

METABOLICAL ENGINEERING OF PICHIA PASTORIS FOR
EXTRACELLULAR THERMOSTABLE GLUCOSE ISOMERASE
PRODUCTION

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EXTRACELLULAR THERMOSTABLE GLUCOSE ISOMERASE
PRODUCTION**

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ABSTRACT

METABOLICAL ENGINEERING OF *Pichia Pastoris* FOR EXTRACELLULAR THERMOSTABLE GLUCOSE ISOMERASE PRODUCTION

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The aim of this study is to develop a metabolically engineered *P. pastoris* strain for production of an active extracellular thermostable glucose isomerase (GI) enzyme by using genetic engineering techniques. For this purpose, research program was performed in two sub-programs. In the first sub-program, *xylA* gene from *Thermus thermophilus* was amplified and inserted into pPICZ α -A expression vector. Thereafter, this pPICZ α -A::*xylA* vector was cloned into *AOX1* locus in *P. pastoris* genome and expressed under alcohol oxidase promoter which is induced by methanol. After constructing the recombinant *P. pastoris* strains, the best producing strain was selected according to the specific enzyme activity assay and SDS-PAGE analyses in batch shaker-bioreactor experiments. The selected recombinant *P. pastoris* clone carrying *xylA* gene in its genome was named as eP20. Using recombinant *P. pastoris* eP20 clone, effects of salt and sorbitol concentration on the

cell growth and recombinant GI activity were investigated. The data obtained from the experiments showed that the maximum cell and GI activity values were obtained in production medium that contained 30 g L⁻¹ sorbitol, 4.35 g L⁻¹ ammonium sulphate, 0.1 M potassium phosphate buffer (pH 6.0), 14.9 g L⁻¹ MgSO₄·7H₂O, 1.17 g L⁻¹ CaSO₄·2H₂O, 1 ml L⁻¹ chloramphenicol and 4.35 ml L⁻¹ PTM1; where, the maximum biomass and recombinant GI activity were calculated, respectively, as 6.3 g L⁻¹ and 3.21 U L⁻¹. Moreover, the research program related with the effect of initial sorbitol concentration shows that optimum initial sorbitol concentration, C_{S0} is 50 g L⁻¹ that resulted a cell concentration and recombinant GI activity which are 7.32 g L⁻¹ and 3.6 U L⁻¹, respectively.

In the second part of the M.Sc. of the study, a pilot scale bioreactor experiment in a working volume of 1 L was performed in controlled bioreactor system. The variations in the cell growth, recombinant GI activity, AOX activity, total protease activity and organic acid concentrations throughout the fermentation were analyzed whereas the specific growth rates, yield coefficients and specific consumption rates were also calculated. The results showed that a pH and oxygen controlled operation enabled an important increase in recombinant GI activity. In this context, recombinant GI activity was increased as 56.1-fold and resulted in 202.8 U L⁻¹ at t=12 whereas the maximum biomass concentration was obtained as 85.2 g L⁻¹ at t=36. In this study, an active thermostable recombinant GI enzyme was produced extracellularly by a yeast cell, i.e. recombinant *P. pastoris*, for the first time.

Keywords: Glucose isomerase, *Pichia pastoris*, extracellular, thermostable

ÖZ

METABOLİK MÜHENDİSLİK YAKLAŞIMIYLA GELİŞTİRİLEN *Pichia pastoris* İLE HÜCRE DIŞI TERMOSTABİL GLUKOZ İZOMERAZ ÜRETİMİ

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Bu çalışmada, metabolik mühendisliği yaklaşımıyla aktif termostabil glukoz izomeraz (GI) enzimi üretebilen *Pichia pastoris* suşunun geliştirilmesi amaçlanmıştır. Bu bağlamda, araştırma program iki ana alt programda yürütülmüştür. İlk kısımda, *Thermus thermophilus*'a ait *xylA* geni çoğaltılmış ve pPICZ α -A ekspresyon vektörüne yerleştirilmiştir. Elde edilen pPICZ α -A::*xylA* vektörü daha sonra *P. pastoris* genomunda bulunan AOX1 lokusuna klonlanmış ve *xylA* geninin metanolle indüklenen alkol oksidaz promoter vasıtasıyla ekspresyonu sağlanmıştır. Elde edilen rekombinant *P. pastoris* suşları içinden, spesifik enzim aktivite analizi ve SDS-PAGE yöntemiyle en iyi üreten suş seçilmiş ve eP20 olarak adlandırılmıştır. Daha sonra, farklı tuz ve sorbitol konsantrasyonlarının hücre çoğalması ve rekombinant GI aktivitesi üzerine etkisi araştırılmıştır. Farklı konsantrasyon değerlerinde tuz içeren üretim ortamlarının kullanıldığı deneyin

sonucunda maksimum hücre derişimi ve GI aktivite değeri 30 g L^{-1} sorbitol, 4.35 g L^{-1} amonyum sülfat, 0.1 M potasyum fosfat tamponu (pH 6.0), 14.9 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.17 g L^{-1} $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 1 ml L^{-1} kloramfenikol ve 4.35 ml L^{-1} PTM1 içeren ortamın optimum olduğu bulunmuştur. Bu deneyde, maksimum hücre konsantrasyonu 6.3 g L^{-1} ve maksimum rekombinant GI aktivitesi 3.21 U L^{-1} olarak belirlenmiştir. Bununla birlikte, başlangıç sorbitol konsantrasyonun (C_{S0}) hücre çoğalması ve rekombinant GI aktivitesi üzerine etkisinin araştırıldığı deneyde, maksimum hücre konsantrasyonu ve rekombinant GI aktivitesi $C_{S0}=50 \text{ g L}^{-1}$ sorbitol içeren ortamda sırasıyla 7.32 g L^{-1} ve 3.6 U L^{-1} olarak elde edilmiştir.

Çalışmanın ikinci alt programında, 1 L 'lik çalışma hacmine sahip pilot ölçek biyoreaktör deneyi gerçekleştirilmiştir. Bu bağlamda, fermentasyon süresince hücre çoğalması, rekombinant GI aktivitesi, AOX aktivitesi, toplam proteaz aktivitesi ve ortamda bulunan organik asit derişimi analiz edilmiştir. Ayrıca, spesifik büyüme hızı, verimler ve spesifik tüketim hızları da hesaplanmıştır. Elde edilen sonuçlar, pH ve oksijenin kontrol edildiği biyoreaktör üretim koşullarının rekombinant GI aktivitesi üzerinde önemli bir artışa sebep olduğunu göstermiştir. Deney sonucunda, rekombinant GI aktivitesinde 56.1-kat bir artış gözlenmiş ve $t=12$ 'de 202.8 U L^{-1} aktivite değeri elde edilmiştir. Bununla birlikte maksimum hücre konsantrasyonu 85.2 g L^{-1} olarak $t=36$ 'da elde edilmiştir. Sonuç olarak, bu çalışmayla birlikte, aktif termostabil rekombinant GI enzimi hücre dışı olarak ilk kez bir maya hücresinde üretilmiştir.

Anahtar Kelimeler: Glukoz izomeraz, *Pichia pastoris*, hücre dışı, termostabil

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TABLE OF CONTENTS

ABSTRACT.....	iv
ÖZ	vi
ACKNOWLEDGMENTS	viii
TABLE OF CONTENTS.....	ix
LIST OF TABLES	xiii
LIST OF FIGURES	xv
NOMENCLATURE	xviii
CHAPTERS	
1. INTRODUCTION	1
2. LITERATURE SURVEY	7
2.1 Enzymes.....	7
2.1.2 Classification of Enzymes.....	8
2.1.3 The Product: Thermostable Glucose Isomerase.....	8
2.1.3.1 Optimal Temperature and pH.....	9
2.1.3.2 Structure	9
2.1.3.4 Metal Ion Requirement and Inhibitors	11
2.1.3.5 Active Site Studies and Reaction Mechanism	12
2.1.3.6 Substrate Specificity	13
2.1.3.7 Genetic Engineering of Glucose Isomerase	15
2.2 Selection of the Host Microorganism: <i>Pichia pastoris</i>	18
2.2.1 Properties	19
2.2.2 <i>Pichia pastoris</i> Expression System.....	20
2.2.2.1 AOX1 and Alternative Promoters.....	21
2.2.2.2 Methanol, Glycerol and Sorbitol Metabolism.....	23
2.2.2.3 Posttranslational Modifications and Protein Secretion	27
2.2.2.4 Proteolytic Degradation	30
2.3 Genetic Engineering Techniques: Methodology.....	32
2.4 Bioprocess Operation Parameters	36
2.4.1 Medium Composition	36

2.4.2 Temperature	37
2.4.3 pH.....	37
2.4.5 Oxygen.....	38
2.5 Computation of Bioprocess Characteristics.....	38
2.5.1 Specific Growth Rate	39
2.5.2 Methanol and Sorbitol Consumption Rates	41
2.5.3 Overall Yield Coefficients	43
3. MATERIALS AND METHODS.....	45
3.1 Chemicals.....	45
3.2 The Microorganism, Plasmids and Maintenance of Microorganisms.....	45
3.3 Growth Media	46
3.3.1 Solid Media.....	46
3.3.2 Precultivation Media.....	47
3.3.3 Production Media.....	48
3.4 Genetic Engineering Techniques	50
3.4.1 Plasmid DNA Isolation from <i>E. coli</i>	50
3.4.2 Agarose Gel Electrophoresis.....	51
3.4.3 DNA Extraction from Agarose Gels.....	51
3.4.4 Primer Design	52
3.4.5 Amplification of <i>xylA</i> Gene	53
3.4.5.1 DNA Purification after PCR	53
3.4.6 Digestion of DNA using Restriction Endonucleases	53
3.4.6 Ligation	54
3.4.7 Transformation into <i>E. coli</i>	55
3.4.8 DNA Sequencing	55
3.4.9 Transfection of <i>Pichia pastoris</i>	56
3.4.10 Isolation of Genomic DNA from Yeast	56
3.5 Recombinant Protein Production	57
3.5.1 Precultivation	57
3.5.2 Thermostable GI Production in Laboratory Scale Air Filtered Shake Bioreactors.....	58
3.5.3 Thermostable GI Production in Pilot Scale Bioreactor.....	58
3.6 Analyses.....	63

3.6.1 Cell Concentration	63
3.6.2 Total Protein Concentration	64
3.6.3 GI Enzyme Assay.....	64
3.6.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	64
3.6.5 Sorbitol Concentration	65
3.6.6 Organic Acid Concentration	66
3.6.7 Protease Activity Assay	67
3.6.8 AOX Activity Assay	67
4. RESULTS AND DISCUSSION	70
4.1 Development of Recombinant <i>P. pastoris</i> carrying <i>xylA</i> Gene	70
4.1.1 Propagation and Purification of pPICZ α A.....	72
4.1.2 Primer Design for Amplification of <i>xylA</i> gene from <i>T. thermophilus</i>	73
4.1.3 Amplification of <i>xylA</i> Gene by Polymerase Chain Reaction (PCR).....	74
4.2 Expression of Thermostable Glucose Isomerase in Recombinant <i>P. pastoris</i> in Laboratory Scale Air-Filtered Shake Bioreactors	84
4.2.1 Microorganism Selection	85
4.2.2 Effects of Salt Concentration on Thermostable GI Production and Cell Growth. 88	
4.2.3 Effects of Sorbitol Concentration on Thermostable GI Production and Cell Growth	91
4.3 Expression of Thermostable Glucose Isomerase in Recombinant <i>P. pastoris</i> in Pilot Scale Bioreactor	93
4.3.1 Cell Growth and Sorbitol Consumption.....	94
4.3.2 Thermostable GI Activity and Production	97
4.3.3 Alcohol Oxidase Activity.....	103
4.3.4 Protease Profile	104
4.3.5 Organic Acid Profiles.....	106
5. CONCLUSION	109
REFERENCES	113
APPENDICES	130
A. BUFFERS AND STOCK SOLUTIONS	130
B. SDS-PAGE SOLUTIONS AND STAINING PROCEDURE	133
C. NUCLEOTIDE SEQUENCES AND PLASMIDS	136
D. PROPERTIES OF DESIGNED PRIMERS	142

E. MOLECULAR WEIGHT MARKERS	145
F. CALIBRATION CURVE FOR BRADFORD ASSAY	146
G. CALIBRATION CURVE FOR D-FRUCTOSE CONCENTRATION	147
H. CALIBRATION CURVE FOR SORBITOL CONCENTRATION	148
I. CALIBRATION CURVE FOR ORGANIC ACID CONCENTRATION	149
J. CALIBRATION CURVE FOR AOX ACTIVITY ASSAY	154

LIST OF TABLES

TABLES

Table 1.1 Industrial enzymes and their uses

Table 2.1 Commercial GI producers.

Table 2.2 Summary of advantages and disadvantages of *P. pastoris* expression system.

Table 2.3 The yield coefficients defined for a bioprocess.

Table 3.1 Strains and plasmids used in this study.

Table 3.2 Composition of solid YPD medium.

Table 3.3 Composition of solid LSLB medium for *E. coli TOP10* carrying pPICZ α -A::xylA.

Table 3.4 Composition of solid LSLB medium for *E. coli BL21 (DE3) PlySs* carrying PRSETA::xylA

Table 3.5 Composition of precultivation medium, BMGY.

Table 3.6 Production medium of *P. pastoris* eP20 for laboratory scale air filtered shake bioreactor experiments.

Table 3.7 Trace salt solution (PTM1) composition.

Table 3.8 BSM medium composition.

Table 3.9 Primers and their target DNA used in this study.

Table 3.10 Reaction mixture content of double digestion.

Table 3.11 Restriction digestion mixture for linearizing of pPICZ α -A::xylA.

Table 3.12 Parameters of Equation 3.1 for Glycerol and Methanol Fed-Batch feeding.

Table 3.13 Conditions for HPLC system for sorbitol analyses

Table 3.14 Conditions for HPLC system for organic acid

Table 4.1 Primers that were used in this study for amplification of xylA gene.

Table 4.2 Thermal-cycler operation parameters for PCR.

Table 4.3 PCR reaction mixture composition.

Table 4.4 Ligation mixture composition.

Table 4.5 Production media containing different salt concentrations.

Table 4.6 Production media containing different sorbitol concentrations.

Table 4.7 Variations in instantaneous specific rates throughout the fermentation.

Table 4.8 Instantaneous specific product formation rates and product formation rates.

Table 4.9 Concentration of organic acids with respect to time during the fermentation process

Table D.1. Properties of designed primers

LIST OF FIGURES

FIGURES

Figure 2.1: 3D structure of glucose isomerase from *Thermus thermophilus*

Figure 2.2 Metal binding sites of GI

Figure 2.3 The reaction catalyzed by GI

Figure 2.4 Suggested complete reaction mechanism of GI

Figure 2.5 Methanol utilization pathway in *Pichia pastoris*

Figure 2.6 Metabolic pathways of glycerol in *Pichia pastoris*

Figure 2.7 Metabolic pathway of sorbitol and mannitol in yeasts.

Figure 2.8 Glycosylation pathways in human and yeast.

Figure 2.9: Schematic drawing of the PCR cycle

Figure 2.10: pPICZ α -A vector

Figure 2.11: Microbial growth phases observed in batch systems.

Figure 3.1 Scale up steps and pilot scale bioreactor system.

Figure 3.2 The predetermined feeding profile for glycerol, calculated for specific growth rate (μ_0) of 0.18 h^{-1} .

Figure 3.3 The predetermined feeding profile for methanol, calculated for specific growth rate (μ_0) of 0.03 h^{-1} .

Figure 4.1 Flowchart of the development of recombinant *P. pastoris* carrying *xylA* gene as the first part of the research plan.

Figure 4.2 Agarose gel electrophoresis image of circular and linear empty pPICZ α -A expression vector.

Figure 4.3 Agarose gel electrophoresis image of amplified *xylA* gene with PCR by using designed primers

Figure 4.4. Agarose gel electrophoresis image of purified PCR products, *xylA* gene.

Figure 4.5 Agarose gel electrophoresis image of *xylA* gene and pPICZ α -A after double digested followed by gel elution.

Figure 4.6 Schematic representation of *xylA* amplification, integration of specific recognition sites by PCR and construction of pPICZ α -A::*xylA* plasmid.

Figure 4.7 Agarose gel electrophoresis image of pPICZ α -A::xylA recombinant vector.

Figure 4.8 Agarose gel electrophoresis image of pPICZ α -A::xylA recombinant vector.

Figure 4.9 Schematic representation of pPICZ α A::xylA integration into *P. pastoris* genome.

Figure 4.10 Agarose gel electrophoresis image of PCR in which genomes of recombinant *P. pastoris* cells were used as templates.

Figure 4.11 SDS-PAGE analyses of two positive colonies.

Figure 4.12 Variation of pH of the cultivation medium.

Figure 4.13 Effects of salt concentration on cell growth.

Figure 4.14 Effects of salt concentration on GI activity.

Figure 4.15 Effect of sorbitol concentration on cell growth.

Figure 4.16 Effect of sorbitol concentration on GI activity.

Figure 4.17 Variation in the cell concentration with respect to cultivation time in the production phase.

Figure 4.18 Sorbitol consumption profile with respect to time.

Figure 4.19 Variation in GI activity throughout the fermentation.

Figure 4.20 Variation of GI activity and sorbitol concentration with respect to time during the fermentation.

Figure 4.20 Variation of GI activity and sorbitol concentration with respect to time during the fermentation.

Figure 4.21 Variation in specific GI activity throughout the fermentation.

Figure 4.22 Variation of GI monomer amount with respect to time during the fermentation process.

Figure 4.23 Variation of GI monomer concentration during the production phase.

Figure 4.24 Variation of AOX activity with respect to time throughout the fermentation.

Figure 4.25 Variation of total protease activity with respect to time throughout the fermentation.

Figure 4.26 Relationship between recombinant GI activity and total protease activity with respect to time during the bioreactor fermentation.

Figure C.1. Schematic representation of pPICZ α -A.

Figure E.1. Molecular weight markers used in this study.

Figure F.1. Standard calibration curve for Bradford assay.

Figure G.1. Standard calibration curve for D-Fructose concentration.

Figure H.1. Standard calibration curve for sorbitol concentration.

Figure D.1 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

Figure D.2 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

Figure D.3 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

Figure D.4 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

Figure D.5 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC

Figure D.6 Calibration curve obtained for acetic acid concentration; analysis was performed by HPLC.

Figure D.7 Calibration curve obtained for oxalic acid concentration; analysis was performed by HPLC.

Figure D.8 Calibration curve obtained for pyruvic acid concentration; analysis was performed by HPLC

Figure J.1. Standard calibration curve for AOX activity assay.

NOMENCLATURE

C	Concentration in the medium	g L^{-1}
DO	Dissolved oxygen	%
Q	Feed inlet rate	L h^{-1}
q	Specific formation or consumption rate	$\text{g g}^{-1} \text{h}^{-1}$
r	Formation or consumption rate	$\text{g g}^{-1} \text{h}^{-1}$
t	Cultivation time	h
T	Temperature	$^{\circ}\text{C}$
U	One unit of an enzyme	
V	Volume of the bioreactor	L
Y	Yield (overall)	g g^{-1}

Greek Letters

μ	Specific growth rate	h^{-1}
μ_t	Total specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Refers to initial condition
A	Refers to activity
C	Refers to concentration
AOX	Refers to alcohol oxidase
m	Refers to melting point
M	Refers to methanol
p	Refers to product
S	Refers to sorbitol or substrate
t	Refers to total
X	Refers to cell

Abbreviations

A	Activity
AOX	Alcohol oxidase
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
FLD	Glutathione-dependent formaldehyhde deydrogenase
GAP	Glyceraldehyde-3-phosphate dehydrogenase
GI	Glucose isomerase
rhGH	Recombinant human growth hormone
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
OD	Optical density
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
<i>xylA</i>	D-xylose isomerase gene

CHAPTER 1

INTRODUCTION

Starting with the Industrial Revolution in the 19th century, the demand to medical, agricultural, energy and food products have been increasing significantly, which also enhanced with the increase in the population. Biochemical processes have been carried out since ancient times (e.g., for the production of wine, bread, cheese, yogurt, beer, and winegar); but it was not until a little more than 150 years ago that Louis Pasteur pointed out the role that living organisms play in these processes. In the years that followed, an increasing number of commercially important biochemicals were produced by the utilization of the activities of various microorganisms. In 1957 several novel bacteria with high productivity were found in Japan, and a new fermentation method called “L-glutamic acid fermentation” commenced in the field of “applied microbiology”, which is presently named as “microbial biotechnology” or in a larger context as “industrial biotechnology”. Since that discovery many investigators had undertaken research programs on the microbial production of other amino acids; moreover, within less than a decade to develop an engineering approach to the subject, i.e., biochemical engineering, the pioneers (Aiba *et al.*, 1965) borrowed heavily from chemical engineering science. Therefore, new production strategies those are combining both biological and engineering disciplines were born. A decade later, a precise definition of the “biotechnology” became a real problem; and in 1982 the OECD definition “Biotechnology is the application of scientific and and engineering principles to the processing of materials by biological agents to provide goods and services” was a real achievement and since then creative abstractions have found new spaces to open new avenues to biochemical industry. In this context, these developments led

biotechnology and bioprocess engineering to improve new methods both in upstream and downstream processes to overcome this increasing demand.

So, as a sub-field of industrial biotechnology, enzyme technology offers new processes and bioproducts that are used as biocatalysts in many fields such as food, detergent, environment, textile and pharmaceutical industries (Table 1.1). Moreover, by courtesy of the developments in molecular genetics, especially, the “breakthrough created innovation” related with the “polymerase chain reaction” in 1988, enabled scientists of the metabolic engineering to produce valuable bioproducts with extreme properties in organisms other than their natural source. These bioproducts obtained from extremophiles such as thermophiles, acidophiles or alkaliphiles therefore have gained attention with respect to their advantages in industrial production. Especially thermostable enzymes –thermozymes- are industrially valuable products due to their resistance to irreversible thermal and chemical denaturation and also the ability to retain its catalytic activity when they are expressed in mesophilic hosts (Zeikus *et al.*, 1998).

Glucose isomerase, E.C. 5.3.1.5 (also known as xylose isomerase or D-xylose ketol-isomerase) is an enzyme that catalyzes the reversible isomerization reaction of glucose to fructose and xylose to xylulose. The isomeration reaction catalyzed by GI is a critical step for the production of high fructose corn syrup (HFCS) which is used in food industry as a sweetener having a sweetness 2-fold higher than glucose and fructose and 1.75-fold higher than sucrose (Bhosale *et al.*, 1996). Presently, GI has a production capacity ca. 100,000 tones/year having a market value of \$1 billion for production of 15 million tones HFCS/year (Demain, 2007).

In HFCS production, the desired amount of fructose in food products and soft drinks is 55%, while only 40-42% fructose can be obtained when isomeration reaction occurs at 58-60°C. Thus, an extra step of chromatography is needed to obtain 55% fructose.

GI was first isolated in 1953 by Hochster *et al.* from *Pseudomonas hydrophila*. (Hochster and Watson, 1963). Thereafter, Schray and Mildvan (1972) and

Hogueangeletti (1975) published their studies about kinetic properties, protein structure and aminoacid composition of GI. Additionally, Farber *et al.* (1989), van Tilbeurgh *et al.* (1992), Asboth and Szaba (2000), Bogumil *et al.* (2000) and Rozanov *et al.* (2009) studied the catalytic mechanism of GI and showed the importance of different metal ions on catalytic activity of GI.

Table 1.1 Industrial enzymes and their uses
(<http://www.anilbioplus.com/aboutus/industrial-enzymes.htm>).

Enzymes	Application Area
Alpha-amylase	Detergent Starch processing Food processing
Cellulase	Detergent Brewing Textile
Xylanase	Paper & Pulp Animal Feed Baking
Protease	Detergent Meat Tanning
Papain	Medicine Leather Food Cosmetics
Glucoamylase	Starch Juice Brewing
Lipase	Detergent Pharmaceuticals Cosmetics Leather

Thermostable GI isolation from a thermophilic microorganism, i.e., *Thermus thermophilus*, was achieved by Dekker *et al.* (1991a). In another study, GI enzyme from *Thermotoga maritima* was isolated and shown to be an industrially important enzyme due to its high optimum working temperatures (Brown *et al.*, 1993). Vieille *et al.* (1995) isolated GI enzyme from *Thermogata neapolitana* and cloned into *E. coli*. To increase economical importance, thermostability properties of GI were

studied in terms of aminoacid composition, hydrophobicity, sulphide bonds, salt bridges by several researches (Hartley *et al.*, 2000; Zhu *et al.*, 1998). Considering these studies other researches tried to improve the thermostability of GI enzyme (Chang *et al.*, 1999; Hartley *et al.*, 2000; Xu *et al.*, 2009).

In terms of recombinant thermostable GI production, *E. coli* is widely used for cloning researches so far. *xylA* gene that encodes GI enzyme isolated from *Clostridium thermohydrosulfuricum* was cloned into recombinant *E. coli* cells and was 20-fold purified afterwards (Dekker *et al.*, 1991b). Same research group achieved to clone *xylA* gene from *T. thermophilus* into *E. coli* and *B. brevis*, and compared production capacities of these two different organisms (Dekker *et al.*, 1992); where, *B. brevis* was shown to be more effective in producing recombinant thermostable GI than that of *E. coli* with a yield of 1 g L⁻¹. Park *et al.* (1997) cloned GI isolated from *Thermus flavus* into *E. coli* and achieved 55-45% fructose conversion. Alternative promoters were also studied in *E. coli* to increase GI production where Mezghani *et al.* (2005) achieved 4-fold higher GI production yield by using *ermE-up* promoter instead of *lac-Z* promoter.

Sarthy *et al.* (1987) isolated GI from *E. coli* and expressed in *Saccharomyces cerevisiae*. However, the enzyme obtained from *S. cerevisiae* was 10³-fold less active than that of *E. coli*. Possible reasons for the inefficient expression in the yeast was speculated as improper folding of recombinant GI, posttranslational modifications, disulfide bridge formation and the internal pH of the yeast cells. However, *xylA* gene from *T. thermophilus* was cloned and expressed successfully into *S. cerevisiae* with a specific activity of 1 U mg⁻¹ mentioning that the natural source of GI has a significant role in functional expression (Walfridsson *et al.*, 1996). Lönn *et al.* (2003) cloned *xylA* *T. thermophilus* into *S. cerevisiae* by chromosomal integration and multicopy plasmids; where, 30 mU mg⁻¹ protein was obtained by multicopy plasmid expression and gave higher yield than that of the chromosomally integrated expression system. *xylA* from *Piromyces sp* was cloned into *S. cerevisiae* (Kuyper *et al.*, 2005); where, the genes related with the conversion of xylulose were also overexpressed to understand the effect of these reactions in xylose fermentation

catalyzed by GI enzyme, and the maximum xylose consumption rate was determined as $1.1 \text{ g xylose g-biomass}^{-1} \text{ h}^{-1}$. Brat *et al.* (2009) cloned *xylA* *Clostridium phytofermentans* into *S. cerevisiae* successfully; and showed that *C. phytofermentans* was the third natural source of GI after *T. thermophilus* and *Piromyces* *sp* whose gene can be cloned and expressed functionally in the yeast with a xylose consumption rate of $0.07 \text{ g xylose g-dry cell}^{-1} \text{ h}^{-1}$. Ha *et al.* (2011) isolated two bacterial *xylA* genes from *Bacteriodes stercoris* HJ-15 and *Bifidobacterium longum* MG1 and cloned into *S. cerevisiae*; however, only *xylA* from *B.stercoris* was functionally expressed in the yeast.

Pichia pastoris is a metylotrophic yeast that is being widely used for heterologous protein production. The ability to grow to very high cell densities on minimal medium is one of the major advantages of *Pichia pastoris* (Cereghino and Cregg, 1999; Cos *et al.*, 2006). Besides this advantage, the ability of expressing foreign proteins at very high levels either intracellularly and extracellularly, stable integration of expression plasmids at specific sites of *P. pastoris* genome either in multiple or single copies and ability of performing many posttranslational modifications such as glycosylation, correct disulfide bond formation and proteolytic processing make *P. pastoris* a promising host for recombinant protein production (Cereghino and Cregg, 1999; 2000; 2002). Alcohol oxidase 1 (*AOX1*) was used for expression of glucose isomerase in this study which is known as one of the tightly-regulated and strongest promoters, which is induced by methanol, but repressed in the presence of glycerol (Cos *et al.*, 2006).

In this study, the aim is to construct a metabolically engineered *Pichia pastoris* strain for production of an active extracellular, thermostable GI enzyme from *Thermus thermophilus* using *AOX1* promoter. In the first part of the study, *xylA* gene was transformed into *P. pastoris* by using pPICZ α -A expression vector and the clones, which produce glucose isomerase enzyme most efficiently, were selected by shaker bioreactor experiments. The effects of sorbitol concentration and salt concentration were investigated in shaker bioreactor experiments. In the second part of the study, a

selected clone was used in a designed bioreactor experiment to investigate the controlled pH and oxygen transfer effects on the glucose isomerase production.

CHAPTER 2

LITERATURE SURVEY

To achieve a metabolically engineered *Pichia pastoris* strain that produces extracellular thermostable glucose isomerase (GI) genetic engineering methods can be used. Besides genetic engineering methods, an overview of the literature on the product glucose isomerase (GI), the host *P. pastoris* and bioprocess operation conditions, are presented considering their influences on the bioprocess development.

2.1 Enzymes

Some proteins bind temporarily to other biomolecules with specific ligands, causing covalent changes in these ligands but staying as unchanged at the end of the reaction; where this protein is called an enzyme and the biomolecule with specific ligands is named as substrates. As a biocatalyst, an enzyme catalyzes a specific biochemical reaction which lowers its activation energy (E_a); thus, increases the rate of the reaction. Most enzymes are proteins, which are composed of aminoacids with an exception of ribozymes, Enzymes have three-dimensional structure which is specific for their substrates. This three –dimensional structure have four distinct types: 1) *Primary structure* : linear aminoacid sequence, 2) *Secondary structure*: highly regular repeating structures which are alpha helix and beta strand stabilized by hydrogen bonds, 3) *Tertiary structure*: the three dimensional structure which is driven by hydrophobic interactions, salt bridges, hydrogen bonds, disulfide bonds and post-translational modifications, 4) *Quaternary structure*: structure that results from the interactions of several proteins or polypeptides, usually called subunits, which is driven by non-covalent interactions and disulfide bonds as in tertiary structure. If two subunits come together for an active protein, that protein is called as

dimer. Proteins which have three subunits are called trimers while those have four subunits are called tetramers. If these subunits are identical, then these multimers are referred with a prefix of *homo*-, while multimers that have different subunits are referred as a prefix of *hetero*- (Raven and Johnson, 2001).

2.1.2 Classification of Enzymes

According to the Enzyme Commission enzymes are divided into six sub-classes. These are oxidoreductases which are responsible for oxidation-reduction reactions; transferases which transfer one group e.g. methyl or glycosyl group from one compound to another; hydrolases which catalyse the hydrolytic cleavage of C-O, C-N, C-C and some other bonds; ligases which are responsible for joining together of two groups to each other; isomerases which catalyse geometric or structural changes within one molecule and lyases which catalyse the cleaving of C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds.

2.1.3 The Product: Thermostable Glucose Isomerase

Glucose isomerase (GI) which is also known as D-xylose isomerase is an isomerase (E.C.5.3.1.5) which catalyzes the reversible interconversion reaction between D-glucose to D-fructose and D-xylose to D-xylulose (Dekker *et al.*, 1991a).

GI is an important enzyme due to its capability of reversible conversion of D-Glucose to D-Fructose which is used in HFCS processes; concordantly reversible conversion of D-Xylose to D-Xylulose which is used in ethanol production. Table 2.1 gives some examples of commercial GI producers.

With the discovery of the isomerization capability of this enzyme from *Pseudomonas hydrophila* by Hochster *et al.* (1957), novel GI producing strains have gained attention. Therefore, the more information was known about structural and kinetic properties of GI such as its stability, active site, three-dimensional structure,

cofactors, inhibitors and substrate specificity, the more improvement had been attained so far. Schray and Mildvan (1972) and Hogueangeletti (1975) published their studies about kinetic properties, protein structure and aminoacid composition of GI. Additionally, Farber *et al.* (1989), van Tilbeurgh *et al.* (1992), Asboth and Szaba (2000), Bogumil *et al.* (2000), Rozanov *et al.* (2009) and Kovalevsky *et al.* (2010) studied the catalytic mechanism of GI and showed the importance of different metal ions on catalytic activity of GI.

Table 2.1 Commercial GI producers (Bhosale *et al.*, 1996).

Manufacturer	Trade Name	Source Organism
Gist Brocades and Anheuser-Busch Inc.	Maxazyme	<i>Actinoplanes missouriensis</i>
Novo-Nordisk	Sweetzyme	<i>Bacillus coagulans</i>
Miles Kali-Chemie	Optisweet	<i>Streptomyces rubiginosus</i>
Finnsugar	Spezyme	<i>Streptomyces rubiginosus</i>
Nagase	Swetase	<i>Streptomyces phaeochromogenes</i> <i>Arthrobacter sp.</i> <i>Streptomyces olivaceus</i>

2.1.3.1 Optimal Temperature and pH

Optimal temperature for thermostable GI ranges from 60°C to 90° depending on the source microorganism while optimal pH value alters between 7.0 and 9.0. Enzymes isolated from thermophilic species show higher stability at high temperatures than that of mesophilic species (Bhosale *et al.*, 1996).

2.1.3.2 Structure

Molecular weight of GI varies between 44,000 and 191,000 Da depending on the source microorganism. GI subunit (monomer) from *Thermus thermophilus* is

composed of 387 aminoacids which has a molecular weight of 44,000 Da. Active GI is a homotetramer with identical subunits, although some GI enzymes from other species are active when they are dimers or rarely trimers while monomeric forms of GI is inactive (Ghatge *et al.*, 1994). The identical subunits are connected with noncovalent bonds.

X-ray crystallography studies showed that tetrameric structure and tertiary fold of each subunit of GI from *T. thermophilus* is similar with the GIs from other microbial sources. Each subunit is composed of two domains. Residues from 1 to 321, Domain I, N-terminal domain, folds as $(\beta/\alpha)_8$ -barrel while residues from 322 to 387, Domain II contacts with Domain I. Chang *et al.* (1999) showed that there are 5 3_{10} helices, 16 α -helices and 10 β -strands in each subunit.

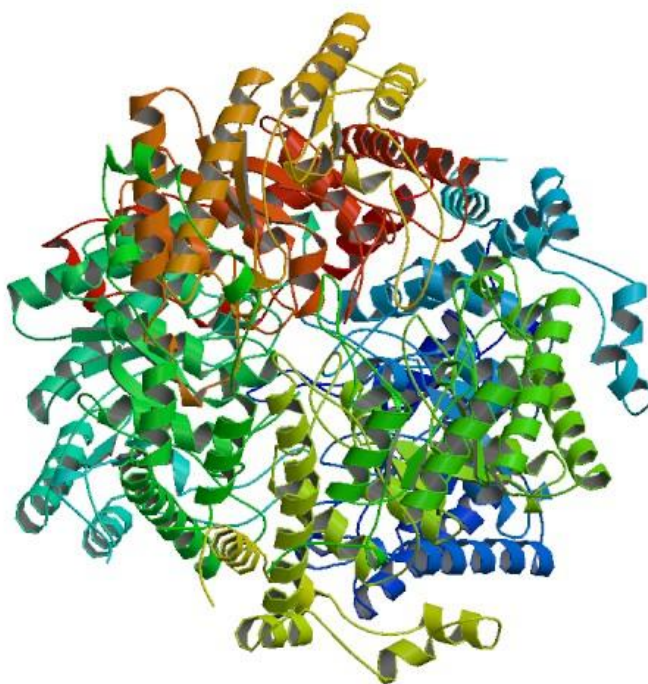


Figure 2.1: 3D structure of glucose isomerase from *Thermus thermophilus* (PDB Accession Number: 1BXB).

