COMPARISON OF BENZALDEHYDE LYASE PRODUCTION CAPACITY IN RECOMBINANT *Escherichia coli* and RECOMBINANT *Bacillus* SPECIES

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ABSTRACT

COMPARISON OF BENZALDEHYDE LYASE PRODUCTION CAPACITY IN RECOMBINANT *Escherichia coli* and RECOMBINANT *Bacillus* SPECIES

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In this study, the benzaldehyde lyase (BAL, EC 4.1.2.38) production in *E. coli* BL21 (DE3) pLySs as intracellular and in *Bacillus* species as extracellular were investigated, and comparison of the production capacity of the enzyme in the developed recombinant microorganisms were compared. For this purpose, firstly, PCR amplified *bal* gene was cloned into pRSETA vector which is under the control of strong T7 promoter and expressed in *E. coli* BL21 (DE3) pLysS strain. With developed recombinant *E. coli* BL21 (DE3) pLySs cells, the effect of bioprocess parameters was systematically investigated. Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as 2.0 kg m^{-3} and 1060 U cm^{-3}, respectively, in the medium containing 20.0 kg m^{-3} glucose, 11.8 kg m^{-3} $(NH_4)_2HPO_4$ and the salt solution. Thereafter, oxygen transfer effects on benzaldehyde lyase production were investigated at air inlet

rate of Q_O/V_R = 0.5 vvm, and agitation rates of N=500 and 750 min^{-1} and at Q_O/V_R = 0.7 vvm, N=750 min^{-1} in pilot scale bioreactor and the highest cell concentration and volumetric BAL activity were found as 1.7 kg m^{-3} and 990 U cm^{-3}, respectively, at 0.5 vvm, 750 min^{-1} condition. Next, the signal DNA sequence of serine alkaline protease (SAP) from *B. licheniformis* DSM 1969 chromosomal DNA (*pre-sub*C) was fused in front of the *bal* by using PCR-based gene splicing by overlap extension (SOE) method. The fusion product of *hybrid* gene first cloned into pUC19 plasmid, thereafter sub-cloned into pBR374 shuttle vector and recombinant plasmid was transferred into various *Bacillus* species. However, no extracellular production of benzaldehyde lyase was observed in none of the developed recombinant *Bacillus* species, probably because of ineffective secretion system, inefficient folding of heterologous protein, degradation of enzyme due to proteolytic activity or high inactivation rate of the enzyme.

Keywords: Benzaldehyde lyase, Production, Recombinant *E. coli*, Recombinant *Bacillus* species, Oxygen Transfer, pRSET, pRB374

ÖZ

BENZALHİT LİYAZ ENZİM ÜRETİM KAPASİTESİNİN REKOMBİNANT *Escherichia coli* VE REKOMBİNANT *Bacillus* TÜRLERİNDE KIYASLANMASI

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Bu çalışmada, *E. coli* BL21 (DE3) pLySs ile hücre içi, *Bacillus* türleriyle hücre dışı benzaldehit liyaz üretimi araştırılmış ve geliştirilen recombinant mikroorganizmaların üretim kapasiteleri kıyaslanmıştır. Bu amaçla, araştırma programı başlıca üç bölümde yürütülmüştür. Çalışmanın ilk bölümünde, polimeraz zincir tepkimesiyle çoğaltılan *bal* geni pRSETA vektörüne T7 promotorunun kontrolü altında bulunacak şekilde klonlanmış ve *E. coli* BL21 (DE3) pLysS hücrelerine transfer edilmiştir. Daha sonra, geliştirilen recombinant *E. coli* BL21 (DE3) pLySs hücreleri kullanılarak biyoproses parametreleri sistematik olarak incelenmiştir. İncelenen koşullarda, en yüksek hücre derişimi ve enzim aktivitesi sırasıyla 2.0 kg m^{-3} ve 1060 U cm^{-3} olarak; 20.0 kg m^{-3} glukoz, 11.8 kg m^{-3} $(NH_4)_2HPO_4$ ve tuz çözeltisi içeren ortamda elde edilmiştir.

Tasarlanan üretim ortamı kullanılarak, biyoreaktör işletim parametrelerinden oksijen aktarımı etkisi, hava giriş hızı Q_0/V_R=0.5 vvm; karıştırma hızları N=500 ve 750 dk^{-1}; ve Q_0/V_R=0.7 vvm, N=750 dk^{-1} koşullarında pilot ölçek biyoreaktörde incelenmiş ve en yüksek hücre derişimi ve enzim aktivitesine sırasıyla 1.7 kg m^{-3} ve 990 U cm^{-3} olarak 0.5 vvm, 750 dk^{-1} koşulunda ulaşılmıştır. *B. licheniformis* (DSM 1969) mikroorganizmasının kromosomal DNA'sından kodlanan, serin alkali proteaz (SAP) enziminin sinyal DNA dizini, *bal* geninin önüne 'gene splicing by overlap extension', (SOE), metoduyla entegre edilmiştir. Bu iki genin birbirine entegrasyonu ile elde edilen *hibrid* gen önce pUC19 plazmidine, daha sonra da pRB374 shuttle plazmidine klonlanmış ve ve rekombinant plazmid Bacillus türlerine transfer edilmiştir. Ancak, yetersiz salgılama sistemi, rekombinant hibrid proteinin uygun katlanmayı gerçekleştirememesi, proteolitik aktiviteye karşı enzimin kendini koruyamaması veya enzim inaktivasyon hızının yüksek olması sebebi ile, geliştirilen rekombinant *Bacillus* türlerinde benzaldehit liyaz enziminin hücre dışı üretimi gerçekleşmemiştir.

Anahtar Kelimeler: Benzaldehit Liyaz, Üretim, Rekombinant *E. coli*, Rekombinant *Bacillus* türleri, Oksijen Aktarımı, pRSET, pRB374

To My Family

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A	Benzaldehyde lyase activity, U cm^{-3}
C_{AA}	Amino acid concentration, kg m^{-3}
C_G	Glucose concentration, kg m^{-3}
C_G^0	Initial glucose concentration, kg m^{-3}
C_{OA}	Organic acid concentration, kg m^{-3}
C_O	Dissolved oxygen concentration, mol m^{-3}; kg m^{-3}
Co_0	Initial dissolved oxygen concentration, mol m^{-3}; kg m^{-3}
C_O^*	Oxygen saturation concentration, mol m^{-3}; kg m^{-3}
C_p	Product formation rate
C_s	Concentration of the substrate
C_X	Cell concentration, kg dry cell m^{-3}
Da	Damköhler number (=OD / OTR_{max}; Maximum possible oxygen utilization rate per maximum mass transfer rate)
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
K_La_0	Physical overall liquid phase mass transfer coefficient; s^{-1}
K_La	Overall liquid phase mass transfer coefficient; s^{-1}
m_0	Rate of oxygen consumption for maintenance, kg oxygen kg^{-1} dry cell weight h^{-1}
m_S	Maintenance coefficients for substrate, kg substrate kg^{-1} dry cell weight h^{-1}
N	Agitation or shaking rate, min^{-1}
P	Product
PCR	Polymerase Chain Reaction
pH_0	Initial pH
Qo	Volumetric air feed rate, $m^3\ min^{-1}$
q_o	Specific oxygen uptake rate, kg kg^{-1} DW h^{-1}
q_s	Specific substrate consumption rate, kg kg^{-1} DW h^{-1}
r	Volumetric rate of reaction (mol $m^{-3}\ s^{-1}$)
RE	Restriction Enzyme
r_{max}	Maximum rate of reaction at infinite reactant concentration

r_0	Oxygen uptake rate, mol m^{-3} s^{-1}; kg m^{-3} h^{-1}
r_P	Product formation rate, kg m^{-3} h^{-1}
r_S	Substrate consumption rate, kg m^{-3} h^{-1}
r_X	Rate of cell growth, kg m^{-3} h^{-1}
S	Substrate
t	Bioreactor cultivation time, h
T	Bioreaction medium temperature, °C
T_{AA}	Total amino acid concentration, kg m^{-3}
T_{OA}	Total organic acid concentration, kg m^{-3}
U	One unit of an enzyme
V_R	Volume of the bioreaction medium, m^3
$Y_{X/S}$	Yield of cell on substrate, kg kg^{-1}
$Y_{X/O}$	Yield of cell on oxygen, kg kg^{-1}
$Y_{S/O}$	Yield of substrate on oxygen, kg kg^{-1}
$Y_{P/X}$	Yield of product on cell, kg kg^{-1}
$Y_{P/S}$	Yield of product on substrate, kg kg^{-1}
$Y_{P/O}$	Yield of product on oxygen, kg kg^{-1}

Greek Letters

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

Abbreviations

Ac	Acetic acid
Ala	Alanine
ATCC	American Type Culture Collection
BAL	Benzaldehyde lyase
bal	Gene of Benzaldehyde Lyase
BGSC	*Bacillus* Genetic Stock Center

Cys	Cysteine
DO	Dissolved oxygen
EC	Enzyme Commission
FP	Forward Primer
Form	Formic Acid
Fum	Fumaric Acid
hybrid	Hybrid Gene
Ile	Isoleucine
Mal	Malic Acid
Met	Methionine
NRRL	Northern Regional Research Center
Ox	Oxalic acid
Orn	Ornithine
OD	Oxygen demand ($=\mu_{max}\ C_X\ /\ Y_{X/O}$; mol $m^{-3}\ s^{-1}$)
OTR	Oxygen transfer rate, mol $m^{-3}\ s^{-1}$
OTR_{max}	Maximum possible mass transfer rate ($=K_La C_O^*$; mol $m^{-3}\ s^{-1}$)
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
*pre-sub*C	Gene of Signal of Peptide of Serine Alkaline Protease
Pro	Proline
Pyr	Pyruvic Acid
RP	Reverse Primer
SOE	Gene Splicing by Overlap Extension Method
Val	Valine
Tyr	Tyrosine

CHAPTER 1

INTRODUCTION

Biotechnology, the use of biochemical and biological materials and processes, has long had a role in chemical technology. By the middle of the twentieth century, most simple organic chemicals were produced synthetically. Advances in molecular biology and genetic engineering during the latter part of the twentieth century have widened the scope of possibilities for the use of biotechnological methods and resulted in increased interest on the part of the chemical industry (Kirk Othmer, 1994). In this sense, the natural catalysts, enzymes, are now used in a wide range in industrial processes as an alternative to chemical catalysts.

The advantages of using enzymes instead of chemical catalysts are directly related to their properties. Many biochemical reactions require an enzyme to proceed at reasonable rates under mild conditions such as neutral pH and low temperatures. Enzymes act in a pH range of about 5-8, typically around 7, and in a temperature range of 20–40^{o}C, preferably at around 30^{o}C. This minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement which are the major challenges in processes where chemical catalysts are used. One more advantage of enzymes is that unlike heavy metals, they are biodegradable and environmentally acceptable. Enzymes are very efficient catalysts; typically the rates of enzyme-mediated processes are 10^{8}-10^{10} times higher than those of the corresponding non-enzymatic reactions and this is far above the values that chemical catalysts are capable of achieving (Faber, 2000).

The polypeptide chain of enzyme is folded in such a way that the active site on the surface of the enzyme acts as a keyhole for a specific substrate which results in specificity property of the enzyme. Actually, there are four main classes of specificity:

1. *Absolute Specificity:* An enzyme exhibits absolute specificity when it acts on only one substrate and catalyses only one reaction
2. *Group Specificity:* Certain enzymes can act on a class of substrates that have a common functional group.
3. *Linkage Specificity:* Certain enzymes are specific for a particular type of chemical bonds.
4. *Steriospecificity:* Certain enzymes can discriminate the differences between the stereoisomer compounds (Scheve, 1984).

Enantioselectivity is the most important property that enzymes display. The synthesis of enantiomerically pure compounds are becoming increasingly more important in the production of pharmaceuticals, agrochemicals (e.g., pesticides, fungicides, herbicides), and flavors (Adam et. al., 1999). All the major biochemical events taking place in an organism are governed by enzymes. Since the majority of them are highly selective with respect to the chirality of substrate, it is obvious that the enantiomers of a given bioactive compound, such as pharmaceutical or an agrochemical, cause different biological effects. The effectiveness of a drug often depends on which enantiomer is used, as does the presence or absence of side effects. For instance, S-naproxen is an important anti-inflammatory drug, while R- enantiomer of naproxen is a liver toxin (Figure 1.1). Therefore, the development of efficient and environmentally acceptable processes for the preparation of enantiomerically pure compounds is essential. Consequently, enantiomerically pure substances can be produced in high enantiomeric excess by utilizing biocatalytic processes since enzymes are chiral materials (Faber, 2000; Hart, 1999).

Enzymes are now widely used for both the biotransformation and synthesis of natural products. Practically every known type of synthetic reaction finds its counterpart in enzyme mediated catalysis. Reactions that form carbon-carbon bonds are among these useful processes. Benzaldehyde lyase (BAL, EC 4.1.2.38) is such an enzyme, which catalyzes cleavage of the carbon-carbon bond of benzoin to form two benzaldehydes in steriospecific manner (Figure 1.2).

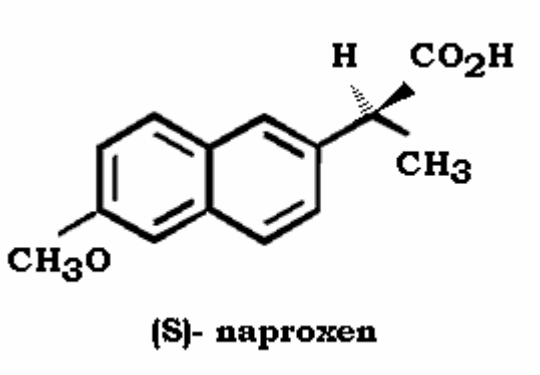


Figure 1.1 S- enantiomers of naproxen molecule.

The steriospecific activity and the thiamine-diphosphate (ThDP) dependency for catalytic activity of the enzyme were firstly reported by Gonzales and Vicuna (1989). In this study, the enzyme was extracted from *Pseudomonas fluorescens* Biovar I grown in the medium containing benzoin as the sole carbon source. Thereafter, Hinrichsen et. al. (1994) was determined the nucleotide sequence containing open reading frame encoding benzaldehyde lyase and cloned the gene to an *Escherichia coli* HB101 using vector pUC18. The sequence was later corrected and the substrate specificity was studied with modified pUC18::*bal* plasmid, where hexa histidine taq was inserted to the C-terminus of the mature gene fragment, by Pohl et. al. (2002). The reversibly catalytic activity of benzaldehyde lyase, where both benzoin and benzaldehyde were substrates, was, firstly, confirmed by Demir et. al. (2001). In this study, the enantioselectivity property of the enzyme, which catalyses both acyloin cleavage and condensation reactions, was proposed; and in further studies the substrate range of the enzyme was expanded (Demir et. al., 2002, 2003, 2004; Dünkelmann et. al., 2002; Sanchez et. al., 2003).

Recently, according to sequence alignments and homology models (Kneen et. al., 2005) and X-ray diffraction analysis (Mosbacher et. al., 2005), the residue similarities between benzaldehyde lyase and other THDP dependent enzymes, like benzoylformate decarboxylase (BFD) and pyruvate decarboxylase (PDC), were declared. Together with the detection of active center and reaction geometry, the homotetramer structure of BAL, having a molecular weight of 4 x 58919 Da., provided a significant knowledge about BAL where better approaches towards the substrate selection can be performed.

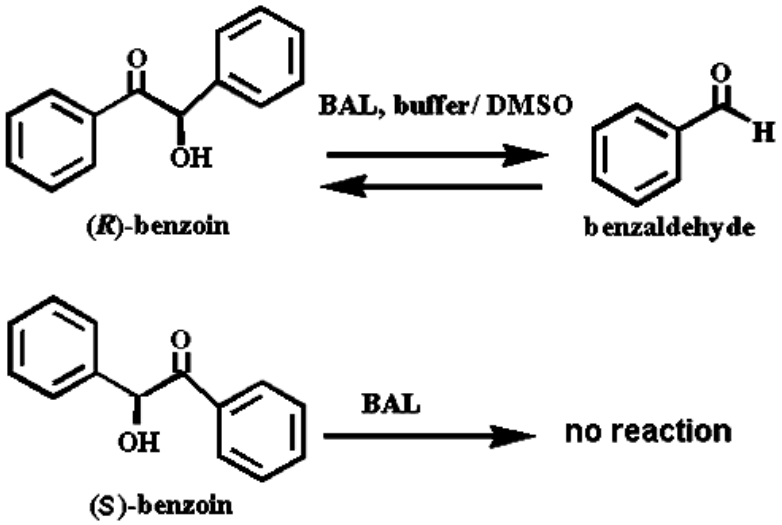


Figure 1.2 Conversion reaction of (R)- and (S)- enantiomers of benzoin molecule.

In a more recent study, Maria et. al. (2005) was investigated the parameters like effects of cofactors, cosolvents, and pH on the stability and the activity of the enzyme. Arranging a reaction medium containing 30% DMSO in the presence of potassium phosphate buffer with 0.5mM Mg^{+2}, 0.5 mM THDP and 1mM DTT at pH pf 9.5 resulted in the highest activity and stability of benzaldehyde lyase.

The effects of bioprocess operation parameters to yield the highest cell concentration and benzaldahyde lyase volumetric and specific activities were investigated by Çalık et. al. (2004, 2006); using the defined medium with glucose and $(NH_4)_2HPO_4$ as the sole carbon and nitrogen sources, respectively, the highest BAL activity was obtained at 0.5 vvm, 500min^{-1} as 860 U cm^{-3} with a cell concentration of 2.3 kg m^{-3} at uncontrolled pH of 7.2, using the recombinant *E. coli* K12 carrying pUC18::*bal* plasmid. In this expression system, the gene of *bal* was under the control *trc* promoter, which is a well known hybrid promoter.

In the current study, as a first part of the study, to maximize the yield and the productivity of benzaldehyde lyase, *E. coli* expression system was improved by cloning the gene of interest into pUC derived expression vector of pRSETA which was designed for high level expression of recombinant proteins by the presence of T7 promoter in *E. coli* as an alternative to modified pUC18::*bal*

plasmid where *trc* promoter was used for inducible production of benzaldehyde lyase; and as a potential producer, *E. coli* BL21 (DE3) pLySs strain was utilized, which was specifically designed for T7 regulated genes. In order to express the gene of interest, it is necessary to deliver T7 RNA polymerase which activates the T7 promoter, and leads to enhanced product delivery. Since the gene of T7 RNA polymerase is under the control of *lac*UV5 promoter and a small portion of *lacZ* gene in chromosomal DNA of *E. coli* BL21 (DE3) pLySs cells, isopropyl β-D-thiogalactoside (IPTG) addition is inducing the polymerase secretion. (http://www.invitrogen.com).

One more advantage of using T7 regulated expression together with *E. coli* BL21 (DE3) strains, derived from *E. coli* B, is its ability to reassimilate the acetate when glucose is depleted, leading to lower level of acetic acid accumulation; and hence, lower acetate inhibition on cell growth together with higher biomass yield (Luli et. al., 1990). Therefore, this microorganism was selected as a host in both batch (Choi et. al., 1997; Christensen et. al, 2002) and fed-batch (Shiloach et. al, 1996; Akesson et. al, 2001; Johnston et. al., 2003) cultivations. However, there is no systematic investigation related with metabolic response of *E. coli* BL21 (DE3) pLySs strain towards the effects of medium components utilized and oxygen transfer conditions in the literature. The only works reporting the effects of bioprocess operation parameters on benzaldehyde lyase production was reported by Çalık et. al. (2004, 2006); where *E. coli* K12 was the host microorganism. In general, the growth characteristics and acetate production level of several *E. coli* strains were compared in wild type (Luli et. al., 1990), and recombinant strains (Shiloach et. al., 1996; Choi et. al., 1997) in where the superiority of *E. coli* BL21 was shown.

In the other studies, where *E. coli* BL21 strain was utilized as host, the dissolved oxygen level was kept constant ranging between 10-30% by either changing the air flow rate (Luli et. al., 1990) or agitation rate in fed-batch processes with an automated control systems (Akesson et. al., 2001; Johnston et. al., 2003) without concerning about the effects of oxygen transfer conditions. Hence, in this study, it was aimed to investigate the effects of bioprocess parameters of medium components in the form of initial glucose and nitrogen sources and oxygen transfer conditions, systematically, on the metabolic response of recombinant *E. coli* BL21 (DE3) pLySs expressing BAL intracellularly.

In the second part of the study, the extracellular secretion of benzaldehyde lyase was aimed. For this, *Bacillus* species were selected as the host microorganisms. To date, production of heterologous proteins by bacteria was achieved using *E. coli* as the host. However, there are some occasions where *E.coli* is not the strain of choice and alternative hosts such as *B. subtilis* may become attractive. The advantages and the disadvantages of *E. coli* and *B. subtilis* as an expression system were summarized in Table 1.1.

Probably, the most attractive feature that *Bacillus* species serve is having a well developed extracellular secretory mechanism. Benzaldehyde lyase is being produced as an intracellular product in *E. coli* strains; whereas expression of the *bal* in *Bacillus* species may be accomplished as extracellular having an advantage of secretion of target proteins directly to the fermentation broth which leads to a natural separation of the product from cell components which is simplifying downstream processing of the protein. However, there are some limitations, as it can be seen in Table 1.1, for utilizing *Bacillus* species as expression system. The major drawbacks of *B. subtilis* as the host are that of having high protease activity and thus the plasmid instability. To overcome these problems, in the current study, it was decided to use a well developed expression system with a plasmid carrying strong, vegetative-phase promoter expressed in *Bacillus* species having low protease activity and/or in protease-deficient strains.

For this purpose, the signal sequence of serine alkaline protease from the chromosomal DNA of *Bacillus licheniformis* DSM 1969 (Çalık et. al., 2003-b) was PCR amplified and fused to the N-terminus the gene coding benzaldehyde lyase according to the gene splicing by overlap extension (SOE) method (Horton et. al., 1993). Signal sequences are known to be a precursor for protein exportation out of the cytoplasm. The gene encoding subtilisin Carlsberg from *Bacillus licheniformis* NCIB 6816 (*sub*C) was firstly reported by Jacobs et. al. (1985- Accession no: X03341); while the gene encoding mature protein of BAL was declared by Pohl, et. al., (2002- Accession no: AX349268). The fused product was cloned into *E. coli*/*B. subtilis* shuttle vector pRB374 (Brückner et. al., 1992) where *B. subtilis veg*II promoter, acting in vegetative phase of growth, is present and expressed in various *Bacillus* species like *B. firmus*, *B. brevis, B. sphaericus* and *B. subtilis apr*$^-$ and *npr*$^-$, *B. subtilis spo*$^-$ and multiple protease deficient strains of *B. subtilis* WB600 and WB700 (Wu et. al., 1991; Ye et. al., 1999).

Table 1.1 Advantages and disadvantages of *E. coli* and *B. subtilis* as a host microorganisms for heterologous protein production (Shuler and Kargı, 2002; Li et. al., 2004; Westers et. al., 2004; Simonen et. al., 1993).

Microorganism	Advantages	Disadvantages
E. coli	Physiology and its genetics are well known enabling manipulations.	It secretes protein into the periplasm and often into inclusion bodies.
	It grows rapidly and gives large yield	It is a pathogenic bacterium and has endotoxins.
	It has simple nutritional requirements.	Acetate production leading to growth inhibiton is high.
B. subtilis	It is considered as a GRAS organism	Proteolytic activity is high.
	There is no inclusion body formation.	It has a high product range leading to competition.
	It has a naturally high secretory capacity and exports proteins directly into the extracellular medium	Plasmid instability is present.
	It has an ability to utilize different substrate.	
	The genome has been fully mapped.	

In the literature, there is no work reporting extracellular delivery of benzaldehyde lyase. However, related with the other biomolecules, Wong et. al (1986-a&b) replaced subtilisin transcriptional regulatory sequence of *Bacillus subtilis* subtilisin with vegetative promoter in order to overcome the protease

problems; and using the constructed system production of TEM β-Lactamase was studied. In later studies, Wang et. al., (1988) was utilized human atrial natriuretic α-factor (hANF) under the control of tandem vegetative promoters of RNA polymerase σ^{43} operon; and finally, Lam et. al. (1998) facilitated the endoglucanase (Eng) and human epidermal growth factor (hEGF) production with *B. subtilis veg*I promoter. In attempting to improve the productivity *in B. subtilis*, efforts were mainly focused on developing protease deficient strains and improving transcriptional elements, especially promoters effective in vegetative phase where proteolytic activity is small.

Hence, in this study, the strategy of assembly of the expression system for intracellular production of BAL in *E. coli* BL21 (DE3) pLySs and extracellular secretion in *Bacillus* species and the production capacity of both recombinant *E. coli* and *Bacillus* species in terms of volumetric benzaldehyde lyase activity were discussed.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

2.1.1 General Characteristics

Enzymes are proteins that are capable of speeding up chemical reactions. The enzyme neither consumed, nor irreversibly altered during this processes. The cells in all living organisms carry out thousands of different chemical reactions. Most of these reactions would normally proceed very slowly without enzymes. Enzymes speed up these necessary reactions and thus make life processes possible (Scheve, 1984).

Enzyme specificity is thought to be a consequence of its elaborate three-dimensional conformation which allows formation of the active site responsible for the catalytic ability of the enzyme (Bailey, 1986). Enzymes of high or low specificity can be selected to suit the desired function. The specificity property reduces interference by undesirable substrates but minimizes the problems of unwanted by-products. By-product formation can be a costly inconvenience in industrial processing. The addition of another process step is always economically undesirable.

Most of the enzymes used on an industrial scale are derived from microbial sources. Enzymes isolated from microorganisms have impressive credentials as catalysts for the synthesis of industrial chemicals. Among the advantages derived from using microbial cells are: 1) potential for reduced catalyst cost; 2) increased enzyme stability; 3) ease of running multi-catalytic

processes; and 4) decreased time for catalyst production (Moses and Cape, 1991).

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

All enzymes are sensitive to pH. A variation in pH can affect the enzyme by altering the conformation of protein structure or ionizing the active site or the substrate (Atkinson and Mavituna, 1991). Enzymes have an optimum temperature at which they work fastest. Usually, enzymes lose activity at quite a low temperature, often slightly above that at which it is typically found. (Bailey, 1986). The temperature optima for enzymes usually lie between 37-47^{o}C, and pH optima range from acidic, i.e., 1.0, to alkaline, i.e., 10.5 (Kirk and Othmer, 1994).

2.1.2 Classification of Enzymes

Enzymes are classified according to a system that was established by the Commission on Enzymes of the International Union of Biochemistry (Atkinson and Mavituna, 1991). This enzyme commission assigned each enzyme a recommended name and four-part distinguishing number (Chaplin, 1990). In this system, enzymes are divided into six major classes according to the reaction types they catalyze. Each of the major classes is further divided into numerical subclasses and sub-subclasses according to the individual reactions involved. The forth number in the classification is the serial number of the enzyme within a subclass (Atkinson and Mavituna, 1991).

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

Table 2.1 International classification of enzymes.

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

2.1.3 Enzyme Activity

The amount of enzyme present or used in a process is difficult to determine in absolute terms (e.g. grams), as its purity is often low and a proportion may be in an inactive, or partially active, state. More relevant parameters are the activity of the enzyme preparation and the activities of any contaminating enzymes. The activity is a measure of enzyme content that is clearly of major interest when the enzyme is to be used in a process. For this reason, enzymes are usually marketed in terms of activity (Chaplin and Bucke, 1990), which was defined by the Commission on Enzymes:

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

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

2.2 Benzaldehyde Lyase

The synthesis of enantiomerically pure compounds are becoming more important in the production of pharmaceuticals, agrochemicals (e.g., pesticides, fungicides, herbicides), and flavors. Of the numerous methods developed in the past decades to obtain optically active compounds, enzymes had an increasing acceptance as a synthetic tool since optically active substances can be produced in high enantiomeric excess from racemic substrates by the help of chiral enzymes (Adam et. al., 1999). Benzaldehyde lyase (BAL, EC 4.1.2.38) is such an enzyme which is used for synthesis of enantio pure 2-hydroxyl ketones which are an important class of compounds in natural product and drug synthesis.

This enzyme catalyzes cleavage of the carbon-carbon bond of benzoin to form two benzaldehydes. BAL also catalyzes the reverse acyloin condensation of benzaldehydes resulting in the synthesis of (R)-benzoins. Only one enantiomer of benzoin acts as substrate when racemic mixture of benzoin is reacted with BAL. In other words, only (R) benzoin is converted into benzaldehyde through BAL catalysis, while (S)-benzoin gives no reaction at all. The catalytic mechanism of the enzyme is schematized in Figure 2.1 (Demir et. al., 2001).

Benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I, which can grow on benzoin as a sole carbon and energy source due to the benzaldehyde lyase activity, was firstly reported by Gonzales et al. (1989). In this study, the purification and the characterization of the enzyme were described and the ability of BAL to catalyze the cleavage of acyloin linkage of benzoin to form benzaldehyde irreversibly was described. It was also stated that benzaldehyde lyase requires THDP for catalytic activity which is maximum at 0.01 mM THDP, while concentrations higher than 0.5 mM were inhibitory. The activity loss was observed when treated with EDTA which can be restored by addition of 1.0 mM concentrations of $MgCl_2$, $MnSO_4$, or $CaSO_4$. The enzyme shows maximal activity between pH 7.5 and 8.5, whereas it is inactive below pH 6.0.

Thereafter, the nucleotide sequence contains open reading frame encoding benzaldehyde lyase and the location of the gene was determined (Hinrichsen et. al., 1994) by cloning the BAL gene into pUC18 plasmid and expressed in *E. coli* HB101.

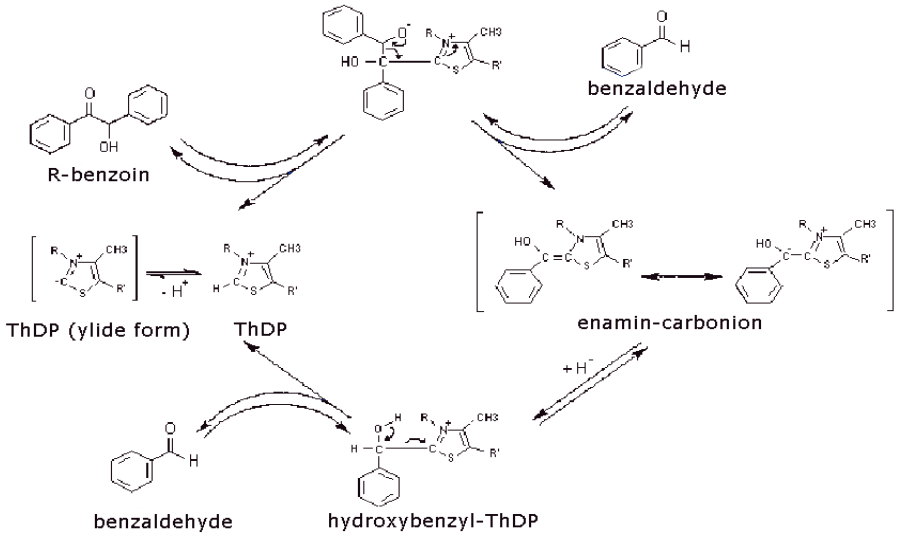


Figure 2.1 Benzaldehyde lyase catalyzed cleavage and synthesis of benzoin. The first step of the catalytic cycle is the attack of ylide form of ThDP on the carbonyl carbon of (R) - benzoin to produce an adduct. The enamine, intermediate product of ThDP dependent enzymes catalyzing the formation of 2-hyroxy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

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

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

hydroxymethylation of aromatic aldehydes with formaldehyde (Demir et. al., 2004) were investigated.

Recently, the enzyme structure has been modeled to show the residue similarities of THDP dependent enzymes like benzoylformate decarboxylase (BFD) and pyruvate decarboxylase (PDC). According to sequence alignments and homology models, the active site residues of BAL were identified and it is found that the glutamine residue, Gln113, present in the active site plays an important role wherein replacement of glutamine residue with alanine or histidine resulted 200 fold activity loss (Kneen et. al., 2005). Thereafter, the X-ray structure of benzaldehyde lyase was established in order to show that BAL belongs to a group of closely related THDP dependent enzymes. The results of X-ray diffraction exposed the active center and reaction geometry together with substrate specificity of benzaldehyde lyase which can later be used to expand the substrate range of the enzyme. It was also affirmed that BAL is a homotetramer, where each subunit binds to one THDP molecule using one Mg^{2+} ion. The enzyme is composed of 4 x 563 amino acid residues and has a molecular weight of 4 x 58919 Da. (Mosbacher et. al., 2005).

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

The studies in the literature reporting the effects of bioprocess operation parameters on benzaldehyde lyase production from *E. coli* K12 were published by Çalık et. al. (2004, 2006). The host microorganism having the highest benzaldehyde lyase productivity was determined as *E. coli* K12 (ATCC 10798)

carrying modified pUC18::*bal* plasmid where inducible hybrid t*rc* promoter is present. Among the investigated media, the highest cell concentration and benzaldehyde lyase activity were obtained as 1.8 kg m^{-3} and 745 U cm^{-3}, respectively, in the medium containing 8.0 kg m^{-3} glucose, 5.0 kg m^{-3} $(NH_4)_2HPO_4$ and the salt solution. Thereafter, the effects of uncontrolled-pH and controlled pH operations and effects of oxygen transfer conditions on benzaldehyde lyase productivity were systematically investigated using the recombinant *E. coli* K12 carrying pUC18::*bal* plasmid. Among the controlled-pH operations, the highest cell concentration and BAL activity were obtained as 2.1 kg m^{-3} and 775 U cm^{-3}, respectively at pH_C 7.0; whereas they were 2.3 kg m^{-3} and 860 U cm^{-3}, respectively at 0.5 vvm, 500 min^{-1} and uncontrolled pH 7.2 condition.

2.3 Genetic Engineering Techniques: Methodology

The techniques of genetic engineering, alternatively known as recombinant DNA technology, brought about a revolution in biotechnology science. Since the industrial enzymes has become more and more important, improving product formation or cellular properties to design and create the optimal biocatalysts through the modification in enzymatic reactions by using recombinant DNA technology lead to the development of genetic engineering techniques. Amplifying the specific DNA regions by Polymerase Chain Reaction (PCR) method, association of two DNA fragments side by side by gene splicing by overlap extension (SOE) method, determination of DNA concentration, SDS-Polyacrylamide analysis of secreted protein and restriction digestion are some basic principles that used in recombinant DNA technology.

2.3.1 Amplification of Specific DNA Region by Polymerase Chain Reaction (PCR) Method:

The PCR exploits certain features of DNA replication. In this method, oligonucleotide primers that are complimentary to opposite strands of short stretches of DNA are synthesized and added to the reaction mixture where heat-resistant DNA polymerase and a mixture of deoxyribonucleoside triphosphates are available (Figure 2.2, step 1). DNA polymerase uses single-stranded DNA as a template for the synthesis of the complimentary new strand. These single-stranded DNA templates can be produced by simply heating

double-stranded DNA to 95°C for two minutes (Figure 2.2, step 2). This breaks the hydrogen bonds. Then the mixture is rapidly cooled to allow the oligonucleotide primers to anneal to the complementary sequences in the DNA molecules (Figure 2.2, step 3). This annealing temperature is a key variable in determining the specificity of a PCR so temperatures and times used vary depending on the sequences to be amplified. Because the polymerase is heat-resistant, DNA polymerase enzyme extends the primers and completes the rest of the DNA strands after heating the reaction mixture to about 72° (Figure 2.2, step 4), the optimum temperature for *Taq* DNA polymerase (Glazer, 1995 and Watson, 1992).

The heat-stable enzyme commonly used is derived from a thermophilic Gram-negative eubacterium, *Thermus aquaticus* (Glazer, 1995). One of these early isolates *Taq* DNA polymerase and its derivatives have a 5' to 3' polymerization depended exonuclease activity. For nucleotide incorporation, the enzyme works best at 75-80°C, depending on the target sequence; its polymerase activity is reduced by a factor of 2 at 60°C and by a factor of 10 at 37°C (Sambrook and Russell, 2001).

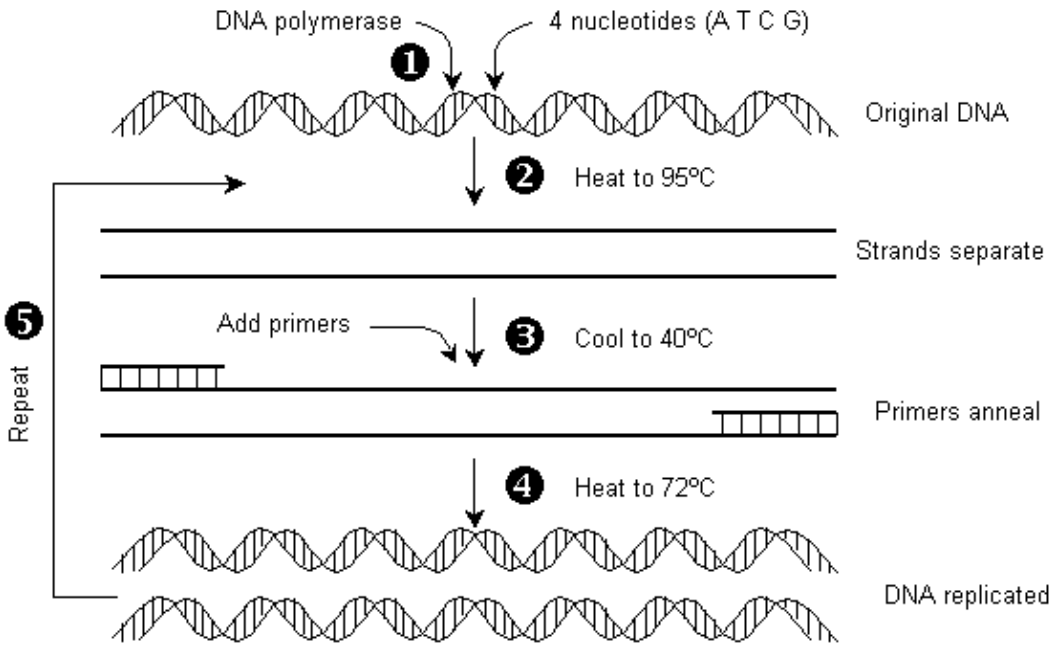


Figure 2.2 The Polymerase Chain Reaction (http://www.biologymad.com)

PCR can be completely automated, so in a few hours a tiny sample of DNA can be amplified millions of times with little effort (Figure 2.3). The net result of a PCR is that by the end of n cycles, the reaction contains a theoretical maximum of 2n double-stranded DNA molecules that are copies of the DNA sequence between the primers (Watson, 1992).

