RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION UNDER DOUBLE PROMOTERS BY *Pichia pastoris*

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RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION UNDER DOUBLE PROMOTERS BY *Pichia pastoris*

Submitted by **DUYGU YALÇINKAYA** in partial fulfilment of the requirements for the degree of **Master of Science in Chemical Engineering Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Halil Kalıpçılar
Head of Department, **Chemical Engineering Dept., METU** _____

Prof. Dr. Pınar Çalık
Supervisor, **Chemical Engineering Dept., METU** _____

Prof. Dr. Tunçer H. Özdamar
Co-supervisor, **Chemical Engineering Dept., Ankara University** _____

Examining Committee Members:

Prof. Dr. Tunçer H. Özdamar
Chemical Engineering Dept., Ankara University _____

Prof. Dr. Pınar Çalık
Chemical Engineering Dept., METU _____

Asst. Prof. Dr. Harun Koku
Chemical Engineering Dept., METU _____

Assist. Prof. Dr. Özgül Persil ÇETİNKOL
Chemistry Dept., METU _____

Assist. Prof. Dr. Eda Çelik Akdur
Chemical Engineering Dept., Hacettepe University _____

Date: 2.06.2017

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: DUYGU YALÇINKAYA

Signature :

ABSTRACT

RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION UNDER DOUBLE PROMOTERS BY *Pichia pastoris*

Yalçınkaya, Duygu

M.Sc., Department of Chemical Engineering

Supervisor: Prof. Dr. Pınar Çalık

Co-Supervisor: Prof. Dr. Tunçer H. Özdamar

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The aim of the M.Sc. thesis is expression of recombinant human growth hormone (rhGH) by *Pichia pastoris* under the double-promoters, namely, glyceraldehyde-3-phosphate dehydrogenase and pyruvate decarboxylase, engineered and constructed by metabolic- and genetic- engineering methods. The DNA fragment including the promoter region of the *P. pastoris* pyruvate decarboxylase (P_{PDC}), *Saccharomyces cerevisiae* α-mating factor (α-MF1) secretion signal sequence, and *hGH* gene, was amplified from the pPDCZαA*::hGH* plasmid and inserted into pPIC3.5K expression vector. Subsequently, the vector pPDC3.5Kα*::hGH* was transfected into HIS4 locus in r-*P. pastoris* which was previously transfected by pGAPZαA*::hGH* vector into GAP locus of the wild-type *P. pastoris* X-33. Following the construction of the r-*P. pastoris* carrying the double-promoter P_{PDC}-P_{GAP}, gene copy number of the construct was determined using quantitative real-time PCR (qRT-PCR). Single copy rhGH expressions under

P_{PDC} and P_{GAP} driven expression systems were compared separately with double copies of rhGH production under the double- promoters in pilot-scale bioreactor experiments at limited-oxygen conditions where constant dissolved oxygen (C_{DO}) was kept at 5%, in semi-batch bioreactors with a continuous feed stream designed with pre-determined $\mu_0 = 0.15\ h^{-1}$ for glucose feeding. Using the constructed r-*P. pastoris* carring the double-promoter, two oxygen transfer conditions were designed at constant dissolved oxygen concentrations of C_{DO} = 5% and 15%, which correspond to, respectively, limited and low-oxygen transfer conditions; and the semi-batch fermentations were carried out with continuous glucose feeding designed with the same pre-determined exponential feed flow rate. The cell, glucose, rhGH, organic acid and ethanol concentrations were determined throughout the fermentations. The highest rhGH concentrations in the semi-batch bioreactor operations, secreted under P_{PDC}, P_{GAP}, and P_{PDC}-P_{GAP} at C_{DO} = 5% limited oxygen transfer condition were as 80, 107, and 179 mg L^{-1}, respectively. The highest rhGH production was obtained with the double promoter expression system when C_{DO} was kept at 15% at t=18 h as 429 mg L^{-1}, and the highest cell concentration was attained at t=18 h as 104 g L^{-1}. The overall cell yield on the substrate, overall product yield on substrate, and overall product yield on the cell calculated as 0.48 g g^{-1}, 2.12 mg g^{-1}, and 4.38 mg g^{-1}, respectively.

In order to investigate the flux distributions in the intracellular bioreaction network under the three expression systems at limited-oxygen transfer conditions; moreover, the effect of oxygen transfer conditions on the double promoter expression system, metabolic flux analyses were carried out. The flux distributions at the crucial branch points, glyceraldehyde-3-phosphate and pyruvate nodes, and futher the ATP generation and cell synthesis fluxes with rhGH production fluxes were inter-dependently evaluated during fed-batch fermentation processes. The formation of the by-products, ethanol, pyruvic acid, acetic acid, and lactic acid, flux distributions in the pyruvate node supported a shift from respiratory to fermentative metabolism in all experiments owing to low-oxygen availability conditions.

Keywords: *Pichia pastoris*; recombinant human growth hormone; secretion; GAP promoter; pyruvate decarboxylase promoter; double promoter, oxygen transfer.

ÖZ

İKİ PROMOTOR KONTROLU ALTINDA *Pichia pastoris* İLE REKOMBİNANT İNSAN BÜYÜME HORMONU ÜRETİMİ

Yalçınkaya, Duygu
Yüksek Lisans, Kimya Mühendisliği Bölümü

Tez Yöneticisi : Prof. Dr. Pınar Çalık
Ortak Tez Yöneticisi :Prof. Dr. Tunçer H. Özdamar

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Bu yüksek lisans tezinin amacı, metabolik mühendislik tasarımı ve gentik mühendisliği metotlarıyla oluşturulan gliseraldehit-3-fosfat dehidrojenaz ve pirüvat dekarboksilaz çift-promotor sistemi altında, *Pichia pastoris* ile rekombinant insan büyüme hormonu (rhGH) üretimidir. *P. pastoris* pirüvat dekarboksilaz gen promotoru (P_{PDC}), *Saccharomyces cerevisiae* α-çiftleşme faktörü prepro sinyal (öncü)-dizini (α-MF1) ile *hGH* geni içeren DNA parçası, pPDCZαA*::hGH* plasmitinden çoğaltılmış ve pPIC3.5K ekspreson vektörüne klonlanmıştır. Oluşturulan pPDC3.5Kα*::hGH* vektörü, pGAPZαA*::hGH* vektörü ile transfekte edilmiş *P. pastoris* X-33 suşunun HIS4 lokusuna entegre edilmiştir. Çift-promotor P_{PDC}-P_{GAP} taşıyan r-*P. pastoris* oluşturulduktan sonra gen kopya sayısı kantitatif polimeraz zincir reaksiyonu (qPCR) ile belirlenmiştir. P_{PDC} ve P_{GAP} altında birer kopya *hGH* geni içeren iki rekombinant sistem ile, aynı suşta çift-promotor P_{PDC}-P_{GAP} altında birer kopya *hGH* geni içeren rekombinant sistem, önceden-belirlenmiş $\mu_0 = 0.15\ h^{-1}$ ile hesaplanmış eksponensiyel sürekli besleme akımı Q(t) ile biyoreaktöre beslenen glukozla, çözünmüş oksijen derişimi C_{DO}= %5 değerine set

edilen oksijen aktarımı koşullarında, yarı-kesikli pilot-ölçek biyoreaktör deneyleriyle kıyaslanmıştır. Çift-promotor P_{PDC}-P_{GAP} sistemi taşıyan r-*P. pastoris* rhGH üretim potansiyeli, iki farklı oksijen aktarım koşulunda, C_{DO} = % 5 oksijen-kısıtlamalı ve 15% düşük-oksijen aktarım koşullarında, aynı yarı-kesikli glukoz besleme stratejisi kullanılarak karşılaştırılmıştır. Deneyler süresince hücre, glukoz, rhGH, organik asit ve etanol derişimleri ölçülmüştür. Oksijen-kısıtlamalı (C_{DO} % 5) koşulda P_{PDC}, P_{GAP}, ve P_{PDC}-P_{GAP} rekombinant-sistemleri altında yapılan üretimlerde salgılanan en yüksek rhGH derişimleri, sırasıyla, 80, 107 ve 179 mg L^{-1} olarak bulunmuştur. Diğer yandan, çift-promotor P_{PDC}-P_{GAP} ekspresyon sistemi ile C_{DO} % 15'te tutulduğunda, en yüksek rhGH üretimi t=18 st'te 429 mg L^{-1} 'ye ulaşmış ve en yüksek hücre derişimi aynı kalma süresinde 104 g L^{-1} olarak bulunmuştur. Toplam substrat tüketimi üzerinden ürün ve hücre verimleri ile toplam hücre üretimine göre ürün verimi sırasıyla 0.48 g g^{-1}, 2.12 mg g^{-1}, ve 4.38 mg g^{-1} olarak hesaplanmıştır.

Hücre-içi tepkime hızları (akıları), ***i)*** kısıtlamalı-oksijen aktarım koşulunda rhGH üretiminde kullanılan P_{PDC}, P_{GAP}, ile P_{PDC}-P_{GAP} rekombinant-sistemlerini kıyaslamak; ve ***ii)*** uygulanan iki oksijen aktarım koşulunun çift-promotor P_{PDC}-P_{GAP} taşıyan r-*P. pastoris* ekspresyon sistemine etkisini, araştırmak için stokiyometrik kütle-korunum temelli matematik modelle hesaplanmış ve hücre-içi akı dağılımları analiz edilmiştir. Gliseraldehit-3-fosfat ile pirüvat dallanma noktalarındaki metabolik akı dağılımları, ATP oluşum, hücre sentezi, ve rhGH üretim akıları iç-etkileşimleriyle değerlendirilmiştir. Düşük oksijen aktarım koşulunda (C_{DO}= % 15), metabolizmanın solunum yolizinden fermentatif yolizine kaydığı doğrulanmıştır.

Anahtar kelimeler: *Pichia pastoris*; hücre-dışına salınım; GAP promotoru; pirüvat dekarboksilaz promotoru; çift-promotor sistemi; rekombinant insan büyüme hormonu; oksijen aktarım koşulları.

To My Family

and

My Beloved fiancé, Burak

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Duygu Yalçınkaya

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NOMENCLATURE

a	Area available for mass transfer	m^2
C	Concentration in the medium	g L^{-1} or mol m^{-3}
C_{DO}	Dissolved oxygen concentration	mol m^{-3}
C_M	Methanol concentration	v/v
C_{Sat}	Saturated dissolved oxygen concentration	mol m^{-3}
D	Diffusivity coefficient	m^2 s^{-1}
DO	Dissolved oxygen	%
H	Henry's constant	L atm mol^{-1}
K_{La}	Overall liquid phase mass transfer coefficient	s^{-1}
N	Agitation rate	min^{-1}
N''	Molar flux	mol s^{-1} m^{-3}
OD	Oxygen demand	mol m^{-3} s^{-1}
OD_{600}	Optical density at 600 nm	
OTR	Oxygen transfer rate	mol m^{-3} s^{-1}
OUR	Oxygen uptake rate	mol m^{-3} s^{-1}
P	Pressure	N m^{-2}
Q	Volumetric flow rate of inlet feed	L h^{-1}
Q_0	Volumetric flow rate of inlet air	L h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
r	Formation or consumption rate	g L^{-1} h^{-1}
t	Cultivation time	h

T	Bioreaction medium temperature	°C
U	One unit of an enzyme	
V	Volume of the bioreactor	L
X	Cell amount (mass)	g
Y	Yield	$g\ g^{-1}$
Y’	Overall yield	$g\ g^{-1}$

Greek Letters

α-MF1	*Saccharomyces cerevisiae* mating factor	
μ	Specific growth rate	h^{-1}
μ_0	Pre-determined specific growth rate	h^{-1}

Subscripts

0	Refers to initial condition
CO_2	Refers to carbon dioxide
M	Refers to melting point
O_2	Refers to oxygen
P	Refers to product
P/S	Refers to product over substrate
P/X	Refers to product over biomass
S	Refers to substrate
X	Refers to cell
X/S	Refers to biomass over substrate

General Abbreviations

ADH	Alcohol dehydrogenase
AGDH	Growth hormone deficiency in adulthood
AIDS	Acquired immunodeficiency syndrome
AOX	Alcohol oxidase
ARG4	Argininosuccinate lyase
BLAST	Basic local alignment search tool
BMGY	Buffered minimal glycerol complex medium
BMMY	Buffered minimal methanol complex medium
bp	Base pair
BR	Bioreactor
BSA	Bovine serum albumin
BSM	Basal salt medium
cDNA	Complementary Deoxyribonucleic acid
CHO	Chineese Hamster ovary
CJD	Creutzfeld-Jacob desease
CRI	Chronic renal insufficiency
CSHP	Chorionic somatomammotropin pseudo gene
DAB	3,3’-Diaminobenzidine
DAS	Dehydroxyacetone synthase
DNA	Deoxyribonucleic acid

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-aided degradation
EPO	Erythropoietin
FDA	Food and drug administration
FLD	Formaldehyde dehydrogenase
GAP/GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GHD	Growth hormone deficiency
GHRH	Growth hormone-releasing hormone
GRAS	Generally regarded as safe
hGH	Human growth hormone
hGH-V	Placental growth hormone
HIV	Human immune deficiency virus
HPLC	High pressure liquid chromatography
HSA	Human serum albumin
ISS	Idiopathic short stature
LB	Luria broth (lysogeny broth)
LSLB	Low-salt Luria broth
MCS	Multiple cloning site
MD	Minimal dextrose

mRNA	Messenger ribonucleic acid
MUT	Methanol utilization pathway
NAD^+	Nicotinamide-adenine dinucleotide
NCBI	National center for biotechnology information
NTC	Non-template control
ORF	Open reading frame
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PDI	Protein disulfide isomerase
PGK	Phosphoglycerate kinase
pI	Isoelectric point
PTM	Post-translational modification
PTM1	Pichia trace minerals medium
PWS	Prader-Willi syndrome
qRT-PCR	Quantitative real-time PCR
rDNA	Recombinant DNA
rhGH	Recombinant human growth hormone
RNA	Ribonucleic acid
RT	Room temperature
SBS	Short-bowel syndrome
SCP	Single cell protein
SD	Standard deviation

SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SOC	Super Optimal broth with Catabolite Repression Medium
SRIH	Somatotropin release-inhibiting hormone
SSA4	Heat shock protein
TCA	Tricarboxylic acid
TE	Tris EDTA
TEF	Translation elongation factor
TF	Transcription factor
TPP	Thiamine pyrophosphate
TS	Turner syndrome
TT	Transcriptional termination
tRNA	Transfer ribonucleic acid
UPR	Unfolded protein response
YEP	Yeast extract peptone
YNB	Yeast Nitrogen Base
YPD	Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION

Historical accounts have indicated that mankind has taken the advantage of fermented food products obtained from microorganisms for thousands of years. At the ancient times fermentation was used to produce food and beverages such as bread, yogurt, cheese, wine and beer. In the recent times, living organisms is being used to produce both endogenous and exogenous biomolecules such as proteins, carbohydrates, lipids, enzymes, amino acids, organic acids, antibiotics besides alcoholic beverages and food. Today, biotechnology is defined by the Organization for Economic Co-operation and Development (OECD) as "the application of science and technology to living organisms as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services." Industrial Biotechnology depending on the rDNA technology as well as bioprocess and hybridoma technologies accelerated with the production of the first pharmaceutical protein namely recombinant human insulin in 1982 (Sekhon, 2010). Recombinant protein production by taking modern medicine into consideration and using them as pharmaceuticals, named biopharmaceuticals, is now an industry with multi-billion dollar market value. The term 'biopharmaceutical' implies recombinant therapeutic proteins, products based on nucleic-acids and more broadly engineered cell or tissue-based products (Walsh, 2010). Nowadays clinical trials are employed for approximately 1300 recombinant proteins and more than 400 recombinant peptides and proteins are marketed upon approval of Food and Drug Administration (FDA). Also, clinical trials have been employed for approximately 1300 recombinant proteins (Sanchez-Garcia et al., 2016). Protein biopharmaceuticals including antibodies (containing fragments like

Fabs, scFvs and nanobodies), interferons, vaccines and hormones such as human growth hormone (hGH), erythropoietin (EPO), insulin become widespread in the treatment of various diseases like cancer, diabetes, and autoimmune diseases. Mainly produced recombinant proteins and their uses were given in Table 1.

Amongst the regulatory elements which are produced in the body, hormones are the most crucial ones. Hormones are biochemical messengers that are produced in special glands and they are transferred to the target cells through the circulatory system by interacting with a receptor. Human growth hormone (hGH) also termed as somatotrophin or somatotropin is a proteohormone which is synthesized and released by the anterior pituitary gland. The most abundant hGH circulated in the bloodstream has a molecular weight of 22 kDa consisting 191 amino acids and transcribed hGH gene is present at the chromosome 17. When the protein is completely translated, the molecule is arranged in a four-helix bundle. hGH is a non-glycosylated protein but contains two disulfide bridges in its structure. hGH induces many biological activities in the body. The first study which was conducted to examine the metabolic effects of growth hormone (GH) was published in 1948 indicated that the GH stimulates fat metabolism and blocks proteolysis in mice (Szego and White, 1948). Thereafter, it was understood that the hormone not only promotes the growth of cartilage, bone and skeletal muscle cells but also stimulates protein synthesis, raises levels of glucose in the bloodstream, increases glycogen stores in the muscles and also stimulates immune system in the body (Walsh, 2007). Growth hormone therapy is now applied in the treatment of many diseases including the GH deficiency both in children and adults, Turner syndrome, chronic renal insufficiency, Prader–Willi syndrome, idiopathic short stature in children, AIDS, human immunodeficiency virus (HIV) syndrome, and lipodystrophy in adults.

Table 1. Some Protein-Based Pharmaceutical Products (Thieman and Palladino, 2004).

SOME PROTEIN-BASED PHARMACEUTICAL PRODUCTS (MOST PRODUCED AS RECOMBINANT PROTEINS)	
Protein	**Application**
Erythropoietin	Treatment of anemia
Interleukins 1, 2, 3, 4	Treatment of cancer, AIDS, radiation- or drug-induced bone marrow suppression
Monoclonal antibodies	Treatment of cancer, rheumatoid arthritis; used for diagnostic purposes
Interferons (α, β, γ, including consensus)	Treatment of cancer, allergies, asthma, arthritis and infectious disease
Colony-stimulating factors	Treatment of cancer, low blood cell count; adjuvant chemotherapy; AIDS therapy
Blood clotting factors	Treatment of hemophilia and related clotting disorders
Human growth factor	Treatment of growth deficiency in children
Epidermal growth factor	Treatment of wounds, skin ulcers, cancer
Insulin	Treatment of types 1 and 2 diabetes mellitus
Insulin-like growth factor	Treatment of type 1 diabetes mellitus
Tissue plasminogen factor	Treatment after heart attack, stroke
Tumor necrosis factor	Cancer treatment
Vaccines	Vaccination against hepatitis B, malaria, herpes

Subsequent to the extraction of bovine anterior pituitary glands by Evans and Long (1921) GH therapy of children suffering from growth hormone deficiency (GHD) was firstly applied with GH, extracted from bovine of porcine in the 1940s. In 1956 GH was firstly isolated from the human pituitary glands and the children suffering from GHD (Li and Papkoff, 1956) were treated by the cadaveric GH until 1985. 1985 has been an end to the use of GH obtained pituitaries of cadavers due to diagnosis of Creutzfeldt–Jacob disease by FDA. In the same year, the first recombinant human GH (rhGH) was produced in *E. coli* and rhGH production was achieved by the murine cells in 1987 (Lindholm, 2006). So, biosynthetic growth hormone has begun to be used in GH therapy and it has been in use ever since. Protropin® was the primary rhGH which was produced in an *E. coli* strain by Genentech approved by FDA and marketed in 1985. However, Protropin was a dissimilar version of the native hGH with an addition of methionine on the N-terminus of the protein. Nowadays, rhGH with identical amino acid sequence to the native hGH is being expressed and marketed by the companies in various strains of *Escherichia coli, Saccharomyces cerevisiae*, and C127 mouse cells. On the other hand, various hGH expression systems including the strains of *Pichia pastoris* (Apte-Deshpande et al., 2009, Çalık et al., 2008, Massahi and Çalık, 2016), *Bacillus subtilis* (Franchi et al., 1991, Özdamar et al., 2009), insect cell/baculovirus system (Yoshimi et al., 1991) and mammalian cells such as Chinese hamster ovary (CHO) cells (Haldankar et al., 1999, Catzel et al., 2003) is being used in the production of rhGH.

Commonly therapeutic proteins are synthesized in the bacteria, *E. coli*, or in mammalian cell lines. Despite the fact that in a simple and cheap media, bacterial expression systems show rapid and robust cell growth they lack various post translational modifications (PTMs) generally found in higher organisms. Conversely, mammalian cells can successfully perform complex PTMs of proteins however long lasting processes, expensive and complex media demand and high risk of viral contamination are the most crucial disadvantages of these expression systems (Vogl et al., 2013). On the other hand, among the other eukaryotic hosts, yeast expression systems combine the advantages of single-celled organisms (i.e.,

fast and robust growth in minimal media) with the ability of proper protein processing (i.e., correct protein folding, PTMs) in the absence of viral DNA as wells as endotoxins (Porro et al., 2005).

Almost 50 years ago, the methylotrophic yeast *Pichia pastoris* was discovered by Koichi Ogata (1969) describing the capability of utilizing methanol as a sole carbon and energy source. Since then the yeast has become the most widely used expression system in the production of recombinant proteins. Along with the growth in simple medium reaching to high cell densities, excretion of the heterologous protein by secreting low levels of endogenous protein to the culture media facilitating the recovery are the primary advantages of *P. pastoris*. Implementation of most of the eukaryotic PTMs including proper protein folding, disulfide bond formation, glycosylation and, proteolytic processing (Cos et al., 2006), and presence of tightly regulated and strong promoter of the alcohol oxidase 1 gene, P_{AOX1} (Cregg et al., 2000), and simplicity of genetic manipulation are the other crucial characteristics of the yeast *P. pastoris* (Macauley-Patrick et al., 2005).

