

BIOPROCESS DEVELOPMENT FOR THERMOSTABLE GLUCOSE
ISOMERASE PRODUCTION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

VAHIDEH ANGARDI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
CHEMICAL ENGINEERING

NOVEMBER 2011

Approval of the thesis:

**BIOPROCESS DEVELOPMENT FOR THERMOSTABLE GLUCOSE
ISOMERASE PRODUCTION**

submitted by **VAHIDEH ANGARDI** in partial fulfillment of the requirements for
the degree of **Doctor of Philosophy in Chemical Engineering Department,**
Middle East Technical University by,

Prof. Dr. Canan Özgen

Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Deniz Üner

Head of Department, **Chemical Engineering**

Prof. Dr. Pınar Çalık

Supervisor, **Chemical Engineering Dept., METU**

Examining Committee Members:

Prof. Dr. Timur Doğu

Chemical Engineering Dept, METU

Prof. Dr. Pınar Çalık

Chemical Engineering Dept, METU

Prof. Dr. Ufuk Bölükbaşı

Chemical Engineering Dept, METU

Prof. Dr. Tunçer H. Özdamar

Chemical Engineering Dept., Ankara University

Prof. Dr. Hayrettin Yücel

Chemical Engineering Dept, METU

Date: 30/ 11 / 2011

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

Name, Last name: Vahideh Angardi

Signature:

ABSTRACT

BIOPROCESS DEVELOPMENT FOR THERMOSTABLE GLUCOSE ISOMERASE PRODUCTION

Angardi, Vahideh

Ph.D., Department of Chemical Engineering

Supervisor: Prof. Dr. Pınar Çalık

November 2011, 239 pages

In this study, process development for glucose isomerase (GI) was aimed. In this context, firstly, thermostable *xyl* genes, PCR amplified from *Thermus thermophilus* and *Pyrococcus furiosus* cells, were recombined to the *E.coli* BL21 (DE3) and *P.pastoris* strains, respectively. But significant increase in the term of GI activity compared with wild type cells only detected in recombinant *E.coli* strain so this strain was selected for further experiments. Then, the effect of different natural and artificial inducers on the production of rGI under control of LacUV5 promoter was investigated in laboratory-scale bioreactors. Lactose was shown to be more efficient in the term of operon induction for long time bioprocesses.

Thereafter, in order to increase thermostable rGI production rate, to achieve high cell density culture of *E.coli* BL21 (DE3) pLysS pRSETA::*xyl*_T as well as to evade acetate accumulation, the effect of exponential feeding strategy of carbon source on the production of thermostable GI enzyme, cell concentration and acetate formation by recombinant *E.coli* BL21 (DE3) pLysS was investigated at four sets of fed-batch bioreactor experiments at three different predetermined specific growth

rates 0.1 h^{-1} (M-0.1), 0.15 h^{-1} (M-0.15), 0.2 h^{-1} (M-0.2) and a glucose based exponential feeding at specific growth rate of 0.15 h^{-1} (G-0.15) were performed by recombinant *E.coli* BL21 (DE3) pLysS cells. The highest biomass was obtained in M-0.15 condition as 9.6 kg m^{-3} at $t=32 \text{ h}$ and the highest rGI activity was achieved in M-0.1 operation as $A=16399 \text{ U L}^{-1}$ at $t=32 \text{ h}$ of bioprocess.

Moreover, peptide ligand with specific affinity toward histidin-tag peptide was selected by phage display technology. Isothermal titration calorimetry and surface plasmon resonance analyses were carried out to determine peptide-peptide interaction properties.

Keywords: Recombinant glucose isomerase, *Escherichia coli*, Molasses, Fed-Batch Operation, Exponential Feeding, peptide, phage display, Peptide-peptide interaction

ÖZ

ISIYA DAYANIKLI GLUKOZ İZOMERAZ ÜRETİMİ İÇİN BİYOPROSES GELİŞTİRİLMESİ

Angardi, Vahideh

Doktora, Kimya Mühendisliği Bölümü

Tez yöneticisi: Prof.Dr.Pınar Çalık

Kasım 2011, 239 Sayfa

Bu çalışmada, glukoz izomeras (GI) üretimi için proses geliştirilmesi amaçlanmıştır. Bu doğrultuda, öncelikle, ısıya dayanıklı *xyl* genleri, PCR ile *Thermus thermophilus* ve *Pyrococcus furiosus* hücrelerinden çoğaltılarak alınmış ve sırasıyla *E.coli* BL21 (DE3) ve *P.pastoris* suşlarına entegre edilmiştir. Fakat, doğal suşlara oranla önemli rekombinant GI aktivitesi artışı sadece rekombinant *E.coli* suşunda belirlenmiştir. Bu sebeple bu suş sonraki çalışmalarda kullanılmak üzere seçilmiştir. Daha sonra, doğal ve yapay indükleyicilerin rGI üzerindeki etkisi LacUV5 promoter kontrolü altında laboratuvar ölçekli reaktörlerde incelenmiştir. Laktozun, operon indükleyici olarak uzun süreli üretimler için daha verimli olduğu bulunmuştur.

rGI üretim miktarının artırılması, *E.coli* BL21 (DE3) pLysS pRSETA::*xyl*_T için yüksek hücre konsantrasyonları elde edilmesi ve ayrıca asetat birikiminin önlenilmesi için karbon kaynağı üstel besleme stratejisine uygun olarak ortama verilmiştir. Üstel besleme stratejisiyle ısıya dayanıklı rGI üretiminde, hücre ve rekombinant *E.coli* BL21 (DE3) pLysS tarafından asetat oluşumu; dört set yarı kesikli biyoreaktör deneyiyle, deney öncesi belirlenmiş üç farklı özgün büyüme hızı ,

0.1 s⁻¹ (M-0.1), 0.15 s⁻¹ (M-0.15), 0.2 s⁻¹ (M-0.2) ve ek olarak 0.15 s⁻¹(G-0.15) özgün çoğalma hızına sahip glukoz temelli üstel besleme stratejisiyle incelenmiştir. En yüksek hücre konsantrasyonu M-0.15 koşulunda 32. saatte 9.6 kg m⁻³ olarak bulunurken, en yüksek rGI aktivitesi M-0.1 koşulunda 32. saatte A=16399 U L⁻¹ olarak elde edilmiştir.

Ayrıca, histidin-tag peptidine doğru afinite gösterebilecek petit lagand seçiminde faj display yöntemi kullanılmıştır. Peptit-peptit etkileşim özelliklerini belirlemek yüzey plazmon rezonans analizli gerçekleştirilmiştir.

Anahtar Kelimeler: Rekombinant glikoz izomeras, *Escherichia coli*, pekmez, yarı kesikli çalışma, üstel Besleme, peptid, faj display, peptit-peptit etkileşimi

To My beloved Family and my spouse

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor Prof.Dr.Pınar Çalık for her continuous support, guidance and help, in all the possible way, throughout this study. Without her encouragements and practical solutions to problems, I would not have achieved this project with such success.

I am also thankful to Prof.Dr.Tunçer H. Özdamar and Prof. Dr. Hayrettin Yücel for accepting to be a member of my progress jury and for all their advices and critics, which made this study have more deepness.

The feeling of a great honor has always been effective for being privileged enough to study at the department of Chemical Engineering, Middle East Technical University. It is always a compliment to be thankful to all of the academic, administrative and technical staff at the department.

I would like to express my special thanks Prof.Dr.İsmail Hakkı Boyacı for giving me the opportunity to use the Food Engineering Department's laboratories in Hacettepe University and to Assist.Prof.Can Özen and Fatma Gül (METU, Molecular Biology and Biotechnology Research Center) for their valuable advices and critics.

I am also thankful to my best friend Işık Haykır for her great friendship and continuous support for years even in sleepless days furthermore to Arzu Kanca and Gülistan Koçer for their help and friendship throughout this study.

I am grateful to countless number of friends, who were beyond being colleagues. Firstly, to our research group in Industrial Biotechnology and Metabolic Engineering Laboratory, for their support, great friendship, advice and encouragement throughout my studies as well as the research group of Prof. Dr. Ufuk Bölükbaşı. Additionally, I would like to thank to lab group of Prof.Dr. İsmail Hakkı

Boyacı specially, to Ceyda Dudak for their friendship and support, making me feel like I was in my own laboratory.

Special thanks also should be given to TÜBİTAK (The Scientific and Technological Research Council of Turkey) and Middle East Technical University (METU) for partial support of this project.

Above all, I would like to deeply thank to my family; Valiyeh Jahandar, Jafar Angardi and my spouse Reza Ettehad, for loving, supporting and encouraging me all through my life. Thanks for existing at all.

TABLE OF CONTENTS

ABSTRACT.....	iv
ÖZ.....	vi
ACKNOWLEDGMENTS.....	ix
TABLE OF CONTENTS.....	xi
LIST OF TABLES.....	xviii
LIST OF FIGURE.....	xxi
NOMENCLATURE.....	xxvii
CHAPTERS	
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	7
2.1 Bioprocess Development	7
2.1.1 Product: Glucose isomerase (GI).....	9
2.1.1.1 Optimal Temperature and pH.....	10
2.1.1.2 Substrate Specificity.....	10
2.1.1.3 Metal Ion Requirement and Inhibitors.....	10
2.1.1.4 Structure of GI.....	11
2.1.1.5 Active Site Studies and Reaction Mechanism.....	12
2.1.1.6 Genetic Engineering of Glucose Isomerase.....	13
2.1.2 Microorganism Selection.....	17
2.1.2.1 <i>Pichia pastoris</i>	17
2.1.2.1.1 General Characteristics.....	17
2.1.2.1.2 <i>P.pastoris</i> Expression System.....	18
2.1.2.1.3 Protein Secretion and Post-Translational Modification in <i>P.pastoris</i>	20
2.1.2.2 <i>Escherichia coli</i>	21
2.1.2.2.1 General Characteristics.....	21
2.1.2.2.2 The Lac Operon.....	22

2.1.2.2.3 T7 Expression System.....	24
2.1.2.2.4 <i>E.coli</i> Fermentations.....	24
2.1.3 Genetic Engineering of the Microorganism: Techniques and Methodology.....	26
2.1.4 Medium Design and Bioreactor Operation Parameters.....	35
2.1.4.1 Medium Composition.....	36
2.1.4.1.1 Molasses.....	40
2.1.4.2 Bioreactor Operation Parameters.....	43
2.1.4.2.1 Temperature.....	43
2.1.4.2.2 pH.....	44
2.1.4.2.3 Oxygen Transfer Rate.....	45
2.1.5 Recombinant Protein Purification.....	46
2.1.5.1 Affinity Based Bio-separation.....	47
2.1.5.1.1 Antibodies.....	48
2.1.5.1.2 Reactive dyes.....	48
2.1.5.1.3 Chelated Metal Ions.....	49
2.1.5.1.4 Aptamers.....	50
2.1.5.1.5 Affinity Tags and Fusion Proteins.....	51
2.1.5.1.6 Phage Display Technology and Combinatorial Peptide Libraries.....	53
2.1.5.1.7 Molecular Modeling.....	55
2.1.5.2 Kinetic Parameters of Interaction.....	55
2.1.5.2.1 Surface Plasmon Resonance (SPR).....	57
2.1.5.2.1.1 SPR Theory.....	58
2.1.5.2.1.2 Surface Modification.....	59
2.1.5.2.1.3 Ligand Immobilization.....	60
2.1.5.2.1.4 Interaction with the Analyte.....	62
2.1.5.2.1.5 Calculation of Affinity Constant Using Raw Data.....	62
2.2 Computation of Bioprocess Characteristics.....	64
2.2.1 Specific Rates and Yield Coefficients.....	64

2.2.2 Oxygen Transfer Characteristics.....	68
3. MATERIALS AND METHODS.....	72
3.1 Chemicals.....	72
3.2 Laboratory Equipment.....	72
3.3 Buffers and Stock Solutions.....	72
3.4 Strains, Plasmids and Maintenance of Microorganisms.....	72
3.5 Growth Media.....	74
3.5.1 The Solid Medium.....	74
3.5.2 The Precultivation Medium.....	75
3.5.3 The Production Medium.....	76
3.5.3.1 Production Medium for <i>P.pastoris</i> Species.....	76
3.5.3.2 Production Medium for <i>E.coli</i> Species.....	77
3.5.3.2.1 Fed-Batch Experiments.....	77
3.5.3.2.2 Pretreatment of Molasses.....	79
3.5.3.2.2 Fed-Batch Operations Abbreviation.....	80
3.6 Analysis.....	81
3.6.1 Cell Concentration.....	81
3.6.2 Glucose Isomerase Activity Measurement.....	81
3.6.2.1 Cysteine-Carbazol-Sulfuric acid method.....	82
3.6.3 Glucose Concentration.....	82
3.6.4 Organic Acid Concentrations.....	83
3.6.5 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate.....	84
3.7 Genetic Engineering Techniques.....	85
3.7.1 Enzymes, Kits, and Molecular Size Markers.....	85
3.7.2 Plasmid DNA Isolation from <i>E. coli</i>	85
3.7.3 Chromosomal DNA Isolation from <i>Thermus thermophilus</i> and <i>Pyrococcus furiosus</i>	85
3.7.4 Agarose Gel Electrophoresis.....	86
3.7.5 DNA Extraction from Agarose Gels	86
3.7.6 Primer Design.....	87

3.7.7 Polymerase Chain Reaction (PCR)	88
3.7.8 Purification of PCR Products.....	89
3.7.9 Restriction Endonucleases Digestion Reaction.....	89
3.7.10 DNA Purification after Digestion.....	90
3.7.11 Ligation Reaction.....	91
3.7.12 DNA Sequencing.....	91
3.7.13 Transformation of plasmid DNA by CaCl ₂ Method to <i>E. coli</i>	91
3.7.13.1 Chemically Competent Cell Preparation.....	93
3.7.13.2 Transformation to <i>E. coli</i> BL21 (DE3) pLysS.....	93
3.7.14 Transfection of <i>Pichia pastoris</i>	94
3.8 Ligand Selection via Phage Display Technique.....	95
3.8.1 Phage Titering.....	97
3.8.2 Phage Amplification for ELISA and Sequencing	99
3.8.2 .1 Phage ELISA Binding Assay with Direct Target Coating.....	99
3.8.2.2 Sequencing of Phage DNA.....	102
3.9 Interaction Measurement by SPR.....	103
3.9.1 Surface cleaning and sensor insertion.....	103
3.9.2 Preparation of active surface.....	103
4. RESULTS AND DISCUSSION.....	106
4.1 Development of Recombinant Microorganisms Producing Thermostable GI.....	106
4.1.1 Development of Recombinant <i>P.pastoris</i> Strains.....	106
4.1.1.1 Primer Design for Generation of <i>xyl_{pext}</i> and <i>xyl_{pint}</i> Gene from <i>P.furiosus</i>	107
4.1.1.2 Amplification of <i>xyl_p</i> Gene by PCR.....	110
4.1.1.2.1 Generation of <i>xyl_{pext}</i> Gene.....	110
4.1.1.2.2 Generation of <i>xyl_{pint}</i> Gene.....	113

4.1.1.3 Propagation and purification of pPICZ α A.....	114
4.1.1.4 Digestion Reaction.....	115
4.1.1.5 Ligation Reaction.....	116
4.1.1.6 Transformation of <i>E. coli</i> cells with Putative Recombinant vectors and Selection of the True Transformants.....	117
4.1.1.7 Digestion and PCR Control of Positive Colonies.....	119
4.1.1.8 Transfection of <i>P. pastoris</i> cells with Recombinant pPICZ α A plasmids.....	120
4.1.1.9 Expression of Glp in Recombinant <i>P. pastoris</i> in Laboratory Scale Air Filtered Shake Bioreactor.....	122
4.1.2 Development of Recombinant <i>E. coli</i> Strain.....	124
4.1.2.1 Primer Design for Generation of <i>xyl_T</i> Gene from <i>T.thermophilus</i>	125
4.1.2.2 Amplification of <i>xyl_T</i> Gene by Polymerase Chain Reaction (PCR).....	127
4.1.2.3 Propagation and purification of pRSETA.....	128
4.1.2.4 Digestion Reaction.....	128
4.1.2.5 Ligation Reaction.....	129
4.1.2.6 Transformation of <i>E. coli</i> cells with Putative Recombinant vectors and Selection of the True Transformants.....	132
4.1.2.7 Digestion and PCR Control of Positive Colonies.....	132
4.1.2.8 Transfection of <i>E.coli</i> BL21 (DE3) Strains with pRSETA:: <i>xyl_T</i>	133
4.2 Expression of Thermostable glucose isomerase in r- <i>E. coli</i> BL21 (DE3) pLysS Strain.....	134
4.2.1 Microorganism Selection.....	134
4.2.2 The Effect of Inducers on Production of GI by r- <i>E.coli</i> BL21 (DE3) pLysS Strain.....	135

VITA.....	235
-----------	-----

LIST OF TABLES

TABLES

Table 1.1 The production rate and world market price for a number of fermentation products	2
Table 2.1 Advantages and disadvantages of <i>Pichia pastoris</i> expression system.....	19
Table 2.2 Recognition sites and cleavage points of restriction enzymes.....	32
Table 2.3 Complex fermentation media frequently used in industry.....	37
Table 2.4 Overall chemical content of beet molasses from European samples.....	42
Table 2.5 Yield coefficients descriptions and their symbols.....	67
Table 3.1 Strains and plasmids used in this study.....	73
Table 3.2 The content of utilized solid medium (YPD) for <i>P. pastoris</i> strains.....	74
Table 3.3 The content of utilized solid medium (LSLB) for <i>E.coli</i> strains.....	74
Table 3.4 The final concentration of antibiotics in the media.....	75
Table 3.5 The content of employed precultivation medium for <i>P. pastoris</i> strain (BMGY)	75
Table 3.6 The composition of the precultivation medium for <i>E.coli</i> strains.....	76
Table 3.7 The composition of the complex production medium of <i>P. pastoris</i> strain (BMMY)	76
Table 3.8 The content of utilized production medium for <i>E.coli</i> species.....	78
Table 3.9 Explanations for the fed-batch operation codes.....	80

Table 3.10 Analyze method applied in determination of organic acid concentration	84
Table 3.11 Primers used in this study and their sequences.....	88
Table 3.12 Components of reaction mixture of restriction digestion with <i>NdeI</i> and <i>EcoRI</i> REs	90
Table 3.13 Components of reaction mixture of restriction digestion with <i>EcoRI</i> and <i>XbaI</i> REs.....	90
Table 3.14 Components of reaction mixture of restriction digestion with <i>AsuII</i> and <i>XbaI</i> REs.....	91
Table 3.15 Components of ligation mixture for construction of recombinant extracellular <i>P.pastoris</i> expression system.....	92
Table 3.16 Components of ligation mixture for construction of recombinant intracellular <i>P.pastoris</i> expression system.....	92
Table 3.17 Components of ligation mixture for construction of recombinant <i>E.coli</i> expression system.....	92
Table 4.1 Enzyme activities at t=12 h of the bioprocess.....	136
Table 4.2 Modifications of Shaker experiment including carbon source and antibiotic (+ Added once, ++ Added twice)	136
Table 4.3 Enzyme activities at t=24 h of the bioprocess.....	137
Table 4.4 Variations in the organic acid concentrations with the cultivation time for fed-batch bioreactor operations.....	147
Table 4.5 The variations in oxygen transfer parameters with cultivation time.....	152

Table 4.6 The variations in the specific rates and the yield coefficients with the cultivation time.....	153
Table 4.7 DNA and amino acid sequences of selected peptides via phage display.....	155
Table 4.8 Properties of peptide ligands selected via phage display.....	156
Table A.1 List of chemicals used in this study and their suppliers.....	188
Table B.1 List of laboratory equipments and their suppliers.....	193
Table I.1 Restriction map of <i>xyl_P</i> gene.....	215
Table I.2 Restriction map of <i>xyl_T</i> gene.....	217
Table J.1 Amino Acid Abbreviations and their codons.....	230
Table J.2 Codon frequency of <i>P. pastoris</i>	231
Table J.3 Codon frequency of <i>E.coli</i> strains.	232

LIST OF FIGURES

FIGURES

Figure 1.1 In vivo and in vitro isomerization reactions catalyzed by GI.....	4
Figure 2.1 Metal binding sites of GI.....	12
Figure 2.2 Steps of interconversion reaction.....	13
Figure 2.3 Complete reaction mechanism of GI.....	16
Figure 2.4 Structure of lac operon.....	23
Figure 2.5 Control of transcription of T7 RNA polymerase.....	24
Figure 2.6 Steps for cloning of the foreign gene in <i>E.coli</i>	27
Figure 2.7 The polymerase chain reaction.....	29
Figure 2.8 pPICZ α A cloning vector.....	30
Figure 2.9 pRSETA cloning vector.....	31
Figure 2.10 Mechanism of ligation reaction.....	33
Figure 2.11 Schematic of ampicillin resistance gene containing cell.....	35
Figure 2.12 Interaction mechanism of antibody with target protein.....	49
Figure 2.13 Illustration of SELEX protocol for aptamer selection.....	50
Figure 2.14 Schematic of fusion protein purification.....	51
Figure 2.15 Phage display technology.....	56

Figure 2.16 Structure of SPR optical sensor.....	59
Figure 2.17 Schematic of SAM.....	61
Figure 2.18 Sensograms of one site binding model at four different ligand concentrations.....	63
Figure 2.19 Variation of dissolved oxygen concentration with time during K_{La} measurement via Dynamic method.....	71
Figure 2.20 Evaluating K_{La} using the Dynamic Method.....	71
Figure 3.1 Schematic presentation of the pilot scale bioreactor system.....	79
Figure 3.2 Expected sensogram for SPR before ligand interaction.....	105
Figure 4.1 Flowchart of the research plan, for the development of the recombinant <i>Pichia pastoris</i> producing extracellular GI_p	108
Figure 4.2 Flowchart of the research plan, for the development of the recombinant <i>Pichia pastoris</i> producing intracellular GI_p	109
Figure 4.3 Agarose gel electrophoresis result. M: 50 bp DNA Marker, Lane 1-2: xyl_p gene amplified in PCR1, Lane 3: xyl_p gene amplified in PCR2, C: negative control.....	111
Figure 4.4 Schematic illustrations of polymerase chain reactions for amplification of extracellular xyl_{pext} gene.....	112
Figure 4.5 Agarose gel electrophoresis result. M: 50 bp DNA Marker, C: negative control, Lane 1: amplified xyl_{pint} gene via PCR using primers GI_{pint} -F and GI_p R.....	113
Figure 4.6 Schematic illustrations of polymerase chain reactions for amplification of extracellular xyl_{pint} gene.....	114

Figure 4.7 Agarose gel electrophoresis results. A) M: λ DNA/Eco91I Marker, Lane 1-2: single digested pPICZ α A. B) M: λ DNA/HindIII Marker, Lane 1-2: undigested pPICZ α A.....114

Figure 4.8 Agarose gel electrophoresis of products of single digestion reactions. A) M1: λ DNA/Eco91I Marker, Lane 1-2: pPICZ α A digested with *Eco*RI. B) M2: λ DNA/ Eco91I Marker, Lane 1-2: pPICZ α A digested with *Xba*I. C) M3: λ DNA/HindIII Marker, Lane 1-2: pPICZ α A digested with *Asu*II.....116

Figure 4.9 Agarose gel electrophoresis of products of double digestion reactions. A) M1: λ DNA/HindIII Marker, Lane 1: *xyl*_{pent} double digested with *Eco*RI and *Xba*I, Lane 2: pPICZ α A vector double digested with *Eco*RI and *Xba*I. B) M2: λ DNA/HindIII Marker, Lane 1-2: pPICZ α A vector double digested with *Asu*II and *Xba*I, Lane 3: *xyl*_{pent} double digested with *Asu*II and *Xba*I.....116

Figure 4.10 Scheme for the development of the expression systems for extracellular and intracellular GI_P production in *P. pastoris* strain. A) Construction of extracellular recombinant pPICZ α A::*xyl*_{pent} vector. B) Construction of intracellular recombinant pPICZ α A::*xyl*_{pent} vector.....118

Figure 4.11 Agarose gel electrophoresis of putative positive colonies. A) M: λ DNA/HindIII Marker; Lane 5 and Lane 12: pPICZ α A vector; Lanes 1, 2, 4, 7, 9, 10 and 11: plasmid isolated from extracellular positive colony containing only one copy of *xyl*_{pent} gene; Lanes 3, 6 and 8: plasmids isolated from extracellular positive colonies containing two copy of *xyl*_{pent} gene. B) M: λ DNA/HindIII Marker; Lanes 4 and 7: pPICZ α A vector; Lanes 1, 2, 3, 5 and 6: plasmids isolated from intracellular positive colony.....119

Figure 4.12 Agarose gel electrophoresis of positive colonies. A) M: λ DNA/HindIII Marker, Lane 1-4: recombinant extracellular pPICZ α A expression vector double digested with *Eco*RI and *Xba*I, Lane 4-8: recombinant extracellular pPICZ α A single digested with *Xba*I. B) M1: λ DNA/HindIII Marker, Lane 1-3: recombinant intracellular pPICZ α A expression vector double digested with *Asu*II and *Xba*I, Lane 4-6: recombinant intracellular pPICZ α A single digested with *Xba*I.....120

Figure 4.13 Agarose gel electrophoresis of PCR control. A) M: 50 bp DNA ladder, Lane 1-4: <i>xyl_{pext}</i> gene amplified from recombinant extracellular pPICZαA expression vectors by PCR with Tag polymerase. B) M: λDNA/HindIII Marker, Lane 1-4: <i>xyl_{pint}</i> gene amplified from recombinant intracellular pPICZαA expression vectors by PCR with Tag polymerase.....	121
Figure 4.14 Agarose gel electrophoresis images of <i>xyl_{pext}</i> and <i>xyl_{pint}</i> genes amplification from genomic DNA of <i>P. pastoris</i> transformants via PCR.....	122
Figure 4.15 GI activity measurements of recombinant <i>Pichia pastoris</i> strains.	124
Figure 4.16 Schematic illustration of primer design for expression in <i>E. coli</i> strains.....	125
Figure 4.17 Flowchart of the research plan, for the development of the r- <i>E. coli</i> producing GI.....	126
Figure 4.18 Agarose gel electrophoresis of chromosomal DNA isolated from <i>T. thermophilus</i>	127
Figure 4.19 Agarose gel electrophoresis image of <i>xyl_T</i>	127
Figure 4.20 Agarose gel electrophoresis of pRSETA extracted from <i>E. coli</i> . XLBlue.....	128
Figure 4.21 Agarose gel electrophoresis of products of digestion reactions. A. M: λDNA/HindIII Marker, Lane 1: pRSETA digested with <i>EcoRI</i> , Lane 2: pRSETA digested with <i>EcoRI</i> and <i>NdeI</i> . B. M: λDNA/HindIII Marker, Lane 1-2: pRSETA digested with <i>NdeI</i> . C. M: λDNA/HindIII Marker, Lane 1: insert DNA digested with <i>EcoRI</i> and <i>NdeI</i> , Lane 2-3: pRSETA digested with <i>EcoRI</i> and <i>NdeI</i>	130
Figure 4.22 Agarose gel electrophoresis image of ligation products.....	130

Figure 4.23 Scheme for the construction of an expression system for GI _T production in <i>E. coli</i> strain.....	131
Figure 4.24 Agarose gel electrophoresis of putative positive colonies. M: λDNA/HindIII Marker, Lane 1 and Lane5: pRSETA vector, Lane 2: plasmid isolated from false 1st positive colony, Lane 3 and Lane 4: plasmids isolated from 2nd and 3rd positive colonies, Lane 6 and Lane 7: plasmids isolated from 4th positive and 5th false positive colonies, respectively.....	132
Figure 4.25 Agarose gel electrophoresis of positive colonies. M1: Eco91 I Marker, Lane 1-3: recombinant pRSETA double digested with <i>Nde</i> I and <i>EcoR</i> I, Lane 4-6: recombinant pRSETA single digested with <i>Nde</i> I, Lane 7-9: <i>xyl_T</i> gene amplified from r-pRSETA by PCR with pfu polymerase, Lane 10-12: <i>xyl_T</i> gene amplified from r-pRSETA by PCR with Tag polymerase, M2: λDNA/Hind.....	133
Figure 4.26 The variation in GI activity for <i>E.coli</i> strains. $C_G = 8.0 \text{ kg m}^{-3}$, $C_N = 5.0 \text{ kg m}^{-3}$, $t = 12 \text{ h}$, $V = 33 \text{ ml}$, $T = 37^\circ\text{C}$, $N = 200 \text{ min}^{-1}$	134
Figure 4.27 Predetermined feeding profiles for M-0.1 (●), M-0.15 (○), M-0.2 (■), G-0.15 (◇) experiments.....	139
Figure 4.28 Variations in the cell concentration with the cultivation time for different feeding rates.....	141
Figures 4.29 Variations in the glucose concentration with the cultivation time for different feeding rates.....	143
Figure 4.30 Variations in the fructose concentration with the cultivation time for different feeding rates.....	143
Figure 4.31 Variations in the glucose isomerase activity with the cultivation time for different feeding rates.....	145
Figure 4.32 Variations in the acetic acid concentration with the cultivation time for different feeding rates.....	149

Figure 4.33 The absorbance of phage clones that bind to GI in ELISA assay.....	154
Figure 4.34 SPR sensogram showing steps involved prior to ligand binding.....	156
Figure 4.35 SPR sensogram for peptide 3 15 at different peptide concentrations....	157
Figure 3.36 The plot of k_{obs} vs peptide 3 15 concentration.....	158
Figure E.1. Calibration curve for fructose concentration.....	209
Figure F.1. Calibration curve for glucose concentration.....	210
Figure G.1 Standard calibration curve for acetic acid.....	211
Figure G.2 Standard calibration curve for citric acid.....	212
Figure G.3 Standard calibration curve for formic acid.....	212
Figure G.4 Standard calibration curve for fumaric acid.....	213
Figure G.5 Standard calibration curve for malic acid.....	213
Figure H.1 A) Lambda DNA/HindIII Marker; B) Gene Ruler 50 bp DNA Ladder.....	214
Figure K.1 Sensogram for peptide 3 14 binding to 6×His at concentration of $C_p=0.2$ mg mL ⁻¹	233
Figure K.1 Sensogram for peptide 3 30 binding to 6×His at concentration of $C_p=0.2$ Mg mL ⁻¹	234

NOMENCLATURE

A	Glucose isomerase activity, U L ⁻¹
C _{AA}	Acetic acid concentration, kg m ⁻³
C _{DO}	Dissolved oxygen concentration, mol m ⁻³
C _{DO} [*]	Oxygen saturation concentration, mol m ⁻³
C _G	Glucose concentration, kg m ⁻³
C _N	(NH ₄) ₂ HPO ₄ concentration, kg m ⁻³
C _X	Cell concentration, kg dry cell m ⁻³
Da	Damköhler number (= OD / OTR _{max} ; Maximum possible oxygen utilization rate per maximum mass transfer rate)
K _L a	Overall liquid phase mass transfer coefficient
N	Agitation or shaking rate, min ⁻¹
Q _o	Volumetric air feed rate, m ³ min ⁻¹
q _o	Specific oxygen uptake rate, kg kg ⁻¹ h ⁻¹
q _s	Specific substrate consumption rate, kg kg ⁻¹ h ⁻¹
t	Bioreactor cultivation time, h
T	Bioreaction medium temperature, °C
U	One unit of an enzyme
V _R	Volume of the bioreaction medium, m ³

$Y_{X/S}$	Yield of cell on substrate, kg kg^{-1}
$Y_{X/O}$	Yield of cell on oxygen, kg kg^{-1}
$Y_{S/O}$	Yield of substrate on oxygen, kg kg^{-1}

Greek Letters

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

Abbreviations

GI	Glucose isomerase
<i>xyl</i>	Gene of glucose isomerase
DO	Dissolved oxygen
EC	Enzyme Commission
OD	Oxygen demand ($=\mu_{\max} C_X / Y_{X/O}$; $\text{mol m}^{-3} \text{s}^{-1}$)
OTR	Oxygen transfer rate, $\text{mol m}^{-3} \text{s}^{-1}$
$\text{OTR}_{\max}$	Maximum possible mass transfer rate, $\text{mol m}^{-3} \text{s}^{-1}$
OUR	Oxygen uptake rate, $\text{mol m}^{-3} \text{s}^{-1}$
PCR	Polymerase chain reaction
HFCS	High-fructose corn syrup

TCA	Tricarboxylic acid
IPTG	Isopropyl β -D-1-thiogalactopyranoside

Subscripts

A	Association
D	Dissociation
fwd	Forward
0	Refers to initial condition
C	Refers to cell
O	Refers to oxygen
R	Refers to bioreaction medium
r	Reverse
X	Refers to cell

CHAPTER 1

INTRODUCTION

Industrial biotechnology, rapidly gaining priority on the schedules of industry, is the application of microbiology, enzyme engineering, metabolic engineering of microorganisms and separation technology in industrial processes for the production of new bio-based substances, materials, and energy (Singh and Ghosh, 2004). Industrial biotechnology proposes significant advantages for economy and environment compared with conventional chemical production methods because an elevated reaction rate, higher conversion efficiency, improved product purity and more cost-effective energy utilization as well as reduced chemical waste formation can be achieved. With the introduction of recombinant DNA technology into industrial fermentation processes, it is possible now to intervene in genetic material of microorganisms, to insert genes from higher organisms into industrial cells and to create new metabolic pathways for the over-production of conservative or new chemical matters with high purity and efficiency. At the present time, bio-based products market represents 5% of the total chemical product sale volume and it has estimated that by 2012, the total fraction will account for 7.7%, corresponding to €135 billion of sales (Soetaert and Vandamme, 2010). Table 1 illustrates the production rate and market price for some of these fermentation products.

Enzymes, biological catalysts, are high molecular weight proteins. They accelerate chemical reactions rates extremely by decreasing the activation energy of enzyme-substrate complex, while they have no impact on the situation of the thermodynamic equilibrium of the reactions (Aehle, 2007). Enzymes have a high degree of substrate specificity and this unique property of enzymes is based on their

three-dimensional structures and on active sites whose chemical groups may be brought into close proximity from different regions of the polypeptide chains. The rapid and specific impact of enzyme on substrate reduces the possibility of side reactions. Enzyme mediated reactions generally go on in aqueous solution under mild temperature and pH ranges (Watson et al., 1987). The main advantage of enzymes compared with conventional catalysts is their biodegradability which makes them environmentally more acceptable. Hence, currently the usage of enzymes as biocatalysts gains importance in industrial biotechnology and their new applications are continuously being investigated (Faber, 2002).

**Table1.1 The production rate and world market price for a number of fermentation products
(Adapted from Soetaert and Vandamme, 2010).**

Product	World production (ton/year)	World market price (€/kg)
Bioethanol	50000000	0.40
High--fructose corn syrup	12000000	16.34
L-Glutamic acid	1500000	1.50
Citric acid	1500000	0.8
L-Lysine	800000	1.5
Lactic acid	250000	1.5
Glucose isomerase	100000	230
Vitamin C	80000	8
Gluconic acid	50000	1.5
Penicillin amidase	40000	-
Antibiotics (bulk products)	30000	150
Xanthan	20000	8
L-Hydroxyphenylalanine	10000	10
Dextran	200	80
Vitamin B ₁₂	3	25000

A thermostable enzyme is one that will not readily subject to destruction or alteration by heat. Mainly, the source of thermostable enzymes and proteins are extremophiles, the microorganisms which can survive in extreme conditions. Implementation of enzymatic reactions at higher temperatures proffers several advantages such as higher reaction rate, higher equilibrium concentration of products and decrease of reaction time (Lamba et al., 2001). Application of thermophiles in industry reduces production expenditures by offering minimal risk of contamination, the possibility of easier volatile products recovery and by altering the rheological properties of cultivation medium such as decreasing viscosity and surface tension. Moreover, when a thermostable enzyme gene from thermophile is cloned into mesophilic microorganism, the recombinant mesophile can produce a large amount of thermostable enzyme extracellularly or intracellularly. Since most of host proteins are temperature-sensitive, the thermostable enzyme would be easily purified by single step of heat treatment (Stern et al., 2001). Enzymes present in thermophiles are also more stable than their mesophilic counterparts, so that they have found considerable potential for many industrial applications.

Xylose/glucose isomerase/D-xylose ketol isomerase (E.C.5.3.1.5) has the physiological function of converting D-xylose to D-xylulose, the first step in the xylose catabolism (Dekker et al., 1991). Since the reversible isomerization of D-glucose to D-fructose is also catalyzed by this enzyme, it is commonly designated as glucose isomerase (GI). Figure 1.1 represents *invivo* and *invitro* reactions catalyzed by GI.

In the nature, interconversion of xylose to xylulose fulfills nutritional requirement of saprophytic bacteria which lives on rotting plant matter. The largest market of glucose isomerase is in food industry as a key enzyme in the favor of high-fructose corn syrup (HFCS) production. HFCS, an equilibrium mixture of glucose and fructose, reveals 1.3 times higher sweetness and regards as cost-effective sweetener compared with sucrose on the basis of its sweetening power. Since, D-fructose is gradually absorbed by the stomach, it does not effect on the blood glucose concentration and it can be employed as an alternative sweetener for diabetic use.

Furthermore, GI is used in combination with other enzymes throughout the fermentations for bioconversion of xylose to ethanol (Bhosale et al., 1996).

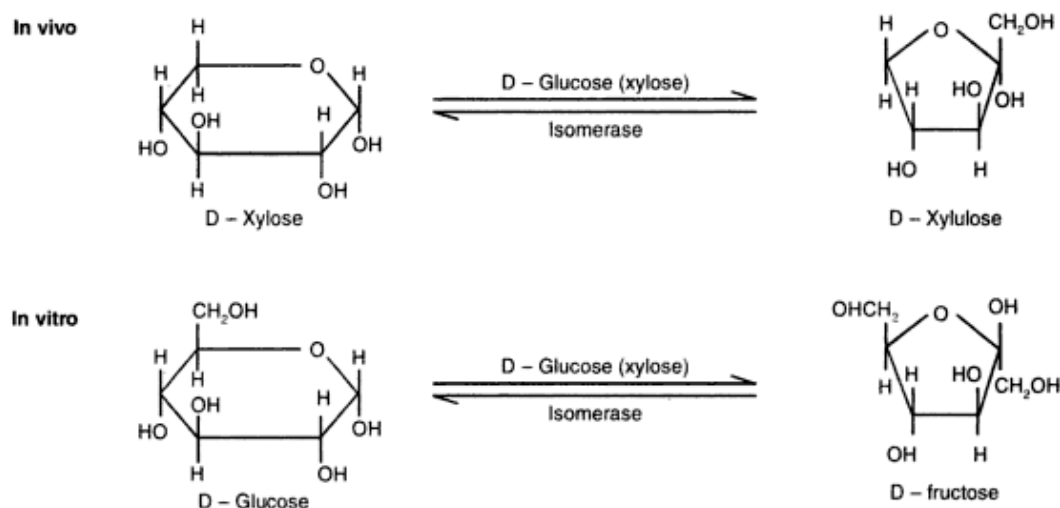


Figure 1.1 In vivo and in vitro isomerization reactions catalyzed by GI.

Since Marshall and Kooi (1957) discovered the reaction of isomerization of D-glucose into D-fructose by cell-free extract of *Pseudomonas hydrophila*, glucose isomerase activity has been identified in over 100 microbial species (Barker and Shirley, 1980; Ulezlo et al., 1986). Currently, most of the offered glucose isomerases in the market have been obtained from mesophilic strains; including *Streptomyces*, *Actinoplanes*, *Flavobacterium*, and *Bacillus* species; thus, the reaction temperature applied in the sweetener production reactions is limited to 60°C by the recent industrial processes.

Carrying out isomerization reaction in the temperature higher than 60°C provides elevated reaction rate and product yield with the reduced viscosity (Lama et al., 2001). Therefore, by considering the technical innovations in the area of molecular biotechnology and lower fructose content resulted from lower reaction temperature, it will be more feasible to clone thermostable *xyl* encoding gene and express it at levels much higher than that naturally produced by wild type cells.

Pichia pastoris, the methylotrophic yeast, offers definite superiority over *Saccharomyces cerevisiae* as a host for heterologous genes over-expression. Extremely high cell density and high level of extracellular or intracellular foreign protein can be achieved by this strain. Multiple copies of the gene of interest can be integrated into the chromosome. It has ability to glycosylate proteins without unacceptable glycosylation or hyperglycosylation which happens in *S. cerevisiae*. Stable integration of expression cassette into specific locations of the *P. pastoris* genome is possible. There is no study in literature related with the engineering of *P. pastoris* for the production of GI.

On the other hand, application of *E.coli*, the trendy prokaryotic gram negative strain, as a recombinant host proposes several benefits as follow: Its genome can be modified easily; control of promoter is straightforward; protease deficient mutant strains are available; modification of plasmid copy number is possible; and heterologous protein accumulation can be enhanced to value as much as 50% of dry cell weight (Soetaert and Vandamme, 2010). It is possible to come across with several studies in literature focusing on the over-expression of *xyl* genes, isolated from natural GI producer cells, via different types of *E.coli* strains (Dekker et al., 1992; Lee et al., 1993; Kaneko et al., 2001; Sariyar et al., 2004; Rhimi et al., 2007).

In this PhD study, it was primarily intended to develop a proficient bioprocess for the over-production of thermostable recombinant GI and to identify a peptide-ligand with particular affinity toward 6× histidine tag that is included in the map of commonly used cloning vectors.

As first attempt for the development of GI producing recombinant microorganism, the extracellular and intracellular recombinant *Pichia pastoris* expression systems were constructed. For this purpose, the hypothetical thermostable *xyl_P* gene, isolated from *Pyrococcus furiosus* cells was amplified in the extension of two PCR reactions: one for generation of extracellular gene, *xyl_{Pext}*, integrated with poly histidine and a Factor Xa recognition site, and the other one for creation of intracellular gene, *xyl_{Pint}*, in which restriction sites were selected and combined to the target sequence in the manner that remove α -Factor signal sequence from pPICZ α A expression vector map during the restriction digestion reaction. The PCR amplified

$xyl_{P_{ext}}$ and $xyl_{P_{int}}$ genes were separately recombined to *P.pastoris*. To be an alternative to *P.pastoris*, *E.coli* expression system was also developed in which the PCR amplified thermostable xyl_T gene from *Thermus thermophilus* with documented activity was recombined to the *E.coli* BL21 (DE3) pLysS strain. In this case, over-expression of target gene at the control of LacUV5 promoter was detected. Furthermore the effect of natural and artificial inducers on the induction of LacUV5 promoter, a mutated version of the bacterial lac promoter, was investigated.

Thereafter, in order to increase cell growth and rGI production as well as to reduce by product formation, mainly acetate, carbon source exponential feeding with predetermined specific growth rate values was performed in pilot scale bioreactors. Finally, phage display methodology was applied to select a ligand with high affinity toward the 6×histidine peptide and intensity of interaction was verified via ELISA and surface plasmon resonance assays.

CHAPTER 2

LITERATURE SURVEY

2.1 Bioprocess Development

With the identification of microorganism or cell type ability in converting substances to valuable products, there are several attempts for development of economical industrial processes. In the light of great diversity in substrates and microorganism types, it is possible to generate different kinds of commercial products from moderately low-priced substance to costly unique chemicals. Although, nowadays with the availability of experienced companies in the area of bioprocess design and scale up, fast implementation of a new processes is possible still there are many hurdles on the road of efficient industrial process development (Doran, 1995; Soetate and Vandamme, 2010). Development of a fermentation process can be roughly divided into four steps. First stage starts with product identification and in the case of pharmaceutical one different therapeutic influence by microbial metabolites should be screened. Also, at this time, new target proteins can be directly identified in the sequenced genomes by using advanced bioinformatic programs. The following step after product identification is proper strain selection or construction via recombinant DNA technology. The main criteria for selection of expression system are the desirability of post-translation modification and secretion, the stability of the protein in question and the projected dose of therapeutic protein per patient. Choice of appropriate fermentation medium and the optimum process conditions are the other important steps in bioprocess development. The cultivation medium should contain a carbon, nitrogen, energy sources in addition to all essential minerals required for growth. It should be readily accessible throughout the year and

causes a minimal of problems in the downstream processing. Complex media, by-product of agricultural sector are frequently used in industry because these media are present in large quantities with relatively cheap price and they contain organic nitrogen source, essential minerals as well as different growth factors (Nielsen and Villadsen, 2003). After laboratory studies which provide a bioprocess compatible for large scale production, bioprocess scale-up starts. In reactor design and scale up the mixing rate, shearing, foaming, mass transfer characteristics should be considered together with pH and heat transfer aspects. Generally, aerobic fermentations' scale-up is accomplished based on the constant oxygen volumetric mass transfer coefficient, k_{La} , or oxygen transfer rate at a pre-determined value of dissolved oxygen concentration (Ju and Chase, 1992). With the information obtained at this stage, the appropriate type of reactor in addition to the best operating mode for production can be decided.

The last step in the bioprocess development is downstream processing that refers to the recovery and purification of bio-products. The recovery of products from fermentation broths is both difficult and expensive; consequently, downstream processing should be considered as the crucial issue throughout the commercial bioprocess development. For example, 60 to 70 % of the selling price of enzymes corresponds to the downstream processing costs. Product cost increases proportional with the application of complicated product recovery methods, because as the number of involved steps in downstream processing decreases the lower operating costs as well as the higher overall product yield can be achieved (Janson and Rayden, 1998). So development of new strategies and techniques has been an indispensable task to produce proteins and enzymes in a highly purified and well-characterized manner (Terpe, 2003). With the advanced achieved in genetic engineering area, it is possible to modify the structure of proteins and improve their purification properties. The amino acid sequences on the either N-terminal or C-terminal end of translated protein can be altered via extra nucleotides insertion into the 3' or 5' end of target DNA sequence to provide a purification fusion. These fusions improve bio-product recovery by stabilizing the proteins and enzymes from proteases attack with helping the protein to form an inclusion body or by introducing

specific purification properties on the protein, making it suitable for immunoaffinity, metal chelate, ion exchange, hydrophobic chromatography, or partition. Alternatively, for well-characterized proteins, specific amino acids may be changed within the protein to introduce patches with a specific affinity for adsorption matrix (Roe, 2001). Diverse domains and peptides can be integrated into target proteins and the criteria which should be considered in the fusion tag selection are as follow:

1. The possibility of one-step adsorption purification;
2. The negligible effect of fusion tag on the structure, configuration and biological function of recombinant target molecule;
3. The accessibility to the native protein after tag removal;
4. The precise assay of a target all through the purification steps;
5. The applicability to the broad-spectrum of diverse proteins and enzymes.

In this study all the key steps in bioprocess development for the recombinant enzyme production were considered.

2.1.1 Product: Glucose isomerase (GI)

Although different enzymes are entailed with different properties on the basis of their particular application, there are some common criteria supposed to be considered during the commercial enzymes production including: reaction rate, optimum temperature and pH for complete activity, half-life, stability in the cultivation media, inhibition mechanisms, and specificity to the substrate (Soetaert and Vandamme, 2010).

Glucose isomerase (E.C.5.3.1.5) with the other name D-xylose isomerase is a biocatalyst which carries out the reversible interconversion reaction of D-glucose to D-fructose along with D-xylose to D-xylulose (Dekker et al., 1991). Since 1957, with the discovery of the glucose-isomerizing capability of the enzyme from *Pseudomonas hydrophila* by Marshall and Kooi (1957), there was a high attempt for identification of novel GI producing strains. The information obtained about the precise properties of GI, like as its three-dimensional structure, stability, active site,

substrate specificity, cofactors and inhibitors occupies a central role in improvement of its characteristics and stability.

2.1.1.1 Optimal Temperature and pH

The most favorable temperature for activity of GI alters from 60 to 90°C depending on the source microorganism producing this enzyme and the presence of Co^{2+} and Mn^{2+} ions increases the optimum temperature value. Generally the enzyme isolated from thermophilic species is more stable at high temperature compared with mesophilic ones (Bhosale et al., 1996). Generally, pH value between 7.0 and 9.0 regarded as optimal pH range for GI activity.

2.1.1.2 Substrate Specificity

D-glucose and D-xylose are the most common substrates which are preferred by the majority of GI producing microorganisms and D-ribose, L-arabinose, L-rhamnose, D-allose as well as 2-deoxyglucose are the other ones. With utilizing substrates containing hydroxyl groups in equatorial state at carbons 3 and 4, maximum isomerization activity of the GI can be achieved, as happens in the case of glucose or xylose application (Chen et al., 1980).

2.1.1.3 Metal Ion Requirement and Inhibitors

Enzymatic cofactors, non-protein component such as vitamins and metal ions, are essential for stable and active conformation of the enzymes. Like other enzyme GI requires several metal ions in order to function properly. Divalent cations with ionic radii less than 0.8 Å such as Mg^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} are known cofactors of GI (Kovalevsky et al., 2010). Different cofactors participate differently in the structure of GI while Mg^{2+} ion acts as strong activator, Co^{2+} and Mn^{2+} ions are effective in GI stabilization by supporting the structured conformation of the enzyme (Gaikwad et al., 1992). Metal ions with larger diameter such as Ag^+ , Hg^{2+} , Cu^{2+} , Zn^{2+} , and Ni^{2+}

inhibit the catalytic activity of GI and xylitol, arabitol, sorbitol, mannitol, lyxose, and tris are the other known inhibitors of GI.

2.1.1.4 Structure of GI

Depending on the strain in which GI extracted or secreted from, molecular weight of the enzyme varies from 44,000 to 191,000 Da. The GI from thermophile *Thermus thermophilus* composed of 387 amino acids with molecular weight of 44,000 Da. Although the extracellular GI from *Bacillus* species has a trimeric structure, GI is generally a tetramer or a dimer of similar or identical subunits connected with noncovalent bonds (Chauthaiwale et al., 1994). Characterization of subunits arrangement and tertiary fold of each subunit of the thermostable GI from *Thermus thermophilus* via X-ray crystallography demonstrated that tetrameric arrangement of this enzyme is basically similar with other GIs. Each monomer is composed of two domains. Domain I (residues 1 to 321) folds into the $(\beta/\alpha)_8$ -barrel. Domain II (residues 322 to 387), lacking β -strands, makes extensive contacts with domain I of an adjacent subunit. There are ten β -strands, 16 α -helices, and five 3_{10} -helices in the composition of the each monomer (Chang et al., 1994). Ghatge et al. (1994) studied the dissociation and unfolding of the tetrameric GI from *Streptomyces* Sp. and revealed that the tetrameric and dimeric forms of enzyme are active whereas the monomeric one is inactive. In the study of Kovalevsky et al. (2010), X-ray crystallography together with neutron diffraction techniques were applied in order to investigate the metal binding sites of GI from *Streptomyces rubiginosus*. This study revealed the necessity of two divalent metal cations for full activity of the enzyme where their locations on the enzyme structure were designated as M1 and M2 respective to the structural and the catalytic metal sites (Figure 2.1).

As the M1 structural metal site prefers Mn^{+2} , Co^{+2} , Cd^{+2} and Pb^{+2} ions, the M2 site shows affinity toward a moderately large variety of divalent metal ions.

2.1.1.5 Active Site Studies and Reaction Mechanism

The identification of amino acids located at or vicinity of the GI active site is important issue for improving GI stability and activity.

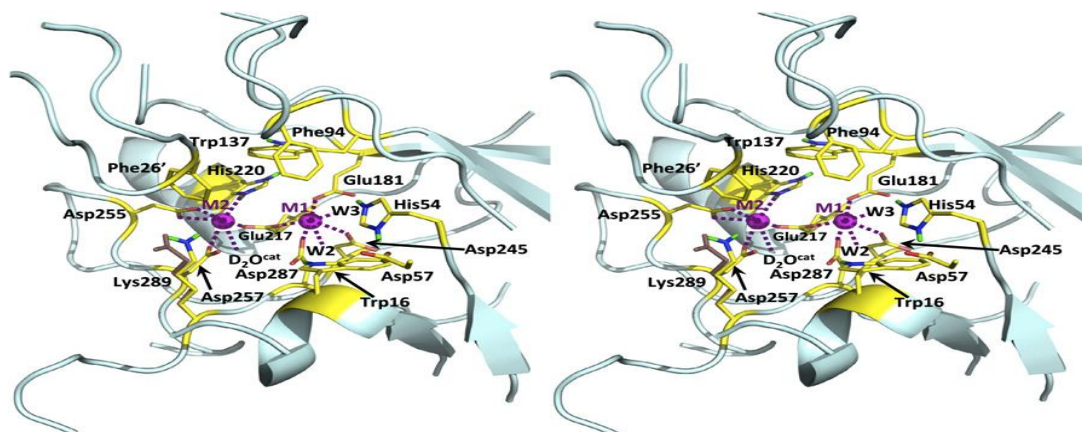


Figure 2.1 Metal binding sites of GI (Kovalevsky et al., 2010).

Batt et al. (1990) studied the effects of site-directed mutagenesis in the active site of GI from *Escherichia coli* and identified two conserved histidine residues (His-101 and His-271) appeared to be vital elements in the enzyme active site. Meilleur et al. (2006) investigated the location of hydrogen atom in GI by neutron crystallography with the aim of attaining information about the mechanism of enzymatic reaction. The neutron structure clearly illustrated that the reaction was initiated via double protonation of His-53 residue located at the active site of the enzyme and continued through an acid catalyzed opening of the sugar ring. According to the study of Kovalevsky et al. (2010), the reversible interconversion reaction of GI proceeds in three steps: ring opening, isomerization and ring closure as it can be seen from Figure 2.2.

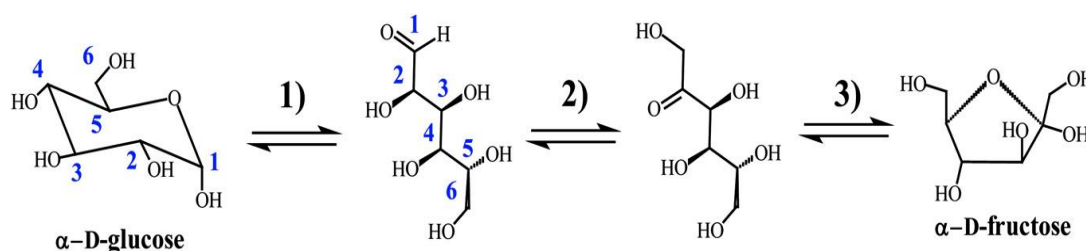


Figure 2.2 Steps of interconversion reaction 1) ring opening, 2) isomerization, 3) ring closure (Kovalevsky et al., 2010).

During this reaction some conformational changes occur in the active site of the enzyme and several amino-acids play critical roles in initiation and implementation of the reaction. In the first stage, the enzyme active region binds to its reactive cyclic sugar substrate and the presence of different arrangements of ordered water molecules facilitates the correct association of the substrate to GI. The next step in the reaction is ring opening in which His-54 transiently gives an extra proton to O5 and takes it after C1-O5 bond is broken. Throughout this step, Lys-289 which was neutral and located away from Asp-257 before ring opening, is protonated and donates H in a hydrogen bond to Asp-257. Then, Lys-183 helps O1 to be placed in the correct position in order to start isomerization step. Finally, in isomerization step catalytic water molecules are in the action. During this step H^+ ion is transferred from C2 and O2 to C1 and O1, respectively. Figure 2.3 illustrates complete reaction mechanism suggested in this study.

2.1.1.6 Genetic Engineering of Glucose Isomerase

The discovery of the glucose-isomerizing capacity of the enzyme from *Pseudomonas hydrophila* by Marshall and Kooi in 1957 was the starting point for many investigations about the GI. After that, GI activity was detected in over 100 microorganisms (Barker and Shirley, 1980; Ulezlo et al., 1986) and the advances occurred in the area of recombinant technology at the same years made it possible to isolate and manipulate *xyl* gene from different strains. Ho et al. (1985) published the first report on the isolation of GI gene from *E.coli*. Then, at the same year Stevis and

Ho (1985) cloned the *E.coli xyl* gene into several *E.coli* plasmids and observed that high expression level of gene was not possible only by insertion of it to high-copy-number plasmid probably, due to strong regulatory affects of natural promoter. The integration of structural gene into *lac* or *tac* promoters resulted in over-production of GI (Stavis and Ho, 1985). Homologous cloning of *xyl* gene under the control of the *tac* promoter induced with isopropylthio β -D-galactopyranoside in *E.coli*, resulted in the production of GI which constituted approximately 28% of the total cell protein (Batt et al., 1986).

Heterologous expression of *xyl* gene from *Bacillus licheniformis* in GI-negative mutant of *E. coli* was achieved by Shin et al. (1985) which result in 20-fold higher GI activity compared with the GI activity of source strain. Chan et al. (1986; 1989) produced recombinant *Schizosaccharomyces pombe* able to utilize D-xylose as a sole carbon source via transforming hybrid plasmid pDB248-XI containing *xyl* gene from *E.coli*. The transformed yeast cells were able to metabolize 10% (w/v) xylose for production of 3.0% (w/v) ethanol. Investigations of D-xylose metabolism by the recombinant *S.pombe* cells revealed that the low GI activity due to proteolytic enzyme degradation was the main limiting step in xylose fermentation by the yeast.

In the study of Lee et al. (1990), the structural gene that encodes thermostable GI in *Clostridium thermosulfurogenes* was cloned in *E.coli* and *Bacillus subtilis* strains and higher amount of thermostable GI was produced by *B. subtilis* (1.54 U mg^{-1}) than that was achieved by recombinant *E.coli* cells (0.46 U mg^{-1}). Then for the first time the nucleotide sequence of *xyl* gene from the hyper-thermophilic *Thermus thermophilus* was determined by Dekker et al. (1991) and as a result of the expression of this gene under the control of the *tac* promoter in *E.coli*, 45-fold higher GI yield obtained. The over production of the thermostable *Thermus thermophilus* HB8 xylose isomerase gene in *E. coli* and *B. brevis* was also reported by this group (Dekker at al., 1992); where, higher soluble GI activity with value of 5600 U L^{-1} was achieved by *B. brevis* than via *E. coli* expression system with the value of 4300 U L^{-1} . Thereafter, Lee et al. (1993) inserted *xyl* gene of *Thermoanaerobacterium saccharolyticum* strain into pUC18 vector. The constructed vector containing the gene of interest was expressed constitutively in *E. coli*. The produced GI was a tetramer

comprised of identical subunits with molecular mass of 200 kDa and the optimum temperature and pH for the enzyme activity were 80°C and 7.0-7.5, respectively.

Another report related with the production of thermostable GI in *E. coli* cells was published by Wuxiang and Jeyaseelan, (1993). A *xylA* gene from thermophilic *Bacillus* sp. was cloned in *E.coli* and as the result of expression 12.8 U mg⁻¹ glucose isomerase activity at 85°C was detected. Moes et al. (1996) constructed eukaryotic recombinant strain of *Saccharomyces cerevisiae* containing the bacterial *xylA* gene of *Clostridium thermosulfirogenes*. Although efficient transcription of the *xylA* gene under the control of the ADH2 promoter was detected via Northern blot analysis, the recombinant strain was unable to produce functional GI enzyme probably due to cytosolic environment of *S. cerevisiae* which prohibits the formation of stable GI tetramer structure and provides the required condition for enzyme degradation even in protease deficient *S. cerevisiae* strains.

In the further studies, production of GI enzyme had been achieved in various *E.coli* cells including: cloning and expression of acid-stable *xyl* gene from *Streptomyces olivaceoviridis* in *E.coli* JM19 (Kaneko et al., 2001); expression and translocation of GI of *T.thermophilus* as a fusion protein in *E. coli* TB1 and ER2508 cells (Sarıyar et al., 2004); expression of thermostable mutant *xyl* gene from *Streptomyces* SK in *E.coli* HB101 strain (Rhimi et al., 2007); as well as insertion of *xylA* gene of *E.coli* K12 into pRAC vector and its expression in *E.coli* BL21 cells (Rozanov et al., 2007).

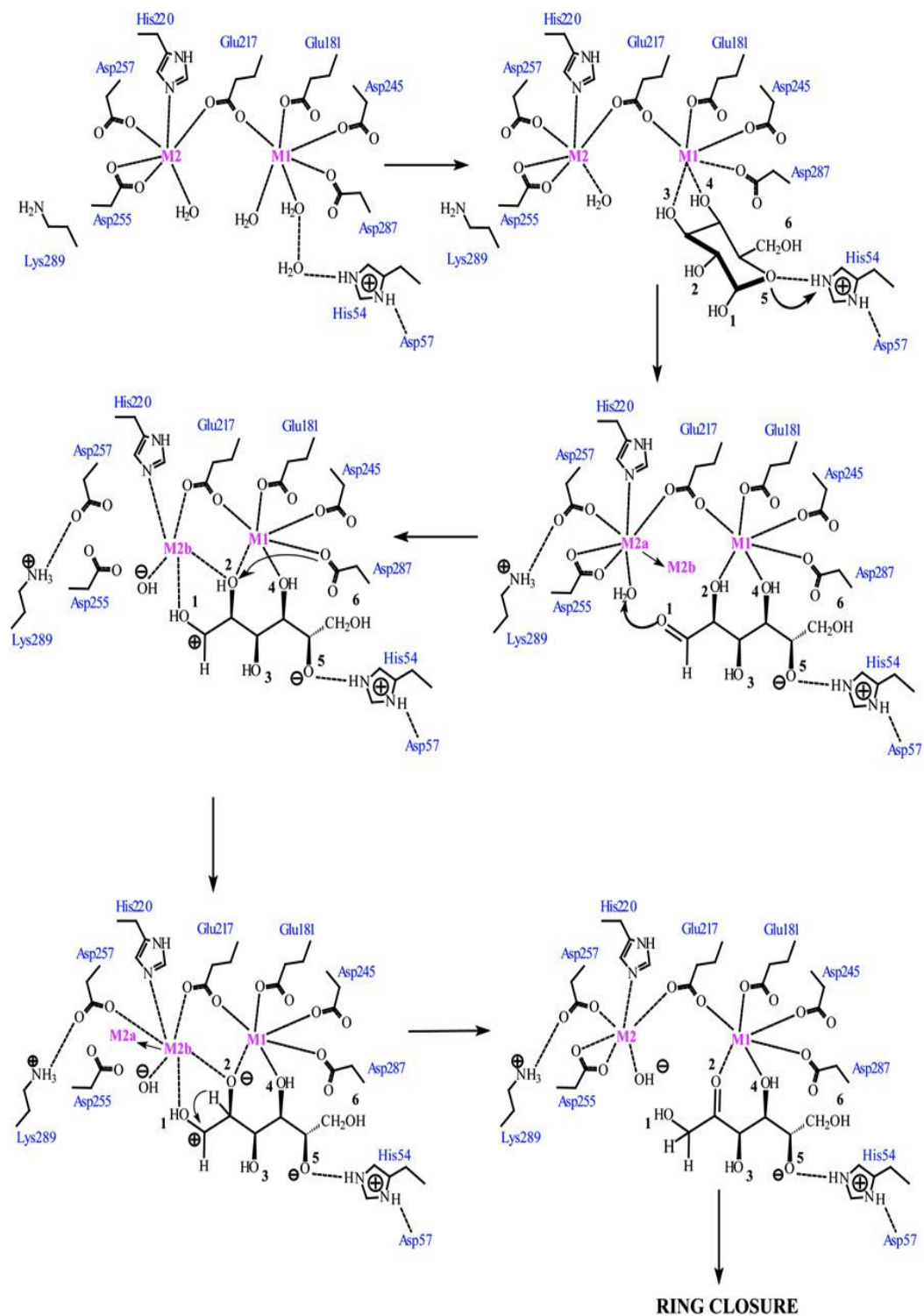


Figure 2.3 Complete reaction mechanism of GI (Kovalevsky et al., 2010).

2.1.2 Microorganism Selection

Selection of appropriate host microorganism and strain improvement for production of the industrial enzyme is one of the critical steps in the development of commercially successful bioprocesses. Several aspects should be considered during the potential host selection to access an industrially feasible bioprocess. Generally, microorganisms are more popular sources in the term of enzyme production compared with plants and animals, because of their fast multiplication rate and ease of culture. The production host should have GRAS (generally recognized as safe) statue, be able to produce large amount of desired enzyme in reasonable time-frame on cheap media, and exhibit possibility for easy scale-up (Kirk and Othamer, 1994; Soetaert and Vandamme, 2010).