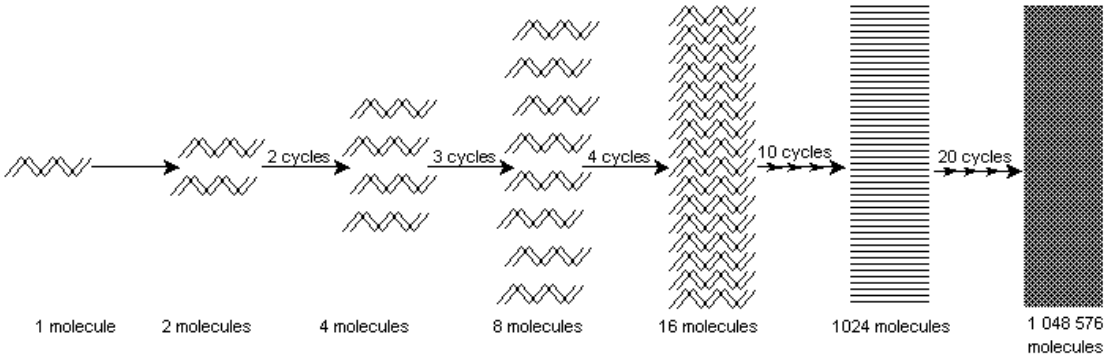


Figure 2.3 The Polymerase Chain Reaction - doubling DNA fragments at each cycle (http://www.biologymad.com)

2.3.2 Gene Splicing by Overlap Extension (SOE) Method:

Engineering of recombinant DNA molecules mostly depend on standard methods like cutting with proper restriction enzymes and ligating to rejoin the DNA fragments and requires specific nucleotide sequences to be recognized by restriction enzymes. Gene splicing by overlap extension method basing on the methodology of recombination of fragments from the genes in separate polymerase chain reaction was firstly reported by Horton et. al (1989) and Ho et. al. (1989). This method provides to join of DNA fragments without any need for the restriction enzymes and is especially very useful where precise recombination in frame as in the case of creating fusion proteins is required. Apart from recombination, specific alterations in the sequences can be performed which allows site directed mutagenesis to be performed simultaneously.

The basic scheme of gene splicing by overlap extension is illustrated in Figure 2.4. First, two PCR products are made in separate reactions; primers a and b produce product AB from gene I and primers c and d are used to amplify fragment CD from gene II. DNA segments are depicted as paired anti-parallel

strands. Primers b and c have had sequences added to their 5’ ends so that the right end of AB matches the sequence at the left end of the CD. When these products are mixed in an SOE reaction, the top strand of AB overlaps with the bottom strand of CD, their 3’ ends being oriented toward each other. This allows them to act as primers on one another to make a recombinant product. The other strands, which point in the wrong directions, do not form product and are not necessary to the reaction (Horton et. al, 1993).

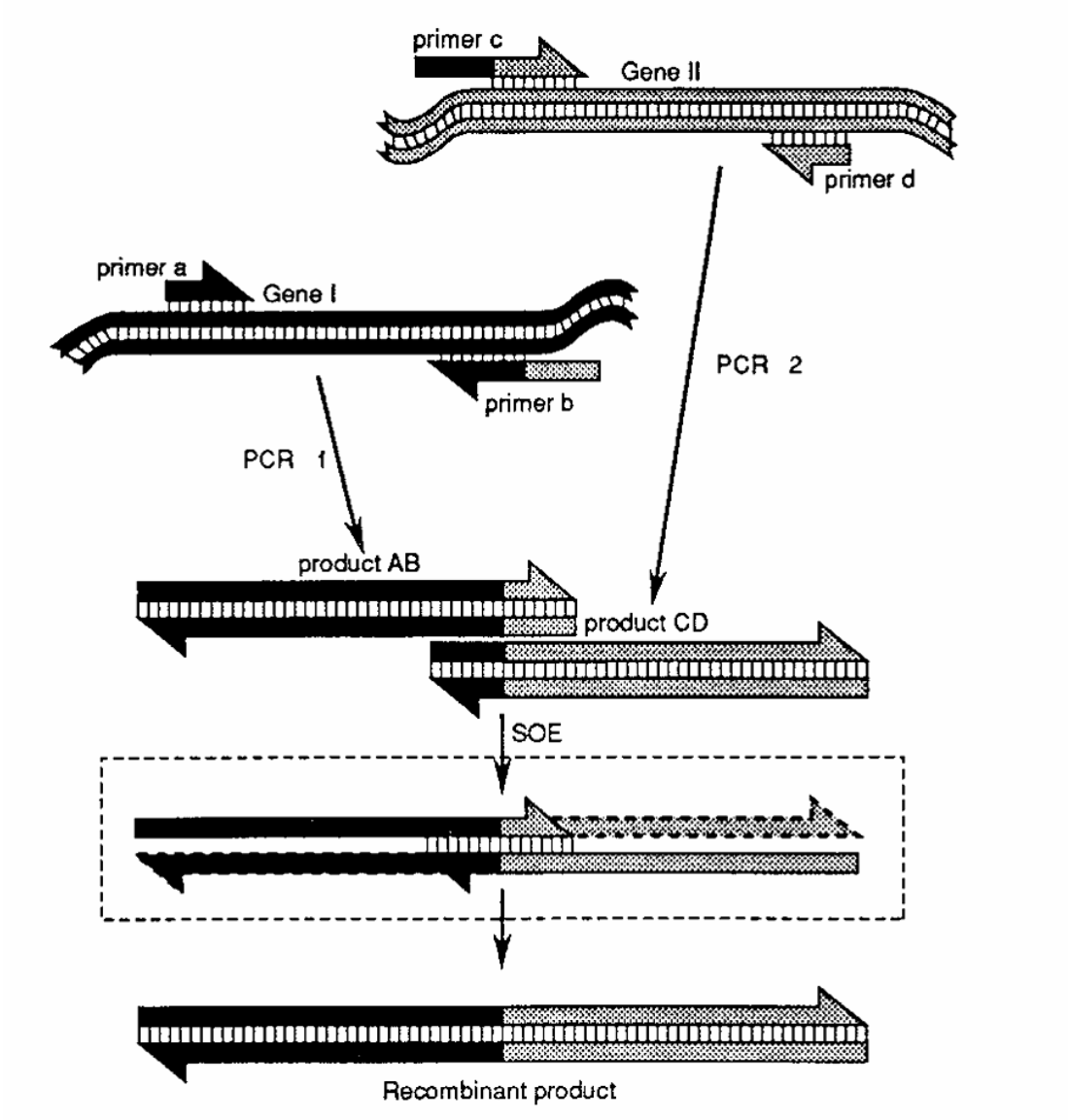


Figure 2.4 Schematic diagram of SOE method (Horton et. al, 1993).

2.3.3 Determination of DNA Concentration

The concentration of DNA can be determined by both gel electrophoresis method or UV spectrophotometer measurements.

DNA molecules are highly negatively charged because of the phosphate ions they contain, so DNA is attracted towards the positive anode when electric current is passed through the gel. However, the polysaccharide matrix of the gel retards the DNA by a process of sieving, so that small fragments move through faster and thus fragments separate according to size. The concentration of agarose ranges between 0.7-1.2% according to the size of the DNA fragments. After loading samples into the wells, electric is applied. At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, higher voltages than 125 volt lead to evaporation in electrophoresis buffer and can damage the gel structure. The different DNA fragments can be visualized by staining with ethidium bromide. This dye binds to the DNA and fluoresces under ultraviolet radiation (Smith and Wood, 1991). Loading the marker in which the weights of each band is known, into the first well, the length of the DNA fragments of interest can be determined (Appendix J).

2.3.4 SDS-Polyacrylamide Gel Electrophoresis of Proteins

Almost all analytical electrophoreses of proteins are carried out in polyacrylamide gels under conditions that ensure dissociation of proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strong anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded onto the gel. The denaturated polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polacrylamide gels in accordance with the size of polypeptide. Unlabeled proteins separated by polyacrylamide gel electrophoresis typically are detected by staining, either with Coomassie Brilliant Blue or with silver salts. In a relatively rapid and straightforward reactions, Coomassie Brilliant Blue binds nonspecifically to proteins but not gel, thereby allowing visualization of proteins as discreet blue bands within the

translucent matrix of the gel. Silver staining, although somewhat more difficult to perform, is significantly more sensitive. The use of silver staining allows detection of proteins resolved by gel electrophoresis at a concentrations nearly 100 fold lower than those detected by Coomassie Brilliant Blue staining (Sambrook, 2001).

2.3.5 Restriction Enzymes

These enzymes serve the bacteria in which they occur as protection from foreign DNA. The enzymes cut foreign DNA from outside the helix, but do not digest the host DNA due to characteristic methylation patterns on the DNA (Scragg, 1988).

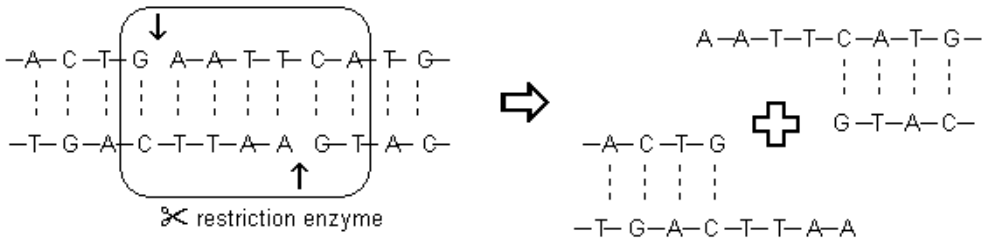


Figure 2.5 The restriction enzyme *Eco*RI and its schematic representation of cleavage of DNA molecule (http://www.biologymad.com).

Restriction enzymes are of several types; the most useful for cloning, the Type II restriction enzymes recognize specific sequences, usually 4-8 bp in length, and cut DNA molecules within these sequences (Kirk and Othmer, 1994). For example the restriction enzyme called *Eco*RI recognizes the double-stranded six-nucleotide sequence of GAATTC and cleavages each strand between the G and A residues as marked in Figure 2.5 by arrows. Cleavage of the DNA at this site produces complimentary single-stranded tails called as sticky ends which can later anneal with complementary single-stranded tails on other DNA fragments. Some restriction enzymes cut straight across both chains to form blunt ends.

The restriction enzymes used in this study and their recognition sequences are listed in Table 2.2.

Table 2.2 Recognition sites and cleavage points of restriction enzymes

Enzyme	Target site
*BamH*I	G^GATCC
*Sac*I	GAGCT^C
*Nde*I	CA^TATG
*Pst*I	CTGCA^G

2.3.6 Cloning and Expression Vectors

Plasmids are circular, double-stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA. These extrachromosomal DNAs exist in a parasitic or symbiotic relationship with their host cell and like the host-cell chromosomal DNA, plasmid DNA is duplicated before every cell division. Plasmids most commonly used in recombinant DNA technology are those that are engineered to optimize their use as vectors in DNA cloning have three common region essential for DNA cloning (Figure 2.6): a replication origin; a marker that permits selection, usually a drug resistance gene; and a region in which exogenous DNA fragments can be inserted (Lodish, 2004).

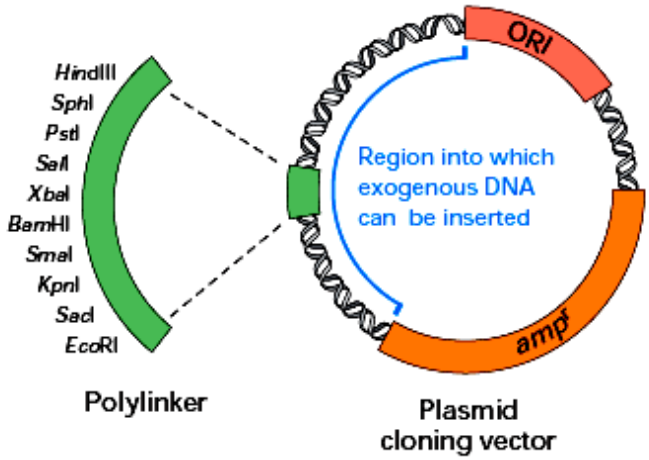


Figure 2.6 Basic components of a plasmid cloning vector that can replicate within an *E. coli* cell (Lodish, 2004)

pUC series vectors are primarily designed for general cloning and sequencing. They can be used as a sub-cloning vector or can also be used for expression purposes.

In previous studies, the gene encoding benzaldehyde lyase enzyme was purified and cloned to pUC18 cloning vector (Hinrichsen et. al., 1994) and the enantioselective synthesis ability of BAL and the bioprocess operation parameters were investigated with various *Escherichia coli* strain using recombinant pUC18::*bal* plasmid (Demir et al., 2001, 2002, 2003 and Çalık et. al., 2004, 2006). The *bal* gene in this modified pUC18::*bal* plasmid was under the control of hybrid *trc* promoter (Figure 2.7) and expressed in different *E. coli* strains. In this study, the pUC19 cloning vector (Figure 2.8) was used for application of sub-cloning for extracellular secretion of the enzyme.

A series of expression vectors are available that are designed to reach the high-level expression of the foreign gene in *E. coli*. One of these genetically engineered expression vector is pRSETA (Figure 2.9). This plasmid has several useful features as a vector such as having T7 promoter to control the expression of the gene of interest; ribosome binding site which has optimum space from the multiple cloning site for efficient translation; multiple cloning site containing 11 restriction enzyme recognition sequence; T7 terminator permitting efficient transcription termination; f1 origin to allow single strand rescue; ampicillin resistant gene; and pUC origin to provide high copy replication and growth in *E. coli* (http://www.invitrogen.com).

For the gene cloning in *Bacillus* species, among the constructed vectors, *E. coli*/*B. subtilis* shuttle vector pRB374 was selected (Figure 2.10). In this designed vector, eleven unique restriction sites flanked by two transcriptional terminators, ampicillin resistant gene for selection in *E. coli* and kanamycin resistant gene for selection in *B. subtilis* are available. The genes cloned into pRB374 from the multiple cloning site are under the control of *B. subtilis veg*II promoter which can initiate transcription in both *B. subtilis* and *E. coli* (Brückner et. al., 1992).

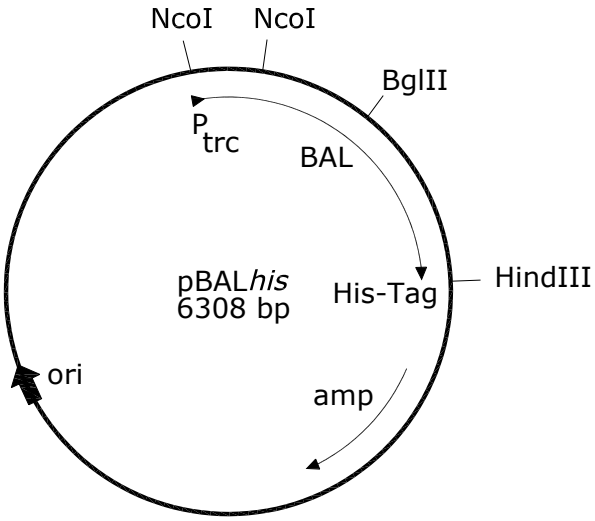


Figure 2.7 Modified pUC18::*bal* plasmid (Pohl et. al., 2002)

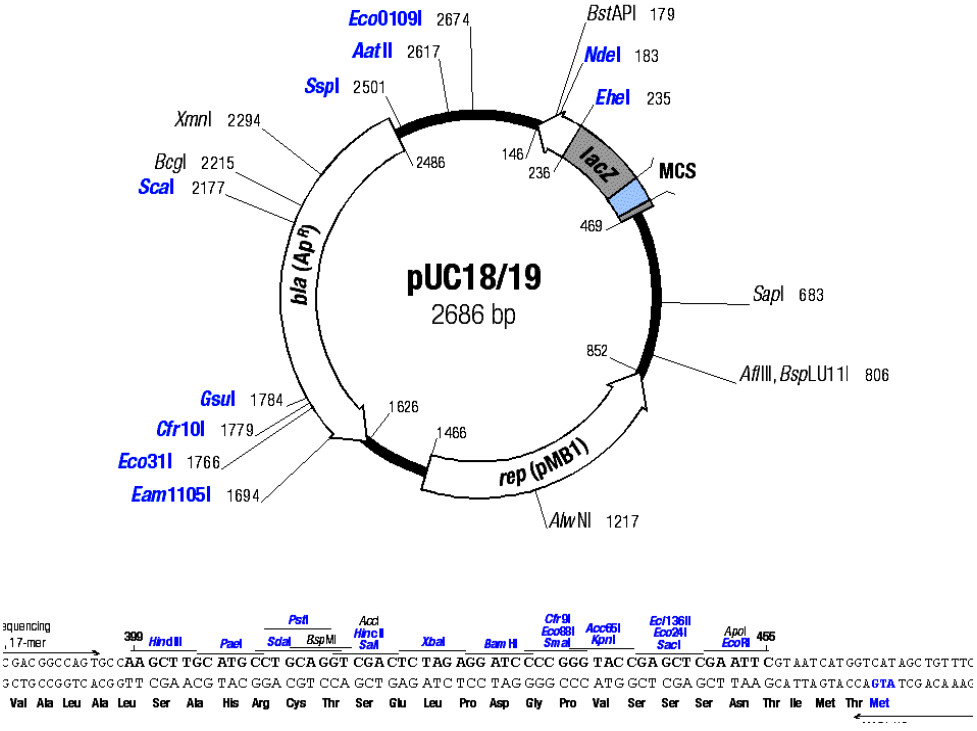


Figure 2.8 pUC18/19 cloning vector (http://www.fermentas.com)

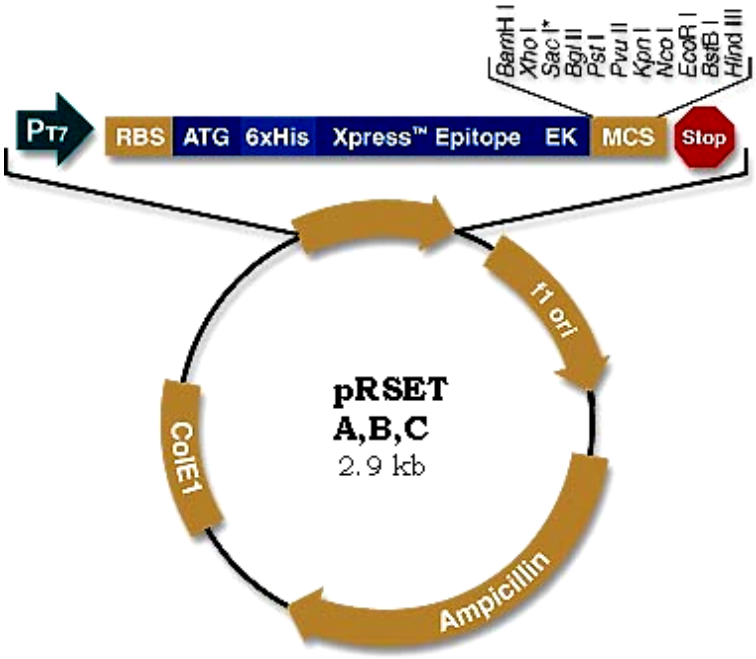


Figure 2.9 pRSET A,B,C expression vectors (http://www.ivitrogen.com).

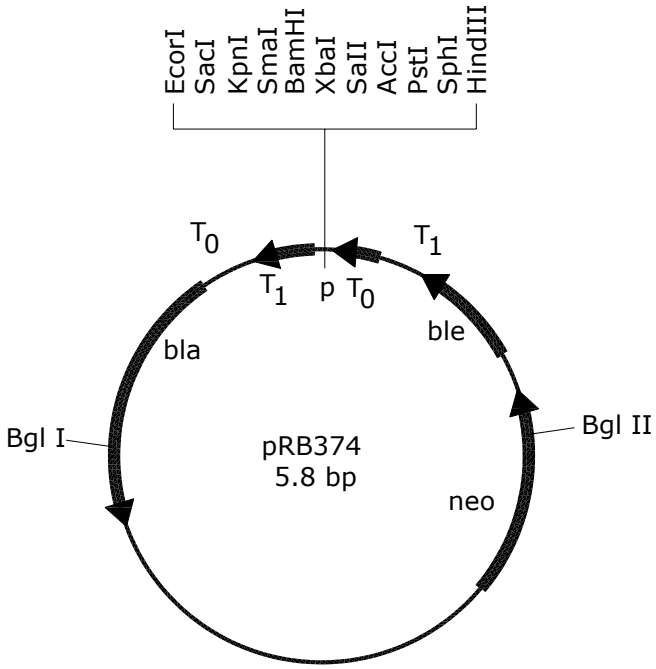


Figure 2.10 pRB374 expression vector (Brückner et. al., 1992).

2.3.7 Ligation Reaction and Transformation

The DNA fragment of interest cut by a restriction enzyme leads to single stranded tails, sticky ends, which have a tendency to anneal with the complementary strand present in the reaction mixture. The addition of vector DNA cut open by the same restriction enzyme results in the annealing of the foreign DNA to the complementary ends of the cut vector. The phophodiester bonds missing between the attached strands (Figure 2.11- indicated by arrows) is covalently bond by DNA ligase. This enzyme catalyzes the condensation of 3'-hyroxyl group with a 5'-phosphate group to add the missing links.

The ligation reaction is the rate limiting step in genetic engineering techniques since this reaction requires the cohesive ends of foreign DNA and open plasmid DNA to attach in correct orientation and anneal while preventing the relegation of opened vector DNA. Therefore, optimum ligation reaction conditions should be determined by both paying attention to foreign DNA and plasmid DNA concentrations. Reclosure of the vector can be minimized by treating the opened vector with phosphatase (Çalık et. al., 1998; Bailey, 1986 and Glazer, 1995).

After ligation, the mixture bearing the desired vector-donor combinations is then moved into the recipient or host cell. In most cases this is done by transformation (Schuler and Kargı, 2002).

There are four different methods for direct introduction by transformation:

1. *Natural transformation:* Foreign DNA is taken up by the bacteria and fused to the chromosomal DNA of the organism. The genetic and physiology of naturally transformable species are not well know, however, with the exceptions of *Bacillus subtilis*.
2. *Artificial transformation:* The most preferable method for *E. coli* cells is to convert the cells into a competent state by resuspension in buffer solutions containing very high concentrations of $CaCl_2$ at 0^oC. The effect of Ca^{+2} on a membrane bilayer with a high content of acidic lipids is to freeze the hydrocarbon interior, presumably by binding tightly to the negatively charged head groups of lipids.

Because the outer membrane of Gram-negative bacteria such as *E. coli* contains a large numbers of acidic groups at a very high density, this membrane becomes frozen and brittle, with cracks through which macromolecules, including DNA, can pass. After DNA is added to the suspension, the cells are heated to 42^{o}C and then chilled. Under these conditions, cells have been found to take a pieces of DNA through the cytoplasmic membrane.

3. *Protoplast transformation:* Enzymes are used to hydrolyze the rigid cell wall to convert the cell into protoplast bounded by the cytoplasmic membrane.
4. *Electroporation:* Applying short electrical pulses of very high voltage is believed to reorient asymmetric membrane components that carry charged groups, thus creating transient holes in the membrane. DNA fragments can then enter through these openings, presumably by spontaneous diffusion (Glazer, 1995).

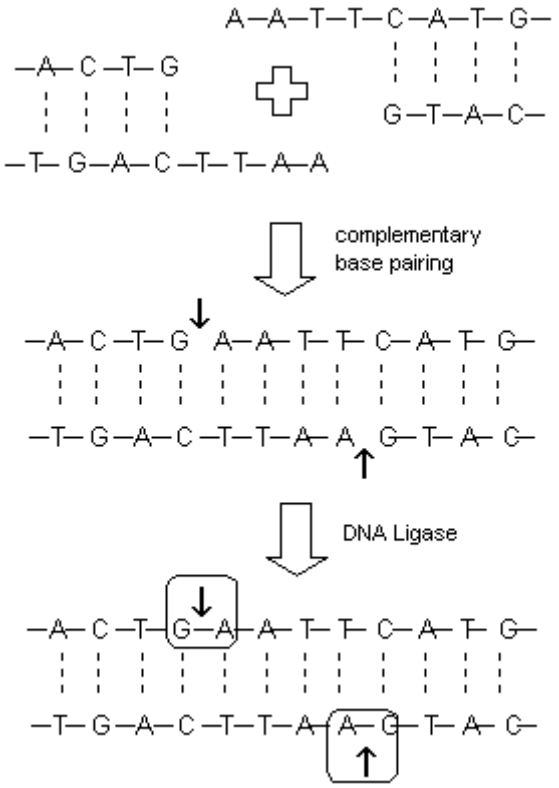


Figure 2.11 Mechanism of ligation reaction (http://www.biologymad.com)

2.3.8 Selection and Screening of Recombinant Plasmids

After transformation, it is important to note that construction of the desired vector-donor DNA usually results in a mixture including some opened or rejoined (without donor DNA) vector molecules, or insertion of DNA contaminants of donor DNA into the vector. Consequently, an efficient method to screen transformants for those with the desired vector-donor DNA combination is important (Schuler and Kargı, 2002).

Antibiotic resistance is most commonly used selection method because it allows for an extended host range and provides more flexibility in growth conditions (Smith, 1995). After transformation, cells possibly carrying the recombinant plasmid with the antibiotic resistance gene, are spread on plates containing a particular antibiotic to select the antibiotic resistant cells. Not all of cells growing on the plates with specific antibiotic carry the desired vector-donor DNA combination. Candidates are further grown and the plasmids they carry are isolated and cut with proper the restriction enzymes to check whether they carry the correct combination.

Insertion of a foreign gene piece of DNA into the vector could be detected by the inability of the cells containing the plasmid with the gene encoding the enzyme β-galactosidase, which can be screened on plates by a color assay. Restriction enzyme recognition sequences in the polylinker region is situated within the fragment of *the E. coli LacZ* gene (*lacZ'*). When the *E. coli* are grown on agar plates containing a colorless compound called X-GAL, cleavage of the X-GAL by the enzyme produces an insoluble blue product. Thus, these *E. coli* colonies with plasmids that do not have an insert of foreign DNA are blue while plasmids with an insertion of foreign DNA yields white colonies because of the shifts in the *lacZ* reading frame which causes β-galactosidase not to be produced (Watson, 1992). By this way, new recombinant cells can be selected. This process is called as blue-white screening.

The expression plasmid, pRB374, carries both kanamycin and ampicillin resistance gene for selection of correct transformants in *Bacillus* species and *E. coli* strains, respectively, from the culture.

Both the cloning vector, pUC19, and expression vector, pRSETA, provide ampicillin resistance for selection. The multiple cloning region of pUC19 vector lies on the *lacZ* gene fragment for screening the recombinants by blue/white colony screening.

2.3.9 Gene Expression and Control Mechanisms

It is important to obtain a good expression from the cloned gene. In bacteria, there exists a control mechanism of gene expression (transcription signals) and their respective proteins (translational signals) (Figure 2.12).

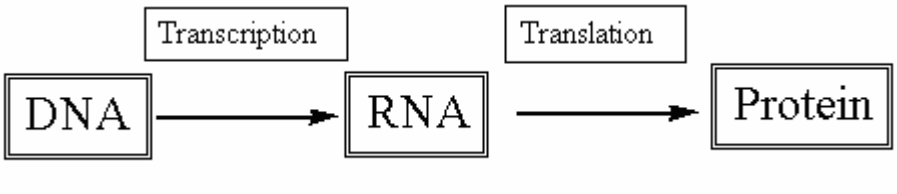


Figure 2.12 Gene expression mechanism.

2.3.9.1 Transcriptional Signals

The genetic analysis of induced enzymes of bacteria provided important information in understanding of mRNA synthesis regulation. mRNA synthesis level is determined by the signals from outside that transmitted by some compounds called inducers. For initiation of transcription, RNA polymerase, which synthesizes the mRNA using DNA molecule as a template should bind to the promoter. Therefore, promoters are the start signals for RNA synthesis (Watson,1992).

2.3.9.1.1 Promoters

The most highly utilized promoters for over-expression have two features in common: they are 1) efficient and 2) repressible. Repressible promoters are superior to unregulated promoters because continuous expression of plasmid-encoded proteins generally puts the host at a selective disadvantage in a

population where some cells have lost the plasmid or acquire deletions and other mutations.

In this study the vector, pRSETA vector, carrying bacteriophage T7 promoter were selected as an alternative to the modified pUC18::*bal* plasmid having *trc* promoter used in previous studies (Demir et. al., 2001, 2002, 2003, 2004; Çalık et. al., 2004, 2006). The *trc* promoter contains the -35 region of the *trp* promoter together with the -10 region of the *lac* promoter (Brosius *et al.*, 1985; Egon *et al.*, 1983; Mulligan *et al.*, 1985). When expression is desired, the *E. coli* are grown to mid-log phase and IPTG (isopropyl-β-D-thiogalactoside) is added to induce expression. Although, it is hard to determine which promoter is the best, the T7 system has become very popular and appears to have become the promoter of choice for high level expression (Smith, 1995). Unlike *trc* promoter, bacteriophage T7 promoter, selected as an alternative, requires T7 RNA polymerase to be activated. Several advantages accompany this system are 1) transcription is completely selective (T7 RNA polymerase will not recognize the host's promoter); 2) T7 RNA polymerase elongates mRNA chains five times faster than *E. coli* RNA polymerase, and the accumulation of the message rises rapidly; 3) Expression of the cloned gene is completely activatable and a repressor is not required.

T7 RNA polymerase can be delivered by induction of a chromosomal copy of the gene. In the former approach, the host strain is a lysogen that carries a copy of T7 RNA polymerase gene on the prophage, along with *lacI*. The T7 RNA polymerase gene is under the control of the *lac*UV5 promoter and repressed until isopropyl β-D-thiogalactosidase IPTG is added to the growth medium. Once sufficient T7 RNA polymerase is produced, it binds to the T7 promoter and transcribes the gene of interest (Smith, 1995).

For extracellular expression of the desired gene in *Bacillus* species, pRB374 expression vector containing *B. subtilis* vegetative promoter, *veg*II, promoter (Brückner et. al., 1992) was selected to encounter the problems mostly appear when heterologous protein are secreted, like proteolytic degradation. The *veg* gene of *Bacillus subtilis* is a useful tool for exploration of the mechanism of transcription, since it has a promoter that is utilized very efficiently in vitro by the major vegetative form of *B. subtilis* RNA polymerase (σ43) (LeGrice et. al., 1986). Therefore, promoters acting efficiently in

vegetative phase are optimal to decrease the proteases secreted to the growth medium. A large number of both prokaryotic and eukaryotic genes have been fused under vegetative promoter and expressed successfully. In the study performed by Wong et. al (1986-a) subtilisin transcriptional regulatory sequence of *Bacillus subtilis* subtilisin was replaced with vegetative promoter, obtained from *B. subtilis* chromosomal DNA, to allow vegetative expression of the *B. subtilis* subtilisin. Thereafter, using the same transcriptional regulatory element and signal peptide sequence, TEM β-Lactamase was produced from the early stages of growth (Wong et. al., 1986-b). In the same manner, using the promoters (tandem vegetative promoters of RNA polymerase σ^{43} operon) active during vegetative growth, human atrial natriuretic α-factor (hANF) production (Wang et. al., 1988) and endoglucanase (Eng) and human epidermal growth factor (hEGF) productions under the control of *B. subtilis veg*I (which represents the RNA polymerase binding site I of the *veg* promoter complex) promoter (Lam et. al., 1998) were successfully achieved.

The transcription system of σ^{43} RNA polymerase from *Bacillus subtilis*, *veg* promoter, was found to initiate transcription in both *B. subtilis* and *E. coli* indicating a closer evolutionary relationship of the expression machinery of these two bacterial species (Peschke et. al., 1985). Therefore, it was stated that genes can be expressed in both hosts provided their ribosomal binding sites (Brückner et. al., 1992).

2.3.9.1.2 Terminators

In the expression systems, it is important to utilize a strong promoter with the terminator behind the expressed gene. Placement of a strong terminator at the 3' end of the gene results in greater stability of the plasmid and greater expression of the recombinant gene. In the absence of the terminator, transcription can proceed into the plasmid, causing over-expression of other genes carried on the vector, potentially causing harm to cell (Smith, 1995).

The expression vector pRSETA contains T7 terminator region for efficient transcription ending (http://www.invitrogen.com), while the shuttle vector for *E. coli* and *B. subtilis*, pRB374, which was derived from pRB273, contains additional transcriptional terminator, T1 of *E. coli rrnB* to improve the versatility (Brückner et. al, 1992).

2.3.9.2 Translational Signals

Translational control which involves several steps affecting the regulation mechanism is the second means of controlling protein synthesis. Expression can be improved at the translational level by the development in the characteristics such as the ribosomal binding site, spacer region between the Shine-Dalgarno sequence and the initiation codon (Smith, 1995). The pRSET vectors are designed properly in where ribosomal binding site (RBS) sequence is optimally spaced from the multiple cloning site for efficient translation of the gene of interest. pRB374 shuttle vector, however, requires genes providing their own RBSs.

2.3.9.3 Protein Secretion in *Bacillus* Species

Among the features that *Bacilli* serve, the heterologous protein secretion to the growth medium is the most attractive feature that made the microorganism so popular for the applications in both industry and research (Smith, 1995).

Although *Bacillus* species, especially *Bacillus subtilis,* is an attractive host for extracellular protein secretion, there are some bottlenecks in the secretory pathway of proteins. The secretory pathway of proteins can be divided into three functional stages: the early stage, involving synthesis of secretory pre-proteins, their interaction with chaperones and binding to the secretory translocase; the second stage, translocation across the cytoplasmic membrane; and the last stage, including removal of the signal peptide, protein refolding and passage through the cell wall. To overcome the bottlenecks through the protein secretion, most of the attempts are concentrated on the construction of *B. subtilis* strains with reduced protease activity. After developing multiply deficient extracellular proteases, WB600 and WB700, protease activity in the medium was reported to be 0.3 % and 0.1% of wild type level, respectively. Thereafter WB800 was generated by inactivating the eighth extracellular protease, WprA (cell wall associated protease) from the chromosomal DNA of WB700 (Li et. al., 2004).

The proteins secreted are synthesized as precursors with an amino terminal extension, which contains the signal sequence or signal peptide. This

peptide distinguishes the exported proteins from the cytoplasmic ones and is needed for targeting of the proteins to the export pathway (Simonen et. al., 1993).

The primary structures of amino-terminal signal peptides show similar three distinct domains. The amino-terminal N-domain of signal peptides contains at least one arginine or lysine residue and suggests interacting with the translocation machinery and negatively charged phospholipids in the lipid bilayer of the membrane during translocation. The H-domain, following the N-domain, is formed by a stretch of hydrophobic residues that seem to adopt an α-helical conformation in the membrane. Helix-breaking glycine or proline residues are frequently present in the middle of this hydrophobic core. The latter residues might allow the signal peptide to form a hairpin-like structure that can insert into the membrane. Helix-breaking residues found at the end of the H-domain, are thought to facilitate cleavage by a specific signal peptidase (SPase). The C-domain, following the H-domain, contains the cleavage site for SPase, which removes the signal peptide from the mature part of the secreted protein during or shortly after translocation. The mature part of the protein is thereby released from the membrane and can fold into its native conformation. Finally, the signal peptide is degraded by signal peptide peptidases (SPases) and removed from the membrane (Figure 2.13). Although different amino-terminal signal peptides tend to be quite similar in general structure, apparently small differences between individual signal peptides can cause cleavage by different SPases, export via different pathways, and transport to different destinations (Tjalsma et. al., 2000).

Ala-X-Ala is the most commonly observed signal peptidase cleavage sequence in *B. subtilis*. In this group of SPase recognition sequence, small aliphatic residues, often alanine, are preferred at positions -1 and -3 relative to the cleavage site in pre-proteins. For the -1 position, alanine, glysine and serine residues, the -3 position can also accommodate larger residues, such as valine, threonine, leucine and isoleucine (Figure 2.14). Especially, an alanine residue at the -1 position relative to the cleavage site seems to be a critical determinant for SPase I-mediated cleavage of pre-proteins in *B. subtilis* (van Roosmalen et. al., 2004).

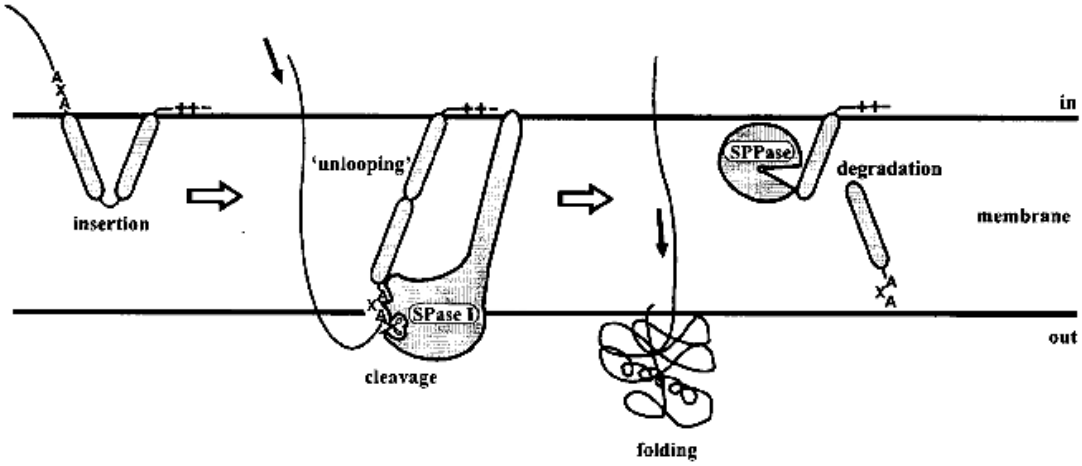


Figure 2.13 Schematic diagram of signal peptide insertion into the cytoplasmic membrane and cleavage by SPase I (Tjalsma et. al, 2000).

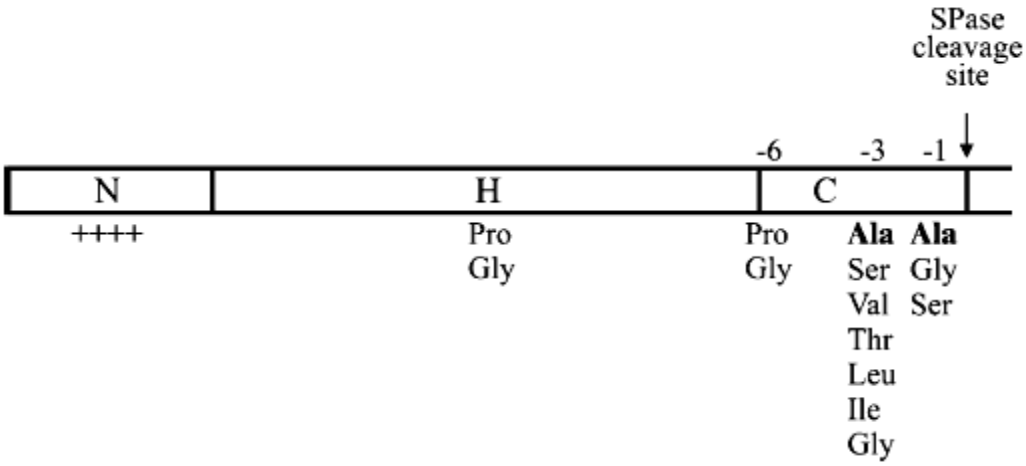


Figure 2.14 Secretory (Sec-type) signal Peptides: positively charged N-terminus (N-region), a central hydrophobic region (H-region), and a polar C-terminal region (C-region) (van Roosmalen et. al.,2004)

Signal peptidases can be a limiting factor for processing of certain precursor proteins (Li et. al., 2004). In attempts to make *Bacillus* species secrete foreign proteins, the joint between *Bacillus* signal peptide and foreign protein is usually made immediately after the signal peptide since extra

residues between the mature portion and signal sequence may affect the stability or activity of the protein (Simonen et. al., 1993)

2.3.9.3.1 Signal Peptide of *B. licheniformis*, *pre-sub*C

The gene encoding subtilisin Carlsberg from *Bacillus licheniformis* NCIB 6816 (*sub*C) was firstly reported by Jacobs et. al. (1985- Accession no: X03341). In this study, the entire nucleotide sequence of the coding sequence as well as 5' and 3' flanking sequences were determined. It was declared that the deduced amino acid sequence reveals an N-terminal signal peptide consisting of 29 residues, a pro-peptide of 76 residues followed by the mature protein comprising 274 residues. Later, the endogenous promoter activity of *sub*C was identified and the 5' and 3' ends of *sub*C transcripts were characterized (Jacobs et. al., 1995). The putative signal sequence of subtilisin Carlsberg has similar properties to other signal sequences belong to the secretory (Sec-type) signal peptides. Jacobs et. al. (1985) has reported that the cleavage site of subC follows the (-3, -1) rule, giving cleavage preferentially after the residues Ala-X-Ala. Based on this rule and other structural considerations, it was predicted that the signal peptidase processing occurs after the residues Ala-Ser-Ala as shown in Table 2.3.