Commercial application of the cell factory of *P. pastoris* began after the approval of FDA and evaluated as GRAS (generally recognized as safe). The first recombinant biopharmaceutical product produced in *P. pastoris* which is approved by FDA is Kalbitor®, a recombinant protein that inhibits kallikrein. Nowadays approximately 70 therapeutic proteins are either on the market or in late phase of the development (Ahmad et al., 2014).

Recombinant hGH production was primarily accomplished by Ecamilla-Trevino et al. (2000) in the yeast *P. pastoris* by a P_{AOX1} driven expression system. Thereby, the studies conducted to produce rhGH under AOX1 promoter by *P. pastoris* has been gathered pace. In order to obtain extracellular rhGH, secretion signals and to enhance the amount of r-protein different concentrations of substrate, methanol, were analyzed by Eurwilaichitr et al. (2002), Orman et al., (2009). Çalık et al. (2008) attached polyhistidinetag with Factor Xa cleavage site to the upstream of the hGH to ease down-stream processes. Besides, together with the studies investigating the effect of co-substrates namely, sorbitol and glycerol, and

surfactants Tween 20 or Tween 80, effect of pre-determined specific growth rate (μ_0), and two different types of *P. pastoris* phenotypes, Mut^+ and Mut^S were researched (Apte-Dashpande et al., 2009, Çalık et al., 2009, Açık, 2009). During rhGH production the optimum pH value was checked up on and determined as 5.0 (Çalık et al., 2010). In order to eliminate the substrate, methanol, being an alternative to the AOX1 promoter, strong and constitutive GAP promoter has commenced to use in studies (Keskin, 2014, Hoxha, 2016, Hüccetoğulları, 2016). Furthermore, expression systems including two novel promoters namely, pyruvate kinase (PYRK) and pyruvate decarboxylase (PDC), were constructed by Massahi (2017). The highest reported rhGH production was performed by Güneş et al. (2014) and 1.3 g L^{-1} was produced under AOX1 promoter driven expression system.

In order to enhance the expression level of heterologous proteins, double promoter expression systems including the strong, inducible, and tightly regulated AOX1 promoter and strong constitutive GAP promoter has been used for the production of variety of recombinant proteins (Wu et al., 2003, He et al., 2008, He et al., 2015, Çalık et al., 2015, Parashar et al., 2016). Subsequent integration of two different expression vectors containing the same gene to be expressed, 1.3 to 6-fold increase was observed in the studies.

In this MSc Thesis, *P. pastoris* double promoter expression system containing two strong constitutive promoters was constructed. In order to attain rhGH production under two constitutive promoters, namely P_{GAP} (glyceraldehyde-3-phosphate dehydrogenase) and P_{PDC} (pyruvate decarboxylase), r-*P. pastoris* strain expressing the rhGH under GAP promoter was used as a parent strain and the constructed pPDC3.5Kα*::hGH* plasmid was transfected into its genome. In order to determine the copy number of the integrant qRT-PCR analysis was performed and the *hGH* expression level of the strain was observed both by shaker and bioreactor experiments. Expression of single copy hGH integrands under P_{GAP} and P_{PDC} with double promoter expression of hGH were compared separately, under limited-oxygen transfer condition, by keeping the DO concentration at 5%, and at a pre-

determined specific growth rate of μ_0=0.15 h^{-1}. Besides, the increase in the oxygen transfer rate (C_{DO}=15%) was also investigated for the expression of rhGH under double promoters. Product formation rates, substrate consumption rates, yield coefficients were determined for each fed-batch bioreactor operations. In addition, organic acid and ethanol concentrations were measured and corresponding changes in the intracellular metabolic fluxes of the strains were designated. In addition, metabolic flux distributions in the two important nodes, GAP and Pyr, in the glycolysis pathway, in the TCA cycle elements, in the fermentative pathway, and changes in the amino acid families were evaluated with ATP generation, biomass sysnthesis, rhGH production fluxes.

CHAPTER 2

LITERATURE SURVEY

2.1 Hormones

Precise control and coordination are required for the numerous metabolic activities that take place in cells and organisms especially in highly developed organisms which are consist of organs and tissues. Specific cells secrete extracellular signals to manipulate and regulate the metabolic activities. Each signal has a unique chemical structure and biologic activity and each target cell has receptors to recognize them. Biochemical messengers synthesized and secreted by special glands, transferred via the circulatory system to regulate the metabolism are termed as hormones (Boyer, 2005).

2.1.1 General Properties of Hormones

In order to maintain homeostasis of the metabolism involving the tissues and the organs of the body several mechanisms are developed. However exclusively two main systems namely, nervous system and the endocrine system are distinguishable (Figure 2.1). Chemical messengers secreted from the axons of neurons into a synapse are called as neurotransmitters. Neuronal signals may travel a fraction of a micrometer to bind their receptor proteins to excite the postsynaptic cell (Raven, 2005). The other chemical signal inside the body is the endocrine system whose signaling mechanism remarkably similar to nervous system except for their anatomic difference. Endocrine glands are responsible for the continuous or periodic production of hormones and the certain glands are the pituitary, thyroid, pineal, adrenal glands, thymus, and pancreas (Pekin, 1979). Biochemical

messengers, hormones, are secreted from the anatomically defined endocrine glands and are transported in the bloodstream from these certain glands either to adjacent cells or to remote tissues or organs. The name hormone was derived from the Greek hormao meaning 'I excite or arouse' (Nussey and Whitehead, 2001). Although transportation rates of hormones are low their activities are durable (Nelson and Cox, 2005). Hormones are not excreted when a proper physiological signal is received; a shift in the concentration of one or more constituent in the blood (calcium, potassium, glucose), conveyance of a neural signal, effect of other hormones or positive or negative feedback control may trigger the endocrine glands. Hormones transmit their message to only their target cells interacting with their receptors which have a ligand-binding, responsible for recognition and hormone binding, and an effector domain, responsible for initiation of the achievement of the biological responses (Norman and Litwack, 1997).

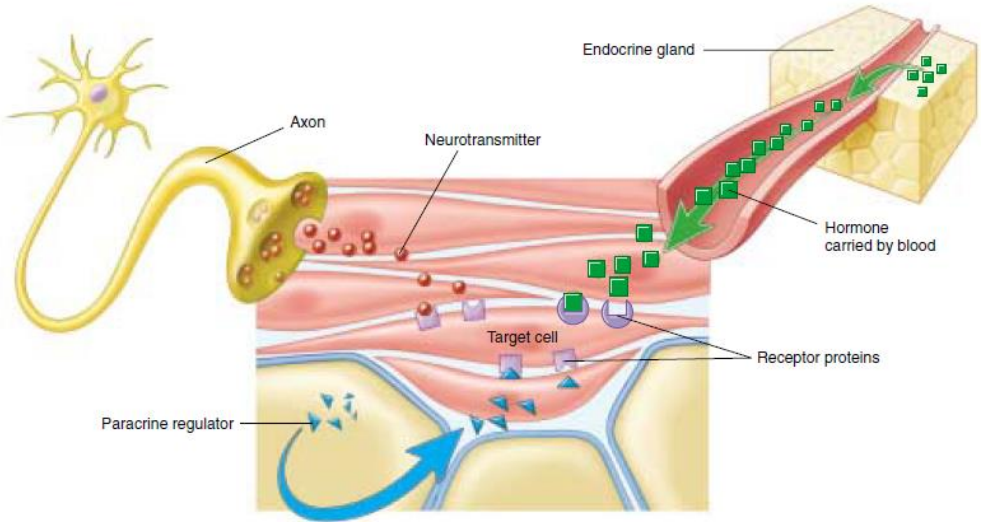

Figure 2.1 Relation between endocrine and nervous system (Raven, 2005).

Hormones can be classified based upon their functions as embryogenesis; growth and development, reproduction and sexual differentiation, adjustment of metabolic activities; fuel allocation, hunger, digestion, eating behavior, mood control, respiration, and movement, and maintenance of interior environment;

electrolyte balance, pressure and volume of the blood (Nelson and Cox, 2005). Low amounts of hormones are essential to mediate these functions and each of the hormones may participate in one or more of these functions and each function may be regulated by various hormones. To illustrate, levels of blood glucose is controlled by the pancreatic peptides, insulin and glucagon as well as growth hormone, cortisol and epinephrine (Nussey and Whitehead, 2001).

2.1.2 Classification of Hormones

There are three primary classes of hormones according to their chemical structures. These groups are the peptide and protein hormones, the steroid hormones and amino acid or fatty acid derived hormones and list of these classes are given in Table 2.1.

2.1.2.1 Peptide Hormones: These are polypeptide chains including 3 to 200 or more amino acid residues. The synthesis begins with the formation of prohormones, longer precursor proteins, on the ribosomes then they packaged and transported in the special secretory vesicles and proteolytic cleavage occurs to acquire active protein. Prolactin, growth hormone, calcitonin, somatostatin, antidiuretic hormone, leptin, insulin, and glucagon are this type of hormones (Nelson and Cox, 2005). Glycoproteins are also polypeptides generally consist of longer than 100 amino acid residues with an attached carbohydrate. These hormones do not enter into the cytoplasm of the target cell because the ligand-binding domain of the receptor is located on the outer surface of the plasma membrane.

2.1.2.2 Steroid Hormones: They are divided into two subgroups as adrenocortical hormones and sex hormones. They are transported through bloodstream binding to specific protein carriers to reach their receptor cells. These lipids deriving from cholesterol are lipophilic (lipid-soluble) and have limited solubility in the plasma membrane due to their hydrophobicity and therefore they can easily pass through the cell. This type of hormones includes cortisol, estradiol, progesterone and testosterone (Nelson and Cox, 2005, Campbell and Reece, 2001).

2.1.2.3 Amine Hormones: They are short chains of amino acids derived from the modification of amino acids tryptophan and tyrosine. The ones that are derived from the amino acid tyrosine which are secreted by the inner portion of the adrenal gland, adrenal medulla, are defined as catecholamines, involve epinephrine and norepinephrine, and the one secreted by the thyroid gland is the hormone thyroxine. Another amine hormone derived from the amino acid tryptophan and secreted by the pineal gland is melatonin (Raven, 2005). Similar to the peptide and glycoprotein hormones catecholamine hormones are hydrophilic. Thus, they cannot pass through the plasma membranes.

Hormones can also be categorized according to their hormonal communication systems which occur in between the gland that the hormone is secreted and the target cell that the massage to be received. Endocrine, coming from the Greek endon, meaning "within," and krinein, meaning "to release", hormones are secreted into bloodstream and transported to the target tissues throughout the body. Paracrine hormones are secreted to the extracellular space without being carried by the blood they regulate their neighboring target. Eicosanoids are this type of hormones. Whereas when the hormone-released cell and the cell comprising receptor on its surface is the same this type of hormone is named as autocrine hormone such as prostaglandins (Nelson and Cox, 2005, Norman and Litwack, 1997).

2.2 Growth Hormone

Growth in animals is a complex physiological mechanism controlled by the endocrine system (Figure 2.2). Animal growth is influenced by the hormones; growth hormone (GH), thyroid hormones, insulin, gonadal steroids namely, androgens and estrogens, and prolactin. However, Growth hormone is generally considered to be the most crucial hormone leading growth and development (Leung, 1988).

Growth hormone (GH), also known as somatotrophin or somatotropin is a polypeptide hormone play a critical role in the growth, development, and in many anabolic processes of vertebrates. GH is generated throughout life, secreted by the

somatotroph cells of the anterior pituitary gland to regulate bone metabolism, body composition, fluid homeostasis, cardiac function, fluid homeostasis, and exercise performance (Juul and Jorgensen, 2000, Sun and Roche, 2005).

2.2.1 Growth Hormone Secretion

Synthesis and secretion of GH is fundamentally managed by hypothalamic peptides; growth hormone-releasing hormone (GHRH), stimulates release of GH, and somatostatin, somatotropin release-inhibiting hormone (SRIH), inhibits secretion of GH (Smith and Thorner, 2000). Amplitudes of pulses are arranged by GHRH whereas frequency and continuum of pulses are controlled by SRIH (Kracier et al., 1988). However, production and secretion of GH is an interactive process with a negative feedback control system which includes brain neurotransmitters such as serotonin, dopamine, and acetylcholine, and insulin-like growth factor I (IGF-I), testosterone, estrogens and glucose (Sun and Roche, 2005).

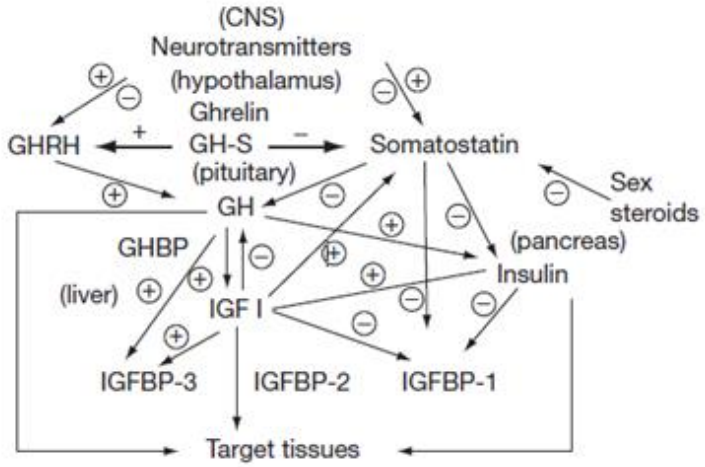

Figure 2.2 The GHRH–GH–IGF-I cascade (Pfaff and Rubin, 2009).

Table 2.1 Major Endocrine Glands and Hormones (Raven, 2005).

Endocrine Gland and Hormone	Target Tissue	Principal Actions	Chemical Nature
POSTERIOR LOBE OF PITUITARY			
Antidiuretic hormone (ADH)	Kidneys	Stimulates reabsorption of water; conserves water	Peptide (9 amino acids)
	Uterus	Stimulates contraction	
Oxytocin	Mammary glands	Stimulates milk ejection	Peptide (9 amino acids)
ANTERIOR LOBE OF PITUITARY			
Growth hormone (GH)	Many organs	Stimulates growth by promoting protein synthesis and fat breakdown	Protein
Adrenocorticotropic hormone (ACTH)	Adrenal cortex	Stimulates secretion of adrenal cortical hormones such as cortisol	Peptide (39 amino acids)
Thyroid-stimulating hormone (TSH)	Thyroid gland	Stimulates thyroxine secretion	Glycoprotein
Luteinizing hormone (LH)	Gonads	Stimulates ovulation and corpus luteum formation in females; stimulates secretion of testosterone in males	Glycoprotein
Follicle-stimulating hormone (FSH)	Gonads	Stimulates spermatogenesis in males; stimulates development of ovarian follicles in females	Glycoprotein
Prolactin (PRL)	Mammary glands	Stimulates milk production	Protein
Melanocyte-stimulating hormone (MSH)	Skin	Stimulates color change in reptiles and amphibians; unknown function in mammals	Peptide (two forms; 13 and 22 amino acids)
THYROID GLAND			
Thyroxine (thyroid hormone)	Most cells	Stimulates metabolic rate; essential to normal growth and development	Iodinated amino acid
Calcitonin	Bone	Lowers blood calcium level by inhibiting loss of calcium from bone	Peptide (32 amino acids)
PARATHYROID GLANDS			
Parathyroid hormone	Bone, kidneys, digestive tract	Raises blood calcium level by stimulating bone breakdown; stimulates calcium reabsorption in kidneys; activates vitamin D	Peptide (34 amino acids)

2.3 Human Growth Hormone

Prime hGH is a non-glycosylated polypeptide chain consisting 191 amino acid residues with a molecular weight of 22 kiloDalton (kDa) (Lewis et al., 1980b). The anionic protein has an isoelectric point (pI) of 5.1 and its empiric formula is $C_{990}H_{1529}N_{262}O_{300}S_7$ (Goeddel et al., 1979). Nucleotide sequence of hGH is given in Figure 2.3.