Kovalevsky *et al.* (2010) investigated the metal binding sites of GI from *Streptomyces rubiginosus* by X-ray crystallography combined with neutron diffraction technique; where, it was shown that two divalent metal cations are

required for full activity of GI. These sites were designated as M1 and M2 whereas M1 refers to structural metal site which prefers Mg^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} and Pb^{2+} ions while M2 is the catalytic metal site which shows a larger variety of affinity of divalent metal cations.

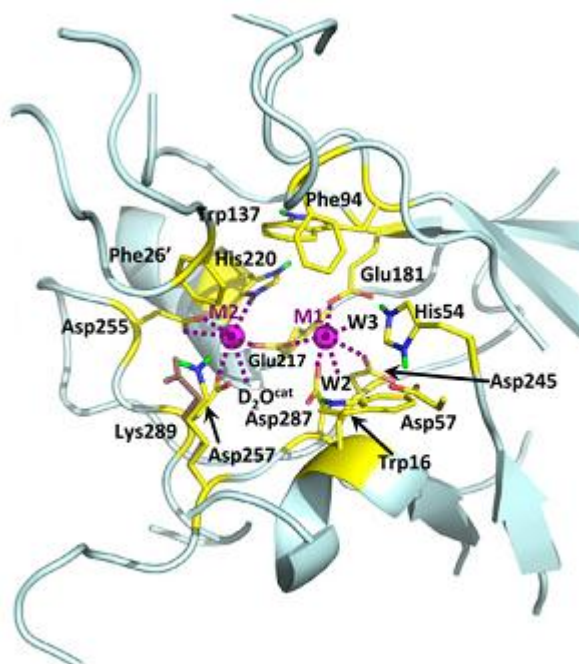


Figure 2.2 Metal binding sites of GI (Kovalevsky *et al.*, 2010). The residues coordinating metal cofactors are represented as magenta spheres and lining of the cavity, Trp16, His54, Phe94, Trp137, and Phe26' are shown.

2.1.3.4 Metal Ion Requirement and Inhibitors

GI requires several metal ions to be active and to function properly like other enzymes. For this purpose, divalent metal cations such as Mg^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} are known as the cofactors of GI (Kovalevsky *et al.*, 2010) while each cofactor acts differently in the structure of GI whereas Mg^{2+} is shown to be the strongest activator. However, Co^{2+} and Mn^{2+} ions are also effective in the stabilization of GI by supporting the conformation (Gaikwad *et al.*, 1992). Metal ions which have larger

diameter more than 0.8 Å inhibit the catalytic activity of GI. These metal inhibitors are Ag^+ , Hg^{2+} , Cu^{2+} , Zn^{2+} , and Ni^{2+} whereas xylitol, arabitol, sorbitol, mannitol, lyxose, and tris are the other known inhibitors of GI (Bhosale *et al.*, 1996).

2.1.3.5 Active Site Studies and Reaction Mechanism

To improve catalytic effect of GI, the identification of location of aminoacids is an important issue. The effects of site directed mutagenesis in the active site of GI from *E. coli* was investigated by Batt *et al.* (1990) and two conserved histidine sites (His-101 and His-271) were identified to be essential for catalytic effect of GI. Meilleur *et al.* (2006) investigated where the hydrogen atoms are located; where, this study showed that isomerization reaction was initiated by double protonation of His-53 residue followed by an acid catalyzed opening of the sugar ring. After these studies, Kovalevsky *et al.* (2010) showed the reversible isomerization reaction proceeds in three steps: ring opening, isomerization and ring closure.

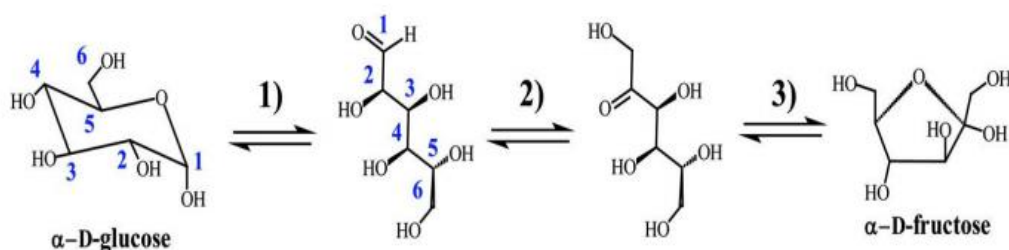


Figure 2.3 The reaction catalyzed by GI is represented with three major steps: (1) ring opening, (2) isomerization, and (3) ring closure (Kovalevsky *et al.*, 2010).

According to this model, conformational changes occur in the active site of the enzyme with the critical help of many aminoacids during the reaction initiation and implementation. In the first step, reactive cyclic sugar substrate and active site of GI bind to each other. In this step, presence of different arrangements of water molecules facilitates the correct association of the substrate to GI. The latter step is

the ring opening where His-53 temporarily gives a proton to O5 and takes it back after the break of C1-O5 bond. While the ring is opening, neutral Lys-289 is protonated and gives a proton to Asp-257. Then, O1 is placed in the correct position to start isomerization reaction by the help of Lys-183. In the last step, isomerization takes place. During this step, proton is transferred from C2 and O2 to C1 and O1, respectively.

2.1.3.6 Substrate Specificity

Maximum isomerization activity of GI is achieved when utilizing substrates containing hydroxyl groups at carbons 3 and 4 in the equatorial position. (Chen, 1980). In this context, common substrates that are preferred by GI are D –Glucose and D-Xylose whereas D-Ribose, L-arabinose, L-Rhamnose and D-Allose are rarely preferred (Bhosale *et al.*, 1996).

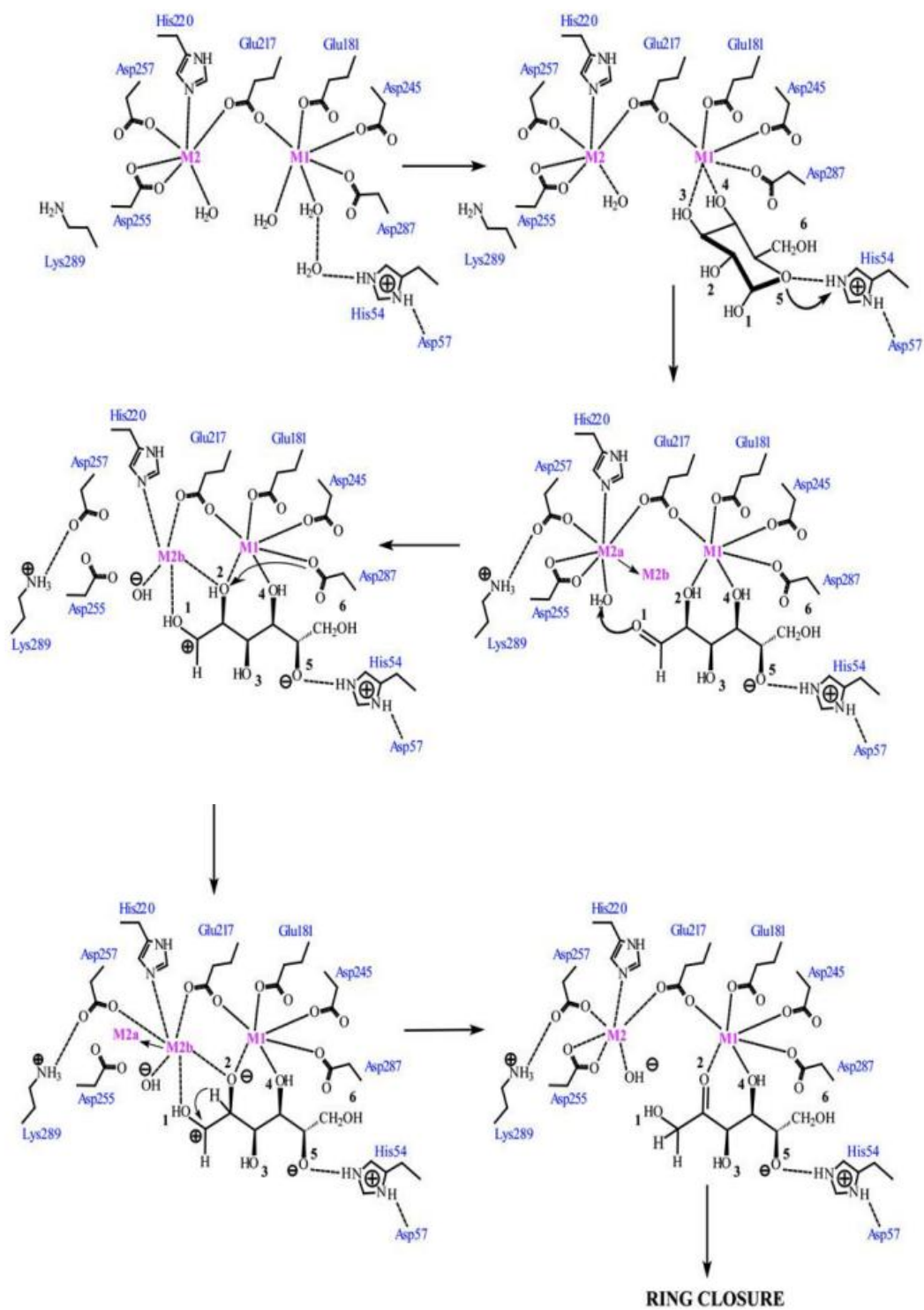


Figure 2.4 Suggested complete reaction mechanism of GI (Kovalevsky *et al.*, 2010)

2.1.3.7 Genetic Engineering of Glucose Isomerase

Besides being a valuable product for industrial application, in order to be an appropriate commercial enzyme, there are several criteria to be taken into consideration such as reaction rate, optimum temperature and pH, stability in different environments, half-life, activation and inhibition mechanisms, selectivity and specificity to the substrate (Soetart and Vandamme, 2010)

After the discovery of isomerizing activity of GI from *Pseudomonas hydrophila* by Hochster *et al.* in 1953, many investigations started and over 100 microorganisms producing GI were detected (Barker and Shirley, 1980; Ulezlo *et al.*, 1986). Concordantly, developing genetic engineering techniques made it possible to manipulate the *xylA* gene, which is responsible of production of GI enzyme from many different strains. The first report was published by Ho *et al.* (1983) about the isolation of GI gene from *E. coli*. In 1985, isolated *E. coli xylA* gene was cloned into several *E. coli* plasmids resulting low expression levels due to strong regulatory effects of natural promoter (Ho and Stevis, 1985). To overcome this problem, *lac* and *tac* promoters were used for over-production of GI and succeeded. Batt *et al.* (1986) cloned *xylA* gene under control of *tac* promoter which is induced by isopropylthio β -D-galactopyranoside in *E. coli*; where, total production of GI reached to 28% of total cell protein. In another study, a non-capable of producing GI *E. coli* strain was used as the host microorganism and *xylA* gene from *Bacillus licheniformis* was cloned into *E. coli* which resulted 20-fold higher GI activity than that of the source microorganism (Shin and Kho, 1985). A comparative study between *E. coli* and *Bacillus subtilis* in terms of their capability of producing active thermostable GI was published by (Lee *et al.*, 1990); where, it was shown that thermostable GI with a higher activity was produced by *B. subtilis* (1.54 U mg^{-1}) compared to that of *E. coli* (0.46 U mg^{-1}). Dekker *et al.* (1991) isolated *xylA* gene from hyper-thermophilic *Thermus thermophilus*, determined its nucleotide sequence, and cloned into *E. coli* under control of *tac* promoter, resulting a 45-fold higher yield comparing to the source microorganism. Again, Dekker *et al.* (1992) cloned *xylA* gene from *T. thermophilus* into *E. coli* and *B. brevis*; where, a higher soluble GI activity value of

5600 U L⁻¹ was obtained with r- *B. brevis* but a lower activity 4300 U L⁻¹ was obtained by r-*E. coli*. Lee *et al.* (1991) isolated a thermostable *xylA* gene from *Thermoanaerobacterium saccharolyticum*, inserted into pUC18 vector and transformed into *E. coli*. The constitutive expression of GI gene was achieved and shown that the GI enzyme encoded by *xylA* was a tetramer composed of identical subunits with a molecular mass of 200 kDa; where, the enzyme characteristics, i.e., the optimum pH and temperature were determined as 7.0-7.5 and 80°C, respectively. Wuxiang and Jeyaseelan (1993) isolated *xylA* gene from thermophilic *Bacillus sp.* and transformed into *E. coli* resulting an active GI with an activity value of 12.8 U mg⁻¹ at 85°C. Further studies for producing active GI enzyme in bacterial strains include cloning and expression of acid-stable *xylA* gene from *Streptomyces olivaceoviridis* in *E. coli JM19* (Kaneko *et al.*,2001); expression of *xylA* gene from *T. thermophilus* as a fusion protein located in periplasmic membrane in *E. coli TB1* and *ER2508* (Sarıyar *et al.*,2004); expression of thermostable mutant *xyl* gene from *Streptomyces SK* in *E. coli HB101* strain (Rhimi *et al.*,2007) and insertion of *xylA* gene of *E. coli K12* into pRAC vector followed by its expression in *E. coli BL21* cells (Rozanov *et al.*, 2007); expression of thermostable GI from *Geobacillus caldoxylosilyticus* TK4 in *E. coli* (Faiz *et al.*,2011).

Chan *et al.* (1986) used *Schizosaccharomyces pombe*, where the hybrid plasmid pDB248-XI containing *xylA* gene from *E. coli*, was transformed into the yeast and the recombinant cells were able to metabolize 10% (w/v) xylose for production of 3% (w/v) ethanol. Further investigations of D-Xylose metabolism of *S.pombe* showed that low GI activity due to proteolytic degradation was the major cause in insufficient xylose fermentation in yeast. Sarthy *et al.* (1987) tried the transformation of *S. cerevisiae* with yeast expression plasmids bearing *xylA* gene from *E. coli*; however, GI activity produced by recombinant *S. cerevisiae* was 10³-fold lower than that of *E. coli*, and speculated the probable reasons as improper folding of the protein, the internal pH of the yeast cells, posttranslational modifications of the protein, and inter- or intra- molecular disulfide bridge formation. Almost a decade later, bacterial *xylA* gene from *Clostridium thermosulfirogenes* was cloned into *Saccharomyces cerevisiae* by Moes *et al.* (1996). Although efficient transcription of

xylA was achieved under the control of ADH2 promoter, *r-S. cerevisiae* was not capable of producing active GI enzyme probably because of cytosolic environment of the yeast cells which prevents GI subunits to form active and stable tetramers that function properly followed by a proteolytic degradation even in protease deficient *S. cerevisiae* strains. First successful study that constructed a yeast strain which produces an active bacterial GI was achieved by Walfridsson *et al.* (1996); where, *T. thermophilus xylA* was cloned and expressed in *S. cerevisiae* under the control of yeast PGK1 promoter showing the highest activity with a value of 1.0 U mg⁻¹ at the optimum temperature, 85°C. The authors explained the success of the yeast strain producing an active GI enzyme with the relatedness between the two organisms taxonomically, and claimed that *T. thermophilus* is more closely related to *S. cerevisiae* than the eubacteria. Later, the expression of bacterial *xylA* genes coding for xylose isomerases from *E. coli* or *Streptomyces coelicolor* in the yeast *Hanensula polymorpha* was investigated (Voronovsky *et al.*,2005); and a mutant *H.polymorpha* which has a deletion of *xyl1* gene encoding xylose reductase and not capable of growing in the xylose medium as the host organism and the promoter of the *H. polymorpha* glyceraldehyde-3-phosphate dehydrogenase gene were used. Voronovsky *et al.* (2005) concluded that, nearly 20% of GI activity (0.15 U mg⁻¹) was achieved by the transformants than that of was achieved by the bacterial host (0.65 U mg⁻¹).

Amongst the recent works, Madhavan *et al.* (2009) cloned *xylA* gene from rumen fungus *Orpinomyces* to *S. cerevisiae*; where, obtained maximum GI activity with a value of 1.90 U mg⁻¹, whereas the ethanol yield was 0.39 (g/g). Tanino,*et al.* (2010) used *xylA* from *Orpinomyces* as the source organism. In addition, GI overexpression cassettes were constructed by the multicopy integration and transformed into *S. cerevisiae* MT8-1; researchers concluded that a successfully GI producing yeast strain was obtained with an ethanol yield of 61.9% on xylose. Lastly, Ha *et al.* (2011) cloned two bacterial xylose isomerase genes from *Bacteroides stercoris* HJ-15 and *Bifidobacterium longum* MG1 into *S. cerevisiae*; where, the transformant with *xylA* from *B. longum* could not assimilate xylose while the transformant with *xylA* from *B. stercoris* was able to grow on xylose with an ethanol yield of 0.31 g/g. this finding

supported the importance of taxonomically relatedness as mentioned by Walfridsson *et al.* (1996).

The overview of the literature shows that extracellular GI production has not been studied successfully yet (Bhosale *et al.*, 1996). First, Iuzuka *et al.* (1971) obtained extracellular GI from *Streptomyces* and claimed in a patent. Chen *et al.* (1979) isolated *Streptomyces flavogriseus* and showed that it can produce extracellular GI enzyme which has an activity of 1.5 U ml^{-1} , whereas it has intracellular GI activity with a value of 3.5 U ml^{-1} . Then, Srinivasan *et al.* (1983) reported extracellular GI production using a sclerotia-forming actinomycete of genus *Chainia* showed with an activity of 4 U ml^{-1} . Chauthaiwale and Rao (1994) reported an alkaliphilic, thermophilic *Bacillus sp.* producing extracellular GI and showed that the requirement for metal cations and the molecular weight are similar as that of the intracellular GIs; where, the maximum specific activity of GI without purification was found as 0.62 U mg^{-1} . Sayyed *et al.*, (2010) reported extracellular GI enzyme by *Aspergillus sp.*; where, the maximum activity of GI was found to be 1.2 U mg^{-1} . Another natural source that produces extracellular GI was reported by Pandidurai *et al.* (2011); where, extracellular GI showed a maximum activity of 41 U ml^{-1} with an optimum temperature value of 37°C . The overview of the literature reveals that, genetically engineered expression systems have not been reported for the production extracellular GI enzyme yet.

2.2 Selection of the Host Microorganism: *Pichia pastoris*

For the development of an industrial bioprocess, selection of the appropriate host microorganism is a critical issue. There are several parameters to be considered when choosing the host microorganism, such as being GRAS (generally recognized as safe), easily accessible, having a high productivity, producing minimum by-products, ability of growing on cheap media, ability of growing at moderate conditions and exhibit possibility for easy scale up (Kirk and Othmer, 1994; Soetaert and

Vandamme, 2010). Mostly, microorganisms are selected rather than animal and plant cells due to their higher growing rates and high productivities.

To this day, production of GI has been achieved in several host organisms including: *Bacillus subtilis* (Lee *et al.*, 1990), *Bacillus brevis* (Dekker *et al.*, 1992), *Streptomyces lividans* (Tan *et al.*, 1990), *Schizosaccharomyces pombe* (Chan *et al.*, 1989), *Saccharomyces cerevisiae* (Moes *et al.*, 1996; Walfridsson *et al.*, 1996) and different *E. coli* strains (Dekker *et al.*, 1992; Lee *et al.*, 1993; Kaneko *et al.*, 2001; Sariyar *et al.*, 2004; Rhimi *et al.*, 2007).

In terms of selection of appropriate microorganism, methylotrophic yeast which utilize methanol as sole carbon source is first reported by Ogata *et al.* (1969). Following the taxonomic studies, *Pichia pastoris* was shown as a potential single cell protein (SCP) producer for animal feed, after the discovery of four genera *Candida*, *Hansenula*, *Pichia* and *Torulopsis* (Lee and Komagata, 1980). In 1970s, Philips Petroleum Company developed the protocols for the growth and media composition of *P. pastoris* (Cereghino and Cregg 2000). This false hypothesis enabled researchers to develop general growth and culture conditions of *P. pastoris*; thereafter in 1980s, Philips Petroleum Company and Salk Institute Biotechnology/ Industrial Associates Inc. (SIBIA, La Jolla, CA) started to study *P. pastoris* as an expression system for the heterologous protein production. Following these attempts, vectors, strains and protocols were developed by SIBIA. In this context, more than 500 recombinant proteins were expressed in *P. pastoris* up today (Plantz *et al.*, 2006, Cos *et al.* 2006). The approval of the first *P. pastoris* produced therapeutic, KALBITOR[®] (Ecalantide) by Food and Drug Administration (FDA, USA) is one of the most important issues for *P. pastoris* in terms of being an industrially feasible microorganism (Walsh 2010).

2.2.1 Properties

Pichia pastoris is a methylotrophic yeast which has unicellular oval shape with the 1-5 μm wide and 5-30 μm long. It has a typical eukaryotic cell wall structure including

30 - 60% polysaccharides (beta-glucan and mannan sugar polymers), 15 - 30 % proteins, 5 - 20% lipids and a small amount of chitin. It has a broad pH range between 3-7 (Cregg *et al.*, 2000) and lives at mild temperatures between 25-35°C (Macauley-Patrick *et al.* 2005). In addition to these general properties, *P. pastoris* is separated from *S. cerevisiae* by its preference respiratory mode of growth instead of fermentative growth. Therefore, by-products such as ethanol and acetic acid that are produced in fermentative growth do not cause such problems as it happens in *S. cerevisiae* (Richter *et al.* 2006).

In this context, *P. pastoris* strains own several advantages of a eukaryotic cell but also the simplicity of a unicellular microorganism as a host organism for heterologous protein production. Table 2.2 summarizes the advantages and disadvantages of this system (Cereghino and Cregg, 1999; Macauley-Partrick *et al.*, 2005).

2.2.2 *Pichia pastoris* Expression System

P. pastoris has become a promising yeast host due to its many advantages over other microorganisms. Criteria that make this system feasible for an industrial application can be summarized as not requiring complex growth medium (Macauley-Patrick *et al.*, 2005), ability to reach high cell densities by using this minimal media (Wegner 1990), being easily manipulated in genetic terms and ability to perform post translational modifications such as glycosylation, disulfide bond formation, proteolytic processing therefore letting proteins to be produced in active forms than in bacterial systems as they are produced in forms of inclusion bodies (Cereghino *et al.* 2002; Jahic *et al.* 2006), ability of secretion of foreign proteins by using signal peptides into extracellular cultivation medium thus simplifying and lowering the cost of downstream processes (Cereghino and Cregg, 2000).

Besides these advantages, the most important factor about *P. pastoris* is that it can use an alcohol oxidase (AOX1) promoter, which is known to be one of the strongest

and tightly regulated eukaryotic promoter (Cereghino and Cregg, 1999). The capability of using this promoter makes *P. pastoris* a popular expression system in over-production of recombinant proteins in industrial applications (Cereghino *et al.*, 2002).

Table 2.2: Summary of advantages and disadvantages of *P. pastoris* expression system

Advantages	Disadvantages
• High yield and productivity • Chemically well defined, simple media • Ability of performing post translational processes • Genetically stable production strains • Low purification cost • High expression levels • GRAS • Broad pH range • Strong promoter (AOX1)	• High levels of proteolytic activity • Ability of non native glycosylation and hyper glycosylation • Long cultivation time • Difficulties in monitoring methanol during the process • Flammable and toxic properties of methanol

2.2.2.1 AOX1 and Alternative Promoters

The methanol metabolism of *P. pastoris* includes two alcohol oxidase genes: AOX1 which was first isolated by Ellis *et al.* (1985) and AOX2 which was discovered by Cregg *et al.* (1989). AOX2 is less active (responsible for 15% of alcohol oxidase activity) than that of AOX1 (responsible for 85% of alcohol oxidase activity) thus resulting the major alcohol oxidase activity detected in *P. pastoris* fermentation systems based on methanol is related to AOX1 which is responsible for nearly 30% of the total cell protein production (Cregg *et al.*, 1989; Cereghino and Cregg, 2000). In terms of nucleotide aminoacid sequences, AOX1 and AOX2 are 90% homologous where the difference in sequence located at 5' the protein coding portion of the two genes resulted in AOX activity differences. In the presence of methanol in the

environment, the AOX1 promoter is induced, whereas it is repressed when there is a repressing carbon source such as glucose, glycerol or ethanol. (Paulová *et al.* 2012). So, AOX1 promoter can be switched on or off by changing the carbon source in the environment and the contamination possibility of unwanted mutants or contaminants can be minimized (Daly and Hearn 2005).

With respect to its methanol-utilizing ability, *P. pastoris* strains can be divide into three phenotypes (Daly and Hearn, 2005):

- **Methanol utilization plus (Mut⁺) phenotype:** Both AOX1 and AOX2 genes are active and cells grow on methanol at wild-type rate with a maximum specific growth rate of 0.14 h⁻¹. They require high amounts of methanol and sensitive to high methanol concentrations (Jungo *et al.* 2006).
- **Methanol utilization slow (Mut^s) phenotype:** AOX1 gene is non-functional, whereas AOX2 is active; therefore, cells grow on methanol slower when compared to wild type. The maximum specific growth rate of Mut^s on methanol is found as 0.04 h⁻¹ (Jungo *et al.*, 2006). Cos *et al.* (2006) showed that Mut^s strains can be advantageous due to diminution of oxygen limitations and difficulties of high cell density cultivation of rapid growth strains.
- **Methanol utilization minus (Mut⁻) phenotype:** Both AOX1 and AOX2 genes are defective thus resulting cells which are unable to grow on methanol.

Although it's an effective expression system, some disadvantages were reported due to usage of methanol; which cannot be suitable for production of certain food products and therapeutics since methanol derived from petrochemical sources as well as storing the large quantities of methanol because of its explosive and flammable properties (Macauley-Patrick *et al.*, 2005; Paulova *et al.*, 2012). Besides problems originated from methanol, other disadvantages of *P. pastoris* expression system such

as long cultivation time, non-native glycosylation and high potential of proteolysis should also be considered.

When these limitations are taken into consideration, alternative promoters were developed by several researchers. Waterham *et al.* (1997) isolated glyceraldehyde-3-phosphate dehydrogenase (GAP) gene which has a constitutive promoter. This promoter allows the cells to grow and be induced in the presence of same carbon source such as glucose or glycerol thus removing the need of switching the cultures from one carbon source to another (Daly and Hearn 2005; Cereghino and Cregg 2000). Another alternative promoter is the glutathione-dependent formaldehyde dehydrogenase (FLD1) (Shen *et al.* 1998); which takes place in the methanol dissimilatory pathway and can be induced with either methanol or methylamine (Cereghino and Cregg, 2000); and, glycerol or glucose can be used as a carbon source and also for induction (Resina *et al.* 2004). The promoter isocitrate lyase (ICL1) reported by Menendez *et al.* (2003), however it needs more attention to be fully understood (Cos *et al.*, 2006). Other alternative promoters are 3-phosphoglycerate kinase (PGK1) (Almeida *et al.*, 2005), a peroxisomal matrix protein (*PEX8*) and a GTPase encoded by *YPT1* (Cos *et al.*, 2006).

2.2.2.2 Methanol, Glycerol and Sorbitol Metabolism

In order to understand the *P. pastoris* expression system under the control of AOX1 gene, methanol metabolism and the related pathways should be considered. Compartmentalized methanol metabolism pathways are presented in Figure 2.5.

AOX (alcohol oxidase), FMD (formate dehydrogenase) and DHAS (dihydroxyacetone synthase) are the key enzymes present in this metabolism and they are produced at high levels in the presence of methanol as sole carbon source (Gellisen *et al.*, 1992). As can be seen from Figure 2.5, methanol utilization starts with oxidation of methanol to formaldehyde and hydrogen peroxide which is catalyzed by AOX enzyme. In order to avoid hydrogen peroxide toxicity, these

reactions take place in peroxisome; where, the formation of formaldehyde is the rate limiting step as well as the regulation is achieved with the increasing amount of AOX enzyme (Couderc and Baratti 1980). The second product, hydrogen peroxide, is then degraded into oxygen and water by peroxisome catalase (Gellisen *et al.*, 1992). Consecutively, some of the formaldehyde is further oxidized either yielding energy by the dissimilatory pathway or generating biomass by the assimilatory pathway (Cereghino and Cregg, 2000). The activation of DHAS is the starting point of the assimilatory pathway. The activation of DHAS condensates formaldehyde with xylulose-5-monophosphate resulting in formation of glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone (DHA). In the next step, GAP enters the TCA cycle yielding further energy whereas NADH and DHA enter xylulose monophosphate cycle yielding xylulose 5-phosphate and biomass. In the dissimilatory pathway, the energy production and detoxification of formaldehyde and formate are catalyzed by NAD^+ -dependent formaldehyde dehydrogenase (FLD) and NAD^+ -dependent formate dehydrogenase (FDH), respectively. These oxidations yield carbon dioxide and NADH followed by the usage in the energy production for growth on methanol (Hartner and Glieder 2006).

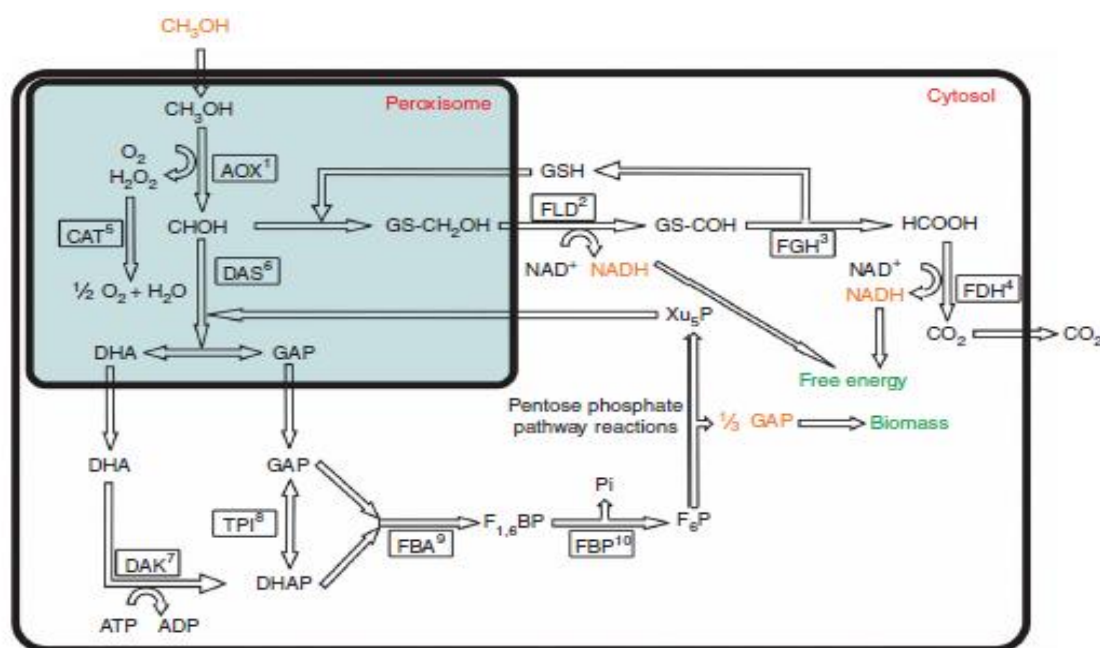


Figure 2.5 Methanol utilization pathway in *Pichia pastoris*. ¹AOX, alcohol oxidase; ²FLD, formaldehyde dehydrogenase; ³FGH, S-ormylglutathione hydrolase; ⁴FDH, formate dehydrogenase; ⁵CAT, catalase; ⁶DAS, dihydroxyacetone synthase; ⁷DAK, dihydroxyacetone kinase; ⁸TPI, triosephosphate isomerase; ⁹FBA, fructose-1,6-bisphosphate aldolase; ¹⁰FBP, fructose-1,6-bisphosphatase; DHA, dihydroxyacetone; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; F_{1,6}BP, fructose-1,6-bisphosphate; F₆P, fructose-6-phosphate; Pi, phosphate; Xu₅P, xylulose-5-phosphate; GSH, glutathione (De Schutter *et al.* 2009).

Another important metabolic pathway is the glycerol metabolism. A schematic representation of glycerol metabolism is given in Figure 2.6. Phosphorylation of glycerol to glycerol-3-phosphate (G3P) under the control of glycerol kinase is the first reaction. Thereafter, G3P is oxidized to dihydroxyacetone phosphate which then enters to the glycolytic pathway, by FDA-dependent glycerol-3-phosphate dehydrogenase resulting in the production of pyruvate. It is then oxidized to acetyl-CoA by pyruvate dehydrogenase. Entering of acetyl-CoA to tricarboxylic acid cycle (TCA) is the trigger of production of cellular constituents and cell wall components (Ren *et al.*, 2003).

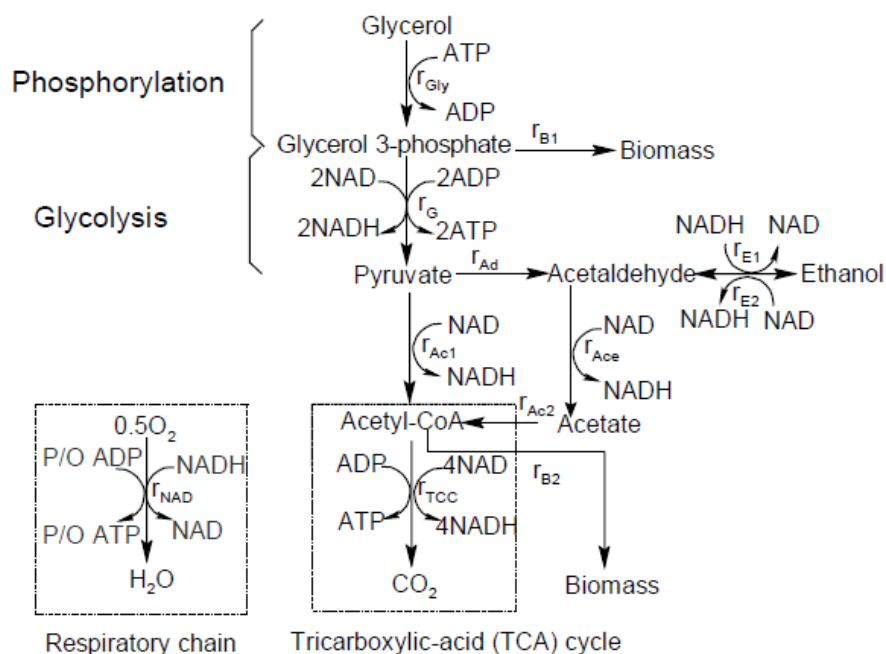


Figure 2.6 Metabolic pathways of glycerol in *Pichia pastoris* (Ren *et al.* 2003)

To improve recombinant protein production by *Pichia pastoris*, multicarbon substrate addition besides the methanol has been investigated (Cregg *et al.*, 1983; McGrew *et al.* 1997; Zhang *et al.* 2003). Due to repression or depression nature of AOX1 gene, glycerol or glucose cannot be used simultaneously with methanol. Therefore, a non-repressing carbon source, sorbitol, has been gained much attention by the researchers (Thorpe *et al.*, 1999; Jungo *et al.* 2007). It was shown that sorbitol utilization starts with the oxidation to fructose by sorbitol dehydrogenase enzyme followed by entering mannitol cycle (Figure 2.7). Fructose is then phosphorylated to fructose-6-phosphate by fructokinase and takes its place in the glycolysis (Walker 1998)

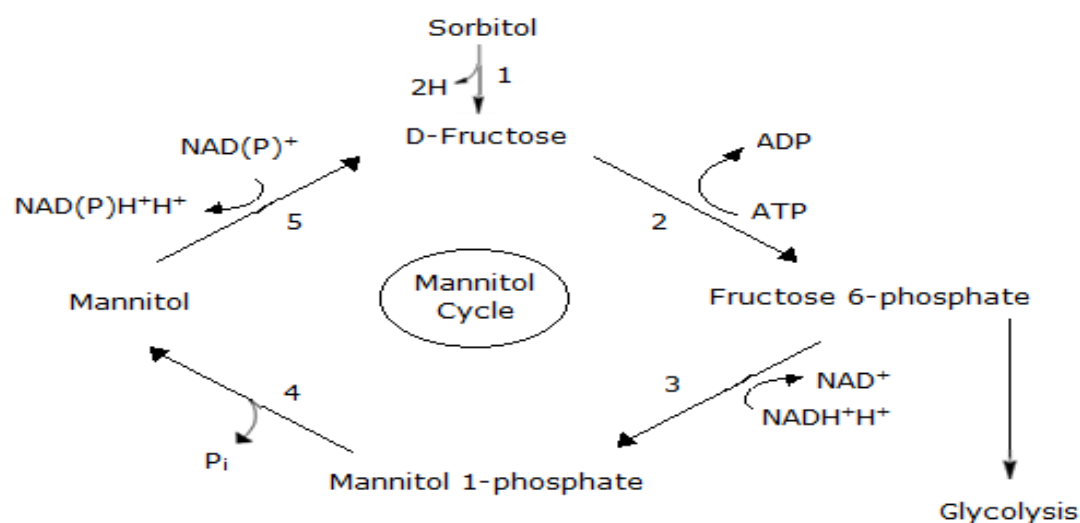


Figure 2.7 Metabolic pathway of sorbitol and mannitol in yeasts. 1, D-glucitol dehydrogenase; 2, fructokinase; 3, mannitol-phosphate dehydrogenase; 4, phosphatase; 5, mannitol dehydrogenase (Walker 1998).