In a resent study published by Westers et. al. (2006), establishment of production system strategy was applied by testing two types of promoters which are P43 and nap promoters; three types of signal peptides which are modified AmyL (Lat, *B. licheniformis* α-amylase) , Pel and SacB; and four different protease deficient strains. Among the investigated strategies nap promoter in combination with Pel signal gave the highest production of human interleukin-3. however, since plasmid instability was observed, second best combination of P43 promoter and *lat* signal sequence was selected. As the host microorganisms, DB104, WB600, WB700 and WB800 were tested and eight-protease deficient strain WB800 resulted in the highest production.

There is no significant evidence for the function of pro-peptide. In the studies using the signal sequence for heterologous protein secretion in *B. subtilis*, only the pre-peptide region is used (Wong et. al., 1986-a&b; Wang et. al., 1988). In the current study, the signal peptide (*pre-sub*C) with its own

ribosomal binding site was used for extracellular production of benzaldehyde lyase.

Table 2.3 Signal peptides of *Bacillus* species. Signal peptides were identified as underlined text, positively charged lysine (K) and arginine (R) residues in the N-domain are indicated in bold letters; the hydrophobic H-domain is indicated in gray shading; and signal peptide cleavage site is indicated by arrows (Tjalsma et. al., 2000; Simonen et. al., 1993)

Protein	Species of origin	Signal Peptide
α-Amylase	*B. subtilis*	MFA**KR**F**K**TSLLPLFAGFLLLFHLVLAGPAA**ASA**↓
α-Amylase	*B. amyloliquefaciens*	MIQ**KRKR**TVSFRLVLMCTLLFVSLPITK**TSA**↓
α-Amylase	*B. licheniformis*	M**K**QH**KR**LYARLLPLLFALIFLLPHSA**AAA**↓
Extracellular protease	*B. subtilis*	M**K**NMSC**K**LVVSVTLFFSFLTIGPL**AHA**↓
Subtilisin E	*B. subtilis*	M**R**S**KK**LWISLLFALTLIFTMAFSNMS**VQA**↓
Subtilisin	*B. amyloliquefaciens*	MRG**KK**VWISLLFALALIFTMAFGSTSS**AQA**↓
Subtilisin Carlsberg	***B. licheniformis***	MM**RKK**SFWLGMLTAFMLVFTMAFSDS**ASA**↓
Pectate lyase (Pel)	*B. subtilis*	M**KK**VMLATALFLGLTPAG**ANA**↓
Levansucrase (SacB)	*B. subtilis*	MNI**KK**FA**K**QATVLTFTTALLAGGATQ**AFA**↓

2.4 Bioprocess Parameters in Enzyme Production

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

In industrial context, the goal is to design and create of optimal system to maximize the yield and productivity of desired products. For this, either genetic manipulation to improve the expression of the product should be performed or the operation conditions of fermentation broth should be optimized. In aerobic bioprocesses, there are some important criteria:

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

2.4.1 Microorganism

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

Benzaldehyde lyase is naturally produced by wild-type *Pseudomonas fluorescens* (Gonzales et. al., 1989). The gene encoding benzaldehyde lyase was firstly cloned to *Escherichia coli* strain by Hinrichsen et. al. (1994), and further studies about the catalysis ability of the enzyme in stereospecific manner were performed by using *E. coli* SG130009 as the host microorganism. In the literature concerning benzaldehyde lyase production, Çalık et. al. (2004, 2006) selected *E. coli* K12 strain as host microorganism among the investigated *E. coli* strains, namely *E. coli* JM109, *E. coli* XL-1 Blue, *E. coli* K12 and *E. coli* GBE 180.

2.4.1.1 *Escherichia coli*

Escherichia coli is extensively used in industry as a host for the production of amino acids and recombinant proteins. The ease of genetic manipulation and wealth of the availability of genetic information coupled with fast growth rate, standardized cultivation techniques and cheap media are reasons for it is popularity (Çalık et. al., 2002-b)

A typical gram-negative cell is *E. coli*. It has an outer membrane supported by a thin peptidoglycan layer. Peptidoglycan is a complex polysaccharide with amino acids and forms a structure somewhat analogous to chain-link fence. A second membrane (the inner or cytoplasmic membrane) exists and is separated from the outer membrane by the periplasmic space. The cytoplasmic membrane contains about 50% protein, 30% lipids, and 20% carbohydrates. The cell envelope serves to retain important cellular compounds and to preferentially exclude undesirable compounds in the environment. Loss of membrane integrity leads to cell lysis and cell death. The cell envelope is crucial to the transport of selected material in and out of the cell (Schuler and Kargı, 2002).

In this study, the BL21 (DE3) pLysS strain was selected for the expression of cloned benzaldehyde lyase gene while *E. coli* K12 and JM109 strains were alternative ones. This strain is modified for high-level protein expression with the T7 regulated genes. This strain carries the DE3 bacteriophage lambda lysogen. This lambda lysogen contains the *lacI* gene, the T7 RNA polymerase gene under the control of the lacUV5 promoter, and a small portion of *lacZ* gene. This *lac* construct is inserted into the *int* gene, which inactivates the *int* gene. Disruption of the *int* gene prevent excision of the phage in the absence of helper phage. The *lac* repressor represses expression of T7 RNA polymerase until addition of IPTG, which allows expression of T7 RNA polymerase.

There is always some basal level expression of T7 RNA polymerase. T7 lysozyme (produced from pLySs) has been shown to bind to T7 polymerase and inhibit transcription. This activity is exploited to reduce basal levels of T7 RNA polymerase. T7 lysozyme is a bifunctional enzyme. In addition to its T7 RNA polymerase binding activity, it also cleavages a specific bond in the

peptidoglycan layer of the *E. coli* cell wall. This activity increases the ease of cell lysis by freeze-thaw cycles prior to purification (http://www.invitrogen.com).

In the literature, *E. coli* BL21 strains were used as a host microorganism in both batch (Choi et. al., 1997; Christensen et. al, 2002) and fed-batch (Shiloach et. al, 1996; Akesson et. al, 2001; Johnston et. al.,2003) cultivations because these strains were reported to be a low acetate producers. In this study *E. coli* BL21(DE3) pLysS was selected as a potential producer of benzaldehyde lyase.

2.4.1.2 The Genus *Bacillus*

The genus, *Bacillus*, are rod-shaped, prokaryotic, gram positive, aerobic or facultative, endospore-forming bacteria. The family's distinguishing feature is the production of endospores, which are highly refractile resting structures formed within the bacterial cells. The ubiquity of *Bacillus* species in nature, the unusual resistances of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein crystals for many insects have attracted ongoing interest in the genus (http://www.textbookofbacteriology.net).

The genus *Bacillus,* exhibits a wide diversity of physiological abilities; although the majority are mesophilic, there are also psychrophilic and thermophilic species (Table 2.4). Moreover, the morphological divisions of *Bacillus* spp. into 1) species that produce oval endospores that do not distend the mother cell, 2) species that produce oval endospores that distend the mother cell, and 3) species that produce spherical endospores is a useful division of genus. Table 2.3 summarizes the physiological classification of *Bacillus* species (Fogarty, 1990; Sonenshein, 1993).

Like most Gram-positive bacteria the surface of the *Bacillus* is complex and is associated with their properties of adherence, resistance and tactical responses. The vegetative cell surface is a laminated structure that consists of a capsule, a proteinaceous surface layer (S-layer), several layers of peptidoglycan sheeting, and the proteins on the outer surface of the plasma membrane. Walls of gram-positive bacteria are dynamically variable and flexible structures that

enclose and protect the underlying cytoplasmic membranes. (http://www.textbookofbacteriology.net; Sonenshein, 1993).

Table 2.4 Allocation of some *Bacillus* species to groups on the basis of phenotypic similarities (Sonenshein, 1993).

Species	Chracteristics of group
Group I *B. alvei* *B. amylolyticus* *B. apiarius* *B. azotofixans* *B. circulans* *B. glucanolyticus* *B. larvae* *B. lautus* *B. lentimorbus* *B. macerans* *b. macquariensis* *B. pabuli* *B. polymyxa* *B. popilliae* *B. psychrosaccharolyticus* *B. pulvifaciens* *B. thiaminolyticus* *B. validus*	All species are facultative anaerobes and grow strongly in absence of oxygen. Acid is produced from variety of sugars. Endospores are ellipsoidal and swell the mother cell. They have fairly complex nutritional requirements, and none are prototrops.
Group II *B. alcolophilus* *B. amyloliquefaciens* *B. anthracis* *B. atrophaeus* *B. carotorum* ***B. firmus*** *B. flexus* *B. laterosporus* *B. lentus* *B. licheniformis* *B. niacini* *B. pantothenicus* *B. pumilus* *B. simplex* ***B. suptilis*** *B. thuringiensis*	All species produce acid from variety of sugars including glucose. Most are able to grow at least weakly in absence of oxygen, particularly if nitrate is present. Spores are ellipsoidal and do not swell the mother cell. These bacteria are therefore an intermediate stage between true facultative anaerobes and strict aerobes.

Table 2.4 –Continued

Species	Chracteristics of group
Group III *(B. alginolyticus)* *B. aneurinolyticus* *B. azotoformans* *B. badius* ***B. brevis*** *(B. chondroitinus)* *B. frudenreichii* *B. gordonae*	These strict aerobes do not produce acid from sugars; names in bracket are exceptions. They produce ellipsoidal spores that swell the mother cell. Most species ("*B. aneurinolyticus*", *B. badius*, *B. brevis* ve "*B. freudenreichii*") have an oxidative metabolism and produce an alkaline reactions in peptone media.
Group IV *B. aminovorans* *B. fusiformis* *B. globisporus* *B. insolitus* *B. marinus* *B. pasteurii* *B. psychrophilus* ***B. sphaericus***	All species produce spherical spores that may swell the mother cell and contain L-lysine or ornithine in cell wall. These bacteria are strickly oxidative and in most cases will not use sugars as a source of carbon or energy, preferring acetate or amino acids such as glutamate as carbon sources..
Group V *B. coagulans* B. flovothermus B. kaustophilus *B. pallidus* *B. schlegelii* *B. smithii* *b. stearothermophilus* *B. thermocloacae* *B. thermodenitrificans* *B. thermoglucusidasius* *B. thermoleovrans* *B. thermoruber*	These thermophilic species all grow optimally at >50°C. Physiologically and morphologically, they are heterogeneous, but most produce ovel spores that swell the mother cell.
Group VI *A. acidocaldarius* *A. acidoterrestiris* *A. sikloheptanius*	Thermophilic, acidophilic species with membraneous ω-alicyclic fatty acids.
Unassigned species *B. benzoervorans* *B. fastidiosus* *B. nagonoensis*	It is not clear how can these species be allocated with conviction to one of these groups.

In the study performed by Çalık et. al. (2003-a), serine alkaline protease production capacity of *Bacillus* species, *namely B. alvei, B amiloliquefaciens, A. badius, B. cereus, B. coagulans, B. firmus, B. licheniformis, B. sphaericus* and *B. subtilis*, have been investigated with both wild-type and recombinant

microorganisms. Performance analyses of the microorganisms at fixed and optimum glucose concentrations together with the by-product distribution were reported. According to the study, the highest cell concentration at fixed glucose concentration (C_{Go}=6 kgm^{-3}) was obtained from r-*B. alvei* followed by r-*B. coagulans* and r-*B. sphaericus*. Those have relatively lower protease activity within the investigated microorganisms while having high enhancement factors (defined as maximum SAP activity of recombinant *Bacillus* species per maximum activity of wild type ones), E_A. Along with the enhancement factors, protease activity and cell concentration values of *B. firmus*, *B. subtilis* and *B. sphaericus* seem to be more prone to be used for foreign heterologous protein secretion.

B. brevis has a cell-wall structure with unique features which give the bacteria high intrinsic capacity for protein secretion indicating that genes of cell-wall proteins are highly active. A further major attraction of *B. brevis* is the low nearly nonexistent level of extracellular proteases. This suggests that *B. brevis* can be an excellent alternative host for heterologous protein secretion (Smith, 1995).

Multiple protease deficient strains of B. subtilis WB600 (Wu et. al., 1991) and WB700 (Ye et. al., 1999) were stated a stepwise improvement in the production of human leukin-3 relative to that of double protease mutated DB104 strain in the study of Westers et. al. (2006), where secretion of functional human interleukin-3 from *Bacillus subtilis* was optimized.

Because of the items mentioned above, in the current study, *B. firmus*, *B. brevis, B. sphaericus* and *B. subtilis* with protease deficient *apr*$^-$ and *npr*$^-$ and *spo*$^-$ and multiple protease deficient WB600 and WB700 strains were selected as the potential producer of benzaldehyde lyase as extracellular.

2.4.1.3 *E. coli* or the Genus *Bacillus*

The demand for foreign gene expression systems is rapidly increasing. Production of heterologous proteins at high levels by bacteria is commonly achieved using *Escherichia coli* as the host. However, the *E. coli* expression system still has disadvantages. For example, it is a pathogenic bacterium and

has endotoxins: it secretes protein into the periplasm and often into inclusion bodies (Li et. al., 2004).

In contrast to the well-known Gram-negative bacterium *E. coli*, the Gram-positive bacterium *B. subtilis* is considered as a GRAS organism (generally recognized as safe). For that reason, the use of *B. subtilis* for the production of food products is highly favored over the use of *E. coli*. The outer cell membrane of most Gram-negative bacteria, like *E. coli*, contains lipopolysaccharides (LPS), generally referred to as endotoxins, which are pyrogenic in humans and other mammals. These endotoxins complicate product purification, because the end-product should be completely endotoxin- free. Furthermore, in comparison to *E. coli*, *B. subtilis* is a more attractive host because it has a naturally high secretory capacity and exports proteins directly into the extracellular medium. The secretion of target proteins leads to a natural separation of the product from cell components simplifying downstream processing of the protein. In addition, it may provide better folding conditions compared to the reducing environment in the cytoplasm, thereby preventing the formation of inclusion bodies. Despite these clear shortcomings of the *E. coli* system, the use of the highly efficient *Bacillus* secretion hosts has remained limited to bulk industrial enzyme production (Westers et. al., 2004). Some problems like proteolysis, competition with other proteins secreted in high level, feedback mechanism and cell wall barrier for secretion have been encountered in attempts to bring about the secretion of foreign proteins from *Bacillus* species. Therefore *Bacillus* species other than *B subtilis* or strains with slightly different cell wall structures can be more suitable hosts for the secretion of proteins (Simonen , 1993).

2.4.1.4 Cell Growth, Kinetics and Yield Factors

Microbial growth can be considered as an increase in the number of individuals in the population as a result of both replication and change in cell size due to the chemical reactions occur inside the cell. In a suitable nutrient medium, organisms extract substrates from the medium and convert them into biomass and metabolic products and excrete them into abiotic phase (Nielsen and Villadsen, 1994; Scragg, 1988)

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

$$\mu = \frac{1}{C_x} \cdot \frac{dC_x}{dt} \tag{2.1}$$

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

When a suitable growth supporting medium is inoculated with cells a characteristic sequence of events termed in the growth cycle takes place. The growth in batch cultivations can be divided into number of distinct phases. Within the different phases of microbial growth, specific growth rate is not constant and varies with respect to phases. Batch cell growth and the metabolic activities are summarized in Table 2.5 (Atkinson and Mavituna, 1991; Shuler and Kargı, 2002).

Table 2.5 Batch Cell Growth Cycle

Phase	Type of growth	Specific growth rate
Lag	Physicochemical equilibration between the organism and the environment following inoculation with very little growth	$\mu \approx 0$
Acceleration	Growth starts to occur	$\mu < \mu_{max}$
Growth	Growth achieves its maximum rate	$\mu \approx \mu_{max}$
Stationary	No net growth occurs as nutrients depleted	$\mu \leq 0$
Death	Loss of viability and destruction by lysis of biomass	$\mu < 0$

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

$$r_X = \frac{dC_X}{dt} = \mu C_X \qquad 2.2$$

Similarly the substrate consumption rate, $-r_S$, and product formation rate, r_P, can be expressed as follows:

$$r_P = \frac{dC_P}{dt} \qquad 2.3$$

$$-r_S = \frac{dC_S}{dt} \qquad 2.4$$

The biomass and product yields, $Y_{X/S}$ and $Y_{P/S}$, respectively, are extremely important parameters since they represent the efficiency of conversion of the substrate into biomass and product. They are defined as the mass of biomass or product formed per unit mass of substrate consumed:

$$Y_{X/S} = \frac{dC_X}{dC_S} = \frac{dC_X/dt}{dC_S/dt} = \frac{r_X}{r_S} \qquad 2.5$$

$$Y_{P/S} = \frac{dC_P}{-dC_S} = \frac{dC_P/dt}{-dC_S/dt} = \frac{r_p}{r_S} \qquad 2.6$$

The usual method of measuring yields is the measure the amounts of biomass and or product formed and substrate consumed over some time period. Then, the overall biomass and product yields can be defined as:

$$\overline{Y}_{X/S} = \frac{\Delta C_X}{\Delta C_S} \qquad (2.7)$$

$$\overline{Y}_{P/S} = \frac{\Delta C_P}{\Delta C_S} \qquad (2.8)$$

where, C_X, C_P and C_S are mass of cell, product and substrate, respectively, involved in metabolism. These are the overall yield coefficients that is the total

biomass or product formed compared with the total substrate consumed over the whole growth cycle.

A list of frequently used yield coefficients is given in Table 2.6.

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

$$q_P = \frac{1}{C_X}\frac{dC_P}{dt} \tag{2.9}$$

Table 2.6 Definition of yield coefficients.

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

$$q_S = \frac{1}{C_X}\frac{dC_S}{dt} \tag{2.10}$$

The maintenance coefficient for oxygen, m_O, represents the amount of oxygen for maintenance. In an aerobic process, oxygen is mainly consumed for three purposes: cell growth, product and by-product formations, and maintenance. The oxygen consumption rate for cell growth can be defined as:

$$-r_{O1} = \frac{dC_X/dt}{\overline{Y}_{X/O}} \tag{2.11}$$

Oxygen consumption rate cell maintenance is defined as:

$$-r_{O2} = m_0 C_X \tag{2.12}$$

If oxygen consumption rate for product formation is omitted, then, total oxygen consumption rate becomes:

$$-r_O = (-r_{O1}) + (-r_{O2}) \tag{2.13}$$

Substituting equations (2.11), and (2.12) into (2.13):

$$-r_O = \frac{dC_X/dt}{\overline{Y}_{X/O}} + m_O C_X \tag{2.14}$$

By using the definition of specific growth rate, μ, equation 2.14 can be rearranged:

$$-r_O = \frac{\mu C_X}{\overline{Y}_{X/O}} + m_O C_X \tag{2.15}$$

Dividing by C_X and μ,and substituting into the equation (2.15), reorganization gives:

$$\frac{-r_O}{C_X \mu} = \frac{1}{Y_{X/O}} = \frac{1}{\overline{Y}_{X/O}} + \frac{m_O}{\mu} \qquad (2.16)$$

Then, the slope of the plot $1/Y_{X/O}$ versus $1/\mu$ gives the value of oxygen consumption rate for maintenance, m_O; and from the intercept, cell yield on oxygen uptake for product formation is omitted, $\overline{Y}_{X/O}$, could be determined.

Similarly, from the slopes of $1/\mu$ versus $1/Y_{X/S}$ plots, m_S, maintenance coefficient for substrate, values can be obtained. m_0 and m_S may differ with the change in bioprocess parameters such as, type of microorganism, type of substrate, pH and temperature.

2.4.2 Medium Design

All living cells require certain nutrients for their growth and development. These nutrients must contain the chemical elements which constitute the cellular materials and structures, as well as those elements which are required for membrane transport, enzyme activity, and for the generation of the energy required for biosynthetic processes (Scragg, 1988). The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation. Nutrients required by cells can be classified in two categories (Shuler and Kargı, 2002):

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

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

Two major types of growth media are defined and complex media. Defined media contain specific amounts of pure chemical compounds with known chemical compositions. Complex media contain natural compounds whose chemical composition is not exactly known. Although, complex medium can provide the necessary growth factors, vitamins, hormones and trace elements; often resulting in higher cell yields, defined medium allows better control over the fermentation leading to reproducible results. Further, recovery and purification of a product is often easier and cheaper in defined media (Shuler and Kargı, 2002).

In the literature, for *E. coli* BL21 strain, LB medium was reported slightly better than M9 medium containing 2.0 kg m^{-3} casamino acid where bovine growth hormone (bGH) expression optimization was aimed (Choi et. al., 1997). In another study performed by Christensen et. al. (2002), glucose and glycerol as carbon substrate were investigated and lower acetate accumulation was observed in glycerol based medium. Furthermore, Çalık et. al. (2004, 2006) designed a glucose based defined medium containing 8.0 kg m^{-3} glucose, 5.0 kg m^{-3} $(NH_4)_2HPO_4$ and salt solution, and using this medium they investigated the effects of bioreactor operation parameters in *E. coli* K12 carrying pUC18::BAL plasmid.

In the study where nine different recombinant *Bacillus* species were examined for higher productivity of serine alkaline protease, the medium containing 6.0 kg m^{-3} glucose, 4.7 kg m^{-3} $(NH_4)_2HPO_4$, 2.0 kg m^{-3} KH_2PO_4 suplimented with 0.04M NaH_2PO_4, and Na_2HPO_4 was used (Çalık et. al., 2003-a).

2.4.3 Bioreactor Operation Parameters

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

2.4.3.1 Temperature

Temperature is one of the most important bioprocess parameters which is normally desired to be kept constant at its optimum value throughout the bioprocess (Nielsen and Villadsen, 1994). The three dimensional structure of enzyme, which is vital for the activity of the molecule, is governed by many forces and interactions such as hydrogen bonding, hydrophobic interactions, and van der Waals forces. At low temperatures the molecule is constrained by these forces; as the temperature increases the thermal motion of the various regions of the enzyme increases until finally the molecule is no longer able to maintain its structure or its activity. Most enzymes have temperature optima between 40-60^{o}C (Kirk-Othmer, 1994).

The temperature effect on benzaldehyde lyase activity was not investigated. The production of *E. coli* is performed at 37^{o}C, which is the bacteria's optimal temperature of growth. Similarly the *Bacillus* species selected were grown at 37^{o}C.

2.4.3.2 pH

Hydrogen ion concentrations (pH) affects the activity of enzymes and therefore the microbial growth rate. The optimal pH for growth may be different from that for product formation. Different organisms have different pH optima, however, for many bacteria, pH optima ranges from 3.0 to 8.0 (Shuler and Kargı, 2002).

In most fermentations, pH can vary substantially. Often the nature of the nitrogen source can be important. Furthermore, pH can change due to the production of organic acids, utilization of acids (particularly amino acids), or the production of bases. Thus, pH control by means of a buffer or an active pH control system is important (Shuler and Kargı, 2002). Nevertheless, some bioprocesses require controlled pH conditions, while others might require uncontrolled pH operations, in order to increase the product yield and selectivity (Çalık et al., 2002-a).

In the study by Çalık et. al. (2006), the effect of controlled and un-controlled pH conditions, as well as of the value of pH in the range of 5.0-7.8,

on benzaldehyde lyase production on a defined medium with glucose as a sole carbon source in batch bioreactors was investigated. Un-controlled pH of 7.2 was reported to be more favorable than controlled pH conditions in terms of enzyme activity and cell concentration. Therefore, in the current study, initial pH of the fermentation medium was arranged as 7.2 with 5M NaOH and 5M H_3PO_4.

For expression in *Bacillus* species, Çalık et. al. (2003-a) adjusted the pH of fermentation medium to 7.25. Thus, initial pH of production medium for growth of *Bacillus* species were arranged as 7.25 with 10M KOH and 5M H_3PO_4.

2.4.3.3 Oxygen Transfer and K_La measurement

Oxygen shows diverse effect on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes. In general, according to cell growth conditions and metabolic pathway analysis, some bioprocesses require high oxygen transfer rate conditions while others require controlled oxygen transfer rates in order to regulate oxygen uptake rates. The evidence shows that dissolved oxygen concentration has been accepted as an important bioreactor operational parameter. The effects of oxygen transfer on the activity and bioprocess residence time profiles of *in vitro* metabolic intermediates and catabolic end products should be investigated in order to understand the details of metabolism and increase the product selectivity. Furthermore, in order to design, scale-up, and operate the bioreactor with adequate mass transfer, the oxygen consumption rates and oxygen transfer coefficients which are the indicators of the mass transfer characteristics of a fermentation process are required; nevertheless, due to the complex composition of the fermentation liquid, it can be difficult to predict these parameters with reasonable accuracy (Çalık et. al., 1998).

Dissolved oxygen is an important substrate in aerobic fermentations and may be a limiting substrate, since oxygen gas is sparingly soluble in water (Shuler and Kargı, 2002). The transfer of oxygen from gas to microorganism takes place in several steps (Figure 2.15), which are 1) transfer from the interior of the bubble to the gas-liquid interface; 2) movement across the gas-liquid interface; 3) Diffusion through the relatively stagnant liquid film surrounding the bubble; 4) Transport through the bulk liquid; 5) Diffusion

through the relatively stagnant liquid film surrounding the cells; 6) Movement across the liquid-cell interface; 7) If the cells are in a floc, clump or solid particle, diffusion through the solid to the individual cell; 8) Transport through the cytoplasm to the site of reaction (Scragg, 1988 and Bailey, 1986).

When cells are dispersed in the liquid, and the bulk fermentation broth is well mixed, the major resistance to oxygen transfer is the liquid film surrounding the gas bubbles; therefore the rate of oxygen transfer from gas to liquid is of prime importance.

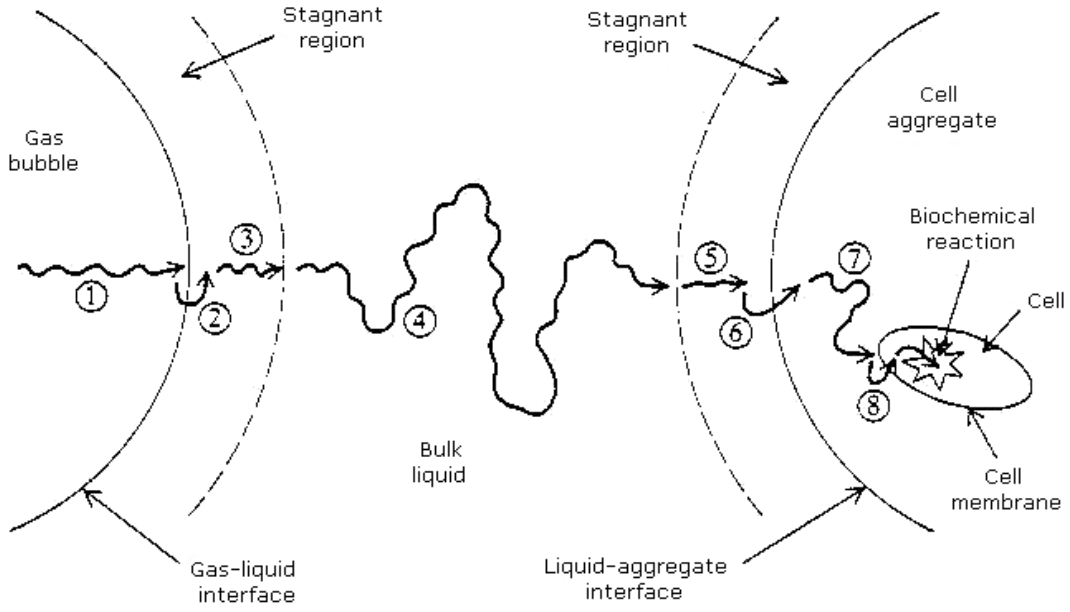


Figure 2.15 Schematic diagram of steps involved in transport of oxygen from a gas bubble to inside a cell. (Bailey, 1986).

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

$$OTR = k_L a(C_O^* - C_O) \qquad (2.17)$$

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

Since solubility of oxygen in aqueous solutions is very low, the liquid phase mass transfer resistance dominates, and the overall liquid phase mass transfer coefficient, K_La, is approximately equal to liquid phase mass transfer coefficient, k_La (Shuler and Kargı, 2002).

Many factors influence oxygen demand; the most important of which are cell species, culture growth phase, and nature of the carbon source in the medium (Bailey, 1986).

The oxygen uptake rate can be defined as:

$$OUR = -r_O = q_O C_X \tag{2.18}$$

where q_O is the specific rate of oxygen consumption and C_X is the cell concentration (Shuler and Kargı, 2002).

Knowledge of K_La behavior allows the operation of bioreactions at conditions where oxygen is not a limiting factor for growth. Numerous methods have been developed for the experimental determination of K_La values. Dynamic method is widely used for the determination of the value of K_La experimentally, and it can be applied during the fermentation process. This method based on a material balance on the oxygen in the liquid phage (Scragg, 1988 and Rainer, 1990):

$$\frac{dC_O}{dt} = K_L a(C_O^* - C_O) - q_0 C_X \tag{2.19}$$

A typical response curve of the dynamic method is given in Figure 2.16.

As shown in figure, at some time t_0, the broth is de-oxygenated by stopping the air flow. During this period, dissolved oxygen concentration, Co_0, drops, and since there is no oxygen transfer (region-II), equation (2.19) reduces to:

$$\frac{dC_O}{dt} = -r_0 \tag{2.20}$$

Using equation (2.20) in region-II of Figure 2.16, oxygen uptake rate, $-r_0$, can be determined. Air inlet is then turned back on, and the increase in C_O is monitored as a function of time. In this period, region-III, equation (2.19) is valid. Combining equations (2.18) and (2.19) and rearranging,

$$C_O = -\frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_0\right) + C_O^* \qquad (2.21)$$

From the slope of a plot of C_O versus $(dC_O/dt - r_0)$, K_La can be determined (Figure 2.17).

The Dynamic Method can also be applied to conditions under which there is no reaction, i.e., $r_0=0$ (Nielsen and Villadsen, 1994). In this case, the broth is de-oxygenated by sparging nitrogen into the vessel. Air inlet is turned back on and again the increase in Co is monitored as a function of time. Modifying equation (2.21)

$$C_O = \frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \qquad (2.22)$$

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

A large number of different empirical correlations for the volumetric mass transfer coefficient K_La have also been presented in the literature. Most of these correlations can be written in the form:

$$K_L a = k\left(\frac{P}{V_R}\right)^{\beta} u_S^{\alpha} \qquad (2.23)$$

where u_s is the superficial gas velocity (m s^{-1}), P/V_R is the power dissipation per unit volume (W m^{-3}) and k is the empirical constant. β and α parameters are specific for the considered system, i.e. for the bioreactor design (Nielsen and Villadsen, 1994).

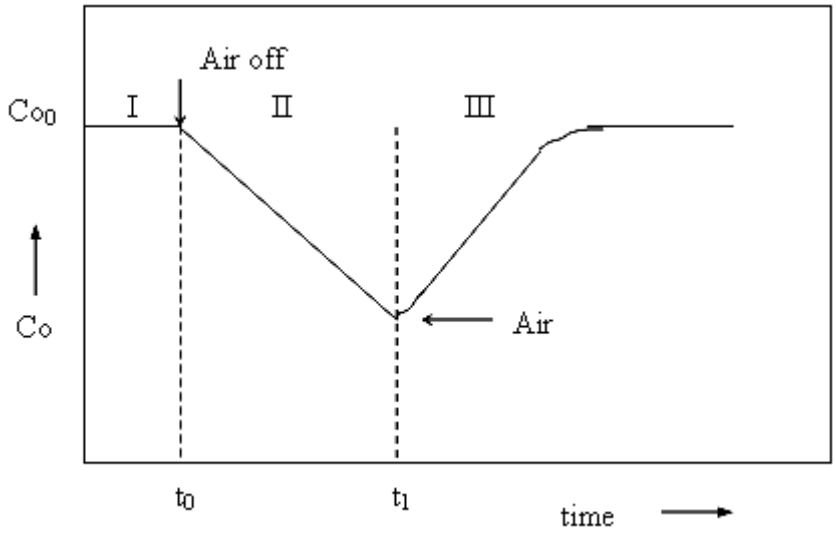


Figure 2.16 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La.

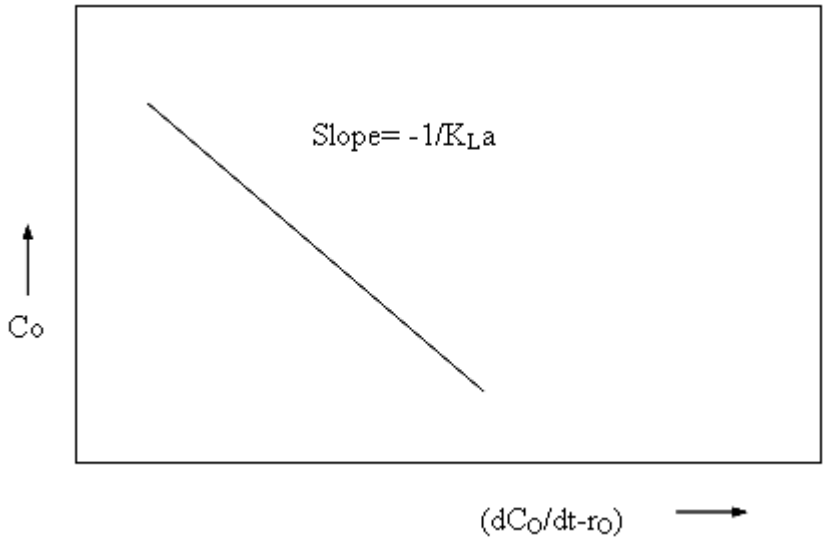


Figure 2.17 Evaluting K_La using the Dynamic Method.

In the study of Çalık et. al (2004), the effects of oxygen transfer on recombinant benzaldehyde lyase production was investigated at six different conditions with parameters, air inlet rate of Q_0/V_R= 0.5 vvm, and agitation rates of N= 250, 375, 500, 625, 750 min^{-1} and at Q_0/V_R= 0.7 vvm, N= 705 min^{-1}, in a pilot scale bioreactor with 1.65 dm^3 working volume. Among the investigated parameters, the highest BAL activity was obtained 0.5 vvm, 500 min^{-1} as 860 U cm^{-3}, where the highest cell concentration (2.3 kg m^{-3}) was obtained. Oxygen transfer characteristics of OUR and K_La values together with the yield and maintenance coefficients and by-product distribution were determined. K_La was increased with cultivation time and agitation rate, with values changing between 0.008-0.046 s^{-1}.

Related with the production of other biomolecules using *E. coli* as the host microorganism, Ryan et. al. (1989) has reported that decrease in air flow rate, reduce the cell productivity while expression level of the β-lactamase was drastically decline at high DO levels in recombinant *E. coli* JM103. Within the investigated conditions with parameters, agitation rates of N= 850 rpm and air inlet rate of Q_0/V_R= 0.068, 3.77 and 9.11 vvm, maximum enzyme activity was reached for the intermediate flow rate.

Similarly, Bhattacharya and Dubey (1997) have investigated the effects of different levels of dissolved oxygen on over-expressing target gene under the control of T7 promoter in *E. coli* K12 by keeping the air flow rates constant at 2.5 vvm but changing agitation rate in each experiment and stated that under sufficient oxygen transfer conditions, target gene expression and cell productivity attained its maximum value while oxygen deficiency resulted in decreased values. In most of the studies performed with *E. coli* dissolved oxygen level was kept constant ranging between 10-30% by either changing the air flow rate (Luli et. al., 1990) or agitation rate in fed-batch processes with an automated control systems (Akesson et. al., 2001; Johnston et. al., 2003).

2.4.3.4 Design by Scale-up

A number of factors of a physical nature change with the scale of the bioreactor. Thus, for different stirrers and different tank geometry the parameter values may change significantly.

The scale-up method strives to attain the identical conditions on the full-scale reactor that were found to be optimum on the pilot plant. This is generally not possible because areas/unit volume must change with scale and mixing conditions also alter. Criteria which are the maintenance of constant power to volume ratio, constant heat transfer coefficient, constant tip speed and constant pumping rate to volume ratio, produce different relationships between the full-scale and pilot scale reactors. They all have the general form:

$$N_2 = N_1\left(\frac{V_2}{V_1}\right)^n \tag{2.24}$$

But n varies between 0 and -1/3 depending on the criterion. It is, therefore, necessary to decide which criteria are most significant for the reaction under consideration.

Constant stirrer power to volume ratio is a suitable criterion when the main duty of the agitator is liquid mixing. The power input for stirring is a major cost for aerobic bioprocesses, and it depends on several factors. Most important are the stirrer rate, N, the impeller diameter, d_s, the density of the fluid, ρ_L, viscosity of the fluid, η. By dimensional analysis, the relationship between power and dimensions for a fixed physical system can be defined as:

$$P = N_p \rho_L \, d_s^{\,5} N^3 \tag{2.25}$$

where N_P is the dimensionless power number. Since we are scaling up at constant power to volume ratio:

$$P_1/V_1 = P_2/V_2 \tag{2.26}$$

and since the scale up is based on geometrical similarity:

$$\frac{D_2}{D_1} = \frac{d_{s1}}{d_{s2}} = \left(\frac{V_2}{V_1}\right)^{1/3} \tag{2.27}$$

Hence, at constant power/unit volume:

$$\mathrm{N}_2 = N_1\left(\frac{V_2}{V_1}\right)^{-2/9} \tag{2.28}$$

Therefore, the stirrer speed must reduce on the larger scale (Nielsen and Villadsen, 1994; Rose, 1981).

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 and Plasmids

For both intracellular and extracellular production of benzaldehyde lyase, microorganisms and plasmids used in this study were listed in Table 3.1.

The recombinant microorganisms are stored in the microbanks (PRO-LAB), by inoculating young colonial growth into cyropreservative fluid present in the vial. After providing the adsorption of microorganisms into the porous beads, excess cryopreservative was aspirated and inoculated cyrovial stored at -70^{o}C.

3.3 The Solid Medium

The recombinant *E. coli* strains and *Bacillus* species, stored on agar slants at 4ºC, were inoculated onto the freshly prepared agar slants under sterile conditions, and were incubated at 37ºC overnight (Table 3.2). According to the antibiotic resistance ability of the microorganisms (kanamycin and ampicillin resistance in pRB374 vector; ampicillin resistance in pRSET vector and chloramphenicol resistance in BL21 (DE3) pLysS cells; lincomycin and erythromycin for WB600 and WB700 cells) proper antibiotics are added to the

agar slant after steam sterilization at 121°C for 20 minutes. The amounts of the antibiotics added to the medium are stated in Table 3.3.