hGH has four alpha helices including two binding domains for the GH receptor. 55% of the secondary structure of hGH is comprised of α-helices providing protein binding to hGH receptors. hGH is defined as a long-chain helical cytokine due to the length of each helices (21 to 30 amino acids). The helices are organized with an up-up-down-down topology and this arrangement constitutes long extended loops between helices 1 and 2 and helices 3 and 4 (Figure 2.4). Helices from the first the fourth include 26, 21, 23 and 30 amino acid residues, respectively. hGH is a quite stable and compact molecule due to the four-helical bundle topology. Inner core of the bundle includes approximately 20 amino acid hydrophobic residues (Okada and Kopchick, 2001). It has two disulfide bonds in its structure. First one links loop to helix 4 (between C53 to C165), and the other disulfide bridge colligates C-terminus to the end of helix 4 (between C182 to C189). Besides, hGH involves minihelical segments in its structure. Minihelices between residues 38–47 and 64–70 colligate helix 1 and 2 and play substantial role in binding of receptor (Pfaff and Rubin, 2009).

```
ttc cca act ata cca cta tct cgt cta ttc gat aac gct atg ctt cgt gct
cat cgt ctt cat cag ctg gcc ttt gac acc tac cag gag ttt gaa gaa gcc
tat atc cca aag gaa cag aag tat tca ttc ctg cag aac ccc cag acc tcc
ctc tgt ttc tca gag tct att ccg aca ccc tcc aac agg gag gaa aca caa
cag aaa tcc aac cta gag ctg ctc cgc atc tcc ctg ctg ctc atc cag tcg
tgg ctg gag ccc gtg cag ttc ctc agg agt gtc ttc gcc aac agc cta gtg
tac ggc gcc tct gac agc aac gtc tat gac ctc cta aag gac cta gag gaa
ggc atc caa acg ctg atg ggg agg ctg gaa gat ggc agc ccc cgg act ggg
cag atc ttc aag cag acc tac agc aag ttc gac aca aac tca cac aac gat
gac gca cta ctc aag aac tac ggg ctg ctc tac tgc ttc agg aag gac atg
gac aag gtc gag aca ttc ctg cgc atc gtg cag tgc cgc tct gtg gag ggc
agc tgt ggc ttc tag ctg ccc ggg tgg cat ccc tgt gac ccc tcc cca gtg
cct ctc ctg gcc
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Figure 2.3 Nucleotide sequence of hGH.

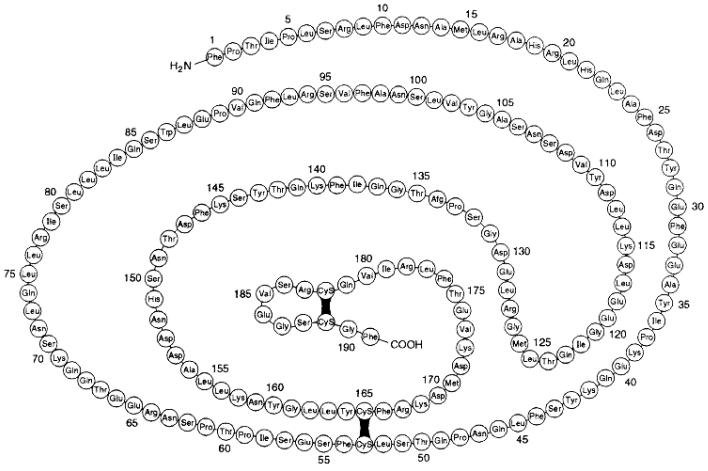


Figure 2.4 Secondory structure of hGH.

The hGH genes localized on the long arm of chromosome 17q22–24 (Pfaff, and Rubin 2009). Circulating human growth hormone (hGH) is a composition of five similar isoforms. Genes that codes for GH is located over 50-kb on the chromosome and these genes are in 5'-3' order hGH-N, (or –1), chorionic

somatomammotropin pseudo gene (CSHP), chorionic somatomammotropin gene hCS-A, hGH-V (or -2) variant of hGH-N, and chorionic somatomammotropin gene hCS-B, quite similar to hCS-A (Sami, 2007). Except CSHP, all genes expressed as 217 amino acid prohormones then are cleaved to 191 amino acid hormones. The hGH-N gene is expressed in the pituitary gland whereas the GH variant gene (hGH-V) is expressed in the placenta. hGH-V, 20-kDa form of hGH-N, differ by 13 amino acids from the primary sequence and substitute hGH-N throughout the second-half of pregnancy (Lewis et al., 1980a, Frankenne et al., 1988). CSHP is defined as pseudogene because it was discovered that the gene is translated but is exposed to an alternative splicing yielding an ultimate nonfunctional protein (Misra-Press et al., 1994). Therefore, in the cluster of these genes hGH-N is the most abundant, the only anabolic growth promoting and the most biologically active molecule. When natural human growth hormone and its variants are compared according to their functions that are related to the structure of hGH revealed that:

i. Biological activity of the hormone is not influenced from the di-sulphide bridges.
ii. Its functions regulated by varied domains of hGH.
iii. N-terminus of the hormone affects the galactopoietic activity.
iv. Hormone receptor binding magnitude may be designated by an only amino acid at a specific position.
v. Hormone receptor binding is affected from the hydrogen bonds and salt bridges of the molecule
vi. The packing of alpha helices bundles is affected by Trp 86.
vii. Stability of hGH depends on the hydrophobic cores of the molecule
viii. hGH has two binding sites namely, SiteI and Site II and they are sterically coupled (Sami, 2007).

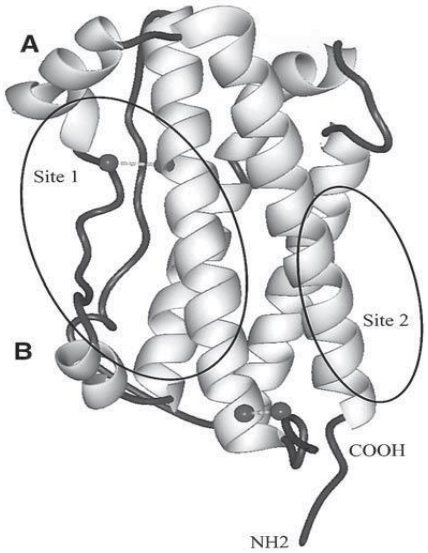


Figure 2.5 Ribbon diagram of hGH (Smith and Thorner, 2000).

2.3.1 Biological Effects of Human Growth Hormone

Growth hormone as one of the major regulator of growth has an important position in the endocrine system. hGH principally attend the anabolic activities. The hormone metabolically affects the skeletal muscle, adipose tissue, and liver with center upon the lipid and carbohydrate metabolism. IGF-I mediating the growth-promoting activity of hGH is generated when hGH binds to its hepatic receptor to regulate the bone and skeletal muscle growth or tissue renewal and repair. Postnatal growth is dependent on GH, while fetal and early postnatal growth is independent of GH (Okada and Kopchick, 2001). During childhood hyposecretion or lack of hGH secretion due to mutation in the gene causes pituitary dwarfism. On the contrary, prior to puberty hypersecretion of hGH yields a phenotype called as gigantism. In adults' skeletal growth have transformed from cartilage to bone, thus excessive hGH secretion inflicted by pituitary adenomas causes deformities in bones and soft tissues which is a condition termed as acromegaly (Raven, 2005).

Predominantly, hGH stimulates lipolysis in the adipose tissue causing an enhanced free fatty acid in the blood stream also by increasing lipoprotein lipase expression hGH stimulates uptake of triglyceride in the liver and muscles. Besides, carbohydrate metabolism may have a bearing upon the hGH along with the

antagonism of insulin action (Vijayakumar et al., 2011). The foremost biological effects of human growth hormone may be summarized as (Charrier and Martal, 1988, Walsh, 2007):

- Enhancement of body growth
- Reduction of adipose mass and enlargement of lean body mass
- Stimulation of growth in all internal organs excluding the brain
- Promoting the immune system
- Stimulation of reticulocytosis in the bone marrow
- Expands extracellular fluid space
- Stimulates erythropoiesis
- Increase of calcium retention and bone mineralization
- Enhancement of muscle and cardiac glycogen stores
- Increment of muscle mass via sarcomere hypertrophy
- Increase of kidney size and renal function
- Stimulation of gluconeogenesis in the liver
- Increment of T4 to T3 deiodination
- Stimulation of amino acid transport
- Stimulation of DNA/RNA synthesis in most cell types
- Stimulation of protein synthesis in most cell types
- Elevation of blood glucose levels: anti-insulin effect
- Mobilization of depot lipids from adipose tissues (lipolytic effect)

2.3.2 Therapeutic Uses of Human Growth Hormone

hGH has a broad range of potential therapeutic uses. It is primarily used for growth hormone deficiency (CGDH) in children and adults to treat pituitary dwarfism. Nowadays, GH treatment is not only used for the GH failure in childhood due to mutations in the specific genes such as GHRHR, GH1, and GH deficiency in adulthood (AGDH) caused by pituitary adenoma or by pituitary surgery or radiotherapy but also hGH has been used for the short stature treatment along with other normal endogenous GH secretion conditions such as, chronic renal insufficiency, Turner syndrome, Prader–Willi syndrome, and more recently, idiopathic short stature in children, AIDS-associated weight loss or cachexia (Cázares-delgadillo et al., 2011; Weiming et al., 2004; Wit et al., 2008). While in the treatment of GH deficient individuals generally low doses are sufficient, in non-GH deficient disorders supra-physiological doses are required on the occasion of resistance of GH and IGF1 (Pavlovic et al., 2008). Necessary doses in terms of conditions with commercially available somatropins are given in Table 2.2.

- **Turner syndrome (TS):** It is a condition occurs in females with defective or completely missing X chromosome in company with the symptoms; short neck, low-set ears, low hairline at the nape, swollen (lymphedema) hands and feet at birth, and soft nails. Individuals with Turner syndrome mostly have normal intelligence however; nonverbal learning disabilities, trouble learning mathematics, behavioral problems are possible. Besides loss of ovarian function and developmental delays (puberty do not start at a normal age) are observed (Cázares-Delgadillo et al., 2011).

- **Prader-Willi syndrome (PWS):** It is a complex genetic disorder due to missing or unexpressed seven genes on chromosome 15. Characteristics of this condition include weak muscle tone (hypotonia), poor growth, and delayed development, feeding difficulties and also chronic overeating (hyperphagia) and obesity. People with Prader-Willi syndrome peculiarly obese people encounter with the form of diabetes, type 2 diabetes mellitus.

Individuals with this syndrome characteristically have mild intellectual impairment and learning disabilities and behavior disorder. Additionally, they have facial particularizations such as narrow forehead, a triangular mouth, and almond-shaped eyes along with short stature; small hands and feet (Hintz, 2004, Cázares-Delgadillo et al., 2011).

- **Chronic renal insufficiency (CRI):** It is inadequate, slowly deteriorating renal function of kidneys to concentrate urine, expel wastes, and conserve electrolytes. The disorder generally arises due to diabetes and high blood pressure, low blood cell count, Alport syndrome, kidney stones and kidney infection, obstructive uropathy, analgesic nephropathy, glomerulonephritis, and polycystic kidney disease. Typically observed symptoms include; loss of appetite, ill feeling, headaches, pruritus, nausea, and loss of weight (Cázares-Delgadillo et al., 2011).

- **Short-bowel syndrome (SBS):** It is a malabsorption or malnutrition disorder due to surgical removal of the small intestine because of the Crohn's disease, intestinal atresias, intestinal volvulus, tumors, trauma or injury inside the small intestine, necrotizing enterocolitis, and gastric bypass surgery (Donohoe and Reynolds, 2010).

- **Idiopathic short stature (ISS):** It is a condition seen in individuals more than 2 standard deviation (SD) below the comparable mean height for their bone age, sex and population without diagnosable disorder in the GH and IGF axis, genetic, endocrine and organ systems (Wit et al., 2008).

Table 2.2 Commercially available somatropins (Cázares-Delgadillo et al., 2011).

Trade name	Company	Host Organism	Indications and usage	Dosage	Administration
Accretropin	Cangene Corporation	*E. coli*	CGDH	0.18–0.3 mg/kg/wk	s.c.
			TS	0.36 mg/kg/wk	
Genotropin	Pharmacia and Upjohn, Pfizer	*E. coli*	CGDH	0.24 mg/kg/wk max,	s.c.
			AGDH	0.08 mg/kg/wk	
			TS	0.33 mg/kg/wk	
			PWS	0.24 mg/kg/wk	
			ISS	Up to 0.47 mg/kg/wk	
Humatrope	Eli Lilly and Co.	*E. coli*	CGDH	0.18 mg/kg/wk	s.c., i.m.
			TS	Up to 0.375 mg/kg/wk	s.c.
			AGDH	max, 0.0125 mg/kg/day	
			ISS	0.37 mg/kg	
Norditropin, Norditropin Nordiflex	Novo Nordisk Inc.	*E. coli*	CGDH	0.024 to 0.034 mg/kg	s.c.
			AGDH	max of 0.016 mg/kg	
			TS	Up to 0.067 mg/kg daily	
Nutropin, Nutropin AQ	Genentech Inc.	*E. coli*	CGDH	Up to 0.3 mg/kg weekly	s.c.
			AGDH	0.006 mg/kg/day	
			CRI	Up to 0.35 mg/kg weekly	
			TS	Up to 0.375 mg/kg/wk	
			ISS	0.3 mg/kg weekly	

Table 2.2 Commercially available somatropins (Cázares-Delgadillo et al., 2011) (Continued).

Trade name	Company	Host Organism	Indications and usage	Dosage	Administration
Omnitrope	Sandoz	*E. coli*	CGDH	0.16 to 0.24 mg/kg weekly	s.c.
			AGDH	max, 0.08 mg/kg/wk	
Serostim	Merck Serono Inc.	C127 mouse cell	AIDS-associated weight loss or cachexia	0.1 to 4 mg daily	s.c., i.m.
Saizen	Merck Serono Inc.	C127 mouse cell	CGDH	0.06 mg/kg	s.c., i.m.
			AGDH	0.005 mg/kg daily	s.c.
Tev-tropin™	TEVA-Pharmaceuticals	*E. coli*	CGDH	0.1 mg/kg 3 times weekly	s.c.
Valtropin	Biopartners	*S. cerevisiae*	CGDH	0.23 mg/kg/ week	s.c.
			AGDH	0.33 mg/day	
			TS	0.053 mg/kg/day	
Zorbtive	Serono/Novartis	C127 mouse cell	SBS	0.1 mg/kg daily	s.c.

***** s.c., subcutaneous; i.m., intramuscular.

Apart from the above-mentioned conditions other therapeutic applications of hGH induction of lactation, counteracting ageing, obesity treatment, induction of ovulation, and body building (Walsh, 2007). In addition to these the hormone is valuable in a wide range of medical applications including bleeding peptic ulcers treatment, repairing the tissues after severe burn, healing of fractures, and injured cartilage. It also has been used for the treatment of juvenile rheumatoid arthritis,

cardiac disease, cystic fibrosis, osteoporosis, thyroid disease, inflammatory bowel disease, or cancer treatment (Schmeck, 1981, Liu et al., 2007).

2.3.3 Recombinant Human Growth Hormone Production

hGH was first described by Evans and Long by applying bovine anterior pituitary extract to rats (1921). The efforts to treat children suffering from GHD began in the mid-1940s. GH, extracted from bovine, and porcine were tried for the therapy however metabolic or biochemical activity could not observed due to species specific features of hormones (Frasier, 1997). Subsequent to extraction of growth hormone from human pituitary glands (Li and Papkoff, 1956), the therapeutic usage of hGH was firstly applied for the treatment of a pituitary dwarf by Raben (1958). Nevertheless, 1 mg of hGH was required to cure one person per day; in other words, more than 360 human pituitaries were required to cure per person per year. Therefore, presence of hGH and corresponding treatment was limited. With the discovery of Creutzfeldt-Jacob, fatal, slow viral and neurological disorder, disease by US Food and Drug Administration (US, FDA) the treatment with pituitary GH was suspended (Walsh, 2007).

Following the determination of biochemical structure of hGH in 1972, hGH production by recombinant DNA technology was accelerated and for the first time the gene was successfully cloned by Goeddel et al. (1979). Recombinant hGH (rhGH) was produced by Genentech (San Francisco, California) in 1981. rhGH production was further improved by the development of the biosynthetic process named as protein secretion technology and in 1985 the first FDA approved biosynthetic growth hormone was produced in *E. coli* under the brand name Protropin (somatrem for injection) having the same 191 amino acid sequence, yet containing an extra amino acid methionine at the N-terminal of the protein (Ford, 1994). Subsequently, eliminating the N-terminal methionine, in 1986 rhGH containing 191 amino acid residues as the native hGH was developed by the company Eli Lilly under the brand name Humatrope.

cDNA of the mature hGH was primarily cloned to the genome of the host microorganism, *P. pastoris*, by Ecamilla-Trevino et al. (2000). The gene was

expressed under the control of P_{AOX1} yielding 49 mg L^{-1} C_{rhGH}. Whether glutamic acid-alanine (glu-ala) spacer was necessary in order to remove the secretion signal alpha mating factor-1 (α-MF) and different cultivations with varied methanol concentrations were investigated by Eurwilaichitr et al. (2002). Mature hGH was obtained when glu-ala was removed and the highest rhGH was produced as 190 mg L^{-1} when induced with C_M=3% (v/v). pPICZαA*::hGH* expression system with a polyhistidinetag (6xHis tag) and Factor Xa cleavage site was constructed by Çalık et al. (2008). In a medium including 1.0% (v/v) methanol and at the end of 24 h induction 115 mg L^{-1} rhGH was obtained. Two different phenotypes of *P. pastoris* namely, Mut^+ and Mut^S, were compared while applying different carbon source feeding strategies under AOX1 promoter by Orman et al., (2009). The highest rhGH production was achieved when BMMY complex medium with a C_{MeOH} of 2% (v/v) was fed and the amount rhGH was found as 160 mg L^{-1}. The effect of feeding rate of methanol on sorbitol consumption was researched by Çalık et al. (2009) by using *P. pastoris* Mut^+ strain at three various specific growth rates (μ). Starting with 50 g L^{-1} sorbitol concentration in each strategy it was revealed that biomass concentration increased as the feeding rate of methanol increased. However lower protease activity and the highest C_{rhGH} (270 mg L^{-1}) was measured in the culture media when methanol was fed to the bioreactor at μ=0.03 h^{-1}. *P. pastoris* Mut^+ and Mut^S strains were also compared by Açık (2009) in two separate cultivation media including glycerol/methanol or sorbitol/methanol. The maximum rhGH expression obtained by using *P. pastoris hGH*-Mut^+ strain by adding sorbitol as co-substrate with μ=0.03h^{-1} as C_{rhGH}=301 mg L^{-1}. Effect of pH on rhGH production was investigated on *P. pastoris hGH*-Mut^+ by Çalık et al. (2010). Four different pH values, 4.2, 5.0, 5.5, and 6.0 were applied and the highest C_{rhGH} was acquired at pH=5.0 as 270 mg L^{-1}. Correspondingly, the strain *hGH*-Mut^+ was further developed by Bozkurt (2012). Among different feeding strategies, rhGH concentration was enhanced to 640 mg L^{-1} at μ=0.03 h^{-1} with constant sorbitol concentration, 50 g L^{-1}, and at the pH value of 5.0. rhGH expressing strain carrying constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter was constructed by Zerze (2012) and 220 mg L^{-1} rhGH was produced. Effect of carbon

sources and pre-determined specific growth rates on rhGH expression under GAP promoter was investigated by Keskin (2014) and the highest C_{rhGH} was obtained as 508 mg L^{-1} at μ_0=0.15 h^{-1} with a glucose concentration of 500 g L^{-1}. Güneş et al. (2014) achieved the highest rhGH concentration as 1.3 g L^{-1} under P_{AOX1} by keeping the methanol concentration constant as 5 g L^{-1} in the media. *P. pastoris* strain carrying pPGAPZαA*::hGH* expression cassette with polyhistidinetag (6xHis) and Factor Xa cleavage site was cultivated in different carbon sources and variable pre-determined μ_0 values were tried by Hoxha (2016) and extracellular rhGH was enhanced to 650 mg L^{-1}. Influence of cell cycle synchronization was investigated by Hüccetoğulları (2016) rhGH concentration was augmented from 220 to 570 mg L^{-1} which was previously obtained by Zerze (2012). Novel promoter approach was developed by Massahi (2017) and two different novel promoters namely, pyruvate kinase (PYRK) and pyruvate decarboxylase (PDC), were used in rhGH production and results were compared to GAP promoter driven expression systems. rhGH concentration was calculated as 122 mg L^{-1} under PDC promoter whereas 54 mg L^{-1} rhGH was produced under GAP promoter.

2.4 Selection of the Host Microorganism

Nowadays biopharmaceuticals have become prominent as being safe, highly effective, being not carcinogenic and having fewer side effects. Up to date, around 400 proteins which are produced by recombinant technologies have been approved as biopharmaceuticals. Through various expression systems, mammalian cell lines, transgenic plants, animal, insect, bacteria and yeast cells have been used and today most of the biopharmaceuticals are recombinant and 45% of them are derived from mammalian cells, 35% of which are produced from CHO (Chinese hamster ovary) cells, 40% produced in bacteria 39% of which are produced in *Escherichia coli* and 15% in yeast *Saccharomyces cerevisiae* (Sanchez and Demain, 2012). Together with the individual characteristics of each, one of the most important and preliminary step in recombinant protein production is the choice of the host microorganism which has capability to proper folding and post-translational modifications. Also, choice of a proper vector, codon optimization of

the gene of interest, epitope tag fusion for purification or observation of the r-protein, choice of signal peptide, lack of proteases, fermentation medium design, optimization of bioprocess parameters are the other certain requirements in r-protein production (Çelik and Çalık, 2011). Advantages and disadvantages of mammalian, bacteria and yeast cells were summarized in Table 2.3 There are several factors that affect the selection of the host microorganism such as being GRAS (generally recognized as safe), giving high yield, growing on cheap media with rapid growth rates, ability to grow at moderate circumstances, quality of the protein, speed of the production, and ease in the scale-up (Soetaert and Vandamme, 2010).

Bacteria generally yield unstable, misfolded, insoluble or biologically inactive eukaryotic proteins and they may have toxic cell wall pyrogens. Mammalian cells on the other hand, have a huge risk of cross contamination due to higher growth rates of bacteria. Also, they have low yield and productivity, and they require complex nutrients (Zhang and Meagher, 2000), (Damasceno, et al., 2012). Yeast cells have superior features over bacteria or mammalian cells which offer certain advantages for the expression of foreign proteins. They are unicellular eukaryotes that can conduct correct folding and glycosylation which may affect the biological activity of the proteins. Besides, faster growth rate, they give higher yields and productivity and they are more economical because they do not need complex nutrients (Walker and Dundee, 2009). *Saccharomyces cerevisiae* was the first selected yeast due to profound knowledge about genetics and physiology (Cregg et al., 1993). Although expression times of this system is quicker and fermentation is cost-effective, *Saccharomyces* has numerous problems like instability of the plasmid during scale-up, low protein yields and the hyperglycosylation of proteins, and allergenic symptoms of the proteins N-linked glycosylation terminated via α-1,3-linked mannose residues. Therefore, this expression system is not ideal for many therapeutic proteins' production (Cregg et al., 1993, Gellissen et al., 2005). The methylotrophic yeast *P. pastoris* has been developed as an alternative expression system to *S. cerevisiae* due to well-

