THE EFFECT OF OXYGEN TRANSFER CONDITIONS ON RECOMBINANT GLUCOSE ISOMERASE PRODUCTION BY PICHIA PASTORIS UNDER GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE PROMOTER

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ABSTRACT

THE EFFECT OF OXYGEN TRANSFER CONDITIONS ON RECOMBINANT GLUCOSE ISOMERASE PRODUCTION BY Pichia pastoris UNDER GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE PROMOTER

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The aim of this study is to investigate effects of oxygen transfer conditions on recombinant glucose isomerase (r-GI) production by Pichia pastoris under glyceraldehyde-3-phosphate dehydrogenase promoter (P\textsubscript{GAP}). Two different sets of operation strategies were investigated in terms of oxygen transfer conditions. In the first one, aeration rate was kept constant at $Q\textsubscript{O}\textsubscript{2}/V = 3\text{ vvm, 6 vvm, and 10 vvm}$ while agitation rate was kept at $N = 900\text{ rpm}$; and in the second one, dissolved oxygen concentration was kept constant at $C\textsubscript{DO} = 5\%, 10\%, 15\%, 20\%\text{ and 40\% air saturation throughout the bioprocesses. In the strategies where oxygen supplementation was relatively high, }Q\textsubscript{O}\textsubscript{2}/V = 6\text{ vvm and 10 vvm, excessive abundance of oxygen at the earlier hours of the bioprocesses limited cell growth and GI activity. Regardless of the oxygen transfer conditions, the cell concentration and glucose isomerase activity profiles showed similar trends in each strategy with
different highest values. The highest cell concentration was achieved when dissolved oxygen concentration was constant at 20% air saturation as 44 g L\(^{-1}\) at t = 9 h, while the highest recombinant GI activity was achieved when dissolved oxygen concentration was kept constant at 15% saturation as 4440 U L\(^{-1}\) at t = 15 h. The highest overall cell yield on substrate was obtained when the dissolved oxygen concentration was kept at 20% air saturation as 0.48 g cell g\(^{-1}\) substrate, which is the theoretical yield used for the calculation of substrate feeding rate. Lower GI activities were obtained by \(P_{\text{GAP}}\) compared to alcohol oxidase 1 promoter (\(P_{\text{AOX1}}\)); however, decrease in fermentation time by more than 3-fold and elimination of methanol usage make \(P_{\text{GAP}}\) a favorable alternative to \(P_{\text{AOX1}}\)-driven expression systems.

**Keywords:** Glucose isomerase, *Pichia pastoris*, oxygen transfer conditions, GAP promoter.
ÖZ

OKSİJEN AKTARIM KOŞULLARININ *Pichia pastoris* İLE GLİSERALDEHİT-3-FOSFAT DEHİROJENAZ PROMOTÖRÜ ALTINDA REKOMBİNANT GLUKOZ İZOMERAZ ÜRETİMİNE ETKİSİ

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Bu çalışmanın amacı oksijen aktarım koşullarının *Pichia pastoris* ile gliseraldehit-3-fosfat dehidrojenaz promotörü (*P*GAP) altında rekombinant glukoz isomeraz (r-GI) üretimine etkilerinin araştırılmasıdır. Oksijen aktarım koşullarının r-GI üretimine etkileri iki deney seti ile incelenmiştir. Birinci deney setinde N = 900 min⁻¹ kararlıtma hızında Qₒ/V = 3 vvm, 6 vvm ve 10 vvm hava giriş hızlarında; ikinci sette Cₒₒ = %5, %10, %15, %20 ve %40 hava doyunluk değerindeki oksijen derişimlerinde araştırılmıştır. Tasarlanan stratejiler arasında yüksek oksijen aktarım hızlarında, Qₒ/V = 6 vvm ve 10 vvm, ortamdaki yüksek oksijen derişimleri hücre çoğalmasını ve r-GI üretimini inhibe etmiştir. Oksijen aktarım koşullarından bağımsız olarak, bütün stratejilerde hücre çoğalma ve r-GI üretim profilleri benzer trendler göstermiş fakat ulaşılan en yüksek değerler farklı olmuştur. En yüksek hücre derişimi çözünmüş oksijen derişimi %20 hava doygunluğunda 44 g L⁻¹ olarak
t = 9 st’te; en yüksek GI aktivitesi %15 hava doygunluğunda 4440 U L⁻¹ olarak t = 15 st’te elde edilmiştir. En yüksek toplam substrat tüketimi üzerinden hücre verimi 0.48 g hücre g⁻¹ glukoz olarak %20 hava doygunluk değerinde elde edilmiştir ve bu değer substrat besleme hızı hesaplamak için kullanılan teorik değere eşittir. Alkol oksidaz 1 promotörü (Pₐₒₓ₁) ile karşılaştırıldığında P₉₈ ile yapılan deneyler sonucunda daha düşük GI aktivitesi elde edilmiş olmasına rağmen fermantasyon süresinin en az üç kat kısalması ve metanol kullanımına gerek olmaması, P₉₈’i Pₐₒₓ₁ yanında önemli bir alternatif haline getirmektedir.

Anahtar Kelimeler: Glukoz izomeraz, Pichia pastoris, oksijen aktarım koşulları, GAP promotör
To my family
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H. Güneş
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<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Unit</th>
</tr>
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<tbody>
<tr>
<td>a</td>
<td>Area</td>
<td>m²</td>
</tr>
<tr>
<td>C</td>
<td>Concentration or Off-bottom clearance of the tank</td>
<td>g L⁻¹, mol L⁻¹ or m</td>
</tr>
<tr>
<td>D</td>
<td>Impeller diameter</td>
<td>m</td>
</tr>
<tr>
<td>Da</td>
<td>Damköhler number</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>Mass flow rate</td>
<td>g h⁻¹</td>
</tr>
<tr>
<td>h</td>
<td>Impeller height</td>
<td>m</td>
</tr>
<tr>
<td>H</td>
<td>Average height of reactor working volume</td>
<td>m</td>
</tr>
<tr>
<td>j</td>
<td>Molar flux</td>
<td>mol m⁻² s⁻¹</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Overall liquid phase mass transfer coefficient</td>
<td>s⁻¹</td>
</tr>
<tr>
<td>N</td>
<td>Agitation rate</td>
<td>rpm</td>
</tr>
<tr>
<td>OUR</td>
<td>Oxygen uptake rate</td>
<td>mol m⁻³ s⁻¹</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxygen transfer rate</td>
<td>mol m⁻³ s⁻¹</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>Partial pressure</td>
<td>atm</td>
</tr>
<tr>
<td>q</td>
<td>Specific formation/consumption rate</td>
<td>mol g⁻¹ h⁻¹</td>
</tr>
<tr>
<td>Q</td>
<td>Volumetric flow rate</td>
<td>m³ h⁻¹</td>
</tr>
<tr>
<td>r</td>
<td>Formation/consumption rate</td>
<td>mol h⁻¹</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
<td>h</td>
</tr>
<tr>
<td>T</td>
<td>Tank diameter or temperature</td>
<td>m or °C</td>
</tr>
<tr>
<td>V</td>
<td>Volume</td>
<td>m³</td>
</tr>
<tr>
<td>Y</td>
<td>Yield</td>
<td>g g⁻¹</td>
</tr>
</tbody>
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Greek Letters

$\eta$  Effectiveness factor (OUR/OD)  
$\mu$  Specific growth rate $h^{-1}$  
$\mu_0$  Pre-determined specific growth rate $h^{-1}$  
$\lambda$  Wavelength nm

Superscripts and Subscripts

*  Value at equilibrium  
0  Initial condition  
AOX  Alcohol oxidase  
DO  Dissolved oxygen  
G  Gas side or glucose  
GAP  Glyceraldehyde-3-phosphate dehydrogenase  
Gly  Glycerol  
i  Interface  
L  Liquid side  
O  Oxygen  
P  Product  
S  Substrate  
X  Cell

Abbreviations

DF  Dilution factor  
FDA  Food and Drug Administration  
GI  Glucose isomerase  
GRAS  Generally recognized/ regarded as safe  
HPLC  High performance liquid chromatography  
OTC  Oxygen transfer condition
P       Promoter
PPP     Pentose phosphate pathway
r       Recombinant
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA     Tricarboxylic Acid
CHAPTER 1

INTRODUCTION

Industrial biotechnology uses microorganisms and enzymes for the production of biomolecules and has a wide range of applications that can be divided into seven subgroups; pharmaceutical biotechnology industry (production of vitamins, vaccines, antibiotics and therapeutic proteins), basic biochemical industry (production of organic acids, amino acids and alcohols), enzyme production industry (production of industrial and restriction enzymes), classical biotechnology industry (yeast production), bioenergy and biofuel industry (production of biogases; ethanol, butanol and biodiesels), agricultural biotechnology and waste treatment by biotechnological processes. As industrial biotechnology uses renewable materials and innovative methodologies to reduce greenhouse gas emission, it is accepted to be a promising approach for lowering the impacts of climate change resulting from chemical engineering industries and related sectors. Along with environmental benefits, it can enhance the performance of industries and product value as it focuses on increasing the efficiency of the processes. Producing biomolecules in organisms other than their native source is now enabled with the developments in recombinant DNA technology. It is done by cloning the gene responsible for the expression of desired biomolecule into the genome of a different organism, known as the host organism. These host organisms are chosen for their ability of overexpression, harmlessness to environment, or for reducing production costs. By being able to choose the optimum host organism, one can increase product value, lower production cost and decrease the environmental pollution caused by the process. As this technology matures,
industrial biotechnology will be able to propose more innovative solutions to climatic and economic problems.

D-glucose isomerase (GI, E.C. 5.3.1.5), also known as D-xylose isomerase or D-xylose ketol-isomerase, is an enzyme catalyzing the reversible isomerization reaction of D-glucose to D-fructose and D-xylose to D-xylulose. Conversion of D-glucose to D-fructose is used in the production of high fructose corn syrup (HFCS), while isomerization of D-xylose to D-xylulose is an important step for the conversion of xylan containing biomass into ethanol. Production of monosaccharides by GI is becoming more popular due to its potential health and medical benefits (Jokela et al., 2002).

According to the report “Industrial Enzymes Market” by Markets and Markets (2014), the global demand for industrial enzyme production would reach $ 6 billion by 2018. According to Turkish Chamber of Food Engineers (2011), HFCS is produced 12.5 million tons since 2006 in the world, and 400 thousand tons are produced since 2010 in Turkey. The world market of HFCS is expected to reach over 153 million tons.

Industrial protein production for commercial purposes is done with the help of genetic and metabolic engineering. Proteins can be expressed in various cell cultures such as mammals, plants, insects, bacteria, molds and yeasts. Large proteins are usually expressed in eukaryotic systems while prokaryotic systems are used for smaller ones. Choosing the right expression system is the most important step for r-protein production. As reported by Sanchez and Demain (2012), 45% of recombinant proteins produced in the USA and Europe come from mammalian cells, 40% from bacteria, whose 39% is from Escherichia coli, and 15% come from yeasts. Although bacterium E. coli is a popular host microorganism, yeast expression systems are gaining more attention due to their ability to do post-translational modifications and secretion which makes post-fermentation purification and modifications easier and also decreases cost (Liu et al, 2012). The most commonly used yeasts for industrial
protein production are *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Pichia pastoris* and *Hansenula polymorpha* (Schmidt, 2004).

Commercial GI market is dominated by *Actinoplanes missouriensis*, *Bacillus coagulans* or *Streptomyces* species which are generally not thermostable. Therefore, commercial isomerization reaction cannot be carried out at temperatures above 60-65°C (Wang *et al.* 2011). Isolating thermostable xylA gene encoding GI from thermostable bacteria such as *Thermotoga neapolitana*, *Thermus thermophilus* or *Thermus flavus* enables the production of thermostable recombinant GI which results in higher enzymatic activity and thermostability (Wang *et al.* 2011).

For the production of thermostable GI, *E. coli* and *Bacillus* species are the most popular expression hosts. Lee *et al.* (1990) cloned thermostable *Clostridium thermosulfurogenes* GI gene into *E. coli* and *B. subtilis* and compared their expression levels. Both expression levels were higher than that in the native source. The highest GI activity was reported to be reached as 1.54 U mg\(^{-1}\) protein in *B. subtilis* and 0.46 U mg\(^{-1}\) protein in *E. coli*. In another study, Dekker *et al.* (1991) cloned thermostable *T. thermophilus* GI gene into *E. coli*. 450 U L\(^{-1}\) GI yield was achieved by the expression in *E. coli*, which is 45-fold higher GI yield compared to the expression in *T. thermophilus*. Thermostable *T. thermophilus* GI encoding xylA gene was cloned to *E. coli* and *B. brevis* by the same research group in another study (Dekker *et al.* 1992). *B. brevis* yielded more than 1 g L\(^{-1}\) thermostable GI and reported to be more productive than *E. coli* in terms of GI production. Park *et al.* (1997) cloned xylA gene from *T. flavus* into *E. coli* and produced thermostable GI whose optimum temperature is 90°C and reached 55% yield from the isomerization reaction of glucose. In a more recent study, Angardi and Çalık (2013) expressed thermostable GI from *T. thermophilus* in *E. coli*. The highest GI activity was obtained as 16400 U L\(^{-1}\). Afterwards, Akdağ and Çalık (2014) achieved 35260 U L\(^{-1}\) thermostable GI activity from *T. thermophilus* by using untreated beet molasses as the substrate for expression in *E. coli*. 
xylA gene from *T. thermophilus* was successfully cloned and expressed in *S. cerevisiae* for ethanolic fermentation of xylose. The highest specific activity was reached as 1 U mg\(^{-1}\) (Walfridsson et al. 1996). Kuyper *et al.* (2005), isolated xylA gene from the fungus *Piromyces* sp. and cloned to *S. cerevisiae* for anaerobic xylose fermentation. In this study, GI activity or concentration was not determined but specific xylose consumption rate was determined as 1.1 g xylose g\(^{-1}\) biomass h\(^{-1}\) which was reported as the highest specific xylose consumption rate among all published data on metabolically engineered *S. cerevisiae* strains. de Figueiredo Vilela *et al.* (2013) expressed *Burkholderia cenocepacia* GI in *S. cerevisiae* for ethanol production. xylA gene insertion increased xylose consumption by 5-fold, and ethanol production more than 1.5-fold.

*P. pastoris* is a methylotrophic yeast reported to be suitable for heterologous protein expression system (Cos *et al.* 2006). Its ability to reach high cell densities on cheap, minimal media, to perform post-translational modifications like glycosylation, disulfide bond formation and proteolytic activity and secrete proteins to extracellular medium have made *P. pastoris* expression systems desirable for recombinant protein production (Cereghino and Cregg, 2000). In their review, Cos *et al.* (2006) reported several different inducible and constitutive promoters of *P. pastoris* which have successfully been used for heterologous protein expression. The most widely studied promoter of *P. pastoris* is alcohol oxidase 1 promoter (P\(_{\text{AOX1}}\)), which is a tightly regulated, inducible promoter. It is fully repressed in the presence of glycerol and derepressed in the presence of methanol. Therefore, in expression systems of *P. pastoris* under P\(_{\text{AOX1}}\), methanol is not only used as the carbon source but also as the inducer of the promoter expressing the target gene (Cereghino and Cregg, 2000; Ata *et al.* 2015; Güneş *et al.* 2015). However, usage of methanol as the carbon source for the fermentation raises environmental and health concerns because of handling issues and toxic nature of methanol and the by-products of fermentation such as formaldehyde and hydrogen peroxide, especially when the targeted protein is going to be used in food or drug related industries. Because of these problematic issues,
using an alternative expression system without the need for methanol usage has gained attention in recent years (Çalık et al. 2015). As a result, glyceraldehyde-3-phosphate dehydrogenase promoter $P_{GAP}$ has become a popular choice for constitutive, methanol-free recombinant protein expressions in $P. pastoris$ (Zhang et al. 2009). Because of the constitutive nature of the $P_{GAP}$, protein expression is coupled with cell growth. This coupling eliminates the need for a several-phased fermentation process, as needed in inducible expressions since a certain cell growth should be achieved to start the induction of the promoter for protein expression (Çalık et al. 2015). In several studies, depending on the fermentation mode and the protein expressed, $P_{GAP}$ is reported as a strong constitutive promoter with comparable expression levels with the ones done under $P_{AOX1}$ (Cos et al. 2005; Zhang et al. 2009; Çalık et al. 2015).

$xyl$A gene from $T. thermophilus$ was firstly cloned into pPICZα-A expression vector in our research group by Ata (2012). Then, pPICZα-A::$xyl$A vector was cloned into $AOX1$ locus of $P. pastoris$ for inducible expression of GI under $AOX1$ promoter. The highest GI activity was reported as 203 U L$^{-1}$ for extracellular production. Afterwards, the effect of codon optimization and different feeding strategies on inducible thermostable GI expression were investigated and reported in Ata et al. (2015). The highest GI activity was determined as 32500 U L$^{-1}$ by keeping methanol concentration in the medium constant at 5 g L$^{-1}$.

In this study, the objective is to investigate the effects of oxygen transfer conditions on recombinant GI production by $P. pastoris$ under $P_{GAP}$ using glucose as the carbon source. In the first three experiments, aeration rate was kept constant at $Q_{0}/V = 3$ vvm, 6 vvm and 10 vvm, while agitation rate was $N = 900$ rpm, and in the other experiments dissolved oxygen concentration in the bioreaction medium was kept constant at 5%, 10%, 15%, 20% and 40%, by using a cascade system to supply air and oxygen enriched air when necessary while agitation rate was $N = 900$ rpm. Fermentation data for each strategy were analyzed in terms of cell concentration, glucose concentration, GI activity, GI monomer concentration, acidic protease
activity and organic acid concentration. Additionally, yield coefficients, the specific substrate consumption rates and oxygen transfer characteristics were calculated in order to understand fermentation characteristics more in-depth and to discuss the response of *P. pastoris* to different fermentation strategies.
CHAPTER 2

LITERATURE SURVEY

Structure and characteristics of the protein, selection of the host microorganism and its promoter, optimum bioprocess operation modes and parameters should be investigated to design an efficient recombinant protein production machinery. In this context, this chapter covers the literature survey done on the recombinant protein to be produced, glucose isomerase, and the host microorganism, *Pichia pastoris*, along with the inspection of bioprocess operation parameters and modes; and their effects on heterologous protein expression by *P. pastoris*.