P.pastoris and *E.coli* are two preferred eukaryotic and prokaryotic expression system for the production of industrial enzymes and proteins, respectively. There are many advantages of using both strains as the host microorganism but in the cases where glycosylation is necessary for stability, activity and accurate folding, production of enzymes or proteins can be carried out by yeast cells. No-glycosylated or normally glycosylated enzymes and proteins which are active without carbohydrate moiety can be expressed successfully in bacterial strains specially *E.coli*. 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) 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).

2.1.2.1. *Pichia pastoris*

2.1.2.1.1 General Characteristics

Pichia pastoris, the methanoltrophic yeast, is a unicellular oval shaped fungus with the 1-5 μm wide and 5-30 μm long. The yeast cell wall is composed of 30 - 60

% polysaccharides (beta-glucan and mannan sugar polymers), 15 - 30 % proteins, 5 - 20 % lipids and a small amount of chitin. Proteins in the yeast cell wall form a complex with the Mannan-Oligo-Saccharides (MOS) component which is known as Mannoprotein complex.

The first attempt for cultivation of this strain on methanol was made by Phillips Petroleum Company during the 1970s and in the following decade as a result of cooperation between this company and Salk Institute of Biotechnology, *P. Pastoris* was introduced as a promising host organism for heterologous protein expression (Cereghino et al., 2000). Nowadays, more than 500 proteins can be produced either extracellularly or intracellularly via recombinant *P.pastoris* host cells (Cos et al., 2006).

Pichia pastoris strain possesses the advantages of eukaryotic cells as well as the simplicity of unicellular microorganisms as host for cloning of heterologous gene. Table 2.1 represents the detailed advantages and disadvantages of this system (Cregg, 1999; Daly and Heam, 2005; Macauley-Partrick et al., 2005).

2.1.2.1.2 *P.pastoris* Expression System

The capability of *P. pastoris* in over-production of recombinant proteins under methanol induced activity of firmly regulated alcohol oxidase 1 (AOX1) promoter, make this strain an attractive host for industrial application (Cereghino et al., 2002). Actually, methanol metabolism in *P.pastoris* is supplied by two alcohol oxidase enzymes: AOX1 and the less active one AOX2. The major alcohol oxidase activity detected on methanol based *P. pastoris* fermentations is related to AOX1 which generally comprises almost 30% of the overall cell protein content (Cereghino and Cregg, 2000).

Depending on the methanol-utilizing ability, *P. pastoris* expression strain is divided into three phenotypes (Daly and Hearn, 2005):

- Wild type methanol utilization phenotype (Mut⁺): both AOX1 and AOX2 are active; rapid growth rate on methanol.

- Methanol utilization slow phenotype (Mut^s): only AOX2 is active; lower growth rate on methanol due to the disruption of AOX1 gene.
- Methanol utilization minus phenotype (Mut⁻): both AOX1 and AOX2 genes are disrupted; cannot grow on methanol.

Table 2.1 Advantages and disadvantages of *Pichia pastoris* expression system.

Advantages	Disadvantages
• High yield • High productivity • Chemically defined media-simple, inexpensive formulation • Product processing like mammalian cells • Stable production strains • Low purification strains • High level of expression of proteins • Eukaryotic post –translational modification • No endotoxin problem • Non-pathogenic • Board pH rang: 3-7 • Ability to utilize methanol • Preference for respiratory growth rather than fermentative • Not overglycosylate • Strong promoter (AOX1)	• Potential of proteolysis, non-native glycosylation • Long time for cell cultivation compared to bacteria • Monitoring methanol during a processes difficult • Since methanol is a petrochemical substance, it may be unsuitable for use in the food industry • Methanol storing in industrial scale is undesirable because it is a fire hazard

There are some restrictions related with the expression of foreign genes in *Pichia pastoris* strain. Since methanol is used for the induction of tightly regulated

AOX1 promoter, its presence in the cultivation medium to induce promoter can be hazardous for production of specific therapeutic proteins, food products and additives. Furthermore, due to the inflammatory property of methanol, its storage and utilization at large quantity can be challenging throughout the large scale and safe bioprocess development (Cereghino and Cregg, 2000). Attributed to these limitations, other alternative promoters such as glyceraldehydes 3-phosphate dehydrogenase gene promoter (GAP), glutathione-dependent formaldehyde dehydrogenase gene promoter (FLD1), isocitrate lyase (ICL1) and 3-phosphoglycerate kinase (PGK1) were developed for over-production of commercial recombinant protein via *P. pastoris* expression system (Waterman et al., 1997; Shen et al., 1998; Menendez et al., 2003; de Almeida et al., 2005).

2.1.2.1.3 Protein Secretion and Post-Translational Modification in *P.pastoris*

The main priority of yeast cells over the bacterial expression systems is the ability of the yeast in performing several post-translational modifications, like: protein folding, glycosylation, development of signal peptides, moreover disulfide bond formation (Cereghino and Cregg, 2000).

Signal sequences, short long peptides, are substantial portions in the structure of extracellular proteins. These peptides functions by prompting the transport mechanism within the cell. Generally, the extracellular protein producers are preferred by investigators and industry because application of these strains reduces the price of downstream processing by making the purification step easier. Alpha-factor prepro-signal from *S. cerevisia* and phosphatase (PHO1) signal are some of the commonly used signal peptides (Macauley-Patrick et al., 2005).

Some proteins especially the ones that are naturally secreted into extracellular medium, include pre- pro region in their structure to facilitate correct folding of proteins during the secretion (Bryan, 2002). In *P. pastoris*, the transport mechanism mediated by α -factor prepro signal sequence, carried out in three steps. At the first stag, protein is reassigned to endoplasmic reticulum where the pre-domain removed

by signal peptidase. Subsequently, the pro-protein is directed toward the golgi compartement and there, pro-domain is removed by means of dibasic endo-peptidase (kex2) activity. Dibasic endo-peptidase, a serine protease, belongs to the pro-hormone convertase family with the ability to identify alkali pairs of amino acid aspects like a Lys–Arg or Arg–Arg. Finally, the recombinant proteins are packed to the secretory vesicles and then sent to the extracellular medium (Daly and Hearn, 2005). In this study α -factor prepro signal was used for the production of recombinant GI in extracellular form.

The other post-translational modification that successfully performed by the yeast cells is glycosylation. Both types of glycosylation, O- and N- linked glycosylation can be performed by *P. pastoris* cells. Generally, glycosylation pattern in yeast is somewhat different from that observed in other mammalian cells, for example, the N-glycans structure in yeast are comprised from heterogeneous high-mannose moieties, where complex or hybrid types are chiefly found in human N-glycans arrangement. So that, utilization of recombinant therapeutic glycoproteins produced by yeast cells can result in some immunogenic effects in human's immune system (De Pourcq et al., 2010). Diversity in protein glycosylation among the yeast strains is also ordinary case as the glycosylated products in *P.pastoris* generally consist of short-length glycosyl chains; the hyper glycosylations is the main problem of *S. cerevisia*. O-linked glycosylation pattern in *Pichia* shows similarity with other yeast strains and O-oligosaccharides are commonly attached to hydroxyl groups of serine and threonine amino acid residues of recombinant proteins.

2.1.2.2. *Escherichia coli*

2.1.2.2.1 General Characteristics

Escherichia coli is gram-negative, facultatively anaerobic, non-sporulating and typically rod shaped bacteria with a cell volume of 0.6-0.7 μm^3 . Different from Gram positive cell wall, *E.coli* has an outer membrane surrounded by a thin layer of peptidoglycan vicinity to the cytoplasmic membrane and the preplasmic space which separates these two layers from each other. Cytoplasmic membrane is composed of a

phospholipids' bilayer and acts an important role in transport of molecules into the cell as well as in energy conservation. The cell wall acts as supporter to hold essential cellular compounds and as barrier to preferentially keep away undesirable molecules from cell environment (Lee, 2009).

Application of *E.coli* as a recombinant host proposes several benefits as follow: its genome can be modified easily, control of promoter is straightforward, protease deficient mutant strains are available, modification of plasmid copy number is possible, and heterologous protein accumulation can be enhanced to value as much as 50% of dry cell weight (Soetaert and Vandamme, 2010). *E. coli* BL21, recognized as a protease-deficient and low acetate producer strain, is widely preferred by researchers in the bioprocess development for production of large variety of enzymes and proteins (Miroux and Walker, 1996; Van de Walle and Shiloach, 1998; Åkesson et al., 1999; Choi et al., 2006; Rozanov et al., 2007).

E. coli BL21 (DE3) pLysS strain, a derivative of *E.coli* BL21, was selected for expression of thermostable *xyl* gene while *E.coli* BL21 (DE3) star and *P.pastoris* X-33 strains were preferred as alternative ones. This expression system contains the DE3 bacteriophage lambda lysogen which carries the *lacI* gene for T7 RNA polymerase under control of the lacUV5 promoter and a small section of *lacZ* gene. The presence of pLysS plasmid in this strain reduces basal expression level of the interested gene via T7 lysozyme production (<http://tools.invitrogen.com>).

2.1.2.2.2 The Lac Operon

The lacUV5 promoter, a mutated version of the lac promoter, is an operon required for the transport and metabolism of lactose in *E.coli* and some other enteric bacteria. It consists of three adjacent structural genes which encode β -galactosidase (*lacZ*), permease (*lacY*) and transacetylase (*lacA*) enzymes as illustrated in Figure 2.4. The lac operon regulation mechanism is managed via several aspects like the accessibility to glucose and lactose. The regulation of lac operon is carried out by a negative control in which binding of repressor to promoter inhibits gene expression. As seen in Figure 2.4, gene encoding for repressor protein (*lacI*) is upstream of the

lac operon. Induction of this operon is possible when repressor effect is prevented by allosteric action. The molecules that cause this action are called inducers and lactose is one the natural inducers of this operon (Klug and Cummings, 2003).

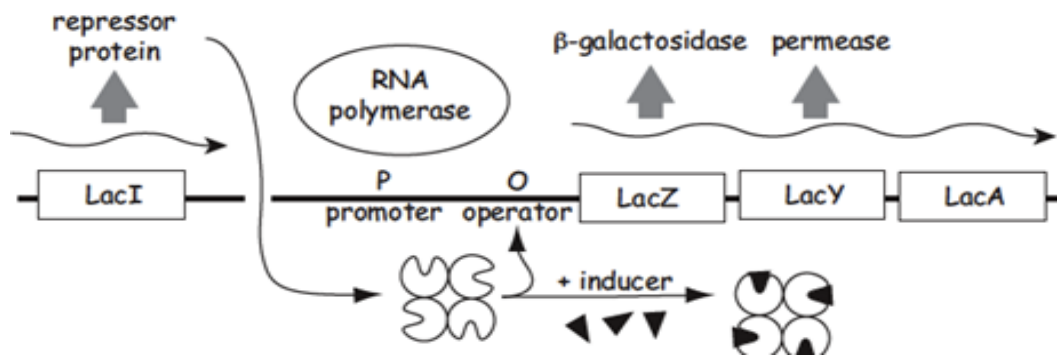


Figure 2.4 Structure of lac operon (<http://ocw.mit.edu/courses/biology/7-03-genetics-fall2004/lecture-notes/lecture16.pdf>).

There are several mechanisms that control regulation of this operon. Regulation initiates as a result of carbon source starvation in the cultivation medium, mainly glucose which is called carbon catabolite repression. When glucose concentration decreases to a certain value, concentration of cAMP increases in the cell. CAP (catabolite activating protein) and cAMP form a complex and bound to the promoter to facilitate expression of lac operon genes. Therefore, lac permease will help lactose to be transported into cell and induce the operon further. After that point induction starts as lactose binds to the repressor and facilitates the transcription (www.oxfordreference.com). Recent studies revealed that induction of lac operon is also possible even in the absence of lactose. This finding provided a market demand for introduction and production of artificial inducers like an isopropyl- β -D-thiogalactopyranoside (IPTG). IPTG, a molecular mimic of allolactose, is transported through cell membrane by diffusion and active transport which is facilitated by lac permease. Facilitated transport of IPTG starts after induction; and it continues until intracellular IPTG levels reach a certain value (Stamatakis and Mantzaris, 2009).

2.1.2.2.3 T7 Expression System

Expression systems are developed in order to express high amount of recombinant products in host cells. These vectors carry all the information necessary to produce the recombinant protein. Studiet and Moffat, (1986) developed T7 expression system in which T7 RNA polymerase is inserted into the genome of host microorganism by using a lambda bacteriophage. As the result, the pET vectors containing T7 promoter, gene of interest and the other elements of an expression vectors such as markers and restriction sites was constructed and transformed into host microorganisms like a *E.coli* BL 21(DE3) strain (http://labs.fhcrc.org/hahn/Methods/biochem_meth/pet.pdf). Lac-UV5 promoter controls production of T7 polymerase in the host cell. Figure 2.5 illustrates operon for the transcription of T7 RNA polymerase in lambda DE3 lysogen.

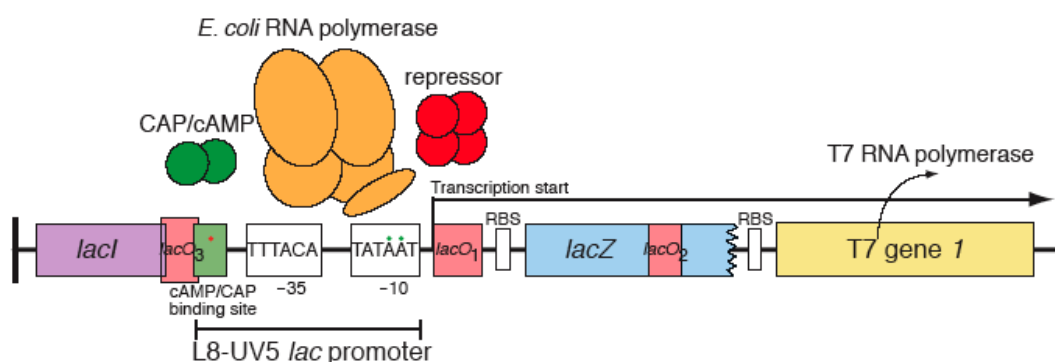


Figure 2.5 Control of Transcription of T7 RNA polymerase.

2.1.2.2.4 *E.coli* Fermentations

The major obstacle in *E.coli* fermentations is the accumulation of acetate as a result of high concentration of carbon source in the cultivation medium under fully aerobic condition and high specific growth rates which is known as over flow metabolism (Akesson et al., 1999). Acetate formation because of mixed-acid

fermentation under anaerobic conditions is also a common case in *E.coli* fermentations. The high concentration of acetate reduces recombinant enzyme production by inhibiting the cell growth and increasing the maintenance energy of cells. Some parameters such as strain selection, medium composition, carbon source supply strategy as well as management of specific growth rate throughout *E.coli* fermentations play critical roles in the design of successful fermentation (Akesson et al., 2001; Van de Walle and Shiloach, 1998).

Acetate accumulation resulted from the variant specific growth rate values, is generally affected from medium composition, fermentation condition and type of strain employed in the fermentation (Han et al., 1992; Lee, 1996). Meyer et al. (1984) investigated effect of complex and defined medium on the acetate formation of *E. coli* K12 D1 fermentation and reported that acetate accumulation within the complex medium happens at the inferior specific growth rate value compared with chemically defined medium. The type of carbon source and its concentration are also effective parameters in the control of acetate formation. It is known that the utilization of glycerol or glucose at the concentration below 0.75 kg m^{-3} can be an effective solution for acetate production problem in *E.coli* cultivations (Holms, 1986; Marison and von Stocker, 1986).

Since excess amount of the carbon source in aerobic *E.coli* cultivations together with high specific growth rate value are the main reasons for over flow metabolism, application of fed-batch operation mode with exponential feeding strategy at the controlled specific growth rate in which the concentration of carbon source is managed at limiting value can resolve acetate formation problem. And high cell density as well as high product yield can be achieved at the end of bioprocesses. Several strategies were developed in the literature with the aim of acetate production reduction in *E.coli* fermentation including different glucose feeding approaches (Paalme et al., 1990; Lee, 1996; Riesenber and Guthke, 1999; Cheng et al., 2003), control of cell growth via substrate limited fed-batch strategies (Shiloach et al., 1995; Lee et al., 1999; Çalık et al., 2009-b), constant dissolved oxygen concentration (Çalık et al. 2009-b; kaya et al. 2009), pH- stat (Wong et al., 1998; Kim at al., 2004) and application of alternative carbon source like glycerol (Pflug et al., 2007). In the

present study, over production of glucose isomerase was achieved by cultivation of recombinant *E. coli* BL21 (DE3) pLysS, a high GI and low acetate producer strain, in which the fermentations were carried out at exponential feeding strategy with different pre-determined specific growth rate values.

2.1.3 Genetic Engineering of the Microorganism: Techniques and Methodology

The genetic engineering techniques are the modern DNA technologies, employed in manipulation and modification of microorganisms genetic materials. Nowadays, the application of genetic engineering methods makes it possible to easily interfere into the organism's genome and to construct new strains with the ability to produce worthy recombinant enzymes and proteins via gene addition, substitution or deletion. Polymerase Chain Reaction (PCR) method, determination of DNA concentration by gel electrophoresis, DNA isolation and extraction, restriction digestion and ligation reaction are some of the principles used in recombinant DNA technology. The typical steps which should be considered during the construction of genetically modified molecules are as follow:

- DNA isolation from cells or tissues, amplification of the target gene by polymerase chain reaction (PCR), and selection of appropriate expression vector.
- Target gene and vector fragmentation by the means of restriction enzymes in order to obtain sticky-ended gene and vector.
- Ligation of target gene and linear vector by DNA ligase enzyme and transformation of constructed recombinant plasmid to a proper host.
- Selection of positive colonies (clones containing the correct orientation of foreign DNA) by plating the transfected cell on the selective media and isolation of positive clones to perform further analyses.

Figure 2.6 illustrates steps for cloning of the foreign gene in *E.coli* as a model microorganism.

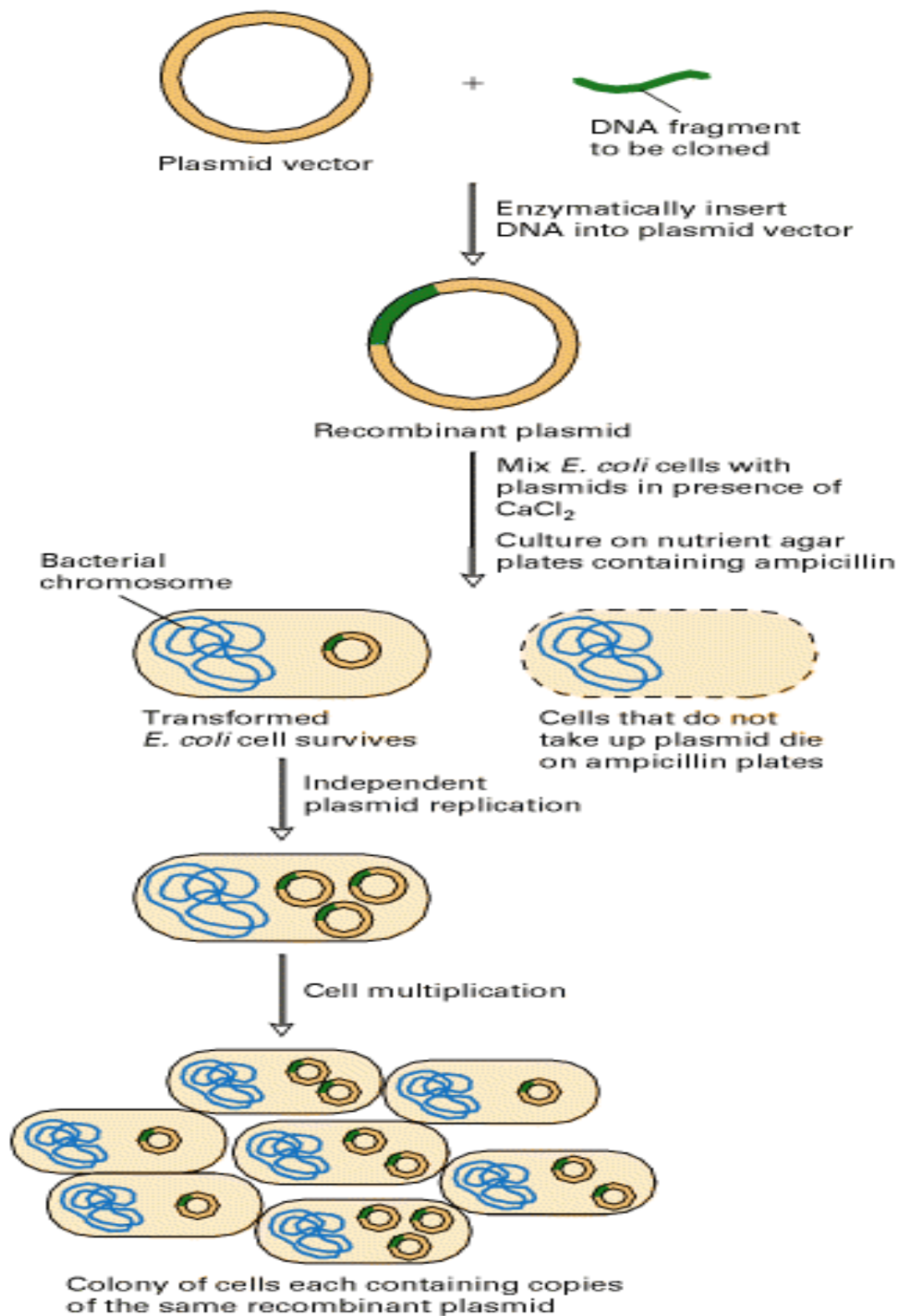


Figure 2.6 Steps for cloning of the foreign gene in *E. coli*.

In order to initiate cloning procedure, firstly, the target gene (eg. *xyl* in this study) should be amplified via polymerase chain reaction (PCR). It is a revolutionary method for in vitro amplification of DNA fragments which was firstly developed by Kary Mullis in the 1980s. This method works on the basis of DNA polymerase thermostable activity to synthesize complementary strand of target gene after denaturation in the presence of oligonucleotides and primers which are specific to either side of the gene of interest. Application of this technology make it possible to generate billions gene copies from a small quantity of the template DNA in order to used in recombinant DNA technology, genetic research areas, agriculture, forensics as well as medicine. Template DNA is amplified in a PCR device in the course of three major time periods which are replicated between 30-40 cycles (Figure 2.7). The replications are carried out in the automated cycler device, with the ability to quickly heat and cool the samples. Every step - denaturation (the double-stranded DNA melts at greater than 90°C and opens into single-stranded DNA), annealing (joining primers and single stranded DNA), and extension (replication of DNA by polymerase at 72 °C) - takes place at the different temperature. The important parameters in the design of a successful PCR program are annealing temperature and time which vary depending on the template sequences.

The use of thermostable DNA polymerase enzymes isolated from thermophilic microorganisms like *Thermus aquaticus*, simplify PCR procedure by enabling the amplification reaction to carried out at higher temperature. Higher reaction temperature results in higher specificity, sensitivity and yield (Saiki et al., 1988). Taq DNA polymerase and pfu DNA polymerase are two popular thermostable enzymes used in DNA amplification reactions. Where Taq DNA polymerase and its derivatives have a 5' to 3' polymerization depended exonuclease activity, Pfu DNA polymerase possesses 3' to 5' exonuclease proofreading activity. For nucleotide incorporation, the enzymes works best at 75-80°C, depending on the target sequence; its polymerase activity is reduced by a factor of 2 at 60°C and by a factor of 10 at 37°C (Cline et al., 1996; Sambrook and Russell, 2001).

Selection of an appropriate carrier plasmid DNA, known as the expression vector, is another crucial step in the construction of recombinant strains. Plasmids are

circular DNA molecules with the ability to replicate autonomously from bacterial chromosomal DNA and vectors are genetically engineered plasmids with optimized property for cloning purpose.

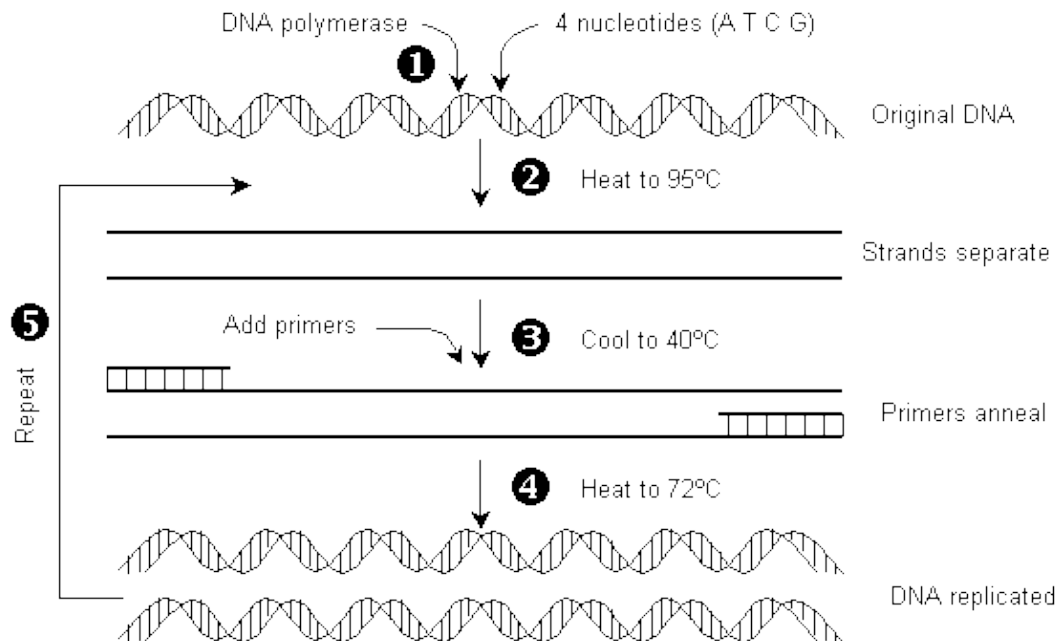


Figure 2.7 The polymerase chain reaction (<http://www.biologymad.com>).

There are several properties which should be offered by the proper cloning vector: it should be able to replicate autonomously; it should be easy isolated and purified; it should be easily introduced into the host cells; it should have suitable marker genes that allow easy detection and/or selection of the transformed host cells and it should contain unique restriction sites for DNA insertion (Walker, 1998).

For the gene cloning in *P.pastoris*, among the different types of yeast vectors, pPICZ α A integrative plasmid was selected (Figure 2.8). This vector includes thirteen unique restriction sites for gene insertion, AOX1 promoter to regulate and induce expression of target gene, Zeocin resistance gene for differentiation of recombinant colonies, α -factor signal sequence to direct recombinant protein secretion, and a polyhistidine (6xHis) peptide to C-terminally tag recombinant protein for easier detection and purification.

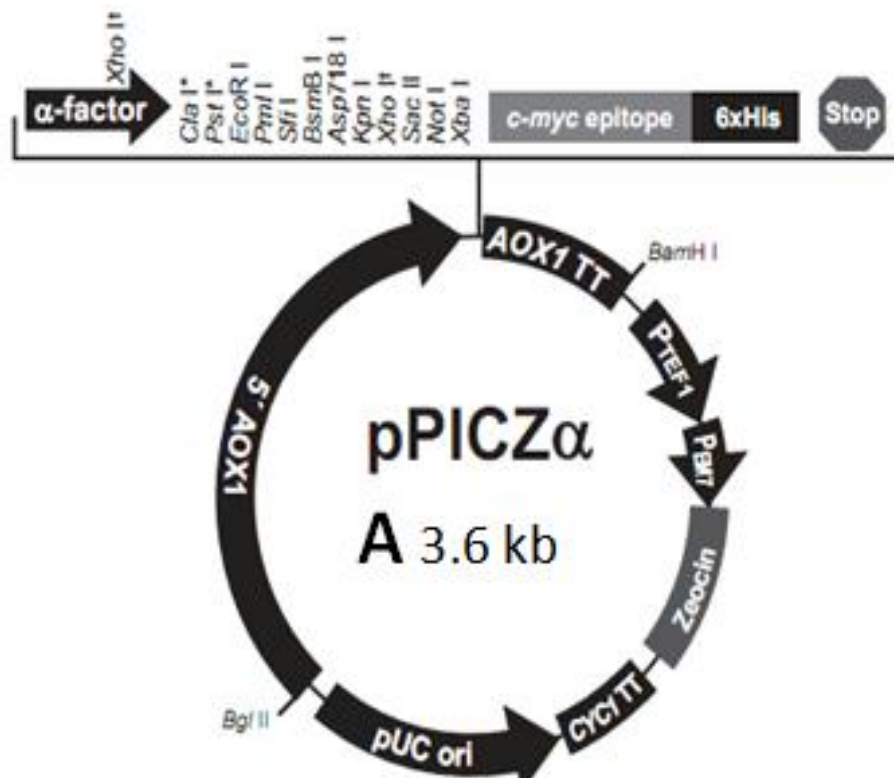


Figure 2.8 pPICZαA cloning vector (<http://www.fermentas.com>).

A series of expression vectors is available designed to reach the high-expression level of the foreign gene in *E.coli*. The pRSETA, selected in this study for expression of thermostable GI, is one of the widely used expression vector for the production of recombinant enzymes and proteins in *E.coli* (Figure 2.9). Regulation of cloned gene expression is controlled by T7 promoter which is recognized by T7 RNA polymerase specifically. pRSET vector also includes T7 gene sequence to give protein stability, multiple cloning site consisted of 11 restriction enzyme recognition sequences, ampicillin resistance gene, N-terminal polyhistidine tag for protein purification and enterokinase cleavage site to remove the fusion tag as well as f1 and pUC regions to allow single strand rescue and high copy replication, respectively (<http://www.invitrogen.com>).

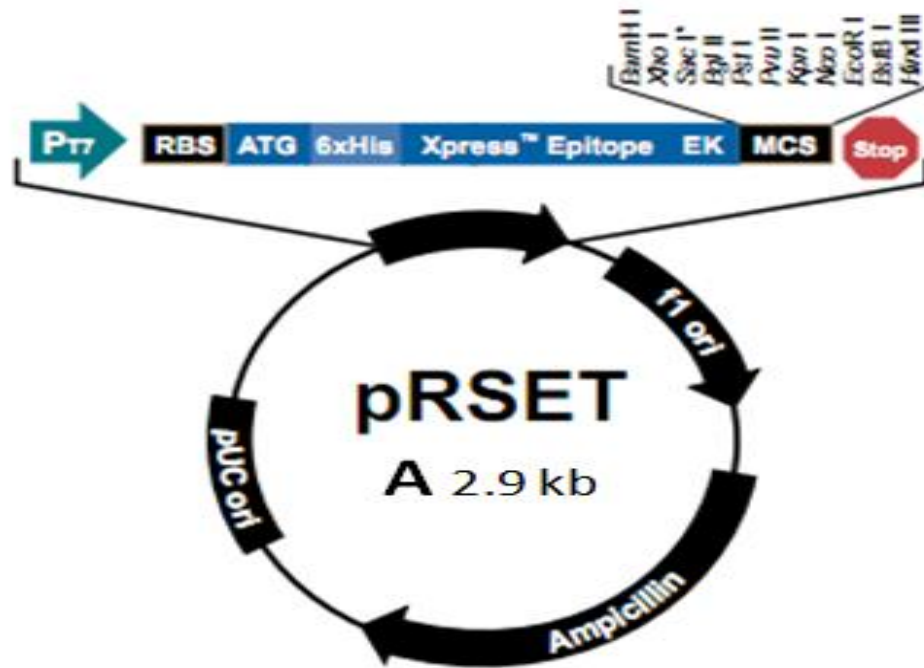


Figure 2.9 pRSETA cloning vector (<http://www.fermentas.com>).

The next step in the construction of recombinant plasmid is the digestion of vector and target gene with desired restriction enzymes. These restriction endonucleases act as a kind of immune system in the bacterial cells protecting them from attack of foreign DNA during viral infections. Restriction enzymes have ability to cut foreign DNA from outside of helix when they do not affect on the host DNA due to characteristic methylation patterns on the DNA (Scragg, 1988). There are four general types of restriction enzymes (Types I, II, III and IV) which are classified based on composition, cofactor requirements, position of restriction site, and the nature of their target sequence.

The type II restriction endonucleases, the most functional ones for recombinant technology, are capable of binding to DNA at any position and traveling along the strand of DNA until recognizing specific sequence where they cut DNA molecules into two fragments. These restriction sequences vary in size from four to twenty nucleotides long and also the nucleotide pattern that is recognized by different restriction enzymes is quite variable, although it is frequently palindromic. Table 2.2 represents the list and recognition sequences of the enzymes used during of

this study. Digestion of selected vector and a target gene by the same restriction enzyme results in the formation of complimentary single-stranded sticky ends in both vector and gene which anneal together during the ligation reaction. The DNA ligase enzyme in a ligation mixture catalyzes the condensation of 3'-hydroxyl group with a 5'-phosphate group by formation of covalent phosphodiester bonds between the complementary ends of the digested vector and target gene (Figure 2.10). The ligation reaction is the main rate limiting step in construction of recombinant expression systems since this reaction requires the cohesive ends of foreign DNA and open plasmid DNA to attach in correct orientation and anneal while preventing the religation of opened vector DNA. So that, the optimum condition for ligation reaction should be supplied by considering target gene and vector concentrations. It is also possible to reduce the probability of vector re-closure by treating the linear vector with phosphatase enzyme (Çalık et al., 1998; Bailey, 1986; Glazer, 1995).

Table 2.2 Recognition sites and cleavage points of restriction enzymes.

Enzyme	Target site
<i>AsuII</i>	TT [^] CGAA
<i>EcoRI</i>	G [^] AATTC
<i>NdeI</i>	CA [^] TATG
<i>SacI</i>	GAGCT [^] C
<i>XbaI</i>	T [^] CTAGA

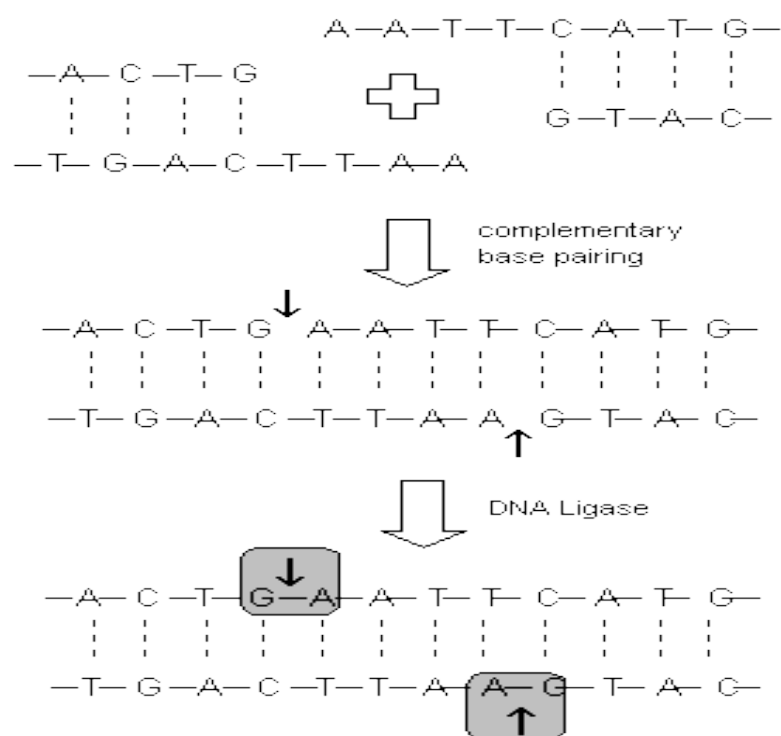


Figure 2.10 Mechanism of ligation reaction (<http://www.biologymad.com>).

Following step after successful ligation is transformation of recombinant expression vector containing gene of interest into the recipient or host cell (Schuler and Kargi, 2002). There are several transforming methods for direct introduction of foreign DNA into intact cells including: natural transformation where foreign DNA is taken up by the bacteria and fused to the chromosomal DNA of the organism, artificial transformation where the permeability of cell wall for foreign DNA is increased by applying heat shock to the cells which are treated by salt solutions like CaCl_2 or LiCl_3 , protoplast transformation in which enzymes are used to hydrolyze the rigid cell wall to convert the cell into protoplast bounded by the cytoplasmic membrane and electroporation where short electrical pulses of very high voltage is applied to create transient holes in the membrane (Glazer, 1995). In this study artificial method was used to transfer pPICZ α A::xyl and pRSETA::xyl plasmids into the *P.pastoris* and *E.coli* cells, respectively. The correct combination of the desired vector with donor DNA is the other important parameter which is supposed to be verified after transformation. Because construction of some opened or rejoined

vector molecules, or vectors containing DNA contaminants of donor DNA is also possible through the ligation reaction (Schuler and Kargı, 2002).

Transformation of an antibiotic resistance gene available in the structure of expression vector introduces a new property to host cells which enable them to withstand the effects of that antibiotic. The antibiotic resistance test is the most commonly used method for selection and screening of recombinant cells (Smith, 1995). In order to differentiate the cells carrying recombinant plasmid from the others by this method, all transformants are spread on plates containing the particular antibiotic and growing cells on this media are selected for further analyses including restriction mapping, PCR, and DNA sequencing (Klug et al., 2006) . Figure 2.11 represents antibiotic resistance selection method schematically. The pPICZ α A and pRSETA vectors used in this study contain zeocin and ampicillin resistance gene, respectively.

Restriction mapping, the description of restriction endonuclease cleavage positions, can be efficiently applied during the recombinant plasmid construction to select proper REs for cloning and to verify the accurate orientation of an interested gene into a vector after the cloning (Dale and Von Schantz, 2003). For this purpose, the constructed recombinant expression vector is treated with REs and fragments size is determined by the mean of gel electrophoresis. Throughout the gel electrophoresis DNA fragments are separated from each other based on their sizes and charges. Staining of fragments by ethidium bromide makes it possible to easily visualized DNA pieces under ultraviolet light and to determine their size by comparing with molecular weight size marker. For PCR control, primers designed for amplification of inserted gene and plasmids isolated from positive colonies are used in PCR reaction to verify the presence of target gene in vector. DNA sequencing is the most consistent method for determining the order of the nucleotide bases in DNA molecules since this method enables us to detect even single base pair mutations which can occur throughout the cloning procedure. All three discussed methods have been applied in this study for selection and screening of recombinant cells.

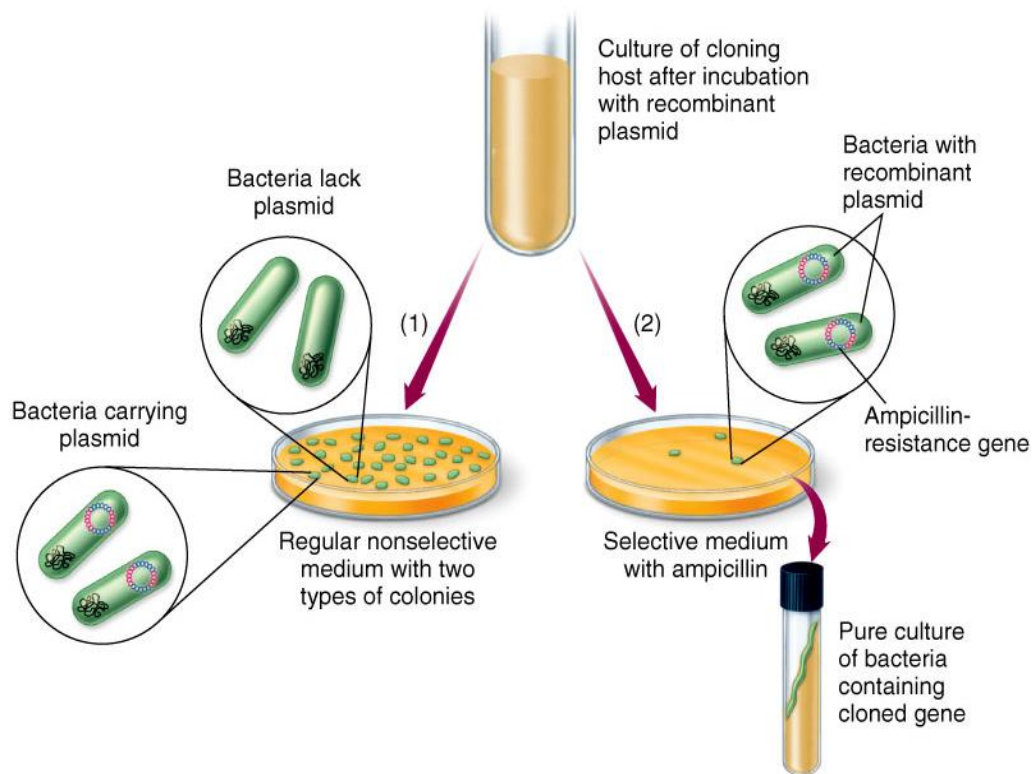


Figure 2.11 Schematic of ampicillin resistance gene containing cell (<http://www.worldofteaching.com>).

2.1.4 Medium Design and Bioreactor Operation Parameters

Any operation dealing with the design and development of processes for the manufacturing of products from raw materials by using microorganisms or their components (e.g. enzymes, chloroplasts), can be termed as a “bioprocess” (Moses and Cape, 1991). The main objective of the commercial bioprocesses is to provide an optimal condition for microorganism growth with the aim of increasing yield and productivity of the desired bio-products. The important criteria that should be considered during aerobic fermentation processes are:

1. Medium composition
2. Bioreactor operation parameters
 - Temperature and pH
 - Dissolved oxygen concentration

2.1.4.1 Medium Composition

Oxygen, carbon, nitrogen, hydrogen and phosphorous are the fundamental elements founded in the structure of common cells and they are employed by the cell in the formation of membranes, nucleic acids, amino acids, proteins as well as the other vital cell organelles. These components, termed as macronutrient, are consumed by the cells in the large quantities and comprise over 1% of the cells dry weight. Micronutrient is the collection of elements which are utilized in inferior quantities and potassium, calcium, magnesium, manganese and iron are some representative aspects of this group. Micronutrients account for 0.1-1% of the cells dry weight and generally function as primary constituents in proteins and enzymes structures. Trace elements are the third group of the components that are consumed in trivial levels (less than 0.1%) and high concentration of these components generally results in cell growth inhibition. But, the determination of sufficient amount of trace elements to promote cell growth without inhibitory effect is a challenging task during the optimal medium design for efficient cell growth and product formation. In additions to the elements mentioned above, there are some organic components essential for cell growth which cannot be synthesize by the cell so they should be added into cultivation medium. These molecules termed as growth factor and vitamins, amino acids and nucleotides are some examples of this collection (http://www.bionewsonline.com/3/what_is_growth_medium.htm).

There are two main categories of cultivation media frequently used in fermentations which are known as defined and complex media. Synthetic or defined medium is prepared by totaling precise quantities of pure inorganic components and generally preferred to determine influence of the definite element on cell growth rate as well as product yield (Kampen, 1997). Nowadays, with a requirement for increased documentation and reproducibility in the fermentation industry, there is a big trend toward of defined media. Also, in the pharmaceutical sector, there is a desire for the defined media utilization; and in these times, application of serum free medium is considered as standard requirement for production of heterologous proteins via mammalian cell cultures. The application of defined media offers

advantages of low cost downstream processing as well as well-documented and reproducible results. Complex or non- synthetic medium is consisting of natural complexes with the no precise information about its chemical composition. Complex cultivation media are frequently proffered in the commercial bio-processes because they are present in large quantities; they are relatively cheap; they often contain an organic nitrogen source, essential mineral, various growth factors, vitamins and trace elements; they usually result in evaluated cell and product yields (Nielsen and Villadsen, 2003; Shuler and Kargı, 2002). Table 2.3 lists some frequently used complex media and their contents.

Table 2.3 Complex fermentation media frequently used in industry (Adapted from Nielsen and Villadsen, 2003).

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

The standard medium for high cell density fermentation of methylotrophic yeast *P.pastoris* is the basalt salt medium (BSM) along with its companion trace salts medium (PTM1). But, due to some difficulties related with utilization of this medium such as unbalances composition, precipitation and high ionic strength, there are several attempts for modification of this medium in literature (Bardy et al., 2001; Thorpe et al., 1999; Jungo et al., 2006). Ammonium hydroxide solution which added gradually into standard cultivation medium serves as both nitrogen source and pH adjustment solution (Cos et al., 2006). The steady addition of ammonium hydroxide

solution at the controlled level provides advantage of avoiding nitrogen accumulation which inhibits cell growth and extends the lag phase and eliminates the high level protease secretion happening as result of nitrogen starvation (Yang et al., 2004).

As other microorganisms, *P.pastoris* prefers some carbon sources for over production of recombinant proteins and enzymes. Methanol, glycerol, sorbitol, glucose, mannitol, and trehalose are commonly used carbon sources for *P.pastoris* fermentations (Brierley et al., 1990; Sreekrishna et al., 1997; Thorpe et al., 1999; Inan and Meagher, 2001). Although methanol is a vital substance for the induction of *P.pastoris* AOX1 promoter, the utilization of methanol at excess concentration as carbon source leads to rapid accumulation of formaldehyde and hydrogen peroxide in intracellular medium as well as product oxidization. Consequently, fed-batch cultures are more prevalent operation modes for the over expression of recombinant proteins by the yeast cells.

Generally production of recombinant proteins via *P.pastoris* expression system carried out in three main steps. At the first stage, defined medium containing excess concentration of glycerol is used in batch cultivation to achieve high cell density. Then, glycerol is fed at limited amount to promote cell growth, to release repression of AOX1 which observed during the batch phase and to reduce the time necessary for the cells to adapt for growth on methanol. Finally, methanol is added into the cultivation at fed-batch mode to induce expression of recombinant gene under the control of AOX1 promoter (Chiruvolu et al., 1997; Tschopp et al., 1987). The other strategy for attaining high cell density, process productivity and reducing the induction time in *Pichia* fermentations is the use of multi-carbon substrate besides methanol. This strategy has been mostly employed for Mut^s strains because of their genetically reduced capacity to assimilate methanol which results in long induction times above 100 h (Ramon et al., 2007). The first report related with utilization of mixed substrates; glycerol/methanol in the fed-batch culture of *P. pastoris* was published by Brierley et al. (1990). Thereafter, mixed substrate feeding strategy was employed by several groups, to achieve high cell density and increased volumetric protein productivity (Cergg et al., 1993; Lowen et al., 1997; McGrew et al., 1997; Katakura et al., 1998; Zhang et al., 2003-b). But, there some other studies

in the literature indicating a partial repression of the AOX1 promoter by glycerol during mixed substrate feeding which may result in lower specific productivity of recombinant protein (Sreekrishna et al., 1997; Hellwing et al., 2001; Xie et al., 2005).

In contrast with glycerol, sorbitol is a non-repressing carbon source for AOX1 promoter which its accumulation during the induction phases does not affect the expression level of recombinant proteins. These advantages make sorbitol as appreciated carbon source for cultivation of Mut⁺ strains other than Mut^S. The first experimental data, showing the advantage of sorbitol supplement for the induction of Mut⁺ strains was given by Inan et al. (2001-b). Then, Roman et al. (2007) investigated the consumption mechanism of sorbitol and methanol in a batch bioreactor and observed sequential consumption of the substrates by the cells. Afterward, in the study of Jungo et al. (2007) sorbitol content in the feed was optimized in a continuous bioreactor experiment and the optimized feeding ratio was used in two fed-batch bioreactor experiments with specific growth of 0.03 h⁻¹ and 0.05 h⁻¹. In the more recent study of Çalık et al. (2010), the effect of methanol exponential feeding at different specific growth rate values on human growth hormone production via recombinant *Pichia pastoris* Mut⁺ strain was investigated. It was reported that at specific growth rate higher than $\mu = 0.03 \text{ h}^{-1}$ sorbitol consumption developed independently from methanol feed rate. The highest rhGH production as 270 mg L⁻¹, was attained at MS-0.03 condition where methanol feeding carried out at specific growth rate of $\mu = 0.03 \text{ h}^{-1}$.

LB (Luria-Bertani) medium is the most commonly used complex starter culture in molecular biology for *E.coli* fermentations. The main components of LB broth are tryptone, yeast extract and sodium chloride. This medium can support *E.coli* growth OD₆₀₀ 2 to 3 under normal shaking incubation condition. In order to achieve high cell culture of *E.coli*, it is vital to add some other components such as phosphorus, sulfur and trace elements into cultivation medium and to increase concentration of macronutrients. But, the use of nutrients at high concentration generally results in inhibition of cell growth and glucose, ammonium, iron, magnesium, phosphorus moreover zinc are some known components inhibiting

E.coli growth at high concentration (Schiloach and Fass, 2005). Therefore, development of the feeding strategy in which the inhibitory nutrients are added gradually into cultivation media can be more advantageous in the term of high cell density achievement. It is also possible to obtain cell density of about 15 kg m⁻³ DCW in *E.coli* fermentations by using defined cultures that contain the maximum non-inhibitive concentration of nutrients (Lee, 1996).

There are several studies in the literature considering the effects of the medium components on *E. coli* growth (Neidhardt et al., 1974; Yee and Blanch, 1993; Choi and Lee, 1997). Furthermore, in the more recent studies of Kaya et al. (2009), a glucose based defined medium including 8.0 kg m⁻³ glucose, 5.0 kg m⁻³ (NH₄)₂HPO₄ and salt solution was developed, and the designed medium utilized to systematically examine influences of different oxygen transfer on the growth and product formation rate of recombinant *E. coli* fermentations. In this study, both glucose based defined medium and molasses based complex medium were employed for over-production of thermostable GI via recombinant *E.coli* BL21 strain.

2.1.4 .1.1 Molasses

Molasses is the most valuable by-product which is obtained at the end of refining process of sugar beets or sugar cane into sugar. The maturity of molasses, its sugar content, and the applied extraction method are effective parameters in the determination of molasses quality. Currently, large variety of commercially important compounds can be produced from molasses based fermentations and ethyl alcohol, citric/glutamic acids, acetone/butanol, several proteins and enzymes are some known examples of these bio-products (Çalık et al., 2003). Utilization of molasses in the industrial bioprocess offers several advantages over the other carbon sources: molasses is relatively cheap; in addition to high sugar concentration, it contains organic non-sugars, essential mineral and vitamins for growth; it is readily available in large quantities (Park and Baratti, 1991; Makkar and Cameotra, 2002). Table 2.4 illustrates the overall chemical content of European beet molasses (Olbrich, 1963).

In the articles, Gerhard et al. (1946) utilized beet molasses based complex medium in favor of succinic acid production via *Aspergillus niger* fermentation. Thereafter, production of succinic acid was achieved on the cane molasses based complex media and high yield of product was reported (Sikander et al., 2002; Agarwal et al., 2006). Related with other products, Roukas (1996) utilized non-sterilized beet molasses to produce ethanol by the mean of free and immobilized *S. cerevisiae* cells in both batch and fed-batch operation modes. The highest ethanol concentration obtained at the end of fed-batch operations of free and immobilized cells was similar with the value of 53 kg m^{-3} where the molasses feed rate and initial carbon source concentration was arranged at the value of 250 ml h^{-1} and 250 kg m^{-3} , respectively. Then, the effect of pretreated beet-molasses on serine alkaline protease production via the batch cultures of *Bacillus* species was determined by Çalik et al. (2003). In this study, the procedure of beet molasses pretreatment was systematically explained.

Dumbrepatil et al. (2008) investigated the effect of different concentrations of molasses cane sugar on lactic acid production in the batch cultivation of *Lactobacillus delbrueckii* and considered that the lactic acid concentration obtained at the end of fermentation proportionally enhanced with the increase of initial molasses concentration. The highest lactic acid concentration with value of 166 kg m^{-3} was attained at initial molasses concentration of 190 kg m^{-3} . Then, in the study of Çalik and Levent (2009-a, 2009-b) molasses-based complex medium was utilized as carbon source in the batch and fed-batch *E. coli* fermentations with the aim of increasing the production of recombinant benzaldehyde lyase and higher BAL activities were attained by using pretreated-beet molasses than the ones reported by defined media. Afterwards, in the master thesis study of Taşpınar (2010), the effect of exponential feeding of pretreated-beet molasses on benzaldehyde lyase production via recombinant *Escherichia coli* BL21 was investigated. Maximum enzyme activity and recombinant cell concentration were reported at exponential feeding strategy with the specific growth rates of 0.15 h^{-1} .

Table 2.4 Overall chemical content of beet molasses from European samples (Olbrich, 1963).

Component	Average composition (%)
Water	16.5
Sucrose	51.0
Glucose and Fructose	1.00
Raffinose	1.00
Organic non-sugars	19.0
Ash components:	11.5
SiO ₂	0.10
K ₂ O	3.90
CaO	0.26
MgO	0.16
P ₂ O ₅	0.06
Na ₂ O	1.30
Fe ₂ O ₃	0.02
Al ₂ O ₃	0.07
CO ₃	3.50
Sulfates as SO ₃	0.55
Cl	1.60
Vitamins (mg/100 g)	
Thiamine (B1)	1.30
Riboflavin (B2)	0.40
Nicotinic acid	51.0
Ca-pantothenate (B3)	1.30
Folic acid	2.10
Pyridoxine-HCl (B2)	5.40
Biotin	0.05

2.1.4 .2 Bioreactor Operation Parameters

Fermentations are generally affected by various parameters including: temperature, pH, ionic strength, nature and composition of the medium, dissolved oxygen, dissolved carbon dioxide, agitation rates, and shear rates in the fermenters. Variation in these factors may show diverse effects on cell and product yield by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999; Soetaert and Vandamme, 2010). Therefore, in order to obtain high productivity in bioprocesses, fermentation should be carried out under controlled condition of oxygen transfer, pH, temperature, and other possible parameters.

2.1.4 .2.1 Temperature

In order to ensure proper growth of microorganism and production of recombinant protein, it is important to keep temperature constant at its optimal value during the fermentation process. Microorganisms do not have any control mechanism to keep their temperature at constant value so that cells are always in thermal equilibrium with their environments (Nielsen et al., 2003). It is known that most of anabolic and catabolic reactions taking place inside the cell are mediated by enzymes.

The catalytic activity of enzymes is based on their three-dimensional structures and on active sites whose chemical groups may be brought into close proximity from different regions of the polypeptide chains. Several forces and interactions such as hydrogen bonding, disulphide bonding, hydrophobic interactions, and van der Waals forces participate in the construction of enzymes' active form. At the desired temperature values, the protein structure is restricted by these forces but as the process temperature increases the thermal motion of the various regions of the enzyme increases until reaching to the point in which molecule is no longer able to maintain its structure or its activity. On the other hand, low temperatures result in lower reaction rate and introduce some limitations for cellular transport processes (Kirk-Othmer, 1994; Watson et al., 1987). In the case of *P.*

pastoris, it has been reported that temperatures above 30°C are not suitable for the production of recombinant proteins due to the secretion of proteases into fermentation media as result of cell death and cell lysis occurred at high temperatures (Inan et al., 1999). Conversely, lower cultivation temperature usually shows a positive effect on the protein yield of *Pichia* fermentation because lower temperature increases stability of recombinant protein by promoting proper folding of proteins and reducing protease release to the fermentation medium (Macauley-Patrick et al., 2005). Although, the higher yields of recombinant proteins at lower temperatures have been stated in several studies (Chen et al., 2000; Li et al., 2001; Sarralegna et al., 2002; Shi et al., 2003), there are other reports in the literature in which lowering the temperature below 30°C does not have a significant impact on the production yields (Inan et al., 1999, Curvers et al., 2001-b; Hong et al., 2002; Kupesulik and Sevela, 2005).

In this study, production of thermostable GI via recombinant *E. coli* and *P. pastoris* cells is performed at the optimal temperature value which is 30°C for *P. pastoris* and 37°C for *E. coli* cell cultures.

2.1.4 .2.2 pH

Hydrogen ion concentration (pH) is the other important bioreactor operation parameter which should be stabilized throughout the fermentations. The maintenance of intracellular pH is vital factor for overall cell functions. Cytoplasmic membrane of microbial cells is equipped with specific enzymes that take active role in the maintaining the intracellular pH at a constant value by pumping protons out of the cell. This transport mechanism consumes Gibbs free energy in the form of ATP and increases cellular maintenance energy demand (Nielsen et al., 2003). Alteration in pH value due to the formation of organic acids, utilization of acids (particularly amino acids), or production of alkali components is a common event during the microbial growth. So that, generally buffer solutions together with active pH controller system are used in bioreactor experiments to keep hydrogen ion concentration constant through the fermentation (Shuler and Kargi, 2002).

P. pastoris has ability to grow across a relatively broad pH range (3.0-7.0) which does not have significant impact on the cell growth. Different studies show that depending on the product types, different pH values can be used as the optimal pH in *Pichia* fermentations because different recombinant proteins can be stable at different pH (Brierley et al., 1994; Clare et al., 1991). The optimum growth pH of *E.coli* strains is 7.0 but this strain has ability to tolerate broad pH range from 4.4 up to 9.0. So, there are several studies in the literature related with diverse protein production by *E.coli* cells in which fermentations were carried out at different pH values (Ryan et al., 1989; Åkesson et al., 2001; Castan et al., 2002; Leon et al., 2003). The effect of controlled and un-controlled pH conditions, as well as of the value of pH in the range of 5.0-7.8, on benzaldehyde lyase productivity of the *E.coli* cells was investigated by Çalık et al. (2006). Un-controlled pH of 7.2 was reported to be more favorable than controlled pH conditions in terms of enzyme activity and cell concentration. Thereafter, in the studies of Kaya et al. (2009) as well as Çalık and Levent (2009-a, 2009-b), pH controlled mode at the value of 7.2 was applied for production of the recombinant enzyme by *E.coli* BL21 strain on glucose based defined and molasses based complex medium, respectively. In the current study, initial pH of the fermentation medium was arranged as 7.2 and its value was carefully monitored and maintained at constant value with 5M NaOH and 5M H₃PO₄ during batch and fed-batch fermentations.

2.1.4 .2.3 Oxygen Transfer Rate

Oxygen requirement of fermentation rely on the microbial species, the concentration of cell, the rheological properties and kind of substrate utilized in cultivation medium. In aerobic fermentation, oxygen supply should be no less than the cells oxygen demand in the other vise inadequate quantity of oxygen restrains microbial cell growth rate (Soetaert and Vandamme, 2010). Throughout the aerobic bioprocesses, variation in the dissolved oxygen concentration manipulates product formation rate as well as cell growth by altering cell's metabolic pathways and fluxes. Generally, there is a high probability for oxygen limitation in viscous

cultivation media and in broths that contain high concentration of oxygen-consuming cells. Type of microorganism, temperature of cultivation broth, and the substrate being oxidized are some key items in the determination of critical dissolved oxygen level (DO) of fermentations. Dissolved oxygen concentrations of 6.4×10^{-5} and $12.8 \times 10^{-5} \text{ kg m}^{-3}$ are the reported crucial value for *S.cervesia* and *E.coli* strains, respectively (Chisti, 1999).

There are several studies in the article demonstrating the affect of oxygen transfer rate on various *E. coli* fermentation products at the diverse fermentation conditions (Ryan et al., 1989; Bhattacha and Dukey, 1997; Castan et al., 2002; Leon et al., 2003; Çalık et al., 2004). On the other hand, there are other researches in which dissolved oxygen level was controlled at constant value (10-30%) either by changing air flow rate (Luli et al., 1990) or agitation rate in fed batch *E.coli* cultivation with an automated controllers (Akesson et al., 2001; Johnston et al., 2003). Related with the optimum oxygen transfer condition for recombinant enzyme production by *E.coli* BL21 strain, Kaya et al. (2009) reported high cell concentration and product formation in the batch fermentation where the dissolved oxygen concentration was controlled at value of 40% throughout the fermentation. Then this optimum value was successfully applied by Çalık and Levent (2009-b) in order to investigate effects of pulse feeding of beet molasses on recombinant benzaldehyde lyase production by *E. coli* BL21 cells.

2.1.5 Recombinant Protein Purification

The other critical step in the bioprocess development for production of recombinant enzyme and protein is recovery and purification of product from cultivation medium. The recovery of products from fermentation broth is both difficult and expensive; therefore downstream processing has long been regarded as the critical factor in the commercial development of the biotechnology. For example, 60 to 70 % of the selling price of enzymes corresponds to the downstream processing costs. Product cost increases with the increase of steps involved in the process, not only due to the capital and operating costs but also due to the overall yield reduction

(Janson and Rayden, 1998). So development of new techniques and methods for the separation and purification of biological macromolecules such as proteins and enzymes has been an important prerequisite for attaining commercially successful bioprocesses (Terpe, 2003).

In recent years, with advances achieved in the area of genetic engineering and recombinant technology, it is possible to change the properties of proteins in order to improve their purification properties. By inserting a segment of the DNA in the either the 3' or 5' end of translated DNA, the amino acid sequences on the either end of protein can be modified to provide a purification fusion. These fusions may be used to simply promote the protein from proteases attack by helping the protein to from an inclusion body or used to impart specific purification properties on the protein, making it suitable for immunoaffinity, metal chelate, ion exchange, hydrophobic chromatography, or partition. Alternatively, for well-characterized proteins, specific amino acids may be changed within the protein sequence to introduce patches with a specific affinity for adsorption matrix (Roe, 2001).

2.1.5.1 Affinity Based Bio-separation

In the last years, the implication of affinity term in the perspective of bio-separation has experienced evolutionary changes. Today, the application of molecular recognition phenomenon is not only constrained to the affinity chromatography areas. At the present time, membrane based purification, precipitation, as well as two-phase/ three-phase extraction methods can be resembled under the affinity based separation title. Major application of the affinity conception is in the area of protein recovery (Mondal et al., 2006). The fundamental steps that should be followed in majority of such applications are:

- Development of affinity microligand through connecting an affinity ligand to a polymeric material after surface activation;
- Affinity adsorption of interested protein by means of designed macroligand;
- Separation of protein-macroligand complex from the remaining aspects;
- Elution of the native protein from the complex.

In addition to the ligands illustrating biological affinity toward the specific protein (receptors and antibodies), a large variety of affinity ligands are currently utilized in the affinity based bio-separation including: dyes, chelated metal ions, peptides selected by phage display technology and ribosomal display methods, combinatorial synthesized peptides, aptamers attained via systematic evaluation of ligand by exponential enrichment (SELEX). Apart from these methods, the application of molecular modeling techniques offers significant facilities in the designing of biomimetic ligands and protein separation (Mondal and Gupta, 2006).

2.1.5.1.1 Antibodies

Antibodies are large Y-shaped proteins which are produced by the immune system in the response of foreign object attack such as bacteria and viruses (Litman et al., 1993). Antibodies have ability to identify and neutralize foreign objects by recognizing a unique part of the foreign targets, termed an antigen (Figure 2.12). Because of this property, antibodies can be used as ligands in affinity based bio-separation. Although, strong and specific interaction can be attained by the antibody, there is some difficulties related with desorption process since the use of harsh elution buffers with low pH values or detergents might be required. These harsh conditions trim down the separation efficiency: by denaturing proteins as well as antibody and by reducing antibody affinity via changing its tertiary structure (Clonis, 1990). In addition to these disadvantages, problems associated with columns coupling by antibodies furthermore their high prices decrease the popularity of antibody application in the separation processes (Revelet et al., 2006).

2.1.5.1.2 Reactive dyes

Reactive dyes are textile dyes that act as a competitive inhibitor for a protein's natural ligand (i.e. co-enzyme, substrate). There are a wide variety of dyes that can be used in large scale as affinity chromatographies. The utilization of dyes provides various advantages as: inexpensive and stable separation, simple immobilization,

mild elution condition and high yield. Generally reactive dyes show affinity toward the proteins that use NADH, NADPH or ATP (<http://www.mnstate.edu/provost/ReactiveDyeChromatogProtocol.pdf>). The application of reactive dyes in affinity based separation area was reported in 80s by several groups (Clonis and Lowe, 1981; Clonis et al., 1987; Denizli et al., 1997; Reyatonetti and Petrotti, 1999; Roy and Gupta 2000). Conversely, the moderate specificity of dyes and low reproducibility are the major drawbacks of these ligands (Clonis, 1990).