established fermentation protocols and strong, tightly-regulated promoters (Cregg et al., 1993). Capability to utilize methanol as a sole carbon and energy source, non-fermentative use of glucose, and efficiently metabolize glycerol to reach high cell densities makes this yeast attractive for bioprocesses (Prielhofer et al., 2015). In addition to these, it has host strains and expression vectors and has the ability to reach high cell densities in a simple and defined medium (Zhang et al., 2000).

Table 2.3 Expression systems by analogy (Pourmir and Johannes, 2012; Vogl et al., 2013).

Prevalently used species	Higher eukaryotes		Yeasts		*E. coli*
	Mammalian cells	Insect cells	*P. pastoris*	*S. cerevisiae*	
Genetic Modifications	Moderate		Simple		Simple
	Low efficiency of stable gene integration, tendency to multiple gene insertions		High transformation efficiencies, maintenance of stable plasmids		Well-established manipulation tools, maintenance of stable plasmids, well understood genetics
Cultivation	Slow growth rates, requirement to complex nutrients, tendency to undergo apoptosis		Rapid growth on simple media to reaching to high cell density, GRAS status		Fastest growth rates, low cost medium
Contaminations	High risk of contamination		Low risk of endotoxins or viral DNAs		Phage infections
Post-translational modifications	Similar to human post-translational modifications		Proteolytic processing, correct folding of eukaryotic proteins, risk of hyperglycosylation of proteins		Limited set of post-translational modifications
Protein yields	High productivity, highly variable expression levels		High yields, high level expression and secretion		High foreign protein expression, low secretion capacity, extensive downstream processing

2.5 *Pichia pastoris* as a Host Microorganism

During recombinant protein production, one of the most crucial prerequisite is the preference of the suitable expression vector and complementary host strain. Ogata et al., (1969) firstly discovered the yeast species that metabolize methanol as sole carbon and energy source. Four yeast genera namely, *Hansenula*, *Candida*, *Pichia*, *Torulopsis* are found to be capable of utilizing monocarbonic compounds and they were named as methylotrophic yeasts. Therewith, methylotrophs, especially *Pichia* genera have been in the spotlight for the production of single-cell protein (SCP) to be used as animal feed (Cos et al., 2006). These specific strains were intensely studied both to investigate their potential for commercial applications and to examine and disclose the specific cell compartments namely, peroxisomes (Faberet al., 1995). In 70's, in order to obtain high cell densities of *Pichia pastoris*, Phillips Petroleum Company developed growth media and fermentation protocols to use in the production of single-cell protein (SCP) from methanol as animal feed (Cereghino and Cregg 2000). However, due to oil crisis in 1970s cost of methane drastically increased and therefore production of SCP from methanol lost its significance. On the other hand, in 1980s with the collaboration of Phillips Petroleum Company and Salk Institute Biotechnology/Industrial Associates Inc (SIBIA, La Jolla, CA, USA) this yeast used for heterologous protein expression both for academic and industrial purposes (Cos et al., 2006) and since then, the methylotrophic yeast *Pichia pastoris* has been extensively used as a host microorganism by expressing more than 500 pharmaceutical compounds and recombinant proteins (Macauley-Patrick et al., 2005). Various, heterologously expressed biopharmaceutical products produced in *Pichia pastoris* have been approved by US Food and Drug Adminstration (FDA) such as Kalbitor® which is a kallikrein inhibitor used in treatment of Hereditary angioedema (Dyax), Insugen® which is human insulin used in treatment of diabetes (Biocon) and Shanferon™ which is interferon-alpha 2b used in Hepatitis C and cancer treatment (Shantha/Sanofi) (http://pichia.com/).

2.5.1 Physiology and Properties of the Yeast *Pichia pastoris*

Yeasts are unicellular fungi which reproduce generally by budding, and sometimes by fission. Their cell sizes differ depending on the species and growth conditions from only 2–3 μm to 20-50 μm in length and between 1 μm and 10 μm in width (Walker, 2009). *Pichia pastoris* is a methylotrophic, mesophilic, and unicellular budding yeast that has oval shape with 5-30 μm in length and 1- 5 μm in width. Along with being homothallic yeast, it is normally stable in the vegetative haploid state unless it is forced to mate (Cereghino and Cregg 2000). *P. pastoris* is also facultative anaerobe and Crabtree negative yeast meaning that under aerobic conditions fermentative product is not formed even if high substrate concentration.

P. pastoris (*Komagataella pastoris)* is taxonomically classified as in the Domain of *Eukaryota*, Supergroup *Opisthokonta*, under the Kingdom Fungi, Subkingdom Eumycota, Phylum Ascomycotina, Class Hemoascomycetes, Order Endomycetales, Family Saccharomycetaceae, and Subfamily Saccharomycetoideae, Genus *Komagataella*, Species *Komagataella pastoris* (Damasceno et al., 2012). Besides, other *Komagataella* genus consist of the strains are *Komagataella pastoris*, *Komagataella phaffii*, *Komagataella populi*, *Komagataella pseudopastoris*, *Komagataella ulmi*, *Ogataea glucozyma*, and *Pichia membranifaciens* (Kurtzman, 2012).

2.5.2 Genetic Organization

Complete genome sequence of strain GS115, DSMZ 70382 and, CBS7435 were published in order to develop systems biology of *P. pastoris* by identifying new promoters and by understanding the intracellular flux distributions, growth properties, and protein secretion under different cultivation conditions. 9.43 Mbp genomic sequence of the strain GS115 of *P. pastoris* is organized in four chromosomes with 5,313 protein-coding genes, 41.6% GC content, 4.90 Mbp genomic sequence of the strain DSMZ 70382 was predicted to have 5,450 ORFs with a GC 41.9% and total size of the strain CBS7435 was determined as 9.35 Mbp

with 5007 protein-coding genes (De Schutter et al. 2009, Mattanovich et al. 2009a, Küberl et al., 2011).

2.5.3 *Pichia pastoris* Host Strains and Expression System

All the *P. pastoris* expression strains are derivatives of the wild-type strain NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL, USA). Commercially available strains reconstituted prototrophic strain X-33, GS115 (*HIS4*), KM71 and KM71H (*AOX1*), protease-deficient strains SMD1168 (*HIS4 PEP4*) and SMD1168H (*PEP4*), and PichiaPink™ (*ADE2*) are the frequently used host strains. In addition, three types of host strains which vary according to their methanol utilization ability (2.6.1.1) and auxotrophic strains were given in 2.9 in detail.

The methylotrophic yeast *P. pastoris* has become favored expression system of a wide variety of heterologous proteins and metabolites. The unique and striking feature of this expression system is having a tightly regulated, strong, methanol-inducible AOX1 promoter and achieving high-level and controlled recombinant protein expression for both laboratory research and industry (Cregg et al.,1993). Also, *P. pastoris* prefers respiratory growth which enables it to reach high cell densities in minimal media. The fermentation is easily scaled-up therefore, parameters such as pH, aeration and carbon source feed rate affecting protein productivity become controllable (Higgins and Cregg, 1990). By combining the advantages of ease of use and manipulation with available protocols, low cost, strongly inducible and constitutive promoters, rapid growth, ability to express high levels of heterologous proteins, ability to perform many eukaryotic co-translational and post-translational modifications such as glycosylation, disulphide bond formation and proteolytic processing (Cregg et al.,2000) and simple purification of secreted recombinant proteins due to the relatively low levels of endogenous protein secretion (Cregg et al.,1993) makes this expression system attractive and promising for recombinant protein production. Advantages and disadvantages of this expression

system was presented in Table 2.4 (Thorpe et al.,1999; Cereghino and Cregg, 1999; Jahic et al., 2006; Macauley-Patrick et al., 2005).

Table 2.4 Advantages and disadvantages of *P. pastoris* expression system.

Advantages	**Disadvantages**
• Rapid growth on chemically well-defined, inexpensive media • High yield and productivity • Strong promoters (P_{AOX1}, P_{GAP}) • High intra- or extracellular level protein expression • Wide pH range • Preference for respiratory growth • Eukaryotic post-translational modifications • Time and effort saving • High transformation efficiencies • Capability of maintaining stable plasmids • Hyperglycosylation is not as much as in *S. cerevisiae.* • Low purification cost • No endotoxin problem • Non-pathogenic	• High level proteolytic activity • Longer cultivation time than bacteria • Non-native glycosylation • Incapability to accomplish complex post-translational modifications • During the bioprocess, it is difficult to track methanol while induction of AOX1 promoter • Flammable and toxic feature of methanol • As a petrochemical byproduct methanol may not be applicable in food industry • Stockpile of methane causes risk of fire hazard in industry

In design of cloning and expression of heterologous proteins, there are several genetic factors taking into consideration (Ahmad et al., 2014). The list was given below and the schematic representation was given in Figure 2.6.

- Promoter selection
- A - T composition of the gene
- Secretion signal peptide characteristics
- Gene dosage
- Promoter-terminator combination
- Codon usage of the expressed gene
- Appropriate selection markers
- Proper protein folding in the ER and Golgi
- Post-translational modifications (PTMs)
- Proteolytic process

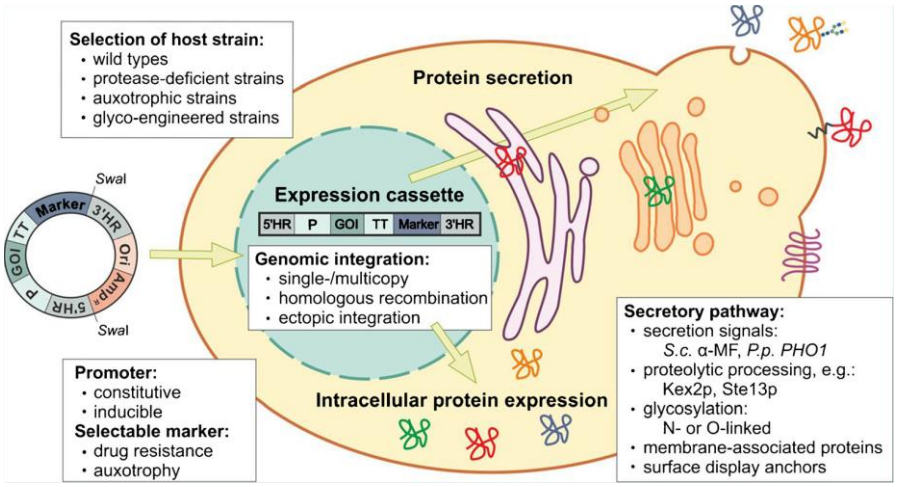


Figure 2.6 Crucial parameters influencing heterologous gene expression in *P. pastoris* (Ahmad et al., 2014).

2.6 Promoters

Gene expression is a process by which the nucleotide sequence of a particular gene utilized in the synthesis of a gene product, generally a protein. The process contains mainly two steps namely, transcription (synthesis of mRNA by RNA polymerase from the DNA) and translation (utilization of mRNA as a template in order to synthesize protein). Transcription of a gene most certainly regulated by the regulatory elements named as enhancers, that affect the strength of the promoters by increasing the RNA polymerase activity, by the certain proteins termed as transcription factors which are required for initiation of transcription, and by the gene promoters (Figure 2.7). Promoters are specific nucleotide sequences located on DNA which are the binding sites of RNA polymerases due to having more affinity to RNA polymerase enzymes. These nucleotide sequences generally present at the upstream of the coding sequence leading the initiation of transcription of a certain gene.

In the course of gene expression, selection of a suitable promoter is a key parameter. If the aim is to augment the expression level of a cloned gene, a vector with a considerably effective promoter (has high affinity for RNA polymerase) is preferred and such promoters are generally called as strong promoters. Nevertheless, when the cloned gene exert toxic effect to the host cell weak promoters are chosen to prevent the cell death. Besides, expression level of the gene can be regulated by utilizing the promoters of the genes which are either inducible or repressible.

2.6.1 Inducible Promoters

Expression of an inducible gene implies to these which can be activated (induced) to enhance the mRNA level and activity of their promoters are affected by the presence of the chemical or physical factors. Therefore, they are switched on/off systems allowing controlled heterologous protein production. Most commonly utilized *P. pastoris* inducible promoters were explained in detail below and novel, inducible *P. pastoris* promoters were listed in Table 2.5.

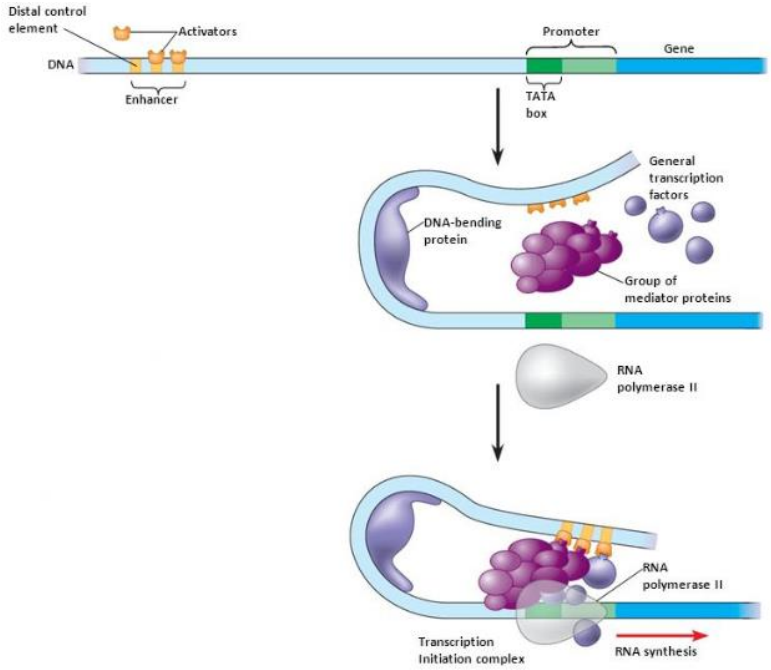


Figure 2.7 Transcription initiation at eukaryotic promoters (2008 Pearson Education, Inc., publishing as Benjamin Cummings).

2.6.1.1 Alcohol oxidase (P_{AOX}) Promoter

Genome of the *P. pastoris* has possession of two alcohol oxidase genes (*AOX 1* and *AOX 2*). In order to understand the expression of those two enzymes in the methanol metabolism of *P. pastoris*, alcohol oxidase I gene (*AOX1*) alcohol oxidase II gene (*AOX2*) were firstly isolated and characterized by Ellis et al. (1985) and by Cregg et al. (1989), respectively. *AOX1* which is the first enzyme in the methanol utilization pathway has strong and tightly regulated AOX1 promoter (P_{AOX1}). P_{AOX1} based gene expression was firstly used by Tschopp et al. (1987) and since then P_{AOX1} has become the most frequently used promoter. In a *P. pastoris* cell, 85% of the alcohol oxidase activity is controlled by *AOX1* promoter while the remaining 15% is regulated by AOX2 promoter (Zhang et al., 2000). When P_{AOX1} is used for heterologous gene expression, utilization of methanol provides induction, biomass growth, and production (Hartner et al., 2008). However, if glucose, glycerol or ethanol used as carbon sources, P_{AOX1} is strongly repressed, the

promoter is de-repressed after depletion of these sources and induced with the methanol addition (Inan and Meagher 2001, Cereghino et al., 2002). This feature makes P_{AOX1} driven expression systems tightly controlled and allow strains to produce heterologous genes which may be toxic to cells (Daly and Hearn 2005). Affinity to methanol and oxygen is lower for alcohol oxidase gene and with methanol feeding, up to 30% of the total intracellular proteins and approximately 5% of the total mRNA of the AOX enzyme can be present cells (Cereghino et al., 2000).

Three *P. pastoris* phenotypes exist with respect to methanol utilization (Daly and Hearn 2005).

Methanol Utilization Phenotypes

- Methanol Utilising Plus (Mut^+) Phenotype: Mut^+ (i.e. GS115) strain with two functional AOX genes is obtained by single crossover integration of a cassette into histidinol dehydrogenase gene, HIS4, or AOX1 locus. This phenotype has wild-type methanol utilization and therefore growth characteristics remain unchanged with a specific growth rate of 0.14 $h^{-1}$and a large quantity of methanol can be required during induction (Cereghino et al., 2002; Jungo et al., 2006; Cos et al., 2006).
- Methanol Utilising Slow (Mut^S) Phenotype: Mut^S (i.e. KM71) strain is constituted by double homologous recombination with an expression cassette in AOX1 locus or by gene insertion inactivating the *AOX1* gene (Romanos et al., 1992, Cregg and Madden 1987). Knock-out of AOX1 gene results in slow growth and slow methanol utilization because cells depend on AOX1 for methanol metabolism. Also, the strain needs alternative carbon sources for growth, it has lower oxygen demand and its specific growth rate decreases to 0.04 h^{-1} (Daly and Hearn 2005, Jungo et al., 2006).
- Methanol Utilising Minus (Mut^-) Phenotype: Mut^- (i.e. MC100-3) strain is generated by disrupting the both *AOX1* and *AOX2* genes. This phenotype cannot use methanol and cannot grow on methanol therefore it requires

alternative carbon sources for growth and r-protein production. As it is expected, their growth rate on methanol is 0 h^{-1} (Jungo et al., 2006).

Although high levels of r-proteins can be produced with the strong promoter AOX1, up to 12 g L^{-1} (Cregg et al. 1993), there are drawbacks in the usage of methanol as a carbon sources in processes. (1) induction with methanol may result in oxidative stress in production strain, that cause reduced cell viability, cell lysis or proteolytic degradation, (2) methanol, as a by-product of petrochemical industry, may be inappropriate for the production of food products due to toxicity, (3) methanol has potential fire hazard due to flammability, (4) during cell growth on methanol, oxygen requirement is far more higher than glucose or glycerol, (5) usage of high amount of oxygen produces notably heat requiring extra effort to control culture in the bioreactor (Hohenblum et al. 2004, Zhan et al., 2016). Consequently, promoters which are not induced by methanol have become more inviting for the production of certain foreign genes.

2.6.1.2 Formaldehyde Dehydrogenase (P_{FLD}) Promoter

Formaldehyde dehydrogenase (*FLD*) is a crucial enzyme in the methanol metabolism and similar to P_{AOX1} by utilizing methanol as a carbon and energy source (Veenhuis et al., 1983). Additionally, *FLD* is implicit in certain alkylated amines metabolism as nitrogen sources. The primary function of *FLD* is to protect the cells from toxic effects of formaldehyde which is formed along with excess methanol (Sibirny et al., 1990). Cells contain at most 20% FLD enzyme of total cellular protein (Gellissen 2000). FLD gene is inducible that utilizes methanol as sole carbon and energy source and methylamine as sole nitrogen source. Using this feature as an advantage, methylamine can be used as an inducer and glucose or glycerol preferable as carbon sources in the production of foreign proteins. FLD1 promoter (P_{FLD1}) is tightly regulated as AOX1 promoter and its transcriptional efficiency is high. Therefore, P_{FLD1} has become an attractive alternative to conduct a methanol-independent recombinant protein expression in *P. pastoris*. Shen et al. (1998) compared activity levels of β-Lactamase (β-lac) under P_{FLD1} and P_{AOX1}

promoters and under FLD1 promoter almost twice β-lac activity (~530 nmol mg^{-1} min^{-1}) was obtained in media that contain methanol as carbon source, methylamine or ammonium sulfate as nitrogen source. In another study effect of nitrogen sources were investigated. FLD1 promoter is ether co-induced by methanol and methylamine or methanol and ammonium and it was found that the activity level of *Rhizopus oryzae* lipase (19 U mL^{-1}) was higher when induced with methylamine (Resina et al., 2005).

2.6.1.3 Isocitrate lyase (P_{ICL1}) Promoter

The gene that encodes isocitrate lyase (*ICL1*) was isolated and characterized by Menendez et al. (2003). Dextranase gene (dexA) from *Penicillium minioluteum* was expressed under the control of P_{ICL1} with different carbon sources. Thus, it was found that the ICL1 promoter induced by ethanol whereas repressed by glucose and depletion of glucose leads to derepression. Being expression of the protein adjusted by carbon source and controlled by culture conditions, P_{ICL1} can be regarded as a good alternative promoter for the expression of the heterologous proteins (Menendez et al., 2003, Cos et al., 2006) nevertheless its expression level was not compared either with P_{AOX1} or P_{GAP}.

2.6.2 Constitutive Promoters

Besides the genes regulated, several genes being essential at any time, are expressed throughout the life of the cell. These kinds of genes are termed as housekeeping or constitutive genes. Likewise, promoters of these genes, constitutive promoters, enable the continuous transcription of a gene while being active in all circumstances. Constitutive promoters which are commonly used in heterologous protein production in *P. pastoris* were explained comprehensively and novel, constitutive *P. pastoris* promoters were listed in Table 2.5.

2.6.2.1 Glyceraldehyde-3-phosphate dehydrogenase (P_{GAP}) Promoter

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a NAD-binding tetrameric protein participates in the glycolysis and gluconeogenesis pathways. GAPDH consists of 333 amino acid residues and has a molecular weight of 35.4 kDa (Waterham et al., 1997). *GAPDH* was firstly isolated from *P. pastoris* genome by Cregg et al. (1989) and it is expressed at high levels under the control of its own promoter P_{GAP}. Although, being strong as P_{AOX1}, P_{GAP} driven *GAPDH* expression levels and r-protein production differs with respect to the carbon sources utilized during cell growth. Under the control of P_{GAP}, β-lactamase activity was investigated with different carbon sources such as glucose, glycerol, methanol and oleic acid by Waterham et al. (1997) and it was found that the highest β-lac activity was obtained by glucose-grown cells whereas the lowest activity was measured by methanol-grown cells. Besides, the activity of the GAP promoter was reduced to one-third when methanol was used in two thirds when glycerol was used as the carbon source (Cereghino and Cregg 2000). In addition, fructose, sorbitol, mannitol, and trehalose could be selected as alternative carbon sources because they get involved in the glycolysis pathway in the carbon metabolism (Çalık et al., 2014).

P_{GAP} is a tightly regulated promoter that yields strong and constitutive protein expression on glucose with a comparable level of AOX1 promoter. Genetic factor optimization and improved fermentation strategies also enable higher expression levels (Çalık et al., 2014). Constitutive expressions are more advantageous than inducible expression systems. They promote continuous transcription, simplify processes and exclude the inducers that have potential hazard (Ahmad et al., 2014). One of the most significant properties of P_{GAP} is the requirement to methanol for induction is extinguished. In addition, a more straight forward growth is obtained because carbon source shift is not necessary anymore (Cereghino and Cregg 2000) and in the cultures cell growth would not be restricted by the oxygen limitation contrary to P_{AOX1} driven systems. Further, by-products of the methanol metabolism, such as formaldehyde and hydrogen peroxide are not accumulated in the culture. On the other hand, constitutive GAP promoter is not