Table 3.1 Strains and plasmids used in this study (NRRL: Northern Regional Research Center; BGSC: Bacillus Genetic Stock Center; ATTC: American Type Culture Collection)

Strains	**Source / Reference**
E.coli	
XL1Blue	Bullock et. al. (1987)
JM109	Yanisch-Peron et. al. (1985)
K12	ATTC 10798
BL21 (DE3) pLySs	Invitrogen Life Technologies (USA)
B. licheniformis	DSM 1969; Çalık et. al. (2003-b)
B. brevis	NRRL NRS 604
B. firmus	NRRL B1107
B. sphaericus	NRRL NRS 732
B. subtilis	
1A751	BGSC
1S19	BGSC
WB600	Wu et. al. ,1991
WB700	Ye et. al., 1999
Plasmids	**Source / Reference**
pUC18::*bal*	Çalık et. al., 2004
pRSETA	Invitrogen Life Technologies (USA)
pUC19	Yanisch-Peron et. al. (1985)
pRB374	Brücknet et. al., 1991
pRSETA::*bal*	This work
pUC19::*hybrid*	This work
pRB374::*hybrid*	This work

Table 3.2 The composition of the solid medium.

Compound	Concentration, kg m^{-3}
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Agar	15.0
pH	7.5

Table 3.3 The final concentration of antibiotics in the solid medium.

Antibiotic	Concentration, kg m^{-3}
Kanamycin	0.010
Ampicillin	0.100
Chloramphenicol	0.035
Erythromycin	0.005
Lincomycin	0.005

3.4 The Precultivation Medium

The recombinant *E. coli* strains and *Bacillus* species, grown in the solid medium, were inoculated into precultivation medium and incubated at 37ºC and N=200 min^{-1} for 12 h in agitation and heating rate controlled orbital shakers (B.Braun, Certomat BS-T) using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. The compositions of precultivation medium were listed in Table 3.4 and Table 3.5. The selective antibiotics were added to the precultivation medium to increase the stability of the plasmid in amounts stated in table 3.2 after sterilization.

Table 3.4 The composition of the precultivation medium of *E. coli* strains.

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

Table 3.5 The composition of the precultivation medium of *Bacillus* species.

Compound	Concentration, kg m^{-3}
Soytryptone	15.0
Peptone	5.0
$MnSO_4.2H_2O$	1.0×10^{-2}
Na_2HPO_4	0.25
$CaCl_2$	0.10

3.5 The Production Medium

The microorganisms inoculated in precultivation medium, were further inoculated in production medium. The reference production medium (RPM) for benzaldehyde lyase production in *E. coli* and starting point of the medium design experiments was chosen to be the medium described in Çalık et. al. (2004, 2006); the composition is given in Table 3.6.

Table 3.6 The composition of the reference BAL production medium.

Component	Concentration, kg m^{-3}
Glucose	8.0
Na_2HPO_4	6.7
KH_2PO_4	3.1
NaCl	0.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2HPO_4$	5.0

Defined medium design was performed in order to reach high levels of expression of the recombinant *E. coli* strains. Furthermore, the effects of the bioprocess operation parameters, i.e., oxygen transfer conditions, were investigated in order to increase the biomass and benzaldehyde lyase production in the recombinant organisms. The investigated parameters are listed in Table 3.7.

Table 3.7 The investigated parameters for benzaldehyde lyase production in *E. coli* strains.

Medium Components		Bioreactor Operation Parameters
Glucose $(NH_4)_2HPO_4$ NH_4Cl $(NH_4)_2SO_4$	Concerted effect of initial glucose and nitrogen source.	Oxygen Transfer Rate Medium Composition

The recombinant *Bacillus* species were inoculated into the production medium whose compositions were given in Table 3.8 (Çalık et. al., 2003-a).

Table 3.8 The composition of the production medium for *Bacillus* species.

Macronutrients	Concentration, kg m^{-3}
Glucose	10.0
$(NH_4)_2HPO_4$	4.71
KH_2PO_4	2.0
Na_2HPO_4	4.3×10^{-2}
NaH_2PO_4	5.63
Micronutrients (Salt Solution)	Concentration, kg m^{-3}
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	1.0×10^{-3}
$ZnSO_4.7H_2O$	0.1×10^{-3}
$MnSO_4.7H_2O$	7.5×10^{-5}
$CuSO_4.5H_2O$	1.0×10^{-5}

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

The pilot scale batch bioreactors (B.Braun Biostat Q4), having a working volume of 0.33-0.750 dm^3, and consisting of temperature, pH, foam and stirring

rate controls, were used for the investigation of oxygen transfer effects. The bioreactor systems contain compact supply units, four autoclaveable bioreactors with a 1.0 dm^3 volume, digital instrumentation system (DCU3) and four rotameters with a central pressure reducer for aeration installed in the supply unit. The culture vessels are jacketed with glass vessels and temperature control is performed with water circulation from the jackets. Cooling water and fed air are provided from Eyela CA 111 model water circulator and external compressor (Larfon), respectively.

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

3.6 Analysis

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

3.6.1 Cell Concentration

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

3.6.2 Benzaldehyde Lyase Activity

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

activity of the intracellular benzaldehyde lyase in r-*E. coli* strains, the cell walls were lyzed at f=10 s^{-1} for 10 minutes using an agitator bead mill (Retsch, MM 200) by using 30% suspension of glass beads in activity buffer of 40 mM Tris-HCl (pH=8.0), 0.02 mM TPP, 0.2 mM $MgCl_2$ buffer. Fresh substrate solutions were prepared daily by mixing 0.5 volume activity buffer with 0.35 volume stock benzoin (0.1mM) and 0.15 volume 15%PEG solution with a final concentrations of 0.035 mM benzoin, 20 mM Tris-HCl (pH=8.0), 0.01 mM TPP, 0.1 mM $MgCl_2$, 7.5% PEG and incubated at 37°C. Stock benzoin solution (0.1mM) was prepared in 15% PEG solution and used after overnight incubation at room temperature. The conversion reaction was carried out at 37°C.

In *Bacillus* species, after removing the cell debris, supernatant was directly added to the substrate solution. The reaction for activity measurement was started by the addition of 20 µl of samples to 3 cm^3 of substrate solution and further incubated for 10 seconds. The enzymatic activity was monitored spectrophotometrically at 250 nm by following the change in the absorbance in ten seconds. The substrate benzoin and the product benzaldehyde have similar absorbance maxima. However, since the two molecules of benzaldehyde are produced per each molecule of (R)-benzoin cleaved, the following formula was used to relate absorbance change to product formed (Gonzalez and Vicuna, 1989):

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

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

3.6.3 Reduced Sugar Concentration

Reduced sugar, glucose, concentration was determined by the glucose oxidation method at 505 nm with a UV spectrophotometer (Boyaci et. al., 2005). D-glucose is oxidized in the presence of glucose oxidase enzyme (equation 3.1) and peroxide formed due to oxidation reaction is further reacted with 4-

aminoantipyrine and phenol in the catalysis of peroxidase to form iminoquinone (equation 3.2) which gives spectro-photometrically observable red color in proportion with glucose concentration.

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

$$H_2O_2 + 4\text{-}\mathrm{Aminoantipyrine} + \mathrm{Phenol} \xrightarrow{\text{peroxidase}} \mathrm{Im}\,inoquinone + H_2O \quad (3.2)$$

The preparation method of analysis solution and standard glucose solutions were given in Appendix C. The calibration curve was obtained from the slope of absorbance versus known standard glucose concentration (Appendix B). The method used in analysis of samples is given below:

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

3.6.4 Organic Acid Concentration

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

Column	Capital Optimal ODS, 5µm
Column dimensions	4.6 x250 mm
System	:Reversed phase chromatography
Mobile phase flow rate	:0.8 ml/min
Column temperature	:30 °C
Detector and wavelength	:Waters 2487 Dual absorbance detector, 254 nm
Injection volume	:5 µl
Analysis period	:15 min

3.6.5 Amino Acids Concentrations

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

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

3.6.6 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

In order to determine the liquid phase mass transfer coefficient and oxygen uptake rate in the benzaldehyde lyase production process, the Dynamic Method (Rainer, 1990), as explained in section 2.4.3.3, was used.

Prior to inoculation of the microorganism to the production medium in the bioreactor, the physical mass transfer coefficient (K_La_0) was determined. After inoculation of the microorganism to the bioreactor, the dynamic oxygen transfer experiments were carried out at certain cultivation times for a short period of time, so that the biological activities of the microorganisms are unaffected. During this period, while the air inlet was totally ceased, the agitation rate was lowered to N=150 min^{-1} in order to lower the effect of surface aeration.

3.7 Genetic Engineering Techniques

3.7.1 Materials

3.7.1.1 Enzymes, Kits, and Molecular Size Markers

Taq DNA polymerase, *Pfu* DNA polymerase, Ribonuclease A (DNase and protease free), T4 DNA ligase, dNTP mixture, ATP and restriction enzymes (BamHI, SacI, PstI and NdeI) and their buffers were purchased from MBI Fermentas.

QIAGEN Plasmid Purification Kit, QIAguick PCR purification Kit and QIAexpress Ni-NTA Spin Columns were obtained from QIAGEN Inc. Gene Elution Kit was purchased from GeneMark Molecular Biology Tools.

Lambda DNA/HindIII Marker, Gene Ruler 50bp DNA ladder and 6X Loading Dye were from MBI Fermentas.

3.7.1.2 Buffers and Solutions

The buffers and the solutions used in this study and their preparations are given in Appendix D.

3.7.2 Determination of DNA Concentration

The concentration of DNA fragments after restriction digestion, PCR amplification or concentration of isolated plasmids after digestion or purification; or DNA molecules treated with any manipulation were analyzed by gel electrophoresis with 0.8-1.7% (w/v) agarose gels according to the weight of the DNA fragment and 1XTBE buffer (for DNA fragments bigger than 1500 bp 0.8% (w/v), for DNA fragments smaller than 500 bp 1.5 % (w/v) agarose gels were used). DNA samples of 10-20µl, mixed with 1/5 volume of 6X loading dye (MBI Fermentas) were applied to the gel which was supplemented by ethidium bromide (Sigma-10 mg/ml) with a final concentration of 0.8µL/ml. At the end of the electrophoresis, bands were visualized with a UV transilluminator and gel photographs were taken using gel imaging and documentation system (UVP BioImaging System, and Hamamatsu Digital CCD Camera).

The molecular weights (Appendix J) and the concentrations of the DNA fragments analyzed were determined by referring to Labworks image acquisition and analysis software (UVP BioImaging System)

3.7.3 PCR Amplification of Target Genes

Primers were designed in accordance with the sequences of *bal* gene and *pre-sub*C gene (Appendix E). Nucleotide sequences of *bal* gene (Accession no: AX349268) and signal peptide sequence of serine alkaline protease gene from *B. licheniformis* (Accession no: X03341) were obtained from National Institutes of Health (ABD), National Library of Medicine, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Restriction enzyme recognition sites were determined (Appendix I) by the help of Restriction Mapper web-page of ABD Molecular Biology Resources (http://www.restrictionmapper.org). After determination of proper restriction enzyme recognition sites for both *bal* and *hybrid* genes (fusion product of *bal* and *pre-sub*C genes), primers were designed for the cloning into appropriate plasmids selected according to the following rules:

1. The nucleotide sequence of the primers should agree with the template region of the DNA that will be amplified.
2. Primers should be 18-45 bases in length.

3. The melting temperatures of two primers should have close values.
4. Primer self-complementarities should be avoided.
5. 3’ of primers should not be complementary, as otherwise primer dimers can be formed
6. 3’ ends of the primer should end with one or two G or C nucleotides in order to increase correct annealing at the site of addition of bases
7. The G+C base composition of primer should be at least 43% (Özçelik, 2003)

The possibility of dimmer formation and self-complimentarity of primers and melting temperature, ΔG, ΔH and ΔS values were checked with a computer program (NAR) and they were illustrated in Appendix H. Designed primers were synthesized in Thermo Hybaid GmbH (Germany) laboratories. The sequences and abbreviations of designed primers were demonstrated in Table 3.9

Table 3.9 Primers deigned for amplification of desired gene fragments.

Name	Sequence	Targeted Gene Fragment
*Nde*I-*bal* Forward Primer	5' CATATGGCGATGATTACAGGC 3'	*bal*
bal Reverse Primer	5’ GCGAGCTCGCTTAGTGATGGTGATGGTGATG 3’	*bal* and *hybrid*
*pre-sub*C Forward Primer	5’ GCGGATCCGCAGCAATCTCCTGTCATTCG 3’	*pre-sub*C and *hybrid*
*pre-sub*C Reverse Primer	5' CCTGTAATCATCGCCATAGCAGAAGCGGAATCG 3’	*pre-sub*C
bal Forward Primer	5’ CGATTCCGCTTCTGCTATGGCGATGATTACAGGC 3’	*bal*

PCR amplification was carried out with thermal cycling program (Techgene, Flexigene). The cycles and the contents of the reaction mixture of 50µl final volume were arranged as follows:

PCR process parameters

1 cycle	T_1 = 94°C,	2-3 min
30 cycle	T_1 = 94°C,	1 min
	T_2 = 55-60°C,	1 min
	T_3 = 72°C,	15 s-2.5 min
1 cycle	T_3 = 72°C,	5-10 min
	T_4 = 4°C,	5 min

Components of reaction mixture of PCR

10XPCR Buffer (with Mg^{++})	5µl
dNTPs (1mM)	10µl
Forward Primer (10µM)	1µl
Reverse Primer (10µM)	1µl
Template DNA	0.03-3µg
dH2O	up to 49 µl
DNA polymerase	2.5 U

3.7.4 Purification of PCR products

The purification of PCR products were performed by using QIAquick PCR Purification Kit according to manufacturer's recommendations. 1 volume of PCR reaction sample was mixed with 5 volumes of PB buffer and placed to QIAquick spin column. After centrifugation, column was washed with PE twice and DNA molecules were eluted in proper amount of water.

3.7.5 Ligation Reaction

PCR amplified genes were cloned into suitable expression vectors from the sticky ends occurred after restriction digestion with altering the gene/vector molar ratio between 3 and 5. The ligation reactions were performed by incubating the reaction mixture of 20µl final volume at 22°C for 16h. The composition of the ligation reaction mixture was arranged as follows:

Insert DNA	~ 60-120 ng
Vector DNA	~ 20-30 ng
Ligation Buffer (with ATP)	2µl
ATP (10 mM)	1.5-2µl
T4 DNA ligase (5 U/ µl)	1µl
dH_2O	up to 19µl
TOTAL	20 µl

3.7.6 Restriction Digestion Reaction

Restriction digestion of the genes of interests were performed by incubating DNA fragments with proper restriction enzymes (REs) and specified buffers of 20µl final volume at 37°C for approximately 2 h. The composition of the restriction digestion reaction mixtures was arranged as given in Tables 3.10, 3.11, 3.12, 3.13. After restriction digestion, the reaction was ended by incubating the mixture at 85°C for 15 min.

Table 3.10 Components of reaction mixture of restriction digestion with *Sac*I RE.

Components	Amounts
DNA fragment	~ 45-200 ng
*Sac*I RE (10U/ µl)	2 µl
1X Buffer *Sac*I$^+$	2 µl
dH_2O	up to 19 µl

Table 3.11 Components of reaction mixture of restriction digestion with *Nde*I RE.

Components	Amounts
DNA fragment	~ 45-200 ng
*Nde*I RE (10U/µl)	1.0 µl
1X Buffer O	2 µl
dH_2O	up to 19 µl

Table 3.12 Components of reaction mixture of restriction digestion with *Pst*I RE.

Components	Amounts
DNA fragment	~ 45-200 ng
*Pst*I RE (10U/µl)	2.0 µl
1X Buffer O	2 µl
dH_2O	up to 19 µl

Table 3.13 Components of reaction mixture of restriction digestion with *BamH*I RE.

Components	Amounts
DNA fragment	~ 45-200 ng
*BamH*I RE (10U/ µl)	2.5 µl
Buffer *BamH*I	2 µl
dH_2O	up to 19 µl

3.7.7 DNA Sequencing

The DNA sequencing is performed by automatic DNA sequencers (Microsynth GmbH, Switzerland) by using the primers designed to control the insertion of target genes.

3.7.8 Transformation of Plasmid DNA by $CaCl_2$ Method to *E.coli*

1. Incubate *Escherichia coli* overnight in LB-solid medium at 37°C,
2. Pick a single colony from a selective plate and inoculate a starter culture of 5 ml LB medium. Grow for 12h (overnight) at 37°C, with vigorous shaking (~200 rpm)
3. Transfer 1ml sample from the precultivation medium to an 100 ml- LB medium and incubate at 37°C and 200 min^{-1} for 3.5 hours,
4. Transfer 10 ml of broth into 30 ml sterile polypropylene tubes; and place on ice for 10 minutes,
5. Separate the microorganisms by centrifugation at 4000 min^{-1}, 4°C for 10 minutes,
6. Separate the supernatant; let the cells dry on a paper tissue for 1 minute,

7. Add 1 ml of 0.1 M $CaCl_2$ solution onto the cells and make a complete solution by vortex, set on ice for 10 minutes,
8. Precipitate the microorganisms by centrifugation at 4000 min^{-1}, 4^{o}C for 10 minutes,
9. Separate the supernatant; let the cells dry on a paper tissue for 1 minute,
10. Add 200 µl of 0.1M $CaCl_2$ solution onto the cells and make a complete solution by vortex, set on ice for 10 minutes,
11. Transfer 200 µl of solution to an eppendorf tube, and add 3 µl of plasmid DNA to this solution. Incubate on ice for 30 minutes,
12. Apply heat-shock to the solution at 42^{o}C for 90 seconds and quickly place the tube on ice for 1 minute,
13. Transfer the cell suspension to sterile culture tubes containing 800 µl of LB medium without antibiotics and incubate at 37^{o}C for 45 minutes with shaking at 140 min^{-1} to recover cells,
14. Transfer 250 µl of the cultured cells onto the center of LB plate containing the desired antibiotic. Immediately spread the cells over the entire surface of the LB plate using a sterile, bent glass rod.
15. Invert the plates and incubate at 37^{o}C overnight. Selected colonies should be visible in 14-24 hours (Sambrook, 2001).

3.7.9 Isolation of Plasmid DNA

1. Pick a single colony from a selective plate and inoculate a starter culture of 30 ml LB medium. Grow for 12h (overnight) at 37^{o}C, with vigorous shaking (~200 rpm)
2. Pour 1ml of culture into microfuge tube and centrifuge at 12000 min^{-1}, 4^{o}C, for 30 s,
3. Remove the supernatant and add again 1ml of culture and repeat the centrifugation step,
4. Remove the supernatant and take off all fluid by micropipette; place the tube on ice,
5. Resuspend the bacterial pellet in 100 µl of ice-cold alkaline lysis solution I one by vigorous vortexing. Make sure that the bacterial pellet is completely dispersed in alkaline lysis solution I,
6. Add 200 µl of freshly prepared alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the content by inverting the tube rapidly for 5 minutes,

7. Add 150 µl of ice-cold alkaline lysis solution III. Close the tube and disperse alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 5 minutes,
8. Centrifuge the bacterial lysate at 12000 min^{-1}, 4°C, for 5 minutes. Transfer the supernatant to a fresh tube.
9. Add 1/10 volumes of NaAc and 2 volumes of EtOH. Mix the solution by vortexing and then allow the mixture to stand for at least 10 minutes at -20°C,
10. Collect the precipitated plasmid DNA by centrifugation at 12000 min^{-1}, 4°C, for 5 minutes.
11. Remove the supernatant gently and stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.
12. Dissolve the plasmid DNA in suitable amount of dH_2O and store the solution at -20°C (Sambrook, 2001).

3.7.10 Purification of Plasmid DNA

Plasmid purification was carried out by using QIAGEN Minipreps Plasmid Purification Kit according to the procedure provided by manufacturer. Bacterial cells incubated overnight were harvested by centrifugation and resuspended in P1 buffer containing RNase A. After cell lysis in P2 buffer, genomic DNA, proteins, cell debris and SDS were precipitated by adding P3 buffer to the mixture and incubated on ice until lyasate become less viscous. Sample was centrifuged to remove the precipitate and supernatant loaded into equilibrated QIAGEN-tip and allowed to enter the resin by gravity. After washing with QC buffer twice, DNA was eluted with QF buffer into clean microcentrifuge tubes. 0.7 volumes isopropanol was added on each sample to precipitate the DNA and centrifuged for 30 min. After washing pellet with 70% ethanol, each pellet was dissolved in proper amount of water.

3.7.11 DNA Extraction from Agarose Gel

DNA fragments analyzed with gel electrophoresis were extracted from the agarose gel by using Gel Elusion Kit (GeneMark). After electrophoresis desired DNA bands were cut and gel slices (up to 350 mg) incubated in 500 µl Binding Solution at 60°C for 15-30 minutes were loaded into spin columns and centrifuged at maximum speed. Columns washed with Washing Solution twice

were transferred into new sterilized microcentrifuge tubes and DNA molecules were eluted in proper amounts of water.

3.7.12 Chromosomal DNA isolation from *Bacillus* Species

Chromosomal DNA of *B. licheniformis* DSM 1969 was isolated according to the method given by Posprech and Neumann (1995) with some modifications.

1. Pick a single colony from plate and inoculate a starter culture of 30 ml LB medium. Grow for 12h (overnight) at 30°C, with vigorous shaking (~200 rpm),
2. Harvest bacterial cells by centrifugation at 3000g, 4°C, for 10 min after removing the supernatant, resuspend the bacterial pellet in 5 ml SET buffer,
3. Add lyzosim (1mg/ml) and incubate at 37°C for 60 min to lysate the bacterial cell wall,
4. Add 1/10 volume of 10%SDS onto lysate, mix and immediately add proteinase-K (0.5mg/ml) over the mixture. Incubate the mixture at 4°C for 2 h with gently shaking,
5. Add 1/3 volume of 5M NaCl and equal volume of chloroform and incubate at room temperature for 30 min. with gently shaking,
6. Centrifuge the two-phase mixture at 4500g for 15 min to assure the separation of each phase. Then, take the water phase carefully with micropipette,
7. Precipitate chromosomal DNA by adding equal volume of room temperature isopropanol. Mix and centrifuge immediately at 4500g for 15 min. Wash DNA pellet with 70% ethanol and centrifuge at 4500g for 15 min. Carefully decant the supernatant without disturbing the pellet.
8. Redissolve the DNA in a suitable volume of water.

3.7.13 Natural Transformation of Plasmid DNA into *Bacillus* Species

Recombinant pRB374 shuttle vector was transformed into *Bacillus* species according to the method described by Özçelik, 2003, with some modifications.

1. Pick a single colony from plate and inoculate a starter culture of 5 ml LB medium. Grow for 12h (overnight) at 37°C, with vigorous shaking (~180 rpm),
2. Transfer 250 µl sample from the precultivation medium to an 50 ml LB medium and incubate at 37 °C and 180 min^{-1} for 3 hours,
3. Harvest bacterial cell by centrifugation at 4000 min^{-1}, 4°C for 15 minutes,
4. Wash separated cells with 3ml 1 mM HEPES (pH=7.0) buffer and with cold electrophoration buffer (25% PEG and 0.1M mannitol) twice. Keep cells cold during washing processes.
5. Resuspend washed cells in 1/200 volume of cold electrophoration buffer (~250 µl) and keep on ice for 10 min.
6. Proceed natural transformation by adding 3 µl (~ 100ng) plasmid DNA into 40 µl competent cells.
7. Transfer transformants into sterile culture tubes containing 200 µl of LB medium without antibiotics and incubate at 37°C for 3 h with shaking at 180 min^{-1} to recover cells,
8. Spread the cells over the entire surface of the LB plate containing proper antibiotic using a sterile, bent glass rod,
9. Invert the plates and incubate at 37°C overnight. Selected colonies should be visible in 14-24 hours.

3.7.14 Purification of 6xHis-tagged Proteins

The purification of 6xHis-tagged Proteins was performed by using QIAexpress Ni-NTA spin columns according to manufacturer's recommendations. 6x-His-tagged benzaldehyde lyase proteins were purified from the *E. coli* under native conditions. Overnight cultivated cells (in 30 ml M9 minimal medium) were harvested by centrifugation at 4000g for 15 min and resuspended in 3 ml activation buffer containing 20 mM Tris-HCl (pH=8.0), 0.01 mM TPP, 0.1 mM $MgCl_2$. Cells were lyzed at f=10 s^{-1} for 10 minutes at the agitator bead mill (Retsch, MM 200) by using 30% suspension of glass beads and incubated for 15 min after addition of 1 ml 4X lysis buffer (Appendix F). Lysate was centrifuged at 10000g for 20 min and supernatant was loaded into previously equilibrated Ni-NTA spin column. Centrifugation was performed at 700g for 3-4 min to ensure the effective binding of lysate with resin. Ni-NTA spin column was washed with 600 µl wash buffer twice with an open lid to ensure that the centrifugation step is completed after 2 min. Pellet proteins were eluted with 200 µl elution buffer.

For protein purification from the *Bacillus* species, 3ml supernatant was incubated with 1 ml 4X lysis buffer and remaining procedure was applied.

3.7.15 SDS Page

The proteins secreted were analyzed with SDS-phage according to the method described by Laemmli, et. al. (1970), with some modifications. Buffers and solutions used in SDS-page analyses were stated in Appendix G.

3.7.15.1 Pouring SDS-polyacrlamide Gels

1. Assemble the glass plates according to the manufacturer's instructions.
2. In an Erlenmeyer flask, prepare appropriate volume of solutions containing the desired concentration of acrylamide for 12% separating gel, using the values given in Appendix G. Mix the solutions in order shown. Polymerization will begin as soon as the NNN'N'-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) have been added.
3. Swirl the mixture rapidly and immediately pour the solution into the gap between the glass plates. Leave sufficient space for the stacking gel. Add some water to overlay the acrylamide solution and leave the gel in a vertical position until polymerization is completed.
4. After 30 min, pour off the water and then remove any remaining water with the edge of paper towel and prepare stacking gel the same as separating gel and pour the stacking gel solution directly onto the surface of the polymerized separating gel. Immediately insert combs into the stacking gel avoiding the air bubbles and place the gel in a vertical position

3.7.15.2 Preparation of Samples and Running the Gel

1. While stacking gel is polymerizing, prepare samples in the appropriate volume of sample buffer and heat them to 100^{o}C for 5min.
2. After polymerization is complete (30 min), mount the gel in electrophoresis apparatus and fill the reservoir with running buffer.

3. Load up 30 µl of each sample into the wells and start running with 20 mA. After the dye front has moved into the separating gel increase the applied current and run until the bromophenol blue reaches the bottom.

3.7.15.3 Staining SDS-Polyacrylamide Gels with Coomassie Brilliant Blue

1. After running is completed, immerse the gel in 5 volumes of staining solution and place on a slowly rotating platform for 4h at room temperature.
2. Remove the stain and save it for future use. Destain the gel by soaking it in the methanol:acetic acid solution without the dye on a slowly rotating platform for 4-8h, changing the destaining solution 3-4 times. After destining store the gel in dH_2O.

3.7.15.4 Staining SDS-Polyacrylamide Gels with Siver Salts

1. After running is completed, fix the proteins by incubating the gel for 4-12 hours at room temperature with gentle shaking in 5 gel volumes of fixing solution.
2. Discard the fixing solution, wash gel three times with 50% ethanol for 20 min.
3. Discard ethanol and rinse the gel with dH_2O three times for 20 s.
4. Discard water and impregnate the gel in silver nitrate solution for 20 min at room temperature with gentle shaking.
5. Discard silver nitrate solution and rinse the gel with dH_2O twice for 20 s.
6. Discard water and add developing solution. Incubate the gel at room temperature with gentle agitation. Stained bans of of protein should appear within a few minutes. Continue incubation until the desired contrast is obtained. Quench the reaction by immersing the gel in stop solution.

CHAPTER 4

RESULTS AND DISCUSSION

This study focuses on the benzaldehyde lyase production in the recombinant *E. coli* as intracellular and in the recombinant *Bacillus* species as extracellular, and comparison of the production capacity of the enzyme in the recombinant microorganisms. For this purpose, the study was carried out in three parts. Firstly, the gene encoding benzaldehyde lyase was cloned and expressed in *E. coli* BL21 (DE3) pLySs strain to improve the production capacity. Thereafter, signal peptide of *B. licheniformis* subtilisin Carlsberg with its own ribosomal binding site was fused into the N-terminus of the gene coding benzaldehyde lyase; and hybrid product was cloned and transferred into *Bacillus* species for the extracellular production of the enzyme. Finally, the expression levels of these recombinant microorganisms were compared.

4.1 Development of the Recombinant Microorganisms for Benzaldehyde Lyase Production by Genetic Engineering Techniques.

4.1.1 Development of the Recombinant *E. coli* Strain

In previous studies (Çalık et. al., 2004, 2006), benzaldehyde lyase was produced from the gene present on modified pUC18::*bal* plasmid under the control of *trc* promoter, which is a well known inducible hybrid promoter. In this study because of the characteristics of strong T7 promoter explained in section 2.3.6, pRSETA expression plasmid carrying T7 promoter was selected as an alternative to modified pUC18::*bal* plasmid.

In order to combine the gene encoding benzaldehyde lyase (*bal* gene) in pRSETA two primers, NdeI- *bal* Forward and *bal* Reverse primers were designed firstly; and thereafter, PCR amplified *bal* gene was cloned into pRSETA. Later the recombinant plasmid was transformed into *E. coli* XL1-Blue for selection of fresh transformants possibly carrying recombinant plasmid; and into *E. coli* BL21 (DE3) pLySs and JM109 for comparison of BAL production, while recombinant pUC18::*bal* was transformed into *E. coli* BL21 (DE3) pLySs to show enhanced delivery of BAL.

4.1.1.1 Primer Design for Generation of *bal* Gene

The recombinant pUC18::*bal* plasmid was used as the template sequence for amplification of *bal* gene, (Appendix E), based on the nucleotide sequence defined by Pohl et. al. (2002). Since the whole nucleotide sequence of the template DNA together with the location of the restriction sites were not known, PCR-based cloning was preferred.

For this, *Nde*I-*bal* Forward, where *Nde*I restriction enzyme recognition sequence was added; and *bal* Reverse, where *Sac*I restriction enzyme recognition sequence was added, the primers were designed according to the rules given in section 3.7.3; and the assembly of the primers designed was illustrated in Figure 4.1.

The thermodynamic properties together with melting temperature, T_m, self complimentary and dimer formation affinities were determined by the use of the computer program, NAR, and these properties were demonstrated in Appendix H. Designed primers (Figure 4.1) were synthesized in Thermo Hybaid GmbH (Germany) Laboratories.

The restriction enzymes that were added to the 5' ends were selected from those of not cutting the *bal* gene according to map of sites for restriction endonucleases obtained from Restriction Mapper web-page of ABD Molecular Biology Resources (http://www.restrictionmapper.org) by introducing the gene sequence of *bal* (Pohl et. al., 2002). The entire list of restriction enzymes both cutting and noncutting the *bal* gene were demonstrated in Appendix I and Table A.2.

❶ **Restriction Enzyme Region + NdeI-*bal* Forward Primer**

***Nde*I (5'CATATG3')**

Sequence: 5' CATATGGCGATGATTACAGGC 3'

❷ **Restriction Enzyme Region + *bal* Reverse Primer**

***Sac*I (5'GAGCTC3')**

Sequence: 5' GCGAGCTCGCTTAGTGATGGTGATGGTGATG 3'

Figure 4.1 Schematic illustration of primer design for expression in *E. coli* strains. 1) *Nde*I RE sequence was associated in front of *bal* Forward Primer as a single-stranded tail. *bal* Forward Primer is complimentary to anti-sense strand of the *bal* gene; 2) *Sac*I RE sequence was associated in front of *bal* Reverse Primer as a singe-stranded tail. *bal* Reverse Primer is complimentary to sense strand of the *bal* gene.

4.1.1.2 Amplification of *bal* Gene by Polymerase Chain Reaction (PCR)

After synthesis of proper single-stranded primers, *bal* gene with *Nde*I and *Sac*I restriction enzyme extensions (final length=1726bp) was amplified with polymerase chain reaction (PCR). PCR exploits certain features of DNA replication. In this method, chemically synthesized oligonucleotide primers, *Nde*I-*bal* Forward and *bal* Reverse primers, were added to the reaction mixture and the temperature of the mixture was heated to 94°C in denaturation step in order to obtain single-stranded template DNA for the synthesis of the complimentary new strand. Then the mixture is rapidly cooled to allow the primers designed to anneal to the complimentary sequences in the DNA molecule.

The annealing temperature and time are central variables in determining the specificity of a PCR. Hence, temperatures and times used vary depending on the sequences to be amplified. Time of amplification of each cycle, which can be calculated from the formula of 1000 bp=1 min, was determined as 2 min to

allow enough time for full amplification. The amplification temperature should lay between the range of (T_m-5) ≤ T_a ≤ (T_m+12). Melting temperature of both primers were arranged as 60.7°C (Appendix H), and thus the finest annealing temperature was determined as 55°C.

Since, the optimum temperature of *Taq* DNA polymerase, a heat-resistant enzyme, was 72°C, after primers were bound to single-stranded DNA molecule, the temperature of the reaction mixture was heated to 72°C in extension step.

The parameters and the final compositions of the polymerase chain reaction mixture were given in Tables 4.1 and 4.2. Two different DNA polymerases, *Taq* and proofreading *Pfu* DNA polymerases, were used to improve the yield and accuracy at annealing temperature, T_a, of 55°C. As it can be seen from Figure 4.2, *Taq* DNA polymerase was superior to *Pfu* DNA polymerase in terms of yield, thus, throughout the cloning of *bal* gene in pRSETA experiments *Taq* DNA polymerase was used in PCR.

In principle, physical and chemical components of PCR can be modified to produce a potential increase in yield, specificity and sensitivity. In all PCR experiments, control reactions, i.e., negative control which contains all reaction components except the template DNA in order to visualize whether any contamination occurred; or positive control which contains PCR product of previously amplified *bal* gene in order to make precise comparison with cloned gene, were included in gel electrophoresis analysis.

Table 4.1 PCR process parameters for amplification of *bal* gene.

1 cycle	T_1 = 94°C,	2 min
30 cycle	T_1 = 94°C,	1 min
	T_2 = 55°C,	1 min
	T_3 = 72°C,	2 min
1 cycle	T_3 = 72°C,	5 min
	T_4 = 4°C,	5 min

Table 4.2 Components of reaction mixture of PCR for amplification of *bal* gene.

Components	Amount	Amount
10XPCR Buffer	5µl	5µl
dNTPs (1mM)	10µl	10µl
FP (10µM)	*Nde*I-*bal* FP: 1µl	*Nde*I-*bal* FP: 1µl
RP (10µM)	BAL RP: 1µl	BAL RP: 1µl
Template DNA	~60ng pUC18::*bal*	~60ng pUC18::*bal*
dH_2O	up to 49 µl	up to 49 µl
DNA polymerase	2.5 U (*Taq*)	2.5 U (*Pfu*)

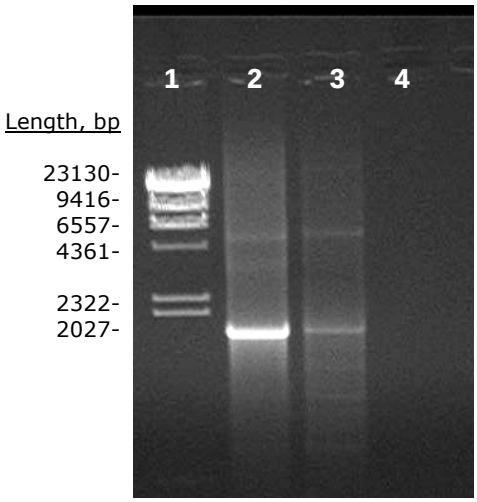


Figure 4.2 Gel electrophoresis image of *bal* gene. 1.well: λDNA/HindIII Marker; 2.well: BAL gene amplified with *Taq* DNA polymerase; 3.well: BAL gene amplified with *Pfu* DNA polymerase; 4.well: Negative control.

The template DNA of pUC18::*bal* plasmid was isolated and purified with Plasmid Purification Kit (QIAGEN) before it was utilized as template, to remove RNA and other proteins, chemicals remaining after isolation, since these materials may inhibit the polymerase and reduce PCR yield.

4.1.1.3 Ligation of *bal* Gene into pRSETA Expression Vector and Transformation in *E. coli* BL21 (DE3) pLySs Strain

PCR amplified *bal* gene was treated with *Nde*I and *Sac*I restriction enzymes (REs) to produce overhanging single-stranded tails- sticky ends, that re-anneal

with complementary single-stranded tails on the pRSETA plasmid which was cut with the same restriction enzymes.

The PCR amplified *bal* gene whose concentration was calculated as 14.5 ng μl^{-1} after purification with PCR Purification Kit (QIAquick), and the pRSETA vector with a concentration of 38.5 ng μl^{-1} after isolated with Plasmid Purification Kit (QIAGEN), were cleaved with *Sac*I and *Nde*I restriction enzymes, separately, in specified buffers (Tables 4.3 and 4.4) of 20µl final volume at 37°C for approximately 2 h and the reaction mixtures were kept at 85°C for 15 min. to denaturize the enzymes used at each step. Between subsequent digestion reactions, processed *bal* gene and linear pRSETA vector were purified by treating with NaAC and EtOH. After separating the fluid by centrifugation from the precipitates, both DNA fragments were dissolved in a suitable amount of water

Table 4.3 Components of reaction mixture of restriction digestion of *bal* gene and pRSETA vector with *Sac*I RE.

	bal gene	pRSETA vector
Components	Amounts	Amounts
DNA fragment	~ 80 ng	~ 120 ng
*Sac*I RE (10U/ µl)	2µl	2µl
Buffer *Sac*I^{+}	2µl	2µl
dH_2O	up to 19µl	up to 19µl

Table 4.4 Components of reaction mixture of restriction digestion of *bal* gene and pRSETA vector with *Nde*I RE.

	bal gene	pRSETA vector
Components	Amounts	Amounts
DNA fragment	~ 65 ng	~ 100 ng
*Nde*I RE (10U/µl)	1.3µl	1.3µl
1X Buffer O	2µl	2µl
dH_2O	up to 19µl	up to 19µl

The ligation reaction is the rate limiting step in genetic engineering techniques since this reaction requires the cohesive ends of foreign DNA and open plasmid DNA to bind in correct orientation. Therefore, optimum ligation

reaction conditions should be determined by increasing the quality and quantity of both the insert DNA and the vector DNA.

Before ligation, the insert DNA, *bal* gene, and the vector DNA, pRSETA, were purified to remove the contaminating reagents and extra bands resulted from the digestion reaction. *bal* gene was purified with PCR Purification Kit (QIAquick); while processed pRSETA vector was purified by extracting the proper band observed in the gel electrophoresis image from the agarose gel with Gene Elusion Kit (GeneMark). At each purification step, after the restriction cleavage, approximately, 20% loss in the concentration of DNA segments were observed; whereas the percentage of loss was much more when samples were purified with PCR purification Kit (30-50%) or with Gene Elusion Kit (40-60%) as it can be seen from Figure 4.3.