2.2.2.3 Posttranslational Modifications and Protein Secretion

The main superiority of yeast cells is the capability of performing several post translational modifications such as correct protein folding, *O*- and *N*-linked glycolysation, development of signal peptides, and disulfide bond formation than those of bacterial hosts (Cregg *et al.* 2000; Macauley-Patrick *et al.* 2005).

Protein Folding and Disulfide Bond Formation

For production of active proteins, folding and disulfide bond formation are critical steps as well as they can be the rate-limiting steps in heterologous protein production (Hohenblum *et al.* 2004). Formation of secondary structures and disulfide bond formation in endoplasmic reticulum (ER) are the two main steps of correct protein folding (Holst *et al.* 1996). As reported in the study of Klausner (1989) protein folding and other post-translational modifications are under strict control thus allowing only properly folded proteins to enter Golgi apparatus. If a secreted protein is misfolded or improperly processed, it accumulates in ER thus causing a stress. In order to reduce that stress misfolded proteins are eliminated by ER-associated

degradation (Wu and Kaufman 2006). However, when there is no stress, the immature proteins are stabilized and aggregation formation is prevented by the help of ER-resident chaperons (Damasceno *et al.*, 2011) as well as the help of protein disulfide isomerase and other folding helper proteins to reduce the stress and increase the heterologous protein secretion (Idiris *et al.* 2010)

Glycosylation

Another post-translational modification performed by yeast cells is glycosylation. Glycosylation is an important process for protein folding, oligomer assembly, structural stability, specific signal transduction, recognition and secretion processes (Wright and Morrison 1997). There are two types of glycosylation: O- and N- linked glycosylation. *P. pastoris* is capable of performing both types, but also having some differences between mammalian cells (Figure 2.8). For instance, *P. pastoris* cells add only mannose residues whereas mammalian cells add other oligosaccharides composed of sialic acid, galactose, and *N*-acetylgalactosamine (Cereghino and Cregg 2000). Thus, some immunogenic effects may be observed in usage of glycoproteins as therapeutic products which are produced by recombinant *P. pastoris* (De Pourcq *et al.*, 2010).

O-linked glycosylation pattern in *Pichia pastoris* is similar when compared to other yeast strains and O-oligosaccharides are commonly attached to hydroxyl groups of serine and threonine amino acid residues of recombinant proteins (Schutter *et al.*, 2009).

N-linked glycosylation pathway is conserved both in yeast and higher eukaryotes. Glycosylation occurs in the lumen of ER after protein translation by adding glycans at the recognition site Asn-X-Ser/Thr (Gemmell and Trimble 1999). The stages of N-linked glycosylation are similar in both yeast and mammalian cells whereas the pattern differs after transport of protein to Golgi apparatus. Yeast cells result with hypermannosylated glycan structures (Gerngross 2004) whereas mammalian cells

depend on additional α -1,2-mannosidase to remove mannose residues (Tulsiani *et al.* 1982).

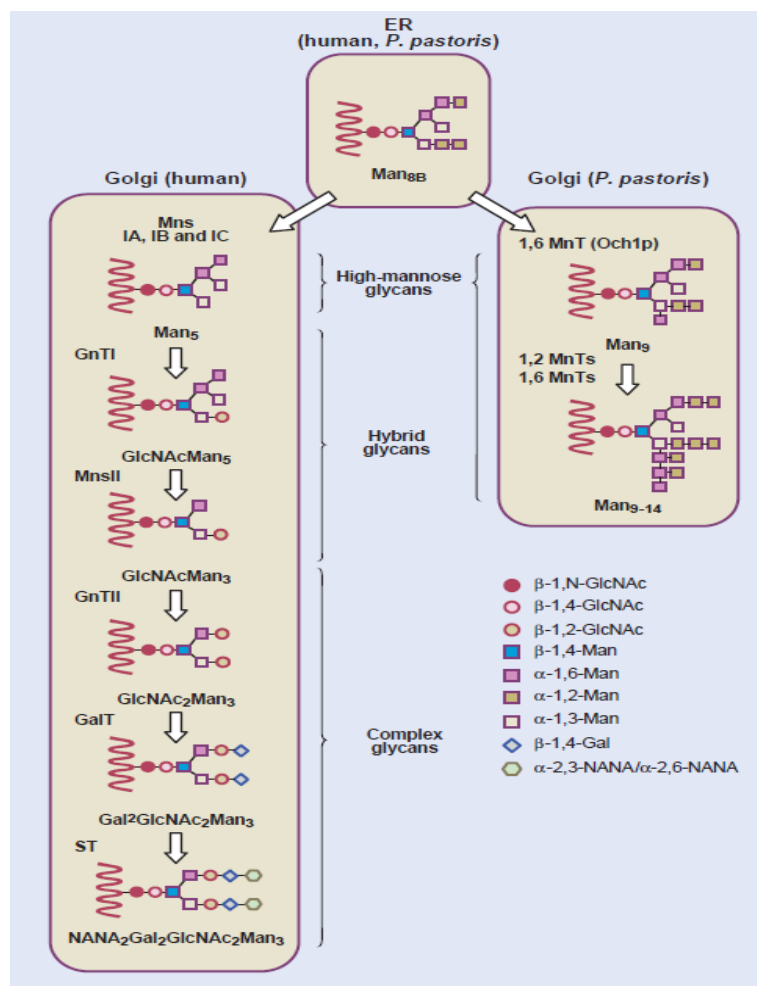


Figure 2.8 Glycosylation pathways in human and yeast. Mns, α -1,2-mannosidase; MnsII, mannosidase II; GnTI, β -1,2-*N*-acetylglucosaminyltransferase I; GnTII, β -1,2-*N*-acetylglucosaminyltransferase II; GalT, β -1,4-galactosyltransferase; SY, sialyltransferase; MnT, mannosyl-transferase (Gerngross 2004).

Oligosaccharide chains are shorter in *P. pastoris* than those of *S. cerevisiae*, thus eliminating the hyperglycosylation problem in *S. cerevisiae*. However, there are many attempts to overcome hypermannosylation problem for therapeutic glycoprotein production in *P. pastoris*. With the advances in glycoengineering, humanization of N-linked glycosylation pathway in *P. pastoris* has been further

investigated so far (Vervecken *et al.* 2004; Jacobs *et al.*, 2009; Damasceno *et al.* 2011).

Secretion Signals

One of the important issues in recombinant protein production is whether the protein should be expressed intracellularly or secreted to the environment. Although characteristics of the protein are important, extracellular production is the most favorable one, due to simplifying and lowering the cost of downstream processes (Daly and Hearn, 2005).

In order to secrete a protein to the environment, a signal sequence is needed. Signal sequences are peptides, which join the structure of extracellular protein to be secreted. For this purpose, protein's own native signal sequence can be used as well as different signal sequences can be chosen such as alpha-mating factor pre-pro leader sequence (α -MF), the acid phosphatase signal sequence (PHO) or the invertase signal sequences (SUC2) (Macauley-Patrick *et al.*, 2005). In *P. pastoris* expression system, the most widely used signal sequence is *S. cerevisiae* α -MF (Sreeknisha *et al.* 1997) leading higher amounts of extracellular recombinant protein than that of native signal sequence (Daly and Hearn 2005). *S. cerevisiae* α -MF includes pre-sequence signal peptide of 19 amino acids and a pro-region of 60 hydrophilic amino acid acids (Julius *et al.* 1984). In *P. pastoris* the secretion mechanism occurs in three stages. At the first stage, protein is reassigned to endoplasmic reticulum where the pre-domain removed by signal peptidase. Thereafter, the pro-protein is transported to Golgi apparatus resulting with the removal of pro-domain is removed by means of dibasic endo-peptidase (*kex2*) activity. At the last step, recombinant protein is packed to the secretory vesicles and then secreted to extracellular environment (Daly and Hearn, 2005).

2.2.2.4 Proteolytic Degradation

Proteolytic degradation is one of the major problems in *P. pastoris* expression system which causes alleviance in the efficient secretion and purification of recombinant

proteins, reduction in protein yield, loss of biological activity, contamination of the product by degradation intermediates in downstream processes (Idiris *et al.*, 2010; Jahic *et al.*, 2003). There are many reasons that cause proteolytic degradation such as heat and pH changes, starvation, change of carbon source and foreign chemicals in the cultivation medium (Hilt and Wolf 1992). For *P. pastoris* expression system, the high oxygen demand of methanol metabolism causes higher amounts of proteases than in glycerol metabolism due to the oxidative stress (Sinha *et al.* 2005; Potvin *et al.*, 2010). Since *P. pastoris* cells can reach high cell densities during the fermentation, vacuolar proteases which can be detected in supernatant of *P. pastoris* cultures together with cell lysis are the main factors in protein degradation (Shen *et al.* 1998).

To overcome this problem many attempts have been performed so far by several researches including controlling cultivation conditions, changing medium composition, addition of protease inhibitors, peptone, casaminoacids and specific aminoacids (Enfors 1992; Gonzalez-Lopez *et al.* 2002). Li *et al.* (2001) decreased temperature from 30°C to 23°C resulting the yield of herring antifreeze proteins from 5.3 mg L⁻¹ to 18 mg L⁻¹. Dragosits *et al.* (2009) showed that the specific productivity of 3H6 Fab fragment was 3-fold higher at 20°C than that of 30°C. Like temperature, pH is also an important parameter for cell growth and recombinant protein production. The decrease of pH of cultivation medium from 5.0 to 4.0 resulted with an increase in expression of CBM (cellulose-binding module)-CALB (cellulose 6A and lipase B) in *P. pastoris* from 40 to 90% (Jahic *et al.* 2003). Potvin *et al.* (2010) also showed that providing of competing enzymatic substrates for proteases other than desired product can be used to prevent the proteolytic degradation.

However, most of these attempts affected limitedly thus increasing the demand for protease deficient strains constructed by genetic manipulations. For this purpose, several protease-deficient strains, SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*), and SMD1168 (*his4 pep4*) have been effectively used in heterologous protein production (Brierley 1998; White *et al.* 1995).

2.3 Genetic Engineering Techniques: Methodology

Construction of metabolically engineered products, which open new avenues in industrial biotechnology, uses the genetic engineering methods which can be summarized as follows:

- The desired gene is amplified with Polymerase Chain Reaction (PCR) by using designed appropriate primers including restriction sites and is cut with restriction enzymes which are required for combining plasmid and the gene.
- After digestion of gene and plasmid, they are ligated by ligase enzyme and the recombinant plasmid including the desired gene is cloned into microorganisms either by plasmid transformation or genomic integration.
- Transformed microorganisms are plated on a medium containing the appropriate antibiotic, whose resistance gene is carried by the plasmid. Therefore only the microorganisms carrying the plasmid can grow on that medium.
- Selected microorganisms from the medium are further analysed by other methods whether they carry the plasmid or not.

The amplification of the desired gene fragment is a crucial step for this procedure. PCR is method for this purpose which amplifies the desired gene *in vitro* by separation the two strands of DNA at high temperatures and incubation with specially designed primers and DNA polymerase enzyme. There are three major steps in PCR those are repeated for 30-40 cycles. These cycles are achieved in an automated thermocycler which can raise and lower the temperature very rapidly within the designed time intervals.. First step is the denaturation step of DNA strand which is carried out at 95°C. Subsequent step is the annealing of specially designed primers to the DNA strand whose temperature greatly depends on the desired gene fragment and the properties of primers. Temperature of this step can vary between 40-65°C. The last step is the elongation which occurs at 72°C by the catalytic effect of DNA polymerases. These DNA polymerases are thermostable enzymes isolated

from hyper thermophilic microorganisms, e.g. *Thermus aquaticus* (Glazer and Nikaido 1995) .

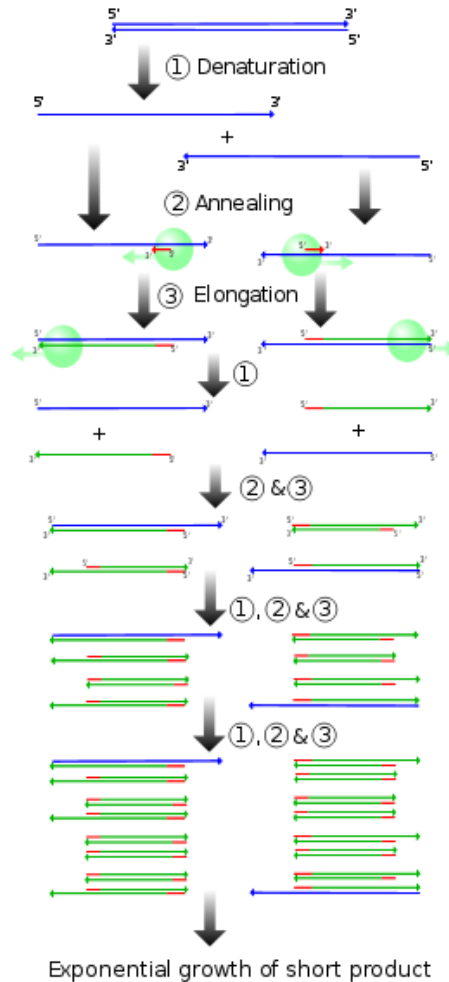


Figure 2.9: Schematic drawing of the PCR cycle. (1) Denaturing at 94–96 °C. (2) Annealing at 45-60 °C (3) Elongation at 72 °C. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses which results with 2^n products where n is the number of cycles.

Another important step of this procedure is the choosing the plasmid. Plasmids are autonomously replicating, circular DNA molecules. In bacteria, they don't integrate

to the bacterial genome and nonessential for cell survival under non-selective conditions (Clug *et al.*, 2006). There are also some plasmids that can integrate to host genome. In recombinant DNA technology, the plasmids have 3 common regions: origin of replication; a marker for selection, e.g. a drug or antibiotic resistance gene and a multiple copy site where exogenous DNA fragment can be inserted (Lodish *et al.* 2003). In this study, pPICZ α -A was used for *Pichia pastoris* transformation. (Figure 2.10). This vector includes Zeocin resistance gene, 13 unique multiple copy sites, AOX1 promoter region to regulate and induce protein expression, α -factor signal sequence for direct extracellular protein secretion, polyhistidine tag (6xHis) and Factor Xa for easy purification of target protein.

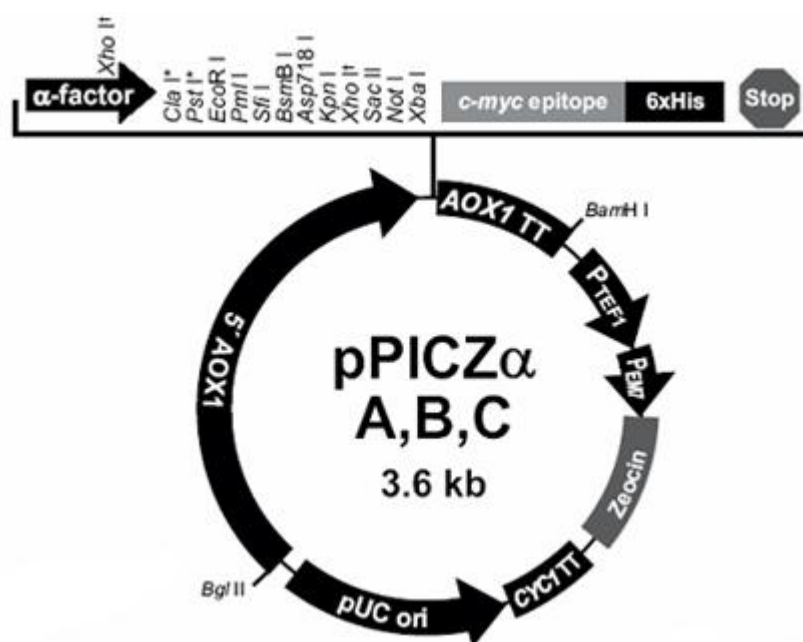


Figure 2.10: pPICZ α -A vector (Invitrogen, catalog number: V195-20).

To insert the desired gene fragment to multiple copy site of plasmid, both fragments (gene and plasmid) should be digested with appropriate restriction enzymes. Normally, restriction enzymes protect the bacteria from foreign DNA strands *in vivo* by recognizing host's own DNA molecule from its methylation pattern; otherwise,

they cut the DNA from outside of the helix. There are three types of restriction enzymes. Type I and Type III restriction enzymes cut DNA at random locations as far as 1000 or more and 25 base pairs from the recognition site, respectively. They also need high ATP energy for this process. As the most useful type of cloning in biotechnology, Type II restriction enzymes cut within the recognition site by recognizing 4-8 bp long base pairs without requiring ATP energy (Kirk and Othmer, 1994).

Restriction of both fragments lead single stranded tails which are called sticky ends. Although some restriction enzymes cut the DNA strand that end up with blunt ends, since sticky ends have a tendency to anneal the complementary strand they are frequently used in this procedure. Thus, incubation of cut gene of interest and plasmid results with the annealing of these two strands. The phosphodiester bonds missing between the annealed strands are bounded covalently by DNA ligase enzyme by condensation of 3'-hydroxyl group with a 5'-phosphate group. Since the ligation reaction needs not only the correct orientation of gene of interest and the plasmid but also the prevention of relegation of opened plasmid, it is the rate limiting step in this procedure.

The step after ligation is the insertion of plasmid into host microorganism. This is done by transformation in most cases. There are 4 main methods of transformation (Glazer, 1995):

- Natural Transformation: Foreign DNA from the environment is taken by the bacteria naturally without any *in vitro* manipulations.
- Artificial Transformation: The cells are got competent by using several chemicals such as CaCl_2 at 0°C so that foreign DNA can easily be taken into the cell.
- Electroporation: The cells are made competent by applying short electrical pulses of high voltage so that transient pores are created in the membrane.
- Protoplast transformation: Rigid cell wall which block the transfer of foreign DNA into cell is hydrolyzed by enzymes e.g lysozyme, so that the cell is converted into protoplasts.

2.4 Bioprocess Operation Parameters

The cells can be considered as micro-bioreactors, because the desired product is produced via intracellular biochemical reaction network inside the cell. Therefore, concentration of secreted protein in bioreactor medium is related with the cell population or density. The oxygen transfer, pH and temperature being the main bioprocess parameters have major effects on product formation in aerobic bioprocesses. These parameters affect metabolic pathways and change metabolic fluxes so that, optimum conditions are required for an industrial feasible production (Çalık *et al.* 1999). In shake bioreactor experiments the parameters of volume, oxygen transfer and substrate addition are limited. In addition, the efficient control of pH and the monitoring of the parameters are not possible (Macauley-Patrick *et al.*, 2005).

In molecular level, heterologous protein expression is affected by many parameters including vector system, promoter choice, codon usage bias, post-translational processes (Niebauer and Robinson 2005). Although general principles for *Pichia pastoris* expression system are provided by Invitrogen, every system needs its own optimization process.

2.4.1 Medium Composition

For extracellular and intracellular heterologous proteins, composition of the medium has diverse effects on cell viability and growth (Kang *et al.* 2000; Chen *et al.* 2000). As provided by Invitrogen Corp. (2002), basal salt medium (BSM) is the common medium that is used for *P. pastoris* expression system. BSM supplies higher concentrations of basic elements whereas trace salt solution, PTM1 provides micronutrients (Invitrogen, 2002). The importance of micronutrients was studied by several researchers. It was shown that at least 200 μM copper is required for maximum activity of laccase produced by recombinant *P. pastoris* (O'Callaghan *et al.* 2002). Boze *et al.* (2001) revealed that the supplementation of basal medium with

seven vitamins and two trace elements increased the yield of porcine follicle-stimulating hormone production as well as cell growth.

In *Pichia pastoris* expression systems ammonium hydroxide is used for both controlling the pH and also as a nitrogen source. Although ammonium hydroxide is required for nitrogen source, the concentration in the medium should be maximum 0.6 M otherwise growth may be inhibited (Yang *et al.* 2004). However, lack of nitrogen increases the proteolytic activity (Kobayashi *et al.* 2000).

2.4.2 Temperature

For biochemical reactions that take place in microbial production, optimum temperature operation condition is the isothermal condition at a determined, constant, optimum temperature, during the fermentation process, since these reactions are liquid phase reactions. Although the effect of temperature varies on different expression systems, it can be generalized that higher temperature may cause denaturation of proteins; whereas low temperature decreases the reaction rates and transport process rates (Nielsen *et al.*, 2003).

Although optimum temperature for the cell growth and production is determined to be as 30°C for *P. pastoris* (Cos *et al.*, 2006), some studies reported that lowering the temperature improves product yield and AOX activity (Jahic *et al.*, 2003; Sarramegna *et al.* 2002), while reduces the cell lysis (Hong *et al.*, 2002; Dragosits *et al.* 2009).

2.4.3 pH

Hydrogen ion concentration (pH) is an important parameter which affects the activity of enzymes consequently the reaction rates, transport mechanisms, cell growth (Chiruvolu *et al.* 1999), the expression rates (Roubin *et al.* 1992), the secretion efficiency (Bae *et al.* 1999) and the proteolytic degradation (Song and Chung 1999).

P. pastoris can tolerate a wide range of pH from 3.0 to 7.0 whereas this range doesn't affect the cell growth significantly (Inan *et al.* 1999) . Several studies show that different pH values can be optimal for different recombinant proteins since every protein can be stable at different pH values (Brierley *et al.*, 1998; Clare *et al.* 1991). Production of human serum albumin was increased significantly by raising the pH of the medium from 5.2 to 6.0 (Sreeknisha *et al.* 1998). In recombinant human growth hormone (rhGH) production by *P. pastoris*, the maximum yield was obtained at pH 5.0 (Çalık *et al.*, 2010); whereas Soyaslan and Çalık (2011) demonstrated that the highest production of rEPO by *P. pastoris* Mut⁺ strain was obtained when cultivation medium was operated at pH 5.0 for the glycerol and glycerol fed-batch stages and at pH 4.5 for induction phase.

2.4.5 Oxygen

Oxygen is also an important parameter for aerobic fermentation processes. Oxygen has diverse effects on product formation because it influences metabolic pathways and metabolic fluxes (Çalık *et al.*, 1999). Therefore, in order to improve the product yield; the effects of oxygen transfer process should be investigated in detail. Although *P. pastoris* is an anaerobic organism, requires oxygen when it grows on methanol; thus, it does not produce ethanol or acetic acid like it does in fermentative growth (Cereghino *et al.*, 2002). Therefore, under the control of AOX1 promoter, the oxygen transfer in *P. pastoris* fermentation process, whereupon the oxygen transfer characteristics, appears as an important bioreactor operation parameter.

2.5 Computation of Bioprocess Characteristics

For an accurate evaluation of the bioprocess, computation of bioprocess characteristics such as specific rates and yields are required. Therefore, computation of specific growth rate, substrate consumption and product formation rates and yield coefficients are presented in this section.

A typical microbial growth (Figure 2.11) can be grouped in four main phases: Lag phase, log phase, stationary phase and death phase (Villadsen *et al.*, 2011). Modeling microbial growth and computation of specific rates are performed assuming that all the cells are in log phase and as if they are identical to each other.

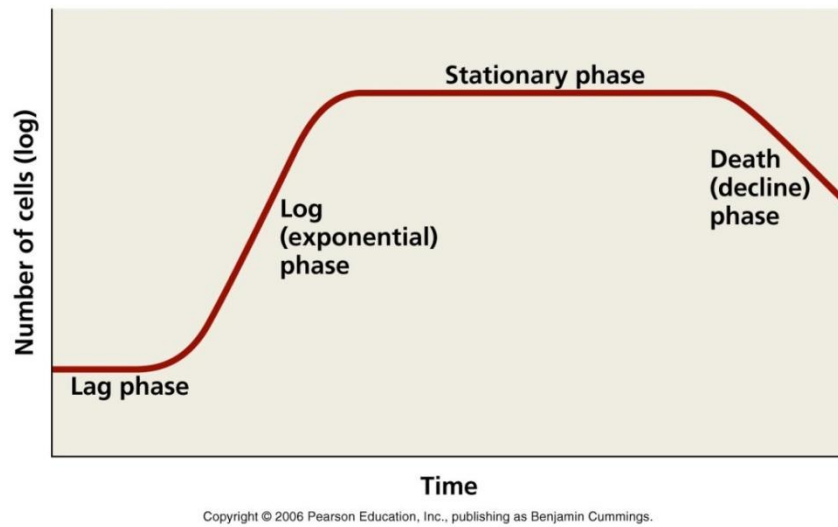


Figure 2.11: Microbial growth phases observed in batch systems.

2.5.1 Specific Growth Rate

Specific growth rate (μ) is an important parameter to understand and control the cell growth. For fed-batch processes, it is derived from the general mass balance;

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

where C_x is the cell concentration (kg m^{-3}), r_x is the cell growth rate ($\text{kg m}^{-3} \text{ h}^{-1}$) and V is the total volume (m^3). The main assumption for Equation 2.1 is that the cells are

generated batch-wise and not lost through sampling. The biomass formation rate (r_x) is defined as;

$$r_x = \mu C_x \quad (2.2)$$

where μ is specific growth rate (h^{-1}) and C_x is the cell concentration. Combining equation (2.1) and (2.2), one can obtain equation (2.3), as follows:

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

Since the operation mode used in this study is fed-batch, the volume changes due to the feeding of methanol and sorbitol with a predetermined rate should be defined. Therefore, the second assumption is that the system has a constant density, which can be obtained from the continuity equation for the semi-batch system, as follows:

$$\frac{dV}{dt} = Q_{\text{in}} - Q_{\text{out}} \quad (2.4)$$

Where Q is the volumetric flow rate ($\text{m}^3 \text{ h}^{-1}$). Since volume change is due to methanol feeding, Q_{out} term is negligible; thus, inserting the equation (2.4) into equation (2.3):

$$\frac{dC_x}{dt} = \left(\mu - \frac{Q}{V} \right) C_x \quad (2.5)$$

Rearranging the equation (2.5):

$$\mu = \frac{dC_x}{dt} \frac{1}{C_x} + \frac{Q}{V} \quad (2.6)$$

2.5.2 Methanol and Sorbitol Consumption Rates

Methanol was fed continuously to the fed-batch operated bioreactor beginning from $t=0$ h. Therefore, the mass balance for methanol can be written as follows:

$$QC_{MO} - 0 + r_M V = \frac{d(C_M V)}{dt} \quad (2.7)$$

in which, the substrate consumption rate, r_M ($\text{kg m}^{-3} \text{h}^{-1}$), can be defined as follows:

$$r_M = q_M C_X \quad (2.8)$$

where, q_M is a specific substrate consumption rate (h^{-1}) and C_X represents the cell concentration. By inserting the equation (2.8) into equation (2.7);

$$QC_M + q_M C_X V = V \frac{dC_M}{dt} + C_M \frac{dV}{dt} \quad (2.9)$$

In this work methanol accumulation was prevented, so that the system can be assumed in quasi-steady state conditions; thus, the last term in equation (2.9) is negligible:

$$C_M \frac{dV}{dt} \sim 0 \quad (2.10)$$

By dividing all the terms by V , the final form of equation (2.9) is:

$$\frac{dC_M}{dt} = \frac{Q}{V} C_{M0} + q_M C_X \quad (2.11)$$

By rearranging the equation (2.11) specific consumption rate of methanol (q_M) (h^{-1}) is obtained;

$$q_M = \frac{Q}{V} \frac{C_{M0}}{C_X} - \frac{1}{C_X} \frac{dC_M}{dt} \quad (2.12)$$

The second substrate sorbitol is fed to the bioreactor batch-wise; therefore, the mass balance equation for sorbitol is as follows:

$$r_S V = \frac{d(C_S V)}{dt} \quad (2.13)$$

in which C_S is the sorbitol concentration ($kg\ m^{-3}$). The substrate consumption rate (r_S) ($kg\ m^{-3}\ h^{-1}$) can be defined as:

$$r_S = q_S C_X \quad (2.14)$$

where q_S is the specific substrate consumption rate (h^{-1}). Combining equation (2.14) and equation (2.13), and then by dividing all terms by V , equation (2.15) is obtained:

$$\frac{dC_S}{dt} = -\frac{Q}{V} C_S + q_S C_X \quad (2.15)$$

The specific sorbitol consumption rate (q_S) is obtained by rearranging the equation (2.15), as follows:

$$q_S = \left(\frac{dC_S}{dt} + \frac{Q}{V} C_S \right) \frac{1}{C_X} \quad (2.16)$$

2.5.3 Overall Yield Coefficients

The yield coefficients that are used for biotechnological processes are presented in Table 2.3. The biomass ($Y_{X/S}$) and product ($Y_{P/S}$) yields are important process values that show the conversion efficiency of the substrate and process efficiency for the product formation. The verbal definition for the first parameter, $Y_{X/S}$ is defined as the ratio of the biomass amount produced per substrate amount consumed within a time interval; and the latter, $Y_{P/S}$ is defined as the ratio of the product amount per substrate amount consumed within the same time interval. Therefore, in mathematical form, these coefficients are defined as follows:

$$Y_{X/S} = -\frac{\Delta X}{\Delta S} \quad (2.17)$$

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \quad (2.18)$$

Where X is biomass, P is product and S is substrate, $Y_{X/S}$ is the overall biomass yield coefficient, $Y_{P/S}$ is the overall product yield coefficient, ΔX is the mass of cell produced, ΔP is the mass of product produced and ΔS is the mass of substrate consumed.

Table 2.3 The yield coefficients defined for a bioprocess.

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

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

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

3.2 The Microorganism, Plasmids and Maintenance of Microorganisms

xylA gene was amplified from PRSETA::*xylA* plasmid (Anghardi, 2011). *Pichia pastoris* X-33 wild type strain and pPICZ α -A shuttle vector carrying Zeocin resistance were purchased from Invitrogen. For propagations of pPICZ α -A shuttle vectors, *E. coli* TOP10 (Invitrogen) were used, respectively. *E. coli* strains carrying shuttle vectors carrying *xylA* gene cloned were named as *E. coli* pPICZ α -A::*xylA*. *P. pastoris* strain carrying *xylA* gene in their genomes was named as *P. pastoris* eP20. For the production of thermostable glucose isomerase, microorganisms and plasmids that are used in this study were summarized in Table 3.1.

Table 3.1 Strains and plasmids used in this study.

Microorganism	Strain	Genotype/Plasmid	Source
<i>E. coli</i>	BL21 (DE3) PlySs	PRSETA:: <i>xylA</i>	Anghardi, 2011
<i>E. coli</i>	TOP10	pPICZ α -A:: <i>xylA</i>	This work
<i>P. pastoris</i>	X-33	Wild type	Invitrogen
<i>P. pastoris</i>	eP20	aox1:: pPICZ α -A- <i>xylA</i>	This work

Microorganisms are stored in microbanks at -55°C for long term storage. For this purpose, young cell colonies were inoculated into cryopreservative fluid present in the microbank vial. The vial was shaken for 5 seconds and waited for 1-2 minutes to assure the adsorption of the cells into the porous beads. Cryopreservative fluid was aspirated before storing the vial at -55°C.

3.3 Growth Media

All growth media were autoclaved at 121°C for 20 minutes for sterilization and appropriate antibiotics were added after cooling to approximately 50°C.

3.3.1 Solid Media

P. pastoris eP20 was grown on solid YPD medium containing 0.15 g L⁻¹ Zeocin. YPD medium without antibiotics was used for inoculation of wild type *P. pastoris* X-33. Composition of solid YPD medium is given in Table 3.2.

Table 3.2 Composition of solid YPD medium.

Compound	Concentration (g L ⁻¹)
Yeast extract	10
Peptone	20
Glucose	20
Agar	20
Zeocin	0.15

0.1 g L⁻¹ ampicillin containing LSLB medium was used for inoculation of *E. coli* BL21 (DE3) PlySs carrying PRSETA::xylA plasmid. *E. coli* TOP10 carrying pPICZα-A::xylA plasmids were inoculated onto LSLB medium containing 0.025 g L⁻¹ Zeocin. *E. coli* strains which didn't carry any plasmids were grown in LSLB

medium without antibiotics. Composition of solid LSLB media is given in Table 3.3 and Table 3.4.

Table 3.3 Composition of solid LSLB medium for *E. coli* TOP10 carrying pPICZ α -A::*xylA*.

Compound	Composition (g L ⁻¹)
Tryptone	10
Yeast extract	5
NaCl	5
Zeocin	0.025
Agar	15

Table 3.4 Composition of solid LSLB medium for *E. coli* BL21 (DE3) PlySs carrying PRSETA::*xylA*.

Compound	Composition (g L ⁻¹)
Tryptone	10
Yeast extract	5
NaCl	5
Ampicillin	0.1
Agar	15

3.3.2 Precultivation Media

After cultivation of recombinant *P. pastoris* eP20 on YPD solid medium for 60 hours, grown cells were inoculated to precultivation medium. *P. pastoris* eP20 was inoculated BMGY as the precultivation medium. Chloramphenicol and biotin were added after sterilization. Composition of BMGY is given in Table 3.5.

Table 3.5 Composition of precultivation medium, BMGY.