appropriate for the production of proteins which are toxic to the cell (Cereghino and Cregg 2000).

In literature, there are several researches indicating that heterologous protein expression is higher with P_{GAP} than of P_{AOX1}. For instance, Menendez et al. (2004) reported 1.3-fold higher fructose releasing exolevanase (LsdB) activity under P_{GAP}, Goodrick et al. (2001) observed similar productivity in a fed-batch fermentation and five-fold higher productivity was achieved in a continuous strategy for the production of human chitinase under P_{GAP}. On the other hand, a certain number of studies suggested that the P_{AOX1} than of P_{GAP}. Five-fold higher productivity of cellobiohydrolase was obtained by Boer et al. (2000) under the control of P_{AOX1}, β- glucuronidase activity was enhanced to 8-fold by using P_{AOX1} (Sears et al., 1998), likewise Debaryomyces castellii phytase was expressed in a fed-batch operation and in 302 U mL^{-1} activity was obtained under the control of P_{AOX1} expression whereas 16.5 U mL^{-1} activity was observed by P_{GAP} (Ragon et al., 2008).

Higher amount of heat generation was measured in methanol combustion than glycerol or glucose combustion. Due to critical influence of temperature on maintenance of product quantity and/or quality, P_{AOX1} driven expression systems requires additional cooling efforts. Therefore, in large-scale (low heat transfer efficiency) heterologous protein productions, P_{GAP} driven expression systems are favored excluding the methanol utilization (Çalık et al., 2015).

2.6.2.2 Phosphoglycerate kinase (P_{PGK1}) Promoter

Being involved in glycolysis and gluconeogenesis, phosphoglycerate kinase (*PGK*) enzyme which generates ATP and 3-phosphoglycerate by transporting a phosphoryl group from1,3-bisphosphoglycerate to ADP is expressed constitutively (de Almeida et al., 2005). Although, in principle, *PGK* expression is constitutive, carbon source alters the strength of the expression since the mRNA levels on glucose is two-fold higher than glycerol. P_{PGK1} was firstly used in the expression of alpha-amylase by de Almeida et al. (2005) and was reported that high

expression levels were achieved. However, studies with three different reporter proteins revealed P_{PGK1} expression is only about 10% of the P_{GAP} (Stadlmayr et al., 2010).

2.6.2.3 Translation elongation factor 1 alpha (P_{TEF1}) Promoter

The translation elongation factor 1-α is liable for the transport of aminoacyl tRNAs to the ribosomes in order to maintain the peptide chain elongation (Slobin, 1980). *TEF1* regulation and expression level of its promoter was primarily investigated by using a lipase fused to a cellulose binding domain as reporter gene (Ahn et al., 2007). It was disclosed that mRNA level is dependent on growth, that is, it is high in the exponential phase and decreases in the stationary phase and approximately twofold higher secreted lipase activity than P_{GAP} was obtained in carbon limited fed-batch cultivations. However, although P_{TEF1} is a strong promoter, expression of the reporter protein varies according to reporter protein and cultivation time when compared with P_{GAP} (Stadlmayr et al., 2010).

2.7 Double Promoter Expression Systems of *Pichia pastoris*

Regulation of transcription of a gene is provided by DNA regions termed promoters. They are crucial regulatory elements controlling strength of the expression and spatial and temporal expression of genes. Therefore, strong promoters are one of the most significant components for recombinant protein production.

Table 2.5 Novel *P. pastoris* promoters.

Gene name	Gene product	Regulation	Reference
DAS	Dihydroxyacetone synthase	Induced by methanol	Tschopp et al. (1987)
PEX8	Peroxisomal matrix protein	Induced by methanol or oleate	Liu et al. (1995), Lin-Cereghino, Cregg (2000)
PHO89 or *NSP*	Sodium-coupled phosphate symporter	Induced by phosphate limitation	Munsterkotter et al. (2000), Lee et al. (2007), Ahn et al. (2009)
ENO1	Enolase	Constitutive	Uemura et al. (1985)
GPM1	Phosphoglycerate mutase	Constitutive	Stadlmayr et al. (2010)
HSP82	Cytoplasmic chaperone (Hsp90 family)	Constitutive	Stadlmayr et al. (2010)
ILV5	Acetohydroxy acid isomeroreductase	Constitutive	Hubmann (2005)
KAR2	ER resident chaperone (Bip)	Constitutive	Stadlmayr et al. (2010)
KEX2	Endopeptidase involved in the processing of secreted proteins	Constitutive	Hubmann (2005)
PET9	ADP/ATP carrier of the inner mitochondrial membrane	Constitutive	Stadlmayr et al. (2010)
SSA4	Heat shock protein	Constitutive	Stadlmayr et al. (2010)
RPS7	Ribosomal protein of the small (40S) subunit	Constitutive	Müller et al. (1998)

Table 2.5 Novel *P. pastoris* promoters (Continued).

Gene name	Gene product	Regulation	Reference
TPI1	Triose phosphate isomerase	Constitutive	Moir and Dumais (1987)
YPT1	GTPase involved in secretion	Constitutive	Sears et al. (1998), Lin-Cereghino, Cregg (2000)
THI11	Protein involved in thiamine biosynthesis	Repressed by thiamin	Stadlmayr et al. (2010)
PIS1	Phosphatidylinositol synthase	Repressed by glycerol	Anderson and Lopes (1996)
PHO8	Repressible alkaline phosphatase	Repressed by high phosphate	Munsterkotter et al. (2000)
MET3	ATP sulfurylase	Repressed by methionine	Delic et al. (2014)
SER1	3-phosphoserine aminotransferase	Repressed by zink	Delic et al. (2014)
THR1	Homoserine kinase	Repressed by L-threonine, Lvaline, L-leucine and L-isoleucine	Delic et al. (2014)

Double promoter expression systems could be applied in several conditions such as:

- Co-expression of two heterologous proteins in a host cell
- Simultaneous expression proteins such as collagen
- Control of gene product concentration by inducing culture medium
- Trace of the foreign gene with simultaneously expressed fluorescent reporter protein
- Evaluation of the influences of two or more proteins on each other
- Establishment of a basis for the constitution of poly-promoter vectors according to the double promoter vectors
- Enhancement of the heterologous protein production

In order to achieve high level heterologous protein production, double promoter expression systems have been widely used in several microorganisms especially in bacteria such as *E. coli* and *B. subtilis* (Ivanov et al., 1993; Winder et al., 2000). For that purpose, several combinations of promoters with different characteristics were employed in foreign protein productions. In the studies, regulations of the promoters (inducible or constitutive), directions of the promoters, and functions of the promoters in different growth conditions (being active in exponential and stationary growth phases), for bacteria, were taken into consideration (Phanaksri et al., 2015; Kang et al., 2010; Wu et al., 2003; Yang et al., 2013).

Among the yeast cells, *S. cerevisiae*, a model, eukaryotic model yeast cell, is commonly used for the investigations of the double promoter expression systems both for the co-expression of the proteins (Kim et al., 2003; Gellissen et al., 1996, Zietsman et al., 2010) and for the enhancement of foreign protein production (Sinah et al., 2012). Various combinations of promoters were tried including, constitutive TEF1 promoter was combined with inducible PGK1 promoter were combined in an

expression vector in opposite directions to produce β-Galactosidase (Partow et al., 2010), as a novel approach *E. coli*, T7 phage promoter and in *S. cerevisiae* CUP1 or MET25 promoters were used in a single vector to produce Glutathione Stransferase (GST) protein by Sinah et al. (2012), constitutive TEF1 promoter and the inducible GAL10 or HXT7 promoters were combined in an expression vector and double expression system with TEF1 and HXT7 promoters yielded higher β-Galactosidase activity in long term bioprocesses ((Vickers et al., 2013).

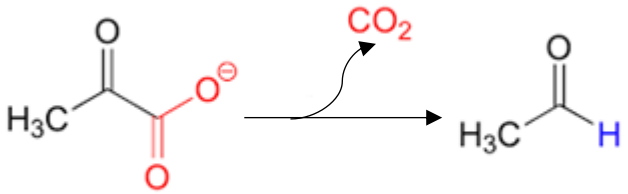
Likewise, double promoter systems were used in protein co-expression and for the development of heterologous protein expression in *P. pastoris*. Two subunits (α and β) of Pea (*Pisum sativum*) mitochondrial pyruvate dehydrogenase (E1) was co-expressed in *P. pastoris* under the control of P_{AOX1} (Moreno et al., 2000), under AOX1 promoter driven expression system productivity of human serum albumin (HSA) and human growth hormone (hGH) fusion protein (HSA/GH) and co-expression either with PDI (Protein disulfide isomerase) or ERO1 (ER Oxidoreductase 1) were investigated by Wu et al. (2014) to increase the secretion capacity of the protein. Since 2003 double promoter expression systems have been utilized in *P. pastoris* for recombinant protein productions. Unlike *S. cerevisiae* two vector systems almost always including inducible (AOX1) promoter and constitutive (GAP) promoter were subsequently transfected into the genome of the yeast (He et al., 2015; X. He et al., 2008; Parashar et al., 2016; Wu et al., 2003). Besides, the only study using two vector systems with the same promoter, AOX1 was reported by Tang et al. (2014). However, up to date there has been no study that stated containing two constitutive promoter expression systems. Expressed genes and their expression levels were summarized in Table 2.6.

Table 2.6 Double promoter expression systems used in heterologous protein production in *P. pastoris*.

Strain	Plasmid	Regulation	Expressed Gene	Expession Level	Reference
GS115	pGAPZαA-hGM-CSF pPIC9K-hGM-CSF	Constitutive expression under P_{GAP} and inducible expression under P_{AOX1}	human granulocyte-macrophage colony-stimulating factor (hGM-CSF)	2-fold higher hGM-CSF expression than constitutive expression	Wu et al. 2003
GS115	pGAPZαA-manA pPIC9K-man-A pPIC9K-manA	Constitutive expression under P_{GAP} and inducible expression under P_{AOX1}	β- mannanase	Almost 1.8-fold higher β-mannanase expression than constitutive expression	He et al. 2008
GS115	pPIC9K-Auman5A pPICZαA-Auman5A	Inducible expression under P_{AOX1}	acidophilic β-mannanase (Auman5A)	2-fold higher Auman5A activity	Tang et al. 2014
GS115	pGAPZαA-rml pPIC9K-rml	Constitutive expression under P_{GAP} and inducible expression under P_{AOX1}	*Rhizomucor miehei* lipase (RML)	Approximately 6-fold higher RML expression compared to both constitutive and inducible expression	He et al. 2015
X-33	pGAPKαA-Amy PICZαA-Amy pPICZαA-Phy pGAPKαA-Phy	Constitutive expression under P_{GAP} and inducible expression under P_{AOX1}	α-amylase and phytase	1.8-fold higher α-amylase expression than inducible expression and 1.3-fold higher phytase than inducible phytase expression	Parashar et al. 2016

2.8 The Gene Pyruvate Decarboxylase

Pyruvate decarboxylase (*PDC*) with E.C. 4.1.1.1 is a thiamine pyrophosphate (TPP) inclusive enzyme that maintains the formation of acetaldehyde from pyruvate by non-oxidative decarboxylation in several mesophilic organisms in presence of cofactor, Mg^{2+} (Eram and Ma, 2013) and throughout the fermentative growth acetaldehyde is utilized in the reoxidation of NADH (Maris et al., 2004). Catalyzing the initial reaction in the fermentative branch, the gene is presented in the mid position of the fermentative and respiratory carbon catabolism (Maris et al., 2004). Activity of the gene varies between the Crabtree-positive and Crabtree-negative yeasts. Approximately, six times more *PDC* activity was detected in Crabtree-positive yeasts in the presence of high amount of sugar (Urk et al., 1990) contrary to Crabtree-negative yeasts due to restriction in the glucose uptake regulated by the H^+-symport systems (van Urk et al., 1989). In addition to sugar concentration, presence of oxygen affects Crabtree-positive and Crabtree-negative yeasts. Crabtree-positive yeasts existence of oxygen and high sugar concentration shifts the metabolism to the tricarboxylic acid (TCA) cycle due to high affinity to *PDC* (van Maris et al., 2004).



pyruvate acetaldehyde

In the genome of *S. cerevisiae*, a Crabtree-positive yeast, six *PDC* genes were identified and three of them (ScPDC1, ScPDC5, and ScPDC6) was stated as responsible for catalytic protein expression whereas two of them (ScPDC2 and ScPDC3) express regulatory proteins. Differently from the yeast *S. cerevisiae*, in Crabtree-negative yeast, *P. pastoris* there is no ethanol production under aerobic conditions. When the genome of *P. pastoris* GS115 was sequenced, a putative (*PpPDC*) gene was identified in the third chromosome (De Schutter et al. 2009,

Mattanovich et al. 2009) of the strain. The gene *PpPDC* was expounded as PAS_chr3_0188 in the genome of GS115 strain and its position along with the direction was introduced in Figure 2.8. Several carbon sources can be used by *P. pastoris*. Besides the ultimate *PDC* activity was observed when glucose was utilized and growth on methanol and ethanol yields insignificant *PDC* activity. Connecting the respiratory and the fermentative pathways in the central carbon metabolism (Figure 2.9) and consequently oxygen dependent *PpPDC* is therefore a significant enzyme (Çalık et al., 2015).

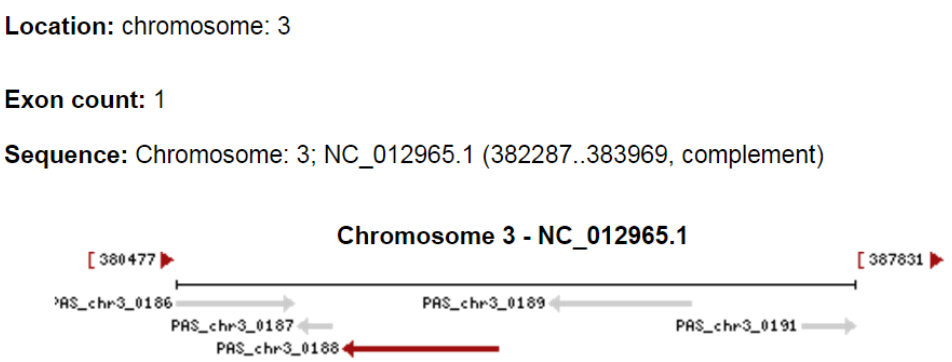


Figure 2.8 The chromosomal position and direction of the *PpPDC* in *P. pastoris* GS115. Red arrow specifies the gene *PpPDC* and grey arrows refer to the adjacent genes (**WWW.NCBI.NLM.NIH.GOV**)

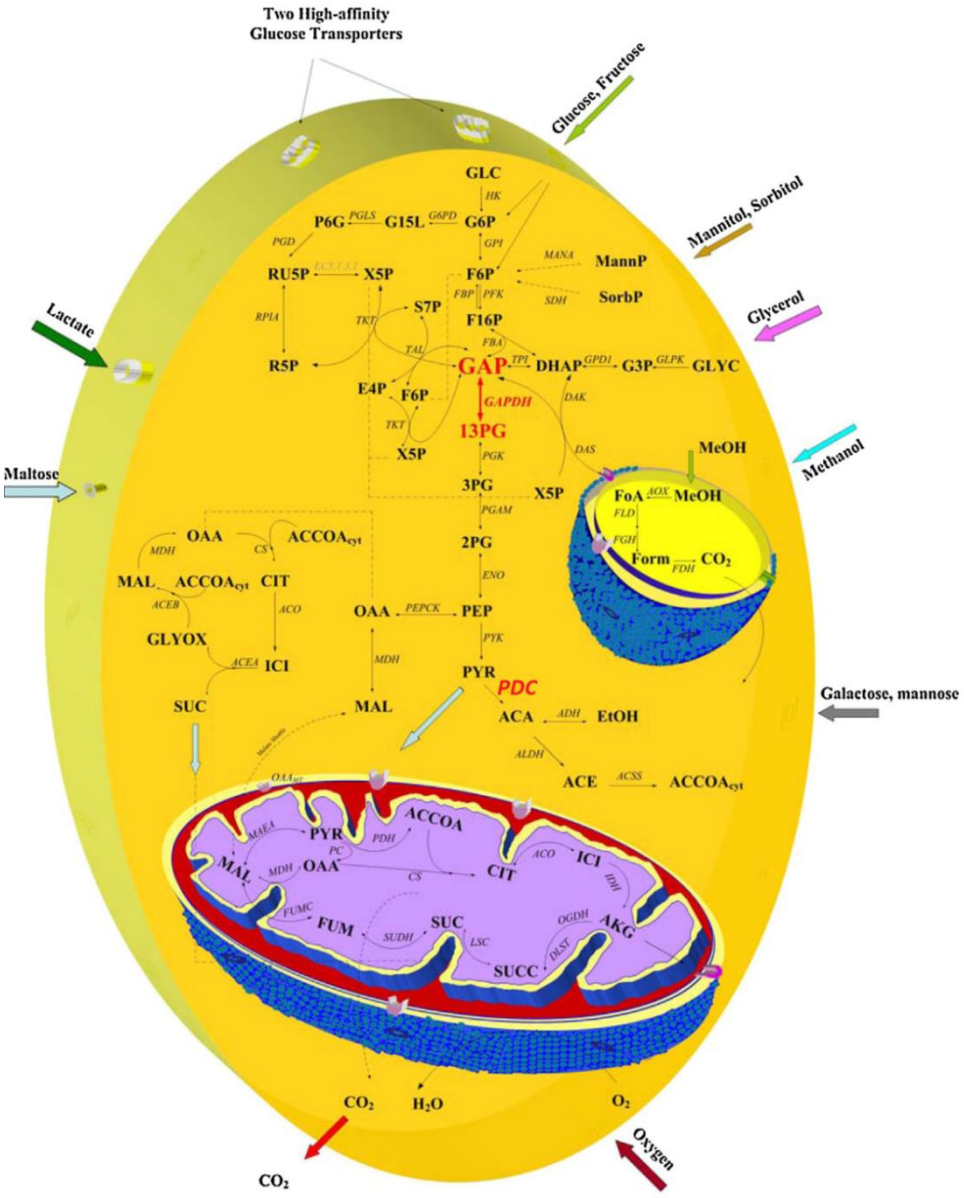


Figure 2.9 Central carbon metabolism of the yeast *P. pastoris* (Çalık et al., 2015).

2.9 Selectable Markers

A selectable marker is a gene which is introduced to host microorganism by an expression vector. It can either be antibiotic resistance or biosynthetic genes. There are several selectable markers that have been developed for *P. pastoris* expression systems and all expression systems have been reproduced by the wild-type NRRL-Y11 430 (Northern Regional Research Laboratories, IL, USA) strain (Macauley-Patrick et al., 2005). Auxotrophic strains, which are formed from histidine biosynthetic pathway, *HIS1*, *HIS2*, *HIS4* (hydroxyhistidinase), *HIS5* and HIS6; from the biosynthetic pathway of arginine *ARG1*, *ARG2*, *ARG3*, *ARG4* (argininosuccinate lyase) from the biosynthetic pathway of uracil URA3 (orotidine 5P-phosphate decarboxylase) and URA5 are the generally preferred as selectable markers (Fickers 2014). Other biosynthetic markers, *ADE1* (PR-amidoimidazolsuccinocarboxamide synthase) (Cereghino et al., 2001), *MET2* (homoserine-O-transacetylase) (Thor et al., 2005) and *FLD1* (formaldehyde dehydrogenase) (Sunga and Cregg, 2004) are essayed. In addition to biosynthetic selection markers bacterial antibiotic resistance genes can be used in *P. pastoris*. Two dominant markers, *Sh ble* from *Streptoalloteichus hindustanus* that bring resistance to the bleomycin-related drug zeocin which cleaves DNA occasioned cell death and *BSD* (blasticidin) which is produced by the bacterium *Streptomyces griseochromogenes* inhibits translation, can be applied for the direct selection of the transformants (Drocourt et al., 1990). Aminoglycoside antibiotic geneticin (G418) resistance gene, which is similar to the bacterial kanamycin *Tn903kan*^{r} gene, is obtained from *Micromonospora rhodorangea*. This antibiotic inhibits the elongation step therefore ceases the polypeptide synthesis. However, this marker cannot be used for direct selection of the transformants, requires additional steps with special auxotrophic strains (Cereghino et al., 2008).

2.10 Gene Dosage

Expression levels of the genes are affected from various genetic and physiological determinants. Although, enhancing gene copy number up to a certain level generally yields higher amounts of heterologous proteins, copy number of the gene to be expressed may be occasionally regarded as a bottleneck due to complete expression pathway of the cell (Macauley-Patrick et al., 2005). Therefore, features of the construct and the foreign protein lead the threshold value.

In order to determine the threshold value various methods are being used. Although one of the most commonly used methods is southern blot analysis (Vassileva et al., 2001; Hohenblum et al., 2004; Sunga and Cregg, 2004; Inan et al., 2005) the analysis needs high amount of DNA and it has the risk of restriction site drop-out. Also, as a rapid method, the relation between the high copy number and resistance to high amount of antibiotic is preferred (Scorer et al., 1994) but this approach only gives an idea about the multi-copy integrations not an exact gene copy number. As a rapid, reliable and precise method, quantitative real-time PCR (qRT-PCR) was firstly developed for the yeast *P. pastoris* by Abad et al. (2010) which enables to quantify the integrated cassettes both absolutely and relatively.

In the production of hepatitis B surface antigen (HbsAg) protein under P_{GAP} in *P. pastoris*, without affecting the transcriptional level, four-fold increase was measured up to four copies of HbsAg gene (Vassileva et al., 2001). Also, increasing number of copies of β-galactosidase was expressed under P_{FLD1} by Sunga and Cregg (2004) and activity of the enzyme was enhanced 17-fold in the existence 22 copies of the gene. Expression level of the gene, human trypsinogen, was enhanced utilizing AOX1 promoter when the copy number was determined as two; however, it was observed that the copy number higher than two yielded lower amount of protein. On the other hand, the same study suggested that the production of recombinant human trypsinogen was not affected from the increasing copy number of the gene, from one to three, in a GAP promoter driven expression system (Hohenblum et al., 2004). Extracellular enhanced green fluorescent protein (*EGFP*) production under AOX1 promoter was examined by Liu et al. (2014) by increasing

the gene copy number from one to six. Contrary to Hohenblum et al. (2004) maximum fluorescence intensity in the culture media was detected at three copies and sharp decrease was observed at six copies.

2.11 Post-translational Modifications and Subsequent Secretion

The foremost advantage of *P. pastoris* over bacterial gene expression systems is having capability to perform various post-translational modifications related to higher eukaryotes such as, signal sequence processing (both pre- and prepro-type), proper folding, disulfide bond formation, lipid addition, O- and N-linked glycosylation (Cregg *et al*. 2000).

Strain engineering for protein secretion is primarily concentrated on four main topics (Idiris et al., 2010):

- Protein folding and quality control mechanism in the endoplasmic reticulum (ER)
- Protein trafficking inside the cells
- Proteolytic degradation minimization
- Post-translational glycosylation engineering

2.11.1 Protein Folding Pathways and Disulfide Bond Formation

In eukaryotes, secretory proteins fold in the ER. Eukaryotes have mechanisms to translocate nascent polypeptides from the cytosol to the ER lumen (Anken and Braakman, 2005). Protein folding commonly begins with secondary structure formation and disulfide bond formation (Holst *et al.* 1996). Usually, secretory pathway is more oxidized than cytosol to carry out disulfide bond development. For proper protein folding and oligomerization proteins include a pro-region whose mRNA recruit to stabilize proteins with forming local secondary structures to resist protein degradation (Daly and Hearn 2005). In addition, ER resident proteins assist to stabilize prevent protein aggregation (Damasceno et al., 2011). Also, folding helpers like the chaperones and protein disulfide isomerase, help to form proper disulfide bonds, and increase the protein secretion (Gasser et

al., 2008). Only the proteins which are properly folded enable to proceed by leaving the ER and going into Golgi apparatus (Klausner 1989). Proteins which are controlled by ER quality control mechanism are sent to the Golgi network within special cargo vesicles. Post-translational modifications take place inside the Golgi. Thereafter, proteins can be delivered to the plasma membrane, to the vacuole or to the endosomal compartments. Proteins to be secreted are packaged into vesicles and transported to the cell surface via the process named as exocytosis. Besides, quality control system of the ER eliminates the unfolded or misfolded proteins. Generally, these proteins and aggregates are retrotranslocated and proteosomally degraded outside the ER by a mechanism named as ER-associated protein degradation (ERAD) (Hoseki et al., 2010). Accumulated malfolded proteins overburden the ER and cause the unfolded protein response (UPR) is active. Both ERAD and UPR may limit the protein secretion (Delic et al., 2014). On the other hand, generally exit of secreted proteins from ER is defined as the rate limiting step through the secretory pathway (Shuster 1991). Protein transport inside the Golgi apparatus performed by vesicles, however delivery system can be inefficient and proteins may be misguided leading vacuolar/lysosomal degradation. Those bottlenecks in the secretion pathway of eukaryotes were presented in Figure 2.10.

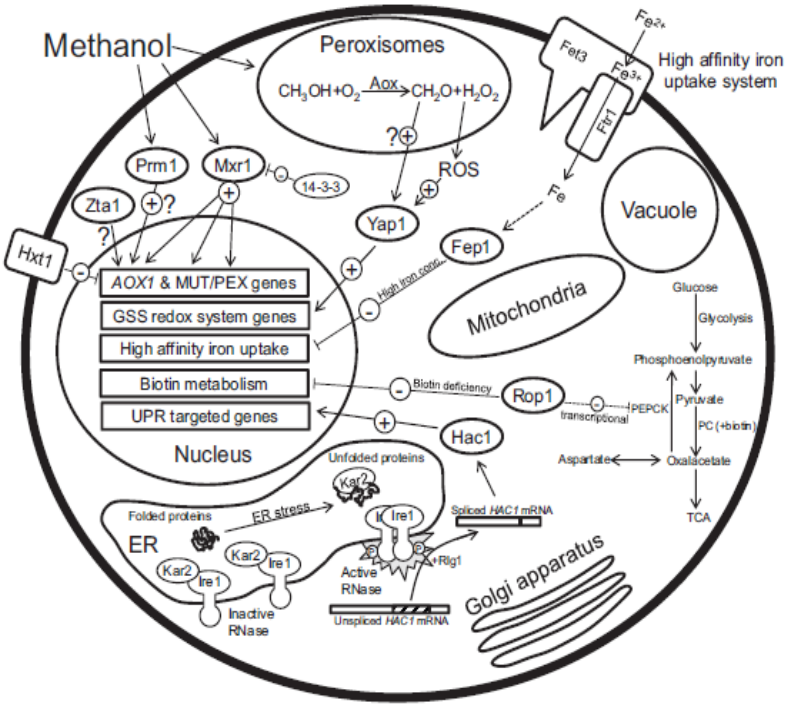


Figure 2.10 Regulatory system containing several cellular processes associated with the heterologous protein production in *P. pastoris*. Scheme includes the oxidative stress response, regulation of the UPR and the high-affinity iron uptake machinery, and biotin metabolism. Positive regulatory effects were shown with a plus sign inside a circle and negative effects were shown with a minus sign inside a circle (Vogl and Glieder, 2013).