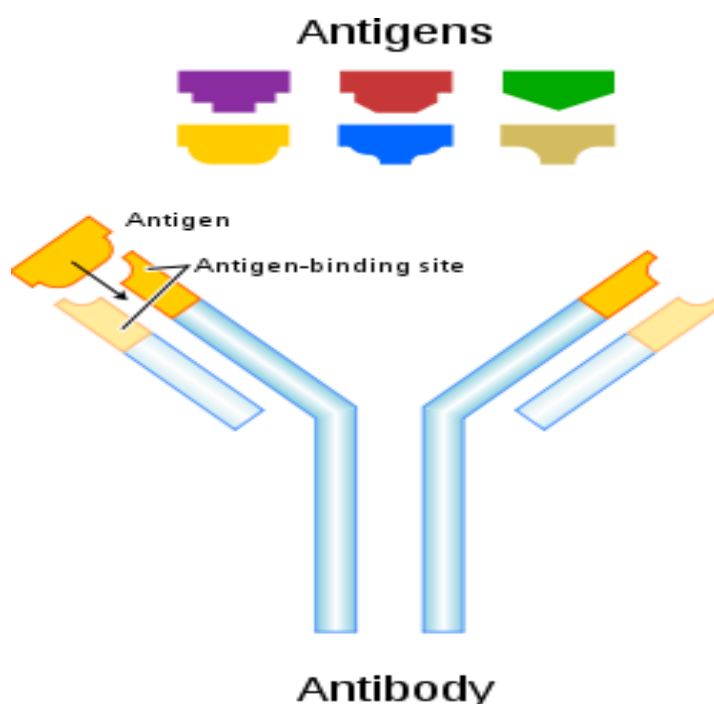


Figure 2.12 Interaction mechanism of antibody with target protein.

2.1.5.1.3 Chelated Metal Ions

Ni^{+2} , Cu^{+2} , Zn^{+2} , Co^{+2} , Mg^{+2} , Fe^{+3} are some metal ions that can be used as ligands for separation of proteins in immobilized metal affinity chromatography. The application of Ni^{+2} in separation of recombinant human prolactin was reported by Ueda et al. (2003). Thereafter, Gupta et al. (2003) investigated the efficiency of different metal ions in the separation of recombinant ovine growth hormone and

reported the higher purification yields 83% and 73.5% with Ni^{+2} and Cu^{+2} ions, respectively.

2.1.5.1.4 Aptamers

Aptamers are artificial nucleic acid ligands, specifically generated against certain targets, such as amino acids, drugs, proteins or other molecules. They function as a nucleic acid based genetic regulatory elements in vivo systems. Artificial ligands are generally isolated from combinatorial libraries of synthetic nucleic acid via SELEX technology (Figure 2.13) where in vitro iterative processes of adsorption, recovery and reamplification are followed (Mairal et al., 2007). There are several studies in the literature related with the application of aptamers for separation of different proteins such as: the purification of recombinant human L-selectin–immunoglobulin G (IgG) fusion protein from Chinese hamster ovary cell conditioned medium (Romig et al., 1999); the separation of cyclic AMP, NAD^+ , AMP, ADP, ADT and adenosine (Deng et al., 2001); and purification of human growth hormone (Çalık et., 2010). The major obstacle in the application of this separation method is the high cost of aptamers.

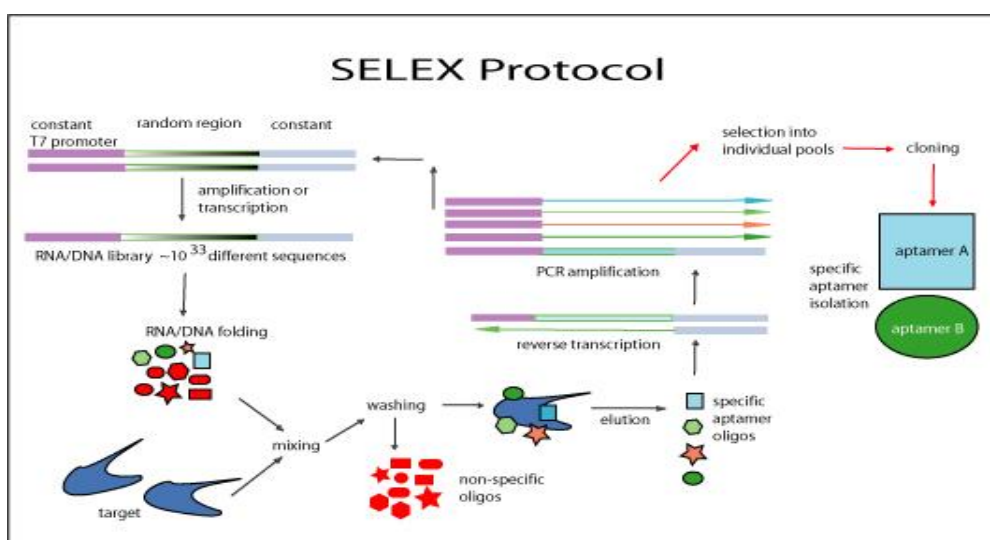


Figure 2.13 Illustration of SELEX protocol for Aptamer selection.

2.1.5.1.5 Affinity Tags and Fusion Proteins

One of the different strategies which have been developed to purify recombinant proteins is the insertion of a very small peptide (tag) to the sequence of target protein. Many different proteins, domains, or peptides can be fused with the target protein and these fusion-tags share the following features: one-step adsorption purification, a minimal effect on tertiary structure and biological activity, easy and specific removal to produce the native protein, simple and accurate assay of the recombinant protein during purification, applicability to a number of different proteins. Although, purification procedure of tagged protein is simple (Figure 2.14), the design of the protein fusion needs inputs from genetic and protein engineering contexts.

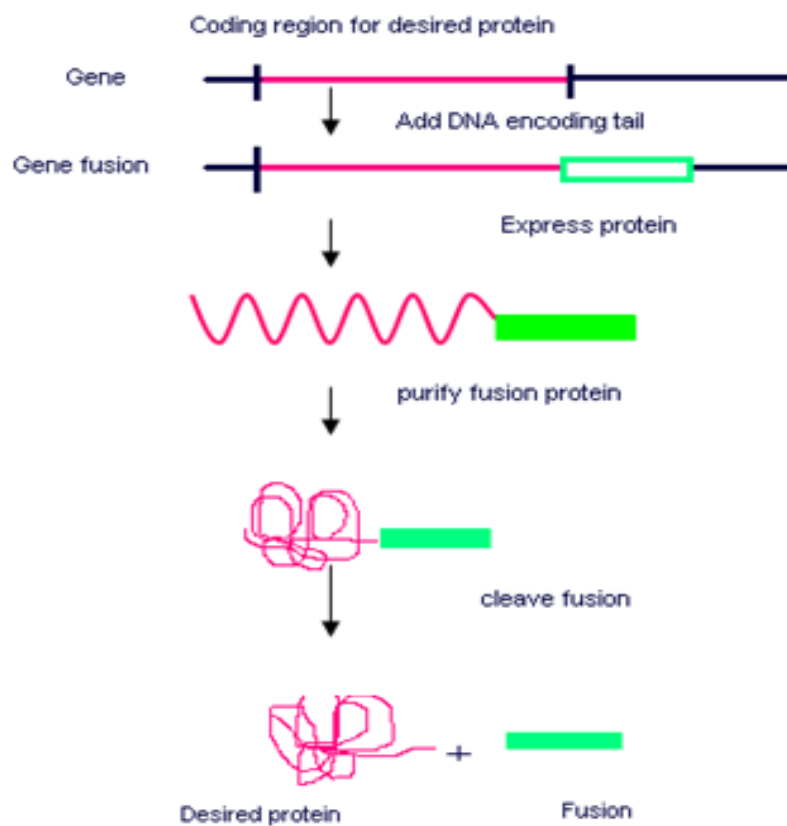


Figure 2.14 Schematic of fusion protein purification.

The most important item in the fusion separation is the end use of target protein. When the protein is being used as a bio-analytical reagent or as protein-enzyme in industry, it is acceptable to leave fusion tag on the protein, if, however, the protein is to be used as a long-term therapeutic product, the fusion protein will probably need to be removed in order to prevent an adverse immunological response. Therefore, by considering the end use of protein the purification steps should be modified. The most commonly used tags are poly-Arg, poly-His, and elastin-like polypeptides (ELPs).

The Arg-tag which consists of five or six arginine residues, was firstly described by Sassenfeld and Brewer (1984). In this study the arginine polypeptide was successfully applied as C-terminal tag in bacteria, resulting in recovery of recombinant protein up to 95% purity and a 44% yield. It was also mentioned that poly arginine might affect the tertiary structure of protein whose C-terminal region is hydrophobic. Hochuli et al. (1988) described a method to purify proteins with histidine tag and successfully purified poly-His-tagged dihydrofolate reductase enzyme by Ni^{+} -NTA matrices. In the study of Chaga et al. (1999-a), Talon was used to purify His-tagged proteins and it was stated that the use of Talon allows the elution of tagged proteins in the mild condition and results in less non-specific protein binding when compared with Ni^{+} -NTA resin. Poly histidine tag based protein purification has been carried out effectively in the several expression systems including bacteria (Chen and Hai, 1994; Rank et al., 2001), yeast (Borsing et al., 1997; Kaslow and Shiloach, 1994), mammalian cell culture (Janknecht et al., 1991; Janknecht and Nordheim, 1992), and baculovirus-infected insect cells (Kuusinen et al., 1995; Schmidt et al., 1998).

Elastin like polypeptides (ELPs) which are artificial biopolymer comprised of the pentapeptide repeat motif of Val-Pro-Gly-Xaa-Gly, undergo a thermosensitive sharp phase transition. Meyer and Chilkoti (1999) developed inverse transition cycling of elastin-like polypeptide fusion proteins as alternative to affinity based chromatography and stated that recombinant proteins tagged with ELPs can be separated by temperature stimulus procedure. Thereafter, Banki et al. (2005) used EPL tag in combination with self-cleaving inteins and introduced a new method for

the purification of recombinant proteins expressed in *E. coli* using self-cleaving elastin-like polypeptide fusion tags without the need for affinity chromatography and proteolytic tag removal.

2.1.5.1.6 Phage Display Technology and Combinatorial Peptide Libraries

Phage display technology employs filamentous fusion phage as a novel vector for the expression of exogenous peptides on the surface of bacteriophages. In 1985 George Smith reported that a foreign protein can be genetically fused to the N-terminus of the minor coat protein pIII of the filamentous phage fd, resulting in display of the protein on the surface of the virion. Since then, a large variety of phage displayed peptide and protein libraries have been constructed (Bass et al., 1990; McCafferty et al., 1990; Barbas et al., 1991; Smith, 1991; Smith and Scott, 1993; Cortese et al. (1996); Hoogenboom 2002, Szardenings 2003). Application of this technique offers various facilities in different subjects such as: identification of receptor-ligands in- vitro and in-vivo systems; bio-separation; gene delivery; and tumor targeting (Arap, 2005).

Related with in-vitro/in-vivo receptor-ligand selection, Huang et al. (1996) successfully used phage peptide library for selection of a ligand that interacts with von Willebrand Factor (vWF) and purified vWF efficiently from human plasma using affinity chromatography on immobilized ligand. Gaskin et al. (2001) described the use of a library of heptapeptide display on the surface of filamentous phage M13 as a potential source of affinity ligands for the purification of *Rhizomucor miehei* lipase. In this study also demonstrated that the interaction of the best ligand with lipase was due to both the heptapeptide sequence and the presence of a part of the phage coat protein. This conclusion was further verified by immobilizing the whole phage on the surface magnetic beads and using the resulted conjugate as an affinity adsorbent. At same year, Jost et al., (2001) used 7-mer phage library and selected THALWHT peptide as ligand for airway epithelial cell. Thereafter, in the study of Chen et al. (2006), the synthetic peptide (CSSSPSKHC) to facilitate transdermal insulin transport through intact skin cells, was selected via in vivo phage display technology.

In the recent study of Soykut et al. (2008) the phage display technology was successfully applied in the identification of peptides binding to staphylococcal enterotoxin B (SEB) which cause food intoxication and as a result three peptides with high affinity to SEB were selected.

Ligand selection toward a specific protein or another peptide via phage display technique is carried out in eight main steps;

- Step 1 (Gene insertion): phage display begins by inserting a diverse set of genes into the genome of phages. During the insertion process each phage receives a different gene.
- Step 2 (protein display): each phage receives only one gene, so each expresses a definite type of peptide or protein. Collectively, the population of phage can display a billion or more proteins as well as peptides, each tied to its own gene.
- Step 3 (library creation): a collection of phages that displaying a population of related but diverse peptides called a library.
- Step 4 (target exposure): the library is exposed to an immobilized target molecule such as a receptor, an enzyme, a peptide, or a protein. When the library is exposed to a target, some members of library will bind to target through an interaction between the displayed molecule and the target itself.
- Step 5 (binding): huge genetic diversity in the displayed peptides increases the likelihood that some phage will bind well or very well to the target.
- Step 6 (amplification): after binding step, the unbound phage will be washed away and specifically-bound phages will be eluted with lowering pH. Amplification of the bound phage in bacteria such as *E.coli* increases the amount of this phage several million-fold, providing enough material for further analyses.
- Step 7 (verification of binding): enzyme linked immunosorbent assay, ELISA, is conducted to determine relative strength of binding and to eliminate phages with low affinity toward the target.

- Step 8 (isolation and sequencing): Finally, the amino acid sequence of the peptide on the selected phage is attained by isolating the phage DNA and sequencing.

Then, the sequenced peptide can be artificially synthesized and used in Surface Plasmon Resonance (SPR) analyzes in order to determine kinetic parameters of the interaction. Figure 2.15 illustrates these steps, schematically.

2.1.5.1.7 Molecular Modeling

In the molecular modeling method, several programs are employed with the aim of simulating protein-ligand interaction at different environmental conditions, to examine any conformational changes that might be occurred during the interaction, to identify the active regions in structure of protein as well as ligand and to design novel affinity ligand. Novel affinity ligand development procedure is based upon determining what structure would precisely fit to an active site, an exposed region or a site where natural ligand binds and attempting to mimick a natural phenomenon of interaction (Labrou, 2003). This type of ligands is known as biomimetric ligands. Erickson et al. (2004) used four docking algorithms (DOCK, Flex X, GOLD and C DOCKER) to analyze a set of 41 protein-ligand complex structure by X-ray and reported that docking accuracy is inversely proportional to changes which a protein undergo as a result of induced fit. So, the application of docking methods in which both protein and ligand are considered as flexible molecules offers more accurate result.

2.1.5.2 Kinetic Parameters of Interaction

Evaluation of the thermodynamic characteristic of affinity based interaction provides insight about the mechanism of interaction and strength of binding. There for, if in affinity based separation the high yield of recovery is aimed, it is important to have idea about the thermodynamic characteristic of interaction. Surface Plasmon

Resonance is one of the widely used techniques for determination of kinetic parameters of interaction and it was effectively employed during this study.

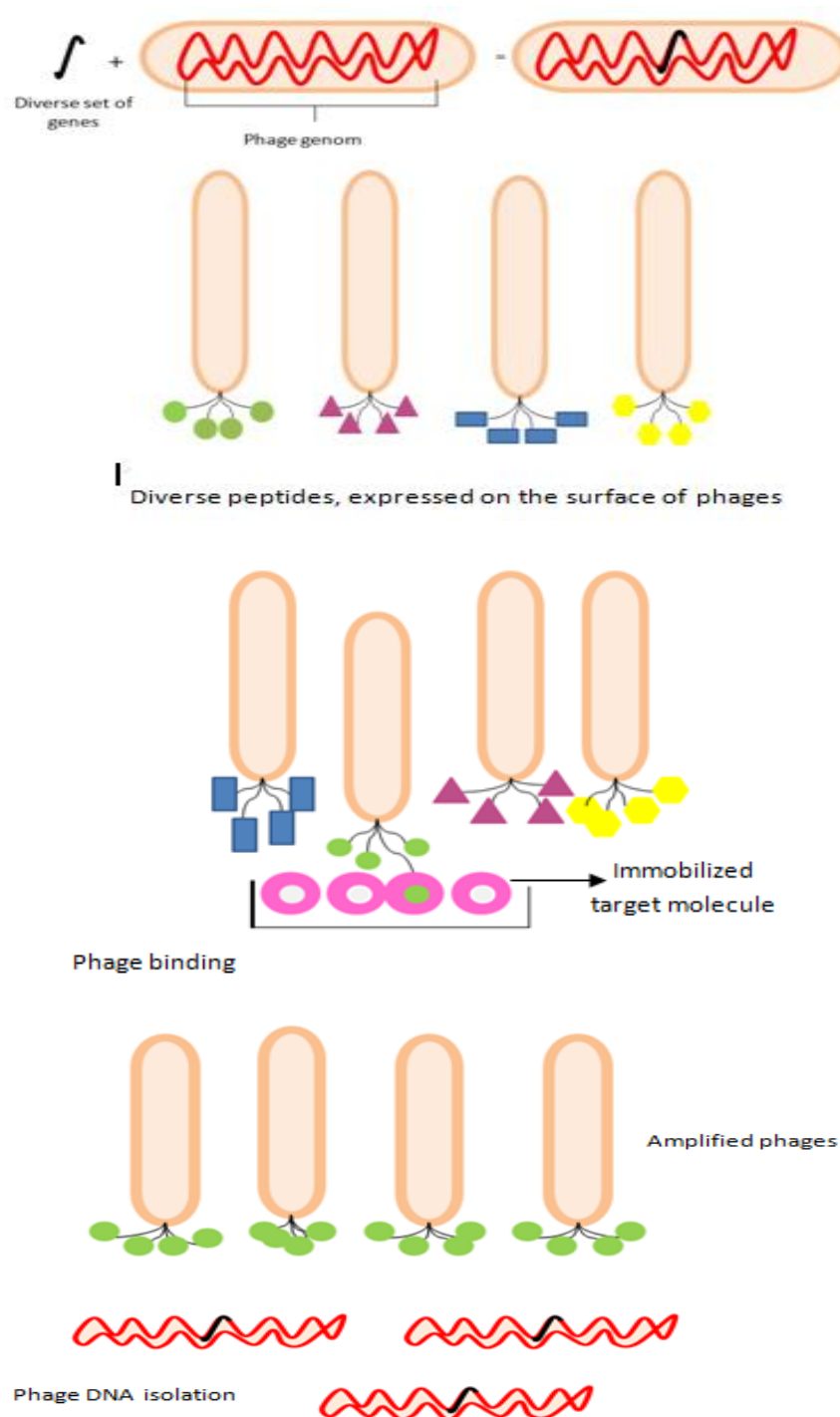


Figure 2.15 Phage display technology.

2.1.5.2.1 Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance is a charge-density oscillation that may exist at the interface of two media with dielectric constants of opposite signs. This technique plays an active role in real-time interaction sensing of biomolecular binding events. Since its first observation by Wood in 1902, the application of SPR technique has increased in the areas of instrumentation development and with the introduction of marketable SPR biosensor in 1990, the quantitative and qualitative characterization of interactions have become possible (Homola et al., 1999). Binding properties, specificity, association/dissociation kinetics and affinity constants are the important parameters that can be accurately determined by this method. Application of this method offers several advantages over the other methods such as:

- Label-free detection technique
- Distinguishes surface-bound material from bulk material
- Monitor molecular interactions in real-time (kinetics)
- Highly-sensitive (Δd_{film} of $\sim 1\text{-}2 \text{ \AA}$ or nanograms of adsorbed mass)
- Works in turbid or opaque samples

Related with biological application of SPR there is a large variety of studies in which almost all type of the interactions like DNA/DNA (Fisher et al., 1994; Yang et al., 1995), protein/protein (Edward et al., 1995; Karlsson al., 1997; Kim et al., 2006; Chen et al., 2009), RNA/DNA (Nelson et al., 2001 and 2002), protein/DNA (Bondeson et al., 1993; Lin et al., 2009; Dexheimer et al., 2010), protein/peptides (Soykut et al., 2008; Jackrel et al., 2009) and protein/ carbohydrates (Mackenzie et al., 1996) characterized by this technology. Furthermore, SPR can be successfully applied in the determination of the thermodynamic constant of the interaction between peptide selected by phage display library and other macro/micro-molecules. Jacobsen et al. (2007) used SPR to characterize the binding affinity of peptides selected by random phage library and urokinase-type plasminogen activator receptor (uPAR) and compared the strength of interaction with uPAR's monoclonal antibody. Similarly, in the study of Soykut et al. (2008), 12-mer peptide ligand for

Staphylococcal enterotoxin B (SEB) was selected and kinetic parameters of binding as well as its thermodynamic features were calculated.

In this study, SPR is utilized to calculate the binding affinity constant and thermodynamic properties of interaction between the peptide selected via phage display technology and artificially synthesized histidine poly-peptide.

2.1.5.2.1.1 SPR Theory

Generally, the SPR optical sensor consists of three main parts, an optical system; a transducing medium which interrelates the optical and (bio)chemical domains, and an electronic system supporting the optoelectronic components of the sensor and allowing data processing (Figure 2.16). SPR utilizes affinity optical sensor in order to measure the refractive index near a sensor surface which alters by changes in mass concentration at the sensor surface. When light is irradiated to the optical prism, a thin metallic film (usually 50 nm gold), the reflectivity of the light becomes almost zero at the angle of incidence where the surface plasma wave of gold surface can couple to the part of the incident light. This angle is called the SPR angle and is very sensitive to the properties of the metal film, film refractivity, and wavelength of the incident (Biacore, 1998; Yamamoto, 2008). In order to perceive any interaction in this system, firstly, one of the interacting partners (the ligand) is immobilized onto the sensor surface while the binding one (the analyte) is fed continuously to the flow cell. As a result of analyte binding to the immobilized ligand and accumulation of analyte on the surface of sensor, the refractive index increases. This change in refractive index is measured in real time, and the results plotted as response or resonance units (RUs) versus time to obtain sensorgram of the interaction. It is important that to record background response when there is a significant difference in the refractive indices of the running and sample buffers. For this purpose, the analyte is injected through a control or reference cell channel, which has no ligand or an irrelevant ligand immobilized to the sensor surface and the background response is subtracted from the sensorgram to obtain the actual binding response (van der Werve et al., 2001).

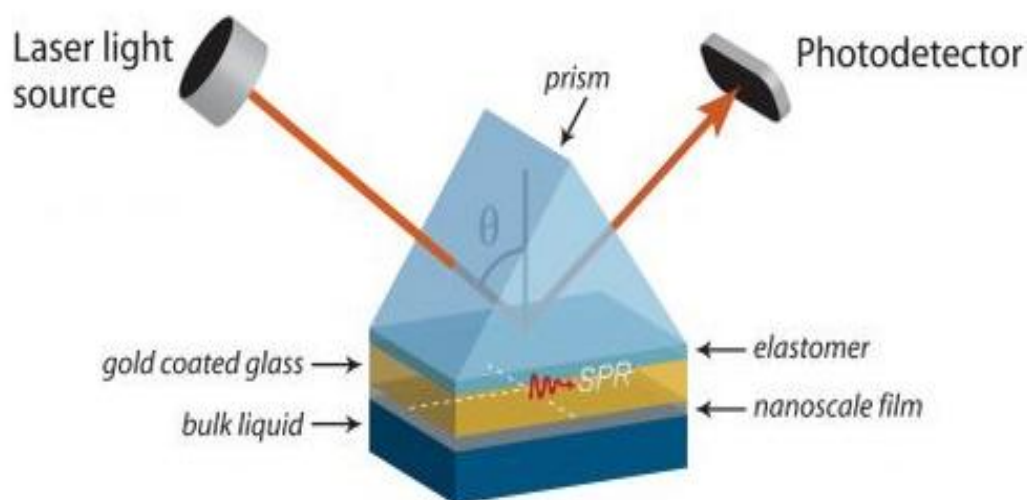


Figure 2.16 Structure of SPR optical sensor (<http://www.bionavis.com/technology/spr/>).

There are some important parameters that should be considered in the design of SPR experiment (Schuck, 1997):

- Selection of a proper immobilization procedure in which ligand retains its native conformation in an accessible manner.
- Determination of proper concentrations of both ligand and analyte.
- Preventing non-specific interactions.
- Eliminating the probability of mass transfer limitation occurrence.

2.1.5.2.1.2 Surface Modification

The use of untreated gold surface in SPR technology generally results in unstructured adsorption of proteins and bioactivity lost (Liedberg et al., 1983). Therefore, attempt for the creation of direct and label-free immobilization techniques is unavoidable. Surface modification technique provides facilities for successful immobilization of interacting species. There are two commonly used surface modification methods involving self-assembled monolayers (SAM) and dextran layer techniques. Self-assembled monolayers (SAMs) are highly ordered chemisorbed organic single layers formed by spontaneous adsorption of an active

amphiphilic surfactant onto a solid surface (Figure 2.17). SAMs are generated via chemisorption of hydrophilic head groups subsequent to a slow two-dimensional assembly of hydrophobic tail groups. The formation of well ordered coating is gradually progressed in the extent of time (8-24 h) when the hydrophobic clusters bring together on the surface of metal film and the hydrophilic tails organize away from the surface. Area of close-packed amphiphilic molecules nucleates and expands until the exterior of the solid is coated with a sole monolayer (Love et al., 2005). SAM formation is induced by using different surface-active organic species such as thiophenols, mercaptoimidazoles, mercaptoanilines, 11-mercaptoundecanoic acid (11-MUA), and 3-mercaptopropanoic acid (Rusmini et al., 2007). Co-adsorption of different surface-active organic components is also a common case in the creation of mixed SAMs. The mechanism of mixed SAMs as well as their chemical compositions and phase behaviors have been widely investigated in the literature. For example, in the studies of Bain et al. (1988) and Folkers et al. (1992), a variety of mixed SAMs with different chain length or different functionality was generated via co-adsorption of different surface-active components. In this study, 11-MUA was used for the generation of active gold surface to facilitate formation of densely packed and well ordered monolayer with reduced steric hindrance (Nam et al., 2004).

2.1.5.2.1.3 Ligand Immobilization

There two general methods for chemical immobilization of protein or ligand to the SAM surface. The first method is based on the biomolecular modification in which the specific reactive groups present on the biomolecule surface of interest are tagged in solution with an appropriate sulfur-containing molecule. The modified biomolecule is then self-assembled onto a solid substrate via the sulfur moiety.

In the second approach, the biomolecules are directly immobilized onto the SAM through the covalent binding to the surface of a monolayer containing free terminal groups such as amines through amide linkage formations or a cross-linking with the surface functional groups.

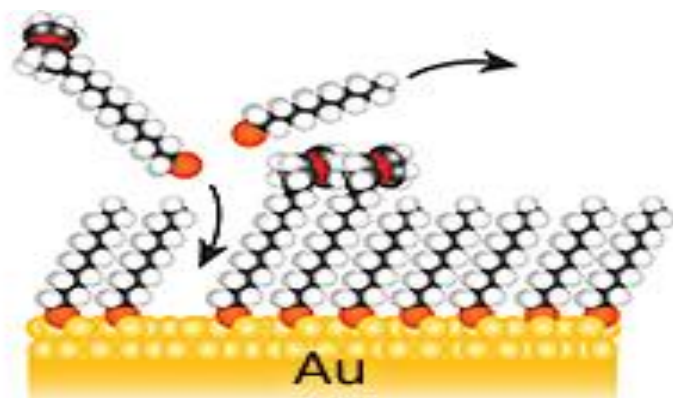


Figure 2.17 Schematic of SAM.

Direct immobilization method is suitable for orientational immobilization of any protein or ligand with moderate purity (>50%) and the pI value above 3.5. Major difficulties related with application of this method are: heterogeneous coupling of protein or ligand on SAMs, adsorption of contaminants and unwanted impurities onto the hydrophobic SAM surface, and complexity in protein regeneration (van der Werve et al., 2001). On the other hand, indirect method offers advantages of consistent and definite orientation of biomolecules on the SAM surfaces and the easier protein regeneration. However, application of this method is restricted to the proteins that contain proper binding sites or tags in their structures for covalent attachment of molecules. Depending on structure and functional group of the protein or ligand to be immobilized, different covalent coupling methods can be selected for different proteins. For example, where biomolecules with disulphide or a free cysteine groups can be easily immobilized by thiol chemistry, aldehyde coupling is widely used for immobilization of glycoproteins (van der Werve et al., 2001). Since both the ligand (6×histidine peptide) and anlyte (peptide selected via phage display) used in this study are peptides without special sites for indirect binding, direct amine coupling method was preferred for immobilization of ligand on SAM surface. For this purpose, firstly, the carboxylic end groups of SAM are activated by treating it with the solution of 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide and N-hydroxysuccinimide (EDC and NHS) with the aim of extremely reactive ester group creation to react with amine functional groups of the ligand via strong amide bonds

formation. Then, the ligand is immobilized on activated surfaces by injecting the known concentration of ligand. Finally, formation of non-specific interaction is prevented by blocking vacant carboxyl groups via treating the surface with ethanolamine (Subramanian et al., 2006; Dudak et al., 2010).

2.1.5.2.1.4 Interaction with the Analyte

Surface equilibration is the other important step subsequent to successful ligand immobilization. For this purpose, the surface will be washed with identical buffer in which the analyte is dissolved. The appropriate analyze buffer should have a low molarity, low salt concentration and a proper pH value for interaction and analyte stability. Thereafter, the interacting component solution is prepared by dissolving the definite amount of analyte in the buffer. The analyte solution with documented concentration is then injected onto sensor surface at constant temperature and flow rate to quantitatively determine the association and dissociation rate constants of interaction. The injection of second control solution (generally buffer) is also recommended in order to rule-out non-specific binding and any other refractive index artifacts. Finally, interaction process is optimized by considering the binding strength and mass transfer limitations at different pH value of the buffer, buffer type, ligand density, analyte concentrations, and injection flow rates (van der Werve et al., 2001).

2.1.5.2.1.5 Calculation of Affinity Constant Using Raw Data

There are several binding models that can be employed for the determination of association and dissociation rate constants. One site binding model is one of the commonly used fitting methods in the calculation of kinetic parameters of interaction that the nature of achieved binding sensorgrams is expected to be like as Figure 2.18 (Gedig, 2008).

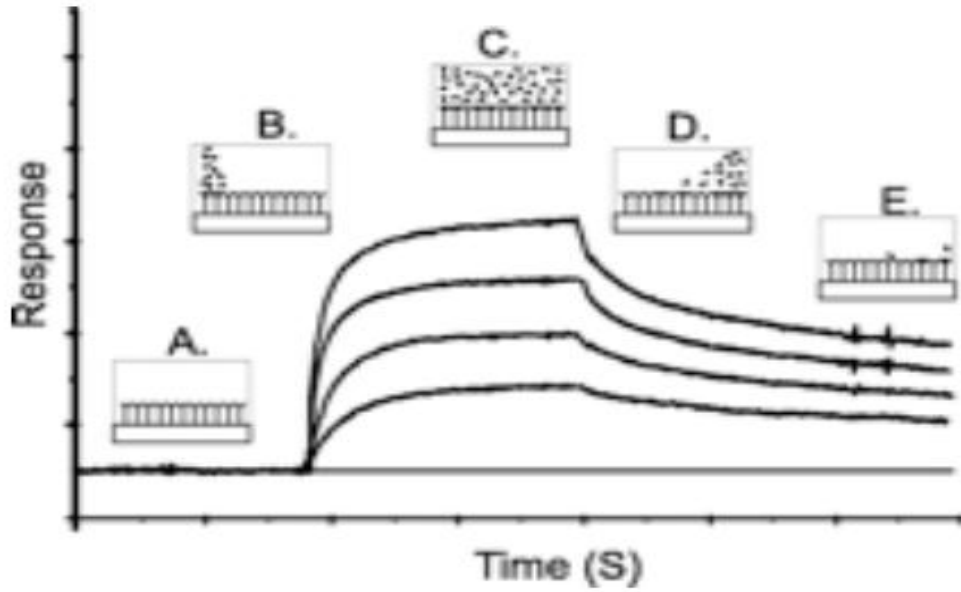


Figure 2.18 Sensorgrams of one site binding model at four different ligand concentrations.

The representative reaction for one site binding model is as follow,



Where k_{fwd} and k_{rev} are the association and dissociation rate constants; P, L and PL stand for the peptide, ligand and peptide-ligand concentration, respectively. Since, the equilibrium association constant, K_A ($L \text{ mol}^{-1}$), and equilibrium dissociation constant, K_D (mol L^{-1}), are inversely proportional with each other their reactions are defined as,

$$K_A = \frac{k_{fwd}}{k_{rev}} = \frac{[PL]}{[P][L]} \quad (2.2)$$

$$K_D = K_A^{-1} = \frac{k_{rev}}{k_{fwd}} = \frac{[P][L]}{[PL]} \quad (2.3)$$

where the terms in brackets indicate species concentrations in mol L^{-1} .

The variation occurred in the SPR equilibrium angle (R_{eq}) is collected and documented by the mean of evaluation software supplied by the equipment. Then, in order to obtain accurate association and dissociation rate constants even in low

strength binding as well as low ligand or analyte concentration, non-linear regression method is applied in the Langmuir binding isotherm equation (2.4);

$$R_{eq} = \left(\frac{[L]}{[L] + K_D} \right) M_{\max} \quad (2.4)$$

where $M_{\max}$ demonstrates the highest binding capacity.

Equation (2.4) re-arranged in the following manner to express the response signal for association (R_t) vs. time.

$$R_{tot} = \left(\frac{k_{fwd}[L]M_{\max}}{k_{fwd}[L] + k_{rev}} \right) (1 - e^{-(k_{fwd}[L] + k_{rev}) \cdot t}) \quad (2.5)$$

In order to simplify the equation (2.5), another parameter is defined as:

$$k_{obs} = k_{fwd}[L] + k_{rev} \quad (2.6)$$

Combining equations (2.6) and (2.5) results in;

$$R_t = \left(\frac{k_{fwd}[L]M_{\max}}{k_{obs}} \right) (1 - e^{-k_{obs} \cdot t}) \quad (2.7)$$

Therefore, k_{obs} can be calculated by fitting R_t vs. time data. With the aim of calculating k_{fwd} and k_{rev} , i.e K_A and K_D , diverse experiments are carried out at different ligand concentrations and obtained data are used with equation 2.6 to calculate k_{fwd} and k_{rev} from the slope and intercept of straight line of k_{obs} vs. $[L]$ plot, respectively. Subsequently, K_A and K_D values are easily calculated from equation (2.3).

2.2 Computation of Bioprocess Characteristics

2.2.1 Specific Rates and Yield Coefficients

Yield coefficients, and the specific rates of cell growth, product formation and substrate consumption are some important parameters which are calculated in fermentation processes in order to accurately characterize and evaluate the

bioprocess. The simple but effective manner for determination of cell growth, product formation as well as substrate consumption rates is concentration measurement of each component during the bioprocess. Generally, the rate of microbial growth is characterized by the specific growth rate since different cell concentration can be achieved at different bioprocesses. The cell growth or biomass formation rate, r_X , is described as the product of cell concentration (C_X) and specific growth rate (μ), i.e.,

$$r_X = \mu C_X \quad (2.8)$$

With the assumption that cells are not lost by sampling during the fermentation, general mass equation for fed-batch cultures take the form of:

$$r_X V = \frac{d(C_X V)}{dt} \quad (2.9)$$

Combining equations (2.8) and (2.9) results,

$$\frac{d(C_X V)}{dt} = \mu C_X V \quad (2.10)$$

In order to simply account volume change in the system due to the molasses feeding, variation in the density of culture is ignored, and from the overall mass balance,

$$\frac{dV}{dt} = Q \quad (2.11)$$

Substituting equation (2.11) into (2.10),

$$\frac{dC_X}{dt} = \left(\mu - \frac{Q}{V}\right) C_X \quad (2.12)$$

Equation (2.12) can be re-written in a following form to calculate specific growth rate as,

$$\mu = \frac{dC_X}{dt} \frac{1}{C_X} + \frac{Q}{V} \quad (2.13)$$

Substrate consumption rate, r_s , the product of cell concentration (C_X) and specific substrate consumption rate (q_s) can be expressed by equation (2.14),

$$-r_S = \frac{dC_S}{dt} = q_S C_X \quad (2.14)$$

In the course of fed-batch bioprocess in which nutrition is continuously fed to the system (molasses in this study), substrate mass balance is demonstrated as,

$$Q C_{S0} + r_S V = \frac{d(C_S V)}{dt} \quad (2.15)$$

By inserting equation (2.14) into (2.15),

$$Q C_{S0} - q_S C_X V = V \frac{dC_S}{dt} + C_S \frac{dV}{dt} \quad (2.16)$$

Generally, in the fed- batch fermentations nutrient feed rate is managed in respect to cell's substrate demand to avoid substrate accumulation. So, it is acceptable to consider system as quasi-steady and neglect the last term on the right hand side of the equation. With this assumption,

$$C_S \frac{dV}{dt} \sim 0 \quad (2.17)$$

equation (2.16) can be simplified by dividing it to V as well as C_X to obtain expression for specific substrate consumption rate as,

$$q_S = \frac{Q}{V} \frac{C_{S0}}{C_X} - \frac{1}{C_X} \frac{dC_S}{dt} \quad (2.18)$$

The term Q/V at equation (2.18) is termed as the extracellular dilution rate. The elimination of this term simplifies fed-batch process into batch operations. To summarize, equations (2.13) and (2.18) in combination with experimental data can be easily used for calculation of μ and q_S , respectively, during the fed-batch operation.

Whenever any particular rate, q_i or r_i is scaled with another rate q_j or r_j the yield coefficient Y_{ij} can be obtained (Nielsen et al., 2003). The biomass yield on substrate, biomass yield on oxygen and substrate yield on oxygen are the other important parameters in characterization of bioprocesses since they demonstrate the

efficiency of conversion of the substrate and oxygen into biomass. A list of frequently used yield coefficients is given in Table 2.5.

Table 2.5 Yield coefficients descriptions and their symbols.

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

The common method for calculation of overall cell yield on substrate is measuring the amounts of biomass formed and substrate consumed over a definite time period. So, the overall biomass yield on substrate can be defined as,

$$\bar{Y}_{X/S} = \frac{\Delta X}{\Delta S} \quad (2.19)$$

Where ΔX is the mass or moles of the cell produced, and ΔS is the mass or moles of the substrate consumed.

This is the overall yield coefficient that illustrates the efficiency of substrate conversion to biomass over the whole growth cycle and it takes constant value for a specific bioprocess. But it should be considered that in batch and fed-batch fermentations, the yield coefficients are not constant throughout the process since

they change with growth rate and metabolic functions of the microorganism (Scragg, 1988). Therefore, it is important to calculate instantaneous yield at a particular point in time. Instantaneous yield can be evaluated as follows:

$$Y_{X/S} = \frac{dC_X}{dC_S} = \frac{dC_X/dt}{dC_S/dt} \quad (2.20)$$

2.2.2 Oxygen Transfer Characteristics

Oxygen is the key substance of aerobic bioprocesses and due to its poor solubility in fermentation broths an incessant delivery of oxygen from the gas phase to the fermentation broth is crucial to minimize the oxidative metabolism of the cells (Nielsen et al., 2003). Oxygen depression in the fermentation of facultative aerobic organisms, such as yeast *S.cerevisiae* or the bacterium *E.coli*, will drastically alter their product formation (Nielsen et al., 2003). Transfer of oxygen from the fermentation medium to the cells carried out in several steps which were explained in details by Scragg, (1988) and Bailey and Ollis (1986). In the fermentation in which the cultivation broth is well-agitated and the cells are submerged in, the most important resistance for efficient oxygen transfer exists in the interphase of liquid and the gas bubbles; consequently transfer of oxygen from gas phase to cultivation broth is rate limiting step. At this condition, oxygen transfer rate (OTR) can be expressed as,

$$OTR = K_L a (C_O^* - C_O) \quad (2.21)$$

In which $k_L a$, the overall volumetric oxygen transfer coefficient, is the product of oxygen transfer coefficient (k_L) and the gas-liquid interfacial area (a). C_O^* and C_O are the terms representing saturated and real dissolved oxygen concentrations, respectively (Nielsen et al., 2003).

Maximum feasible oxygen transfer rate, the rate term attained at the saturated dissolved oxygen concentration, can be simply calculate via the following equation,

$$OTR_{max} = K_L a C_O^* \quad (2.22)$$

Furthermore, the expression for oxygen uptake rate (OUR) which illustrates the oxygen demand of cells through the fermentation is defined as,

$$OUR = -r_o = q_o C_x \quad (2.23)$$

where q_o symbolizes the specific oxygen consumption rate. There are several methods for the experimental determination of K_La . The Dynamic Method (Bandyopadhyay and Humprey, 1967), a commonly used approach due to its accuracy and simplicity, is based on a material balance for oxygen in the liquid phase (Scragg, 1988 and Rainer, 1990);

$$\frac{dC_o}{dt} = OTR - OUR = K_La(C_o^* - C_o) + r_o \quad (2.24)$$

A typical response curve of the Dynamic Method is given in Figure 2.19. In order to calculate K_La by this method, at the first step, cultivation medium is de-oxygenated by interrupting the air supply and decreasing agitation rate to diminish oxygen diffusion into medium. At some point in this definite time, dissolved oxygen concentration goes down to its minimal value and equation (2.24) reduces to:

$$\frac{dC_o}{dt} = r_o \quad (2.25)$$

This equation is used in region-II of the Figure 2.19, to determine oxygen uptake rate ($-r_o$). At the next step (region-III), air flow is re-supplied to medium, and the raise in dissolved oxygen concentration is monitored and documented with respect to time. Equation (2.24) can be accurately applied at this region and as a result of combining the equation (2.25) with (2.24) and re-arranging, K_La can be calculated from the following equation.

$$C_o = -\frac{1}{K_La} \left(\frac{dC_o}{dt} - r_o \right) + C_o^* \quad (2.26)$$

For this purpose, C_o is plotted versus $dC_o/dt - r_o$ in which K_La can be easily determined from the slope of plot (Figure 2.20). This procedure is also successfully employed to calculate physical mass transfer coefficient, K_La_0 , in the production

medium at the absence of microorganism by considering $r_o=0$ (Nielsen et al., 2003). In this situation equation (2.26) is simplified as,

$$C_O = -\frac{1}{K_L a} \frac{dC_O}{dt} + C_O^* \quad (2.27)$$

And again the slop of C_O versus dC_O/dt plot presents physical mass transfer coefficient.

In order to compare the relative rates of maximum oxygen transfer and biochemical reactions and find the rate limiting step of the bioprocess, the maximum possible oxygen utilization rate (OD=Oxygen Demand) should be calculated. Maximum possible oxygen utilization rate is defined as (Çalık et al., 2000),

$$OD = \frac{\mu_{max} C_X}{Y_{X/O}} \quad (2.28)$$

Generally, in the aerobic fermentations effectiveness factor (η) together with Damköhler number (Da), defined as oxygen uptake rate per maximum possible oxygen and the maximum possible utilization rate per maximum transfer rate, respectively, are calculated to interpret oxygen limitations throughout the bioprocess. The associated expression for η and Da are given in equations (2.29) and (2.30), respectively;

$$\eta = \frac{OUR}{OD} \quad (2.29)$$

$$Da = \frac{OD}{OTR_{max}} \quad (2.30)$$

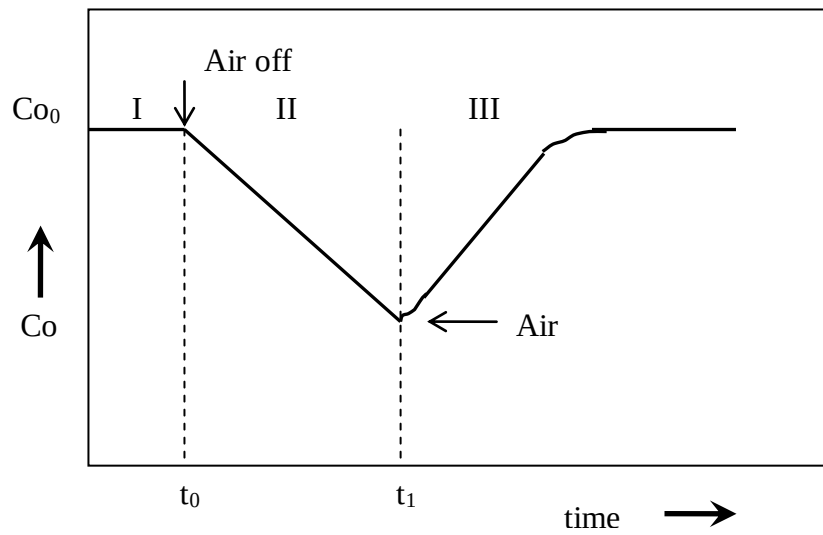


Figure 2.19 The representative plot of dissolved oxygen concentration vs time during K_La determination via Dynamic method.

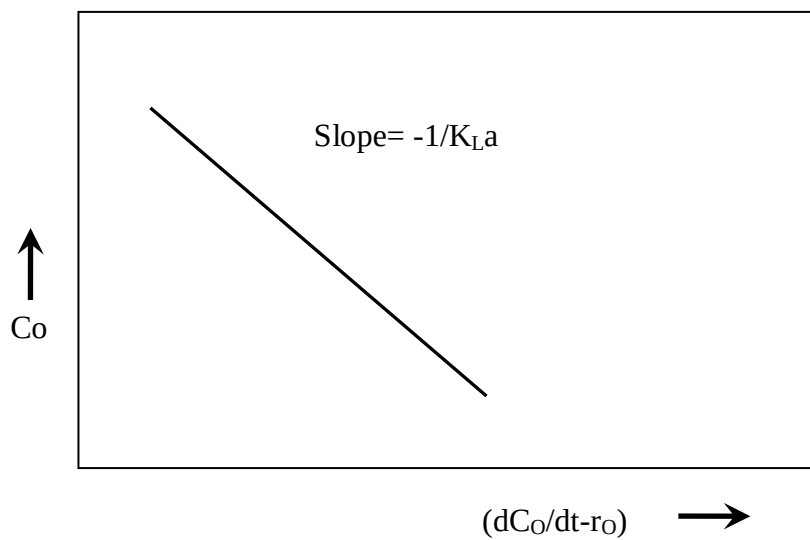


Figure 2.20 Calculation of K_La from the slope of C_o vs $(dC_o/dt - r_o)$ plot.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals were analytical grade except otherwise affirmed, and all used chemicals are listed in Appendix A.

3.2 Laboratory Equipment

The list of laboratory equipment used in this study is given in Appendix B.

3.3 Buffers and Stock Solutions

The buffers and stock solutions used in this study are given in Appendix C. The sterilization of solutions was performed via autoclaving for 20 min at 121°C or by filter sterilization through 0.25/0.45 µm filters (Sartorius AG, Germany) depending on the type of substances dissolved in.

3.4 Strains, Plasmids and Maintenance of Microorganisms

The *xyl* genes employed for cloning to *P.pastoris* and *E.coli* strains were amplified from chromosomal DNA of *Pyrococcus furiosus* and *Thermus thermophilus* HB8, respectively. Both *T.thermophilus* HB8 DSM-579 and *P. furiosus* DSM-3638 strains purchased from cell culture collection of DSMZ (Braunschweig, Germany). *E. coli* BL21 (DE3) pLysS /Star and XL1-Blue strains

purchased from Invitrogen Life Technologies (Carlsband, CA, USA), used for expression of *xyl* gene and plasmid propagation in *E.coli*, respectively. *P.pastoris* X-33 wild type strain as well as pPICZαA shuttle vector carrying Zeocin resistance were also purchased from Invitrogen. The recombinant *E. coli* strain expressing *T.thermophilus xyl_T* gene was named as *E. coli* V11. And recombinant *P.pastoris* strains which produce GI enzyme in the extracellular and intracellular manners, were named as *P.pastoris* V55 and V57, respectively. The strains and plasmids used in this study are summarized in Table 3.1. The sequences and schematic representation of the plasmids are given in Appendix D. For long-term storage of microorganisms, the microbanks (PRO-LAB) were made, for this purpose young colonial growth cells were inoculated into the vial containing cryopreservative solution and gently mixed to facilitate adsorption of cells into the porous beads dispersed in the solution. Thereafter, excess cryopreservative solution was removed from vial by pipetting it out and inoculated cryovial stored at -80°C.

Table 3.1 Strains and plasmids used in this study.

Genus	Species	Strain	Genotype/plasmid	Source
<i>Escherischia</i>	<i>coli</i>	XLBlue	Wild type	Invitrogen
<i>Escherischia</i>	<i>coli</i>	BL21 (DE3)	pLysS	Invitrogen
<i>Escherischia</i>	<i>coli</i>	BL21 (DE3)	star	Invitrogen
<i>Escherischia</i>	<i>coli</i>	XLBlue	pRSETA:: <i>xyl_T</i>	This study
<i>Escherischia</i>	<i>coli</i>	XLBlue	pRSETA:: <i>xyl_P</i>	This study
<i>Escherischia</i>	<i>coli</i>	V 11	pLysS, pRSETA:: <i>xyl_T</i>	This Study
<i>Escherischia</i>	<i>coli</i>	ER2738	Wild type	Phage display kit
<i>Thermus</i>	<i>thermophilus</i>	HB8 DSM 579	Wild type	DSM
<i>Pyrococcus</i>	<i>furiosus</i>	DSM 3638	Wild type	DSM
<i>Pichia</i>	<i>pastoris</i>	X-33	Wild type	Invitrogen
<i>Pichia</i>	<i>pastoris</i>	V55	pPICZαA:: <i>xyl_{Pext}</i>	This study
<i>Pichia</i>	<i>pastoris</i>	V57	pPICZαA:: <i>xyl_{Pint}</i>	This study

3.5 Growth Media

3.5.1 The Solid Medium

Recombinant *P. pastoris* strains were cultivated on YPD Agar slants (Table 3.2) by incubating them at 30°C for 48-72 h. The freshly prepared LSLB Agar slants were used for propagation of recombinant *E.coli* strains stored in microbanks at -80°C. The Agar plates, inoculated with recombinant *E.coli* cells, were incubated at 37°C for 12 h (Table 3.3). According to the antibiotic resistance capability of the microorganisms (Ampicillin resistance in pRSET vector; Chloramphenicol resistance in BL21 (DE3) pLysS cells; Zeocin resistance in pPICZαA vector), the proper concentration of antibiotics were added to solid medium after steam sterilization at 121°C for 20 min. Table 3.4 represents the list and concentrations of the antibiotics supplied to the cultivation media for maintain of recombinant plasmids.

Table 3.2 The content of utilized solid medium (YPD) for *P. pastoris* strains.

Compound	Concentration, kg m ⁻³
Yeast extract	10.0
Peptone	20.0
Glucose	20.0
Agar	20.0
pH	07.0

Table 3.3 The content of utilized solid medium (LSLB) for *E.coli* strains.

Compound	Concentration, kg m ⁻³
Yeast extract	05.0
Soytryptone	10.0
NaCl	05.0
Agar	15.0
pH	0.70

Table 3.4 The final concentration of antibiotics in the media.

Antibiotic	Concentration, kg m ⁻³
Ampicillin	0.100
Chloramphenicol	0.035
Zeocin for <i>E.coli</i>	0.025
Zeocin for <i>P. pastoris</i>	0.100

3.5.2 The Precultivation Medium

The recombinant *P. pastoris* and *E.coli* species, propagated in the solid medium, were inoculated into precultivation medium. The cell growth is carried out in orbital shakers (B.Braun, Certomat BS-1) with agitation and heating rate controller using air-filtered Erlenmeyer flasks at optimum growth condition which is T=30°C, N=225 min⁻¹ and t=24 h, for *P. pastoris* species and T=37°C, N=200 min⁻¹ and t=12 h for *E.coli* strains. The compositions of precultivation medium for *P.pastoris* and *E.coli* strains were given in Tables 3.5 and 3.6, respectively. The selective antibiotics were also added to the precultivation medium in amounts mentioned in Table 3.4 after sterilization.

Table 3.5 The content of employed precultivation medium for *P. pastoris* strain (BMGY).

Compound	Concentration, kg m ⁻³
Yeast extract	10.0
Peptone	20.0
Potassium phosphate buffer pH 6.0	0.1 M
YNB	13.4
Biotin	4×10 ⁻⁵
Glycerol	10.0

Table 3.6 The composition of the precultivation medium for *E.coli* strains.

Compound	Concentration, kg m ⁻³
Soytryptone	10.0
Yeast extract	05.0
NaCl	10.0

3.5.3 The Production Medium

3.5.3.1 Production Medium for *P.pastoris* Species

The propagated recombinant *P. pastoris* cells in precultivation medium were harvested by centrifugation at 4000 rpm for 10 min⁻¹ at room temperature and resuspended in BMMY production medium (Table 3.7). Although, the addition of zeocin to production medium was not essential due to genomic integration of the recombinant gene, chloramphenicol was added in order to protect the cultivations from contaminants. The laboratory scale experiments for microbial growth and medium design were executed in air filtered, baffled Erlenmeyer flasks 250 ml in size that had working volume capacities of 50 ml and the recombinant cells incubated at T=30°C and N=225 min⁻¹ for t=24 h.

Table 3.7 The composition of the complex production medium of *P. pastoris* strain (BMMY).

Compound	Concentration, kg m ⁻³
Yeast extract	10.0
Peptone	20.0
Potassium phosphate buffer pH 6.0	0.1 M
YNB	13.4
Biotin	4×10 ⁻⁵
Methanol	10.0

3.5.3.2 Production Medium for *E.coli* Species

The recombinant *E.coli* strain proliferated in the precultivation medium was subsequently transferred into the production medium at the inoculation ratio of 1:10. The laboratory scale experiments for cell growth were conducted in air filtered, Erlenmeyer flasks with the size of 150 ml and working volume of 33 ml where the recombinant *E.coli* cells incubated at $T=37^{\circ}\text{C}$ and $N=200\text{ min}^{-1}$ for $t=12\text{ h}$.

The pilot scale 3.0 dm^3 fermentor (Braun CT2-2), having a working volume of $1\text{--}2.0\text{ dm}^3$ and including temperature, pH, foam, stirring rate and dissolved oxygen control units was used during the batch and fed-batch experiments. The efficient temperature control and sterilization provided in bioreactor by using an external cooler, steam generator and a jacket around the bioreactor. The fermentor was agitated with two four-blade Rushton turbines and consisted of four baffles and a sparger. Air was supplied through a compressor and oxygen through a pure oxygen tube. The inlet air was enriched with oxygen passing thorough a mass flow controller when needed. This bioreactor system (Figure 3.1) was used to investigate the effects of oxygen transfer and different feeding strategies on the recombinant thermostable GI production by *E.coli* BL21 (DE3) pLysS::xyl_T. The pilot scale cultivations were performed at $T=37^{\circ}\text{C}$, and $\text{DO}=40\%$ which was cascaded to agitation as well as air flow rate. The pH of the medium maintained at $\text{pH}=7.2$ value through the bioprocesses with 5 M KOH and 5 M H_3PO_4 .

3.5.3.2.1 Fed-Batch Experiments

The glucose based defined medium and molasses based complex medium having an initial volume of 1L used for fed-batch production of r-GI. Substrate feeding was initiated after $t=8\text{--}9\text{ h}$ of batch phase and the pH of cultivation was controlled at a constant value of $\text{pH}=7.2$ throughout the processes. With the aim of developing an efficient exponential feeding strategy for valuated recombinant enzyme production, the influence of different specific growth rates at the values of 0.1, 0.15, and 0.2 was systematically examined on the r-GI production. Glucose or

pretreated beet-molasses was exponentially fed into the bioreactor in accordance with the subsequent equation (Weigand et al., 1979).

$$F(t) = \frac{C_{x0}V_0\mu_0}{C_{s0}Y_{x/s}} \exp(\mu_0 t) \quad (3.1)$$

Where μ_0 is the pre-determined specific growth rate, V_0 is the preliminary volume of cultivation medium before starting to feed, C_{x0} symbolizes the cell concentration attained at the end of batch phase, C_{s0} represents the concentration of substrate intended to feed, and $Y_{x/s}$ is cell yield on substrate. In the fed- batch phase, the pre-fixed parameters were taken as: $Y_{x/s}=0.32$ and 0.3 (Çalık and Levent, 2009-b; Kaya et al., 2009) with $C_{s0}=250$ and 200 kg m^{-3} , respectively for molasses and glucose based exponential feeding profiles. The chemical compositions of production media used at the batch mode for molasses based and glucose based fermentations were specified in Table 3.8.

Table 3.8 The content of utilized production medium for *E.coli* species.

Compound	Concentration, kg m^{-3}	
	Molasses based	Glucose based
Molasses	30	0.0
Glucose	0.0	8.0
Na_2HPO_4	6.7	6.7
KH_2PO_4	3.1	3.1
$(\text{NH}_4)_2\text{HPO}_4$	5.0	5.0
NaCl	0.5	0.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5	0.5
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.2×10^{-5}	0.2×10^{-5}
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1	0.1

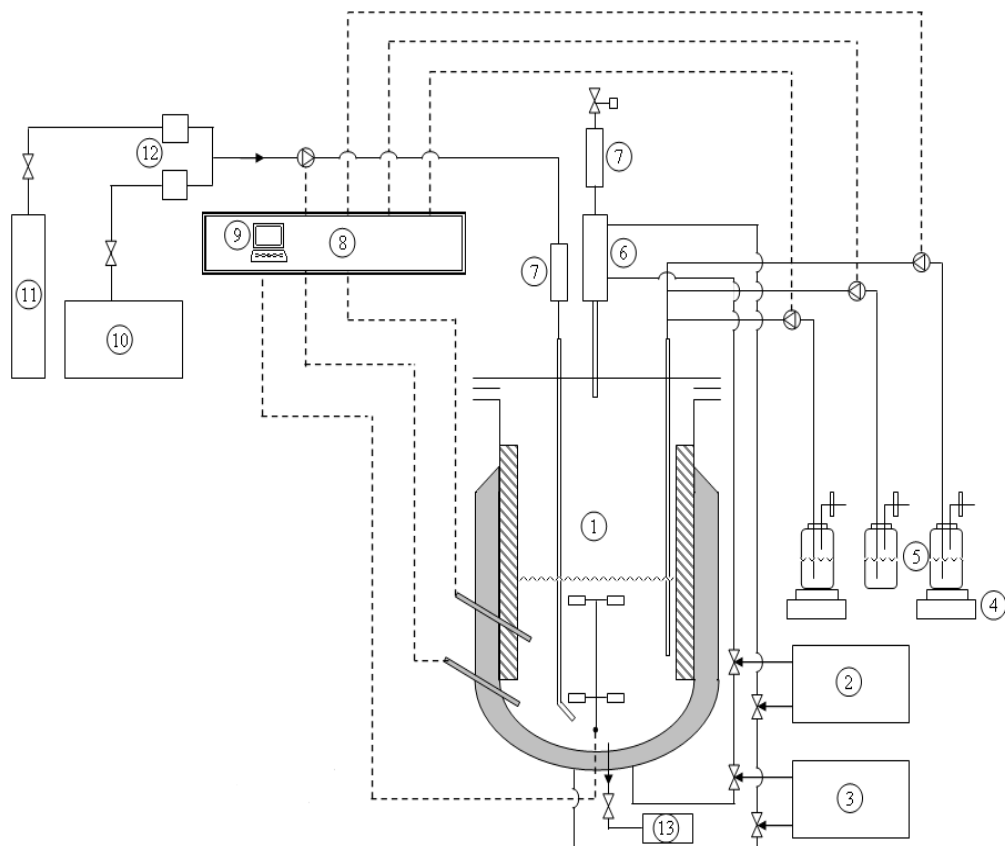


Figure 3.1 Illustration of employed pilot scale bioreactor system: (1) Fermentor, Biostat CT2-2 (2) Cooler (3) Steam generator (4) balances (5) substrate, antifoam and base reseviors (6) Exhaust cooler (7) Air filters (8) regulator (9) Biostat CT Software (10) Air compressor (11) Pure O₂ reservoir (12) Digital mass flow meters (13) Sampling valve (Çelik, 2008).

3.5.3.2.2 Pretreatment of Molasses

Beet molasses complex medium with the composition of: 50% sucrose, 18% water, 12% organic non-sugar compounds including nitrogen element in their structure, 10% inorganic components, 8% organic non-sugar matters with no nitrogen element in their structure, 1.2% raffinose, 0.3% invert monosaccharide like a glucose and fructose, 0.3% galactinol and 0.2% kestose and pH=7.5 was provided from Ankara Beet Sugar Factory (Turkey). Acid hydrolysis method (Çalık et al. 2003) was systematically employed for chemical modification of molasses as follows:

- Dilution: 125 g of beet molasses was dissolved in ultra pure water to obtain a 250 ml solution.
- Centrifugation: Two rounds of 20 min centrifugation at 6,000 rpm and temperature of +4°C were performed to precipitate insoluble matters and separate impurity.
- HCl hydrolysis: The pH of the beet molasses was reduced to pH=1.8 by addition of 37% HCl at room temperature following with incubation in the water bath at 90°C for 3 h in order to efficiently hydrolysis the disaccharide (sucrose) and trisaccharide (raffinose) available in molasses to their monomer such as glucose, fructose and galactose.
- pH adjustment: The solution of 5 M KOH was gradually added into pretreated molasses to increase pH=7.2, the desired pH value for microbial growth.

3.5.3.2.3 Fed-Batch Operations Abbreviation

For straightforward description of the bioprocesses, the conducted fed-batch experiments were coded as listed in Table 3.9.

Table 3.9 Explanations for the fed-batch operation codes.

Exponential Feeding		
Operation Code	Feed kind	Predetermined specific growth rate, μ , h ⁻¹
G-0.15	glucose	0.15
M-0.10	molasses	0.10
M-0.15	molasses	0.15
M-0.20	molasses	0.20

3.6 Analysis

In order to collect data that precisely describe the conducted experiments, samples were taken from cultivation media at particular times in the course of the fermentations. Subsequent to cell concentration measurement, the samples were centrifuged at 6000 rpm for 10 min at 4°C to separate supernatant from the cell pellets. In recombinant *E. coli* and *P.pastoris* strains producing r-GI intracellularly, the cell pellet was utilized to measure glucose isomerase activity after the cell wall lysis; where supernatant was utilized throughout extracellular r-GI_p activity determination moreover in glucose, fructose and organic acid concentrations measurement assays.

3.6.1 Cell Concentration

Cell concentration on the bases of dry weights was determined via a UV-Vis spectrophotometer (Thermo Spectronic, Heλiosα) at 600nm and to obtain more accurate data 1ml of sample taken from cultivation medium was diluted with dH₂O to read OD₆₀₀ in the acceptable range of 0.1-0.8. The calibration curves for converting measured optical density of *E. coli* and *P.pastoris* to cell concentration, C_x(kg m⁻³) are given in equations (3.2) and (3.3), respectively.

$$C_x = \frac{OD_{600}}{2.8782} \times \text{Dilution Rate} \quad (3.2)$$

$$C_x = 0.275 \times OD_{600} \times \text{Dilution rate} \quad (3.3)$$

3.6.2 Glucose Isomerase Activity Measurement

GI activity determination method is based on spectrophotometric measurement of the conversion of D-glucose into D-fructose. One unit of GI activity was defined as the formation of 1μmol of D-fructose per minute at 80°C. The GI activity measurement method can be summarized as:

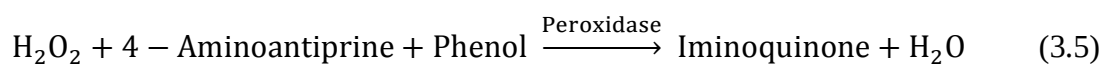
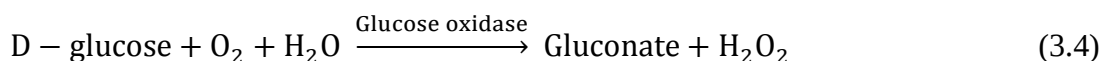
1. Definite amount of samples were taken from culture broth,
2. Cells were precipitated by centrifugation at 6000 rpm for 10 min,
3. Supernatant was removed and crude cell extracts were resuspended in 970 μ l of 0.1 M potassium phosphate buffer.
4. The cell wall lysis at $f=10\text{ s}^{-1}$ for 10 min was performed at the agitator bead mill (Retsch, MM 200) via adding glass beads into cell suspension.
5. After cell wall disruption, 50 μ l of the intracellular enzyme solution was taken from the mixture and added to 50 μ l of freshly prepared activity buffer, containing 0.4 M D-glucose and 10 mM MnCl_2 in 0.02 M potassium buffer pH 7.0 at 80°C.
6. The reaction mixture with 100 μ l final volume was incubated at 80°C water bath for 10 min and the isomerisation reaction was terminated after 10 min through denaturizing thermostable recombinant enzyme by adding 60 μ l of reaction mixture in to the tube containing 540 μ l of 0.1M HCl.
7. The cysteine-carbazol-sulfuric acid method (Dische and Borenfreund, 1951) was utilized to determine concentration of D-fructose formed at the end of reaction.

