INVESTIGATION OF THERMOSTABLE RECOMBINANT GLUCOSE ISOMERASE PRODUCTION BY SUCROSE UTILIZING ESCHERICHIA COLI

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INVESTIGATION OF THERMOSTABLE RECOMBINANT GLUCOSE ISOMERASE PRODUCTION BY SUCROSE UTILIZING ESCHERICHIA COLI

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

INVESTIGATION OF THERMOSTABLE RECOMBINANT GLUCOSE ISOMERASE PRODUCTION BY SUCROSE UTILIZING ESCHERICHIA COLI

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The aim of this M.Sc. thesis is to investigate the production of thermostable glucose isomerase (GI; EC 5.3.1.5) by metabolically engineered sucrose-utilizing *Escherichia coli* in the designed molasses-based production media. Throughout the experiments the cell growth, sucrose consumption, recombinant GI activity, and by-product concentrations were analyzed. For this purpose, in the first part of this thesis, pRSETA plasmid carrying the thermostable GI encoding gene from *Thermus thermophilus* (*xylA*) was isolated and pRSETA::*xylA* plasmid was transformed into *Escherichia coli* W. Thereafter, the highest GI producing strain was selected using different carbon sources; and the selected strain was named as *E. coli* W-26.

In the second part, the production of recombinant glucose isomerase was investigated firstly, in a program using laboratory scale shake-bioreactor experiments; and then by four sets of pilot scale bioreactor experiments. The recombinant GI production capacity of *E. coli* W-26 was tested on different carbon sources, i.e., glucose, sucrose, and molasses, at different concentrations in shake bioreactors. Growth in 32 g L^{-1} molasses-based medium resulted in higher recombinant GI activity and cell concentrations than those obtained in glucose and sucrose based media. Based on these results, four set of pilot scale semi-batch bioreactor

experiment were designed where complex substrate molasses acted as the carbon and nitrogen source. In this context, four feeding strategies were designed using pulse-or exponential- feeding strategies, and the influences on the cell growth, GI production, and by-product formations were investigated with the same pre-determined specific growth rate at μ=0.05 h^{-1}. The highest cell concentration was obtained as 18.4 g L^{-1} at t=26 h and the highest recombinant GI activity was achieved as 35264.5 U L^{-1} at t=16 h of bioprocess in BR4 operation by two molasses pulses with $(NH_4)_2HPO_4$ and antibiotic addition at t=5 h and t=8 h; shifting to semi-batch operation at t=11 h by feeding molasses based medium with an exponential volumetric feeding rate calculated using the pre-determined μ=0.05 h^{-1}.

Keywords: Thermostable glucose isomerase, *Escherichia coli* W, Sucrose, Molasses, Feeding strategy

ÖZ

SUKROZ TÜKETEBİLEN ESCHERICHIA COLI İLE ISIYA DAYANIKLI REKOMBİNANT GLUKOZ IZOMERAZ ÜRETİMİNİN ARAŞTIRILMASI

Akdağ, Burcu

Yüksek Lisans, Biyoteknoloji Bölümü

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Bu yüksek lisans tezinin amacı, tasarlanan melas temelli üretim ortamında, metabolik olarak değiştirilmiş sukroz kullanan *Escherichia coli* ile termostabil glukoz izomeraz (GI: EC 5.3.1.5) üretiminin araştırılmasıdır. Deneyler süresince pilot ölçekli biyoreaktörde hücre çoğalması, sukroz tüketimi, rekombinant GI aktivitesi ve yan ürün konsantrasyonları analiz edilmiştir. Bu amaçla, tez çalışmasının birinci bölümünde, *Thermus thermophilus* (*xylA*)'un termostabil GI'yı kodlayan geni taşıyan pRSETA izole edilmiş ve izole edilen pRSETA::*xylA* plazmitinin *E. coli* W hücrelerine transformasyonu gerçekleştirilmiştir. Daha sonra farklı karbon kaynaklarının kullanılarak, en fazla GI enzimi üreten suş seçilmiştir ve seçilen suş *E. coli* W-26 olarak adlandırılmıştır.

İkinci bölümde, laboratuvar ölçekli çalkalamalı biyoreaktör ve pilot ölçekli biyoreaktör deneyleri ile rekombinant glukoz izomeraz üretimi iki ardışık programla araştırılmıştır. *E. coli* W-26, rekombinant GI üretim potansiyeli, üç karbon kaynağı, glukoz, sukroz ve melas ile değişik konsantrasyonlarda araştırılmıştır. Karbon kaynakları ve derişimlerinin etkisi, laboratuvar ölçekli çalkalamalı biyoreaktör deney programında araştırılmış, kalma süresine karşı hücre derişimleri ve GI enzim aktiviteleri ölçülmüştür. 32 g

L^{-1} melas içeren ortamda, glukoz ve sukroz içeren ortamlardan daha yüksek hücre derişimi ve GI aktivitesi elde edildiğinden başlangıç karbon kaynağı konsantrasyonu olarak kullanılmasına karar verilmiştir. Bu sonuçlara dayanarak, kompleks melas substratının karbon ve azot kaynağı olarak davrandığı dört set pilot ölçekli yarı-kesikli biyoreaktör deneyi tasarlanmıştır. Bu kapsamda puls eklemeli ya da eksponansiyel besleme stratejileri kullanılarak dört besleme stratejisi tasarlanmış; hücre çoğalması, GI üretimi ve yan ürün oluşumları üzerindeki etkileri önceden belirlenmiş aynı özgül çoğalma hızı, μ=0.05 st^{-1} ile araştırılmıştır. En yüksek hücre derişimi, t=26 st'te 18.4 g L^{-1} ve en yüksek rekombinant GI aktivitesi, t=16 st'te 35264.5 U L^{-1} olarak BR4 koşulunda; 5. ve 8. saatlerde $(NH_4)_2HPO_4$ ilavesi ile birlikte verilen iki melas eklemesinin ardından 11. saatten sonra yarı-kesikli işletim ile μ=0.05 st^{-1} özgül çoğalma hızı kullanılarak hesaplanan eksponansiyel besleme debisi ile melas çözeltisi sürekli beslenerek elde edilmiştir.

Anahtar kelimeler: Termostabil glukoz izomeraz, *Escherichia coli* W, Sukroz, Melas, Besleme stratejisi

To my family

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October 03, 2013 Burcu Akdağ

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NOMENCLATURE

A	Glucose isomerase activity	U L^{-1}
$\overline{Y}$	Overall yield	g g^{-1}
C	Concentration	g L^{-1}
C_X	Cell concentration	g dry cell L^{-1}
N	Agitation rate	min^{-1}
Q	Volumetric air feed rate	L h^{-1}
q	Specific formation or consumption rate	gg^{-1} h^{-1}
r	Formation or consumption rate	g g^{-1} h^{-1}
t	Cultivation time	h
T	Bioreactor liquid medium temperature	°C
U	One unit of an enzyme	U
V	Volume of the bioreactor medium	L
Y	Yield (overall)	g g^{-1}

Greek Letters

λ	Wavelength	nm
μ	Specific growth rate	h^{-1}
ρ	Density	g L^{-1}

Abbreviations

csc	chromosomally encoded sucrose catabolism
DCW	Dry Cell Weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E. coli W	*Escherichia coli* Waksman
EC	Enzyme Commission
GI	Glucose isomerase
GRAS	Generally Recognized As Safe
HFCS	High-fructose corn syrup
HPLC	High pressure liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KIP	Potassium phosphate buffer
LB	Luria-Bertani
NRRL	Northern Regional Research Laboratory
OD	Optic density
PCR	Polymerase chain reaction

TCA	Tricarboxylic acid
xylA	Gene of glucose isomerase

Subscripts

0	Refers to initial condition and stock concentration
O	Refers to oxygen
R	Refers to bioreaction medium
S	Substrate
X	Refers to cell

CHAPTER 1

INTRODUCTION

Biotechnology is defined as "The application of science and technology to living organisms as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services" in 1982 by OECD. The role of these living organisms in making daily products like bread, cheese, wine and fermented meat has been known since ancient times. Today, basic biotechnology has been evolving with modern technologies such as recombinant DNA and cloning technology, metabolic engineering, microbiology and other cell culture technologies. Therefore, utilization of microorganisms and biotechnology applications have positive effect on treatments of many diseases, providing green technology, dealing with environmental problems and production of industrial goods like antibiotics, amino acids and enzymes (Ratledge and Kristiansen, 2006). Some of the important bio-products and their annual production amounts are given Table 1.1.

Enzymes are biological catalysts which reduce activation energy leading to increase in reaction rate. They are produced by living cells and their catalyzing metabolisms take place under mild pH and temperature conditions. Moreover, unique three dimensional structures of enzymes provide them high specificity to their substrates. Enzymes are divided into six sub-classes according to the Enzyme Commission (E.C). These are oxidoreductases which catalyze the oxido-reduction reactions; transferases which transfer one group from one compound to another; hydrolases which are responsible for hydrolytic cleavage of C-O, C-N and C-C; ligases that catalyze ligation of two groups to each other's; isomerases which catalyze geometric changes in one molecule and lyases that are responsible for the cleaving C-C, C-O, C-N, and other bonds by leaving double bonds or double bonds adding to groups.

Glucose isomerase (E.C.5.3.1.5) is a type of isomerase which catalyzes the isomeration reaction of glucose to fructose reversibly. It also converts D-xylose to D-xylulose. It is also known with another name as Xylose isomerase due to this function. Glucose isomerase (GI) has a crucial role on the production of High Fructose Corn Syrup (HFCS). HFCS is glucose-fructose mixture which has been used as sweetener with large volumes in food industry. In addition, GI is used in bioconversion of xylose to ethanol by combination with other enzymes throughout the fermentations. Thereafter, the produced ethanol can be used as an energy source as an alternative fuel (Bhosale *et al*., 1996). The starting point of the investigations of the glucose isomerase capacity is the discovery of

isomerizing activity of GI from *Pseudomonas hydrophila* by Hochster *et al*. in 1953. Several host microorganisms such as *Bacillus subtilis* (Lee *et al*., 1990), *Bacillus brevis* and *Escherichia coli* (Dekker *et al*., 1992), *Streptomyces lividans* (Tan *et al*., 1990), *Schizosaccharomyces pombe* (Chan *et al*., 1989), *Saccharomyces cerevisiae* (Moes *et al*., 1996) have been used for GI production. The first report related to the isolation of GI encoding gene from *Escherichia coli* was published by Ho *et al*. (1983). In addition, there are several studies in which GI encoding gene from natural producer strains were over-expressed in different types of *Escherichia coli* strains (Dekker *et al*., 1992; Lee *et al*., 1993; Kaneko *et al*., 2001; Sarıyar *et al*., 2004; Rhimi *et al*., 2007; Angardi and Çalık, 2013).

Table1.1 The annual production amount of fermentation products (Adapted from Soetaert and Vandamme, 2010)

Product	World production (ton/year)
Bioethanol	50000000
High--fructose corn syrup	12000000
L-Glutamic acid	1500000
Lactic acid	250000
Glucose isomerase	100000
Vitamin C	80000

Most recently, mainly mesophilic strains such as *Streptomyces*, *Actinoplanes*, *Flavobacterium, Bacillus* and *Escherichia coli* are used for glucose isomerase production processes. In the industrial processes, the sweetener production reactions were limited to 60^{o}C owing to characteristics of these mesophilic strains. However, Lamba *et al*. (2001) stated that the yield and the reaction rate of the product can be increased at the temperature which was higher than 60^{o}C in the isomeration reaction. Thermostable enzymes are favorable in the applications of industrial biotechnology due to decreasing of reaction time, having higher reaction rate, reducing contamination risk and being more stable than mesophilic enzymes (Lamba *et al*., 2001; Sterner *et al*., 2001). The thermostable enzyme sources are extremophiles which can survive and grow at extremely high temperatures. The nucleotide sequence of *xylA* gene from hyper-thermophilic *Thermus thermophilus* was determined by Dekker *et al*. (1991). They also reported that a 45-fold higher GI activity than their previous study was obtained by expressing *xylA* gene in *Escherichia coli* under *tac* promoter. Thermostable GI production capacities of *Escherichia coli* and *B. brevis* were compared by Dekker *et al*. (1992) and it was shown that *B. brevis* is more appropriate than *E. coli* as a host organism for expression of *xylA* gene of *T. thermophilus*. GI activity was achieved as 5,600 U L^{-1} by *B. brevis* that was 1.3-fold higher than that obtained by *Escherichia coli*. In addition, GI encoding gene of another thermophile microorganism,

Thermogata neapolitana was isolated and cloned into *Escherichia coli* (Vieille *et al.,* 1995). 55-45% fructose conversion was achieved via *xyl* isolation from *Thermus flavus and* transformation of *E. coli* by Park *et al.* (1997). In the PhD thesis of Angardi (2011), thermostable *xylT* gene of *Thermus thermophilus* inserted to strongly-regulated prokaryotic pRSETA expression vector. This metabolically engineered plasmid was transferred into *Escherichia coli* BL21 (DE3) pLysS and the highest glucose isomerase activity was obtained as 16,400 U L^{-1} (Angardi and Çalık, 2013).

Escherichia coli is one of the widely used host microorganism for the production of recombinant enzymes, due to having well-characterized system and being known its whole genome (Yee and Blanch, 1993; Lee, 1996). *Escherichia coli* strains are divided into five groups which are A, B1, B2, D and E according to phylogenetic properties of them. While B2, D and E groups are known as pathogenic strains; groups A and B1 have the status of Generally Recognized as Safe (GRAS). *Escherichia coli* W (NRRL B-766) in group B1 is the only safe strain that can utilize sucrose as a carbon source due to having csc regulon sites: i) a regulator (cscR) ii) a sucrose transporter (cscB) iii) an invertase (cscA) iv) a fructokinase (cscK) (Bockmann *et al*., 1992). Sucrose is the most important and abundant carbohydrate which is the transport molecule in plants and readily-available feedstock for industrial bioprocesses (Archer *et al*., 2011). Because sucrose is cheaper and greener than glucose (decreasing gas emissions in the production of ethanol from sucrose), the utilization of sucrose makes the bio-processes more feasible than the usage of other carbon sources (Archer *et al*., 2011). Furthermore, *Escherichia coli* W strain has several advantages as a host microorganism in biotechnological processes such as having high growth rates, being low acetate producer, having high tolerance against environmental impacts (high temperature, high acidic conditions, high ethanol concentrations etc.) and not encoding any antibiotic resistance genes (Lee and Chang, 1993; Nagata, 2001; Gleiser and Bauer, 1981).

In this M.Sc. thesis, it was aimed to investigate the thermostable glucose isomerase production capacity of metabolically engineered *Escherichia coli* W. In the first part of the study, pRSETA carrying the thermostable GI encoding gene of *Thermus thermophilus* (*xyl*) (Angardi and Çalık, 2013) was transformed into *E. coli* W. *E. coli* W-26 carrying pRSETA::*xylA* plasmid having the highest glucose isomerase production capacity was chosen according to the results of the microorganism selection experiments. Thereafter, laboratory scale bioreactor experiments were carried out with different carbon sources in order to determine the initial conditions of the pilot scale bioreactor experiments. Molasses based pilot scale bioreactor experiments with pulse and continuous feeding strategies leading to higher thermostable glucose isomerase activity by recombinant *Escherichia coli* W carrying pRSETA::*xylA* plasmid were conducted. Moreover, cell growth, GI activity, bioreactor characteristics and by-product formation were investigated in these bioreactor operations.

CHAPTER 2

LITERATURE SURVEY

2.1 Glucose Isomerase

Glucose isomerase (E.C.5.3.1.5) is a type of enzyme which belongs to isomerases that catalyzes the isomeration reaction of glucose to fructose and it also converts D-xylose to D-xylulose, reversibly (Dekker *et al*., 1991).

GI catalyzes the critical step of the production of high fructose corn syrup (HFCS). Furthermore, GI is used for conversion of xylose to ethanol in association with other enzymes throughout the bio-fermentations (Bhosale *et al*., 1996).

Table 2.1 GI Producing Companies (Bhosale *et al*., 1996)

Company	Trade Name	Microorganism source
Gist Brocades and Anheuser-Busch Inc.	Maxazyme	*Actinoplanes missouriensis*
Novo-Nordisk	Sweetzyme	*Bacillus coagulans*
Finnsugar	Spezyme	*Streptomyces rubiginosus*
Nagase	Swetase	*Streptomyces phaeochromogenes* *Arthrobacter sp.* *Streptomyces olivaceus*

Novel GI producing strains have attained importance after the invention of the isomerization capability of GI produced from *Pseudomonas hydrophila* by Hochster *et al*. (1957), and by Marshall and Kooi (1957). Moreover, 3-D structure, stability, substrate specificity, active site, inhibitor and co-factor properties of GI was obtained to discover structural and kinetic characteristics of GI (Schray and Mildvan, 1972; Hogue-Angeletti, 1975; Asboth and Szaba 2000, Kovalevsky *et al*.,2010).

Although different enzymes show different properties, there are several mutual criteria supposed to taken into consideration in the production of commercial enzymes, such as optimum temperature and pH, reaction rate, stability, half-life, substrate specificity and inhibition mechanisms (Soetaert and Vandamme, 2010).

2.1.1 Optimal Temperature and pH

The optimal temperature and pH values depend on the source of microorganism and alter from 60°C to 90°C and from pH 7.0 to 9.0, respectively. The enzymes which are isolated from thermophilic species indicate better stability at high temperatures when compared to mesophilic strains (Bhosale *et al*., 1996).

2.1.2 Structure

Molecular weight of glucose isomerase is between 44,000 and 191,000 Da which depends on the source microorganism. The subunits (monomers) are composed of 387 amino acids which has molecular weight of 44,000 Da (from *Thermus thermophiles*). There are ten β -strands, 16 α -helices, and five 310-helices in the compound of the each monomer (Chang *et al*., 1999). Each of these is consisted of two domains; Domain I, N-terminal domain (residues 1 to 321) folds into the (β/α) 8-barrel while Domain II (residues 322 to 387) contacts with Domain I.

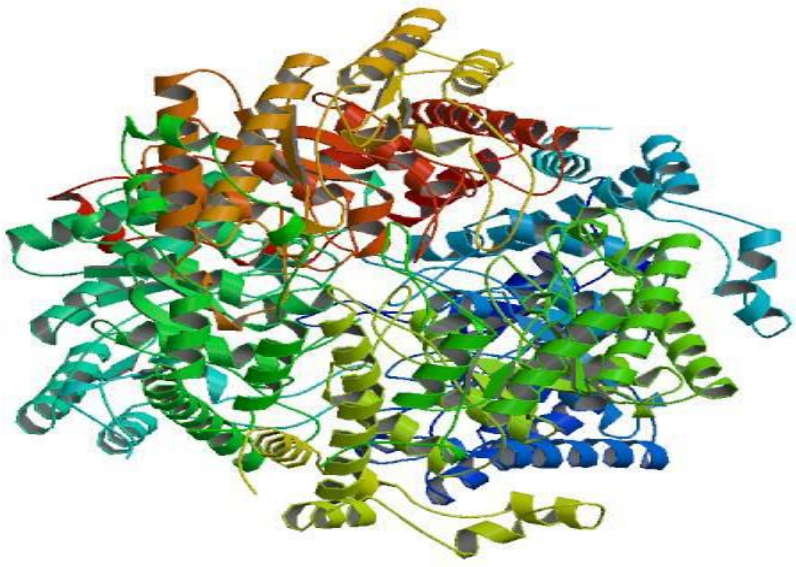

Figure 2.1 3D structure of glucose isomerase from *Thermus thermophilus* (PDB Accession Number: 1BXB)

The similarity of tetrameric structure of GI from *T. thermophiles* with GIs from other microbial sources was understood by the X-ray crystallography studies. Although monomeric GI form is always inactive, homotetrameric GIs with identical subunits are generally active as dimeric and trimeric forms as well (Ghatge *et al*., 1994; Chauthaiwale *et al*., 1994).

The metal binding sites of GI of *Streptomyces rubiginosus* was investigated by the applying mutual technique of X-ray crystallography and neutron diffraction by Kovalevsky *et al*. (2010). By the help of this study, necessity of two divalent metal cations for full GI activity was shown. Due to the position on the GI structure, the metal sites were named as M1 and M2; while M1 site prefers Mn^{+2}, Mg^{+2}, Co^{+2} and Pb^{+2} ions, M2 structural metal site shows various affinity of divalent-metal cations.

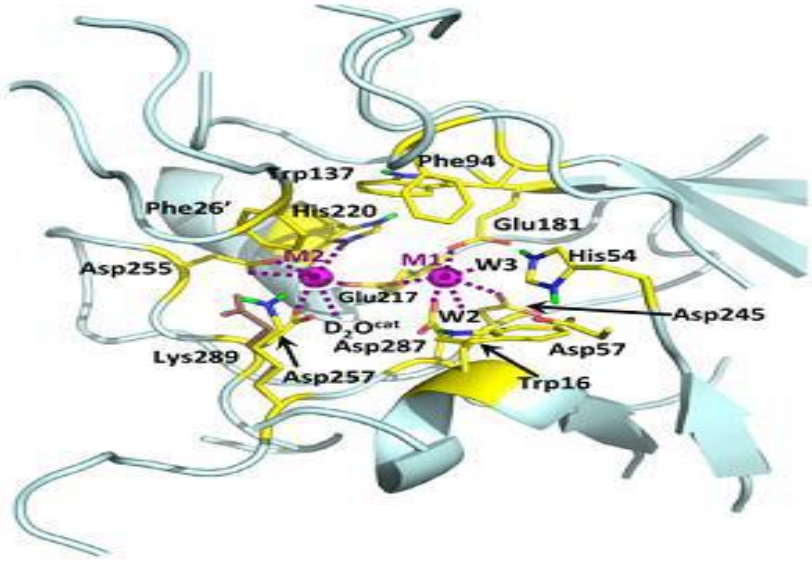

Figure 2.2 Metal binding sites of GI (Kovalevsky *et al*., 2010)

2.1.3 Metal Ion Requirement and Inhibitors

Cofactors are nonprotein components such as metal ions and vitamins, are necessary for enzyme stabilization and activation. Like other enzymes, GI also needs divalent metal cations such as Mn^{+2}, Mg^{+2}, Co^{+22} as cofactor **(**Kovalevsky *et al*., 2010). Metal ions which have larger diameter like Ag^{+}, Hg^{+2}, and Ni^{+2} are known as inhibitors of GI. In addition sorbitol, mannitol, xylitol, arabitol and tris are other known inhibitors of GI (Bhosale *et al*., 1996).

2.1.4 Active Site Studies and Reaction Mechanism

Two histidine residues (His-101 and His-271) those located in the active site of the glucose isomerase were proven to be crucial for the activity of the enzyme by site directed mutagenesis studies (Batt, 1990). In order to determine the mechanism of enzymatic reaction pathway, Meilleur *et al*. (2006) specified the position of the hydrogen atoms. The study of them demonstrated that initiation of isomerization reaction by double protonation of His-53 residue continued through an acid catalyzed opening of the sugar ring. With respect to these studies the reversible isomerase reaction of GI was demonstrated that it proceeds in three steps: i) ring opening, ii) isomerization and iii) ring closure, as it can be seen from Figure 2.3 (Kovalevsky, 2010).