2.11.2 O- and N-linked Glycosylation

Glycosylation is one of the most prevalent and complex post-translational modifications that can be accomplished by the yeast *P. pastoris* (Goochee et al., 1991). Nearly 0.5–1.0% of native proteins in the genome of eukaryotes are glycosylated (Daly and Hearn, 2005). Glycosylation takes place inside the lumen of the ER. In eukaryotes glycosylation of proteins has two main roles. Firstly, *N*-linked glycosylation contribute in the protein quality mechanism of the ER and secondly, glycan modification and extension in the Golgi gain properties to protein solubility, in vivo half-life or biological activity (Delic et al., 2014). Besides, *P. pastoris* is a more attractive host than *S. cerevisiae* because; glycosylated proteins have shorter glycosyl chains (Bretthauer and Castellino 1999). The two types of

glycosylation namely, *O*- and *N*-linked glycosylation, can be successfully performed by *P. pastoris* somewhat different from mammalian cells. While *P. pastoris* can only add mannose, mammalian cells can remove mannose residues and incorporate other oligosaccharides such as, fructose, galactose, N-acetyl-galactosamine, N-acetyl-glucosamine and also sialic acid by Golgi enzymes (Cereghino and Cregg 2000, Li et al., 2001). Therefore, the recombinant therapeutic proteins that produced by *P. pastoris* may have an adverse immunogenic effect (De Pourcq et al, 2010).

O-linked (serine and threonine side chain) glycosylation differs from higher eukaryotes to lower ones. Eukaryotic cells attach O-oligosaccharides onto the hydroxyl groups of serine and threonine residues of the recombinant protein (Schutter *et al.* ,2009). In mammalian cells O-linked oligosaccharides consist of various sugars whereas the yeast *P. pastoris* can only add mannose (Man) residues (Cereghino et al., 2000).

In literature, *N*-linked (asparagine-linked) glycosylation has been tried for different host organisms like yeast, plants, insects, fungi and animal cells (Jacobs and Callewaert 2009). Its pathway is preserved not only in higher eukaryotes but also in yeast. In eukaryotes, *N*-linked glycosylation commences on the ER's cytoplasmic side by attaching glycans to the Asn-Xaa-Ser/Thr site (Gemmill and Trimble 1999). Being similar in eukaryotes *N*-linked glycosylation pattern differs in mammalian and yeast cells. While the mammalian Golgi apparatus can remove mannose residues via the enzyme α-1-2-mannosidase or add to obtain high-mannose type ($Man_{5-6}GlcNAc_2$) oligosaccharides (Tulsiani *et al.,* 1982) hypermannosylated glycans can be observed in yeast cells (Cereghino et al., 2000).

Because of hGH is a non-glycosylated protein, dispose of the glycosylation or trimming the glycosyl chains would not cause any problem during rhGH production in *P. pastoris* (Trevino et al., 2000, Massahi and Çalık 2015).

2.12 Secretion Signals

P. pastoris can successfully produce recombinant proteins either intracellularly or extracellularly. Secretion of relatively lower levels of endogenous proteins into environment provides convenience to downstream processing (Cereghino et al., 2000). Direction of proteins into secretory pathway requires a specific signal sequence and it conducts ER translocation. Signal peptides are hydrophobic and the selection direct the post-translational secretion mechanism to different translocon pores (Davis et al., 1996). Secretion signal sequences are generally short amino-terminal pre-sequences, and they have a charged amino-terminal section accompanied by 6 to 15 hydrophobic residues with a cleavage site in order to be recognized by signal peptidase (Romanos et al., 1992). Although different types of signal peptides including *S. cerevisiae* acid phosphatase signal sequence (PHO), invertase signal sequence (SUC2), and *P. pastoris* endogenous acid phosphatase (PHO1) signal have been tried and succeed in the secretion of heterologous proteins, the most commonly used secretion signal is the *S. cerevisiae* α-mating factor pre-pro-signal which is the precursor of mating pheromone (Fitzgerald and Glick 2014). This signal sequence consists of 19-residue signal (pre) sequence terminates the cleavage site of the signal peptide by signal peptidase within the ER. Pre-region of the peptide is followed by 66-amino acid pro sequence. Pro-domain of the signal peptide is removed inside the Golgi apparatus by dibasic Kex2 endoprotease between Arg-Lys and Glu-Ala repeats are cleaved by the Ste13 protein (Brake et al., 1984, Fuller et al., 1988).

2.13 Proteolytic Degradation

Especially in high cell density fermentations, proteolysis becomes a significant problem due to the proteases resident in the secretory pathway (Werten and de Wolf 2005), secretion of host-specific proteases into culture (Kang et al., 2000) or due to cell lysis (Sinha et al. 2005) causing trouble in the production of heterologous proteins, decrease in yield, deprivation in biological activity, and product contamination because of the similar physicochemical and/or affinity characteristics of the intermediates (Kobayashi et al., 2000, Jahic, 2003). Besides,

methanol metabolism of *P. pastoris* requires high oxygen that causes oxidative stress and heat-shock response induces the protease amount (Hilt and Wolf, 1992, Sinha *et al*. 2005)

There are several strategies that have been constituted in order to overcome the proteolysis namely, adjustment of the fermentation parameters, such as specific growth rate, pH, and temperature, usage of protease deficient strains (Gleeson et al., 1998), addition of soytone and lowering the salt concentration (Zhao et al. 2008), and addition of protease inhibitors (Macauley-Patrick et al. 2005). On the other hand, changing medium composition such as, nitrogen and carbon sources affect the proteolytic activity because soluble proteases are mostly located inside the vacuoles and their levels vary by nutrition (Hansen et al., 1977).

Fermentation parameters affect the heterologous protein production. Being able to live at a wide pH range (3.0–7.0) allows the pH to be adjusted to solve the protease problem without affecting the growth rate (Chiruvolu et al., 1998, Sreekrishna et al., 1997). In order to stabilize the recombinant protein, different pH values are evaluated. For instance, optimum pH values for recombinant mouse epidermal factor and human serum albumin were found as 6.0 by Clare et al. (1991) and Kobayashi et al. (2000), on the other hand, in the production of insulin-like growth factor-I and cytokine growth-blocking peptide, ideal pH was determined as 3.0 (Brierley et al., 1994, Koganesawa et al., 2002).

Another approach is to reduce the cultivation temperature. At high temperatures proteins become unstable, folding problems arises and dead cells releases more proteases (Hong et al., 2002). Decreasing temperature from 30°C to 23°C increases the cell viability and increases the yield of herring antifreeze proteins from 5.3 mg L^{-1} to 18 mg L^{-1} (Li et al., 2001). When temperature was reduced to 20°C and methanol concentration was decreased to 0.5%, it was observed that the laccase activity was enhanced (Hong et al., 2002). In order to prohibit oxygen limitation temperature-limited fed-batch (TLFB) technique was used by Jahic *et al*. (2003). By that technique cell viability and fusion protein

concentration were increased whereas protease activity in the culture supernatant was lowered.

By changing the specific growth rate proteolysis has also been diminished. Keeping methanol concentration at growth rate-limiting amounts leads to a significant reduction in the hirudin proteolytic degradation (Xiang and Yuan 2002).

Protein degradation is further diminished by addition of amino acid rich supplements, such as peptone or casamino acids or specific protease inhibitors (Macauley-Patrick et al., 2005, Clare et al., 1991). Peptone is regarded as competing and alternative substrate for proteases. These substrates can repress induction of proteases result in nitrogen limitation. Brankamp et al. (1995) supplied peptone and casamino acids to the cultivation media and achieved higher yield of anticoagulant-antimetastatic protein ghilanten. On the other hand, it was found that in *P. pastoris* there are three types of proteases expressing a single-chain antibody (scFv) and they are namely, aspartic, cysteine and serine-type proteases. Thus, it was detected that addition of serine protease inhibitor reduces total protease activity 53% and addition of aspartic protease inhibitor reduces total protease activity 30% (Shi et al., 2003). However, high cost of peptone and specific protease inhibitors are not favorable especially in large scale productions (Jahic at al., 2006).

In some cases, optimization of the cultivation parameters cannot be enough to solve the proteolysis problem and manipulation in the expression host strain could be the only solution (Li et al. 2010). Protease-deficient strains namely, SMD1163 (ΔHIS4 Δpep4 ΔHIS1), SMD1165 (ΔHIS4 ΔPRB1) and SMD1168 (Δhis4 Δpep4) have been used in the expression of protease-sensitive proteins and they increase the yield and productivity (Gleeson et al. 1998). In these strains the genes encoding proteinase A (*PEP4*) and/or proteinase B (*PRB1*) are disrupted. However, protease-deficient strains have lower viability and lower specific growth rates, they are not active as wild-type strains and their transformation is more difficult (Cereghino and Cregg 2000). Besides, when both protease-deficient and wild-type strains expressing herring antifreeze protein were compared, it was observed that protein secretion began earlier with the wild-type strain than the

protease-deficient strain but higher amount of protein was obtained by the protease-deficient strain (12.8% as opposed to 4% from the wild-type) (Li et al., 2001).

2.14 Fundamental Parameters in Bioprocess Design

Microorganisms can be regarded as the microbioreactors inside the bioreactors (Çalık et al., 1999) and their growth is practically proportional to the concentration of the heterologous protein in the medium. Limitations while controlling the volume, dissolved oxygen concentration, substrate amount in shake-flask culture make bioreactors more attractive to produce high level r-protein (Macauley-Patrick et al., 2005). Therefore, productivity of the heterologous protein is not only depends on the genetic engineering techniques but also bioprocess parameters such as fermentation medium composition, oxygen transfer characteristics of the medium resulting from the aeration and agitation rate, medium temperature and, pH. Since these prominent parameters vary by foreign protein and genotype of the strain, there is no single fixed condition and each factor should be optimized for each bioprocess (Cos et al., 2006) despite the *Pichia* fermentation process guidelines provided by Invitrogen Co.

2.14.1 Medium Composition

Including biosynthetic precursors by diminishing the demand to generate biosynthetic precursors and using them directly in anabolic pathways while conserving the metabolic energy, results in more vigorous cell growth in complex rich media than in mineral media. Thus, variability on cell growth, viability of the cell, and implicitly heterologous protein production are highly affected by the composition of the culture medium (Li et al., 2007). As in other cells, *P. pastoris* also require carbon and nitrogen sources for their growth. The most widely used carbon sources are glucose and glycerol whereas nitrogen sources are yeast nitrogen, peptone, and yeast extract (Li et al., 2007). Complex media such as yeast extract peptone dextrose medium (YPD) or buffered complex glycerol medium (BMGY) and buffered complex menthol medium (BMMY) are frequently applied either in the selection of *P. pastoris* transformants or in the production of

heterologous proteins, respectively (Gonçalves et al., 2013). Further, peptone and casamino acids may be added to the fermentation medium in order to avoid the proteolytic degradation of the heterologous protein as explained in 2.13.

However, utilization of complex media including yeast extracts and peptones results in variations in the process. In order to standardize and optimize the production medium and process, to obtain repeatable results, and to obtain parallel expression levels of the desired protein elimination of the complex components is required (Macauley-Patrick et al., 2005). Therefore, for each *P. pastoris* strain a defined medium is generated with supplemented specific components for instance in the growth of wildtype X-33 strain minimal medium is used and in the growth of GS115 and KM71H strains minimal medium enriched with histidine and arginine are utilized, respectively (Lin et al., 2007). In *P. pastoris* expression systems the most commonly employed defined medium is the basalt salt medium (BSM) provided by Invitrogen Co. is used to achieve high cell density fermentation. Nevertheless, this medium at pH greater than 5.0 may pose problems such as precipitation, unbalanced composition, ionic strength, OD measurement interference, and starvation of nutrients (Cos et al., 2006). Variants of BSM media have been developed coupling with the trace salt solution PTM1. Stratton et al. (1998) lowered the amount of ferrous sulfate and zinc chloride nearly 3-fold. The problem in that formulation was the precipitation of salts reducing the dissolved mineral concentration as well as turbidity. On the other hand, in alternative formulations a non-precipitate-forming compound, sodium hexametaphosphate, was replaced with phosphoric acid (Oehler et al., 1998) or basal salt concentration was reduced to one quarter by Brady et al. (2001). These variations have been indicated to be influential due to redounded cell growth rate and biomass yield. Yeast nitrogen base (YNB) is another chemically defined medium used in the *P. pastoris* expression systems. The medium comprises not only nitrogen sources as ammonium sulfate and asparagine but also includes trace elements, mineral salts, and vitamins. Furthermore, in a chemically defined medium using glycerol as the main carbon source ramped up the biomass production (Ghosalkar et al., 2008).

The methylotrophic yeast *P. pastoris* can utilize methanol as a sole carbon and energy source. Moreover, sugars such as glucose, fructose, galactose, trehalose, maltose, sugar alcohols such as, sorbitol and mannitol or fermentation products glycerol, ethanol, and lactate organic acids such as acetic acid and lactic acid, and citric acid can be used for energy production. However, unlike *S. cerevisiae*, *P. pastoris* is incapable of metabolizing sucrose and galactose due to lack of invertase enzyme and GAL2 gene that codes galactose transporter (Inan and Meagher 2001, Xie et al., 2005, Çalık et al., 2015). Commonly used nitrogen source, ammonium hydroxide, has a massive impact on cell growth, protein expression and proteolytic activity (Kobayashi et al., 2000).

All the defined media are supplemented with micronutrients like Fe, Mn, Cu and biotin, normally used in the PTM1 trace salts composition recommended by Invitrogen. Mg, Ca, and certain transition metals are the primary structural components of metalloenzymes in cell metabolism. During recombinant protein production metal supplements affect carbon fluxes (Plantz et al., 2006), besides elimination or reducement of non-essential metal ions avails to reduce the production cost, metal effluent removal, and equipment deterioration, facilitate downstream processing and dissuasion of changed cell physiology due to excess metals (Brady et al., 2001, Plantz et al., 2007). The requirements of the medium possibly rely on the protein to be expressed, as well as the negative effects of excess metal may be encountered related to cell growth and productivity. For recombinant laccase production, the addition of at least 200μM copper (O'Callaghan et al., 2002), and the addition of 100 μM ferric ion significantly enhances expression level of recombinant porcine lactoferrin in *P. pastoris* (Wang et al., 2002). The inclusion of Triton X-114 to the fermentation medium increased the efficiency of rPIN-a (recombinant puroindoline-a) production ten-fold (Issaly et al., 2001).

In industry, undefined and complex media are generally employed due to economic reasons. Minimizing the production cost is one of the most critical parameters for industrial fermentations. Despite variable compositions, agricultural wastes or byproducts of processes are frequently chosen for yeast growth for

instance molasses, glucose syrups, malt worth, cheese whey, sulfite liquor and grape juice (Schaechter, 2009).

2.14.3 Temperature

Optimization of the fermentation temperature is one of the most crucial physical operation conditions. Temperature affects yeast growth, specific productivity of the recombinant protein, and directs carbon fluxes towards protein production. Metabolic activities of microorganisms are affected from the environmental conditions because they cannot adjust their internal temperature (Donati, 2007). The effect of temperature on each expression system is varied. Optimal temperature can get under control of byproduct formation and excess biomass production while directing carbon flux to heterologous protein formation. When generalized, at low temperatures reaction's transport process rates decreases, low temperature maintains the cells in secretion phases in the cell cycle and reduces folding stress in the cells and allows more properly folded protein (Gasser et al., 2008). Nevertheless, it was indicated by Invitrogen that temperatures higher than 32°C may damage proteins or may cause cells death and high temperatures may increase protease activity. Although the optimum temperature of heterologous protein production of *P. pastoris* is regarded as 30°C (Cos et al., 2006), it relies on the nature of the heterologous protein. Lowering the temperature to 20-25°C has been determined to enhance yield of the heterologous protein in several circumstances. More specifically, Li et al. (2001) reduced the fermentation temperature to 23°C for the production of recombinant Herring Antifreeze Protein (hAFP) and obtained fully biologically active hAFP, Lin et al. 2008 compared three different process temperatures, 20-25-30°C, and achieved the highest protein yield at the lowest temperature, during induction phase keeping temperature at 20°C provided 2.5 to 10-fold increase in the yield of recombinant dengue virus type 2 envelope domain III (Batra et al., 2010), double and triple yield values of antibody fragment Fab 3H6 was attained at 25°C and 20°C, respectively compared to 30°C fermentation temperature and 8-fold higher volumetric productivity was reported by Anasontzis and Penã (2014) at 25°C rather than 30°C in recombinant galactose

oxidase production. In spite of more proper folding and secretion at low temperatures, several hyper thermophilic enzymes were enhanced at higher temperatures such as 40°C (Gasser et al., 2008).

2.14.4 pH

pH is defined as the potential of hydrogen, determines the acidity and alkalinity of the solution. pH is one of the most pivotal monitored parameters which is measured during bioprocesses because it affects the cell growth rate, transport mechanisms, stability of the proteins, activity of the enzymes, protein expression rates, efficiency of the secretion, and proteolytic degradation (Fickers, 2014). Microorganisms have a steady intracellular pH independent of the extracellular medium and difference in Gibbs free energy is used to sustain the proton gradient throughout the cell membrane (Nielsen and Villadsen, 1994). Yeast growth is generally inhibited when cultivation medium is acidified by organic acids such as acetic and lactic acid than is acidified by mineral acids such as hydrochloric acid. Differential ion uptake, secretion of organic acids such as succinate and acetate, proton secretion during nutrient transport, carbon dioxide formation and dissociation are acidifying the media during fermentation (Walker, 2009). *P. pastoris* can grow on a broad acidic pH range between 3.0 and 7.0; generally, pH value 3.5 to 5.5 is more preferable (Inan et al., 1999) due to slight effect on the cell growth rate allowing freedom to adjust the pH while minimizing the proteolytic activity and stability of the protein (Macauley-Patrick et al., 2005).

Optimum pH value of the medium varies according to the nature of the heterologous protein and strain dependent. Therefore, various pH values are designated as optimum for different proteins (Fickers, 2014). Inan et al. (1999) found that specific yield and activity of the (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) mostly affected from the change in pH and its optimum value was found as 7.0. For the production of Fc fusion protein in *P. pastoris* five different pH values (from 3.0 to 7.2) was investigated and highest protein production was obtained at pH of 7.2 (Lin et al., 2007). pH 6.0 is determined as optimum in the