Compound	Composition (g L ⁻¹)
Yeast extract	10
Peptone	20
Potassium phosphate buffer (pH=6.0)	0.1 M
Yeast Nitrogen Base (YNB) (w/o aminacids)	13.4
Biotin	4 x 10 ⁻⁵
Glycerol	10
Chloramphenicol (34 mg ml ⁻¹)	1 ml L ⁻¹

3.3.3 Production Media

After precultivation, *P. pastoris* eP20 was inoculated into production medium. Composition of production media for laboratory scale air filtered shake bioreactor experiments and trace element solution, PTM1, into which *P. pastoris* eP20 cells were inoculated are given in Table 3.6 and 3.7, respectively. Production medium was a defined medium reported by Jungo *et al* (2006) with the following modification. Instead of ammonium chloride, ammonium sulphate was added as the N source. PTM1 was sterilized by filtering and added after sterilization. Basal salt medium (BSM) was used as the production medium for pilot scale bioreactor experiments for *P. pastoris* eP20 cells. Composition of BSM is given in Table 3.8.

Table 3.6 Production medium of *P. pastoris* eP20 for laboratory scale air filtered shake bioreactor experiments.

Compound	Composition (g L⁻¹)
Methanol	1 ml
Sorbitol	30
Ammonium sulphate	4.35
Potassium phosphate buffer (pH=6.0)	0.1 M
MgSO₄·7H₂O	14.9
CaSO₄·2H₂O	1.17
Chloramphenicol (34 mg ml⁻¹)	1 ml
PTM1	4.35 ml

Table 3.7 Trace salt solution (PTM1) composition

Compound	Composition (g L⁻¹)
CuSO₄·5H₂O	6
NaI	0.08
MnSO₄·H₂O	3
Na₂MoO₄·2H₂O	0.2
H₃BO₃	0.02
ZnCl₂	20
FeSO₄·7H₂O	65
CoCl₂	0.5
H₂SO₄	5
Biotin	0.2

Table 3.8 BSM medium composition.

Compound	Composition (g L ⁻¹)
85% H ₃ PO ₄	26.7 ml
CaSO ₄ ·2H ₂ O	1.17
MgSO ₄ ·7H ₂ O	14.9
KOH	4.13
K ₂ SO ₄	18.2
Glycerol	40

3.4 Genetic Engineering Techniques

3.4.1 Plasmid DNA Isolation from *E. coli*

Plasmid DNA from *E. coli* cells was isolated by Alkaline Lysis Midiprep Method reported by Sambrook and Russell (2001). Solution compositions used for isolation of plasmid DNA were given in Appendix A. For this purpose single colony from selective medium was inoculated to LSLB medium containing appropriate antibiotics and cultivated overnight at 37°C. After overnight cultivation, the culture was transferred to 15 ml sterile falcon and centrifuged at 2000 g for 10 minutes at 4°C and supernatant was removed. Cell pellet was resuspended in 200 µl ice-cold Alkaline Lysis Solution I by vigorous vortexing and mixture was transferred to a 1.5 ml eppendorf tube. 400 µl freshly prepared Alkaline Lysis Solution II was added and the content was mixed by inverting 5-6 times. Thereafter, 300 µl ice cold Alkaline Lysis Solution III was added to the mixture and mixed by inverting 5-6 times. Eppendorf tube was stored on ice 3-5 minutes. The mixture was centrifuged at 16100 g for 5 minutes at 4°C and 600 µl of supernatant was transferred to a fresh eppendorf tube. An equal volume of phenol:chloroform was added to the solution and mixed by vortexing followed by a centrifugation step at 16100 g for 2 minutes at 4°C. The aqueous upper layer was transferred to a fresh eppendorf tube. 600 µl isopropanol was added followed by vigorous vortexing and the mixture was stored at room temperature for 2 minutes. The mixture was centrifuged at 16100 g for 5 minutes at

room temperature and supernatant was removed. The tube was allowed to stand on an inverted position for the fluid to drain away. After approximately 4-5 minutes, 1 ml of 70% ethanol was added and centrifuged at 16100 g for 2 minutes at room temperature. Supernatant was removed carefully. The remaining any ethanol beads were removed by evaporation at room temperature for 10-15 minutes by avoiding dehydration of the pellet. After evaporation of ethanol, the pellet was dissolved in 100 μ l TE buffer (pH:8.0) containing 20 μ g/ml RNase A and stored at -20°C until further use.

3.4.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate, visualize and extract the DNA strands. For this purpose, 0.8 g of agarose was dissolved in 100 ml 1X TBE buffer and heated until boiling point by using a hot plate. After cooling it to approximately 50°C, 3.5 μ l of ethidium bromide from a 10 mg ml⁻¹ stock solution was added. The gel was poured into a gel tray and suitable comb was inserted. After waiting for 10-15 minutes, cooled gel was taken and used in a tank filled with 1X TBE buffer. Loaded DNA samples were run with the DNA ladder for size estimation at 90 V for 45-60 minutes. Separated DNA strands were visualized under UV illumination at 302 nm.

3.4.3 DNA Extraction from Agarose Gels

DNA extraction was performed by using Gel Elution Kit (GeneMark) in the case of a single DNA band was to be purified. For this purpose, the visualized DNA fragment after electrophoresis was excised from the gel by using a clean, sharp scalpel (up to 350 mg) and purified according to manufacturer's instructions. Purified DNA fragment was eluted in proper amount of Elution Buffer supplied by the manufacturer at the end of the protocol and stored at -20°C until use.

3.4.4 Primer Design

Primers were designed in accordance with the GI sequence (Dekker *et al*, 1991). Restriction sites were determined by the help of Restriction Mapper website of USA Molecular Biology Resources (www.restrictionmapper.org). Codon usage bias of *P. pastoris* was taken into account, where possible. Possibility of homo and heterodimer formation, loop formation and melting points of the primers were determined by using a program called OligoAnalyzer. These primers were used for insertion of the gene into pPICZ α -A. The primers and their target sequences used in this study were given in Table 3.9. Thermodynamic properties of designed primers are given in Appendix D.

Table 3.9 Primers and their target sequences used in this study.

Name	Abbreviation	Target Nucleotide Sequence
GI Forward Primer	EGI-F	<i>xylA</i>
GI Reverse Primer	EGI-R	<i>xylA</i>
AOX Forward Primer	AOX-F	<i>AOX1</i>
AOX Reverse Primer	AOX-R	<i>AOX1</i>

Designed primers were dissolved in sterile water with a concentration of 100 pmol μl^{-1} and stored at -20°C for stock. Then, they were diluted 50 times to 5 pmol μl^{-1} resulting 5 μM primer solutions that were used in PCR experiments.

3.4.5 Amplification of *xylA* Gene

PCR was used for amplification of DNA. For this purpose, primers that are specific to the sequences which are given in Table 3.9 were designed and used in the amplification process. Annealing temperature and MgSO₄ concentration were optimized as the PCR process parameters. PCR reaction was carried out by a thermocycler (Techgene, Flexigene).

3.4.5.1 DNA Purification after PCR

PCR product was purified by using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. Purified DNA was eluted in 50 µl of Elution Buffer supplied by the manufacturer and stored at -20°C for further use.

3.4.6 Digestion of DNA using Restriction Endonucleases

Restriction digestion of the genes and the plasmids were performed by incubating the DNA fragments with proper restriction enzymes at 37°C for 2 hours. After double digestion, the reaction was terminated by incubating the mixture at 80°C for 20 minutes. Reaction mixture content of double digestion is given in Table 3.10.

Table 3.10 Reaction mixture content of double digestion.

Component	Amount
DNA fragment	45-200 ng
10X Tango Buffer	4 µl
<i>EcoR</i> -I (10 U µl ⁻¹)	0.5 µl
<i>Xba</i> -I (10 U µl ⁻¹)	1 µl
Water	Up to 20 µl

For transfection of *P. pastoris*, a linearized plasmid was used. Restriction digestion reaction mixture for linearization was given in Table 3.11. Restriction digestion of the plasmid was performed by incubating the DNA fragments with proper restriction enzyme at 37°C for 2 hours and reaction was terminated by heat inactivation at 65 °C for 20 minutes. Digestion was verified by agarose gel electrophoresis. Vector was extracted by phenol/chloroform and ethanol precipitated by the addition of 1/10 volume 3 M sodium acetate and 2.5 volume 100% ethanol. Pellet was washed by 80% ethanol and dissolved in 10 µl of sterile water. Concentration of plasmid was adjusted as 200 ng µl⁻¹ for efficient transfection.

Table 3.11 Restriction digestion mixture for linearizing of pPICZα-A::xylA.

Component	Amount
pPICZα-A::xylA	30-50 µg
10X Buffer <i>Pme</i> I	5 µl
<i>Pme</i> I (5 U µl⁻¹)	2 µl
Water	Up to 50 µl

3.4.6 Ligation

The amplified gene by PCR was cloned into suitable expression vectors from its sticky ends obtained by restriction digestion by optimizing molar ratio as 1:1 or 3:1.

$$100 \text{ ng vector} \times \frac{\text{Size of insert (bp)}}{\text{Size of vector (bp)}} \times \text{Molar ratio} = \text{amount of insert (ng)}$$

The ligation reaction was performed by incubation of the ligation mixture at 22°C for 2 hours and the reaction was terminated by incubating the mixture at 65°C for 20

minutes. Ligation product stored at -20°C until further use. 10 µl of this reaction mixture was used for *E. coli* transformation.

3.4.7 Transformation into *E. coli*

Transformation of *E. coli* was achieved by using CaCl₂ method described by Sambrook and Russell (2001). The procedure started with the incubation of *E. coli* TOP10 strain at 37°C for 16-20 hours on LB solid medium. A single colony from this medium was picked and inoculated to 50 ml LB Broth. After incubation for 3.5-4 hours (until the OD₆₀₀ reaches approximately 0.35-0.4) at 37°C with shaking at 200 rpm, the cells were transferred to a 50 ml ice-cold falcon and stored on ice for 10 minutes. Thereafter, the cells were harvested by centrifuging at 2700 g for 10 minutes at 4°C and supernatant was removed. Pellet was resuspended in 30 ml ice-cold 80 mM MgCl₂-20 mM CaCl₂ by swirling. Mixture was centrifuged at 2700 g for 10 minutes at 4°C and supernatant was decanted. Pellet was resuspended in 2 ml ice cold 0.1 M CaCl₂ by vortexing. 200 µl of this solution was transferred to a sterile ice cold 50 ml falcon tube. 10 µl of ligation mixture (Section 3.4.6) was added to the solution and stored on ice for 30 minutes. The tube was transferred to 42°C water-bath for heat shock for exactly 90 seconds and rapidly transferred them on ice and stored for 1-2 minutes. After addition of 800 µl of LB broth, the mixture was incubated at 37°C for 45 minutes in water-bath. 25-200 µl of the cultured cells were transferred to LSLB plates containing appropriate antibiotics and was immediately spread over the entire surface. The plates were incubated at 37°C on an inverted position for 16-20 hours.

3.4.8 DNA Sequencing

After transformation of *E. coli*, inserted GI sequence was verified by DNA sequencing in Central Laboratory in METU.

3.4.9 Transfection of *Pichia pastoris*

Transfection of *P. pastoris* to be used in recombinant GI production was performed using LiCl method according to the manufacturer's instructions (Invitrogen, 2002).

For this purpose, *P. pastoris* X-33 was inoculated on YPD plate and incubated for 48 hours at 30°C. A single colony was inoculated to 50 ml YPD medium and incubated to an OD₆₀₀ of 0.8 to 1.0 at 30°C 200 rpm for 12-14 hours. The cells were harvested and centrifuged at 4000 g for 5 minutes at room temperature, supernatant was removed and cells were washed with 25 ml sterile water and centrifuged at 1500 g for 10 minutes at room temperature. Pellet was resuspended in 1 ml 100 mM filter-sterilized LiCl, transferred to a 1.5 ml eppendorf tube and centrifuged at maximum speed for 15 seconds. LiCl was removed with a pipette and cells were resuspended in 400 µl 100 mM filter-sterilized LiCl. For each transformation, 50 µl of cell suspension was dispensed into 1.5 ml eppendorf tubes and immediately centrifuged at maximum speed for 15 seconds. LiCl was removed with a pipette. For transformation of the cells, 240 µl of %50 PEG, 36 µl of 1 M LiCl, 10 µl of 5 mg/ml single-stranded DNA and 30-50 µg linearized plasmid DNA in 50 µl sterile water were added to each tube in the given order and vortexed vigorously until the cell pellet was completely mixed. The tube was incubated at 30°C for 30 min without shaking followed by a heat shock in a water-bath at 42°C for 20-25 min. Cells were pelleted by centrifuging at 6000-8000 rpm for 15 seconds, transformation solution was removed and pellet was gently resuspended in 1 ml YPD and incubated at 30°C with shaking. After 2 hours of incubation, 25-100 µl of the medium was spread on YPD plates containing 150 mg ml⁻¹ Zeocin and incubated for 2-3 days at 30°C.

3.4.10 Isolation of Genomic DNA from Yeast

Isolation of genomic DNA was done according to the method described by Burke *et al.* (2000) with slight modifications.

Cells were incubated on YPD plates (Table 3.2) plates for 60-72 hours and used directly for genomic DNA isolation. A single colony was transferred into a 2 ml eppendorf tube and dissolved in 0.2 ml yeast lysis buffer, 0.2 ml phenol:chloroform:isoamylalcohol (25:24:1) and 0.3 g acid-washed glass beads were added. Mixture was vortexed vigorously for 4 minutes. 0.2 ml TE Buffer (pH: 8.0) was added and total mixture was centrifuged at 4000 g for 2 minutes at room temperature. Aqueous phase was transferred to a fresh 2 ml eppendorf tube, mixed with 1 ml 100% EtOH by inversion and centrifuged at maximum speed for 2 minutes. Supernatant was discarded. Remaining pellet was resuspended in 0.4 ml TE buffer (pH:8.0) and treated with 10 μ l of 10 mg ml⁻¹ RNase A. Mixture was incubated at 37°C for 5 minutes. After incubation, 1 ml 100% EtOH and 14 μ l of 3 M sodium acetate were added and DNA was pelleted by centrifugation at maximum speed for 2 minutes. Supernatant was removed and DNA pellet was air dried. Air dried DNA was dissolved in 50 μ l of TE buffer (pH: 8.0) and stored at -20°C until further use.

3.5 Recombinant Protein Production

Thermostable GI production by metabolically engineered *P. pastoris* was carried out using laboratory scale air filtered shake bioreactors and pilot scale bioreactor.

3.5.1 Precultivation

Chosen *P. pastoris* eP20 producing thermostable GI from microbanks was plated onto YPD solid medium (Table 3.2) and incubated for 48-60 hours at 30°C. A single colony was inoculated into precultivation medium, BMGY (Table 3.5) and was shaken at 225 rpm at 30°C for 16-18 hours to reach an OD₆₀₀ 2-6. The cells were harvested by centrifugation at 1500g for 10 minutes at 4° and resuspended in production medium (either in laboratory scale air filtered shake bioreactors or pilot scale bioreactor) so that initial OD₆₀₀=1 was obtained.

3.5.2 Thermostable GI Production in Laboratory Scale Air Filtered Shake Bioreactors

Thermostable GI production in batch cultures were carried out in air filtered and baffled Erlenmeyer flasks with a volume of 250 ml containing 50 ml production medium at 30°C. (Table 3.6). Cells were induced by addition of 1% (v/v) methanol every 24 hours.

3.5.3 Thermostable GI Production in Pilot Scale Bioreactor

Pilot scale 3 L bioreactor (Braun CT2-2) having a working volume of 1 L where temperature, pH, foam, stirring rate, feed inlet rate and dissolved oxygen can be controlled was used. Figure 3.1 represents scheme of bioreactor system. Bioreactor system had an external cooler, steam generator and a jacket for sterilization and to control the temperature. Rushton turbines with four blades were used for stirring. The bioreactor consisted four baffles and a sparger for homogeneous oxygen distribution. Air was supplied through a compressor but also with a pure oxygen tube. In the case of insufficient oxygen conditions in the bioreactor, inlet air was enriched by passing pure oxygen through a mass flow controller. Feed solutions were transferred aseptically by using inlet ports and peristaltic pumps. They were placed on balances to measure the amount fed inside the bioreactor.

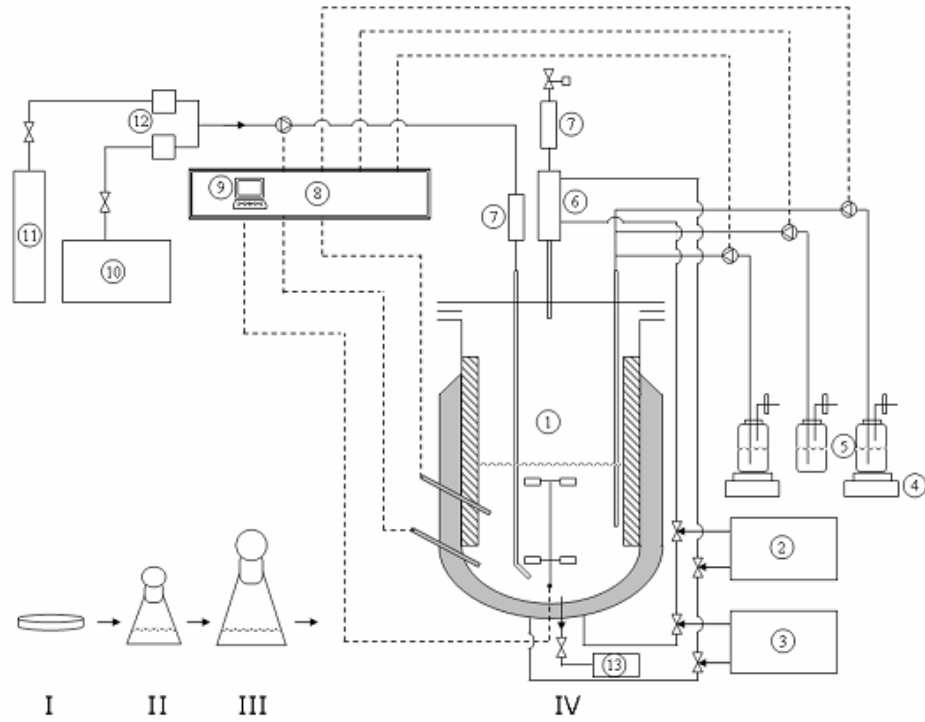


Figure 3.1 Scale up steps and pilot scale bioreactor system. I: Solid medium inoculated from stock culture; II: 1st Precultivation medium, $V=10\text{ mL}$; III: 2nd Precultivation medium, $V=50\text{ mL}$; IV: Pilot scale bioreactor system, $V_0=1\text{ L}$, which is composed of (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O₂ tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008).

3.5.3.1 Bioreactor Operation Parameters

Temperature was kept constant at 30°C by using an external cooler and a steam generator. 25% ammonia solution was used to adjust pH as well as it was used as a nitrogen source (Çelik *et al.*, 2009). The pH of the medium was controlled by using PI controller of the bioreactor system with the parameters of $X_P=30\%$, $T_I=30\text{ s}$ and 10% opened base-pump-valve. Agitation rate was kept at 900 rpm for homogeneous and efficient oxygen and substrate transport. Dissolved oxygen (DO) was kept at

20% to prevent oxygen limitation. Whenever the air is insufficient for oxygen supply, pure oxygen was fed and air was enriched by pure oxygen under control of mass flow controller. Excess of foam was removed by adding small amounts of 10% antifoam solution when required.

3.5.3.2 Production Phases in Fed-Batch Pilot Scale Bioreactor Operations

A standart protocol (Stratton, Chiruvolu, and Meagher 1998) which is modified by Çelik *et al.*, 2009 was used for production of thermostable GI production. After precultivation step as described in Part 3.5.1, the harvested cells were transferred to bioreactor such that an initial $OD_{600}=1$ was obtained in the BSM (Table 3.7) . After transfer of cells, a four-phase production protocol was used:

- **Glycerol batch phase (GB):** This phase lasted approximately 12-15 hours to reach an $OD_{600}=30$ which corresponds to cell concentration $C_X=10.0 \text{ g L}^{-1}$. The major aim of this phase was to increase the cell concentration. As the carbon source, glycerol was used instead of methanol since cells can grow on glycerol faster than that of methanol. The initial concentration of glycerol was adjusted to 40 g L^{-1} since higher glycerol concentrations may inhibit cell growth (Cos *et al.*, 2006).
- **Glycerol Fed-Batch Phase (GFB):** In this phase, 50% glycerol containing 12 mL L^{-1} PTM1 was fed with a pre-determined exponential feeding profile with a specific growth rate $\mu=0.18 \text{ h}^{-1}$ in order to prevent glycerol accumulation and to increase cell concentration. This phase lasted until the cell concentration reached to an $OD_{600}=80-100$ ($C_X=24-30 \text{ g L}^{-1}$). It took approximately 7-8 hours to reach the desired cell concentration.
- **Methanol Transition Phase (MT):** This phase helped the cells to adapt the carbon source switching from glycerol to methanol. In this phase, Methanol (100% methanol containing 12 mL L^{-1} PTM1) was fed to the bioreactor with a volumetric feed rate of 3.6 ml h^{-1} per liter initial fermentation volume during 4 hours according to the suggestions of Invitrogen Pichia Fermentation Process Guideline (Invitrogen 2002).

- **Methanol Fed-Batch Phase (MFB):** This phase was the phase where the GI was produced in significant amounts. For this purpose, thirty minutes before switching to MFB, sorbitol (500 g L⁻¹ sterile solution) was fed batch-wise to the system so that initial concentration of sorbitol, C_{S0}=50 g L⁻¹ was obtained as well as 100% methanol including 12 mL L⁻¹ PTM1 was fed to the system with a pre-determined exponential feeding profile where specific growth rate, $\mu=0.03 \text{ h}^{-1}$.

The predetermined exponential feed rate was determined using equation 3.1, calculated for a constant specific growth rate.

$$F(t) = \frac{\mu_0 V_0 C_{x0}}{Y_{X/S} C_{S0}} \exp(\mu_0 t) \quad (3.1)$$

where μ_0 is the desired growth rate (h⁻¹), V_0 is the initial volume (L), C_{x0} (g L⁻¹) is the initial cell concentration, $Y_{X/S}$ (g g⁻¹) is the cell yield on the substrate, C_{S0} is feed substrate concentration. Parameters of equation 3.1 for glycerol and methanol are given in Table 3.12.

Table 3.12 Parameters of Equation 3.1 for Glycerol and Methanol Fed-Batch feeding.

Parameter	Glycerol	Methanol
	Fed-Bach Feeding	Fed-Batch Feeding
$\mu_0 \text{ (h}^{-1}\text{)}$	0.18	0.03
$Y_{X/S} \text{ (g g}^{-1}\text{)}$	0.5*	0.42**
$C_{S0} \text{ (g L}^{-1}\text{)}$	790	1130

* Cos *et al.*, 2005, ** Jungo *et al.*, 2006

The pre-determined feeding profiles for glycerol and sorbitol are plotted as Figure 3.2 and Figure 3.3, respectively; in accordance with the parameters given in Table 3.12.

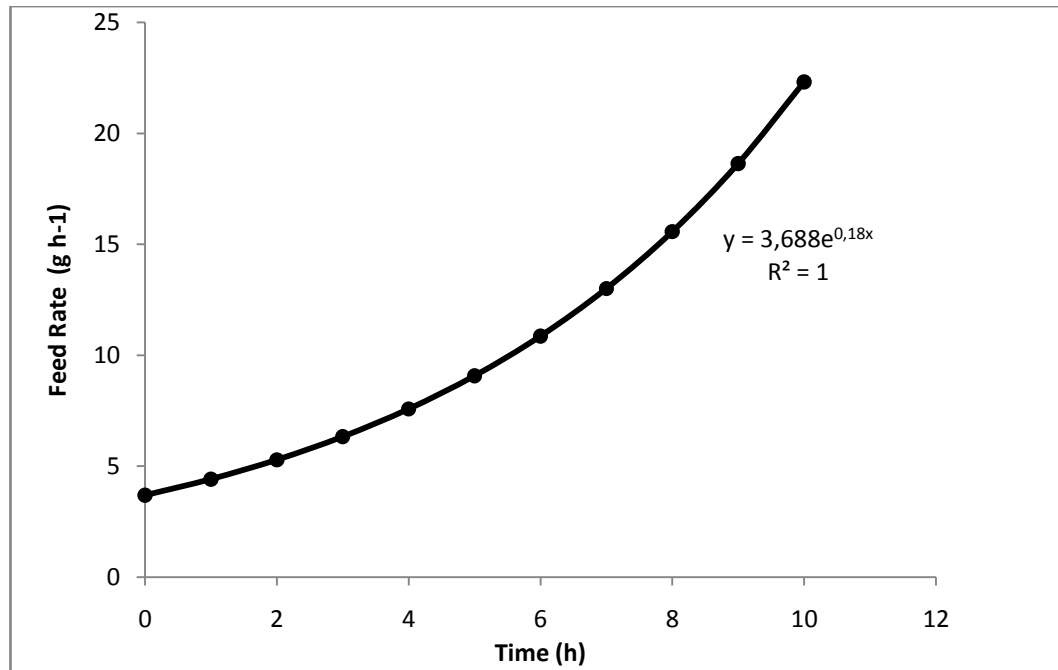


Figure 3.2 The predetermined feeding profile for glycerol, calculated for specific growth rate (μ_0) of 0.18 h^{-1} .

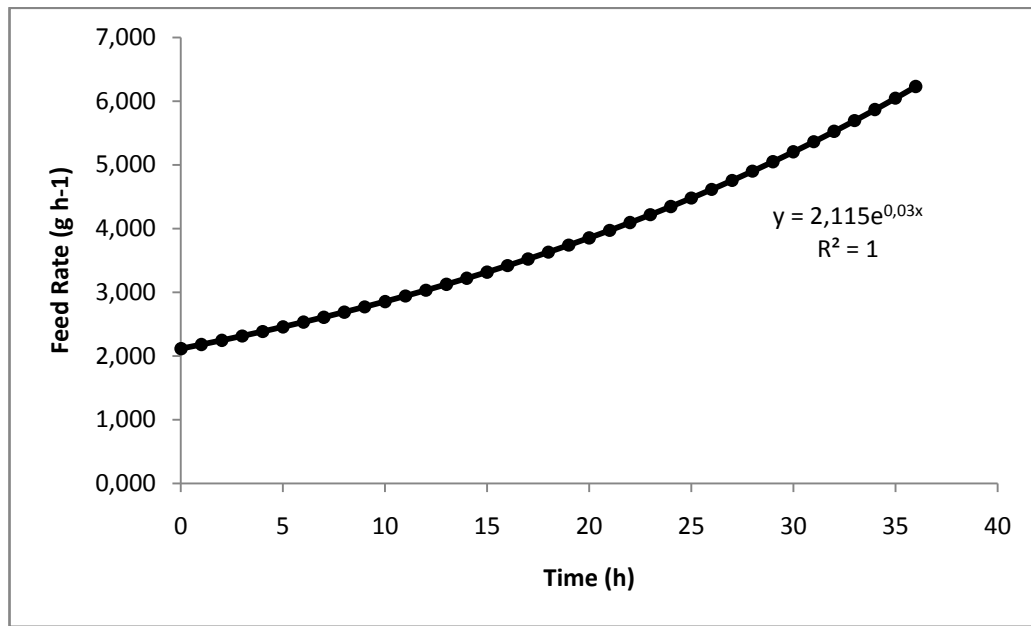


Figure 3.3 The predetermined feeding profile for methanol, calculated for specific growth rate (μ_0) of 0.03 h^{-1} .

3.6 Analyses

3.6.1 Cell Concentration

Cell concentration was measured by using UV-Vis Spectrophotometer at 600 nm. Samples which were taken from cultivation medium was diluted with dH_2O to an OD_{600} in the range of 0.1-0.8. Cell concentration, C_x (g L^{-1}) was calculated by using the following equation.

$$C_x = 0.3 * OD_{600} * \text{Dilution Factor} \quad (3.2)$$

3.6.2 Total Protein Concentration

Total protein concentration of samples was calculated by using Bradford assay (Bradford, 1976). For this purpose, 20 μ l sample was mixed with 1 ml Bradford reagent (BioRad) and incubated for 10 minutes at room temperature. The absorbance was read at 595 nm and total protein concentration was calculated by using the calibration curve given in Appendix F.

3.6.3 GI Enzyme Assay

For GI activity assays, cells were centrifuged at 1500 g at 4°C for 10 minutes. Supernatant was used for extracellular GI activity assays.

To determine GI activity, 50 μ l of supernatant was used and added to 50 μ l freshly prepared activity buffer containing 0.4 M D-Glucose and 10 mM MnCl_2 in 0.02 M potassium phosphate buffer (pH:7.0). The mixture was incubated at 80°C for 10 minutes and reaction was terminated by adding 60 μ l of reaction mixture to 540 μ l 0.1 M HCl. 1 U of GI activity was defined as the formation of 1 μ mol D-Fructose per minute under certain reaction conditions. Produced D-Fructose was analyzed by using cysteine-carbazol-sulfuric acid method as described by Dische and Borenfreund (1951). According to the method, 1.8 ml 70% (v/v) sulfuric acid, 60 μ l of freshly prepared 1.5% cysteine in 37% HCl and 60 μ l of freshly prepared 0.12% (w/v) carbazol in 95% EtOH was added in the given order after reaction termination. The mixture was vortexed and incubated for 30 minutes at room temperature. Produced fructose was determined with UV-Vis Spectrophotometer by measuring the absorbance at 560 nm. Calibration curve for formation of D-Fructose was given in Appendix G.

3.6.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970). 20 μ l of supernatant samples with 10 μ l loading dye were treated at 95°C for 5 minutes by using a

thermocycler and immediately chilled on ice for 5 minutes. A prestained marker was run simultaneously at 40 mA of constant current. Staining procedure and buffers used are given Appendix B.

3.6.5 Sorbitol Concentration

Sorbitol concentration was measured by High Performance Liquid Chromatography (HPLC) (Waters, Alliance 2695). This method is based on reverse phase HPLC and the concentrations were calculated by considering the chromatogram based on the standart solutions' chromatograms. Samples that were analyzed were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to system. Dilutions were made by mobile phase. Table 3.13 shows the parameters of HPLC for calculating the concentrations of sorbitol. Calibration curve for sorbitol concentration is given in Appendix H.

Table 3.13 Conditions for HPLC system for sorbitol analyses

Column	Capital Optimal ODS, 5µm
Column dimensions	4.6x250 mm
System	Reversed phase chromatography
Mobile phase	5 mM H ₂ SO ₄
Mobile phase flow rate	0.5 mL/min
Column temperature	30°C
Detector type and wavelength	Waters 2414 Refractive Index detector, 214 nm
Detector temperature	30°C
Injection volume	5 µL
Analysis period	10 min
Space time	5 min

3.6.6 Organic Acid Concentration

Organic acid concentrations were measured by High Performance Liquid Chromatography (HPLC) (Waters, Alliance 2695). This method is based on reverse phase HPLC and the concentrations were calculated by considering the chromatogram based on the standart solutions' chromatograms. Samples that were analyzed were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to system. Dilutions were made by mobile phase. Table 3.14 shows the parameters of HPLC for calculating the concentrations of sorbitol and methanol. Calibration curves for organic acids are given in Appendix I.

Table 3.14 Conditions for HPLC system for organic acid analysis (İleri and Çalık 2006).

Column	Capital Optimal ODS, 5µm
Column dimensions	4.6x250 mm
System	Reversed phase chromatography
Mobile phase	3.12% NaH ₂ PO ₄ (w/v) and %0.62x10 ⁻³ (v/v) H ₃ PO ₄
Mobile phase flow rate	0.8 mL/min
Column temperature	30°C
Detector type and wavelength	Waters 2487 Dual absorbance detector, 210 nm
Detector temperature	30°C
Injection volume	5 µL
Analysis period	15 min
Space time	5 min

3.6.7 Protease Activity Assay

Casein was used to measure the proteolytic activity. For this purpose, culture broth was centrifuged for 10 minutes at 1500g at 4°C and supernatant was used. Hammerstein casein (2 ml of 0.5% w/v) in 0.05 M sodium acetate buffer (pH 5.0) for acidic protease activity measurement, 0.05 M sodium phosphate buffer (pH 7.0) for neutral protease activity measurement or 0.05 M borate buffer (pH 10.0) alkali for protease activity measurement was mixed with 1 ml diluted sample. Hydrolyzation reaction was carried out at 37°C for 20 minutes. The reaction was terminated by addition of 10% trichloroacetic acid (TCA) and reaction mixture was centrifuged at 10500g for 10 minutes at 4°C. After incubation of samples at room temperature for 5 minutes, the absorbance of supernatant was measured at 275 nm in UV-Vis spectrophotometer. 1 U protease activity was defined as the formation of 4 nmole tyrosine per minute (Moon and Parulekar, 1991). Calibration equation for calculation of protease activity by using the absorbance is given in the Equation 3.3:

$$A = \left(\frac{\text{Absorbance}}{0.8 \times 1 / \mu\text{mol} \cdot \text{cm}^{-3}} \right) \left(\frac{1\text{U}}{4\text{nmolmin}} \right) \left(\frac{1}{20\text{ min}} \right) \left(\frac{1000\text{nmol}}{1\mu\text{mol}} \right) \left(\frac{\text{Dilution}}{\text{Ratio}} \right) \quad (3.3)$$

3.6.8 AOX Activity Assay

Since AOX is the first enzyme of methanol metabolism of *Pichia pastoris*, the specific activity of the AOX has to be determined to evaluate the fermentation system. Alcohol oxidase (AOX) and horseradish peroxidase (HRP) were used in a bi-enzymatic assay to monitor the oxidation of methanol to formaldehyde. For this purpose, phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) were chosen to measure the concentration of H₂O₂ produced by AOX. The first step of the reaction series starts with the oxidation of methanol to H₂O₂ and formaldehyde which is catalyzed by AOX. Secondly, two moles of H₂O₂ reacts with the one mole

of PSA and one mole of 4-AAP, yielding one mole of quinoneimine dye one mole of hydrogensulfate and three moles of water. The reaction which take place in this reaction system is given below.



Quinoneimine dye has a characteristic magenta color with a maximum absorbtion around 490 nm. Therefore, the activity of AOX was determined by monitoring the associated increase in absorbance at 490 nm with UV-Vis spectrophotometer. As can be seen from the rate equations above, this increase is proportional to the rate of formation of H₂O₂ and as a consequence to the rate of methanol consumption.

To determine the activity of AOX, the cells should be lysed since AOX is an intracellular enzyme. For this purpose, 1 mL of fermentation medium was centrifuged (10 min, 12500 rpm, +4°C) and supernatant was removed. Intracellular medium was extracted by using Yeast Lysis Buffer (Appendix A). First, 500 µl of Yeast Lysis Buffer was added to the pellet and vortexed for 20 seconds followed by incubation on ice for 30 seconds which is repeated for 3 times in series. Next, the same procedure was applied by adding approximately 0.5 g glass beads. Subsequently, the cells were centrifuged at 3000 rpm for 2 min at +4°C and the supernatans were taken away from the cells and centrifugated again at 12500 rpm for 5 min at +4°C. Supernatant was used for AOX activity assay.

All kinetic studies were performed at 25°C using a standard assay reaction mixture which contained 0.4 mM 4-AAP, 25 mM PSA and 2 U ml⁻¹ HRP in 0.1 M phosphate buffer (pH 7.0). One unit acitivity of AOX was defined as the formation of 1 µmol H₂O₂ per minute at 25°C (Azevedo *et al.*, 2004).