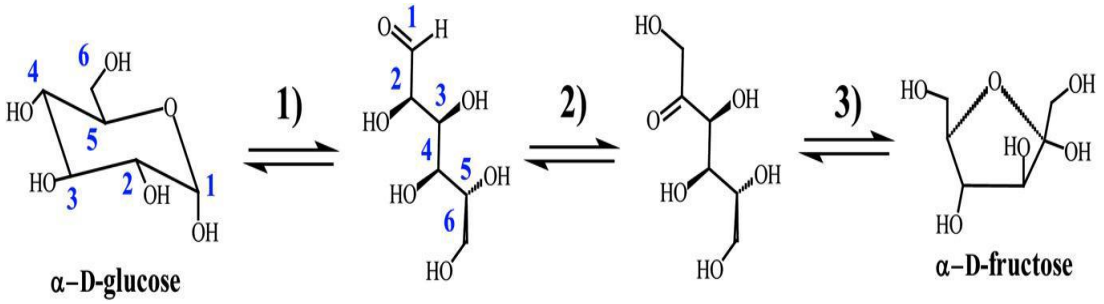

Figure 2.3 Catalyzed reaction mechanisms by glucose isomerase; 1) ring opening, 2) isomerization, and 3) ring closure (Kovalevsky *et al*., 2010)

In this model, several conformational changes take place at the active site of the enzyme throughout the reaction initiation and implementation by the critical help of many amino acids. The reaction is initiated by binding of reactive cyclic sugar substrate and active region of enzyme to each other. In this step, a diverse arrangement of water molecules presence eases the true participation of the substrate to GI. In the second step, the ring opening occurs in which His-54 transiently leads an extra proton to O5 and takes the proton back after the break of C1-O5 bond. During the ring opening reaction, neutral Lys-289 is protonated and donates a proton within a hydrogen bond to Asp-257. Then, in order to start isomeration step, O1 is placed in the correct location by the help of Lys-183. In the final step, isomeration reaction occurs with transfer of H^+ ion from C2 and O2 to C1 and O1, respectively. The reaction mechanism of glucose isomerase is shown in Figure 2.4.

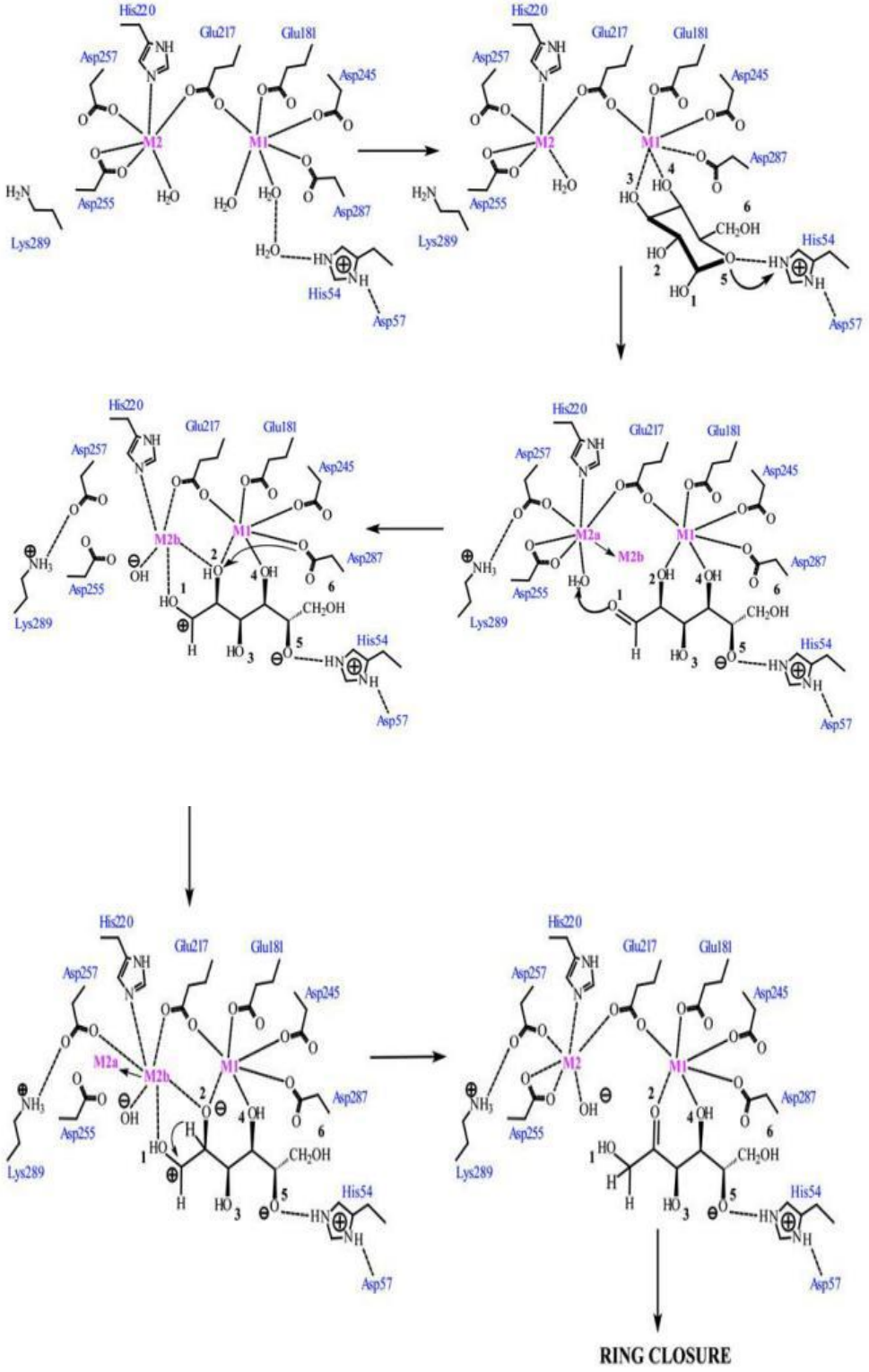

Figure 2.4 Complete reaction mechanism recommended in this study (Kovalevsky *et al*., 2010)

2.1.5 Substrate Specificity

While GI producing microorganisms generally prefer D-glucose and D-xylose to have higher isomeration activity; D-ribose, L-arabinose, L-rhamnose and D-allose are rarely preferred (Bhosale *et al*., 1996). The maximum isomeration activity of GI is achieved by substrate utilization which contains hydroxyl groups at carbons 3 and 4 in the equatorial state (Chen, 1980).

2.1.6 Genetic Engineering of Glucose Isomerase

The starting point of the investigations of Glucose isomerase is the discovery of isomerizing activity of GI from *Pseudomonas hydrophila* by Hochster *et al*. in 1953 and it is followed by the detection of GI activity in over 100 microorganisms (Barker and Shirley, 1980; Ulezlo *et al*., 1986). Furthermore, improvements in recombinant DNA technology occurred at the same years and isolation and manipulation of *xylA* gene from different strains became possible.

The first report which was about the isolation of GI gene from *Escherichia coli* was published by Ho *et al*. (1983). In 1955 Stevis and Ho (1985) cloned the isolated *Escherichia coli xylA* gene into *E. coli* plasmids and low expression levels was observed due to strong regulatory effects of natural promoter. This problem was solved by the integration of structural gene into lac or tac promoters (Stevis and Ho, 1985).

Shin *et al*. (1985) isolated *xylA* gene from *B. licheniformis* and cloned into GI-negative mutant of *Escherichia coli* strain and achieved 20-fold higher enzyme activity than the source microorganism's GI activity. In the study of Wuxiang and Jeyaseelan (1993), *xylA* gene from thermophilic *Bacillus sp*. was isolated and *Escherichia coli* transformation was carried out with the result of the detection of 12.8 U mg^{-1} glucose isomerase activity at 85°C. Batt *et al.* (1986) investigated *xylA* gene controlled by *tac* promoter which was induced by isopropylthio β-D-galactopyranoside in *E. coli*; brought about the production of GI attained to 28% of total cell protein.

Dekker *et al.* (1991) obtained 45-fold higher yield than the source microorganism by isolating *xylA* gene from hyper-thermophilic *T. thermophilus* followed by determination of its nucleotide sequence and cloning into *Escherichia coli* under control of tac promoter. Moreover, GI activities of recombinant *xylA* from *T. thermophilus* expressed in *Escherichia coli* and *B. brevis* were compared to one another by Dekker *et al.* (1992); where *r-B. brevis* had 5600 U L^{-1} and *r- Escherichia coli* had 4300 U L^{-1} of GI activity.

Lee *et al*. (1990) published the study between *Escherichia coli* and *B. subtilis* in order to compare their thermostable GI producing capability by using the *xylA* gene from *C. thermosulfirogenes*. *B. subtilis* showed higher enzyme activity (1.54 Umg^{-1}) than *Escherichia coli* (0.46 U mg^{-1}). Again Lee *et al.* (1993) inserted the *xylA* gene from *Thermoanaerobacterium saccharolyticum* into pUC18 and transformed into *Escherichia coli*. After the expression of GI gene in *E. coli*, the product was identified as a tetramer

composed of identical subunits with molecular weight of 200 kDa. Moreover its optimal temperature and pH were 80°C and pH 7.0-7.5, respectively.

There are more researches about the production of active GI by cloning *xylA* genes, which was obtained from diverse sources, into appropriate *Escherichia coli* strains (Kaneko *et al*., 2001; Sarıyar *et al*., 2004; Rhimi *et al*., 2007; Rozanov *et al*., 2007). In the master thesis of Ata, 2012; *xylA* gene which was obtained from *Thermus thermophilus* that encodes glucose isomerase was inserted to pPICZα-A expression vector. This pPICZα-A::*xylA* plasmid was integrated to AOX1 locus in the *P. pastoris* genome to design a recombinant *P. pastoris* strain which produce an active thermostable GI from *T. thermophilus.* In the PhD thesis of Angardi (2011), thermostable *xylT* gene of *Thermus thermophilus* was recombined into strongly regulated prokaryotic pRSETA expression vector and pRSETA::*xylT* obtained was transferred into *E. coli* BL21 (DE3) pLysS to investigate glucose isomerase activity capacity. In the literature, the highest GI activity was reported as 41,000 U L^{-1} with *Enterobacter agglomerans* in the xylose and peptone containing fermentation medium by Pandidurai *et al*. (2011).

In addition, there are several studies aimed to use yeast cells for the production of GI. Chan *et al*. (1986; 1989) developed recombinant *S. pombe* where the pDB248-XI including *xylA* gene from *E. coli* was transformed into yeast cells and they produced 3% (w/v) ethanol by utilizing 10% (w/v) xylose. In 1996, Moes *et al*. cloned the *xylA* gene from *C. thermosulfirogenes* into *S. cerevisiae*. Although influential transcription of the *xylA* gene with the control of the ADH2 was carried out, the recombinant *S. cerevisiae* was lacking ability of producing GI in an active form. Because of cytosolic environment of *S. cerevisiae* that represses GI subunits to form active and stable tetramers that function correctly and followed by proteolytic degradation. Walfridsson *et al*. (1996) achieved the first successful construction of yeast strain, *S. cerevisiae*, which produced bacterial glucose isomerase, *T. thermophilus xylA*, in the active form.

2.2 Microorganism

From prokaryotic systems to eukaryotic systems, different microorganisms are applied for enzyme productions with the strain improvement methods such as mutation, selection, and genetic recombination (Aehle, 2007, Parekh *et al*., 2000). One of the critical steps for the achievement of successful bioprocesses is appropriate host microorganism selection and strain improvement in development of the interested product commercially (Nielsen *et al*., 2003). Comparing with plant and animal sources; microorganism are more popular for recombinant product production host due to having fast multiplication rate and ease of culture.

Potential host has to enhance the productivity with carrying some properties such as; reducing the cost of the process, high product activity and feasibility for industrial bioprocesses, growing on cheap media, and easily scaling-up (Kirk and Othamer, 1994; Soetaert and Vandamme, 2010). In addition, the host microorganism must have GRAS (generally recognized as safe) statue.

One of the most preferred host microorganisms for the production of industrial enzymes and proteins is *E. coli* because of achieving high yields and good protein secretion, being easy to control gene expression, being suitable for extensive selection of cloning vectors, having high productivity on low cost productions. In contrast, there are several disadvantages of *E. coli* such as deficiency of post-translational modifications, containing high endotoxins and aggregation of proteins (Nielsen *et al*., 2003).

2.2.1 General Characteristics of *Escherichia coli*

Escherichia coli is facultatively anaerobic rod shaped non-sporulating and gram-negative bacterium with a cell volume of 0.6-0.7 μm^3. The bacterium was identified by Theodor Escherich.

E. coli is one of the best studied model microorganism for recombinant product processes and its applications has several advantages as follow: possibility of easy genome modification, well characterized system by the help of the knowledge of its whole genome, possibility of mutations to have protease deficient strains (Soetaert and Vandamme, 2010). In addition, it is one of the most important host organism in biotechnology and for industrial applications due to; growing rapidly on simple media with high cell concentrations and obtaining heterologous protein rate can be increased to 50% of dry cell weight (Soetaert and Vandamme, 2010; Glazer and Nikaido, 1995; Choi *et al*., 2006; Yee and Blanch, 1993; Lee, 1996).

On the other hand, acetate formation and accumulation is the main problem for *E. coli* cultivation due to high carbon source concentration and high specific growth rates with aerobic fermentation condition which is called over flow metabolism (Akesson *et al*., 1999). Recombinant protein production is negatively affected by high acetate concentration as a result of cell growth inhibition and increasing cells maintenance energy. It is also affected by fermentation composition, medium conditions and type of used strains (Han *et al*., 1992; Lee, 1996). Acetate accumulation problem in *E. coli* fermentations is solved by keeping glucose concentration below 0.75 g L^{-1} (Holms, 1986; Marison and von Stocker, 1986). In addition, maintaining limited carbon source concentration with semi-batch (fed-batch) operation at controlled specific growth rate by exponential feeding is the other solution for acetate accumulation. Also there are several strains which their own metabolism tend to produce low acetate levels or some of them are genetically modified to obtain low-acetate-producer. In the master thesis of Taşpınar (2010) and the PhD thesis of Angardi (2011), the low acetate producer strain, *E. coli* BL21 (DE3) pLysS was used. In the latter study, *E. coli* W was used as a host microorganism and it was reported that *Escherichia coli* Waksman is low acetate producer strain.

The feeding methods effect on cell concentration, recombinant protein production and acetate formation for diverse *E. coli* strains were investigated by comparing all modes of operations; thus the exponential feeding strategy was reported as the best strategy for controlling acetate accumulation lower than the critical level for inhibitory value above 5 g L^{-1} (Luli and Strohl, 1990; Yee and Blanch, 1993; Lee, 1996; Choi *et al*., 2006).

In *E. coli* fermentations, it is possible to obtain high volumetric productivities because of high cell densities with high expression level of *E. coli* (Shuler and Kargı, 1992; Choi *et al*., 2006). Currently, above 100 g DCW L^{-1} of *E. coli* can be achieved by using fed-batch cultivations techniques (Lee, 1996; Shiloach and Fass, 2005). However, high specific growth rate and excess amount of carbon source cause the major problem for *E. coli*; the accumulation of acetate (Åkesson *et al*., 1999). Since cell growth is inhibited by high amount of acetate, the recombinant protein production is reduced as well (Luli and Strohl, 1990; Han *et al*., 1992; Åkesson *et al*., 2001). By the help of the fermentation parameters such as selecting appropriate strain, determination of medium composition, applying cultivation condition and choosing carbon source feeding strategy are the important ways of reducing acetate formation (Van de Walle and Shiloach, 1998; Åkesson *et al*., 2001).

2.2.2 Host Microorganism: *E. coli* W

In the industrial application, manufacturing of bio-products and chemicals using sucrose utilized by *E. coli* strains is not common. Some of *E. coli* strains are able to utilize sucrose as a sole carbon source. *Escherichia coli* Waksman (*E. coli* W) is one of these strains. *E. coli* W (ATCC 9637, NRRL B-766) was firstly isolated from soil by Selman A. Waksman in 1943 (Archer *et al*., 2011) This strain is the only safe strain that can utilize sucrose as a carbon source among other *E. coli* strains which are regarded as GRAS. The W strain uses chromosomally encoded sucrose catabolism (csc) regulon which consists of cscA (invertase), cscB (permease), cscK (fructokinase), and cscR (repressor) to utilize the sugar (Bruschi *et al*., 2012). Figure 2.5 demonstrates the uptake metabolism of sucrose in *E. coli* W.

Sucrose is a significant molecule used in plants through carbohydrate transportation. The most advantageous feature of this molecule for industrial bioprocesses is its availability. In addition, since sucrose is cheaper and more environment-friendly than other carbon sources by decreasing gas emissions during ethanol production, it is frequently preferred. Owing to these properties of this abundant disaccharide, the processes in which sucrose is used become more feasible than the processes in which other carbon sources are used (Archer *et al*., 2011; 133). On the other hand; fast growing rate, low acetate production, high tolerance to environmental impacts such as high temperature, high activity and high concentrations of ethanol, and lack of any antibiotic resistance gene are the advantages of the strain W (Archer *et al*., 2011).

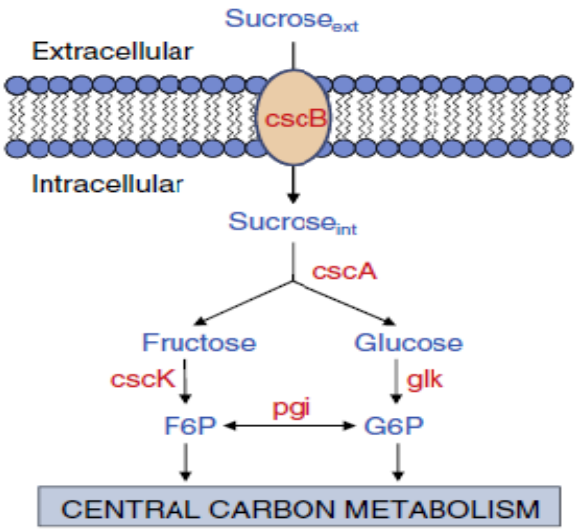

Figure 2.5 Sucrose utilization mechanisms in *E. coli* W **(**Bruschi *et al*., 2012)

The transport of the sucrose through the cell membrane is initiated by cscB in the beginning of the sucrose uptake. Then, cscA is involved in the processes in order to hydrolyzing the cell sucrose into fructose and glucose. Afterwards, these monosaccharides are phosphorylated by cscK and glk (glucokinase) to obtain Fructose-6-Phosphate (F6P) and Glucose-6-Phosphate (G6P), respectively. Where, F6P enters glycolysis directly; G6P is converted to fructose-6-P by pgi (phosphoglucoisomerase) before entering glycolysis or enters the pentose phosphate pathway directly. At low sucrose concentrations, cscR inhibits the transcription of the csc genes (Bruschi *et al*., 2012).

2.3 Genetic Engineering Techniques and Methodology

The genetic engineering applications such as gene addition, substitution or deletion make possible to construct new strains and to obtain valuable enzymes and proteins by them. Polymerase Chain Reaction (PCR) applications for amplification of the target gene, gel electrophoresis, DNA isolation and, microorganism and plasmid selection are some of the genetic engineering methods which are performed in recombinant DNA technology.

In the genetic engineering experiments of this study, plasmid isolation, agarose gel electrophoresis, wild type *E. coli* transformation of metabolically engineered plasmid and microorganism selection was achieved. There are important criteria that should be considered while choosing suitable cloning vector such as replicating autonomously, containing proper marker genes and having unique restriction sites for insertion of DNA (Walker, 1998). In this study, pRSETA expression vector which was widely used for recombinant protein production in *E. coli* was selected (Figure 2.6). This pRSETA expression vector is consisted of ampicillin resistance gene, T7 gene sequence, multiple

cloning site included 11 restriction enzyme recognition sequences, N-terminal poly-histidine tag, f1 and pUC regions (http://www. invitrogen.com).

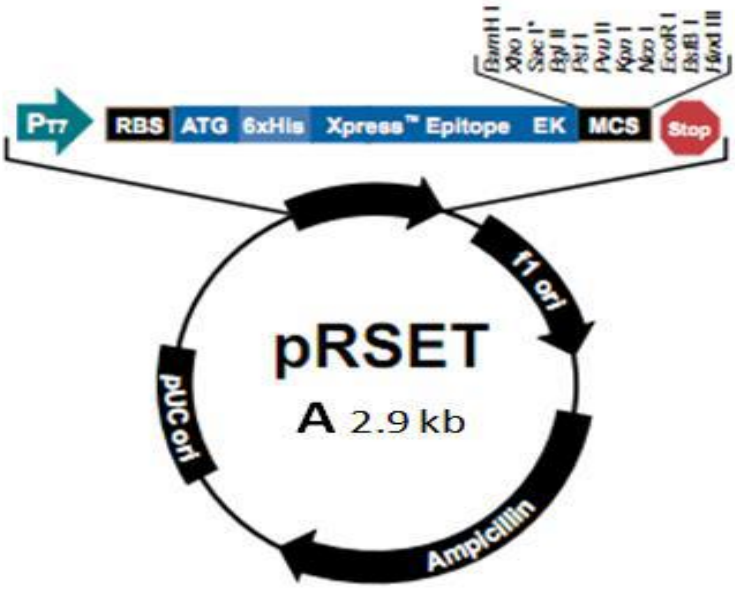

Figure 2.6 pRSETA cloning vector (Angardi, 2011)

Angardi (2011) designed pRSETA::*xylA* for the production of thermostable glucose isomerase. After this recombinant plasmid isolation, transformation was carried out into the host cell; wild type *E. coli* W. There are four main methods for the introduction of foreign DNA into host cells (Glazer, 1995);

- Natural transformation: Foreign DNA is taken up by the bacteria and fused to the chromosomal DNA of the organism without any *in vitro* treatment.

- Artificial transformation: Cell wall permeability is increased by using several chemicals such as $CaCl_2$ or $LiCl_3$ with heat shock (0°C) to be taken foreign DNA into the cell easily.

- Protoplast transformation: Enzymes are used to hydrolyze the rigid cell wall to convert the cell into protoplast to transfer of foreign DNA into cell.

- Electroporation: Short electrical pulses of high voltage are applied to the cells to make them component by obtaining transient pores in their membrane.

In this master thesis, artificial method ($CaCl_2$ Method) was used to transfer pRSETA::*xylA* plasmid into the *E .coli* cells. After transformation, selection of recombinant strains was carried out by the help of the antibiotic resistance gene. All of the transformants were spread on plates containing the particular antibiotic in order to distinguish the cells carrying true

plasmid from the other cells. Then, growing cells on this media were selected for further analyses such as PCR, agarose gel electrophoresis and DNA sequencing (Klug *et al*., 2006). The steps of cloning of the foreign gene in *E. coli* as a model microorganism are illustrated in Figure 2.7.