2.1. Enzymes

The biological catalysts, enzymes, increase the rate of biochemical reactions by interacting with the reactants and products to provide a more thermodynamically favorable pathway for transformation of reactants to products. To visualize, the rate of enzymatically catalyzed reactions usually $10^6$-$10^{12}$ times more than that of the uncatalyzed reactions and several orders of magnitude more than the corresponding ones that are chemically catalyzed (Voet *et al.* 2013).

Enzymes are divided into six subgroups according to the type of the reaction they catalyze. These subgroups are oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase. Oxidoreductases catalyze oxidation/reduction reactions in which hydrogen and oxygen are gained or lost; transferases are responsible for the transfer of functional groups such as amino acid and phosphate groups; hydrolases catalyze hydrolysis reactions; lyases remove groups of atoms without hydrolysis;
isomerase catalyze isomerization reactions; and ligases are synthesis enzymes (Palmer et al. 2007).

Enzymes have three-dimensional substrate-specific structures. These three-dimensional structures have four types; primary, secondary, tertiary and quaternary structures. When amino acids have a linear sequence, then the structure is called primary structure; when the repeating structure has alpha helices and beta strands which are stabilized by hydrogen bonds, the structure is known as secondary structure; when the structure of the protein is determined by salt bridges, hydrogen bonds, hydrophobic interactions, disulfide bonds and post-translational modifications, the structure is tertiary structure, and when the structure results from the interactions of several proteins or polypeptides, known as subunits, and is determined by noncovalent interactions and disulfide bonds, the structure is known as quaternary structure. If the protein consists of two subunits, it is called as dimer; if there are three subunits, the protein is trimer; and if the protein is composed of four subunits, it is called as tetramer. If the subunits are the same polypeptides or proteins, then the multimers get the prefix homo-, if these subunits are different from one another, then they have the prefix hetero- (Raven et al, 2013).

2.2. Product: Thermostable Glucose Isomerase

Glucose isomerase (GI, E.C. 5.3.1.5), also known as D-xylose isomerase or D-xylose ketol-isomerase is an isomerization enzyme which catalyzes the reversible reaction of D-glucose to D-fructose and D-xylose to D-xylulose (Figure 1). The former reaction is important for high fructose corn syrup (HFCS) production while the latter is an essential step of ethanol production from biomass containing xylan.

Marshall and Kooi discovered an enzyme in Pseudomonas hydrophila that is capable of isomerization of glucose into fructose in 1957, and the microorganisms carrying glucose isomerase producing strains gained attention for their potential to be used in HFCS production (Bhosale et al. 1996). As the research on GI widened, it is seen that it is widely distributed in prokaryotes; especially bacteria and actinomycetes.
have high capacity to produce GI. While several *Bacillus* species are good producers of GI, only fungus that is known to be capable of GI production is *Aspergillus oryzae* (Bhosale *et al.* 1996). In addition, GI production in the yeasts *Candida utilis* (Wang *et al.* 1980) and *Candida boidinii* (Vongauvanlert and Tani, 1988) has been reported. Some commercial GI producers are given in Table 1.

![Diagram of isomerization reaction catalyzed by GI.](image)

**Figure 1.** Isomerization reaction catalyzed by GI. (1) Ring opening, (2) isomerization, (3) ring closure (Kovalevsky *et al.* 2010).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Trade Name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinoplanes missouriensis</em></td>
<td>Maxazyme</td>
<td>Gist Brocades and Anheuser-Busch Inc.</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>Sweetzyme</td>
<td>Novo-Nordisk</td>
</tr>
<tr>
<td><em>Streptomyces rubiginosus</em></td>
<td>Optisweet</td>
<td>Miles Kali-Chemie</td>
</tr>
</tbody>
</table>

**Table 1.** Commercially important GI producers (Bhosale *et al.* 1996)

2.2.1. Structure and Characteristics of Glucose Isomerase

GI from *T. thermophilus* is a homotetramer with identical, four subunits in its active form. Identical subunits are connected to each other with noncovalent bonds. Depending on the source microorganism, molecular weight of GI can vary between 44 and 191 kDa. Subunits of GI from *T. thermophilus* are composed of 387 amino acids and have a molecular weight of 44 kDa (Dekker *et al.*, 1991) (Table 2).
Chang et al. (1999) determined the crystal structure of thermostable GI from *T. thermophilus* and reported that the tertiary fold of the subunits are similar to glucose isomerases from other source organisms. The monomers are composed of two domains. Domain I, residues from 1 to 321, folds into \((\beta/\alpha)_8\)-barrel, while Domain II, residues from 321 to 387, contacts with Domain I of the adjacent subunit. Each monomer of GI has ten \(\beta\)-strands, sixteen \(\alpha\)-helices, and five 310-helices (Figure 2).

To understand the structural factors affecting thermostability of GI from *T. thermophilus*, they compared the structure with two other GIs from *Arthrobacter B3728* and *Actinoplanes missouriensis* whose optimum reaction temperatures are 80°C and 75°C, respectively. Increase in ion pairs and ion pair networks, decrease in large inter-subunit cavities, removal of potential deamidation/isoaspartate formation sides and shortened loops have been speculated to increase thermostability of GI from *Thermus thermophilus* compared to two other GIs from *Arthrobacter B3728* and *Actinoplanes missouriensis*.

Depending on the host microorganism, optimum temperature for thermostable GI ranges between 60°C and 90°C. Enzymes from thermophilic hosts are reported to be more stable at high temperatures compared to those from mesophilic sources (Bhosale et al., 1996). Hlima et al. (2012) showed that the optimum temperature for GI from *Streptomyces* sp. SK is 80°C; while optimum temperature of GI from *Actinoplanes missouriensis* was reported to be 60°C (Lambeir et al. 1992). Using GI from thermophilic hosts are more desirable since increasing temperature is important because of its positive effect on the equilibrium concentration of fructose, and a higher reaction rate (Hlima et al. 2012).

Like temperature, optimum pH for GI activity depends on the host organism while it typically ranges between 6.5 and 8.5. Hlima et al. (2012) reported optimum pH for GI from *Streptomyces* sp. SK to be 6.5 and pH of the isomerization reaction with GI from *T. thermophilus* was reported as 7.0 by Dekker et al. (1991); also, Mishra and Debnath (2002) found the optimum pH to vary between 7.0 and 8.5 depending on the buffer solution used.
Table 2a. Sequence of GI from *T. thermophilus* (National Center for Biotechnology Information GenBank, Accession Number: D90256.1).

```
GTGTACGAGCCAAACCAGGAGCACAGGTTTACCTTTTGCCCTTTGGACTGTTGGCAATGTG  60
GGCCGTGATCCCTTCGGGAGCGCCGGTTTTACGTGTTGGTTCAT  120
AAGCTGGCGAGCTGGGACCAGACATGAGGGCTCTTCAAGAAGCTCCTGATGAA  180
GGGCTTCACAGCGCCGGACCCTTTGGTTGGCCCTATGCCCTGGAGAGAGCTGGAG  240
ACCATGGACCTGGGCGACAGCTTGGGGGCCGAGATCTACGTGCTGTGTGGCGCGAGG  300
GGAGCTGAGTTGAGGAGCCGCCAGGGCAAGGCGCCGAAGTCTGGGACTGGGTGCGGGAGGCG  360
CTGAACTTCATGCCGCTACCGCAGAAGGATACCGTTACGTTGGGTCCCCCTGCCAGAGCTGGCCGGGCTTAACTGGTTCCACGGCTGGCAGGCTCTCGACGCCGGGAAGCTT  480
TCGGAGAACCCTCAAGGCGCCCTTTTTCTGGTTGACCTCTCGGAAGCTCCGCTACCGAG  540
GGCCCCGGCCACTTTTGACGGCCCAACGCGCTCGTACCAGAGGAGAAGGGGTTTGGCCCGCTCCAGTAG  600
TTGGGGTGCCCTACTGCCGAGAAGGCCCGGGCCCTTGCCCTGGCGGTGGAGGCTTCCCCCTGCCGTACCGAGGACGAAGAAGGGGTTTGGCCGGCTGCATGCGTACCTACCTGATCTTAAAGGAAAGGGCTGAAGCCTTCCGC  660
GAGGATCCCCTTACGCCGAGGACCAGGGATACGGGTACCGGTTTGCCCTCGAG  720
CCCAAGCCTACCGCAGCCGGCAAGCCGGACATTTACTTCGCCACCCTGGGAGCCATGCTCCGCC  780
TTCGAGAGGCTGATCGCTACTCTACCTGTCTCTGGAAGGCTGGCGGTGGAGACCTCCTGGGGGTGGCGGGGG  840
GCCCTTTGGGCGCCCTACTCCGGAGAAGGCGGCGGAGCCTCAAGCGGCGGAGCTTG  900
GAGGATCCCAGGCTAGGTGCTCTGGTTCTGACTATCAAGAAGATCTGCGGCCTTG  960
GCCCTTTGGGCGCGAGAAGGCGGCGGAGCCTCAAGCGGCGGAGCTTG  1020
CTCGAGGAGCGGCCGCTGGCTGGGTATTATGCCCTGGGACAGCCTTGCCGAGTGGGCGAGTGGGAGGTGGAGAGGATCCCCTTACGCCGAGGACCAGGGATACGGGTACCGGTTTGCCCTCGAG  1140
TACCTCCTGGGTTGCGGGG  1164
```
Table 2b. Codon-optimized sequence of GI from *T. thermophilus*. Altered codons are in black. Altered Kex2 cleavage sites are highlighted in yellow (Ata et al. 2015; National Center for Biotechnology Information GenBank Accession Number: AB981659).

| Codon-optimized sequence of GI from T. thermophilus. Altered codons are in black. Altered Kex2 cleavage sites are highlighted in yellow (Ata et al. 2015; National Center for Biotechnology Information GenBank Accession Number: AB981659). |
|---|---|
| ATG | TACGAGCCAACCAGAGCACAGA | ATTTACCTTTGGGTCTTGGACTGTGAGTATGTG | 60 |
| GTGAGA | GATCCAATCCGGAGCTGTGAAGAGAGA | AGTGGACCCAGTGGTACGGTCTTGGATGTTCAT | 120 |
| AAGCTG | GCTGAGCCTTGGTGCCCCTAGCTGGTTA | AACTCCACGAGGACAGCTGAGAAGAGGCTTTGAGTGAATGAGA | 180 |
| GTACCTC | TCCATCAGGAAGAGACACCAGATCGTGAGAAGAAGATTA | AGTCAAGAAGGCTTTGAGTGAATGAGA | 240 |
| GGTGCC | CTACCTCTCAGGAGAGAACCAGATCGTGAGAAGAAGATTA | AGTCAAGAAGGCTTTGAGTGAATGAGA | 300 |
| ACCATGGC | ACCCTGGTGAGCAGAGCTTGGTGCCCCTAGCTGGTTA | AACTCCACGAGGACAGCTGAGAAGAGGCTTTGAGTGAATGAGA | 360 |
| GGAGCTGAGGTGAAGG | ACTGGTAAAGGGCAGAGGCCTGGTACGGTCTTGGATGTTCAT | AACTCCACGAGGACAGCTGAGAAGAGGCTTTGAGTGAATGAGA | 420 |
| CTGAACCTC | ATGCCTGGCCGCTACGCCGAGACCGAGATAGGTATGGAGTTG | CTAAGAAGCAGCAGTGGTCACCTGCTTTCAAGGAC | 480 |
| CCAAGC | CCTTAACTCAGGAGAGAACCAGATCGTGAGAAGAAGATTA | AGTCAAGAAGGCTTTGAGTGAATGAGA | 540 |
| TTTATCTACCTGGGAAGAGCACACCAGATCGTGAGAAGAAGATTA | AGTCAAGAAGGCTTTGAGTGAATGAGA | 600 |
| ACCATGGC | ACCCTGGTGAGCAGAGCTTGGTGCCCCTAGCTGGTTA | AACTCCACGAGGACAGCTGAGAAGAGGCTTTGAGTGAATGAGA | 660 |
| GTGCC | CTACCTCTCAGGAGAGAACCAGATCGTGAGAAGAAGATTA | AGTCAAGAAGGCTTTGAGTGAATGAGA | 720 |
| ACCATGGC | ACCCTGGTGAGCAGAGCTTGGTGCCCCTAGCTGGTTA | AACTCCACGAGGACAGCTGAGAAGAGGCTTTGAGTGAATGAGA | 780 |
| TCTGAGAAC | TTAGAGGAGCT | CTTTTCTTCTTGAGGACTTGGTGAATTCTCCAGTCT | 840 |
| GGTCCAAGA | CACTTGACGCCCAAGCCCTAGAACCAGAGGAGAGGAGAGGAG | AGTGGTCTTGGACCAGGCTTTGAGTGAATGAGA | 900 |
| TCCGCG | AGAGGTGCATCAGGAGAGAACCAGAGGAGAGGAGAGGAG | AGTGGTCTTGGACCAGGCTTTGAGTGAATGAGA | 960 |
| GAGGATCCA | GAGGTCACCCAGGGCTTGGTGCCCCTAGCTGGTTA | AACTCCACGAGGACAGCTGAGAAGAGGCTTTGAGTGAATGAGA | 1020 |
| GCTCTTTCG | GTTTTTCTGAGGAGAGAACCAGATCGTGAGAAGAAGATTA | AGTCAAGAAGGCTTTGAGTGAATGAGA | 1080 |
| TTAGGAGGCAAGAAGAAGAAGGTTATGGCCCTGGAA | AGAACCAGGAGAGGAGAGGAGAGGAG | 1140 |
| TACTTGCTGGGTGTTGAGG | | | 1164 |
Kovalevsky et al. (2010) investigated the metal ion roles on the isomerization reaction catalyzed by GI from *Streptomyces rubiginosus* by X-ray crystallography and neutron diffraction. Requirement for two divalent metal cations was reported to obtain a fully active GI. These metal binding sites were named as M1 and M2; where M1 is the structural metal sites for Mn$^{2+}$, Mg$^{2+}$, Pb$^{2+}$, Co$^{2+}$ and Cd$^{2+}$ ions and M2 is the catalytic metal site showing larger variety of affinity for divalent metal cations, which may or may not inhibit the enzyme (Figure 3). Bhosale et al. (1996) stated that

**Figure 2.** Crystal structure of glucose isomerase from *T. thermophilus*, determined by X-ray crystallography. Protein chains are colored from the N-terminal to C-terminal using a rainbow color gradient (RCSB Protein Data Bank, Accession Number: 1BXB).
ions with larger diameter than 0.8 $\AA$ inhibit the enzyme activity; i.e., Hg$^{2+}$, Ag$^+$, Cu$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$.

Figure 3. Metal binding sites of GI from *S. rubiginosus* (Kovalevsky *et al.* 2010). The residues coordinating metal cofactors, M1 and M2, are shown in magenta circles and lining cavity, Trp16, His54, Phe94, Trp137, and Phe26’ are shown.

Identification of the location of the amino acids is essential to improve the catalytic activity of GI. Batt *et al.* (1990) identified the essential histidine residues in the active site of GI from *E. coli*, which are His-101 and His-271. Afterwards, Meilleur *et al.* (2006) located hydrogen atoms and showed that the initiation of reaction is
done by double protonation of His-53 at the active site and the reaction continued with the opening of the sugar ring via acid catalysis. As shown in Figure 1, isomerization reaction of glucose to fructose was proposed to be in three steps: ring opening, isomerization and ring closure (Kovalevsky et al. 2010).

Complete reaction mechanism proposed by Kovalevsky et al. (2010) is given in Figure 4. Some conformational changes take place in the active site during the time course of the reaction. For the initiation and implementation of the reaction, some amino acids play essential roles. In the first step, active site of the enzyme and reactive sugar cycle bind together where different arrangements of water molecules help the substrate to correctly bind to GI active site. The ring opening takes place in the next step where His-53 gives a proton to O5 temporarily, and takes it back after C1-O5 bond breaks. Within this step, neutral Lys-289 gets protonated and gives a proton to Asp-257. Then the isomerization step begins with the correct placement of O1 by Lys-183. During isomerization, a proton in transferred to C1 and O1 from C2 and O2, respectively.

Effect of codon optimization on the glucose isomerase production by P. pastoris was investigated by Ata et al. (2015). About 30% of the codons were changed according to codon usage bias of P. pastoris and Arg356, Arg365, Arg366 and Arg367 were replaced with Lys to prevent the cleavage of GI by Kex2 protease. (Table 2b) and 2.4-fold higher GI activity was obtained. It was also noted that the codon optimization did not affect optimum pH or temperature of thermostable GI.

2.3. Selection of the Host Microorganism

Selection of the host microorganism is a crucial step for recombinant protein production. Depending on the targeted protein; several parameters should be considered when selecting the host microorganism such as high productivity, accessibility, limited production of by-products, being GRAS (generally recognized as safe), ability to do overexpression; post-translational modifications; intra- or extracellular production; and to grow in moderate conditions (Kirk and Othmer,
Generally microorganisms are preferred for industrial protein production over plant and animal cell lines due to their ease of cultivation and fast rate of multiplication.

