} s^{-1}	OUR x 10^3 mol m^{-3} s^{-1}	OD x 10^3 mol m^{-3} s^{-1}	Da	η
			BR1				
0	0.022	4.91	8.18	4.91	5.38	0.66	0.91
2	0.018	4.07	6.78	4.07	9.90	1.46	0.41
6	0.010	2.29	3.81	2.29	14.01	3.67	0.16
8	0.017	3.86	6.43	3.86	119.86	18.64	0.03
			BR2				
0	0.018	3.95	6.59	3.95	4.74	0.72	0.83
2	0.017	3.75	6.26	3.75	4.74	0.76	0.79
4	0.011	2.38	3.97	2.38	6.35	1.60	0.38
6	0.014	3.20	5.33	3.20	20.94	3.93	0.15
8	0.012	2.72	4.53	2.72	80.32	17.72	0.03
			BR3				
0	0.018	4.03	6.72	4.03	4.04	0.60	0.98
2	0.018	4.03	6.72	4.03	7.04	1.05	0.57
4	0.020	4.44	7.41	4.44	14.29	1.93	0.31
6	0.019	4.27	7.12	4.27	44.55	6.26	0.10
			BR4				
0	0.013	2.80	4.66	2.80	3.10	0.67	0.90
2	0.012	2.65	4.41	2.65	5.52	1.25	0.48
4	0.023	7.91	13.18	7.91	15.15	1.15	0.52
6	0.018	4.02	6.70	4.02	29.88	4.46	0.14

4.1.6. Specific Growth Rate and Yield Coefficients

The specific growth rates, μ, specific oxygen uptake rates, q_O, specific substrate consumption rate, q_S, and the yield coefficients, $Y_{X/S}$, $Y_{X/O}$, $Y_{S/O}$, were calculated throughout the fermentations and they are given in Table 4.3 for batch bioreactor operations.

For all the operations, μ values were decreased with the cultivation time and reached to zero. Therefore the highest specific growth rate values were obtained at the beginning of the fermentations as 1.14, 1.04, 1.71, and 1.13 h^{-1} for BR1, BR2, BR3 and BR4, respectively. The maximum specific growth rate of BR3 experiment was the highest while the other three were similar, which is due to the higher cell concentrations at BR3 operation between t=0 and 2 h.

Specific oxygen uptake and substrate consumption rates were also decreased with the cultivation time. Substrate consumption was calculated for the total of glucose and fructose consumptions. The highest q_O was achieved in BR3 and the highest q_S value was achieved in BR4 operations. The highest specific oxygen uptake rates were calculated as 3.87, 3.10, 3.93, and 2.47 g g^{-1} h^{-1} for BR1, BR2, BR3, and BR4 operations, respectively. The maximum specific substrate utilization rates were obtained as 3.99, 5.08, 4.20, and 5.31 g g^{-1} h^{-1} for BR1, BR2, BR3, and BR4 operations, respectively.

The yield coefficients should be determined for a better understanding of the efficiency of the bioprocesses. The cell yields on substrate were increased at the beginning of the fermentations and then reached a maximum value before the stationary phase occurred. The maximum $Y_{X/S}$ values were attained as 0.39, 0.67, 0.52 and 0.59 g g^{-1} for BR1, BR2, BR3 and BR4 operations, respectively. The highest $Y_{X/S}$ was achieved in BR2 operation which has the maximum cell concentration, as expected, since the substrate consumptions of the cases having same initial molasses concentrations were similar to each other. Additionally, the

highest overall cell yield on substrate was achieved in BR2 operation as 0.35 g g^{-1} again due to the highest cell concentrations of this case. Whereas the lowest overall cell yield on substrate was attained in BR1 operation, since it has similar cell concentrations with BR3 and BR4 operations while having higher initial molasses concentrations. Cell yield on oxygen, $Y_{X/O}$, values were increased at the beginning of the fermentations and then they decreased generally when the cell growth reached to the stationary phase. $Y_{S/O}$ values, which is the substrate metabolized per amount of oxygen used, were altered during the bioprocesses. The highest $Y_{X/O}$, and $Y_{S/O}$ values were obtained in BR2 and BR4 operations, respectively.

Table 4.3. Variations in the specific growth rate and yield coefficients in batch bioreactor operations.

t h	μ h^{-1}	q_O $g\ g^{-1}\ h^{-1}$	q_S $g\ g^{-1}\ h^{-1}$	$Y_{X/S}$ $g\ g^{-1}$	$Y_{X/O}$ $g\ g^{-1}$	$Y_{S/O}$ $g\ g^{-1}$
			BR1			
0	1.141	3.866			0.30	3.58
2	0.514	0.976	3.997	0.08	0.53	4.09
4	0.521		1.781	0.18		
6	0.204	0.093	0.613	0.39	2.20	6.61
8	0.040	0.129	0.184	0.24	0.31	1.43
			overall	0.23		
			BR2			
0	1.043	3.098			0.34	5.60
2	0.990	0.954	5.075	0.06	1.04	5.32
4	0.469	0.141	0.985	0.36	3.32	6.98
6	0.191	0.090	0.306	0.61	2.12	3.40
8	0.042	0.062	0.080	0.67	0.69	1.30
			overall	0.35		
			BR3			
0	1.709	3.932			0.44	6.65
2	0.716	0.890	4.201	0.07	0.80	4.72
4	0.389	0.318	0.822	0.42	1.23	2.59
6	0.120	0.162	0.224	0.52	0.74	1.38
8	0.006		0			
			Overall	0.26		
			BR4			
0	1.128	2.474			0.46	9.70
2	0.599	0.719	5.310	0.05	0.83	7.39
4	0.653	0.794	1.098	0.26	0.82	1.38
6	0.168	0.135	0.192	0.59	1.24	1.42
8	0	0.147	0.051	0.08	0	0.35
			overall	0.29		

4.2. Development of Molasses Feeding Strategy for BAL Production

In order to increase the cell growth and recombinant enzyme production, fed-batch cultivations were conducted using the optimal batch production medium. The effects of molasses feeding rate on benzaldehyde lyase production by recombinant *E. coli* were investigated with exponential feeding at different specific growth rates and a combined strategy in which exponential feeding was followed by constant feeding. The fed-batch experiments are coded to make it easy to describe the operations, and they are given in Table 4.4. All of the fed-batch operations were conducted after an initial batch phase. Feeding was started when glucose and fructose concentrations in the medium were reached to zero and the cell growth was begun to slow down. The feed rate was controlled automatically by the bioreactor control unit and verified with a balance that placed under the bottle of the feed substrate solution.

Table 4.4. Explanations for the fed-batch operation codes.

Exponential Feeding	
Operation Code	Predetermined specific growth rate, μ, h^{-1}
μ-0.1	0.1
μ-0.15	0.15
μ-0.2	0.2
Combined Exponential and Constant Feeding	
Operation Code	Experiment
μ-0.2+	Exponential at μ=0.2 h^{-1} followed by constant
μ-0.2+(antibiotics)	μ-0.2+ operation with antibiotic addition to the feed solution

Exponential feeding experiments were carried out at three specific growth rates as 0.1, 0.15, and 0.2 h^{-1}, with feeding pretreated molasses to the bioreactor according to equation (3.1). The predetermined feeding profiles are shown in Figure 4.7. These predetermined specific growth rates were selected considering the favored specific growth rate range for *E. coli* in the literature. Specific growth rates between 0.1 and 0.3 h^{-1} were generally preferred in *E. coli* cultivations to avoid acetate accumulation (Lee, 1996). In the literature, Wong et al. (1998) investigated the effects of specific growth rates between 0.1 and 0.3 h^{-1} for a recombinant *E. coli* BL21 strain and μ=0.15 h^{-1} was reported as the best case in terms of cell growth. Besides, for different strains of *E. coli* and/or for different products, the optimal growth rates could be varied. Yee and Blanch (1993) used μ=0.2 h^{-1} for recombinant trypsin production by *E. coli* X90; whereas Khalilzadeh et al. (2003) used μ=0.12 h^{-1} for human interferon-gamma production by *E. coli* BL21 (DE3) and reached 100 g L^{-1} cell densities. Additionally, Meyer et al. (1984) reported that with complex media acetate formation occurs at the specific growth rates above 0.2 h^{-1}.

The combined exponential and constant feeding strategy was developed to overcome the overfeeding problem in exponential feeding strategy running at high specific growth rates and to reduce acetate accumulation. 10 hours of exponential feeding at μ=0.2 h^{-1} was followed by constant feeding at the rate of the 10^{th} hour of the exponential profile which is 21.6 g h^{-1} (Figure 4.7). In the literature, Lin et al. (2001) developed a similar feeding strategy for *E. coli* K12, in which the exponential feeding part was conducted at μ=0.27 h^{-1} for 9.1 hours, it was continued with constant feeding at that rate for a while (2.5 h), then the constant feed rate was lowered. Also Sandén et al. (2003) applied a strategy for *E. coli* AF1000 producing β-galactosidase as exponential feeding with μ=0.5 h^{-1} for 4.16 hours, then feed rate was kept constant.

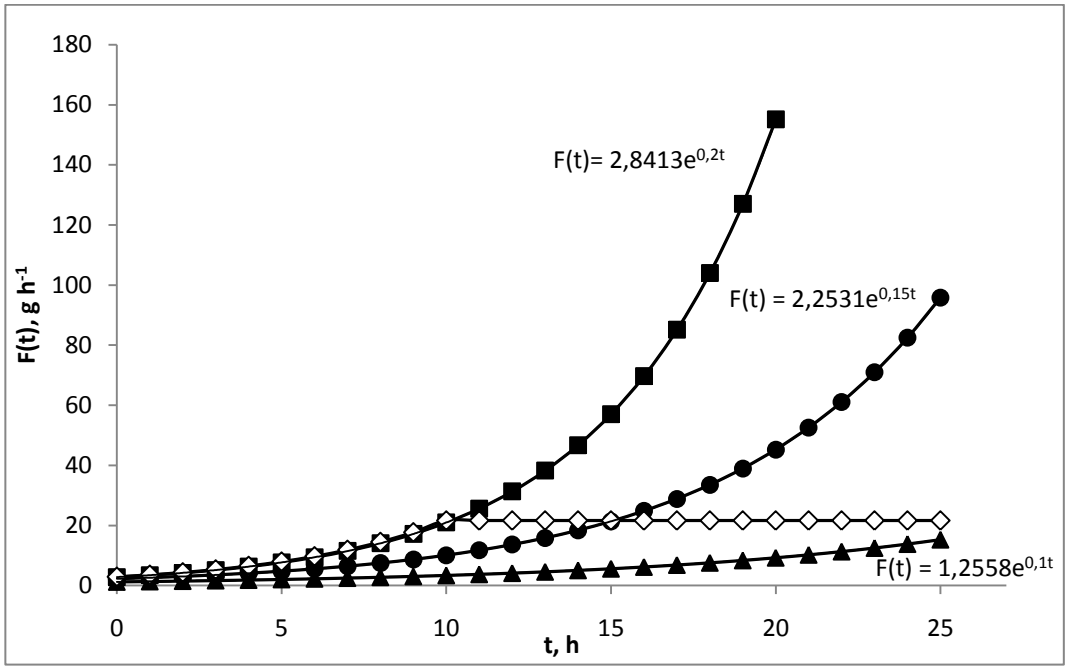

Figure 4.7. Predetermined feeding profiles for μ-0.1 (▲), μ-0.15 (●), μ-0.2 (■), μ-0.2+ (◇) experiments. t=0 is the time that feeding was started.