3. 6.2.1 Cysteine-Carbazol-Sulfuric acid method

After the reaction termination, 1.8 ml of 70% (v/v) Sulfuric acid, 60 μ l of freshly prepared 1.5%(w/v) Cystein-HCl, and 60 μ l of freshly prepared 0.12%(w/v) carbazol in 95% ethanol were correspondingly added into the reaction tube. Finally, formation of D-fructose from D-glucose after 30 min incubation at room temperature was measured colorimetricly by using a UV-Vis Spectrophotometer at the absorbance of 560 nm. The detected absorbance was converted to concentration using calibration curve for D-fructose given in Appendix E.

3.6.3 Glucose Concentration

Glucose analysis kit (Biasis, Ankara) functioning on the basis of glucose oxidation method was applied in order to measure glucose concentrations, C_G (kg m^{-3}), via UV-Vis Spectrophotometer at wave length of 505 nm (Boyaci et. al., 2005). In the first step of the assay, glucose oxidase enzyme was used to carry out the oxidation reaction of D-glucose and water into gluconate and peroxide (equation 3.4). In the second step, the created peroxide is reacted with phenol and 4-aminoantipyrine under the catalytic activity of peroxidase enzyme to produce red colored iminoquinone component equimolar with glucose (equation 3.5).



The analysis kit contains: a glucose assay reactive mixture including glucose oxidase, peroxidase and 4-aminoantipyrine; buffer solution to provide phenol together with potassium dihydrogen phosphate substances. Ultra pure water used for dilution of samples with the probable glucose concentration, higher than 2 kg m^{-3} . The 0.05 ml of each sample was added to the freshly prepared mixture of 0.05 ml glucose assay reactive solution, 0.40 ml assay buffer and 2.05 ml distilled water; and incubated for 20 min at 37°C water bath. Finally, the absorbance of formed red color was spectrophotometrically measured at 505 nm. With the aim of relating the detected absorbances to glucose concentration, standard solutions at the different glucose concentration below the value of 2 kg m^{-3} were prepared and analyzed simultaneously with sample sets to develop accurate standard glucose calibration curve as given in Appendix F.

3.6.4 Organic Acid Concentrations

Reversed phase HPLC system (Waters HPLC, Alliance 2695, Milford, MA) was employed in the determination of organic acid concentrations during the bioprocesses. The Capital Optimal ODS-5Xm column (Capital HPLC, West Lothian,

UK) was installed for concentration measurement in which a mobile phase (3.12% (w/v) NaH_2PO_4 and $0.62 \times 10^{-3}\%$ (v/v) H_3PO_4) containing filtrated samples was passed through the analyze column under the conditions given in Table 3.10.

In order to develop standard calibration curves for detected organic acids, solution of pure organic acids were prepared at different concentrations and analyzed via HPLC system and the areas of observed peaks were plotted vs. their concentrations for each definite organic acid. Afterward, the standard curve of each organic acid was specifically employed to convert the peak's area of related organic acid into concentration. The standard calibration curves of the organic acids, detected during the fed-batch experiments, and their equations were listed in Appendix G.

Table 3.10 Analyze method applied in determination of organic acid concentration.

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

3.6.5 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

The Dynamic method as clarified in the section 2.2.2 was used to calculate liquid phase mass transfer coefficients and oxygen uptake rates in the course of the rGI production. In this order, dynamic oxygen transfer assays were conducted at characteristic cultivation times with short duration to prevent negative effect of oxygen starvation on biological functions of the cells. Throughout this period the air

delivery to cultivation medium was completely blocked and the stirring rate was decreased to $N=50 \text{ min}^{-1}$ to minimize the effect of surface aeration.

3.7 Genetic Engineering Techniques

3.7.1 Enzymes, Kits, and Molecular Size Markers

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

Plasmid Purification Kits and PCR Purification Kits were provided from Fermentase Inc. Gene Elution Kit was purchased from Gene Molecular Biology Tools. Lambda DNA/HindIII Markers, Gene Ruler 50 bp DNA ladders and 6X Loading Dyes were the other materials obtained from MBI Fermentas.

3.7.2 Plasmid DNA Isolation from *E. coli*

Plasmid DNA (i.e. pRSETA and pPICZ α A vectors) from *E. coli* cells was extracted using Fermentas Mini-Prep, according to manufacturer's instructions. For this purpose, a single colony from selective solid medium was inoculated into 5 ml selective liquid medium and grown overnight at 37°C and 200 rpm in 30 ml universal tube. At the end of the protocol, 30-50 μl of dH₂O was used to elute DNA from spin column and the extracted plasmid DNA was stored at -20 °C.

3.7.3 Chromosomal DNA Isolation from *Thermus thermophilus* and *Pyrococcus furiosus*

A 5-ml culture of *Thermus thermophilus* HB8 was harvested at the OD₆₀₀ of 1.8 and the cell pellet was resuspended in 0.5 ml STE buffer. RNase A was added to a final concentration of 100 mg ml⁻¹, SDS to 8.5 mg ml⁻¹, and proteinase K to 100 mg ml⁻¹. Incubation for 2 h at 37°C was followed by five times phenol: chloroform:

isoamylalcohol extractions, ethanol precipitation, and resuspension in 30 μ l of sterile dH₂O.

Related with *Pyrococcus furiosus*, a 50-ml of cell culture was harvested at 6000 rpm for 15 min and the cell pellet was resuspended in 0.8 ml STE buffer and transferred to the 2 ml eppendorf containing 0.1 ml of 10% SDS. The sample was mixed gently by inverting tube 5-6 times and 0.01 ml of RNase A in addition to 0.05 ml Proteinase K was orderly added to the mixture and incubated for 15 min at room temperature and 1 h at 55°C, respectively. The DNA isolation procedure was followed by six times phenol: chloroform: isoamylalcohol extractions, ethanol precipitation, and resuspension in 40 μ l of sterile dH₂O.

3.7.4 Agarose Gel Electrophoresis

In order to visualize and to determine size and concentration of DNA fragments after any treatment, gel electrophoresis was used during the study. 0.8% (w/v) agarose gel was prepared by dissolving 0.4 g agarose in 50 ml 1×TBE buffer and heated until boiling point. After cooling to approximately 55°C, ethidium bromide (Sigma-10 mg ml⁻¹) was added to solution at a final concentration of 0.8 μ l ml⁻¹ and the gel poured into a suitable gel tray. An appropriate comb was inserted and the gel was allowed to solidify. The gel tank was filled with 1×TAE buffer. 10-20 μ l of DNA samples, mixed with 1/5 volume of 6x loading dye were applied into the wells, as well as the DNA ladder (Appendix H) for size estimation. Electrophoresis was performed at 100 V for 60 min and the DNA fragments were visualized by using UV transsilluminator. Gel photographs were taken and stored with gel imaging system and documentation system, respectively (UVP BioImaging System, and Hamamatsu Digital CCD Camera).

3.7.5 DNA Extraction from Agarose Gels

To extract a specific DNA fragment from agarose gel after gel electrophoresis, Gel Elution Kit (GeneMark) was used. For this purpose, the desired

DNA fragment visualized above UV illuminating board was separated from the gel and was cut into slices (up to 350 mg). Weighted gel slices were incubated in 500 µl Binding Solution at 60°C for 30-60 min up to complete melting of gel slices and the solution were loaded into spin column. The procedure was followed according to manufacturer's instructions. At the end of the protocol, 30-50 µl of dH₂O was used to elute DNA from spin column. Extracted DNA from salt content was stored at -20°C for further use.

3.7.6 Primer Design

Three different set of primers were designed in this study which their sequences are given in Table 3.11. Restriction enzyme recognition sites were determined (Appendix I) by the help of Restriction Mapper web-page of ABD Molecular Biology Resources (<http://www.restrictionmapper.org>). In the selection of nucleotide sequences corresponding to start and stop codons, the codon usage by *E.coli* and *P.pastoris* strains (Appendix J) was also taken into account, wherever it was possible.

The possibility of primer dimer structure, self-complimentarity formation, the thermodynamic properties, G+C base composition, and melting temperature of primers were controlled by the Oligo Explorer 1.2 program. The results of this program, together with thermodynamic properties of designed primers are given in Appendix I. The sequences for restriction enzyme recognition are underlined. Extra nucleotides were added to the 5' end of restriction enzyme recognition sites, since site recognition by the enzyme close to the end of DNA fragment could otherwise be problematic.

Primers GI_{Pext}-F1 and GI_{Pext}-F2 designed in accordance with the sequences of *xyl_P* gene from *P.furiosus* (Accession no: NC_003413.1) to adjoin *Eco*RI restriction site (6 bp), 6xHis-Tag sequence (18 bp) and Factor Xa recognition sequence (12 bp) to the 5' end of target sequence during amplification to produce rGI extracellularly. Since an addition of 36 bases was required at the 5' end *xyl_P* sequence, two relatively short forward primers were designed instead of a single long primer. In order to

obtain GI from *P. furiosus* in the form of intracellular enzyme, another forward primer (GI_{Pint}-F) was designed in the manner that α -factor signal sequence was removed from expression vector sequence and *Asu*II restriction site (6 bp) was included at the 5' end. The reverse primer (GI_P-R) with the *Xba*I restriction site and (TCA) stop codon was employed in the amplification of GI_P both for extracellular and intracellular gene recombinations.

Primers GI_T-F and GI_T-R were designed to amplify the sequence of *xyl_T* gene from *T. thermophilus* (Accession no: D90256) and add *Nde*I restriction site (6 bp) to the 5' end of *xyl_T* sequence during amplification. The reverse primer was designed in manner that, at the 3' end of the *xyl_T* sequence, stop codon (TGA) and *Eco*R I restriction site (6 bp) is included.

Table 3.11 Primers used in this study and their sequences.

Name	Sequence
GI _T -F	5' CGCATATGTACGAGCCCAAACCGGAGCACAGG 3'
GI _T -R	5' GGAATTCTTCACCCCCGCACCCCCAGGAG 3'
GI _{Pext} -F1	5' CACCACATTGAAGGGAGAATGAAAGTTGGAGTTAGC 3'
GI _{Pext} -F2	5' GGAATTCATCACCATCACCACCACATTGAAGGGAGA 3'
GI _{Pint} -F	5' GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA 3'
GI _P -R	5' GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG 3'

3.7.7 Polymerase Chain Reaction (PCR)

PCR amplification of DNA was carried out by thermal cycling program (Techgene, Flexigene). The reaction mixture of the 50 μ l final volume was prepared on ice including the following matters:

10x PCR Reaction buffer (with Mg^{2+})	: 5 μl
dNTP (1 mM stock)	: 10 μl
Forward primer (10 μM stock)	: 2 μl
Reverse primer (10 μM stock)	: 2 μl
Template DNA	: 100 ng (1 μl)
Taq DNA polymerase (1U μl^{-1})	: 1 μl
Sterile dH_2O	: to 50 μl

The PCR program used was:

94°C	3 min	}	×1 cycle

94°C	1 min	}	x 30 cycles
60°C	1.5 min		
72°C	1-1.5 min		

72°C	5-10 min	}	×1 cycle
4°C	∞		

3.7.8 Purification of PCR Products

The purification of PCR products was performed by Fermentas PCR purification kit according to manufacturer's instructions to purify DNA from salt, PCR enzyme, primers, and nucleotides. Finally 20-50 μl of dH_2O was used to recover DNA from spin columns.

3.7.9 Restriction Endonucleases Digestion Reaction

Restriction digestion of plasmid DNA and template gene were carried out by incubating DNA fragments with proper restriction enzymes (REs) in buffers of 20 μl final volume at 37°C for approximately 4-9 h. The composition of the restriction

digestion mixtures was given in Tables 3.12, 3.13 and 3.14. After digestion reaction, the mixtures were incubated at 65°C for 20 min to deactivate the REs.

3.7.10 DNA Purification after Digestion

After restriction endonuclease digestion, Fermentas PCR purification kit was used again to purify DNA from REs and buffer according to manufacturer's instructions. At the end of the protocol, purified DNA was eluted with 20-50 µl of dH₂O.

Table 3.12 Components of reaction mixture of restriction digestion with *EcoRI* and *XbaI* REs.

Components	Amounts
DNA fragment	~ 50-200 ng
<i>EcoRI</i> RE (10U µl ⁻¹)	1 µl
<i>XbaI</i> RE (10U µl ⁻¹)	1 µl
10X Buffer Tango	2µl
Sterile dH ₂ O	up to 20 µl

Table 3.13 Components of reaction mixture of restriction digestion with *AsuII* and *XbaI* REs.

Components	Amounts
DNA fragment	~ 50-200 ng
<i>AsuII</i> RE (10U µl ⁻¹)	1 µl
<i>XbaI</i> RE (10U µl ⁻¹)	1 µl
10X Tango	2 µl
Sterile dH ₂ O	up to 20 µl

Table 3.14 Components of reaction mixture of restriction digestion with *NdeI* and *EcoRI* REs.

Components	Amounts
DNA fragment	~ 50-200 ng
<i>NdeI</i> RE (10U μl^{-1})	1 μl
<i>EcoRI</i> RE (10U μl^{-1})	1 μl
10X Buffer O	2 μl
Sterile dH ₂ O	up to 20 μl

3.7.11 Ligation Reaction

PCR amplified gene or insert DNA was cloned into suitable expression vector from the sticky ends obtained after restriction digestion reaction. The amount of insert DNA to be added to the reaction mixture was calculated such that insert: vector ratio of 1:3 was achieved, as given in the below equation.

$$100\text{ng vector} \times \frac{\text{size of insert}(\text{bp})}{\text{size of vector}(\text{bp})} \times \frac{3}{1} = \text{amount of inset}(\text{ng}) \quad (3.6)$$

The ligation mixture was carried out by incubating of 10 μl final reaction volume at 16°C for 16 h and 6 μl of the mixture was used for transformation. The composition of ligation mixtures was in Tables 3.15, 3.16 and 3.17. A control reaction was setted up containing all the reagents listed above except the insert DNA.

3.7.12 DNA Sequencing

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

3.7.13 Transformation of plasmid DNA by CaCl₂ Method to *E. coli*

For propagation of plasmids after the ligation reaction, *E. coli* XLBlue chemically competent cells (section 3.7.13.1) were used. One 230 μl vial was thawed

on ice for 10 min. 6 µl of the ligation mixture was added and gently mixed by tapping.

Table 3.15 Components of ligation mixture for construction of recombinant extracellular *P.pastoris* expression system.

Components	Amounts
10X ligation buffer	1 µl
Insert DNA (841 bp)	70-120 ng
Double digested vector DNA (3536 bp)	100 ng
T4 DNA ligase	1 µl
Sterile dH ₂ O	up to 10 µl

Table 3.16 Components of ligation mixture for construction of recombinant intracellular *P.pastoris* expression system.

Components	Amounts
10X ligation buffer	1 µl
Insert DNA (804 bp)	70-120 ng
Double digested vector DNA (3263 bp)	100 ng
T4 DNA ligase	1 µl
Sterile dH ₂ O	up to 10 µl

Table 3.17 Components of ligation mixture for construction of recombinant *E.coli* expression system.

Components	Amounts
10X ligation buffer	1 µl
Insert DNA (1164 bp)	100-150 ng
Double digested vector DNA (2762 bp)	100 ng
T4 DNA ligase	1 µl
Sterile dH ₂ O	up to 10 µl

The vial was incubated on ice for 30 min, then heat-shock was applied in 42°C water-bath for exactly 90 sec and quickly placed on ice for 1 min. 800 µl of prewarmed LB medium was added to the vial inside laminar flow cabinet and the vial was incubated at 37°C for exactly 45 min with shaking. 50-250 µl of transformation mixture was spread on prewarmed LSLB agar with selective antibiotic and incubated at 37°C overnight. Selected colonies should be visible in 14-16 h (Sambrook, 2001).

3.7.13.1 Chemically Competent Cell Preparation

1. Incubate wild type *E.coli* XLBlue on LB-solid medium for 12 h at 37°C,
2. Pick a single colony from a selective plate and inoculate a starter culture of 5 ml LB medium. Grow for 12-15 h at 37°C, with vigorous shaking at 200 min⁻¹,
3. Inoculate 100 ml-LB broth by 1 ml of starter culture and cultivate at T=37°C and N= 250 min⁻¹ for t=3-3.5 h,
4. Transfer 50ml of broth into 50 ml sterile centrifuge tubes; and hold the tubes on the ice for 10 min,
5. Precipitate the cells via centrifugation at 6000 rpm, 4°C for 10 min,
6. Remove the cells from supernatant and let them dry on paper tissue for 1 min,
7. Transfer 5 ml of filter sterilized 0.1 M CaCl₂ cold solution into each tube and re-suspend the cell pellet in the solution by pipetting and place on ice for 10 min,
8. Repeat 5th step of the protocol,
9. Add 1 ml of filter sterilized 0.1 M CaCl₂ solution to each tube and once more re-suspend the cells and hold on ice for 10 min,
10. Transfer 200 µl of competent cell to storage tubes; add glycerol 13 % (v/v) to each tube and store at -80°C for further use.

3.7.13.2. Transformation to *E. coli* BL21 (DE3) pLysS

The propagated plasmid DNA was isolated from *E.coli* XLBlue by Fermentase plasmid isolation kit and isolated plasmid DNA was transformed by CaCl_2 method into *E. coli* BL21 (DE3) pLysS strain. The procedure in the section 3.7.13.1 was repeated to prepare *E. coli* BL21 (DE3) pLysS strain competent cells.

3.7.14 Transfection of *Pichia pastoris*

Transfection of *P. pastoris* was performed using LiCl method according to manufacturer's instructions (Invitrogen, catalog V195-20).

YPD plate was inoculated with *Pichia pastoris* X-33 and incubated for 48 h in 30°C incubator. 5 ml YPD was inoculated with a single colony and grown overnight to saturation in 30°C shaker. 50 ml culture of YPD was inoculated using preculture, to an initial OD_{600} of approximately 0.1 and incubated at 30°C with shaking to an OD_{600} of 0.8 to 1.0 (approximately 10^8 cells ml^{-1} ; 6-7 h). During this period, the plasmid DNA to be integrated into the genome had to be digested at a single site. Therefore, pPICZ α A::xyl_{Pext} and pPICZ α A::xyl_{Pint} plasmids were digested with *Sac* I at 37°C for 6 h. Full digestion was verified by running 2 μl of sample on agarose gel and then purified. The concentration of the plasmid DNA was adjusted to 0.1-0.2 $\mu\text{g } \mu\text{l}^{-1}$ and verified by agarose gel electrophoresis. When the OD_{600} reached 0.8 - 1.0, the cells were harvested at 4000 rpm for 5 min, washed with 25 ml of sterile water, and centrifuged at 1500 rpm for 10 min at room temperature. The cell pellet was resuspended in 1 ml of 100 mM LiCl, transferred to a 1.5 ml microcentrifuge tube, centrifuged at maximum speed for 15 sec. LiCl was removed with a pipette and the cells were resuspended in 400 μl of 100 mM LiCl. For each transformation, 50 μl of the cell suspension was dispensed into a 1.5 ml microcentrifuge tube, immediately centrifuged at maximum speed for 15 sec and LiCl was removed with a pipette. To each tube for transformation, 240 μl of 50% PEG, 36 μl of 1 M LiCl, 10 μl of 5 mg ml^{-1} single-stranded DNA and 5-10 μg plasmid DNA in 50 μl sterile water were added in the order given and vortexed vigorously until the cell pellet was completely mixed. The tube was incubated at 30°C for 30 min without shaking, and then heat shocked in a water bath at 42°C for

25 min. The cells were pelleted by centrifugation at 6000 rpm for 15 sec, gently resuspended in 1 ml of YPD and incubated at 30°C with shaking. After 2 h of incubation, 25-100 µl was spread on YPD + Zeocin plates and incubated for 2-3 days at 30°C.

3.8 Ligand Selection via Phage Display Technique

Phage display technique was applied to select ligand with affinity toward the 6×His peptide. Phage display was started by surface panning (direct target coating) procedure as follow:

Day One

1. A solution of 6×His peptide with the concentration of 250 µg ml⁻¹ was prepared in 0.1M NaHCO₃, pH 8.6 buffer.
2. 150 µl of the 6×His solution was added to one well of 96-microtiter well ELISA plate and mixed gently by pipetting in order to wet the well surface completely.
3. Overnight incubation at 4°C in refrigerator was performed to coat the well surface completely with 6×His peptide.
4. Inoculate *E.coli* ER2738 in 10ml of LB medium and shaken at 200 rpm, 37°C for 4-8h.

Day Two

5. The coating solution was discarded from the well by slapping the ELISA plate surface prostratly on a clean paper towel. The well was completely loaded with blocking buffer and was incubated at 4°C for no less than 1 h.
6. The blocking buffer was poured off as in step 5. TBST (TBS+0.1% [v/v] Tween-20) buffer was used to wash the well 6 times by filling the well with TBST, discarding the solution, and slapping the plate prostratly on a clean paper towel each time.
7. Phage library was diluted by adding 15 µl of phage library into coated well containing 135 µl of TBS buffer and the plate was covered with parafilm and shaken gently for 1 h at room temperature.

8. The plate was slapped onto a clean paper towel to pour off the non-binding phage.
9. TBST buffer was once more utilized to wash the well for 10 times.
10. For elution of the bound phages, 110 μl of elution buffer, consisted of 0.2 M Glycine-HCl (pH 2.2) and 1 kg m^{-3} BSA, was added into well and the solution was transferred in a microcentrifuge tube, containing 15 μl of 1 M Tris-HCl, pH 9.1 to neutralize the elution mixture.
11. A small quantity ($\sim 10 \mu\text{l}$) of the eluate was titered as described in the section 3.8.1 and the remaining part was amplified by adding the eluate to the 20 ml of *E.coli* culture from step 4 (should be early-log at this point) and incubating with vigorous shaking at 37°C for 4.5 h.

Note: At this point, the remaining eluate can be stored overnight at 4°C and amplified in the next day. For this purpose, inoculate *E.coli* ER2738 in 10 ml of LB medium and incubate with shaking overnight at 37°C. The next day, the overnight culture was diluted in 20 ml of LB in a 250 ml Erlenmeyer flask at the ratio 1:100. The unamplified eluate was added in flask and incubated with vigorous shaking at 37°C for 4.5 h. Then proceed to step 12.

12. The cell pellets were precipitated by centrifugation at 4°C, 12000 rpm for 10 min. The supernatant was transferred to a fresh centrifuge tube and re-rotate (discard the precipitate).
13. The 80% of upper supernatant was transferred to a new tube and 1/6 volume of 20% PEG/2.5 M NaCl solution was added into tube to allow overnight precipitation of the phage at 4°C.

Day Three

14. The PEG precipitation step was completed by centrifugation the tube at 12,000 rpm for 30 minutes at 4°C. The supernatant was gently removed; the centrifugation step was repeated for 5 minutes and the remaining supernatant was removed via pipetting.

15. The phage pellet was dissolved in 1 ml of TBS buffer; the suspension was transferred to a new sterile centrifuge tube, and was rotated at 13,200 rpm for 5 min at 4°C to precipitate residual cells.
16. The supernatant was transferred once more into new microcentrifuge tube and solution of 20% PEG/2.5 M NaCl was added in it at the volumetric ratio of 1/6; the tube was placed on ice for 1h and then rotated at 13,200 rpm for 10 min; the supernatant was discarded and the tube was centrifugated for a short time; The remaining supernatant was gently discarded with a micropipette.
17. The phage pellet was re-suspended in 200 µl of TBS buffer and the solution was rotated for additional 1 min to precipitate residual insoluble matters. The supernatant was transferred into a fresh tube. The resulted eluate was amplified phage from first round of panning which named as AF1. For long time storage, 50 µl of amplified was transferred to the storage tube containing 50 µl of sterile glycerol and store at -20°C.
18. The amplified eluate was titered as described in section 3.8.1.
19. The well was coated for the second round of panning with 6×His peptide as it was done in steps 1-3.

3.8.1 Phage Titering

- 10 ml of LB medium inoculated with a single colony of *E.coli* ER2738 strain and incubated at 37°C with shaking for 4-8 h.
- Top agar was melt in microwave and distributed in the quantity of 3ml into sterile tubes, one per expected phage dilution; the tubes were maintained at 60°C to prevent their solidification.
- LB/IPTG/Xgal plates one per expected dilution were pre-warmed by incubating them at 37°C at least for 1 h before use.
- Serial dilutions of phage were carried out in TBS buffer. The dilution rang of 10^8 - 10^{11} and 10^1 - 10^4 was used for amplified phage and unamplified panning eluates, respectively. Fresh aerosol resistance sterile

pipette tips were utilized for each dilution to eliminate possibility of any cross-contamination.

- As soon as the cell growth of *E.coli* ER2738 culture (step 1) approach to mid-log phase, the cultivation was dispensed in the volume of 200 μ l into sterile microfuge tubes, one for each phage dilution.
- 10 μ l of each phage dilution was added to the microfuge tube containing *E.coli* culture; the tube's contents were quickly mixed, and incubate at room temperature for 5 min to let infection of *E.coli* cells by the phage.
- Infected *E.coli* cells were transferred in to the culture tubes containing Top Agar at 55°C. Each culture tube was briefly mixed by vortex and immediately decanted on a pre-warmed LB/IPTG/Xgal plate with gentle rotating to spread top agar evenly on plate surface. After solidification, the plates were over nightly incubated at 37°C.
- Blue colored plaques formed on the plates were counted and multiplied with dilution factor for that plate to get phage titer in plaque forming units (pfu) per 10 μ l.

Day Four and Five

20. The blue plaque from the titering plates in step 18 was counted and the phage titer was calculated. This value was used to decide on the input phage volume for the second round of panning.
21. The second round of panning was carried out by reiterating steps 4-17 using particular amount of amplified eluate from first round as input phage, and increasing the Tween concentration in the TBST buffer to 0.5% (v/v).
22. The resulted second round amplified eluate was tittered on LB/IPTG/Xgal plates as described in section 3.8.1.
23. A new well was coated again with target for the third round of panning as in steps 1-3.

Day Six

24. The third and final panning round was carried out by employing the second round amplified eluate at an input titer equivalent to what was used in the first round (step 7), again using 0.5% Tween during the washing steps.

25. The unamplified third round eluate was tittered on LB/IPTG/Xgal plates.
26. In order to prepare sufficient quantity of phage for sequencing and ELISA assays, phage amplification procedure was performed as it was explained in section 3.8.2.

3.8.2 Phage Amplification for Sequencing and ELISA Assays

To obtain sufficient quantity of phages for DNA sequencing and ELISA assays, it is necessary to amplify phage, either from individual plaques or from the eluted pool. Phage amplification procedure can be summarized as:

1. An overnight culture of *E.coli* was diluted in LB medium at the ratio 1:100. For each clone to be characterized, 1ml of dilute culture was dispensed into culture tubes. 2 clones from first and second rounds and 39 clones from third round were used for amplification and consensus binding sequence detection.
2. A sterile injector needle was utilized to slab the phage plaques from fresh tittering plates that contain plaques <100. Well separated plaques was selected to ensure that each plaque include a single DNA sequence and transferred to the tubes containing the dilute culture.
3. Inoculated tubes were incubated at 37°C with vigorous shaking for 4-4.5 h.
4. The cultures were transferred to microcentrifuge tubes and rotated at 12,000 rpm for 30 s. The supernatants were carried to new tubes and re-rotated. 80% of the upper supernatants were once more transferred to fresh tubes and for long time storage amplified phage stocks were diluted at the ratio of 1:1 with sterile glycerol and stored at -20°C.

3.8.2. 1 Phage ELISA Binding Assay with Direct Target Coating

The following ELISA protocol can be used for rapid determination of whether a selected phage clone binds to the target, without the need for an antibody specific for target. In this procedure the wells of microtiter ELISA plate were coated with the target at 250 µg ml⁻¹, and each amplified phage clone was added to the plate

in the manner that each well contains equal number of phages. Bound phages were then detected with anti-M13-HRP conjugate monoclonal antibody (Fitzgerald Inc). This method cannot determine whether the selected phage binds with high or low affinity because the amount of target coated on the plate is not quantifiable and is present at sufficiently high amount to permit multivalent binding to phage. The method can distinguish true target binding from binding to the plastic wells surface and is also useful for qualitative determination of relative binding affinities for a number of selected clones in parallel. The phage ELISA binding assay was carried out as follow:

1. Firstly, the plaque amplification was carried out. After the first centrifugation, in step 4, save the phage-containing supernatant at 4°C.
2. An overnight culture of *E.coli* was diluted at the ration of 1:100 in 20 ml LB medium for each clone to be characterized.
3. 5 µl of phage stock from step 1 was added to a 20 ml culture for each clone and was incubated at 37°C for 4-4.5 h with vigorous shaking.
4. The culture was transferred to a microcentrifuge tube, and was rotated at 12,000 rpm for 10 min at 4°C. The supernatant was carried out to a new tube and re-rotated.
5. The 80% of upper supernatant was transferred to a new tube and 1/6 volume of 20% PEG/2.5 M NaCl solution was added into tube to allow overnight precipitation of the phage at 4°C.
6. The PEG precipitation was centrifuged a 12,000 rpm for 30 min at 4°C. The supernatant was discarded , the tube was re-spinned, and the residual supernatant was removed with a pipette. The phage pellet should be a white finger print sized smear on the side of the tube.
7. The pellet was re-suspended in 1 ml of TBS, the suspension was transferred to a microcentrifuge tube, and was rotated at 13,200 rpm for 5 min at 4°C to pellet residual cells.
8. The supernatant was transferred into new microcentrifuge tube, 1/6 volume of 20% PEG/2.5 M NaCl solution was added in it, and the tube was incubated on ice for 1h. The tube was rotated at 13,200 rpm for 10 min at 4°C, the

supernatant was discarded, re-spined briefly, and the residual supernatant was removed with a micropipette.

9. The pellet was suspended in 200 μl of TBS buffer and in order to obtain same concentration for each clone, all phage concentration was brought to 10^{13} pfu ml^{-1} .
10. For each clone to be characterized two wells of ELISA plate were coated with 250 $\mu\text{g ml}^{-1}$ of target in 0.1 M NaHCO_3 , pH=8.6. Additionally 4 extra wells were allocated for control wells and one of them coated with target. To obtain efficient binding the plate was incubated overnight at 4°C in a refrigerator. Control wells contents were arranged as:
 - C1. Blocking buffer+ Phage+ Antibody+ Substrate (to test binding of phage to BSA coated well)
 - C2. Target+ Blocking buffer+ Antibody+ Substrate (to test antibody specify to phage)
 - C3. Blocking buffer+ Substrate (zero base for colorimetric absorbance measurement)
 - C4. Blocking buffer+ Antibody+ Substrate (to test binding of antibody to BSA-coated well)
11. The unbound target solution was discarded from well by firmly slapping it face down onto a clean paper towel. The wells were completely filled with blocking buffer including control wells (C1-C4) and plate was incubated at 4°C for at least 1 h.
12. The blocking buffer was poured off. TBST (TBS+0.1% [v/v] Tween-20) buffer was used to wash the wells 6 times by filling the wells with TBST, discarding the solution, and slapping the plate face down on a clean paper towel each time.
13. The serial dilutions in TBS buffer for the each phage were prepared to bring phages concentration into same value (1×10^{10} pfu ml^{-1}).
14. 100 μl of each diluted phage was transferred to two wells coated with target in step 10. Also, one of the phage was added to C1 control well and the other

control wells were filled with TBST buffer to prevent well drying. The plate was incubated with shaking for 1-2 h at room temperature.

15. TBST buffer was again used for wells washing for 6 times.
16. HRP-conjugated anti-M13 monoclonal antibody was diluted in 1:20 ratio in blocking buffer and 200 µl of it was added to each well including C1-C2-C4. To prevent drying of C3 control well, it was filled with TBST buffer and the plate was incubated with shaking for 1 h at room temperature.
17. The wells were washed 6 times again with TBST.
18. Immediately prior to the detection step, HRP substrate was arranged by adding 36 µl of 30% H₂O₂ into the 21 ml of ABST stock solution.
19. 200µl of substrate mixture was added to each well including all control wells. The plate was incubated for 20-60 min at 37°C with gentle agitation.
20. Microplate reader at 405 nm (BIO-TEKEL 808, Biotek Instruments, Winooski, VT, USA) was used to read the plate. For each phage concentration, the signals obtained with and without target protein was compared.

3.8. 2.2 Sequencing of Phage DNA

1. The plaque amplification was carried out as described before. After the first centrifugation, in step 4, 60 µl of each selected phage stock solution was transferred to microcentrifuge tube.
2. 60 µl of Phenol/ Chloroform/ Isoamylalcohol mixture was added to each tube. The solution was mixed by vortex and after 1min, mixing was repeated.
3. The tubes were centrifuged at 13200 rpm for 15 min and three phases formation was detected in each tube.
4. The upper aqueous phase was transferred to fresh tube. To precipitate DNA ethanol at the ratio 1:3 and 3M sodium acetate pH 7.1 were added to each tube.
5. The tubes were incubated at -20°C for 12-15 h. Then, tubes were centrifuged at 13200 rpm for 15 min at 4°C to collect DNA.

6. The supernatant was gently discarded and the tubes were held in an inverted position on a clean paper towel to let all of fluid to drain away.
7. 40 μl of TE buffer was used to re-suspend DNA and the sample tubes were sent for DNA sequencing.

3.9 Interaction Measurement by SPR

SPR was used with the aim of obtaining the kinetic parameters of interaction. The general method which was developed by Texas Instrument is slightly modified for peptide-peptide binding analysis and the details of procedure are given as follows.

3.9.1 Surface cleaning and sensor insertion

Firstly, the gold surface of sensor was cleaned by applying 20 μl of piranha solution (a mixture of 96% H_2SO_4 and 30% H_2O_2 in the ratio of 3:1) without touching the gold surface. After few minutes, the surface was completely rinsed with deionized water and was dried by passing nitrogen gas flow through the gold surface. Then, the sensor was inserted into the SPR cell and the cell was tightly closed to prevent any possible leakage of flow during the analysis time. Spreeta 5 software was used for the analysis of samples. After carrying out the dry calibration, pure ethanol with flow rate of 50 $\mu\text{l min}^{-1}$ was passed through the sensor surface for 20 min. Finally, filter sterilized deionized water was passed at the same flow rate with ethanol through the surface and after stabilization of signals for each channel the wet calibration was done.

3.9.2 Preparation of active surface

The 10 mM solution of mercaptoundecanoic acid (11-MUA $\text{C}_{11}\text{H}_{22}\text{O}_2\text{S}$) in ethanol was used to form Self-Assembled Monolayer (SAM) on the gold surface by chemisorptions. For this purpose, the MUA solution was passed through the gold

surface at flow rate of $7 \mu\text{l min}^{-1}$ for 18 h. The formation of ordered and well packed layer was achieved by keeping the gold surface under 11-MUA flow overnight. After SAM formation, the surface was washed first with pure ethanol then with deionized water at flow rate of $50 \mu\text{l min}^{-1}$. In order to form active surface for immobilization of peptide, amine coupling method which is mostly used as primary method was selected. As the standard protocol for amino coupling EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/ N hydroxysuccinimide) mixture was used such that in the mixture the concentrations were 0.2 M and 0.05 M for EDC and NHS, respectively (Van der Werve and Barclay, 1996). The surface of three channels was treated by this mixture at a flow rate of $10 \mu\text{l min}^{-1}$ for an hour to form free carboxyl groups which would bind to amine groups of peptide. Unbound EDC/NHS was removed by passing water at a flow rate of $20 \mu\text{l min}^{-1}$, until steady response diagram was achieved. Then, the selected peptide solution selected for immobilization was prepared in TBS buffer and loaded to two lines of sensor chip at flow rate of $20 \mu\text{l min}^{-1}$ where the third line was selected as control channel and this line was treated by water at the same flow rate. Adsorption of particles on the chip surface caused an increase in the response units. The feeding was continued until response units reach to steady state condition (no longer increasing) so the saturation of surfaces was guaranteed by this way. The unbound or weakly bound peptides were removed from the chip surface by washing the channels surface and the decrease in response unit of sensorgram was detected. Again, until obtaining steady state response, passing of water was continued. The difference between response unit values before and after washing indicated whether the peptides were bounded or not. Then, the free and unoccupied carboxyl groups were blocked by passing the solution of 1 M Ethanolamine at a flow rate of $20 \mu\text{l min}^{-1}$ and a significant increase in the response unit was detected. Once more, the excess ethanolamine was washed and at the end of these steps expected sensorgram was as image shown in Figure 3.2.

After obtaining steady condition, the channels were treated with analysis buffer at flow rate of $20 \mu\text{l min}^{-1}$ until the flat lines of response were achieved. Then the peptide ligand solution was prepared at two different concentrations and injected at flow rate of $20 \mu\text{l min}^{-1}$ to the channels. As the ligands reach to the surface

the increase in response units of two line was detected and association was completed when the plateau was reached. Finally, buffer was passed through the channels in order to observe the dissociation profile. The structure of dissociation and the times it took for complete dissociation were the important factors in determination of the strength of binding. According to the association and dissociation profiles, the binding properties were calculated.

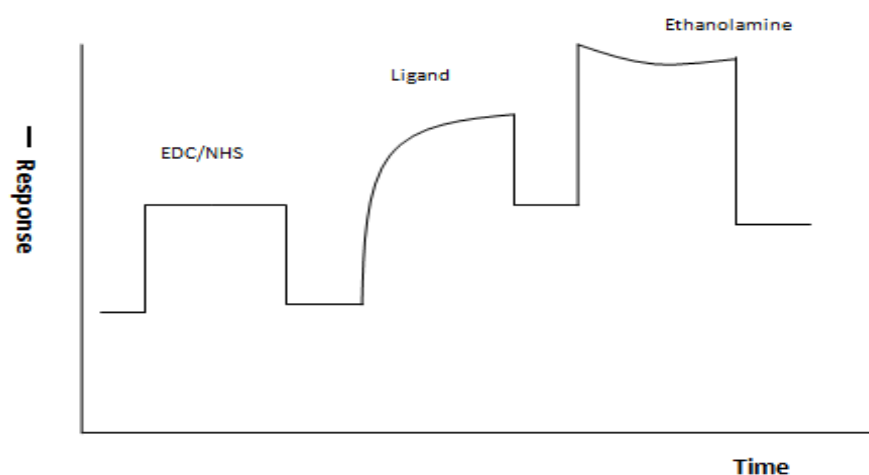


Figure 3.2 Expected sensorgram for SPR before ligand interaction.

CHAPTER 4

RESULTS AND DISCUSSION

In this study, glucose isomerase genes (*xyl*) from thermophiles *Pyrococcus furiosus* and *Thermus thermophilus* were expressed in *P. pastoris* and *E.coli* strains, respectively, in order to over-produce the thermostable enzyme. In this frame, the study was carried out in three main stages. In the first phase of the study, the recombinant microorganisms producing thermostable rGI were developed and the most appropriate expression system for the enzyme over-production was selected. In the second part, the effect of exponential feeding strategy of carbon source on the production of rGI enzyme, cell concentration as well as by-product formation by the selected expression system was investigated at four sets of fed-batch bioreactor experiments at the three different predetermined specific growth rate values. Moreover, phase display library assay was conducted to select peptide sequence with specific affinity toward the 6×His peptide and the affinity, specificity as well as binding property of interaction were verified by surface plasmon resonance analyses.

4.1 Development of Recombinant Microorganisms Producing Thermostable GI

4.1.1 Development of Recombinant *P. pastoris* Strains

As first attempt for the development of GI producing recombinant microorganism, the extracellular and intracellular recombinant *Pichia pastoris* expression systems were constructed. For this purpose, hypothetical thermostable *xyl_P* gene of *Pyrococcus furiosus* strain, which shows ~75% similarity with other thermostable *xyl* genes, was amplified via PCR. The amplification stage was carried

on the basis of two different approaches, one for generation of extracellular gene in which poly-histidine tag, a target site for the Factor Xa protease together with *EcoRI* and *XbaI* restriction sites were added to wild type sequence of *xyl_p* gene in the extension of two PCR reactions. The other set was subsequently performed to achieve high quantity of intracellular *xyl_p* gene. So that, *AsuII* and *XbaI* restriction sites were selected and combined to the target sequence in order to remove α -Factor signal sequence from pPICZ α A expression vector map throughout the restriction digestion reaction. The PCR products, *xyl_{pext}* and *xyl_{pint}* genes, were separately cloned to the pre-treated pPICZ α A vectors which were digested with correlated restriction enzymes. The accurate construction of recombinant expression vectors, both extracellular system, pPICZ α A:: *xyl_{pext}* , and intracellular one, pPICZ α A:: *xyl_{pint}*, was confirmed by the mean of DNA sequencing, restriction digestion as well as PCR amplification control experiments. The constructed recombinant expression systems were transformed into *Pichia pastoris* cells where two colonies of positively transfected cells from each extracellular and intracellular recombinant *P.pastoris* strains were selected for further studies. Production of recombinant thermostable GI_p enzyme either in the extracellular manner via strains *P. pastoris* pPICZ α ::*xyl_{pext1}* and *P. pastoris* pPICZ α ::*xyl_{pext2}* strains or in the intracellular manner by strains *P. pastoris* pPICZ α ::*xyl_{pint1}* and *P. pastoris* pPICZ α ::*xyl_{pint2}* were verified by evaluating GI_p activities of these cells with that of wild type *P. pastoris* cells. The experimental steps for the construction of both extracellular and intracellular *Pichia* expression systems were schematically summarized in Figures 4.1 and 4.2, respectively.

4.1.1.1 Primer Design for Generation of *xyl_{pext}* and *xyl_{pint}* Gene from *P.furiosus*

Two forward (GI_{pext}-F1 and GI_{pext}-F2) primers were designed in accordance to the sequence of *xyl_p* gene from *P.furiosus* for the amplification of extracellular *xyl_p* (*xyl_{pext}*) gene. *EcoRI* restriction site (6 bp), 6xHis-tag sequence (18 bp) and Factor Xa protease recognition sequence (12 bp) were combined to the 5' end of *xyl_p* sequence. Since an addition of 36 bases was required at the 5' end of *xyl_p* sequence, two relatively short forward primers were designed instead of a single long primer.

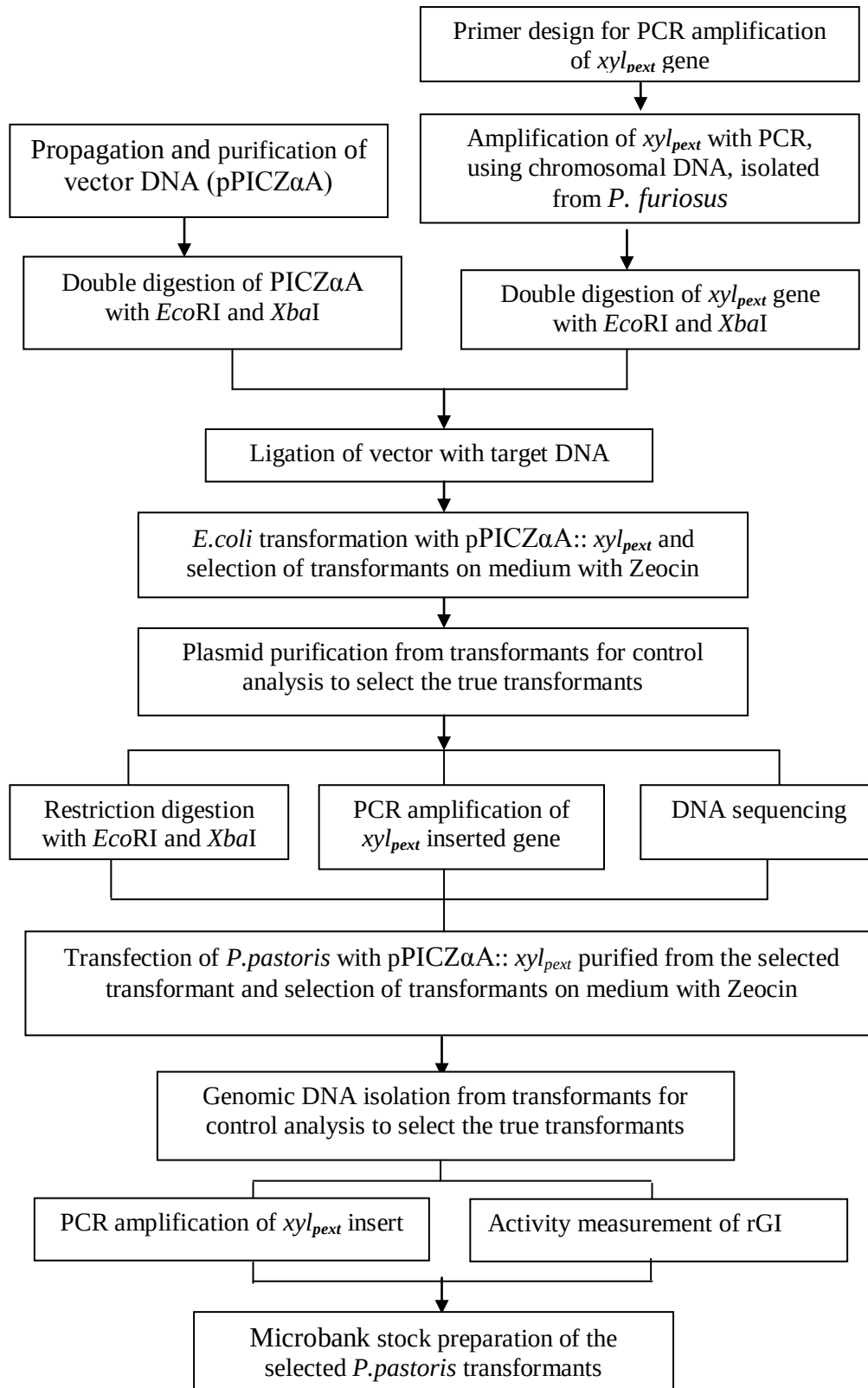


Figure 4.1 Flowchart of the research plan, for the development of the recombinant *Pichia pastoris* producing extracellular GI_p.

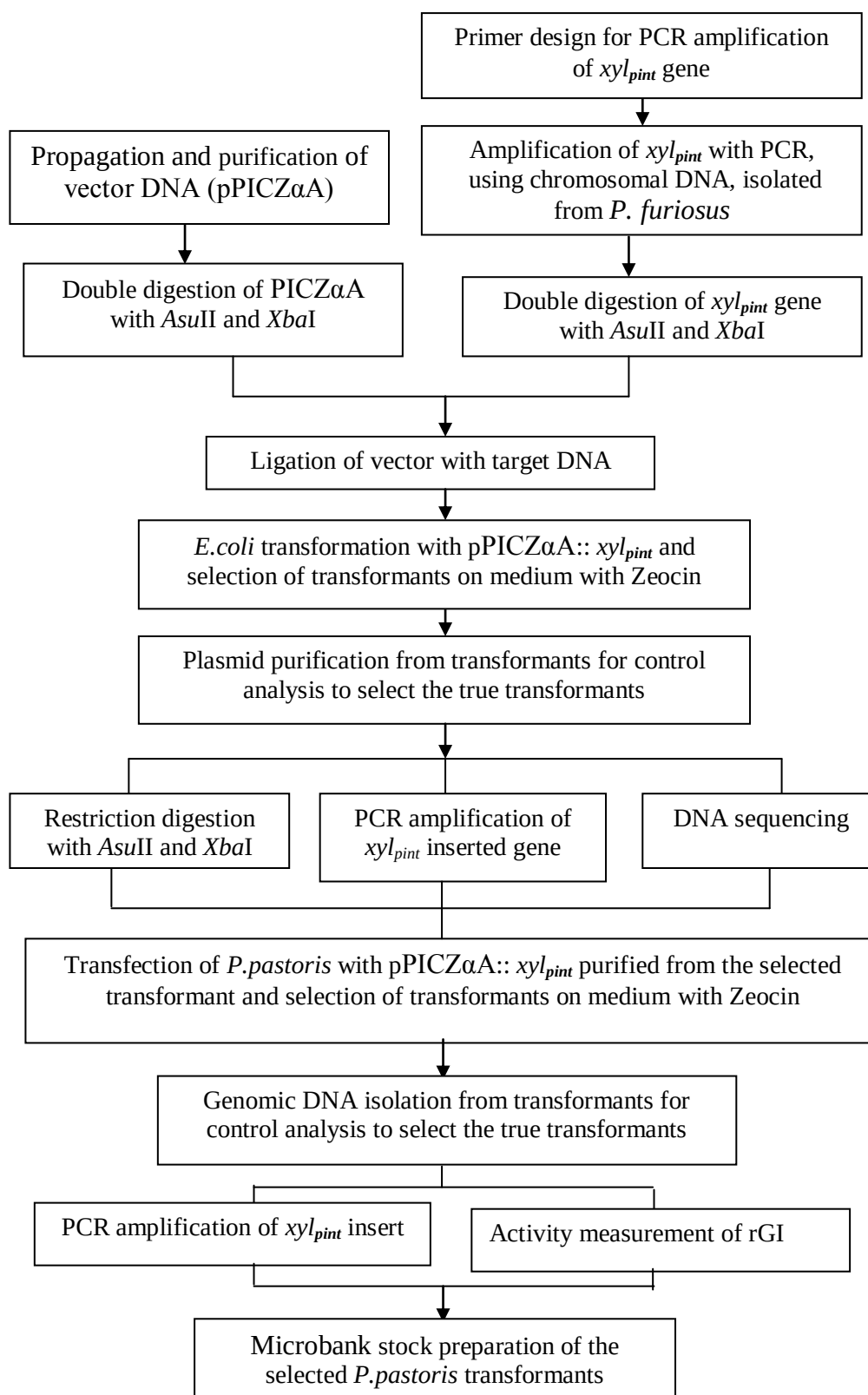


Figure 4.2 Flowchart of the research plan, for the development of the recombinant *Pichia pastoris* producing intracellular GI_p.

Related with the generation and amplification of the intracellular *xyl_p* gene (*xyl_{pint}*) only one forward primer, GI_{pint}-F, was designed in order to add *Asu*II restriction site to the 5' end of *xyl_p* sequence. The reverse primer (GI_p-R) was designed in the manner that, at the 3' end of the *xyl_p* sequence, stop codon and *Xba*I restriction site (6 bp) are incorporated and it was utilized in the generation of both *xyl_{pext}* and *xyl_{pint}* genes throughout the study. The sequences of all designed primers are given in Table 3.11.

4.1.1.2 Amplification of *xyl_p* Gene by PCR

The key parameters in the designing of successful PCR program for specific amplification of target gene are the annealing temperature and time which vary depending on the sequences to be amplified. Required time for each cycle was calculated by the formula of 1000 bp=1 min and 1.5 min was determined as the sufficient annealing time for full length amplification and the finest annealing temperature was determined as 60°C. Since a heat-resistant DNA polymerase (*Taq* DNA polymerase) utilized for the amplification reaction, the extension temperature was arranged to 72°C which is the optimum temperature for *Taq* DNA polymerase activity. The parameters and the final compositions of the polymerase chain reaction mixture were given in the section 3.7.7.

In all conducted PCR experiments, negative control reaction which restrains all reaction matters excluding the template DNA was also performed in order to check incidence of any contamination during the PCR reaction.

4.1.1.2.1 Generation of *xyl_{pext}* Gene

After synthesis of proper single-stranded primers, *xyl_{pext}* gene with *Eco*RI and *Xba*I restriction enzyme extensions (final length=851 bp) was amplified with PCR. In order to add polyhistidine-tag as well as Factor Xa recognition site to the N-terminal end of *xyl_p*, two PCR processes were carried out. In the first set of PCR, 1µl of the chromosomal DNA isolated from hyper thermophile *P. furiosus* strain (section 3.7.3) was used as template to elongate 5' end of *xyl_p* gene with 2×histidine

constituent and Factor Xa recognition site using the primer set GI_{pext}-F1 and GI_p-R. The amplification reaction was performed at the condition mentioned in section 3.7.7 with some modifications where pfu polymerase enzyme was used instead of Tag DNA polymerase to increase the fidelity of polymerization reaction and the annealing time was reduced to 1min. The purified PCR product was run on the agarose gel and the 832 bp band was obtained (Figure 4.3, Lane1-2).

In the second round of the PCR, 1µl of the purified PCR product from the first set was used as template with the primers GI_{pext}-F2 and GI_p-R to add four remaining histidine residues of polyhistidine-tag as well as *EcoRI* and *XbaI* restriction sites to the gene sequence. Once more, the purified PCR product was run on the agarose gel and the 851 bp band was obtained (Figure 4.3, Lane 3). The schematic illustration of two different PCR assemblies was shown on Figure 4.4.

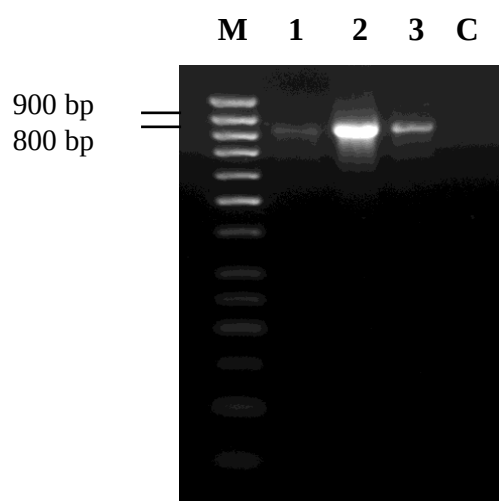
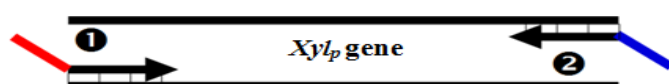


Figure 4.3 Agarose gel electrophoresis result. M: 50 bp DNA Marker, Lane 1-2: *xyl_p* gene amplified in PCR1, Lane 3: *xyl_p* gene amplified in PCR2, C: negative control.

PCR SET A:



1) **2×His+ Factor Xa** + complimentary to anti-sense strand of the *Xyl_p* gene

2×His+ Factor Xa (CACCACATTGAAGGGAGA)

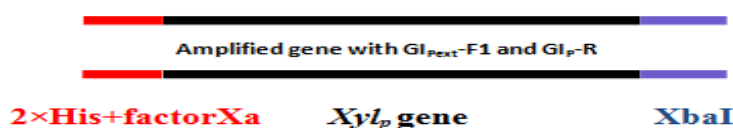
Sequence: 5' CACCACATTGAAGGGAGAATGAAAGTTGGAGTTAGC3'

2) **Restriction Enzyme Region**+ complimentary to sense strand of the *Xyl_p* gene

XbaI(TCTAGA)

Sequence: 5' GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG3'

PCR 1
↓



PCR SET B:



3) **Restriction Enzyme Region +4×His**+complimentary to anti-sense strand of PCR1 product

EcoRI (GAATTC) + 4×His

Sequence: 5'GGAATTCCATCACCATCACCAACACATTGAAGGGAGA3'

4) **Restriction Enzyme Region**+ complimentary to sense strand of the *Xyl_p* gene

XbaI(TCTAGA)

Sequence: 5' GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG3'

PCR 2
↓



Figure 4.4 Schematic illustrations of polymerase chain reactions for amplification of extracellular *xyl_{pext}* gene.

4.1.1.2.2 Generation of *xyl_{pint}* Gene

For the generation of recombinant *P.pastoris* strain that expresses *xyl_p* gene intracellularly, *Asu*II and *Xba*I restriction sites were selected and integrated to the 5' and 3'ends of the *xyl_p* gene, respectively, intended for deletion of α -Factor signal sequence from pPICZ α A expression vector map during the restriction digestion reaction. In this frame, 1 μ l of the *P. furiosus* chromosomal DNA was utilized as template with the primer set of GI_{pint}-F and GI_p-R through the PCR. The purified PCR product was run on the agarose gel and the 826 bp band was obtained (Figure 4.5, Lane 1). The sequences and assembly of primer set for generation and amplification of the intracellular *xyl_p* gene (*xyl_{pint}*) was illustrated in Figure 4.6.

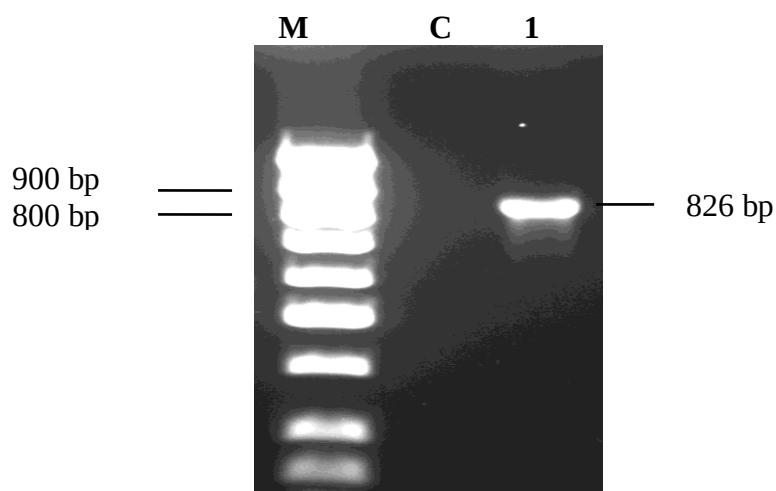
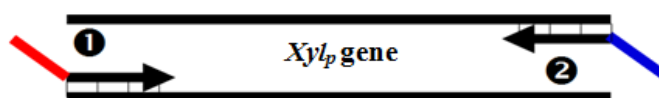


Figure 4.5 Agarose gel electrophoresis result. M: 50 bp DNA Marker, C: negative control, Lane 1: amplified *xyl_{pint}* gene via PCR using primers GI_{pint}-F and GI_p-R.



1) **Restriction Enzyme Region** + complimentary to anti-sense strand of the *Xyl_p* gene

AsuII (TTCGAA)

Sequence: 5' GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA 3'

2) **Restriction Enzyme Region** + complimentary to sense strand of the *Xyl_p* gene

XbaI (TCTAGA)

Sequence: 5' GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG 3'

Figure 4.6 Schematic illustrations of polymerase chain reactions for amplification of extracellular *xyl_{pint}* gene.

4.1.1.3 Propagation and purification of pPICZαA

The pPICZαA vector was propagated in *E.coli* Top10 cells. The plasmid was isolated from the cultivation of a single colony (section 3.7.2) and digested once to visualize its actual size (3593 bp) in agarose gel electrophoresis. Undigested plasmid run faster on agarose gel and is not useful in visualizing the actual size of the plasmid. Figure 4.7-A and 4.7-B represent digested and undigested gel electrophoresis images of pPICZαA vector, respectively.

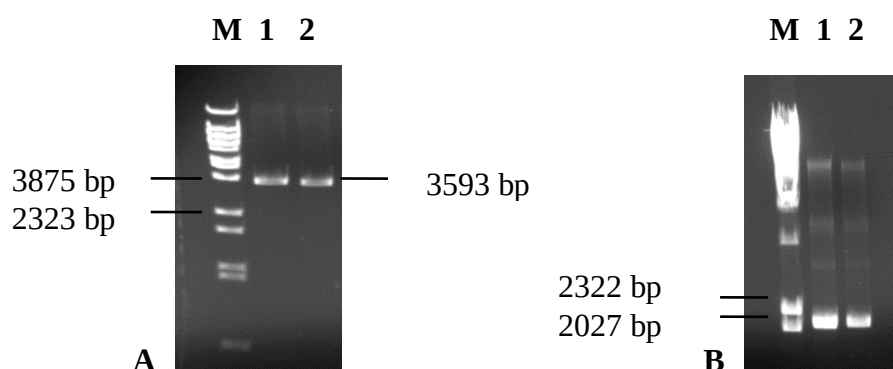


Figure 4.7 Agarose gel electrophoresis results. A) M: λDNA/Eco91I Marker, Lane 1-2: single digested pPICZαA. B) M: λDNA/HindIII Marker, Lane 1-2: undigested pPICZαA.

4.1.1.4 Digestion Reaction

Restriction enzymes which were utilized in the construction of extracellular and intracellular *Pichia* expression systems can be summarized as follow:

- *EcoRI* and *XbaI* restriction sites are at either ends of the multiple cloning site of the vector pPICZ α A, so there would not be any extra nucleotides in the sequence.
- *AsuII* and *XbaI* restriction site was selected for intracellular *xyl_p* expression to delete α -Factor signal peptide sequence from the pPICZ α A vector in the manner that there would not be any extra nucleotides in the sequence.
- They have no restriction sites in the sequence of *xyl_p* gene as verified by NEB cutter V2.0 software (<http://tools.neb.com/NEBcutter2/index.php>).

Seven set of digestion reactions were carried out prior to the ligation step:

1. pPICZ α A digestion with *EcoRI* for control
2. pPICZ α A digestion with *XbaI* for control
3. pPICZ α A digestion with *AsuII* for control
4. pPICZ α A double digestion with *XbaI* and *EcoRI* for extracellular ligation
5. *xyl_{Pext}* double digestion with *XbaI* and *EcoRI* for extracellular ligation
6. pPICZ α A double digestion with *XbaI* and *AsuII* for intracellular ligation
7. *xyl_{Pint}* double digestion with *XbaI* and *AsuII* for intracellular ligation

As illustrated in wells 1 - 2 of Figures 4.8. A-C as well as Figures (4.9.A Lane: 2 and 4.2.B, Lane: 1-2) single and double digestions of the vector DNA were successful for both intracellular and extracellular cases. The single digestion at either end of the insert cannot be visualized directly by agarose gel electrophoresis since the fragment of DNA digested off by the enzyme is only a couple of nucleotides. Before ligation, the double digested insert DNAs (*xyl_{Pext}* and *xyl_{Pint}*) along with the double digested vector DNAs, pPICZ α As, were purified to remove the reaction reagents and extra band resulted from the digestion reaction (Figures 4.9.A and 4.9.B). *xyl_{Pext}* and *xyl_{Pint}* genes were purified by PCR purification kit (section 3.7.10);

while processed pPICZαA vectors were purified by extracting the desired band from the agarose gel (section 3.7.5).

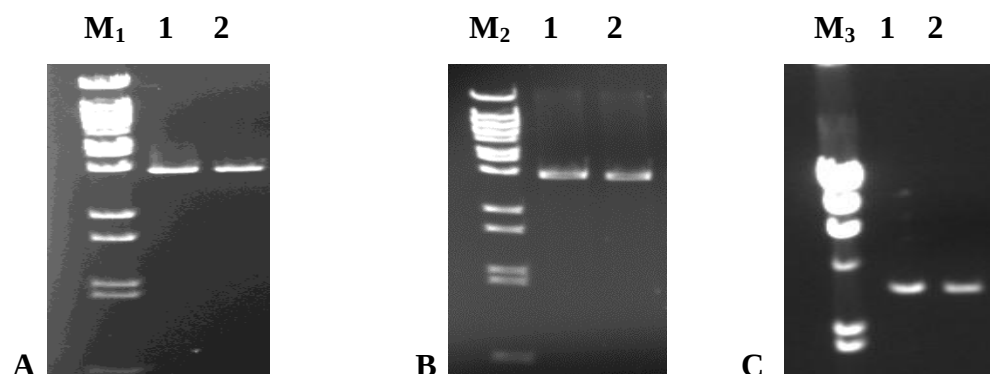


Figure 4.8 Agarose gel electrophoresis of products of single digestion reactions. A) M₁: λDNA/Eco91I Marker, Lane 1-2: pPICZαA digested with *EcoRI*. B) M₂: λDNA/ Eco91I Marker, Lane 1-2: pPICZαA digested with *XbaI*. C) M₃: λDNA/HindIII Marker, Lane 1-2: pPICZαA digested with *AsuII*.