For recombinant GI production, *E. coli* and *Bacillus* species are the most widely-used expression systems (Bhosale *et al.* 1996). Other microorganisms used for GI production are as follows: *Schizosaccharomyces pombe* (Chan *et al.* 1986), *Streptomyces lividans* (Tan *et al.* 1990), *S. cerevisiae* (Moes *et al.* 1996; Walfridsson *et al.* 1996), and *P. pastoris* (Ata *et al.* 2015).

Yeast are eukaryotic cells that can reach high cell densities in cheap, defined media, do post-translational modifications such as glycosylation, and disulfide bond formation and they can do extracellular protein expression. Because of these advantages, yeasts are suitable hosts for heterologous protein expression (Cos *et al.* 2006).

### 2.4. The Host Microorganism: *Pichia pastoris*

*P. pastoris* is a facultative anaerobe methylotrophic yeast, widely used in biochemical and genetic research as well as industrial biotechnology (Kurtzmann, 2009). It is a eukaryote with oval-shaped cells which are 1 – 5 μm wide and 5 – 30 μm long. It lives in moderate temperatures (Macauley-Patrick *et al.* 2005) and has a broad pH range between 3 – 7 (Cereghino and Cregg, 2000). Cos *et al.* (2006) reported that over 600 proteins have been expressed in *P. pastoris* up today.

In 1970s, *P. pastoris* was firstly introduced by Philips Petroleum for the production of single cell proteins (SCP) as animal feed additive due to its ability to reach high cell densities in the presence of methanol as the only carbon source. They developed the first protocols for growth and media composition. But the oil crisis in 1973 increased methanol prices and made this process unfeasible (Ahmad *et al.* 2014).

Afterwards, Cregg *et al.* (1985) developed *P. pastoris* expression system for heterologous protein expression under strong and tightly regulated inducible alcohol oxidase 1 promoter, P_{AOX1}.
Figure 4. Complete reaction mechanism, as proposed by Kovalevsky et al. (2010).
From that day, *P. pastoris* expression system has been used for several different recombinant protein productions. So far, one of the most important breakthroughs in terms of *P. pastoris* being important for pharmaceutical industries is the FDA (Food and Drug Administration) approval of the recombinant pharmaceutical kallikrein inhibitor, Kalbitor® (Ahmad *et al.* 2014; Thomson, 2010).

*P. pastoris* has many advantages over other microorganisms. Due to its ability to perform post-translational modifications that higher eukaryotes perform, *P. pastoris* can be used to express proteins as biologically active molecules that would become inactive inclusion bodies in bacterial systems. It provides rapid growth in defined media with few endogenous protein secretion, has a good protein secretion capacity, and it is cheaper than using higher eukaryotes. Due to its physiological similarity to *S. cerevisiae*, which is the most widely-used yeast for protein expression, its metabolic manipulation and expression protocols are simple and readily available (Cereghino *et al.* 2002; Jahic *et al.* 2006).

However, there are some disadvantages of *P. pastoris* expression systems such as high proteolytic activity and non-native glycosylation. There are also some additional disadvantages resulting from using $P_{AOX1}$ and $P_{AOX2}$ promoters and methanol usage as the carbon source. Its usage for food and pharmaceutical protein production is controversial because of flammable and toxic properties of methanol; also handling and transportation of large amounts of methanol is expensive and troublesome (Çalık *et al.* 2015).

### 2.4.1. Promoters of *Pichia pastoris*

Promoters are the regions of DNA that starts the transcription of a particular gene. The first enzyme in the methanol assimilation pathway of *P. pastoris* is alcohol oxidase. There are two alcohol oxidase genes in *P. pastoris*, $P_{AOX1}$ and $P_{AOX2}$. $P_{AOX1}$ is responsible for 90% of the production of alcohol oxidase enzyme in the cell while the remaining 10% is produced from $P_{AOX2}$. They are both used for heterologous protein expressions by *P. pastoris*; $P_{AOX1}$ yielding higher expression levels than $P_{AOX2}$.
(Cos et al. 2006). \( P_{AOX1} \) and \( P_{AOX2} \) promoters are tightly regulated and inducible. They are induced in the presence of methanol and repressed in the presence of glycerol. Other than \( P_{AOX1} \) and \( P_{AOX2} \), there are other promoters that are induced with methanol, such as the promoters of dihydroxyacetone synthase (\( P_{DAS} \)) (Tschopp et al. 1987) and formaldehyde dehydrogenase (\( P_{FLD1} \)) (Shen et al. 1998). Other important inducible promoters of \( P. pastoris \) are of high affinity glucose transporter (\( P_{G1} \), induced with glucose limitation), isocitrate lyase (\( P_{ICL1} \), induced with ethanol) (Menendez et al. 2003); alcohol dehydrogenase (\( P_{ADH1} \), induced with glycerol and ethanol); enolase (\( P_{ENO1} \), induced with glycerol); and glycerol kinase (\( P_{GUT1} \), induced with glucose, glycerol and ethanol) (Cregg and Tolstorukov, 2012).

Contrary to inducible promoters; when protein expression is done with constitutive promoters, protein production is accomplished along with cell growth; therefore, the need for carbon source shift is eliminated. The most commonly used constitutive promoter of \( P. pastoris \) is glyceraldehyde-3-phosphate dehydrogenase promoter, \( P_{GAP} \). Some of novel constitutive promoters of \( P. pastoris \) are reported as: promoter of translation elongation factor 1 (\( P_{TEF1} \)), (Ahn et al. 2007); 3-phosphoglycerate kinase (\( P_{PGK1} \)), (de Almeida et al. 2005); and putative aldehyde dehydrogenase (\( P_{G6} \)). (Prielhofer et al. 2013). Although \( P_{TEF1} \), \( P_{G1} \), \( P_{DAS} \) and \( P_{FLD1} \) have been reported to reach expression levels similar to \( P_{GAP} \) and \( P_{AOX1} \); \( P_{AOX1} \) is still the most widely used promoter of \( P. pastoris \), followed by \( P_{GAP} \).

2.4.1.1. Promoter of Glyceraldehyde-3-phosphate Dehydrogenase: \( P_{GAP} \)

Because of the potential hazards of using methanol as the carbon source, researchers have been focusing on alternative promoters that would utilize safer carbon sources for environment and health. One of the promoters of \( P. pastoris \) by which methanol is not used as the carbon source is the promoter of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encoding gene. GAPDH is a homotetrameric protein with 333 amino acid residues, located in glycolysis pathway of \( P. pastoris \) metabolism (Figure 5).
Several heterologous proteins have been expressed at high levels under the promoter of GAPDH encoding gene, $P_{GAP}$, through fine-tuning bioreactor operation parameters, fermentation strategies and genetic manipulation. Glyceraldehyde-3-phosphate dehydrogenase gene was firstly isolated by Waterham et al. (1997) and its promoter was used for the expression of $\beta$-lactamase by using glucose as the carbon source. Higher expression levels were reached with $P_{GAP}$ than with $P_{AOX1}$. In this expression system, proteins are expressed along with cell growth as long as the targeted protein is not toxic to the cell since toxic proteins would inhibit cell proliferation (Çalık et al. 2015). Generally, the highest expression levels were reached when glucose is used as the carbon source. In some cases, glycerol can be used as the carbon source and comparable expression levels with glucose can be reached.

2.4.1.2. Comparison of glyceraldehyde-3-phosphate dehydrogenase and alcohol oxidase 1 promoters

Several research groups compared the performances of $P_{GAP}$- and $P_{AOX1}$-driven expression systems. Some authors reported that $P_{GAP}$ expression system is more efficient than $P_{AOX1}$; while others reported the opposite. As mentioned before, Waterham et al. (1997) compared $\beta$-lactamase expression levels of $P_{GAP}$ and $P_{AOX1}$ and reported that $P_{GAP}$ is a successful alternative to $P_{AOX1}$ as they attained 1.7-fold higher enzyme activity by $P_{GAP}$-driven expression.

Similarly, Menéndez et al. (2004) reported fructose-releasing exo-levanase (LsdB) expression level of $P.~pastoris$ with $P_{GAP}$ and $P_{AOX1}$ and obtained 26.6 U ml$^{-1}$ LsdB under $P_{GAP}$, corresponding to 1.3-fold higher LsdB than obtained under $P_{AOX1}$. Moreover, they achieved more than 3.1-fold higher productivity while the process time is shortened by almost 2.5-fold. Although similar productivities were obtained for the production of human chitinase; higher protein titers were reported by Goodrick et al. (2001). On the other hand; Boer et al. (2000) obtained 5-fold higher cellobiohydrolase
Figure 5. Glycolysis/Gluconeogenesis pathway of *P. pastoris*, GAPDH enzyme indicated with pink (GenomeNet Database, KEGG)
production with $P_{AOX1}$ than $P_{GAP}$. Similarly, Vassileva et al. (2001) and Sears et al. (1998) obtained 1.4- and 8-fold higher recombinant protein expressions under $P_{AOX1}$, respectively.

2.4.2. Central Carbon Metabolism of *Pichia pastoris*

Overexpression of heterologous proteins directly affects the metabolism of the cells. *P. pastoris* can produce energy by fermentation of sugars; *i.e.*, fructose and glucose, and sugar alcohols; *i.e.*, mannitol and sorbitol, and by oxidation of fermentation products; glycerol, ethanol and lactate. Being a methylotrrophic yeast, *P. pastoris* can utilize sources with only one carbon atom, such as methanol. Unlike *S. cerevisiae*, *P. pastoris* lacks invertase gene so it cannot metabolize sucrose (Çalık et al. 2015). Schematic representation of central carbon metabolism of *P. pastoris* is given in Figure 6.

There is a specific order for the uptake of the carbohydrates with intermittent lag phases as the genes responsible for transporters are tightly regulated by the glucose concentration in the medium. Limited glucose uptake rates prevent *P. pastoris* metabolism from overflow, resulting in lower cell yield and targeted product formation.

Although glucose and glycerol enter the carbon metabolism from glycolysis pathway, they have different regulations. In *S. cerevisiae*, glucose addition to the cultivation medium in which cells are growing on glycerol causes rapid changes in the pattern of protein phosphorylation and transcriptional state of the genome. Zaman et al. (2008) reported that more than 40% of the genes change their expressions by at least 2-fold minutes after the addition of glucose to the medium containing glycerol for cell growth. The comparative effect of glucose and glycerol has not been investigated yet in *P. pastoris*; however, its response can be expected to be similar to some extend with *S. cerevisiae*.

Heyland et al. (2011) investigated the effect of recombinant protein overexpression on the cells’ metabolism growing on glucose. TCA cycle is found to have the highest
response to recombinant protein overexpression since protein synthesis is an energy consuming process. Metabolic burden on the cell metabolism resulting from protein overexpression is compensated by decreasing the cell yield on ATP, specific cell growth rate, glucose consumption rate and byproduct formation rate. The limiting metabolic process is found to be the synthesis of energetically costly amino acids.

2.4.3. Post-Translational Modifications and Protein Secretion

One of the most important reasons for yeasts to be favorable host microorganisms is their ability to do some post-translational modifications such as correct protein folding, disulfide bond formation, glycosylation, and signal peptide development.

When heterologous proteins are overexpressed, they usually result in misfolded, inactive protein aggregates. Lack of disulfide bond formation and saturation of the cellular folding machinery can be the reasons for misfolded protein formation (Baneyx, 1999; Schlieker et al. 2002).

If a protein is misfolded, it causes stress to the cell by accumulating in the endoplasmic reticulum (ER). To reduce the stress, misfolded proteins are degraded by ER-associated degradation. When the stress is prevented, immature proteins are stabilized by the ER-resident chaperones and the help of disulfide isomerase and other proteins that facilitate protein folding to reduce stress and increase recombinant protein secretion (Idiris et al. 2010).

Some proteins require some hydrocarbons to be attached to certain amino acid groups for the correct folding, stability and solubility. This process is known as “glycosylation” and involves several enzymes to work concomitantly. There are two types of glycosylation (O- and N-linked) and P. pastoris is capable of doing both of them with some differences from mammalian cells. P. pastoris cells only add mannose residues while in mammalian cells oligosaccharides composed of galactose, N-acetylglactosamine and sialic acid can be added as well (Cereghino and Cregg, 2000).
Although the mode of production, extracellular or intracellular, mainly depends on the characteristics of the protein, extracellular production is usually more favorable because it reduces the cost of downstream processing. A signal sequence is necessary
in order to secrete proteins to the extracellular environment. Native signal sequences of proteins can be used as well as different signal sequences like alpha-mating factor pre-pro leader sequence (α-MF) (Macauley-Patrick et al. 2005).

In *P. pastoris*, secretion of the proteins with α-MF is conducted in three stages. First, protein is transported to ER where pre-domain is removed by signal peptidase, in the second stage pro-domain is removed in Golgi apparatus by dibasic endo-peptidase (*kex2*) activity. Lastly, the protein is packed to secretory vesicles and secreted to extracellular medium (Daly and Hearn, 2005).

### 2.4.4. Proteolytic Degradation

Degradation of the produced proteins by proteases is a major problem in *P. pastoris* expression system because it causes reduction in protein yield, protein activity, and contamination of the product by degradation intermediates (Idiris *et al.* 2010). Some reasons for high proteolytic activity are change in pH and temperature, carbon source shift, and foreign chemicals in the fermentation medium. Since *P. pastoris* can reach high cell concentrations, especially in fed-batch operations, accumulation of proteases in extracellular medium together with cell lysis are the main factors of proteolytic degradation in *P. pastoris* expression systems (Shen *et al.* 1998).

To prevent proteins from proteolytic degradation, adding casamino acids is known to be an effective method (Pal *et al.* 2006). Goodrick *et al.* (2001) speculates that in fed-batch processes, proteins produced are exposed to higher and higher concentration of proteolytic enzymes as fermentation time passes. Stabilization of the recombinant proteins and avoidance of proteolytic activity can be achieved by addition of casamino acids. Similarly, Pal *et al.* (2006) and Khasa *et al.* (2007) obtained higher yields of cell and recombinant protein concentration by the addition of casamino acids. However, development of protease-deficient strains to avoid proteolytic degradation is known to be the most effective strategy when changing the fermentation parameters fail to solve this problem.
2.5. Medium Design

Compared to inducible expression systems, where the inducer is also the carbon source in most cases, there are more variety of carbon sources in constitutive expression systems. Glucose, glycerol, methanol and oleic acid have been reported as the carbon sources for $P_{GAP}$-driven expression systems (Zhang et al. 2009); and fructose, sorbitol, mannitol and trehalose are also possible carbon sources as they can also be utilized by the carbon metabolism in glycolysis pathway (Čalık et al. 2015).

Waterham et al. (1997) compared glucose, glycerol, oleic acid and methanol as carbon sources and the maximum activity of $\beta$-lactamase was achieved in glucose; 73%, 45%, and 36% of activity with glucose was achieved with glycerol, oleic acid and methanol, respectively. Pal et al. (2006) compared the effect of glucose, glycerol and sorbitol for human granulocyte-macrophage colony-stimulating factor (hGM-CSF) production in shake-flasks. The results showed that maximum cell density was reached with glycerol but the highest recombinant protein concentration was achieved with glucose. Similarly, glucose yielded higher recombinant protein yield than glycerol in shake-flask experiments, as reported by Fei et al. (2009). On the other hand, Goodrick et al. (2001) reported higher recombinant human chitinase production with glycerol while cell concentration was higher with glucose in continuous expression. Then, Garcia-Ortega et al. (2013) used a strategy for human antigen-binding fragment (Fab) production such that glycerol was used as the carbon source for the batch phase, then the process was shifted to fed-batch with glucose as the carbon source. This strategy was reported as the most successful combination of the carbon sources for Fab production. By these results, it can be interpreted that there are several factors affecting the efficiency of the carbon source, such that the target protein, fermentation mode or the aim of fermentation; i.e., aiming high cell concentration or recombinant protein yields. Therefore, the choice of the carbon source should be done according to these parameters.

As mentioned earlier, casamino acids can be used both to increase product yield and prevent proteolytic degradation. As reported by Fei et al. (2009), instead of casamino
acids, addition of precursor amino acids to the fermentation medium can increase the targeted protein yield as well. In these cases, amino acid composition of the targeted protein should be considered since rate-limiting amino acid may differ for each protein production. Proteolytic degradation of the recombinant protein should be taken into consideration as well as the amino acid composition. Addition of amino acids to the fermentation medium facilitates the metabolic network of *P. pastoris* to increase cell concentration and recombinant protein production (Çalık *et al.* 2015).

### 2.6. Bioreactor Operation Conditions

After the designation of the expression system and feed medium components, bioreactor operation modes; *i.e.*, pH, temperature and oxygen transfer conditions should be investigated for optimum conditions.

#### 2.6.1. pH

As a rule of thumb, pH of the fermentation medium should not be close to the isoelectric point (pI) of the targeted protein in order to avoid precipitation. Therefore, pH value that the fermentation should be carried is dependent on the desired protein. Although *P. pastoris* is known to survive at a wide range of pH; values between 5.0 and 5.5 under pH-controlled conditions are reported to be optimum for recombinant protein expression and maintaining cell viability. In some cases, pH = 6.0 has also been reported to be the optimum such as methionine adenosyltransferase (MET) (Hu *et al.* 2008), *Yarrowia lipolytica* lipase LIP2 (Wang *et al.* 2012), and D-amino acid oxidase (Zheng *et al.* 2006) production.