The reaction mixture containing 3 mL standard assay reaction mixture, 30 µL HRP, 375 µL methanol and 75 µL sample was mixed in a cuvette and the increase in the absorbance at 500 nm was monitored for four minutes and recorded in every 30 sec intervals for 4 minutes. To convert absorbance to specific AOX activity the equation 3.4 was formed using calibration curve given in Appendix J.

$$C_{AOX} \left(\frac{U}{gCDW} \right) = 15.67 \left(\frac{UmL^{-1}}{Absorbance} \right) * OD_{500} * \frac{1}{C_X} \quad (3.4)$$

CHAPTER 4

RESULTS AND DISCUSSION

In this M.Sc. thesis, an expression system for the production of extracellular thermostable glucose isomerase (GI; EC 5.3.1.5) enzyme by *Pichia pastoris*, was designed and investigated. In the first part of the study, based on the metabolic engineering design an original expression system for *P. pastoris* was constructed. In this context, *xylA* gene that encodes GI enzyme was cloned into the pPICZ α -A expression vector and then transferred into *P. pastoris* genome. The transformants were verified by PCR, and the best producing recombinant strain was selected by enzyme activity assay and SDS-PAGE experiments. In the second part of the study, effects of sorbitol and salt concentrations on recombinant GI activity were investigated in batch shaker-bioreactor experiments. Using the data obtained from shaker-bioreactor experiments, a pilot-scale bioreactor experiment was designed and conducted; and the fermentation characteristics were analyzed.

4.1 Development of Recombinant *P. pastoris* carrying *xylA* Gene

The research program for the development of recombinant *P. pastoris* strain producing extracellular thermostable GI was carried out mainly in three steps, as presented schematically in Figure 4.1. First, the pPICZ α -A expression vector was isolated and the *xylA* gene that encodes thermostable GI was amplified by PCR with the help of specially designed primers. In the second step, the amplified *xylA* gene was fused into pPICZ α -A expression vector by primer extension which carries an α -MF signal for the secretion of protein, AOX1 promoter region to be induced with methanol and Zeocin resistance gene. Lastly, transformed pPICZ α -A::*xylA* was integrated into the genome of *P. pastoris* and expression of active GI was achieved.

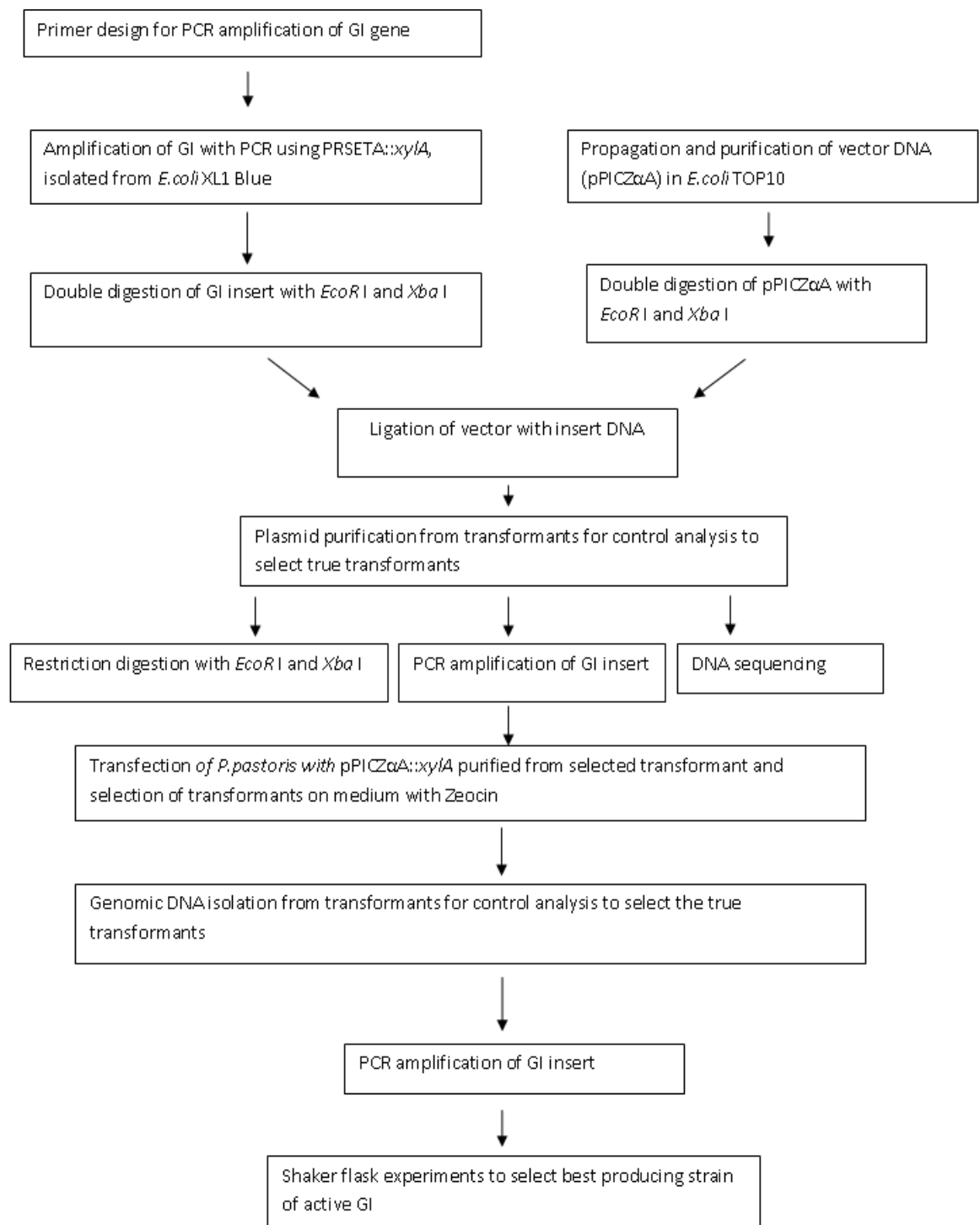


Figure 4.1 Based on the metabolic engineering desing (Figure 4.6), schematic presentation of the construction of recombinant *P. pastoris* carrying *xylA* gene for extracellular thermostable glucose isomerase production.

4.1.1 Propagation and Purification of pPICZ α A

The lyophilized pPICZ α A vector was dissolved in sterile water at 10 ng μ l⁻¹ concentration and used in transformation of *E. coli*. From a single colony, microbanks were prepared. The plasmid used in this study was purified from this colony and single digested to visualize its actual size since circular (undigested) plasmids run faster when compared to linearized ones (Figure 4.2).

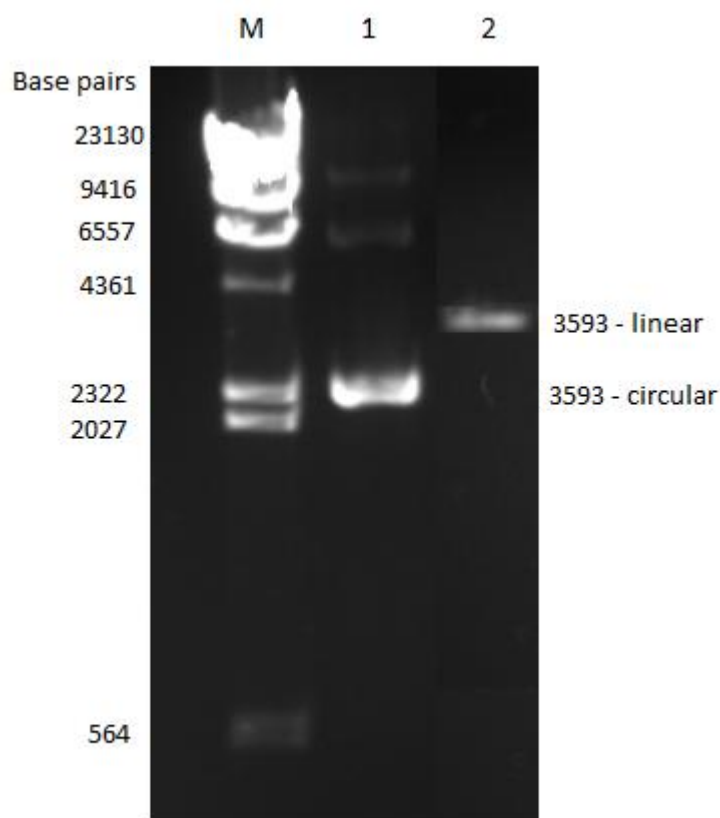


Figure 4.2 Agarose gel electrophoresis image of circular and linear empty pPICZ α -A expression vector. M:Marker, Lane 1: circular pPICZ α -A, Lane 2: linear pPICZ α -A.

4.1.2 Primer Design for Amplification of *xylA* gene from *T. thermophilus*

For amplification of *xylA* gene, PRSET-A::*xylA* (Anghardi, 2011) was used as the template in PCR amplification of *xylA* gene. For this purpose, *E. coli* XL1 Blue cells carrying PRSETA::*xylA* plasmid were inoculated onto LSLB plates (Table 3.4) from microbank stocks for plasmid extraction. Extracted plasmids were used for amplification of *xylA* gene as the template.

For the amplification of *xylA* gene, two primers were designed in accordance with the nucleotide sequence obtained from Dekker *et al.* (1991a). In this context, one forward primer and one reverse primer were designed. Forward primers included an *EcoR* I restriction enzyme recognition site and a 27 bp long nucleotide sequence from *xylA* sequence starting from 5' end. However, the original start codon, GTG was changed as ATG, which is a more yeast-like start codon for efficient transcription (Walfridsson *et al.*, 1996). Reverse primer was designed as it contained *Xba* I restriction enzyme recognition site as well as a 27 bp long nucleotide sequence from *xylA* gene sequence. While designing the primers, thermodynamic properties, melting temperatures (T_m), dimer and loop formation were taken into consideration with the help of OligoAnalyzer program. Primers used for amplification of *xylA* gene in this study were given in Table 4.1. Properties of designed primers were given in Appendix D. Besides primers those were designed, primers for *AOX1* regions were used to verify the location of insertion of the gene.

Table 4.1 Primers that were used in this study for amplification of *xylA* gene. Underlined sequences are restriction enzyme recognition sites.

Name	Sequence
GI Forward Primer	5'-GGAATTCATGTACGAGCCCAAACCAGAGCA CAGG-3'
GI Reverse Primer	5'-CGTCTAGATCACCCCGCACCCCCAGGAAGT ACTC-3'
AOX1 Forward Primer	5'-GACTGGTTCCAATTGACAAGC-3'
AOX1 Reverse Primer	5'-GCAAATGGCATTCTGACATCC-3'

4.1.3 Amplification of *xylA* Gene by Polymerase Chain Reaction (PCR)

After designing the primers specific for *xylA* gene from *T. thermophilus* as well as including recognition sites for restriction enzymes *EcoR* I for forward primer and *Xba* I for reverse primer was amplified by PCR. Annealing temperature was optimized and selected as 44°C for 1 cycle and 50°C for 29 cycles. The temperature of the first cycle was determined lower than the other cycles, to increase the probability of annealing the primer to the *xylA* sequence; and then, the temperature of remaining cycles were higher, i.e. 50°C, to prevent the non-specific bindings. As the time of amplification is also a parameter besides the temperature that both depend on the DNA sequence to be amplified, the time of elongation was determined as 3 minutes to allow full amplification by taking into consideration *Pfu* DNA Polymerase Manual (Fermentas), where 0.5-1 min/kb elongation time is recommended. Thermal-cycler optimized operation parameters for the PCR are presented in Table 4.2.

Another important factor that influences the PCR efficiency is salt concentration. Higher salt concentrations increase the binding efficiency of primers as well as cause

non-specific binding problems. Therefore, three different MgSO_4 concentrations were investigated and the best salt concentration was found to be 3 mM (Figure 4.3). The optimized PCR reaction composition is presented in Table 4.3.

Table 4.2 Thermal-cycler operation parameters for the PCR

Number of Cycles	Temperature	Time
1 cycle	94°C	3 min
1 cycle	94°C	1 min
	44°C	1 min
	72°C	3 min
29 cycle	94°C	1 min
	50°C	1 min
	72°C	3 min
1 cycle	72°C	10 min

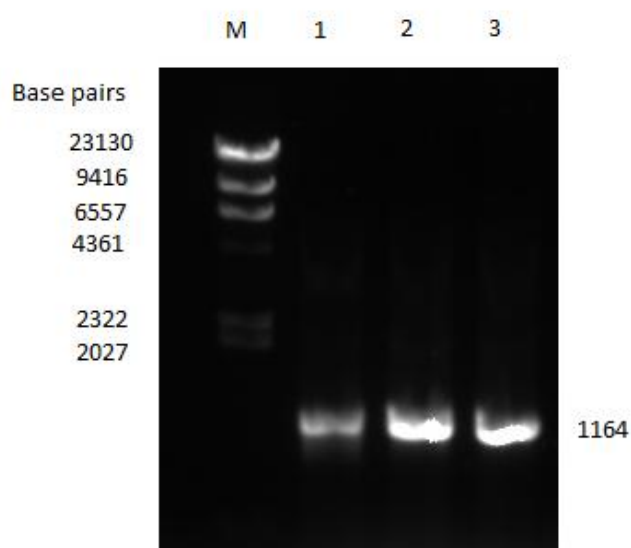


Figure 4.3 Agarose gel electrophoresis image of amplified *xylA* gene with PCR by using the designed primers (Expected size:1164 bp). M: Marker, Lane 1: MgSO_4 : 2 mM, Lane 2: MgSO_4 : 3 mM, Lane 3: MgSO_4 :4 mM.

Table 4.3 PCR reaction mixture composition.

Component	Amount
10X PCR Reaction Buffer (without Mg^{++})	5 μ l
dNTP (2 mM)	10 μ l
MgSO ₄ (25 mM)	6 μ l
Forward Primer (5 mM)	5 μ l
Reverse Primer (5 mM)	5 μ l
Template DNA (0.01-1 μ g)	0.5-5 μ l
<i>Pfu</i> DNA polymerase (2.5 U μ l ⁻¹)	0.5 μ l
Water	Up to 50 μ l

PCR amplified *xylA* gene under the conditions mentioned in Tables 4.2 and 4.3 was then purified as described in Section 3.4.5.1. The purified PCR products were run on agarose gel and visualized (Figure 4.4).

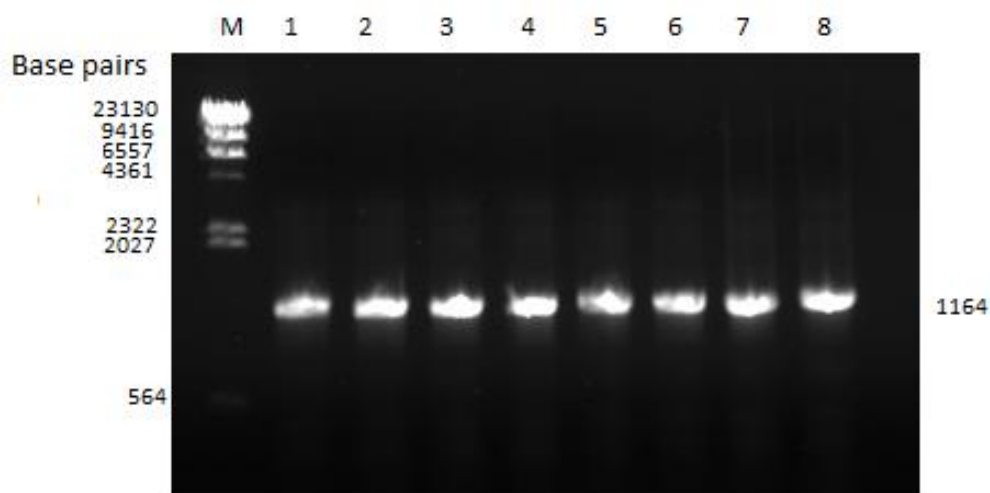


Figure 4.4. Agarose gel electrophoresis image of purified PCR products; *xylA* gene. M:Marker; whereas, the other lanes show purified *xylA* gene.

4.1.4 Restriction Enzyme Digestion of *xylA* and pPICZ α -A

Restriction enzymes used in this study were selected by taking into consideration the following criteria:

- *EcoR* I and *Xba* I restriction enzymes were selected as in they are in the multiple cloning site of the pPICZ α -A so that there wouldn't be any extra nucleotides in the *xylA* sequence.
- By using www.restrictionmapper.org website, it was verified that none of the restriction enzymes were included in *xylA* gene sequence.

PCR amplified *xylA* gene which was purified with PCR Purification Kit (Fermentas) and pPICZ α A vector isolated by Alkaline Lysis Method were double digested by *EcoR* I and *Xba* I restriction enzymes in a final volume of 20 μ l at 37°C for 2 hours (Table 3.12). Reaction mixtures were kept at 65°C for 20 minutes for thermal inactivation of restriction enzymes. After digestion, *xylA* gene and pPICZ α A vectors were run on agarose gel electrophoresis and extracted by Gene Elution Kit (GeneMark). The amounts of the DNA were determined both by spectrophotometric and intensity measurements of the bands on agarose gel.

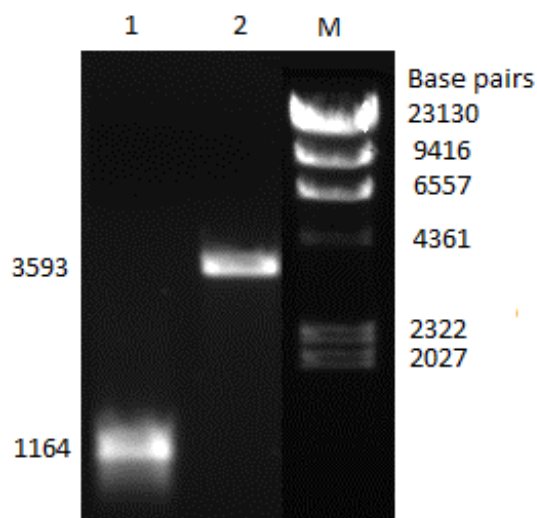


Figure 4.5 Agarose gel electrophoresis image of *xylA* gene and pPICZ α -A, after double digestion followed by gel elution. Lane 1: *xylA*, Lane 2: pPICZ α -A, M:Marker.

4.1.6 Ligation of *xylA* Gene and pPICZ α -A

After extraction of DNA fragments, ligation reactions were performed as described in Section 3.4.6. Ligation was succeeded at a molar ratio of 3:1 in a ligation reaction composition that is given in Table 4.4. Ligation mixture kept at 22°C for 1 hour and reaction was terminated by incubation at 65°C for 20 minutes. The product of the ligation reaction is named as pPICZ α -A::*xylA* (Figure 4.6).

Table 4.4 Ligation mixture composition.

Component	Amount
Double digested insert DNA	30-50 ng
Double digested vector DNA	100 ng
T4 DNA Ligase (5 U μl^{-1})	0.5 μl
Water	Up to 20 μl

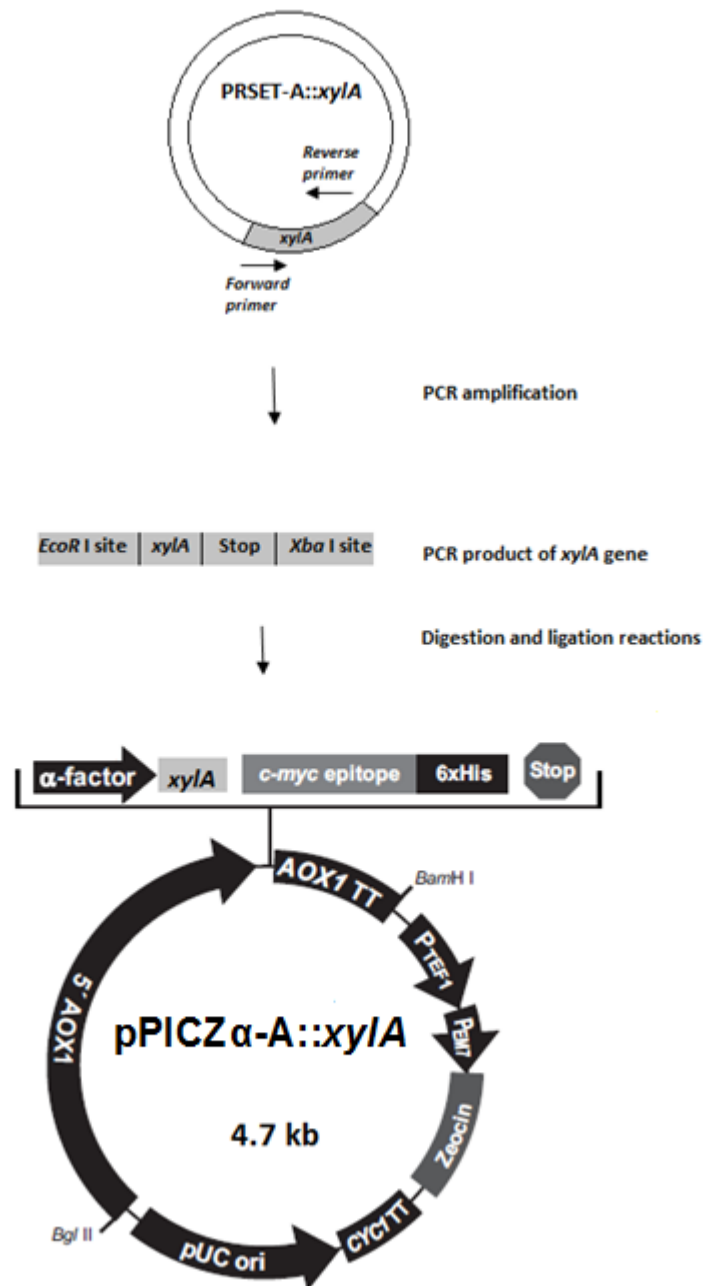


Figure 4.6 Schematic representation of *xylA* amplification, integration of specific recognition sites by PCR and construction of pPICZα-A::*xylA* plasmid.

4.1.7 Transformation of pPICZ α -A::xylA into *E. coli* TOP10 cells and Selection of True Transformants

The ligation products were transformed into *E. coli* TOP10 strain by CaCl₂ method as described in Section 3.4.7; and fresh transformants were plated on LSLB Agar (Table 3.3) and incubated for 18-20 hours. The colonies obtained were transferred onto new plates and plasmids were isolated by using Alkaline Lysis Method, which is described in Section 3.4.1. Isolation of plasmids from these new colonies showed that 12 isolated plasmids were larger than empty pPICZ α -A vector; although only three of them can be seen in Figure 4.7. These three plasmids were then used for further analyses.

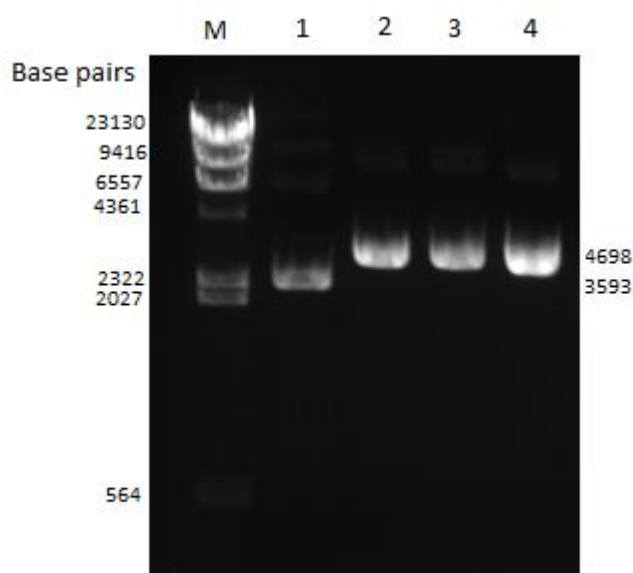


Figure 4.7 Agarose gel electrophoresis image of pPICZ α -A::xylA recombinant vector. M: Marker, Lane 1: circular empty pPICZ α -A vector, Lane 2,3,4: circular recombinant pPICZ α -A::xylA vectors.

Consequently, these plasmids were single digested by using *EcoR* I and double digested by using *EcoR* I and *Xba* I for verifying their actual size. And then, the plasmids were used as templates in PCR by using the primers designed for *xylA* gene.

They were also verified with using them as PCR templates by using the primers designed for *xylA* gene; whereas also verified by using as PCR templates by using the *AOX1* primers.

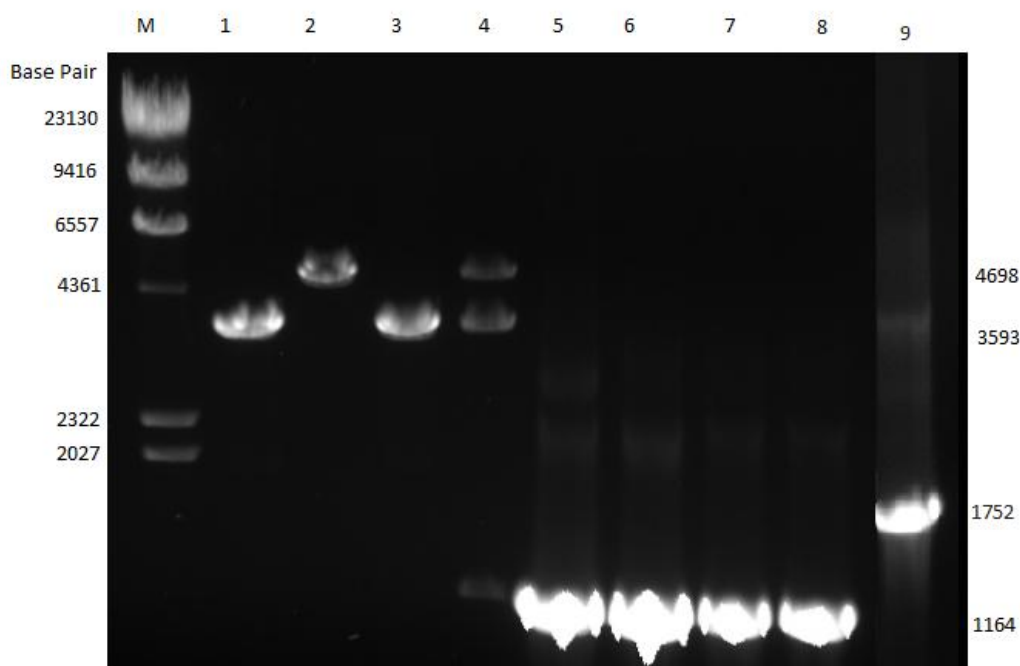


Figure 4.8 Agarose gel electrophoresis image of pPICZ α -A::*xylA* recombinant vector. M: Marker, Lane 1: *EcoR* I digested pPICZ α -A (expected size: 3593 bp), Lane 2: *EcoR* I digested pPICZ α -A::*xylA* (expected size: 4698 bp), Lane 3: *EcoR* I/*Xba* I digested pPICZ α -A (expected size: 3534 bp), Lane 4: *EcoR* I/*Xba* I digested pPICZ α -A::*xylA* (expected sizes: 4698 bp for undigested pPICZ α -A::*xylA*, 3593 bp for double digested pPICZ α -A::*xylA*, 1164 bp for *xylA* gene), Lane 5: PCR product of *EcoR* I digested pPICZ α -A::*xylA*, Lane 6: PCR product of *EcoR* I/*Xba* I digested pPICZ α -A::*xylA*, Lane 7: PCR product of circular pPICZ α -A::*xylA*, Lane 8: PCR product of PRSET-A::*xylA* as positive control, Lane 9: PCR product of pPICZ α -A::*xylA* with *AOX1* forward and *AOX1* reverse primers (expected size: 1752 bp).

The sizes of the bands were visualized and controlled (Figure 4.8) which confirmed successful construction of the recombinant plasmid. The DNA sequence of the insert DNA was also analyzed by automatic DNA sequencers (METU Central Laboratory, Ankara) using *xylA* gene and *AOX1* primers. The results revealed that the cloning is successful.

4.1.8 Transfection of *P. pastoris* cells with pPICZ α A::*xylA* plasmid

For transfection of *P. pastoris*, pPICZ α A::*xylA* vector was first linearized from its *AOX1* promoter region so that double integration event could occur at *AOX1* locus at the genome of *P. pastoris*. A schematic illustration of integration of *xylA* gene into *P. pastoris* genome is presented in Figure 4.9. For this purpose, *Pme* I enzyme was chosen as a single cutter for linearization. pPICZ α A::*xylA* was single digested with *Pme* I enzyme for 2 hours; the reaction was terminated by heat inactivation at 65 °C for 20 minutes. Digestion was verified by agarose gel electrophoresis. Vector was extracted by phenol/chloroform and ethanol precipitated by the addition of 1/10 volume 3 M sodium acetate and 2.5 volume 100% ethanol. Pellet was washed by 80% ethanol and dissolved in 10 μ l of sterile water. The concentration of plasmid was adjusted to a value within 600 – 1000 ng μ l⁻¹ for efficient transfection.

Transfection of *P. pastoris* was performed by using LiCl method as described in section 3.4.9. After 72 hours, selected colonies were inoculated to YPD plates (Table 3.2), and then used for genomic DNA isolation to be used in the controls by PCR. Out of 23 colonies, two of them showed positive results (Figure 4.10).

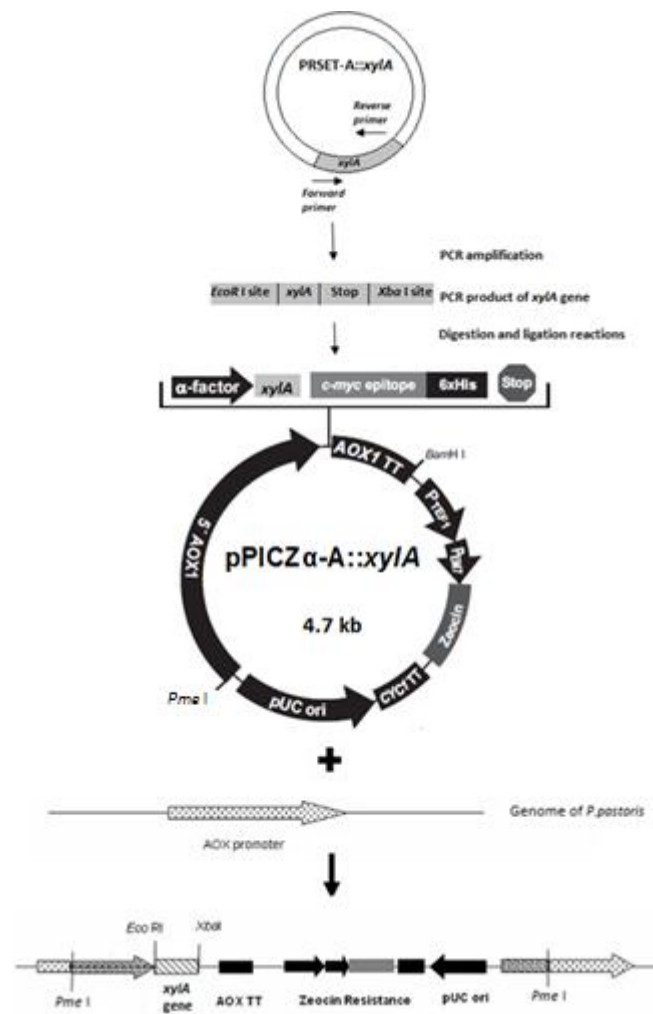


Figure 4.9 Schematic representation of pPICZαA::xylA integration into *P. pastoris* genome. The recombinant plasmid which is digested by *Pme*I from *AOX1* promoter region resulted with a linearized plasmid having homologous regions to the *AOX1* promoter region in the genome of *P. pastoris* at the both ends. After integration of the plasmid to the genome, there are two functional copies of the *AOX1* promoter followed by *xylA* gene and Zeocin resistance gene in the genome.

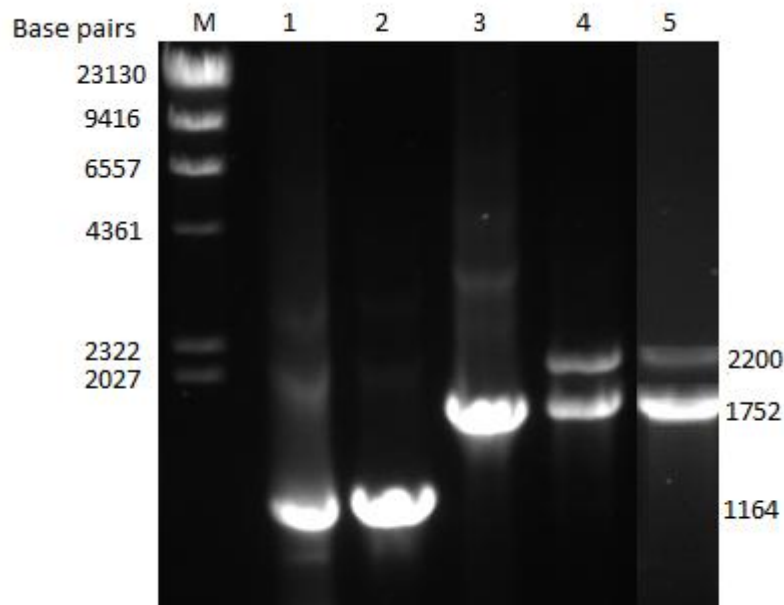


Figure 4.10 Agarose gel electrophoresis image of PCR in which genomes of recombinant *P. pastoris* cells are used as templates. M: Marker, Lane 1 and Lane 2: PCR product of *xylA* gene insert of genomic DNA of *P. pastoris* transformants by using forward and reverse primers designed for *xylA*, Lane 3: PCR product of pPICZαA::*xylA* as positive control by using AOX1 forward and reverse primers., Lane 4 and Lane 5: PCR product of *xylA* gene insert of genomic DNA of *P. pastoris* transformants by using AOX1 forward and reverse primers.

4.2 Expression of Thermostable Glucose Isomerase in Recombinant *P. pastoris* in Laboratory Scale Air-Filtered Shake Bioreactors

After developing recombinant *P. pastoris* strain carrying *xylA* gene, in the second sub-program of the research plan, the best producing strain was selected and used in the bioreactor experiments. In laboratory-scale shake-bioreactor experiments effects of sorbitol and salt concentrations were investigated.

4.2.1 Microorganism Selection

After insertion of pPICZ α A::xylA plasmid into *P. pastoris* genome, twenty three colonies were selected and tested by PCR (Section 4.1.7). The results show that two of them carry xylA gene; and named as eP1 and eP20. In order to choose the best producing strain, these colonies were plated on solid YPD medium (Table 3.2). After 60 hours, they were inoculated into precultivation medium and incubated at 30°C at 200 rpm for 16-18 hours using air filtered 150 ml Erlenmeyer flasks which contained 25 ml precultivation medium (Table 3.5). Thereafter, the cells were harvested and centrifuged at 1500 g at 4°C for 10 minutes and resuspended in production medium (Table 3.6) so that an initial OD₆₀₀=1.0 was obtained. The recombinant cells were incubated at 30°C at 200 rpm for 72 hours using air-filtered and baffled V=250 ml bioreactors which contained 50 ml production medium. The cultures were induced by 1% (v/v) methanol every 24 hours and samples were taken for enzyme activity assays and SDS-PAGE to choose the best producing strain.

The best producing strain carrying xylA gene was chosen by using both SDS-PAGE and the enzyme activity assay. SDS-PAGE was used to determine the total GI subunit production of the recombinant *P. pastoris* since SDS-PAGE procedure requires the proteins to be denaturated. Besides, as the activity is also an important parameter for the enzyme, the activity assays were also performed to select the best producing strain.