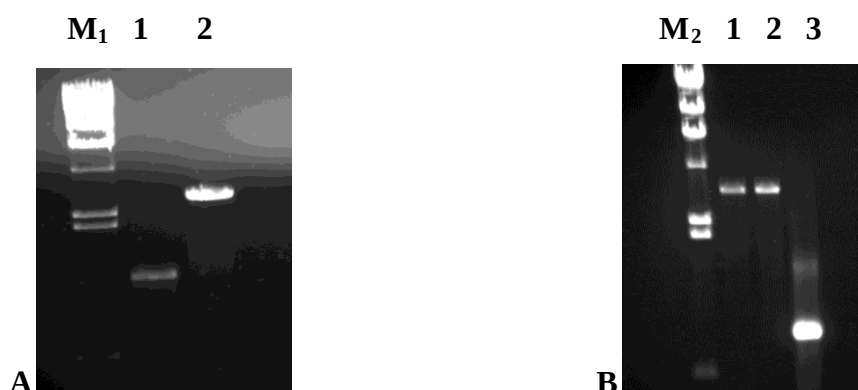


Figure 4.9 Agarose gel electrophoresis of products of double digestion reactions. A) M₁: λDNA/HindIII Marker, Lane 1: *xylP_{ext}* double digested with *EcoRI* and *XbaI*, Lane 2: pPICZαA vector double digested with *EcoRI* and *XbaI*. B) M₂: λDNA/HindIII Marker, Lane 1-2: pPICZαA vector double digested with *AsuII* and *XbaI*, Lane 3: *xylP_{int}* double digested with *AsuII* and *XbaI*.

4.1.1.5 Ligation Reaction

The ligation reaction was performed by mixing the double digested insert DNA with double digested vector DNA as explained in section 3.7.11. The ligation

mixture for development of extracellular expression vector was prepared at Gene/vector ratio of 5 by mixing 0.5 μl of the processed xyl_{pext} gene at the concentration of 238 $\text{ng } \mu\text{l}^{-1}$ with 1.5 μl of digested vector pPICZ α A at the concentration of 75 $\text{ng } \mu\text{l}^{-1}$. In the case of intracellular expression vector construction, 1.5 μl of treated xyl_{pint} gene with the concentration of 150 $\text{ng } \mu\text{l}^{-1}$ along with 5 μl of digested pPICZ α A vector at the concentration of 20 $\text{ng } \mu\text{l}^{-1}$ were added to the ligation mixture containing T4 DNA ligase enzyme, buffer and ATP. The prepared ligation mixtures were incubated at 16°C for 16 h and the artifacts of the ligation reactions were the putative pPICZ α A:: xyl_{pext} and pPICZ α A:: xyl_{pint} plasmids (Figure 4.10).

4.1.1.6 Transformation of *E. coli* cells with Putative Recombinant vectors and Selection of the True Transformants

Following to the completion of ligation step, 6 μl of each putative pPICZ α A:: xyl_{pext} and pPICZ α A:: xyl_{pint} recombinant vectors was transformed into *E. coli* XLBlue strain via CaCl_2 method as described in section 3.7.13.

The fresh transformants were grown on LSLB-agar plates supplemented with 25 $\mu\text{g ml}^{-1}$ Zeocin for 12-15 h. In the both cases, lots of single colonies were detected on the selective media. Ten and five single colonies were randomly selected to verify proper insertion of target gene into extracellular and intracellular expression vector, respectively. Selected colonies were grown in the 7 ml of LSLB + Zeocin medium for plasmid isolation. All of the selected colonies gave band bigger than the pPICZ α A vector (Figure 4.11.A-B) in gel images, where three extracellular expression vectors containing two copy of inset were also detected (Figure 4.11.A, Lane: 3, 6 and 8). The isolated plasmids were utilized in restriction enzyme digestion and PCR analyzes to check the accurate length insertion as well as correct orientation attachment of DNAs.

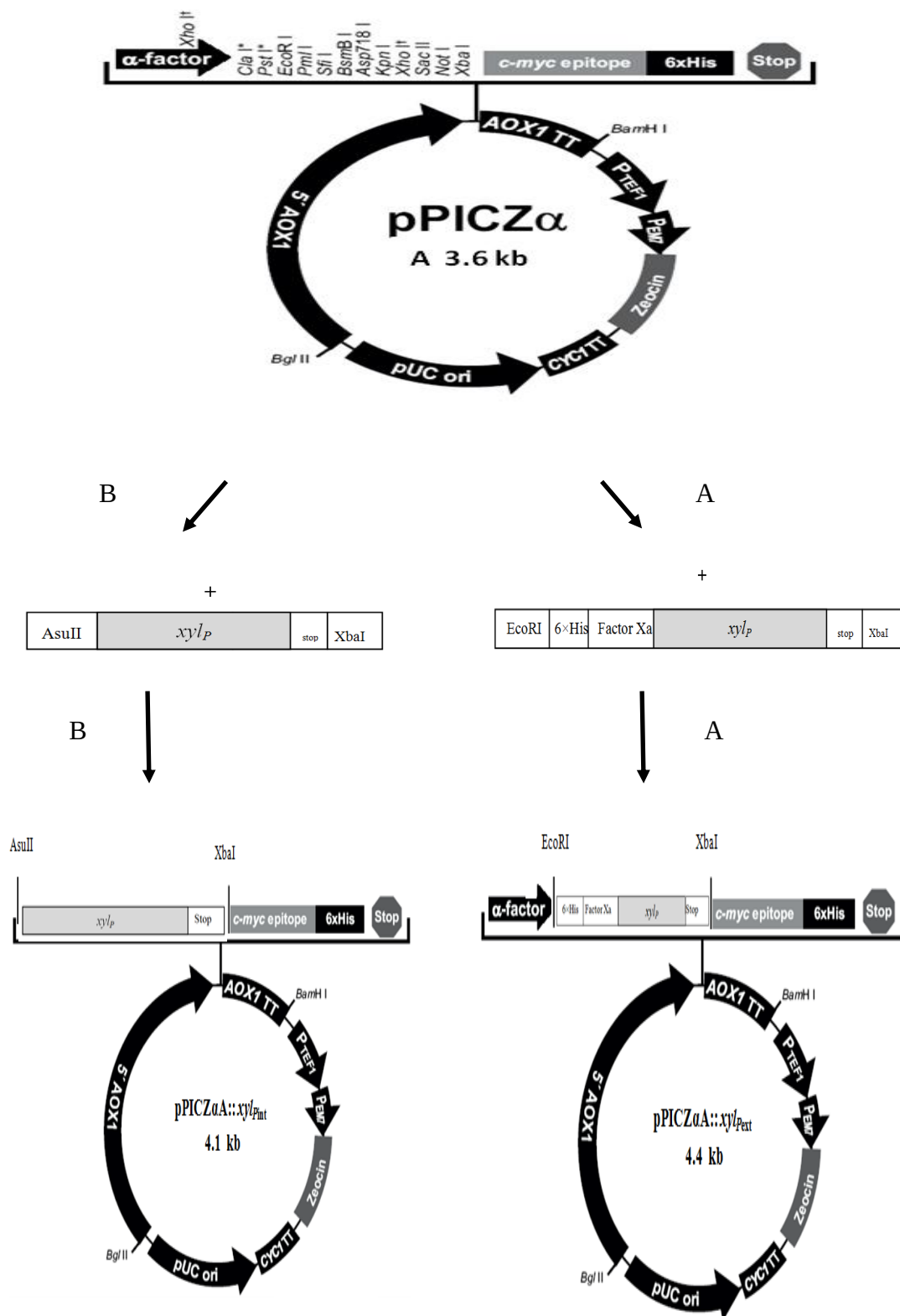


Figure 4.10 Scheme for the development of the expression systems for extracellular and intracellular GI_P production in *P. pastoris* strain. A) Construction of extracellular recombinant pPICZαA::xyl_{Pext} vector. B) Construction of intracellular recombinant pPICZαA::xyl_{Pint} vector.

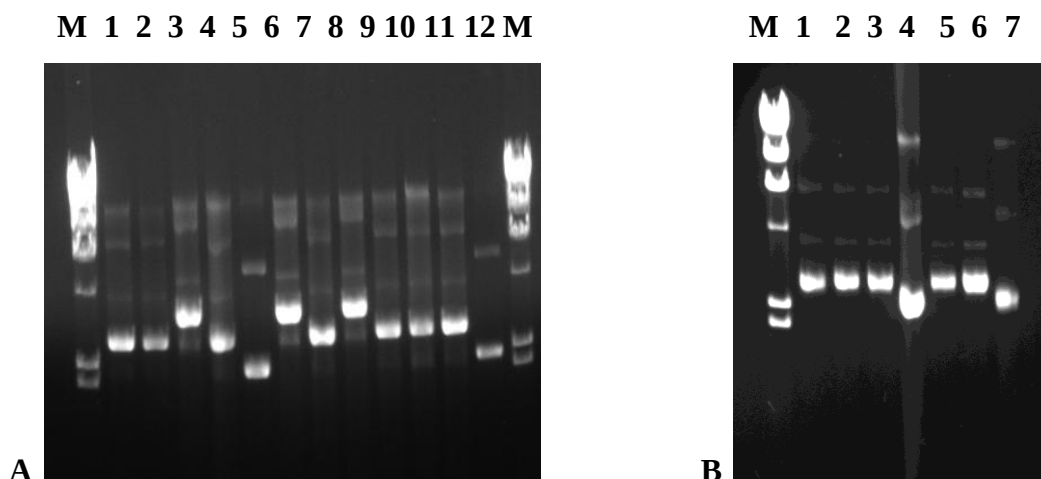


Figure 4.11 Agarose gel electrophoresis of putative positive colonies. A) M: λ DNA/HindIII Marker; Lane 5 and Lane 12: pPICZ α A vector; Lanes 1, 2, 4, 7, 9, 10 and 11: plasmid isolated from extracellular positive colony containing only one copy of xyl_{pext} gene; Lanes 3, 6 and 8: plasmids isolated from extracellular positive colonies containing two copy of xyl_{pext} gene. B) M: λ DNA/HindIII Marker; Lanes 4 and 7: pPICZ α A vector; Lanes 1, 2, 3, 5 and 6: plasmids isolated from intracellular positive colony.

4.1.1.7 Digestion and PCR Control of Positive Colonies

Four putative extracellular and three putative intracellular recombinant pPICZ α A expression vectors were purified according to the protocol given in Section 3.7.2. The purified extracellular expression vectors were first double digested with *Eco*RI and *Xba*I REs and then verified by single digestion with *Xba*I. As illustrated in Figure 4.12.A, all colonies gave a band in the exact size of xyl_{pext} insert gene (lane: 1-4) as a result of double digestion and gave a band in the size of extracellular vector and xyl_{pext} gene combination as a result of single digestion (lane: 5-8). Due to insertion of two sequences of xyl_{pext} gene in 3rd colony, two bands visualized in the gel electrophoresis image of single digestion (lane: 7). In the case of intracellular expression vectors, double and single digestion controls were carried out by the *Asu*II and *Xba*I REs and again a band in the exact size of xyl_{pint} insert gene and a band in the size of intracellular vector and xyl_{pint} gene combination were obtained as the result of double and single digestions, respectively (4.12.B).

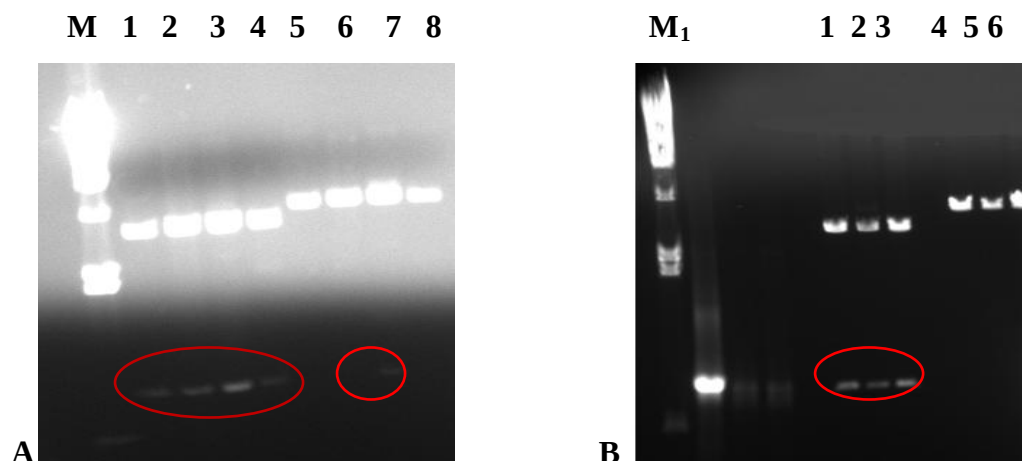


Figure 4.12 Agarose gel electrophoresis of positive colonies. A) M: λ DNA/HindIII Marker, Lane 1-4: recombinant extracellular pPICZ α A expression vector double digested with *EcoRI* and *XbaI*, Lane 4-8: recombinant extracellular pPICZ α A single digested with *XbaI*. B) M₁: λ DNA/HindIII Marker, Lane 1-3: recombinant intracellular pPICZ α A expression vector double digested with *AsuII* and *XbaI*, Lane 4-6: recombinant intracellular pPICZ α A single digested with *XbaI*.

The plasmid isolated from positive colonies were also controlled by PCR, using GI_{pe_{ext}}-F₂ and GI_P-R primers for verification of *xyl*_{pe_{ext}} gene insertion, and GI_{pi_{nt}}-F with GI_P-R to check insertion of *xyl*_{pi_{nt}} gene. As illustrated in Figure 4.13.A-B, in both cases successful amplification of inset gene was detected at the end of PCR. Finally, the nucleotide sequence of insert DNAs were further controlled by automatic DNA sequencers, (Refgen, Turkey) and the results confirmed that the clonings were successful for both extracellular and intracellular expression vectors development. The DNA sequences of pPICZ α ::*xyl*_{pe_{ext}} and pPICZ α ::*xyl*_{pi_{nt}} are given in Appendix D.

4.1.1.8 Transfection of *P. pastoris* cells with Recombinant pPICZ α A plasmids

With the aim of genomic integration of pPICZ α A::*xyl*_{pe_{ext}} and pPICZ α ::*xyl*_{pi_{nt}} plasmids into at the AOX1 locus, the plasmids were linearized at its AOX promoter region so that double integration event can occur at AOX locus. *SacI* restriction

enzyme was employed as a single-cutter RE for linearization of pPICZ α A::xyl_{pe} and pPICZ α ::xyl_{pi} plasmids.

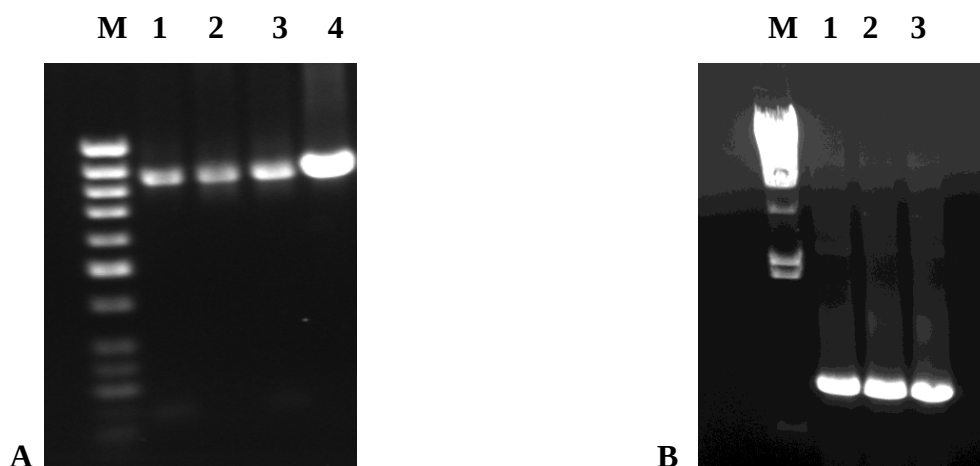


Figure 4.13 Agarose gel electrophoresis of PCR control. A) M: 50 bp DNA ladder, Lane 1-4: xyl_{pe} gene amplified from recombinant extracellular pPICZ α A expression vectors by PCR with Tag polymerase. B) M: λDNA/HindIII Marker, Lane 1-4: xyl_{pi} gene amplified from recombinant intracellular pPICZ α A expression vectors by PCR with Tag polymerase.

Full digestion of recombinant plasmids were achieved by incubating plasmids along with *Sac*I in the appropriate buffer for 6 h at 37°C and full digestion verified by agarose gel electrophoresis. Then the digestion product was purified by the extraction from agarose gel (section 3.7.5). The concentration of the digested and purified plasmids was arranged to 0.2 μg μl⁻¹ to increase efficiency of cell transfections.

Transfection was performed as explained in the section 3.7.14. After 48 h of incubation at 30°C, five and four single colonies were randomly selected from extracellular and intracellular transfected *Pichia* cells, respectively for further controls. The selected colonies were inoculated onto YPD + Zeocin (0.100 kg m⁻³) plates for short term storage and then inoculated into 10 ml YPD + Zeocin medium. Their genomic DNA was isolated to be used in controls by PCR (Figure 4.14). All of the selected recombinant colonies showed positive results at the end of PCR.

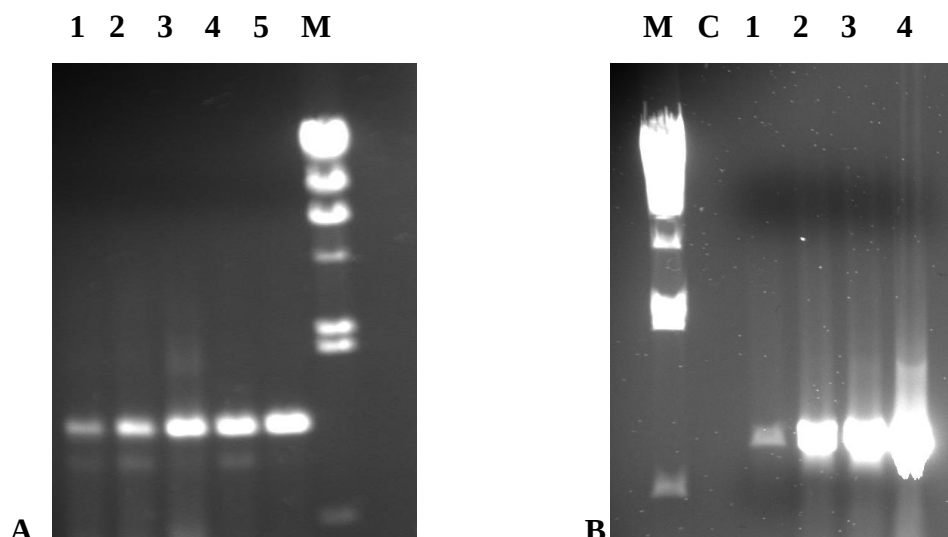


Figure 4.14 Agarose gel electrophoresis images of *xyl_{Pext}* and *xyl_{Pint}* genes amplification from genomic DNA of *P. pastoris* transformants via PCR. A) Lane 1-5: PCR amplification of *xyl_{Pext}* (expected size=851 bp) from five extracellular colonies by using *GI_{Pext}-F₂* and *GI_P-R* primers, M: λ DNA/HindIII Marker. B) M: λ DNA/HindIII Marker, C: negative control, Lane 1-4: PCR amplification of *xyl_{Pint}* (expected size=826 bp) from four intracellular colonies by using *GI_{Pint}-F* and *GI_P-R* primers.

4.1.1.9 Expression of *GI_P* in Recombinant *P. pastoris* in Laboratory Scale Air Filtered Shake Bioreactors

After developing recombinant *P. pastoris* strains expressing hypothetical *xyl_P* gene of thermophilic *P. furiosus* strain in both extracellular and intracellular manners, the GI activity of recombinant cells were compared with each other to select the most suitable strains producing the thermostable rGI enzyme at high expression levels. In this frame, two colonies of positively transfected cells from each extracellular and intracellular recombinant *P.pastoris* strains were selected and grown in the fresh solid medium. Then, grown colonies were transferred into precultivation medium and incubated at $T=30^{\circ}\text{C}$ and $N=225\text{ min}^{-1}$ for $t=24\text{ h}$ in agitation and heating rate controlled orbital shakers using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 10 ml. Then, they were harvested by centrifugation at 4000 rpm, 10 min at room temperature and resuspended in BMMY production medium (including 1.0 % (v/v) methanol). The recombinant cells were incubated at $T=30^{\circ}\text{C}$ and $N=225\text{ min}^{-1}$ for $t=42\text{ h}$ using air-filtered, baffled Erlenmeyer flasks 250

ml in size that had working volume capacities of 50 ml. Every 24 h, 1.0% methanol was added to the production medium and 1 ml of production medium from each flask was taken and GI activity of the samples were measured by cysteine-carbazol-sulfuric acid method. As illustrated in Figure 4.15, the maximum extracellular GI_P activity was obtained via cultivation of *P. pastoris* pPICZα::xyl_{Pext2} strain as 210 U L⁻¹. The highest value for intracellular GI_P activity was attained as 342 U L⁻¹ by *P. pastoris* pPICZα::xyl_{Pint2} recombinant cells, where the cultivation of wild type strain in the same condition and medium result in GI activity at the value of 107 U L⁻¹. However, compared with GI activity of thermophilic wild type microorganisms such as *Bacillus thermoantarcticus* with the reported activity of 1660 U L⁻¹ (Çalık et al., 2008) or other prokaryotic recombinant strains like an *E.coli* TB1 as 1595 U L⁻¹ (Sarıyar et al., 2004), it was concluded that both designed extracellular and intracellular recombinant *P. pastoris* systems were not very successful in the term of GI production.

There are two probable explanations for detection of inferior GI activity in *P. pastoris* expression system. The first one is based on the sequence of xyl_P gene. The amino acid sequence of hypothetical GI_P only shows ~ 75% similarity with the protein sequence of other thermostable GI enzymes so there is possibility for recombination of inactive xyl gene. The other explanation is based on the selected expression system. There is another study under the progress in our research group in which the thermostable xyl gene from *Thermus thermophilus* with the documented activity was recombined both extracellularly and intracellularly to the pPICZαA expression vector. Again the similar results were detected and the trivial GI activity at the value of 178 U L⁻¹ and 274 U L⁻¹ were attained by recombinant *P. pastoris* pPICZα::xyl_{Text} and *P. pastoris* pPICZα::xyl_{Tint} strains, respectively (Figure 4.15, pink bars). Most likely, the cytosolic environment of the yeasts cells prohibits the formation of stable GI tetramer structure which results in proteolytic degradation of the enzyme even in protease deficient yeast strains. Although, based on the these findings it is reasonable to conclude that *P. pastoris* is not the most promising host for the over-production of tetrameric or dimeric GI, the doubt on the active function of hypothetical GI from *Pyrococcus furiosus* cannot be eliminated definitely.

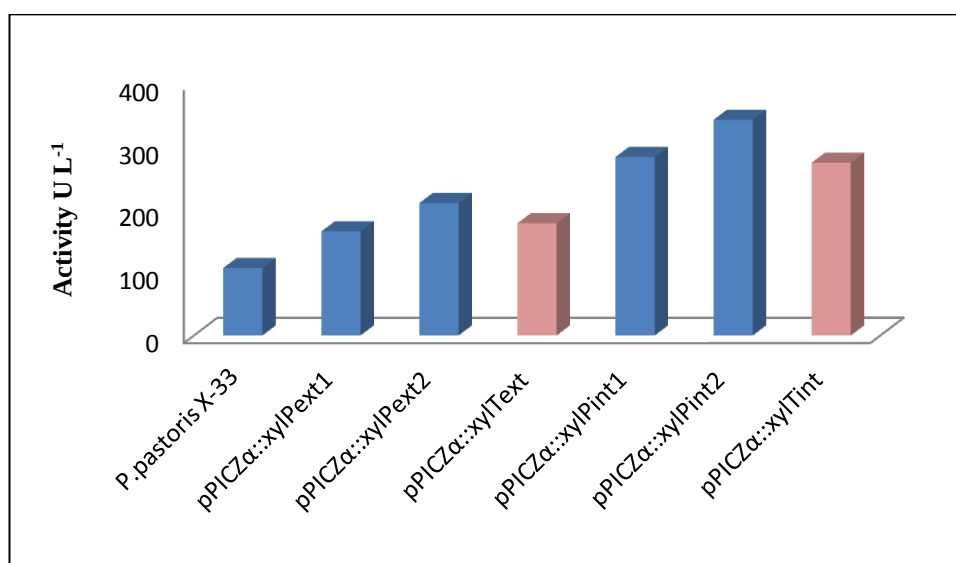


Figure 4.15 GI activity measurements of recombinant *Pichia pastoris* strains.

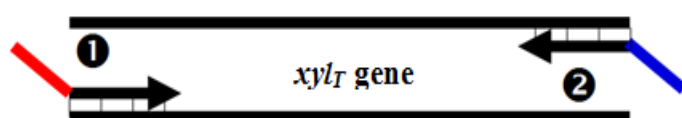
4.1.2 Development of Recombinant *E. coli* Strain

On the basis of these results, at the second attempt for the over-production of thermostable GI enzyme, the recombination procedures were tried with the different *xyl* gene and host. In this favor, the PCR amplified thermostable *xyl_T* gene of *Thermus thermophilus* strain with the documented activity was fused with *NdeI* and *EcoRI* restriction sites under the catalytic activity of *Taq* DNA polymerase. After the subsequent digestion reactions with selected RE enzymes, *xyl_T* gene was ligated to pRSETA vector and the generated expression vector, pRSETA::*xyl_T*, was transformed into *E.coli* XL1 Blue strain with the aim of propagating the recombinant plasmid. The proper length insertion and correct orientation attachment of target gene were verified by restriction digestion, PCR amplification and DNA sequencing. The colony that gave the exact match of sequence with the wild type *xyl_T* was transformed into *E.coli* BL21 (DE3) pLysS strain and *E.coli* BL21 (DE3) star strains. *E.coli* BL21 (DE3) pLysS carrying recombinant pRSETA::*xyl_T* plasmid was selected as the glucose isomerase producer, due to the highest GI production capability of this strain in the glucose based cultivation medium as 1940 U L⁻¹. The experimental steps

which were carried out through the construction of recombinant *E.coli* expression system producing rGI, were schematically summarized in Figure 4.17.

4.1.2.1 Primer Design for Generation of *xyl_T* Gene from *T.thermophilus*

The following forward (GI_T-F) and reverse (GI_T-R) primers were designed in accordance to the sequence of *xyl_T* gene of *T.thermophilus* in order to add NdeI (6 bp) and EcoRI (6 bp) restriction sites to the 5' and 3' ends of target sequence during the PCR amplification (Figure 4.16).



1) **Restriction Enzyme Region+ GI_T -F Primer**

NdeI (CATATG)

Sequence: 5' CGCATATGTACGAGCCCAAACCGGAGCACAGG 3'

2) **Restriction Enzyme Region+ GI_T -R Primer**

EcoRI (GAATTC)

Sequence: 5' GGAATTCTTCACCCCCGCACCCCCAGGAG 3'

Figure 4.16 Illustration of primer design for expression of the *xyl_T* in *E. coli* strains. 1) *NdeI* RE sequence was associated in front of GI forward primer as a single-stranded tail. GI_T-F primer is complimentary to anti-sense strand of the *xyl_T* gene; 2) *EcoRI* RE sequence was associated in front of GI reverse primer as a single-stranded tail. GI_T-R primer is complimentary to sense strand of the *xyl_T* gene.

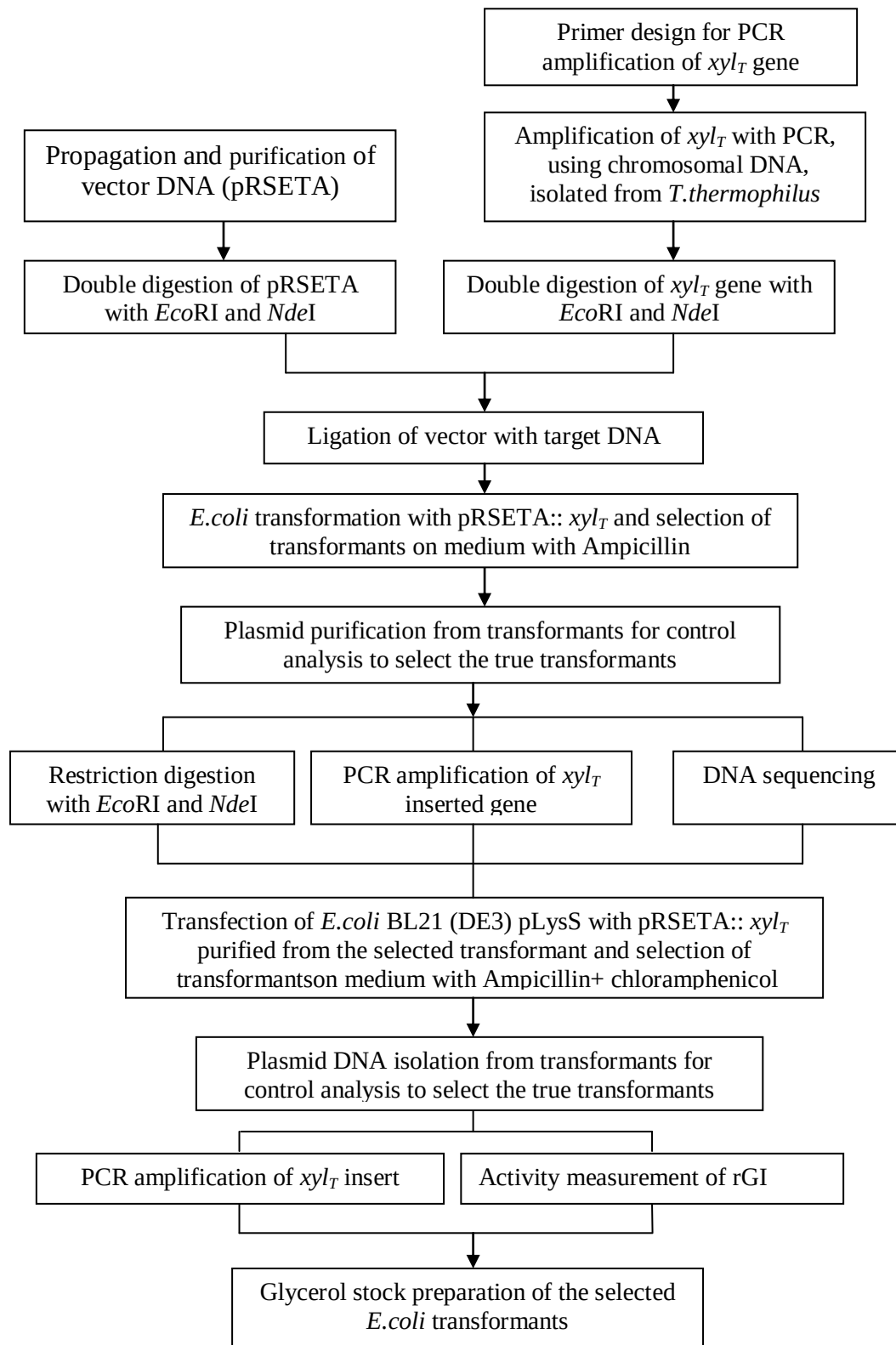


Figure 4.17 Flowchart of the research plan, for the development of the r- *E. coli* producing GI_T.

4.1.2.2 Amplification of *xyl_T* Gene by Polymerase Chain Reaction (PCR)

Subsequent to synthesis of appropriate single-stranded primers, *xyl_T* gene with *Nde*I and *Eco*RI restriction enzyme extensions (final length=1177bp) was amplified with PCR. In this frame, artificially synthesized oligonucleotides, GI_T-R and GI_T-F primers were added to the reaction mixture containing chromosomal DNA isolated from *T.thermophilus* (Figure 4.18) as template and the temperature of the mixture was raised to 94°C in denaturation step to obtain single-stranded template DNA for the synthesis of the complimentary new strand.

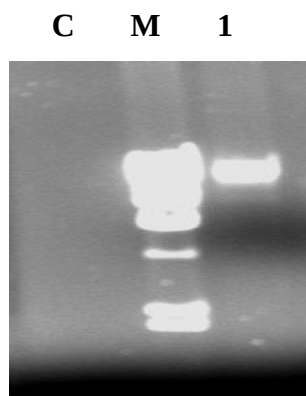


Figure 4.18 Agarose gel electrophoresis of chromosomal DNA isolated from *T. thermophilus*. C: Negative control, M: λDNA/HindIII Marker, Lane 1: chromosomal DNA.

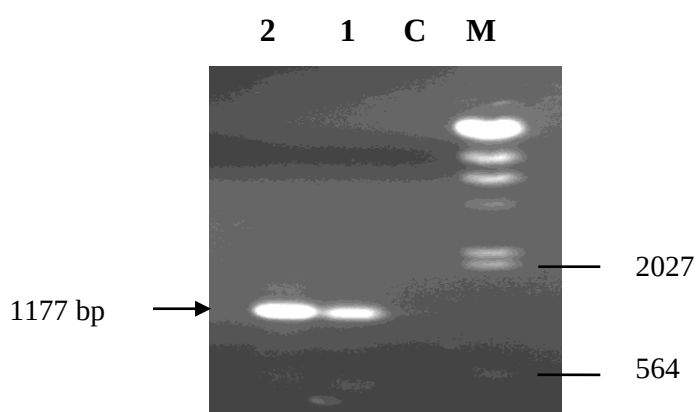


Figure 4.19 Agarose gel electrophoresis image of *xyl_T* gene. M: λDNA/HindIII Marker, C: Negative control, Lane 1-2: *xyl_T* gene amplified by PCR, using *T.thermophilus* chromosomal DNA as template and the primer set GI_T-F and GI_T-R primers.

Then the mixture is rapidly cooled to provide a suitable condition for annealing of primers to the complementary sequences in the DNA molecule. Figure 4.19 represents agarose gel electrophoresis image of *xyl_T* gene amplification by PCR.

4.1.2.3 Propagation and purification of pRSETA

The pRSETA vector was propagated in *E.coli* XLBlue cells. The plasmid was purified from a single colony and digested once to visualize its actual size (2900 bp) in agarose gel electrophoresis (Figure 4.20). Undigested plasmid runs faster on agarose gel and is not useful in visualizing the actual size of the plasmid.

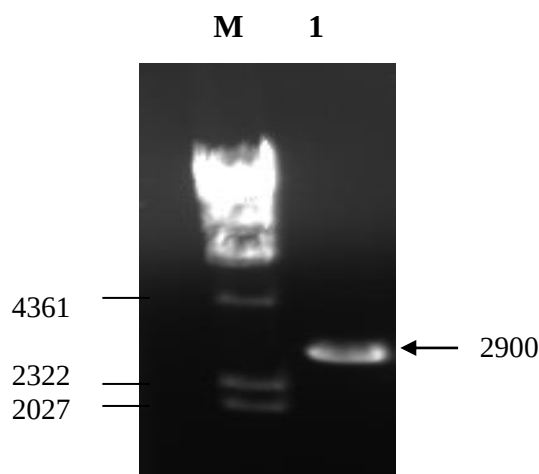


Figure 4.20 Agarose gel electrophoresis of pRSETA extracted from *E. coli* XLBlue. M: λ DNA/HindIII Marker, Lane 1: single digested pRSETA vector.

4.1.2.4 Digestion Reaction

The following parameters were considered in the selection of restriction enzymes to insert the *xyl_T* gene into the pRSETA vector:

- *EcoRI* and *NdeI* restriction sites are at either ends of the multiple cloning site of the vector pRSETA, so there would not be any extra nucleotides in the sequence.

- They have no restriction sites in the sequence of *xyl_T* gene as verified by NEB cutter V2.0 software (<http://tools.neb.com/NEBcutter2/index.php>).
- Both *EcoRI* and *NdeI* are relatively inexpensive and readily available enzymes.

Four sets of digestion reactions were carried out prior to the ligation step:

1. pRSETA digestion with *NdeI* for control,
2. pRSETA digestion with *EcoRI* for control,
3. pRSETA double digestion with *NdeI* and *EcoRI* for ligation,
4. Insert DNA double digestion with *NdeI* and *EcoRI* for ligation.

As illustrated in wells 1 - 2 of Figures 4.21.A-B, digestion of the vector DNA was successful at both ends. The single digestion at either end of the insert cannot be visualized directly by agarose gel electrophoresis (Figure 4.21.C, lanes 1) since the fragment of DNA digested off by the enzyme is only a couple of nucleotides. Before ligation, the double digested insert DNA, *xyl_T*, and the double digested vector DNA, pRSETA, were purified to remove the reaction reagents and extra band resulted from the digestion reaction. *xyl_T* gene was purified by PCR purification kit (section 3.7.10); while processed pRSETA vector was purified by extracting the desired band from the agarose gel (section 3.7.5). After each purification step, DNA concentration loss was detected; while the loss percentage with Gene Elution Kit was higher when compared with PCR purification Kit.

4.1.2.5 Ligation Reaction

The ligation reaction was performed by mixing the double digested insert DNA with double digested vector DNA as explained in section 3.7.11. The ligation reaction mixtures were prepared at different Gene/vector ratio of 3-7 by mixing the processed *xyl_T* gene with the concentration of 208 ng μl^{-1} , and digested vector pRSETA with the concentration of 92 ng μl^{-1} and the reaction was carried out at 16°C for 16 h. The artifacts of the ligation reaction were the putative pRSETA::*xyl_T*

plasmids (Figure 4.22). The assembly of the recombinant molecule, pRSETA::xyl_T, was schematically illustrated in Figure 4.23.

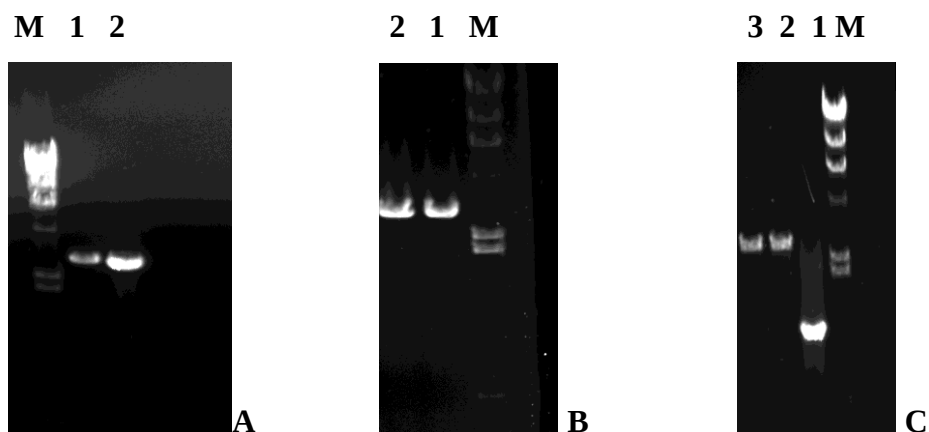


Figure 4.21 Agarose gel electrophoresis of products of digestion reactions. A. M: λ DNA/HindIII Marker, Lane 1: pRSETA digested with *Eco*RI, Lane 2: pRSETA digested with *Eco*RI and *Nde*I. B. M: λ DNA/HindIII Marker, Lane 1-2: pRSETA digested with *Nde*I. C. M: λ DNA/HindIII Marker, Lane 1: insert DNA digested with *Eco*RI and *Nde*I, Lane 2-3: pRSETA digested with *Eco*RI and *Nde*I.

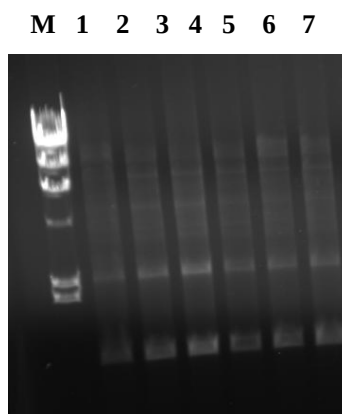


Figure 4.22 Agarose gel electrophoresis of ligation products. M: λ DNA/HindIII, Lane 1-8: different Gene/vector ratio of 3-7, respectively.

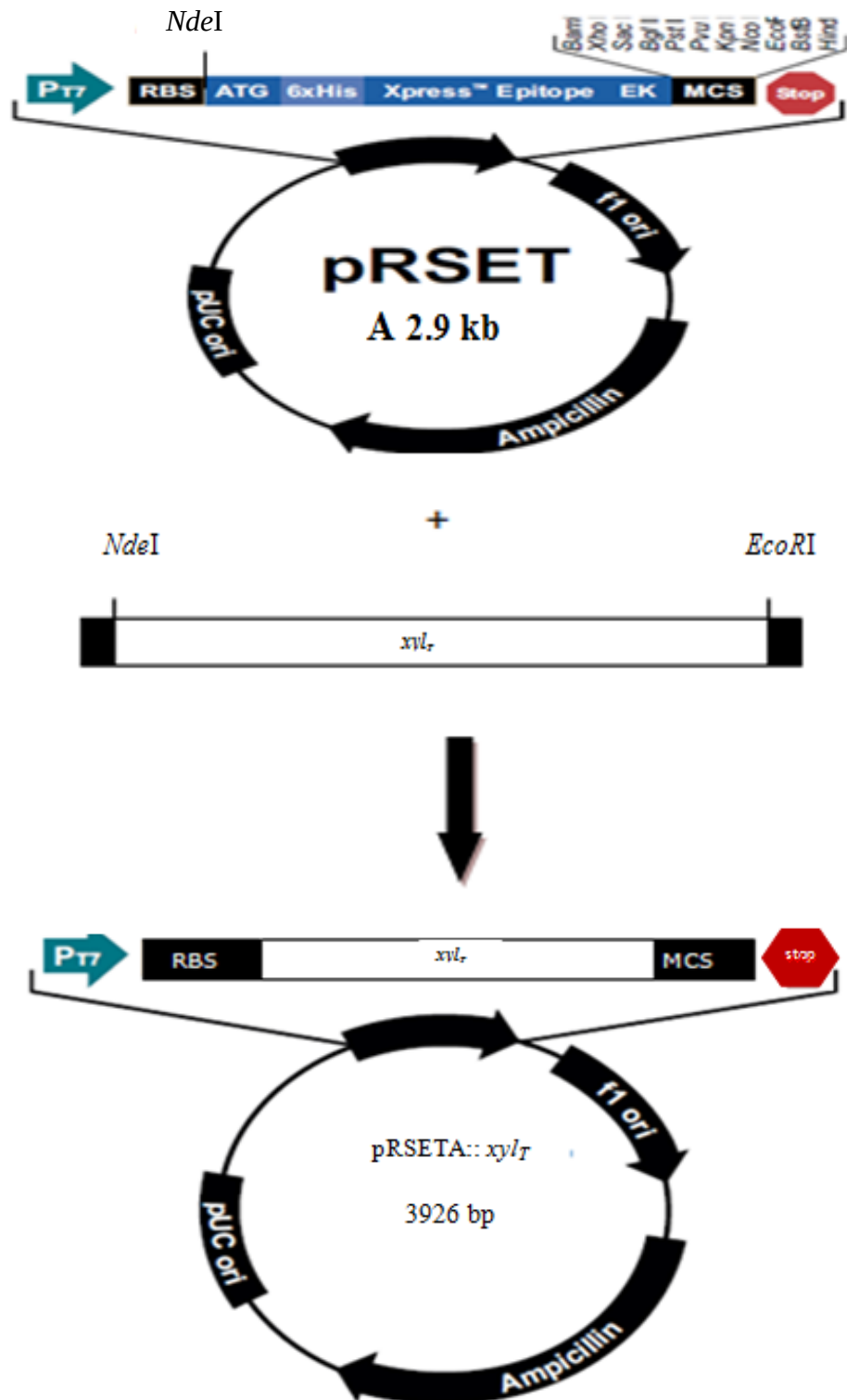


Figure 4.23 Scheme for the construction of an expression system for GI_T production in *E. coli* strain. 1.177 kbp DNA fragment of PCR amplified xyl_T gene with $NdeI$ and $EcoRI$ endings was associated to pRSETA plasmid from $NdeI$ and $EcoRI$ restriction sites.

4.1.2.6 Transformation of *E. coli* cells with pRSETA::xyl_T and Selection of the True Transformants

Subsequent to the ligation reaction, the putative recombinant plasmids were propagated by transferring 6 µl of ligation products into *E.coli* XLBlue strain via CaCl₂ method as described in section 3.7.13 and fresh transformants were grown on LB agar plates supplemented with 100 µg ml⁻¹ ampicillin for 12-15 h. Five single colonies were randomly selected and grown in 7 ml LB + Ampicillin medium for plasmid isolation. Only three colonies among the selected ones gave band bigger than the pRSETA vector (Figure 4.24) and these isolated plasmids were used to test the proper length insertion and correct orientation attachment of the DNA by restriction enzyme digestion and PCR assays.

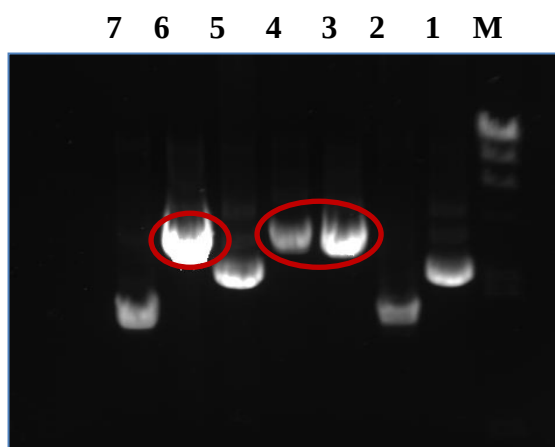


Figure 4.24 Agarose gel electrophoresis of putative positive colonies. M: λDNA/HindIII Marker, Lane 1 and Lane5: pRSETA vector, Lane 2: plasmid isolated from false 1st positive colony, Lane 3 and Lane 4: plasmids isolated from 2nd and 3rd positive colonies, Lane 6 and Lane 7: plasmids isolated from 4th positive and 5th false positive colonies, respectively.

4.1.2.7 Digestion and PCR Control of Positive Colonies

The plasmids purified according to the protocol given in section 3.7.2, firstly, were single digested with *Nde*I. Digestion control was followed by double digestion with *Eco*R I and *Nde* I. As seen in Figure 4.25 (lanes: 1-6), all three colonies gave bands in the precise size of xyl_T insert gene as well as the gene and vector

combination in consequence of double and single digestion reactions, respectively. The selected three colonies were also controlled by PCR, using GI_T-F and GI_T-R primers, and all three colonies gave a band in the size of 1164 bp as expected (Figure 4.25, lanes: 7-12). As final control, the sequences of isolated plasmids from positive colonies were determined by automatic DNA sequencers (section 3.7.12) and one of the three colonies gave the precise sequence (Appendix D) when analyzed by Basic Local Alignment Search tool (BLAST). The colony that gave the exact match of sequence was named as *E. coli* pRSETA::*xyl_T*, and its microbank stock was prepared for long time storage.

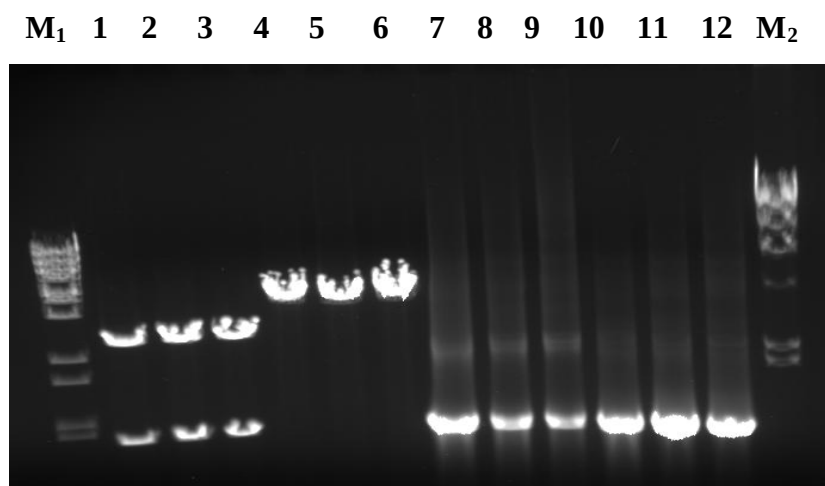


Figure 4.25 Agarose gel electrophoresis of positive colonies. M₁: Eco91 I Marker, Lane 1-3: recombinant pRSETA double digested with *Nde* I and *Eco*R I, Lane 4-6: recombinant pRSETA single digested with *Nde*I, Lane 7-9: *xyl_T* gene amplified from r-pRSETA by PCR with pfu polymerase, Lane 10-12: *xyl_T* gene amplified from r-pRSETA by PCR with Tag polymerase, M₂: λ DNA/HindIII Marker.

4.1.2.8 Transfection of *E.coli* BL21 (DE3) Strains with pRSETA::*xyl_T*

The propagated recombinant pRSETA::*xyl_T* plasmid was transformed into *E.coli* BL21 (DE3) pLysS strain and *E.coli* BL21 (DE3) star strains by CaCl₂ method as described in section 3.7.13 and transfected cells were cultivated in glucose-based production medium (Table 3.8) induced with 1mM Isopropyl β -D-1-

thiogalactopyranoside (IPTG) at $t=4.0$ h to differentiate the strain with the highest rGI production capability. For this purpose, the samples from production mediums were taken at $t=12$ h and GI activity of them were measured as described in the section 3.6.2.

As it is seen from Figure 4.26, the highest GI activity was observed in *E.coli* BL21 (DE3) pLysS pRSETA:: *xyI*_T strain in production medium as 1940 U L^{-1} and 10.3-fold increase in GI activity was observed when compared with wild type *E.coli* BL21 GI activity. Therefore *E.coli* BL21 (DE3) pLysS carrying the pRSETA::*xyI*_T was selected as potential producer of GI and named as V11 strain.

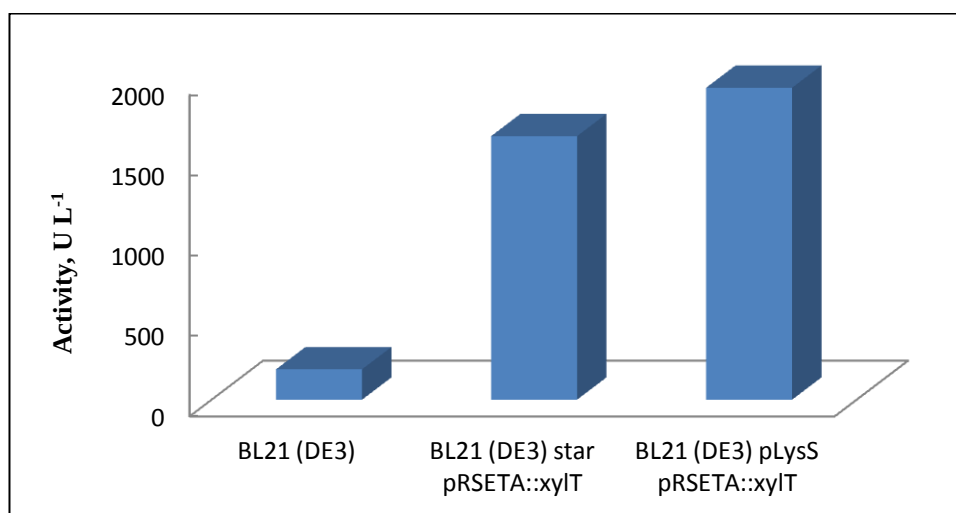


Figure 4.26 The variation in GI activity for *E.coli* strains. $C_G = 8.0 \text{ kg m}^{-3}$, $C_N = 5.0 \text{ kg m}^{-3}$, $t=12$ h, $V=33$ ml, $T=37^\circ\text{C}$, $N=200 \text{ min}^{-1}$.

4.2 Expression of Thermostable glucose isomerase in r- *E. coli* BL21 (DE3) pLysS Strain

4.2.1 Microorganism Selection

In order to select proper recombinant microorganism for over-production of thermostable glucose isomerase, the rGI activity obtained from *E.coli* BL21 (DE3) pLysS, pRSETA::*xyI*_T strain was compared with that of obtained from *P.pastoris*

pPICZ α ::*xyl*_{pinT}. The GI production by *E.coli* BL21 (DE3) pLysS strain carrying pRSETA::*xyl*_T plasmid was resulted in 5.7- fold increase in the enzyme activity. So that, the *E.coli* expression system was favored as the potential producer of rGI and the effect of feeding strategy and bioprocess parameters on the thermostable rGI production was investigated by using this strain.

4.2.2 The Effect of Inducers on Production of GI by r- *E. coli* BL21 (DE3) pLysS Strain

After selection of the appropriate recombinant microorganism, the effect of natural and artificial inducers (lactose, arabinose and IPTG) at concentration of 1mM on T7 expression system of *E.coli* BL21 (DE3) pLysS, pRSETA::*xyl*_T was examined. T7 expression system is based on the production of T7 RNA polymerase which is under control of LacUV5 promoter. The important point in induction of the Lac UV5 promoter is the intracellular concentration of inducer so inducers were supplied to cultivation medium before cell growth approaching to its saturation value. For this purpose, glucose based recombinant *E.coli* cell cultures were induced with lactose, arabinose, IPTG as well as combination of lactose and IPTG at t=4 h hour of bioprocess and one flask was left as control without inducer. The induced cell cultures were incubated at T=37°C and N=200 min⁻¹ for t=24 h in agitation and heating rate controlled orbital shakers using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. Table 4.1 represents rGI activity of samples taken in 12nd hour of bioprocess. The highest activity of rGI at t=12 h was obtained in the medium containing IPTG as inducer. Since IPTG is non degradable inducer, it is generally preferred in the bioprocesses. It is also known that IPTG can be transported through the cell membrane by diffusion without need to lac permease enzyme. Therefore, at this level it is logical to have a higher enzyme activity for IPTG induced cells. The lower enzyme activity for IPTG+ lactose induced cells may be is due to saturation of the lac permease which is a functional enzyme for lactose transportation.

Table 4.1 Enzyme activities at t=12 h of the bioprocess.

Sample	Activity (U L ⁻¹)
IPTG	1472
Lactose	1342
IPTG+ Lactose	1431
Arabinose	1308
None	0801

After taking sample at t=12 h, some modifications were performed in the cultivation media which were listed at Table 4.2. Then, the bioprocess was continued and in order to investigate the effects of additional carbon source and antibiotic on the enzyme production, samples were taken from the flasks at t=24 h of bioprocess.

Table 4.2 Modifications of Shaker Experiment including carbon source and antibiotic (+ Added once, ++ Added twice).

Flask No	t=4 h	Modification t=12 h	Final situation
1	IPTG	5 g/L glucose	IPTG ⁺
2	IPTG	IPTG + 5g/L glucose + Amp	IPTG ⁺⁺ , Amp ⁺
3	IPTG	IPTG + 5g/L glucose	IPTG ⁺⁺
4	IPTG	5 g/L glucose+ Amp	IPTG ⁺ , Amp ⁺
5	IPTG+ Lactose	IPTG+ Lactose+5g/L glucose	IPTG ⁺⁺ , Lactose ⁺⁺
6	Lactose	Lactose + 5g/L glucose	Lactose ⁺⁺
7	Arabinose	Arabinose + 5g/ L glucose	Arabinose ⁺⁺
8	None	5g/ L glucose	none

Table 4.3 shows activities of glucose isomerase of samples taken at t=24 h. The comparison between enzyme activities of flask no 1 and 3 showed that extra IPTG addition did not have a significant effect on the activity per cell mass. Since IPTG is not consumed by cell, its intracellular concentration remains constant and when reaches to the saturation value, any increase in the extracellular concentration does not effect on its transportation. Thus, no matter what the extracellular concentration is, expression level induction could not be increased with the addition of extra IPTG to the cultivation medium.

Table 4.3 Enzyme activities at t=24 h of the bioprocess

Sample No	Sample Modification	Activity (U L ⁻¹)	Activity/cell (U mg ⁻¹)
1	IPTG +	1809	1.285
2	IPTG++, Amp+	1305	0.726
3	IPTG++	2072	1.255
4	IPTG+, Amp+	2153	1.256
5	IPTG++, Lactose++	1467	0.981
6	Lactose++	2192	1.408
7	Arabinose++	1329	0.930
8	none	1691	0.103

Another modification performed in the cultivation media, was the addition of extra ampicillin with the aim of investigating the possibility of any plasmid lost during the process. However, it was seen that activity values for 1st and 4th samples were very close to each other, which indicates that there was no plasmid loss.

The modification corresponds to the sample 5 was adding up of couple value of IPTG and Lactose into the medium. Recombinant enzyme activity was not also very high for this sample. It may be suggested that for all IPTG+ Lactose cases, transportation and intracellular reactions of these samples may be counteracting.

Sample 6 was the one in which lactose was coupled and used as only inducer; and it was seen that the highest GI activity per volume and per cell was achieved in this case. It is known that Lac operon works on principle of lactose metabolism in the absence of favorable carbon source. High activity of this sample may be due to the natural action of lactose on this operon. After $t=12$ h, when glucose was nearly consumed by the cells, lactose became an available carbon source for the cell. Therefore, carbon catabolite repression was relieved. Since lactose could be metabolized by the cell, it did not accumulated in the cell and its transport continued during the bioprocess. So, it can be concluded that, although IPTG was more efficient in the operon induction at first hours, the use of natural inducer lactose might be more advantageous for long time bioprocess. The application of other analogues of lactose such as galatose in the induction of LacUV5 promoter is also common in the literature (Çalık and Levent, 2009). For example, by employing the molasses based complex medium, the need for IPTG addition can be eliminated due to availability of galactose in this medium (Çalık and Levent, 2009).

Effect of arabinse on the induction of lac promoter was also investigated in flask 7 and the activity result illustrated that this sugar was not as thriving as lactose in the term of promoter induction. Finally, the activity result of flask 8 represented that lacUV5 promoter has always a basal level of expression even without inducer. Because, after some time when glucose was completely depleted by the cells, cAMP derepression of lacUV5 promoter results in the basal level of T7 RNA polymerase expression. Therefore, it is logical to detect T7 RNA polymerase expression and enzyme activity with the cells that were not induced during the bioprocess.

4.3 Development of Carbon Source Feeding Strategy for rGI Over-Production

In order to increase the cell growth and thermostable rGI production, fed-batch cultivations were conducted using the production medium indicated in Table 3.8. The effects of carbon source feeding rate on thermostable GI production by recombinant *E. coli* BL21 (DE3) pLysS pRSETA::*xyI*_T strain were investigated at

four sets of fed-batch bioreactor experiments at the predetermined specific growth rates of $\mu=0.1$, 0.15, and 0.2 h^{-1} where dissolved oxygen ($\approx 40\%$) cascade to air-flow and agitation rates. In order to make description of the operation modes easy, the fed-batch experiments were coded as demonstrated in Table 3.9.

All of the fed-batch operations were conducted after an initial batch phase and feeding was started when glucose and fructose nearly consumed by the cells. The feed rate was controlled automatically by the bioreactor control unit and verified with a balance that placed under the bottle of the feed substrate solution.

Exponential feeding experiments were carried out at three predetermined specific growth rates of 0.1, 0.15, and 0.2 h^{-1} , with the feeding of glucose or pretreated molasses to the bioreactor according to equation (3.1). The predetermined feeding profiles were shown in Figure 4.27. The specific growth rate values were decided based on the favored specific growth rate range for *E. coli* growth in the literature.

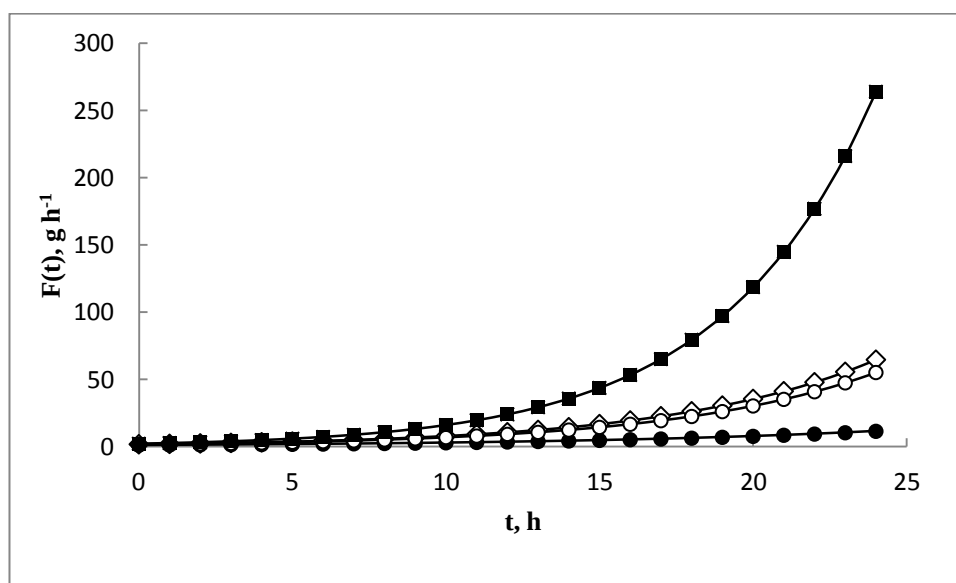


Figure 4.27 Predetermined feeding profiles for M-0.1 (●), M-0.15 (○), M-0.2 (■), G-0.15 (◇) experiments. $t=0$ h is the time where feeding was started.

In general, to evade acetate accumulation in *E.coli* fermentations, specific growth rate values between 0.1 and 0.3 h⁻¹ were favored in the literature (Lee, 1996; Wong et al., 1998). The optimum specific growth rate for recombinant *E.coli* cultivations alters based on product and strain types. For example, where $\mu=0.2$ h⁻¹ was reported as optimum growth rate for recombinant trypsin production by *E. coli* X90; Khalilzadeh et al. (2003) employed $\mu=0.12$ h⁻¹ for the production of human interferon-gamma by recombinant *E. coli* BL21 (DE3) cells. Additionally, Meyer et al. (1984) reported that in the case of complex media utilization, acetate formation occurs at the specific growth rates above 0.2 h⁻¹. Also in the master study of Taşpınar (2010), $\mu= 0.15$ h⁻¹ was reported as the optimum specific growth rate for recombinant BAL production via *E. coli* BL21 (DE3) pLysS strain.

4.3.1 Effects of Feeding Strategy on Cell Growth

The effect of molasses and glucose based exponential feeding strategies on the cell concentration variations with the cultivation time were given in Figure 4.28. For all the cases, the cell concentrations attained in the batch phase (t=0-9 h) were nearly the same. Feeding was started when cell growth was reach to the stationary phase; and with feeding initiation, a considerable increase in density of the cells was detected for all cases. Among the exponential feeding experiments, the highest cell concentration was obtained in M-0.15 condition as 9.6 kg m⁻³ at t=32 h. The maximum cell concentrations for M-0.2, M-0.1 and G-0.15 experiments were achieved as 9.18 kg m⁻³ (t=26 h), 8.69 kg m⁻³ (t=32 h) and 6.4 kg m⁻³ (t=20 h), respectively. At the M-0.2 operation, as a result of higher feeding rate of molasses, the maximum cell concentration was attained earlier than the other operation conditions. So it was concluded that cell growth rate increases as the feeding rate increases.

As it was obvious from Figure 4.28 , the cell concentration at M-0.2 operation mode was the highest cell profile until t=26 h ; however, after that time the stationary phase occurred, and the cell concentrations remained below that obtained in M-0.15 case, probably due to the excess amounts of substrate present in the medium.

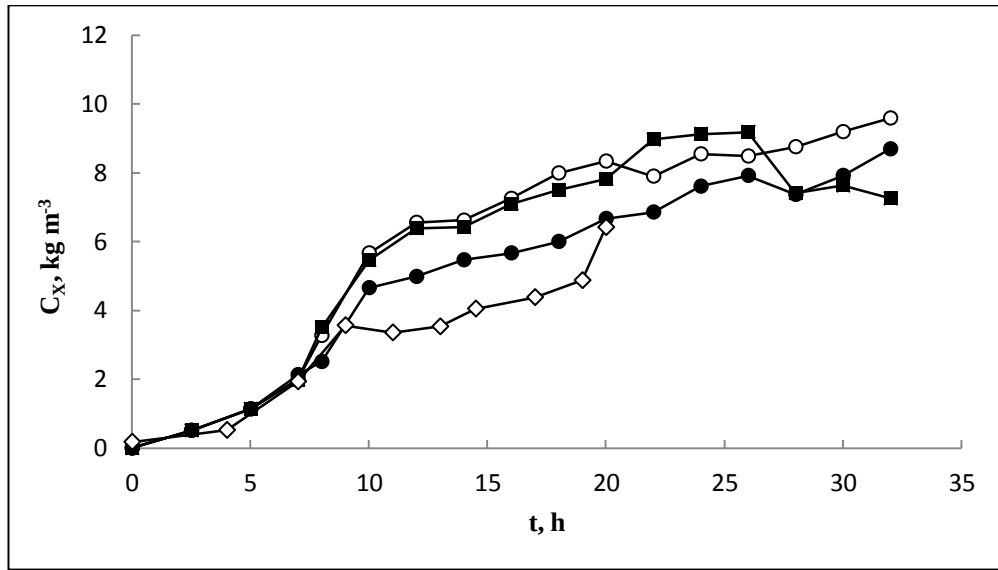


Figure 4.28 Variations in the cell concentration with the cultivation time for different feeding rates; M-0.1 (●), M-0.15 (○), M-0.2 (■), G-0.15 (◇).