4.2.1. Effects of Feeding Strategy on Cell Growth

The variations in the cell concentrations with the cultivation time are given in Figure 4.8 for fed-batch operations. For all the cases, the cell concentrations attained in the batch phase (t=0-9 h) were nearly the same. Feeding was started when cell growth was about to reach to the stationary phase, and when feeding was started the cell growth rates were instantly increased for all cases.

Among the exponential feeding experiments the highest cell concentration was obtained in μ-0.15 condition as 21.7 g L^{-1} at t=34 h. Whereas, in μ-0.1 and μ-0.2 experiments the maximum cell concentrations were close to each other, which were obtained as 15.9 g L^{-1} (t=34 h) and 16.2 g L^{-1} (t=26 h), respectively. At μ-0.2

operation, due to the higher feeding amounts of molasses, the maximum cell concentration was attained earlier than the other two experiments. Moreover, increasing cell growth rates were observed with increasing feeding rates. After 14th hour of the bioprocesses the cell concentrations of each operation were significantly differed from each other (Figure 4.8). The cell growth in μ-0.2 experiment was higher than that obtained in the other two operations until t=26 h; however, after that time the stationary phase occurred, and the cell concentrations remained below that obtained in μ-0.15 case, probably due to the excess amounts of substrate present in the medium. According to the cell concentration results of exponential feeding experiments, μ-0.15 case can be referred as the most favorable operation since the maximum cell concentration achieved in this feeding rate is 1.35–fold higher than that attained in μ-0.1 and μ-0.2.

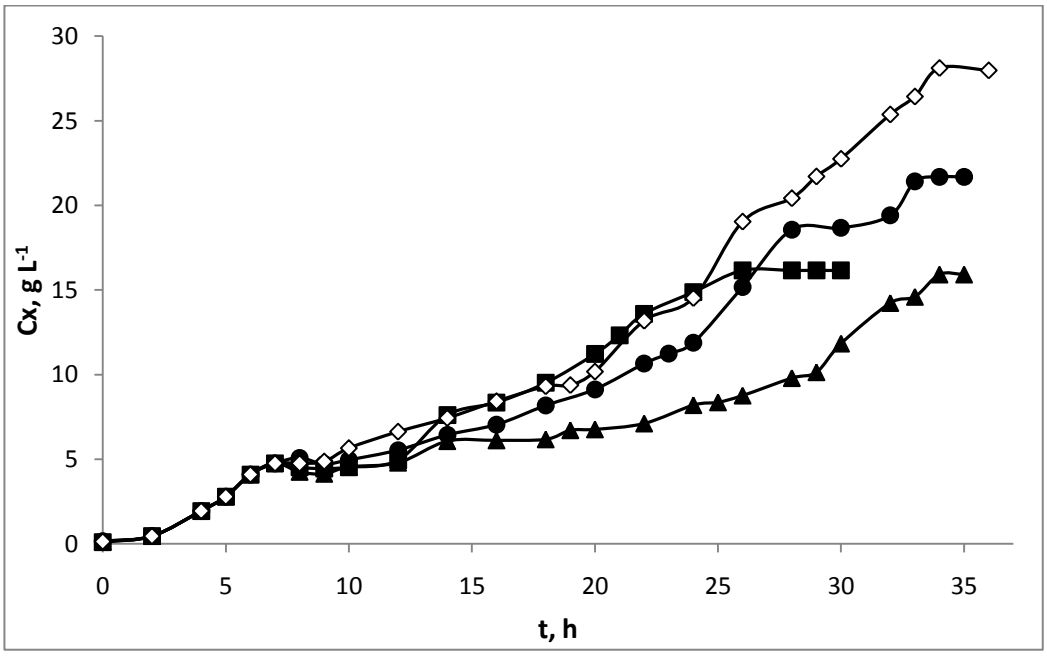

Figure 4.8. Variations in the cell concentration with the cultivation time for different feeding rates; μ-0.1 (▲), μ-0.15 (●), μ-0.2 (■), μ-0.2+ (◇).

In the exponential feeding phase of the combined exponential and constant feeding strategy, μ-0.2+, the specific growth rate was selected as $\mu=0.2\ h^{-1}$ to reach higher cell concentrations at the beginning of the fermentation, and with stopping exponentially increased feed rate at an appropriate time, it was aimed to avoid the accumulation of the substrate in order to overcome the problems occurred in μ-0.2 experiment. As can be seen from Figure 4.8, with this feeding strategy, the cell growth was continued for 34 hours and reached to a maximum cell concentration of 28.1 g L^{-1}, which was 1.3-fold higher than that obtained in μ-0.15 operation. Besides, inhibition of the cell growth, which was observed in μ-0.2 experiment, was prevented with μ-0.2+ operation. From the cell concentration profiles, it can be concluded that the combined exponential and constant feeding strategy have been successfully applied in terms of the cell growth.

In the literature, the maximum cell concentration of *E. coli* producing BAL was reported as 8.04 g L^{-1} by Çalık and Levent (2009b). They used the same molasses based medium as in the present study, and the maximum cell concentration was achieved by using a pulse feeding strategy according to the glucose and fructose concentrations in the medium. In this study, the maximum cell concentration for BAL production by *E. coli* was obtained, which was 3.5-fold higher than that achieved by Çalık and Levent (2009b).

4.2.2. Effects of Feeding Strategy on Substrate Consumption

The concentration of the carbon source in the medium directly affects the cell growth, by-product and product formation. The limited amounts of the glucose and fructose may result in lower cell concentrations and production, whereas the excess amounts will lead to acetate production and will decrease the cell growth and product formation due to acetate inhibition. Therefore, substrate feeding rate should be optimized in a fed-batch process.

In all the fed-batch experiments applied in this study, glucose was never detected in the fermentation medium after t=6 h. Therefore only the variations of fructose concentration with the cultivation time are given in Figure 4.9. With the increase in the feed rate, fructose accumulation in the fermentation broth was observed. Also, when fructose accumulation was sharply increased, a decrease in the cell growth rate was occurred. In μ-0.2 operation, a significant increase in fructose concentration was detected after t=26 h, and at the end of the fermentation it was reached to a concentration of 8.9 g L^{-1}, which was too high when compared to μ-0.1 and μ-0.15 cases and can explain the early stationary phase in this experiment. During the exponential growth phase, fructose concentrations in the medium did not change significantly.

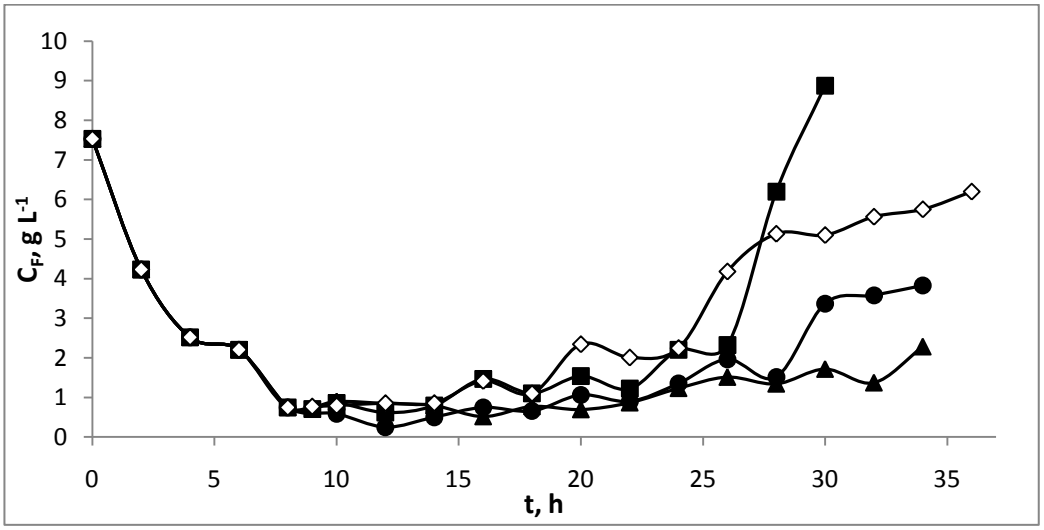

Figure 4.9. Variations in the fructose concentration with the cultivation time for different feeding rates; μ-0.1 (▲), μ-0.15 (●), μ-0.2 (■), μ-0.2+ (◇).

Since glucose was never detected in the medium throughout the fermentations, the presence of fructose can be favored, as the depletion of the substrate may generate stressful conditions which will induce protease formation. Furthermore, if glucose has been existed in the medium, considerable increase in fructose concentrations could be observed.

Exponential feeding strategy supplies substrate with a predetermined feeding profile and the substrate concentration in the fermentation medium is not taken into consideration which can lead to substrate overfeeding; thus reducing the feed rate systematically may be a solution for this problem (Wong et al., 1998). In the present study, it was seen that feeding with high specific growth rate resulted in considerable accumulation of fructose and caused inhibition of the cell growth. Therefore, in μ-0.2+ condition with lowering the feed rate before high amounts of fructose accumulation was begun, the total accumulation of fructose was lowered and inhibition of the cell growth was prevented.

4.2.3. Effects of Feeding Strategy on BAL Production

The variations in benzaldehyde lyase activities and in the specific benzaldehyde lyase activities with the cultivation time and with fed-batch operations having different feeding rates are given in Figure 4.10 and Figure 4.11, respectively.