#### 2.6.2. Temperature

In general, while higher temperatures result in protein denaturation, lower temperatures cause lower reaction rates. Therefore, bioprocesses should be carried out at controlled, isothermal and optimum temperatures. Although 30°C has been reported as the optimum temperature for *P. pastoris* by many studies (as cited in Cos *et al.* 2006; Çalık *et al.* 2015), there are some studies done in lower temperatures to increase the solubility of oxygen to enhance oxygen transfer rates at an expense of
lower reaction rates and metabolic fluxes. Dragosits et al. (2009) investigated the
effect of decreasing the fermentation temperature to 20°C on the proteome of _P.
pastoris_. In this study, while fluxes through TCA cycle were reduced, a 3-fold
increase in recombinant protein production was observed.

2.6.3. Dissolved Oxygen Concentration

Oxygen concentration in the fermentation medium is related with its dynamics with
cultivation time. Oxygen mass balance equation in the bioreactor liquid medium can
be expressed as a verbal equation as follows:

\[(\text{oxygen}_{\text{in}}) - (\text{oxygen}_{\text{out}}) + (\text{oxygen \ uptake rate}) = (\text{accumulation of oxygen})\]  (1)

Difference of the first two terms is equal to oxygen transfer rate (OTR) and it is
balanced with oxygen uptake rate \((r_{O} = \text{OUR})\) of _P. pastoris_ cells. OUR is related
with cell metabolism and the dynamics of intracellular reaction network. OTR and
OUR are important fermentation characteristics for recombinant protein production
rather than dissolved oxygen concentration \((C_{DO})\). \(C_{DO}\) only shows the instantaneous
accumulation of oxygen in the production medium and can be measured by an online
oxygen probe attached to the bioreactor. Since oxygen transfer into the cell strongly
affects cell metabolism and intracellular metabolic fluxes, to fine-tune bioreactor
performance according to the physiology of the cells, the regulatory effect of oxygen
transfer requires further investigation (Çalık et al. 2000)

In _P. pastoris_, dissolved oxygen is generally kept at 20-30% saturation by supplying
air with a cascade system with constant agitation speed (Gasser et al. 2006; Zheng et
al. 2006; Zhang et al. 2007; Fei et al. 2009; Çalık et al. 2015). Air is supplemented
along with pure oxygen when feeding only air is not enough to meet desired
dissolved oxygen concentration levels because oxygen consumption rate increases
with increasing cell concentration.

_P. pastoris_ fermentation strategies can also be dependent on constant oxygen transfer
rate. In these processes, air flow rate is adjusted in a manner such that the aeration
rate (volume air per reactor volume per minute, vvm) is kept constant throughout the
process. By its nature, this kind of process does not take the oxygen demand of the cells into account but supplies a constant oxygen flow per unit volume at all times.

Hu et al. (2008) reported MAT production by limited glycerol feeding with keeping dissolved oxygen (DO) at 50%, 25% and 0%. Lower DO values improved specific glycerol uptake rate ($q_{\text{Gly}}$) and specific growth rate ($\mu$). Keeping DO at 0% did not depress, but increased respiratory activity of the cells, resulting in higher $q_{\text{Gly}}$ values. Baumann et al. (2008), reported similar results for Fab production with choosing glucose as the carbon source. Compared to limited-aerobic (10.91% DO) and fully-aerobic (20.97% DO) fermentation; hypoxic (5.97-8.39% DO) conditions resulted in 2-fold decrease in biomass, significant ethanol formation and 2.5-fold increase in specific product formation rate ($q_p$). Similar to Hu et al. (2008), they showed that limitation on oxygen does not repress glucose consumption but results in a switch to an alternative metabolic pathway leading to increased specific substrate consumption rate and by-product formation as $P$. pastoris behaves facultative anaerobically in the presence of glucose. With these findings, they developed a feedback control system to keep ethanol concentration in the medium at 1% (v/v) for three different protein production while keeping $C_{\text{DO}}$ at hypoxic levels and compared this new strategy with standard cultivation where glucose was fed with a constant rate of $F = 161.7 \text{ g h}^{-1}$ while keeping oxygen concentration above 20% saturation. Compared to standard cultivation conditions, hypoxic fed-batch strategy and keeping ethanol concentration constant resulted in 13% higher product titer, reduced fermentation time by more than 3-fold and biomass concentration decreased by almost 1.5-fold. Again 2.5-fold increase in $q_p$ was observed and $\mu$ remained the same. They speculated that since glycolytic fluxes are higher in hypoxic conditions; glycolytic genes and promoters such as $P_{\text{GAP}}$ are upregulated.

Later on, Baumann et al. (2010) examined the effect of $C_{\text{DO}}$ on the interaction between the transcription of the genes and heterologous protein expression in $P$. pastoris cells. They used the data from the previous study (Baumann et al. 2008) and investigated the cellular adaptations on low oxygen concentrations and recombinant
protein production. Under hypoxia, most of the abundant proteins were involved in amino acid metabolism, glycolysis and general stress response while the proteins involved in TCA cycle, oxidative stress response and vitamin metabolism were expressed at lower levels. The increase in the transcription level of glycolytic genes under hypoxia supports their speculation in the previous study that Fab productivity increased under hypoxia as a result of the upregulation of the glycolytic genes, and the genes under the control of glycolytic promoters, such as P_{GAP}.

Since *P. pastoris* is a Crabtree-negative yeast, it is more vulnerable to changes in the availability of dissolved oxygen compared to the Crabtree-positive yeast *S. cerevisiae* (Baumann *et al.* 2010). Therefore, parameters related with oxygen transfer of microbial processes; such as oxygen transfer characteristics; oxygen uptake rate (OUR), oxygen transfer rate (OTR), and volumetric mass transfer coefficient (K_{L,a}), along with bioreactor hydrodynamics for recombinant protein production by *P. pastoris* should be systematically studied in order to gain further insight about the bioprocess characterization.

### 2.7. Oxygen Transfer Characteristics

As oxygen has low solubility in fermentation media (nearly 10 ppm), oxygen transfer is usually the rate limiting step for bioprocesses and determined the overall reaction rate.

The process of oxygen transfer from gas phase as an air bubble to the solid phase (into the cell) is divided into nine steps as explained by Whitman’s (1923) two film theory (Garcia-Ochoa *et al.* 2010) as follows (Figure 7):

1. Transfer from the bubble to the gas liquid interface,
2. Movement along the gas-liquid interface,
3. Diffusion through the liquid film around the bubble,
4. Movement through the bulk liquid (fermentation broth),
5. Diffusion through the stagnant liquid film around the cell,
(vi) Movement along the liquid-cell interface and – if cells are within a flock, clump or a solid particle – diffusion through the solid and then to the cell,

(vii) Transfer through the cytoplasm towards the reaction site,

(viii) Reactions involving $O_2$ consumption and $CO_2$ formation,

(ix) Transfer of the gases formed as a result of the reaction in the reverse direction.

Transportation of oxygen across the bulk liquid gets faster when the cells are homogeneously suspended in the well-mixed fermentation medium without any aggregation. Then, the second part of step (vi) is negligible and nonpolar molecules can be transported through the cell membrane by passive transportation (Campbell and Reece, 2011). Resistance to the transportation along the cytoplasm to the reaction site is generally neglected because of the small size of the microorganism (Nielsen and Villadsen, 1994). Stagnant liquid around the gas bubble represents the greatest limitation to mass transfer. In such a system, mass transfer can be modelled by Whitman’s (1923) two film theory (Figure 8):

$$j^0 = k_G \cdot (p_G - p_i) = k_L \cdot (C_{DO,i} - C_{DO})$$

(2)

$j^0$ is the molar flux of oxygen across the gas-liquid interface ($mol m^{-2} s^{-1}$), $k_G$ and $k_L$ are local (gas side and liquid side, respectively) mass transfer coefficients, $p_G$ is the partial pressure of oxygen in gas bubbles, $C_{DO}$ is the dissolved oxygen concentration in the liquid and subscript $i$ represents the interface.

When overall mass transfer is considered, equation (2) can be written as:

$$J^0 = K_G \cdot (p_G - p^*) = K_L \cdot (C^*_{DO} - C_{DO})$$

(3)
where \( p^* \) is the oxygen pressure in equilibrium with the liquid phase and \( C_{Do}^* \) is the oxygen saturation concentration in the bulk liquid in equilibrium with the bulk gas phase, \( K_G \) and \( K_L \) are overall mass transfer coefficients according to Henry’s law, \( p^* = H \cdot C_{Do}^* \).

Combining equations (2) and (3):

\[
\frac{1}{K_L} = \frac{1}{H \cdot K_G} + \frac{1}{k_L} \tag{4}
\]

Since solubility of oxygen in liquid broths is low, Henry’s constant is large, therefore first term on the right hand side becomes negligible. Therefore overall mass transfer coefficient can be taken as equal to local mass transfer coefficient in liquid side, \( K_L = k_L \). Oxygen transfer rate, \( \text{OTR}, N_{O_2} \) can be obtained by multiplying overall molar flux, \( J^0 \), with interfacial area per unit volume of liquid, \( a \):

\[
\text{OTR} = N_{O_2} = J^0 \cdot a = K_L \cdot a \left( C_{Do}^* - C_{Do} \right) \tag{5}
\]

Due to the difficulties of measuring \( k_i \) and \( a \) separately, the product of them, \( k_i a \) is reported and this parameter is named as volumetric mass transfer coefficient. Determination of this parameter is crucial as it is the key element to quantify the effects of dissolved oxygen and oxygen transfer characteristics on bioprocess performance.

Differential mass balance for dissolved oxygen in the bioreactor can be written as:

\[
K_L a \cdot V \left( C_{Do}^* - C_{Do} \right) - q_{O_2} \cdot C_x \cdot V = \frac{d(C_{Do} \cdot V)}{dt} \tag{6}
\]

If the reactor volume is constant and well-mixing is established, equation (6) becomes:

\[
K_L a \left( C_{Do}^* - C_{Do} \right) - q_{O_2} \cdot C_x = \frac{dC_{Do}}{dt} \tag{7}
\]
Figure 7. (a) Oxygen transfer from gas bubble into the cell (b) $C_{DO}$ profile along the oxygen transfer process.
Figure 8. Schematic representation of the gas-liquid interface according to two film theory (Garcia-Ochoa and Gomez, 2009).

where \( \frac{dC_{DO}}{dt} \) term is the rate of oxygen accumulation in the liquid phase, 

\[ K_L a \left( C^*_{DO} - C_{DO} \right) \] term is OTR and \( q_{O_2} \cdot C_X \) term is OUR, where \( q_{O_2} \) is specific oxygen uptake rate and \( C_X \) is cell concentration.

\[ OTR + OUR = \frac{dC_{DO}}{dt} \] (8)

2.7.1. Experimental Determination of Volumetric Mass Transfer Coefficient

There are several methods to determine \( K_L a \) experimentally, which can be divided into two subgroups by one of which \( K_L a \) is measured in the absence of microorganisms or with nonviable cells to eliminate the effect of biochemical reactions (\( OUR = 0 \)); and in the other one, with the presence of microorganisms that
can utilize oxygen \((OUR \neq 0)\) (Garcia-Ochoa and Gomez, 2009). Some of these techniques are sodium sulfite oxidation method, oxygen balance method, static gassing-out method and glucose oxide method (Rainer, 1990).

OTR, OUR and \(K_La\) are usually measured by different methods for the same experiment but these values can be obtained simultaneously by applying dynamic method which is based on the respiratory activity of the microorganisms. In this method, firstly gas flow rate is set to zero and agitation rate decreased to the lowest possible value to eliminate surface aeration. \(C_{DO}\) is monitored by the oxygen probe. By eliminating the transfer of oxygen into the bioreactor, OTR is set equal to zero and equation (8) simply becomes:

\[
OUR = \frac{dC_{DO}}{dt} \quad (9)
\]

Before reaching critical \(C_{DO}\) level, oxygen supply is turned on again and agitation speed is set to normal bioreactor operation conditions to maintain the same oxygen transfer characteristics. In this time interval, \(C_{DO}\) increases until it reaches its steady value (Figure 9). By using the calculated OUR value from equation (9), \(K_La\) can be determined by using equation (8). Knowing \(C_x\) and calculating \(q_{O_2}\) from OUR, one can integrate equation (8) to get:

\[
q_{O_2} \cdot C_x \cdot \Delta t + \Delta C_{DO} = K_La \cdot \int_{t_1}^{t_2} \left( C_{DO}^* - C_{DO} \right) \cdot dt \quad (10)
\]

Equation (10) can be used to calculate \(K_La\) several times during one bioprocess by solving this equation for each data set of \(C_{DO}\) vs. time. Alternatively, Equation (8) can be used to plot the graph of \(C_{DO}\) vs. \(\frac{dC_{DO}}{dt} - r_{O_2}\), whose slope gives \(\frac{1}{K_La}\) (Figure 10).
The presence of microorganisms in the reaction medium as solid particles represents a physical resistance to mass transfer of oxygen. Therefore, $K_La_0$ is also determined by using the same method in order to interpret the effect of this resistance. This method is applied before cells are inoculated to the bioreaction medium. The only difference of this method from measuring $K_La$ is the supplementation of nitrogen to decrease $C_{DO}$ as oxygen cannot be utilized in the absence of the viable microorganisms. Therefore, equation (7) simplifies to:

$$K_La_0\left(C^*_c - C_{DO}\right) = \frac{dC_{DO}}{dt} \quad (11)$$

### 2.7.2. Other Parameters Involving Oxygen Transfer Characteristics

Although determination of OUR, OTR and $K_La$ are considered to be the major parameters to understand oxygen transfer characteristics of bioprocesses; other parameters such as maximum possible oxygen transfer rate ($OTR_{max}$), maximum possible oxygen uptake rate ($OUR_{max}$, also known as oxygen demand, $OD$), Damköhler number (Da), and effectiveness factor ($\eta$) need to be calculated to gain a deeper insight of oxygen transfer characteristics and mass transfer limitations in bioprocesses (Çalık et al. 1998).

Maximum oxygen transfer rate can be obtained when the driving force, concentration difference, is the highest. For that reason, dissolved oxygen concentration in the fermentation medium should be equal to zero. Therefore:

$$OTR_{max} = J_{0,max} \cdot a = K_La \cdot C^*_c \quad (12)$$
**Figure 9.** Change in $C_{DO}$ during the application of dynamic method (Garcia-Ochoa and Gomez, 2009).

**Figure 10.** Determination of $K_La$ by dynamic method.
Maximum oxygen utilization rate is equal to:

\[ OUR_{\text{max}} = -r_{O_2,\text{max}} = \frac{C_X \cdot \mu_{\text{max}}}{Y_{X/O_2}} \]  \hfill (13)

where \( \mu_{\text{max}} \) is equal to maximum specific cell growth rate and \( Y_{X/O_2} \) represents the oxygen yield coefficient on cell, which is equal to mass of cell generated per unit mass of oxygen consumed.

Damköhler number (Da) is a dimensionless number defined as the ratio of reaction rate to mass transfer rate which characterizes if there is a reaction or mass transfer limitation in the system. In bioprocesses, a modified Damköhler number is defined as the ratio of maximum possible oxygen utilization rate to maximum oxygen transfer rate:

\[ Da = \frac{OUR_{\text{max}}}{OTR_{\text{max}}} \]  \hfill (14)

To calculate the efficiency of the oxygen uptake rate facilitated by the bioprocess, the ratio of \( OUR \) to \( OUR_{\text{max}} \) is defined as the effectiveness factor (\( \eta \)):

\[ \eta = \frac{OUR}{OUR_{\text{max}}} \]  \hfill (15)

In literature Çelik et al. (2009) used these parameters to determine the oxygen transfer characteristics of recombinant human erythropoietin (rHuEPO) production by \( P.\) pastoris using \( P_{AOX1} \) and sorbitol as the co-substrate and Çalık et al. (1998, 2000) for serine alkaline protease (SAP) production by \( B.\) licheniformis.

2.8. Influence of Geometrical Parameters of the Bioreactor on Mass Transfer Coefficient and Mixing

Hydrodynamic conditions of the bioreactors strongly affect the gas-liquid mass transfer in bioprocesses which are determined by the properties of the culture, operation temperature and pressure, the presence of viable cells that can consume oxygen and geometrical characteristics of the bioreactor (Garcia-Ochoa and Gomez,
Stirred tank bioreactors are widely used for bioprocesses as they provide excellent heat and mass transfer rates and mixing. Many parameters affect the mass transfer and mixing conditions but the most important ones are the stirrer speed, baffle type, impeller type, diameter and number.

Lu et al. (1997) investigated the effects of the width and number of baffles in stirred tanks with Rushton turbine impellers and showed that there is an optimum number of baffles for the improvement of mixing and mixing time. For the vessels having impeller over vessel diameter (D/T) ratio larger than 0.2; when the baffle number was more than 8, mixing was interrupted by the sparging gas through the impeller and this increases mixing time. They found the optimum number of baffles to be 4 for mixing and oxygen dispersion.

Karimi et al. (2013) used three different impellers (Rushton turbine, pitched 4-blades and pitched 2-blades) with six configurations to investigate their effects on oxygen transfer. The results were reported to be in favor of twin Rushton turbine with 23% to 77% improvement on $K_{La}$. Compared to pitched 2- and 4-blades, Rushton turbine impellers were found to break and disperse air bubbles more effectively since they have larger cross sectional area.

Schaepe et al. (2013) tested the effects of different impeller types, diameters and different baffles on $K_{La}$. Rushton turbines and 6 blade concave disk impellers were used with different impeller diameters (D = 75 mm and D = 105 mm). When the effect of different number of impellers are compared, it was found that 2 impellers stimulated higher $K_{La}$ values compared to 3 impellers. The results showed that mixing times are shorter with 2 impeller configuration. Because of the formation of flow compartments around impellers, top-to-bottom mixing gets retarded by fluid recirculation between impellers as number of impellers increases (Sieblist et al. 2011). Therefore, 2 impeller configuration is favorable both for better oxygen transfer and for mixing. To take the baffle size and type into account, three baffles were investigated; 15 mm and 30 mm standard baffle and 30 mm C-baffle. Higher
power consumptions were required with larger baffle sizes when the agitation rate was kept constant but their “energetic efficiency” with respect to mass transfer rates is smaller compared to narrower baffles. It is also important to note that antifoam agent consumption was significantly lower (-30%) with 30 mm baffle rods. Reducing the amount of antifoam agent used is important as they cause problems in downstream processing.