The high concentration of molasses in the cultivation medium increases viscosity of medium and introduces higher mass transfer resistance for successful oxygen transfer into cells.

In the literature, Dekker et al. (1992) developed a recombinant *E.coli* BL21 (DE3) strain expressing thermostable *xyl* gene of *Thermus thermophilus* and obtained the cell concentration of 2 kg m^{-3} with the batch cultivation of this strain in the M9ZB medium induced with IPTG at mid-logarithmic phase. Related with production of other bioproducts with recombinant *E.coli* BL21 (DE3) pLysS strain, Çalık and Levent (2009-b) reported the maximum cell concentration of 8.04 kg m^{-3} in molasses based complex medium via applying a pulse feeding strategy. Also in the master study of Taşpınar (2010), the molasses based exponential feeding strategy at $\mu=0.15 \text{ h}^{-1}$ was used for production of rBAL enzyme by *E.coli* BL21 (DE3) pLysS cells and the cell concentration of 21.7 kg m^{-3} was obtained. The lower cell concentration which was achieved in this study at the same feeding condition and medium can be explained by the fact that different product have different effects on the cell metabolism.

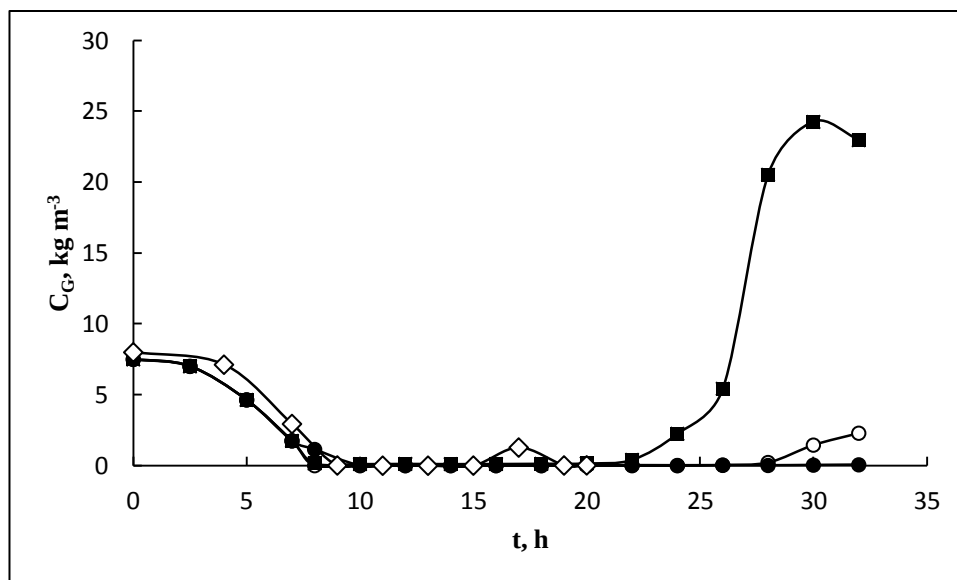
4.3.2 Effects of Feeding Strategy on Substrate Consumption

Control of carbon source concentration in the cultivation medium is one of the crucial design parameters in bioprocesses, since the carbon source concentration directly affects the cell growth, by-product and product formation. Where the lack of sufficient carbon source results in lower cell and product yield, the excess amounts will lead to formation of by-product as well as decreasing cell growth rate and enzyme production due to by-product inhibition. The variations in glucose and fructose concentrations with the cultivation time were shown in Figures 4.29 and 4.30, respectively. In the batch mode of the bioprocesses after $t=3$ h, a sharp decrease was observed in glucose concentration profile for all operation conditions and with glucose depletion in the medium, the cell growth reached to its stationary phase where the carbon source feeding was initiated.

In the all fed-batch conditions except at the end hours of M-0.2, there was no significant accumulation of glucose in fermentation media. In M-0.2 condition, accumulation of glucose occurred after $t=22$ h and its concentration in fermentation broth increased with the increase in feed rate. The final glucose concentration in M-0.2 condition was detected as 22.9 kg m^{-3} , resulted in the early stationary phase in this operation. In M-0.2 and M-0.15 operation modes, a significant increase in fructose concentration was detected after $t=20$ h, and at the end of the fermentation it was reached to a concentration of 26.74 kg m^{-3} and 13.72 kg m^{-3} , respectively. The glucose and fructose profiles of M-0.15 condition demonstrated the preference of glucose to fructose by the cells during the cultivation time. In M-0.1 mode, there was no significant fructose accumulation during the bioprocess time indicating in more effective substrate management in this condition.

Generally, batch cultivations of *E. coli* BL21 were carried out at the medium containing higher initial glucose concentrations between 20.0 up to 40.0 kg m^{-3} which resulted in the accumulation of acetate in the fermentation medium (Luli et. al., 1990; Shiloach et. al., 1996). However, in the fed-batch processes, the lower glucose concentration was usually favored in order to increase cell density, product formation

as well as avoiding from acetate accumulation (Luli et al., 1990; Shiloach et al., 1996; Johnston et al., 2003; Çalık and Levent, 2009-b).



Figures 4.29 Variations in the glucose concentration with the cultivation time for different feeding rates; M-0.1 (●), M-0.15 (○), M-0.2 (■), G-0.15 (◇).

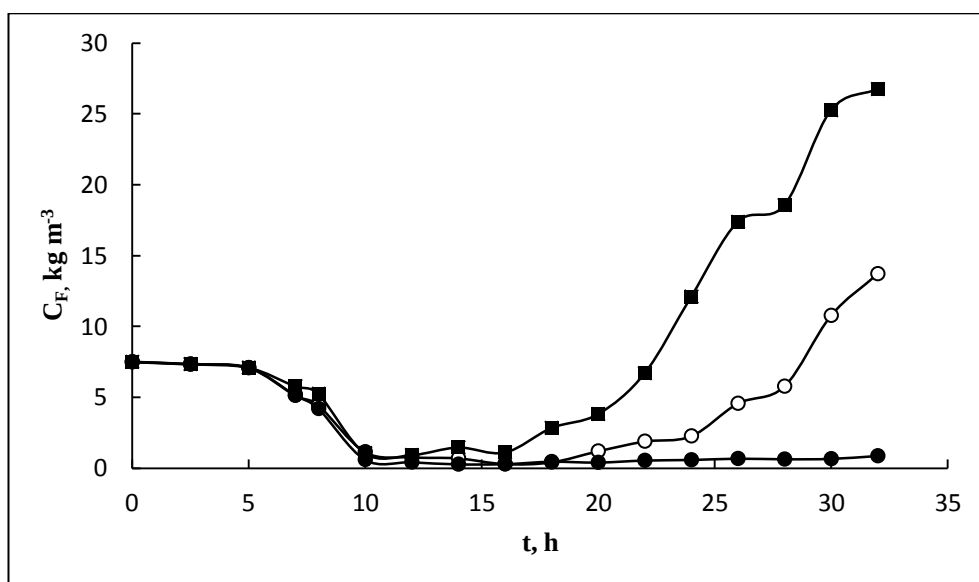


Figure 4.30 Variations in the fructose concentration with the cultivation time for different feeding rates; M-0.1 (●), M-0.15 (○), M-0.2 (■).

4.3.3 Effects of Feeding Strategy on GI Production

The variations in glucose isomerase activities with the cultivation time and the effects of feeding strategy on the enzyme activities were illustrated in Figure 4.31. In all molasses based experiments, GI activities were increased significantly after starting to feed. The highest GI activity was obtained at M-0.1 operation mode at $t=30$ h as 16399 U L^{-1} . The limiting substrate concentration and the better substrate control, achieved in this condition resulted in higher GI activity. On the other hand, the lowest GI volumetric activity as 1753 U L^{-1} was examined at G-0.15 among the investigated feeding strategies. By comparing the highest GI activity of M-0.1 and M-0.15 conditions with that of G-0.15 operation, 9.3 and 5.0-fold increase was absorbed, respectively. So, it was concluded that molasses based feeding strategies were more successful in the terms of cell concentration and recombinant enzyme production. The higher cell concentration and enzyme activities detected in the molasses based operations can be explained by the composition of molasses which contains natural sugar inducers such as galactose. Since noise value due to stochasticity in gene expression is much lower for natural inducer, it is logical to obtain higher cell and product concentration for molasses based feeding conditions. Although, GI activities between $t=12-22$ h at M-0.2 and M-0.15 conditions were comparable with each other, due to the inhibitory effect of substrate accumulation in M-0.2 experiment after $t=22$ h, a sharp decrease in GI activities was detected. In the literature, Dekker et al. (1992) produced thermostable *Thermus thermophilus* GI in *Escherichia coli* BL21 (DE3) and reported the maximum GI activity of 4300 U L^{-1} . As can be seen, molasses based exponential feeding strategy at $\mu=0.1 \text{ h}^{-1}$ lead to a 3.81-fold increase in the GI compared with the reported value in the literature. When the maximum GI activity of current study was compared with the one of our pervious study (Çalık et al., 2008) in which maximum activity of 1840 U L^{-1} obtained via batch cultivation of wild type thermophilic *Bacillus antacticusin* on xylan based complex medium, 8.91-fold increase in the enzyme activity was observed.

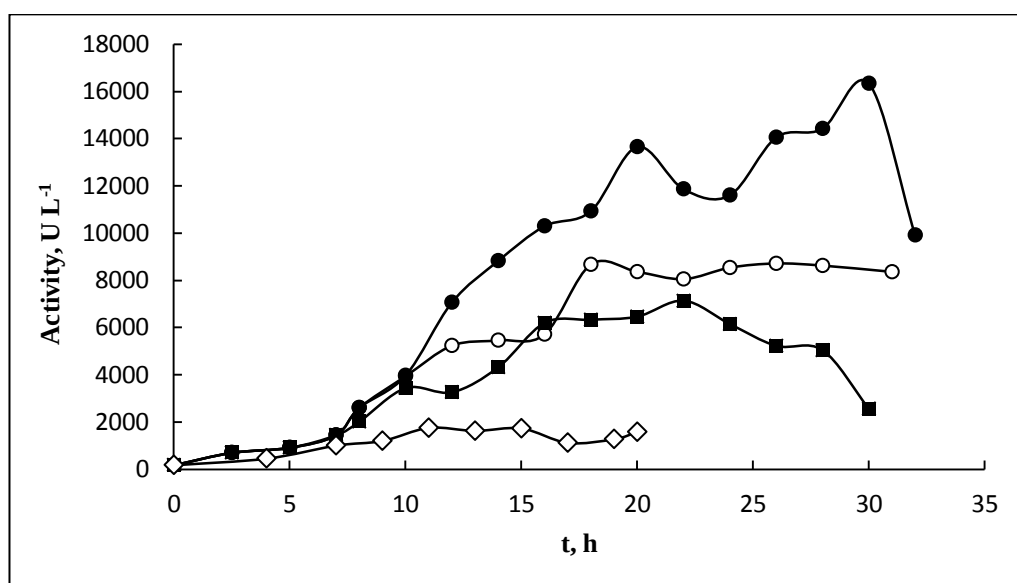


Figure 4.31 Variations in the glucose isomerase activity with the cultivation time for different feeding rates; M-0.1 (●), M-0.15 (○), M-0.2 (■), G-0.15 (◇).

4.3.4 Organic acid concentration profiles

One of the major obstacles in *E. coli* fermentation is onset of acetic acid production which is known to inhibit cell growth and recombinant protein production due to the accumulation of undissociated (protonated- CH_3COOH) form of acetic acid in the medium (Shiloach et al., 1996; Johnston et al., 2003; Akesson et al., 2001; Luli et al., 1990; Çalık et al., 2004 and 2006). The undissociated form of acetic acid has lipophilic property with ability to freely permeate the cell membrane and accumulate in the medium. When a fraction of undissociated acid present extracellularly re-enters the cell, it dissociates (ionized- CH_3COO^-) at the relatively higher intracellular pH. When this process continues, the intracellular pH decreases and hence the ΔpH component of the promotive force collapses. Thus, apart from energy consumption for rapid growth, *E. coli* cells were required for additional energy to maintenance the optimum intracellular pH which results in reduction of cellular efficiency (Stephanopoulos, 1998; Luli et al., 1990). The *E. coli* BL21 (DE3) pLysS strain utilized in this study is a derivative form of *E. coli* B which is

known as a low acetate producing strain (Luli et al., 1990; Shiloach et al., 1996). Acetate formation in *E. coli* fermentations occurs when the excess carbon source, especially glucose, combined with oxygen limitation and it is known as Crabtree effect or overflow metabolism (Doelle et al., 1982).

The concentrations of the organic acids detected in the fermentation medium were given in Table 4.4. Acetic, formic, fumaric, citric and malic acids were the organic acids detected in the fermentation broths. In all operation conditions, except M-0.1, similar trends were observed and with the increase in the cultivation time, organic acid concentrations increased. In the M-0.1 operation mode, firstly, secreted organic acid concentrations in the extracellular medium were increased with time but because of substrate limitation in this condition, organic acids penetrated into assimilatory pathways and their concentrations decreased at the remaining hours of the bioprocess.

Total organic acid concentrations detected at the end hour of fed-batch phase were 1.82, 15.09, 25.07 and 2.82 kg m⁻³ for M-0.1, M-0.15, M-0.2 and G-0.15 operations, respectively. Presence of the organic acids of the TCA cycle, i.e., citric, formic and malic acids indicates the inadequate oxygen utilization, which leads to repression of the TCA cycle enzymes. The variation in the main by-product concentration, acetic acid concentration, with cultivation time was shown in Figure 4.32. The maximum acetic acid concentrations for the fed batch phases were achieved as 1.72, 12.32, 18.14, and 1.34 kg m⁻³, for M-0.1 (t=18 h), M-0.15 (t=32 h), M-0.2 (t=32 h), and G-0.15 (t=20 h) operations, respectively.

By comparing Figures 4.32, 4.30 and 4.29, it can be seen that high level of substrate accumulation especially glucose in fermentation medium resulted in the secretion of acetic acid at excessive amount. It is also known that the excess amount of substrate leads to formation of by-product as well as decreasing cell growth rate and enzyme production due to overflow metabolism which explains observed reduction in GI activity and cell growth rate at end hours of M-0.2 condition.

Table 4.4 Variations in the organic acid concentrations with the cultivation time for fed-batch bioreactor operations.

t	Concentrations, kg m ⁻³					Total
	Acetic acid	Formic acid	malic acid	Citric acid	Fumaric acid	
M-0.1						
8	0.1459	0.1068	0.0949	0.1879	0.0766	0.6121
10	0.1979	0.2841	0.2841	0.1984	0.0799	1.0444
12	0.5073	0.4302	0.3367	0.1462	0.0838	1.5042
14	0.7431	0.8556	0.4308	0.1148	0.0876	2.2319
16	0.9661	0.8878	0.5058	0.1148	0.0887	2.5632
18	1.7220	0.9477	0.8399	0.0738	0.0908	3.6742
20	1.2783	1.0250	0.9661	0.0738	0.0925	3.4357
22	1.2759	1.2759	1,1016	0	0.1081	3.7615
24	0.3927	1.1886	1.2756	0	0.1215	2.9784
26	0.1455	1,2482	1.1566	0	0.1313	2.6816
28	0	0.9435	1.0530	0.0209	0.1275	2.1449
30	0	0.7814	0.8897	0.0835	0.1164	1.8710
32	0	0.7523	0.8676	0.0939	0.1036	1.8174
M-0.15						
8	0.1820	0.0461	0.0352	-	0.0375	0.3615
10	0.4316	0.0875	0.3410	0.0209	0.0386	0.9563
12	0.6641	0.1068	0.3478	0.0522	0.0429	1.4102
14	0.6668	0.1242	0.3511	0.0626	0.0461	1.4400
16	0.6721	0.3032	0.3633	0.0317	0.0456	1.4565
18	0.5895	0.3134	0.3986	0.0939	0.0481	1.5012
20	0.5964	0.3438	0.4235	0.1044	0.0762	1.6376
22	0.6400	0.3711	0.4724	0.1044	0.0870	1.8933
24	0.7376	0.4371	0.5271	0.1775	0.0913	2.1512
26	3.8531	0.5895	0.6473	0.2819	0.0929	5.6561
28	6.7228	0.6177	0.7494	0.3624	0.0928	8.7284
30	8.1264	0.7809	0.8376	0.6368	0.1102	10.518
32	12.323	0.8010	0.9057	0.8665	0.1324	15.094

Table 4.4 Variations in the organic acid concentrations with the cultivation time for fed-batch bioreactor operations (*continued*).

t	Concentrations, kg m ⁻³					Total
	Acetic acid	Formic acid	malic acid	Citric acid	Fumaric acid	
M-0.2						
8	0.5411	0.5960	0.1523	0.0417	0.0569	1.3880
10	0.6079	0.6277	0.2901	0.0626	0.0640	1.6523
12	0.9669	0.7362	0.3016	0,0731	0.0824	2,1602
14	1.0004	0.7508	0.3366	0.1253	0.0829	2.2960
16	1.1872	1.0265	0.3624	0.1462	0.0996	2.8219
18	1.5817	1.0571	0.3977	0.1775	0.1004	3.3144
20	5.8458	1.0601	0.4348	0.1879	0.1119	7.6405
22	8.3144	1.3223	0.9217	0.2714	0.1148	10.945
24	8.9224	1.6564	1.4111	0.3027	0.1368	12.429
26	13.651	1.7332	1.7983	0.3236	0.1823	17.688
28	14.310	1.8104	2.3205	0.5115	0.3196	19.272
30	17.774	2.6428	2.6914	0.6999	0.3228	24.131
32	18.146	2.8866	2.8655	0.8456	0.3299	25.073
G-0.15						
4	0.1905	0	0	0	0.0003	0.1908
7	0.3795	0.0004	0.1615	0.0209	0.0003	0.5626
9	0.4836	0.0905	0.2274	0.0731	0.0004	0.8750
11	0.5432	0.1225	0.2307	0.1566	0.0041	1.0571
13	0.7213	0.1684	0.2686	0.2819	0.0042	1.4444
15	0.8286	0.2334	0.0344	0.4280	0.0050	1.5294
17	1.0059	0.3428	0.0351	0.5117	0.0053	1.9008
20	1.3467	0.4928	0.3601	0.6159	0.0059	2.8214

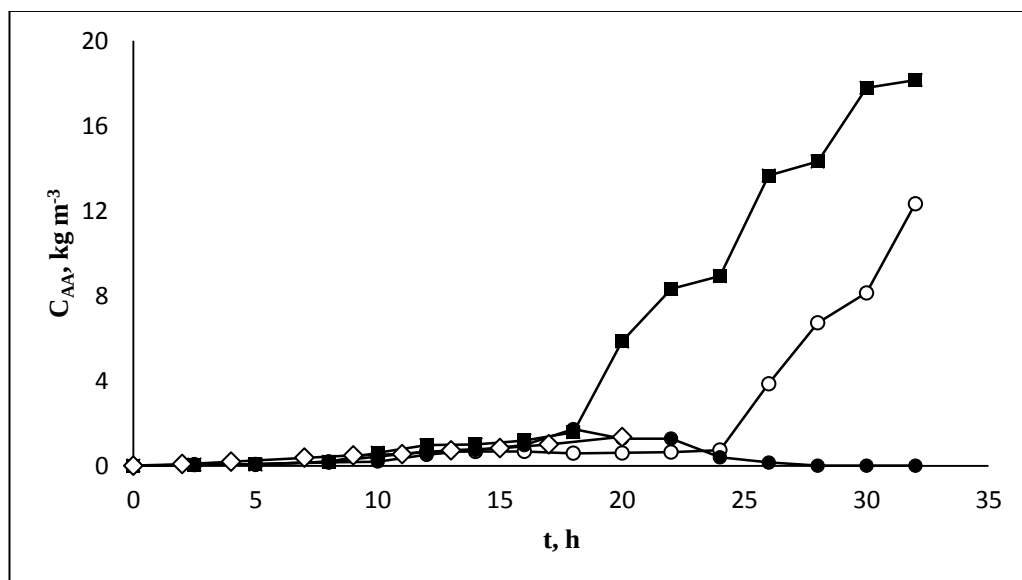


Figure 4.32 Variations in the acetic acid concentration with the cultivation time for different feeding rates; M-0.1 (●), M-0.15 (○), M-0.2 (■), G-0.15 (◇).

4.3.5 Oxygen Transfer Characteristics

During rGI production by *E.coli* in fed-batch culture using different substrate feeding rates, the dynamic method was applied to determine the variations in volumetric mass transfer coefficient (K_La), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate (OD), Damköhler number (Da) and maximum possible oxygen transfer rate ($OTR_{max}=K_La \times C_{DO}^*$). The values of these parameters were given in Table 4.5. At all the operation conditions, K_La values were decreased during the batch phase of the bioprocess with increasing cell concentrations and when feeding was started again an increase in K_La values were detected.

Generally, when higher glucose and fructose amounts were present in the medium, higher K_La values were obtained. During experiments K_La values were oscillated between 0.009 and 0.033 s⁻¹. The highest K_La values were observed as 0.02, 0.016, 0.018 and 0.033 s⁻¹ for M-0.1, M-0.15, M-0.2, and G-0.15, respectively. The maximum K_La value was obtained at the end of G-0.15 operation as 0.033 s⁻¹ where the lowest cell concentration was also detected between investigated

conditions. Temperature, air flow rate, agitation rate, presence of fine particles in the mass transfer zone and the rheological properties of the fermentation medium are some key items affecting on the K_La throughout the bioprocess. The variation in the cultivation medium composition, as result of feeding and by-product formation, can significantly change the bubble size and liquid film resistance around the gas bubble during the fed-batch cultivations which explains increase in K_La values after starting to feed.

The oxygen transfer rate ($OTR = K_La \times (C_{DO}^* - C_{DO})$) is proportional to the difference between the equilibrium concentration and the dynamic dissolved oxygen concentration in the medium. Since dissolved oxygen in the process was kept constant throughout the process, the driving force for oxygen transfer was the same for all conditions as a result K_La values were became the only effective parameter for oxygen transfer rates. So, a similar trend with K_La values was detected for OTR variations and the maximum values of 4.57 and 4.15 $\text{mol m}^{-3} \text{s}^{-1}$ were obtained at $t=18$ h in M-0.15 and $t=20$ h in G-0.15 conditions, respectively. Since dissolved oxygen in the process was kept constant throughout the process, the OUR values were the same as the OTR values. The highest OUR values were obtained as 4.57, 3.53, 3.99 and 4.15 $\text{mol m}^{-3} \text{s}^{-1}$ for M-0.1, M-0.15, M-0.2 and G-0.15, respectively. In order to analyze the relative effects of mass transfer and biochemical reaction on the thermostable glucose isomerase production by recombinant *E. coli* BL21 (DE3) pLysS; modified Damköhler number (Çalık et al., 2000), Da , which is the ratio of the maximum possible oxygen utilization rate (OD) to the maximum oxygen transfer rate ($OTR_{\max}$), was calculated during the bioprocesses. As obvious from Table 4.5, for all the fed-batch operations except first hours of G-0.15, Da values ($=OD/OTR_{\max}$) were higher than 1 ($Da \gg 1$) indicating that mass transfer was the rate limiting step and maximum possible oxygen utilization rate was significantly lower than at bulk phase condition. The maximum Da values were obtained as 49.3, 8.76, 9.62 and 20.80 for M-0.1, M-0.15, M-0.2, and G-0.15, respectively.

Effectiveness factor, η , which is the ratio between the observed oxygen uptake rate and maximum possible oxygen utilization rate, was also calculated in the absence of any mass transfer resistance. Throughout the fermentations, the η values

were lower than 1 showing that the oxygen consumption was below the maximum oxygen demand. This indicates that oxygen transport was limited for all conditions due to the high cell concentrations and high by-product secretion. The maximum η values were achieved at first hours of bioprocesses as 0.27 for molasses based operations and 0.61 for glucose based fed-batch operation where the lowest Da values were also obtained.

4.3.6 Specific Growth Rate and Yield Coefficients

The variations in specific growth rate, μ , specific oxygen uptake rate, q_o , specific substrate utilization rate, q_s , and yield coefficients shown in Table 4.6, were calculated as described by Çalık et al. (2006). For all the operations, μ values were not strictly constant at the desired value and generally the values were below the predetermined specific growth rate. This was probably due to the inhibitory effects of by-products formation as a result of insufficient oxygen transfer during the cultivations time in cell. The highest specific growth rates were obtained as 0.04, 0.19, 0.18, and 0.09 h^{-1} for M-0.1, M-0.15, M-0.2, and G-0.15 conditions, respectively. Specific substrate utilization rates (q_s) were generally increased throughout the fed-batch phase of fermentations. As the feeding rates increased the maximum values of specific substrate consumption rate increased and it reach to highest value in M-0.2 operation as 1.90 $\text{kg kg}^{-1} \text{h}^{-1}$.

Specific oxygen uptake rates (q_o) showed a decreasing profile with the cultivation time at the all conditions and the highest values were attained at the first hour of batch phase as 0.77, 0.79, 0.66, and 0.52 $\text{kg kg}^{-1} \text{h}^{-1}$ for M-0.1, M-0.15, M-0.2, and G-0.15 conditions, respectively. The overall specific cell yields on substrate ($Y_{X/S}$) changed inversely with increasing feed rate among the molasses based operation conditions and the highest value was obtained at M-0.1 as 0.25 kg kg^{-1} , as result of the increased efficiency of carbon source utilization for product formation in this condition. The cell yield on oxygen ($Y_{X/O}$) and yield of substrate on oxygen ($Y_{S/O}$) values were altered during fermentations. While, the highest $Y_{X/O}$ values were

1.28, 1.85, 1.07 and 2.15 kg kg⁻¹ for M-0.1, M-0.15, M-0.2, and G-0.15; the highest Y_{S/O} values obtained as 2.95, 3.14, 5.92 and 5.05 kg kg⁻¹.

Table 4.5 The variations in oxygen transfer parameters with cultivation time.

Condition	t (h)	K _L a (s ⁻¹)	OTR ^x 10 ³ (molm ⁻³ s ⁻¹)	OTR _{max} ^x 10 ³ (molm ⁻³ s ⁻¹)	OUR ^x 10 ³ (molm ⁻³ s ⁻¹)	OD ^x 10 ³ (molm ⁻³ s ⁻¹)	Da	η
M-0.1	2	0.015	3.45	5.74	3.45	12.53	2.18	0.27
	4	0.014	3.07	5.12	3.07	20.32	3.96	0.15
	6	0.011	2.53	4.21	2.53	16.03	3.81	0.16
	12	0.016	3.62	6.03	3.62	160.79	26.68	0.02
	16	0.018	4.15	6.92	4.15	341.02	49.30	0.01
	18	0.020	4.57	7.62	4.57	238.54	31.32	0.02
M-0.15	2	0.016	3.53	5.89	3.53	12.85	2.18	0.27
	4	0.013	2.88	4.80	2.88	19.06	3.96	0.15
	9	0.009	2.08	3.47	2.08	30.38	8.76	0.02
M-0.2	2	0.013	2.96	4.93	4.93	10.76	2.18	0.27
	4	0.012	2.72	4.53	4.53	17.96	3.96	0.15
	6	0.016	3.55	5.91	5.91	22.53	3.81	0.16
	10	0.018	3.99	6.87	6.87	64.09	9.62	0.06
G-0.15	4	0.019	2.39	3.98	3.98	3.89	0.97	0.61
	8	0.018	2.30	3.84	3.84	21.52	5.61	0.11
	14	0.009	1.09	1.81	1.81	47.84	26.43	0.02
	18	0.016	1.88	3.30	3.30	27.05	8.19	0.07
	20	0.033	4.12	1.69	1.69	143.02	20.80	0.03

Table 4.6 The variations in the specific rates and the yield coefficients with the cultivation time.

Condition	t (h)	μ (h ⁻¹)	q_o (kgkg ⁻¹ h ⁻¹)	q_s (kgkg ⁻¹ h ⁻¹)	$Y_{x/s}$ (kgkg ⁻¹)	$Y_{x/o}$ (kgkg ⁻¹)	$Y_{s/o}$ (kgkg ⁻¹)
M-0.1	2	0.40	0.77	0.51	0.69	0.52	0.66
	4	0.22	0.31	0.91	0.24	0.71	2.95
	6	0.21	0.18	0.24	0.18	1.28	1.77
	12	0.03	0.08	0.08	0.41	0.39	0.96
	16	0.02	0.08	0.08	0.22	0.21	0.93
	18	0.03	0.09	0.08	0.35	0.32	0.89
	22	0.02	-	0.11	0.13	-	-
	26	0.02	-	0.14	0.14	-	-
	32	0.04	-	0.23	0.19	-	-
	Overall				0.25		
M-0.15	2	0.40	0.79	0.51	0.69	0.50	0.64
	4	0.22	0.29	0.91	0.24	0.75	3.14
	9	0.19	0.05	0.36	0.21	1.85	3.10
	12	0.07	-	0.11	0.04	-	-
	16	0.04	-	0.17	0.31	-	-
	18	0.05	-	0.16	0.13	-	-
	22	0.01	-	0.24	0.16	-	-
	26	0.01	-	0.34	0.05	-	-
	30	0.02	-	0.39	0.11	-	-
	Overall				0.13		
M-0.2	2	0.40	0.66	0.51	0.69	0.60	0.77
	4	0.22	0.27	0.91	0.24	0.80	3.22
	6	0.21	0.25	0.24	0.18	0.91	1.26
	10	0.18	0.08	0.50	0.36	1.07	5.92
	12	0.07	-	0.48	0.15	-	-
	18	0.03	-	0.26	0.10	-	-
	22	0.06	-	0.47	0.13	-	-
	26	0.01	-	0.74	0.01	-	-
	30	0.01	-	1.74	0.01	-	-
	Overall				0.10		
G-0.15	4	0.89	0.52	0.41	0.34	1.71	0.79
	8	0.13	0.11	0.70	0.42	1.34	5.05
	14	0.09	0.03	0.15	0.04	1.10	4.14
	18	0.06	0.05	0.07	0.45	2.15	1.28
	20	0.01	0.07	0.24	0.17	0.56	3.21
	Overall				0.45		

4.4 Determination of Peptide-Ligand with High Affinity toward 6×Histidine Peptide

4.4.1 Experimental Screening of Ligand via Phage Display Method

12-mers phage display peptide library consisted of $\sim 2.7 \times 10^9$ electroporated sequences at high copies was employed for experimental selection of peptide ligand toward 6×histidine tag by means of the procedure described in section 3.8. After three rounds of panning and amplification, 41 phage clones were randomly selected. ELISA assay was also conducted in order to verify the exclusivity of selected peptide toward the target and to discriminate the phage clones with highest affinity. For this purpose, equal concentration, 10^{11} pfu/ml, of each selected clone was incubated in directly target coated ELISA plates and the assay was carried out as described in section 3.8. The absorbances of nine high affinity phage clones were given in decreasing manner in Figure 4.33.

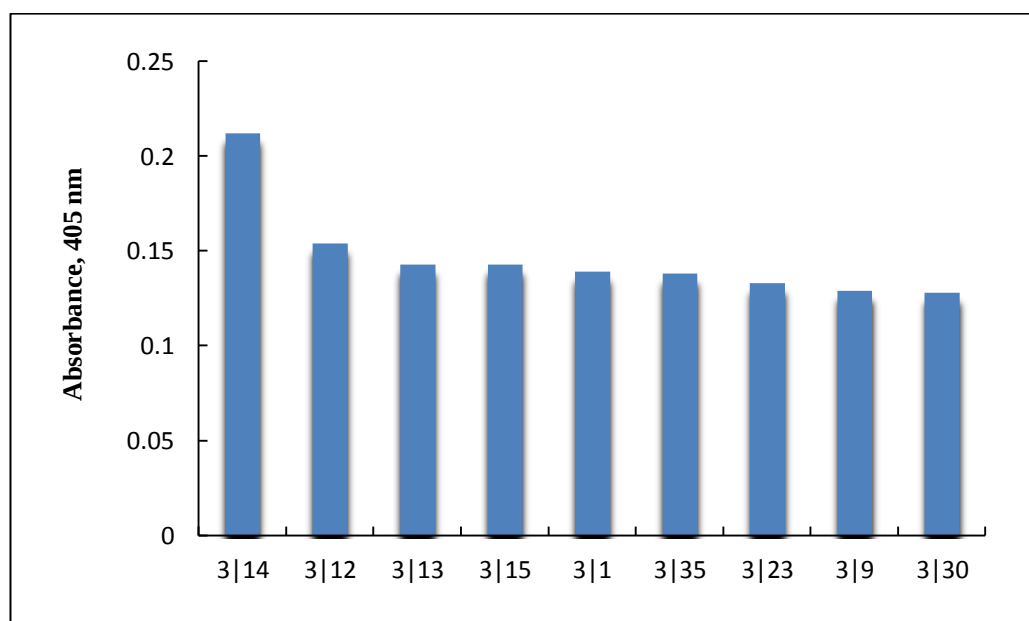


Figure 4.33 The absorbance of phage clones that bind to GI in ELISA assay.

The sequences of selected peptides were determined by automated cycle sequencing with dye-labeled dideoxynucleotides and they were read correspond to the anticodon strand of the template and the amino acid sequence of selected peptides was determined (Table 4.7).

Table 4.7 DNA and amino acid sequences of selected peptides via phage display.

Peptide Code	DNA Sequence	Amino Acid Sequence
3 14	CAUGCUAUUUAUCCGCGUCAG	HAIYPRQ
3 12	UCUGCUGGGAUUUAUCUUUAU	SAGIYLY
3 13	AAUCUGGUGACGCUGAUGAAU	NLVTLMN
3 15	GGGAAGCCUAUGCCUCCGAUG	GKPMPPM
3 1	UGGCCUACGCUGCAGUGGGCG	WPTLQWA
3 35	ACUUUUCAUGCGAUGUUUCCU	TFHAMF
3 23	UCUUUUGUUCUCCCUAAUUAU	SFVLPYY
3 9	UCUAUUCUGCCGUAUCCUUAU	SILPYPY
3 30	CUGGCGCGUGAGCCUACGUCG	LAREPTS

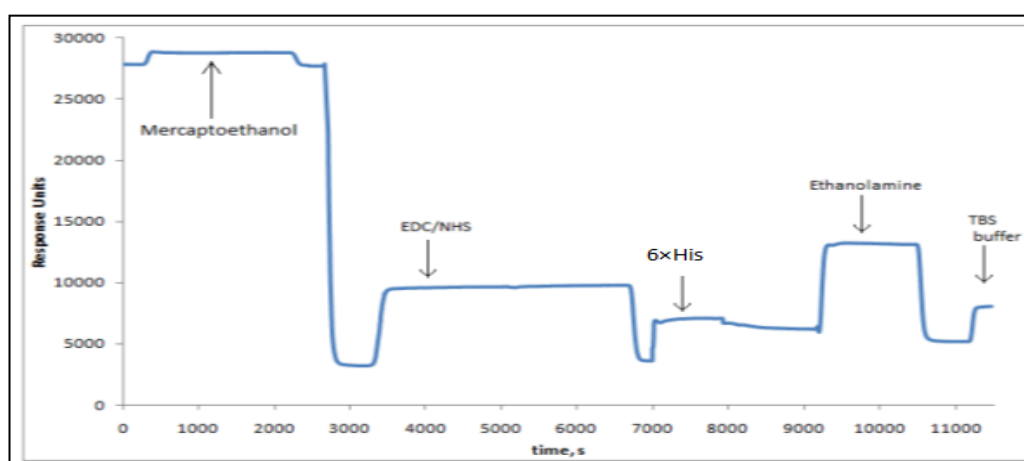
As obvious from Table 4.7, different sequences for phage clones were obtained as the result of the DNA sequencing analyses. The amino acid sequences of the selected peptides were used with Peptide Property Calculator program (https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi) with the aim of determining the isoelectric point (pI) , net charge at pH=7.0 and the percentage of hydrophilic as well as hydrophobic residues for each peptide (Table 4.8). Since all of the interacting analytes are prepared in the aqueous phase, peptides 3|14, 3|15 and 3|30 were selected based on the ELISA results and hydrophilic residues percentage. Then, these peptides were artificially synthesized and employed in surface plasmon resonance analyses to verify their affinity toward the histidine tag and to calculate the kinetic parameters of interactions.

Table 4.8 Properties of peptide ligands selected via phage display.

Peptide Code	Amino Acid Sequence	Molecular Weight	Net Charge	pI	Hydrophilic Residues %	Hydrophobic Residues %	Others (%)
3 14	HAIYPRQ	884.00	2	9.3	29	29	42
3 12	SAGIYLY	785.89	0	6.0	0	57	43
3 13	NLVTLMN	803.97	0	6.0	0	57	43
3 15	GKPMPPM	756.98	1	9.7	14	29	57
3 1	WPTLQWA	901.02	0	6.0	0	43	57
3 35	TFHAMF	850.00	1	7.5	14	43	43
3 23	SFVLPYY	888.02	0	6.0	0	71	29
3 9	SILPYPY	851.99	0	6.0	0	57	43
3 30	LAREPTS	772.85	0	6.3	29	14	57

4.4.2 Surface Plasmon Resonance Studies

SPR experiments were carried out according to the protocol described at the section 3.9. The similar sensorgram was observed for all experimental runs before ligand binding all through the preparation of Self-Assembled Monolayer surface, activation of surface by EDC/NHS and vacant surface blockation with ethanolamine (Figure 4.34).

**Figure 4.34 SPR sensorgram showing steps involved prior to ligand binding.**

Throughout the SPR experiments carried out by peptides 3|14 and 3|30, it was observed that both peptides bind to the control surface (surface without 6×His) with similar or higher affinity toward the surface occupied by immobilized 6×His peptide (Appendix K). As peptide 3|15 showed specific affinity to 6×His in preliminary SPR experiment, different concentrations of this peptide were prepared and utilized in additional SPR analyses in order to calculate the kinetic parameters of binding. Figure 4.35 illustrates the results of the binding analysis of peptide 3|15 at different concentrations of 0.15, 0.2, 0.3 and 0.5 mg ml⁻¹.

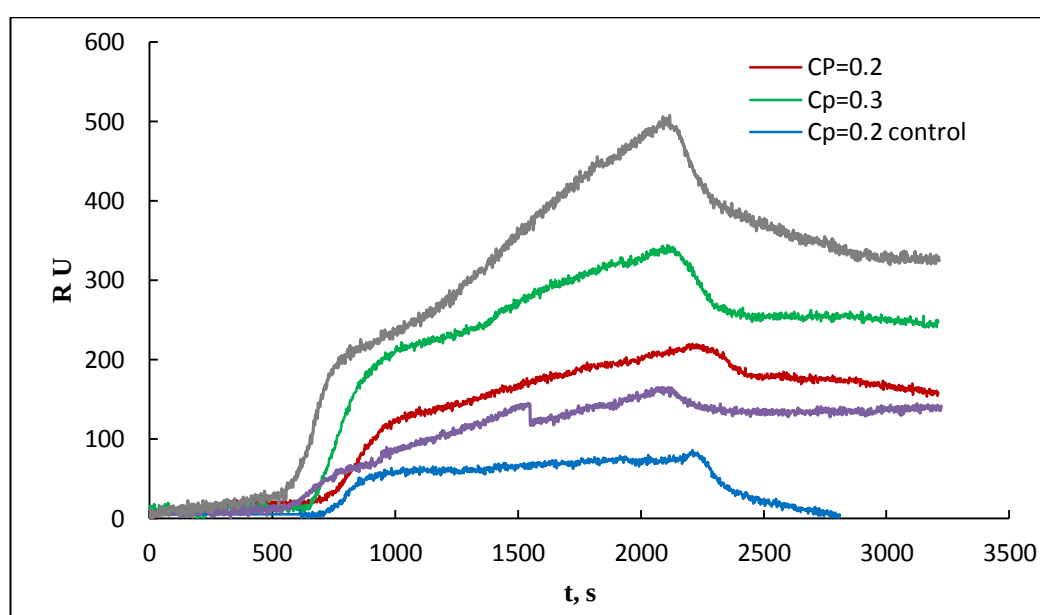


Figure 4.35 SPR sensorgram for peptide 3|15 at different peptide concentrations in increasing order of Cp= 0 (control), 0.15, 0.2, 0.3 and 0.5 mg mL⁻¹.

The simple monolayer Langmuir adsorption model (equation 2.7) was employed for calculating the affinity constant K_A and K_D by fitting adsorption part of each sensorgram to this model. The obtained k_{obs} values from the curve fitting, were plotted with respect to the ligand concentration as shown in Figure 4.36. The plotted linear line was fitted to equation (2.6) in which K_A and k_D values calculated as $3.2 \times 10^3 \text{ M}^{-1}$ and $3 \times 10^{-4} \text{ M}$, respectively.

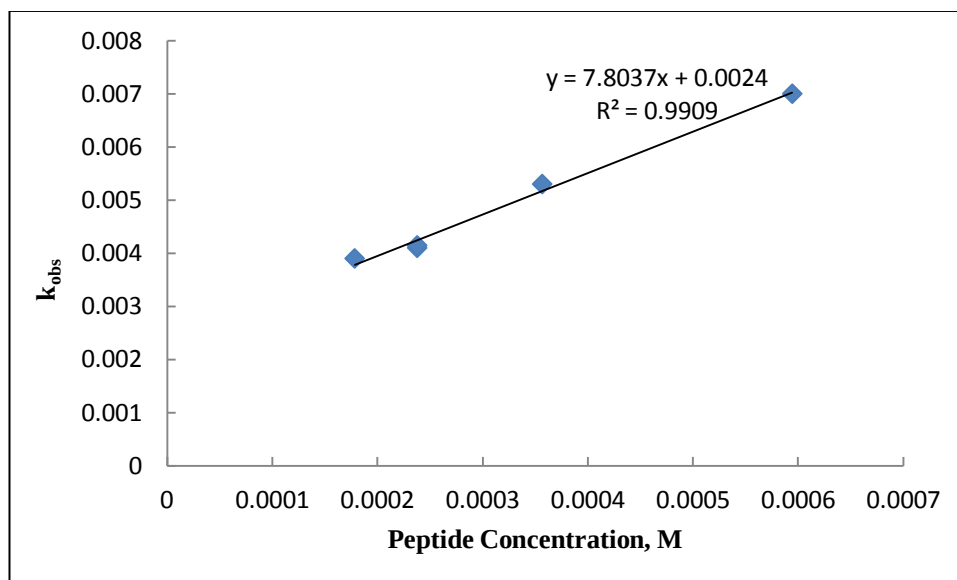


Figure 4.36 The plot of k_{obs} vs peptide 3|15 concentration.

CHAPTER 5

CONCLUSIONS

In this study, it was aimed to develop a bioprocess for over expression of thermostable *xyl* gene, and to select a proper peptide ligand with the specific affinity toward 6×histidine peptide.

As first attempt for the development of GI producing recombinant microorganism, the extracellular and intracellular recombinant *Pichia pastoris* expression systems were systematically assembled via genetic engineering techniques.

For extracellular enzyme production, the hypothetical thermostable *xyl_P* of *Pyrococcus furiosus* was amplified and integrated with *EcoRI* restriction site, a target site for the Factor Xa protease and poly-histidine tag at 5' end as well as *XbaI* site at the 3' location by the mean of two relatively short forward (GI_{Pext}-F1, GI_{Pext}-F1) and one reverse (GI_P-R) primers in PCR. Subsequent to restriction digestion reactions, *xyl_{Pext}* gene was inserted to pPICZαA vector and constructed pPICZαA::*xyl_{Pext}* was expressed in *P.pastoris* strain.

Production of recombinant thermostable GI_P enzyme either in the extracellular or intracellular manner was verified by evaluating GI_P activities of recombinant cells with the wild type *P. pastoris*. The highest extracellular GI_P activity as 209 U L⁻¹ was obtained via cultivation of *P. pastoris* pPICZα::*xyl_{Pext2}* strain. The highest value for intracellular GI_P activity was attained as 342 U L⁻¹ by *P. pastoris* pPICZα::*xyl_{Pint2}* recombinant cells, where the cultivation of wild type strain in the same condition and medium resulted in GI activity at the value of 107 U L⁻¹.

The inferior GI activities detected in the *P. pastoris* expression systems were elucidated by two probable explanations. The first possible reason was the selection

of inappropriate expression system and the other probability was the recombination of inactive *xyl* gene.

So at the second approach, the over-production of recombinant GI enzyme was promised with recombination of thermostable *xyl_T* gene of *Thermus thermophilus* strain with the documented activity into strongly regulated prokaryotic pRSETA expression vector and the generated recombinant plasmid, pRSETA::*xyl_T*, was transformed into *E.coli* BL21 (DE3) pLysS and *E.coli* BL21 (DE3) star strains for expression.

E.coli BL21 (DE3) pLysS carrying recombinant pRSETA::*xyl_T* plasmid was selected as the glucose isomerase producer, due to the highest GI production capability of this strain in the glucose based cultivation medium as 1940 U L⁻¹.

The thermostable GI_T activity of the recombinant *E.coli* BL21 (DE3) pLysS pRSETA::*xyl_T* cells was compared with the one of recombinant *P. pastoris* pPICZα::*xyl_{Tint}* strain and 9.33 -fold increase in the recombinant enzyme activity was observed.

The effect of natural and artificial inducers (lactose, arabinose, IPTG and their combinations) at concentration of 1mM on T7 expression system of *E.coli* BL21 (DE3) pLysS pRSETA::*xyl_T* was examined in the glucose based *E.coli* production medium at T=37°C, N=200 min⁻¹, V=33 cm³. The highest rGI activities at t=12 and 24 h were achieved by IPTG and lactose induced cells as 1472 and 2193 U L⁻¹, respectively. It was considered that the application of cheap natural inducers such as lactose was more advantageous and economical for induction of recombinant *E.coli* cells throughout the long bioprocesses.

In the next stage, the effect of exponential feeding strategy of carbon source on the production of thermostable GI enzyme, cell concentration and acetate formation by recombinant *E.coli* BL21 (DE3) pLysS was investigated at four sets of fed-batch bioreactor experiments at the predetermined specific growth rates of $\mu=0.1$, 0.15, and 0.2 h⁻¹.

At all operation modes, feeding of carbon source either glucose or pretreated-beet molasses containing IPTG or galactose inducers, respectively, initiated after eight hours batch phase with the *E.coli* production medium (Table 3.8). The fed-

batch pilot scale operations were performed at $T=37^{\circ}\text{C}$, and $\text{DO}=40\%$ cascade to air flow and agitation rate where the pH of the medium maintained at $\text{pH}=7.2$ value through the bioprocesses with 5 M KOH and 5 M H_3PO_4 . The feed rate was controlled automatically by the bioreactor control unit and verified with a balance that placed under the bottle of the feed substrate solution.

Among the exponential feeding experiments the highest cell concentration was obtained in M-0.15 condition as 9.6 kg m^{-3} at $t=32 \text{ h}$. The maximum cell concentrations for M-0.2, M-0.1 and G-0.15 experiments were achieved as 9.18 kg m^{-3} ($t=26 \text{ h}$), 8.69 kg m^{-3} ($t=32 \text{ h}$) and 6.4 kg m^{-3} ($t=20 \text{ h}$), respectively.

At the M-0.2 operation, as a result of higher feeding amounts of molasses, the maximum cell concentration was attained earlier than the other operation conditions. However, due to the inhibitory effect of excess carbon source on the cell growth at the aerobic fermentation, the cell growth stationary phase was also observed earlier; and at the end of bioprocess, the cell concentration of M-0.2 mode remained below the value obtained by M-0.15 condition.

The highest cell concentration of M-0.15 was evaluated against the maximum cell concentration of G-0.15 and 1.5-fold increase was attained indicating the priority of molasses based exponential feeding application in design of high cell density culture of recombinant *E.coli* BL21 (DE3) pLysS.

In the batch phase of the bioprocesses after $t=3 \text{ h}$, a sharp decrease was observed in glucose concentration profile for all operation conditions and with depletion of glucose in the medium the cell growth reached to its stationary phase where the carbon source feeding was initiated.

Throughout the fed-batch part except at the end hours of M-0.2, there was no significant accumulation of glucose in the fermentation media. In M-0.2 condition, accumulation of glucose occurred after $t=22 \text{ h}$ and its concentration in fermentation broth increased with the increase in feed rate. The final glucose concentration in M-0.2 condition was detected as 22.9 kg m^{-3} , resulted in the early stationary phase in this operation.

In M-0.2 and M-0.15 operation modes, a significant increase in fructose concentration was detected after $t=20 \text{ h}$, and at the end of the fermentations it was

reached to the respective concentrations of 26.74 kg m^{-3} and 13.72 kg m^{-3} . The glucose and fructose profiles of M-0.15 condition demonstrated the preference of glucose to fructose by the cells during the cultivation time. In M-0.1 mode, there was no significant fructose accumulation during the bioprocess indicating in more effective substrate management at this condition.

The highest GI activity was obtained in M-0.1 operation mode at $t=30 \text{ h}$ as 16399 U L^{-1} . The limiting substrate concentration and the better substrate control, achieved in this condition resulted in higher GI activity. On the other hand, the lowest GI volumetric activity as 1753 U L^{-1} was examined at G-0.15 among the investigated feeding strategies.

The accumulation of glucose at the end hours of G-0.15, M-0.15 and M-0.2 operation modes resulted in reduction of catabolite transcriptional activator protein (CAP) expression necessary for the induction of lacUV5 promoter and as consequence lower rGI activity detection.

The highest GI activity of M-0.1 and M-0.15 conditions was compared with that of G-0.15 operation, 9.3 and 5.0-fold increase was absorbed, respectively. So it was concluded that molasses based feeding strategies were more successful in the terms of cell concentration and recombinant enzyme production.

The presence of galactose, one of the monomers of trisaccharide raffinose in the beet molasses content, resulted in higher GI activities since the noise value due to stochasticity in gene expression is lower for natural inducer.

Acetic, formic, fumaric, citric in addition to malic acids were the organic acids detected in the fermentation broths. In all operation conditions, except M-0.1, similar trends were observed and with the increase in the cultivation time, organic acid concentrations increased.

In the M-0.1 operation mode, firstly, secreted organic acid concentrations in the extracellular medium were increased with time but because of substrate limitation in this condition, organic acids penetrated into assimilatory pathways and their concentrations decreased at the remaining hours of bioprocess.

Total organic acid concentrations detected at the end hour of fed-batch phases were 1.82 , 15.09 , 25.07 and 2.82 kg m^{-3} for M-0.1, M-0.15, M-0.2 and G-0.15

operations, respectively. Presence of the organic acids of the TCA cycle, i.e., citric, formic and malic acids designates the inadequate oxygen utilization, which leads to repression of the TCA cycle enzymes.

Acetic acid was the major secreted organic acid in all operation conditions. The maximum acetic acid concentration for the fed batch phases was achieved as 1.72, 12.32, 18.14, and 1.34 kg m⁻³ respective to M-0.1 (t=18 h), M-0.15 (t=32 h), M-0.2 (t=32 h), and G-0.15 (t=20 h) operations.

The highest acetic acid concentration as 18.14 kg m⁻³ was detected at the end hour of M-0.2 strategy where the highest fructose and glucose accumulation was also observed and resulted in earlier stationary phase of cell growth as well as significant reduction in the rGI activity.

At M-0.1 operation mode, the acetic acid concentrations ($C_{AA}=0-1.7$ kg m⁻³) oscillated within tolerable limits as a result of more efficient feeding management achieved in this condition.

At all the operation conditions, K_{La} values were decreased during the batch phase of bioprocess with increasing cell concentrations and when feeding was started again an increase in K_{La} values were detected. Generally, when higher glucose and fructose amounts were present in the medium, higher K_{La} values were obtained. The highest K_{La} values were observed as 0.020, 0.016, 0.018 and 0.033 s⁻¹ for M-0.1, M-0.15, M-0.2, and G-0.15, respectively. The maximum K_{La} was obtained at the end of G-0.15 operation with the value of 0.033 s⁻¹ where the lowest cell concentration was also detected.

A similar trend with K_{La} values was detected for OTR variations and the maximum values of 4.57 and 4.15 mol m⁻³ s⁻¹ were obtained at t=18 h in M-0.15 and t=20 h in G-0.15 conditions, respectively. Since dissolved oxygen level was kept constant throughout the process, the OUR values were the same as the OTR values. The highest OUR values were obtained as 4.57, 3.53, 3.99 and 4.15 mol m⁻³ s⁻¹ for M-0.1, M-0.15, M-0.2 and G-0.15, respectively.

For all the fed-batch operations except the first hours of G-0.15, Da values were higher than 1 indicating that mass transfer was the rate limiting step and maximum possible oxygen utilization rate was significantly lower than the value can

be achieved at the bulk phase. The maximum Da values were obtained as 49.3, 8.76, 9.62 and 20.80 and for M-0.1, M-0.15, M-0.2, and G-0.15, respectively.

Throughout the fermentations, the η values were lower than 1 showing that the oxygen consumption was below the maximum oxygen demand. The maximum η values were achieved at first hours of bioprocesses as 0.27 for molasses based operations and 0.61 for glucose based fed-batch operation.

For all the operations, specific growth rate values were not strictly constant at the desired value and generally the values were below the predetermined specific growth rate. This was probably due to the inhibitory effects of by-products formation as a result of insufficient oxygen transfer during the cultivations time. The highest specific growth rates were obtained as 0.04, 0.19, 0.18, and 0.09 h⁻¹ for M-0.1, M-0.15, M-0.2, and G-0.15 conditions, respectively.

Specific substrate utilization rates were generally increased throughout the fed-batch phase of fermentations. As the feeding rates increased the maximum values of specific substrate consumption rate increased and it reach to highest value in M-0.2 operation as 1.90 kg kg⁻¹ h⁻¹.

Specific oxygen uptake rates showed a decreasing profile with the cultivation time at all the conditions and the highest values were attained at the first hour of batch phase as 0.77, 0.79, 0.66, and 0.52 kg kg⁻¹ h⁻¹ for M-0.1, M-0.15, M-0.2, and G-0.15 conditions, respectively.

The maximum overall specific cell yield on substrate ($Y_{X/S}$) was also attained at M-0.1 condition indicating the elevated efficiency of carbon source utilization for product formation.

To conclude, the molasses based exponential feeding strategy with 0.1 predetermined specific cell growth rate (M-0.1) was selected as optimum feeding strategy for the over-production of recombinant GI by *E.coli* BL21 (DE3) pLysS::xyl_T cells, and as the result of effectual feeding strategy supplied in this condition, the lower amount of carbon source was utilized, the lower acetic was formed and the highest GI activity was detected.

In the third part of this study, the peptide ligands that show affinity toward 6×His were selected from phage display peptide library and the kinetic parameters of peptide-peptide interaction were determined via surface plasmon resonance.

In SPR studies, the peptide 3|15 with the amino acid sequence of GKPMPPM, demonstrated selective binding profiles toward 6×His tag at different peptide concentrations up to 0.5 mg mL⁻¹.

The single site Langmuir adsorption model was employed for the calculation of affinity constants and by fitting the adsorption part of each sensorgram to this model, K_D value was calculated as 3×10⁻⁴ M.

REFERENCES

- Aehle, W. (ed), 2007. Enzymes in industry: production and applications. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
- Agarwal, L., Isar, J., Meghwanshi, G.K., Saxena, R.K., 2006. A cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by *Escherichia coli*. Jour. of App. Microbio., 100: 1348–1354.
- Arap, M.A., 2005. Phage display technology - Applications and innovations. Genet. and Molecul. Bio., 28: 1-9 .
- Akesson, M., Hagander, P., Axelsson, J.P., 2001. Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. Biotech. and Bioeng., 73: 223-230.
- Akesson, M., Karlsson, E.N., Hagander, P., Axelsson, J.P., Tocaj A., 1999. On-line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. Biotech. and Bioeng., 64: 590-598.
- Bailey, E. J., Ollis, F. D., 1986. Biochemical Engineering Fundamentals, 2nd ed., Mc Graw-Hill, Inc., Singapore.
- Bandyopadhyay, B., Humprey, A.E., 1967. Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. Biotechnology and Bioengineering, 9; 533-544.
- Banki, M. R., Feng, L., Wood, D., 2005. Simple bioseparations using self-cleaving elastin-like polypeptidetags. Nature Methods, 787: 1-3.
- Barbas, C.F., Kang, A.S., Lerner, R.A. and Benkovic, S.J.1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. USA., 88:7978-7982.
- Barker, S.A and Shirley, J.A., 1980. Glucose oxidase, glucose dehydrogenase, glucose isomerase, β -galactosidase and invertase in economic microbiology. London, New York, Toronto, Sydney, San Francisco: Academic press.

Bass, S., Greene, R., and Wells, J.A., 1990. Hormone phage: An enrichment method for variant proteins with altered binding properties. *Proteins* 8:309-314.

Batt, C. A., A. C. Jamieson, and M. A. Vandeyar. 1990. Identification of essential histidine residues in the active site of *Escherichia coli* xylose (glucose) isomerase. *Proc. Natl. Acad. Sci. USA* 87:618–622.

Bhattacharya, K. S., Dubey, K. A., 1997. Effects of Dissolved Oxygen and Oxygen Mass Transfer on Overexpression of Target Gene in Recombinant *E.coli*, *Enzy. and Microb. Techno.*, 20, 355-360.

Bhosale, S., Rao, M., Deshpande, V., 1996. Molecular and industrial aspects of glucose isomerase. *Microbiology Reviews*, 60: 280-300.

BiologyMad A-Level biology. <http://www.biologymad.com/>. Last accessed: June 2010.

Bondeson K., Frostell-Karlsson A., Fägerstam L., Magnussin G., 1993. Lactose repressor operator-DNA interactions: kinetic analysis by surface plasmon resonance biosensor. *Analyt. Biochem.*, 214: 245-251.

Borsing, L., Berger, E.G., Malissard, M., 1997. Expression and purification of His-tagged beta-1,4 galactosyltransferase in yeast and in COS cells. *Biochem. Biophys. Res. Commun.*, 240:586–590.

Brady, C.P., Shimp, R.L., Miles, A.P., Whitmore M., Stowers A.W., Stowers, A.W., 2001. High-level production and purification of P3P2MSP1₁₉ an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. *Pro. Expre. and Pur.*, 23: 468-475.

Brierley, R.A., Bussineau, C., Kosson, R., Melton, A., Siegel, R.S., 1990. Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme, *Ann. N.Y. Acad. Sci.* 589: 350-362.

Bryan, P. N., 2002. Prodomains and protein folding catalysis, *Chem. Rev.*, 102: 4805-4815.

Castan, A., Nasman, A., Enfors, S-O., 2002. Oxygen enriched air supply in *Escherichia coli* processes: production of biomass and recombinant human growth hormone. *Enzyme and Microbial Technology*, 30: 847-854.

Cereghino, J. L., Cregg, J. M., 2000. Heterologous protein expression in the methylotropic yeast *Pichia Pastoris*, FEMS Microbio. Rev., 24: 45-66.

Cereghino, G. P. L., Cereghino, J. L., Ilgen, C., Cregg, J. M., 2002. Production of recombinant proteins in fermenter cultures of the *Pichia Pastoris*. Biotechno., 13:329–332.

Chaga, G., Hopp J., Nelson, P., 1999a. Immobilized metal ion affinity chromatography on Co²⁺-carboxymethylaspartate-agarose superflow, as demonstrated by one-step purification of lactate dehydrogenase from chicken breast. Biotechnol. Appl. Biochem., 29:19–24.

Chan, E., Ueng P. P., and Chen, L. F., 1989. Metabolism of D-xylose in *Schizosaccharomyces pombe* cloned with a xylose-isomerase gene. Appl. Microbiol. Biotechnol. 31:524–528.

Chang, C., Park, B.C., Lee, D-S., Suh, S.W., 1999. Crystal structures of thermostable Xylose isomerases from *Thermus caldophilus* and *Thermus Thermophilus*: possible structural determinants of thermostability. J. Mol. Biol. 288: 623-634.

Chauthaiwale, J. V., and M. B. Rao. 1994. Production and purification of extracellular D-xylose isomerase from an alkaliphilic, thermophilic *Bacillus* sp. Appl. Environ. Microbiol. 60: 4495–4499.

Chen, W. P. 1980. Glucose isomerase. Process Biochem. 15: 36–41.

Chen, Y., Jin, M., Egborge, T., Coppola, G., Andre, J., Calhoun, D.H., 2000. Expression and characterization of glycosylated and catalytically active recombinant human α -galactosidase A produced in *Pichia pastoris*. Prot. Expres. and Purifi., 2000: 472-484.

Chen, B.P., Hai, T., 1994. Expression vectors for affinity purification and radiolabeling of proteins using *Escherichia coli* as host. Gene, 139:73–75.

Chen, Y., Shen, Y., Guo, X., Zhang, C., Yang, W., Ma M., Liu, S., Zhang, M., Wen L-P., 2006. Transdermal protein delivery by coadministered peptide identified via phage display. Nat. Biotech. Lett. 24 : 455-460.

Chen L., Wang Q., Hou W., 2009. The utilization of BSA-modified chip on the investigation of ligand/protein interaction with surface plasma resonance. Afr. Jour. of Biotech., 8:7148-7155.

Cheng, L-C., Hor, L-I., Wu, J-Y, Chen, T-L., 2003. Effect of specific growth rate on the production of a recombinant nuclease by *Escherichia coli*. *Bioch. Engin. Jour.*, 14: 101-107.

Choi, J.H., Keum, K.C., Lee S.Y., 2006. Production of recombinant proteins by high cell density culture of *Escherichia coli*. *Che. Engin. Scien.*, 61: 876-885.

Chiruvolu, V., Cregg, J.M. and Meagher, M.M., 1997. Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. *Enzy. and Micro. Technol.*, 21: 277-283.

Clare, J.J., Rayment, F.B., Ballantyne, S.P., Sreerkrishna, K., Romanos, M.A., 1991. High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *BioTechnology*, 9: 455–460.

Cline J., Braman J.C. and Hogrefe, H.H., PCR fidelity of Pfu DNA polymerase and other thermostable DNA polymerases. *Nucl. Acids Resear.*, 24: 3546–3551.

Clonis, Y., 1990. *Process Affinity Chromatography. Separation processes in Biotechnology.* Asenjo J.A. (Ed.). 1st Ed., Marcel Dekker Inc., New York.

Clonis Y.D., Lowe C.R., 1981. Affinity chromatography on immobilized triazine dyes. Studies on the interaction with multinucleotide dependent enzymes. *Bioch. et Biophys. Acta.*, 659: 86–98.

Clonis Y.D., Stead V., Lowe C.R., 1987. Novel cationic triazine dyes for protein purification. *Biotech. Bioeng.* 30: 621–627.

Cortesea, R., Monacia, P., Luzzagoa, A., Santinia, C., Bartolia, F., Corteseb, I., Fortugnoa, P., Galfrea, G., Nicosiaa, A., Felicia, F., 1996. Selection of biologically active peptides by phage display of random peptide libraries. *Cur. Opin. in Biotech.*, 7: 616-621.

Cos, O., Ramón R., Montesinos J.L. and Valero, F., 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. *Micro. Cell Fact.* 5: 1-20.

Cregg, J.M., 1999. *Gene expression systems*, Edited by Joseph M. Fernandez and James P. Hoeffler, Academic Press, San Diego, California.

Curvers, S., Linnemann, J., Klauser, T., Wandrey, C. and Takors, R., 2001-b. Recombinant protein production with *Pichia pastoris* in continuous fermentation - Kinetic analysis of growth and product formation. *Chemie Ingenieur Technik*, 73: 1615-1621.