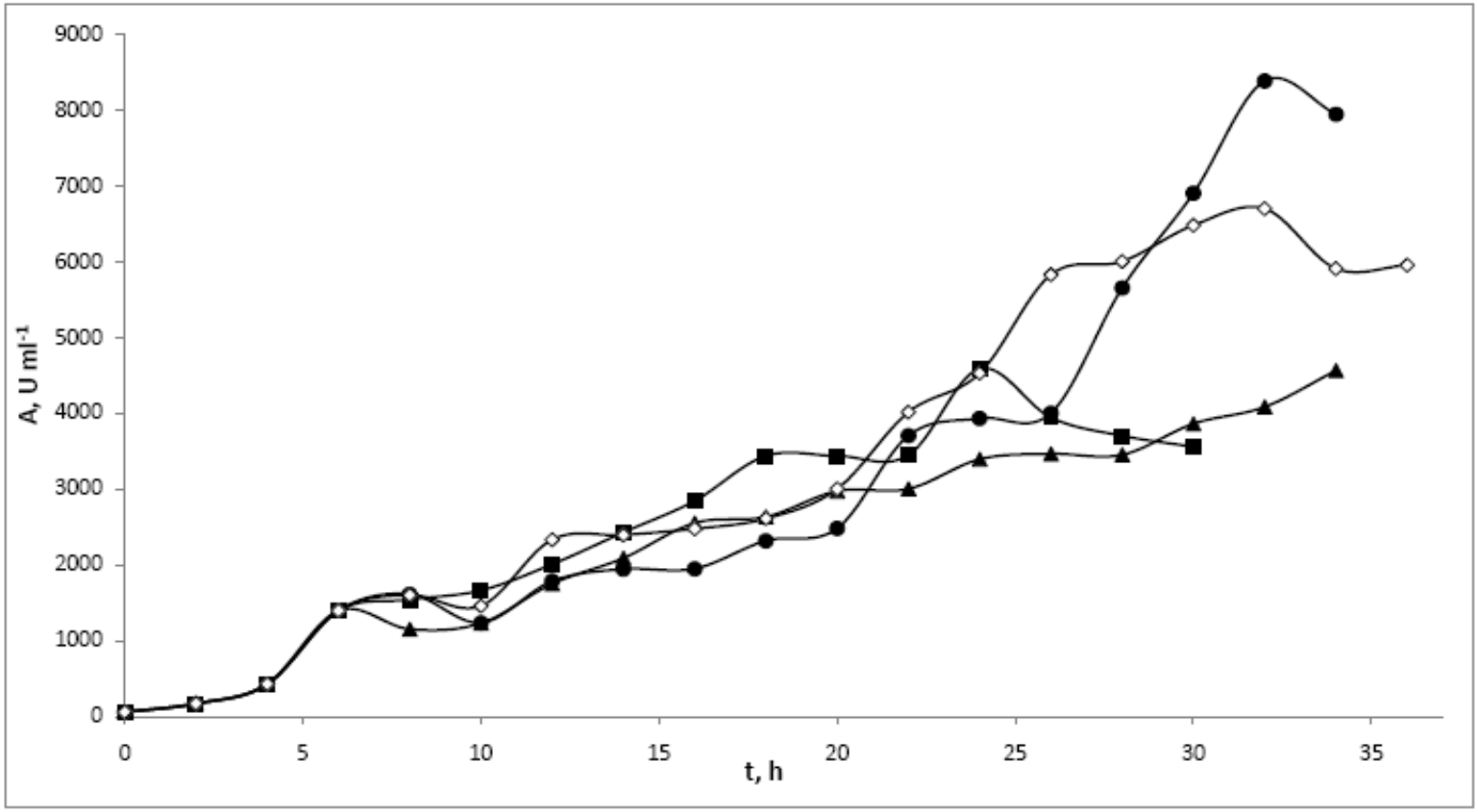

Figure 4.10. Variations in benzaldehyde lyase activity with the cultivation time for different feeding rates; μ-0.1 (▲), μ-0.15 (●), μ-0.2 (■), μ-0.2+ (◇).

BAL activities were increased significantly after the feeding was started in all operations. In the beginning of the bioprocess BAL activities of μ-0.2 operation were higher than that of the μ-0.1 and μ-0.15 operations; moreover at μ-0.2 operation the maximum activity was achieved as 4584 U ml^{-1} at t=24 h which was higher than the other two operations until t=24 h; and then it decreased considerably. In μ-0.1 operation, BAL activities were increased throughout the fermentation and gave a maximum as 4561 U ml^{-1} at t=34 h, which was almost the same as the maximum activity of μ-0.2 operation. For the first 20 h of the fermentations, enzyme activities of μ-0.15 operation were close to the activities of μ-0.1 case, which then sharply increased and reached a maximum of 8379 U ml^{-1} at t=32 h. This value was the highest BAL activity in this study. In the literature, Çalık and Levent (2009b) reported the maximum BAL activity by *E. coli* as 2315 U ml^{-1} with the same molasses based medium and using a pulse feeding strategy. As can be seen, exponential feeding strategy at μ=0.15 h^{-1} lead to a 3.62-fold increase in the highest BAL activity reported in the literature.

In the combined exponential and constant feeding strategy, although the highest cell concentration was achieved among the other feeding experiments, the maximum BAL activity could not reached to that of μ-0.15 experiment and was measured as 6695 U ml^{-1} at t=32 h. However, the maximum BAL activity of μ-0.2+ operation was higher than the maximum of μ-0.2 experiment. The enzyme activities of μ-0.2+ experiment may remain low because of acetate inhibition, since fructose concentrations were significantly higher than that of μ-0.15 operation after t=28 h, and the enzyme activities were decreased after t=30 h in this operation. Moreover, the high cell concentration and low enzyme production in μ-0.2+ operation might be a result of the plasmid instability which can occur because of the high dilution rates and long cultivations times. The stability of the plasmid was also investigated and discussed in Section 4.2.4.

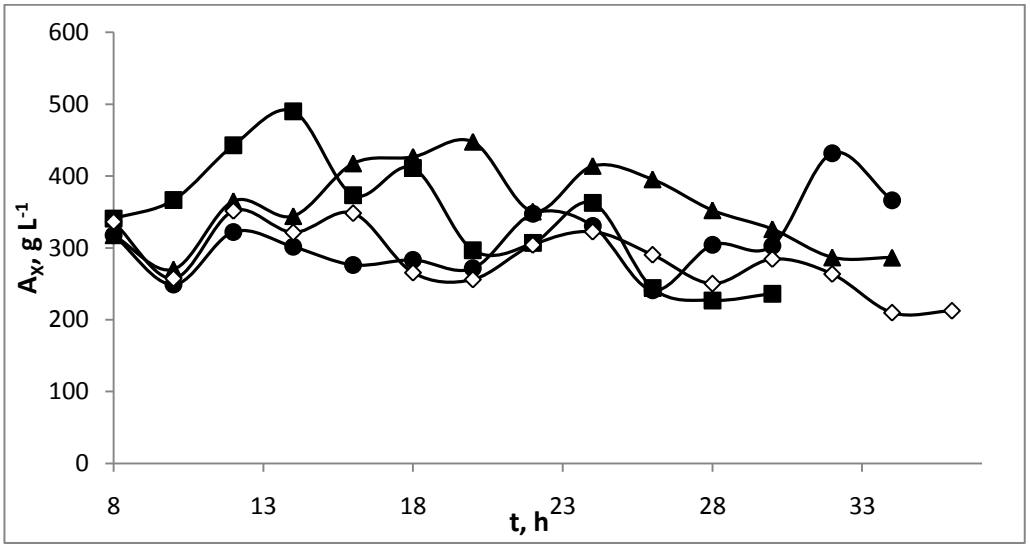

Figure 4.11. Variations in specific benzaldehyde lyase activity with the cultivation time for different feeding rates; μ-0.1 (▲), μ-0.15 (●), μ-0.2 (■), μ-0.2+ (◇).

The specific BAL activities were generally increased at the beginning of the feeding phase and at the end of the fermentations a decrease was occurred. For the first hours of the fed-batch phase, the specific BAL activities of μ-0.2 condition were significantly higher due to the higher enzyme activities at that period. In μ-0.1 case, the specific activities were remained higher than the other three cases between t=16 and 32 h, as a result of showing similar enzyme activities while having lower cell concentrations. For μ-0.15 operation specific BAL activities were not changed significantly at the beginning of the fed-batch phase, which then increased and gave a maximum at t= 32 h.

4.2.4. Investigation of the Plasmid Stability

Plasmid stability is an important parameter for recombinant productions. With high number of generations in fed-batch and continuous cultures, plasmid loss

with time occurs (Sandén et al., 2003). When cells lost their plasmids, a good cell growth profile with low product formation can be expected. Since this was the case for μ-0.2+ condition, plasmid stability was investigated by adding antibiotics into the feed molasses solution. By feeding antibiotics into the fermentation medium, growing the cells having plasmids was aimed due to the antibiotic resistance genes of the plasmid containing cells.

The variations in the cell growth and in BAL activities with the cultivation time for μ-0.2+ and μ-0.2+(antibiotics) experiments are given in Figure 4.12 and Figure 4.13, respectively. As can be seen from the figures, neither cell growth nor BAL production were effected considerably with feeding an antibiotic added molasses solution. In μ-0.2+(antibiotics) operation the maximum cell concentration and BAL activity were achieved as 26.3 g L^{-1} and 6768 U ml^{-1}, respectively.

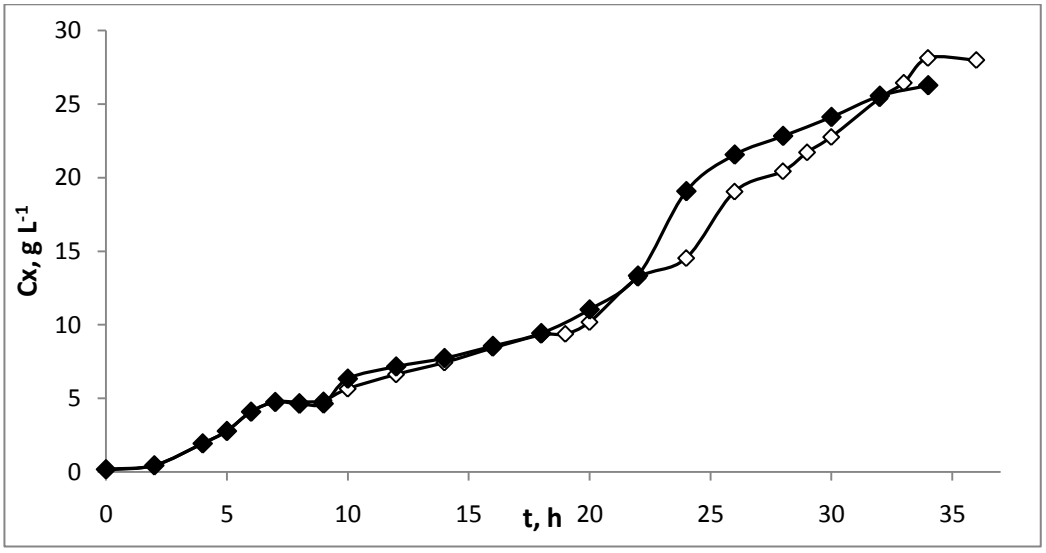

Figure 4.12. Variations in the cell concentration with the cultivation time for the combined exponential and constant feeding operations with or without antibiotics in the feed solution; μ-0.2+ (◇), μ-0.2+(antibiotics) (◆).