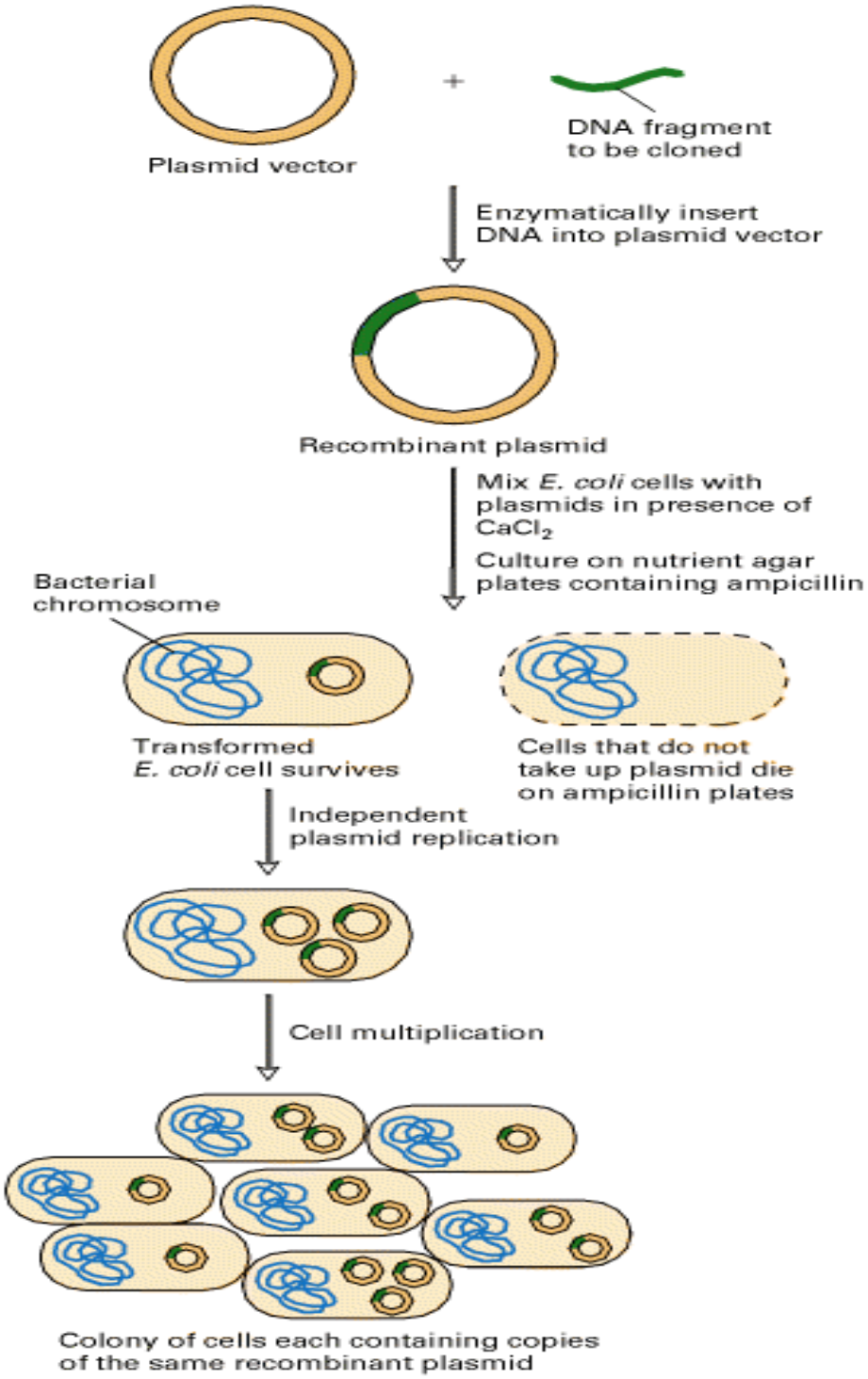

Figure 2.7 Steps for cloning of the foreign gene in *E. coli* (Angardi, 2011)

2.4 Medium Composition and Bioreactor Operation Parameters

A bioreactor is any device which is employed to carry out biochemical conversions of substances to products. In addition, the bioreactor supplies an optimal conducive environment to functioning of the biocatalyst. Therefore, bioreactors are the important part of bio-based productions such as production of biomass and metabolites, bio-transforming or degrading wastes driven by microorganisms, enzymes, and animal and plant cells.

In the design of a successful fermentation; strain selection, carbon source feeding strategy, medium composition, and specific growth rate are important parameters which also play critical roles in *E. coli* fermentations (Akesson *et al*., 2001; Van de Walle and Shiloach, 1998).

There are several criteria that should be optimized to increase productivity and yields of the commercially valuable bio-products such as medium composition and bioreactor operation parameters (temperature, pH, dissolved oxygen concentration).

2.4.1 Medium Composition

Medium composition determination is a very critical step for fermentation process design (Nielsen *et al*., 2003). Macronutrients (carbon, nitrogen, hydrogen and phosphorous), micronutrients (potassium, calcium, magnesium, manganese and iron), organic components (vitamins, amino acids, nucleotides and other growth factors) and trace elements, which are needed for cell growth, maintenance, biosynthesis of cellular substances, products of cell operation, including source of energy should be contained in fermentation media (Kampen, 1997; Nielsen *et al*., 2003).

Defined media and complex media are the types of growth media. Defined medium (synthetic medium) is prepared by exact amounts of pure inorganic components. Owing to the advantages of easy reproducibility, low foaming tendency, product recovery, and simple purification; defined medium is generally preferred to determine the effects of specific elements on cell growth rate and product formation and yields (Kampen, 1997). On the other hand, complex medium is composed of natural complexes but its chemical composition is not known precisely. Due to the advantages of presence in large quantities; relative cheapness; including nitrogen source, essential mineral, various growth factors, vitamins and trace elements thus resulting in evaluated cell and product yields, complex medium generally is preferred in industrial fermentations (Shuler and Kargı, 2002).

One of the most commonly used growth media is LB (Luria-Bertani) for *E. coli* cultivations. LB consists of tryptone, yeast extract and sodium chloride. Cell density of *E. coli* can be up to OD_{600} 2 to 3 under appropriate environmental incubation conditions. To achieve higher density, nutrient concentrations must be increased by considering inhibition concentrations of the substances. High concentrations of glucose, ammonium, iron, magnesium, zinc and phosphorus have inhibitory effect on *E. coli* growth (Schiloach and Fass, 2005).

In defined medium cultivations, containing maximum non-inhibitory nutrient concentrations, cell concentrations of *E. coli* can reach approximately 15 g DCW L^{-1} (Schiloach and Fass, 2005).

Table 2.2 General complex fermentation media (Nielsen and Villadsen, 2003)

Medium	Contents
Corn steep liquor	Lactate, amino acids, minerals, vitamins
Corn starch	Starch, glucose
Barley malt	Starch, sucrose
Molasses	Sucrose, raffinose, glucose, fructose
Pharmamedia	Carbohydrates, minerals, amino acids, vitamins
Serum	Amino acids, growth factors
Whey	Lactose, proteins
Yeast extract	Peptides, amino acids, vitamins

In the literature, the effect of medium components on *E. coli* growth characteristics has been demonstrated in many studies (Neidhardt *et al*., 1974; Yee and Blanch, 1993; Choi and Lee, 1997). In addition, Kaya *et al*. (2009) developed a glucose based medium containing 8 g L^{-1} glucose, 5 g L^{-1} $(NH_4)_2HPO_4$ and salt solution to investigate the effects of different oxygen transfer rate on cell growth and product formation of recombinant *E. coli* for benzaldehyde lyase production bioprocesses.

Appropriate feeding strategy and well-developed medium are the important parameters for obtain high cell concentration of *E. coli* (Lee, 1996; Schiloach and Fass, 2005). Experimental and trial-and-error-based processes are necessary for the development of the cultivation medium; therefore media optimization is an experiment-intensive process (Lee, 1996).

Table 2.3 Diverse complex carbon source compounds (Atkinson and Mavituna, 1991)

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

Angardi (2011) investigated molasses-based medium containing 30 g L^{-1} molasses, 5 g L^{-1} $(NH_4)_2HPO_4$ and salt solutions with exponential feeding to develop production of recombinant thermostable glucose isomerase with *E. coli* BL21 (DE3) pLysS strain. Diverse pre-determined specific growth rates of molasses feedings were examined and μ=0.1 h^{-1} value was reported as the best condition for glucose isomerase activity. In addition, glucose isomerase activity with molasses feed were compared to glucose feed with the same pre-determined specific growth rate and it was shown that complex medium had a positive effect on enzyme activity. In the study of Angardi, it was shown that molasses based production medium does not need any additive for the induction of enzyme production due to having galactose in it, but sucrose and glucose based production media need inducer; IPTG (Isopropyl β-D-1-thiogalactopyranoside).

2.4.1.1 Sucrose

Sucrose is a white, odorless, crystalline powder which is commonly known as table sugar or saccharose and it is best known for its role in food industry due to its sweet taste. The sucrose molecule is a disaccharide which is composed of glucose and fructose which are linked via an ether bond (glycosidic linkage) between C1 on the glucosyl subunit and C2 on the fructosyl unit. Sucrose is an organic compound which is obtained from sugar cane or sugar beets with the molecular formula of $C_{12}H_{22}O_{11}$ (Figure 2.8).

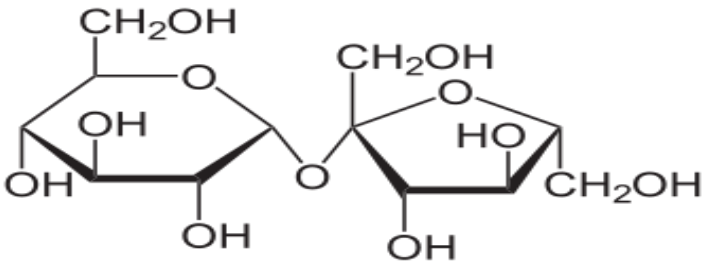

Figure 2.8 Sucrose structure

In the mid-1980's sucrose and cheap sucrose-containing substrates such as molasses potential was first recognized for industrial *E. coli*-based bioprocesses (Garcia, 1985; Scholle *et al*., 1987). Sucrose is the most abundant disaccharide on earth and it is the cheapest readily-available carbon source that can be used as a feedstock (Koutinas *et al*. 2004). Since the price of the carbon feedstock is the most important parameter of large-scale bioprocesses for the production of industrial bio-products and chemicals, sucrose have been becoming a highly attractive feedstock because of its low price (Rude and Schirmer, 2009). In addition, sucrose has a positive influence on protection of cells and protein structure from environmental stresses such as oxidation, heat, and acid, so it can be an appropriate carbon source to enhance bio-product formation (Kilimann *et al*. 2006; Molina-Hoppner *et al*. 2004; Prestrelski *et al*. 1993; Van Opstal *et al*. 2003).

Also glucose from hydrolyzed corn starch was used for *E. coli*-based bioprocesses as a carbon and energy source. However, it was reported that sucrose from sugarcane has superior environmental performance than glucose from corn and sucrose from sugar beet as residual sugarcane biomass can be burned to provide energy to the system (Renouf *et al*., 2008). Moreover, the growth of sugarcane in tropical regions is relatively easier than other sugar feedstock (Renouf *et al*., 2008). Thus, sucrose from sugarcane is emerging as a preferred carbon source in comparison with glucose.

However, many of widely used *Escherichia coli* K-12, B, and C cannot utilize sucrose, and less than 50% of the wild-type strains can catabolize sucrose as a sole carbon source including *E. coli* W, EC3132, and O157:H7 (Jahreis *et al*. 2002). *E. coli* W is able to utilize because it has chromosomally encoded sucrose catabolism (csc) regulon as mentioned Section 2.2.2.

2.4.1.2 Molasses

The main and the most valuable by-product of the sugar industry is sugar beet molasses. Molasses can be used for the production of a lot of commercially valuable compounds such as ethyl alcohol, citric/glutamic acids, acetone/butanol, baker's yeast, several proteins and enzymes (Çalık *et al*., 2003).

There are several advantages to utilize molasses rather than to use other carbon sources in the industrial bio-product fermentations. Molasses is cheaper, has high sugar concentration, contains organic non-sugars, essential minerals and vitamins for growth, is easily available in large amounts and can be used for industrial ethanol production in its own sugarcane factories (Park and Baratti, 1991; Kirk and Othmer, 1994).

In the literature, citric acid was produced by utilizing beet molasses and cane molasses based complex medium via *Aspergillus niger* fermentation and it was reported that citric acid production were increased by using complex media rather than defined media (Gerhard *et al*., 1946; Sikander *et al*., 2002).

In addition, ethanol was produced with the value of 53 kg m^{-3} with non-sterilized beet molasses by immobilized and free *S. cerevisiae* cells in fed-batch operation modes (Roukas, 1996). Afterwards, pre-treated beet molasses effect on serine alkaline proteases production was concluded using *Bacillus* species with in batch operations by Çalık *et al*. (2003).

The effect of different molasses concentrations on lactic acid production was investigated by Dumbrepatil *et al*. (2008) by using *Lactobacillus delbrueckii in* batch feeding modes. In order to attain 166 g L^{-1} lactic acid, initial molasses concentration of 190 g L^{-1} was used.

Table 2.4 Content of beet molasses from European samples (Olbrich, 1963)

Component	Average composition (%)
Water	16.5
Sucrose	51.0
Glucose and Fructose	1.00
Raffinose	1.00
Organic non-sugars	19.0
Ash components:	11.5
SiO_2	0.10
K_2O	3.90
CaO	0.26
MgO	0.16
P_2O_5	0.06
Na_2O	1.30
Fe_2O_3	0.02
Al_2O_3	0.07
CO_3	3.50
Sulfates as SO_3	0.55
Cl	1.60
Vitamins (mg/100 g)	
Thiamine (B1)	1.30
Riboflavin (B2)	0.40
Nicotinic acid	51.0
Ca-pantothenate (B3)	1.30
Folic acid	2.10
Pyridoxine-HCl (B2)	5.40
Biotin	0.05

Çalık and Levent (2009-a, 2009-b) reported that the medium with pretreated-beet molasses had higher BAL activity when compared to defined medium both in batch and fed-batch *E. coli* fermentations.

The effect of exponential feeding on recombinant benzaldehyde lyase production (BAL) via using pretreated-beet molasses by *E. coli* was examined in the master thesis of Taşpınar (2010); thus pre-determined specific growth rate of 0.15 h^{-1} was reported as the best value for maximum BAL activity and cell growth. Afterwards, recombinant GI production was developed with exponential feeding via using pretreated-beet molasses by *E. coli* in the PhD thesis of Vahideh (2011); whereby pre-determined specific growth rate of 0.1 h^{-1} was reported as the best value for maximum GI activity as A=16,400 U L^{-1}. In this master thesis, un-treated molasses was used as favor carbon source for glucose isomerase production by *E. coli* W-26 in pilot scale bioreactors.

The molasses ingredients which was used in the current study and was obtained from Ankara Sugar Factory, is given in Table 2.5.

Table 2.5 Molasses composition of the current study (Ankara Sugar Factory)

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

2.4.2 Bioreactor Operation Parameters

For aerobic fermentations; temperature, pH, and dissolved oxygen concentration are the most important bioreactor operation conditions which effect product yield and cell growth in biofermentor.

2.4.2.1 Temperature

One of the most critical bioreactor operation conditions which should be optimized and kept constant is temperature. Temperature affects cell growth rates, yield coefficients and recombinant protein production. While low temperature causes decrease in cell growth and reaction rate; high temperature causes protein denaturation and cell death (Shuler and Kargı, 1992, Nielsen *et al*., 2003; Kirk-Othmer, 1994; Watson *et al*., 1987). In this work, *E. coli* cells were performed at 37°C, which is the optimal temperature point for *E. coli*, to produce of thermostable GI.

2.4.2.2 pH

pH is another important parameter for bioreactor operation which should be controlled during the fermentation, due to its effect on the enzyme activity, transport mechanism and growth of cells (Chiruvolu *et al*. 1999). For overall cell functions it is critical to keep pH in optimum conditions, which can be changed by nitrogen source utilization and by-product formation during the fermentation, so buffer solutions are used to keep constant the hydrogen ion concentrations through the bioprocess (Shuler and Kargı, 2002).

The change of the optimum pH values depends on the type of the microorganism. For several bacteria, the optimum growth pH is between pH 3 and pH 8; pH 7 is the optimum value for *E. coli* strains (Shuler and Kargı, 1992). In the literature, there are several studies which were carried out to produce diverse proteins by *E. coli* cells in which cultivations were conducted at different pH values (Åkesson *et al*., 2001; Castan *et al*., 2002; Leon *et al*.,

2003). Ryan *et al.* (1989), Çalık *et al*. (2006), investigated the effect of medium pH on diverse products and growth of *E. coli.*

Investigation of controlled and un-controlled pH conditions' effect on BAL activity and cell concentrations by using recombinant *E. coli* was carried out at the pH values between pH 5.0 and pH 7.8 by Çalık *et al*. (2006). It was reported that the un-controlled pH 7.2 is the most favorable value. In addition, in the studies of Kaya *et al.* (2009), Çalık and Levent (2009-a, 2009-b), working value at controlled pH 7.2 for BAL production with *E. coli* BL21 strain were applied with molasses based medium. In the master thesis of Taşpınar (2010) and PhD thesis of Angardi (2011), *E. coli* strains were worked at pH=7.2 and pH values were kept constant with the use of 5 M KOH and 5 M H_3PO_4. In the current study, initial pH of the fermentation medium was arranged as pH 7.2 and this value was tried to maintain at that level.

2.4.2.3 Oxygen

Another important parameter for aerobic bioprocesses is dissolved oxygen (DO). Due to the low solubility of oxygen in water, DO can be a limiting constituent in aerobic fermentations. Product formation rate and cell growth are influenced by oxygen concentrations due to effect on metabolic pathways (Çalık *et al*., 1999). Therefore oxygen transfer process should be examined in detail to develop the yields of products. The manipulation of air supplied rate to the system and the changing agitation rate are the ways for the control of the dissolved oxygen concentrations.

Type of the microbial species, cell concentrations, temperature and type of the substrate utilization in the fermentation media are the important parameters for determination of the oxygen requirement for bioprocesses. Since the low oxygen concentrations limit the cell growth rate in the aerobic fermentations, the adequate amount of oxygen should be supplied to the cultivation medium (Soetaert and Vandamme, 2010).

In the literature, there are several researches which showed the effect of various oxygen transfer rates on diverse *E. coli* fermentations products (Ryan *et al*., 1989; Bhattacha and Dukey, 1997; Çalık *et al*., 2004). Kaya *et al*. (2009) reported that the optimum oxygen transfer rate for the *E. coli* BL21 strain to produce recombinant enzyme production with high cell concentration and product formation was managed by keeping DO level at 40% throughout the fermentation. This optimum value was applied into pulse feeding of molasses in order to produce benzaldehyde lyase production by Çalık and Levent (2009-b). In the master thesis of Taşpınar (2010) and PhD thesis of Angardi (2011), pilot scale bioreactors with *E. coli* cultivations were tried to maintain the oxygen level at D0=40%.

2.4.3 Modes of Operation

In a bioprocess the most significant parameter in terms of biomolecule synthesis is the design of a medium adjusting characteristic of oxygen transfer and pH conditions for an aerobic microorganism to function properly. The prerequisites for improving the production

capacity in a bioprocess are to have knowledge about the genetic mechanism and to identify the network of intracellular metabolic reaction (Çalık *et al*., 2003). After that, the way of increasing the product yield and selectivity is investigation and evaluation of bioreactor operation conditions and parameters that affect the metabolic pathways.

2.4.3.1 Batch Fermentation

In the batch operation, all production nutrients should be added to bioreactor medium before cultivation begins. Only oxygen, acid/base and antifoam are supplied to bioreactors during the cultivation due to oxygen requirement for aerobic fermentation, pH adjustment and decreasing level of foam (Yamané and Shimizu, 1984; Harada *et al*., 1997).

Because batch operation has minimum contamination risk and has low mutation possibility; it is preferred in industrial biotechnology to produce commercial bio-products. In addition, in batch operations, entire substrate conversion can be achieved thus high productivity can be obtained (Winkler, 1990; Nielsen *et al*., 2003). On the other hand, the quality difference in product formation is one of the disadvantages of this operation (Shuler and Kargı, 1992).

2.4.3.2 Continuous Fermentation

In the continuous mode of operation, during the fermentation, all fresh nutrients are added to the bioreactor medium continuously and the fermented broth is removed from the bioreactor medium with the same volume in order to maintain steady-state operation conditions and to keep the culture volume constant (Yamané and Shimizu, 1984; Harada *et al*., 1997). Continuous mode is operated without feedback control; but pH, cell and nutrient concentration are controlled. In order to investigate the physiological state of the strains, fermentation with continuous operations can be helpful due to the optimized media formulation (Harada *et al*., 1997).

Continuous fermentations have several advantages such as automation, constant product quality with high productivity and steady-state operation. On the other hand, necessity of cell adaptation to a different production system and difficulty of maintaining the bioreactor system cause increase in cost. In addition, mutation is one of the major problems in this operation mode (Nielsen *et al*., 2003).

2.4.3.3 Semi-batch (Fed-batch) Fermentation

Fresh nutrients are fed intermittently to the bioreactor throughout cultivation in fed-batch operations. However, the cultivated medium kept in the bioreactor during the process which is different from continuous mode operation (Yamané and Shimizu, 1984; Harada *et al*., 1997).

The basic feature of fed-batch processes is that the substrate concentrations or additional nutrients which are added into the fermentation medium can be controlled by arranging the feed rate that directly affects the target goods productivity (Yamané and Shimizu, 1984; Winkler, 1990). Fed-batch mode of operation has various advantages of both batch and continuous fermentation systems combination (Nielsen *et al*., 2003) as avoiding substrate inhibition, catabolite repression, and glucose effect (Harada *et al*., 1997). Moreover, optimization of the desired product can be easily controlled in this mode (Nielsen *et al*., 2003). Since the effects of the chosen feeding method on cell concentration, cell productivity and product formation; feeding method selection is very critical for bioprocesses (Lee, 1996). The classification of fed-batch cultivations according to the mode of fed is given in Table 2.6. The feeding methods can be divided in two groups; without and with feedback control.

Table 2.6 Feeding methods in fed-batch operations (Yamané and Shimizu, 1984)

Without Feedback Control	
Intermittent addition	Pulse nutrient feeding
Constant rate	Feeding nutrient at a predetermined constant rate
Exponentially increased rate	Feeding nutrient at an exponential rate with a predetermined constant rate
Optimized	Feeding nutrient with combined strategy
With Feedback Control	
Indirect feedback control	DO, pH, cell concentration, etc.
Direct feedback control	Substrate concentration control (constant or optimal)

In intermittent addition feeding strategy; nutrients are fed to the fermentation system with pulses given in order to maintain the substrate concentration at the same level. In this feeding type, high biomass can be obtained over time if the substrate is fed progressively to the system.

In constant feed rate strategy, substrates or nutrients are added to the bioreactor with a constant pre-determined rate. In this type of feeding, because of the high cell fermentation and high level of production volume, the specific growth rate and cell growth rate decreases gradually with time (Lee, 1996).

Optimized feeding is based on combination of feeding strategies containing fed-batch feeding with linear and exponential feeding (Christiansen *et al*., 2003).

In exponential feeding, substrates are supplied to the fermentation medium exponentially with a predetermined constant specific growth rate (μ, h^{-1}) and in this condition; it is possible to obtain high cell growth for a long period of time and constant substrate concentration in the medium (Yamané and Shimizu, 1984; Lee, 1996). On the other hand, the specific growth rate may deviate unexpectedly in some cases and additional control mechanisms may be necessary (Lee, 1996).

Feedback control of fed-batch operations is optimized directly or indirectly. Indirect feedback control is based on the measurement of physical parameters such as pH, DO, CO_2 evolution rate or cell concentration changes. In this indirect control system, the substrate utilization characteristic of microorganisms is the main parameter to optimize the fermenter production medium. As an example; when substrate in the cultivation medium is consumed, the level of DO shows sharply rises. In this situation, necessary amount of substrate is added by pre-determined feeding rate to keep the DO at the desired range. However, in direct feedback control in fed-batch operations; the control is achieved by measurement of the substrate concentration in the fermentation medium (Yamanè and Shimizu, 1984; Lee, 1996). In both indirect and direct feedback control operations; substrate concentration is kept constant or optimal level.