After subsequent RE digestion, the cleaved DNA fragments were mixed to allow annealing to form the recombinant molecule by complementary base pairing. The enzyme, T4 DNA ligase, then, chemically bond these annealed fragments into an intact recombinant plasmid by sealing the gaps and covalently binding the two strands. The ligation reaction was proceed at different Gene/Vector ratio of 3 and 5 by mixing the processed *bal* gene, having a concentration of 8.4 ng/µl, and cleaved pRSETA vector, having 26.7 ng/µl concentration (Table 4.5). The reaction mixtures of 20µl final volume were incubated at 22^{o}C for 16 h and the reaction was stopped by incubating the mixtures at 75^{o}C for 10 min.

Table 4.5 pRSETA:: *bal* ligation reaction conditions.

Ligation Reaction	G/V=3	G/V=5
Insert DNA	~ 60 ng	~ 120 ng
Vector DNA	~ 20 ng	~ 24ng
Ligation Buffer (with ATP)	2µl	2µl
T4 DNA ligase (5u/µl)	1µl	1µl
ATP (10mM)	1.5µl	1.5µl
dH_2O	up to 19µl	up to 19µl
TOTAL	20 µl	20 µl

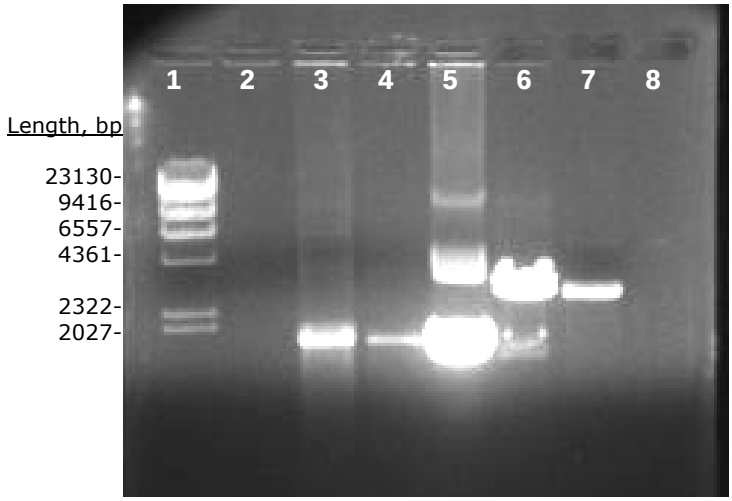


Figure 4.3 Gel electrophoresis image of *bal* gene and pRSETA vector after restriction digestion; 1.well: λDNA/HindIII Marker; 3.well: *bal* gene cut with *Sac*I and *Nde*I; 4.well: Purified *bal* gene cut with *Sac*I and *Nde*I; 5.well: isolated pRSETA vector. 6.well: pRSETA vector cut with *Sac*I and *Nde*I; 7.well: Purified pRSETA vector cut with *Sac*I and *Nde*I.

The assembly of the recombinant molecule, pRSETA::*bal*, was schematically illustrated in Figure 4.4.

Next, the ligation samples were transformed into *E. coli* XL1-Blue strain by $CaCl_2$ method described in section 3.7.9 and fresh transformants were grown on LB-agar medium supplemented with 100 µg/ml ampicilin for 12-18 h. The selected putative recombinant colonies were further incubated for plasmid DNA isolation. Only one colony among the selected colonies gave a band bigger than the pRSETA vector and this plasmid was tested out to control whether the DNA fragment inserted was in proper length and it was attached in correct orientation.

The putative recombinant plasmid was, firstly, checked with PCR by being used as template and the gel electrophoresis image (Figure 4.5) showed that *bal* gene is present in the plasmid. The orientation was controlled with subsequent restriction digestions of the putative recombinant plasmid with *Sac*I and *Pst*I restriction enzymes. The components of restriction digestion of *bal* gene *Pst*I was given in Table 4.6. *Pst*I is cutting the *bal* gene from the 938th nucleotide and thus remaining part which is 778bp in length was expected to be observed in the reaction medium. The gel electrophoresis results prove that *bal* gene was inserted into the pRSETA plasmid in correct orientation (Figure 4.5).

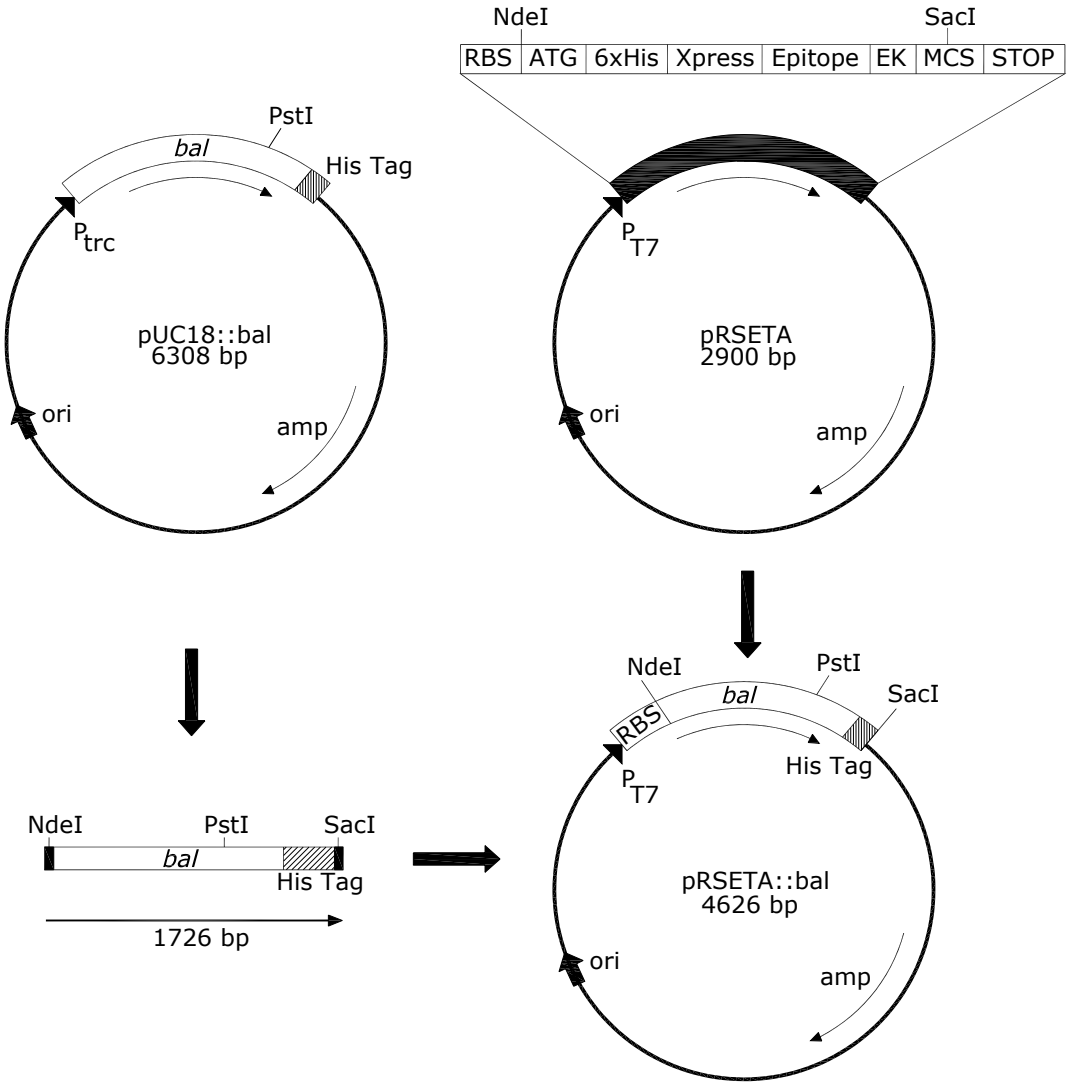


Figure 4.4 Scheme for the construction of an expression system for BAL production in *E. coli* strains. 1.7 kbp DNA fragment of PCR amplified *bal* gene with *Nde*I and *Sac*I endings was associated to pRSETA plasmid from *Nde*I and *Sac*I restriction sites. The insertion and transcription directions were indicated by arrows; Hatched region indicates the location of poly-histidine tag used to the N-terminus of *bal* gene; P, promoter; RBS, ribosomal binding site.

The DNA sequence of insert DNA was further controlled by automatic DNA sequencers, (Microsynth GmbH, Switzerland) and results proved that the cloning was successful. The recombinant pRSETA plasmid was transformed into *E. coli* BL21 (DE3) pLySs strain by $CaCl_2$ method and expression was achieved under 1.0 mM IPTG induction.

Table 4.6 Components of reaction mixture of restriction digestion of the putative recombinant pRSETA vector with *Sac*I and *Pst*I REs.

*Sac*I Digestion		*Pst*I Digestion	
Components	Amounts	Components	Amounts
DNA fragment	~ 120 ng	DNA fragment	~ 150 ng
*Sac*I RE (10U/ µl)	2µl	*Pst*I RE (10U/µl)	1.8µl
Buffer *Sac*I^{+}	2µl	Buffer O	2µl
dH_2O	up to 19µl	dH_2O	up to 19µl

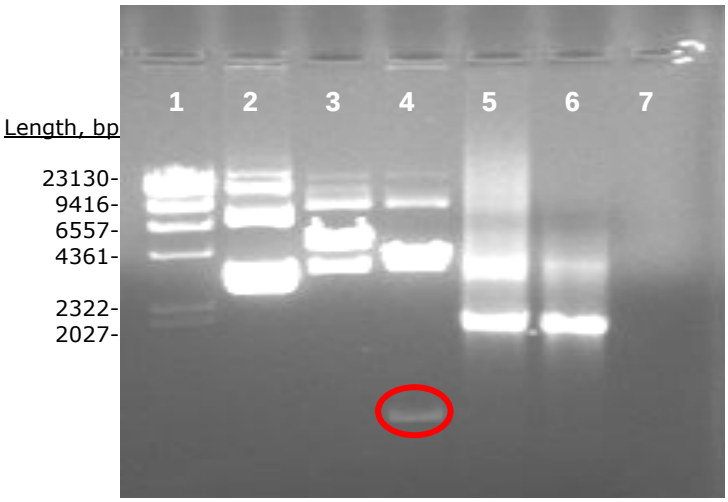


Figure 4.5 Gel electrophoresis image of the recombinant pRSETA vector before and after restriction digestion and PCR product of r-pRSETA plasmid. 1.well: λDNA/HindIII Marker; 2.well: r-pRSETA plasmid; 3.well: linear r-pRSETA cut with *Sac*I; 4.well: r-pRSETA cut with *Sac*I and *Pst*I; 5.well: *bal* gene amplified from r-pRSETA; 6.well: Positive control (*bal* gene); 7. well: Negative control.

4.1.1.4 SDS-Page Analysis of Benzaldehyde Lyase Excreted

The recombinant cells carrying r-pRSETA::*bal* plasmid grown in the reference production medium (RPM) given in Table 3.5 for 12 h were harvested by centrifugation and supernantant was removed. 6x-His-tagged benzaldehyde lyase proteins excreted intracellularly were purified from *E. coli* under native conditions with QIAexpress Ni-NTA spin columns.

Analytical electrophoreses of benzaldehyde lyase was carried out in polyacrylamide gel by dissociating the protein into its individual polypeptide subunits by treating the intracellular mixture of the recombinant *E. coli* BL21 (DE3) pLySs strain with reducing agent SDS followed by heat exposure. The enzyme is composed of 4 x 563 amino acid residues and has a molecular weight of 4 x 58919 Da. (Mosbacher et. al., 2005). After electrophoresis, seperated proteins were stained with Coomassie Brilliant Blue (Figure 4.6). The enzyme migrated in sodium dodecyl sulfate- containing polyacrylamide gel as a single polypeptide with a molecular weight of ~59kDa matching with molecular weight observed for previously utilized *E. coli* K12 strain carrying r-pUC18::*bal* plasmid.

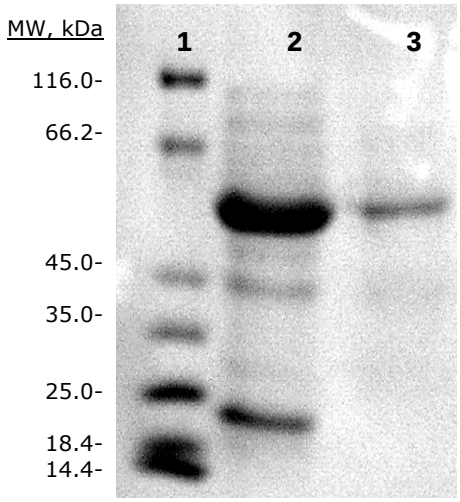


Figure 4.6 SDS-page image of benzaldehyde lyase enzyme: 1.well: Protein Marker; 2.well: purified benzaldehyde lyase produced from *E. coli* BL21 (DE3) pLySs strain carrying pRSETA::*bal*; 3.well: purified benzaldehyde lyase produced from *E. coli* K12 strain carrying pUC18::*bal*.

4.1.2 Development of the Recombinant *Bacillus* Species

In the second part of this study, extracellular production of benzaldehyde lyase was aimed. For this, an expression system was built. Signal peptides are known to be a precursor for protein transformation from cytoplasm through out of the cell. Therefore, signal peptide of subtilisin Carlsberg from chromosomal DNA of *Bacillus licheniformis* DSM 1969 (*pre-sub*C gene) was amplified by polymerase chain reaction with the help of oligonucleotide primers, *pre-sub*C Forward and *pre-sub*C Reverse primers. Next, the gene encoding benzaldehyde lyase (*bal* gene), was amplified with *bal* Forward and *bal* Reverse primers. These

two DNA fragments were associated to each other by gene splicing by overlap extension (SOE) method resulting in *hybrid* gene (*pre-sub*C::*bal*). Further, the fused *hybrid* gene was cloned into pUC19 and sub-cloned into pRB374 *E. coli* / *B. subtilis* shuttle vector from *BamH*I and *Sac*I restriction sites. Later, the engineered plasmid was transformed into firstly, *E. coli* XL1-Blue for selection of fresh transformants possibly carrying recombinant plasmid, then into *Bacillus* species of *B. brevis* NRRL NRS 604, *B. firmus* NRRL B1107, *B. sphaericus* NRRL NRS 732 (NRRL = Northern Regional Research Center), *B subtilis* BGSC-1A751 and *B. subtilis* BGSC-1A1 (BGSC = *Bacillus* Genetic Stock Center), WB600 (Wu et. al., 1991) and WB700 (Ye et. al., 1999) by natural transformation, and expressed under the control of strong *veg*II promoter which provides expression during vegetative phase of the growth.

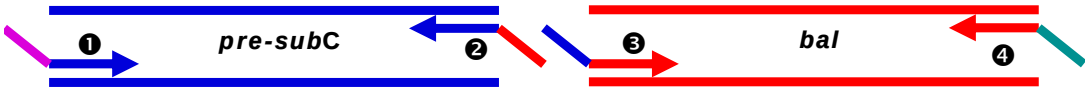
4.1.2.1 Primer Design for Generation of *bal*, *pre-sub*C and *hybrid* Genes

The gene encoding subtilisin Carlsberg from *Bacillus licheniformis* NCIB 6816 (*sub*C) was firstly reported by Jacobs et. al. (1985- Accession no: X03341). The signal peptide of serine alkaline protease, *pre-sub*C gene, was obtained from the chromosomal DNA of *B. licheniformis* DSM 1969 (Çalık et. al., 2003-b) and the recombinant pUC18::*bal* plasmid was used as the template sequence (Pohl, et. al., 2002-Accession no: AX349268) for amplification of *bal* gene.

Complete DNA sequences of the templates were attained from National Institutes of Health (ABD), National Library of Medicine, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and nucleotide sequences were illustrated in Appendix E. Three more primers were designed since the *bal* Reverse Primer used for *bal* gene obtained in section 4.1.1.1 was the same. Four different primers, two for *pre-sub*C gene, others for *bal* gene were used to create the *hybrid* gene. At the beginning of the forward primer of *pre-sub*C, the *BamH*I restriction enzyme recognition sequence was added. As it can be seen from Figure 4.7, extra sequences were added to the 5' end of oligonucleotide primers 2 and 3 in order to provide overlapping ends to the amplified fragments. By this way, in the following denaturation and reannealing processes, strand from the two fragments will act as primers on each other and extension of overlap by DNA polymerase will result in the recombinant hybrid gene. Similarly, to obtain the mature protein gene encoding *bal* from the donor plasmid, the forward primer with complimentary strand to *pre-sub*C and *bal*

reverse primer designed previously with *Sac*I recognition sequence were used (Figure 4.7).

The possibility of dimer and self-complimentary formation affinities and thermodynamic properties of primers were checked with a computer program named NAR and results were summarized in Appendix H. The melting temperature of each primer was arranged to be around 60°C since closer annealing temperatures were required for enhanced amplification of gene of interest.



❶ **Restriction Enzyme Region + *pre-sub*C Forward Primer**
***BamH*I (5'GGATCC3')**
Sequence: 5' GCGGATCCGCAGCAATCTCCTGTCATTCG 3'

❷ **Complimentary Strand to *bal* + *pre-sub*C Reverse Primer**

Sequence: 5' CCTGTAATCATCGCCATAGCAGAAGCGGAATCG 3'

❸ **Complimentary Strand to *pre-sub*C + *bal* Forward Primer**

Sequence: 5' CGATTCCGCTTCTGCTATGGCGATGATTACAGGC 3'

❹ **Restriction Enzyme Region + *bal* Reverse Primer**
***Sac*I (5'GAGCTC3')**
Sequence: 5' GCGAGCTCGCTTAGTGATGGTGATGGTGATG 3'

Figure 4.7 Schematic illustration of primer design for expression in *Bacillus* species. 1) *BamH*I RE sequence was associated in front of pre-subC Forward Primer as a single-stranded tail. pre-subC Forward Primer is complimentary to anti-sense strand of the *bal* gene; 2) Overlapping extension complimentary to BAL anti-sense strand was added to 5' end of pre-subC Reverse Primer, which is complementary to sense strand of pre-subC gene; 3) Overlapping extension complimentary to pre-subC sense strand was added to 5' end of BAL Forward Primer, which is complementary to anti-sense strand of bal gene; 4) *Sac*I RE sequence was associated in front of BAL Reverse Primer as a singe-stranded tail. BAL Reverse Primer is complimentary to sense strand of the *bal* gene.

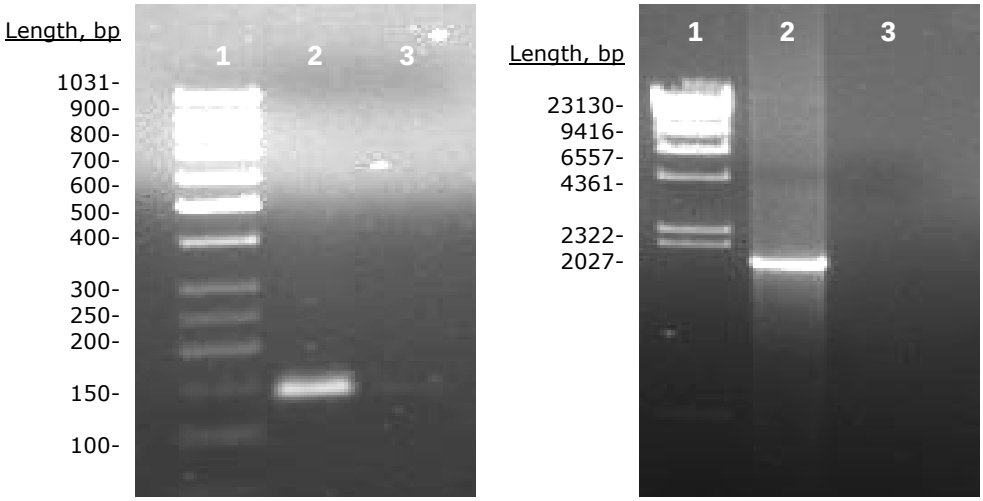
4.1.2.2 Amplification of *bal*, *pre-sub*C and *hybrid* Genes by Polymerase Chain Reaction (PCR) and SOE Method

In the genetic applications performed for *Bacillus* species, the signal sequence of subtilisin Carlsberg gene present on chromosomal DNA (cDNA) of *Bacillus licheniformis* DSM 1969, *pre-sub*C, with *BamH*I recognition sequence (final length=141bp) was isolated as demonstrated in Figure 4.8-a. In the second step the mature protein gene encoding BAL with *Sac*I sequence (final length=1739bp) was obtained from the recombinant pUC18::*bal* plasmid (Figure 4.8-b). These two sequences were associated with gene splicing by overlap extension method (SOE). Complimentary primers, *pre-sub*C Reverse Primer and *bal* Forward Primer, were used to generate two DNA fragments having overlapping ends. These fragments were combined in a subsequent fusion reaction in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand (Ho et. al., 1989). The resulting fusion product was further be amplified in 3rd PCR (Figure 4.9) with a final length of 1846bp.

The annealing temperature and time were determined in accordance with the length of each DNA fragment and PCR components and parameters were illustrated in Tables 4.7 and 4.8. *pre-sub*C and *bal* genes were amplified with proofreading *Pfu* DNA polymerase not to allow any additional nucleotide insertion between the signal peptide and the mature part of *bal* gene since frame shift occurred due to additional residues can alter the 3-D conformation of the expressed protein or decrease the efficiency of signal peptidase act during cleavage of signal peptide from mature part (Simonen et. al., 1993).

Taq DNA polymerase, on the other hand, has an activity to add 3' A-overhangs to each end of the PCR product and thus not recommended for precise combination of two DNA fragments. *Hybrid* gene was produced with *Taq* DNA polymerase since it was previously shown that *Taq* DNA polymerase has superior efficiency in terms of yield and 3' A-overhangs does not cause any frame shift for fused *hybrid* gene.

The annealing temperature of pre-*sub*C, *bal* and *hybrid* genes were kept at 60^{o}C for accurate binding of primers.



(a) (b)

Figure 4.8 Gel electrophoresis image of *pre-sub*C and *bal* genes. (a): 1.well: Low Range Marker; 2.well: *pre-sub*C gene amplified with *Pfu* DNA polymerase; 3.well: Negative control. (b): 1.well: λDNA/HindIII Marker; 2.well: *bal* gene amplified with *Taq* DNA polymerase; 3.well: Negative control.

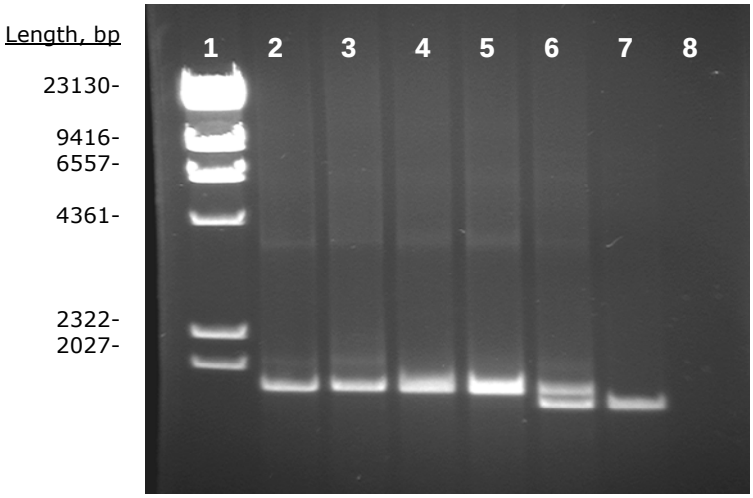


Figure 4.9 Gel electrophoresis image of *hybrid* gene. 1.well: λDNA/HindIII Marker; 2-5.well: *hybrid* gene amplified with *Taq* DNA polymerase; 6.well: *hybrid*+*bal* genes for control; 7.well: Positive control (*bal* gene) control; 8.well: Negative control.

Table 4.7 PCR process parameters for amplification of *pre-sub*C, *bal* and *hybrid* genes.

		Genes to be amplified		
		*pre-sub*C gene	*bal* gene	*hybrid* gene
1 cycle	T_1 = 94°C,	2 min.	3 min.	3 min.
30 cycle	T_1 = 94°C,	1 min.	1 min.	1 min.
	T_2 = 60°C,	1 min.	1 min.	1 min.
	T_3 = 72°C,	15 s.	2 min.	2.5 min.
1 cycle	T_3 = 72°C,	5 min.	10 min.	10 min.
	T_4 = 4°C,	5 min.	5 min.	5 min.

Table 4.8 Components of reaction mixture of PCR for amplification of *pre-sub*C, *bal* and *hybrid* genes

	Genes to be amplified		
	*pre-sub*C gene	*bal* gene	*hybrid* gene
Components	Amount	Amount	Amount
10XPCR Buffer	5µl	5µl	5µl
dNTPs (1mM)	10µl	10µl	10µl
FP (10µM)	pre-subC FP: 1µl	BAL FB: 1µl	pre-subC FP: 1µl
RP (10µM)	pre-subC RP: 1µl	BAL RP: 1µl	BAL RP: 1µl
Template DNA	~3 µg cDNA	~60 ng pUC18::*bal*	~30 ng *pre-sub*C + ~30 ng *bal* genes
dH_2O	up to 49 µl	up to 49 µl	up to 49 µl
DNA polymerase	2.5 U *Pfu*	2.5 U *Pfu*	2.5 U *Taq*

Starting with pure template DNA can eliminate the any kind of contamination especially when fresh PCR products were directly used as template since reaction mixture can contain many components like salts, proteins, metals and organic solvents which can inhibit the polymerase efficiency and reduce PCR yield. Also having lots of redundant substances in the reaction mixture can mask the required amplifiable templates resulting in reduced yield.

Therefore, *pre-sub*C and *bal* genes were purified with PCR Purification Kit (QIAquick) before utilized as template DNA.

4.1.2.3 Ligation of *hybrid* Gene into pUC19 Cloning Vector and Transformation in *E. coli* XL1-Blue Strain

PCR amplified and purified *hybrid* gene (C_{hybrid} = 28.2 ng $\mu l^{-}1$) and isolated pUC19 vector (C_{pUC19} = 59.8 ng μl^{-1}) were digested with, firstly, *Sac*I, then with *BamH*I restriction enzymes in reaction mixture of 20 µl final volume whose components were listed in Tables 4.9 and 4.10, subsequently, for two hours at 37°C. The order of subsequent RE cleavage reaction was determined according to the salt concentration of specified buffers of REs. Since the salt concentration of Buffer *BamH*I (~10 mM) was superior than that of Buffer *Sac*I^{+} (~ 10 mM), both hybrid gene and vector DNAs were firstly treated with *Sac*I, then *BamH*I. After restriction digestion, reaction mixtures were kept at 85°C for 15 min. to end the reaction. Between each subsequent restriction digestion, DNA fragments were purified by precipitating with NaAc and EtOH. Thereafter, digested and purified *hybrid* gene (C_{hybrid} = 9.1 ng μl^{-1}) was cloned into purified linear pUC19 cloning vector (C_{pUC19} = 15.4 ng μl^{-1}) treated with the same restriction enzymes from the multiple cloning site present onto *LacZ* gene fragment according to the ligation reaction conditions illustrated in Table 4.11. Purified gene fragments were associated by keeping the Gen/Vector ratio at 3 and at 22°C for 16 h.

After transformation of ligation product into *E. coli* XL1-Blue strain by $CaCl_2$ method, transformants were incubated on LB-agar medium supplemented with 100 µg/ml ampicilin, IPTG and X-GAL for 12-18 h. Putative colonies were selected according to the blue-white screening method and incubated overnight in LB Broth supplemented with 100 µg/ml ampicilin at 37°C for plasmid DNA isolation. The assembly of the recombinant molecule, pUC19::*hybrid*, was schematically illustrated in Figure 4.10.

Table 4.9 Components of reaction mixture of restriction digestion of *hybrid* gene and pUC19 cloning vector with *Sac*I RE.

	Hybrid gene	pUC19 plasmid
Components	Amounts	Amounts
DNA fragment	~ 60 ng	~ 200 ng
*Sac*I RE (10U/ µl)	2.5µl	2.5µl
Buffer *Sac*I^{+}	2µl	2µl
dH_2O	up to 19µl	up to 19µl

Table 4.10 Components of reaction mixture of restriction digestion of *hybrid* gene and pUC19 cloning vector with *BamH*I RE.

	Hybrid gene	pUC19 plasmid
Components	Amounts	Amounts
DNA fragment	~ 45 ng	~ 180 ng
*BamH*I RE (10U/ µl)	2.5µl	2.5µl
Buffer *BamH*I	2µl	2µl
dH_2O	up to 19µl	up to 19µl

Table 4.11 pUC19:: *hybrid* ligation reaction conditions.

Ligation Reaction	G/V=3
Insert DNA	~ 90 ng
Vector DNA	~ 30 ng
Ligation Buffer (with ATP)	2µl
T4 DNA ligase (5u/µl)	1µl
ATP (10mM)	2µl
dH_2O	up to 19µl
TOTAL	20 µl

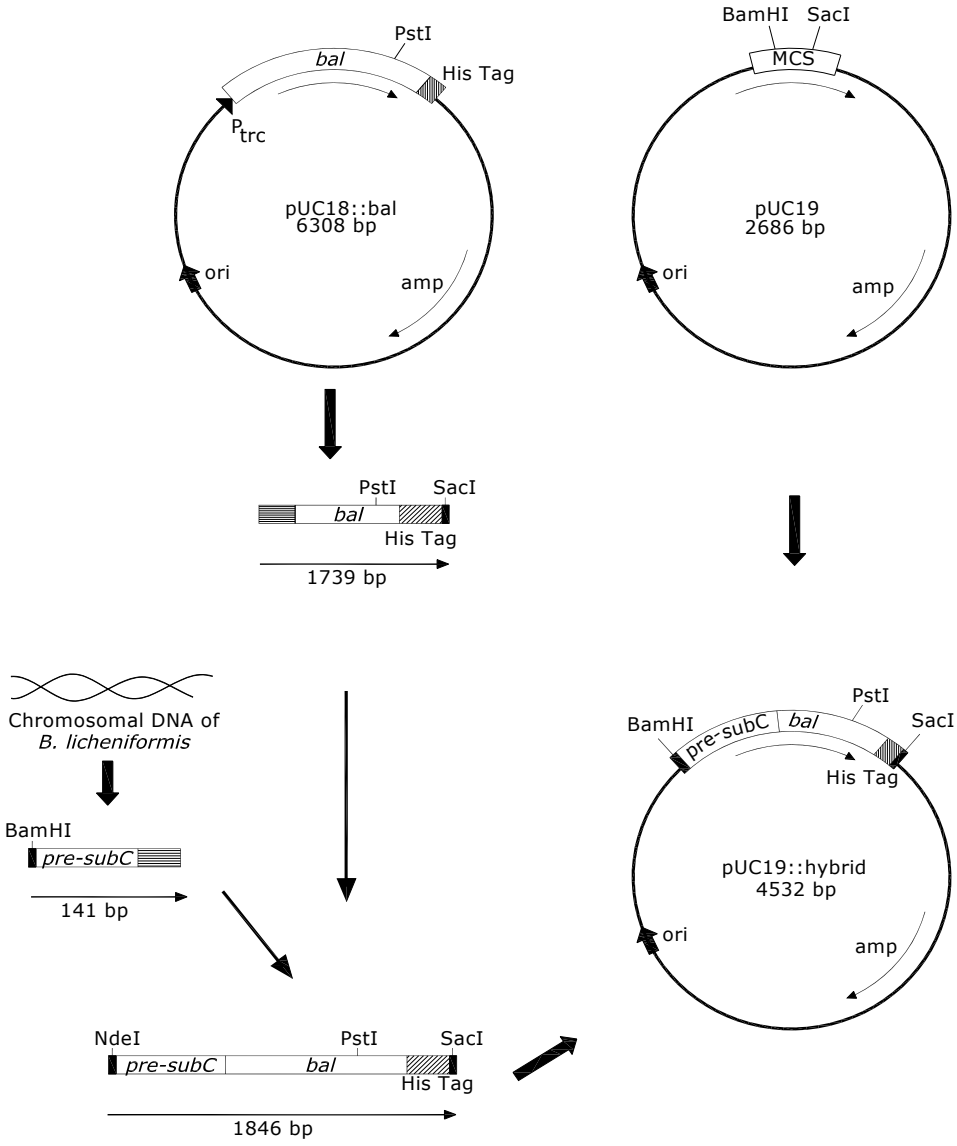


Figure 4.10 Scheme for the construction of the recombinant pUC19::*hybrid*. 141 bp DNA fragment of signal peptide of serine alkaline protease with *BamH*I ending and complementary sequence to *bal* gene (horizontally hatched region) was associated to 1739 bp DNA fragment of *bal* gene having *Sac*I end and complementary sequence to *pre-sub*C gene leading to 1846 bp DNA fragment of *hybrid* gene. *Hybrid* gene *(pre-subC::bal)* with *Sac*I and *BamH*I endings was cloned into pUC19 plasmid from *Sac*I and *BamH*I restriction sites. The insertion and transcription directions were indicated by arrows; Cross hatched region indicates the location of poly-histidine tag used to the N-terminus of *bal* gene; P, promoter; MCS, multiple cloning site.

The putative recombinant pUC19::*hybrid* plasmids were analyzed by restriction digestion to confirm the insertion of the *hybrid* gene to the cloning plasmid. The putative recombinant plasmids were treated, firstly, with *Sac*I and *BamH*I restriction enzymes and a gene portion in the same length of *hybrid* gene was observed. The cut putative recombinant plasmids were further digested with *Pst*I restriction enzyme, which is known to cut the *hybrid* gene from the middle leading to two gene fragments of 1058bp and 778bp. The gel electrophoresis results verified that the *hybrid* gene was cloned into pUC19 cloning vector in correct orientation (Figure 4.11).

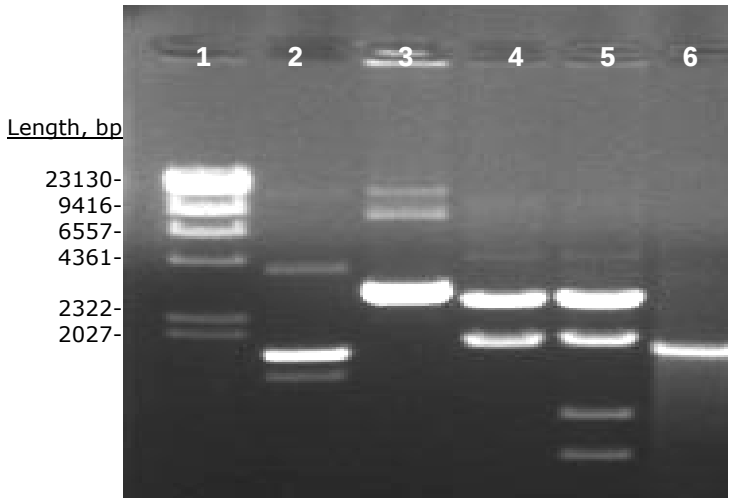


Figure 4.11 Gel electrophoresis image of the recombinant pUC19 vector before and after restriction digestion. 1.well: λDNA/HindIII Marker; 2.well: pUC19 plasmid; 3.well: r-pUC19 plasmid; 4.well: linear r-pUC19 cut with *Sac*I and *BamH*I; 5.well: r-pUC19 cut with *Sac*I, *BamH*I and *Pst*I; 6.well: Positive control (BAL gene).

4.1.2.4 Ligation of *hybrid* Gene into pRB374 Expression Vector and Transformation in *Bacillus* Species

The *hybrid* gene sub-cloned into pUC19 cloning vector was isolated from r-pUC19 plasmid by subsequent digestion of the plasmid with *Sac*I and *BamH*I (Tables 4.12 and 4.13) and purified from agarose gel. Similarly, isolated pRB374 *E. coli*/*B. subtilis* shuttle vector was cut with the same REs and purified by Gene Elusion Kit (Figure 4.13).

The cut *hybrid* gene (C_{hybrid} = 8.2 ng μl^{-1}) was ligated to purified linear pRB374 plasmid (C_{pRB374} = 12.0 ng μl^{-1}) from the cohesive ends by the help of T4 DNA ligase and reaction was performed at 22^0C for 16h (Table 4.14). The ligation mixture was transformed into *E. coli* XL1-Blue by $CaCl_2$ method and putative colonies carrying r-pRB374 plasmid with ampicilin resistance gene were picked from solid medium and isolated for further control experiments.

The assembly of the recombinant molecule, pRB374::*hybrid*, was schematically illustrated in Figure 4.12.

Similar tests performed with r-pUC19 plasmid were carried out to ensure the insertion of the gene in correct orientation. The putative recombinant pRB374::*hybrid* plasmids were explored to the RE digestions and PCR by being used as template DNA. The gel electrophoresis images (Figure 4.14) show that cloning was successful.

The DNA sequence of the insert DNA was further controlled by automatic DNA sequencers (Microsynth GmbH, Switzerland) and results proved that cloning was successful.

The recombinant pRB374::*hybrid* plasmids were further transformed into *Bacillus* species of *B. firmus*, *B. brevis*, *B. sphaericus* and *B. subtilis* with protease deficient *apr*$^-$and *npr*$^-$, *spo*$^-$, and multiple protease deficient WB600 and WB700 strains by natural transformation. The recombinant colonies carrying r-pRB374::*hybrid* plasmids were grown on agar slants supplemented with 10 µg/ml kanamycin.

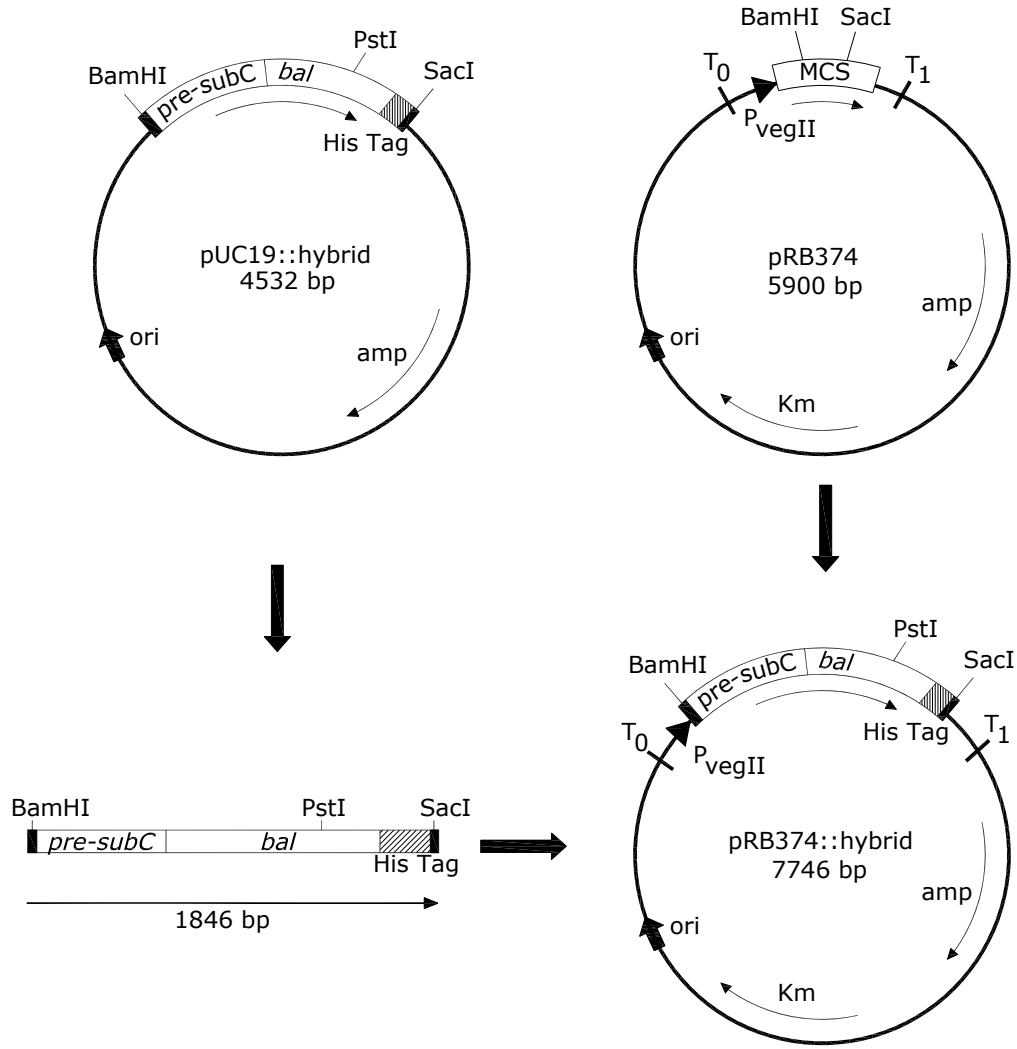


Figure 4.12 Scheme for the construction of an expression system for BAL production in *Bacillus* species. 1846 bp DNA fragment of *hybrid* gene (*pre-sub*C::*bal*) with *Sac*I and *BamH*I endings was cloned into pRB374 plasmid from *Sac*I and *BamH*I restriction sites. The insertion and transcription directions were indicated by arrows; Cross hatched region indicates the location of poly-histidine tag used to the N-terminus of *bal* gene; P, promoter; MCS, multiple cloning site.