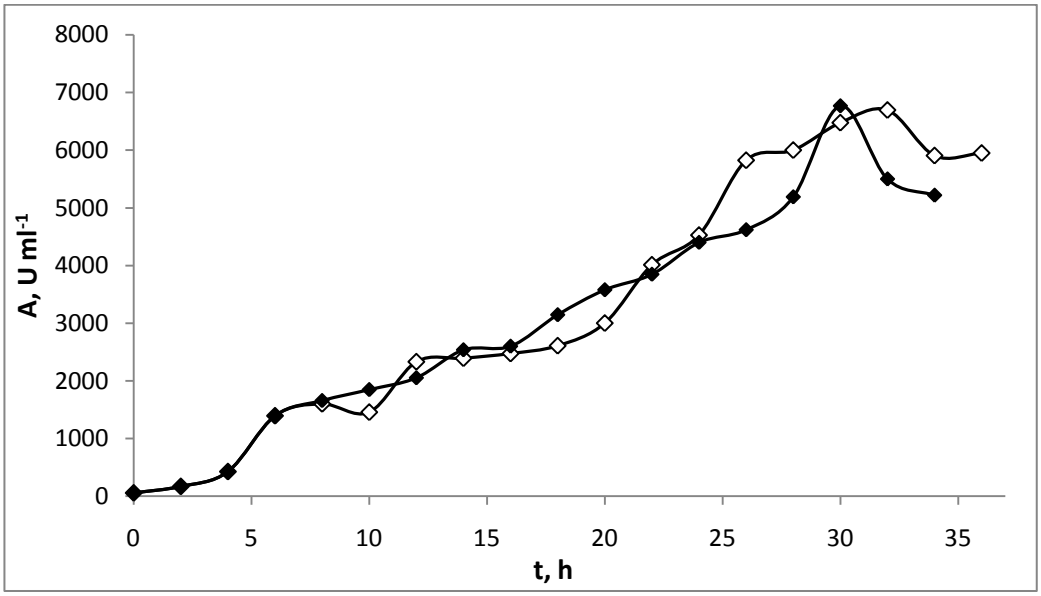

Figure 4.13. Variations in benzaldehyde activity with the cultivation time for the combined exponential and constant feeding operations with or without antibiotics in the feed solution; μ-0.2+ (◇), μ-0.2+(antibiotics) (◆).

It can be concluded that plasmids were stable and the low product formation was not due to the plasmid loss. The possible reasons for that may be inhibition by by-product acetate; and/or degradation by proteases caused by nutrient, other than the carbon source, limitation.

4.2.5. Effects of Feeding Strategy on Organic Acid Concentration Profiles

The concentrations of the organic acids detected in the fermentation medium are given in Table 4.5, and the variations in the total organic acid concentrations with the cultivation time are shown in Figure 4.14 for fed-batch experiments. The concentrations were given from t=8 h since the batch phase results were the same as BR2 operation. Acetic, formic, succinic, citric, fumaric, and oxalic acids were detected in fed-batch operations.

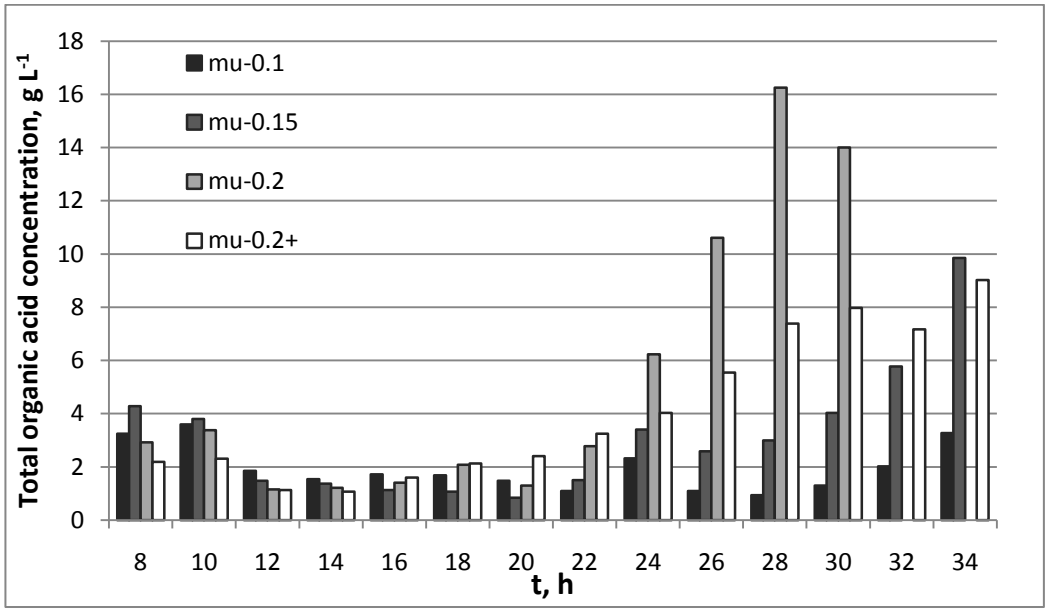

Figure 4.14. Variations in the total organic acid concentrations with the cultivation time for different feeding rates; in an order as μ-0.1, μ-0.15, μ-0.2, μ-0.2+.

The maximum total organic acid concentrations for the fed batch phases were achieved as 3.27, 9.85, 16.25, and 9.68 g L^{-1}, for μ-0.1 (t=34 h), μ-0.15 (t=34 h), μ-0.2 (t=28 h), and μ-0.2+ (t=36 h) operations, respectively. For increasing amounts of molasses feeding, increase in the maximum organic acid concentrations was observed. Since acetic acid was the major by-product, the variations in its concentrations with the cultivation time are given in Figure 4.15. The increased acetic acid concentrations in the batch phase were decreased with the fed-batch phase after t=12 h. For all the cases, acetic acid concentrations were nearly constant at low concentrations between 0.2 to 0.7 g L^{-1} until t=20 h. This is probably because of low fructose accumulation in the fermentation medium at that period. With the accumulation of fructose in high levels, significant increases in acetic acid concentrations were observed. If Figure 4.9 and Figure 4.15 examined

together, it can be seen that acetate formation profiles were parallel to fructose accumulation profiles. Also, the increases in fructose and acetate concentrations affected the cell growth.

There are some explanations for acetate production due to the overflow metabolism where AcetylCoA is directed to acetate then entering TCA cycle (Åkesson et al., 1999). One of them is that the respiratory system, where NADH is reoxidized, has a limited capacity. Since the AcetylCoA flux to the TCA cycle results in NADH production, when the respiratory saturates to avoid accumulation of NADH, redirection of AcetylCoA flux to acetate would be necessary. Another explanation is that the TCA cycle has a limited capacity and this limitation is reached before respiration saturates (Majewski and Domach, 1990). With the saturation of TCA cycle, AcetylCoA flux would again be directed to acetate due to the increasing glucose uptake (Åkesson et al., 1999).

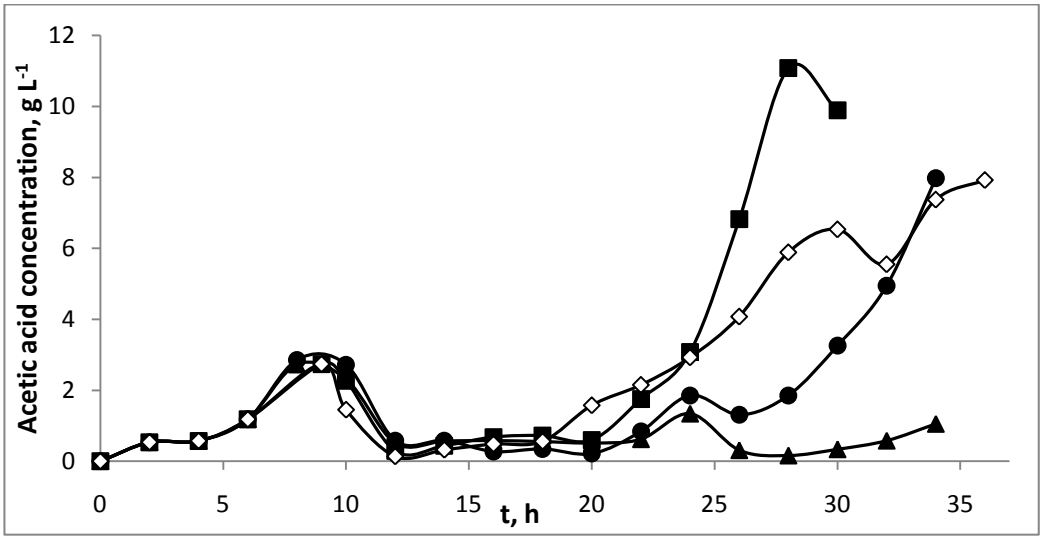

Figure 4.15. Variations in acetic acid concentrations with the cultivation time for different feeding rates; μ-0.1 (▲), μ-0.15 (●), μ-0.2 (■), μ-0.2+ (◇).

Table 4.5. Variations in the organic acid concentrations with the cultivation time for fed-batch bioreactor operations.