2.9. Computation of Bioprocess Characteristics

Computation of bioprocess characteristics in terms of specific rates and yields is of the utmost importance. Here, the calculation procedures and derivations of the equations regarding specific cell growth and substrate consumption rates, as well as yield coefficients are given in this subsection.

2.9.1. Specific Cell Growth Rate

Microbial growth can be divided into four phases: lag phase, exponential phase, stationary phase, and death phase (Figure 11).

Lag phase is the time when the cell culture adapts itself to the new environment and synthesize essential biomolecules for cell growth. In this phase, cells get more mature and gain the ability of division. This phase should be kept as short as possible by preparing production medium similar to precultivation medium to make the adaptation to the new environment easier.

Exponential phase is the time that the cell concentration increases with constant specific growth rate. The growth rate is defined as the number of new microorganisms appearing per unit time and it is proportional to the present population by equation (16) (Bailey and Ollis, 1986):

\[ \mu \cdot C_x = \frac{dC_x}{dt} \]  

(16)
Figure 11. Typical microbial growth curve of a batch culture.

For the cells in the exponential phase, natural logarithm of the cells versus time curve is plotted. The slope of this curve gives the maximum number of division per cell per unit time, $\mu_{\text{max}}$.

Exponential growth decelerates as the nutrients deplete and inhibitory by-products accumulate. When the exponential phase is finished, stationary phase starts. In this phase, cell concentration remains more or less the same as the cell formation and death rates are almost equal. In the processes where the target product is the secondary metabolites, stationary phase is desired where the exponential phase is only for the biomass accumulation and production of primary metabolites.

If the cells are not harvested during the stationary phase; death phase starts and the cell number rapidly decreases in a logarithmic trend because of the high death rate.

In fed-batch (semi-batch) processes, cells are inoculated into the medium at the start-up. If cells are assumed not to be lost by sampling, the process can be assumed to be batch-wise in terms of cells.
\[ r_x \cdot V = \frac{d(C_x \cdot V)}{dt} \]  
\hspace{1cm} (17)

where \( V \) is the working volume and \( r_x \) is the cell generation rate which can be demonstrated as:

\[ r_x = \mu \cdot C_x \]  
\hspace{1cm} (18)

Substitution of equation (18) into equation (17) and application of chain rule gives:

\[ \mu \cdot C_x \cdot V = V \frac{dC_x}{dt} + C_x \frac{dV}{dt} \]  
\hspace{1cm} (19)

In fed-batch processes, volume increases with increasing time due to substrate feeding and this volume change can be shown as:

\[ Q_s = \frac{dV}{dt} \]  
\hspace{1cm} (20)

where \( Q_s \) is the volumetric flow rate into the reactor.

Substitution of equation (20) into equation (19) gives:

\[ \mu \cdot C_x \cdot V = V \frac{dC_x}{dt} + C_x \cdot Q_s \]  
\hspace{1cm} (21)

Rewriting equation (21):

\[ \left( \mu - \frac{Q_s}{V} \right) C_x = \frac{dC_x}{dt} \]  
\hspace{1cm} (22)

By using equation (22), specific cell growth rate can be calculated in the rearranged form:

\[ \mu = \frac{dC_x}{dt} \cdot \frac{1}{C_x} + \frac{Q_s}{V} \]  
\hspace{1cm} (23)

### 2.9.2. Specific Substrate Consumption Rate

For substrate point-of-view, the process is conducted in a fed-batch manner. Therefore, following mass balance equation can be used for the substrate.
\[ Q_s \cdot C_{s_0} + r_s \cdot V = \frac{d(C_s \cdot V)}{dt} \] (24)

where \( C_s \) is the substrate concentration in the fermentation medium, \( Q_s \) is volumetric substrate feeding rate, \( C_{s_0} \) is the concentration of substrate in the feeding medium (stock concentration) and \( r_s \) is the substrate consumption rate which can be expressed as:

\[-r_s = q_s \cdot C_X \] (25)

where \( q_s \) is the specific substrate consumption rate.

Substitution of equation (25) into equation (24) results in:

\[ q_s = \left( Q_s \cdot C_{s_0} - \frac{d(C_s \cdot V)}{dt} \right) \frac{1}{C_X \cdot V} \] (26)

Applying chain rule and rearranging the terms:

\[ q_s = \left( \frac{Q_s \cdot C_{s_0}}{V} - \frac{dC_s}{dt} \cdot \frac{V}{dt} \right) \frac{1}{C_X} \] (27)

The process can be regarded as pseudo-steady state in terms of substrate; therefore \( \frac{dC_s}{dt} \) can be taken as zero \( \left( \frac{dC_s}{dt} \approx 0 \right) \).

Substitution of equation (20) into equation (27) results in:

\[ q_s = \frac{Q_s}{C_X \cdot V} \left( C_{s_0} - C_s \right) \] (28)

To be able to calculate specific substrate consumption rate \( (q_s) \), volumetric substrate flow rate \( (Q_s) \) should be known.

By taking \( \frac{dC_s}{dt} \approx 0 \), rate of substrate utilization is related only to cell formation:
where $Y_{X/S}$ is the cell yield on substrate.

In this case, equation (25) can be rewritten by using equation (29):

$$q_s = \frac{r_x}{Y_{X/S} \cdot C_x}$$

(30)

Now equation (30) can be substituted into equation (28):

$$\frac{r_x}{Y_{X/S}} = \frac{Q_s}{V} \left(C_{S_0} - C_s\right)$$

(31)

Using equation (18) and rearranging:

$$Q_s \left(C_{S_0} - C_s\right) = \frac{V \cdot \mu \cdot C_x}{Y_{X/S}}$$

(32)

Knowing that the differentiation of equation (19) results in:

$$C_x \cdot V = C_{X_0} \cdot V_0 \cdot \exp(\mu \cdot t)$$

(33)

Substitution of equation (33) into equation (32):

$$Q_s = \frac{\mu_0 \cdot C_{X_0} \cdot V_0}{Y_{X/S} \cdot (C_{S_0} - C_s)} \cdot \exp(\mu_0 \cdot t)$$

(34)

Equation (34) is used for calculating the feeding profile of the substrate. This feeding strategy assumes that cells are always in the exponential phase and does not account for stationary phase of the cells. As glucose feeding increases exponentially regardless of cell concentration, when cells enter stationary phase glucose starts to accumulate in the medium and feeding becomes inefficient.
2.9.3. Yield Coefficients

In bioprocesses quantity of the biomass or product formation can be described by yield coefficients. These yield coefficients can be used to express the substrate or process efficiency for product and biomass formation. Overall yield coefficients are used to describe the formation of the products per the utilized substrate for the entire process (Equation (35)) while instantaneous yield coefficients are used to describe the instantaneous efficiency of the bioprocess (Equation (36)).

\[
Y_{x/s} = \frac{\Delta C_x / \Delta t}{-\Delta C_s / \Delta t} \\
Y_{p/s} = \frac{\Delta C_p / \Delta t}{-\Delta C_s / \Delta t}
\]  
(35)

\[
Y_{x/s} = \frac{r_x}{-r_s} = \frac{dC_x / dt}{-dC_s / dt} \\
Y_{p/s} = \frac{r_p}{-r_s} = \frac{dC_p / dt}{-dC_s / dt}
\]  
(36)

In aerobic processes, yield coefficients of cell and product on oxygen can also be calculated (Equation (37)).

\[
Y_{x/o} = \frac{r_x}{-r_o} \\
Y_{p/o} = \frac{r_p}{-r_o}
\]  
(37)
CHAPTER 3

MATERIALS AND METHOD

3.1 Chemicals
Chemicals used in this study were analytical grade and obtained from Sigma, Merck
and Fluka.

3.2 Buffers and Stock Solutions
Preparation and formulation of the buffers and stock solutions are presented in
APPENDIX A. Depending on the characteristics of the solution, sterilization was
done either by autoclaving at 121°C for 20 minutes or by filtering through filters with
0.20 or 0.45 μm pore diameter (Sartorius Stedim Biotech GmbH, Germany)

3.3 Microorganism
P. pastoris X-33 strain, carrying pGAPZα-A::xylA_Cd_Opt was used as GI producer.
(Güneş and Çalık, submitted). Codon optimized xylA sequence was obtained from
pPICZα-A::xylA_Cd_Opt plasmid constructed by Ata et al. (2015). The shuttle vector
pGAPZα-A was obtained from Invitrogen and propagated in E. coli DH5α cells,
grown in low salt Luria broth (LSLB) and purified. The purified pGAPZα-A::xylA_Cd-
Opt plasmid was digested with PagI (Fermantas). Linearized plasmid was used for
transfection of P. pastoris wild type X-33 host strain, using lithium chloride
transformation method (Invitrogen).
3.4 Growth Media

All growth media were sterilized by autoclaving at 121°C for 20 minutes, then let to cool to about 50°C and necessary antibiotics, Zeocin™ or chloramphenicol, were added.

3.4.1 Solid Medium

*P. pastoris* cells carrying pGAPZα-A::xylA<subImageContext>cd-opt</sub> gene, stored at -80°C in microbanks, were inoculated onto YPD agar plates whose composition is given in Table 3. Inoculated plates were incubated at 30°C for about 48 hours and stored at +4°C.

**Table 3.** Composition of YPD agar solid medium

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>20 g</td>
</tr>
<tr>
<td>Glucose*</td>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Zeocin™</td>
<td>1 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>

* Sterilized by a filter with 0.20 μm pore diameter and added to the medium after autoclaving.

3.4.2 Precultivation Medium

*P. pastoris* cells carrying pGAPZα-A::xylA<subImageContext>cd-opt</sub> gene, grown on YPD agar plates were inoculated into the precultivation medium (buffered glycerol complex medium, BMGY), whose composition is given in Table 4 (Invitrogen). The precultivation was performed in 250 ml baffled, air-filtered shake bioreactors with 50 ml working volume for 15-20 hours. An orbital shaker (Sartorius, Germany) was used with agitation speed of 200 rpm and temperature of 30°C.
3.4.3 Production Media

Production of glucose isomerase with *P. pastoris* under P<sub>GAP</sub> was done in two phases. In the first phase, basal salts medium (BSM) was used where glycerol is the carbon source (Table 5). In the second phase, 50% (w/v) glucose solution was used. In both phases, production medium also contains *Pichia* trace minerals (PTM1) (Table 6).

Table 4. Composition of the precultivation medium, BMGY

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>20 g</td>
</tr>
<tr>
<td>Potassium phosphate buffer (pH=6.0)</td>
<td>0.1 mol</td>
</tr>
<tr>
<td>Yeast nitrogen base (YNB)</td>
<td>13.4 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Biotin*</td>
<td>4·10&lt;sup&gt;-5&lt;/sup&gt; g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 g</td>
</tr>
<tr>
<td>Chloramphenicol (from 34 mg ml&lt;sup&gt;-1&lt;/sup&gt; stock)*</td>
<td>1 ml</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O to 1 L</td>
<td></td>
</tr>
</tbody>
</table>

* Added after autoclaving.

3.5. Recombinant Glucose Isomerase Production

3.5.1. Precultivation

*P. pastoris* cells carrying pGAPZα-<small>A::xyIA<sub>Cd-Opt</sub></small> gene are taken from the microbanks stored at -80°C, and transferred onto YPD-Agar plates. Plates were incubated at 30°C for 48 h in an incubator (Nüve, Turkey). Afterwards, a single colony was chosen and inoculated into 50 ml BMGY for precultivation. Precultivation was conducted at 200 rpm, 30°C for 15-20 h in laboratory scale baffled air-filtered shake flasks. Precultivation continued until OD<sub>600</sub> = 2-8, when cells are known to be in the exponential phase of their growth. When the desired cell concentration was reached, they were centrifuged at 1500 g and 4°C for 10 minutes. By this way, cells were separated from the supernatant containing precultivation medium. These cells were
dissolved in water and fed to the bioreactor containing BSM such that the initial OD$_{600}$ in the bioreactor is equal to 1.

**Table 5.** Composition of basal salts medium, BSM

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>85 % H$_3$PO$_4$</td>
<td>26.7 ml</td>
</tr>
<tr>
<td>CaSO$_4$.2H$_2$O</td>
<td>1.17 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>14.9 g</td>
</tr>
<tr>
<td>KOH</td>
<td>4.13 g</td>
</tr>
<tr>
<td>K$_2$SO$_4$</td>
<td>18.2 g</td>
</tr>
<tr>
<td>Glycerol (100 %)</td>
<td>41.95 ml</td>
</tr>
<tr>
<td>Chloramphenicol (from 34 mg ml$^{-1}$ stock )*</td>
<td>1 ml</td>
</tr>
<tr>
<td>10% Antifoam*</td>
<td>1 ml</td>
</tr>
<tr>
<td>PTM1*</td>
<td>4.35 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>

*Added after autoclaving.

**Table 6.** Composition of *Pichia* trace salts, PTM1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Composition (per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>6 g</td>
</tr>
<tr>
<td>NaI</td>
<td>0.08 g</td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>3 g</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.02 g</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>20 g</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>65 g</td>
</tr>
<tr>
<td>CoCl.6H$_2$O</td>
<td>0.916 g</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>5 ml</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>
3.5.2. Production in Laboratory Scale Bioreactor

For the production of glucose isomerase, a 3 L laboratory scale bioreactor (Braun CT2-2) was used with a working volume of 1-1.5 L. In this reactor; temperature, pH, dissolved oxygen, foam, agitation and feed inlet rates can be controlled. Schematic representation of the bioreactor is given in Figure 12.

3.5.2.1. Bioreactor Operation Parameters

Temperature was kept at 30±0.1°C using the PI controller of the bioreactor and by an external cooler, steam generator and a jacket. Agitation was done by a six-bladed Rushton turbine. To keep dissolved oxygen and pH in the bioreaction medium under control, oxygen and pH (Hamilton, Switzerland) probes were used. Oxygen supplement of the bioreactor was provided by an air compressor and a sparger. In the cases when supplying only air to keep the dissolved oxygen concentration at a desired value is not enough; air was enriched with increasing amounts of pure oxygen passing through a digital mass flow controller. pH of the bioreaction medium was maintained at 5.5±0.1 in the batch phase, and at 5.0±0.1 in the semi-batch phase using 25% NH₃OH. Foaming in the bioreactor was kept under control by adding 0.01% (v/v) antifoam solution (Y-30 emulsion, Sigma) to BSM at the beginning of batch phase, and minor amounts of 10% antifoam solution was added manually if needed throughout the bioprocess. Base and antifoam solutions and the feed solution of semi-batch phase were supplied through the inlet port of the bioreactor by peristaltic pumps.
Figure 12. Schematic representation of lab scale bioreactor system together with precultivation steps. I. Solid medium containing inoculated cells from microbank. II. Precultivation medium II. Lab 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 O2 tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008)
Table 7. Geometric properties of the bioreactor

<table>
<thead>
<tr>
<th>Property</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tank diameter</td>
<td>$T = 12 \text{ cm}$</td>
</tr>
<tr>
<td>Impeller diameter</td>
<td>$D = T/2.4$</td>
</tr>
<tr>
<td>Impeller height</td>
<td>$h = T/12$</td>
</tr>
<tr>
<td>Off-bottom clearance</td>
<td>$C = T/6$</td>
</tr>
<tr>
<td>Average height of working volume</td>
<td>$H = 0.667T$</td>
</tr>
</tbody>
</table>

3.5.2.2. Production Phases

Cells harvested from the precultivation medium are inoculated to the bioreactor in such a manner that OD$_{600}$ is equal to 1, which is equivalent to 0.275 g L$^{-1}$ cell concentration. As mentioned in previous sections, production of glucose isomerase under $P_{\text{GAP}}$ was done in two phases; glycerol batch and glucose semi-batch phases.

**Glycerol Batch (GB) Phase:** The first phase was conducted batch-wise where BSM is used as the bioreaction medium. In this phase, cell growth is the main objective. Therefore, glycerol was used as the carbon source as it is known to enable higher cell densities than glucose (Çalık et al. 2015) This phase continued until all glycerol was depleted, which is signaled by cell concentration reaching 15-20 g L$^{-1}$. Therefore cell growth was monitored for an indication of glycerol depletion, which takes about 18-20 h.

**Glucose Fed-Batch (GFB) Phase:** The main objective of the second phase of the production is to produce recombinant GI together with cell growth. Therefore, a non-repressive carbon source, glucose, was used for expressions under the constitutive $P_{\text{GAP}}$. The feed solution consists of 50% (w/v) glucose and 12 ml L$^{-1}$ PTM1. Carbon source was supplied by the feed solution while a basic solution, 25% NH$_3$OH, was
used as nitrogen source, as well as to adjust pH. Glucose solution containing PTM1 was fed to the bioreactor continuously, according to predetermined exponential feed rate. The equation used to calculate the feeding rate using predetermined exponential cell growth rate is given in Equation (34) while the parameters of the equation are given in Table 8.