recombinant mouse epidermal factor and galactose oxidase productions in which lower than pH 6.0 value yields unstable galactose oxidase (Clare et al., 1991, Macauley-Patrick et al., 2005). A33 single-chain Fv antibody fragment and mouse and rat collagen gelatins were expressed at different pH values by Damasceno et al. (2004) and by Werten et al. (1999). Despite lower growth rates, the highest yields were achieved at pH 3.0 while reducing the proteolytic degradation. A33scFv production attained 4.88 g L^{-1} and gelatin production reached 14.8 g L^{-1}. Optimum pH of the medium used in the production of recombinant single-chain Fv antibody (scFv) fragment is within the range of 6.5–8.0 and scFv production reached 25 mg/L at the incubation temperature of 15°C (X. Shi et al., 2003). Recombinant glycerol kinase (GK) production was optimized by Terrazas et al. (2014), the highest GK activity of 1.57 U mL^{-1} was achieved at 30° C and pH 6.0.The highest rEPO production was achieved as 0.16 g L^{-1} at pH 5.0 during glycerol batch and glycerol fed-batch phases and at pH 4.5 for methanol induction phase (Soyaslan and Çalık 2011) as well as optimum pH of the cultivation medium for the production of recombinant human growth hormone (rhGH) was determined as 5.0 and the highest rhGH concentration was achieved as 0.27 g L^{-1} (Çalık et al., 2010).

2.14.5 Oxygen Transfer in Cellular Systems

In aerobic fermentations oxygen is the supreme parameter because oxygen availability and transfer can limit the productivity of the fermentation. It is crucial in respiratory growth as well as influential as oxidizing agent (Talaro, 2005). Cell growth is highly affected by oxygen availability designated by its mass transfer coefficient is a consequence of aeration and adequate mixing. There are several chemical and physical factors affecting the oxygen transfer rate such as composition of the medium, hydrodynamics of gas bubbles, temperature, speed of the stirrer, and partial pressure of oxygen. The pathway for oxygen transport was represented in Figure 2.8 (Bailey, 1986).

Transport resistances can be listed as;

- Diffusion from interior gas to gas-liquid interference
- Movement through the gas- liquid interference
- Diffusion through the relatively unmixed liquid region surrounding the bubble
- Transport through the bulk liquid to unmixed liquid region surrounding the cells
- Movement through the liquid film associated with the microorganism
- Transport across the liquid-cell interface
- Diffusion into cells. If the cells are in a floc, mycelia, clump or solid particle
- Transport into intracellular reaction site

However, some of these resistances can be eliminated. Since the resistance due to low solubility of oxygen in aqueous solutions gas film resistance within the bubble can be ignored relative to the liquid film surrounding the bubble and the major resistance in the oxygen transfer is the liquid film around the bubbles. Also, oxygen transfer resistance in the bulk liquid is small in a perfectly mixed reactor due to low concentration gradients. Resistance at the liquid-cell interface is usually negligible. In the case of individual cells, they are suspended in liquid instead of a clump, step seven vanishes. Because size of the single cell is very small, hence they have large interfacial area, relative to the gas bubbles their impact on oxygen transfer is commonly neglected. On the other hand, if cells are in clumps, oxygen transfer resistance depends on the size of the clump. Due to short distances within the cell intracellular mass transfer resistance is generally negligible.

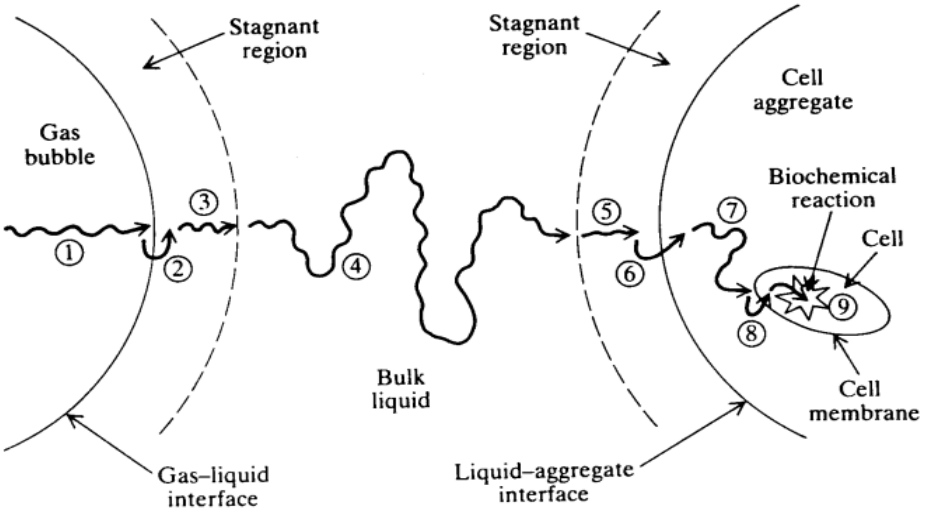


Figure 2.11 Schematic representation of oxygen transport steps from a gas bubble to inside a cell (Baily, 1986).

Gas-liquid mass transfer generally modelled according to two film theory (Whitman, 1923). The theory is governed by the molecular diffusion thus diffusive molar flux through the film can be explained by Fick's Law in Equation 2.1.

$$N^{"} = -D\frac{\partial C}{\partial y} \qquad (2.1)$$

where $N^{"}$ is the molar flux (mol s^{-1} m^{-3}), D is the diffusivity and molar flux is proportional to the concentration gradient. In oxygen transfer the rate limiting step is the diffusion across the liquid layer surrounding the bubble. The film theory can be simplified by assuming mass transfer occurrance in a thin film with a thicknessδ. Therefore, assuming perfect mixing of liquid and gas phases in the bioreactor the oxygen transfer rate equation at the liquid interface is described as:

$$k_L = \frac{D}{\delta} \qquad (2.2)$$

$$N^{"} = k_G(p_G - p_{sat}) = k_L(C_{sat} - C_L) \qquad (2.3)$$

and

$$N^{"} = k_G(p_G - p_i) = k_L(C_i - C_L)$$

where oxygen transfer rate (OTR) oxygen transfer rate per unit volume (mol s^{-1} m^{-3}), k_L is the liquid-phase mass transfer coefficient (m s^{-1}), a is the gas-liquid interfacial area per unit volume (m^2 m^{-3}), C_{sat} is the saturated dissolved oxygen concentration and C is the oxygen concentration in the medium (mol m^{-3}), index i used for the values at the gas-liquid interface. Due to low solubility of oxygen difference between the concentrations is rather small. k_La is assumed to have no local variations and depends on the agitation speed, gas flow rate, and partial pressure of oxygen. Oxygen transfer rate in the fermentor must be higher than the oxygen consumption rate (OUR) of the cells failing this result in anaerobic fermentation. Oxygen demand is affected from the species of the cell, carbon source in the medium, and growth phase of the culture.

Saturated dissolved oxygen concentration is proportional to partial pressure of oxygen in the gas and the relation is expressed by Henry's law:

$$P_{O_2} = H \cdot C_{satO_2} \qquad (2.4)$$

where H is the Henry's law constant depends on the temperature and it is calculated by the equation 2.5 (Royce and Thornhill 1991).

$$H = 1000 \cdot \exp(12.74 - \frac{133.4}{T-206.7}) \qquad (2.5)$$

Also, $C_{O_2} = DO\% \cdot C_{satO_2}$

Then the driving force can be calculated by the following equation:

$$C_{satO_2} - C_{O_2} = \frac{P \cdot y_{O_2}}{H}(1 - DO\%) \qquad (2.6)$$

When molar fluxes are combined the obtained equation:

$$\frac{1}{K_L} = \frac{1}{HK_G} + \frac{1}{k_L}$$

Since oxygen is slightly soluble in water H is very large. Thus, the gas phase resistance can beneglected and the overall mass transport coefficient becomes equal to the local coefficient: $K_L = k_L$ (Garcia-Ochoa and Gomez, 2009).

Dissolved oxygen (DO) concentration is dynamically evaluated by the mass balance equation as:

$$\frac{dC_{O_2}}{dt} = OTR - OUR \qquad (2.7)$$

$$OUR = -r_O = q_{O_2} \cdot C_X \qquad (2.8)$$

$$OTR = k_L a(C_{sat} - C)$$

where $-r_O$ is the oxygen consumption rate (mol m^{-3} s^{-1}), q_{O_2} is the specific oxygen consumption rate (mol kg^{-1} s^{-1}) and C_X is the cell concentration (kg m^{-3}).

Oxygen as a growth factor is utilized by the cells to obtain fatty acids for cell membrane and for sterol biosynthesis (Walker, 2009). During the fermentation process DO is one of the most crucial parameter that affect cell growth and heterologous protein expression by affecting metabolic pathways and altering the metabolic fluxes (Çalık et al., 1999). When P_{AOX} driven expression system is used for the expression of the target protein in *P. pastoris* methanol feeding rate is directly proportional to the C_{DO} because oxygen is used both for the respiration and for the initial oxidation of methanol to formaldehyde (Jungo, 2007). Generally, during the induction phase dissolved oxygen level was kept around 20 – 30% of saturation to maintain fully aerobic conditions (Jahic et al., 2006, Gasser et al.,

2006). Avoiding methanol accumulation by adjusting the methanol feed to the extent dissolved oxygen in the supernatant yielded 2.5 higher *Rhizopus oryzae* lipase (ROL) productivity in the bioreactor (Minning et al., 2001). However, cultivations performed by methanol feeding causes a powerful oxidative stress in methylotrophic yeasts that may be reduced by addition of by the addition of antioxidants (Xiao et al., 2006).

Throughout the high cell density fermentations oxygen requirement of the yeast *P. pastoris* is elevated because the yeast give preference to respiratory pathway instead of fermentative pathway (Chen et al., 2007). Being a facultative anaerobe metabolic pathways of *P. pastoris* tend towards the by-product formation when cultured on glucose. During heterologous protein production under P_{GAP} driven expression systems, effect of C_{DO} was investigated by researchers. Under hypoxic conditions, Hu et al. (2008) stated that the cell concentration was enhanced on the contrary to Baumann at al. (2008). Investigating three different dissolved oxygen concentrations as normoxic (21%), oxygen-limiting (11%) and hypoxic (8%) Baumann et al. (2010) stated that core metabolism of *P. pastoris* to hypoxia is proportional to the metabolic, transcriptomic, and proteomic fluxes by means of a strong transcriptional regulation in pentose phosphate pathway, glycolysis and TCA cycle. Furthermore, lower oxygen alters the lipid metabolism, protein folding and trafficking, and stress responses particularly the unfolded protein response. Also, hypoxic conditions in fed batch fermentation by P_{GAP} driven expression system lead to an enhanced the specific productivity as well as the volumetric productivity of the secreted protein (Baumann et al., 2008). Keeping C_{DO} at 5, 10, 15, 20 and 40% effect of glucose isomerase production under P_{GAP} was investigated (Güneş and Çalık, 2016). Similar to the studies conducted by Baumann et al. decrease in DO causes a downtrend in biomass however, maintaining C_{DO} at 15% was reported to yield highest volumetric activity and specific enzyme activity rather than the limited-oxygen conditions. Thus, reducing the dissolved oxygen in the fermentation broth alters the expression levels of the heterologous protein produced in *P. pastoris* under P_{GAP}.

Oxygen demand of the system depends on the cell concentration in the bioreactor. High cell densities may cause limited or low-oxygen conditions. Therefore, in recombinant protein productions oxygen transfer conditions were investigated to control OUR by OTR, and oxygen transfer coefficients (K_La) are required (Çalık et al., 2014). OTR, OUR, and K_La values have been computed to have opinion for the effect of methanol feeding on oxygen transfer parameters (Çelik et al., 2009, Çalık et al., 2009), effect of glycerol feeding under oxygen transfer limitation conditions (Oliveira et al., 2005), modelling the oxygen transfer to determine the biomass (Liang and Yuan, 2007).

2.15 Evaluation of Bioprocess Characteristics

Although cell growth is a complex process, it complies with the conservation of mass and energy. During the calculations of kinetic data of bioprocess, parameters including specific growth rate of the cell, substrate consumption and product formation rates are required to evaluate the productivity and yield. In order to evaluate the performance of the bioprocesses, to attain absolute values, and to compare the results accurately, specific rates along with the yield coefficients of each semi-batch operations are computed by the mass balances of cell, substrate and product (Çalık et al., 2016, Öztürk et al., 2016).

2.15.1 Mass Balance Equation for the Cell in Semi-Batch Reactor

Cell growth principally implies to cell proliferation meaning the increase in number due to cell division. Together with the assumption of there is no input or output of cells throughout the fermentation, material balance equation of the cells is written as (Çalık et .al, 2016):

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

where r_x is the cell formation rate in $(gL^{-1}h^{-1})$, V is working volume of the fermentation in $[L]$, C_X is concentration of cell (gL^{-1}), t is the cultivation time.

The relation between cell concentration and the cell growth rate can be characterized with a first order equation:

$$r_X = \mu C_X \qquad (2.10)$$

where μ (h^{-1}) is specific growth rate of the cell. If the working volume of the bioreactor is assumed as constant during the fermentation, ($V_{Total-samples} \ll V(t)$ and when the Equation 2.10 is substituted into the Equation 2.9 then the new equation takes the form of:

$$\frac{d(C_X V)}{dt} = \mu C_X V \qquad (2.11)$$

Due to semi-batch operation mode (feeding of glucose according to a predetermined specific growth rate), the volume of the bioreaction medium increases. Assuming that the cultivation media has a constant density equation of continuity for the semi-batch operation, the equation can be written as:

$$\frac{dV}{dt} = Q_{in} - Q_{out} \qquad (2.12)$$

where Q is the feed volumetric flow rate ($L\ h^{-1}$). Since semi-batch processes has no outlet stream, Q_{out} is zero and thus, Equation 2.12 becomes:

$$\frac{dV}{dt} = Q_{in} \qquad (2.13)$$

The derivative on the left-hand side of the Equation 2.11 can be written as:

$$C_X \frac{dV}{dt} + V \frac{dC_X}{dt} = \mu C_X V \qquad (2.14)$$

When the Equation 2.14 is rearranged, μ term for the semi-batch processes can be defined as:

$$\mu = \frac{1}{C_X}\frac{dC_X}{dt} + \frac{1}{V}\frac{dV}{dt} \quad (2.15)$$

Eventually, when the Equation 2.13 is inserted into Equation 2.15, the equation for the specific growth rate of the cell is as follows:

$$\mu = \frac{1}{C_X}\frac{dC_X}{dt} + \frac{Q}{V} \quad (2.16)$$

2.15.2 Mass Balance Equation for Substrate in Semi-Batch Reactor

In the semi-batch bioprocesses, feed stream of the substrate into the bioreactor is permanent whereas effluent stream is absent. Thus, the mass balance equation of the substrate can be written as (Çalık et .al, 2016):

$$\frac{d(C_S V)}{dt} = Q_S C_{S_0} + r_S V \quad (2.17)$$

where r_S is utilization rate of substrate ($g\ h^{-1}$), C_{S_0} is the concentration of substrate in the stock medium ($g\ L^{-1}$), and Q_S is the volumetric feed flow rate ($L\ h^{-1}$). Specific substrate consumption rate can be defined with a first-order kinetic equation in terms of the cell concentration as the following equation:

$$-r_S = q_S C_X \quad (2.18)$$

where q_S is the specific substrate consumption rate ($g\ g^{-1}\ h^{-1}$). When the derivative term in Equation 2.17 is separated and Equation 2.18 is substituted into the Equation 2.17, the equation used in the calculation of q_S becomes:

$$q_S = \frac{1}{C_x}\left(\frac{Q_S}{V}C_{S_0} - \frac{C_S}{V}\frac{dV}{dt} - \frac{dC_S}{dt}\right) \quad (2.19)$$

If it is assumed that the all substrate fed into the system is utilized in the bioreactor ($(dC_S/dt) = 0$), and volume of the bioreactor is taken as constant, during the semi-

batch bioprocess, quasi-steady state assumption for the substrate can be valid (Öztürk et al., 2016). Besides, r_S, substrate consumption rate can be defined with regard to yield of cell on substrate, $Y_{X/S}$:

$$r_S = \left(r_x / Y_{X/S}\right) \tag{2.20}$$

When Equation 2.20 is substituted in Equation 2.17 the equation becomes:

$$Q_S C_{S_0} - \frac{r_X V}{Y_{X/S}} = C_S \frac{dV}{dt} \tag{2.21}$$

If Equations 2.10 and 2.13 are inserted in Equation 2.21 the equation takes the form of:

$$Q_S C_{S_0} - \frac{\mu C_X V}{Y_{X/S}} = C_S Q_S \tag{2.22}$$

By integrating the Equation 2.9 the linear form of equation is obtained as:

$$C_X V = C_{X_0} V_X e^{\mu t} \tag{2.23}$$

When the equations 2.22 and 2.23 are combined volumetric feed flow rate Q_S becomes:

$$Q_S = \frac{\mu C_{X_0} V_0}{Y_{X/S}(C_{S_0} - C_S)} e^{\mu t} \tag{2.24}$$

Assuming the complete substrate consumption in the bioreactor or taking $C_{S_0} \gg C_S$, Equation 2.24 can be rearranged as:

$$Q_S = \frac{\mu C_{X_0} V_0}{Y_{X/S}(C_{S_0})} e^{\mu t} \tag{2.25}$$

2.15.3 Mass Balance Equation for Product in Semi-Batch Reactor

The product is formed in the cells batchwise meaning that the intracellularly synthesized products are secreted into the cultivation medium and therefore there is neither input nor output stream in the system. Thus, material balance of the generated product can be written as (Çalık et .al, 2016):

$$\frac{d(C_P V)}{dt} = r_P V \qquad (2.26)$$

where r_P is the rhGH generation rate, that can be described with a first-order kinetic equation in terms of the cell concentration as the following equation:

$$r_P = q_P C_X \qquad (2.27)$$

where, q_P is the specific rhGH generation rate ($g\ g^{-1}\ h^{-1}$). When Equation 2.27 is substituted into Equation 2.26, and the rearranged equation of q_P as follows:

$$q_P = \frac{1}{C_X}\left(\frac{dC_P}{dt} + \frac{C_P}{V}\frac{dV}{dt}\right) \qquad (2.28)$$

2.15.4 Overall and Instantaneous Yield Coefficients

Efficiency of the reactions inside the bioreactor (substrate conversion and product generation) is determined by the yield values. Yield coefficients are the ratio of rate of generation to rate of consumption. Their definitions are supplied in Table 2.7 and mathematical notations of instantaneous yield coefficients as follows (Doran,1995):

$$Y_{X/S} = \frac{r_X}{-r_S} = \left(\frac{dC_X/dt}{-dC_S/dt}\right) \qquad (2.29)$$

$$Y_{P/S} = \frac{r_P}{-r_S} = \left(\frac{dC_P/dt}{-dC_S/dt}\right) \qquad (2.30)$$

Besides, overall yield coefficients can be evaluated for time interval, these values can be calculated as:

$$Y'_{X/S} = \frac{r_X}{-r_S} = \frac{\Delta C_X/\Delta t}{-\Delta C_S/\Delta t} \quad (2.31)$$

$$Y'_{P/S} = \frac{r_P}{-r_S} = \frac{\Delta C_P/\Delta t}{-\Delta C_S/\Delta t} \quad (2.32)$$

Common yield coefficients utilized to determine the performance of the bioprocesses are supplied with their symbols, definitions and units in Table 2.7.

Table 2.7 Definition of yield coefficients.

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

2.16 Metabolic Flux Analysis

Being a micro-bioreactor inside the bioreactor, state of bioprocesses are mostly rely on microorganisms (Çalık et al., 1999). Metabolic flux is described as "the rate at which material is processed through a pathway" (Stephanopoulos et al., 1998). In a biological system dispersion of metabolic flux is crutial procuring distinctive mark which enables to control the *in vivo* physiological statement of the system (Smolke, 2009). By the help of bioreaction network analysis, or metabolic flux analysis (MFA), conversion of nutrients to products, by-products, energy, and oxidative cofactors is revealed for overall metabolic process and especially at the key branch points (Stephanopoulos et al., 1998, Çalık and Özdamar, 2002).

Stoichiometric models have been utilized to predict the path of the metabolic fluxes under certain conditions at a particular time (MFA). Yet, the model neglects the "dynamic intracellular behavior" by assuming pseudosteady state (PSS) for the intracellular metabolites due to the scantiness of intracellular quantification methods (Llaneras and Picó, 2008) and by depending upon the high turnover of the intracellular metabolites preventing them to accumulate inside the cell (Çelik et al., 2010).

The stoichiometric matrix "A" of metabolic fluxes is constructed by presuming a metabolic flux including "m" metabolites at rows and "n" reactions at columns to determine the stoichiometric coefficients of metabolites in every reaction by taking into consideration the biochemistry of the microorganism.

$$\boldsymbol{A} = [\dots]_{m\times n} \qquad (2.33)$$

Thereafter the mass-flux-balance equations can be written for metabolites as the linear equation as given below (Çalık et al., 1999):

$$\boldsymbol{A}\times\boldsymbol{r}(t) = \boldsymbol{C}(t) \qquad (2.34)$$

where, $\boldsymbol{r}(t)$ is the vector of the fluxes and $\boldsymbol{C}(t)$ is the vector of accumulation of metabolites. The $\boldsymbol{C}(t)$ term composed of two sub-vectors:

$$\boldsymbol{C}(t) = \boldsymbol{C_1}(t) + \boldsymbol{C_2}(t) \qquad (2.35)$$

$\boldsymbol{C_1}(t)$ and $\boldsymbol{C_2}(t)$ stand for the extracellular and intracellular metabolite accumulation vectors, respectively. Due to dynamic behavior of the cells the pseudo steady state assumption is considered valid and $\boldsymbol{C_2}(t) = 0$ in equation (2.35):

$$\boldsymbol{A}\times\boldsymbol{r}(t) = \boldsymbol{C_1}(t) \qquad (2.36)$$

According to the results metabolic fluxes in the reaction network are estimated for the key branches of the pathways (Çalık and Özdamar, 2002).

If $m > n$, the model provides the exact solution. The solution of equation (2.34) can be find out by a constrained least-squares approach by minimizing the sum of squares of residuals from the stoichiometric mass balance. However, if m < n, the "n-m" degrees of freedom for the vectorial equation making the system underspecified. At these circumstances the metabolic network can be determined by minimizing or maximizing the objective function, therewith the optimum metabolic pathway utilization that accomplish the aim. The mathematical formulation for the objective function Z is determined as follows (Çalık et al, 1999):

$$Z = \Sigma \alpha_i r_i \qquad (2.37)$$

where Z is a linear combination of the fluxes r_i, and α_i is the coefficient of the component-i in the stoichiometric equation of the corresponding reaction.

MFA have been performed in several studies by taking into consideration various objectives at different *P. pastoris* strains. A macrokinetic model was applied a *P. pastoris* strain that express HSA under P_{AOX1} to identify ATP and NADH during the growth phases. By the help of the model macrokinetic bioreaction rates were obtained and estimated cell growth and protein production were attained with admissible accuracy (Ren et al., 2003). In order to profile metabolic fluxes of *P. pastoris* strain expressing ROL under P_{AOX1}, biosynthetically directed fractional (BDF) ^{13}C-labeling was carried out and amino acid biosynthesis and central carbon metabolism of the yeast was investigated when glycerol was utilized as the carbon source. It was stated that the regulation of central carbon metabolism in *P. pastoris* resembles to the those in *S. cerevisiae* more than to those *P. stipitis* (Sola et al., 2004). Effect of co-substrates (glycerol and methanol) were investigated by (Sola et al., 2007) by employing BDF ^{13}C-labeling method. It was reported that prevelant amino acids were synthesized when the cells cultivated solely on glycerol and dilution rates of methanol effects the production of key intermediates in the pentose phosphate pathway (PPP). By applying stoichiometry-