	Concentrations, g L^{-1}						
t	**Acetic acid**	**Formic acid**	**Succinic acid**	**Citric acid**	**Fumaric acid**	**Oxalic acid**	**Total**
				μ-0.1			
10	2.3083	0.0544	0.8979	0.2937	0.0034	0.0321	3.5898
12	0.5099	0.0474	0.8388	0.4203	0.0049	0.0337	1.8548
14	0.5669	0.0379	0.5400	0.3605	0.0042	0.0299	1.5394
16	0.5801	0.0372	0.5487	0.5124	0.0060	0.0364	1.7208
18	0.5521	0.0478	0.6195	0.4103	0.0048	0.0417	1.6762
20	0.5110	0	0.5750	0.3490	0.0041	0.0356	1.4746
22	0.6191	0.0389	0.3934	0	0	0.0383	1.0897
24	1.3392	0.0650	0.4044	0.4591	0.0054	0.0494	2.3225
26	0.3081	0.0524	0.2884	0.3945	0.0046	0.0447	1.0926
28	0.1586	0.0311	0.2037	0.5060	0	0.0342	0.9336
30	0.3348	0.0545	0.4287	0.4158	0.0048	0.0641	1.3027
32	0.5779	0.0692	0.7779	0.5078	0.0059	0.0802	2.0188
34	1.0516	0.1044	0.6211	1.3276	0	0.1599	3.2646
				μ-0.15			
10	2.7191	0.0533	0.8535	0.1435	0.0041	0.0267	3.8001
12	0.5832	0.0423	0.5794	0.1792	0.0570	0.0356	1.4767
14	0.5831	0.0661	0.5313	0.1485	0.0055	0.0383	1.3727
16	0.2733	0.0656	0.4936	0.1994	0.0604	0.0391	1.1313
18	0.3460	0.0614	0.3068	0.2294	0.0707	0.0513	1.0656
20	0.2147	0.0515	0.3000	0.1369	0.0773	0.0565	0.8369
22	0.8392	0.0739	0.3100	0.1224	0.0851	0.0739	1.5044
24	1.8539	0.1172	0.9166	0.1336	0.1118	0.2711	3.4042
26	1.3099	0.0821	1.0034	0	0.1153	0.0748	2.5854
28	1.8563	0.1172	0.7748	0.0634	0.0897	0.0875	2.9889
30	3.2586	0.2082	0.2187	0.1047	0.1357	0.1011	4.0269
32	4.9510	0.1907	0.2697	0.0733	0.1797	0.1041	5.7685
34	7.9791	0.2398	1.3545	0	0.1448	0.1340	9.8521

Table 4.5. Variations in the organic acid concentrations with the cultivation time for fed-batch bioreactor operations *(continued)*.

	Concentrations, g L^{-1}						
t	**Acetic acid**	**Formic acid**	**Succinic acid**	**Citric acid**	**Fumaric acid**	**Oxalic acid**	**Total**
				μ-0.2			
10	2.2703	0.0554	0.8824	0.0936	0.0514	0.0272	3.3802
12	0.2886	0.0474	0.5932	0.1396	0.0579	0.0285	1.1551
14	0.4394	0.0525	0.4720	0.1598	0.0547	0.0376	1.2161
16	0.6823	0.0827	0.3170	0.2089	0.0637	0.0517	1.4063
18	0.7213	0.0958	0.8896	0.2221	0.0887	0.0595	2.0771
20	0.6017	0.0965	0.1126	0.3303	0.0945	0.0595	1.2949
22	1.7512	0.1602	0.1623	0.5193	0.1077	0.0803	2.7811
24	3.0808	0.2423	0.6855	1.9648	0.1469	0.1076	6.2278
26	6.8223	0.3541	0.8824	2.2038	0.1501	0.1936	10.6062
28	11.0792	0.3858	1.2844	2.9966	0.2543	0.2516	16.2518
30	9.8870	0.3607	1.1844	2.2498	0.1735	0.1497	14.0050
				μ-0.2+			
10	1.4499	0.0432	0.7160	0.0669	0	0.0262	2.3022
12	0.1355	0.0622	0.7930	0.0976	0	0.0363	1.1246
14	0.3209	0.0528	0.5428	0.1204	0	0.0370	1.0740
16	0.4826	0.0925	0.7751	0.1972	0	0.0543	1.6017
18	0.5583	0.0885	1.2261	0.1885	0	0.0606	2.1219
20	1.5752	0.1509	0.6103	0	0.0014	0.0689	2.4068
22	2.1508	0.1458	0.8958	0	0	0.0595	3.2519
24	2.9269	0.2178	0.7657	0	0.0029	0.1100	4.0232
26	4.0751	0.2486	1.1002	0	0.0034	0.1214	5.5487
28	5.8879	0.3109	1.0536	0	0.0048	0.1311	7.3883
30	6.5349	0.3118	0.9815	0	0.0051	0.1377	7.9710
32	5.5503	0.3278	1.1521	0	0.0073	0.1365	7.1741
34	7.3738	0.3476	1.1631	0	0.0088	0.1336	9.0269
36	7.9251	0.3602	1.2416	0	0.0139	0.1377	9.6784

In μ-0.2 operation acetate concentration became inhibitory after t=25 h, which explains the early stationary growth phase and the decrease in BAL activity after t=26 h for this case. It can be concluded that, exponential feeding with a high specific growth rate resulted in overfeeding; excess amounts of the carbon source was detected in the medium, which induced acetate formation by overflow mechanism. Similarly, for μ-0.15 experiment acetic acid concentration reached to inhibitory level after t=32 h, where a decrease in BAL activity was observed. In μ-0.1 operation acetate concentrations were remained below the inhibitory level throughout the fermentation, a maximum concentration of 1.3 g L^{-1} was achieved in this operation, which was also lower than the maximum of the batch phase. From the cell growth and BAL activity profiles of μ-0.1 operation, it can be seen that no inhibition was occurred.

4.2.6. Oxygen Transfer Characteristics of Fed-Batch Operations

The oxygen transfer parameters ie., oxygen uptake rate (OUR), oxygen transfer coefficient (K_La), oxygen transfer rate (OTR), oxygen utilization rate (OD), Damköhler number (Da), and effectiveness factor (η) were calculated throughout the fermentations, and the variations in these parameters for fed-batch experiments are given in Table 4.6.

For all the fed-batch operations, K_La values did not change significantly at the times where by-product concentrations were attained nearly constant for each experiment. Generally, K_La values were increased at the beginning of the feeding phases and then decreased with increasing cell concentrations and by-product secretion to the extracellular medium. When cell growth was stopped an increase in K_La values were obtained. Also, for higher feed rates higher K_La values were generally obtained. Probably due to the higher organic acid production, higher fructose accumulation and higher amounts of molasses fed into the system which makes changes in the rheological properties of the broth. During the fed-batch experiments K_La values were oscillated between 0.006 and 0.042 s^{-1}. The highest

K_La values were observed as 0.026, 0.023, 0.042, and 0,035 s^{-1} for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. The maximum K_La value was obtained as 0.042 s^{-1} at t=22 h in μ-0.2 operation, which has the highest molasses feeding rate and the highest organic acid concentrations.

The oxygen uptake rate depends on the biomass production and substrate consumption rates. Since dissolved oxygen in the process was kept constant throughout the process, the OUR values were the same as the OTR values. The highest OUR values were obtained as 5.78, 17.60 and 11.71 mol m^{-3} s^{-1} for μ-0.1, μ-0.2, and μ-0.2+, respectively. With the increase in feed rate OUR values were increased and gave its maximum in μ-0.2 operation. OTR is proportional to K_La, thus showed a similar trend, and give a maximum value of 17.60 mol m^{-3} s^{-1} at t=22 h in μ-0.2 operation. OTR values were generally higher for the increased feed rates, which is probably due to the increase in the difference between the saturated and dissolved oxygen concentrations with increasing feed rates.

To find the rate limiting step of the bioprocess, Damköhler number, which is the ratio of the maximum possible oxygen utilization rate to the maximum oxygen transfer rate, was calculated. For all the fed-batch operations, Da values were mostly higher than 1 that means mass transfer resistances dominate biochemical reaction resistance. The maximum Da values were obtained as 2.79, 1.54, 2.59, and 2.85 for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. For all the conditions, high feeding rates have lead to an increase in oxygen demand which cannot be satisfied by the maximum possible oxygen transfer rate, explaining the results of Da values higher than 1. However, Da values were not significantly higher than 1.

Table 4.6. Variations in the oxygen transfer parameters in fed-batch operations.

t h	K_La s^{-1}	$OTR*10^3$ mol m^{-3} s^{-1}	$OTR_{max}*10^3$ mol m^{-3} s^{-1}	$OUR*10^3$ mol m^{-3} s^{-1}	$OD*10^3$ mol m^{-3} s^{-1}	Da	η
			μ-0.1				
10	0.019	4.11	6.86	4.11	15.38	2.24	0.27
12	0.015	3.22	5.37	3.22	4.06	0.76	0.79
14	0.022	4.89	8.16	4.89	9.00	1.10	0.54
18	0.013	2.77	4.62	2.77	12.91	2.79	0.22
22	0.015	3.25	5.42	3.25	5.95	1.10	0.55
24	0.012	2.55	4.24	2.55	5.06	1.19	0.50
26	0.006	1.43	2.38	1.43	3.13	1.32	0.46
30	0.018	3.99	6.65	3.99	4.26	0.64	0.94
32	0.016	3.56	5.94	3.56	4.97	0.84	0.72
34	0.026	5.78	9.64	5.78	10.93	1.13	0.53
			μ-0.15				
10	0.011	2.50	4.17	2.50	6.41	1.54	0.39
12	0.014	3.21	5.34	3.21	7.18	1.35	0.45
14	0.019	4.25	7.08	4.25	10.86	1.54	0.39
16	0.016	3.58	5.97	3.58	8.72	1.46	0.41
18	0.023	5.18	8.64	5.18	12.19	1.41	0.43
			μ-0.2				
10	0.027	10.74	17.91	10.74	46.32	2.59	0.23
12	0.028	8.11	13.51	8.11	10.44	0.77	0.78
14	0.026	10.36	17.27	10.36	18.52	1.07	0.56
16	0.023	10.26	17.10	10.26	36.06	2.11	0.29
18	0.011	3.23	5.38	3.23	8.57	1.59	0.38
20	0.025	11.98	19.97	11.98	26.34	1.32	0.46
22	0.042	17.60	29.33	17.60	52.40	1.79	0.34
28	0.034	10.69	17.82	10.69			

Table 4.6. Variations in the oxygen transfer parameters in fed-batch operations *(continued)*.

t h	K_La s^{-1}	$OTR*10^3$ mol m^{-3} s^{-1}	$OTR_{max}*10^3$ mol m^{-3} s^{-1}	$OUR*10^3$ mol m^{-3} s^{-1}	$OD*10^3$ mol m^{-3} s^{-1}	Da	η
			μ-0.2+				
10	0.032	8.24	13.73	8.24	19.15	1.40	0.43
12	0.032	8.04	13.40	8.04	13.16	0.98	0.61
14	0.032	8.07	13.45	8.07	14.68	1.09	0.55
16	0.027	11.38	18.97	11.38	22.49	1.19	0.51
18	0.025	10.37	17.28	10.37	24.22	1.40	0.43
20	0.025	10.40	17.33	10.40	12.02	0.69	0.87
22	0.021	11.71	19.52	11.71	17.66	0.91	0.66
26	0.032	9.50	15.84	9.50	13.09	0.83	0.73
28	0.029	8.10	13.50	8.10	26.87	1.99	0.30
30	0.023	6.57	10.94	6.57	13.28	1.21	0.50
32	0.035	9.88	16.46	9.88	20.55	1.25	0.48
34	0.025	7.08	11.80	7.08	33.61	2.85	0.21

Throughout the fermentations, the effectiveness factors were lower than 1 showing that the oxygen consumption was below the maximum oxygen demand. This indicates that oxygen transport was limited for all conditions due to the high cell concentrations and high by-product secretion. The maximum η values were 0.94, 0.45, 0.78, and 0.87 for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively.