Table 4.12 Components of reaction mixture of restriction digestion of r-pUC19 and pRB374 plasmids with *Sac*I RE.

	r-pUC19	pRB374
Components	Amounts	Amounts
DNA fragment	~ 150 ng	~ 200 ng
*Sac*I RE (10U/ µl)	2.0µl	2.0µl
Buffer *Sac*I^{+}	2µl	2µl
dH_2O	up to 19µl	up to 19µl

Table 4.13 Components of reaction mixture of restriction digestion of r-pUC19 and pRB374 plasmids with *BamH*I RE.

	r-pUC19	pRB374
Components	Amounts	Amounts
DNA fragment	~ 120 ng	~ 160 ng
*BamH*I RE (10U/ µl)	2.5µl	2.5µl
Buffer *BamH*I	2µl	2µl
dH_2O	up to 19µl	up to 19µl

Table 4.14 pRB374:: *hybrid* ligation reaction conditions.

Ligation Reaction	G/V=3
Insert DNA	~ 90 ng
Vector DNA	~ 30 ng
Ligation Buffer (with ATP)	2µl
T4 DNA ligase (5u/µl)	1µl
ATP (10mM)	2µl
dH_2O	up to 19µl
TOTAL	20 µl

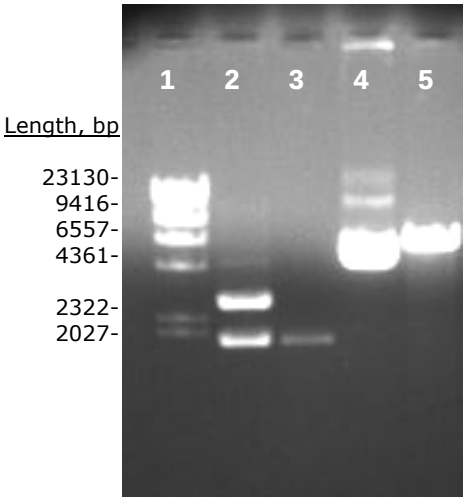


Figure 4.13 Gel electrophoresis image of the recombinant pUC19 and pRB374 plasmids before and after purification. 1.well: λDNA/HindIII Marker; 2.well: linear r-pUC19 cut with *Sac*I and *BamH*I; 3.well: purified *hybrid* gene with gene elusion kit; 4.well: linear pRB374 plasmid cut with *Sac*I and *BamH*I; 5.well: purified linear pRB374 plasmid with gene elusion kit.

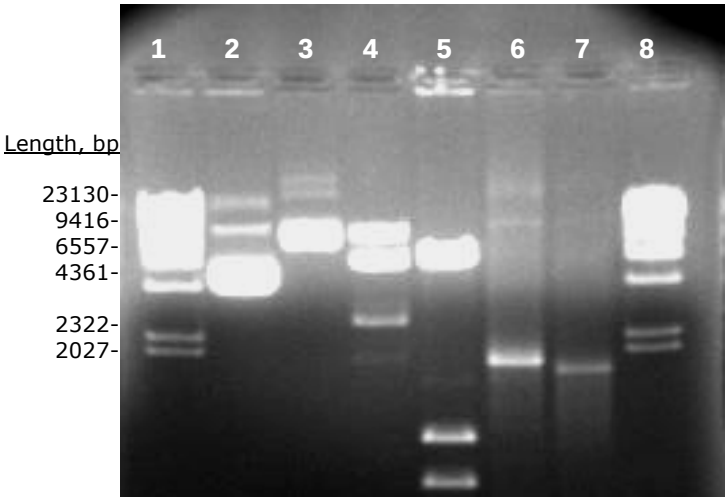


Figure 4.14 Gel electrophoresis image of the recombinant pRB374 plasmid before and after restriction digestion and PCR results. 1.well: λDNA/HindIII Marker; 2.well: pRB374 plasmid; 3.well: r-pRB374 plasmid; 4.well: linear r-pRB374 cut with *Sac*I and *BamH*I; 5.well: r-pRB374 cut with *Sac*I, *BamH*I and *Pst*I; 6.well: *hybrid* gene amplified from r-pRB374; 7. well: *bal* gene amplified from r-pRB374; 8. well: λDNA/HindIII Marker.

4.2 Expression of Benzaldehyde Lyase in r- *E. coli* BL21 (DE3) pLySs Strain

According to the research program for production of benzaldehyde lyase, the recombinant pRSETA::*bal* and pUC18::*bal* plasmids were transformed into various *E. coli* strains and the effects of bioprocess operation parameters were investigated in mainly three parts. After selecting the *Escherichia coli* strain and proper recombinant plasmid having the highest benzaldehyde lyase production capacity, the production medium was designed in terms of carbon and nitrogen sources; and by using designed medium, bioreactor operation parameters, i.e., oxygen transfer conditions together with the oxygen transfer characteristics in pilot scale bioreactor were examined.

4.2.1 Microorganism Selection

The recombinant plasmids pRSETA::*bal* and pUC18::*bal* were transformed into *E. coli* JM 109 and *E. coli* BL21 (DE3) pLySs; and *E. coli* K12 and *E. coli* BL21 (DE3) pLySs, respectively, by $CaCl_2$ method and grown in reference production medium (Table 3.6) induced with 1mM IPTG at t=4 h to find out the microorganism having the highest benzaldehyde lyase production capacity.

As it is seen from Figure 4.12, the highest benzaldehyde activity was observed in *Escherichia coli* BL21 (DE3) pLySs strain carrying the pRSETA::*bal* plasmid in reference production medium as 910 U cm^{-3}, followed by *E. coli* BL21 (DE3) pLySs carrying pUC18::*bal*, *E. coli* K12 carrying pUC18::*bal* and by *E. coli* JM 109 carrying pRSETA::*bal* with the activity values of 760, 745 and 375 U cm^{-3}, respectively. As it was clearly seen from the Figure 4.15, *E. coli* BL21 (DE3) pLySs strain was resulted in higher productivity with both recombinant plasmids, which was previously observed in the studies of Choi et. al. (1997), where the highest bovine growth hormone production was found in *E. coli* BL21 strain among the investigated *E. coli* strains and in the study of Shiloach et. al., (1996), where *E. coli* BL21 (λDE3) was selected as the host strain for the recombinant protein expression. Therefore, in the first part of the study of expression of benzaldehyde lyase in *Escherichia coli*, BL21 (DE3) pLySs strain carrying the pRSETA::*bal* was selected as potential producer of the benzaldehyde lyase (Figure 4.15).

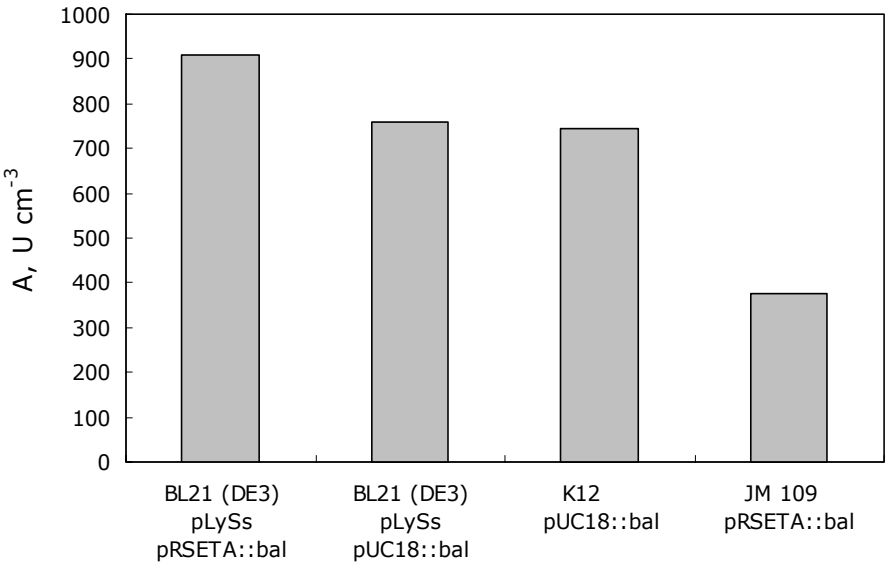


Figure 4.15 The variations in benzaldehyde lyase activity for various *E. coli* strains carriying different expression vectors. C_G^o = 8.0 kg m^{-3}, C_N^o = 5.0 kg m^{-3}, t=12 h, V=33 cm^3, T=37°C, N=200 min^{-1}.

4.2.2 Medium Design

After the selection of the microorganism, a defined medium was designed in order to increase the benzaldehyde lyase production and the cell growth rate. The medium described in Çalık et. al (2006) was decided to be the starting point for medium design and named as RPM (reference production medium), in this perspective, the effects of the concentrations of inorganic nitrogen sources, i.e., $(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$; carbon source, i.e., glucose; and their concerted effects; were investigated in order to compare the results with that of Çalık et. al. (2006). The experiments were conducted in agitation (N=200 min^{-1}) and heating rate (T=37°C) controlled laboratory scale bioreactors.

In the RPM (Table 3.6), the benzaldehyde lyase volumetric activity and cell concentration were found as 910 U cm^{-3} and 1.5 kg m^{-3}, respectively (Figure 4.15).

4.2.2.1 The Effect of Inorganic Nitrogen Sources

The effect of inorganic nitrogen sources of NH_4Cl, $(NH_4)_2HPO_4$, and $(NH_4)_2SO_4$ was investigated at the initial concentrations of NH_4Cl, (kg m^{-3}): 5.0, 5.5, 6.0, 6.5; $(NH_4)_2HPO_4$, (kg m^{-3}): 4.0, 4.5, 5.0, 5.5, 6.0; and $(NH_4)_2SO_4$, (kg m^{-3}): 4.5, 5.0, 5.5 by omitting the nitrogen source from the RPM medium. Glucose concentration, C_G^o, was kept at 8 kg m^{-3}. It was observed that both cell concentration and benzaldehyde activity values were lowest when $(NH_4)_2SO_4$ was used, because of the inhibitory effect of $SO_4^=$ on cell metabolism. These results are in agreement with the results of Çalık et. al. (2006). The highest cell concentration and benzaldehyde lyase activity were attained when $(NH_4)_2HPO_4$ was utilized as the inorganic nitrogen source as 1.5 kg m^{-3} and 955 U cm^{-3}, respectively; and both the cell growth and enzyme activity values tended to decrease at higher $(NH_4)_2HPO_4$ concentrations which can be explained by inhibitory effect of high nitrogen content on both variables (Table 4.15).

In the study of Çalık et. al. (2006), the optimum nitrogen content for maximum BAL production was declared to be $(NH_4)_2HPO_4$ with a concentration of 5.0 kg m^{-3} when initial glucose concentration was 8.0 kg m^{-3}. The volumetric activity and cell concentration in this medium was reported as 1.8 kg m^{-3} and 745 U cm^{-3}, respectively. Relative to the findings of Çalık et. al., (2006), in this study, 1.3-fold increase in BAL activity was obtained, in spite of the 1.2-fold decrease in the cell growth; thus, BAL specific activity increased 1.6-fold.

4.2.2.2 The Effect of Initial Glucose Concentrations

The most frequently applied energy source for cellular growth is glucose since it can be metabolized easily by microorganisms. After determination of the nitrogen source, the effect of initial glucose concentration on benzaldehyde lyase production at C_N^o=4.5 kg m^{-3} was examined by altering the amount of glucose of C_G^o=6.0, 8.0, 10.0, 12.0, 15.0, 18.0, 20.0 and 25.0 kg m^{-3}. Cell concentration and benzaldehyde lyase activity profiles had a similar trend in response to initial glucose concentration and gave slight distinction with respect to glucose concentration. As the initial glucose concentration increases, cell concentration and benzaldehyde lyase activity first increased and then each gave a maximum when the initial glucose concentration was 8.0 kg m^{-3} (Figure 4.16).

Table 4.15 The variations in cell concentration and benzaldehyde lyase activity with the initial nitrogen source concentration. C_G^o = 8.0 kg m^{-3}, C_N^o = 5.0 kg m^{-3}, t=12 h, V=33 cm^3, T=37°C, N=200 min^{-1}.

Component	kg m^{-3}	C_x, kg m^{-3}	A, U cm^{-3}
NH_4Cl	5.0	1.2	695
	5.5	1.3	840
	6.0	1.3	875
	6.5	1.3	905
$(NH_4)_2HPO_4$	4.0	1.4	945
	4.5	**1.5**	**955**
	5.0	1.5	910
	5.5	1.5	905
	6.0	1.5	900
$(NH_4)_2SO_4$	4.5	1.1	675
	5.0	1.1	736
	5.5	1.2	760

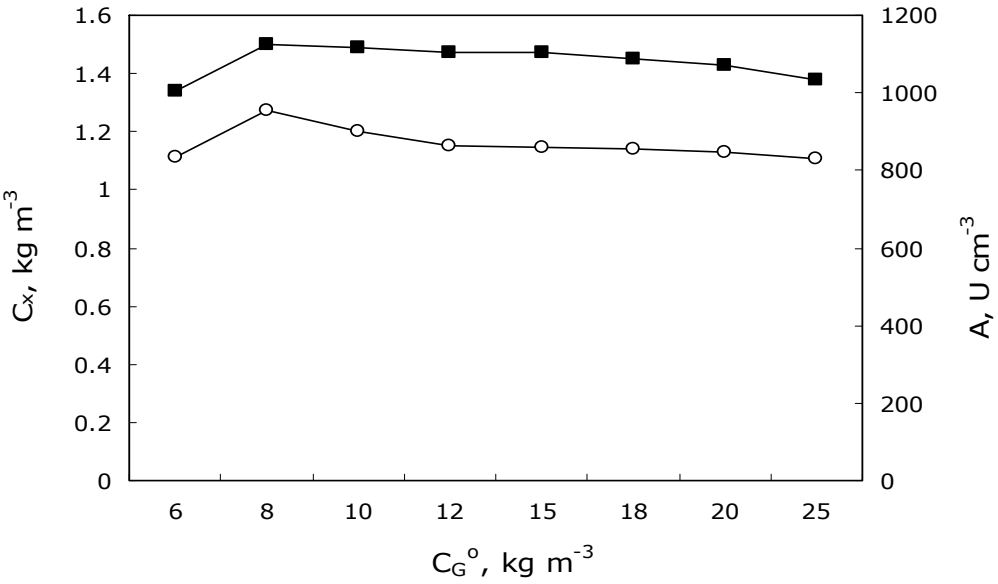


Figure 4.16 The variations in cell concentration and benzaldehyde lyase activity with the initial glucose concentration. C_N^o = 4.5 kg $m^{-}3$, t=12 h, V=33 cm^3, T=37°C, N=200 min^{-1}. Cell concentration: (■); Benzaldehyde lyase activity: (○).

Both cell concentration and benzaldehyde lyase activity decreased at higher initial glucose concentrations and previous optimum condition of C_G^o= 8.0 kg m^{-3} and C_N^o= 4.5 kg m^{-3} gave the highest cell concentration of C_x=1.5 kg m^{-3} and benzaldehyde lyase activity of A=955 U cm^{-3} at t=12 h of the bioprocess (Figure 4.16), similar to the findings of Çalık et. al. (2006), where the recombinant *E. coli* K12 was selected as the host. It seems that, initial glucose concentration of 8.0 kg m^{-3} at low nitrogen source concentrations is ideal for optimum cell growth and, hence the BAL yield in the recombinant *E. coli* strains.

4.2.2.3 The Concerted Effects of Initial Glucose Concentrations and $(NH_4)_2HPO_4$

The effect of initial glucose concentration was further studied at constant C_G^o/C_N^0=1.7 to examine the concerted effect of glucose together with $(NH_4)_2HPO_4$ concentrations. As it is seen in Table 4.16, higher glucose concentration of 20 kg m^{-3} and $(NH_4)_2HPO_4$ concentration of 11.8 kg m^{-3} resulted as the optimum condition in terms of the cell growth and benzaldehyde lyase productivity, having values of cell concentration C_x=2.0 kg m^{-3} and benzaldehyde lyase activity of A=1060 U cm^{-3}. Although, higher values of both glucose and nitrogen source have an inhibitory effect on the cell growth, under the combined excess condition, the maximum productivity was obtained.

Table 4.16 The variations in cell concentration and benzaldehyde lyase activity with initial glucose concentration at constant C_G^o/C_N^0. C_G^o/C_N^0=1.7, t=12 h, V=33 cm^3, T=37°C, N=200 min^{-1}.

Glucose kg m^{-3}	$(NH_4)_2HPO_4$ kg m^{-3}	C_x, kg m^{-3}	A, U cm^{-3}
6	3.5	1.3	900
8	4.5	1.5	955
10	5.9	1.6	965
12	7	1.7	975
15	8.8	1.7	1000
18	10.5	1.8	1040
20	**11.8**	**2.0**	**1060**
25	14.7	1.7	970

4.2.2.4 The Optimized Medium

As a consequence, among the investigated media, the highest cell concentration and benzaldehyde lyase activity was obtained as 2.0 kg m^{-3} and 1060 U cm^{-3}, respectively, in the medium containing 20.0 kg m^{-3} glucose, 11.8 kg m^{-3} $(NH_4)_2HPO_4$ and the salt solution. The activity obtained in the optimized medium was 1.2-fold higher than the activity obtained in the reference production medium (Table 3.6).

4.2.3 Bioreactor Operation Parameters

By using *Escherichia coli* BL21 (DE3) pLysS and the designed medium, the effect of oxygen transfer was studied together with the fermentation and oxygen transfer characteristics of the bioprocess, in the pilot scale bioreactors.

4.2.3.1 Oxygen Transfer Effects

In aerobic processes, oxygen is a key substrate for the cell growth and, therefore, continuous transfer of oxygen from the gas phase to liquid phase is crucial. The effect of oxygen transfer was studied systematically for benzaldehyde lyase production in the recombinant *E. coli* BL21 (DE3) pLySs strain by altering the parameters of air inlet rate, Q_O/V_R, and agitation rate, N at the stated conditions in Table 4.17.

This research program was carried out at three different conditions with the parameters, air inlet rate of Q_O/V_R = 0.5 vvm and agitation rates of N=500 and 750 min^{-1}, and at Q_O/V_R = 0.7 vvm and N=750 min^{-1}, while medium components were C_G^o= 20.0 kg m^{-3}, C_N^o= 11.8 kg m^{-3} and salt solution. The oxygen transfer condition at Q_O/V_R = 0.5 vvm and N=500 min^{-1}, was named as 'low oxygen transfer' condition (LOT); following operation at Q_O/V_R = 0.5 vvm and N=750 min^{-1} was named as 'medium oxygen transfer' condition (MOT); and the last operation at Q_O/V_R = 0.7 vvm, N=750 min^{-1} was named as 'high oxygen transfer' condition (HOT) according to the amount of oxygen supplied to the bioreactor systems. Moreover, to find out the effects of initial glucose and inorganic $(NH_4)_2HPO_4$ concentrations on fermentation and oxygen transfer characteristics, optimum glucose concentration of C_G^o= 8.0 kg m^{-3} at the constant $(NH_4)_2HPO_4$ concentration of C_N^o= 4.5 kg m^{-3} together with air inlet rate of Q_O/V_R = 0.5 vvm,

and the agitation rate of N=750 min^{-1} was tested, and named as 'medium oxygen transfer*' condition (MOT*).

The investigation of the effects of oxygen transfer on benzaldehyde lyase production was performed in batch-bioreactor systems, equipped with temperature, pH, foam, air inlet and stirring rate controls with V_R =550 cm^3 working volume.

Table 4.17 Oxygen transfer conditions and their abbreviations.

Medium Components C_G^o, kg m^{-3}; C_N^o, kg m^{-3}	Oxygen Transfer Condition Applied N, min^{-1}; Q_O/V_R, vvm	Abbreviation
C_G^o =20; C_N^o=11.8	N=500, Q_O/V_R = 0.5	LOT
C_G^o =20; C_N^o=11.8	N=750, Q_O/V_R = 0.5	MOT
C_G^o =20; C_N^o=11.8	N=750, Q_O/V_R = 0.7	HOT
C_G^o =8.0; C_N^o=4.5	N=750, Q_O/V_R = 0.5	MOT*

4.2.3.1.1 Dissolved Oxygen and pH Profiles

The variations in the dissolved oxygen and medium pH with the cultivation time, agitation rate and air inlet rates applied and medium components are respectively illustrated in Figures 4.17 and 4.18.

Dissolved oxygen (DO) level in the fermentation broth is an indicator of oxygen limitations occurred due to exceeding rate of oxygen consumption over the oxygen supply. At all oxygen transfer conditions due to the high oxygen demand of the cells at the beginning of the bioprocess, a generous decrease was observed in the dissolved oxygen profiles; and system's DO level remained under 20% (C_{DO}< 0.04 mol m^{-3}) of the saturation at all oxygen transfer conditions until t=10 h and profiles gradually increased, in general, until the end of the bioprocess at MOT and HOT conditions; while it was below 10 % (C_{DO}< 0.02 mol m^{-3}) of the saturation at LOT condition throughout the process (Figure 4.15). It is known that the ionic strength of the fermentation media can affect the transport of certain nutrients in and out of cells, the metabolic functions of cells, and the solubility of certain substrates, such as dissolved oxygen (Shuler and Kargı, 2002). Thus, the highest decrease rate in the medium pH and insufficient oxygen

level of LOT condition, where the increase in H^+ ion concentration in the fermentation broth was the highest (Figure 4.18), can be explained by concerted effect of these parameters to each other. Conversely, DO level of MOT* condition decreased to 20-30% (C_{DO}= 0.04-0.06 mol m^{-3}) of saturation of dissolved oxygen concentration until t=11h and steadily increased until the process ending.

In the study of Çalık et. al. (2004), it was shown that physical K_La_o values were increasing with the amount of oxygen supplied to the bioreactors ranging between 0.007-0.020. When K_La_o values of present study were compared with that of Çalık et. al. (2004), LOT, MOT and MOT* conditions are corresponding to the MOT1 condition (Q_O/V_R = 0.5vvm, N=500 min^{-1}) approximately; whereas HOT condition is matching with HOT1 (Q_O/V_R = 0.5 vvm, N=750 min^{-1}) as defined in the study of Çalık et. al. (2004). As it was explained in section 2.4.3.3 and 2.4.3.4, there are lots of parameters, mostly specific for the system used, affecting the volumetric mass transfer coefficient, K_La. Assuming an ideal bioreactor, where complete mixing takes place instantaneously, overall value of K_La depends on specific power input, P/V_R, and the superficial gas velocity, u_s. The superficial gas velocity increases when the cross sectional area of the tank decreases, if the same vvm-value is maintained. The power input mostly depends on the stirrer rate, N, and the impeller diameter, when the density and the viscosity of the fermentation broth are not changing significantly (Nielsen and Villadsen, 1994). At MOT* condition, where the density and the viscosity of the broth were assumed to be almost the same with that of used by Çalık et. al. (2004), the only parameter affecting the physical mass transfer coefficient, K_La_o, was the volume of the bioreactor together with the impeller diameter. Therefore, when the equation 2.28 was applied to find out the corresponding agitation rate, N, to the bioreactor system used by Çalık et. al. (2004), where the working volume was V_R=1.65 dm^3, the scaled up stirring rate was found to be approximately 590 min^{-1}, if the fermentation was carried out in the bioreactor used in the study of Çalık et. al. (2004). According to this result, MOT* condition corresponds to somewhere between MOT1 (Q_O/V_R = 0.5 vvm, N=500 min^{-1}) and MOT2 conditions (Q_O/V_R = 0.5 vvm, N=625 min^{-1}) as defined in the study of Çalık et. al. (2004). These results verify the effect of different stirrers and different tank geometries on the physical K_La_o values. In other words, obtaining smaller K_La_o values corresponding to aeration conditions was an expected result, since agitation rate of 750 min^{-1} applied in the current study could be substituted with agitation rate of 590 min^{-1} in the bioreactor used in the study of Çalık et. al.

(2004). However, in dissolved oxygen profiles, similar trends with that of observed by Çalık et. al. (2004) was not obtained with the corresponding conditions. In stead, at all oxygen transfer conditions oxygen limitation was effective. As a consequence, it was concluded that *E. coli* BL21 (DE3) pLySs strain's oxygen demand is much higher than that of *E. coli* K12 to maintain the oxidative metabolism.

Furthermore, in the study of Çalık et. al. (2004), in the low oxygen transfer conditions, the medium pH was decreased to 5.5 while in the other oxygen sufficient conditions, the pH decrease was not so dramatic. As a result of the higher oxygen demand of *E. coli* BL21 (DE3) pLySs, all of the four oxygen transfer conditions applied act as limited oxygen transfer conditions, thus, similar trends in both DO level and medium pH was observed like the findings of Çalık et. al. (2004) at low oxygen transfer condition where *E. coli* K12 strain was the selected host. Therefore, it can be concluded that, both recombinant *E. coli* K12 and *E. coli* BL21 (DE3) pLySs strains showed similar response to oxygen limitation and low oxygen concentrations in the medium; whereas *E. coli* BL21 requires more oxygen to overcome oxygen limitation in the medium.

For many organisms, there exists a permanent regulatory force on the system to maintain the intracellular pH relativelly constant by pumping protons in or out of the cell membrane which leads to fluctuations in environmental pH. The initial pH value was pH_0=7.2 at all the conditions. The pH of the fermentation broth was inclined to decrease gradually with the cultivation time (Figure 4.15) among the investigated oxygen transfer parameters; and reduction rate of pH was the highest at LOT condition, while it was almost the same at MOT and HOT conditions.

Although it is known that utilization of ammonium ions as sole nitrogen source decreases the medium pH because of the hydrogen ions released after metabolism of ammonia (Shuler and Kargı, 2002), at MOT* condition, the pH of the medium was decreased with the highest rate when compared with that of the MOT condition where glucose and nitrogen sources were in excess. At MOT* both glucose and oxygen limitations were observed which may alter the metabolic network towards the by-product formation, e.i., organic and amino acids.

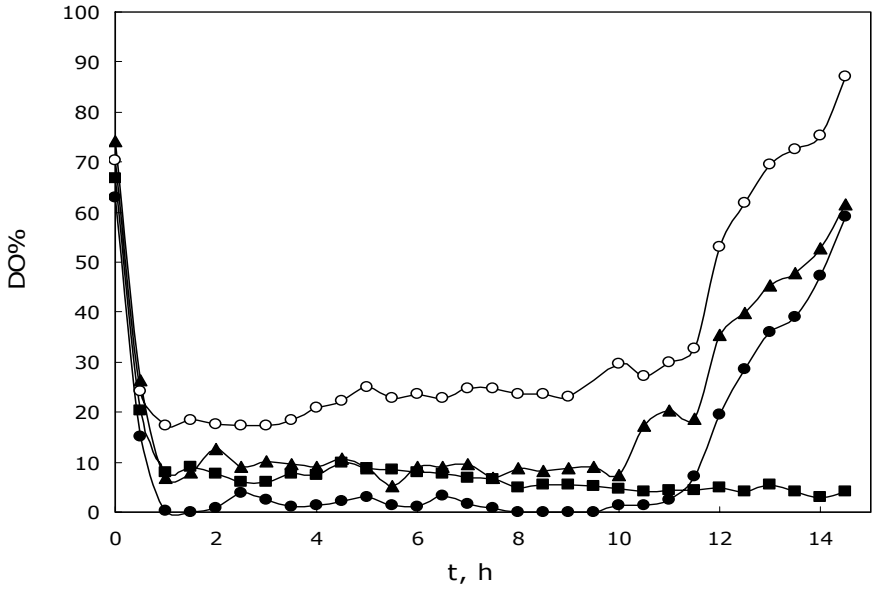


Figure 4.17 The variations in the dissolved oxygen concentration with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

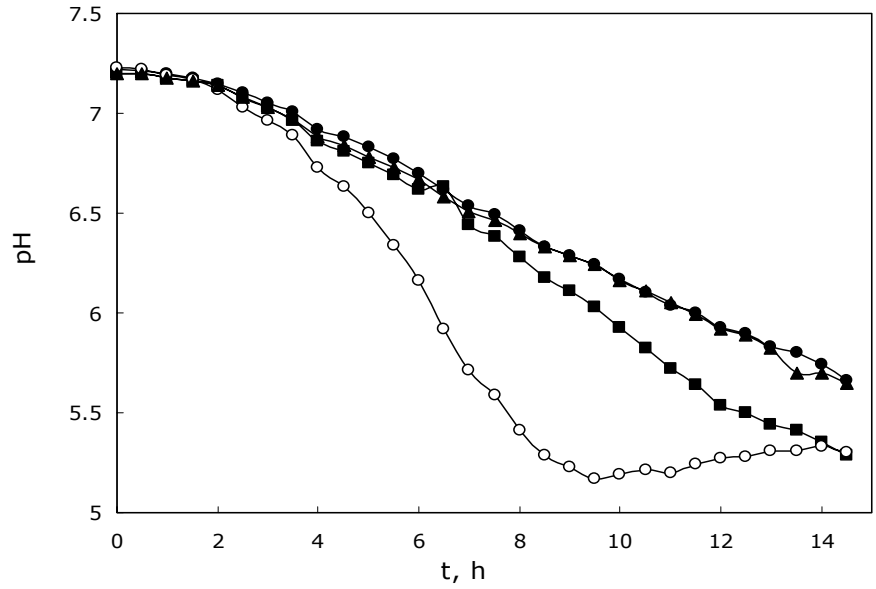


Figure 4.18 The variations in medium pH with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

4.2.3.1.2 Cell Growth Profiles

The variations in the cell concentration with cultivation time agitation and air inlet rates, and medium components are given in Figure 4.19.

Until t=4 h, cell formation did not change significantly with respect to the oxygen transfer conditions applied and medium used. On the other hand, after t=4 h cell profiles altered considerably throughout the bioprocess. In general, cell growth of all conditions reached to stationary phase after t=8 h when cell formation rate decreased. The highest cell concentration with a value of 1.7 kg m^{-3} was obtained at MOT condition which is lower than the cell concentration of 2.0 kg m^{-3} obtained at laboratory scale bioreactor with working volume of 33 cm^3. The lowest cell concentration of 1.5 kg m^{-3} was observed at LOT condition among the investigated oxygen transfer conditions.

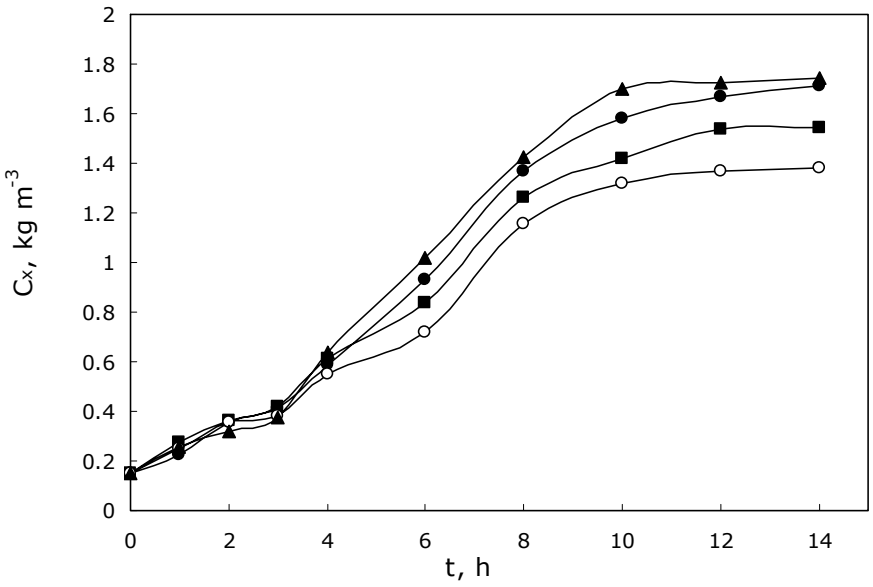


Figure 4.19 The variations in cell concentration with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

Having similar trend with others, medium containing 8.0 kg m^{-3} glucose and 4.5 kg m^{-3} $(NH_4)_2HPO_4$, which is MOT* condition, resulted in lower cell concentration with a value of 1.4 kg m^{-3} than the one obtained from laboratory scale bioreactor with 33 cm^3 working volume (C_x=1.5 kg m^{-3}). Under these circumstances, since oxygen has low solubility in aqueous solutions, oxygen

supply seems to be insufficient for maintenance of oxidative metabolism which results in lower cell growth.

In the study of Çalık et. al. (2004), among the investigated oxygen transfer conditions, the condition with agitation rate of N=500 min^{-1}, air inlet rate of Q_O/V_R = 0.5vvm, and medium composed of 8.0 kg m^{-3} glucose and 5.0 kg m^{-3} $(NH_4)_2HPO_4$ were reported to be the optimum condition in terms of the cell growth with a value of 2.3 kg m^{-3} in the recombinant *E. coli* K12 carrying pUC18::*bal* plasmid at pilot scale bioreactor with a working volume of 1.65 dm^3.

4.2.3.1.3 Glucose Concentration Profiles

The effect of oxygen transfer conditions and medium content used on glucose consumption throughout the process is given in Figure 4.20.

At t=0-2 h of the bioprocess, glucose concentration values were decreased sharply at all the conditions. Between t=2-8 h, until the microbial growth curves reached to their stationary phase, glucose consumption rate was decreased following an increase after t=8 h for applied oxygen transfer conditions.

Glucose was depleted after t=8 h of the process at MOT* condition and an increase in the dissolved oxygen profile was observed at the same time period. When MOT* condition was compared with the findings of Çalık et. al. (2004), where glucose consumption rate was lower, it can be concluded that, *E. coli* BL21 (DE3) pLySs strain requires more glucose as a carbon source to maintain the optimum state of cell growth than *E. coli* K12 does. Similarly, in most of the batch processes, where *E. coli* BL21 was the selected microorganism, higher initial glucose concentrations of 20.0 kg m^{-3} (Luli et. al., 1990), or that of 40.0 kg m^{-3} (Shiloach et. al., 1996) were preferred in production mediums. In fed-batch processes, however, glucose feeding strategies were arranged in order to keep the cell growth at critical level where acetate accumulation starts with automated feeding systems (Johnston et. al., 2003; Akesson et. al., 200) where lower initial glucose concentration up to 2 kg m^{-3} were preferred (Luli et. al., 1990; Shiloach et. al., 1996).

Among the investigated oxygen transfer parameters, the highest consumption rate was observed at MOT condition, where the cell concentration

was the highest; whereas, the lowest glucose consumption rate was attained at HOT condition, where cells require less amount of energy since oxygen limitation is not significant as other conditions.

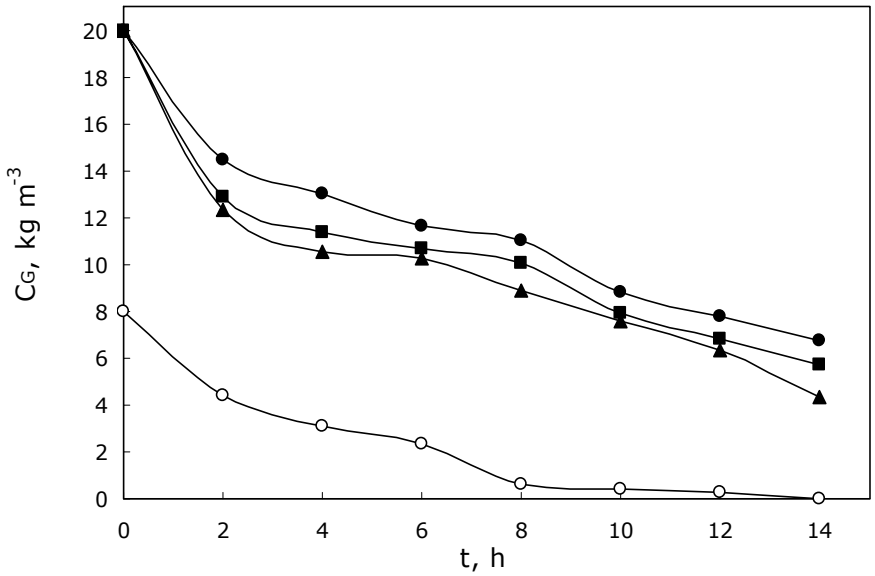


Figure 4.20 The variations in glucose concentration with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

4.2.3.1.4 Benzaldehyde Lyase Activity Profiles

The variations of benzaldehyde lyase volumetric activity with the cultivation time at the oxygen transfer conditions applied and medium used are respectively given in Figure 4.21.

The highest benzaldehyde lyase volumetric activity was obtained at MOT condition (A=990 U/cm^3), where the benzaldehyde lyase activity was also higher than all the other conditions throughout the process; and the lowest benzaldehyde lyase volumetric activity was obtained at LOT as A=680 U cm^{-3} among the investigated oxygen transfer conditions at t=12 h. For all conditions, activity values decreased strictly when cultivation time was lengthened to 14^{th} hour.