First, SDS-PAGE analysis was performed. For this purpose, 20 μ l of supernatants collected at t=24, 48 and 72 h were used (Section 3.6.4). Figure 4.11 shows the results of SDS-PAGE analysis of the two colonies at different times.

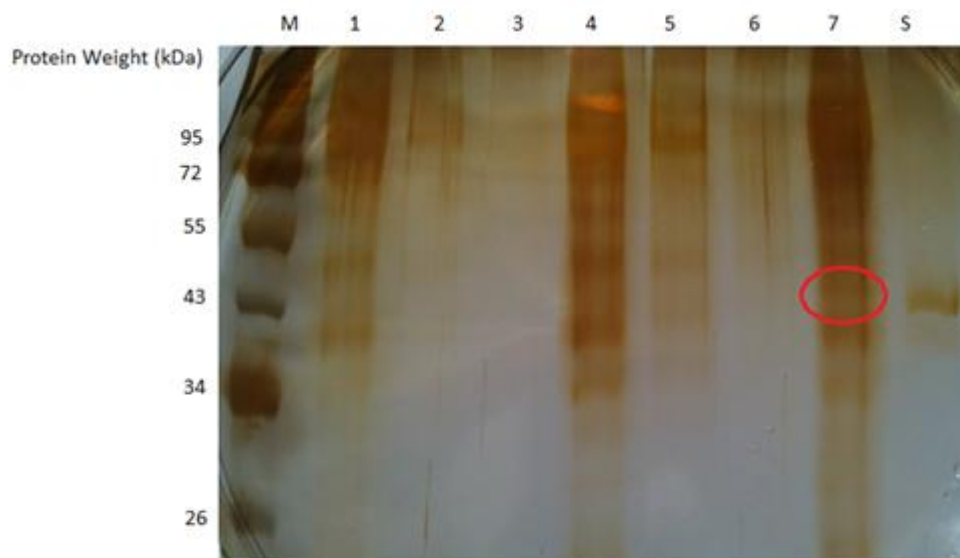


Figure 4.11 SDS-PAGE analyses of the two positive colonies, eP1 and eP20. M: Marker; Lane 1: *P. pastoris* strain carrying empty pPICZαA vector as negative control; Lane 2, Lane 3 and Lane 4: Supernatants of eP1 at y=24, 48 and 72 h, respectively; Lane 5, Lane 6 and Lane 7: Supernatants of eP20 at y=24, 48 and 72 h, respectively; S: Standard.

As can be seen from Figure 4.11, only in the supernatant of eP20, little amounts of GI is observed. There is no band in the negative control, as well as in eP1, which couldn't produce a visible amount of GI. One possible reason of this result might be the proteolysis, because of various yeast proteases which hydrolysis GI enzyme synthesized in the cell (Ohya *et al.*, 2002). Moreover, the length of induction time has an influence on the proteolysis; that means, increasing the residence time for the hydrolysis of GI enzyme which is known as the degradation of the enzyme (Daly and Hearn, 2005). Thus, I can conclude that high amounts of proteases because of high cell concentrations might have caused rapid degradation of GI in eP1.

The results obtained from the enzyme activity assay which were performed after SDS-PAGE analysis showed that the maximum activity obtained by eP20 was 3 U L^{-1} at $t=24 \text{ h}$ whereas no activity was observed in supernatant of eP1. This result is consistent with the SDS-PAGE analyses, since no visible amount of GI was seen in

cultivation medium of eP1. The possible reason for the low activity value of GI might be the low pH values in the cultivation media. To verify this, pH of the medium was measured every 24 hours. The results showed that *P. pastoris* cells have a tendency to lower the pH of the fermentation medium throughout the cultivation (Figure 4.12). Since pH cannot be controlled in air-filtered shaker bioreactors, the pH decreased to a value of 2.4 at t=72 h. On the other hand, it was reported that the minimum pH that GI from *T. thermophilus* in recombinant microorganisms can tolerate is 5.0 (Xu *et al.* 2009). Therefore, I can conclude that low GI activity values are obtained in shaker-bioreactors, where pH control cannot be controlled; as a pH value of 4.7 at t=24 h, further a pH value of 2.47 after 24 hours was observed because of uncontrolled pH operation.

Therefore, eP20 strain is chosen as the recombinant *P. pastoris* strain and further investigations were performed by using eP20.

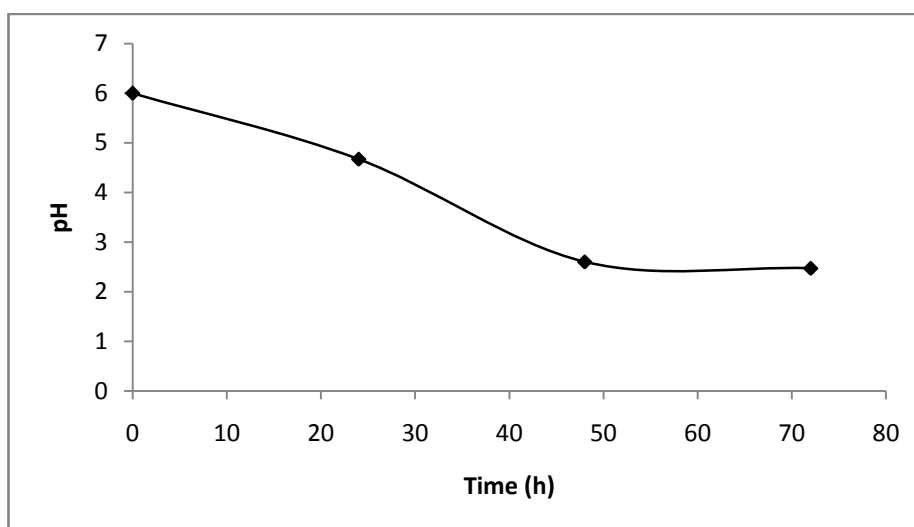


Figure 4.12 Variation of pH with the cultivation time in shaker-bioreactors.

4.2.2 Effects of Salt Concentration on Thermostable GI Production and Cell Growth

After selection of the best producing *P. pastoris* strain, eP20, effects of salt concentration on the recombinant GI production and cell growth were investigated. For this purpose, eP20 cells from YPD solid medium (Table 3.2) were inoculated into the precultivation medium after 60 hours and incubated at 30°C at 200 rpm for 16-18 hours using air filtered V=150 ml shaker-bioreactors flasks which contained 25 ml precultivation medium (Table 3.5). Thereafter, the cells were harvested and centrifuged at 1500 g at 4°C for 10 minutes and resuspended in the production medium containing different concentrations of salts so that an initial OD₆₀₀=1.0 was obtained. These production media contained components at the same concentrations given in Table 3.6 except PTM1, MgSO₄·7H₂O and CaSO₄·2H₂O. Table 4.5 shows the PTM1, MgSO₄·7H₂O and CaSO₄·2H₂O concentrations in five different production media. The cultures were induced by 1% (v/v) methanol.

Table 4.5 Production media containing different salt concentrations.

Production Medium No.	Concentration (g L ⁻¹)		
	PTM1	MgSO ₄ ·7H ₂ O	CaSO ₄ ·2H ₂ O
1	4.35 ml	14.9	1.17
2	0.87 ml	2.5	0.5
3	0.435 ml	2.5	0.5
4	0.2175 ml	2.5	0.5
5	4.35 ml	14.9	0.5

To investigate the effect of salt concentration, five different salt concentrations were used in the production media. As given in Table 4.2, MgSO₄·7H₂O concentration in the production medium-5 was not changed since Mg²⁺ is activator of GI

(Kovalevsky *et al.*, 2010; Bhosale *et al.*, 1996). $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ and PTM1 concentrations were decreased since Ca^{+2} and components in PTM1 such as Zn^{2+} and Cu^{2+} are inhibitors of GI (Bhosale *et al.*, 1996). Figure 4.13 exhibit the cell growth of eP20 in the production media containing different concentrations of salts. The maximum cell concentration was obtained as 6.3 g L^{-1} in the production medium-1 at $t=48 \text{ h}$, while the cells that were cultivated in other production media other than production medium-1 almost reached to the same cell concentrations as 6.2, 5.8, 5.7, and 6.1 g L^{-1} , respectively, in the production media- 2,3,4 and 5. Figure 4.13 reveals that the cell growth is not highly dependent on the salt concentration in air-filtered shaker-bioreactors, although the cell concentration was slightly higher in the production medium-1 compared to the other media. Nevertheless, these results support the importance of micronutrients as shown by several researchers (O'Callaghan *et al.*, 2002; Boze *et al.* 2001).

Variations of GI activity with the cultivation time under different salt concentrations are presented in Figure 4.14. Unlike the cell growth, GI activity is affected by the salt concentration. This can be explained as the active site of GI is affected by different metal ions (Gaikwad *et al.*, 1992; Bhosale *et al.*, 1996). The highest GI activity is obtained in production medium-1 at $t=30 \text{ h}$ with a value of 3.21 U L^{-1} . As can be seen in Figure 4.14, decreasing the salt concentration negatively affected the GI activity which means different ions in PTM1 besides $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ help GI to function properly. The maximum values of GI activity were obtained at $t=30 \text{ h}$ in the production media-1 and 5; whereas, production media-2 to 4 were resulted the maximum GI activity values at $t=24$. However values of GI activity at $t=24 \text{ h}$ and $t=30$ were not considerably higher than each other, supporting the data obtained in Section 4.2.1, where the maximum GI activity value was attained at $t=24$.

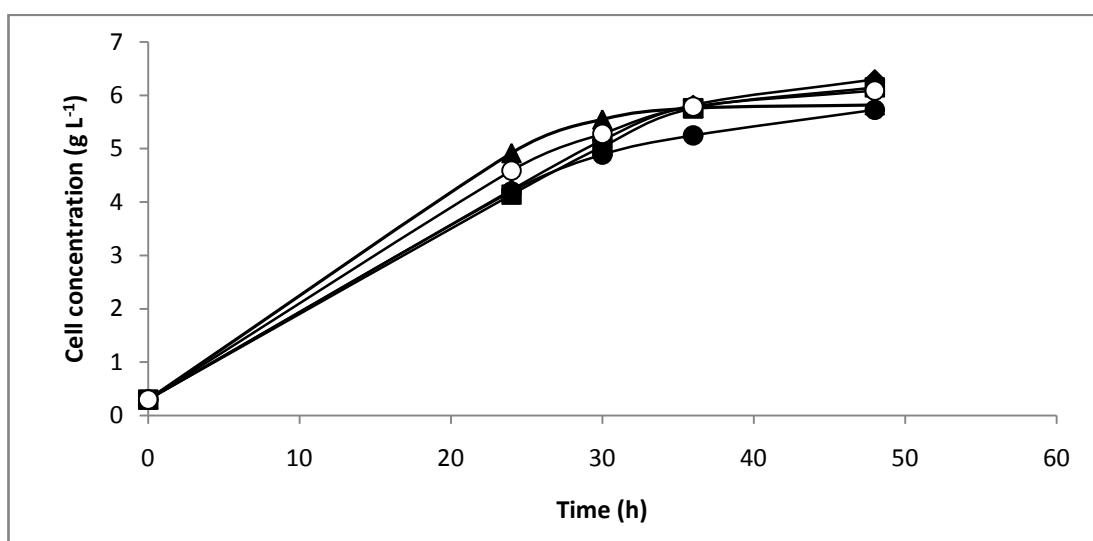


Figure 4.13 Effects of salt concentration on the cell growth. Salt concentrations; production medium-1 (♦), production medium-2 (■), production medium-3 (▲), production medium-4 (●), production medium-5 (○). Production media numbers are explained in Table 4.2.

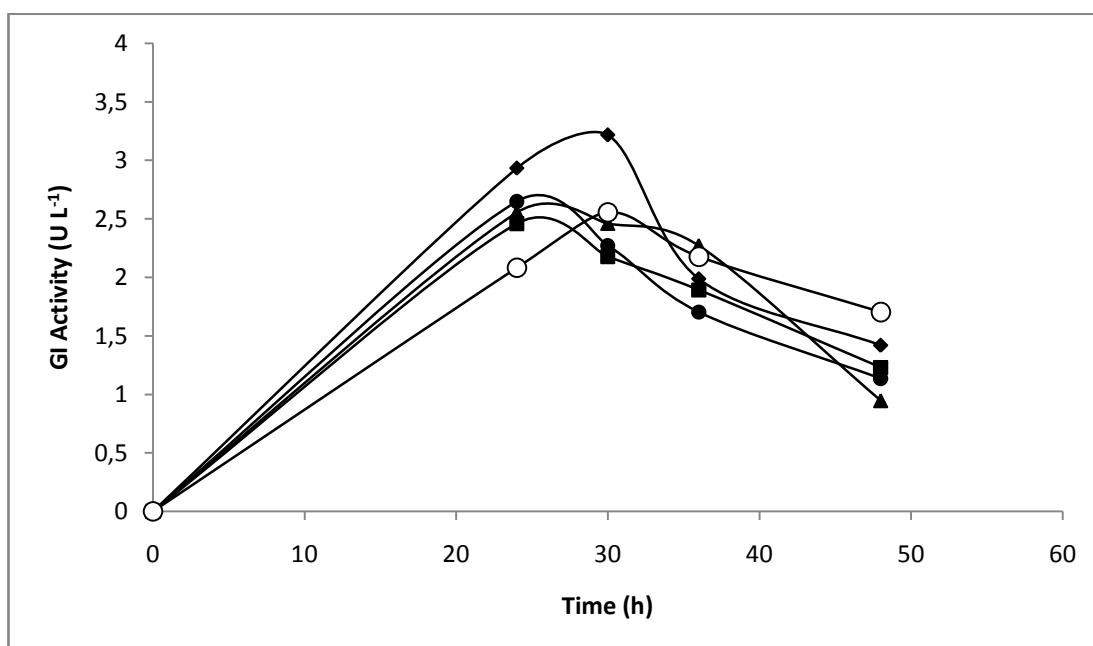


Figure 4.14 Effects of salt concentration on GI activity. Different salt concentrations; production medium 1 (♦), production medium 2 (■), production medium 3 (▲), production medium 4 (●), production medium (○). Numbers of production media refer to Table 4.2.

4.2.3 Effects of Sorbitol Concentration on Thermostable GI Production and Cell Growth

After the research program related with the salt concentration, effects of sorbitol concentration as the co-substrate on the cell growth and GI activity were investigated. For this purpose, eP20 cells from YPD solid medium (Table 3.2) were inoculated into the precultivation medium (Table 3.5) for 60 hours, and incubated at 30°C at 200 rpm for 16-18 hours using air-filtered 150 ml shaker-bioreactors which contained 25 ml precultivation medium. Thereafter, the cells were harvested and centrifuged at 1500 g at 4°C for 10 minutes and resuspended in the production medium containing different concentrations of sorbitol so that an initial $OD_{600}=1.0$ was obtained. The cultures were induced by 1% (v/v) methanol. Table 4.6 shows the different production media containing different concentrations of sorbitol as the co-substrate. Other components of production medium are at the same concentrations as given in Table 3.6.

Table 4.6 Production media containing different sorbitol concentrations.

Production Medium No	Sorbitol Concentration (g L ⁻¹)
1	0
2	10
3	30
4	50

Figure 4.15 illustrates the effect of sorbitol concentration on the cell growth. From the figure, it is clear that addition of sorbitol to the medium promotes the cell growth; where the maximum cell concentration obtained as $C_x=7.3$ g L⁻¹ in production medium-4 that contains 50 g L⁻¹ sorbitol. By decreasing the sorbitol concentration, the maximum cell concentration values also decrease. The maximum cell concentrations were 4.2, 5.9, and 6.8 g L⁻¹, respectively, in production media containing no-sorbitol, 10.0, and 30.0 g L⁻¹ sorbitol.

Like the cell growth profiles, GI activity increased with increasing sorbitol concentration. As can be seen from Figure 4.16, the maximum GI activity was achieved as 3.6 U L^{-1} at $t=27 \text{ h}$ in the production medium-4 which contains an initial sorbitol concentration of 50 g L^{-1} . Although sorbitol was reported as an inhibitor of GI (Bhosale *et al.*, 1996), the presence of Mg^{+2} protects GI from sorbitol inhibition (Sanchez and Smiley 1975). However, the differences between values of the maximum GI activities obtained were not significantly higher, thus showing that sorbitol is not the most important parameter in air-filtered shaker-bioreactors as the pH and oxygen transfer are not under control. The other production media containing no-sorbitol, 10.0 , and 30.0 g L^{-1} sorbitol resulted in maximum GI activity values, respectively, as 2.7 U L^{-1} at $t=24 \text{ h}$, 2.9 U L^{-1} at $t=24 \text{ h}$, and 3.2 U L^{-1} at $t=27 \text{ h}$. This research program also reveals that sorbitol as the co-substrate promotes the cell growth and recombinant protein production as reported by Çelik *et al.*, 2009 and Jungo *et al.*, 2007.

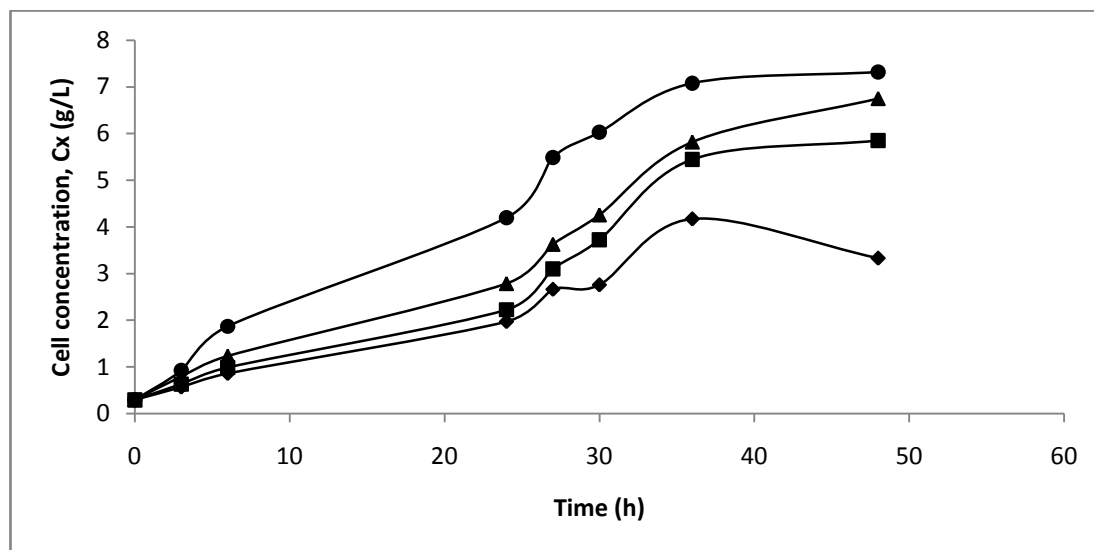


Figure 4.15 Effects of sorbitol concentration on the cell growth. Initial sorbitol concentrations; no sorbitol (♦), 10 g L^{-1} (■), 30 g L^{-1} (▲), 50 g L^{-1} (●).

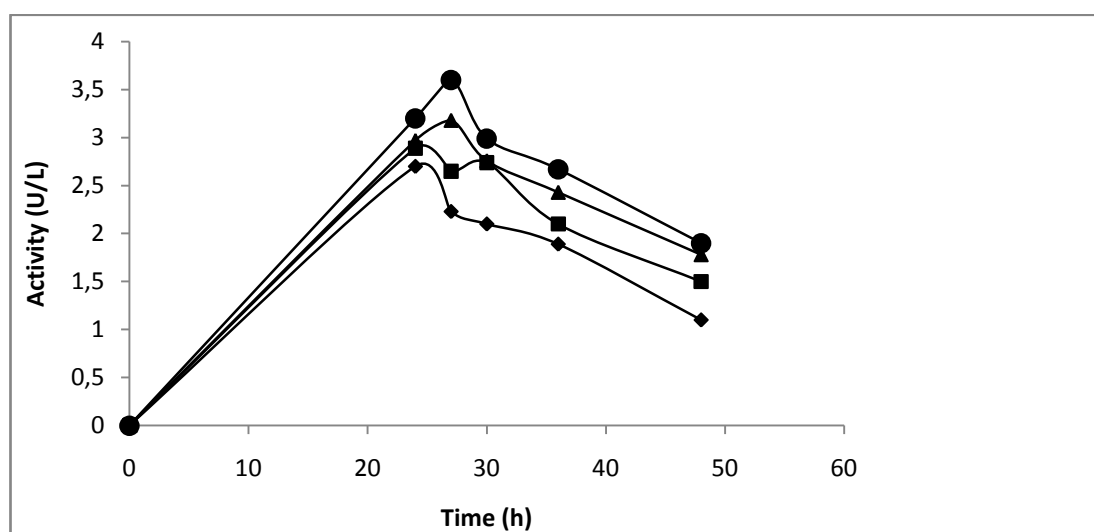


Figure 4.16 Effects of sorbitol concentration on GI activity. Initial sorbitol concentrations; no sorbitol (♦), 10 g L⁻¹ (■), 30 g L⁻¹ (▲), 50 g L⁻¹ (●).

4.3 Expression of Thermostable Glucose Isomerase in Recombinant *P. pastoris* in Pilot Scale Bioreactor

Based on the research programs achieved in shaker-bioreactors, a pilot-scale bioreactor experiment was programmed. The pilot-scale V=3dm³ bioreactor consisted of a system of working volume 1.0-2.2 dm³ with temperature, pH, foam, oxygen and stirring rate controls.

By using the data obtained in Sections 4.2.2 and 4.2.3, the pilot-scale bioreactor experiment conditions were set up. eP20 strain was inoculated into the precultivation medium (Table 3.5) and incubated at 30°C at 200 rpm for 20-22 hours using baffled 250 ml shaker-bioreactors which had working volumes of 50 ml. Thereafter, the cells were harvested at 1,500 g at 4°C for 10 minutes and resuspended in the production medium given in Table 3.8 until the cell concentration corresponding to OD₆₀₀=1.0 was obtained. After inoculation of the cells into the bioreactor, a four-phase experiment was performed as given in detail, in Section 3.5.3.2. The bioreactor temperature, pH and dissolved oxygen conditions were set up to, respectively, 30°C, 5.0 and 20%. Sorbitol was added batch-wise at C_{S0}=50 g L⁻¹, as determined in Section 4.2.3; as it was mentioned that 50 g L⁻¹ sorbitol is the non-inhibitory limit

concentration for the cell growth and recombinant protein production (Çalık *et al.*, 2010).

4.3.1 Cell Growth and Sorbitol Consumption

The variations in the cell concentration with the cultivation time in the production phase are given in Figure 4.17. As can be seen from the figure, the maximum cell concentration was $C_x=85.2 \text{ g L}^{-1}$ at $t=36 \text{ h}$. Due to the addition of the co-substrate sorbitol besides methanol, the lag phase was shortened than that of the single substrate methanol operation as reported by Çalık *et al.* (2010). Sorbitol consumption with the cultivation time is given in Figure 4.18. From Figure 4.18, it can be seen that sorbitol was consumed with a decreasing profile and was totally utilized at $t=15 \text{ h}$ as reported by Çalık *et al.* (2010). Moreover, since the utilization of sorbitol began at the beginning of induction phase after the addition of sorbitol batch-wise, methanol and sorbitol consumptions occur simultaneously in the cells, as was also reported by Çalık *et al.*, (2009), Çelik *et al.* (2009), Jungo *et al.* (2007). A characteristic decrease in the growth rate at $t=18 \text{ h}$ is observed, as the co-substrate sorbitol was consumed totally at $t=15 \text{ h}$ where the bioprocess proceeds with sole carbon source methanol. Thereafter, the cell concentration increased throughout the bioprocess. In the previous study by Çalık *et al.* (2010) for recombinant human growth hormone (rhGH) production by *P. pastoris* where the same feeding strategy was used, that is the addition of sorbitol at the beginning of the production phase, resulted in the maximum cell concentration at $t=24 \text{ h}$ as $C_x=42.3 \text{ g L}^{-1}$; whereas in this work, in recombinant GI enzyme production by *P. pastoris* the cell concentration at $t=24 \text{ h}$ is $C_x=62.8 \text{ g L}^{-1}$ which is 1.5-fold higher. Thus, in general it is clear that recombinant *P. pastoris* strains that produce different recombinant proteins have different growth profiles despite the same feeding strategy is used.

Table 4.7 emphasizes the calculated instantaneous specific growth and sorbitol consumption rates; where, until $t=12 \text{ h}$ both substrates, i.e., methanol and sorbitol, were used, and then the bioprocess proceeds only with the substrate methanol. Since sorbitol was totally consumed within 15 hours, the specific growth rate (μ) is calculated as $\mu_{MS}=0.053 \text{ h}^{-1}$ which shows that specific growth rate for sorbitol is

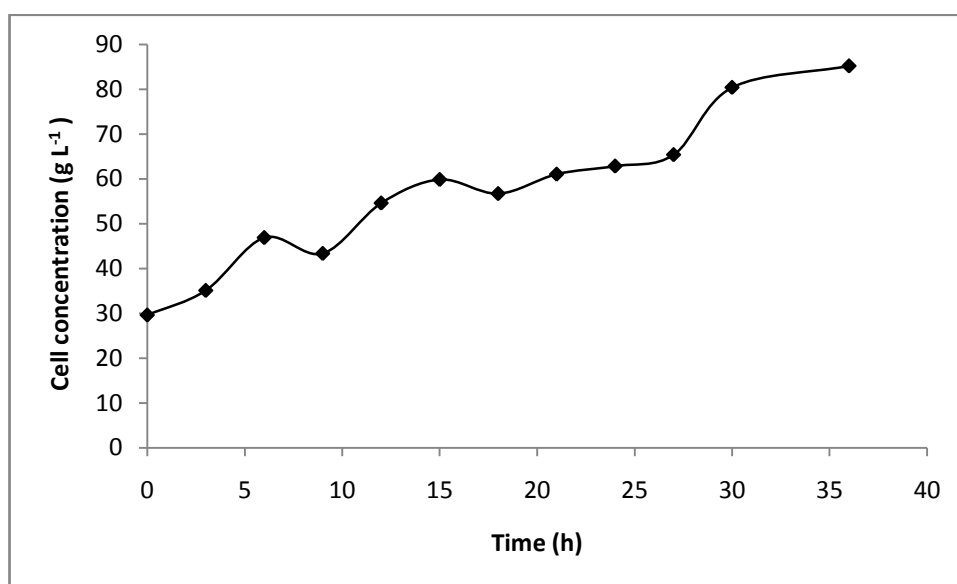


Figure 4.17 Variation in the cell concentration with respect to cultivation time in the production phase.

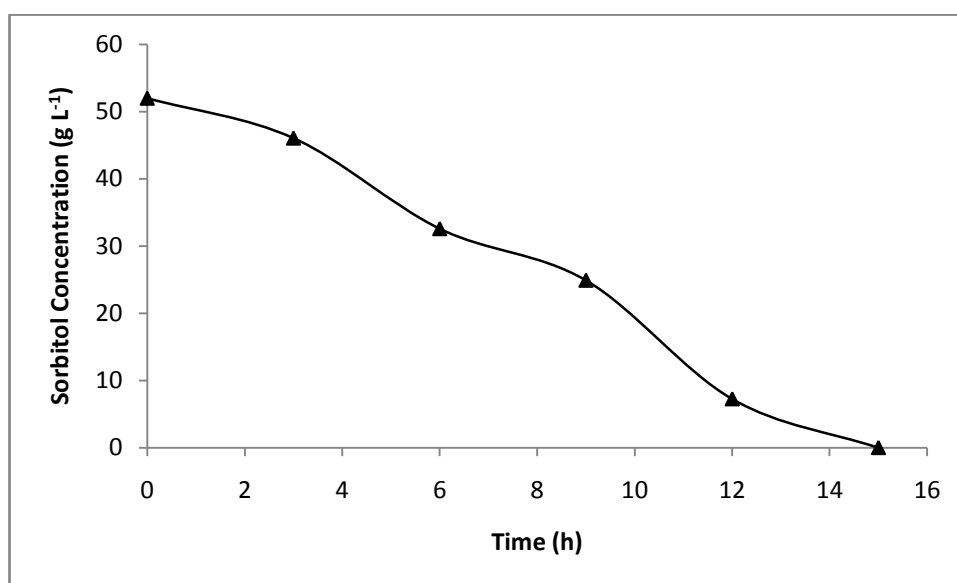


Figure 4.18 Sorbitol consumption profile with respect to time.

$\mu_s=0.023 \text{ h}^{-1}$. After the consumption of sorbitol in the bioreactor at $t=15 \text{ h}$, the overall specific growth rate between $t=15\text{-}36 \text{ h}$ was calculated as $\mu_M=0.025 \text{ h}^{-1}$ which is

consistent with the pre-determined methanol feeding profile which is $\mu_M=0.030 \text{ h}^{-1}$. Therefore, total specific growth rate μ_{MS} is the combination of specific growth rates on methanol (μ_M) and sorbitol (μ_S). Table 4.7 reveals that the specific growth rates are higher when sorbitol is present in the medium; in other words, after the consumption of sorbitol, instantaneous specific growth rates decrease showing that sorbitol presence in the medium enhances the cell growth.

Table 4.7 The variations in the instantaneous specific growth rate, specific sorbitol consumption rate and sorbitol consumption rate throughout the fermentation.

t h	μ_t h^{-1}	q_s $\text{g g}^{-1} \text{h}^{-1}$	r_s $\text{g L}^{-1} \text{h}^{-1}$
0	0.064	0.062	1.84
3	0.084	0.028	0.98
6	0.032	0.052	2.44
9	0.033	0.073	3.16
12	0.054	0.027	1.47
15	0.01		
18	0.008		
21	0.021		
24	0.016		
27	0.050		
30	0.033		
36	0.038		

The yield coefficients including the overall yield of biomass generated per mass of sorbitol consumed ($Y_{X/St}$), overall yield of biomass generated per mass of total methanol consumed ($Y_{X/Mt}$), were calculated for the analysis of the bioprocess. Thus, $Y_{X/St}$ and $Y_{X/Mt}$ were calculated, respectively, as 0.18 g g^{-1} and 0.25 g g^{-1} ; for comparison of the overall yield values, amongst, $Y_{X/St}=0.15$ for rhGH production by *P. pastoris* was reported by Çalık *et al.* (2010) is indeed a close value.

4.3.2 Thermostable GI Activity and Production

The aim of the pilot scale bioreactor program is to determine the GI enzyme production capacity of the recombinant *P. pastoris* eP20 strain carrying *xylA* gene in its genome, in a controlled bioreactor system. Figure 4.19 shows the calculated enzyme activity values with the cultivation time.

As can be seen from Figure 4.19, the maximum GI activity is obtained at $t=12$ h which is 202.86 U L^{-1} . After $t=12$ h, a sharp decrease in enzyme activity is observed after the depletion of sorbitol. The positive effect of sorbitol concentration on GI activity was first shown in Section 4.2.3 which supports the data obtained in the pilot scale bioreactor system. Thus, following the total consumption of sorbitol, GI activity starts to decrease (Figure 4.20). A possible reason of the decrease in GI activity with the cultivation time might be the increase in proteolytic activity of *P. pastoris* cells throughout the bioprocess which is a problem for *P. pastoris* expression systems (Potvin *et al.*, 2010). Therefore, protease activities in the supernatants of the samples were analyzed and the results are presented in Section 4.3.4.

The variations in the specific GI activity of recombinant *P. pastoris* eP20 with the cultivation time are presented in Figure 4.21. Figure 4.21 reveals that the maximum specific GI activity was obtained as 1.51 U mg^{-1} protein at $t=12$ h. As reviewed in Section 2.1.3.7 the bacterial systems such as *E. coli* or *Bacillus sp.* produce more active recombinant thermostable GI enzymes; however, those systems synthesize only intracellular GI enzymes. Therefore I can conclude that, the extracellular GI enzyme produced by the constructed recombinant *P. pastoris* eP20 clone carrying *xylA* gene in its genome, is produced extracellular thermostable recombinant GI

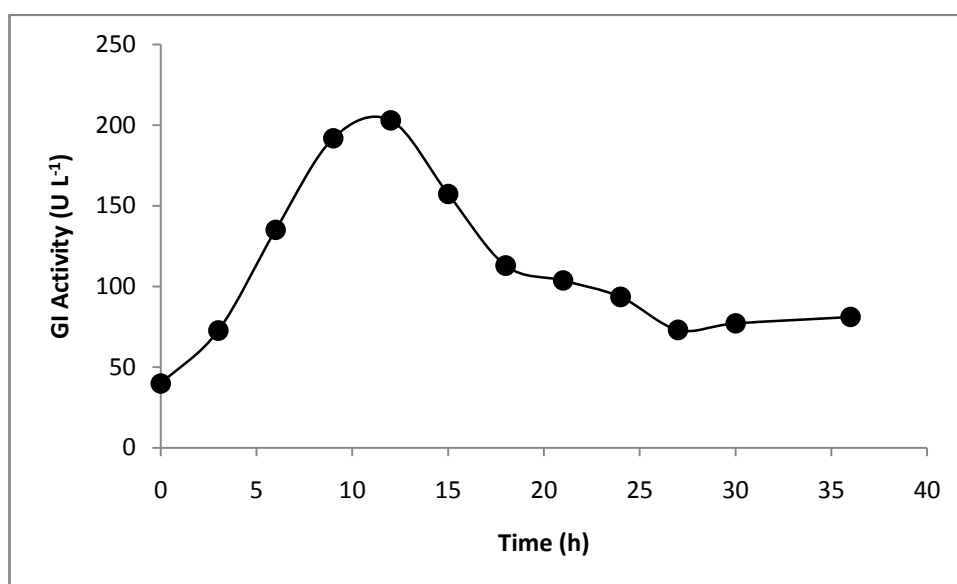


Figure 4.19 The variations in GI activity with the cultivation time

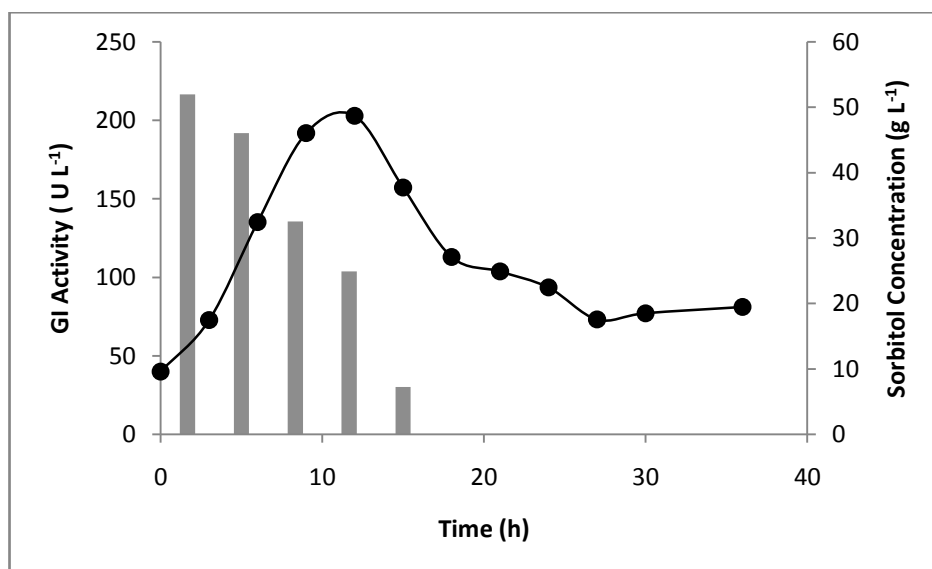


Figure 4.20 The variations of GI activity and sorbitol concentration with the cultivation time. Bar graph represents sorbitol concentration; whereas, (■) represents GI activity (U L⁻¹)

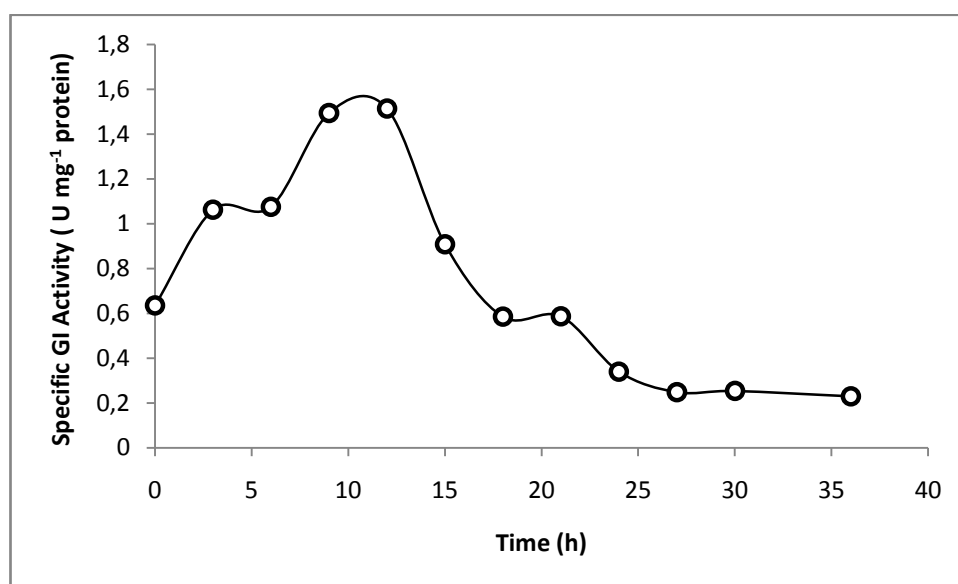


Figure 4.21 Variation in specific GI activity throughout the fermentation.

enzyme for the first time; moreover based on the present literature, the highest thermostable extracellular recombinant GI activity is obtained by *P. pastoris* eP20 eP20 clone.