4.2.7. Specific Growth Rate and Yield Coefficients of Fed-Batch Operations

The variations in specific growth rates, μ, specific oxygen uptake rates, q_O, specific glucose, fructose and total substrate consumption rates, q_G, q_F, q_S, and the yield coefficients, $Y_{X/S}$, $Y_{X/O}$, $Y_{S/O}$, with the cultivation time and with different feeding rates are given in Table 4.3.

For all the operations, μ values were not constant at the desired value and also they are below the predetermined specific growth rate. This is probably due to the limitations in oxygen transfer and growth inhibition by acetate production. They affected the ideal conditions that are necessary to attain cell growth with a constant predetermined specific growth rate. The highest specific growth rates were obtained as 0.102, 0.133, 0.160, and 0.139 h^{-1} for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. The highest μ values increased with increasing feed rates which can be expected.

Specific glucose, fructose and total substrate consumption rates were generally increased throughout the fermentations. The highest specific total substrate consumption was achieved in μ-0.2 operation as 0.90 g g^{-1}, and the highest q_S values were decreased with decreasing feed rates. Furthermore, the highest specific oxygen consumption rate was obtained in μ-0.2 operation as 0.273 g g^{-1} and it was generally higher for higher feed rates.

The yield coefficients are important since they represent the efficiency of the bioprocess. The overall cell yields on substrate were obtained as 0.52, 0.17, 0.18, and 0.34 g g^{-1} for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. It can be seen that the highest overall $Y_{X/S}$ was attained in μ-0.1 operation since it has the lowest substrate feed while cell concentration was comparable. Highest cell concentrations of μ-0.2+ operation also resulted in a higher $Y_{X/S}$ value than the other two operations. $Y_{X/O}$ and $Y_{S/O}$ values were altered during fermentations. The highest $Y_{X/O}$ values were 2.49, 1.93, and 1.64 g g^{-1} for μ-0.1, μ-0.2, and μ-0.2+, respectively. Whereas the highest $Y_{S/O}$ values were 0.68, 1.33, and 0.66 g g^{-1} for μ-0.1, μ-0.2, and μ-0.2+, respectively.

Table 4.7. Variations in the specific growth rate and yield coefficients in fed-batch operations.

t h	μ h^{-1}	q_O $g\ g^{-1}\ h^{-1}$	q_F $g\ g^{-1}\ h^{-1}$	q_G $g\ g^{-1}\ h^{-1}$	q_S $g\ g^{-1}\ h^{-1}$	$Y_{X/S}$ $g\ g^{-1}$	$Y_{X/O}$ $g\ g^{-1}$	$Y_{S/O}$ $g\ g^{-1}$
				μ-0.1				
10	0.028	0.104	0.034	0.028	0.062	3.95	0.26	0.05
12	0.081	0.078	0.038	0.032	0.070	0.43	1.02	0.07
14	0.056	0.093	0.045	0.031	0.076	1.84	0.59	0.15
16	0.007	0.061	0.039	0.038	0.076	0.04		
18	0.024	0.052	0.038	0.045	0.084	0.09	0.42	0.14
20	0.039		0.048	0.051	0.099	0.39		
22	0.059	0.053	0.039	0.058	0.097	0.38	1.04	0.36
24	0.055	0.036	0.041	0.061	0.102	0.79	1.41	0.55
26	0.052	0.019	0.066	0.069	0.135	0.31	2.43	0.17
28	0.086		0.069	0.074	0.144	0.38		
30	0.102	0.039	0.073	0.074	0.147	0.73	2.41	0.02
32	0.082	0.029	0.064	0.074	0.138	0.57	2.49	0.35
34	0.065	0.042	0.050	0.079	0.129	0.44	1.26	0.68
					Overall	0.52		
				μ-0.15				
10	0.061	0.058	0.083	0.049	0.132	0.43	1.01	0.58
12	0.070	0.067	0.063	0.059	0.122	0.43	1.00	0.07
14	0.063	0.076	0.049	0.068	0.117	0.80	0.77	0.25
16	0.067	0.059	0.078	0.083	0.161	0.38	1.05	0.09
18	0.071	0.073	0.086	0.096	0.181	0.43	0.87	0.13
20	0.078		0.107	0.114	0.220	0.32		
22	0.078		0.122	0.129	0.250	0.32		
24	0.113		0.129	0.151	0.280	0.22		
26	0.133		0.151	0.154	0.305	0.43		
28	0.074		0.143	0.162	0.305	0.29		
30	0.048		0.179	0.207	0.385			
32	0.087		0.241	0.247	0.487	0.04		
34	0.109		0.265	0.271	0.536	0.08		
					Overall	0.17		

Table 4.7. Variations in the specific growth rate and yield coefficients in fed-batch operations *(continued)*.

t h	μ h^{-1}	q_O $g\ g^{-1}\ h^{-1}$	q_F $g\ g^{-1}\ h^{-1}$	q_G $g\ g^{-1}\ h^{-1}$	q_S $g\ g^{-1}\ h^{-1}$	$Y_{X/S}$ $g\ g^{-1}$	$Y_{X/O}$ $g\ g^{-1}$	$Y_{S/O}$ $g\ g^{-1}$
				μ-0.2				
10	0.049	0.273	0.094	0.067	0.162	0.21	0.17	0.10
12	0.160	0.189	0.095	0.091	0.186	0.26	0.82	0.02
14	0.118	0.157	0.060	0.088	0.148	1.40	0.71	0.18
16	0.066	0.142	0.108	0.118	0.226	0.31	0.40	0.07
18	0.089	0.039	0.149	0.151	0.300	0.23	1.93	0.05
20	0.111	0.123	0.183	0.185	0.368	0.27	0.74	0.02
22	0.096	0.149	0.206	0.218	0.425	0.22	0.45	0.08
24	0.083		0.262	0.280	0.542	0.09		
26	0.058		0.292	0.353	0.645	0.06		
28	0.044	0.082	0.396	0.505	0.900			1.33
					Overall	0.18		
				μ-0.2+				
10	0.089	0.168	0.053	0.059	0.112	1.65	0.51	0.03
12	0.072	0.140	0.072	0.074	0.146	0.72	0.48	0.02
14	0.068	0.125	0.079	0.097	0.176	0.38	0.48	0.15
16	0.066	0.156	0.119	0.126	0.245	0.38	0.36	0.05
18	0.062	0.128	0.142	0.167	0.308	0.18	0.37	0.19
20	0.113	0.118	0.158	0.180	0.338	0.15	0.81	0.19
22	0.090	0.102	0.136	0.134	0.270	0.37	0.71	0.02
24	0.139		0.083	0.122	0.205	0.11		
26	0.096	0.055	0.047	0.083	0.129	1.04	1.46	0.66
28	0.049	0.046	0.067	0.079	0.146	0.06	0.73	0.25
30	0.070	0.033	0.064	0.068	0.132	0.30	1.64	0.14
32	0.068	0.045	0.053	0.060	0.113	0.36	1.18	0.14
34	0.037	0.029	0.047	0.052	0.099	0.36	0.80	0.20
36	0.011		0.043	0.051	0.094			
					Overall	0.34		

CHAPTER 5

CONCLUSIONS

In this study, it was aimed to investigate the effects of the designed feeding strategies on benzaldehyde lyase production by *Escherichia coli* BL21 (DE3) pLySs carrying the recombinant pRSETA::*bal* plasmid using pretreated molasses as the carbon and energy source. In order to design the feeding strategies, the effects of medium composition on the cell growth, product and by-product formations, and oxygen transfer were evaluated with batch bioreactor operations. In order to increase BAL production and the cell concentration, fed-batch operations were conducted using exponential feeding strategy at three different specific growth rates. Moreover, a combined exponential and constant feeding strategy was developed to overcome the overfeeding problem in exponential feeding at high specific growth rates with lowering the feed rate gradually. Furthermore, the plasmid stability for long term fermentations was examined by antibiotic addition to the feed solution.

Effect of medium composition in batch phase of the fermentation process was investigated using two different molasses concentrations, nitrogen limitation and medium without $CoCl_2$. The highest cell concentration and BAL activity were obtained as 5.07 g L^{-1}, and 1611 U ml^{-1} at t=8 h, respectively, in BR2 operation containing 30 g L^{-1} pretreated molasses, and 5 g L^{-1} $(NH_4)_2HPO_4$.

In order to achieve high cell concentrations and BAL activities, the effects of molasses feeding rate were investigated with fed-batch operations. In this context, exponential feeding strategies operating at the specific growth rates as 0.1, 0.15, and 0.2 h^{-1}, and a combined strategy in which 10 hours of exponential feeding at μ=0.2 h^{-1} was followed by constant feeding having a feed rate of 21.6 g h^{-1}. In the fed-batch fermentations, 9 hours of batch operation phase with the optimized medium was followed by fed-batch operation phase using the pre-determined feeding profiles.