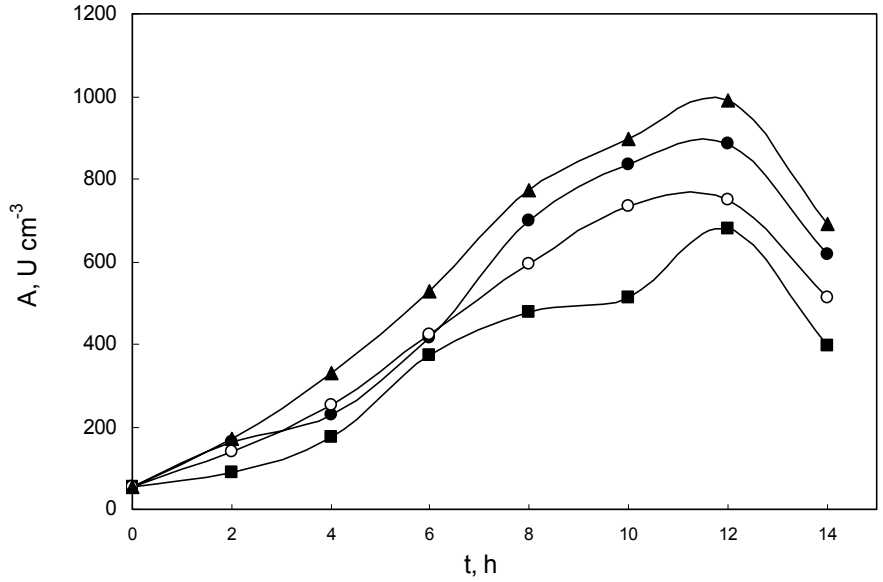


Figure 4.21 The variations in the benzaldehyde lyase volumetric activity with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

Lower activity value of 750 U cm^{-3} was reached when glucose and $(NH_4)_2HPO_4$ concentrations were lower (MOT* condition) than MOT condition, where the cell growth was also lower. This may be a result of glucose depletion at the end of exponential phase of growth and increased proteolytic activity. Although, *E. coli* BL21 (DE3) pLySs is protease deficient strain, glucose depletion can lead to increase in protease production which may result in lower productivity. In the study of Choi et.al. (1997), a decrease in expression level of bovine growth hormone was observed due to the starvation for a required nutrient in *E. coli* BL21 strain. Thus, it can be concluded that the protease degradation caused by nutrient depletion can account for this decrease.

Although, utilization of lower glucose and inorganic nitrogen amounts is more preferable in industrial applications, since in production, the aim is to obtain the maximum volumetric benzaldehyde lyase activity and concentration; it is necessary to increase the concentration of the benzaldehyde lyase producing cells. Therefore, it was concluded that for benzaldehyde lyase production, the bioreactor should be operated at medium oxygen transfer conditions, where the highest amount of cell was generated.

4.2.3.1.5 Organic and Amino Acid Concentration Profiles

The variations in the organic acids detected in the fermentation broth with cultivation time and oxygen transfer conditions were demonstrated in Table 4.18.

Apparently, for all oxygen transfer conditions, acetic acid is the major by-product in the fermentation medium. One of the main challenges in total productivity in *E. coli* cultivation is onset of acetic acid production which is known to inhibit the cell growth and the recombinant protein production (Shiloach et.a l., 1996; Johnston et., al., 2003; Akesson et., al., 2001; Luli et. al., 1990; Çalık et. al., 2004, 2006). The reason is the accumulation of undissociated (protonated- CH_3COOH) form of acetic acid in the medium. Since undissociated state of acetate is lipophilic, it can freely permeate the cell membrane and accumulate in the medium. When a fraction of undissociated acid present extracellularly re-enters the cell, it dissociates (ionized- CH_3COO^-) at the relatively higher intracellular pH. When this process continues, the intracellular pH decreases and hence the ΔpH component of the promotive force collapses. Thus, apart from energy consumption for rapid growth, *E. coli* requires energy for maintenance of optimum intracellular pH which leads to inhibition on cellular efficiency (Stephanopoulos, 1998; Luli et. al., 1990).

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

For all oxygen transfer conditions applied, acetic, formic, fumaric, oxalic, malic, maleic and pyruvic acids were detected in the fermentation broth in general increasing amounts of those through t=10 h. Presence of the organic acids of TCA cycle, i.e., fumaric and malic acid may be a consequence of inadequate oxygen supply which leads to repression on TCA cycle enzymes. Similarly, in the study of Çalık et. al. (2004), under oxygen limiting conditions the TCA cycle organic acids of α-ketoglutaric acid and succinic acid were found in the medium showing insufficient operation of TCA cycle under low OT conditions and

higher acetate accumulation, up to 2.5 kg m^{-3}, was observed, where the recombinant *E. coli* K12 was the host microorganism. Facultatively aerobic bacterium *E. coli* can drastically change its product formation under deprived oxygen conditions. Since for all oxygen transfer conditions applied, oxygen was the rate limiting step, oxygen limitation is the major cause for acetate accumulation and TCA cycle organic acids.

The highest acetate accumulation occurred in MOT condition, where the highest cell concentration and the highest glucose consumption were observed, as 1.09 kg m^{-3} at t=10 h; followed by LOT condition as 0.90 kg m^{-3} and HOT condition as 0.84 kg m^{-3}.

All oxygen transfer conditions showed similar trend in terms of acetate accumulation in fermentation broth. Until t=10 h acetate amount in the medium increased gradually, thereafter, a slight decrease was observed (Figure 4.22). The change in the metabolism can be explained by utilization pathway of the acetate with respect to glucose uptake rate. At all conditions, the glucose consumption rate was decreased between t=2-8 h, while a slight increase was observed after t=10 h, where the cell growth was reached to stationary phase. At the beginning of the bioprocess, increases in acetate amount in response to the cell growth, where glucose consumption rate is decreasing, occurs until acetate amount in the medium attained to critical value, around 1 kg m^{-3}. The decrease in glucose uptake rate leads to lower growth and consequently reduces the acetate production (Shiloach et. al., 1996).

Table 4.20 summarizes the organic acid and amino acids produced throughout the bioprocess at MOT* condition. As in the case of other oxygen transfer conditions studied, acetic, formic, oxalic, maleic and pyruvic acids together with TCA cycle organic acids of fumaric and malic acids were detected in the fermentation broth, indicating the improper activity of TCA cycle enzymes due to the oxygen limitation. Similar to previous conditions acetate is the major by-product, also, the highest acetate accumulation was found at this condition ($C_{Acetic\ Acid}$ = 1.27 kg m^{-3}) at t=10 h. Apart from the effect of limiting oxygen, glucose was depleted at t=8 h resulting in higher amounts of organic acids at t=10 h. Although acetic acid concentration was higher when compared with MOT condition, in terms of all other organic acids detected in the medium of MOT* condition, especially pyruvic acid which was the second major by-product,

tended to decrease to the lower values than those of found in MOT condition after t=10 h. This can be the reason for pH increase observed in fermentation broth after t=10 h. It was, also, reported that *E. coli* B, ancestor of BL21 (DE3) pLySs, was classified as strain that produce acetate and then reassimilate it once glucose is depleted (Luli et. al., 1990).

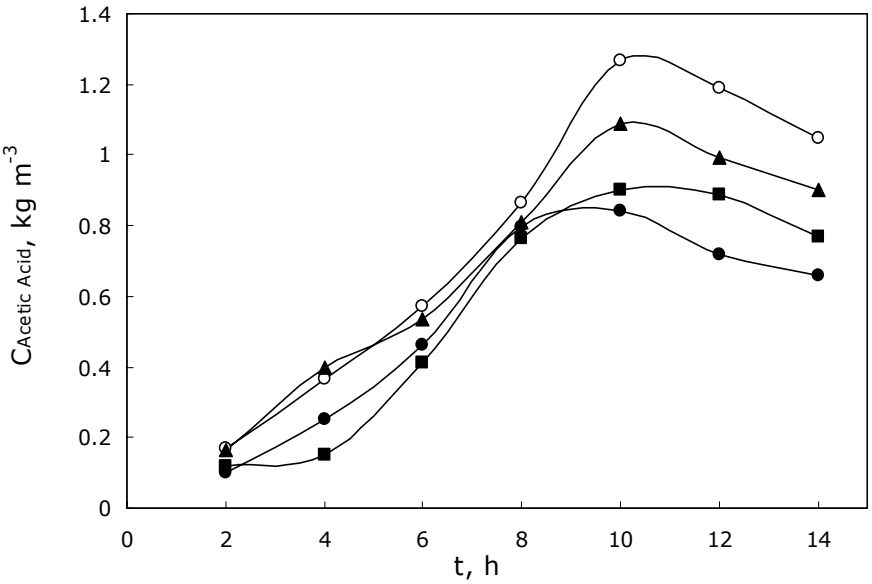


Figure 4.22 The variations in acetic acid concentration with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

The variations in amino acids with cultivation time with respect to oxygen transfer conditions and medium components used were listed in Tables 4.19 and 4.20. Most microorganisms have the metabolic machinery to synthesize all essential amino acids from carbon and nitrogen sources for the production of proteins. Alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, phenilalanine, and ornithine are the amino acids that were detected in the medium, but in very low amounts. The highest total amino acids produced was at HOT condition at t=12 h as T_{AA}= 10.9x10^{-3} kg m^{-3}, followed by MOT condition as a T_{AA}= 9.1x10^{-3} kg m^{-3} and LOT condition as T_{AA}= 8.8x10^{-3} kg m^{-3}. On the other hand, the total amino acid concentration at MOT* condition was obtained at t=14 h as T_{AA}= 3.9x10^{-3} kg m^{-3}, being the lowest one. For all conditions studied, ornithine was the major amino acid produced, which indicates that the demand for ornithine should be lower than the produced amount, thus ornithine was excreted to the fermentation broth.

Table 4.18 The variations in organic acid concentrations with cultivation time and oxygen transfer conditions.

LOT, C_{OA} ($kg.m^{-3}$)							
Time (h)	2	4	6	8	10	12	14
Ac	0.1192	0.1531	0.4119	0.7650	0.9020	0.8876	0.7708
Form	-	0.0186	0.0337	0.0052	0.0136	-	-
Fum	-	-	-	-	-	0.0003	-
Ox	0.0117	0.0047	0.0017	-	-	-	-
Mal	0.0008	0.0020	0.0174	0.0648	0.0573	0.0550	0.0803
Male	0.0008	0.0009	0.0011	0.0019	0.0013	0.0013	0.0010
Pyr	-	-	0.0012	-	0.0132	0.4188	0.0603
T_{OA}	**0.1325**	**0.1792**	**0.4670**	**0.8369**	**0.9875**	**1.3631**	**0.9124**
MOT, C_{OA} ($kg.m^{-3}$)							
Time (h)	2	4	6	8	10	12	14
Ac	0.1663	0.3970	0.5376	0.8080	1.0872	0.9949	0.9020
Form	-	0.0265	0.0049	0.0074	-	-	-
Fum	-	-	-	-	0.0002	0.0002	0.0002
Ox	0.0139	0.0088	0.0036	-	-	-	0.0027
Mal	-	0.0043	0.0281	0.0239	0.0725	0.0639	0.0506
Male	0.0010	0.0017	0.0019	0.0007	0.0018	0.0014	0.0011
Pyr	-	-	0.0020	0.0058	0.1201	0.3281	0.4550
T_{OA}	**0.1812**	**0.4383**	**0.5780**	**0.8458**	**1.2818**	**1.3886**	**1.4116**
HOT, C_{OA} ($kg.m^{-3}$)							
Time (h)	2	4	6	8	10	12	14
Ac	0.1005	0.2523	0.4640	0.7964	0.8409	0.7200	0.6599
Form	-	0.0120	0.0192	0.0106	-	-	-
Fum	-	-	-	-	0.0002	-	-
Ox	0.0134	0.0029	0.0017	-	-	-	0.0021
Mal	0.0010	0.0014	0.0121	0.0553	0.0597	0.0381	0.0283
Male	0.0010	0.0007	0.0010	0.0016	0.0014	0.0009	0.0008
Pyr	-	-	0.0008	0.0067	0.1498	0.3176	0.4418
T_{OA}	**0.1159**	**0.2693**	**0.4988**	**0.8706**	**1.0520**	**1.0767**	**1.1330**

Table 4.19 The variations in amino acid concentrations with cultivation time oxygen transfer conditions.

LOT, $C_{AA}x10^3$ (kg.m^{-3})							
Time (h)	2	4	6	8	10	12	14
Ala	-	-	-	-	-	-	-
Pro	-	-	0.7239	-	-	-	0.7217
Tyr	-	-	-	-	-	0.5236	-
Val	1.1998	1.2163	-	-	1.2581	-	-
Met	-	-	0.0362	0.1063	-	-	-
Cys	-	0.5934	-	-	-	-	-
Ile	0.1748	-	0.0745	-	0.1259	0.0648	0.1064
Phe	0.0317	0.3840	-	-	-	-	-
Orn	3.0343	2.0720	2.7502	2.9801	3.3201	8.2513	7.3944
T_{AA}	**4.4405**	**4.2657**	**3.5848**	**3.0864**	**4.7042**	**8.8397**	**8.2226**
MOT, $C_{AA}x10^3$ (kg.m^{-3})							
Time (h)	2	4	6	8	10	12	14
Ala	-	-	-	-	-	0.2113	-
Pro	0.4271	-	-	-	0.5489	-	-
Tyr	-	0.4476	0.3304	0.5908	-	-	-
Val	0.7782	-	-	-	-	0.7921	-
Met	-	-	-	0.0086	0.0314	-	-
Cys	-	-	0.6516	-	-	0.2121	-
Ile	0.0870	-	-	0.0671	0.0763	0.0309	-
Phe	-	-	-	-	-	-	-
Orn	2.3374	3.4522	2.5158	2.8963	4.6216	7.8485	4.5045
T_{AA}	**3.6297**	**3.8999**	**3.4979**	**3.5628**	**5.2782**	**9.0949**	**4.5045**
HOT, $C_{AA}x10^3$ (kg.m^{-3})							
Time (h)	2	4	6	8	10	12	14
Ala	0.2621	-	-	-	-	-	-
Pro	-	-	0.7508	0.7302	0.5081	3.7514	-
Tyr	-	-	-	-	-	-	0.2861
Val	0.8283	0.9984	-	-	-	0.8611	-
Met	-	-	0.0376	-	0.0306	-	0.0511
Cys	-	0.8376	-	-	-	-	-
Ile	0.1087	-	0.0722	0.0662	0.1173	0.0997	0.0365
Phe	-	-	-	-	-	-	-
Orn	1.7692	0.7920	2.6539	3.1526	3.6177	6.1750	6.5917
T_{AA}	**2.9683**	**2.6279**	**3.5145**	**3.9489**	**4.2737**	**10.8872**	**6.9655**

Table 4.20 The variations in organic acid and amino acid concentrations with cultivation time at MOT* condition.

MOT*, C_{OA} (kg.m^{-3})							
Time (h)	2	4	6	8	10	12	14
Ac	0.1692	0.3676	0.5701	0.8632	1.2670	1.1900	1.0467
Form	-	-	0.0114	-	-	-	-
Fum	-	-	-	-	0.0003	-	-
Ox	0.0150	0.0021	0.0205	-	-	-	-
Mal	0.0009	0.0033	0.0391	0.0681	0.0733	0.0349	0.0574
Male	0.0011	0.0017	0.0018	0.0018	0.0019	0.0009	0.0015
Pyr	-	0.0005	0.0031	0.0981	0.3511	0.2204	0.2247
T_{OA}	**0.1862**	**0.3752**	**0.6460**	**1.0312**	**1.6936**	**1.4463**	**1.3303**
MOT*, C_{AA}x10^3 (kg.m^{-3})							
Time (h)	2	4	6	8	10	12	14
Ala	-	-	-	-	-	-	0.2684
Pro	-	0.5653	-	-	-	0.4840	-
Tyr	0.5382	-	-	-	0.4332	-	-
Val	-	-	-	1.2796	-	-	0.7365
Met	-	0.0438	0.1096	-	-	-	-
Cys	0.2316	-	-	0.5637	0.3472	-	-
Ile	-	0.0923	0.1024	-	-	0.0912	0.0548
Phe	0.0734	-	-	-	0.1379	-	-
Orn	1.4325	1.1056	1.3757	1.3020	1.9828	1.5546	2.7990
T_{AA}	**2.2756**	**1.8070**	**1.5876**	**3.1452**	**2.9011**	**2.1298**	**3.8587**

4.2.3.1.6 Oxygen Transfer Characteristics

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r_0, and oxygen transfer coefficient, K_La. At t<0 h, the physical oxygen transfer coefficient K_La_o was measured in the medium in the absence of the microorganism. The variations in K_La, oxygen uptake rate, oxygen transfer rate and the enhancement factor E ($=K_La/K_La_o$) throughout the bioprocesses are given in Table 4.21.

Volumetric mass transfer coefficients, K_La values and subsequently the enhancement factor, E, found were increased through the end of the process at MOT, HOT and MOT* conditions studied. K_La depends on agitation rate, temperature, rheological properties of the fermentation medium and presence of

fine particles in the mass transfer zone. The presence of salts, other medium components and by-products formed can significantly alter the bubble size and liquid film resistance around the gas bubble. Taking concerted effect of the dynamic shear stress caused by eddies and viscous stress into account, since all other parameters remained constant, it can be concluded that observed increase in K_La, could be the result of the decrease in viscosity of the medium, which, also, explains higher K_La values obtained for MOT* condition when compared with MOT condition. Among the LOT, MOT and HOT conditions, the volumetric mass transfer coefficient showed an increase by increasing the concerted effect of the parameters air inlet rate (Q_O/V_R) and agitation rate (N), as expected, since the severity of oxygen requirements depends on the oxygen transfer conditions applied.

The dynamic method was applied to the conditions where there is no reaction, i.e., $q_o=0$ and physical K_La, K_La_o, values were determined to investigate the influence of operating parameters, e.g, the stirring speed and the gas flow rate, on the volumetric mass transfer condition in model media. K_La_o values increased with the increase in the oxygen transfer condition, as expected, and E ($=K_La/K_La_o$) values varied between 1.02 and 1.60. Enhancement factor is a measure of relative rates of mass transfer and chemical reaction and presence of particles in the mass transfer zone. The low E values obtained, shows that slow reaction is accompanied with mass transfer, as expected in most of the fermentation processes accomplished in stirred bioreactors (Çalık et. al., 2004).

Oxygen transfer rate, which is proportional to difference between the equilibrium concentration and the dynamic dissolved oxygen concentration in the medium, had a tendency to decrease through the end of the process at MOT and HOT conditions; while oxygen consumption increased subsequently, having a higher value than OTR's through the end of the process where the cell formation and the substrate consumption rates, i.e. metabolic activity of the cells, were high; whereas OUR was lower than OTR at t=0.5 h at LOT condition. Comparing the MOT and MOT* operations, it could be seen that at the very beginning of the cell growth both OTR and OUR values were higher at MOT*; while they were superior at MOT condition at the end of the process where both cell formation and benzaldehyde activity were higher.

In Table 4.21, the maximum possible oxygen utilization rate (OD), the maximum possible mass transfer rate (OTR_{max}), Damköhler number (Da), and effectiveness factor (η) values were given in order to find the relative effects of mass transfer and biochemical reaction on the benzaldehyde lyase production process by the recombinant *E. coli* BL21 (DE3) pLySs.

It is clear in Table 4.21 that, at all operation conditions, mass-transfer resistances, Damköhler number (Da), which is the ratio of the maximum rate of oxygen consumption in the bioprocess to its maximum mass transfer rate ($=OD/OTR_{mx}$) were effective through the end of the process ($Da>>1$) indicating that mass transfer is the rate limiting step and maximum possible oxygen utilization rate is significantly lower than at bulk phase condition. At the very beginning of the process (t=0.5 h), however, Da value is smaller than 1 designating the low mass transfer resistance where consumption proceeds at the same rate as in the bulk phase. With the increase in agitation rate, Da, that is the oxygen limitation, decreases.

Effectiveness factor, η, which is the ratio between the observed oxygen uptake rate and maximum possible oxygen utilization rate obtained in the absence of any mass transfer resistance, was 1.0 at LOT condition and close to 1.0 at MOT and MOT* conditions; whereas it was 0.66 at HOT condition at t=0.5 h. These results are indicating that cells were consuming oxygen at almost maximum possible oxygen utilization rate at beginning of the process at LOT, MOT and MOT* conditions; while observed oxygen consumption was lower than transferred oxygen amount at HOT condition indicating ineffective utilization of oxygen supplied to the system for cellular metabolism. The effectiveness factor values were, in general, dropped to zero at the end of the process, where mass transfer resistances were the greatest.

4.2.3.1.7 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_o, the specific substrate utilization rate, q_s, and the yield coefficients with the cultivation time are given in Table 4.22.

Table 4.21 The variations in oxygen transfer parameters with cultivation time, oxygen transfer conditions and medium used.

Condition	t (h)	K_La (s^{-1})	E K_La/K_La_0	$OTRx10^3$ ($molm^{-3}s^{-1}$)	$OTR_{max}x10^3$ ($molm^{-3}s^{-1}$)	$OURx10^3$ ($molm^{-3}s^{-1}$)	$ODx10^3$ ($molm^{-3}s^{-1}$)	Da OD/OTR_{mx}	η OUR/OD
LOT	0.5	0.012	1.50	1.94	2.44	1.10	1.10	0.45	1.00
	12.5	-	-	-	-	-	-	-	-
MOT	0.5	0.012	1.32	1.80	2.44	1.20	1.33	0.55	0.90
	12.5	0.013	1.43	1.59	2.64	2.20	523.24	198.27	0.00
HOT	0.5	0.013	1.02	2.27	2.68	1.00	1.51	0.56	0.66
	12.5	0.015	1.15	2.17	3.05	1.60	147.63	48.48	0.01
MOT*	0.5	0.013	1.38	2.00	2.64	1.30	1.54	0.58	0.85
	12.5	0.015	1.60	1.16	3.05	1.60	403.16	132.40	0.00

At all operation conditions applied, the specific growth rate (μ), in general, decreased after t>3 h, as expected (Figure 4.23); and the highest specific growth rate (μ) was obtained as μ_{max}=0.57 h^{-1} at t=0.5 h at LOT condition, followed by MOT condition with a value of 0.52 h^{-1} at t=0.5 h, where the highest cell concentration was obtained. Even slight increases were observed at either t=1 h or t= 3 h at HOT and MOT conditions, respectively, the maximum specific growth rate values were obtained when t= 0.5 h. The highest decrease rate between t=3-6 h was viewed at MOT condition; but the specific growth rates remained almost the same after t>6 h at all conditions.

The maximum specific growth rate observed in the study of Çalık et. al. (2004) was reported as 1.19 h^{-1} at t=1 h at HOT1 (N=750min^{-1}, Q_O/V_R = 0.5 vvm) condition and the specific growth rate values were decreased with the cultivation time where decrease rate was higher at high oxygen transfer conditions. Since the specific cell growth rate was almost 2 fold lower in the recombinant *E. coli* BL21 (DE3) pLySs because of the oxygen limitation observed at all conditions, lower cell concentration values, were obtained relative to the findings of Çalık et. al. (2004).

The specific oxygen uptake rates (q_O) and the specific substrate utilization rates (q_S) decreased with the cultivation time at all the oxygen transfer conditions having the highest values of 0.67 and 18.71 kg kg^{-1} h^{-1} at MOT operation at t=0.5 h, respectively; while they were 0.74 and 8.84 kg kg^{-1} h^{-1}, respectively, for MOT* condition at t=0.5 h. The highest decrease rate of the specific oxygen uptake rate was, also, obtained in MOT*, probably because of the lowest cell formation attained at this condition.

The specific substrate utilization rates of LOT, MOT, and HOT conditions were approximately four fold higher than the values claimed in Çalık et. al. (2004) at the beginning of the process; while they were almost the same at the end of the process. Therefore, it can be suggested that glucose demand is much more in *E. coli* BL21 (DE3) pLySs cells than *E. coli* K12 ones, since, even in MOT* condition, where the initial glucose concentration was the same as that of used in Çalık et. al. (2004), higher q_S values were found at the beginning of the process.

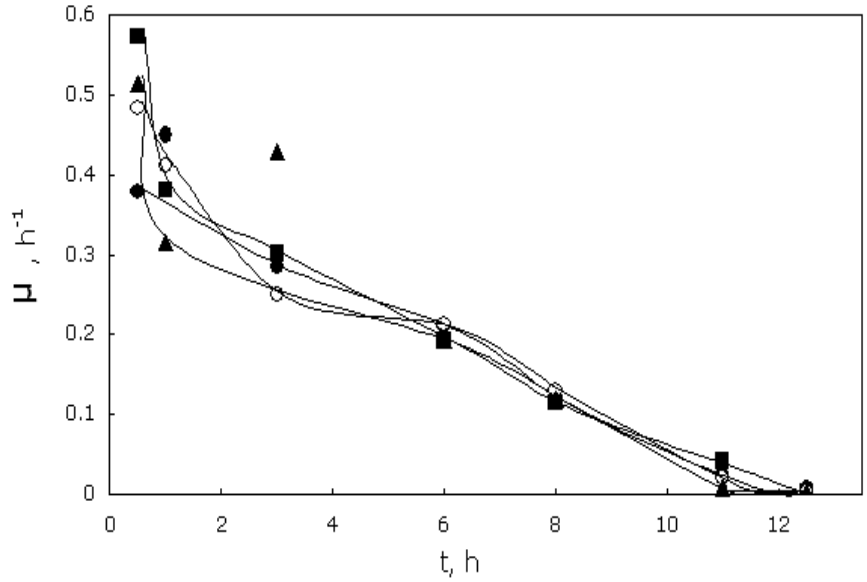


Figure 4.23 The variations in the specific growth rate with the cultivation time, agitation and air inlet rates applied and medium contents. T=37°C, V_R= 550 cm^3: LOT (■); MOT (▲); HOT (●); MOT* (○).

The yield coefficients, which represent the overall distribution and the efficiency of conversion reactions, were given in Table 4.21. The specific cell yield on substrate ($Y_{X/S}$), in general, increased with cultivation time until t=6 h and decreased gradually through the end of the process, where the cell growth reached to stationary phase. Although, almost the same trend of $Y_{X/S}$ values were observed at LOT and MOT conditions throughout the process, the highest value of 0.50 kg kg^{-1} h^{-1} at t=6 h was found at LOT operation. On the other hand, the specific cell yield on substrate at MOT* operation was increased until t=11 h, then decreased drastically, most probably because, although glucose was depleted after t=8, cell concentration remained the same.

The cell yield on oxygen values decreased with the cultivation time and the highest $Y_{X/O}$ value was obtained at LOT condition at t=0.5 h as 0.97 kg kg^{-1}. The lowest $Y_{X/O}$ value was obtained at MOT and MOT* operations at t=12.5 h as 0.02 kg kg^{-1} indicating the inefficient use of the oxygen through biochemical reaction network, probably because of the oxygen limitation. At MOT* operation the reduction rate of the specific cell yield on oxygen was smaller than the rate at MOT condition signaling that less energy was used for the cell formation at MOT*.

Correspondingly, the amount of substrate metabolized per the amount of oxygen used ($Y_{S/O}$) decreased with the cultivation time. The highest $Y_{S/O}$ was obtained as 28.17 kg kg^{-1} at LOT condition at t=0.5 h; while the lowest $Y_{S/O}$ was obtained as 0.30 kg kg^{-1} at MOT* condition at t=12.5 h. The decrease with cultivation time is indicating the increase in energy requirement or decrease in the efficiency of energy metabolism with cultivation time (Çalık et. al., 2006). The amount of substrate consumed per amount of oxygen utilized, $Y_{S/O}$, values were higher in *E. coli* BL21 (DE3) pLySs cells relative to *E. coli* K12 cells (Çalık et. al., 2004) at the beginning of the process, while relatively much smaller values were observed at the end of the process. Therefore, there can be suggested that *E. coli* BL21 (DE3) pLySs cells require much more energy for effective glucose utilization to maintain the optimum state of the cell growth than *E. coli* K12 does.

4.3 Expression of Benzaldehyde Lyase in r- *Bacillus* Species

According to the research program for production of benzaldehyde lyase, the recombinant pRB374::*hybrid* plasmid, where fused gene fragment was under the control of *veg*II promoter (which was claimed to enhance secretion in exponential phase of the cell growth) was transformed into various *Bacillus* species and extracellular expression capacities of these recombinant microorganisms were investigated.

The recombinant plasmid transformed into *B. brevis* NRRL NRS 604, *B. firmus* NRRL B1107, *B. sphaericus* NRRL NRS 732 (Northern Regional Research Center), *B subtilis* BGSC-1A751 and *B. subtilis* BGSC-1S19 (*Bacillus* Genetic Stock Center), WB600 and WB700 (Wu et. al., 1991; Ye et. al., 1999) by natural transformation and expression capacities of these microorganisms were aimed to be compared in order to obtain the best system for benzaldehyde lyase production.

The recombinant microorganisms were incubated for 24 h in the defined medium given in Table 3.7. The cells were separated by centrifugation and the supernatant was used for SDS-page, TLC analyses and the activity assays. However, benzaldehyde lyase activity was not observed by any of the recombinant *Bacillus* species carrying the recombinant plasmid pRB374::*hybrid*.

Table 4.22 The variations in the specific rates and the yield coefficients with the cultivation time, oxygen transfer conditions and medium used.

Condition	t (h)	μ (h^{-1})	q_o ($kgkg^{-1}h^{-1}$)	q_s ($kgkg^{-1}h^{-1}$)	Yx/s ($kgkg^{-1}$)	Yx/o ($kgkg^{-1}$)	Ys/o ($kgkg^{-1}$)
LOT	0.5	0.57	0.59	16.69	0.03	0.97	28.17
	1	0.38	-	12.97	0.03	-	-
	3	0.30	-	1.79	0.17	-	-
	6	0.25	-	0.38	0.50	-	-
	8	0.19	-	0.55	0.21	-	-
	11	0.11	-	0.36	0.11	-	-
	12.5	0.04	-	0.18	0.01	-	-
	OVERALL				0.11		
MOT	0.5	0.52	0.67	18.71	0.03	0.77	27.81
	1	0.32	-	14.87	0.02	-	-
	3	0.43	-	2.40	0.18	-	-
	6	0.35	-	0.40	0.48	-	-
	8	0.19	-	0.48	0.25	-	-
	11	0.12	-	0.36	0.02	-	-
	12.5	0.01	0.15	0.29	0.01	0.02	1.98
	OVERALL				0.11		
HOT	0.5	0.38	0.61	14.63	0.03	0.62	23.92
	1	0.45	-	12.30	0.04	-	-
	3	0.28	-	1.78	0.16	-	-
	6	0.30	-	0.54	0.39	-	-
	8	0.21	-	0.52	0.23	-	-
	11	0.12	-	0.32	0.08	-	-
	12.5	0.03	0.11	0.15	0.04	0.06	1.38
	OVERALL				0.13		
MOT*	0.5	0.48	0.74	8.84	0.05	0.65	11.89
	1	0.41	-	7.12	0.06	-	-
	3	0.25	-	1.74	0.14	-	-
	6	0.23	-	0.87	0.24	-	-
	8	0.21	-	0.43	0.30	-	-
	11	0.13	-	0.05	0.43	-	-
	12.5	0.02	0.13	0.04	0.06	0.02	0.30
	OVERALL				0.17		

After harvesting the cells, both intracellular and extracellular samples were loaded to polyacrylamide gels and stained with silver nitrate to detect any minor amount of the recombinant protein, if it exists (see Appendix K). However, neither intracellulary nor extracellularly, any band with proper molecular weight was detected due to the high intra- and extra-cellular proteins other than the benzaldehyde lyase present in the samples. After

Although, *Bacillus subtilis* and related *Bacillus* species are known as an attractive host for studying the expression and secretion of foreign proteins, proteolytic activity present in the microorganisms was thought to be a reason of no production of benzaldehyde lyase. Therefore, six and seven protease deficient derivatives of *B. subtilis*, WB600 and WB700 (Wu et. al., 1991; Ye et. al., 1999) were used as the host microorganism. These multiple protease deficient strains were previously declared to provide a stepwise improvement in the production of human leukin-3 relative to that of obtained in double protease mutated DB104 strain in the study of Westers et. al. (2006), where secretion of functional human interleukin-3 from *Bacillus subtilis* was optimized. In this study, approximately 100 mg dm^{-3} human interleukin-3 production was reported, even with eight protease deficient WB 800 strain. However, there was no benzaldehyde lyase production in these modified microorganisms. Therefore, it was concluded that the problem may be due to the expression system built.

In the study of Westers et. al. (2006), the effect of different types of promoters, signal peptides and their concerted effects were investigated in different *B. subtilis* cells. Among the investigated promoters of P43, which is a well known vegetative phase promoter, and *nap*; signal peptides of Lat, Pel and SacB; *nap* promoter in combination with Pel signal gave the highest production of the recombinant biomolecule while another combination of both promoters with SacB signal peptide did not result in productive secretion of human interleukin-3, although efficient secretion of straphylokinase under guidance of this signal peptide has been reported previously (Ye et. al., 1999). Therefore, the reason for inefficient expression can be due to the improper selection of promoter and signal peptide combination for benzaldehyde lyase.

Moreover, the intracellular fused protein, composed of signal peptide and mature part of *bal* gene, production was investigated by incubating the *E. coli* XL1-Blue cells carrying pRB374::*hybrid* plasmid in LB broth. Previously, it was

stated that the transcriptional elements of pRB374 plasmid were functional in both *E. coli* and *B. subtilis* (Brückner et. al., 1992). Nevertheless, no activity was observed in both activity assay and TLC analyses where carboligation of benzaldehyde reaction was carried out. For intracellular production, newly synthesized proteins can fold either properly or improperly. The improperly folded proteins may form inclusion bodies or be degraded by proteases (Wong et. al., 1995). Thus, it was claimed that signal peptide extension in N-terminus of *bal* gene had an effect on the active site of the benzaldehyde lyase which leaded to activity loss or improper folding of the enzyme leaded to proteolytic degradation in the cell.

The signal peptidase cleavage site of signal peptide of gene encoding subtilisin Carlsberg from *Bacillus licheniformis* NCIB 6816 (*pre-sub*C), which was firstly reported by Jacobs et. al. (1985), was claimed to come after Ala-Ser-Ala residues remaining a 29 amino acid residue of pre-peptide. In the study performed by Tjalsma et. al. (2000), the predicted signal peptides of *B. subtilis* and amino acid residues around (putative) cleavage sites were examined and, according to the predictions, an alanine was found to be the most abundant residue (27%) at position +1 of the mature protein, but all other residues, with the exception of cytein and proline, seem to be allowed at this position. In original operon of serine alkaline protease gene fragment, Ala residue follows the Ala-Ser-Ala structure; whereas in current study the b*al* gene coding mature portion of benzaldehyde lyase starts with Met residue. Therefore, having Ala residue immediately after the cleavage site, may lead a better expression level of benzaldehyde lyase.

As recently stated, Mosbacher et. al. (2005) was studied the X-ray structure of benzaldehyde lyase to show that BAL belongs to a group of closely related TPP dependent enzymes. According to the results, it was proposed that BAL is a homotetramer, where each subunit is composed of 4 x 563 amino acid residues and has a molecular weight of 4 x 58919 Da. This may be the most crucial reason for not observing BAL activity in the fermentation broth, where volume of the bulk liquid is incomparable with the volume of the cell. Therefore, coalescence probability of each monomer in such a huge environment, even if all other factors like salts, by-products produced or other chemicals present in the medium are assumed to be negligible, to form tetramer structure may be the limiting factor on BAL activity.

In the study of Maria et. al. (2005), it was shown that even the active form of BAL in homotetramer structure losses its activity almost completely within 3 h in water, and hence, carboligation reactions were performed in potassium phosphate buffer supplemented with TPP, Mg^{2+}, DTT as a cofactor and 30% DMSO as a cosolvent. Hence, the production rate may be much smaller than the inactivation rate of enzyme since enzyme losses its active form within 3 h in aqueous solutions like fermentation broth. Furthermore, since inactive form of BAL, monomers, are less stable and sensible, expressed amount, if secreted, may directly degraded by proteases even in protease deficient strains since many form of both intracellular and extracellular proteases are present in the cell.

Apart from the reasons mentioned, the addition of poly-His tag, designed for facilitated purification, can lead to modified processing of the N-terminus of the protein as it was the case in the study of Ledent et. al. (1997). In this study, it was proposed that the addition of poly-His C-terminal extension to the β-lactamase of thermophilic *Bacillus licheniformis* strain modified the site of action of the signal peptidase resulted in the unexpected decrease in the productivity. Thus, the interaction between the 6xHis tag and signal peptide may inhibit both signal peptidase acting site and poly-His tag activity in a concerted way by altering the 3-D confirmation.

To overcome the obstacles faced with extracellular secretion of benzaldehyde lyase, the transcriptional apparatus can be improved either by altering the signal peptide or promoter used. Apart from the transcriptional improvement, benzaldehyde lyase secretion may be provided by eliminating the C-terminus hexa histidine tag extension, since hexa histidine tag extension may be the reason of the malfolding of the recombinant molecule leading to inefficient Spase cleavage activity to remove the signal peptide from the mature protein. Most importantly, through protein engineering, target protein can be re-designed to have better stability and facilitated affinity to form the tetramer structure out of the cell.

CHAPTER 5

CONCLUSION

In this study, benzaldehyde lyase production capacity in recombinant *E. coli* BL21 (DE3) pLySs as intracellular and in recombinant *Bacillus* species of *B. brevis*, *B. firmus*, *B. sphaericus*, *B subtilis apr*$^-$ and *npr*$^-$, *B. subtilis spo*$^-$, *B. subtilis* WB600 and *B. subtilis* WB700, as extracellular were aimed to be compared. For this purpose, the gene encoding BAL with poly-His tag extension was cloned and expressed in *E. coli* BL21 (DE3) pLySs strain under the control of strong T7 promoter; and at optimum defined mediums designed, effects of bioreactor operation parameters, i.e., oxygen transfer conditions and medium components used together with oxygen transfer characteristics of the bioprocess were investigated in the pilot scale bioreactor. Thereafter, for extracellular production signal peptide of *B. licheniformis* subtilisin Carlsberg with its own ribosomal binding site was fused to the N-terminus of the gene coding benzaldehyde lyase; and fused product was cloned and transformed in *Bacillus* species. In this context the following conclusions were drawn:

1. For higher expression of benzaldehyde lyase in *E. coli*, genetically engineered expression vector of pRSETA, providing T7 promoter to control the expression of the gene of interest, was selected as an alternative to modified pUC18::*bal* plasmid where *trc* promoter is present. The gene encoding BAL enzyme, (Accession no: AX349268), was amplified by using modified pUC18::*bal* plasmid as template with the help of the primers, *Nde*I-*bal* Forward and *bal* Reverse primers, under the catalytic activity of *Taq* DNA polymerase with PCR. After subsequent restriction digestion with *Nde*I and *Sac*I restriction enzymes, *bal* gene was fused to pRSETA

plasmid and recombinant plasmid was transformed into the *E. coli* BL21 (DE3) pLySs strain for expression.

2. For extracellular expression of BAL in *Bacillus* species, signal peptide of *B. licheniformis* subtilisin Carlsberg with its own ribosomal binding site was fused to the N-terminus of *bal* gene according to SOE method with the help of the primers designed, *pre-sub*C Forward and *pre-sub*C Reverse primers for amplification of *pre-sub*C gene (Accession no: X03341), *bal* Forward and *bal* Reverse primers for amplification of the *bal* gene. Fused product of the *hybrid* gene was sub-cloned into the pUC19 plasmid, and further ligated to pRB374, *E. coli*/ *B. subtilis* shuttle vector, after restriction digestion with proper restriction enzymes. The recombinant pRB374::*hybrid* plasmids were transformed into various *Bacillus* species for expression.
3. The *Escherichia coli* BL21 (DE3) pLySs carrying recombinant pRSETA::*bal* plasmid was selected as the benzaldehyde lyase producer, due to the highest BAL production capacity of this strain in RPM medium as 910 U $cm^{-3.}$
4. Among the investigated media, the highest cell concentration and the benzaldehyde lyase activity were obtained as 2.0 kg m^{-3} and 1060 U cm^{-3}, respectively, in the medium containing 20.0 kg m^{-3} glucose, 11.8 kg m^{-3} $(NH_4)_2HPO_4$ and the salt solution; T=37°C, N=200 min^{-1}, V=33 cm^3.
5. The effects of oxygen transfer on benzaldehyde lyase production in *E. coli* were investigated within low-, medium-, and high-oxygen transfer rates at three different conditions with the parameters, air inlet rate of Q_O/V_R = 0.5 vvm, and agitation rates of N=500 and 750 min^{-1} and at Q_O/V_R = 0.7 vvm, N=750 min^{-1} in a pilot scale bioreactor with 550 cm^3 working volume.
6. For all oxygen transfer conditions, no oxygen accumulation was observed; in other words oxygen supply was the rate limiting step. Therefore, lower benzaldehyde lyase activities together with cell concentrations were observed for all conditions applied relative to values obtained in laboratory scale bioreactors. Highest cell concentration and volumetric BAL activity values were 1.7 kg m^{-3} and 990 U cm^{-3}, respectively, at MOT condition where the highest glucose consumption was observed.