Çalık, P., Angardi V., Haykır N.I., and Hakkı Boyacı, I., 2009. Glucose isomerase production on a xylan-based medium by *Bacillus thermoantarcticus*. *Biochem. Engin.Jour.*, 43: 5-8.

Çalık, G., Berk, M, Boyacı, F.G., Çalık, P., Takaç, S., Özdamar, T.H., 2001. Pretreatment processes of molasses for the utilization in fermentation processes. In: Hofman M, Thonart P (eds) *Focus in biotechnolog : engineering and manufacturing for biotechnology*. Kluwer Academic, The Netherlands, 4: 21–28

Çalık, P., Balci, O., Ozdamar, T.H., 2010. Human growth hormone specific aptamer identification using oligonucleotide ligand evolution method. *Prot. Expres. and Purif.* 69:21-28.

Çalık, P., Çalık, G., Özdamar, T.H., 1998. Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis*: use of citric acid as the carbon source. *Enz. and Micro. Techno.*, 23:451–461.

Çalık, P., Çalık, G., Takaç, S., Özdamar, T.H., 1999. Metabolic Flux Analysis for Serine Alkaline Protease Fermentation by *Bacillus licheniformis* in a Defined Medium: Effects of the Oxygen Transfer Rate. *Biotec. and Bioeng.*, 64: 151-167.

Çalık, P., Çalık, G., Özdamar, T.H., 2000. Oxygen transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis*. *Biotec and Bioeng.*, 69: 301-311.

Çalık, P., İnankur, B., Elif Ş. Soyaslan, E. Ş., Şahin, M., Taşpınar, H., Açık, E., Bayraktar, E., 2010. Fermentation and oxygen transfer characteristics in recombinant human growth hormone production by *Pichia pastoris* in sorbitol batch and methanol fed-batch operation. *Jour. of Chem. Tech. and Biotech.*, 85: 226–233.

Çalık, P., Levent H., 2009a. Effects of pretreated beet molasses on benzaldehyde lyase production by recombinant *Escherichia coli* BL21 (DE3) pLysS. *Jour. of Appl. Microbio.*, 107: 1536-1541.

Çalık, P., Levent, H., 2009-b. Effects of pulse feeding of beet molasses on recombinant benzaldehyde lyase production by *Escherichia coli* BL21 (DE3). App. Microbio.and Biotechno., 85: 65-73.

Çalık, G., Pehlivan, P., Kalender, N., Calık, P., Özdamar, T.H., 2003. Utilization of pretreated molasses for serine alkaline protease production with recombinant *Bacillus* species. Chem. Engin. Com., 190: 630-644.

Çalık, P., Yılğör, P., Ayhan, P., Demir, A.S., 2004. Oxygen transfer effects on recombinant benzaldehyde lyase production. Chemical Engineering Science, 59: 5075-5083.

Çalık, P., Yılğör, P., Demir, A.S., 2006. Influence of controlled-pH and uncontrolled-pH operations on recombinant benzaldehyde lyase production by *Escherichia coli*. Enz. and Micro. Tech., 38: 617-627.

Çelik, E., 2008. Bioprocess development for therapeutical protein production. PhD Thesis, Middle East Technical University, Turkey.

Dale, J.W. and Von Schantz, M., 2007. From genes to genomes: concepts and applications of DNA technology, 2nd Ed. John Wiley & Sons Ltd, England.

Daly, R. and Hearn, M. T. W., 2005. Expression of heterologous proteins in *Pichia Pastoris*: a useful experimental tool in protein engineering and production, J. Mol. Recognit., 18: 119–138.

de Almeida, J.R.M., de Moraes, L.M.P., Torres, F.A.G., 2005. Molecular characterization of the 3-phosphoglycerate kinase gen (PGK1) form the methylotrophic yeast *Pichia pastoris*. Yeast, 22: 725-737.

de Pourcq, K., de Schutter, K., Callewaert, N., 2010. Engineering of glycosylation in yeast and other fungi:current state and perspectives. Appl. Microbiol. Biotechnol., 87:1617–1631.

Dekker, K., Sugiura, A., Yamagata, H., Sakaguchi, K., Udaka S., 1992. Efficient production of thermostable *Thermus thermophilus* xylose isomerase in *Escherichia coli* and *Bacillus brevis*. Appl. Microbiol. Biotechnol. 36: 727-732.

Dekker, K., yamagata, H., Sakaguchi, K., Udaka, S., 1991. Xylose (Glucose) isomerase gene from the thermophile *Thermus thermophilus*: cloning, sequencing,

and comparison with other thermostable xylose isomerases. *Journal of Bacteriology*, 173(10): 3078-3083.

Deng, Q., German, I., Buchanan, D., and Kennedy, R.T., 2001. Retention and separation of adenosine and analogues by affinity chromatography with an aptamer stationary phase. *Anal. Chem.* 73:5415-5421.

Denizli, A., Salih, B., Kavakli, C., Piskin, E., 1997. Dye-incorporated poly (EGDMA-HEMA) microspheres as specific sorbents for aluminum removal. *Jour. of Chromatog .B*, 698: 89–96.

Dexheimer, T.S., Stephen, A.G., Fivash, M.J., Fisher, R.J., Pommier, Y., 2010. The DNA binding and 3'-end preferential activity of human tyrosyl-DNA phosphoesterase. *Nuc. Acids Res.*, 38: 2444-2452.

Doelle, H. W., Ewings, K. N., Hollywood, N. W., 1982. Regulation of Glucose Metabolism in Bacterial Systems, *Adv. Biochem. Eng.*, 23, 1-35.

Doran, M.P., 1995. *Bioprocess engineering principles*. Academic Press Ltd., San Diego.

Dudak, F.C., Boyaci, I.H. and Orner, B. P., 2011. The Discovery of Small-Molecule Mimicking Peptides through Phage Display. *Molecules*, 16:774-789.

Dumbrepatil, A., Adsul, M., Chaudhari, S., Khire, J. and Gokhale D., 2008. Lactic acid from molasses in batch fermentation. *Appl. Environ. Microbiol.*, 74: 333–335.

Edwards, P.R., Gill, A., Pollard-Knight, D.V., Hoare, M., Buckle, P.E., et al, 1995. kinetics of protein-protein interactions at the surface of an optical biosensor. *Analy. Biochem.*, 231:210-217.

Erickson, J.A., Jalaie, M., Robertson, D.H., Lewis, R.A., Vieth, M., 2004. Lessons in molecular recognition: The effects of ligand and protein flexibility on molecular docking accuracy. *Jour. of Medic. Chem.*, 47: 45-55.

Faber, K., 2000. *Biotransformations in Organic Chemistry*, 4th Ed., Springer, Germany.

Fermentas Molecular Biology Tools. <http://www.fermentas.com>. Last accessed: January 2011.

- Fisher, R.J., Fivash, M.J., 1994. Surface plasmon resonance based methods for measuring the kinetics and binding affinities of biomolecular interactions. *Curr. Opin. in Biotech.* 5:389-395.
- Folkers, J.P., Laibinis, P.E., Whitesides, G. M., Deutch, J., 1994. Phase behavior of two-component self-assembled monolayers of alkanethiolates on gold. *J. Phys. Chem.*, 98: 563–571.
- Gaikwad, S.M., Rao M.B., Deshpande, V.V., 1992. D-Glucose/xylose isomerase from *Streptomyces*: differential roles of magnesium and cobalt ions. *Enzyme and Microbial Technology*, 14: 317-320.
- Gaskin, D.J., Starck, K., Turner, N.A., Vulfson, E.N., 2001. Phage display combinatorial libraries of short peptides: ligand selection for protein purification. *Enz. Microb. Technol.*, 28:766.
- Gedig E., 2008. Surface Chemistry in SPR Technology, *Handbook of Surface Plasmon Resonance.*, 1st Ed. , Ed by. Schasfoort R.B.M., Tudos A.J., The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge UK.
- Gerhardt, P., Dorrell, W. W. and Baldwin, I. L., 1946. Citric acid fermentation of beet molasses. *J. Bacteriol.*, 52: 555–564.
- Glazer, A. N., Nikaido, H., 1995. *Microbial Biotechnology: Fundamentals of Applied Biotechnology*, W.H. Freeman and Company, USA.
- Ghatge, M. S., S. U. Phadtare, A. M. Bodhe, and V. V. Deshpande., 1994. Unfolding and refolding of glucose/xylose isomerase from *Streptomyces* sp.NCIM 2730. *Enzyme Microb. Technol.*, 16:323–327.
- Han Y., Gras S., Forde G.M., 2009. Binding properties of peptidic affinity ligands for plasmid DNA capture and detection. *AIChE Jour.*, 55: 505-515.
- Han, K., Lim, H.C., Hong J., 1992. Acetic acid formation in *Escherichia coli* fermentation. *Biotec. and Bioeng.*, 39: 663-671.
- Hellwig, S., Emde, F., Raven, N.P.G., Henke, M., Van der Logt, P., Fischer, R., 2001. Analysis of single chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentation. *Biotech. and Bioeng.*, 74:344–352.

Ho, N. W. Y., S. Rosenfeld, P. Stevis, and G. T. Tsao., 1983. Purification and characterisation of the D-xylose isomerase gene from *E. coli*. *Enzyme Microb. Technol.*, 5:417–420.

Ho, N. W. Y., and P. E. Stevis. 1985. Overproduction of D-xylose isomerase in *Escherichia coli* by cloning the D-xylose isomerase gene. *Enzyme Microb. Technol.* 7:592–596.

Holms, W.H., 1986. The central metabolic pathways of *Escherichia coli*: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. *Cur. Top. in Cel. Reg.*, 28: 69-105.

Homola, J., Yee, S.S., Gauglitz, G., 1999. Surface plasmon resonance sensors: review. *Sen. and Actuat. B: Chem.*, 54: 3-15.

Hong, F., Meinander, N.Q., Jonsson, L.J., 2002. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotech. and Bioeng.*, 79: 438-449.

Hoogenboom, H.R., 2002. Overview of antibody phage-display technology and its applications. *Methods Mol Biol* 178:1-37.

Huang, P.Y., Baumbach, G.A., Dadd, C.A., Buettner, J.A., Masecar, B.L., Hentsch, M., Hammond, D.J., Carbonell, R.G., 1996. Affinity purification of von Willebrand factor using ligands derived from peptide libraries. *Bioorg. Med. Chem.*, 4: 699-708.

Inan, M., Chiruvolu, V., Eskridge, K.M., Vlasuk, G.P., Dickerson, K., Brown, S., Meagher, M.M., 1999. Optimization of temperature-glycerol-pH conditions for a fedbatch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. *Enz. and Micro. Techno.*, 24: 438-445.

Inan, M., Meagher M. M., 2001-a. The effect of ethanol and acetate on protein expression in *Pichia Pastoris*. *Jour. of Bioscien. and Bioengin.*, 92: 337-341.

Inan, M., Meagher, M.M., 2001-b. Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. *Jour. of Bioscien. and Bioengin.*, 92: 585-589.

Invitrogen (2002). *Pichia* fermentation process guidelines. www.invitrogen.com. Last accessed: October 2009.

Invitrogen (2002). pRSETA cloning vector user manual Cat. no.V351-20. www.invitrogen.com/content/sfs/manuals/prset_man.pdf. Last accessed: June 2010.

Jackrel, M. E., Valverde, R., Regan, L., 2009. Redesign of a protein–peptide interaction: Characterization and applications. *Prot. Scien.*, 18: 762–774.

Jacobsen, B., Gardsvoll, H., Funch, G.J., stergaard, S., Barkholt, V., Ploug M., 2007. One-step affinity purification of recombinant urokinase-type plasminogen activator receptor using a synthetic peptide developed by combinatorial chemistry. *Prot. Exp.and Purif.*, 52: 286-296.

Janknecht, R., de Martynoff, G., Lou, J., Hipkind, R., Nordheim, A., Stunnenberg, H.G., 1991. Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccina virus. *Proc. Natl. Acad. Sci. USA*, 88: 8972–8976.

Janknecht, R., Nordheim, A., 1992. Affinity purification of histidine tagged proteins transiently produced in HeLa cells. *Gene*, 121: 321–324.

Janson J.C., 2011. *Protein Purification, Principles, High Resolution Methods and Applications*, 3th Ed., Wiley and sons, Inc., New Jersey.

Johnston, A. W., Stewart, M., Lee, P., Cooney, J. M., 2003. Tracking the Acetate Threshold Using DO-Transient Control During Medium and High Cell Density Cultivation of Recombinant *Escherichia coli* in Complex Media, *Biotech. and Bioeng.*, 84: 315-323.

Jost, P.J., Harbottle, R.P., Knight, A., Miller, A.D., Coutelle, C., Schneider, H., 2001. A novel peptide, for targeting of human epithelia. *FEBS Lett.*, 489:263-269.

Ju, L-K. and Chase, G.G., 1992. Improved scale-up strategies of bioreactors. *Biopro.Eng.*, 8: 49-53.

Jungo, C., Rerat, C., Marison, I.W., von Stockar, U., 2006. Quantitative caracterization of the regulation of the synthesis of alcohol oxidase and of the expression of recombinant avidin in a *Pichia pastoris* Mut⁺ strain. *Enz. Microb. Technol.* 39: 936–944.

Jungo, C., Schenk, J., Pasquier, M., Marison, I.W., von Stockar, U., 2007. A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*. *Jour. of Biotech.*, 131: 57-66.

Kaneko, T., Saito, K., Kawamura, Y., Takahashi, S., 2001. Molecular cloning of acid-stable glucose isomerase gene from *Streptomyces olivaceoviridis* E-86 by a simple two-step PCR method, and its expression in *Escherichia coli*.

Kampen, W.H., 1997. Nutritional requirements in fermentation processes. In: Vogel, H.C., Todaro C.L. (eds), *Fermentation and biochemical engineering handbook: principles, process design, and equipment*, Second edition. Noyes Publications, New Jersey, 122-168.

Karlsson, R., Falt, A., 1997. Experimental design for kinetic analysis of protein-protein interaction with surface plasmon resonance biosensors. *Jour. Of Immunolog. Meth.*, 200:121-133.

Kaslow, D.C., Shiloach, J., 1994. Production, purification and immunogenicity of a malarian transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified using nickel-NTA agarose. *Bio/Tech.*, 12:494-499.

Karlsson R., Falt A., 1997. Experimental design for kinetic analysis of protein-protein interaction with surface plasmon resonance biosensors. *Jour. of Immun. Met.*, 200: 121-133.

Katakuga, Y., Zhang, W., Zhuang, G., Omasa, T., Kishimoto, M., Goto, Y., Suga, K.I., 1998. Effect of methanol concentration on the production of human beta-2-glycoprotein I domain V by a recombinant *Pichia pastoris*: A simple system for control of methanol concentration using a semiconductor gas sensor. *Jour. of Ferm. and Bioeng.*, 86:482-487.

Kaya Çeliker, H., Angardi, V., Çalık, P., 2009. Regulatory effects of oxygen transfer on overexpression of recombinant protein by *Escherichia coli* BL21 (DE3). *Biotech. Jour.*, 4: 1066-1076.

Kim, B.S., Lee, S.C., Lee, S.Y., Chang, Y.K., Chang, H.N., 2004. High cell density fed-batch cultivation of *Escherichia coli* using exponential feeding combined with pH-stat. *Biopro. and Bi Jour. of Gen. Microbio.*, 1993., 139: 1227-1234.

Kim M., Park K., Jeong E.J., Shin Y.B., Chung B.H., 2006. Surface Plasmon resonance imaging analysis of protein-protein interactions using on-chip expressed capture protein. *Analyt. Biochem.*, 226:175-181.

Kirk, R.E., Othmer, D.F., 1994. *Encyclopedia of chemical technology*, 4th Ed. The Interscience Encyclopedia Inc., New York.

Klug, W. S., Cummings, M. R., Spencer, C. A., 2006. Concepts of genetics, 8th Ed., Pearson Prentice Hall, New Jersey.

Klug, W. and Cummings, M., 2003. Concepts of genetics. New Jersey: Prentice Hall.

Konig H., Claus, H., Varma, A., 2010. Prokaryotic cell wall compounds structure and biochemistry. Springer Heidelberg Dordrecht London New York.

Kovalevsky, A. et al. (2010). Metal ion roles and the movement of hydrogen during reaction catalyzed by d-xylose isomerase: a joint x-ray and neutron diffraction study. Cell Press, 688-699.

Kupesulik, B. and Sevelle, B., 2005. Optimization of specific product formation rate by statistical and formal kinetic model description of an HSA producing *Pichia pastoris* Mut^S strain. Chem. and Biochem. Eng. Quart., 19 : 99-108.

Kuusinen, A., Arvola, M., Oker-Blom, C., Kein_men, K., 1995. Purification of recombinant GluR-D glutamate receptor produced in Sf21 insect cells. Eur. J. Biochem., 233:720–726.

Labrou N.E., 2003. Design and selection of ligands for affinity chromatography. Jour. of Chromatog. B. 790: 67–78.

Lamba, L., Nicolaus, B., Calandrelli, V., Romano, I., Basile, R., Gambacarta, A., 2001. Purification and characterization of thermostable xylose (glucose) isomerase from *Bacillus thermoantarcticus*. Jour. of Indus. Microb. and Biotech., 27:234-240.

Lee S. Y., 2009. Systems Biology and Biotechnology of *Escherichia Coli*. Springer Science Business Media , Korea.

Lee, C., L. Bhatnagar, B. C. Saha, Y. Lee, M. Takagi, T. Imanaka, M. Bagdasarian, and J. G. Zeikus. 1990. Cloning and expression of the *Clostridium thermosulfurogenes* glucose isomerase. Appl. Environ. Microbiol. 56:2638–2643.

Lee, S.Y., 1996. High cell-density culture of *Escherichia coli*. Tren. in Biotech., 14: 98-105.

Lee, J., Lee, S.Y., Park, S., Middelberg, A.P.J., 1999. Control of fed batch fermentations. Biotechnol. Adv., 17:29–48.

Leon, A.D., Hernandez, V., Galindo, E., Ramirez, O.T., 2003. Effects of dissolved oxygen tension on the production of recombinant penicillin acylase in *Escherichia coli*. *Enzy.and Microb. Techno.*, 33: 689-697.

Li, A., Crimmins, D.L., Luo, Q., Hartupee, J., Landt, Y., Ladenson, J.H., Wilson, D., Anant, S., Dieckgraefe, B.K. (2003). Expression of a novel regenerating gene product, Reg IV, by high density fermentation in *Pichia pastoris* : production, purification, and characterization. *Pro. Expres. and Purif.*, 31: 197-206.

Liedberg, B., Nylander, C., Lundström, 1983. Surface plasmon resonance for gas detection and biosensing. *Sens. and Act.*, 4: 299-304.

Lin, K., Wey, M., Kan, L., Shiuan, D., 2009. Characterization of the interactions of lysozyme with DNA by surface plasmon resonance and circular dichroism spectroscopy. *Appl. Biochem. Biotech.*, 158: 631-641.

Litman, G.W., Rast, J.P., Shamblott, M.J., Haire, R.N., Hulst, M., Roess W., Litman R.T., Hinds-Frey K.R., Zilch, A., Amemiya C.T., 1993. Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. *Mol. Biol. Evol.* 10 : 60–72.

Loewen, M.C., Liu, X., Davies, P.L. and Daugulis, A.J., 1997. Biosynthetic production of type II fish antifreeze protein: fermentation by *Pichia pastoris*. *Appl. Microb. Biotech.*, 48: 480-486.

Love, J.C., Estroff, L.A., Kriebel, J.K., Nuzzo, R.G., Whitesides, G.M., 2005. Self assembled monolayers of thiolates on metals as a form of nanotechnology. *Chem. Rev.*, 105:1103-1116.

Luli, W. G., Strohl, R. W., 1990. Comparison of Growth, Acetate Production, and Acetate Inhibition of *Escherichia coli* Strains in Batch and Fed-Batch Fermentations, *Appl. and Enviro. Microbio.*, 56, 1004-1011.

Macauley-Patrick, S., Fazenda, M. L., McNeil, B., and Harvey, L. M., 2005. Heterologous protein production using the *Pichia Pastoris* expression system. *Yeast*, 22: 249–270.

MacKenzie, C. R.; Hiram, T.; Deng, S.; Bundle, D. R.; Narang, S. A.; Young, N. M., 1996. Quantitative analysis of bacterial toxin affinity and specificity for glycolipid receptors by surface plasmon resonance. *J. Biol. Chem.*, 271: 1527-1533.

Makkar, R.S., Cameotra, S.S., 2002. An update on the use of unconventional substrates for biosurfactant production and their new applications. *Applied Micro. and Biotech.*, 58 :428-434.

Marial, T., Özlep, V.C., Sanchez, P.L., Mir, M., Katakis, I., Sullivan, C.K., 2008. Aptamers: molecular tools for analytical applications. *Anal. Bioanal. Chem.*, 390: 989-1007.

Marshall, R. O., and Kooi, E. R., 1957. Enzymatic conversion of D-glucose to D-fructose. *Science* 125:648–649.

Marison, I.W., von Stocker U., 1986. The application of a novel heat flux calorimeter for studying growth of *Escherichia coli* W in aerobic batch culture. *Biotech. and Bioeng.*, 28: 1780-1793.

McCafferty, J., Griffiths, A.D., Winter, G., and Chiswell, D.J., 1990. Phage antibodies: Filamentous phage displaying antibody variable domains. *Nature* 348:552-554.

McGrew, J.T., Leiske, D., Dell, B., Klinke, R., Krasts, D., Wee, S., Abbott, N., Armitage, R. and Harrington, K., 1997. Expression of trimeric CD41 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants. *Gene*, 187: 193-200.

Meilleur, F., Snell, E. H., Van der Woerd, M. J., Russell A. J., Dean A. A., 2006. A quasi-Laue neutron crystallographic study of D-xylose isomerase. *Eur. Biophys. J.*, 35:601–609.

Menendez, J., valdes, I., Cabrera, N., 2003. The ICL1 gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. *Yeast*, 20:1997-1108.

Meyer, D.E., Chilkoti, A., 1999. Purification of recombinant proteins by fusion with thermally-responsive polypeptides. *Nat. Biotechnol.*, 17: 1112–1115.

Meyer, H.P., Leist, C., Fiechter, A., 1984. Acetate formation in continuous culture of *Escherichia coli* K12 D1 on defined and complex media. *Jour. of Biotech.*, 1: 355-358.

Moes, C.J., Isak S. Pretorius, I.S., van Zyl, W.H., 1996. Cloning and expression of the *Clostridium Thermosulfurogenes* D-xylose isomerase (xyk4) in *Saccharomyces Cerevisiae*. *Biotech. Let.*, 18: 269-274.

Mondal, K., Gupta, M.N., 2006. The affinity concept in bioseparation : Evolving paradigms and expanding range of applications. *Biomolecular Engineering* 23: 59-76.

Moses, V., Cape, R. E., 1991. *Biotechnology: The Science and the Business*, Harwood Academic Publishers, Switzerland.

Miroux, B. and Walker J.E., 1996. Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* 260: 289–298.

Mitopem courseware Massachusetts Institute of Technology, Lecture notes (2004). <http://ocw.mit.edu/courses/biology/7-03-geneticsfall2004/lecturenotes/lecture16.pdf>. Last accessed: July 2010.

Nam Y., Chang J.C., Wheeler B.C., Brewer G.J., 2004. gold-coated microelectrode array with thiol linked self assembled monolayers for engineering neuronal cultures. *IEEE Trans. on Biom. Eng.*, 51:158-165.

Neidhardt, F.C., Bloch, P.L., Smith, D.F., 1974. Culture medium for enterobacteria. *Jour.of Bact.*, 119: 736-747.

Nelson, B. P., Grimsrud, T.E., Liles, M. R., Goodman, R.M.,and Corn, R.M., 2001. Surface Plasmon Resonance imaging measurements of DNA and RNA hybridization adsorption onto DNA microarrays. *Anal. Chem.*, 73: 1-7.

Nelson, B. P., Liles, M. R., Kendra B. Frederick, K.B., Corn, R.M., and Goodman, R.M., 2002. Label-free detection of 16S ribosomal RNA hybridization on reusable DNA arrays using surface Plasmon resonance imaging. *Environm. Microbio.* 4: 735–743.

Nielsen, J., Villadsen, J., Lidén, G., 2003. *Bioreaction engineering principles*, Second edition. Kluwer Academic/Plenum Publisher, New York.

Novagen pET system manual, TB055 8th Edition (1999). http://labs.fhcrc.org/hahn/Methods/biochem_meth/pet.pdf. Last accessed: July 2010.

Oxford Reference Online. www.oxfordreference.com. Last accessed: September 2010.

Paalme, T., Tiisma, K., Kahru, A., Vanatalu, K., Vilu, R., 1990. Glucose-limited fed-batch cultivation of *Escherichia coli* with computer-controlled fixed growth rate. *Biotech. and Bioeng.*, 35: 312-319.

Park, S.C., Baratti, J., 1991. Batch fermentation kinetics of sugar beet molasses by *Zymomonas mobilis*. *Biotech. and Bioeng.*, 38: 304-313.

Patel N., Davies M. C., Hartshorne M., Heaton R. J., Roberts C.J., Tendler S. J. B., Williams P. M. 1997. Immobilization of protein molecules onto homogeneous and mixed carboxylate-terminated self-assembled monolayers *Langmuir*.13: 6485-6490.

Pflug, S., Richter S.M., Urlacher, V.B., 2007. Development of a fed-batch process for the production of the cytochrome P450 monooxygenase CYP102A1 from *Bacillus megaterium* in *E. coli*. *Jour. of Biotechno.*, 129:481–488.

Ramon, R., Ferrer, P., Valero, F., 2007. Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*. *Jour. of Biotech.*, 130: 39-46.

Rank, K.B., Mildner, A.M., Leone, J.W., Koeplinger, K.A., Chou, K.C., Tomasselli A.G., Henrikson, R.L., Sharma, S.K., 2001. [W206R]-procaspase 3: an inactivatable substrate for caspase 8. *Protein Expr. Purif.*, 22, 258–266.

Ravelet, C., Grosset, C., Peyrin E., 2006. Liquid chromatography, electrophoresis and capillary electrophoresis applications of DNA and RNA aptamers. *Jour. of Chromat. A*. 1117 :1-10.

Reya-Tonetti G., Perotti, N.I., 1999. Rapid screening of textile dyes employed as affinity ligands to purify enzymes from yeast. *Biotech. Appl. Biochem.* 29:151–156.

Rhimi, M., Ben Messaoud, E., Borgi M. A., Ben khadra, K., Bejar, S., 2007. Co-expression of l-arabinose isomerase and d-glucose isomerase in *E. coli* and development of an efficient process producing simultaneously d-tagatose and d-fructose. *Enz. Micro. Tech.*, 40: 1531–1537.

Riesenberg, D., Guthke, R., 1999. High cell-density cultivation of microorganisms. *App. Microbio.*, 51: 422–430.

Roe, S., 2001. Protein purification applications: a practical approach. Oxford ; New York : Oxford University Press.

Romig, T.S., Bell, C., Drolet, D.W., 1999. Aptamer affinity chromatography combinatorial chemistry applied to protein purification. *Jour. of Chromatog. B.* 731:275-284.

Roy, I., Gupta, M.N., 2000. Purification of alkaline phosphatase from chicken intestine by expanded-bed affinity chromatography on dye-linked cellulose. *Biotech. Appl. Biochem.* 32: 81–87.

Rozanov, A. S., Zagrebelny, S.N, and Beklemishev, a.B., 2007. Cloning of *Escherichia coli* K12 xylose isomerase (glucose isomerase) gene and Studying the Enzymatic Properties of Its expression Product. *App. Bioch. Micro.*, 45(1): 31–37.

Roukas, T., 1996. Ethanol production from non-sterilized beet molasses by free and immobilized *Saccharomyces cerevisiae* cells using fed-batch culture. *Jour. of Food Eng.*, 27: 87-96.

Rusmini F., Zhong Z., Feijen J., 2007. Protein Immobilization strategies for protein biochips. *Biomacromolecules.* 8:1775-1789.

Ryan, W., Parulekar, S.J., Stark, B.C., 1989. Expression of β -lactamase by recombinant *Escherichia coli* strains containing plasmids at different sizes - effects of pH, phosphate, and dissolved oxygen. *Biotech. and Bioeng.*, 34: 309-319.

Saiki, R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., G.T., Mullis, K.B., Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239: 481-491.

Sambrook, J., Russell, D.W. *Molecular Cloning, A laboratory manual* Vol. 1, 2, 3, 3rd Ed., Cold Spring Harbor, USA. 2001.

Sassenfeld, H.M. and Brewer, S.J., 1984. A Polypeptide fusion designed for the purification of recombinant proteins. *Nat. Biotech.*, 2:76 - 81 (1984)

Sarıyar, B., Özkan, P., Kırdar, B., Hortaçsu, A., 2004. Expression and translocation of glucose isomerase as a fusion protein in *E. coli*. *Enz. Micro. Tec.*, 35:105–112.

Sarramegna, V., Demange, P., Milon, A., Talmont, F., 2002. Optimizing functional versus total expression of the human m-opioid receptor in *Pichia pastoris*. *Protein Expression and Purification*, 24: 212-220.

Schmidt, M., Tuominen, N., Johansson, T., Weiss, S.A., Keinamen, K., Oker-Blom, C., 1998. Baculovirus-mediated large-scale expression and purification of a poly Histidine- tagged Rubrella virus capsid protein. *Prot. Expr. Purif.*, 12: 323–330.

Scientific Publications - Work Done by Microbiology Reader Bioscreen C., 2005. www.bionewsonline.com/3/what_is_growth_medium.htm. Last accessed: March 2011.

Schuck, P., 1997. Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules. *Ann. Rev. of biophy. and biomol. Struc.*, 26: 541-566.

Scragg, A. H., 1988. *Biotechnology for Engineers: Biological Systems in Technolog. Proces.ses*, Ellis Horwood Ltd., England.

Shen, S., Sulter, G., Jeffries, T.W., Cregg, J.M., 1998. A strong nitrogen sourceregulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. *Gene*, 216: 93-102.

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S. M., Hegedus, D., 2003. Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. *Pro. Expres. and Purif.*, 28: 321-330.

Shiloach, J., Kaufman, J., Guillard, A.S., Fass, R., 1996. Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by *Escherichia coli* BL21 (λ DE3) and *Escherichia coli* JM109. *Biotech. and Bioeng.*, 49: 421-428.

Shiloach, J., Fass, R., 2005. Growing *E. coli* to high cell density - A historical perspective on method development. *Biotechnology Advances*, 23: 345-357.

Shin, M., and Y. H. Kho., 1985. Expression of glucose isomerase gene from *Bacillus licheniformis* in *Escherichia coli*. *Korean J. Microbiol.*, 23:138–146.

Shuler, M. L., Kargi, F., 2002. *Bioprocess Engineering: Basic Concepts*, 2nd Ed., Prentice Hall Inc., USA.

Sikander, A., Ikram, U-H., Qadeer, M.A., Javed, I., 2002. Production of citric acid by *Aspergillus niger* using cane molasses in a stirred fermentor. *Techn. Note.*, 5: 258-272.

Singh, R., Ghosh, S.K., 2004. Industrial biotechnology, 1st Ed., Global vision publishing house, Dehli-93, India.

Smith, A., 1995. Gene Expression in Recombinant Microorganisms, 1st Ed., Marcel Dekker Inc., USA.

Smith, G.P., 1991. Surface presentation of protein epitopes using bacteriophage expression systems. Curr. Opin. Biotechnol., 2:668-673.

Smith, G.P. and Scott, J.K., 1993. Libraries of peptides and proteins displayed on filamentous phage. Meth. Enzymol., 217:228-257.

Soetart, W., and Vandamme, J., 2010. Industrial biotechnology sustainable growth and economic sucess. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Soykut, E.A., Dudak, F.C., Boyaci, I.H., 2008. Selection of *staphylococcal enterotoxin B* (SEB) binding peptide using phage display technology. Biochem. and Biophy. Resear. Comm., 370:104-108.

Sreekrishna, K., Brankamp, R.G., Kroop, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A., Birkenberger, L.A., 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene, 190: 55-62.

Stamatakis, M, and Mantzaris, N.V., 2009. Comparison of deterministic and stochastic models of the lac operon genetic network. Biophy. Jour., 96: 887-906.

Stadlmayra, G., Mecklenbräukera, A., Rothmüllera, M., Maurera, M., Sauera, M., Mattanovicha, D., Gassera, B., 2010. Jour. of Biotech., 150: 519-529.

Stephanopoulos, G. N., Aristidou, A. A., Nielsen, J., 1998. Metabolic Engineering: Principles and Methodologies, Academic Press, UK.

Sterner, R., Liebl W., 2001. Thermophilic adaptation of proteins. Critical Reviews in Biochemistry and Molecular Biology, 36(1):39-106.

Studier, F.W., Moffatt, B.A., 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Jour. of Mol. Bio., 18:113-30.

Subramanian, A., Irudayaraj, J., Ryan, T., 2006. A mixed self-assembled monolayer based surface plasmon immunosensor for detection of *E.coli* O157:H7, Biosen. and Bioelec., 21: 998-1006.

Szardenings, M., 2003. Phage display of random peptide libraries: Applications, limits, and potential. J. Rec. Sign. Transd. Res., 23:307-349.

Tan, H.R., He, S., Zhuang, Z.H., Xue, Y.G., Zhang, G.J., 1990. Cloning and expression of *E. coli* glucose isomerase gene in *Streptomyces lividans*. Yi Chuan Bao, 17: 390-397.

Taşpınar, H., 2010. Exponential feeding strategy development for Benzaldehyde lyase production by recombinant *Escherichia coli*. M.Sc Thesis, Middle East Technical University, Turkey.

Terpe, K., 2002. Overview of tag fusions: from molecular and biochemical fundamentals to commercial systems. Appl. Microbiol. Biotechnol., 60:523-533.

Thorpe, E.D., d'Anjou, M.C., Daugulis, A.J., 1999. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnol. Lett. 21, 669–672.

Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A., Gingeras, T.R., 1987. Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. Nucl. Acids Res., 15: 3859-3876.

Ueda, E.K.M., Gout, P.W., Morganti, L., 2003. Current and Prospective applications of metal ion–protein binding. Jour. of Chromatog. A. 988: 1–23.

Ulezlo, I.V., Ananychev, A.V., Bezborodov, A.M., 1986. Glucose isomerase (xylose ketol isomerase). Uspekhi Biologicheskoy Khimii (Moscow), 27:136-163.

Van de Walle, M., Shiloach, J., 1998. Proposed mechanism of acetate accumulation in two recombinant *Escherichia coli* strains during high density fermentation. Biotech. and Bioeng., 57: 71-78.

Van der Merwe P.A., Ed by Harding, S., and Chowdhry P .Z., 2001. Surface Plasmon Resonance in Protein-Ligand interactions: hydrodynamics and calorimetry. Practical Approach series, Oxford University.

Van der Werve, P.A., Barclay, A.N. 1996. Analysis of cell-adhesion molecule interactions using surface plasmon resonance. *Cur. Opin. in Imm.* 8: 257-261.

Xie, J., Zhou, Q., Du, P., Gan, R. and Ye, Q., 2005. Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiostatin production. *Enz. and Micro. Techn.*, 36: 210-216.

Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V., Cregg, J.M., 1997. Isolation of the *Pichia pastoris* glyceraldehydes-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene*, 186:37-44.

Watson, J.D., Hopkins N.H., Roberts, J.W., Steitz, J.A., Weiner, A.M., 1987. *Molecular biology of the gene*, 4th Ed. The Benjamin/Cummings Publishing Company, California.

Weigand, W.A., Lim H.C., Creagan, C.C., 1979. Optimization of a repeated fed-batch reactor for maximum cell productivity. *Biotec. and Bioeng. Symp.*, 9: 335-348.

Wobbe, L., Zimmermann, D., Wibrock, M., Urman, S., Sewald, K., Malesevic Sewald, N., 2006. Intergrin $\alpha 5 \beta 1$: a new purification strategy based on immobilized peptides. *Jour. of Pept. Res.*, 66:22-29.

Wong, H.H., Kim, Y.C., Lee, S.Y., Chang, H.N., 1998. Effect of post-induction nutrient feeding strategies on the production of bioadhesive protein in *Escherichia coli*. *Biotech. and Bioeng.*, 60: 271-276.

World of Teaching, (2010). <http://www.worldofteaching.com>. Last accessed: December 2010.

Wuxiang, L., and K. Jeyaseelan., 1993. High level expression of a thermostable *Bacillus xylose* (glucose) isomerase in *Escherichia coli*. *Biotechnol. Lett.*, 15:1101–1106.

Yamamoto, M., 2008. Surface plasmon resonance (SPR) theory: tutorial. Department of Energy and Hydrocarbon Chemistry, Kyoto University, Kyoto-Daigaku-Katsura, Nishikyo-ku, 615-8510, Japan: 1-33.

Yang W., Wu H., Barbas C.F., 1995. Surface plasmon resonance based kinetic studies of zinc finger-DNA interactions. *Jour. of Immu. Met.*, 183: 175-82.

Yang, J., Zhou, X.S., Zhang, Y.X., 2004. Improvement of recombinant hirudin production by controlling NH^{+4} concentration in *Pichia Pastoris* fermentation. Biotech. Lett., 26: 1013-1017.

Ye, Q., Li, X., Yan, M., Cao, H., Xu, L., Zhang, Y., Chen, Y., Xiong, J., Ouyang, P., Ying, H., 2010. High-level production of heterologous proteins using untreated cane molasses and corn steep liquor in *Escherichia coli* medium. Appl. Micro. and Biotech., 87: 517-525.

Yee, L., Blanch, H.W., 1993. Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*. Biotec. and Bioeng., 41: 781-790.

Zhang, W., Bevins, M.A., Plantz, B.A., Smith, L.A., 2000a. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of Botulinum Neurotoxin, Serotype A. Biotech. and Bioeng., 70 : 1-8.

APPENDIX A

CHEMICALS AND SUPPLIERS

Table A.1 List of chemicals used in this study and their suppliers.

Chemical	Supplier
ABST	Sigma
Acetonitrile, HPLC grade	Sigma
Agarose (electrophoresis grade)	Sigma
Ampicillin	Sigma
Antifoam Y-30 emulsion	Sigma
Arabinose	Sigma
Di-Ammonium hydrogen phosphate	Merck
Ammonium sulphate	Merck
Antibody, M13, fd, F1 filamentous phages-HRP	Fitzgerald
Bacto agar	Difco
Bacto peptone	Difco
D-Biotin	Sigma
Bovine Serum albumin	Sigma
Boric acid	Acros Organics
Calcium chloride 2-hydrate	BDH
Boric acid	Acros Organics

Table A.1 List of chemicals used in this study and their suppliers (continued).

Chemical	Supplier
Calcium chloride 2-hydrate	BDH
Carbazol	Sigma
Choloramphenicol	Sigma
Chloroform	Lab-Scan
Cobalt Chloride	Sigma
D-glucose monohydrate	Merck
D-Fructose	Sigma
DNA ladder – Lambda DNA HindIII	Fermentas
dNTPs	Fermentas
Ethanol	Riedel-deHaën
Ethanolamine	Sigma
Ethidium bromide	Sigma
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	Sigma
Calcium chloride 2-hydrate	BDH
Carbazol	Sigma
Choloramphenicol	Sigma
Chloroform	Lab-Scan
Cobalt Chloride	Sigma
D-glucose monohydrate	Merck
D-Fructose	Sigma
DNA ladder – Lambda DNA HindIII	Fermentas
dNTPs	Fermentas

Table A.1 List of chemicals used in this study and their suppliers (continued).

Chemical	Supplier
Ethanol	Riedel-deHaën
Ethanolamine	Sigma
Ethidium bromide	Sigma
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	Sigma
Glass beads	Sigma
Glycerol	Merck
Glycine	Sigma
Glucose isomerase	Genecor
6×Histidine peptide	Genescript
Hydrochloric acid	Merck
Hydrogen peroxide	Sigma
N-hydroxysuccinimide	Sigma
Isoamyl alcohol	Sigma
Isopropyl β -D-1 thiogalactopyranoside (IPTG)	Sigma
Lactose	Difco
L-cystein hydrochloride	Sigma
Magnesium chloride 6-hydrate	Merck
Magnesium sulfate 7-hydrate	Merck
Manganese chloride	Sigma
Manganese sulfate anhydrous	Sigma
Mercaptoundecanoic acid	Aldrich
Methanol	BDH
Microbank	Pro-Lab

Table A.1 List of chemicals used in this study and their suppliers (continued).

Chemical	Supplier
Molasses	Ankara sugar plant
Peptide 3 15	Genescript
Phenol: Chloroform: Isoamyl alcohol 25:24:1	Fluka
Ph.D. 12TM phage display peptide library	Sacem
Polyethylene glycol, 3350	Sigma
Polyethylene glycol, 8000	Sigma-Aldrich
Potassium chloride	Merck
Potassium dihydrogen orthophosphate	Merck
di-Potassium hydrogen orthophosphate	Merck
Potassium hydroxide	Merck
Primers	Fermentas
Proteinase K	Fermentas
RNaseA	Fermentas
Restriction enzymes	Fermentas
Sodium acetate	Sigma
Sodium chloride	Riedel-deHaën
Sodium citrate	Merck
Sodium dihydrogen orthophosphate, anhydrous	Merck
Sodium dodecyl sulphate (SDS)	Sigma
di-Sodium hydrogen orthophosphate, anhydrous	Merck
Sodium hydroxide	Merck
Sulphuric acid	Merck
Soytryptone	Merck
T4 DNA polymerase	Fermentas

Table A.1 List of chemicals used in this study and their suppliers (*continued*).

Chemical	Supplier
Trizma Base	Sigma
Tryptone	Merck
Tween 20	Sigma
X-gal	Fermentas
Yeast extract	Fluka

APPENDIX B

LABORATORY EQUIPMENT AND SUPPLIERS

Table B.1 List of laboratory equipments and their suppliers.

Equipment	Supplier
Autoclave	HVE-50, Hirayama (Japan)
Balances	Sartorius (Germany)
Centrifuges	5415 R, Eppendorf (Germany) Z323K, Hermle (Germany)
Deep freezers	Legaci, Revco (USA)
Gel documentation system	UVP Bioluminescence imaging system (USA)
Horizontal gel-electrophoresis	BioRad (USA)
Hybridization oven	HB-1D, Techne (UK)
HPLC	Waters (USA)
Incubators	GenLab (UK) Nüve (Turkey)
Laminar flow cabinet	Nuaire (USA)
Micropipets	Gilson (France)
Microplate reader	Bio-TEK EL 808 (USA)
Orbital shakers	C25, C25KC, New Brunswick Scientific (USA) Certomat BS-T, BS-I, B.Braun (Germany)
PCR machine	GeneAmp-9700, Applied Biosystems (USA)

Table B.1 List of laboratory equipments and their suppliers (continued).

Equipment	Supplier
pH meter	Sartorius (Germany)
Refrigerators	Thermo Electron-Revco (USA)
Spectrophotometer	Helios alpha, ThermoSpectronics (UK) Ce2021, Cecil (UK)
SPR	Spreeta sensor (USA)
Ultrafiltration cell	Amicon, Millipore (USA)
Vacuum evaporator	SPD111V, Savant (USA)
Vertical gel-electrophoresis	BioRad (USA)
Vortex	Labnet (USA)
Water baths	GFLTM Thermolab (Germany)
Water purification system	Millipore (USA)

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

Blocking buffer	0.1 M NaHCO_3 (pH 8.6), 5mg/ml BSA. Filter sterilized and stored at 4°C.
0.12%(w/v) carbazol	0.12 g carbazol was dissolved in 100ml dH_2O and used freshly.
1.5%(w/v) Cystein HCl	0.75 g Cystein was dissolved in 50ml dH_2O and used freshly.
6 x DNA gel-loading buffer	0.25 % Bromophenol blue, 0.25 % xylene cyanol FF, 40 % sucrose in dH_2O . Stored at room temperature.
0.5 M EDTA, pH 8.0	18.61 g Ethylenediaminetetra acetic acid disodium salt dihydrate was dissolved in 80 ml dH_2O . NaOH was added until EDTA was dissolved. The final pH was further adjusted to pH 8.0 and the final volume was adjusted to 100 ml. The buffer was autoclaved and stored at room temperature.
0.2 M Glycine-HCl buffer	0.2 M Glycine-HCl (pH 2.2), 1mg/ml BSA. Filter sterilized and stored at 4°C.
GI Activity Buffer	0.4M D-glucose and 10mM MnCl_2 in 0.02M potassium buffer pH 7.0.

PTG/Xgal Stock solution	1.25 g IPTG and 1g Xgal were mixed in 25ml DMF. Solution was stored at -20°C.
PEG/NaCl Solution	20% (w/v) polyethylene glycol-8000, 2.5M NaCl. Autoclaved, mixed well to combine separated layers while still warm. Stored at room temperature.
0.1 M potassium phosphate, pH 7.0	3.85 ml of 1M KH_2PO_4 , 6.15 ml of 1M K_2HPO_4 was dissolved in dH_2O and the volume made up to 100 ml. The solution pH was controlled, autoclaved and stored at room temperature.
5 x TBE	54 g Tris base and 27.5 g of boric acid were dissolved in 20 ml of 0.5 M EDTA, pH8.0, made up to 1 liter with sterile dH_2O and stored at room temperature. Before use, the stock solution was diluted 1:5 with dH_2O .
1 M Tris-Cl, pH 8.0	12.1 g Tris base was dissolved in 80 ml dH_2O and the pH was adjusted to 8.0 by adding concentrated HCl. The buffer volume was arranged to 100 ml, autoclaved and stored at room temperature.
RNaseA stock solution	RNaseA was dissolved at a concentration of 10 mg/ml in 50 mM potassium acetate (pH 5.5) and boiled for 10 min. Stored at -20 ° C.
3 M Sodium acetate, pH 5.2	24.6 g sodium acetate was dissolved in 80 ml dH_2O and the pH was adjusted to 5.2 with 3M acetic acid. The buffer was filter sterilized and stored at 2-8°C.
Single-stranded carrier DNA	500 mg of high MW DNA (Sigma) was added to 100 mL TE buffer pH 8.0 and stored at -20°C. Before use, the DNA solution was boiled for 15 min and kept on ice.

70% (v/v) Sulfuric acid	70 ml sulfuric acid was gradually added to 30 ml dH ₂ O.
TE Buffer, pH 8.0	1 ml of 1M Tris-Cl (pH 8.0), 200 µl of 0.5 M EDTA (pH 8.0) was added to 80 ml dH ₂ O and the volume was made up to 100 ml. The buffer was autoclaved and stored at room temperature.
TBS buffer	50 mM Tris-HCl (pH 7.5),150 mM NaCl. Autoclaved and stored at room temperature.
TBS-T solution	0.1% Tween-20 was added into 1xTBS solution. Prepared on the day of use.
1 M Tris-HCl, pH 9.1	12.1g Tris base was dissolved in 80 ml dH ₂ O and pH was adjusted to 9.1 with 6N HCl. The buffer was made up to 100ml with dH ₂ O. The buffer was autoclaved and stored at 2-8°C.
Yeast Lysis Solution	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl-pH 8.0, 1mM Na ₂ EDTA. The solution was autoclaved and stored at room temperature.

APPENDIX D

DNA SEQUENCES AND PLASMIDS

Sequence of pPICZaA (3593 bp)

agatctaacatccaaagacgaaagggtgaatgaaacctttttgccatccgacatccacaggtccattc
tcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacct
ccactcctcttctcctcaacacccactttttgccatcgaaaaaccagcccagttattgggcttgattgg
agctcgctcattccaattccttctattaggtactaacacccatgactttattagcctgtctatcctgg
ccccctggcgagggttcattggtttgtttatttccgaatgcaacaagctccgcattacacccgaacatca
ctccagatgagggctttctgagtgtgggtcaaatagtttcatgttccccaatggcccaaaactgac
agtttaaacgctgtcttggaaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttg
ttcgttgaaatgctaacggccagttgggtcaaaaagaaacttccaaaagtcggcataccgtttgccttg
tttggatttgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtcctctctatcgcttc
tgaaccccggtgcacctgtgcgaaacgcaaatggggaaacacccgctttttggatgattatgcattg
tctccacattgtatgcttccaagattctgggtgggaatactgctgatagcctaactgcatgatcaaaa
tttaactgttctaaccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctt
tttttttatcatcattattagcttactttcataattgcgactgggtccaattgacaagctttttgattt
taacgacttttaacgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgagatttcct
tcaatttttactgctgttttattcgcagcatcctccgcattagctgctccagtcacactacaacaga
agatgaaacggcacaaattccggctgaagctgtcatcggttactcagatttagaaggggatttcgatg
ttgctgttttgccattttccaacagcacaaataacgggttattgtttataaataactactattgccagc
attgctgctaagaagaaggggtatctctcgagaaaagagagggctgaagctgaattcacgtggcccag
ccggccgtctcggtacgtcgagccgcggcgccagctttctagaacaaaaactcatctca
gaagaggatctgaatagcgccgtcgaccatcatcatcatcattgagttttagtccttagacatga
ctgttcctcagttcaagttgggcacttacgagaagaccggctcttgctagattctaataagaggatgt
cagaatgccatttgccctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagt
ataggattttttttgtcattttgtttcttctcgtagcagcttgctcctgatcagcctatctcgcagct
gatgaatatcttgtggttaggggtttgggaaaatcattcgagtttgatgtttttcttggattttccac
tctcttcagagtacagaagattaagtgcagaccttcgtttgtgaggatccccacacaccatagcttc
aaaatgtttctactccttttttactcttccagattttctcgactccgcgcacatcgccgtaccacttca
aaacaccaagcacagcataactaaattttccctctttctcctctaggggtgctgtaattaccggtac
taaagggtttggaaaagaaaaagagaccgcctcgtttctttttcttcgtcgaaaaaggcaataaaaaat
ttttatcacgtttctttttcttgaaatttttttttttagttttttctctttcagtgacctccattga
tatttaagtttaataaaacggtcttcaattttctcaagtttcagtttcatttttcttgttctattacaact
ttttttacttcttgttccattagaaagaaagcatagcaatctaataaggggcggtgttgacaattaa
tcatcggcatagtatatcggcatagtataatacgacaaggtgaggaactaaaccatggccaagttgac
cagtgccgttccgggtgctcaccgcgcgcgacgtcgccggagcggtcgagttctggaccgaccggctcg
ggttctcccggaacttcgtggaggacgacttcgcgggtgtggtccgggacgacgtgacctgttcatc
agcgcggtccaggaccaggtggtgcccggacaacaccctggcctgggtgtgggtgcgcggcctggacga
gctgtacgcagtggtcgagggtcgtgtccacgaacttcggggacgcctccggggccggccatgaccg
agatcgggcagcagccgtggggggcgagggttcgccctgcgcgacccggccggcaactgcgtgcacttc
gtggccgaggagcaggactgacacgtccgacggcgcccccacgggtcccaggcctcggagatccgtccc
ccttttcttttgcgatcatgtaattagttatgtcacgcttacattcacgccctccccccacatcc
gctctaaccgaaaaggaaggagtttagacaacctgaagtctaggtccctatttttttttatagttat

gttagtattaagaacggttatttatatttcaaatttttctttttttctgtacagacgcgtgtacgcat
gtaacattatactgaaaaccttgcttgagaagggttttgggacgctcgaaggctttaatttgcaagctg
gagaccaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcggtt
tttccataggctccgccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacc
cgacaggactataaagataaccaggcggtttccccctggaagctccctcggtgcgctctcctgttccgacc
ctgcegttaccgataacctgtccgcctttctcccttcgggaagcggtggcgctttctcaatgtcacg
ctgtagggtatctcagttcgggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttc
agcccgaaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcg
ccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttctt
gaagtgggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagccag
ttaccttcggaaaaagagttggtagctcttgatccggcaaaacaaaccaccgctggtagcggtggtttt
tttgtttgaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctac
ggggtctgacgctcagtggaacgaaaactcacgttaagggattttgggtcatgagatc

Multiple Cloning Site of pPICZaA

```

      5' end of AOX1 mRNA
      |
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA
      |
      5' AOX1 priming site
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
      |
1187 GAG AAA AGA GAG GCT GAA GCT 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 CTGTTCTCA GTTCAAGTTG GGCACCTACG AGAAGACCGG TCTTGCTAGA
      |
      3' AOX1 priming site
1411 TTCTAATCAA GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTGTAT
      |
      3' polyadenylation site
1471 ACTTTTTTAT TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT

```

Sequence of pPICZaA::xyl_{pent} plasmid

agatctaacatccaaagacgaaagggttgaaacacccccccatccgacatccacaggtccattc
tcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacct
ccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattgg
agctcgctcattccaattccttctattaggtactaacaccatgactttattagcctgtctatcctgg
ccccctggcgagggttcattgtttgtttatttccgaatgcaacaagctccgcattacacccgaacatca
ctccagatgagggctttctgagtgtgggtcaaatagtttcatgttccccaaatggcccaaaactgac
agtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttg
ttcgttgaaatgctaacggccagttgggtcaaaaagaaacttccaaaagtcggcataaccgtttgtcttg
tttggattgattgacgaatgctcaaaaataatctcattaatgcttagcgcagttctctatcgcttc
tgaaccccggtgcacctgtgcccgaacgcaaaggggaaacacccgctttttggatgattatgcattg
tctccacattgtatgcttccaagattctgggtgggaatactgctgatagcctaacgttcatgatcaaaa
tttaactgttctaaccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacttt
tttttttatcatcattattagcttactttcataattgcgactgggtccaattgacaagcttttgattt
taacgacttttaacgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgagatttctt
tcaatttttactgctgtttttattcgcagcatcctccgcattagctgctccagtcaacactacaacaga
agatgaaacggcacaattccggctgaagctgtcatcggttactcagatttagaaggggatttcgatg
ttgctgttttgccattttccaacagcacaataacgggttattgtttataaataactactattgccagc
attgctgctaagaagaaggggtatctctcgagaaaagagagggctgaagctgaattc**caccatcacca**
tcaccatattgaagggagaatgaaagttggagtttagctcgctctattcgataacgctatgcttcgtgct
catcgcttcatcagctggcctttgacacctaccaggagtttgaagaagcctatatcccaaagggaaca
gaagtattcattcctgcagaacccccagacctccctctgtttctcagagtctattccgacacctcca
acagggaggaaacacacagaaatccaacctagagctgctccgcattctccctgctgctcatccagtcg
tggttgagagcccgctgcagttcctcaggagtgctctcgccaacagcctagtgtacggcgctctgacag
caacgtctatgacctcctaaaggacctagaggaaggcatccaaacgctgatggggaggctggaagatg
gcagcccccgactgggcagatcttcaagcagacctacagcaagttcgacacaaaactcacacaacgat
gacgcactactcaagaactacgggctgctctactgcttcaggaaggacatggacaaggtcgagacatt
cctgcgcacgtgcagtgccgctctgtggaggggcagctgtggcttctagtctagaacaaaaactcatc
tcagaagaggatctgaatagcgccgctcgacctcatcatcatcattgagtttgtagccttagaca
tgactgttccctcagttcaagttgggcacttacgagaagaccggtcttgctagattctaataagagga
tgtcagaatgccatttgctgagagatgcaggcttcatttttgatacttttttatttgtaacctatat
agtataggattttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgca
gctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcc
cactcctcttcagagtacagaagattaagtgcagaccttcgtttgtgaggatccccacacaccatagc
ttcaaaatgtttctactccttttttactcttccagattttctcggaactccgcgcacatcgccgtaccact
tcaaaacacccaagcacagcataactaaattttccctctttcttctctaggggtgtcgtaattaccg
tactaaagggtttggaaaagaaaaagagaccgctcgtttctttttcttcgtcgaaaaaggcaataaa
aatttttatcacgtttctttttcttgaaattttttttttagttttttctctttcagtgacctccat
tgataatttaagttaataaacgggtcttcaatttctcaagtttcagtttcatttttcttggttctattaca
actttttttacttcttggttcattagaaagaaagcatagcaatctaataagggggcgggtgttgacaat
taatcatcggcatagtatatcggcatagtataatacagacaagggtgaggaactaaaccatggccaagtt
gaccagtgccgttccgggtgctcaccgcgcgcagctcgccggagcggctcgagttctggaccgaccggc
tcgggttctcccggaacttcgtggaggacgacttcgccgggtgtgggtccgggacgacgtgacctgttc
atcagcgcggtccaggaccaggtggtgccggacaacaccctggcctgggtgtgggtgcgcggcctgga

cgagctgtacgccgagtggtcggaggctcgtgtccacgaacttccgggacgcctccgggcccggccatga
 ccgagatcggcgagcagccgtggggggcgggagttcgcctcgcgcgacccggccggcaactgcgtgcac
 ttcgtggccgaggagcaggactgacacgtccgacggcgccacgggtcccaggcctcggagatccgt
 cccctttttcctttgtcgatatcatgtaattagttatgtcacgccttacattcacgccctccccccaca
 tccgctctaaccgaaaaggaaggagttagacaacctgaagtctaggtccctatttatTTTTTTtagt
 tatgttagtattaagaacgttatTTTatatTTTcaaTTTTTctTTTTTTctgtacagacgcgtgtacg
 catgtaacattatactgaaaaccttgcttgagaaggTTTTTgggacgctcgaaggctttaatttgcaag
 ctggagaccaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggc
 gTTTTccataggctccgccccctgacgagcatcacaaaaatcgacgctcaagtcagagggtggcgaa
 acccgacaggactataaagataaccaggcgTTTTCCCCtggaagctccctcgtgcgctctcctggtccg
 accctgccgcttacgggataacctgtccgcctTTTctcccttcgggaagcggtggcgctTTTctcaatgctc
 acgctgtaggtatctcagttcgggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacCCCCcg
 ttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgactta
 tcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcgggtgtacagagtt
 cttgaagtgggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagc
 cagttaccttcggaaaaagagttggtagctcttgatccggcaaaacaaaccaccgctggtagcgggtggt
 TTTTTgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttc
 tacggggtctgacgctcagtggaacgaaaactcacgttaagggaTTTTTgggtcatgagatc

Note:

Underlined sequences are restricted enzyme recognition sites (*EcoRI* and *XbaI*). Highlighted sequences are complementary parts of primers on *xyI_p* gene. Sequence denoted by thick characters is His-tag and Factor Xa recognition site.

Sequence of pPICZaA::xyl_{pin} plasmid (4069 bp)

agatctaacatccaaagacgaaagggttgaaacacccccccatccgacatccacaggtccattc
tcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacct
ccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattgg
agctcgctcattccaattccttctattaggtactaacaccatgactttattagcctgtctatcctgg
ccccctggcgagggttcattgtttgtttatttccgaatgcaacaagctccgcattacacccgaacatca
ctccagatgagggccttctgagtgtgggtcaaatagtttcatgttccccaaatggcccaaaactgac
agtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttgg
ttcgttgaaatgctaacggccagttgggtcaaaaagaaacttccaaaagtcggcataaccgtttgtcttg
tttggatttgattgacgaatgctcaaaaataatctcattaatgcttagcgcagttctctctatcgcttc
tgaaccccggtgcacctgtgcccgaacgcaaattggggaaacacccgcttttttgatgattatgcattg
tctccacattgtatgcttccaagattctgggtgggaatactgctgatagcctaacgttcatgatcaaaa
tttaactgttctaaccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacttt
tttttttatcatcattattagcttactttcataattgcgactgggtccaattgacaagcttttgattt
taacgacttttaacgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgaaagttgga
gttagcataatccccactttttaaatgaaagaaaaacccttgccatatttagcagatatcaaagt
aaaaaattatgatttcgtccagatatccccacgcttgggagttattaaaaacggaaccgtgatag
aagaaaagttgaaagaaatagagacaataactaaaggggtgttgaaatagattacatagttagaatgcc
gtttcgttgaaatttaagggacaacgtctactactcaagacattttaaagttgccaaggcggtccttgga
tggtgctattaagcttgagcaaaaaacaatagttatgcaaagtggaaaaactggaaggttagacttag
agattgactcaataaaagctttggctgatatagcagaaaactttggaataaatattgcacttgaaaac
acgttttagtgtaaggatactctctacgtaataagacaacgtgaataaggacaacgttggatttgctct
ggatgtagctcatgcattcttaagtgccaggggggatgaaaataggctcttagaagatgttagactag
gagtcgagaaaaacaataatccttttagtccacgataattttggaataatgttccccaggttagagcca
gaagatgccctagcttacgggggtgggagatctacaccttctcccaggggaaggaaagataccatttg
aaagataataagactcttcaaagatataccaattctcctaaggtaaaagacgttaagacttttgaaa
acctccccctcaaaatctgatctgcttcagagattgatgaggtgatttctagaacaaaaactcatctca
gaagaggatctgaatagcgccgtcgaccatcatcatcatcattgagtttgtagccttagacatga
ctgttcctcagttcaagttgggcacttacgagaagaccggctcttgctagattctaataagaggatgt
cagaatgccatttgctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagt
ataggattttttttgtcattttgtttcttctcgtagcgttgcctgatcagcctatctcgagct
gatgaatatcttggttaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttccac
tctcttcagagtacagaagattaagtgcagaccttcgtttgtgaggatccccacacaccatagcttc
aaaatgtttctactccttttttactcttcagattttctcggaactccgcgcacgcgtaccacttca
aaacaccaagcacagcatactaaattttccctcttctcctcctaggggtgcgttaattaccggtac
taaagggtttggaaaagaaaaagagaccgcctcggtttctttttcttcgtcgaaaaaggcaataaaat
ttttatcacgtttctttttcttgaaatttttttttagttttttctctttcagtgacctccattga
tatttaagtttaataaacggtcttcaatttctcaagtttcagtttcatttttcttggtctattacaact
tttttacttcttggtcatttagaaagaaagcatagcaatctaataagggcggtgttgacaattaa
tcatcggcatagtatatcggcatagtataatacgacaaggtgaggaactaaaccatggccaagttgac
cagtgccgttccgggtgctcaccgcgcgcgacgtcgccggagcggtcgagttctggaccgaccggctcg
ggttctccgggacttcgtggaggacgacttcgccgggtgtggtccgggacgacgtgacctgttcac
agcgcggtccaggaccaggtggtgccggacaacacctggcctgggtgtgggtgcgcggcctggacga
gctgtacgcgcgagtggtcgagggtcggtgtccacgaacttcggggacgcctccgggcccggccatgaccg

agatcggcgagcagccgtgggggagggttcgccctgcgcgacccggccggcaactgcgtgcacttc
 gtggccgaggagcaggactgacacgtccgacggcgcccccacgggtcccaggcctcggagatccgtccc
 ccttttcctttgtcgatatcatgtaattagttatgtcacgcttacattcacgccctccccccacatcc
 gctctaaccgaaaaggaaggagtttagacaacctgaagtctaggtccctatttatttttttatagttagt
 gttagtattaagaacggttatttatatttcaaatttttcttttttttctgtacagacgcgtgtacgcat
 gtaacattatactgaaaaccttgcttgagaagggttttgggacgctcgaaggctttaatttgcaagctg
 gagaccaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcggtt
 tttccatagggtccgccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacc
 cgacaggactataaagataaccaggcggtttccccctggaagctccctcgtgcgctctcctgttccgacc
 ctgccgcttaccggataacctgtccgcctttctcccttcgggaagcggtggcgctttctcaatgctcacg
 ctgtaggtatctcagttcgggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttc
 agcccgaccgctgcgccttatccggtaactatcgctcttgagtccaacccggtaagacacgacttatcg
 cactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttctt
 gaagtgggtggcctaactacggctacactagaaggacagtatgttggtatctgcgctctgctgaagccag
 ttaccttcggaaaaagagttggtagctcttgatccggcaacaaaccaccgctggtagcggtggtttt
 tttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatctttttctac
 ggggtctgacgctcagtggaacgaaaactcacgttaagggttttgggtcatgagatc

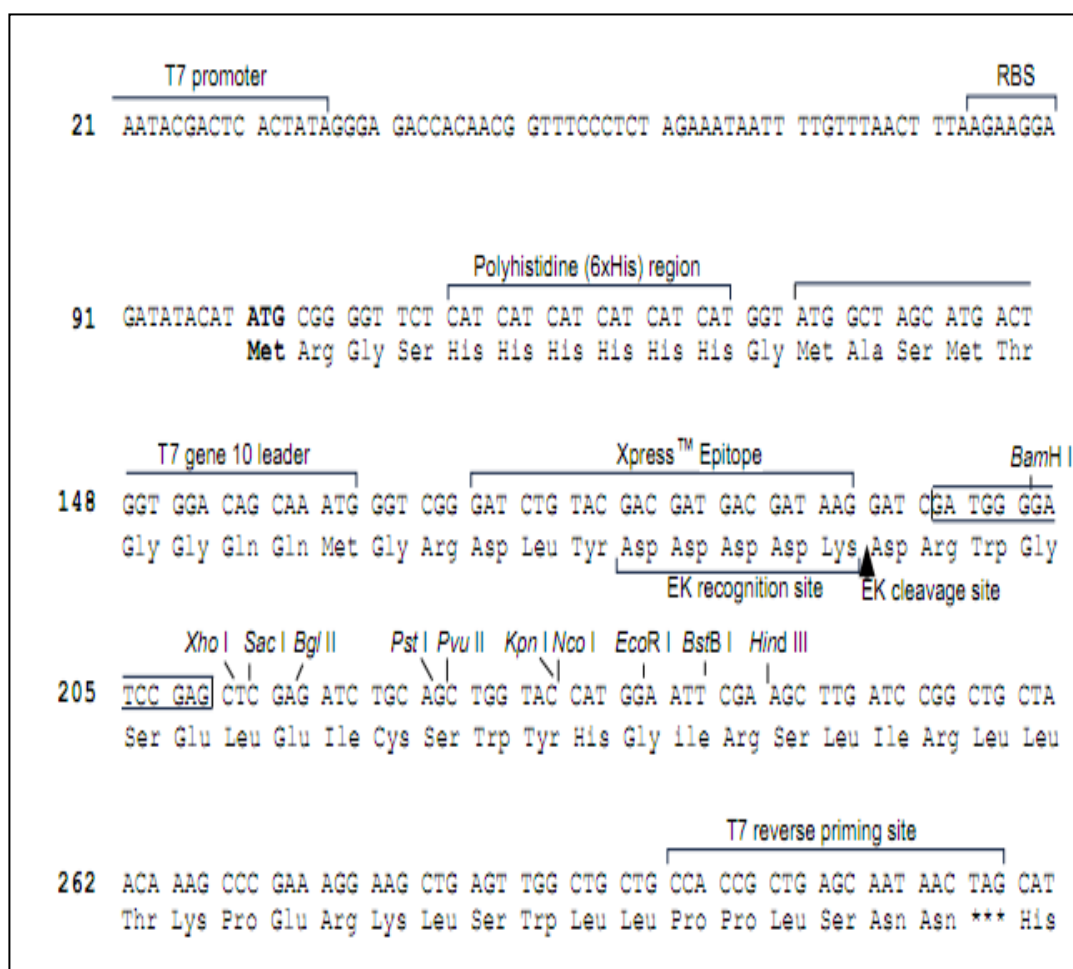
Note:

Underlined sequences are restricted enzyme recognition sites (*Asu*II and *Xba*I). Highlighted sequences are complementary parts of primers on *xyl_P* gene.