In fed-batch operations, the highest cell concentrations were achieved as 15.9, 21.7, 16.2, and 28.1 g L^{-1} for μ-0.1, μ-0.15, μ-0.2 and μ-0.2+ operations, at t=34, 34, 26, and 34 h, respectively. In terms of cell growth, μ-0.2+ operation seems to be successfully applied since the inhibition of cell growth in μ-0.2 case was prevented and also the highest cell concentration among the exponential feeding experiments, which is attained in μ-0.15 operation, was increased. The highest BAL activities were achieved as 4561 U ml^{-1}, 8379 U ml^{-1}, 4584 U ml^{-1}, and 6695 U ml^{-1} for μ-0.1 (t=34 h), μ-0.15 (t=32 h), μ-0.2 (t=24 h), and μ-0.2+ (t=32 h) operations, respectively. In the combined exponential and constant feeding strategy, although the highest cell concentration was achieved amongst the fed-batch bioreactor operations, the maximum BAL activity could not reach to that of μ-0.15 condition. Additionally, the cell concentrations and BAL activities in μ-0.2 operation were higher than the other two exponential feeding operations for the first 26 hours of the fermentation; which shows that high feeding amounts results in increased cell growth and enzyme production, however, after a certain point of time the accumulation of the carbon source significantly increases and causes acetate accumulation in inhibitory levels. Also, μ-0.2 and μ-0.2+ operations showed similar profiles for the first 10 hours of the fed-batch phases in terms of the cell growth and BAL activity, which is expected.

The highest BAL activity of μ-0.15 condition, which is 8379 U ml^{-1}, is the maximum BAL activity reported in the literature and also the highest cell concentration of μ-0.2+ operation, which is 28.1 g L^{-1}, is the maximum cell concentration of *E. coli* producing BAL.

During the fed-batch phases, glucose was never detected in the medium, while fructose was accumulated throughout the fermentations. By increasing the feed rates, considerable amounts of fructose accumulation was observed at the end of the fermentations. The highest fructose accumulation was observed in μ-0.2 operation as 8.9 g L^{-1} which was higher compared to the highest fructose concentrations of μ-0.1 and μ-0.15 cases and with μ-0.2+ operation fructose accumulation was lowered.

For all the pilot scale bioreactor operations, acetic, formic, succinic, citric, fumaric, and oxalic acids were detected in the fermentation medium. Presence of the TCA cycle organic acids as succinic, citric and fumaric may be due to the inadequate oxygen supply which resulted in repression on the TCA cycle enzymes or their improper activity. The total maximum organic acid concentrations were detected as 3.27, 9.85, 16.25, and 9.68 g L^{-1} in μ-0.1 (t=34 h), μ-0.15 (t=34 h), μ-0.2 (t=28 h), and μ-0.2+ (t=36 h) operations, respectively. The highest total organic acid concentrations were increased with increasing feed rates.

Acetic acid was the major organic acid in all the conditions, as expected. When fructose accumulation was sharply increased, an increase in acetate accumulation was observed and thus a decrease in cell growth rate was occurred. In μ-0.2 and μ-0.15 operations acetate concentration became inhibitory after t=25 h and t=32 h, respectively, where a decrease in the cell growth rate and BAL activity was observed for both two cases. However, in μ-0.1 operation acetate concentrations were remained below the inhibitory level throughout the fermentation, and

correspondingly any drop in BAL activity and any significant decrease in cell concentration were not observed.

At higher feeding rates higher K_La values were generally obtained. The highest K_La values were 0.026, 0.023, 0.042, and 0,035 s^{-1} for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. The highest OUR values were obtained as 5.78, 17.60, and 11.71 mol m^{-3} s^{-1} for μ-0.1, μ-0.2, and μ-0.2+, respectively. With the increase in the feed rate the OTR values were increased and gave its maximum in μ-0.2 operation as 17.60 mol m^{-3} s^{-1}. In general, high feeding rates increased the oxygen demand which cannot be satisfied by the maximum possible oxygen transfer rate, therefore Da values higher than 1 were observed. The maximum Da values were obtained as 2.79, 1.54, 2.59, and 2.85 for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively.

The highest specific growth rates were obtained as 0.102, 0.133, 0.160, and 0.139 h^{-1} for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. The highest μ values increased with increasing feed rates which can be expected. The highest specific substrate consumption and the highest specific oxygen consumption rate were attained in μ-0.2 operation as 0.90 g g^{-1} and 0.273 g g^{-1}, respectively; also they generally increased with increasing feed rates. The overall cell yields on substrate were obtained as 0.52, 0.17, 0.18, and 0.34 g g^{-1} for μ-0.1, μ-0.15, μ-0.2, and μ-0.2+, respectively. Moreover, the highest $Y_{X/O}$ values were attained as 2.49, 1.93, and 1.64 g g^{-1} and the highest $Y_{S/O}$ values were attained as 0.68, 1.33, and 0.66 g g^{-1} for μ-0.1, μ-0.2, and μ-0.2+ operations, respectively.

Furthermore, the plasmid stability was investigated due to the reason of attaining high cell concentrations having low BAL activities in μ-0.2+ operation. In this program, the fermentation was conducted with adding antibiotics in the feed solution and by using the feeding method of μ-0.2+ operation. It was seen that the plasmid was stable since the cell concentrations and BAL activities did not changed with those obtained in μ-0.2+ operation.

As a conclusion, μ-0.15 operation is the favorable fed-batch mode of operation, since the highest BAL production was attained. Exponential feeding at high specific growth rate (μ-0.2) increased the cell growth and product formation at the beginning of the fermentation but then caused overfeeding and acetate inhibition; while exponential feeding at low specific growth rate (μ-0.1) resulted in insufficient cell growth and enzyme production and increased the fermentation time when compared to the other two operations. On the other hand, the highest cell concentration was achieved in μ-0.2+ operation, whereas the highest enzyme activity obtained was much lower than that of μ-0.15 operation. The combined exponential and constant feeding strategy also has advantages since the growth inhibition occurred in μ-0.2 operation due to the high substrate feed rate can be eliminated by lowering the feed rate. As a further investigation, a fed-batch operation strategy as exponential feeding at a high specific growth rate at the beginning and then switching to a feed rate with a lower specific growth rate could be proposed to avoid fructose accumulation and thus to reduce acetate production. Moreover, after a period of fed-batch operation by feeding only molasses solution, addition of salt solutions to the feed could also be considered.

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APPENDIX A

CALIBRATION OF *Escherichia coli* CONCENTRATION

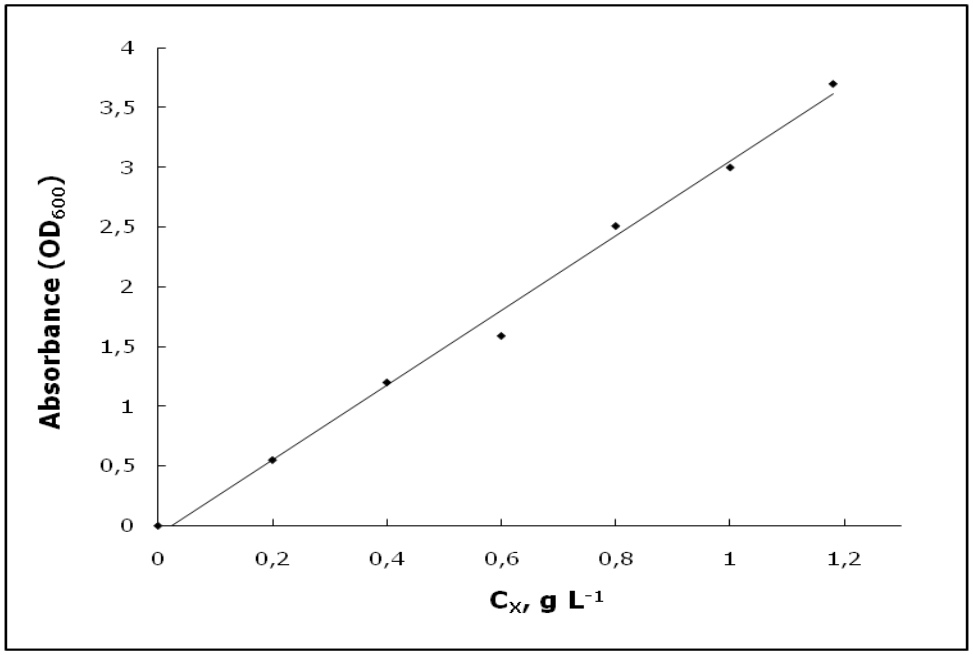

Figure A.1. Calibration curve for determination of *Escherichia coli* concentration.

Slope of the curve, m=2.8782 1/(g CDW L^{-1})

$$C_X = \frac{OD_{600}}{2.8782}\ x\ Dilution\ Ratio \qquad \text{(A.1)}$$

APPENDIX B

CALIBRATION OF GLUCOSE CONCENTRATION

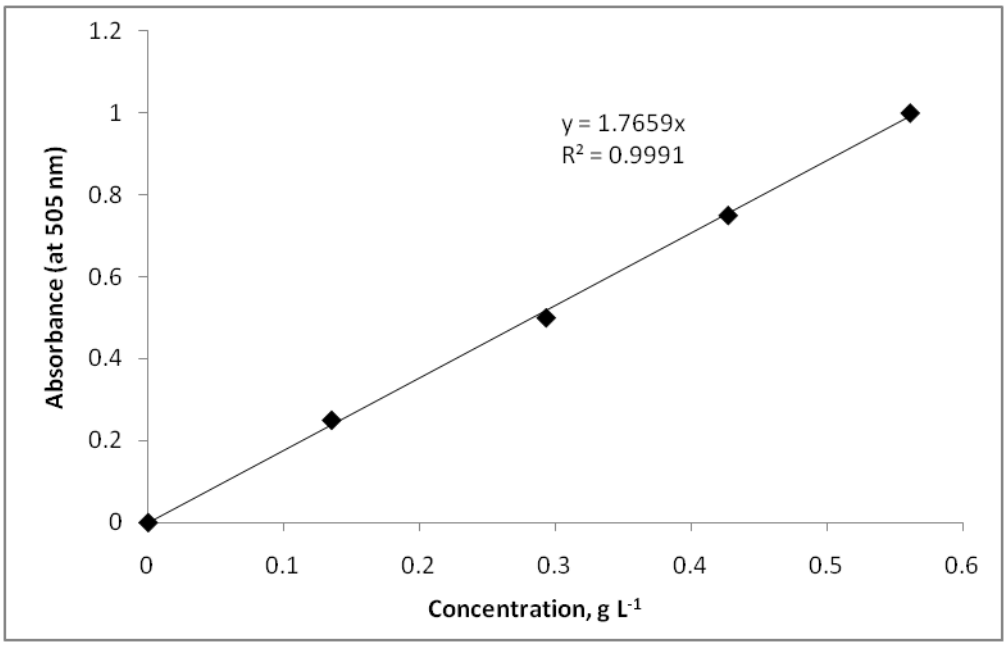

Figure B.1. Calibration curve for glucose concentration.