7. At all oxygen transfer conditions investigated, DO level of the systems were remained under 20% (C_{DO}< 0.04 mol m^{-3}) of the saturation until t=10 h; where it was under 30% (C_{DO}= 0.06 mol m^{-3}) of saturation of dissolved oxygen concentration until t=11h at MOT* condition.
8. The medium pH was gradually decreased starting from 7.2 at all oxygen transfer conditions, while the highest rate of decrease in pH was at LOT condition. At MOT* condition, where initial glucose and $(NH_4)_2HPO_4$ concentrations were 8.0 kg m^{-3} and 4.5 kg m^{-3}, respectively, the medium pH decreased to 5.17 at t=9.5 h, then increased slowly at the same period of glucose depletion occurred.
9. Organic acid concentrations at all operation conditions were, in general, increased by cultivation time while acetic acid was the major by-product in the fermentation medium. The highest acetate accumulation occurred in MOT condition, where the highest cell concentration and the highest glucose consumption were observed, as 1.09 kg m^{-3} at t=10 h; followed by LOT condition as 0.90 kg m^{-3} and HOT condition as 0.84 kg m^{-3} among the investigated oxygen transfer conditions; whereas it was 1.27 kg m^{-3} at t=10 h at MOT* condition. Relative to the acetic acid accumulation occurred in the recombinant *E.coli* K12 cells carrying the modified pUC18::*bal* plasmid, lower acetic acid formation was observed in *E.coli* BL21 (DE3) pLySs strain under oxygen limiting conditions.
10. At all oxygen transfer conditions applied and medium components used, acetic, formic, fumaric, oxalic, malic, maleic and pyruvic acids were detected in the fermentation broth. Presence of the organic acids of TCA cycle, i.e., fumaric and malic acid in the medium indicates inefficient activity of TCA cycle enzymes due to the oxygen limitation.
11. At all conditions, amino acids of alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, phenilalanine, and ornithine were detected in the broth with an increasing trend by cultivation time, but in very low amounts. The highest total amino acids produced was at HOT condition at t= 12 as T_{AA}= 10.9x10^{-3} kg m^{-3}, while the lowest one was T_{AA}= 3.9x10^{-3} kg m^{-3} obtained at MOT* condition, most probably because of the nutritional exhaustion after t=10 h.

12. K_La and consequently the enhancement factor E varied between 0.012-0.015 and 1.02-1.60, respectively; having the highest values at MOT*condition. In general, K_La and E values got higher through the process most probably because of the decrease in the viscosity due to nutrition consumption; and, low enhancement factor E values indicate that the mass transfer rate is much higher than the reaction rate.
13. Oxygen transfer rate was reduced with cultivation time at MOT and HOT conditions; while oxygen consumption increased subsequently, having a higher value than OTR's through the end of the process where the cell formation and the substrate consumption rates were high; whereas OTR was lower than OUR at t= 0.5 h in LOT condition. At MOT* operation relatively higher OTR and OUR values were obtained than MOT condition at t=0.5 h; while they were superior in MOT condition at the end of the process.
14. At all operations mass transfer resistances were effective (Da>>1) through the end of the process; while they were smaller than 1 at the beginning of the processes, signaling the low mass transfer resistance. Effectiveness factor, η, was 1.0 at LOT condition and close to 1.0 at MOT and MOT* conditions; whereas it was 0.66 at HOT condition at t=0.5 h indicating observed oxygen consumption was lower than transferred oxygen amount at HOT condition. The effectiveness factor values were, in general, dropped to zero at the end of the process, where mass transfer resistances were the greatest.
15. According to the overall view of oxygen consumption in the recombinant *E.coli* BL21 (DE3) pLySs cells, this microorganism requires higher amounts of oxygen supplied to the system for efficient cell growth, glucose utilization and heterologous foreign protein secretion than *E. coli* K12 strain.
16. The highest specific growth rate (μ) was obtained as $\mu_{max}=0.57\ h^{-1}$ at t=0.5 h at LOT condition. Since the specific cell growth rate was lower in the recombinant *E. coli* BL21 (DE3) pLySs because of the oxygen limitation observed at all conditions, lower cell concentration values relative to the recombinant K12 cells carrying the modified pUC18::*bal* plasmid were observed.

17. The yield coefficients of specific cell yield on substrate ($Y_{X/S}$), specific cell yield on oxygen ($Y_{X/O}$), and specific substrate yield on oxygen ($Y_{S/O}$) values were calculated according to empirical data and $Y_{X/S}$ values showed an increasing trend until t=6 h then decreased gradually; while $Y_{X/O}$ and $Y_{S/O}$ reduced with the cultivation time. The recombinant *E. coli* BL21 (DE3) pLySs cells utilize higher amount of glucose as a carbon source per oxygen consumed relative to the recombinant K12 cells at the beginning of the process; while, relatively much smaller $Y_{S/O}$ values were observed at the end of the processes indicating the recombinant *E. coli* BL21 (DE3) pLySs cells require much more energy for effective glucose utilization to maintain the optimum state of cell growth than the recombinant *E. coli* K12 does.
18. Extracellular secretion of the benzaldehyde lyase was not successful with the expression system built where the *hybrid* gene was under the control of *veg*II promoter in various *Bacillus* species. Unsuccessful expression may be resulted from the following reasons:

- Proteolytic activity present in *Bacillus* species may degrade the benzaldehyde lyase transcribed.
- Transcriptional elements of the signal peptide and the promoter selection may not be proper for efficient secretion of benzaldehyde lyase.
- Signal peptidase efficiency may be reduced or lost due to disruption in 3-D formation because of unexpected influence of C-terminus poly-His tag extension to the signal peptide.
- The production rate may be much smaller than the inactivation rate of the enzyme since the enzyme losses its active form within 3 h in aqueous solutions like fermentation broth.
- Since active form of the benzaldehyde lyase is in tetramer form, inefficient folding of monomers secreted to the fermentation broth, if they were secreted, may result in not observing the benzaldehyde lyase activity. Moreover, malfolding of the enzyme may enhance the proteolytic degradation.

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APPENDIX A

Calibration of Cell Concentration

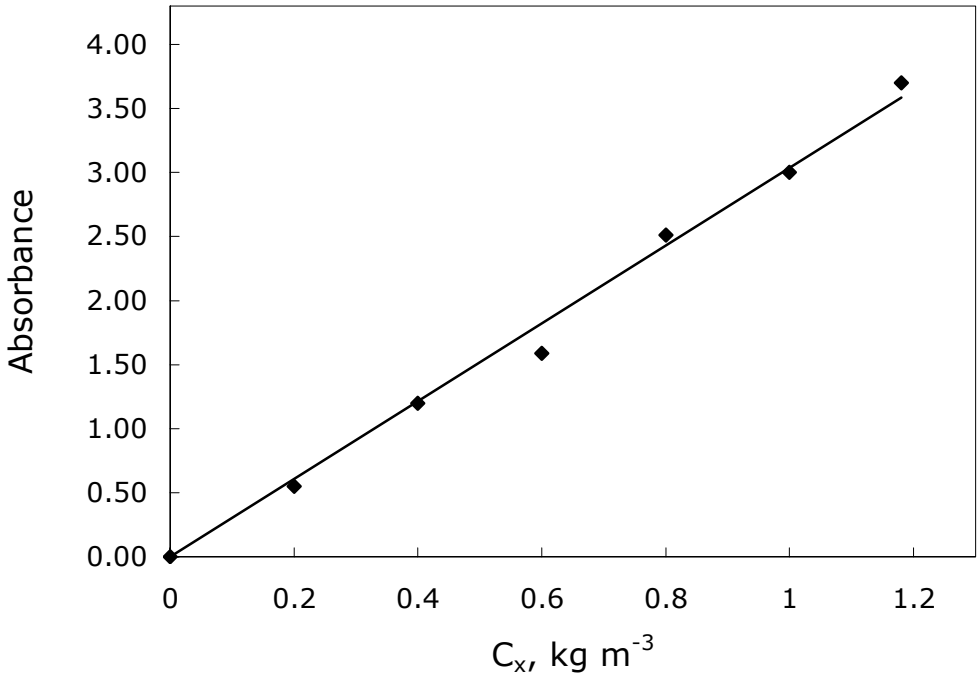

Figure A.1 Calibration curve for cell concentration

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

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

APPENDIX B

Calibration of Reduced Sugar Concentration

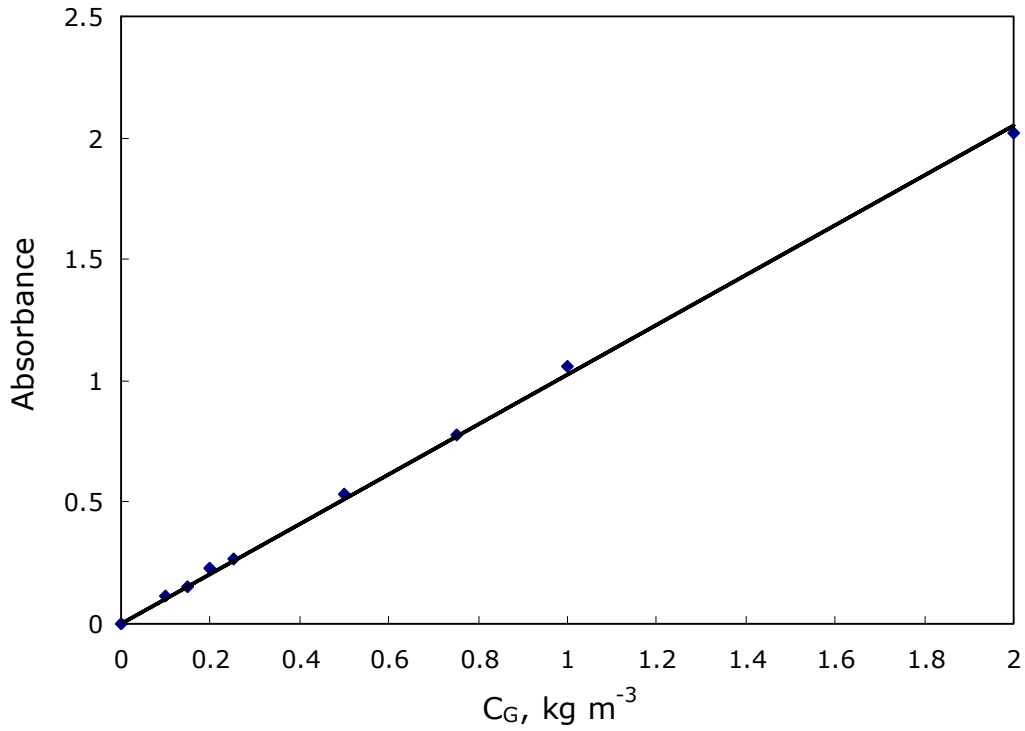

Figure A.2 Calibration curve of the glucose analysis solution

Slope of the calibration curve, m=1.0249 1/kg m^{-3} (λ=505 nm)

$$C_G = \frac{\text{Absorbance}}{1.0249} \times \text{Dilution Rate}$$

APPENDIX C

Preparation of Buffers for Glucose Analyses

Preparation of Analysis Solution

1. Dilute the mixture containing glucose oxidase (900U), preoxidase (500U), and 4-aminoantiphrine (10mM) to final volume of 100 ml by 150 mM phosphate buffer at pH=7.5 consisting of phenol (4mM) and additives.

2. Mix thouroughly for 1 minute.

Preperation of Standard Glucose Solution

1. Prepare 10 ml 5g/L standard glucose solution using glucose standard and 150 mM phosphate buffer (pH=7.5) consisting of phenol (4mM) and additives.

2. Using this solution, prepare 0.25, 0.50, 0.75, and 1.0 g/L standard solutions.

APPENDIX D

Preparation of Buffers and Solutions Used in Genetic Engineering Experiments

LB

Soytryptone	10 kg m^{-3}
Yeast exract	5 kg m^{-3}
NaCl	10 kg m^{-3}

LBA

Soytryptone	10 kg m^{-3}
Yeast exract	5 kg m^{-3}
NaCl	10 kg m^{-3}
Agar	15 kg m^{-3}

ALKALINE LYSIS SOLUTION I

Glucose	50 mM
Tris-HCl (pH=8.0)	25 mM
EDTA	10 mM

ALKALINE LYSIS SOLUTION II

NaOH	0.2 N
SDS	% 1

ALKALINE LYSIS SOLUTION III

Potassium Acetate	5 M
Acetic Acid	11.5 (v/v)

SET

NaCI	75 mM
EDTA	25 mM

10X TBE

Tris	108 kg m^{-3}
Boric Acid	55 kg m^{-3}
EDTA	9.3 kg m^{-3}

TSE

Tris HCl, pH= 8.0	10 mM
NaCl	300 mM
EDTA	10 mM

SOLUTION A

Tris HCl, pH= 8.1	10 mM
EDTA	10 mM
NaCl	50 mM
Sacchorose	8% (w/v)

SOLUTION B

SDS	1% (w/v)
NaOH	0.2 M

SOLUTION C

Potassium Acetate	5 M
Acetate Acid	60 ml
dH_2O	Up to 100 ml

Antibiotics	Stock Solutions	
	Concentration	Storage
Ampicillin	50 mg/ml in H_2O	-20°C
Chloramphenicol	34 mg/ml in ethanol	-20°C
Kanamycin	10 mg/ml in H_2O	-20°C
Erythromycin	10 mg/ml in H_2O	-20°C
Lincomycin	30 mg/ml in H_2O	-20°C

APPENDIX E

Gene Sequence of *pre-sub*C and *bal* genes

1- Gene Sequence of *pre-sub*C gene including its RBS sequence:

(Accession No:X03341)

ctgaataaagaggaggagagtgagtaatgatgaggaaaaagagtttttggcttggga
tgctgacggccttcatgctcgtgttcacgatggcattcagcgattccgcttctgct

2- Gene Sequence of *bal* gene including C-terminal poly-His tag extension:

(Accession No:A349268)

atggcgatgattacaggcggcgaactggttgttcgcaccctaataaaggctggggtc
gaacatctgttcggcctgcacggcgcgcatatcgatacgatttttcaagcctgtctc
gatcatgatgtgccgatcatcgacacccgccatgaggccgccgcagggcatgcggcc
gagggctatgcccgcgctggcgccaagctgggcgtggcgctggtcacggcgggcggg
ggatttaccaatgcggtcacgcccattgccaacgcttggctggatcgcacgccggtg
ctcttcctcaccggatcgggcgcgctgcgtgatgatgaaaccaacacgttgcaggcg
gggattgatcaggtcgccatggcggcgcccattaccaaatgggcgcatcgggtgatg
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ccacgcgggccggtgttgctggatctgccgtgggatattctgatgaaccagattgat
gaggatagcgtcattatccccgatctggtcttgtccgcgcatggggccagacccgac
cctgccgatctggatcaggctctcgcgcttttgcgcaaggcggagcggccggtcatc
gtgctcggctcagaagcctcgcggacagcgcgcaagacggcgcttagcgccttcgtg
gcggcgactggcgtgccggtgtttgccgattatgaagggctaagcatgctctcgggg
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gatgccgcgccagatctcgtgctgatgctgggggcgcgctttggccttaacaccggg
catggatctgggcagttgatcccccatagcgcgcaggtcattcaggtcgaccctgat
gcctgcgagctgggacgcctgcagggcatcgctctgggcattgtggccgatgtgggt
gggaccatcgaggctttggcgcaggccaccgcgcaagatgcggcttggccggatcgc
ggcgactggtgcgccaaagtgacggatctggcgcaagagcgctatgccagcatcgct
gcgaaatcgagcagcgagcatgcgctccacccctttcacgcctcgcaggtcattgcc
aaacacgtcgatgcaggggtgacggtggtagcggatggtgcgctgacctatctctgg
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gcaggccgccgcacgatccttgtgaccggcgatggctcggtgggctatagcatcggt
gaatttgatacgctggtgcgcaaacaattgccgctgatcgtcatcatcatgaacaac
caaagctgggggggcgacattgcatttccagcaattggccgtcggccccaatcgcgtg
acgggcacccgtttggaaaatggctcctatcacggggtggccgccgcctttggcgcg
gatggctatcatgtcgacagtgtggagagcttttctgcggctctggcccaagcgctc
gcccataatcgccccgcctgcatcaatgtcgcggtcgcgctcgatccgatcccgccc
gaagaactcattctgatcggcatggacccccttcggatctcatcaccatcaccatcac
taa

APPENDIX F

Preperation of Buffers for 6xHis-Tagged Protein Purification

Lysis buffer (1 liter)

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4.H_20$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
10 mM imidazole	0.68 g imidazole (MW 68.08 g/mol)
Adjust pH to 8.0 using NaOH	

Wash buffer (1 liter)

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4.H_20$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
20 mM imidazole	1.36 g imidazole (MW 68.08 g/mol)
Adjust pH to 8.0 using NaOH	

Elution buffer (1 liter)

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4.H_20$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
250 mM imidazole	17.00 g imidazole (MW 68.08 g/mol)
Adjust pH to 8.0 using NaOH	

APPENDIX G

Preparation of Related Buffers for SDS-Polyacrylamide Gel Electrophoresis

1- Preparation of Separation and Stacking Gel for SDS-Polyacrylamide Gel Electrophoresis

	Stacking Gel	Seperating Gel		
	5%	7.5%	10%	12%
30% acrylamide mix	1.67 ml	2.5 ml	3.33 ml	4 ml
dH_2O	5.68 ml	4.85 ml	4.05 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5 ml	2.5 ml	2.5 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml	-	-	-
10% (w/v) SDS	100 µl	100 µl	100 µl	100 µl
10% (w/v) ammonium persulfate	60 µl	50 µl	50 µl	50 µl
TEMED	15 µl	10 µl	10 µl	10 µl

2- Preparation of Materials Used for Staining of SDS-Polyacrylamide Gels with Coomassie Brilliant Blue

A. Staining Solution:
Dissolve 0.25g Coomassie Brilliant Blue in 100 ml methanol: acetic acid solution.
B. Methanol: Acetic Acid Solution:
Combine 900 ml of methanol:H_2O (500 ml of methanol and 400 ml of H_2O) and 100 ml of glacial acetic acid.

3- Preparation of Materials Used for Staining of SDS-Polyacrylamide Gels with Silver Salts

A. Fixer

Mix 150 ml methanol + 36 ml acetic acid + 150 µl 37% formaldehyde and complete to 300 ml with distilled water. This solution can be used several times.

B. 50% Ethanol

Mix 600 ml pure ethanol + 600 ml distilled water. This solution should always be prepared freshly.

C. Pretreatment Solution

Dissolve 0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 400 ml distilled water by mixing with a glass rod. Take 8 ml and set aside for further use in developing solution preparation.

D. Silver Nitrate Solution

Dissolve 0.8 g silver nitrate in 400 ml distilled water and add 300 µl 37% formaldehyde.

E. Developing Solution

Dissolve 9 g potassium carbonate in 400 ml distilled water. Add 8 ml from pretreatment solution and 300 µl 37% formaldehyde.

F. Stop Solution

Mix 200 ml methanol + 48 ml acetic acid and complete to 400 ml with distilled water.

APPENDIX H

Thermodynamic Properties of Designed Primers Together with Dimer and Self-Complimentary Formation Affinities

Table A.1 Thermodynamic properties of designed primers.

Name	Length	GC%	T_m (°C)	ΔG kcal/mol	ΔH kcal/mol	ΔS eu
NdeI-BAL Forward Primer	21	47.6	60.7	-34.4	-60.3	-415.3
BAL Reverse Primer	31	54.8	60.7	-54.7	-233.5	-592.3
*pre-sub*C Forward Primer	29	58.6	59.9	-59.4	-255.6	-651.9
*pre-sub*C Reverse Primer	33	51.5	58.8	-61.2	-264.6	-675.1
BAL Forward Primer	34	52.9	60.7	-64.3	-275.7	-701.8

The self complimentary and dimer formation affinities are given below:

NdeI-bal Forward Primer:

Sequence: 5' **CATATG**GCGATGATTACAGGC 3'

- **NdeI RE Sequence**
- 18 nt complimentary to anti-sense strand of *bal* gene

Dimer formation:

```
                          5' CATATGGCGATGATTACAGGC 3'
                             ||||||
                3' CGGACATTAGTAGCGGTATAC 5'
```

Self-complementarity:

```
          5'  CATATG
              |||
3' CGGACATTAGTAGCG
```

bal-Reverse Primer:

Sequence: 5' GC**GAGCTC**GCTTAGTGATGGTGATGGTGATG 3'

- **SacI RE Sequence**
- 21 nt complementary to sense strand of *bal* gene

Dimer formation:

```
                                                5' GCGAGCTCGCTTAGTGATGGTGA 3'
                                                   ||||||||||
                                3' GTAGTGGTAGTGGTAGTGATTCGCTCGAGCG 5'
```

Self-complementarity:

```
                          5'  GCGAG┐
                              |||  |
     3' GTAGTGGTAGTGGTAGTGATTCGCTC┘
```

***pre-sub*C Forward Primer:**

Sequence: 5′ GC**GGATCC**GCAGCAATCTCCTGTCATTCG 3′

- **BamHI RE Sequence**
- 19 nt complementary to anti-sense strand of *pre-sub*C gene

Dimer formation:

```
                                                   5' GCGGATCCGCCTGAATAAAGA 3'
                                                      ||||||||||
                                  3' GTGAGAGGAGGAGAAATAAGTCCGCCTAGGCG 5'
```

Self-complementarity:

```
                          5'  GCGGA┐
                              |||  |
     3' GTGAGAGGAGGAGAAATAAGTCCGCCT┘
```

***pre-sub*C Reverse Primer:**

Sequence: 5′CCTGTAATCATCGCCATAGCAGAAGCGGAATCG 3′

- 16 nt analog to *pre-sub*C

Dimer formation:

```
                          5' CCTGTAATCATCGCCATAGCAGAAGCGGAATCG 3'
                                    |||    ||    |||
                               3' GCTAAGGCGAAGACGATACCGCTACTAATGTCC 5'
```

Self-complementarity:

```
                       5'  CCTGTAATCAT┐
                           |||  ||   C|
          3' GCTAAGGCGAAGACGATACCG┘
```

***bal*-Forward Primer:**

Sequence: 5′ CGATTCCGCTTCTGCTATGGCGATGATTACAGGC 3′

- 18 nt analog to *bal*

Dimer formation:

```
                      5' CGATTCCGCTTCTGCTATGGCGATGATTACAGGC 3'
                                 |||  ||       ||  |||
                            3' CGGACATTAGTAGCGGTATCGTCTTCGCCTTAGC 5'
```

Self-complementarity:

```
                    5'  CGATTCCGCTTCTG┐
                        |||      |
       3' CGGACATTAGTAGCGGTATC┘
```

APPENDIX I

Map Sites of Restriction Endonucleases

Restriction enzymes that are cutting the *bal* gene fragment are listed in Table A.2; whereas that of those noncutting the *bal* gene as follows:

Noncutter restriction enzymes of *bal* gene: AarI, AatII, AclI, AflII, AgeI, AloI, AlwNI, ApaI, ApaLI, AscI, AsuII, AvrII, BalI, BamHI, BbvCI, BciVI, BfiI, BplI, Bpu10I, BsaAI, BsaXI, BseRI, BsmI, Bsp1407I, BspLU11I, BstEII, BstXI, BtsI, DraII, DrdI, Eam1105I, Eco31I, Eco57I, Eco57MI, EcoRI, EcoRII, EcoRV, Esp3I, FalI, FseI, FspAI, GsuI, Hin4I, HindIII, HpaI, KpnI, MluI, MmeI, NaeI, NdeI, NheI, NotI, NruI, PI-PspI, PI-SceI, PacI, PfoI, PleI, PmaCI, PmeI, PpiI, PpuMI, PshAI, PsiI, PsrI, PvuI, PvuII, RsrII, SacI, SacII, SanDI, ScaI, SexAI, SfiI, SgfI, SmaI, SmlI, SnaBI, SpeI, SrfI, SspI, StuI, SwaI, TaqII, TatI, TfiI, Tth111I, VspI, XbaI, XhoI

Table A.2 Restriction enzymes that of cuting *bal* gene.

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BsrBI	CCGCTC	6	blunt	1	615
BtrI	CACGTC	6	blunt	1	1146
OliI	CACNNNNGTG	6	blunt	1	280
XmnI	GAANNNNTTC	6	blunt	1	1661
ApoI	RAATTY	6	five_prime	1	1369
AvaI	CYCGRG	6	five_prime	1	735
BclI	TGATCA	6	five_prime	1	348
BglII	AGATCT	6	five_prime	1	810
BsmAI	GTCTC	5	five_prime	1	115
ClaI	ATCGAT	6	five_prime	1	89
EcoNI	CCTNNNNNAGG	6	five_prime	1	43
Ksp632I	CTCTTC	6	five_prime	1	292
NcoI	CCATGG	6	five_prime	1	359
SapI	GCTCTTC	7	five_prime	1	292
SgrAI	CRCCGGYG	8	five_prime	1	279
StyI	CCWWGG	6	five_prime	1	359
BseMII	CTCAG	5	three_prime	1	650
BseSI	GKGCMC	6	three_prime	1	1489
BsgI	GTGCAG	6	three_prime	1	58
DraIII	CACNNNGTG	6	three_prime	1	1518
EciI	GGCGGA	6	three_prime	1	625
PstI	CTGCAG	6	three_prime	1	935
Sse8387I	CCTGCAGG	8	three_prime	1	935

Table A.2, continued

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
TspGWI	ACGGA	5	three_prime	1	1063
TspRI	CASTG	5	three_prime	1	1563
Eco47III	AGCGCT	6	blunt	2	1066, 1592
HindII	GTYRAC	6	blunt	2	903, 1554
AccI	GTMKAC	6	five_prime	2	902, 1553
AflIII	ACRYGT	6	five_prime	2	329, 1143
AvaII	GGWCC	5	five_prime	2	971, 1677
BspHI	TCATGA	6	five_prime	2	117, 1415
BspMI	ACCTGC	6	five_prime	2	879, 1119
FokI	GGATG	5	five_prime	2	1186, 1552
HgaI	GACGC	5	five_prime	2	510, 935
MfeI	CAATTG	6	five_prime	2	1393, 1456
NarI	GGCGCC	6	five_prime	2	191, 367
SalI	GTCGAC	6	five_prime	2	901, 1552
BaeI	ACNNNNGTAYC	7	three_prime	2	1367, 1400
Hpy99I	CGWCG	5	three_prime	2	1150, 1468
MboII	GAAGA	5	three_prime	2	279, 1666
PflMI	CCANNNNNTGG	6	three_prime	2	263, 1458
BsaBI	GATNNNNATC	6	blunt	3	581, 1408, 1544
MslI	CAYNNNNRTG	6	blunt	3	280, 1148, 1414
AcyI	GRCGYC	6	five_prime	3	191, 367, 927
BsrDI	GCAATG	6	three_prime	3	251, 1133, 1441
BsrI	ACTGG	5	three_prime	3	29, 696, 1036
NspI	RCATGY	6	three_prime	3	166, 732, 1105
SduI	GDGCHC	6	three_prime	3	287, 632, 1489
SphI	GCATGC	6	three_prime	3	166, 732, 1105
XcmI	CCANNNNNNNNNTGG	6	three_prime	3	201, 264, 981
BbvI	GCAGC	5	five_prime	4	296, 728, 1068, 1106
TseI	GCWGC	5	five_prime	4	309, 741, 1081, 1094
TspDTI	ATGAA	5	three_prime	4	335, 514, 731, 1432
Cfr10I	RCCGGY	6	five_prime	5	279, 465, 618, 699, 1336
XhoII	RGATCY	6	five_prime	5	477, 810, 859, 1050, 1687

Table A.2, continued

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BglI	GCCNNNNNGGC	6	three_prime	5	77, 364, 462, 682, 935
BseYI	CCCAGC	6	five_prime	6	49, 198, 825, 921, 1284, 1430
Tsp45I	GTSAC	5	five_prime	6	213, 243, 1044, 1158, 1332, 1479
BccI	CCATC	5	five_prime	7	390, 982, 1168, 1254, 1336, 1534, 1706
BsePI	GCGCGC	6	five_prime	7	80, 305, 452, 655, 832, 884, 1289
CfrI	YGGCCR	6	five_prime	7	167, 616, 956, 1015, 1298, 1460, 1520
HphI	GGTGA	5	three_prime	7	285, 405, 435, 1170, 1378, 1687, 1693
BcgI	CGANNNNNNTGC	6	three_prime	8	160, 194, 678, 712, 892, 926, 1605, 1639
HaeII	RGCGCY	6	three_prime	8	194, 211, 370, 670, 677, 1068, 1285, 1594
FauI	CCCGC	5	five_prime	10	148, 190, 213, 217, 333, 454, 749, 754, 1617, 1655
SfaNI	GCATC	5	five_prime	12	396, 416, 738, 789, 812, 900, 947, 996, 1085, 1140, 1370, 1625
TauI	GCSGC	5	three_prime	18	20, 154, 157, 169, 367, 445, 618, 688, 805, 1012, 1028, 1217, 1319, 1322, 1401, 1525, 1528, 1579

Restriction enzymes that are cutting the *pre-sub*C gene fragment are listed in Table A,3; whereas that of those noncutting the bal gene as follows:

Noncutter restriction enzymes of *pre-sub*C gene: AarI, AatII, AccI, AclI, AcyI, AflII, AflIII, AgeI, AloI, AlwNI, ApaI, ApaLI, ApoI, AscI, AsuII, AvaI, AvaII, AvrII, BaeI, BalI, BamHI, BbvI, BbvCI, BcgI, BciVI, BclI, BfiI, BglI, BglII, BplI, Bpu10I, BsaAI, BsaBI, BseMII, BsePI, BseSI, BseYI, BsgI, BsmAI, Bsp1407I, BspHI, BspLU11I, BspMI, BsrI, BsrBI, BsrDI, BstEII, BstXI, BtrI, BtsI, CfrI, Cfr10I, ClaI, DraII, DraIII, DrdI, Eam1105I, EciI, Eco31I, Eco47III, Eco57I, Eco57MI, EcoNI, EcoRI, EcoRII, EcoRV, Esp3I, FalI, FauI, FseI, FspAI, GsuI, HaeII, HgaI, Hin4I, HindII, HindIII, HpaI, HphI, Hpy99I, KpnI, Ksp632I, MboII, MfeI, MluI, MmeI, NaeI, NarI, NcoI, NdeI, NheI, NotI, NruI, NspI, OliI, PI-PspI, PI-SceI, PacI, PflMI, PfoI, PleI, PmaCI, PmeI, PpiI, PpuMI, PshAI, PsiI, PsrI, PstI, PvuI, PvuII, RsrII, SacI, SacII, SalI, SanDI, SapI, ScaI, SduI, SexAI, SfiI, SgfI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SrfI, Sse8387I, SspI, StuI, StyI, SwaI, TaqII, TatI, TauI, TseI, Tsp45I, TspGWI, TspRI, Tth111I, VspI, XbaI, XcmI, XhoI, XhoII, XmnI

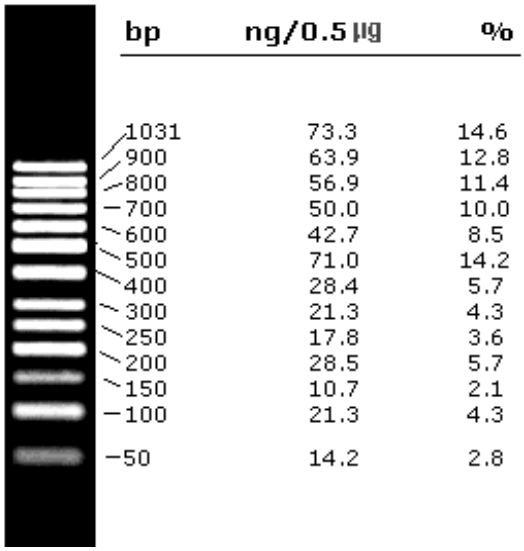
Table A.3 Restriction enzymes that of cuting *pre-sub*C gene.

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
MslI	CAYNNNNRTG	6	blunt	1	75
BccI	CCATC	5	five_prime	1	80
FokI	GGATG	5	five_prime	1	68
SfaNI	GCATC	5	five_prime	1	46
TfiI	GAWTC	5	five_prime	1	99
BsmI	GAATGC	6	three_prime	1	90
TspDTI	ATGAA	5	three_prime	1	59
BsaXI	ACNNNNNCTCC	6	three_prime	2	7, 37
BseRI	GAGGAG	6	three_prime	2	25, 28

APPENDIX J

Discrete DNA Fragments of Markers and Molecular Weights of Discrete Proteins of Protein Marker

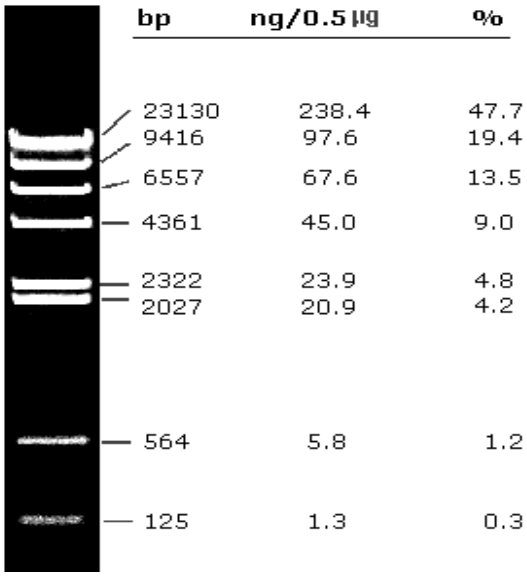
O'GeneRuler 50bp DNA Ladder



bp	ng/0.5 µg	%
1031	73.3	14.6
900	63.9	12.8
800	56.9	11.4
700	50.0	10.0
600	42.7	8.5
500	71.0	14.2
400	28.4	5.7
300	21.3	4.3
250	17.8	3.6
200	28.5	5.7
150	10.7	2.1
100	21.3	4.3
50	14.2	2.8

Figure A.3 Discrete DNA fragments of O'GeneRuler 50bp DNA Ladder in bp.

Lambda DNA HindIII Marker



bp	ng/0.5 µg	%
23130	238.4	47.7
9416	97.6	19.4
6557	67.6	13.5
4361	45.0	9.0
2322	23.9	4.8
2027	20.9	4.2
564	5.8	1.2
125	1.3	0.3

Figure A.4 Discrete DNA fragments of Lambda DNA HindIII Marker in bp.

Protein	Source	Molecular weight, kDa
β-galactosidase	*E.coli*	116.0
Bovine serum albumin	bovine plasma	66.2
Ovalbumin	chicken egg white	45.0
Lactate dehydrogenase	porcine muscle	35.0
Restriction endonuclease Bsp98I	*E.coli*	25.0
β-lactoglobulin	bovine milk	18.4
Lysozyme	chicken egg white	14.4

Figure A.5 Molecular weights of discrete proteins of Protein Molecular Weight Marker in kDa.

APPENDIX K

SDS-page Images of Intracellular and Extracellular Samples Taken from *Bacillus* Species

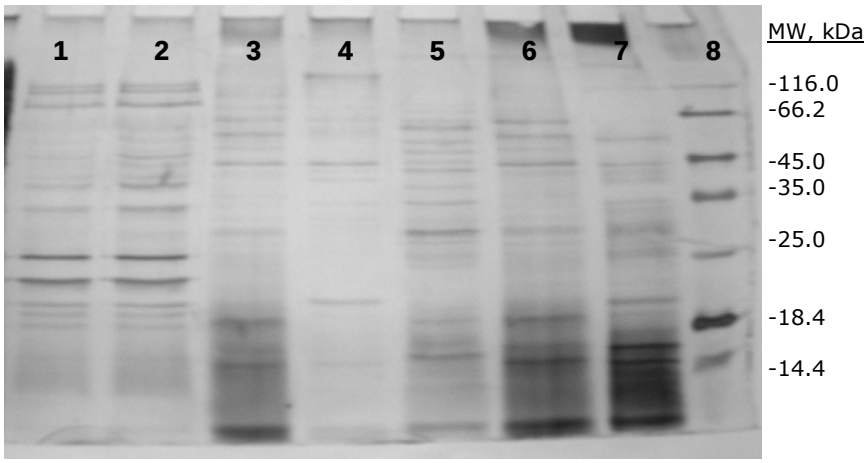


Figure A.6 SDS-page image of extracellular samples taken from *Bacillus* species. 1.well: WB700; 2. well: WB600; 3.well: *B. subtilis* apr$^-$ and npr$^-$; 4. well: *B. subtilis* spo$^-$; 5. well *B. brevis*; 6. well: *B. sphaericus*; 7.well: *B. firmus*; 8.well: Protein Marker.

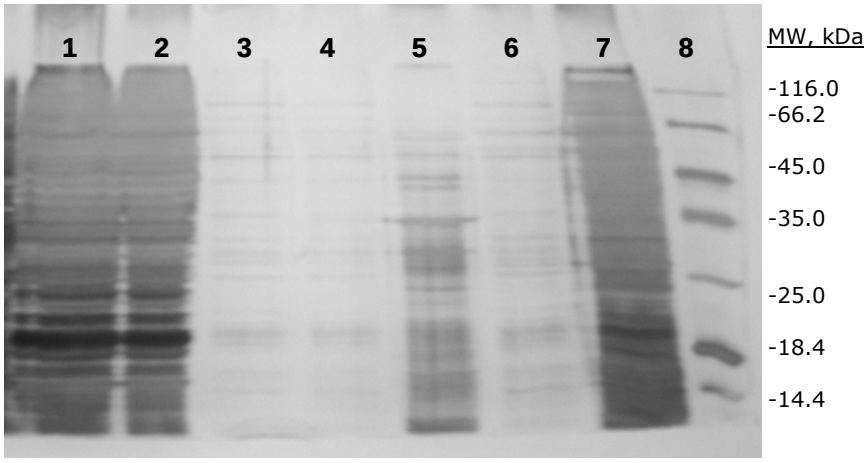


Figure A.7 SDS-page image of intracellular samples taken from *Bacillus* species. 1.well: WB700; 2. well: WB600; 3.well: *B. subtilis* apr$^-$ and npr$^-$; 4. well: *B. subtilis* spo$^-$; 5. well *B. brevis*; 6. well: *B. sphaericus*; 7.well: *B. firmus*; 8.well: Protein Marker.