It is indeed important to note that using thermophilic microorganisms producing recombinant thermostable enzymes at high temperatures increase the cost; whereas, producing thermostable enzymes by constructing recombinant microorganisms producing at mild temperature conditions decrease the cost. In a general context, the activity value obtained in this work, is lower than those of the natural sources which produce extracellular GI having activity values, which reported by Chen *et al.*(1979) as 1500 U L⁻¹, Srinivasan *et al.*(1983) as 4000 U L⁻¹, and Pandidurai *et al.*(2011) as 41000 U L⁻¹; whereas, the specific activity value obtained in this work is higher than that of Chauthaiwale and Rao (1994) as 0.62 U mg⁻¹.

In a wider context, the production of GI enzyme with other yeast cells which express active GI enzyme intracellularly can also be compared with the results of this work. In the literature, intracellular GI with high value of specific GI activity were obtained by Walfridsson *et al.* (1996), Voronovsky *et al.*, (2005) and Madhavan *et al.* (2009),

respectively, as 1.0, 0.19 and 1.90 U mg⁻¹; alas, intracellular enzymes need high cost for downstream processes.

Total GI subunit production was investigated by using SDS-PAGE (Figure 4.22). After determining the total protein present in the extracellular medium by Bradford Protein Assay (Section 3.6.2), it is found that approximately 75% of the total extracellular protein was composed of GI subunits. Figure 4.23 exhibits the variation of GI subunit concentration throughout the cultivation time which shows maximum GI subunit concentration was achieved at t=30 h with a value of C_p=213 mg L⁻¹ whereas it is slightly higher than the concentration at t=24 h.

The instantaneous specific product formation rates and product formation rates are given in Table 4.8. Average product formation rates for the entire fermentation process are calculated as r_{CGI}=4.33*10⁻³ g L⁻¹ h⁻¹ and r_{AGI}=12.87 U L⁻¹ h⁻¹. Table 4.8 exhibits that the maximum specific GI subunit production rate was obtained at the beginning of the production phase with a value of 0.32 g g⁻¹ h⁻¹ when there was sorbitol present in the medium. Moreover, maximum AOX activity was also obtained at the beginning of the production phase as mentioned detailed in Section 4.3.3. This result shows that the specific GI subunit production rate is closely related with the AOX activity. Although total GI subunit concentration increased with respect to time, the specific GI subunit production rates decreases similar to profile of AOX activity (Figure 4.24). Similar to this, maximum specific GI production rate in terms of activity was obtained at the beginning of the production phase at t=3 with a value of q_{AGI}=0.458 U g⁻¹ h⁻¹. Similar to cell growth rate and specific GI subunit production rate, maximum specific GI production rate in terms of activity was obtained when sorbitol was present in the extracellular medium. Moreover, specific GI production rate in terms of activity shows a similar profile as obtained in AOX activity (Figure 4.24) which indicates higher AOX activity is related with higher specific production rates of GI subunit concentration and activity value.

The overall yields for production of recombinant GI subunit, Y_{P/X} and Y_{P/S} are calculated as 3.87 mg g⁻¹ and 1.52 mg g⁻¹, respectively.

Table 4.8 The instantaneous specific product formation rates and product formation rates.

Time (h)	$q_{\text{CGI}} \cdot 1000$ $\text{g g}^{-1} \text{h}^{-1}$	$r_{\text{CGI}} \cdot 1000$ $\text{g L}^{-1} \text{h}^{-1}$	q_{AGI} $\text{U g}^{-1} \text{h}^{-1}$	r_{AGI} $\text{U L}^{-1} \text{h}^{-1}$
0	0.32	9.52	0.37	11.03
3	0.27	9.54	0.46	16.08
6	0.01	0.05	0.43	20.28
9	0.01	0.22	0.28	11.95
12	0.10	5.57	0.09	5.01
15	0.10	6.06		
18	0.02	1.37		
21	0.18	11.39		
24	0.18	11.74		
27	0.05	3.08		
30	0.02	1.88		

Approximately the same recombinant protein concentration was achieved as well as 270 mg L⁻¹ rhGH was produced in the study of Çalık *et al* (2010). However, it should be noted that activity is a more important parameter than the concentration for enzyme production. In this context, comparison of GI subunit concentration with GI activity shows that the amount of total GI subunit concentration increased with respect to time although GI activity decreased after t=12 h. One possible reason for this is the increasing proteolytic activity which is mentioned in Section 4.3.4. Increasing protease activity in the supernatant may have inhibited the homotetramer structure formation. Another possible reason is that the overproduction stress of recombinant GI production on *P. pastoris* cells. It was reported that secretion of recombinant proteins in yeast cells including *P. pastoris* may cause several stresses on the host cell including trouble in folding and disulfide bond formation (Hohenblum *et al.*, 2003). Therefore, possible folding problem in formation of homotetramer structure might have caused non-functional GI subunits.

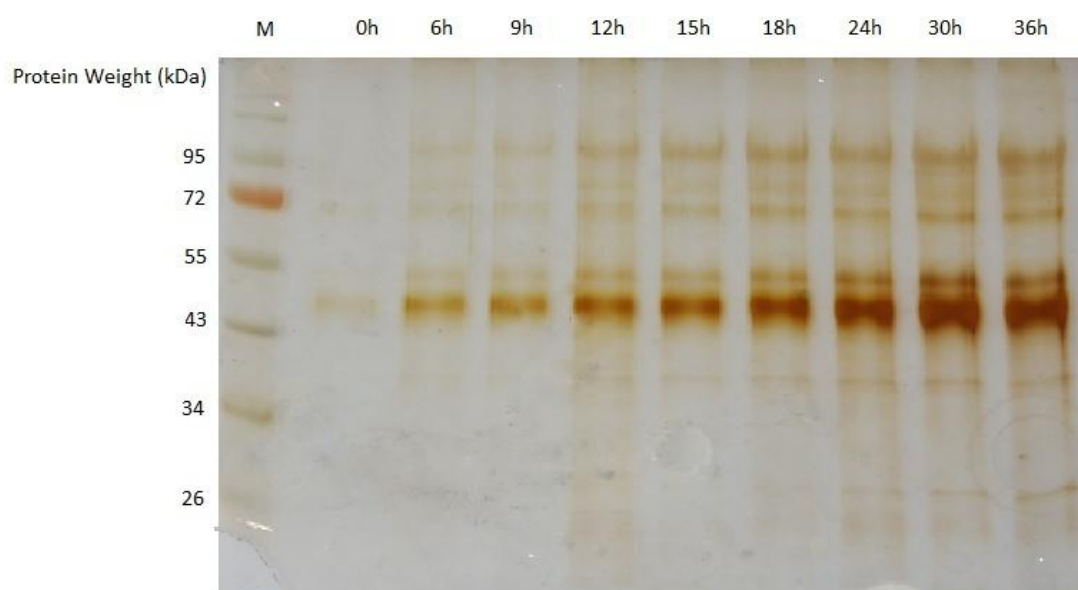


Figure 4.22 The variations of GI subunit amounts with the cultivation time. M: Marker. Other lanes refer to time of sampling.

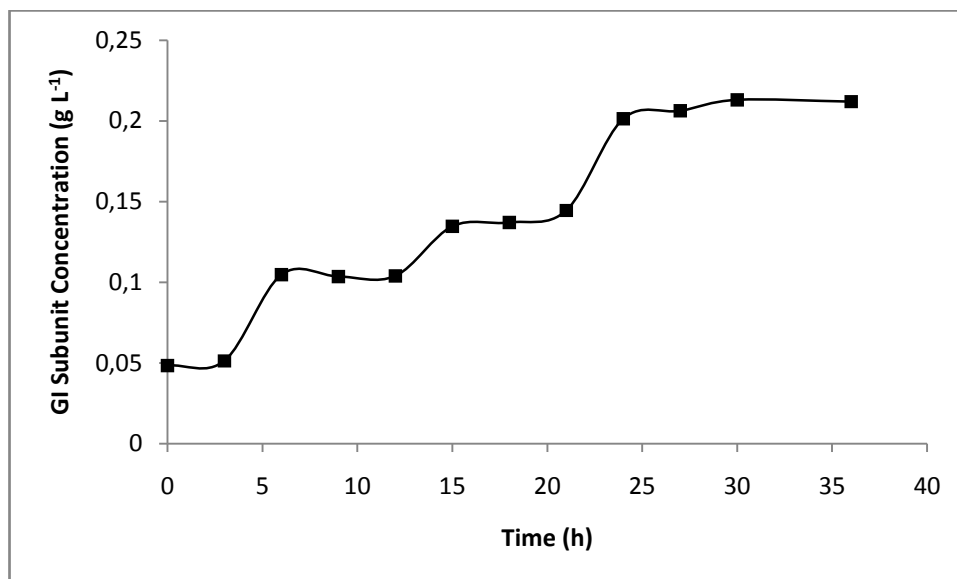


Figure 4.23 The variations of GI subunit concentration with the cultivation time.

4.3.3 Alcohol Oxidase Activity

Since thermostable GI production is under control of AOX promoter, AOX activity of the cells can help to understand better the relationship between AOX and recombinant GI production. AOX activities with respect to time throughout the fermentation are given in Figure 4.24.

From the Figure 4.24, the highest AOX activity was calculated as $61.1 \text{ U g CDW}^{-1}$ at $t=3$. After reaching highest AOX activity value at $t=3$ h, following the depletion of sorbitol in the medium, the AOX activity values start to decrease and never increase to higher levels as obtained when sorbitol was in the cultivation medium. This can be explained by the shifting of metabolism in a medium that contains only methanol as the carbon source after $t=15$ h. This shifting probably caused a lag phase in the cells so that AOX activities were lowered which can be seen in cell growth profile.

Obtained data from pilot scale bioreactor experiment exhibits that AOX activity profile did not show a similar trend than that of Çalık *et al.* (2010). In the mentioned study, maximum AOX activity was obtained at $t=15$ h of a value of $40.7 \text{ U g cell}^{-1}$ when sorbitol was totally consumed by *P. pastoris* cells producing rhGH reaching. But in this study, maximum AOX activity was obtained earlier in the production phase, due to modification of methanol transition (MT) phase, which was performed by feeding methanol to the system at a rate of $2.8 \text{ g L}^{-1} \text{ h}^{-1}$ for 4 hours instead of giving a pulse of methanol feed, $C_{M0}=1.5 \text{ g L}^{-1}$ for 6 hours in the study of Çalık *et al.* (2010); therefore, higher AOX activities were obtained in the beginning of production phase.

The reason for the fluctuation of the AOX activities is related with the sampling hours. Methanol profiles were calculated for every 3 hours as well as samples were taken every 3 hours. According to the calculated methanol feeding profile, methanol was not fed to the system continuously every 3 hours. When these times coincide with the sampling hours, AOX activities obtained from these samples resulted with lower values of AOX activities since no methanol was fed to the medium at that time.

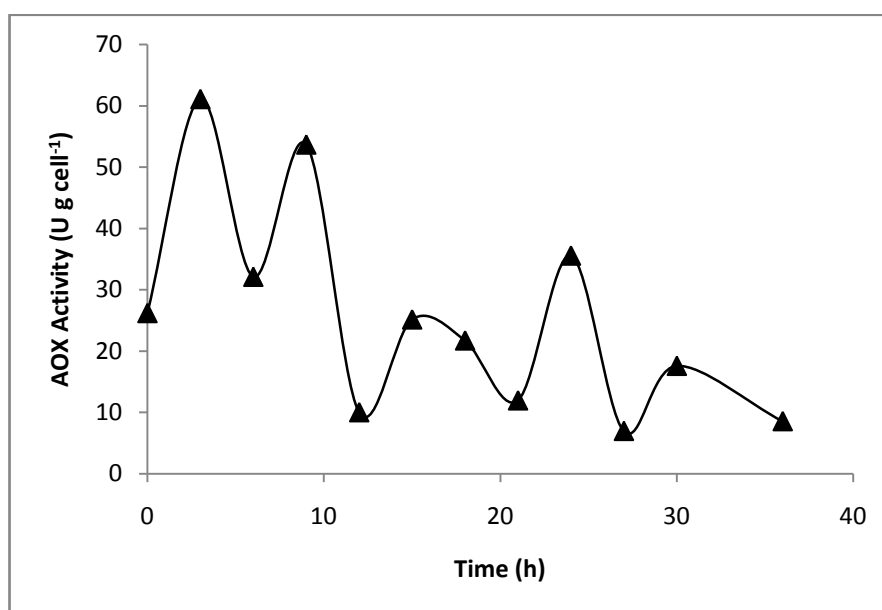


Figure 4.24 The variations of AOX activity with cultivation time.

4.3.4 Protease Profile

As mentioned before, proteolytic activity in supernatant in *P. pastoris* expression systems is a disadvantage. Thus, total protease activity including acidic, neutral and basic proteases in the extracellular medium was investigated to see the effect of proteolytic activity on recombinant thermostable GI production. In Figure 4.25 total proteolytic activity variation is given. As given in the figure, total proteolytic activity starts at minimum level which is 0.027 g L^{-1} and reaches to maximum level at $t=36 \text{ h}$ which is 0.093 g L^{-1} with an increasing profile.

Figure 4.26 shows the relationship between recombinant GI production and total proteolytic activity. As can be seen from the figure, maximum recombinant thermostable GI activity was obtained earlier in the production phase at $t=12 \text{ h}$ where relatively minimum proteolytic activity was observed. Following the depletion of sorbitol in the medium, GI activity starts to decrease while total protease activity increases. Thus, I can conclude that proteolytic activity in the extracellular medium is a limiting step that prevents reaching to higher levels of recombinant GI activity in *P. pastoris* expression systems.

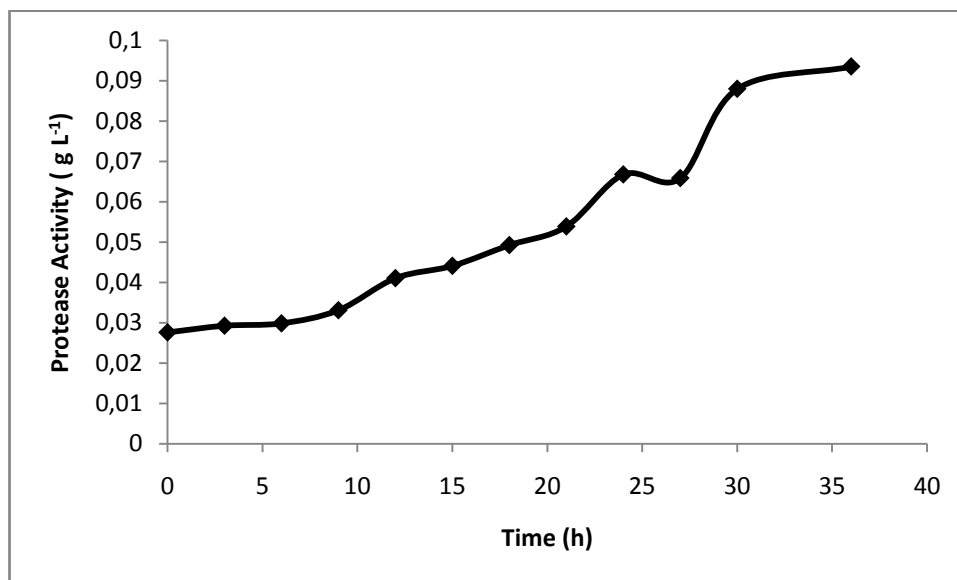


Figure 4.25 The variations of total protease activity with the cultivation time.

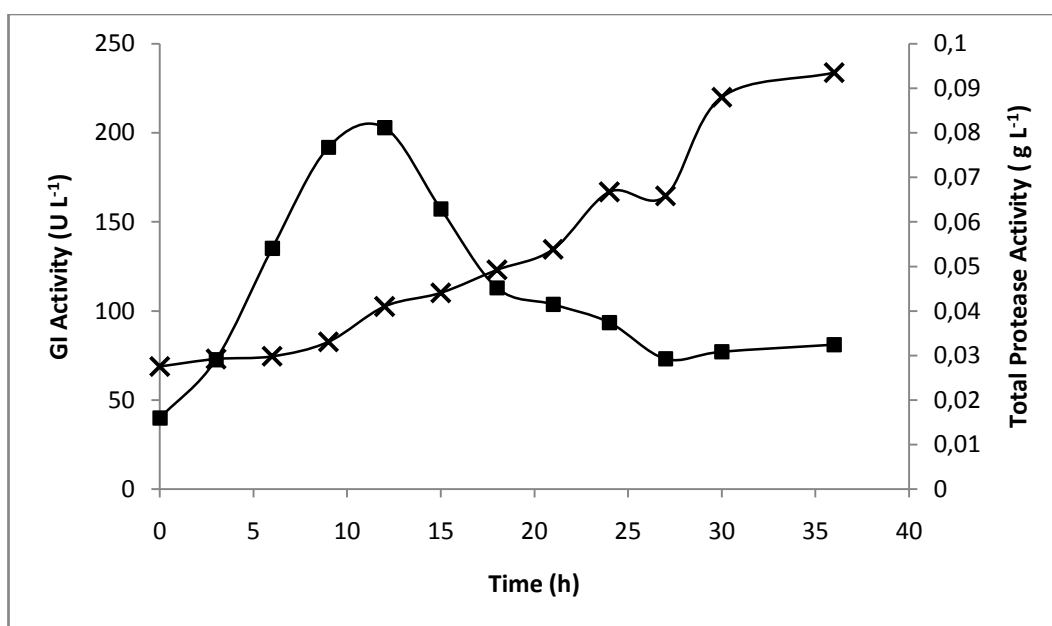


Figure 4.26 Relationship between recombinant GI activity and total protease activity with the cultivation time during the bioreactor fermentation. GI activity, U L⁻¹ (■); total protease activity, g L⁻¹ (X).

4.3.5 Organic Acid Profiles

Organic acid profile can give a better understanding of the intracellular reaction network thus the overall profile of the fermentation. Table 4.9 exhibits the variation of several organic acids during the fermentation process.

When considering the methanol utilization pathway in *P. pastoris*, formic acid concentration gives a better understanding of bioprocess profile. In *P. pastoris*, methanol utilization catalyzed by AOX resulted in production of hydrogen peroxide and formaldehyde, which are toxic to the cells (Zhang *et al.*, 2000). Afterwards, some of the formaldehyde is converted to cell components whereas some portion of formaldehyde is oxidized to formic acid. Another reason for formation of formic acid is the oxygen limitation (Charoenrat *et al.*, 2006). Moreover, lactic acid concentrations can help to decide, whether there is an oxygen limitation throughout the bioprocess, since lactic acid formation occurs when cells prefer fermentative pathway rather than respiratory pathway. From Table 4.5, it is obvious that lactic acid was present in the medium during the production phase which increased dramatically after $t=24$. As expected, formic acid concentration had a significant increase after $t=24$, too. Therefore, it can be said that oxygen limitation affected the fermentation process during the production phase. Moreover, it should be noted that, dissolved oxygen concentration was never achieved to hold at 20% although it was set to 20% which was always below the set point. Thus, oxygen fed to the system was insufficient despite the fact that maximum air enriched with pure oxygen was fed to the bioreactor within the limits of the equipment used.

Table 4.9 Concentrations of organic acids with the cultivation time throughout the fermentation. All concentrations are given in g L⁻¹.

Time (h)	0	6	12	15	18	24	30	36
Formic Acid	0.0213	0.0376	0.0295	0.0363	0.0451	0.0478	0.3969	0.4202
Fumaric Acid	---	---	---	---	---	0.0005	0.0023	0.0046
Pyruvic Acid	0.0014	0.0016	0.0023	0.0021	0.0058	0.0061	0.0234	0.0381
Acetic Acid	---	---	0.0366	0.0227	0.0428	0.0542	0.1683	0.3950
Citric Acid	---	---	0.0082	0.0094	0.0094	0.0165	0.0463	0.0873
Succinic Acid	---	---	---	---	0.0467	0.0900	0.5450	1.0359
Oxalic Acid	0.0010	0.0013	0.0012	0.0011	0.0024	0.0055	0.0196	0.0341
Lactic Acid	0.0671	0.0758	0.0896	0.0838	0.1013	0.1130	0.3815	0.8830

Other organic acids such as fumaric acid, pyruvic acid, acetic acid, citric acid, succinic acid and oxalic acid are metabolites of TCA cycle. Pyruvic acid and oxalic acid were detected in the extracellular medium during the production phase as well as they started to accumulate more after $t=24$ h. Acetic acid and citric acid were not detected in the cultivation medium until $t=12$ h, which means that these metabolites were consumed efficiently in the TCA cycle. Similar to other organic acids mentioned before, concentrations of acetic and citric acid reached to significantly higher levels after $t=24$ h. Fumaric acid and succinic acid were not detected until $t=24$ h and $t=18$ h, respectively; which showed a similar trend after $t=24$ h and started to accumulate in the extracellular medium. These results show that, organic acids were not consumed efficiently in the TCA cycle after $t=24$ h and started to accumulate. Thus, since TCA cycle is related with the respiratory pathway, it can be said that oxygen limitation became dominant in the bioprocess after $t=24$ h although limitation was observed during the bioprocess as mentioned in the formic acid and lactic acid case.

CHAPTER 5

CONCLUSION

The aim of this M.Sc. thesis is to produce extracellular thermostable glucose isomerase (GI; EC 5.3.1.5) enzyme by metabolically engineered *P. pastoris*. In this context, the research program was based on two main sub-programs. In the first research program, based on a metabolic engineering design a recombinant *P. pastoris* strain that produce an active thermostable GI from *T. thermophilus* was constructed. For this purpose, *xylA* gene that encodes GI was inserted to pPICZ α -A expression vector and this to pPICZ α -A::*xylA* plasmid was integrated to AOX1 locus in the *P. pastoris* genome. With enzymatic activity assay and SDS-PAGE analysis, best producing strain was selected and named as eP20. Thereafter, effects of salt and sorbitol concentrations on the cell growth and recombinant thermostable GI activity were investigated in air filtered shake bioreactors. In the second part of the research program, a pilot scale bioreactor experiment was performed to determine the profile of the cell growth and recombinant GI activity under pH, oxygen and temperature controlled conditions with a pre-determined feeding profile for methanol with the addition of sorbitol as co-substrate batch-wise.

The results obtained from the first research program are given below:

- Based on the metabolic engineering design, for extracellular expression of *xylA* gene, the forward and reverse primers that are specific for *xylA* gene were designed. The forward and reverse primers were 34 bp oligonucleotides which include *EcoR* I and *Xba* I restriction sites, respectively. *xylA* gene was amplified by using these designed primers with PCR. Thereafter, the PCR product obtained was cloned into pPICZ α -A expression vector after restriction of *EcoR* I and *Xba* I into *E. coli*

TOP10 cells. After the selection of the true transformant by using Zeocin as a selective antibiotics, pPICZ α -A::xylA plasmid which was linearized by *Pme* I restriction enzyme was cloned into AOX1 locus of *P. pastoris* genome. By the specific enzymatic assay and SDS-PAGE analysis, best producing strain was selected and named as eP20.

- Different production media which contained different salt concentrations were investigated to demonstrate the effects of salt concentration on the cell growth and recombinant GI production. The results show that lowering concentrations of PTM1, MgSO₄·7H₂O and CaSO₄·2H₂O negatively affected both the cell growth and recombinant GI. The maximum cell concentration was obtained as 6.3 g L⁻¹ in the production medium containing 30 g L⁻¹ sorbitol, 4.35 g L⁻¹ ammonium sulphate, 0.1 M potassium phosphate buffer (pH 6.0), 14.9 g L⁻¹ MgSO₄·7H₂O, 1.17 g L⁻¹ CaSO₄·2H₂O, 1 ml L⁻¹ chloramphenicol and 4.35 ml L⁻¹ PTM1. Similar to the cell production, the maximum recombinant GI activity was obtained as 3.21 U L⁻¹ at t=24 h in the production medium-1. This result shows that the presence of the ions in the cultivation medium positively affect recombinant GI activity, similar to previous findings.
- Investigation of the effect of sorbitol concentration as a non-inhibiting co-substrate on the cell growth and recombinant GI activity shows that optimum initial sorbitol concentration was 50 g L⁻¹, which yielded biomass concentration and recombinant GI activity as 7.3 g L⁻¹ and 3.6 U L⁻¹, respectively.

In the second research program, a pilot scale bioreactor experiment was performed. The pilot scale bioreactor consisted of a system of working volume $V=1\text{ dm}^3$ with temperature, pH, foam, stirring rate and dissolved oxygen controls, was conducted at the temperature set to 30°C, pH set to 5.0, and dissolved oxygen set to 20%. At the beginning of the production phase (MFB), sorbitol was fed to the system batch-wise at an initial concentration of $C_{S0}=50\text{ g L}^{-1}$. Methanol was fed into the bioreactor with a pre-determined feeding profile which was calculated by using constant specific

growth rate, $\mu=0.03 \text{ h}^{-1}$. The results obtained from this experiment set are given below:

- Sorbitol fed to the system was totally consumed within 15 hours.
- The maximum biomass concentration was obtained as $C_x=85.2 \text{ g L}^{-1}$ at $t=36 \text{ h}$. According to the cell growth profile no long lag phase was observed due to addition of sorbitol batch-wise. The overall specific growth rate between $t=0-15$ where both sorbitol and methanol were present in the medium was calculated as $\mu_{MS}=0.53$. After the depletion of sorbitol, the overall specific growth rate between $t=15-36 \text{ h}$ was calculated as $\mu_M=0.025 \text{ h}^{-1}$. $Y_{X/St}$ and $Y_{X/Mt}$ were calculated as 0.18 g g^{-1} and 0.25 g g^{-1} , respectively.
- The maximum recombinant GI activity was obtained as 202.8 U L^{-1} at $t=12 \text{ h}$ which decreased after the depletion of sorbitol in the bioreactor production medium. The specific activity was calculated as 1.51 U mg^{-1} protein at $t=12$. At the end of the bioprocess, the highest extracellular thermostable GI activity value was obtained in the literature.
- The extracellular thermostable GI enzyme is produced by the constructed recombinant *P. pastoris* eP20 clone carrying *xylA* gene in its genome for the first time; moreover based on the present literature, the highest thermostable extracellular recombinant GI activity is obtained by *P. pastoris* eP20 clone.
- Maximum GI monomer concentration was obtained as 213 mg L^{-1} at $t=30 \text{ h}$. The overall yields for recombinant GI monomer, $Y_{P/X}$ and $Y_{P/S}$ are calculated as 3.87 mg g^{-1} and 1.52 mg g^{-1} , respectively.
- According to SDS-PAGE results, the amount of total GI monomers increased with respect to the cultivation time, although GI activity decreased after $t=12 \text{ h}$ possibly because of the increase in proteolytic activity of the cells and overload of recombinant GI production.
- The maximum AOX activity was obtained as $61.1 \text{ U g CDW}^{-1}$ at $t=3 \text{ h}$. After the consumption of sorbitol in the bioreactor, it was never observed AOX activities as high as obtained when sorbitol was present in the medium.
- An increasing protease activity profile with respect to the cultivation time time was obtained during the fermentation process. The maximum total

protease activity was observed as 0.67 g L^{-1} at $t=36$. A sharper increase in proteolytic activity and sharper decrease in GI activity was observed at $t=21$ which shows close relationship between total proteolytic activity and recombinant GI production.

- Organic acid profiles show that oxygen limitation take place during the production phase with an increasing trend after $t=24$ h. Lactic acid was detected in the extracellular medium from $t=0$ h to $t=36$ h although its concentration increased to higher levels after $t=24$ h and reached to the maximum at $t=36$ h as 0.8830 g L^{-1} . Another organic acid, formic acid which takes place in methanol utilization pathway, was detected in the cultivation medium between $t=0$ - 36 h, whereas increased to its maximum concentration of 0.4202 g L^{-1} at $t=36$ h. This accumulation after $t=24$ shows that oxygen limitaiton is a limiting parameter especially after $t=24$ since formic acid formation is also related with the oxygen limitation in the cells. Other organic acids which are utilized in the TCA cycle such as fumaric acid, pyruvic acid, acetic acid, citric acid, succinic acid and oxalix acid were detected at higher levels after $t=24$, although they were detected at relatively low concentrations at the beginning of the production phase. The accumulation of these organic acids indicate that they couldn't be used in the TCA cycle efficiently after $t=24$ h, which also means an oxygen limitation is present in the fermentation process after $t=24$ h.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Yeast Lysis Buffer for Genomic DNA Isolation	Dissolve 2 g of Triton-X 100, 1 g of SDS, 5.84 g of NaCl, 0.1 moles of Tris-Cl (pH=8.0) and 0.338 g of Na ₂ EDTA in 1 L of distilled water.
Alkaline Lysis Solution I	Prepare 100 ml batches including 50 mM glucose, 25 mM Tris-Cl and 100 mM EDTA in distilled water. Autoclave and store at 4°C.
Alkaline Lysis Solution II	Prepare 10 ml batches including 0.2 M NaOH and 1% SDS (w/v) in distilled water. Freshly prepare and store at room temperature.
Alkaline Lysis Solution III	Prepare 100 ml batches including 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of dH ₂ O. Store it at room temperature and transfer to an ice bucket before use.
0.5 M Ethylenediaminetetraacetic acid EDTA (pH:8.0)	Dissolve 18.61 g EDTA in 80 ml distilled water. Adjust pH to 8.0 and bring final volume up to 100 ml. Autoclave and store at room temperature.

1 M Tris-Cl, pH 8.0	Dissolve 12.1 g Tris base 80 mL dH ₂ O and adjust the pH to 8.0 by adding concentrated HCl. Make up the volume up to 100 ml. Autoclave and store at room temperature.
10X TE Buffer (pH 8.0)	Prepare 100 mM Tris-Cl (pH=8.0) and 10 mM EDTA (pH=8.0) in distilled water. Autoclave and store at 4°C. Dilute it 10 times before use.
5X TBE Buffer	Dissolve 54 g of Tris base, 27.5 g of boric acid and 20 ml of EDTA (0.5 M) in 950 mL distilled water. Adjust pH to 8.0 and make it up to 1 L. Dilute it 5 times before use.
1 M Potassium Phosphate Buffer (pH=6.0)	Dissolve 56.48 g KH ₂ PO ₄ , 14.8 g K ₂ HPO ₄ in 500 ml distilled water. Autoclave and store at room temperature.
Antifoam	Prepare 10% (v/v) antifoam solution with distilled water. Autoclave before use.
Base	25% NH ₃ OH (Sigma). No need to sterilize.
Yeast Lysis Buffer for AOX Activity Assay	Prepare 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 10% glycerol in 50 ml distilled water. Add 1 protease inhibitor cocktail tablet. Filter sterilize and store at +4°C for maximum 3 months.

0.1 M Potassium Phosphate Buffer (pH 7.0)	Dissolve 13.61 g of KH_2PO_4 in 100 ml distilled water. Dissolve 17.41 g of K_2HPO_4 in distilled water. Mix 38.5 ml of KH_2PO_4 and 68.5 ml K_2HPO_4 . Control the pH and store at room temperature. The buffer was autoclaved and stored at room temperature.
0.05 M Sodium Acetate Buffer (for acidic proteases)	Dissolve 0.713 mL acetic acid in 25 mL distilled water. Dissolve 2.052 g sodium acetate in 50 mL distilled water. Titrate sodium acetate solution with acetic acid solution to pH 5.0 to a final volume of 50 mL. Dilute to 500 mL before use. Autoclave and store at $+4^\circ\text{C}$.
0.05 M Sodium Phosphate Buffer (for neutral proteases)	Dissolve 6.70 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 50 mL distilled water. Dissolve 3.90 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 50 mL distilled water. Titrate till pH 7.0 to a final volume of 50 mL. Dilute to 500 mL before use. Autoclave and store at room temperature.
Borate Buffer (for alkali proteases)	Dissolve 2.381 g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ in 250 ml distilled water and adjust pH to 10. Bring up the final volume to 500 ml. Filter sterilize and store at $+4^\circ\text{C}$.

APPENDIX B

SDS-PAGE SOLUTIONS AND STAINING PROCEDURE

10% (w/v) Ammonium persulphate (APS)	Add 0.1 g APS to 1 mL dH ₂ O, freshly prepared.
30% Acrylamide-bis	Dissolve 14.6 g acrylamide and 0.4 g N'N'- bis-methylene-acrylamide in 50 ml distilled water. Store at 2-8°C in the dark.
10% (w/v) SDS	Dissolve 5 g of SDS in 50 ml distilled water. Store at room temperature.
1.5 M Tris-HCl (pH 8.8)	Dissolve 26.3 g Tris base in 150 mL dH ₂ O and adjust pH to 8.8 with 6 N HCl. Bring up the final volume to 200 mL with distilled water. Autoclave and store at 2-8°C.
0.5 M Tris-HCl (pH 6.8)	Dissolve 12.2 g Tris base in 150 mL dH ₂ O and adjust pH to 6.8 with 6N HCl. Bring up the final volume to 200 mL with distilled water. Autoclave and store at 2-8°C.
Stacking Gel Buffer (5%)	Mix 2.8 mL dH ₂ O, 0.85 mL 30% Acrylamide-bis, 1.25mL 0.5M Tris-HCl (pH 6.8), 50µL 10% SDS, prior to gel preparation add 25µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine (TEMED).