Exponential feeding methods have been used for *E. coli* bioprocesses to achieve maximum amounts of biomass in a short time where the specific growth rate is generally selected between 0.1 and 0.3 h^{-1} (Kim *et al*., 2004). Most recently, Angardi and Çalık (2013) obtained GI activity of A = 16,400 U L^{-1} at t = 30 h under molasses based exponential feeding strategy with the pre-determined specific growth rate of $\mu = 0.1\ h^{-1}$.

2.5 Bioprocess Kinetics and Characteristics

To evaluate the microbial biotechnological (fermentation) process, calculation of the yield coefficients, specific formation- and consumption- rates of the cell and substrate; mathematical modeling of the bioprocess is required by construction of the mass-balance equations for the cell, substrate, and the product.

2.5.1 Mass Equation for the Cell

Microbial growth can be regarded as an increase in the cell population via biochemical reactions taken place inside the cell. The cell growth is characterized by the specific growth rate, μ (h^{-1}). The general mass balance for the cell can be constructed as stated in Equation-2.1 by assuming that cells are generated batch-wise and not lost during sampling.

$$r_x V = \frac{d(C_x V)}{dt} \tag{2.1}$$

where r_X is the biomass formation rate, C_X is the cell concentration, (g L^{-1}), V is the bioreactor working volume, (L) , and t is the cultivation time, (h). The biomass formation (cell growth) rate, r_X can be defined as follows:

$$r_x = \mu C_x \tag{2.2}$$

By inserting the Equation-2.2 into Equation-2.1 one can derive the Equation-2.3, as follows:

$$\mu C_x V = \frac{d(C_x V)}{dt} \tag{2.3}$$

Volume of the liquid culture is changed owing to the substrate feeding, hence for simplicity, the density of the liquid medium is assumed to be constant, and from overall mass balance:

$$\frac{dV}{dt} = Q \tag{2.4}$$

where, Q is the volumetric flow rate (m^3 h^{-1}).

Rearranging the Equation-2.3 with Equation-2.4, the specific growth rate for fed-batch operation is derived as follows:

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

2.5.2 Mass Balance Equation for the Substrate (s)

Mass balance for the feeding substrate in fed-batch bioreactor system:

$$Q_S\, C_{S_0} + r_S\, V = \frac{d\left(C_S\, V\right)}{dt} \tag{2.6}$$

The substrate consumption rate, r_S, can be defined as the product of specific substrate consumption rate, q_S, (h^{-1}), and the cell concentration, C_X, (g L^{-1}) as stated in Equation-2.7:

$$r_S = q_S\, C_X \tag{2.7}$$

Inserting Equation-2.7 into Equation-2.6, the following equations are derived to define the specific substrate consumption rate:

$$Q_S\, C_{S_0} + q_S\, C_X\, V = C_S\, \frac{dV}{dt} + V\, \frac{dC_S}{dt} \tag{2.8}$$

$$q_S = \frac{1}{C_X}\left(\frac{C_S}{V}Q + \frac{dC_S}{dt} - \frac{Q_S}{V}C_{S_0}\right) \tag{2.9}$$

In the fed-batch operation generally quasi-steady state assumption is valid for the substrate C_S ($dC_S/dt = 0$) and the substrate consumption rate (r_S) is also defined as in terms of the biomass formation rate (r_X) and the yield coefficient $Y_{X/S}$ in the following equation,

$$-r_S = \left(r_X / Y_{X/S}\right) \tag{2.10}$$

and, inserted in Equation-2.6:

$$Q_S C_{S_0} - \frac{r_X V}{Y_{X/S}} = C_S \frac{dV}{dt} \tag{2.11}$$

By inserting the terms, $r_X=\mu C_X$ and $dV/dt=Q_S$ into Equation-2.11;

$$Q_S C_{S_0} - \frac{\mu C_X V}{Y_{X/S}} = C_S Q_S \tag{2.12}$$

The solution of the differential Equation-2.1 together with the kinetic Equation-2.2 is:

$$C_X V = C_{X_0} V_0 e^{\mu t} \tag{2.13}$$

By inserting Equation-2.13 into 2.12, the volumetric feed rate of the substrate(s) is defined as follows:

$$Q_S = \frac{\mu C_{X_0} V_0}{Y_{X/S}(C_{S_0} - C_S)} \exp(\mu t) \tag{2.14}$$

The term, C_S can be neglected in Equation-2.14 due to $C_{So} >> C_S$ and the volumetric feed rate of the substrate can be simplified as follows:

$$Q_S = \frac{\mu C_{X_0} V_0}{Y_{X/S} C_{S_0}} \exp(\mu t) \tag{2.15}$$

The mass of cell produced per unit mass of substrate consumed known as also the cell yield on substrate, $Y_{X/S}$ (g g^{-1}) is defined as follows:

$$Y_{X/S} = \frac{r_X}{-r_S} = \frac{dC_X/dt}{-dC_S/dt} \tag{2.16}$$

Moreover, the yield coefficient, $Y_{X/S}$ is determined for a finite period of time presenting as the overall yield coefficient as given in Equation-2.17:

$$\overline{Y}_{X/S} = \frac{\Delta C_X/\Delta t}{-\Delta C_S/\Delta t} \tag{2.17}$$

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

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

3.2 The Microorganism, Plasmids and Maintenance of Microorganisms

pRSETA::*xylA* plasmid (Angardi and Çalık, 2013) isolated from recombinant *Escherichia coli* BL21 (DE3) pLysS strain carrying pRSETA::*xylA* plasmid (Angardi, 2011) was transformed to *Escherichia coli* W. Microorganisms and plasmids that were used in the production of thermostable glucose isomerase study were summarized in Table 3.1.

Young cell colonies were inoculated into cyropreservative fluid present in a vial which contains porous beads. The vial was shaken for five seconds and allowed to sit for 1-2 minutes to assure the adsorption of cells into the porous beads. Cyropreservative fluid was aspirated before storing the vial at -80°C. The microorganism has been stored in the microbanks (PRO-LAB) at -80°C for long term storage.

Table 3.1 Used strains and plasmids in this study

Microorganism	Strain	Genotype/Plasmid	Source
E. coli	BL21 (DE3) pLysS	pRSETA::*xylA*	Angardi, 2011
E. coli	WAKSMAN	Wild Type	NRRL (B766)
E. coli	W-26	pRSETA::*xylA*	This work

3.3 Growth Media

Solid, pre-cultivation and production media were autoclaved for sterilization at 121°C for twenty minutes and appropriate antibiotics were added after cooling to approximately 50°C. Antibiotic types and their concentrations are given in Table 3.2. The wild type *E. coli* W was inoculated to growth medium without any antibiotics. For *E. coli* BL21 (DE3) pLysS strain, chloramphenicol and ampicillin were added to cultivation media under sterile conditions and for recombinant *E. coli* W, ampicillin (100 mg/ml) was added to growth medium after sterilization (Ampicillin resistance in pRSETA expression vector; chloramphenicol resistance in BL21 (DE3) pLysS cells, Angardi, 2011).

Table 3.2 Concentrations of antibiotics in growth media

Microorganism	**Antibiotic**	**Concentrations g L^{-1}**
E. coli W wild type	-	none
E. coli W-26	Ampicillin	0.100
E. coli BL21 (DE3) pLysS (Angardi, 2011)	Ampicillin Chloramphenicol	0.100 0.007

3.3.1 Solid Medium and Growth Conditions

The wild type *E. coli* W and recombinant strains were grown on Luria-Bertani (LB) Agar. The Agar plates, inoculated with *E. coli* strains, were incubated at 37°C for 24 hours with appropriate antibiotics. The compositions of solid medium (LB Agar) is given in Table 3.3.

Table 3.3 Ingredients of solid medium (LB Agar)

Strain	***E. coli* W wild type**	***E. coli* W-26**	***E. coli* BL21 (DE3) pLysS**
Compound	**Concentration, g L^{-1}**		
Soytryptone	10.000	10.000	10.000
Yeast extract	5.000	5.000	5.000
NaCl	10.000	10.000	10.000
Agar	15.000	15.000	15.000
Ampicillin	-	0.100	0.100
Chloramphenicol	-	-	0.007

3.3.2 Pre-cultivation Medium and Growth Conditions

After propagation on the solid medium, cells were inoculated into Luria-Bertani (LB) broth. Cells were incubated by using air-filtered Erlenmeyer flasks with a size of 150 ml and having a working volume of 30 ml at 37°C with N=200 min^{-1} for 12 hours in an agitation rate and temperature controlled orbital shaker (B. Braun, Certomat BS-T). Ingredients of the pre-cultivation medium, LB Broth, are given in Table 3.4.

Table 3.4 Ingredients of pre-cultivation medium (LB Broth)

Strain	***E. coli* W wild type**	***E. coli* W-26**	***E. coli* BL21 (DE3) pLysS**
Compound	**Concentration, g L^{-1}**		
Soytryptone	10.000	10.000	10.000
Yeast extract	5.000	5.000	5.000
NaCl	10.000	10.000	10.000
Ampicillin	-	0.100	0.100
Chloramphenicol	-	-	0.007

3.3.3 The Production Medium

After 12 hours of proliferation in the precultivation medium, microorganisms were transferred to the production medium with the inoculation ratio of 1:10.

Molasses based cultivation medium does not need any IPTG (Isopropyl β-D-1-thiogalactopyranoside) for the induction of enzyme production due to having galactose in it; however, as sucrose and glucose based production media need IPTG, IPTG was added to production medium at t=4 h of the fermentation with 0.1 M final concentration. The ingredients of pilot scale and laboratory scale production media were given in Table 3.5 and Table 3.6, respectively.

Table 3.5 Ingredients of pilot scale production medium

Compound	Concentration, g L^{-1}
Molasses	32.0
NaCl	0.5
$MgSO_4.7H2O$	0.5
$ZnSO_4.7H2O$	0.2×10^{-5}
$MnSO_4.7H2O$	0.1
Na_2HPO_4	6.7
KH_2PO_4	3.1
$(NH_4)_2HPO_4$	5.0
Ampicillin	0.1

Table 3.6 Ingredients of laboratory scale production medium

	Glucose		Sucrose		Molasses	
Media	**8 g L^{-1}**	**16 g L^{-1}**	**8 g L^{-1}**	**16 g L^{-1}**	**16 g L^{-1}**	**32 g L^{-1}**
Compound	**Concentration, g L^{-1}**					
NaCl	0.5	0.5	0.5	0.5	0.5	0.5
$MgSO_4.7H2O$	0.5	0.5	0.5	0.5	0.5	0.5
$ZnSO_4.7H2O$	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}
$MnSO_4.7H2O$	0.1	0.1	0.1	0.1	0.1	0.1
Na_2HPO_4	6.7	6.7	6.7	6.7	6.7	6.7
KH_2PO_4	3.1	3.1	3.1	3.1	3.1	3.1
$(NH_4)_2HPO_4$	5	5	5	5	5	5
Glucose	8	16	-	-	-	-
Sucrose	-	-	8	16	-	-
Molasses	-	-	-	-	16	32
Ampicillin*	0.1	0.1	0.1	0.1	0.1	0.1
IPTG (0.1 M)	√	√	√	√	-	-

*Ampicillin is not added to the production medium into wild type *E. coli* W medium

3.3.3.1 Properties and Preparation of Molasses

Beet molasses provided from Ankara Beet Sugar Factory (Turkey)contained 50% sucrose, 18% water, 12% organic non-sugar compounds including nitrogen element, 10% inorganic components, 8% organic non-sugar matters with no nitrogen, 1.2% raffinose, 0.3% invert monosaccharide like glucose and fructose, 0.3% galactinol and 0.2% kestose and its pH is7.5.

Since most of the *E. coli* strains cannot utilize sucrose, it is generally needed to employ acid hydrolysis pretreatment to obtain fructose and glucose from sucrose as described by Çalık *et al* (2003). However, as *E. coli* W was able to utilize sucrose as a carbon source, molasses was just diluted and centrifuged. The procedure is given below:

- *Dilution*: 240 g beet molasses was diluted to a final volume of 500 ml with water.
- *Centrifugation*: Centrifugation was performed at 6000 g and +4^{o}C for 20 minutes to precipitate insoluble matters and separate impurity.
- *Sterilization*: Sterilization was carried out at 121°C for 20 minutes and this pretreated molasses was ready to use in fermentation experiments.

3.4 Genetic Engineering Techniques

3.4.1 Plasmid DNA Isolation from *E. coli*

Plasmid DNA isolation was performed according to Alkaline Lysis Midiprep Method reported by Sambrook and Russell (2001). Solution compositions used for the isolation of plasmid DNA are given in Appendix A.

Steps for this purpose are as follows;

1. Pick a single colony from agar plate.
2. Inoculate 10 ml of LB medium, containing appropriate antibiotics, with a single colony from selective medium overnight at 37 °C.
3. Transfer the culture to 15 ml sterile falcon and centrifuge at 2000 g for 10 minutes at 4°C.
4. Remove supernatant and take off all fluid by micropipette.
5. Resuspend the pellet by ice-cold Alkaline Lysis Solution I in 200 µl by vigorous vortexing. The bacterial pellet should be completely dispersed in alkaline lysis solution I, transfer the mixture to a 1.5 ml eppendorf tube.
6. Add 400 µl freshly prepared Alkaline Lysis Solution II. Mix the content by inverting 5-6 times. Store it on ice.
7. Add 300 µl ice cold Alkaline Lysis Solution III to the mixture. Mix it by inverting 5-6 times. Store it on ice for 3-5 minutes.
8. Centrifuge at 15,000 g for 5 minutes at 4°C.
9. Transfer 600 µl of supernatant to a fresh eppendorf tube.
10. Add an equal volume of phenol: chloroform. Mix by vortexing.

11. Centrifuge at 15000 g for 2 minutes at 4°C.
12. Transfer aqueous upper layer to a fresh eppendorf tube.
13. Add 600 µl isopropanol at room temperature. Mix it by vortexing, and store the mixture at room temperature for 2 minutes.
14. Centrifuge at 15,000 g for 5 minutes at room temperature. Remove supernatant.
15. Stand the tube on an inverted position for approximately 4-5 minutes to allow the fluid to drain away.
16. Add 1 ml of 70% ethanol. Centrifuge at 15000 g for 2 minutes at room temperature
17. Remove the supernatant carefully.
18. Remove any beads of ethanol by evaporation at room temperature for 10-15 minutes.
19. Dissolve the pellet in 100 µl TE buffer (pH: 8.0) containing 20 µg/ml RNase A. Vortex gently.
20. Store at -20° C for further use.

3.4.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used in order to visualize and to determine the size and concentration of DNA strands. 0.8% (w/v) agarose gel was dissolved in 100 ml 1XTBE buffer and heated until boiling point. After cooling it to approximately 50°C-55°C, 5 µl of ethidium bromide from 10 mg/ml stock solution was added before it was poured into gel tray with inserting suitable comb.

The gel tank was filled with 1xTBE buffer and after waiting for 10-15 minutes, 10-20 µl of DNA samples, mixed with 1:5 volume of 6x loading dye were applied into the wells, as well as the DNA ladder for size estimation at 90 V for 45-60 minutes. At the end of the electrophoresis, the DNA fragments were visualized under UV illumination at 302 nm (UVP Bio Imaging System and Hamamatsu Digital CCD Camera). The concentrations of the DNA fragments were determined using UVP Bio lmaging Systems Labworks Image Acquisition and Analysis Software.

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

3.4.3.1 Chemically Competent Cell Preparation

Transformation of *E. coli* was achieved by using $CaCl_2$ method described by Sambrook and Russell (2001). The steps of this method are as follows;

1. Incubate wild type *Escherichia coli* Waksman on LB agar for 12 hours at 37°C.
2. Pick a single colony from a selective plate and inoculate a starter culture of 5 ml LB medium. Grow for 12-15 hours at 37°C, with vigorous shaking at 200 min^{-1}.
3. Take 1 ml sample from the pre-cultivation medium and transfer it into 100 ml LB medium, cultivate at 37°C and 200 rpm for 2.5 hours.
4. Transfer 50 ml of broth into 50 ml sterile polypropylene tubes; and place the tubes on ice for 10 minutes.
5. Centrifuge the cells at 4000 rpm for 10 minutes at 4°C.

6. Remove the cells from supernatant; place the tubes on a paper tissue for 1 minute to dry the cells.
7. Add 30 ml of filter-sterilized $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$-20 mM $CaCl_2$) into each tube and make a complete solution by pipetting, place on ice for 10 minutes.
8. Centrifuge at 4000 rpm, for 10 minutes at +4°C.
9. Remove the cells from supernatant; place the tubes on a paper tissue for a minute to dry the cells.
10. Add 2 ml of filter-sterilized 0.1M $CaCl_2$ solution onto the cells and make a complete solution by pipetting and hold on ice for 10 minutes.
11. Transfer 200 µl of competent cell to eppendorf tubes for storage. Store at -80°C for further use.

3.4.3.2 *E. coli* Transformation

The steps of transformation process are as follows;

1. Transfer 200 µl of competent cell solution to a sterile ice-cold 50 ml falcon tube.
2. Add 10 µl from ligation mixture to this solution. Then, store the tubes on ice for 30 minutes.
3. Apply heat-shock to the solution at 42°C for exactly 90 seconds and rapidly place the tube on ice for 1-2 minutes.
4. Transfer the cell suspension to sterile tubes which contained 800 µl of pre-warmed LB medium without antibiotics and incubate at 37°C for 45 minutes with shaking at 200 rpm.
5. Transfer 25-200 µl from the cultured cells onto pre-warmed LB agar plate with the appropriate antibiotic. With a sterile glass rod, spread the cells over the LB agar plate immediately and incubate at 37°C overnight (Kaya, 2006). Selected colonies should be visible in 14-16 hours (Sambrook, 2001).

3.5 Microorganism Selection

After transformation process was carried out, agarose gel electrophoresis was needed to perform in order to check plasmid content of recombinant strains. The true strains, which were carrying the engineered plasmid, were examined with diverse carbon sources in order to select the highest glucose isomerase activity producer strain. In the microorganism selection experiments, laboratory scale air filtered shake bioreactors were used before scaling-up.

3.6 Recombinant Protein Production

Thermostable GI production by *E. coli* W carrying pRSETA::*xylA* plasmid was conducted in laboratory-scale air filtered shake bioreactors and pilot scale bioreactors.

3.6.1 Preparation of the Cells for the Enzyme Production

The selected *E. coli* W carrying pRSETA::*xylA* plasmid which was over-producing thermostable GI was stocked in microbanks at -80°C. The stocked microbank was plated onto LB Agar medium (Table 3.3) and incubated for 24 hours at 37°C. After 24 hours, a single colony from the agar plate was inoculated into pre-cultivation medium (LB Broth, Table 3.4). *E. coli* W cells which were proliferated in the precultivation medium at N=200 min^{-1} and 37°C for 12 hours, transferred to the production medium (Table 3.5 and Table 3.6) with 1:10 ratio either for laboratory scale air filtered shake bioreactors or for pilot scale bioreactors. All of the growth media contained appropriate antibiotics as represented in Table 3.2.

3.6.2 Thermostable GI Production in Laboratory Scale Air Filtered Shake Bioreactors

Production of GI was performed in 150 ml air filtered Erlenmeyer flasks with 30 mL working volume at 37°C with N=200 min^{-1}. *E. coli* W-26 containing pRSETA::*xylA* plasmid was inoculated into solid medium at 37°C. After 24 hours, pre-cultivation was started with a single colony of inoculum. Then, cells were inoculated in 8 g L^{-1} glucose, 16 g L^{-1} glucose, 8 g L^{-1} sucrose, 16 g L^{-1} sucrose, 16 g L^{-1} molasses and 32 g L^{-1} molasses-containing production media (Table 3.5) to develop pilot scale production conditions. IPTG was added at the 4^{th} hour of the fermentation in glucose and sucrose based production media for induction of GI (Angardi, 2011).

3.6.3 Thermostable GI Production in Pilot Scale Bioreactor

In the pilot scale bioreactor experiments, GI production was carried out in the 3 L bioreactor (Braun CT2-2) with a working volume of 1 L. The bioreactor contained pH, temperature, stirring, foam, feed inlet rate and dissolved oxygen control systems. In addition, the system had an external cooler, steam generator and a jacket for control of the temperature and sterilization. Air was given via a compressor whereas feed solutions, antifoam, acid and base were added to the system with inlet ports. Homogeneous oxygen distribution was managed by the help of a sparger and four baffles. Stirring was provided by Rushton turbines with four blades. A schematic representation of the bioreactor production is given in Figure 3.1.

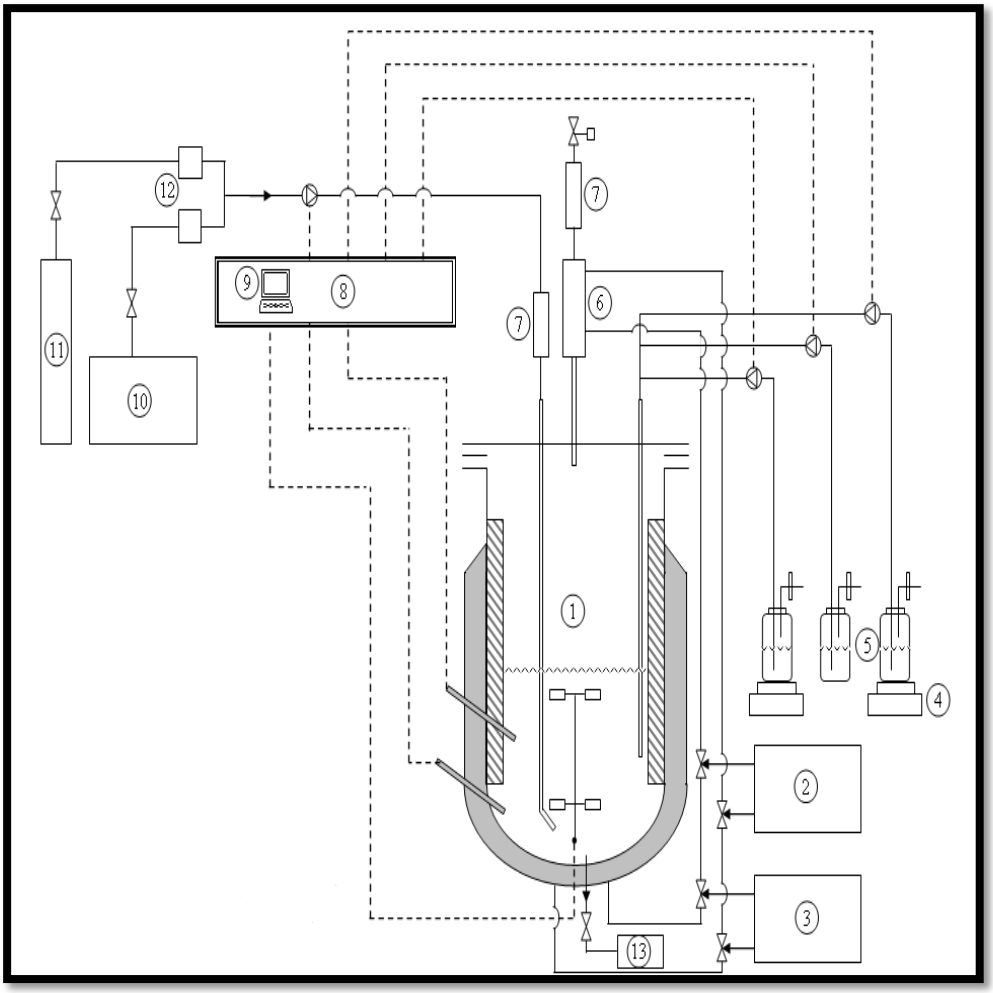

Figure 3.1 Schematic presentation of the pilot scale bioreactor system: (1) Bio-reaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11)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

3.6.3.1 Bioreactor Operation Parameters

Temperature was kept constant at 37°C by using an external cooler and a steam generator. 5M H_3PO_4 and 5M NaOH solution were used to adjust pH. Agitation rate was kept at 750 rpm for efficient oxygen and substrate transport. Dissolved oxygen (DO) was kept at 40%. Excess of foam was removed by 10% antifoam solution when needed.