According to the equation obtained from the plot:

$$C_G = \frac{Absorbance}{1.7659}\ x\ Dilution\ Ratio \qquad \text{(B.1)}$$

APPENDIX C

CALIBRATION OF FRUCTOSE CONCENTRATION

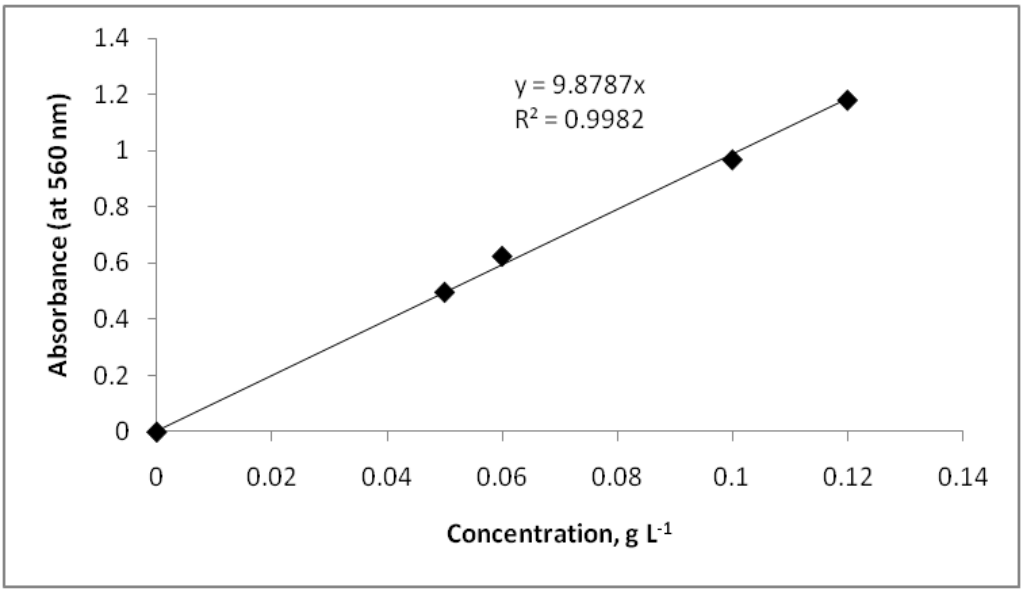

Figure C.1. Calibration curve for fructose concentration.

According to the equation obtained from the plot:

$$C_F = \frac{Absorbance}{9.8787}\ x\ Dilution\ Ratio \qquad \text{(C.1)}$$

APPENDIX D

CALIBRATION OF ORGANIC ACID CONCENTRATIONS

The calibration curves for the determined organic acids in the fermentation broth are given in the below figures. Concentrations were determined using the equations written in the plots.

- **Acetic Acid**

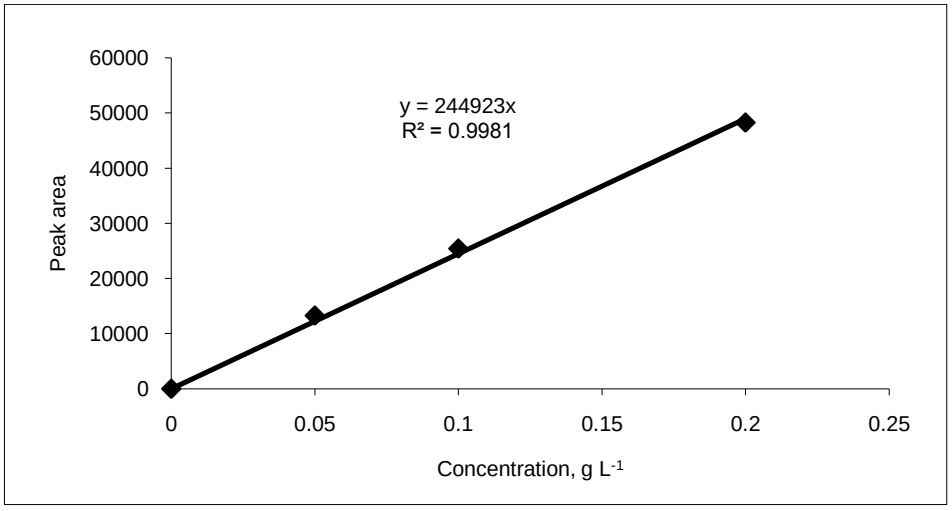

Figure D.1. Standard calibration curve for acetic acid.

- **Succinic Acid**

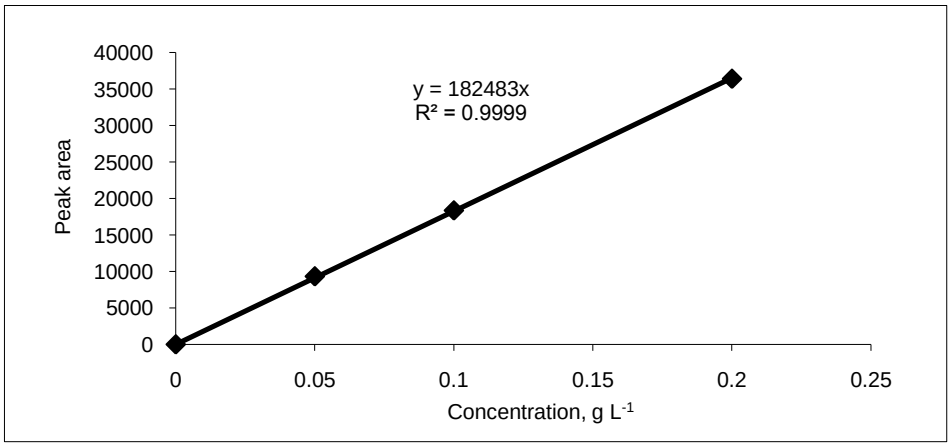

Figure D.2. Standard calibration curve for succinic acid.

- **Citric Acid**

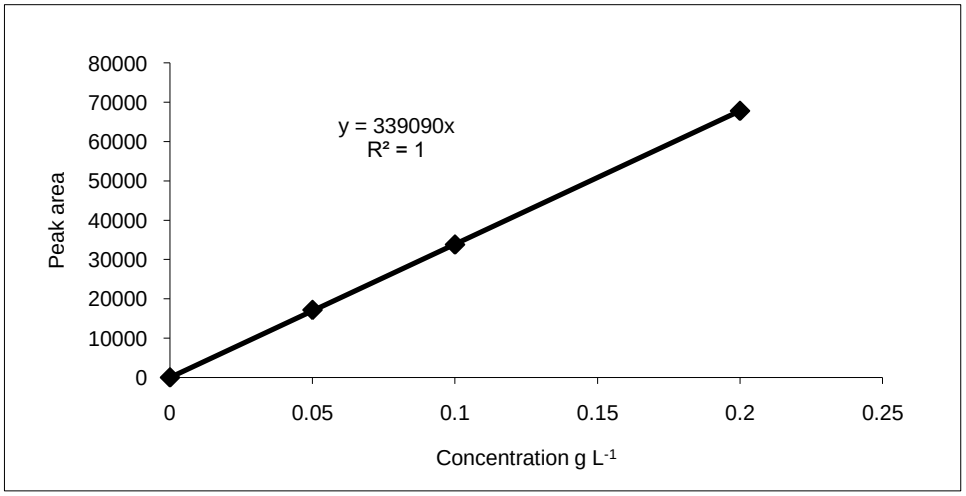

Figure D.3. Standard calibration curve for citric acid.

- **Fumaric Acid**

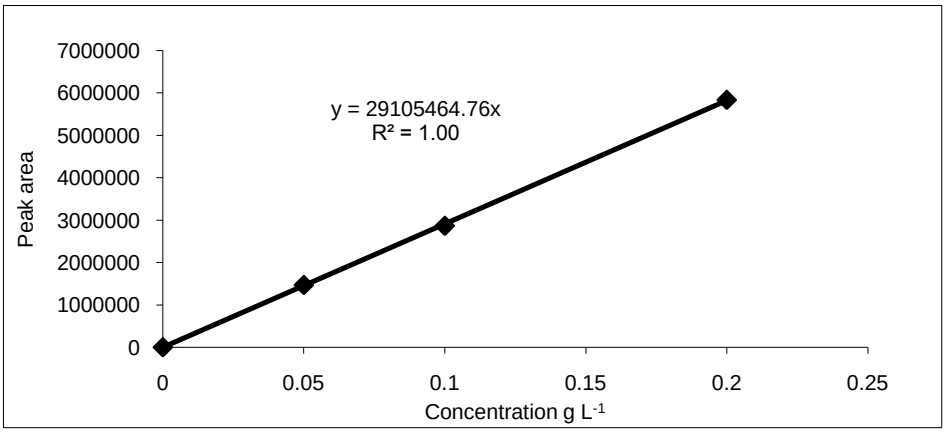

Figure D.4. Standard calibration curve for fumaric acid.

- **Oxalic Acid**

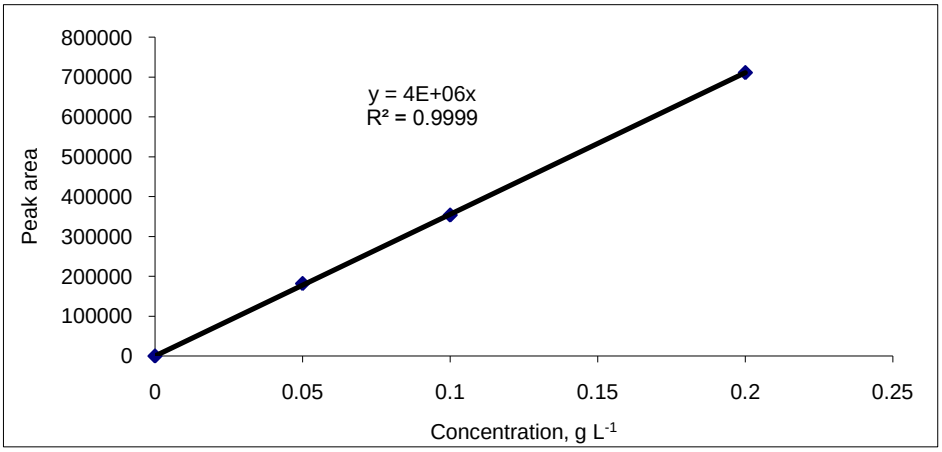

Figure D.5. Standard calibration curve for oxalic acid.