Sequence of pRSETA (2897bp)

gatctcgatcccgcgaaattaatacgactcactatagggagaccacaacggtttccctctagaaataa
ttttgtttaactttaagaaggagatatatacatatgcgggttctcatcatcatcatcatggtatgg
ctagcatgactggtggacagcaaattgggtcgggatctgtacgacgatgacgataaggatcgatgggga
tccgagctcgagatctgcagctggtaccatggaattcgaagcttgatccggctgctaacaaagcccga
aaggaagctgagttggctgctgccaccgctgagcaataactagcataaacccttggggcctctaaacg
ggtcttgaggggttttttgcgtgaaaggaggaactatatccggatctggcgtaataagcgaagaggcccg
caccgatcgcccttcccaacagttgcgagcctgaatggcgaatgggacgcgccttgtagcggcgcat
taagcgcggcggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccctagcgcggcgt
cctttcgctttcttcccttccctttctcgccacggttcgcccgttttccccgtcaagctctaaatcgggg
gctcccttttaggttccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggatgag
gttcacgtagtgggccatcgccctgatagacggtttttcgccctttgacggttgagtgccacggttctt
aatagtggactcttgttccaaactggaacaacactcaaccctatctcggctctattcttttgatttata
agggatttttgcgatttgcgctatttggttaaaaaatgagctgatttaacaaaaatttaacgcgaatt
ttaacaaaaatattaacgcttacaatttaggtggcacttttgcgggaaatgtgcgcggaaccctattt
gtttatttttctaatacattcaaatatgtatccgctcatgagacaataaaccctgataaatgcttcaa
taatatgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggc
attttgccttctgtttttgctcaccacagaaacgctggtgaaagtaaaagatgctgaagatcagttgg
gtgcacgagtggttacatcgaaactggatctcaacagcggttaagatccttgagagttttcgccccgaa
gaacgttttccaatgatgagcacttttaaaagtctgctatgtggcgcggtattatcccgatttgacgc
cgggcaagagcaactcggtcgcccgcatacactattctcagaatgacttggttgagtactcaccagtca
cagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgtgccataaccatgagtgat
aacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcacaa
catgggggatcatgtaactcgcttgatcgttgggaaccggagctgaatgaagccataccaaacgacg
agcgtgacaccacgatgcctgtagcaatggcaacaacggttgcgcaaacatttaactggcgaaactactt
actctagcttcccggaacaattaatagactggatggaggcggataaaagttgcaggagcacttctgcg
ctcgcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtta
tcattgcagcactggggccagatggttaagccctcccgatcgtagttatctacacgacggggagtcag
gcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggttaact
gtcagaccaagtttactcatatatacttttagattgatttaaaacttcatttttaatttaaaaggatct
aggtgaagatcctttttgataatctcatgacaaaaatcccttaacgtgagttttcgttccactgagcg
tcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgtaactctgctgctt
gcaacaaaaaaaccacgctaccagcggtggtttgtttgcccggatcaagagctaccaactctttttc
cgaaggtaactggcttcagcagagcgagataaccaataactgttcttctagtgtagccgtagttaggc
caccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatacctgttaccagtggctgc
tgccagtggcgataaagtctgtgtcttaccgggttggtgactcaagacgatagttaccggataaggcgacg
ggtcgggctgaacgggggttctgtgcacacagcccagcttgagcgaaacgacctacaccgaactgaga
tacctacagcgtgagctatgagaaagcgccacgcttcccgaaaggagaaaggcgacaggtatccggt
aagcggcagggctcggaacaggagagcgacgagggagcttccagggggaaacgccttggtatctttata
gtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcagggggcgagc
ctatggaaaaacgccagcaacgcggcctttttacgggttcttgcccttttgcgtggccttttgcacat
gttctttctgcgttatccctgattctgtggataaccgtattaccgcctttgagtgcgctgataccg
ctcgccgcagccgaacgacgagcgcgagcagtgatgagcgcggaagcgcgaagagcgcccaatacgc
aaaccgcctctccccgcgcgttggccgattcattaatgcag

Multiple Cloning Site of pRSETA



Sequence of pRSETA-*xyI*_T (3926 bp)

gatctcgatcccgcgaaattaatacgaactcactatagggagaccacaaacggtttccctctagaaataa
ttttgtttaactttaagaaggagatatatcatatgtacgagcccaaaccggagcacagggtttacctttg
gcctttggactgtgggcaatgtgggcccgtgatcccttcggggacgcggttcgggagaggctggacccg
gtttacgtgggttcataagctggcggagcttggggcctacggggtaaaccttcacgacgaggacctgat
cccgcgggggcagcgccctcctcaggagcgggaccagatcgtgagggcgcttcaagaaggctctcgtatgaaa
ccggcctcaagggtcccatgggtcaccgccaacctcttctccgaccctgctttcaaggacggggccttc
acgagcccgacccttgggttcgggcctatgccttgccgaagagcctggagaccatggacctgggggc
agagcttggggccgagatctacgtgggtctggccgggcccgggagggagctgaggtggaggccacgggca
aggcccggaaggctctgggactgggtgcccggaggcgctgaacttcatggccgcctacgccgaggaccag
ggatacgggtaccgggtttgccctcgagcccaagcctaacgagccccggggggacatttacttcgccac
cgtggggagcatgctgcctttattcataccctggaccggccccgagcgcttcggcctgaacccccgagt
tcgcccacgagaccatggccgggcttaactttgtccacgcccgtggcccaggctctcgacgcccgggaag
cttttccacattgacctcaacgaccaacggatgagccggtttgaccaggacctccgcttcggctcggga
gaacctcaaggcggcctttttcctgggtggacctcctggaaagctccggctaccaggggcccccgccact
ttgacgcccacgccttgctaccgaggacgaagaagggtttgggccttcgcccagggctgcatgcgt
acctacctgatcttaaaggaaagggtgaagccttccgcgaggatcccagaggtcaaggagcttcttgc
cgcttactatcaagaagatcctgcggccttggcccttttgggcccctactcccgcgagaaggccgaag
ccctcaagcggggcgagcttccctcagaggccaagcggcgccggggttatgccctggaacgcctggac
cagctggcggtggagtacctcctgggggtgcccggggtgagaattcgaagcttgatccggctgctaac
aaagcccgaagggaagctgagttggctgctgccaccgctgagcaataactagcataacccttggggc
ctctaaacgggtcttgaggggttttttgcgtgaaaggaggaactatatccggatctggcgtaatagcga
agagggccgcaccgatcgcccttcccaacagttgcgcagcctgaatggcgaatgggacgcgcctgtga
gcggcgcattaagcgcggcggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgcccta
gcgcccgcctcctttcgctttcttcccttcccttctcgccacgttcgcccggctttccccgtcaagctct
aaatcgggggctcccttttagggttccgatttagtgctttacggcacctcgacccccaaaaacttgatt
agggtgatgggtcacgtagtgggcatcgccctgatagacgggtttttcgccctttgacgttggagtcc
acgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcggtctattcttt
tgatttataagggtttttgcccatttcggcctattgggttaaaaaatgagctgatttaacaaaaattta
acggaatttttaacaaaaatattaacgcttacaatttaggtggcacttttcgggggaaatgtgcgcggaa
cccctattttgtttatttttctaaatacattcaaataatgtatccgctcatgagacaataaccctgataa
atgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattccctt
ttttgcggcattttgccttccgtttttgctcaccagaaacgctggtgaaagtaaaagatgctgaag
atcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagtttt
cgccccgaagaacgttttccaatgatgagcacttttaagttctgctatgtggcgcggtattatccccg
tattgacgcggggcaagagcaactcggtgcgcgcatacactattctcagaatgacttggttgagtact
caccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgtgcccataacc
atgagtataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttt
tttgacacatgggggatcatgtaactcgccctgatcggttgggaaccggagctgaatgaagccatac
caaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaacatttaactggc
gaactacttactctagcttcccggcaacaattaatagactggatggaggcggaataaagtgcaggacc
acttctgcgtcggcccttccggctggctgggtttattgctgataaatctggagccggtgagcgtgggt
ctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacg
gggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagca
ttggtaactgtcagaccaagtttactcatatatacttttagattgattttaaacttcatttttaattta
aaaggatctaggtgaagatcctttttgataatctcatgaccaaatacccttaacgtgagttttcggtc
cactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgtaat
ctgctgcttgcaaaacaaaaaaaccaccgctaccagcgggtgggtttgtttgccggatcaagagctacca
ctctttttccgaaggtaactggcttcagcagagcgcagataccaaataactgttcttctagtgtagccg

tagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatacctgttacc
 agtggctgctgccagtggcgataagtcgtgtcttaccgggttggaactcaagacgatagttaccggata
 aggcgcagcggctcgggctgaacggggggttcgtgcacacagcccagcttggagcgaacgacctacacc
 gaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaaggcggacag
 gtatccggttaagcggcagggctcggaacaggagagcgcacgagggagcttccaggggaaacgcctggt
 atctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgatgctcgtcaggg
 gggcggagcctatggaaaaacgccagcaacgcggcctttttacggttcttgcccttttgctggccttt
 tgctcacatgttctttcctgcttattccctgattctgtggataaccgtattaccgcctttgagtga
 ctgataccgctcgccgcagccgaacgaccgagcgcagcagtcagtgaagcgaagagcgc
 ccaatacgcgaacgcctctccccgcgcgttggccgattcattaatgcag

Note:

Underlined sequences are restricted enzyme recognition sites (*NdeI* and *EcoRI*). Highlighted sequences are complementary parts of primers on *xyl_T* gene.

APPENDIX E

CALIBRATION OF FRUCTOSE CONCENTRATION

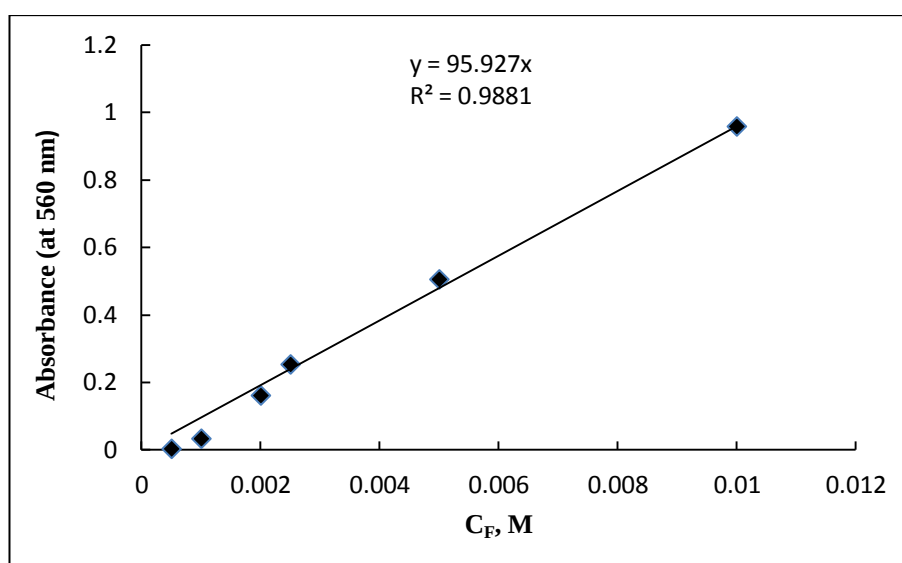


Figure E.1. Calibration curve for fructose concentration.

According to the equation obtained from the plot:

$$C_F = \frac{\text{Absorbance}}{95.92} \times \text{Dilution Ratio} \quad (\text{E.1})$$

APPENDIX F

CALIBRATION OF GLUCOSE CONCENTRATION

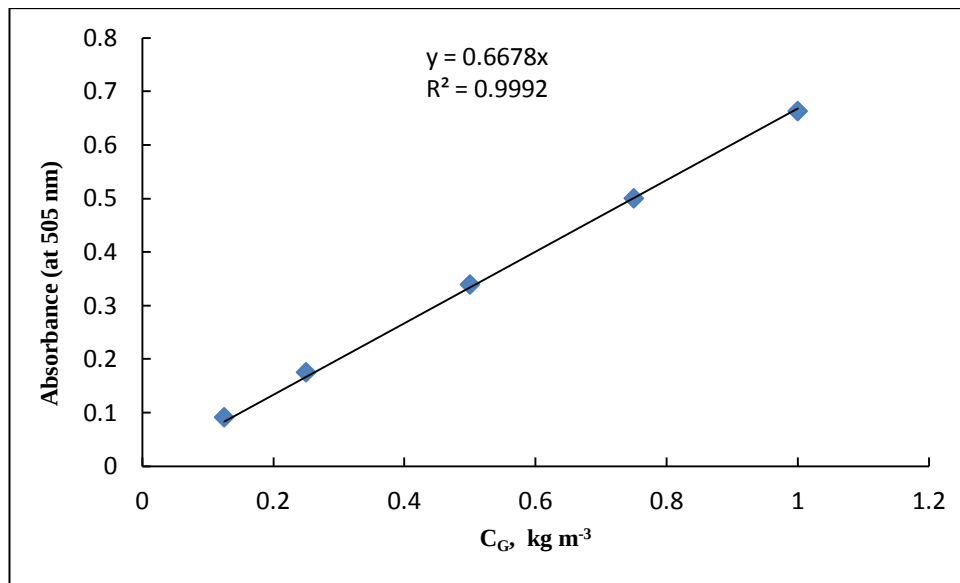


Figure F.1. Calibration curve for glucose concentration.

According to the equation obtained from the plot:

$$C_G = \frac{\text{Absorbance}}{0.6678} \times \text{Dilution Ratio} \quad (\text{F.1})$$

APPENDIX G

CALIBRATION OF ORGANIC ACID CONCENTRATIONS

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

Acetic Acid

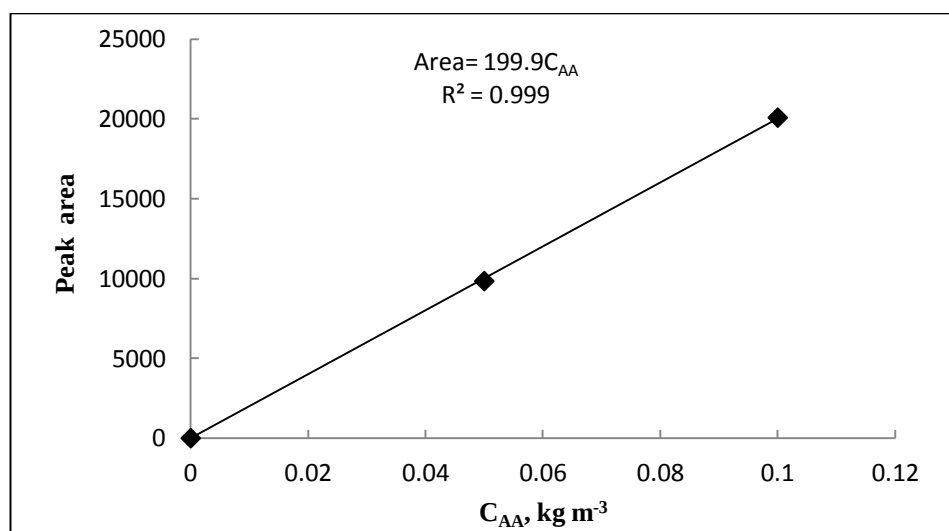


Figure G.1 Standard calibration curve for acetic acid

Citric Acid

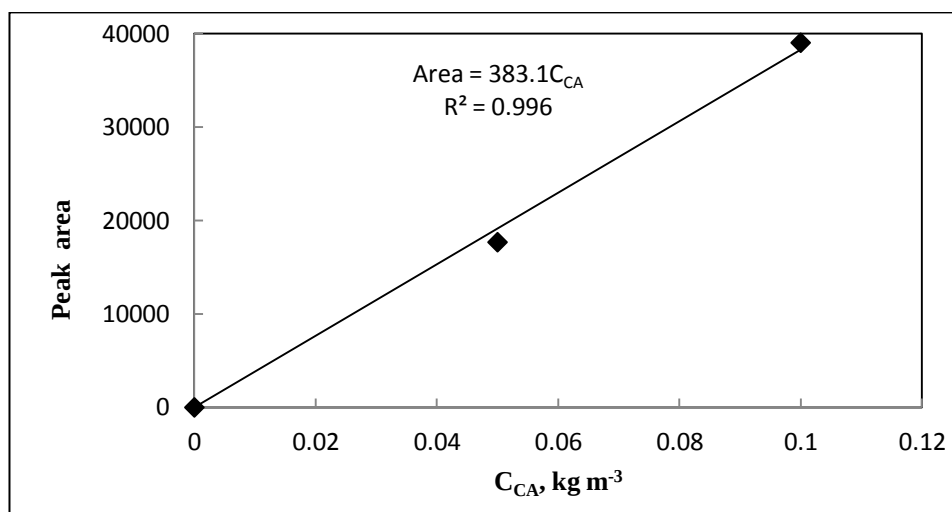


Figure G.2 Standard calibration curve for citric acid.

Formic Acid

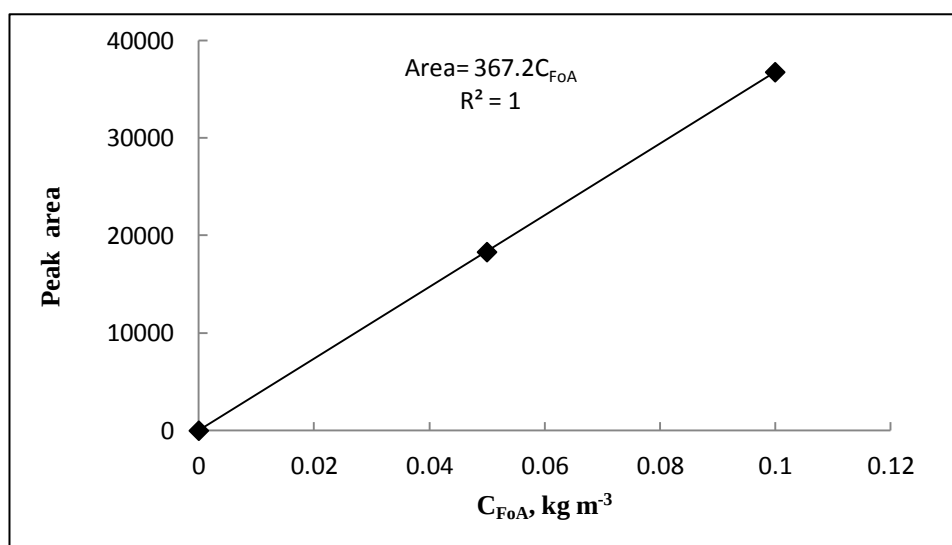


Figure G.3 Standard calibration curve for formic acid.

Fumaric Acid

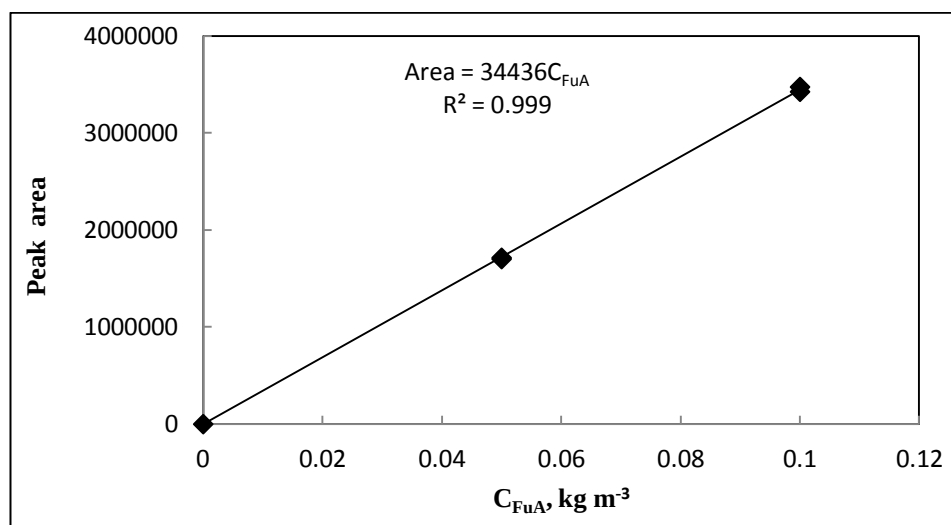


Figure G.4 Standard calibration curve for fumaric acid.

Malic Acid

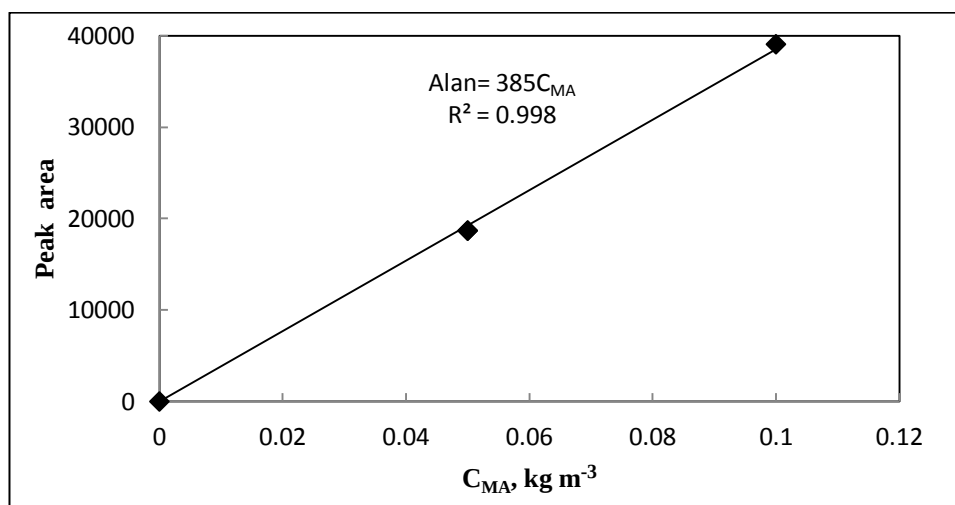


Figure G.5 Standard calibration curve for malic acid.

APPENDIX H

MOLECULAR WEIGHT MARKERS

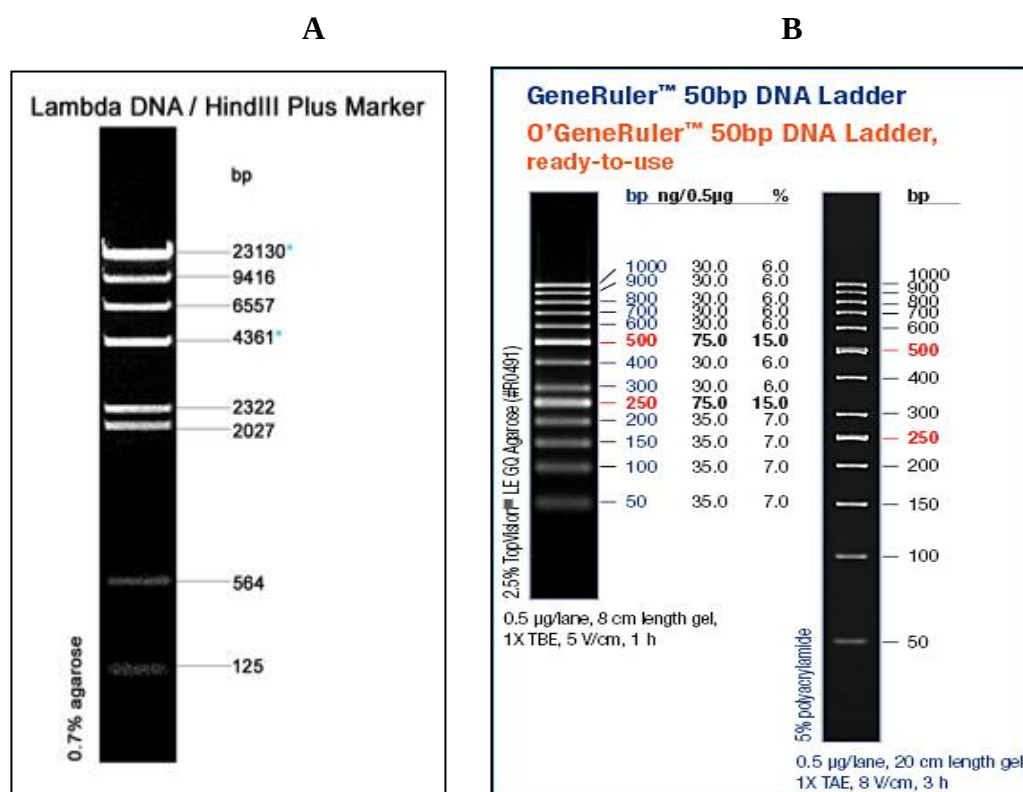


Figure H.1 A: Lambda DNA/HindIII Marker; B: Gene Ruler 50 bp DNA Ladder.

APPENDIX I.1

RESTRICTION ENZYME RECOGNITION SITES

Conformation: linear

Overhang: five_prime, three_prime, blunt

Minimum Site Length: 5 bases

Maximum Number of Cuts: all

Included: all commercial, prototypes only

Noncutters: AarI, AatII, AbsI, AcyI, AgeI, AjuI, AlfI, AloI, AlwNI, ApaI, ApaLI, ArsI, AscI, AsuII, AvaI, AvaII, AvrII, BaeI, BalI, BamHI, BbvI, BbvCI, BccI, BcgI, BclI, BfiI, BglI, Bpu10I, BsaBI, BseMII, BsePI, BseRI, BseYI, BsgI, Bsp1407I, BspHI, BspMI, BsrBI, BsrDI, BstEII, BstXI, BtgZI, BtrI, BtsI, CfrI, Cfr10I, ClaI, CspCI, DraII, DraIII, DrdI, EciI, Eco31I, Eco47III, EcoNI, EcoP15I, EcoRI, Esp3I, FauI, FseI, FspAI, GsuI, HaeII, HgaI, HindII, HpaI, HphI, Hpy99I, KpnI, MauBI, MfeI, MluI, MslI, NaeI, NarI, NcoI, NdeI, NheI, NmeAIII, NotI, NruI, NspI, OliI, PacI, PfoI, PmaCI, PmeI, PpiI, PpuMI, PshAI, PsiI, PI-PspI, PspXI, PsrI, PstI, PvuI, PvuII, RsrII, SacI, SacII, SalI, SanDI, SapI, ScaI, PI-SceI, SexAI, SfiI, SgfI, SgrAI, SgrDI, SmaI, SpeI, SphI, SrfI, Sse8387I, StuI, SwaI, TaqII, TatI, Taul, TfiI, TseI, TsoI, Tsp45I, TspRI, TstI, VspI, XbaI, XhoI, XmnI

TableI.1 Restriction map of *xyl_P* gene

Name	Sequence	Overhang	Cut Positions
BsaAI	YACGTR	blunt	447
EcoRV	GATATC	blunt	72
SnaBI	TACGTA	blunt	447
SspI	AATATT	blunt	405
AccI	GTMKAC	five_prime	242
AclI	AACGTT	five_prime	473
AflIII	CTTAAG	five_prime	507
AflIII	ACRYGT	five_prime	419

Table I.1 Restriction map of *xyl_P* gene (continued).

Name	Sequence	Overhang	Cut Positions
BsmAI	GTCTC	five_prime	163
SfaNI	GCATC	five_prime	618
StyI	CCWWGG	five_prime	269
Tth111I	GACNNNGTC	five_prime	238
XhoII	RGATCY	five_prime	651
BciVI	GTATCC	three_prime	429
BseSI	GKGCMC	three_prime	516
Eam1105I	GACNNNNNGTC	three_prime	556
Eco57I	CTGAAG	three_prime	769
Eco57MI	CTGRAG	three_prime	769
HaeIV	GAYNNNNNRTC	three_prime	575
MmeI	TCCRAC	three_prime	456
PflMI	CCANNNNNNTGG	three_prime	117
SduI	GDGCHC	three_prime	516
TspGWI	ACGGA	three_prime	149
HindIII	AAGCTT	five_prime	295, 368
PasI	CCCWGGG	five_prime	516, 667
SmlI	CTYRAG	five_prime	249, 507
BarI	GAAGNNNNNTAC	three_prime	665, 697
BdaI	TGANNNNNNTCA	three_prime	766, 800
BplI	GAGNNNNNCTC	three_prime	343, 375
BsmI	GAATGC	three_prime	215, 502
BsrI	ACTGG	three_prime	214, 339
FalI	AAGNNNNNCTT	three_prime	262, 294
Hin4I	GAYNNNNNVTC	three_prime	542, 574
XcmI	CCANNNNNNNNTGG	three_prime	276, 592
EcoRII	CCWGG	five_prime	515, 611, 666
FokI	GGATG	five_prime	295, 502, 535
PleI	GAGTC	five_prime	352, 565, 698
TspDTI	ATGAA	three_prime	16, 53, 539
BsaXI	ACNNNNNCTCC	three_prime	647, 677, 710, 740
MboII	GAAGA	three_prime	160, 553, 637, 699
AgsI	TTSA	three_prime	159, 190, 225, 415

Name: *xyl_T* gene restriction map

Overhang: five_prime, three_prime, blunt

Minimum Site Length: 5 bases

Maximum Number of Cuts: all

Included: all commercial, prototypes only

Noncutters: AarI, AatII, AccI, AclI, AflII, AflIII, AjuI, AlfI, AloI, AlwNI, ApaLI, ApoI, AscI, AsuII, AvrII, BaeI, Ball, BarI, BccI, BcgI, BclI, BglI, BplI, BsaBI, BsePI, BseYI, BsgI, BsmI, Bsp1407I, BspHI, BspMI, BtgZI, BtrI, BtsI, ClaI, CspCI, DrdI, Eam1105I, EcoNI, EcoP15I, EcoRI, EcoRV, Esp3I, FseI, FspAI, HaeIV, Hin4I, HindII, HpaI, MauBI, MfeI, MluI, MslI, NaeI, NdeI, NheI, NotI, NruI, OliI, PacI, PasI, PleI, PmaCI, PmeI, PpiI, PshAI, PsiI, PI-PspI, PsrI, PstI, PvuI, RsrII, SacI, SalI, SanDI, ScaI, PI-SceI, SexAI, SfaNI, SfiI, SgfI, SgrAI, SgrDI, SnaBI, SpeI, SrfI, Sse8387I, SspI, StuI, SwaI, TaqII, TatI, TfiI, TsoI, TspRI, Tth111I, VspI, XbaI, XcmI

TableI.2 Restriction map of *xyl_T* gene

Name	Sequence	Overhang	Cut Positions
BsrBI	CCGCTC	blunt	198
Eco47III	AGCGCT	blunt	628
PvuII	CAGCTG	blunt	1128
SmaI	CCCGGG	blunt	559
XmnI	GAANNNTTC	blunt	718
AbsI	CCTCGAGG	five_prime	1081
AgeI	ACCGGT	five_prime	524
BamHI	GGATCC	five_prime	963
BbvCI	CCTCAGC	five_prime	425
BbvI	GCAGC	five_prime	898
BglII	AGATCT	five_prime	392
Bpu10I	CCTNAGC	five_prime	425
BstEII	GGTNACC	five_prime	261
FokI	GGATG	five_prime	759
HindIII	AAGCTT	five_prime	715
NarI	GGCGCC	five_prime	1095
PfoI	TCCNGGA	five_prime	818
SapI	GCTCTTC	five_prime	343
TseI	GCWGC	five_prime	911
Tsp45I	GTSAC	five_prime	261
BciVI	GTATCC	three_prime	508

Table I.2 Restriction map of *xyl_T* gene (continued).

Name	Sequence	Overhang	Cut Positions
BfiI	ACTGGG	three_prime	474
BsrDI	GCAATG	three_prime	60
BsrI	ACTGG	three_prime	469
BstXI	CCANNNNNNTGG	three_prime	369
DraIII	CACNNNGTG	three_prime	690
Eco57I	CTGAAG	three_prime	968
GsuI	CTGGAG	three_prime	376
HphI	GGTGA	three_prime	255
KpnI	GGTACC	three_prime	525
MmeI	TCCRAC	three_prime	305
PflMI	CCANNNNNNTGG	three_prime	690
SacII	CCGCGG	three_prime	178
TspGWI	ACGGA	three_prime	759
BsaAI	YACGTR	blunt	111, 399
BsmAI	GTCTC	five_prime	352, 652
Eco31I	GGTCTC	five_prime	352, 652
PspXI	VCTCGAGB	five_prime	535, 1081
XhoI	CTCGAG	five_prime	535, 1081
AgsI	TTSAA	three_prime	222, 294
ApaI	GGGCCC	three_prime	844, 1033
BdaI	TGANNNNNNTCA	three_prime	201, 235
BseMII	CTCAG	three_prime	205, 416
EciI	GGCGGA	three_prime	142, 1084
Eco57MI	CTGRAG	three_prime	376, 968
FalI	AAGNNNNNCTT	three_prime	784, 816
Hpy99I	CGWCG	three_prime	163, 709
NmeAIII	GCCGAG	three_prime	414, 528
NspI	RCATGY	three_prime	594, 918
SphI	GCATGC	three_prime	594, 918
TstI	CACNNNNNNNTCC	three_prime	413, 445
AcyI	GRCGYC	five_prime	707, 857, 1095
CfrI	YGGCCR	five_prime	406, 491, 665
HgaI	GACGC	five_prime	88, 715, 865
NcoI	CCATGG	five_prime	257, 362, 662
PpuMI	RGGWCCY	five_prime	165, 252, 765
XhoII	RGATCY	five_prime	392, 963, 1005
BseSI	GKGCMC	three_prime	184, 844, 1033
Cfr10I	RCCGGY	five_prime	241, 524, 617, 752
MboII	GAAGA	three_prime	267, 360, 895, 1015

Table I.2 Restriction map of *xyl_T* gene (continued).

Name	Sequence	Overhang	Cut Positions
TspDTI	ATGAA	three_prime	106, 251, 477, 595
SmlI	CTYRAG	five_prime	247, 535, 790, 1060, 1081
TauI	GCSGC	three_prime	496, 799, 991, 1015, 1095
BsaXI	ACNNNNNCTCC	three_prime	10, 40, 262, 292, 413, 443
AvaI	CYCGRG	five_prime	535, 557, 622, 643, 905, 967, 1081
DraII	RGGNCCY	five_prime	137, 165, 252, 302, 765, 840, 841, 1030
FauI	CCCGC	five_prime	170, 182, 191, 464, 853, 1048, 1058, 1148
SduI	GDGCHC	three_prime	11, 23, 184, 316, 542, 557, 844, 1033
AvaII	GGWCC	five_prime	99, 165, 201, 252, 318, 366, 507, 615, 765, 813
EcoRII	CCWGG	five_prime	353, 368, 509, 611, 695, 761, 806, 818, 836, 1109

APPENDIX I.2

DIMMER STRUCTURE, SELF-COMPLIMENTARITY FORMATION AND THE THERMODYNAMIC PROPERTIES OF DESIGNED PRIMERS

GI_T-F

Name : GI_T-F
Primer : 5'-CGCATATGTACGAGCCCAAACCGGAGCACAGG-3'
Reverse : 3'-GGACACGAGGCCAAACCCGAGCATGTATACGC-5'
Length : 32 nt

T_m (basic) : 102,0 °C
T_m (salt) : 77,6 °C
T_m (NN) : 75,6 °C

GC % : 59,4 %
dG : -70,6 kCal/mol

3'-tail GC % : 71,4 %
3'-tail dG : -12,6 kCal/mol

Molecular weight : 9899,4 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 2,81 µM
and contains 27,8 µg ssDNA

GI_T-F self annealing:

5'-CGCATATGTACGAGCCCAAACCGGAGCACAGG-3'

||||

3'-GGACACGAGGCCAAACCCGAGCATGTATACGC-5'

dG: -5,76 kcal/mol

5'-CGCATATGTACGAGCCCAAACCGGAGCACAGG-3'

|||||

3'-GGACACGAGGCCAAACCCGAGCATGTATACGC-5'

dG: -4,74 kcal/mol

5'-CGCATATGTACGAGCCCAAACCGGAGCACAGG-3'

: |||| :

3'-GGACACGAGGCCAAACCCGAGCATGTATACGC-5'

dG: -2,93 kcal/mol

```
5'-CGCATATGTACGAGCCCAAACCGAGCACAGG-3'
      ||| :           : :::
3'-GGACACGAGGCCAAACCCGAGCATGTATACGC-5'
dG: -1,39 kcal/mol
```

GI_T-F loops:

```
5'-CGCATATGTACGAGCCCA
      ||| :          )
3'-GGACACGAGGCCAA
dG: -0,97 kcal/mol
```

GI_T-R

Name : GIT-R
Primer : 5'-GGAATTCTTCACCCCCGCACCCCCAGGAG-3'
Reverse : 3'-GAGGACCCCCACGCCCCCACTTCTTAAGG-5'
Length : 29 nt

T_m (basic) : 96,0 °C
T_m (salt) : 77,5 °C
T_m (NN) : 76,8 °C

GC % : 65,5 %
dG : -68,7 kCal/mol

3'-tail GC % : 71,4 %
3'-tail dG : -12,2 kCal/mol

Molecular weight : 8830,7 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 3,47 µM
and contains 30,6 µg ssDNA

GI_T-R self annealing:

```
5'-GGAATTCTTCACCCCCGCACCCCCAGGAG-3'
      |||||
3'-GAGGACCCCCACGCCCCCACTTCTTAAGG-5'
dG: -5,54 kcal/mol
```

```
5'-GGAATTCTTCACCCCCGCACCCCCAGGAG-3'
      ||| :::
3'-GAGGACCCCCACGCCCCCACTTCTTAAGG-5'
dG: -0,62 kcal/mol
```

GI_T-R loops:

5'-GGAAT
||| T
3'-GAGGACCCCCACGCCCCCACTTC
dG: -0,20 kcal/mol

GI_{pept}-F1

Name : GI_{pept}-F1
Primer : 5'-CACCACATTGAAGGGAGAATGAAAGTTGGAGTTAGC-3'
Reverse : 3'-CGATTGAGGTTGAAAGTAAGAGGGAAGTTACACCAC-5'
Length : 36 nt

T_m (basic) : 104,0 °C
T_m (salt) : 74,3 °C
T_m (NN) : 69,6 °C

GC % : 44,4 %
dG : -71,2 kCal/mol

3'-tail GC % : 42,9 %
3'-tail dG : -9,2 kCal/mol

Molecular weight : 11268,3 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 2,36 µM
and contains 26,6 µg ssDNA

GI_{pept}-F1 self annealing:

5'-CACCACATTGAAGGGAGAATGAAAGTTGGAGTTAGC-3'
: ::: :: : : :: ||| :
3'-CGATTGAGGTTGAAAGTAAGAGGGAAGTTACACCAC-5'
dG: -2,35 kcal/mol

5'-CACCACATTGAAGGGAGAATGAAAGTTGGAGTTAGC-3'
|||| ::::
3'-CGATTGAGGTTGAAAGTAAGAGGGAAGTTACACCAC-5'
dG: -1,20 kcal/mol

GI_{pept}-F1 loops:

5'-CACCACATTGAAGGG
: ||| :: : A
3'-CGATTGAGGTTGAAAGTAAG
dG: -1,93 kcal/mol

5'-CACCACATTGAAG
 ||||)
 3'-CGATTGAGGTTGAAAGTAAGAGG
 dG: -0,78 kcal/mol

GI_{pext}-F2

Name : GI_{pext}-F2
 Primer : 5'-GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
 Reverse : 3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
 Length : 37 nt

Tm (basic) : 110,0 °C
 Tm (salt) : 76,7 °C
 Tm (NN) : 72,5 °C

GC % : 48,6 %
 dG : -79,1 kCal/mol

3'-tail GC % : 57,1 %
 3'-tail dG : -12,7 kCal/mol

Molecular weight : 11382,4 g/mol

1 ml of the primer solution with an
 absorbance of 1 at 260 nm is 2,44 µM
 and contains 27,8 µg ssDNA

GI_{pext}-F2 self annealing:

5'- GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
 |||||
 3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
 dG: -12,20 kcal/mol

5'-GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
 ||| : : ::
 3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
 dG: -2,85 kcal/mol

5'-GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
 : :: ||| : : :: ::
 3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
 dG: -1,72 kcal/mol

5'-GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
 :: ||| : :: ::
 3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
 dG: -1,72 kcal/mol

5'-GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
 ||| :: : :: ::
 3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
 dG: -0,62 kcal/mol

```

          5'-GGAATTCCATCACCATCACCACCACATTGAAGGGAGA-3'
              |||      :      ::      :      :::
3'-AGAGGGAAGTTACACCACCACTACCACTACCTTAAGG-5'
dG: 1,02 kcal/mol

```

GI_{ext}-F2 loops:

```

5'-GGAATTCCATCACCATCACC
      ||| :      )
      3'-AGAGGGAAGTTACACCA
dG: -2,43 kcal/mol

```

```

5'-GGAATTCCATCACCATCAC
      : :: ||| :   C
      3'-AGAGGGAAGTTACACCA
dG: -1,30 kcal/mol

```

```

5'-GGAATTCCATCACCATCACCAC
      : :: |||      C
      3'-AGAGGGAAGTTACA
dG: -1,30 kcal/mol

```

```

      5'-GGAATTCCATCACCATC
          ||| ::      A
3'-AGAGGGAAGTTACACCACC
dG: -0,20 kcal/mol

```

```

          5'-GGAATTCCATCACCA
              |||      :      )
3'-AGAGGGAAGTTACACCACCACT
dG: 1,44 kcal/mol

```

GIP_{int}-F

Name : GIPint-F
Primer : 5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
Reverse : 3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'
Length : 33 nt

T_m (basic) : 94,0 °C
T_m (salt) : 71,4 °C
T_m (NN) : 67,5 °C

GC % : 42,4 %
dG : -63,3 kCal/mol

3'-tail GC % : 28,6 %
3'-tail dG : -8,5 kCal/mol

Molecular weight : 10287,7 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 2,65 µM
and contains 27,2 µg ssDNA

GIP_{int}-F self annealing:

5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
|||||
3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'
dG: -6,74 kcal/mol

5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
: ||| : : :
3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'
dG: -1,78 kcal/mol

5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
: ||| : : :
3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'
dG: -0,62 kcal/mol

5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
: ||| : : : :
3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'
dG: -0,29 kcal/mol

5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
||| : : : :
3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'
dG: -0,29 kcal/mol

5'-GCCTTCGAAACGATGAAAGTTGGAGTTAGCATA-3'
||| : : : :
3'-ATACGATTGAGGTTGAAAGTAGCAAAGCTTCCG-5'

dG: 0,22 kcal/mo

GI_{pint}-F loops:

5'-GCCTTCGA
 : ||| A
3'-ATACGATTGAGGTTGAAAGTAGCA
dG: -1,36 kcal/mol

5'-GCCTTCGAAA
 : |||)
3'-ATACGATTGAGGTTGAAAGTAGC
dG: -0,20 kcal/mol

5'-GCCTTCGAAACGAT
 ||| G
3'-ATACGATTGAGGTTGAAA
dG: 0,13 kcal/mol

5'-GCCTTCGAAACGATGAA
 : ||| : A
3'-ATACGATTGAGGTTG
dG: 0,13 kcal/mol

5'-GCCTTCGAAA
 ||| : C
3'-ATACGATTGAGGTTGAAAGTAG
dG: 0,64 kcal/mol

GI_p-R

Name : GI_p-R
Primer : 5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
Reverse : 3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
Length : 39 nt

T_m (basic) : 112,0 °C
T_m (salt) : 75,7 °C
T_m (NN) : 69,6 °C

GC % : 43,6 %
dG : -77,7 kCal/mol

3'-tail GC % : 42,9 %
3'-tail dG : -9,3 kCal/mol

Molecular weight : 11965,8 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 2,37 µM
and contains 28,3 µg ssDNA

GI_p-R self annealing:

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
|||||
3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
dG: -7,35 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
|||| :
3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
dG: -3,60 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
|||| :
3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
dG: -2,46 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
||| :: :: :: ::
3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
dG: -2,46 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
: ||| : : :: :
3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
dG: -1,72 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'

: : ||| :: :: : :
 3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
 dG: -1,72 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
 : : ||| :: : :
 3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
 dG: -1,72 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
 : ||| : : : : :: :
 3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
 dG: -1,38 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
 ||| :::
 3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
 dG: -1,38 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGCAGATCAG-3'
 ||| :: : : :: ::
 3'-GACTAGACGAAGTCTCTAACTACTCCACTAAAGATCTCG-5'
 dG: -0,58 kcal/mol

GI₂-R loops:

5'-GCTCTAGAAATCACCTCATCAATCTCTGA
 |||| A
 3'-GACTAGACG
 dG: -3,18 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTGAAGC
 ||||)
 3'-GACTAGA
 dG: -3,18 kcal/mol

5'-GCTCTAGAAATCACCTCATCAATCTCTG
 |||| A
 3'-GACTAGACGA
 dG: -2,04 kcal/mol

5'-GCTCTAGAAATCACCT
 ||| :: : :)
 3'-GACTAGACGAAGTCTCTAACTAC
 dG: -2,04 kcal/mol

5'-GCTCTAGAAATCACCTCATCAA
 : : |||)
 3'-GACTAGACGAAGTCTCT
 dG: -1,30 kcal/mol

5'-GCTCTAGAAATCACCTCAT

```

      :      |||      C
3'-GACTAGACGAAGTCTCTAA
dG: -1,30 kcal/mol

5'-GCTCTAGAAATCACCTCATCAAT
      :      : |||      C
      3'-GACTAGACGAAGTCT
dG: -1,30 kcal/mol

5'-GCTCTAGAAATCACCTCA
      : |||      :      : T
3'-GACTAGACGAAGTCTCTAAC
dG: -0,96 kcal/mol

      5'-GCTCTAGAAATCACC
      |||      )
3'-GACTAGACGAAGTCTCTAACTACT
dG: -0,96 kcal/mol

5'-GCTCTAGAAATCACCTCATCAA
      |||      ::      : T
      3'-GACTAGACGAAGTCTC
dG: -0,17 kcal/mol

```

APPENDIX J

AMINO ACID CODONS AND ABBREVIATIONS

Table J.1 Amino Acid Abbreviations and their codons.

Amino acid	One-letter code	Three-letter-code	Codons
Alanine	A	Ala	GCU, GCC, GCA, GCG
Cysteine	C	Cys	UGU, UGC
Aspartic Acid	D	Asp	GAU, GAC
Glutamic Acid	E	Glu	GAA, GAG
Phenylalanine	F	Phe	UUU, UUC
Glycine	G	Gly	GGU,GGC,GGG,GGG
Histidine	H	His	CAU, CAC
Isoleucine	I	Ile	AUU, AUC, AUA
Lysine	K	Lys	AAA, AAG
Leucine	L	Leu	CUU, CUC, CUA, CUG
Methionine	M	Met	AUG (start codon)
Asparagine	N	Asn	AAU, AAC
Proline	P	Pro	CCU, CCC, CCG,CCA,
Glutamine	Q	Gln	CAA, CAG
Arginine	R	Arg	CGU, CGC,CGA, CGG
Serine	S	Ser	AGU, AGC
Threonine	T	Thr	ACU, ACC, ACA, ACG
Valine	V	Val	GUU, GUC, GUA, GUG
Tryptophan	W	Trp	UGG
Tyrosine	Y	Tyr	UAU, UAC
Stop Codons			UGA, UAA, UAG

Table J.2 Codon frequency of *P. pastoris* (<http://www.kazusa.or.jp/codon/>, Last accessed: October 2009).

Codon	Usage	Codon	Usage	Codon	Usage	Codon	Usage
UUU	23.5	UCU	23.9	UAU	14.8	UGU	08.0
UUC	20.2	UCC	16.6	UAC	18.7	UGC	04.4
UUA	15.3	UCA	14.6	UAA	00.9	UGA	00.3
UUG	32.1	UCG	06.9	UAG	00.5	UGG	10.3
CUU	15.6	CCU	15.0	CAU	11.3	CGU	06.8
CUC	07.4	CCC	06.4	CAC	09.2	CGC	02.1
CUA	10.9	CCA	17.5	CAA	24.2	CGA	04.3
CUG	15.4	CCU	03.9	CAG	14.7	CGG	01.9
AUU	31.0	ACU	22.5	AAU	23.0	AGU	12.3
AUC	20.1	ACC	14.6	AAC	25.8	AGC	07.4
AUA	11.4	ACA	13.4	AAA	29.6	AGA	20.6
AUG	18.6	ACG	06.1	AAG	35.4	AGG	06.5
GUU	27.6	GCU	30.0	GAU	35.9	GGU	27.6
GUC	15.5	GCC	17.2	GAC	26.5	GGC	08.2
GUA	09.6	GCA	15.1	GAA	39.0	GGA	19.7
GUG	12.4	GCG	03.8	GAG	30.4	GGG	05.8

Table J.3 Codon frequency of *E.coli* strains (<http://www.faculty.ucr.edu/~mmaduro/codonusage/codontable.htm>, Last accessed: January 2010).

Codon	Usage	Codon	Usage	Codon	Usage	Codon	Usage
UUU	55.1	UCU	13.3	UAU	54.4	UGU	40.9
UUC	44.9	UCC	15.0	UAC	45.6	UGC	59.1
UUA	11.0	UCA	10.8	UAA	TER	UGA	TER
UUG	13.0	UCG	16.9	UAG	TER	UGG	100
CUU	09.7	CCU	13.7	CAU	56.8	CGU	39.0
CUC	10.4	CCC	11.2	CAC	43.2	CGC	42.2
CUA	03.1	CCA	18.6	CAA	33.4	CGA	05.5
CUG	52.8	CCU	56.5	CAG	66.6	CGG	09.0
AUU	51.2	ACU	14.8	AAU	40.9	AGU	13.9
AUC	44.4	ACC	46.8	AAC	59.1	AGC	30.0
AUA	04.4	ACA	10.5	AAA	75.4	AGA	01.7
AUG	100	ACG	27.8	AAG	24.6	AGG	01.5
GUU	27.6	GCU	14.5	GAU	62.8	GGU	32.9
GUC	15.5	GCC	27.6	GAC	37.2	GGC	43.2
GUA	09.6	GCA	19.6	GAA	68.3	GGA	09.2
GUG	12.4	GCG	38.2	GAG	31.7	GGG	14.7

APPENDIX K

SURFACE PLASMON RESONANCE ASSAYS

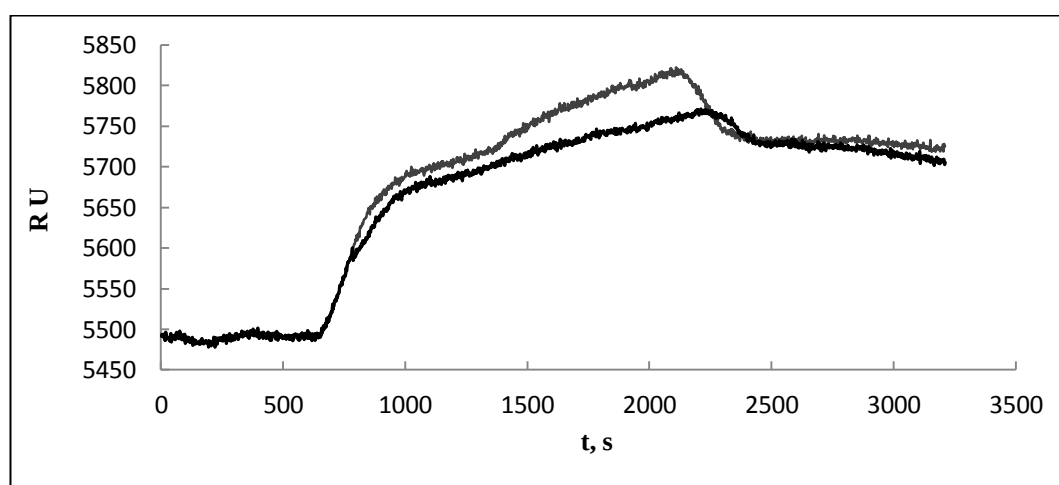


Figure K.1 Sensorgram for peptide 3|14 binding to 6×His at concentration of $C_p=0.2 \text{ mg mL}^{-1}$, upper curve: control, lower curve: actual run.

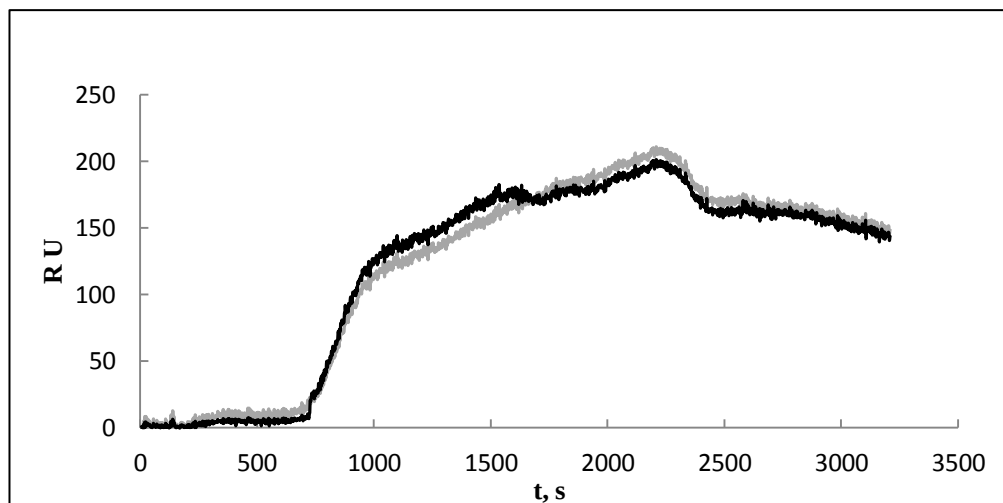


Figure K.2 Sensorgram for peptide 3|30 binding to 6×His at concentration of $C_p=0.2 \text{ mg mL}^{-1}$,
 blak curve: actual run, gray curve: control

CURRICULUM VITAE

VAHIDEH ANGARDI, Ph.D.

PERSONAL DATA

Date of Birth: June 28, 1978
Place o Birth: MAHABAD-IRAN
Marital Status: Married
Home Address: Sahit Osman Avcı M. 72 S. No: 7 D Blok 2 Goksu kent Sit.,
06770 Ankara-Turkiye
Work Address: Middle East Technical University, Chemical Engineering
Department, Biotechnology and Bioengineering Laboratory
06531 Ankara-Turkey
Telephone:
Office : +90 (312) 210 4381-210 4354
Cellular : +90 544 319 3267
Fax: +90 (312) 210 4883
E-mail: e145169@metu.edu.tr, Vahideh_anghardi@yahoo.com
Web: www.che.metu.edu.tr/~pcalik/groupmembers.htm

EDUCATION

2007-2011 Middle East Technical University, Ankara, Turkey

Doctor of Philosophy in Chemical Engineering, summer 2011

Major: Industrial Biotechnology

Cumulative GPA: 3.57 out of 4.0(Honor)

Ph.D. Dissertation: Bioprocess Development for Thermophilic Glucose
Isomerase Production

2004-2007 Middle East Technical University, Ankara, Turkey

Master of Science in Chemical Engineering

Major: Industrial Biotechnology

Cumulative GPA: 3.42 out of 4.0

M.Sc. Thesis: Influence of Oxygen Transfer on Benzaldehyde Lyase
Production by Recombinant *Escherichia coli* BL21 (DE3) pLysS, 2007

1997-2001 SAHAND University of Technology, Tabriz, Iran

Bachelor of Science in Chemical Engineering

Cumulative GPA: 13.72 out of 20.0

1992-1996 SEMA High School, Tabriz, Iran

Major: Mathematics & Physics

Cumulative GPA: 17.45 out of 20.0

WORK EXPERIENCE

2005-2011 Middle East Technical University Chemical Eng. Department, Ankara, Turkey

I worked as a graduate assistant in METU Chemical Engineering Department, Industrial Biotechnology Research Projects. Major interests are; Microbiology, Biochemistry, Enzyme Engineering, pharmaceutical Biotechnology, Fundamentals of Biochemical Engineering, Metabolic Engineering, Bioseparation, and Recombinant DNA Technology.

I participated in three research projects sponsored by the Scientific and Technological Research Council of Turkey.

2008-2011 Middle East Technical University Chemical Eng. Department, Ankara, Turkey

I worked as a teaching assistant of Topics in Chemical Engineering, in METU Chemical Engineering department, Ankara, Turkey (I am currently working as teaching assistant to train new MSc and PhD students who start their thesis experimental part, in biotechnology lab.)

2002-2004 Herbi Daru (pharmacy factory), Tabriz, Iran

Worked as an quality control engineer

Summer 2000 Zahravi pharmacy factory, Tabriz, Iran

Summer Practice

SPECIAL SKILLS AND QUALIFICATIONS

- Computer Skills
 - *Operating System* MS Windows, MS DOS
 - *Language* C++, Matlab 2009a
 - *Software* MS Office (Word, Excel, and Power Point), and Mathcad
- Laboratory Skills: Highly professional in operating the equipments and perform analysis: Bioreactor, High Performance Liquid Chromatography (HPLC), High Performance Capillary Electrophoresis (HPCE), UV

spectrophotometer, PCR ,RT-PCR, UV transilluminator, Gel electrophoresis, SDS-page, Phage display technology, SPR, ITC, MALDI-TOF MS, and Microarray Training, Genetic engineering techniques

- Ability to work well under pressure, handle multiple tasks and fast learner
- Work well both independently and as a part of a team

Participated Projects

- *Prof. Dr. Pınar Çalık*, “Bioprocess development for human growth hormone production by *pichia pastoris*”, METU- Scientific and Technological Research Council of Turkey, January 2010 – December 2011 (still on progress) Project No: 109R015
- *Prof. Dr. Pınar Çalık*, “Bioprocess development for Erythropoietin production”, METU- Scientific and Technological Research Council of Turkey, March 2008 - December 2009 Project No: MAG 107M258
- *Prof. Dr. Pınar Çalık*, “The production of recombinant enzymes by considering the principles of biochemical reactions + metebolik + genetic engineering”, METU- Scientific and Technological Research Council of Turkey, April 2005- February 2008 Project No: 104M258

REFERENCES

- **Prof. Dr. Pınar Çalık**, Chemical Engineering Department, Middle East Technical university 06531 Ankara – Turkey, +90 (312) 210 4385 pcalik@metu.edu.tr
- **Prof. Dr. Tunçer H. Özdamar**, Chemical Engineering Department, Ankara University 06531 Ankara – Turkey, Tel: +90-312-203 3440 - Fax: +90-312-212 15 46, ozdamar@eng.ankara.edu.tr
- **Prof. Dr. Birol M.R. Demiral**, Head of Center of Excellence for EOR Schlumberger Chair in Petroleum Engineering Universiti Teknologi Petronas Bandar Seri Iskandar, 31750 Tronoh, Perak Darul Ridzuan Malaysia, D/L: +605 368 7071, H/P: +6012 917 6814, Fax: +605 365 5670, birol_demiral@petronas.com.my

PUBLICATION:

- Hande Kaya-Çeliker, **Vahideh Angardi**, Pınar Çalık ” Regulatory effects of oxygen transfer on overexpression of recombinant benzaldehyde lyase production by *Escherichia coli* BL21 (DE3)” Biotechnology Journal; Volume 4, Issue 7, Apr 15 2009

- Pınar Çalık, **Vahideh Angardi**, Nazife Işık Haykır and Ismail Hakkı Boyacı “ Glucose isomerase production on a xylan-based medium by *Bacillus thermoantarcticus*” Biochemical Engineering Journal Volume 43, Issue 1, 15 January 2009, Pages 8-15
- **Vahideh Angardi**, Hande Kaya, Hande Levent, Pınar Çalık “Benzaldehyde Lyase Production for Catalysis of Cleavage Reactions” Inonu University, National, Eighth National Chemical Eng. Congress 26-29 August 2008
- **Vahideh Angardi**, P. Çalık “Effect of feeding rate on intracellular reaction network of glucose isomerase producing *Escherichia coli* BL21 (DE3) pLysS” ECCE / ECAB 2011 to be held in Berlin/Germany from September 25-29
- **Vahideh Angardi**, P. Çalık “Effects of exponential feeding of carbon source on recombinant glucose isomerase production by *Escherichia coli* BL21(DE3)” Biochemical Engineering Journal

HONORS AND ACTIVITIES

Prizes and Awards

- METU Graduate Courses Performance Award 2007-2008 Academic Year
- The Scientific and Technological Research Council of Turkey PhD Student Research Scholarship, January 2010 – December 2011 Project No: 109R015
- The Scientific and Technological Research Council of Turkey PhD Student Research Scholarship March 2008 - December 2009 Project No: MAG 107M258
- The Scientific and Technological Research Council of Turkey M.Sc Student Research Scholarship, April 2005- February 2008 Project No: 104M258
- METU International successful PhD students’ tuition fee payment exemption scholarship, September 2007-present (International PhD students, who are working 8 hours per week in their departments. The approval of the Department Chairmanship is required for those students.)

OTHER ACTIVITIES

- Participated in the “Microarray Training and Data Analysis Course” Nov. 24-25, 2008 METU Central Laboratory, Ankara, Turkey

Languages:

- Azeri : Mother Tongue
- English : Advanced Level
- Persian : Advanced Level
- Turkish : Advanced Level
- Arabic : Basic Level