All of the bioreactor experiments were started with the concentration of 32 g L^{-1} molasses. In exponential feeding, predetermined specific growth rate, μ was 0.05 h^{-1} for all of the bioreactors. Molasses pulses and molasses pulses with $(NH_4)_2HPO_4$ addition were given to bioreactor to increase the molasses and nitrogen source concentration to its initial values. 0.2 g L^{-1} ampicillin was added into all of the feeding molasses media.

3.7 Storage of Samples

Samples were taken from the cultivation medium every two hours. Each 1 mL sample was centrifuged at 13200 rpm for 10 minutes at +4°C. After supernatant was removed carefully, pellets were kept at -80°C for further analysis. In this study, in order to prevent a decrease in enzyme activity, different storage conditions were tested according to master thesis of Namaldı (2005). Pellets were re-suspended in 0.5 % (w/v) and 2% (w/v) maltodextrin solution which was prepared in potassium phosphate buffer at pH=7.0. Thereafter, they were kept at -80°C for prolonged storage.

3.8 Analysis

Samples were taken from cultivation media every two hours of the fermentations to measure cell concentrations, glucose isomerase activities, sucrose/glucose and organic acid concentrations. Subsequent to cell concentration measurement, the samples were centrifuged at 13500 rpm for 10 minutes at +4°C to separate supernatant from the cell pellets. Cell precipitation was used for the determination of the enzyme activity, and supernatant was stored at -80°C for further analysis such as sucrose, glucose, and organic acid concentrations measurement assays.

3.8.1 Cell Concentration

Cell concentration was measured by using UV-Vis Spectrophotometer at 600 nm (Thermo Spectronic, Heiλosα) on the basis of dry cell weights. Samples taken from the fermentation medium were diluted to an OD_{600} in the range of 0.1-0.8

The calibration curve for converting measured optical density of *E. coli* cells and to cell concentration, Cx (g L^{-1}) is given in equation (3.1).

$$C_X = \frac{OD_{600}}{2.8782} \times \text{Dilution Rate} \tag{3.1}$$

3.8.2 Glucose Isomerase Activity Assay

GI activity was determined by the measurement of the conversion of D-glucose into D-fructose. One unit of GI activity was defined as the formation of 1μmol of D-fructose per minute at 80°C. The steps of GI activity assay are as follows;

1. Take certain amount of samples from culture broth.
2. Centrifuge cells at 6000 rpm for 10 minutes.
3. Remove supernatant; resuspend the cell extracts in 970 μl of 0.1 M potassium phosphate buffer.

4. Lyse the cell wall at f=10 s^{-1} for 10 minutes performing at the agitator bead mill (Retsch, MM 200) via adding glass beads into cell suspension.
5. Take 50µl of enzyme solution from the mixture. Add 50µl of freshly prepared activity buffer, containing 0.4 M D-glucose and 10 mM $MnCl_2$ in 0.02 M potassium buffer pH 7.0 at 80°C.
6. Incubate 100 µl of the reaction mixture at 80°C water bath for 10 minutes and terminate the isomerization reaction after 10 minutes through denaturizing thermostable recombinant enzyme by adding 60 µl of reaction mixture into a tube containing 540 µl of 0.1M HCl.
7. Perform the cysteine-carbazole-sulfuric acid method (Dische and Borenfreund, 1951) to determine the concentration of D-fructose formed at the end of reaction.

Cysteine-Carbazole-Sulfuric acid method is performed as follows;

After the reaction termination, add 1.8 ml of 70% (v/v) sulfuric acid, 60 µl of freshly prepared 1.5% (w/v) cysteine-HCl, and 60 µl of freshly prepared 0.12% (w/v) carbazole in 95% ethanol correspondingly. Then, incubate the tubes for 30 minutes at room temperature for formation of D-fructose and measure its concentrations by UV-Vis Spectrophotometer at the absorbance of 560 nm. Use calibration curve to measure D-fructose concentration for activity assay which is given in Appendix B.

3.8.3 Sucrose Concentration

To determine sucrose concentration in the molasses, sucrose analysis kit (Biasis, Ankara) was used. The method bases on three steps: The first one is the sucrose oxidize to D-glucose and D-fructose with α-glucosidase catalysis. Then, the amount of D-glucose is measured by the glucose oxidation method with UV-Vis spectrophotometer at wave length of 505 nm (Boyaci et. al., 2005). The same methods are employed with Glucose Analysis kit (Biasis, Ankara).

Sucrose analysis kit includes a sucrose analysis reactive, a glucose analysis reactive and a glucose analysis buffer. The sucrose analysis reactive consists of sodium citrate buffer, invertase and sodium azide; however, the glucose analysis reactive consists of glucose oxidase, peroxidase and 4-aminoantipirin, and also the glucose analysis buffer solution includes potassium dihydrogen phosphate in phenol. Dilutions of samples were made with water to have sucrose concentration lower than 4 g L^{-1}.

$$\text{Sucrose} + H_2O \xrightarrow{\alpha-\text{Glucosidase}} D-\text{glucose} + \text{Fructose} \quad (3.2)$$

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

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

Assay was started with oxidation reaction of sucrose and water into D-glucose and fructose, by using α-glucosidase (Equation 3.2) at 55°C for 20 minutes, then the second oxidation occurred from D-glucose and water to gluconate and peroxide which was catalyzed by glucose oxidase (3.3). Thereafter , the created peroxide was reacted with 4-aminoantipyrine and phenol in the catalysis of peroxidase to form iminoquinone at 37°C water bath for 20 minutes which gives spectrophotometrically observable red color in proportion with glucose concentration (Equation 3.4). At the end of the reaction, measurement was carried out by UV Spectrophotometer at 505 nm with diverse sucrose samples. Samples which were below 4 g L^{-1} were analyzed to achieve standard curves as shown in Appendix C.

3.8.4 Organic Acid Concentrations

High Performance Liquid Chromatography (HPLC) (Waters, Alliance 2695) with a Capital Optimal ODS-5Xm column (Capital HPLC, West Lothian, UK) was employed in the determination of organic acid concentrations during the bioprocesses. The method is based on reversed phase HPLC, using a mobile phase with 3.12% (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4 for the calculation of the concentrations by considering the chromatogram based on the standard solutions chromatograms. Samples were filtered with 45 μm filters (ACRODISC CR PTFE) and then loaded on the HPLC system.

The organic acid concentrations were determined by the calculation of the related peak in the HPLC chromatogram. The conversions of the peak's areas were calculated using the standard calibration curves of standard organic acids. The standard calibration curves for the detected organic acids were given in Appendix D.

Table 3.7 HPLC conditions for determination of organic acid analysis (İleri and Çalık 2006)

Column	Capital Optimal ODS, 5 μm
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Mobile phase	3.12% NaH_2PO_4 (w/v) and 0.62x10^{-3}% (v/v) H_3PO_4
Mobile phase flow rate	0.8 ml min^{-1}
Column temperature	30°C
Detector type and wavelength	Waters 2487 Dual absorbance detector, 210 nm
Injection volume	5 μl
Analysis period	15 min

CHAPTER 4

RESULTS AND DISCUSSION

In this M.Sc. thesis, the objective was to investigate the production of thermostable glucose isomerase (GI; EC 5.3.1.5) by metabolically engineered *Escherichia coli* which catalyze the isomerization reaction of glucose to fructose reversibly. In the first part of the study, the plasmid pRSETA::*xylA* (Angardi and Çalık, 2013) was isolated and then transformed in *E. coli W*. *E. coli* W-26 carrying pRSETA::*xylA* plasmid having the highest glucose isomerase production capacity was chosen according to the results of the microorganism selection experiments in which different carbon sources were used. In this part, the storage conditions were also optimized.

In the second part, recombinant GI production by *E. coli* W-26 was investigated in the designed laboratory scale shake-bioreactor experiments and by pilot scale bioreactor experiments. Firstly, three different carbon sources (glucose, sucrose and molasses) in different concentrations were examined in laboratory scale shake-bioreactor experiments. According to the results of the laboratory scale shake bioreactor experiments, molasses based pilot scale bioreactor experiments were carried out.

At the pre-determined specific growth rate of μ=0.05 h^{-1}, the effects of molasses-based medium and molasses pulses with and without nitrogen sources on cell growth, GI production and by-product formation were exponentially investigated.

4.1 Microorganism Selection and Storage

4.1.1 Isolation of pRSETA containing *xylA*

The plasmid isolation of pRSETA containing *xylA* was achieved by alkaline lysis method, as mentioned in Section 3.4.1. In order to determine the size of the plasmid and to determine the true plasmid, the isolated circular plasmid was run with the marker (Lambda DNA/HindIII Marker) and the plasmid without *xylA* gene in agarose gel. The image of the agarose gel electrophoresis in comparison with the isolated plasmids with marker and the plasmid without *xylA* gene is given in Figure 4.1.

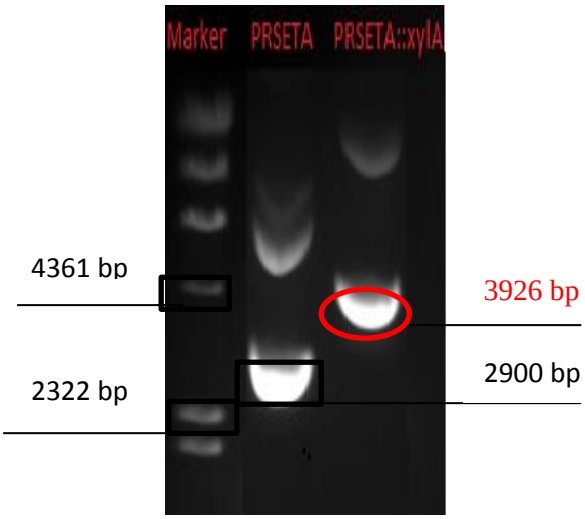

Figure 4.1 Agarose gel electrophoresis; Marker: Lambda DNA/HindIII, pRSETA: Empty Vector, pRSETA::*xylA*: True Plasmid

It was observed in Figure 4.1 that; the plasmid pRSETA::*xylA* was slower than pRSETA because of its size due to *xylA* in it, as expected. In addition, the size of the tested plasmids verified that plasmid isolation was performed straightly.

4.1.2 *E. coli* W Transformation with pRSETA::*xylA*

pRSETA::*xylA* plasmid was transformed into *E. coli* W using $CaCl_2$ method (Section 3.4.3). After transformation of *E. coli*, ten of the selected single colonies which were able to grow on the LB agar containing ampicillin were incubated in pre-cultivation medium to obtain their plasmids by alkaline lysis method. Then, the ten plasmid preps were run through agarose gel with marker and empty vector to check whether they had the true plasmid or not. The images of isolated plasmids were presented in Figure 4.2.

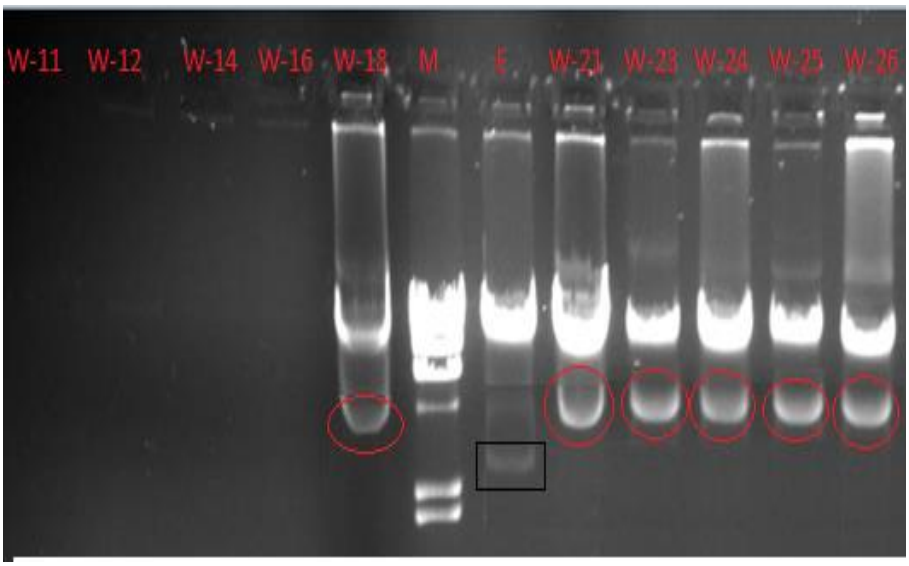

Figure 4.2 Agarose gel electrophoresis; M: Marker (Lambda DNA/ HindIII); E: Empty vector pRSETA; W-18, W-21, W-23, W-24, W-25, W-26: pRSETA::*xylA*

It was observed in Figure 4.2 that; while W-18, W-21, W-23, W-24, W-25 and W-26 had the pRSETA::*xylA*; W-11, W-12, W-14 and W-16 did not carry the plasmid. In order to select the strain which over-produced GI; W-18, W-21, W-23, W-24, W-25 and W-26 were tested on different media.

4.1.3 Microorganism Selection

E. coli W-18, W-21, W-23, W-24, W-25 and W-26 carrying the recombinant plasmid, were investigated in the media including different carbon sources in order to determine the most efficient strain. Firstly, single colonies were inoculated to solid medium and incubated for 24 hours. Then, 30 ml LB broth with 100 mg L^{-1} ampicillin in 150 ml Erlenmeyer flasks were used as pre-cultivation medium at 37°C for 12 hours in shake bioreactors at N=200 min^{-1}. After pre-cultivation, the cells were transferred to the production media with the inoculation ratio of 1:10.

Two experiments were carried out as mentioned:

i. In the first experiment, the wild type *E. coli* W and the recombinant strains W-18, W-21, W-23, W-24, W-25, and W-26 were compared. After growth on solid medium and inoculation to pre-cultivation medium, 8 g L^{-1} sucrose and 16 g L^{-1} molasses were used as carbon sources in the production medium. IPTG was added to sucrose containing medium at the 4^{th} hour to induce the enzyme production. Based on the cell concentrations and enzyme activity data, W-24 and W-26 were chosen to perform the second experiment.

ii. In the second experiment, 8 g L^{-1} sucrose, 16 g L^{-1} sucrose, 16 g L^{-1} molasses and 32 g L^{-1} molasses were used in the production medium with strains of W-24 and W-26. Cell concentrations and GI activities were measured and the strain W-26 was chosen for further experiments.

4.1.3.1 Microorganism Selection via Cell Concentrations

Samples were taken at the 12th hour from the production medium to measure the cell concentrations. The cell concentrations were calculated with the Equation 3.1 as given in Section 3.8.1.

i. The cell concentrations of the recombinant cells W-18, W-21, W-23, W-24, W-25, and W-26, and the wild type *E. coli* W in 8 g L^{-1} sucrose and 16 g L^{-1} molasses containing production medium are presented in Figure 4.3.

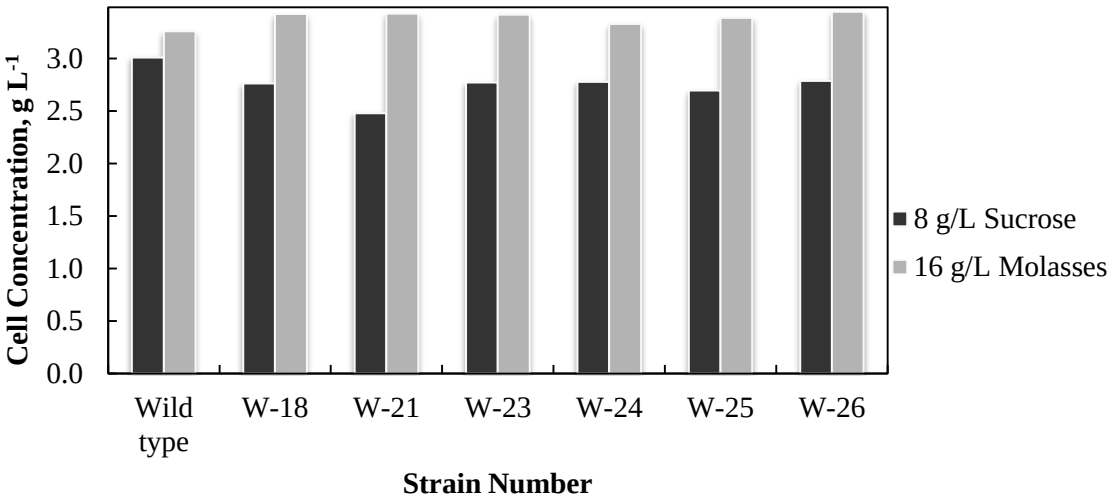

Figure 4.3 Variations in the cell concentrations in 8 g L^{-1} sucrose and 16 g L^{-1} molasses containing media for W-18, W-21, W-23, W-24, W-25, and W-26, and the wild type *E. coli* W at t=12 h

As seen in Figure 4.3 all of the strains produced nearly similar cell concentrations including wild type *E. coli* W. The highest cell concentration was achieved by W-26 as 3.44 g L^{-1} with 16 g L^{-1} molasses containing production medium. The highest cell concentration in sucrose containing medium was 2.79 g L^{-1} by the same recombinant strain while wild type *E. coli* W had the cell concentrations of 3.26 g L^{-1} and 3.01 g L^{-1} at 16 g L^{-1} molasses and 8 g L^{-1} sucrose containing production medium, respectively. Because of the molasses ingredients such as amino acids, organic acids, vitamins and minerals, all the strains which

were grown on the molasses-containing performed had higher cell concentrations compared to sucrose containing production medium.

ii. In order to select the best strain in terms of enzyme production, another experiment set was carried out. For this purpose, W-24 and W-26 were conducted in 8 g L^{-1} and 16 g L^{-1} sucrose, and 16 g L^{-1} and 32 g L^{-1} molasses containing production media. Concentrations of W-24 and W-26 cultures were presented in Figure 4.4.

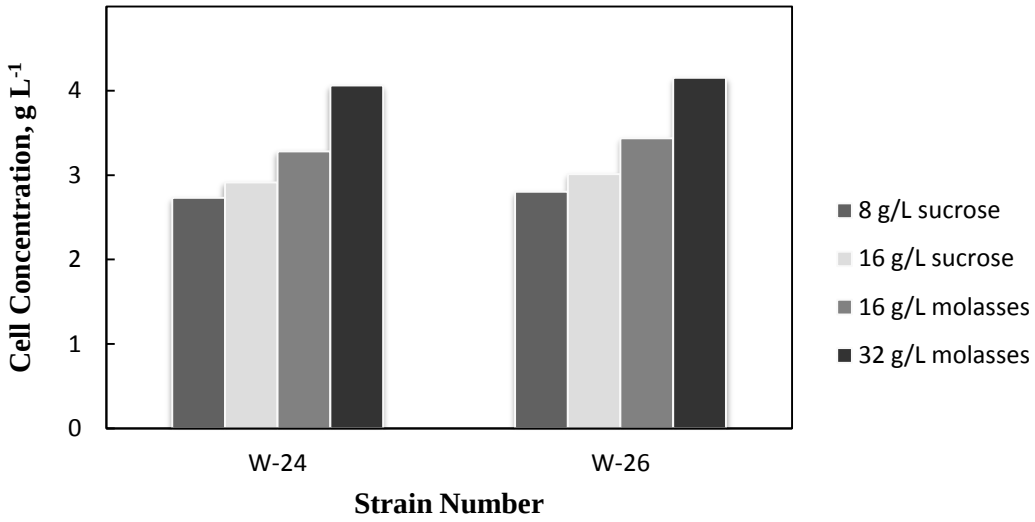

Figure 4.4 Variations in the cell concentrations in 8 g L^{-1}- 16 g L^{-1} sucrose, and 16 g L^{-1}- 32 g L^{-1} molasses containing production media of W-24 and W-26 at t=12 h

It was determined that the cell concentrations of W-24 and W-26 were similar to each other in all the carbon sources. The highest cell concentrations on both strains were found as ca. 4 g L^{-1} after t=12 h in 32 g L^{-1} molasses containing medium. Therefore it was concluded that molasses has positive effect on the cell growth.

4.1.3.2 Microorganism Selection via GI Activity

The data of the cell concentrations was not enough to determine the best GI producing strain. Therefore, selection of the recombinant strain was carried out with consideration of enzyme activities as well.

i. Glucose isomerase activity of the selected strains W-18, W-21, W-23, W-24, W-25, and W-26, and the wild type *E. coli* W grown in 8 g L^{-1} sucrose- and 16 g L^{-1} molasses containing production media are presented in Figure 4.5.

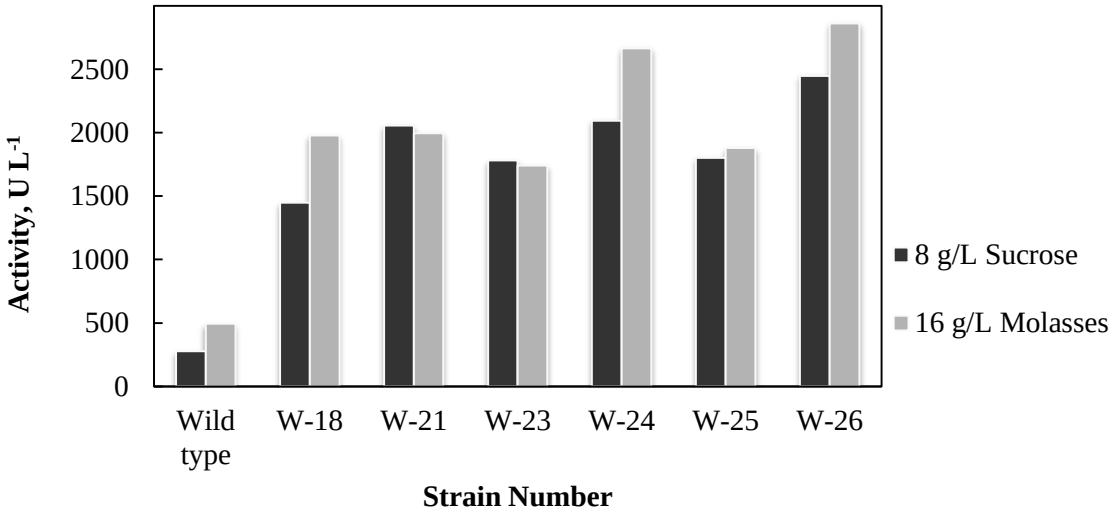

Figure 4.5 Variations in glucose isomerase activity in 8 g L^{-1} sucrose and 16 g L^{-1} molasses containing media of W-18, W-21, W-23, W-24, W-25, and W-26, and the wild type *E. coli* W at t=12 h

Although all of the strains produced similar cell concentrations (Figure 4.4), glucose isomerase activities were different. While W-21, W-23 and W-25 strains produced nearly same enzyme activities on different type of carbon sources; W-24 and W-26 strains demonstrated higher GI activities on molasses containing medium. In addition, higher enzyme activities were obtained with W-24 and W-26 in both 8 g L^{-1} sucrose- and 16 g L^{-1} molasses containing conditions. When compared to the wild type *E. coli* W, it was determined that W-24 and W-26 produced approximately 5.40- and 5.80-fold higher activities on molasses containing medium, respectively.

ii. In the second microorganism selection experiment set, W-24 and W-26 were used because of their higher activities than other selected recombinant strains in the first experiment with more diverse production medium conditions. The glucose isomerase activities of W-24 and W-26 strains in 8 g L^{-1} and 16 g L^{-1} sucrose, and 16 g L^{-1} and 32 g L^{-1} molasses containing media are presented in Figure 4.6.