Table 8. Values of the parameters in Equation 34

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucose feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_0$</td>
<td>0.15 h$^{-1}$</td>
</tr>
<tr>
<td>$Y_{S/X}$</td>
<td>0.48 g cell g$^{-1}$ glucose</td>
</tr>
<tr>
<td>$C_{S_0}$</td>
<td>500 g L$^{-1}$</td>
</tr>
</tbody>
</table>

3.5.2.3. Feeding Strategies Applied in Bioreactor Experiments

In all of the experiments, precultivation and the first phase of the bioprocesses were carried out exactly the same while different strategies were applied in the second phase of the bioprocesses. In those experiments, glucose feeding was done the same using predetermined exponential feeding rate of $\mu_0 = 0.15h^{-1}$, as optimized in a previous study (Keskin, 2014) while different oxygen transfer strategies were applied in each experiment. Strategies followed can be divided into two groups: experiments based on constant oxygen transfer condition (OTC), and constant dissolved oxygen concentration ($C_{DO}$). All of the strategies applied are given in Table 9.
Table 9. Strategies applied in bioreactor experiments

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Dissolved Oxygen (C_{DO}), %</th>
<th>Aeration Rate (Qo/V), vvm</th>
<th>Agitation Rate, (N) rpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. OTC₁</td>
<td>Variable</td>
<td>3</td>
<td>900</td>
</tr>
<tr>
<td>2. OTC₂</td>
<td>Variable</td>
<td>6</td>
<td>900</td>
</tr>
<tr>
<td>3. OTC₃</td>
<td>Variable</td>
<td>10</td>
<td>900</td>
</tr>
<tr>
<td>4. CDO₁</td>
<td>5</td>
<td>Variable</td>
<td>900</td>
</tr>
<tr>
<td>5. CDO₂</td>
<td>10</td>
<td>Variable</td>
<td>900</td>
</tr>
<tr>
<td>6. CDO₃</td>
<td>15</td>
<td>Variable</td>
<td>900</td>
</tr>
<tr>
<td>7. CDO₄</td>
<td>20</td>
<td>Variable</td>
<td>900</td>
</tr>
<tr>
<td>8. CDO₅</td>
<td>40</td>
<td>Variable</td>
<td>900</td>
</tr>
</tbody>
</table>

3.6 Analyses

During the experiments, a sample from the bioreaction medium was taken in every three hours. Firstly, cell concentration was determined from this sample, then it was centrifuged at 1500 g and +4°C for 10 minutes to separate pellets and supernatant. Supernatants were used to determine glucose concentration, GI activity and monomer concentration by SDS-PAGE. Some of the supernatant was then filtered with filters having 0.20 μm pore diameter and these filtrates were used in organic acid concentration determination via HPLC.
3.6.1. Cell Concentration

A UV-Vis Spectrophotometer (Thermo Spectronic, Heliosα) was used at 600 nm to determine *P. pastoris* cell concentration as dry cell weight per liter. In order to stay in the accurate measurement range of the UV-Vis Spectrophotometer (0.1-0.8 Å), samples were usually diluted with distilled water. Following equation was used to calculate cell concentration from OD$_{600}$ (Orman, 2007):

$$C_X = 0.275 \cdot OD_{600} \cdot DF$$  \hspace{1cm} (38)

Where $C_X$ cell concentration in DCW L$^{-1}$ is, $OD_{600}$ is the optical density determined at 600 nm and DF is the dilution factor.

3.6.2. Glucose Concentration

Glucose concentration was determined by using a glucose analysis kit (Biasis, Ankara) based on glucose oxidation method (Boyacı, 2005). In this method, a series of reactions takes place where the conversion of glucose to gluconate and hydrogen peroxide is the first step of the reaction in the presence of glucose oxidase catalyst. Then, hydrogen peroxide reacts with 4-aminoantipyrine and phenol with peroxidase catalysis, to produce iminoquinone and water. The concentration of the last product, iminoquinone, having a reddish-pink color and being equimolar with glucose in the reaction medium, was measured by UV-Vis Spectrophotometer at 505 nm.

$$D - glucose + O_2 + H_2O \xrightarrow{Glucose Oxidase} Gluconate + H_2O_2$$

$$H_2O_2 + 4 - a \text{ minoantipyrine} + \text{Phenol \xrightarrow{Peroxidase} Iminoquinone + H}_2O$$

While conducting the experiment, first 2 ml of dH$_2$O was added to reaction tubes, followed by the addition of 400 µl of buffer solution containing phenol and potassium dihydrogen phosphate. Then, 50 µl of sample solution was added to the tubes. Lastly, 50 µl of glucose reactive mixture containing glucose oxide, 4-aminoantipyrin and peroxidase, was added to the tubes and reaction started with the addition of the glucose reactive mixture. Reaction tubes were placed in a water bath.
at 37°C for 20 minutes to obtain the red color resulted from the formation of iminoquinone. OD$_{505}$ of iminoquinone was measured with UV-Vis Spectrophotometer at 505 nm. Standard glucose solutions were used to obtain the calibration curve given in APPENDIX B to determine the glucose concentration of the reaction samples in g L$^{-1}$.

3.6.3. Glucose Isomerase Activity

Supernatants of samples centrifuged at 1500 g, +4°C for 10 minutes were used to determine the extracellular glucose isomerase activity.

To determine the GI activity, 50 μl supernatant was added to 50 μl freshly prepared activity buffer of 0.02 M potassium phosphate buffer (pH=7.0) at 80°C, containing 0.4 M D-glucose and 10 mM MnCl$_2$. The solution was incubated at 80°C for 10 minutes. After 10 minutes, the reaction was stopped by terminating by adding 60 μl of this mixture to 540 μl 0.1 M HCl solution. D-fructose produced was analyzed by the method described by Dische and Borenfreund (1951), known as carbazole-cysteine-sulfuric acid method. In this method, 1.8 ml 70% (v/v) sulfuric acid was added to test tubes containing the reaction mixture and 0.1 M HCl. Then 60 μl of freshly prepared 1.5% cysteine in 37% HCl and 60 μl of freshly prepared 0.12% (w/v) carbazole in ethanol was added to the mixture in this order. The mixture was incubated at room temperature for 30 minutes and vortexed occasionally. After 30 minutes, produced D-fructose concentration was determined by measuring the absorbance at 560 nm, using a UV-Vis Spectrophotometer. 1 U of GI activity is defined as the formation of 1 μmol D-fructose per minute under this reaction conditions. Equation (39) was used to calculate GI activity in U L$^{-1}$ from the absorbance of D-fructose.

$$A(UL^{-1}) = \frac{Abs(560nm) - 0.014}{50.94} \cdot 10^5$$  \hspace{1cm} (39)

3.6.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE technique was used to determine the monomer concentration of GI secreted to extracellular medium. For that reason, 20 μl supernatants of the samples
were added to 10 μl loading buffer. Because of the homotetrameric physiology of GI, firstly heat denaturation was performed using a thermocycler at 95°C for 5 minutes to obtain GI monomers. SDS-PAGE procedure was conducted as described by Laemmli (1970); samples ran simultaneously with a prestained protein marker at a constant current of 40 mA. Silver staining was used to stain the gels. The staining procedure is given in APPENDIX C.

3.6.4. Protease Activity Assay

Extracellular acidic protease activity was determined by using supernatants of the samples. 2 ml 0.5% (w/v) Hammerstein casein in 0.05 M sodium acetate buffer (pH=5.0) was mixed with 1 ml diluted sample and incubated at 37°C for 20 minutes. After this time period, 10% trichloroacetic acid (TCA) was added to terminate the reaction. The solution was then centrifuged at 10500 g, +4°C for 10 minutes. After centrifugation, solution was incubated at room temperature for 5 minutes and the absorbance was measured at 275 nm in UV-Vis Spectrophotometer. 1 U of protease activity was defined by Moon and Parulekar (1991) as the formation of 4 nmol tyrosine per minute. The equation (40) was used in the calculation of protease activity.

\[
A(UL^{-1}) = \left( \frac{\text{Abs}(275nm)}{0.8} \right) \left( \frac{1U}{4\text{nmol min}^{-1}} \right) \left( \frac{1}{20\text{min}} \right) \left( \frac{1000\text{nmol}}{1\mu\text{mol}} \right) (DF) \tag{40}
\]

3.6.5. Organic Acid Concentration

Organic acid concentrations were measured by a High Performance Liquid Chromatography (HPLC, Waters, Alliance 2695). Concentration of each organic acid was calculated by using the calibration curves plotted by analyzing standard solutions’ chromatograms. Filtered samples were used and diluted at least 5 times prior to analysis. Table 10 shows the operating conditions of HPLC for organic acid concentration analysis. Calibration curve for each organic acid is given in APPENDIX D.
Table 10. HPLC operation conditions for organic acid analysis (İleri and Çalık, 2006)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Capital Optimal ODS</td>
</tr>
<tr>
<td>Column dimensions</td>
<td>4.6 mm x 250 mm x 5 μm</td>
</tr>
<tr>
<td>System</td>
<td>Reversed phase chromatography</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>3.12% (w/v) NaH$_2$PO$_4$, 0.62 x 10$^{-3}$ (v/v) H$_3$PO$_4$</td>
</tr>
<tr>
<td>Flow rate of mobile phase</td>
<td>0.8 ml min$^{-1}$</td>
</tr>
<tr>
<td>Column temperature</td>
<td>30°C</td>
</tr>
<tr>
<td>Detector type and wavelength</td>
<td>Waters 2487 Dual Absorbance Detector, 210 nm</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>30°C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>5 μl</td>
</tr>
<tr>
<td>Analysis period</td>
<td>15 min</td>
</tr>
<tr>
<td>Delay time</td>
<td>5 min</td>
</tr>
</tbody>
</table>
CHAPTER 4

RESULTS AND DISCUSSION

In this study, effects of oxygen transfer conditions on thermostable glucose isomerase production by recombinant *Pichia pastoris* under $P_{GAP}$ was systematically investigated. Eight different experiments were conducted which can be divided into two subgroups: (i) experiments done with constant oxygen transfer condition (OTC) and (ii) experiments done with constant dissolved oxygen concentration in the fermentation medium ($C_{DO}$). In the first set of experiments, aeration rate was kept constant at $Q_{O}/V = 3, 6$ and $10$ vvm by altering the air flow rate into the bioreactor according to volume change while agitation speed was $N = 900$ rpm. In the second set of experiments, dissolved oxygen was kept constant at $C_{DO} = 5\%, 10\%, 15\%, 20\%$ and $40\%$ saturation by a cascade system to supply air and, if necessary, oxygen-enriched air. In all of the experiments, glucose was used as the carbon source and it was fed with a precalculated exponential feed-stream flow rate (Equation (34)) using predetermined specific growth rate of $\mu_0 = 0.15h^{-1}$. Designed strategies were investigated and compared with each other in terms of the cell and glucose concentration, GI and protease activities, and organic acid concentrations. Oxygen transfer characteristics of each strategy were also investigated along with the bioprocess characteristics such as specific consumption rates and yield coefficients.

In all of the experiments, precultivation and glycerol batch phase of the production were done according to the methods given in Sections 3.5.1 and 3.5.2. Since the difference in the strategies are in the second phase of the production, glucose fed-batch phase, all of the comparisons were given for that phase and, although it is $t \approx
20 h of the overall production phase, $t = 0$ h is taken as the beginning of this phase for the sake of simplicity in presentation and discussion of the results.

Variation of dissolved oxygen concentration in the fermentation medium with cultivation time is given in Figure 13. For constant OTC processes, it can clearly be seen that there is a strong connection between $C_{\text{DO}}$ and $C_X$. As cell concentration increases, $C_{\text{DO}}$ decreases and eventually drops to zero. Afterwards, when cells enter stationary phase, $C_{\text{DO}}$ starts to increase again, even reaching higher concentrations than the starting values. For constant $C_{\text{DO}}$ experiments, it was possible to keep $C_{\text{DO}}$ constant at the desired value with $\pm 0.02$ mmol L$^{-1}$ (~4 – 5%) deviation while this deviation is slightly higher in CDO$_5$. Maintaining 40% air saturation is not possible by only air supplementation, even at the earlier hours of the bioprocess. Introducing pure oxygen along with air causes some fluctuations, which causes deviations in $C_{\text{DO}}$ levels. $\pm 0.02$ mmol L$^{-1}$ deviation might be acceptable for most of the processes but it is rather too much deviation for CDO$_1$ strategy. A more robust control system is necessary if the process is to be conducted at low $C_{\text{DO}}$ levels.

**Figure 13.** Variation of dissolved oxygen concentration in the fermentation medium with cultivation time. OTC$_1$ (solid line), OTC$_2$ (dashed line), OTC$_3$ (long dashed and dotted line).
Figure 14. Variation of dissolved oxygen concentration in the fermentation medium with cultivation time. CDO₁ (dashed and dotted line), CDO₂ (square dotted line), CDO₃ (solid line), CDO₄ (long dashed line), CDO₅ (round dotted line).

4.1. GI Production with Constant Oxygen Transfer Condition

Effects of constant OTC were investigated by three experiments (Table 9) and the operation conditions were compared with each other in terms of cell concentration, glucose accumulation, GI and protease activities.

The variations in cell concentration with cultivation time at constant OTC are presented in Figure 13. Among the applied strategies, the highest cell concentration was obtained in OTC₁ as 35 g L⁻¹; whereas, the highest cell concentration decreased to 28 g L⁻¹ both in OTC₂ and OTC₃ with the increase in air flow rate. This can be explained by the excess amount of oxygen present in the fermentation medium, especially at the earlier hours of the process. High oxygen concentrations induce oxidative stress which can result in metabolism shift and retard cell growth (Baumann et al. 2011).
Figure 15. Variations in cell concentration with the cultivation time in constant OTC bioprocesses. OTC₁ (●), OTC₂ (■), OTC₃ (▲).

Glucose started to accumulate in the medium after t = 12 h in OTC₃, t = 9 h in OTC₁, and t = 6 h in OTC₂ (Figure 16). As stated by Baumann et al. (2008) oxygen limitation did not directly decrease substrate consumption. Glucose concentration in the fermentation medium is related with specific glucose uptake rate of the cells. As a result of high oxygen availability, the highest glucose uptake rate of the cells was obtained in OTC₃.

GI activity profiles of OTC₁, OTC₂ and OTC₃ are given in Figure 17. The overall highest GI activity was achieved with OTC₁ strategy as 3100 U L⁻¹ while the highest GI activity obtained in OTC₃ is considerably low, 1200 U L⁻¹. GI activity mainly depends on GI monomer production and the correct folding of GI subunits to obtain active GI homotetramer structure. As P_{GAP} is located in the glycolysis pathway, glucose isomerase production is directly related with the response of glycolysis pathway to environmental changes.
Figure 16. Variations in glucose concentration with the cultivation time for constant OTC bioprocesses. OTC$_1$ (●), OTC$_2$ (■), OTC$_3$ (▲).

In that context, oxygen limitation is reported to increase regulation of glycolytic genes and consequently the activity of P$_{GAP}$ (Baumann et al. 2010). High oxygen concentrations resulting from high oxygen transfer conditions throughout the process may cause oxidative stress (Gasser et al. 2008) and decrease GI activity through unfolded protein response (UPR). Although GI is secreted as monomer subunits, it seems that it could not form its active conformation in the extracellular medium. Extreme pH, high osmolarity and dissolved oxygen concentration may result in problems during the formation of active GI configuration.

The highest acidic proteolytic activity was obtained as 143 U mL$^{-1}$ in OTC$_3$, by which the lowest GI activity was attained (Figure 18). This was the expected result since protease activity is inversely related to GI activity. For OTC$_1$ and OTC$_2$, a direct correlation could not be drawn between GI and protease activity since GI activity was higher in OTC$_1$ and protease activity was lower in OTC$_2$ because proteolytic activity is not the only factor affecting GI activity (Figure 19). However,
if proteolytic degradation could have been prevented, higher GI activities would have been obtained by using OTC₁ strategy.

**Figure 17.** Variations in GI activity with the cultivation time for constant OTC bioprocesses. OTC₁ (●), OTC₂ (■), OTC₃ (▲).

**Figure 18.** Variations in protease activity with the cultivation time for constant OTC bioprocesses. OTC₁ (●), OTC₂ (■), OTC₃ (▲).
Figure 19. Comparative representation of the variations of protease (solid lines) and GI (dashed lines) activities with the cultivation time for constant OTC bioprocesses. OTC1 (●), OTC2 (■), OTC3 (▲).

4.2. GI Production with Constant Dissolved Oxygen Concentration

Figure 20 shows the cell concentration profiles of CDO1, CDO2, CDO3, CDO4 and CDO5. Among the strategies based on dissolved oxygen concentration, the highest cell concentration was achieved with CDO4 as 44 g L\(^{-1}\) at t = 9 h. At all C\(_{DO}\) conditions, the bioprocesses entered stationary phase between t = 9 – 12 h. Lower dissolved oxygen concentrations resulted in lower cell concentrations.

The highest glucose accumulation was observed in CDO1 strategy (88 g glucose L\(^{-1}\)), with CDO5 being a close second with 85 g glucose L\(^{-1}\) at t=15 h of the bioprocesses (Figure 21). Although the cell concentrations were relatively higher with higher DO, the glucose consumption rate decreased with increasing DO. The least glucose accumulation was obtained by CDO4 strategy which was expected since the parameters of the glucose feeding strategy were optimized at dissolved oxygen concentration of 20% air saturation. As the cells were obviously not in the exponential growth phase after t = 12 h, glucose accumulation rate is the highest.
between $t = 12 - 15$ h. If the processes had been conducted for 12 h, negligible amounts of glucose would have been accumulated in all CDO strategies except for CDO$_1$. Since the lowest cell concentration was obtained in CDO$_1$, high concentrations of glucose in the fermentation media were expected.

![Graph showing cell concentration variations with cultivation time for CDO bioprocesses.](image)

**Figure 20.** Variations in cell concentration with the cultivation time for CDO bioprocesses. CDO$_1$ (o), CDO$_2$ (□), CDO$_3$ (x), CDO$_4$ (◊), CDO$_5$ (Δ).