Resolving Gel Buffer (12%)	Mix 3.4 mL distilled water, 4 mL 30% Acrylamide-bis, 2.5 mL 1.5 M Tris-HCl (pH 8.8), 100 µL 10% SDS, prior to gel preparation add 50 µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine (TEMED).
4X SDS-PAGE Sample Loading Buffer	Mix 200 mM Tris-HCl (pH 6.8); 40% glycerol; 6% SDS, 0.013% Bromophenol blue, 10% 2-mercaptoethanol. Distribute into microcentrifuge tubes and store at -20°C.
5X SDS-PAGE Running Buffer	Dissolve 15 g Tris Base, 72 g glycine, 5 g SDS, in 1L distilled water. Store at 2-8°C and dilute 5 times with dH ₂ O before use
Fixer Solution	Mix 100 mL methanol, 24 mL glacial acetic acid, 150 µL formaldehyde and complete to 200 mL with distilled water. Can be used up to 1 month. Store in the dark.
Pretreatment Solution	Dissolve 0.05 g sodium thiosulphate (Na ₂ S ₂ O ₃ .5H ₂ O) in 400 ml distilled water Take 2 mL and set aside for further use in developing solution preparation. Freshly prepared.
Silver Nitrate Solution	Dissolve 0.2 g silver nitrate in 400 mL distilled water and add 75 µL 37% formaldehyde. Store in the dark. Freshly prepared.
Developing Solution	Dissolve 2.25 g potassium carbonate in 100 mL distilled water. Add 2 mL from pretreatment solution and 75 µL 37% formaldehyde. Freshly prepared.

Stop Solution

Mix 50 mL methanol and 12 mL acetic acid. Bring up the final volume to 100 ml with distilled water. Store in the dark. Freshly prepared.

Staining Procedure for SDS-PAGE

Step	Solution	Time of Treatment	Comments
Fixing	Fixer	>1 hr	Overnight incubation is acceptable.
Washing	%50 Ethanol	3 x 20 min	Should be fresh.
Pre-treatment	Pretreatment Solution	1 min	Should be fresh.
Rinse	Distilled water	3 x 20 sec	Time should be exact.
Impregnate	Silver nitrate solution	20 min	Should be fresh.
Rinse	Distilled water	3 x 20 sec	Time should be exact.
Developing	Developing Solution	~5 min	Some distilled water can be added after a few minutes to slow down the reaction. Time is determined by observation of color development.
Stop	Stop solution	>10 min	Gels can be stored in this solution.

APPENDIX C

NUCLEOTIDE SEQUENCES AND PLASMIDS

Sequence of Glucose Isomerase (1164 bp)

gtgtacgagcccaaaccggagcacaggtttacctttggcctttggactgtgggcaatgtggg
ccgtgatcccttcggggacgcggttcgggagaggctggaccggtttacgtggttcataagc
tggcggagcttggggcctacggggtaaaccttcacgacgaggacctgatcccgcggggcacg
cctcctcaggagcgggaccagatcgtgaggcgcttcaagaaggctctcgatgaaaccggcct
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cgagcccggacccttgggttcgggcctatgccttgcggaagagcctggagaccttgacctg
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cctacgccgaggaccagggatacgggtaccggtttgcctcgagcccaagcctaacgagccc
cggggggacatttacttcgccaccgtggggagcatgctcgcctttattcataacctggaccg
gcccgagcgcttcggcctgaaccccaggttcgcccacgagacctggccgggcttaactttg
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cggatgagccggtttgaccaggacctccgcttcggctcgggagaacctcaaggcggcctttt
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ttactatcaagaagatcctgcggccttggcccttttggggccctactcccgcgagaaggccg
aagccctcaagcgggaggagcttcccctcgaggccaagcggcgccggggttatgcctggaa
cgcttgaccagctggcggtggagtacctcctgggggtgcgggggtga

Sequence of pPICZ- α A (3593 bp)

agatctaacatccaaagacgaaagggttgaaatgaaacctttttgccatccgacatccacaggt
ccattctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccggttgca
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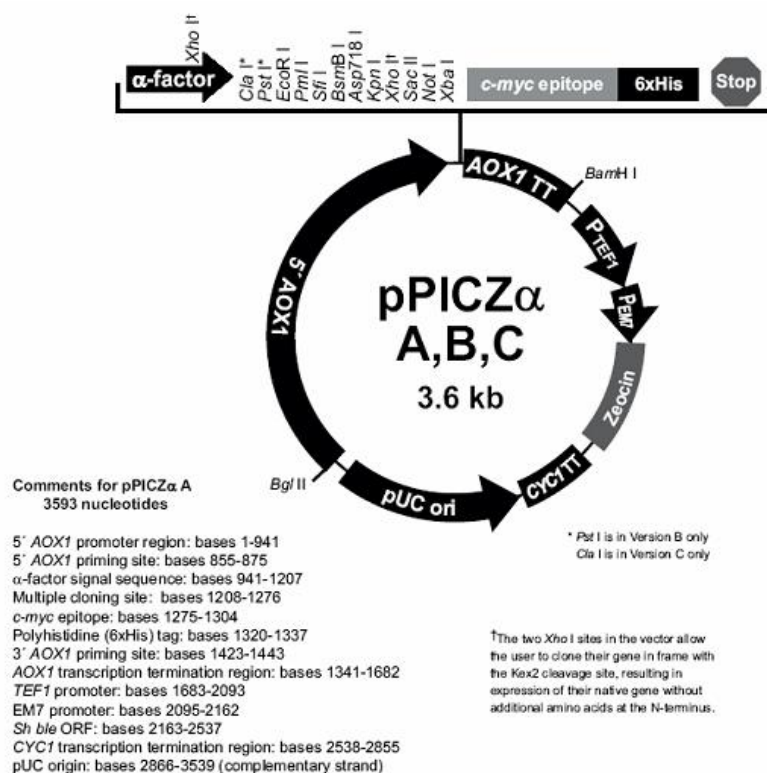


Figure C.1. Schematic representation of pPICZα-A (Invitrogen, 2001)

Multiple Cloning Sites of pPICZa-A

```

      5' end of AOX1 mRNA                                5' AOX1 priming site
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

871 CAAGCTTTTG ATTTTAACGA CTTTAAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT

931 ATTCGAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala

983 TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA
      Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala

      α-factor signal sequence
1034 CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
      Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe

1085 GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe

      Xho I*
1136 ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu

      Kex2 signal cleavage      EcoR I      Pml I      Sfi I      BsmB I Asp718 I
1187 GAG AAA AGA GAG GCT GAA GC GAATTCAC GTGGCCCAG CCGGCCGTC TCGGATCGGT
      Glu Lys Arg Glu Ala Glu Ala
      Ste13 signal cleavage
      Kpn I Xho I      Sac II Not I      Xba I      c-myc epitope
1244 ACCTCGAGCC GCGGCGGCC GCCAGCTTTC TA GAA CAA AAA CTC ATC TCA GAA GAG
      Glu Gln Lys Leu Ile Ser Glu Glu
      polyhistidine tag
1299 GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTAGCC
      Asp Leu Asn Ser Ala Val Asp His His His His His His ***

1351 TTAGACATGA CTGTTCTCTCA GTTCAAGTTG GGCACTTACG AGAAGACCGG TCTTGCTAGA

      3' AOX1 priming site
1411 TTCTAATCAA GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT

      3' polyadenylation site
1471 ACTTTTTTAT TTGTAACTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT

```

Sequence of pPICZ- α A::xylA (4700 bp)

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Underlined sequences are restriction enzyme recognition sites: *EcoR* I and *Xba* I, respectively.

Bold sequence is the nucleotide sequence of *xylA*.

APPENDIX D

PROPERTIES OF DESIGNED PRIMERS

Table D.1. Properties of designed primers

Name	Length (bp)	GC (%)	T _m (°C)	ΔG(kcal/mol)
EGI-F	34	52.9	72.6	-72.7
GI-R	35	61.8	73.3	-81.8

EGI-F self annealing:

5'-GGAATTCATGTACGAGCCCCAAACCAGAGCACAGG-3'
|||||
3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'
dG: -5,54 kcal/mol

5'-GGAATTCATGTACGAGCCCCAAACCAGAGCACAGG-3'
: |||| :
3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'
dG: -2,93 kcal/mol

5'-GGAATTCATGTACGAGCCCCAAACCAGAGCACAGG-3'
: |||| :
3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'
dG: -2,29 kcal/mol

5'-GGAATTCATGTACGAGCCCCAAACCAGAGCACAGG-3'
: ||| : : :: :
3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'
dG: -1,39 kcal/mol

EGI-F loops:

5'-GGAATTCATGTACGAGCCCA
: ||| :)
3'-GGACACGAGACCAA
dG: -0,97 kcal/mol

GI-R self annealing:

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'
|||||
3'-CTCATGAAGGACCCCCACGCCCCCACTAGATCTGC-5'
dG: -7,35 kcal/mol
5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'
|||||
3'-CTCATGAAGGACCCCCACGCCCCCACTAGATCTGC-5'
dG: -6,69 kcal/mol

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'
: |||| :
3'-CTCATGAAGGACCCCCACGCCCCCACTAGATCTGC-5'
dG: -3,30 kcal/mol

GI-R loops:

None!

GI-R - EGI-F annealing:

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'

: : : |||

3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'

dG: -2,93 kcal/mol

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'

||| : :

3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'

dG: -1,45 kcal/mol

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'

||| : : : : :

3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'

dG: -1,38 kcal/mol

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'

:: : : : |||

3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'

dG: -1,38 kcal/mol

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'

:: |||

3'-GGACACGAGACCAAACCCGAGCATGTACTTAAGG-5'

dG: -1,38 kcal/mol

5'-CGTCTAGATCACCCCCGCACCCCCAGGAAGTACTC-3'

: : :: : :: ||| :

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dG: -0,62 kcal/mol

APPENDIX E

MOLECULAR WEIGHT MARKERS

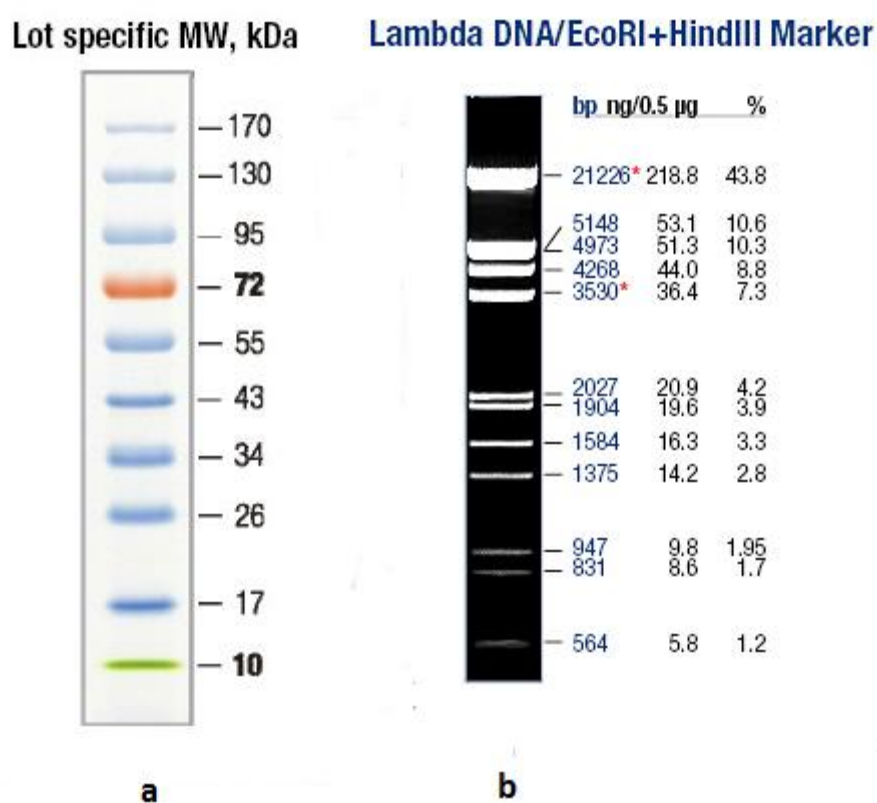


Figure E.1. Molecular weight markers used in this study. **(a)** PageRuler Prestained Protein Ladder used in SDS-PAGE, **(b)** Lambda DNA/*EcoR* I *Hind*III Marker used in Agarose Gel Electrophoresis.

APPENDIX F

CALIBRATION CURVE FOR BRADFORD ASSAY

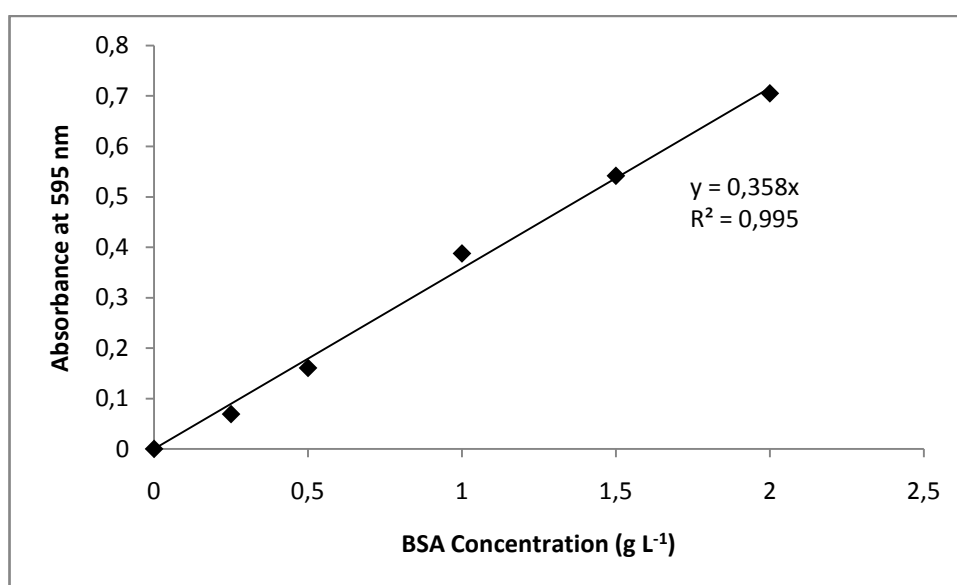


Figure F.1. Calibration curve for Bradford assay.

APPENDIX G

CALIBRATION CURVE FOR D-FRUCTOSE CONCENTRATION

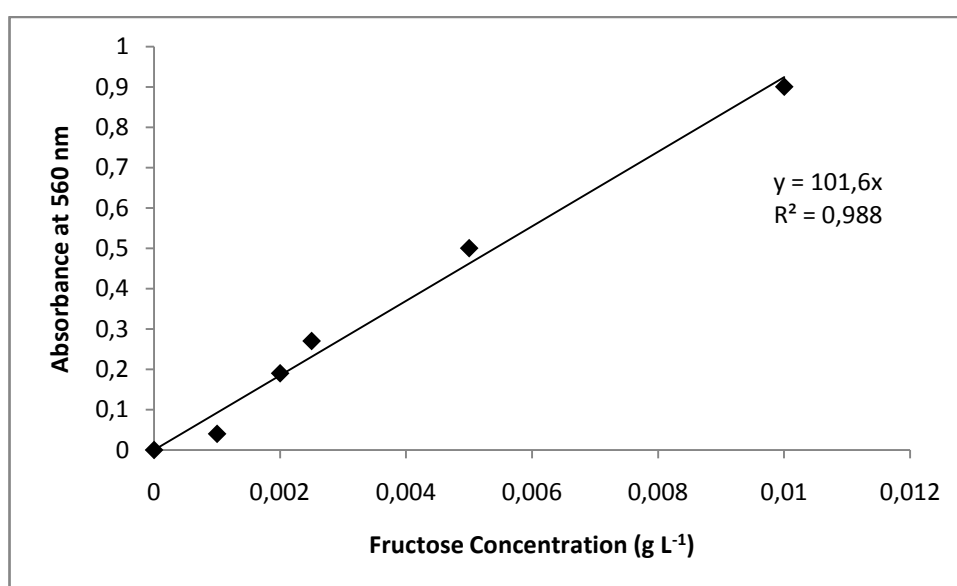


Figure G.1. Calibration curve for D-Fructose concentration.

APPENDIX H

CALIBRATION CURVE FOR SORBITOL CONCENTRATION

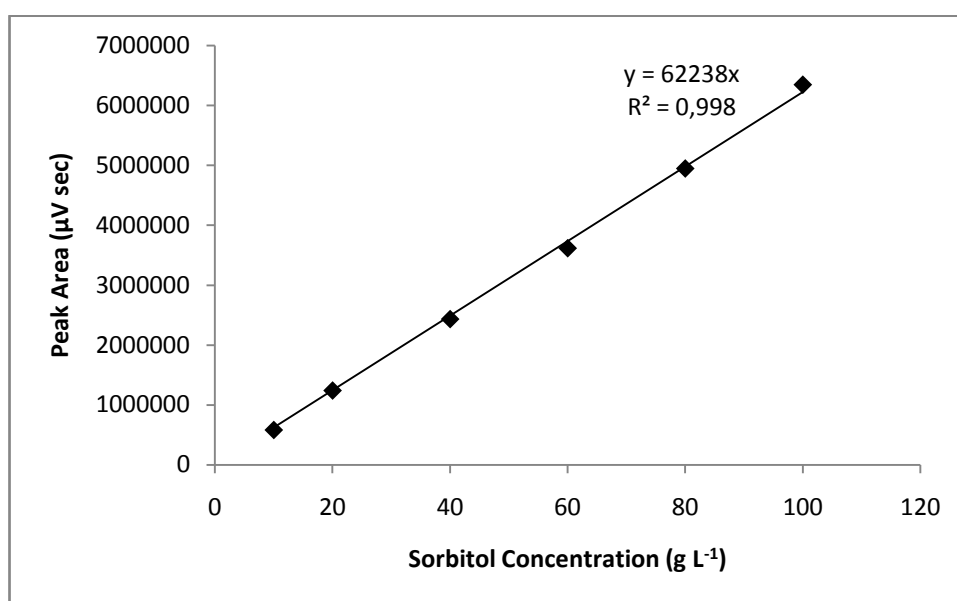


Figure H.1. Calibration curve for sorbitol concentration; analysis was performed by HPLC.

APPENDIX I

CALIBRATION CURVE FOR ORGANIC ACID CONCENTRATION

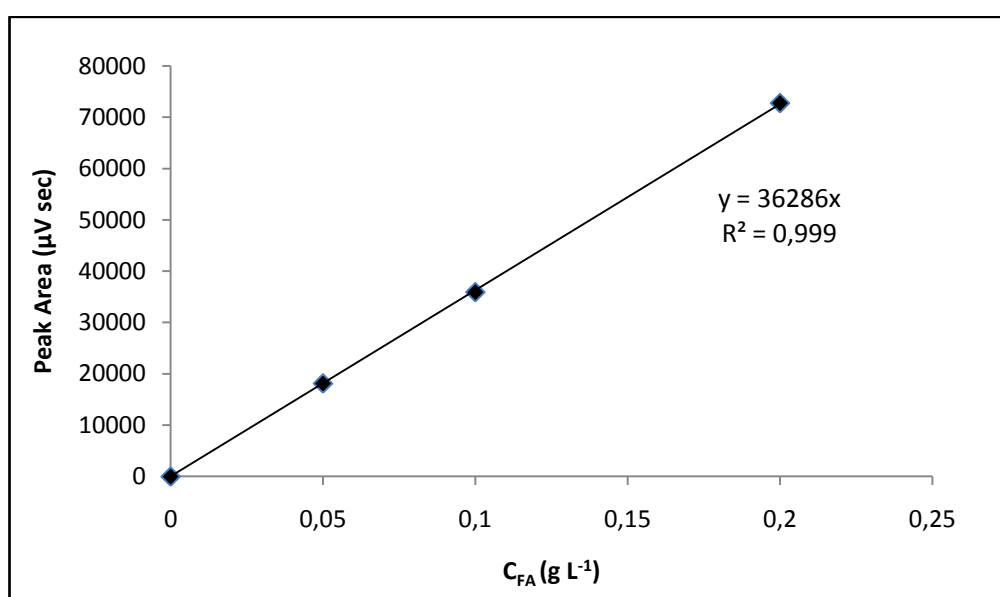


Figure D.1 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

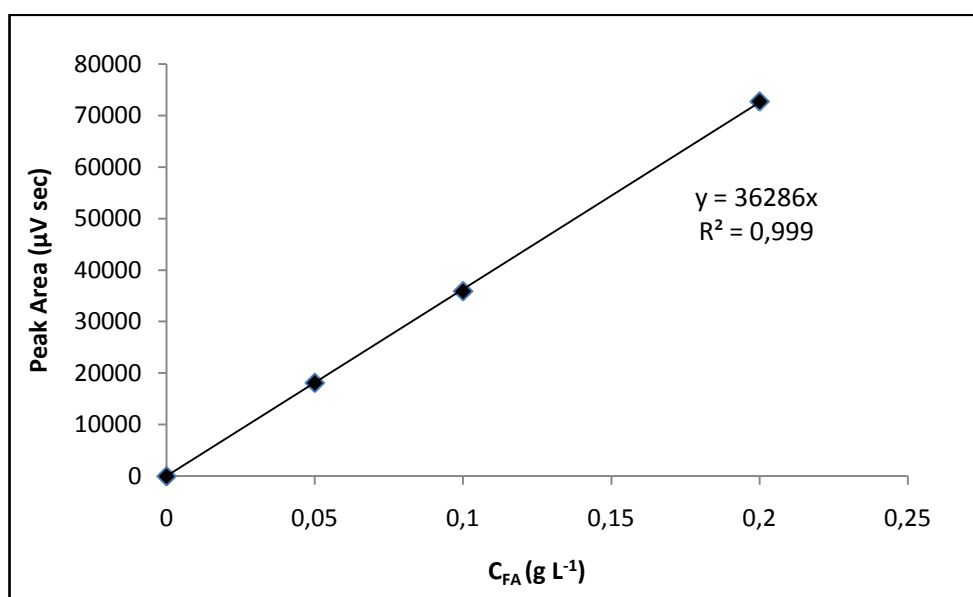


Figure D.2 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

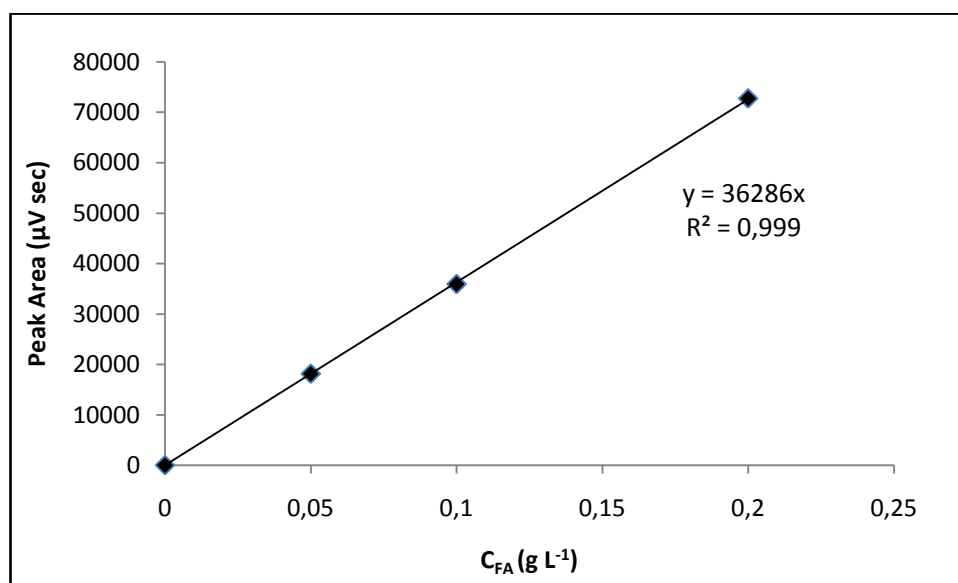


Figure D.3 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

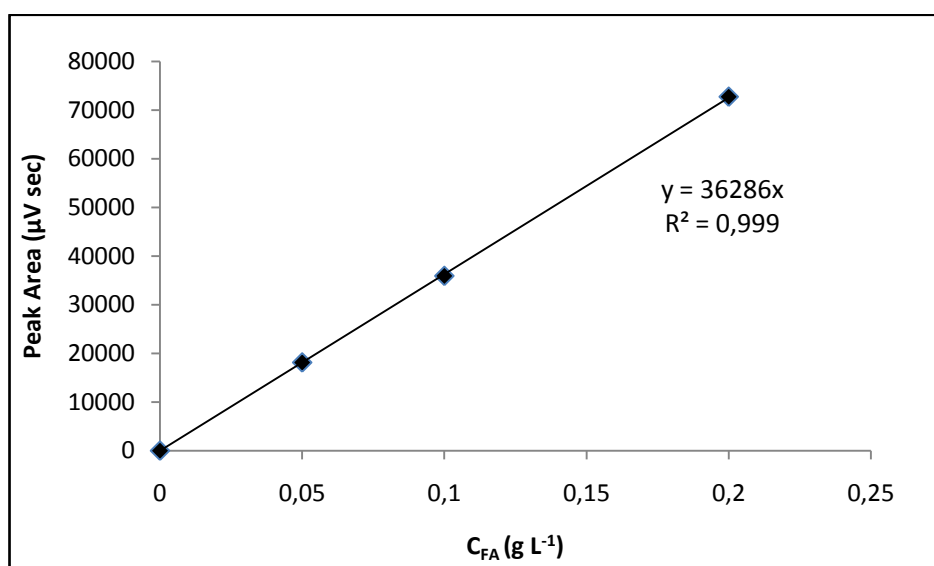


Figure D.4 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

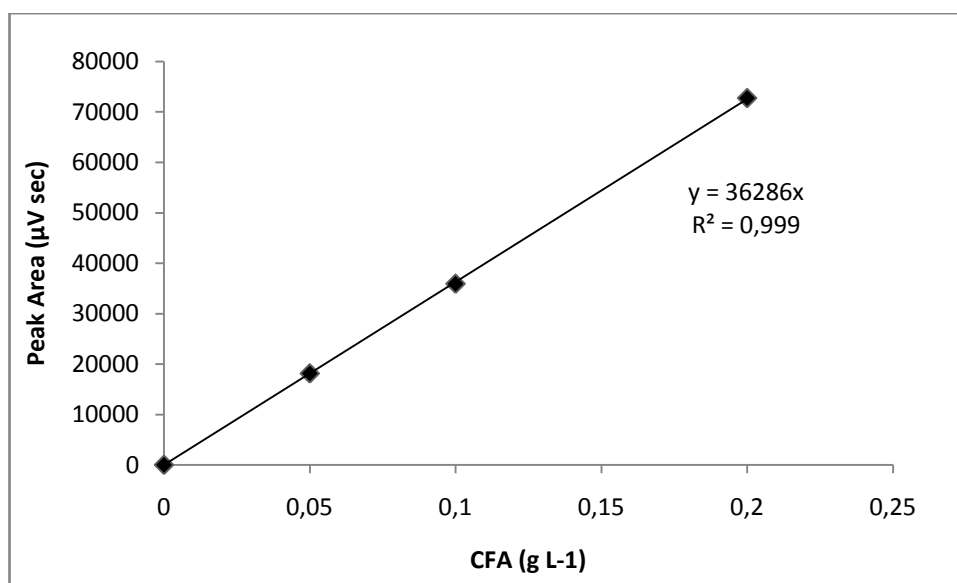


Figure D.5 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC

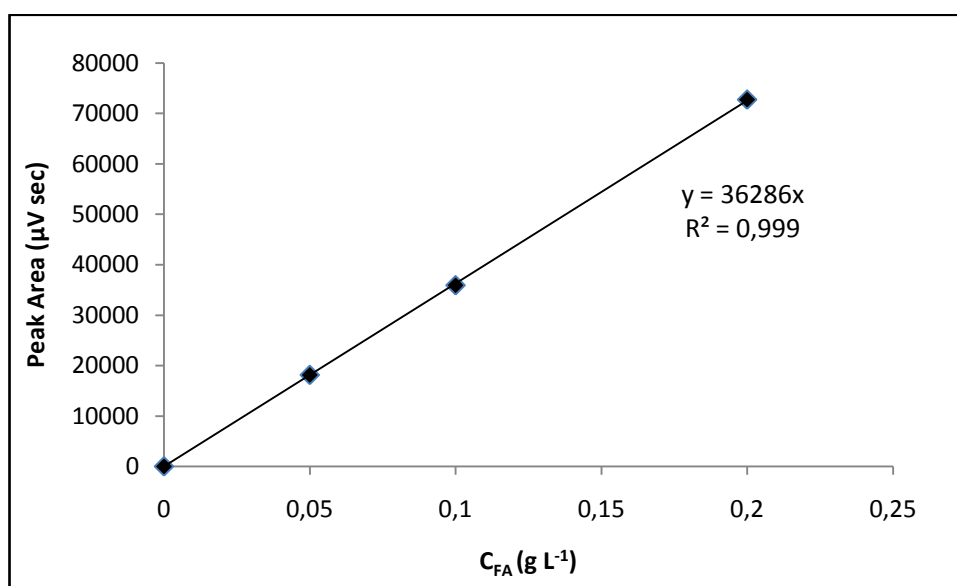


Figure D.6 Calibration curve obtained for acetic acid concentration; analysis was performed by HPLC.

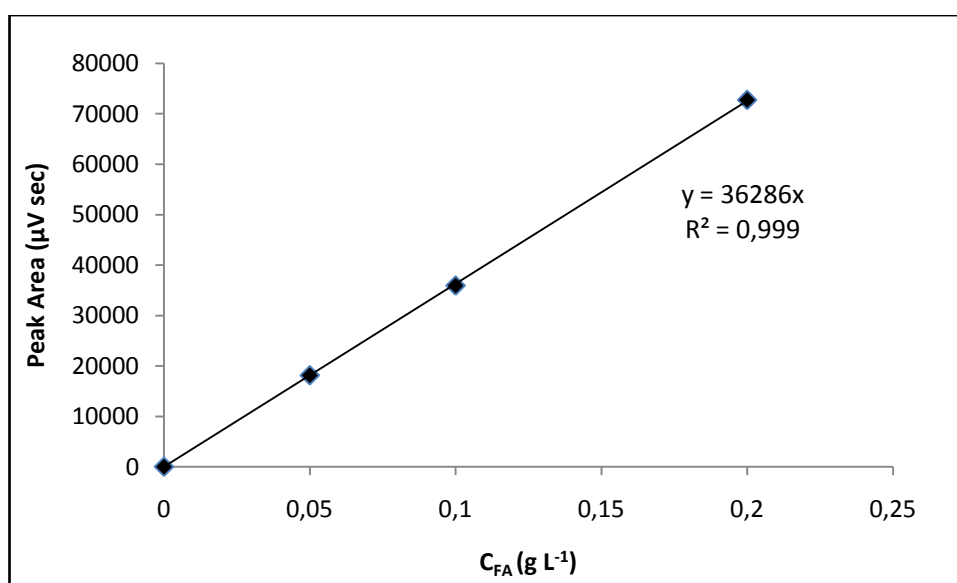


Figure D.7 Calibration curve obtained for oxalic acid concentration; analysis was performed by HPLC.

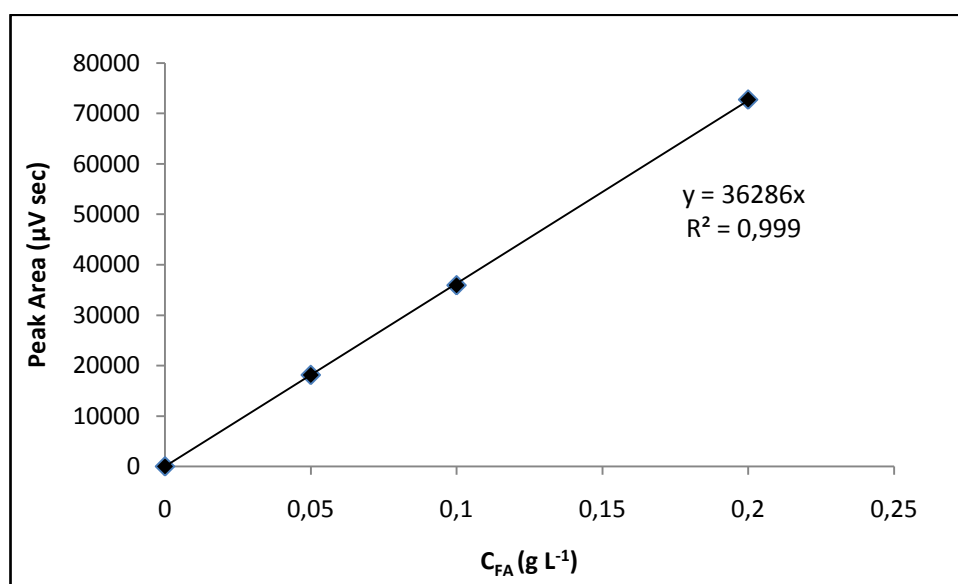


Figure D.8 Calibration curve obtained for pyruvic acid concentration; analysis was performed by HPLC

APPENDIX J

CALIBRATION CURVE FOR AOX ACTIVITY ASSAY

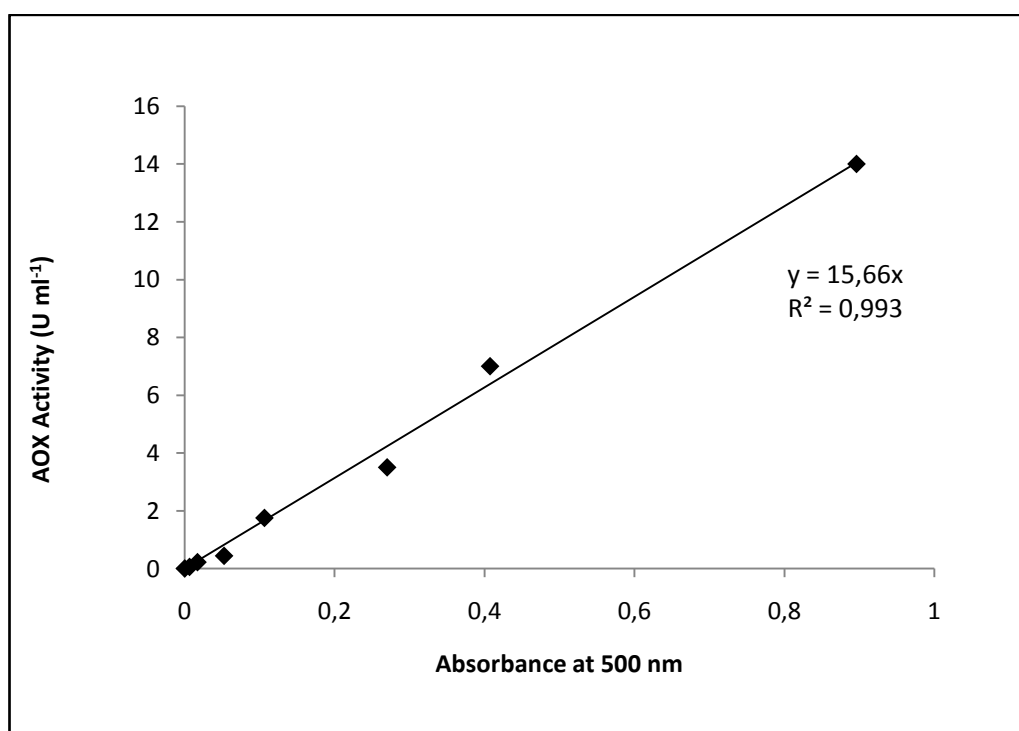


Figure J.1. Calibration curve for AOX activity assay.