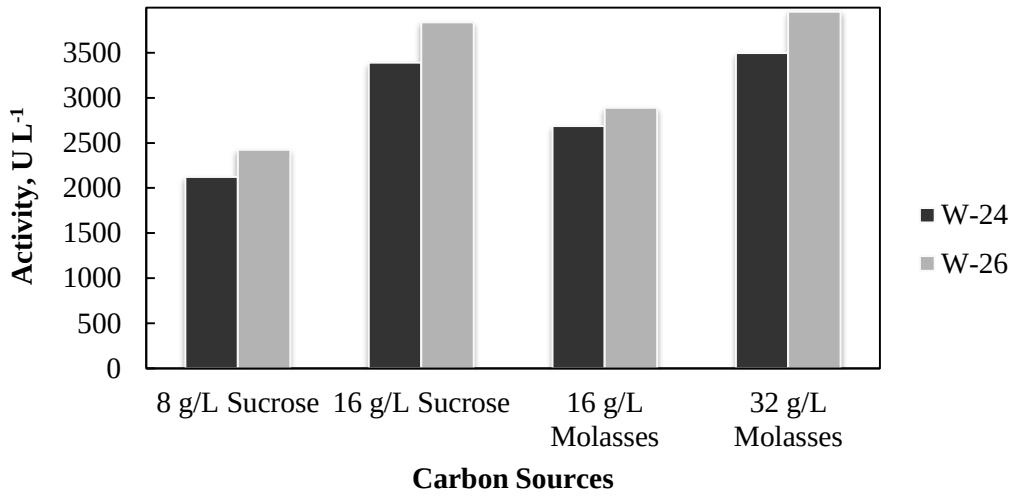

Figure 4.6 Variations in glucose isomerase activity in 8 g L^{-1}- 16 g L^{-1} sucrose, and 16 g L^{-1}- 32 g L^{-1} molasses containing production media of W-24 and W-26 at t=12 h

As can be observed in Figure 4.6, activities were positively affected by the presence of molasses in the production medium at all conditions. Thus, it is concluded that increasing concentrations increased GI activities. For all of the experiment sets, higher enzyme activities were measured by the strain W-26, so *E. coli* W-26 strain was selected for the production of glucose isomerase in laboratory scale and pilot scale bioreactor experiments.

4.1.4 Storage Conditions

To keep glucose isomerase activity at high levels, different concentrations of maltodextrin (Namaldı, 2005) in KIP (potassium phosphate buffer, pH=7.0) were tested. As a result 0.5 % (w/v) maltodextrin solution in KIP buffer was used instead of 2% (w/v) maltodextrin solution in KIP buffer due to higher glucose isomerase activity. When compared to normal storage conditions, it was found that dissolving pellets in 0.5 % (w/v) maltodextrin performed ca. 16% higher activity, although dissolving pellets in 2% (w/v) maltodextrin had no positive effects on preserving the enzyme activity.

4.2 Recombinant GI Production

Thermostable glucose isomerase production was developed by laboratory scale shake bioreactor experiments after genetic experiments of *E. coli* W by transferring pRSETA vector carrying glucose isomerase encoding gene from *Thermus thermophilus* (Angardi, 2011). Then, four sets of molasses-based pilot scale bioreactor experiments were conducted.

4.2.1 Laboratory Scale Recombinant GI Production

The selected W-26 strain was inoculated on solid medium at 37°C. After incubation for 24 hours, pre-cultivation was started with a single colony of the selected strain. Production of GI was performed in 150 ml Erlenmeyer flasks with a working volume of 30 mL at 37°C with N=200 min^{-1}. Different carbon sources at different concentrations were used to produce recombinant glucose isomerase and to develop pilot scale enzyme production conditions as follows: 8 g L^{-1} glucose, 16 g L^{-1} glucose, 8 g L^{-1} sucrose, 16 g L^{-1} sucrose, 16 g L^{-1} molasses and 32 g L^{-1} molasses containing media.

4.2.1.1 Cell Growth Results in Laboratory Scale Shake Bioreactors

The effect of the carbon sources on the cell concentration at t=12h of the bioprocess is presented in Figure 4.7. Both glucose and sucrose containing media had similar effects on the cell concentrations; the cell concentrations were found as 2.64 g L^{-1} and 2.70 g L^{-1} at 8 g L^{-1} glucose-and sucrose containing media and 3.03 g L^{-1} and 3.05 g L^{-1} at 16 g L^{-1} glucose- and sucrose containing media, respectively. For each carbon source it was observed that increasing carbon concentration has positive effect on the cell growth. In addition, although 16 g L^{-1} molasses which approximately includes 8 g L^{-1} sucrose, higher cell concentrations were obtained by the medium containing 16 g L^{-1} molasses. The maximum cell growth was 4.19 g L^{-1} in 32 g L^{-1} molasses containing medium, which was 1.37-fold higher than that of 16 g L^{-1} sucrose containing production medium.

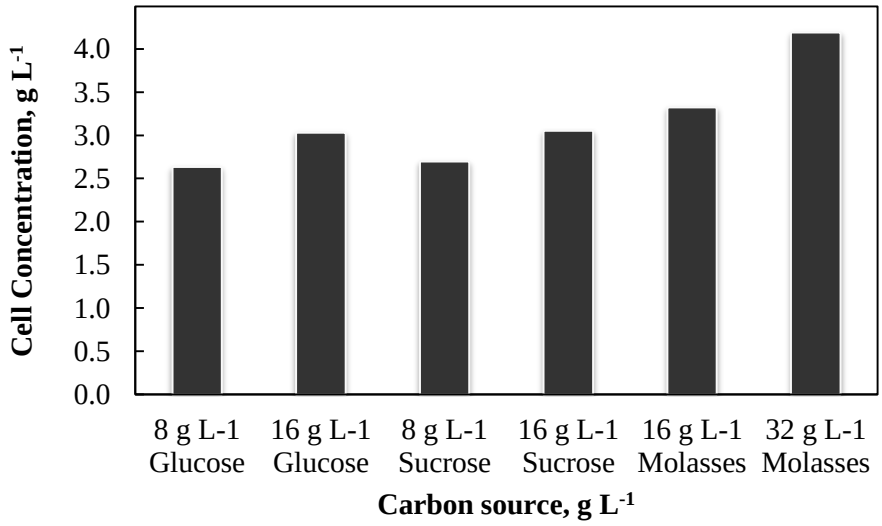

Figure 4.7 Variations in cell concentrations of W-26 in different carbon sources in laboratory scale shake bioreactor experiments at t=12 h

4.2.1.2 Activity Results of Laboratory Scale Shake Bioreactors

At the end of the shake bioreactor experiments, 1 mL samples were removed and centrifuged. After centrifugation, supernatant was removed; pellets were dissolved in 0.5% (w/v) maltodextrin in KIP buffer. This re-dissolved solution was centrifuged again before activity measurements. The GI activity assay was performed as described in Section 3.8.2. The effects of carbon sources on enzyme activities are given in Figure 4.8.

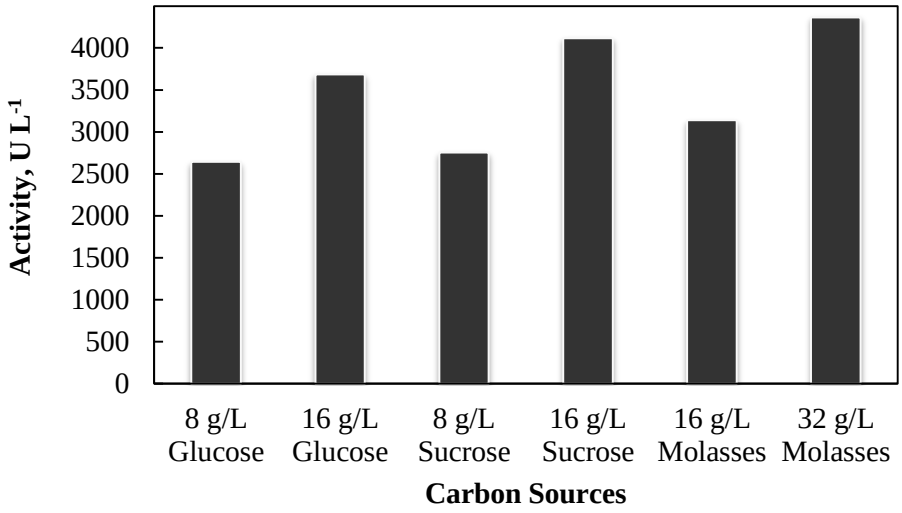

Figure 4.8 Variations in glucose isomerase activity of W-26 on different carbon sources in laboratory scale shake bioreactor experiments at t=12 h

The lowest GI activity was 2644.5 U L^{-1} at 8 g L^{-1} glucose concentration, 1.65-fold lower than the maximum activity (Figure 4.8). In addition, 8 g L^{-1} sucrose containing medium performed similar activity with a value of 2754.5 U L^{-1}. It is concluded that increase in carbon source concentrations increases glucose isomerase activity. 16 g L^{-1} glucose, 16 g L^{-1} sucrose and 32 g L^{-1} sucrose containing media showed 1.40-, 1.49- and 1.39-fold higher activities than 8 g L^{-1} glucose, 8 g L^{-1} sucrose and 16 g L^{-1} molasses containing media, respectively. The highest GI activity was obtained at 32 g L^{-1} molasses containing medium as 4364.1 U L^{-1} due to higher cell concentration and molasses ingredients. The presence of galactose in the beet molasses content is resulted in higher GI activities due to being natural inducer to gene expression of GI (Angardi, 2011). When compared the cell growth results of laboratory scale bioreactors (Figure 4.7) to activity results (Figure 4.8), it can be concluded that the trend is similar.

4.2.2 Pilot Scale Thermostable Glucose Isomerase Production

Laboratory scale experiments showed that higher cell concentrations results in higher enzyme activity which was achieved by the use molasses-based media when compared to sucrose or glucose-based media. In order to increase glucose isomerase production, four pilot scale bioreactor experiments were conducted. All of the bioreactors experiments were started with the following same three steps: i) Cell growth on solid medium for 24 hours at 37°C, ii) Pre-cultivation at 37°C for 12 hours at N=200 min^{-1} iii) Transfer of the cells to pilot scale bioreactor with the inoculation ratio of 1:10. The pilot scale bioreactor, which has a 1.0-2.2 dm^3 working volume, consisted of pH, temperature, stirring rate, foam and oxygen controls. The conducted semi-batch experiments were abbreviated and summarized in Table 4.1.

Table 4.1 The semi-batch operation codes explanations

Code	Type of feed
BR1	Phase I. Batch, $C_{S0,molasses}$=32 g L^{-1}, $C_{N0,(NH4)2HPO4}$=5 g L^{-1} (t =0-5 h), Phase II. Semi-batch operation by exponential molasses feeding at μ_0=0.05 h^{-1} starting at t=5 h until t=28 h, (pH=7.2)
BR2	Phase I. Batch, C_{S0}=32 g L^{-1} molasses, $C_{N0,(NH4)2HPO4}$=5 g L^{-1} (t =0-5h), Phase II. Pulse feeding of molasses to obtain C_{S0} (32 g L^{-1} molasses) at t=5 h Phase III. Semi-batch operation by exponential molasses feeding at μ_0=0.05 h^{-1} starting at t=8 h until t=28 h, (pH=7.2)
BR3	Phase I. Batch, C_{S0}=32 g L^{-1} molasses, $C_{N0,(NH4)2HPO4}$=5 g L^{-1} (t =0-5h), Phase II. Pulse feeding of molasses to obtain C_{S0} (32 g L^{-1} molasses) at t=5 h and t=8 h Phase III. Semi-batch operation by exponential molasses feeding at μ_0=0.05 h^{-1} starting at t=11 h until t=28 h, (pH=7.2)
BR4	Phase I. Batch, C_{S0}=32 g L^{-1} molasses, $C_{N0,(NH4)2HPO4}$=5 g L^{-1} (t =0-5h) Phase II. Pulse feeding of molasses containing $(NH_4)_2HPO_4$ to obtain C_{S0} (32 g L^{-1} molasses) and C_{N0} (5 g L^{-1} $(NH_4)_2HPO_4$) at t=5h and t=8h Phase III. Semi-batch operation by exponential molasses feeding at μ_0=0.05 h^{-1} starting at t=11 h until t=28 h, (pH=7.2)

In order to make a comparison, all the pre-steps of each bioreactor experiments were conducted similarly. All bioreactors were started with the same initial cell concentration. In addition to initial antibiotic concentration, supplementary ampicillin having the concentration of 0.2 g L^{-1} (two times of regular concentration) was added to the feed to maintain plasmid stabilization during the exponential and pulse feedings.

After pulse feeding, the bioprocess shifted to semi-batch operation in which the feeding profiles were calculated by using Equation 2.14 with the pre-determined values of the cell growth at μ=0.05 h^{-1}. Since the starting times of semi-batch operations were not the same under each operation and the measured cell concentrations were different at the beginning of each semi-batch operation, Q (t) graphs were different, as expected.

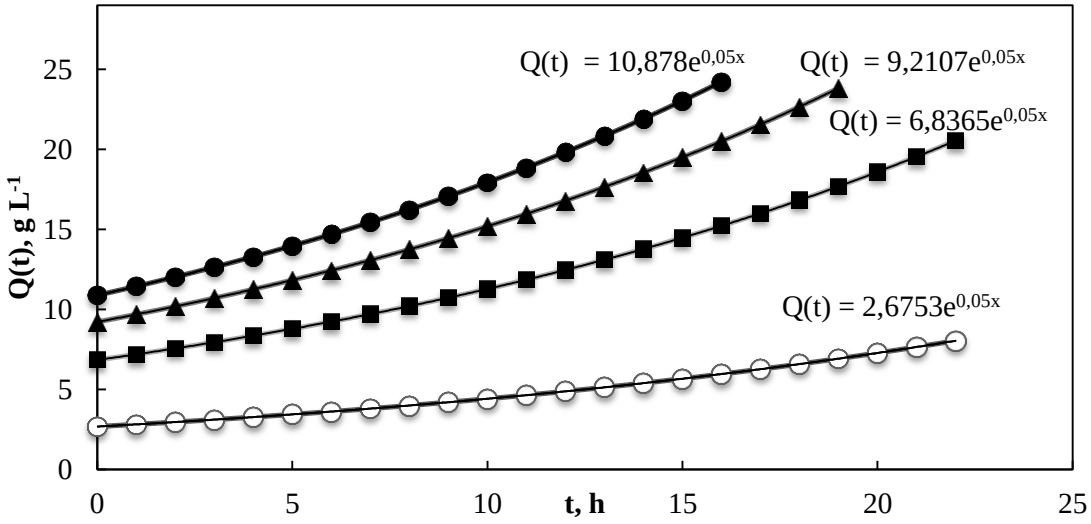

Figure 4.9 The pre-determined feeding profile for molasses, calculated for the specific growth rate (μ) of 0.05 h^{-1}: BR4 (●), BR3 (▲), BR2 (■), BR1 (○) (t=0 indicates the starting time of the semi-batch operations)

4.2.2.1 Feeding Strategy Effects on Substrate Consumption

All of the bioreactors were carried out with molasses based media but for detection of carbon sources, the substrate consumption graphics showed the sucrose concentration in the molasses, which means 32 g L^{-1} molasses has 16 g L^{-1} sucrose in it. The concentrations of sucrose/molasses were measured by sucrose analysis kit every two hours. Samples were diluted at appropriate rates according to directions in sucrose analysis kit manual.

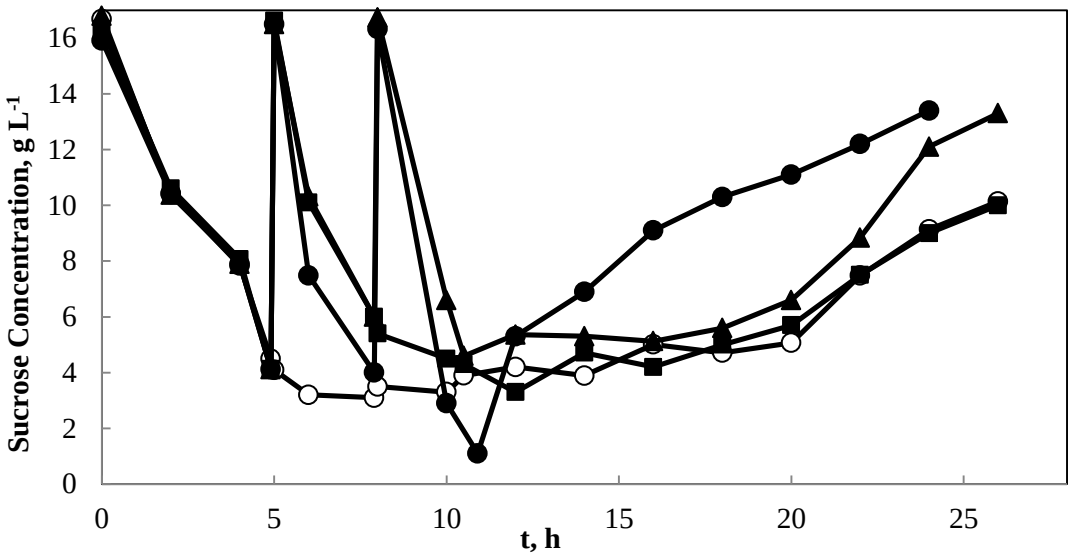

Figure 4.10 Variation in sucrose concentrations of all bioreactors BR4 (●), BR3 (▲), BR2 (■), BR1 (○)

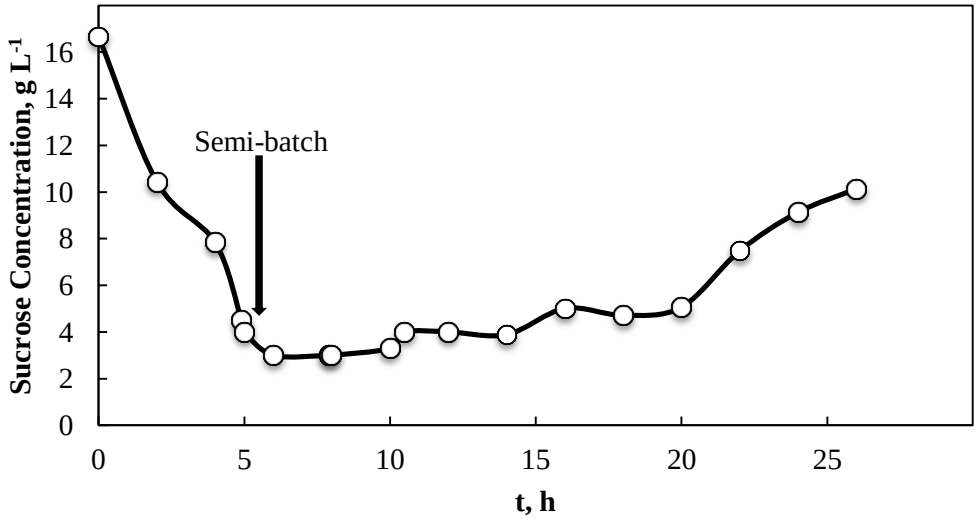

Figure 4.11 Variation in sucrose concentrations of BR1

The initial concentration of sucrose in molasses was 16.7 g L^{-1} in BR1 and it gradually decreased during the first five hours. After t=5 h, fed-batch operation was started with μ=0.05h^{-1}. Since it was reported that *E. coli* W cannot grow under low sucrose concentration, it was aimed to keep the sucrose concentration at about 5 g L^{-1}. During the next 15 hours, sucrose concentration was kept at around 5 g L^{-1}, but after t=20 h, sucrose accumulation started with attaining to stationary phase of cells.

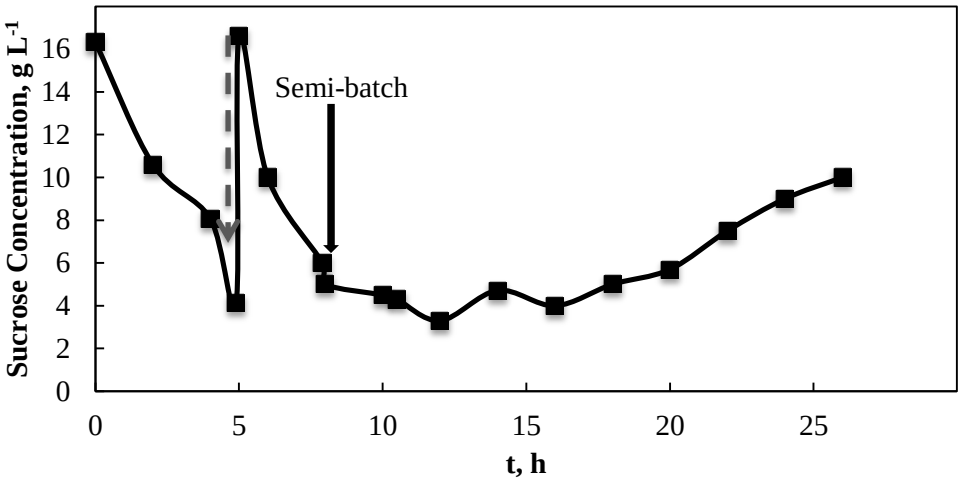

Figure 4.12 Variation in sucrose concentrations of BR2 (The dashed line indicates the molasses pulse.)

In BR2, the initial sucrose concentration in molasses-based production medium was 16.3 g L^{-1}. During five hours of the batch mode of fermentation, the sucrose was utilized similarly as in BR1 operation, with nearly the same cell concentrations as presented in Figure 4.11. After 5 hours, molasses with ampicillin was given as pulse-feeding to medium to reach 16 g L^{-1} sucrose concentration in the bioreactor. At t=8 h, the sucrose concentration decreased to ca. 5 g L^{-1}, and fed-batch operation was started with the same pre-determined specific growth rate (μ=0.05 h^{-1}) as in the strategy of BR1. At t=20 h, sucrose accumulated in fermentation medium.

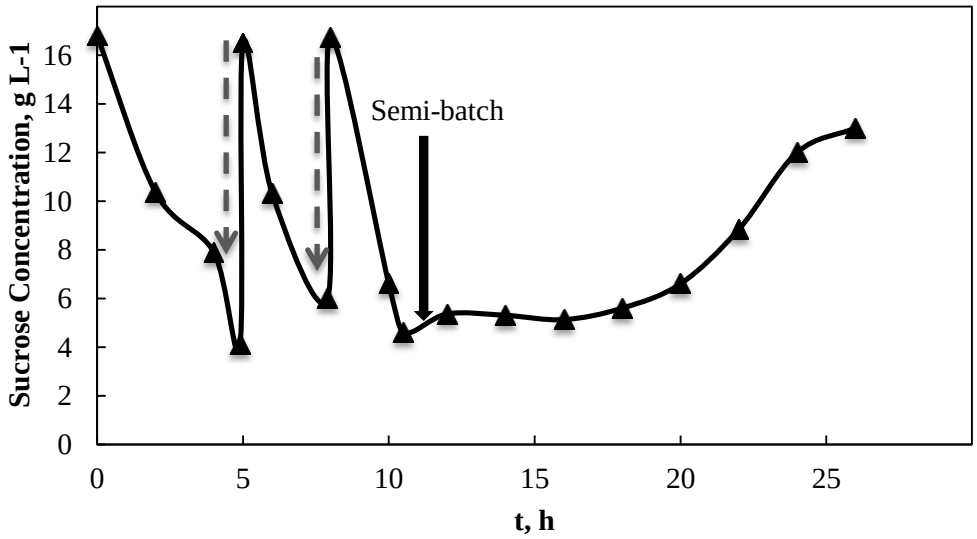

Figure 4.13 Variation in sucrose concentrations of BR3 (The dashed lines indicate the molasses pulses.)