The highest GI activity was obtained when DO was kept constant at 15%, as 4440 U L$^{-1}$. The lowest GI activity was obtained with the highest CDO, *i.e.*, CDO$_5$. High oxygen supply to meet the oxygen demand may have caused oxidative stress on the cells and may have been resulted in unfolded or misfolded protein response. Similar to CDO$_5$, keeping DO constant at 20% (CDO$_4$) also resulted in rather low GI activity.
Figure 21. Variations in glucose concentration with the cultivation time for CDO bioprocesses. CDO\(_1\) (o), CDO\(_2\) (□), CDO\(_3\) (x), CDO\(_4\) (◊), CDO\(_5\) (Δ).

Similar to constant OTC strategies, relatively low protease activities were obtained in DO-stat strategies (Figure 24). The highest protease activity was obtained as 147 U mL\(^{-1}\) with CDO\(_5\) while the second highest protease activity was 146 U mL\(^{-1}\) with CDO\(_1\). The highest GI activity was obtained when proteolytic activity was minimum (CDO\(_3\)), nonetheless, in the case of CDO\(_5\), GI activity was not relatively high when proteolytic activity was almost as low as in CDO\(_3\). It is clearly known that GI production is inversely proportional to proteolytic activity, however, a straightforward conclusion on the correlations between protease activity and GI activity could not be drawn (Figure 25).
Figure 22. Variations in GI activity with the cultivation time for CDO bioprocesses. CDO\(_1\) (o), CDO\(_2\) (□), CDO\(_3\) (x), CDO\(_4\) (◊), CDO\(_5\) (Δ).

Figure 23. Comparison of maximum activity reached in each CDO bioprocess.
Figure 24. Variations in protease activity with the cultivation time for CDO bioprocesses. CDO\(_1\) (o), CDO\(_2\) (□), CDO\(_3\) (x), CDO\(_4\) (◊), CDO\(_5\) (Δ).

Figure 25. Comparative representation of variations in protease (solid lines) and GI (dashed lines) activities with the cultivation time for CDO bioprocesses. CDO\(_1\) (o), CDO\(_2\) (□), CDO\(_3\) (x), CDO\(_4\) (◊), CDO\(_5\) (Δ).
4.3. Comparison of Constant Oxygen Transfer Condition and Constant Dissolved Oxygen Concentration Strategies

To systematically investigate the effect of oxygen transfer conditions of GI activity and production, findings of all designed strategies were compared and the results are discussed throughout this section.

4.3.1. Cell Concentration and Specific Cell Growth Rate

Glucose fed-batch phase was started when glycerol is depleted in the production medium (BSM). This depletion was understood by monitoring the cell concentration since it reaches about 15-20 g L\(^{-1}\) when glycerol is depleted. Therefore, glucose fed-batch phase was started more or less at the same cell concentration in all experiments. This is also important to be able to start the production while cells are at the same phase.

As shown in Figure 26, for the first three hours of the processes, the cell concentrations were approximately the same. These first three hours can be considered as the adaptation of the cells to the change of the carbon source to glucose. Since the initial cell concentrations were the same, their response to the shift of the carbon source can be evaluated to be roughly the same.

In all strategies, the highest cell concentrations were reached at \(t = 9\) h of the glucose fed-batch phase; the highest of which is 44 g L\(^{-1}\) obtained in \(C_{DO4}\) strategy. Since the predetermined specific growth rate was optimized according to the oxygen transfer conditions such that \(C_{DO} = 20\%\) at all times (Keskin, 2014), obtaining the highest cell growth rate in \(CDO4\) strategy was the expected result.

Although the cell concentration profiles were similar for the first three hours, the specific cell growth rate (\(\mu\)) values differ for each strategy (Figure 27) even though the predetermined specific growth rate was the same in all strategies. The reason for this phenomenon is the behavior of the cells after the third hour of the process. Different increments in the increase of the cell concentrations after the third hour resulted in dissimilar differential values for the calculation of the specific cell growth
rate. Since overall cell concentrations were lower in constant OTC strategies, lower specific cell growth rates were obtained at the earlier hours of those bioprocesses.

Since the processes lasted as short as 15 h, the highest cell concentrations are lower than the cell concentrations obtained in the other strategies. Guan et al. (2013) obtained 75 g L\(^{-1}\) cell concentration after 60 h of fermentation; whereas, 150 g L\(^{-1}\) cell concentration was reached after 90 h of fermentation by Pepeliaev et al. (2011). On the other hand, Khasa et al (2007) obtained 33.5 g L\(^{-1}\) after 40 h.

![Graph showing variations in cell concentration with cultivation time, comparison of all experiments. OTC\(_1\) (●), OTC\(_2\) (■), OTC\(_3\) (▲), CDO\(_1\) (○), CDO\(_2\) (□), CDO\(_3\) (×), CDO\(_4\) (◊), CDO\(_5\) (Δ).](image)

At the earlier hours of the bioprocesses, \(\mu\) values changed between \(\mu = 0.13 - 0.16\) h\(^{-1}\) for CDO strategies while it was between \(\mu = 0.07 - 0.09\) h\(^{-1}\) for constant OTC strategies. Keeping in mind that predetermined specific cell growth rate was \(\mu = 0.15\) h\(^{-1}\), the cells are theoretically expected to grow with that rate throughout the bioprocess. In CDO strategies, the cells tend to obey that predetermined value more than constant oxygen transfer condition strategies.
Figure 27. Variations in the specific growth rate with the cultivation time. OTC₁ (●), OTC₂ (■), OTC₃ (▲), CDO₁ (○), CDO₂ (□), CDO₃ (◊), CDO₄ (●), CDO₅ (Δ).

4.3.2. Glucose Concentration and Specific Glucose Consumption Rate

Since the cell growth rates were closer to the theoretical value, no glucose accumulation was observed at the beginning of all strategies. The highest glucose concentration was obtained as 171 g L⁻¹ in OTC₂ strategy. Relatively higher glucose accumulations were observed in constant OTC strategies.

Glucose accumulation typically started when cells entered stationary phase, between t = 9 - 12 h except for OTC₂, where the highest glucose accumulation was detected. Interestingly, although OTC₂ and OTC₃ strategies showed similar cell growth trends, OTC₂ resulted in two-fold more glucose accumulation compared to OTC₃, because of higher oxygen transfer rate in OTC₃. The least glucose accumulation was obtained by CDO₄ as it is the most optimum oxygen condition for the parameters of the feeding profile (Figure 28).
Specific substrate consumption rates \((q_S)\) were similar at the beginning of all strategies since the parameters affecting were similar. The highest specific substrate consumption rate was obtained at \(t = 15\) h as \(q_S = 0.83\) g glucose g\(^{-1}\) cell h\(^{-1}\) with CDO\(_4\) strategy. \(q_S\) values increased in CDO\(_3\) and CDO\(_2\) while they were more or less constant in CDO\(_2\) around \(q_S = 0.30\) g glucose g\(^{-1}\) cell h\(^{-1}\) and decreased with cultivation time in other strategies.

**Figure 28.** Feeding profile of glucose solution according to Equation 34.
Figure 29. Variations in glucose concentration in the fermentation medium with the cultivation time. OTC<sub>1</sub> (●), OTC<sub>2</sub> (■), OTC<sub>3</sub> (▲), CDO<sub>1</sub> (○), CDO<sub>2</sub> (□), CDO<sub>3</sub> (x), CDO<sub>4</sub> (◊), CDO<sub>5</sub> (Δ).

Figure 30. Variations in the specific substrate consumption rate with the cultivation time. OTC<sub>1</sub> (●), OTC<sub>2</sub> (■), OTC<sub>3</sub> (▲), CDO<sub>1</sub> (○), CDO<sub>2</sub> (□), CDO<sub>3</sub> (x), CDO<sub>4</sub> (◊), CDO<sub>5</sub> (Δ).
4.3.3. GI Activity and Specific GI Activity

Figure 31 shows variation in GI activity with the cultivation time for each strategy applied. The highest GI activity was obtained when dissolved oxygen concentration was kept constant at 15% air saturation, CDO₃, as 4440 U L⁻¹, being 1.4-fold higher than the second highest activity, which was obtained by CDO₁. Comparable results were obtained for the strategies CDO₁, CDO₂ and OTC₁. Lower activity values obtained in the strategies where oxygen concentrations were relatively higher; OTC₂, OTC₃, CDO₄ and CDO₅. High oxygen transfer rate may cause oxidative stress within the cells, which eventually results in UPR and disturbs secretion of the proteins to extracellular medium (Sjöblom et al. 2008).

![Figure 31. Variations in GI activity with the cultivation time. OTC₁ (●), OTC₂ (■), OTC₃ (▲), CDO₁ (○), CDO₂ (□), CDO₃ (x), CDO₄ (◊), CDO₅ (Δ).](image)

Specific GI activity, q_GI, is defined as GI activity obtained per gram cell. This value is evaluated to gain an insight about the active GI production capacity of the cells. q_GI values for each strategy are given in Figure 32. When specific activities were compared, CDO₁, CDO₃ and OTC₂ showed very similar peak values, followed by

77
OTC₁ and CDO₂. The lowest specific activities were obtained from OTC₃, CDO₄ and CDO₅. The highest specific activity obtained was 126 U g⁻¹ cell by CDO₃ strategy.

In *P. pastoris* fermentations, dissolved oxygen concentration is conventionally kept constant at moderate conditions, around 20 – 30% saturation but recent findings showed that low oxygen availability and even hypoxia can enhance cells’ metabolic pathway for recombinant protein production (Baumann *et al.* 2008). Results obtained in this study also support Baumann and her co-workers’ findings in terms of recombinant protein production and oxygen availability.

![Variations in the specific GI activity with the cultivation time. OTC₁ (●), OTC₂ (■), OTC₃ (▲), CDO₁ (○), CDO₂ (□), CDO₃ (●), CDO₄ (◊), CDO₅ (Δ).](image)

**Figure 32.** Variations in the specific GI activity with the cultivation time. OTC₁ (●), OTC₂ (■), OTC₃ (▲), CDO₁ (○), CDO₂ (□), CDO₃ (●), CDO₄ (◊), CDO₅ (Δ).

Ata *et al.* (2015) investigated the effect of feeding strategies on GI production by *P. pastoris* under *P*ₐₒₓ₁ and obtained 32500 U L⁻¹ activity at *t* = 53 h while the specific activity was ca. 270 U g⁻¹ cell when methanol concentration in the medium was kept constant at 5 g L⁻¹. Under *P*ₐₒₓ₁, GI activity was 7-fold and specific GI activity was 2-fold higher than the highest values obtained in this study, with an expense of longer cultivation time. Although 7-fold low GI activities were obtained under *P*_ₐₙₚ,
performances of individual cells, as evaluated by specific GI activity, only 2-fold lower. Performance of individual cells can be enhanced by fine-tuning the bioreactor operation parameters; which would result in higher specific GI activities. If more efficient feeding strategies are enabled in the future; comparable or even higher productivities can be obtained by $P_{GAP}$, than $P_{AOX1}$ in more than 3-fold shorter fermentation times.

Akdağ and Çalık (2014) investigated the effect of untreated beet-molasses feeding strategies on GI production by sucrose-utilizing *E. coli*. The highest GI activity was obtained as 35270 U L$^{-1}$ at $t = 17$ h. Although high GI activities were obtained by *E. coli* at comparably early hours of the process; intracellular production of GI makes *E. coli* an undesirable host microorganism when downstream processing is taken into consideration.

### 4.3.4. GI Monomer Concentration: SDS-PAGE

To determine the effect of oxygen availability on GI monomer concentration, SDS-PAGE analysis was done. Figure 33 shows the comparison of GI monomers produced in each strategy on a silver stained SDS-PAGE gel. When the intensities are compared, it is seen that although GI activity is almost 2-fold higher in CDO$^3$, monomer concentration of GI produced by CDO$^4$ is almost the same as that produced by CDO$^3$. This result indicates a problem with the formation of active homotetramer structure of the GI monomer after secretion to the extracellular medium. It can clearly be deducted that even though cells have a higher capacity of GI production with CDO$^4$, monomers produced and secreted remain as subunits instead of forming correct GI homotetramer conformation when dissolved oxygen concentration in the fermentation medium is high.

In OTC$^3$ strategy, the cell concentration, GI activity and secreted GI monomer concentration remained at comparatively low levels. Although high substrate consumption rates were obtained, attaining low specific growth rate and low productivity can be the consequence of intracellular protein accumulation due to high
oxidative stress that might have been resulted from high oxygen transfer rates (Hohenblum et al. 2003).

Figure 33. Silver stained SDS-PAGE gel images of the applied strategies at t = 15 h. From left to right: Marker, OTC1, OTC2, OTC3, CDO1, CDO2, CDO4, CDO5, and CDO3.

4.3.5. Proteolytic Activity

Protease activities with respect to the cultivation time are given in Figure 34. Only acidic protease activity was evaluated since bioprocesses were conducted within pH = 5.0 – 5.5. Analogous profiles were obtained in each process. The highest proteolytic activity was obtained as 147 U mL\(^{-1}\) in CDO4; being almost the same as OTC3. Evaluating proteolytic activity is important for the fermentation processes since the secretion of protease enzymes degrades produced recombinant proteins and decreases the final product yield.
In all strategies, the highest GI activities were obtained during the last hour of the cultivation. Even though cells enter stationary and/or death phase, GI production continues to increase and proteolytic degradation is negligible.

4.3.6. Organic Acid Concentrations

Organic acid concentrations were analyzed for each strategy and given in Figure 35 through Figure 42 and tabulated in APPENDIX E. The most excreted organic acid was acetic acid, which was present in various concentrations in every bioprocess. Pyruvic acid was hardly detected in all processes. In low-to-moderate oxygen transfer conditions: gluconic, lactic and the organic acids involved in TCA cycle show higher tendency of accumulation.

Gluconic acid could not be detected in the strategies where moderate oxygen transfer conditions were applied. It accumulated to rather high concentrations in OTC₁, OTC₂ and CDO₁ strategies. Gluconic acid is used by pentose phosphate pathway (PPP) of
*P. pastoris* when oxygen and substrate availability is at moderate conditions. During starvation and hypoxia, gluconic acid cannot enter PPP and starts to accumulate. Therefore, incomplete glucose oxidation can be the reason for gluconic acid accumulation.

Lactic acid is produced during oxygen limitation because of the shift of pathway from fully-aerobic to anaerobic conditions. Lactic acid mostly accumulated in relatively lower oxygen transfer conditions whereas no lactic acid accumulation was observed in C_{DO4} and C_{DO5} strategies. Generally, lactic acid started to accumulate when DO dropped to almost zero for constant OTC strategies whereas it could be detected at all times with increasing concentration for CDO strategies where C_{DO} is comparatively low.

Pyruvic acid was almost never detected, indicating that fluxes through pyruvate node are high enough to prevent accumulation. Acetic acid was almost always detected in every strategy with increasing concentrations with cultivation time.

Other organic acids; citric-, malic-, succinic- and fumaric acids are involved in TCA cycle. Oxygen is essential for TCA cycle to be able to work properly. Similar to other oxygen-dependent metabolites, they accumulated less in higher oxygen availability, and more in rather low oxygen availability due to the low efficiency of aerobic respiration.
Figure 35. Variations in organic acid concentrations with the cultivation time for OTC\textsubscript{1} strategy.

Figure 36. Variations in organic acid concentrations with the cultivation time for OTC\textsubscript{2} strategy.
Figure 37. Variations in organic acid concentrations with the cultivation time for OTC\textsubscript{3} strategy.

Figure 38. Variations in organic acid concentrations with the cultivation time for CDO\textsubscript{1} strategy.
Figure 39. Variations in organic acid concentrations with the cultivation time for CDO$_2$ strategy.

Figure 40. Variations in organic acid concentrations with the cultivation time for CDO$_3$ strategy.
Figure 41. Variations in organic acid concentrations with the cultivation time for CDO$_4$ strategy.

Figure 42. Variations in organic acid concentrations with the cultivation time for CDO$_5$ strategy.
Organic acid concentrations in the fermentation medium should be examined to gain a further understanding of the bioprocesses and possible limitations of the targeted protein production. Availability of organic acids is of utmost importance as most of the pathways, especially central carbon metabolism, is directly affected by their presence. Accumulation of these organic acids should be controlled by fine-tuning the bioreactor operation conditions for recombinant protein production since it may decrease the fluxes towards the recombinant protein production; furthermore, result in the waste of raw materials and lower specific cell growth rates as cell concentration cannot increase further.

4.3.7. Oxygen Transfer Characteristics

In order to investigate the effect of oxygen transfer characteristics on GI production, volumetric mass transfer coefficient, oxygen transfer rate, oxygen uptake rate, maximum possible oxygen transfer and uptake rates, Damköhler number, and effectiveness factor were calculated. Variation of oxygen transfer characteristics are given in Table 11. Parameters involved in oxygen transfer could only be calculated for the first six hours of the bioprocesses. Due to high demand of oxygen resulting from high cell concentrations, it was not possible to apply dynamic method to evaluate the parameters for the proceeding hours.

Volumetric mass transfer coefficient depends on superficial gas velocity, power input to the broth and medium properties such as viscosity. Therefore, $K_{La}$ can be considered the same when aeration and agitation rates, and the broth composition are the same. By this assumption, all CDO experiments, at least for the zeroth hour, should perform the same oxygen transfer characteristics as aeration and agitation rates are almost the same, as well as cell concentration and fermentation medium composition. When Table 11 is analyzed, it is seen that this assumption is somewhat correct. In the case of constant OTC experiments, since air flow rate differs among the strategies, different $K_{La}$ values were expected. Peculiarly, exactly the same $K_{La}$ values were obtained at $t = 0$ h for constant OTC strategies. For the consecutive
hours, the highest $K_{L,a}$ values were obtained in OTC$_3$ strategy as 0.054 s$^{-1}$ and 0.083 s$^{-1}$ for $t = 3$ h and $t = 6$ h of the process, respectively. As cell concentration increases, volumetric uptake rate of oxygen, OUR, increases. The highest OUR was obtained from OTC$_3$ at $t = 6$ h as 0.029 mol m$^{-3}$ s$^{-1}$.