BR3 operation was started with the sucrose concentration of 16.8 g L^{-1}. The first pulse was given at t=5 h as in the strategies of BR1 and BR2, when the sucrose concentration was 4.12 g L^{-1}. After the first pulse of molasses, sucrose concentration was increased to 16.50 g L^{-1} and the second pulse was given after 3 hours of batch operation. The sucrose concentration was increased from 6.00 g L^{-1} to 16.73 g L^{-1} after the second molasses pulse was given. The semi-batch operation was started with the decrease in sucrose concentration to 4-5 g L^{-1}. After 11 hours of semi-batch feeding, at t=20 h, significant substrate accumulation started.

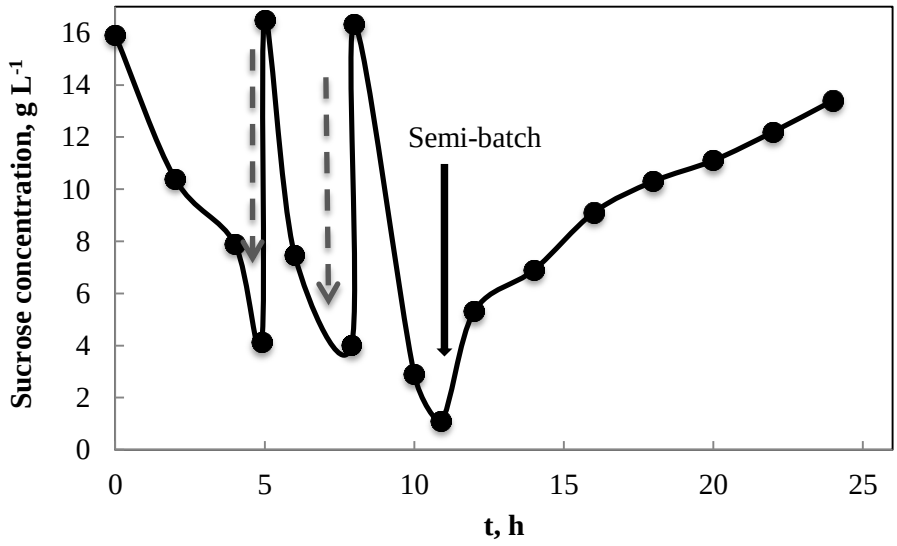

Figure 4.14 Variation in sucrose concentrations of BR4 (The dashed lines indicate the molasses pulses.)

Since the highest enzyme activity was obtained in BR3 operation compared to BR1 and BR2 conditions, effect of $(NH_4)_2HPO_4$ addition was investigated in BR4. $(NH_4)_2HPO_4$ initial concentration was 5 g L^{-1} in all bioreactor experiments. The additional $(NH_4)_2HPO_4$ supplied to the bioreactor was determined based on the carbon/nitrogen source which was approximately 4:1 for each pulse. Thus, after determining the amount of the carbon source consumed, the amount of concentration that was added was calculated based on the ratio. Nitrogen source together with molasses was supplied to the system.

Because, cell concentrations in BR4 were higher than BR3, carbon source was utilized faster than that of BR3. As a result, at the time of the second pulse-feeding (t=8 h), and the time of the shift of the semi-batch operation (t=11 h), the sucrose concentrations were lower than BR3 as 4.00 g L^{-1} and 1.06 g L^{-1}, respectively.

The semi-batch operation was started at t=11 h, as BR3 was started. On the other hand, at the time of semi-batch operation was started, the sucrose concentration was lower than desired value which was not a favorable condition for the growth of *E. coli* W. Sucrose accumulation started as soon as semi-batch feeding was started. Because of this undesired situation, cell growth and glucose isomerase production rates are decreased with the cultivation time (Figure 4.15 and Figure 4.16).

In addition, in semi-batch bioreactor operations the amounts of consumed sucrose are in Table 4.2. As can be seen in Table 4.2, sucrose was never consumed completely by the cells. These results are consistent with the results observed in Figure 4.10, 4.11, 4.12, 4.13 and 4.14. In all bioreactor experiments, there was sucrose consumption above 65% and the highest consumption was determined as 80.26% in BR3 operation.

Table 4.2 Semi-batch bioreactor operation characteristics based on the substrate sucrose

Strategies	Batch operatio cultivation time prior to semi-batch operation (t), h	Initial sucrose for batch operation at t=0 (m_{S0}), g	Maximum GI		Overall values within t=0 – t=t_{max}		
			Cultivation time (t_{max}), h	Activity A_{GI}, U L^{-1}	Total sucrose fed (m_S), g	Total sucrose consumed Δm_S, g	Consumption ratio of sucrose fed, %
BR1	5	16.70	16	15839.5	14.43	26.33	66.74
BR2	8	16.30	16	19020.4	17.03	29.33	75.34
BR3	11	16.80	16	28102.3	25.83	35.53	80.26
BR4	11	15.90	16	35264.5	28.25	35.05	67.29

4.2.2.2 Feeding Strategy Effects on the Cell Growth

The variations in the cell concentration with the cultivation time are given in Figure 4.15. As can be seen from the figure; all of the cell concentrations are similar values until t=6 h. BR2 and BR3 cell concentration values which are close to each other during the fermentations, whereas the cell concentrations of BR3 were slightly higher than that of the BR2.

Minimum cell concentration was obtained in BR1 operation while the highest cell concentration value was achieved in BR4 operation. Besides, the profile of the cell concentration in BR4 is different than the other bioreactor operation profiles after t= 6 h. Although the number of pulses and the pre-determined specific growth rate of BR4 and BR3 were exactly the same, the reason of obtaining a different cell concentration profile in BR4 was the addition of $(NH_4)_2HPO_4$ into BR4 molasses pulses. In addition, the maximum cell concentration of BR4 was attained earlier than BR3 experiment, with a higher peak value.

The lowest final cell concentration was determined in BR1 operation as 12.92 g L^{-1}; whereas in BR2 and BR3 experiments the maximum cell concentrations were close to each other 16.0 g L^{-1} (t=24 h) and 16.3 g L^{-1} (t=28 h), respectively. Among the exponential feeding strategies the highest cell concentration was obtained in BR4 as 18.4 g L^{-1} at t=26 h. So, it can be concluded that higher cell growth rates obtained in BR2 BR3 are due to increased number of molasses pulses.

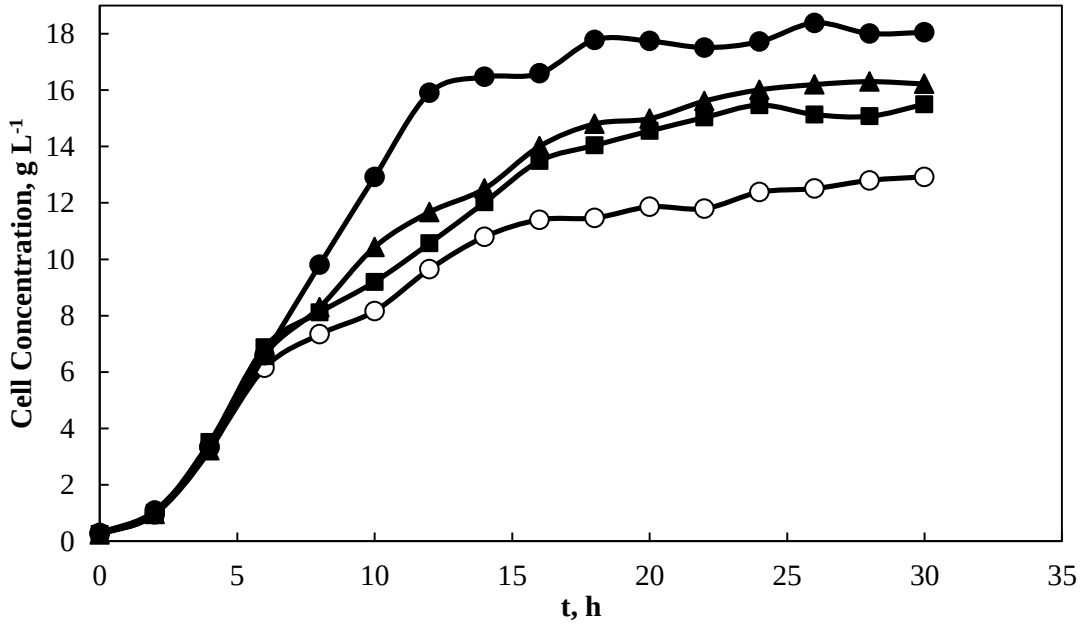

Figure 4.15 Variations in the cell concentrations in pilot scale bioreactors with the cultivation time with different feeding strategies by recombinant *E. coli* W-26: BR4(●), BR3(▲), BR2(■), BR1(○)

According to the cell concentration results of exponential feeding experiments, BR4 was referred as the favorable operation since the maximum cell concentration achieved in this feeding strategy was 1.42-, 1.15- and 1.13-fold higher than that attained in BR1, BR2 and BR3, respectively.

From our research group Angardi (2011) reported 9.6 g L^{-1} *E. coli* BL21 (DE3) pLysS cell concentration in a semi-batch operation using also hydrolyzed molasses based medium, at growth rate of μ=0.15 h^{-1}. In this MSc thesis, with the same recombinant plasmid pRSETA::*xylA*, in the semi-batch strategy BR4 1.92-fold higher cell concentration was achieved.

4.2.2.3 Feeding Strategy Effects on GI Activity

In order to determine GI production capacity of *E. coli* W-26, pilot scale bioreactor experiments were conducted. The variations in glucose isomerase activities with the cultivation time are given in Figure 4.16.

In BR1, BR2, BR3 and BR4 GI activities reached their maxima at t=16 h; where the GI values were close until t=4 h. In all of the four molasses based experiments, GI activities were increased significantly after molasses feeding was started by semi-batch operation as mentioned in Table 4.1.The activity levels of BR1 and BR2 were closer during the process compared to the other operations; whereas BR2 values were higher than BR1 values after t=4 h due to the pulse molasses feeding. The highest GI activities in BR1 and BR2 were measured at t= 16 h of the bioprocess as 15839.5 U L^{-1} and 19020.4 U $L^{-1,}$ respectively.

The activity level of BR3 increased linearly between, t=5 h which was the cultivation time of the first pulse, and t=16 h which was the cultivation time of the maximum GI activity level. Moreover, BR3 reached the maximum GI activity level of BR2 and BR1 two and four-hours earlier, respectively. GI activities increased significantly after the pulse-feedings were started at all bioreactor experiments.

At BR4 operation, glucose isomerase activity is higher than other designed bioreactor experiments after pulse feedings due to addition of nitrogen source ($(NH_4)_2HPO_4$). GI activity values of BR4 increased more rapidly than other processes after t=5 h, and BR4 produced higher GI values.

The maximum glucose isomerase activity measured in BR4 operation is 35264.5 U L^{-1} at t=16 h. Moreover the maximum level of BR4 was 1.25-, 1.85- and 2.23-fold higher than those of BR3, BR2 and BR1, respectively. When compared to Figure 4.15; it can be seen that when the cell growth reached to the stationary phase, GI activities decrease after the maximum. All enzyme activities were decreased after their peak values probably because of protease secretion. Also sucrose was accumulated after 20 hours of the fermentation, so substrate inhibition might have effect on the enzyme activity.

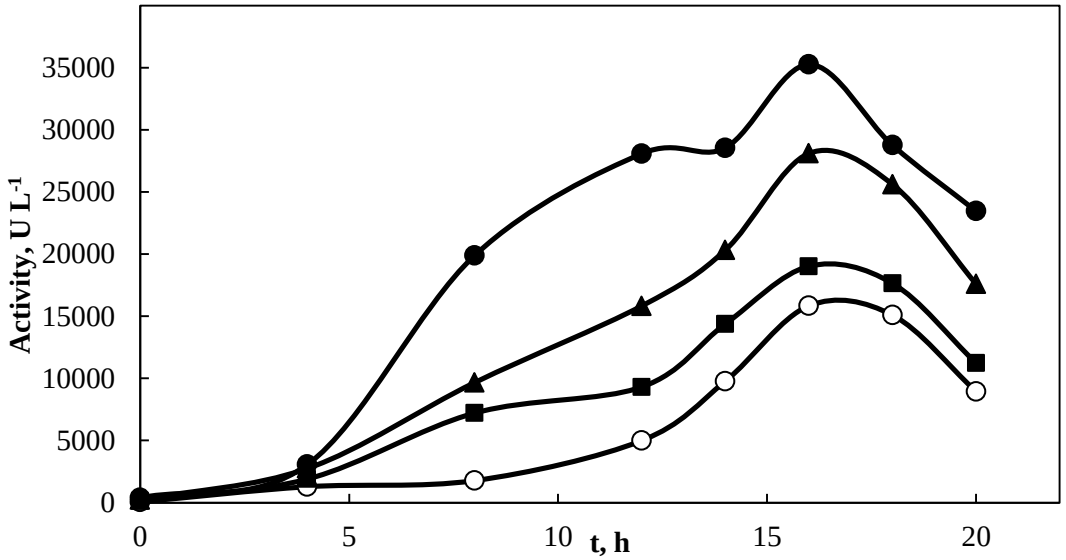

Figure 4.16 Variations in GI activity in pilot scale bioreactors with the cultivation time for different feeding strategies by recombinant *E. coli* W-26:BR4(●), BR3(▲), BR2(■), BR1(○)

In the study of Angardi (2011), in which *E. coli* BL21 (DE3) was used, the maximum GI activity was achieved as 16,400 U L^{-1} with the same expression vector and the same production medium containing hydrolyzed molasses but at a different specific growth rate, i.e. μ=0.1 h^{-1}. In this thesis, in which sucrose utilizing *E. coli* W was used, 2.15-fold higher GI activity than that of the study of Angardi (2011) which was using *Escherichia coli* BL21 (DE3) pLysS was achieved by exponential feeding of un-hydrolyzed molasses after pulse-feed together with $(NH_4)_2HPO_4$ addition.

4.2.2.4 Feeding Strategy Effects on Organic Acid

The concentrations of the organic acids detected in the fermentation medium were given in Table 4.3 and Figure 4.17 for every four hours. Organic acid profiles help to figure out the intracellular reaction networks of recombinant *Escherichia coli* processes profoundly. Acetic, oxalic, formic, fumaric, malic and citric acids were the organic acids that were detected in the fermentation broths**.** In all operation conditions, nearly similar trends were observed and organic acid concentrations increased with the cultivation time. For all bioreactor experiments; acetic acid, oxalic acid, formic acid, fumaric acid, malic acid and citric acid which are mostly involved in TCA cycle reactions were detected in the fermentation medium.

Total organic acid concentrations detected at the final hour of semi-batch phase were 14.40, 15.08, 18.46 and 20.92 g L^{-1} for BR1, BR2, BR3 and BR4 operations, respectively. For increasing numbers of molasses pulse-feeding, increase in the maximum organic acid concentrations was observed. Presence of organic acids of the TCA cycle, i.e., citric, formic and malic acids indicates the inadequate oxygen utilization, which leads to repression of the

TCA cycle enzymes. The maximum acetic acid concentrations for the fed batch phases were measured as 5.12, 6.14, 7.48 and 9.22 g L^{-1}, for BR1, BR2, BR3 and BR4 operations, respectively. Higher acetic acid concentrations were observed for higher cell concentrations, in which oxygen demands were higher, therefore oxygen supply may become inadequate.

By comparison with sucrose consumption figures (Figure 4.10, 4.11, 4.12, 4.13, 4.14); it can be clearly seen that increasing levels of substrate accumulation resulted in acetic acid secretion with high amounts. Hence, this overflow metabolism decreases the rates of cell growth and enzyme production.

Accumulation of undissociated (protonated- CH_3COOH) form of acetic acid in the cultivation medium is one of the biggest problem in *E. coli* fermentation because it decreases the formation of recombinant product and inhibits cell growth (Shiloach *et al*., 1996; Akesson *et al*., 2001; Çalık *et al*., 2004 and 2006). There are various explanations of acetate production occurred by the overflow metabolism in which Acetyl-coA is directed to acetate followed by entering in the TCA cycle (Åkesson *et al*., 1999). One of them is the limited capacity of the respiratory system where NADH is reoxidized. When the respiratory system saturates to avoid accumulation of NADH, the flux of acetyl-coA to the TCA cycle ends up with NADH production so redirection of acetyl-coA flux to acetate would be required. The other explanation is the limited capacity of the TCA cycle, which is reached before the saturation of respiration (Majewski and Domach, 1990). Because of the increasing glucose uptake, Acetyl-coA flux would again be directed to acetate when the TCA cycle saturates (Åkesson *et al*., 1999). In order to overcome this problem low acetate producing strains are developed and preferred to use in industrial applications. In addition, *E. coli* W was reported as a low acetate producer strain like other *E. coli* B group strains (Shiloach *et al*., 1996).

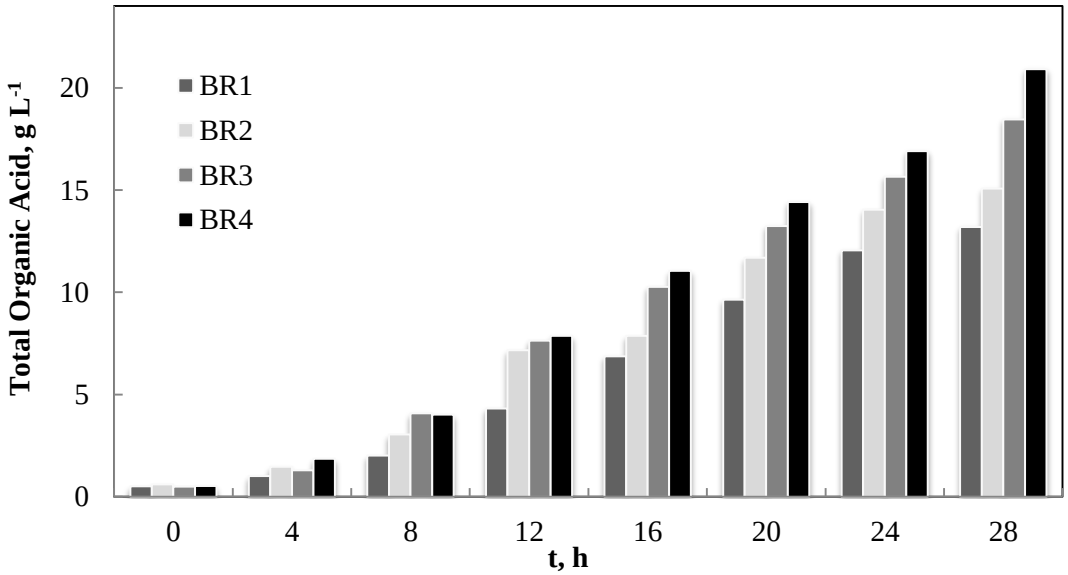

Figure 4.17 Variations in total organic acid concentration with the cultivation time for bioreactor operations

Table 4.3 Variations in the organic acid concentrations with the cultivation time for bioreactor operations

	Concentrations, g L^{-1}					
t	**Acetic acid**	**Oxalic acid**	**Formic acid**	**Fumaric acid**	**Malic acid**	**Citric acid**
			BR1			
0	0.1023	0.0197	0.0541	0.0015	0.2124	0.1283
4	0.5159	0.0802	0.1071	0.0023	0.2567	0.0598
8	0.9721	0.0819	0.2234	0.0016	0.6978	0.0519
12	2.1040	0.1281	0.4792	0.0039	1.5420	0.0743
16	3.4477	0.2103	1.1985	0.0051	1.8856	0.1333
20	3.8095	1.7845	1.5643	0.0073	2.3028	0.1840
24	5.0496	1.7845	2.1296	0.0135	2.6278	0.4521
28	5.1157	1.7845	2.4781	0.0122	2.8415	0.9631
			BR2			
0	0.0990	0.0213	0.0467	0.0003	0.2782	0.1731
4	0.5558	0.0814	0.1095	0.0008	0.2717	0.4558
8	1.5476	0.1028	0.2256	0.0025	0.4858	0.6864
12	3.5460	0.1383	0.8180	0.0057	1.7801	0.8885
16	4.1488	0.1320	2.5086	0.0079	1.0846	0.0000
20	4.8925	1.9144	2.6549	0.0126	2.0908	0.1300
24	5.9031	2.3113	2.8729	0.0139	2.7753	0.1685
28	6.1414	3.7874	2.1288	0.0218	2.7924	0.2062

Table 4.3 Variations in the organic acid concentrations with the cultivation time for bioreactor operations (continued)

	Concentrations. g L^{-1}					
t	**Acetic acid**	**Oxalic acid**	**Formic acid**	**Fumaric acid**	**Malic Acid**	**Citric acid**
			BR3			
0	0.0945	0.0182	0.0596	0.0010	0.1757	0.1588
4	0.6490	0.0838	0.3575	0.0024	0.1705	0.0473
8	1.3480	0.1130	1.4633	0.0048	0.3521	0.8081
12	4.2675	0.1786	0.9752	0.0115	1.1523	1.0612
16	4.5692	0.7250	2.2861	0.0177	1.3428	1.3282
20	5.2897	1.5489	2.9925	0.0169	1.8659	1.5366
24	6.3123	3.4643	2.4759	0.0341	1.6139	1.7659
28	7.4821	3.1052	3.3979	0.0470	2.7295	1.7030
			BR4			
0	0.0917	0.0239	0.0446	0.0005	0.1602	0.2189
4	0.5558	0.0972	0.1054	0.0007	0.6309	0.4823
8	1.9548	0.1114	0.3028	0.0020	0.9829	0.6804
12	4.9460	0.1866	0.3935	0.0034	1.2814	1.0630
16	5.9125	0.2546	2.1178	0.0041	1.4098	1.3585
20	6.6053	1.1795	2.6383	0.0042	2.4011	1.5927
24	7.5701	2.3929	2.8175	0.0077	2.3567	1.7675
28	9.2159	3.1551	3.9816	0.0084	2.7258	1.8349

4.2.2.5 Specific Growth Rate and Yield Coefficient

The variations in bioprocess characteristic based on the fermentation efficiency were calculated for pilot scale bioreactor experiments. Specific growth rate (μ), specific substrate utilization rate (q_S) and yield coefficients represented in Table 4.4 and they were calculated as described by Çalık *et al*. (2006).