The ratio of the oxygen uptake rate to oxygen demand is defined as the effectiveness factor. Closer its value gets to 1, more efficient the oxygen uptake process is. The highest effectiveness factor was obtained in CDO$_5$ strategy as $\eta = 0.77$ at $t = 0$ h. Except from CDO$_5$, at the beginning of the processes, all strategies showed similar efficiencies around 0.50 - 0.60 and typically decreased with cultivation time because of higher demand of oxygen.

In the cases where oxygen supply is high, Da is less than 1 for the first hours of the bioprocesses. As time passes and oxygen demand increases, Da becomes more than 1 in all strategies.

Specific oxygen uptake rate is defined as the mass of oxygen up-taken by unit cell per time. While $q_O$ values increased with time for constant OTC processes, they stayed somewhat the same through the time course for CDO processes. This was the expected result since in CDO strategies, oxygen supply is controlled by the dissolved oxygen concentration readily in the broth, which indirectly connected to cell concentration. As cell concentration increases, oxygen supply increases accordingly, resulting in constant oxygen uptake rate by individual cells. On the other hand, oxygen is supplied at a constant rate, regardless of the cell concentration in constant OTC processes; resulting in increased $q_O$ values.
Table 11. Variations of oxygen transfer characteristics with cultivation time.

<table>
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<th>Time</th>
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<td>8.51</td>
<td>14.1</td>
<td>20.0</td>
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<td>11.0</td>
<td>19.7</td>
</tr>
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<td>107</td>
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<td>30.7</td>
</tr>
<tr>
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<td>12.6</td>
<td>28.9</td>
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<td>15.9</td>
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<tr>
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<td>45.3</td>
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<td>$Da$</td>
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<td>1.86</td>
<td>1.30</td>
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<td>0.042</td>
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<tr>
<td>$OUR \times 10^3$ mol m$^{-3}$ s$^{-1}$</td>
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<td>10.8</td>
<td>14.5</td>
</tr>
<tr>
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<td>14.1</td>
<td>34.3</td>
</tr>
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<td>0.76</td>
<td>0.42</td>
</tr>
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<td>$Da$</td>
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</tr>
<tr>
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<table>
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<td>8.70</td>
<td>13.7</td>
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<tr>
<td>$OTR_{\text{max}} \times 10^3$ mol m$^{-3}$ s$^{-1}$</td>
<td>3.70</td>
<td>10.4</td>
<td>16.7</td>
</tr>
<tr>
<td>$OUR \times 10^3$ mol m$^{-3}$ s$^{-1}$</td>
<td>3.14</td>
<td>8.70</td>
<td>13.7</td>
</tr>
<tr>
<td>$OUR_{\text{max}} \times 10^3$ mol m$^{-3}$ s$^{-1}$</td>
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<td>11.6</td>
<td>30.0</td>
</tr>
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<td>0.59</td>
<td>0.75</td>
<td>0.46</td>
</tr>
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<td>$Da$</td>
<td>1.45</td>
<td>1.12</td>
<td>1.80</td>
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Table 11. Continued.

<table>
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</thead>
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<tr>
<td></td>
<td>0 h</td>
<td>3 h</td>
<td>6 h</td>
<td></td>
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<td>$K_{l,a}$ s^{-1}</td>
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<tr>
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<td>11.1</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>$OUR \cdot 10^3$ mol m^{-3} s^{-1}</td>
<td>5.92</td>
<td>8.67</td>
<td>12.7</td>
<td></td>
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<td>12.9</td>
<td>20.2</td>
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<tr>
<td>Da</td>
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<td>1.16</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>$q_O$ g oxygen g^{-1} cell h^{-1}</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>$K_{l,a}$ s^{-1}</td>
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<td>0.03</td>
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<td>11.1</td>
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<td>0.04</td>
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</tbody>
</table>
4.3.8. Yield Coefficients

Yield coefficients were calculated for the exponential phase of the bioprocesses. Overall and instantaneous yield with respect to each strategy are given in Table 12.

Since the highest cell concentration was reached in CDO₄ strategy, the highest overall $Y_{XS}$ is obtained by CDO₄ as well. Since the amount of glucose fed is the same for all experiments and the amount of the glucose utilized can be assumed to be the same for the first nine hour of the processes, the only parameter affecting the cell yield on substrate is the cell concentration. It is important to note that theoretical cell yield on glucose is 0.48 g g⁻¹ (Cos et al. 2005) when $C_{DO}$ is kept constant at 20% air saturation. Indeed $Y_{XS} = 0.48$ g g⁻¹ was achieved by that strategy.

The highest $Y_{X/O}$ value was obtained by OTC₂ strategy as 5.45 g g⁻¹ at the beginning of the bioprocess. The highest $Y_{X/O}$ values were all obtained at the beginning of the production due to increasing OUR values at the proceeding hours of the bioprocesses.
Table 12. Instantaneous and overall yield coefficients

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Time (h)</th>
<th>( Y_{XS} ) (g cell g(^{-1}) substrate)</th>
<th>( Y_{X/O} ) (g cell g(^{-1}) oxygen)</th>
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<td>1.12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.23</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td><strong>0.33</strong></td>
<td>-</td>
</tr>
<tr>
<td>OTC(_2)</td>
<td>0</td>
<td>0.21</td>
<td>3.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.24</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.18</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.20</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
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<td><strong>0.19</strong></td>
<td>-</td>
</tr>
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<td>0.19</td>
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</tr>
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<td>1.29</td>
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<td></td>
<td>9</td>
<td>0.01</td>
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<tr>
<td>Overall</td>
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<td>2.10</td>
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<td>0.45</td>
<td>2.25</td>
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<td>1.22</td>
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<tr>
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<td>-</td>
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Table 12. Continued.

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<th>Strategy</th>
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<th>(Y_{X/S}) g cell g(^{-1}) substrate</th>
<th>(Y_{X/O}) g cell g(^{-1}) oxygen</th>
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<tbody>
<tr>
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<td>1.79</td>
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<td></td>
<td>9</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td><strong>0.23</strong></td>
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<td>-</td>
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CHAPTER 5

CONCLUSION

For aerobic organisms, oxygen transfer is essential and optimization of oxygen transfer conditions play a very important role to obtain high productivities. *P. pastoris* fermentations are conventionally conducted at 20 - 30% air saturation and more recent findings show that lower oxygen concentrations enabled higher product titers. The disagreement on oxygen transfer conditions in *P. pastoris* fermentations prompted us to investigate the effect of oxygen transfer conditions on glucose isomerase production by *P. pastoris* under P<sub>GAP</sub>. To analyze the response of *P. pastoris* cells to oxygen transfer conditions; cell concentration, glucose accumulation, GI activity and monomer concentration, proteolytic degradation and organic acid profiles were presented. Designed and applied strategies were also analyzed in terms of oxygen transfer and bioprocess characteristics such as specific formation and consumption rates, as well as overall and instantaneous yields.

Among all strategies, the highest cell concentration was obtained in CDO<sub>4</sub> as 44 g L<sup>-1</sup> at t = 9 h. Since the parameters determining the feeding rate of glucose was optimized according to 20% air saturation of the fermentation medium, obtaining the highest cell density by CDO<sub>4</sub> strategy was the expected result. Overall lowest cell concentrations were obtained in OTC<sub>2</sub> and OTC<sub>3</sub> strategies. It may be speculated that the excessive abundance of oxygen at the earlier hours of the bioprocesses result in a shift in metabolic pathway and represses cell growth.

During the exponential phase of cell growth, glucose accumulation was prevented since the glucose uptake rate was equal to glucose feeding rate; except for OTC<sub>2</sub>. The
The highest specific glucose consumption rate was obtained as $q_S = 0.80$ g glucose g$^{-1}$ cell h$^{-1}$ by CDO$_4$ strategy.

The highest GI activity was achieved in CDO$_3$ strategy as 4440 U L$^{-1}$, which is 1.4-fold higher than the second highest GI activity achieved. The highest specific GI activity was also obtained by CDO$_3$ strategy as 126 U g$^{-1}$ cell. Lower activity values were obtained in the strategies where oxygen had higher abundance, such as OTC$_2$, OTC$_3$, CDO$_4$ and CDO$_5$ strategies. Lower oxygen availability probably increases transcription levels of the genes involved in glycolysis pathway which may be the reason for the increased productivities with low-to-moderate oxygen transfer conditions under $P_{GAP}$. Although the highest cell concentrations were reached at $t = 9$ h of each strategy, GI activity continued to increase even at the stationary phase, reaching its peak at $t = 15$ h. When the duration of bioprocesses was extended, GI activity decreased as well as cell concentration (data not shown), therefore the bioprocesses were kept as short as fifteen hours.

By CDO$_1$ strategy, the second highest GI activity was obtained. However, there is considerable amount of glucose accumulation due to high rates of glucose feeding. Since glucose accumulation may cause substrate inhibition to cells, glucose feeding rate should be decreased in order to prevent accumulation. If the parameters of predetermined exponential feeding equation are optimized, better results might be obtained in terms of both cell growth and GI activity. It is important to note that keeping dissolved oxygen concentration at low saturation levels such as 5% is challenging and a robust control system for the air flow is necessary such as a PID control system.

SDS-PAGE analysis showed that even though CDO$_4$ had almost the same GI monomer concentration as CDO$_3$, the activity obtained from CDO$_3$ strategy was almost 2-fold higher than that from CDO$_4$. Reasons for obtaining low GI activity in spite of having high monomer concentration should be studied deeper to overcome the problem and obtain higher productivities.
Organic acids involved in TCA cycle showed high abundance in the strategies with low-oxygen availability, which was the expected result as oxygen is essential for TCA cycle to work properly. Pyruvic acid was hardly detected during fermentation, indicating the fluxes going into and out of pyruvate node are high enough to prevent accumulation. Although organic acid concentrations give an insight about the central metabolism of the cells, doing metabolic flux analysis would be more beneficial in order to reduce speculation levels. Along with metabolic flux analysis, proteome and transcriptome studies would be valuable to have a deeper insight of the up and downregulations within the cells with changing oxygen transfer conditions.

The bioprocesses were analyzed in terms of oxygen uptake efficiencies. The highest oxygen uptake efficiency was achieved by CDO5 strategy as 0.77 at the beginning of the bioprocess. Also in other strategies, the highest levels of efficiencies were obtained at the earlier hours of the processes and their values ranged between 0.50 – 0.60.

When overall cell yields on substrate were calculated for each strategy, it is seen that only CDO4 reached the $Y_{X/S}$ value used as the parameter of the substrate feeding equation. Different overall yields were obtained for different oxygen transfer strategies, indicating that $Y_{X/S}$ parameter, along with other parameters, should be optimized in the feeding equation according to the oxygen transfer strategy to be applied.

Compared to $P_{AOX1}$-driven expression systems, using $P_{GAP}$ for GI production considerably decreased cultivation time. Although as high GI activities could not be reached, constitutive nature of $P_{GAP}$, shortening of the cultivation time and elimination of methanol usage make $P_{GAP}$ a favorable alternative to $P_{AOX1}$ for recombinant protein production under $P. pastoris$. 


Bhosale, S. H., Mala, B.L., Deshpande, V.V., 1996. “Molecular and industrial aspects of glucose isomerase.” Microbiological Reviews 60(2):280-300.


metabolism to bioreactor operation parameters.” *Biochemical Engineering Journal* 95:20-36.


Sears, I.B., O’Connor, J., Rossanese, O.W., Glick, B.S., A versatile set of vectors for constitutive and regulated gene expression in Pichia pastoris.” Yeast 14:783-790.


APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Fermentation Media

1 M Potassium phosphate buffer, pH= 6.0
56.48 g KH$_2$PO$_4$ and 14.8 g K$_2$HPO$_4$ were dissolved in dH$_2$O and the volume is completed to 500 ml. The solution is sterilized by autoclave and stored at room temperature.

Antifoam solution
100 ml, 10% antifoam solution is prepared with dH2O and sterilized by autoclave.

Base
25% NH$_3$OH. No need to sterilize.

SDS-PAGE Solutions

Resolving gel
6 ml BioRad FastCast® resolving gel solution, 50 μl ammonium persulfate and 5 μl N,N,N’,N’-tetramethylethylenediamine are mixed and poured into

Stacking gel
3 ml BioRad FastCast® stacking gel solution, 25 μl ammonium persulfate and 5 μl N,N,N’,N’-tetramethylethylenediamine.

4X SDS-PAGE Sample loading buffer
200 mM Tris-HCl, pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue; 10% 2- mercaptoethanol. Store at -20°C.
**5X SDS-PAGE Running buffer**

15 g Tris Base, 72 g glycine, 5 g SDS, dH₂O to 1 liter. Store at +4°C.

**Silver Staining Solutions**

**Fixer**

Mix 100 ml methanol, 24 ml acetic acid, 100 μl 37% formaldehyde and complete to 200 mL with dH₂O. Store at room temperature up to 1 month.

**Pretreatment**

Dissolve 0.05 g Na₂S₂O₃·5H₂O in 250 ml distilled water by mixing with a glass rod. Take 2 ml and set aside for further use in developing solution preparation.

**Silver nitrate**

Dissolve 0.2 g silver nitrate in 100 ml dH₂O and add 75 μl 37% formaldehyde.

**Developing**

Dissolve 2.25 g potassium carbonate in 100 mL dH₂O. Add 2 ml from pretreatment solution and 75 μl 37% formaldehyde.

**Stop**

Mix 50 ml ethanol, 12 ml acetic acid and dilute to 100 ml with dH₂O.

**Protease Assay Solutions**

**0.05 M Sodium acetate buffer**

Mix 0.713 ml acetic acid in 25 ml dH₂O. Dissolve 2.052 g sodium acetate in 50 ml dH₂O. Titrate sodium acetate solution with acetic acid solution until pH = 5.0 and dilute to 500 ml. Autoclave and store at +4°C.
Figure 43. Calibration curve for glucose concentration

The graph shows a linear relationship between absorbance at 565 nm and glucose concentration, given by the equation:

\[ y = 0.5716x \]

with \( R^2 = 0.9995 \).
# SILVER STAINING PROCEDURE

<table>
<thead>
<tr>
<th>Step</th>
<th>Time of Treatment</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing</td>
<td>&gt; 1 h</td>
<td>Overnight treatment is acceptable.</td>
</tr>
<tr>
<td>Washing</td>
<td>3 x 20 min</td>
<td>Solution must be freshly prepared.</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>1 min</td>
<td>Solution must be freshly prepared.</td>
</tr>
<tr>
<td>Rinsing</td>
<td>3 x 20 s</td>
<td>Timing must be exact.</td>
</tr>
<tr>
<td>Impregnation</td>
<td>20 min</td>
<td>Solution must be freshly prepared.</td>
</tr>
<tr>
<td>Rinsing</td>
<td>3 x 20 s</td>
<td>Timing must be exact.</td>
</tr>
<tr>
<td>Developing</td>
<td>As long as the desired bands can be seen. (~5 min). Time should be determined by observation of the staining.</td>
<td>dH₂O can be added after few minutes to slow down the reaction.</td>
</tr>
<tr>
<td>Stop</td>
<td>&gt;10 min</td>
<td>Gels can be stored in this solution.</td>
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</table>
APPENDIX D

CALIBRATION CURVES FOR ORGANIC ACIDS

Figure 44. Calibration curve for gluconic acid concentration determination by HPLC.

$y = 16612x$  
$R^2 = 0.9995$
Figure 45. Calibration curve for malic acid concentration determination by HPLC.

Figure 46. Calibration curve for citric acid concentration determination by HPLC.
Figure 47. Calibration curve for lactic acid concentration determination by HPLC.

Figure 48. Calibration curve for succinic acid concentration determination by HPLC.
Figure 49. Calibration curve for fumaric acid concentration determination by HPLC.
**APPENDIX E**

**ORGANIC ACID CONCENTRATIONS**

Table 13. Organic acid accumulation in the bioreaction medium with cultivation time.

<table>
<thead>
<tr>
<th>Organic Acid Concentration, g L⁻¹</th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>12 h</th>
<th>15 h</th>
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<tbody>
<tr>
<td><strong>Gluconic Acid</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Malic Acid</strong></td>
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<td>0.72</td>
<td>0.79</td>
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<td>0.26</td>
<td>0.38</td>
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<td>0.52</td>
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<td>0.70</td>
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<td>-</td>
<td>0.01</td>
<td>0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<p>| <strong>OTC₂</strong>                          |      |      |      |      |      |
| <strong>Gluconic Acid</strong>                 | -    | 1.90 | 2.55 | 4.56 | 3.61 |
| <strong>Malic Acid</strong>                    | 0.36 | 1.65 | -    | -    | -    |
| <strong>Citric Acid</strong>                   | -    | 0.01 | 0.01 | 0.02 | 0.02 |
| <strong>Pyruvic Acid</strong>                  | -    | -    | -    | -    | -    |
| <strong>Lactic Acid</strong>                   | -    | 0.19 | 0.35 | 0.69 | 0.69 |
| <strong>Acetic Acid</strong>                   | -    | 1.18 | 1.57 | 2.30 | 3.11 |
| <strong>Succinic Acid</strong>                 | -    | -    | 0.68 | 0.71 | 0.77 |
| <strong>Fumaric Acid</strong>                  | -    | -    | -    | 0.02 | 0.03 |</p>
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<tr>
<th></th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>12 h</th>
<th>15 h</th>
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Table 13. Continued.

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