Table 4.4 Variations in fermentation characteristics

Feeding Strategies	t (h)	μ (h^{-1})	q_S ($g\ g^{-1}\ h^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)
BR1	4	0.39	0.70	0.44
	8	0.07	0.08	0.20
	12	0.07	0.06	0.22
	16	0.01	0.06	0.07
	20	0.01	0.04	0.04
	24	0.01	0.06	0.05
	28	0.01	0.08	-
BR2	4	0.42	0.04	0.99
	8	0.07	0.33	0.11
	12	0.07	0.14	0.08
	16	0.04	0.14	0.08
	20	0.02	0.12	0.02
	24	0.00	0.14	0.02
	28	0.01	0.15	-
BR3	4	0.44	0.17	0.92
	8	0.12	0.11	-
	12	0.04	0.18	0.12
	16	0.04	0.15	0.07
	20	0.01	0.12	0.01
	24	0.01	0.13	0.01
	28	0.00	0.18	0.00
BR4	4	0.41	0.22	0.91
	8	0.16	0.16	-
	12	0.06	0.05	0.16
	16	0.02	0.11	0.00
	20	0.00	0.16	0.00
	24	0.01	0.20	0.01
	28	0.00	0.20	-

Generally, the specific growth rate values were below the predetermined μ (0.05 h^{-1}) and these values were not constant at the for all bioreactor conditions. This decrease might be due to inhibitory effects of by-product formation as a result of insufficient oxygen level through the all fermentations. The ideal conditions which are needed to attain cell growth with a constant predetermined specific growth rate is negatively affected due to this by-product formation, especially acetate accumulation. The highest specific growth rates after initiating of semi-batch were obtained as 0.07, 0.07, 0.04, and 0.06 h^{-1} for BR1, BR2, BR3 and BR4 operations, respectively.

q_S (Specific substrate consumption rate, h^{-1}) were mostly increased during the semi-batch phase of fermentations. The maximum value of specific substrate consumption rate increased as the number of pulse-feeding increased and the highest value of 0.20 g g^{-1} h^{-1} was obtained in BR4 operation.

The values of overall specific cell yields on substrate ($Y_{X/S}$) were calculated as 0.42, 0.45, 0.37 and 0.47 g g^{-1} for BR1, BR2, BR3and BR4 operations, respectively. Since all of the values were similar to each other; it can be concluded that substrate utilization occurred with the same efficiency in all fermentation conditions.

CONCLUSIONS

In this study, it was aimed to investigate the production of thermostable glucose isomerase (GI; EC 5.3.1.5) using metabolically engineered sucrose utilizing *Escherichia coli*. In the first part of this thesis work, pRSETA::*xylA* (Angardi and Çalık, 2013) was isolated and transformation of *Escherichia coli* W was carried out. Then, the best producing strain was selected by using different carbon sources in order to over-produce GI. In addition, the storage condition was optimized. The results obtained from the first part of this study are given below:

- After the transformation of pRSETA::*xylA E. coli* W, putative colonies were selected and the size of the plasmid isolated, was verified by agarose gel electrophoresis.

- For microorganism selection, different types of carbon sources in different concentrations were investigated in two sets of experiments. The strain that had 5.80–fold higher glucose isomerase activity than that of wild type *E. coli* W was selected as the best GI producer strain, named *E. coli* W-26 and used in further experiments.

- 0.5% (w/v) and 2% (w/v) maltodextrin in KIP buffer were tested for pellet storage conditions. It was shown that the pellets dissolved in 0.5% (w/v) maltodextrin solution had 16% higher remaining activity when compared to pellets without any treatment; whereas dissolving pellets in 2% (w/v) maltodextrin had no positive effects on preservation of the enzyme activity.

In the second part of the study, the production of recombinant glucose isomerase by W-26 was investigated by laboratory scale shake bioreactor experiment and four sets of pilot scale bioreactor experiments. Different types of carbon sources (glucose, sucrose and molasses) in different concentrations were examined in laboratory scale shake bioreactor experiments which were then followed by pilot scale bioreactor experiments. Basically, the effects of molasses based medium and molasses pulses with/without $(NH_4)_2HPO_4$ were investigated with the same pre-determined specific growth rate of μ=0.05 h^{-1} on cell growth, GI production and by-product formation. The results obtained from the second part of this study are given below:

1. Laboratory scale shake bioreactor experiments were performed by cultivation of *E. coli* W-26 containing pRSETA::*xylA* in six different media containing 8 g L^{-1} and 16 g L^{-1} glucose, 8 g L^{-1} and 16 g L^{-1} sucrose and 16 g L^{-1} and 32 g L^{-1} molasses. Similar cell growth profile and values were obtained in sucrose and glucose based media. In the production with 32 g L^{-1} molasses containing medium; the maximum

cell concentration was found as 1.37-fold higher than 16 g L^{-1} sucrose containing medium after 12 hours. It was concluded that glucose isomerase activities demonstrated similar trend with cell growth; as increased carbon source concentrations caused increased glucose isomerase activity. The media containing 16 g L^{-1} glucose, 16 g L^{-1} sucrose, and 32 g L^{-1} molasses had 1.40-, 1.49-, and 1.39-fold higher activities than the media containing 8 g L^{-1} glucose, 8g L^{-1} sucrose and 16 g L^{-1} molasses, respectively. Hence, 32 g L^{-1} molasses containing medium was chosen as the initial condition of pilot scale bioreactor experiments due to the cell concentration and glucose isomerase activity values below;

a. The maximum cell concentration was measured as 4.19 g L^{-1} at t=12 h.
b. The highest enzyme activity was obtained as 4364.1 U L^{-1} after 12 hours.

2. In accordance with the cell growth and activity data of laboratory scale shake bioreactor experiment, 32 g L^{-1} molasses was chosen as the initial carbon concentration for the four sets of pilot scale bioreactor experiments. Among four different bioreactor experiments, it was determined that BR1 had the lowest cell concentration at each time after t=5 h. On the other hand, the maximum cell concentrations in BR2 and BR3 experiments were close to each other, which were obtained as 16.0 g L^{-1} at t=24 h and 16.3 g L^{-1} at t=28 h, respectively. The maximum cell concentration was found as 18.4 g L^{-1} in BR4 and this value was 1.42-, 1.15- and 1.13-fold higher than that achieved in BR1, BR2 and BR3, respectively.

3. GI activities were increased significantly after the pulse-feedings were given for all bioreactor experiments. Measured GI activity values of BR1 and BR2 were closer to each other during the process in comparison with the other operations; but, GI activity obtained in BR2 was slightly higher than that of BR1. The highest GI activity of BR1 and BR2 were measured at t=16 h as 15839.5 U L^{-1} and 19020.4 U L^{-1}, respectively. In BR3, GI activity values increased linearly between t=4 h and t=16 h, namely the time of the first pulse feed and the peak value, respectively. Glucose isomerase activities demonstrated higher increase in BR4 than other bioreactor experiments after pulse feedings with addition of $(NH_4)_2HPO_4$. Beginning with the t=4 h, values of BR4 began to increase more rapidly than the other processes and during the fermentation BR4 values had always higher values. Moreover, the peak level of GI activity obtained in BR4 was 1.25-, 1.85- and 2.23-fold higher than those of BR3, BR2 and BR1, respectively. It was concluded that BR4 was the optimum feeding strategy due to the cell concentration and glucose isomerase values below;

a. The highest cell concentration was 18.4 g L^{-1} at t=26 h.
b. The maximum glucose isomerase activity was 35264.5 U L^{-1} at 16^{th}.

4. All of the bioreactor experiments were started with 32 g L^{-1} molasses containing production medium where the sucrose concentration was assumed as 16 g L^{-1} in it. After pulse feeding was given, semi-batch feeding was initiated and in all of the bioreactor experiments, sucrose started to accumulate after 20 hours, except for

BR4. In BR4 operation, sucrose started to accumulate as soon as semi-batch operation was initiated.

5. Acetic, oxalic, formic, fumaric, malic and citric acids were the organic acids detected in the fermentation broths. Total organic acid concentrations measured at the last hour of fermentations were 14.40, 15.08, 18.46 and 20.92 g L^{-1} for BR1, BR2, BR3 and BR4 operations, respectively. Also the maximum acetic acid concentrations were obtained as 5.12, 6.14, 7.48 and 9.22 g L^{-1}, for BR1, BR2, BR3 and BR4 operations, respectively.

6. It was determined that the highest specific growth rates were mostly below the predetermined μ=0.05 h^{-1}; after initiation of semi-batch fermentation. The values of 0.07, 0.07, 0.04, and 0.06 h^{-1} were obtained from BR1, BR2, BR3 and BR4 operations, respectively. Specific substrate consumption rate were generally increased throughout the semi-batch phase of bio-processes and the highest value was obtained in BR4 operation as 0.20 g g^{-1} h^{-1}. In addition, it was found that the values of the maximum qS increased by the increased number of molasses-pulses given. Overall specific cell yields on substrate ($Y_{X/S}$) were similar to each other; as 0.42, 0.45, 0.37 and 0.47 g g^{-1} for BR1, BR2, BR3and BR4 operations, respectively.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Alkaline Lysis Solution I	Prepare 100 ml batches including 50 mM glucose, 25 mM Tris-Cl and 100 mM EDTA in distilled water. Autoclave and store at 4°C.
Alkaline Lysis Solution II	Prepare 10 ml batches including 0.2 M NaOH and 1% SDS (w/v) in distilled water. Freshly prepare.
Alkaline Lysis Solution III	Prepare 100 ml batches including 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of dH2O. Store it at room temperature and transfer to an ice bucket before use.
0.12%(w/v) carbazole	0.12 g carbazole was dissolved in 100ml dH2O and used freshly.
1.5%(w/v) Cysteine HCl	0.75 g Cysteine was dissolved in 50ml dH2O and used freshly.
6 x DNA gel-loading buffer	0.25 % Bromophenol blue, 0.25 % xylenecyanol FF, 40 % sucrose in dH2O.

GI Activity Buffer	0.4M D-glucose and 10mM MnCl2 in 0.02M potassium buffer pH 7.0.
0.1 M potassium phosphate, pH 7.0	3.85 ml of 1M KH2PO4, 6.15 ml of 1M K2HPO4 was dissolved in dH20 and the volume made up to 100 ml. The solution pH was controlled, autoclaved and stored at room temperature.
RNaseA stock solution	RNaseA was dissolved at a concentration of 10 mg/ml in 50 mM potassium acetate (pH 5.5) and boiled for 10 minutes. Stored at -20 ˚ C.
70% (v/v) Sulfuric acid	70 ml sulfuric acid was gradually added to 30 ml dH2O.
10X TE Buffer (pH 8.0)	Prepare 100 mM Tris-Cl (pH=8.0) and 10 mM EDTA (pH=8.0) in distilled water. Autoclave and store at 4°C. Dilute it 10 times before use.
5X TBE Buffer	Dissolve 54 g of Tris base, 27.5 g of boric acid and 20 ml of EDTA (0.5 M) in 950 mL distilled water. Adjust pH to 8.0 and make it up to 1 L. Dilute it 5 times before use.
Antifoam	Prepare 10% (v/v) antifoam solution with distilled water. Autoclave before use.

APPENDIX B

CALIBRATION OF D-FRUCTOSE CONCENTRATION

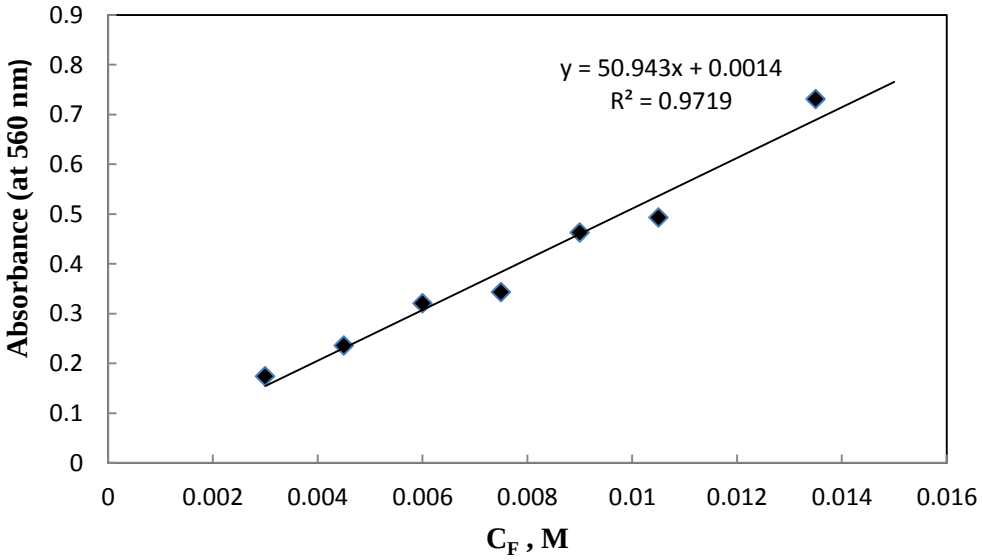

Figure B.1 Calibration of D-Fructose concentration

According to the equation obtained from the plot:

$$Activity\ (UL^{-1}) = \frac{C_F\ (\mu M)}{reaction\ time(min)} \qquad (\boldsymbol{B.1})$$

$$C_F(\mu M) = \frac{(Absorbance - 0{,}0014))}{50{,}943}\ x\ Dilution\ Ratio\ x\ 10^6 \qquad (\boldsymbol{B.2})$$

$$Activity\ (UL^{-1}) = \frac{C_F(\mu M)}{10\ (min)} \qquad \textbf{(B.3)}$$

APPENDIX C

CALIBRATION CURVE FOR SUCROSE CONCENTRATION

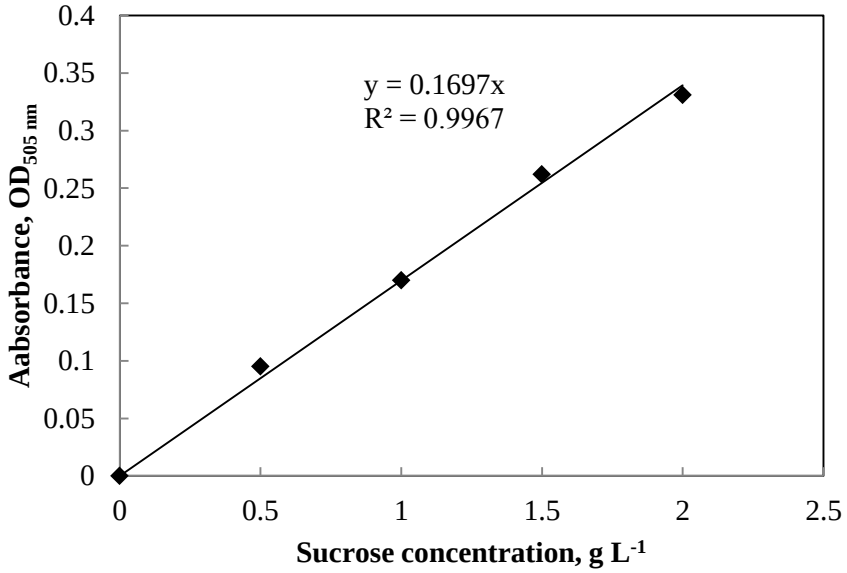

Figure C.1 Calibration curve for sucrose concentration

APPENDIX D

CALIBRATION CURVES FOR ORGANIC ACID

- **Acetic Acid**

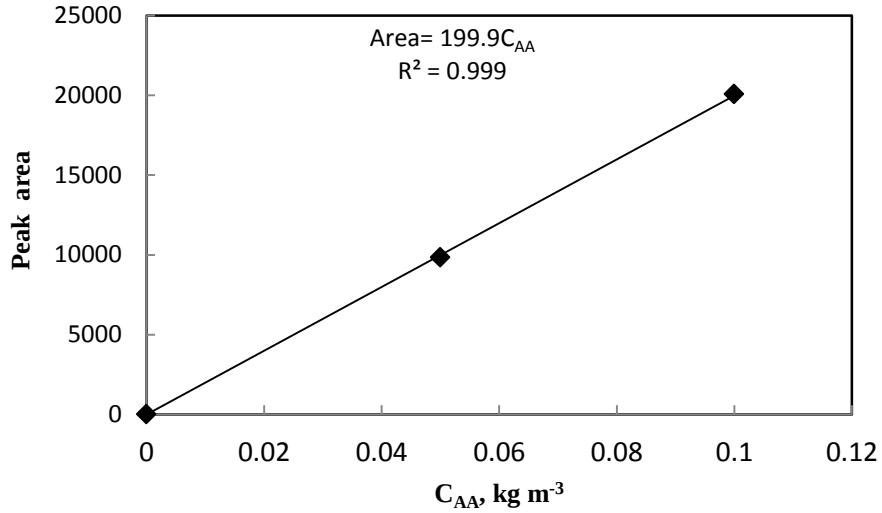

Figure D.1 Standard calibration curve for acetic acid.

- **Citric Acid**

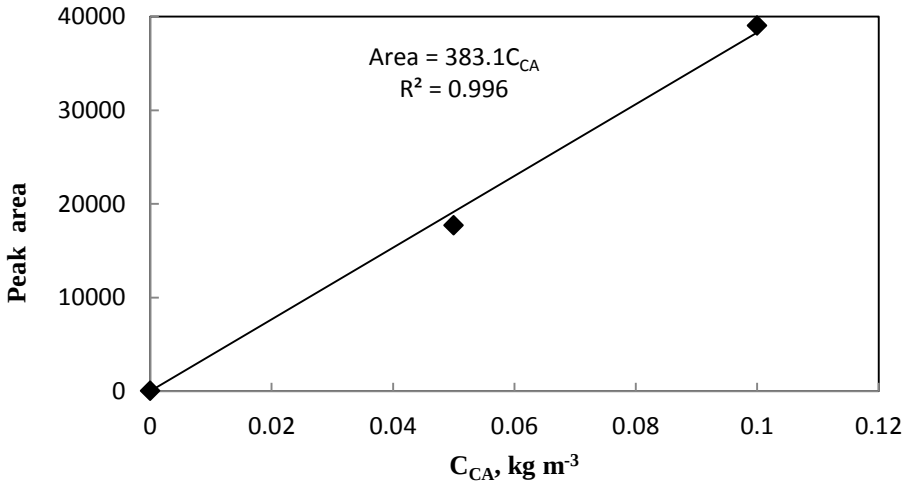

Figure D.2 Standard calibration curve for citric acid.

- **Formic Acid**

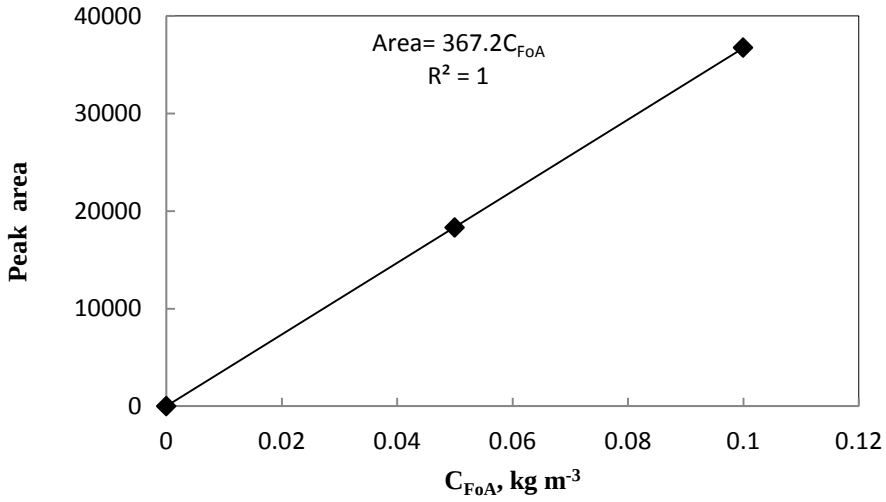

Figure D.3 Standard calibration curve for formic acid.

- **Fumaric Acid**

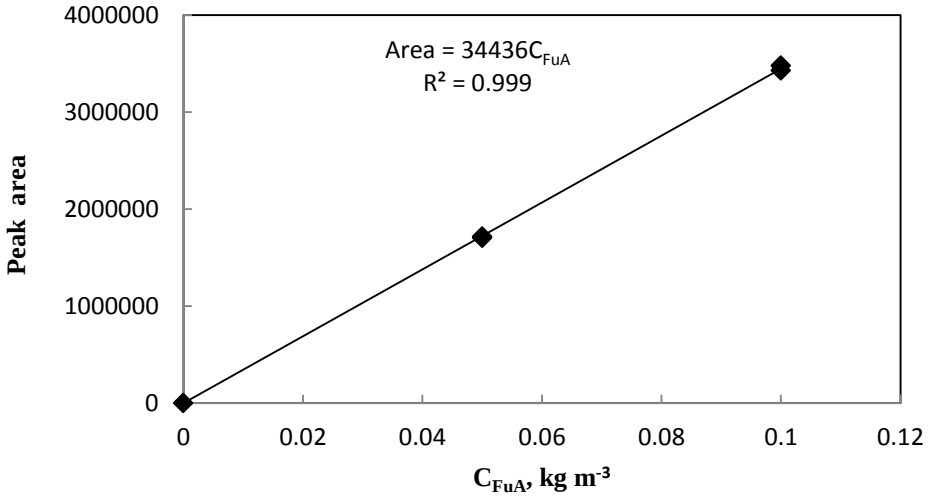

Figure D.4 Standard calibration curve for fumaric acid.

- **Malic Acid**

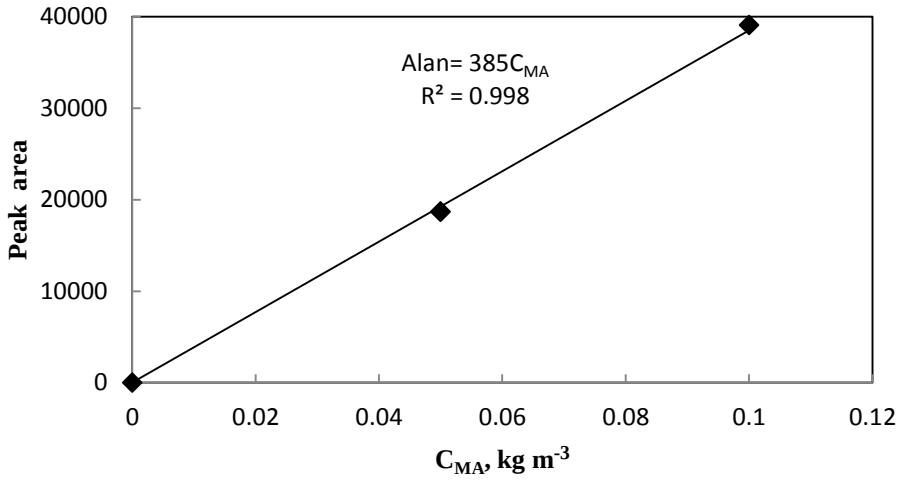

Figure D.5 Standard calibration curve for malic acid.

- **Oxalic Acid**

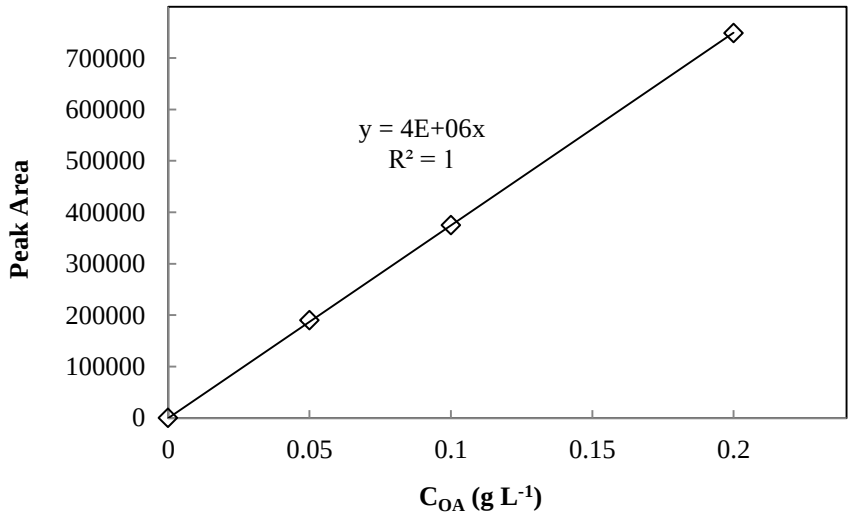

Figure D.6 Standard calibration curve for oxalic acid.