based model, effect of dual carbon sources (methanol/sorbitol) on intracellular metabolic fluxes along the central carbon metabolism of r-*P.pastoris* expressing human erythropoietin (rHuEPO) were inspected by Çelik et al. (2010). In order to observe the variations in intracellular fluxes in *P. pastoris* strain expessing r-protein under GAP promoter was investigated by Heyland et al. (2010) when glucose was utilized as sole carbon and energy source in batch and fed-batch fermentations. The physiological adaptation of *P. pastoris* strains (both wild-type, X-33, and expressing r-Fab under P_{GAP}) to different oxygen conditions were studied by Baumann et al (2010). Another research which was conducted by Heyland et al. (2011) purposed to quntify the physiology of cells producing varied levels of r-protein in batch cultivations by considering the foreign gene expression leads metabolic burden on the microorganism. It was revealed that P. pastoris balances the requirement to additional resources by lowering the by-product formation and promoting energy production by means of the TCA cycle. The stoichiometry-based model was used to investigate the effect of substrate (methanol) and co-substrate (sorbitol) feeding rates on the bioreaction network in r-P. pastoris to enhance the rhGH production under P_{AOX1} (Çalık et al., 2011). Central carbon metabolism fluxes of r-*P. pastoris* expressing *Rhizopus oryzae* lipase (Rol) in chemostat cultivations including glucose:methanol 80:20 (w/w) mix as carbon source at a detemined dilution rate was analyzed. When compared to control strain, enhanced glycolytic, TCA cycle and NADH regeneration fluxes, and higher methanol dissimilation rates were determined by MFA (Jordà et al., 2012). To identify the central carbon metabolism together with the PPP in chemostat fermentations including methanol-glucose carbon sources, metabolomics and ^{13}C flux analysis were performed by Jordà et al. (2013). MFA was applied to transient continuous cultures to study the impact of methanol/sorbitol co-feeding process to quantify the metabolic flux network of r-P. pastoris strain producing β-galactosidase (Niu et al., 2013). Besides, three different chemostat cultures including 80:20, 60:40 and 40:60 (w:w) glycerol/methanol mixtures at two different dilution rates were compared to determine the redistribution of central carbon metabolism fluxes. It was reported that the carbon flux distribution was less influenced from the ratio of carbon sources

(Jordà et al., 2014). Hybrid MFA combining the traditional parametric estimation of central carbon fluxes with non-parametric statistical modeling of product-related quantitative or qualitative measurements as a function of central carbon fluxes was developed by Isidro et al. (2016) and effect of 26 variations of the BSM+PTM1 medium on central carbon metabolism was investigated in r-*P. pastoris* expressing single-chain antibody fragment.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Chemicals used in this research were analytical grade and supplied from Sigma Aldrich Co., Difco Laboratories, Fluka Co., and Merck & Co. Inc.

3.2 Enzymes, Kits, Buffers, Media and Stock Solutions

All kits and enzymes were purchased from Thermo Fisher Scientific Inc. The SYBR Green was acquired from Bio-Rad and G-418 Solution was procured from Roche. Protocols and formulations of the kits, buffers, and solutions were given in Appendix A. Each preparation was performed by using ultra purified water and all media and solutions were heat-sterilized by autoclaving at 121°C for 20 minutes or by sterilizing filtration through 0.20 or 0.45 μm filters (Sartorius AG, Gottingen, Germany) depending on the type of the substances of the solutions. They were stored at either at 4°C or at room temperature.

3.3 The Microorganism, Plasmids and Maintenance of Microorganisms

The plasmid pPIC3.5K carrying the bacterial kanamycin resistance gene which provides resistance to Geneticin (G-418) in eukaryotes was kindly provided by Prof.Dr. Mehmet İnan, Akdeniz University. Wild type *P. pastoris* X-33 strain and *E. coli* DH5α strain carrying pGAPZαA shuttle vectors providing resistance to Zeocin were purchased from Invitrogen, Carlsbad, CA, USA. *P. pastoris* pGAPZαA*::hGH*/X-33 was developed by Massahi and Çalık (2015) and used as parent strain in this study. P_{PDC} concomitantly *S. cerevisiae α* mating factor secretion signal downstream of the promoter and *hGH* gene was amplified from *E. coli* strains carrying pPDCZαA*::hGH* plasmids (Massahi, 2017) to construct and

amplify the pPDC3.5Kα*::hGH* plasmid. Double promoter expression system harboring P_{GAP} and P_{PDC} expressing the rhGH was accomplished by transfecting the vector pPDCZαA*::hGH* into pGAPZαA*::hGH*/X-33 competent cells and named as GAP-PDC*::hGH*. Schematic representations and sequences of the plasmids were presented in Appendix C.

All wild type and recombinant microorganisms used in this research are conserved in microbanks (PRO-LAB) which are cryovials system including treated beads and cryopreservative solution in the vials. Inoculated cells were adhered onto the porous bead surfaces, excess cryopreservative solution were aspirated as fluid-free as possible and the cyrovials stored at -80°C. The inoculated bead was used by direct inoculation onto solid suitable medium.

3.4 Growth Media for Bacterial and Yeast Cultivation

Wild type *E. coli* DH5α strain was cultivated on solid low-salt LB (LSLB) agar without antibiotic. Recombinant *E. coli* DH5α strain carrying pPDCZαA*::hGH* plasmids were inoculated onto LSLB agar slants comprising 0.025 g L^{-1} Zeocin whereas recombinant *E. coli* DH5α strain carrying pPDC3.5Kα*::hGH* plasmids were inoculated onto LSLB agar slants containing 0.1 g L^{-1} ampicillin. All *E. coli* strains preserved in microbanks were inoculated overnight at 37°C and adscititious antibiotic concentrations were invariable in LSLB liquid media.

Recombinant *P. pastoris* pGAPZαA*::hGH*/X-33 strain was incubated onto YPD agar including 0.1g L^{-1} Zeocin and recombinant GAP-PDC*::hGH* strain was grown both on YPD medium consisting 0.1g L^{-1} Zeocin and YPD medium comprising 0.25 g L^{-1} G-418. Both *P. pastoris* strains stored in microbanks were inoculated at 30°C for 48-60 hours.

The liquid mediums used for cell inoculation does not contain agar and media compositions of LSLB and YPD solid media was given in Table 3.1 and Table 3.2, and Table 3.3.

Table 3.1 Composition of solid LSLB medium used for *E. coli* DH5α strain carrying pPDCZαA*::hGH* plasmids

Compound	**Concentration (g L^{-1})**
Tryptone	10
Yeast extract	5
NaCl	5
Zeocin	0.025
Agar	15

Table 3.2 Composition of solid LSLB medium used for *E. coli* DH5α strain carrying pPDC3.5Kα*::hGH* plasmids.

Compound	**Concentration (g L^{-1})**
Tryptone	10
Yeast extract	5
NaCl	5
Ampcillin	0.1
Agar	15

Table 3.3 Composition of solid YPD medium used for recombinant *P. pastoris* pGAPZαA*::hGH*/X-33 strain.

Compound	**Concentration (g L^{-1})**
Yeast extract	10
Peptone	20
Glucose	20
Zeocin	0.1
Agar	20

3.5 Preparation and Analysis of Nucleic Acids

Genetic engineering, alternatively known as recombinant DNA technology or genetic modification is interested in the production of products which are obtained by the metabolic action of microorganisms. The development of genetic engineering techniques has made recombinant proteins more attractive. Despite the presence of diverse and complex techniques, basic principles of genetic manipulation are straightforward and they can be explained as follows:

- The desired DNA fragment is amplified with Polymerase Chain Reaction (PCR) by using primers that include restriction sites.
- Amplified region and the appropriate plasmid are cut by the suitable restriction enzymes.
- Digested gene and plasmid are ligated by ligase enzyme and recombinant plasmid containing the gene is transformed into host microorganism.
- Transformed microorganisms are plated on a medium including the proper antibiotic resistance gene carried by the plasmid. Only the cloned microorganisms carrying the plasmid can grow on the media and can readily be selected.
- However, the selected microorganisms are also controlled using other genetic engineering techniques whether they carry the plasmid or not.

3.5.1 Plasmid DNA Isolation from *E. coli*

GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific Inc.) was used for isolation of plasmid DNA of *E. coli* pPDCZαA*::hGH* which was utilized as template in PCR. Plasmid DNA isolation procedure given by Thermo Fisher Scientific for bacteria is as follows:

1. LSLB plates containing zeocin were prepared single colony was inoculated onto medium and cultivated overnight at 37°C.

2. Single colony cells were transferred and incubated for 12-16 hours at 37°C and 200 rpm in LSLB medium in 10 mL of 50 mL sterile falcons. 2.5 µL Zeocin were added per 10 mL of medium.
3. The all subsequent steps were performed at room temperature and all centrifugations which were carried out in a microcentrifuge performed at 14000 rcf.
4. Cells were harvested by centrifugation at 6800 rcf for 2 minutes in a standard tabletop centrifuge to remove the LSLB medium.
5. Pellets were resuspended in 250 µL Resuspension Solution, containing RNase, (stored in 4°C) by pipetting up and down and were transferred to a sterile 1.5 mL microcentrifuge tube.
6. 250 µL Lysis Solution was added and mixed thoroughly by inversion 4-6 times to release the plasmids without affecting the genomic DNA.
7. No longer than five minutes 350 µL Neutralization Solution was immediately added and mildly mixed by inversion 4-6 times to precipitate cellular debris and to suspend the plasmid DNA in the solution.
8. Microcentrifuge tube was centrifuged in a microcentrifuge for 5 minutes to precipitate cell debris and chromosomal DNA.
9. Approximately 500 µL supernatant was transferred to GeneJet spin column which includes a silica matrix providing plasmid DNA binding.
10. Spin column was centrifuged for 1 minute at 14000 rcf and flow-through was discarded
11. 500 µL Wash Solution including ethanol was added to column both to precipitate the plasmid DNA and to remove any remaining contaminants.
12. Spin column was centrifuged for 1 minute at 14000 rcf and flow-through was discarded.
13. Same wash procedure was repeated.
14. In order to remove residual solution, ethanol, additional centrifugation was applied for 1 minute.
15. Column was transplanted to a new 1.5 mL tube

16. 50 µL Elution Buffer was leaved into the center of the column releasing the plasmid DNA from silica matrix and incubated for 2 to 4 minutes.
17. Column was centrifuged for 2 minute at 14000 rcf
18. Additional and same elution procedure was applied.
19. Final solution containing purified plasmid DNA was stored at -20°C.

3.5.2 Agarose Gel Electrophoresis

Agarose gels permit identification, separation; extraction and visualization of nucleic acids rely on the charge migration. Agarose Gel Electrophoresis is an effective method for DNA strands from 0.5 to 25 kb (Voytas, 2001). Separation, according to size of DNA fragment is achieved by the migration of negatively charged molecules by means of an agarose matrix with an electric field towards anode. Agarose gels are prepared between 0.7 % and 2 % based on the intended purpose and size of the DNA fragments and to allow visualization ethidium bromide can be supplemented to the electrophoresis buffer. Approximate length of the DNA fragments can be estimated by comparison with the DNA ladders.

In this study agarose gels including 0.8 % agarose in 1X TAE Buffer was prepared by heating the solution until boiling on a hot plate. Following cooling to around 50°C ethidium bromide with a final concentration of 0.035 µg mL^{-1} was added. Gel was solidified on a gel tray and then transferred in gel electrophoresis tank containing 1X TAE buffer. Appropriate DNA ladder and samples were loaded into the sample wells and were run at 90V for 40-60 minutes achieving optimal separation and visualized upon illumination with UV light at 302 nm.

3.5.3 Primer Design

Primers used in this study were designed with their possible homodimer, heterodimer and loop formation, and melting temperatures by using the program OligoAnalyzer 3.1 (eu.idtdna.com/calc/analyzer) and purchased from Sentromer DNA Technologies (Istanbul, Turkey). Restriction endonucleases were prefixed

and suffixed to the primers which were used for insertion into pPIC3.5K plasmids. Thermodynamic features of designed primers were given in Appendix B.

Forward Primer

PDC-Fw **5'-** AGACGTCGGGACAAGCACACGATTA **-3'**

AatII Complementary to P_{PDC}

Reverse Primer

hGH-Rv **5'-** CATACCGGTCTAGAAGCCACAGCTG **-3'**

AgeI Complementary to *hGH*

The final concentration of designed primers was set to be as 100 pmol μL^{-1} by dissolving in sterile water and stored at -20°C. Diluted aliquots including 5 µM primer solutions were utilized in PCR experiments.

3.5.4 Polymerase chain reaction – PCR

One of the most essential steps in this procedure is the amplification of the required specific DNA fragment from a complex pool of DNA. PCR is an *in vitro* DNA amplification and it is simple, crucial, elegant, and straightforward enzymatic assay. Template DNA, suitable primers, the availability of the four deoxynucleotide triphosphates (dNTPs), DNA polymerase, and correct buffer composition are required by the PCR assay. DNA polymerases are the enzymes that create PCR products by linking nucleotides. DNA polymerases are thermostable enzymes which have been isolated from hyperthermophilic microorganisms, such as *Thermus aquaticus* or *Pyrococcus furiosus* (Chien et al., 1976, Glazer and Nikaido 1995). Primers are short defined sequences complementary to the template DNA and they amplify the exact DNA fragment. dNTPs consist of the four bases namely, adenine, thymine, cytosine, and guanine (A, T, C, G) which are fundamentally building blocks that are utilized by the DNA polymerase to create new DNA strand. When DNA duplex is heated the two strands of DNA is separated and effectively duplicated by the primers and DNA polymerase enzyme. Automated thermocycler

which is a programmable heating system can quickly raise or lower the temperature and can hold during the desired period at the desired temperature (Weier & Gray, 1988). The three basic steps in PCR which are repeated for 25 to 35 cycles were given below:

- **Denaturation step** is the separation of the two strands of the DNA double helix to form single strands carried out at high temperatures (94-96°C).
- **Annealing step** is binding the primers to the single stranded DNA by lowering the temperature to optimum annealing temperature which varies with the properties of primers (40-65°C).
- **Elongation step** is the synthesis of the nascent DNA strand most commonly occurs at 72°C.

Amplification of DNA fragment including PDC promoter region, α-MF secretion signal and hGH gene was carried out with the thermal cycling machine Bio-Rad iCycler (CA, USA). Isolated pPDCZαA*::hGH* plasmids were used as templates in PCR. Annealing temperature and salt concentration were determined as the optimum PCR process conditions. In order to control the amplification reaction 1-5 μL of mixture was analyzed by agarose gel electrophoresis. Thermal cycler operation conditions and mixture constituents were given in Table 3.4 and Table 3.5, respectively.

Table 3.4 PCR reaction operation parameters.

Number of Cycles	Temperature	Time
1 cycle	94°C	3 minutes
1 cycle	94°C	1 minute
	58°C	1 minute
	72°C	3 minutes
29 cycles	94°C	1 minute
	61°C	1 minute
	72°C	3 minutes
1 cycle	72°C	10 minutes

Table 3.5 PCR reaction mixture composition.

Component	Amount
10X PCR Reaction Buffer (without Mg^{++})	5 µL
$MgSO_4$ (25 mM)	6 µL
dNTP Mix (2 mM)	10 µL
Forward primer (5 µM)	2 µL
Reverse primer (5 µM)	0.2 µM
Template DNA (50 pg - 1 µg)	0.5-5 µL
Pfu DNA Polymerase (2.5 U μL^{-1})	1 µL
Nuclease-free water	variable
Total volume	50 µL

3.5.5 Purification of PCR Products

In order to obtain pure PCR products GeneJET PCR Purification Kit was used which removes primers, unincorporated nucleotides, salts, enzymes, dNTPs and other reaction mixtures. PCR products and linearized plasmids were purified

according to the protocol provided by the manufacturer's instructions. End PCR products were eluted in elution buffer and they were stored at -20 °C for further usage.

3.5.6 Restriction Digestion of DNA

Restriction endonucleases (restriction enzymes) are bacterial enzymes which cleave double-stranded DNA at particular, target base sequences named as recognition sites. Bacteria use these enzymes to degrade the bacteriophages and the viral DNA by recognizing certain nucleotides. The feature of restriction enzymes are used in many applications such as cutting both the vector and the DNA fragment with the same restriction enzymes and reuniting them to form a new vector, linearization of plasmids, or cleaving genomic DNA to analyze genes.

In this study restriction sites were identified by using Restriction Mapper website of USA Molecular Biology Resources (www.restrictionmapper.org). Restriction digest of PCR products and plasmids were achieved by double digestion with AgeI (BshTI) and AatII restriction enzymes subsequent to purification of PCR products and isolated plasmids. Reaction mixtures of double digestion of both PCR products and plasmid DNA were given in Table 3.6 and Table 3.7, respectively.

Table 3.6 Reaction mixture of double digested PCR products.

Component	**Amount**
10X Buffer Tango	2 µL
AatII	1 µL
AgeI (BshTI)	4 µL
PCR reaction mixture	10 µL (~0.1-0.5 µg of DNA)
Nuclease-free water	13 µL
Total volume	30 µL

Table 3.7 Reaction mixture of double digested plasmids.

Component	Amount
10X Buffer Tango	2 µL
AatII	0.5 µL
AgeI (BshTI)	2 µL
DNA (0.5-1 µg µL-1)	1 µL
Nuclease-free water	14.5 µL
Total volume	20 µL

Prepared reaction mixtures were mix gently and spun down for a few seconds. Restriction digestion of PCR products and plasmids were accomplished by incubating the mixtures at 37°C for 2 hours and enzymes were thermally inactivated by heating for 20 minutes at 65°C.

3.5.7 Purification of Digested DNA

For the purification of double digested PCR products GeneJET PCR Purification Kit was used whereas in order to purify the double digested amplicons, DNA was extracted from the agarose gel by the help of GeneJET Gel Extraction Kit. To that end, DNA were run on agarose gel to separate the DNA fragments by electrophoresis and requested DNA fragments were excised by slicing with a razor blade or a clean scalpel up to 400 mg DNA slices. Then the protocol which was provided by the manufacturer was applied.

3.5.8 Ligation Reaction

The amplified, digested and purified PCR products and isolated, digested and purified vectors are ligated from their sticky ends by the help of enzyme in a molar ratio of insert DNA over vector varied between 1:1 and 5:1. Ligation reaction composition was given in Table 3.8.

Table 3.8 Reaction mixture of ligation.

Component	**Amount**
10X T4 DNA Ligase buffer	2 µL
Linear vector DNA	20-100 ng
Insert DNA	1:1 to 5:1 molar ratio over vector
T4 DNA Ligase	1 U
Nuclease-free water	varied
Total volume	20 µL

In this research, insert DNA over vector ratio was optimized as 3:1. Required amount of insert DNA added to the reaction mixture was determined by the following equation:

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

The reaction was maintained by incubating the ligation mixture at 22°C for 1 hour and the enzyme T4 DNA Ligase was heat inactivated at 65°C for 10 minutes to terminate the reaction. Ligation mixture was preserved at -20°C for further usage in *E. coli* transformation.

3.5.9 Transformation of Plasmid DNA into *E. coli*

In order to propagate the plasmids wild type *E. coli* DH5α cells chemically competent cells preparation and transformation was performed by $CaCl_2$ method defined by Sambrook and Russell (2001). The competent cell facture protocol is as follows:

1. Wild type *E. coli* DH5α strain was incubated on LB solid medium at 37°C for 16 to 20 hours.
2. Single colony was picked and inoculated in a 250 mL erlenmeyer flask in 50 mL of LB-Broth at 37°C with shaking at 200 rpm.
3. When OD_{600} reached to 0.35-0.4, after approximately 3.5-4 hours incubation, the cells were transferred into precooled, sterile 50 mL chilled falcon and embedded on ice for 10 minutes.
4. The cells were collected by centrifugation at 4°C and 2700 rcf for 10 minutes and supernatant was discarded.
5. The pellet was resuspended in 30 mL ice-cold 80mM $MgCl_2$-20mM $CaCl_2$ (filter-sterilized) solution by swirling.
6. The cells were harvested by centrifugation at 4°C and 2700 rcf for 10 minutes and supernatant was removed.
7. The pellet was resuspended in 2 ml ice cold 0.1M $CaCl_2$ (filter-sterilized) solution by pipetting.
8. In this way competent cells have been prepared and they can either be used directly in transformation or can be stored at -80°C including 12% glycerol aliquots.

The transformation protocol continues as follows:

9. 50 µl of the mixture was transferred into precooled 1.5 mL chilled Eppendorf tube using ice-cold micropipette tips.
10. 1-5 µl containing 1 pg to 100 ng of ligation mixture (plasmid DNA) was added to cell mixture. Tube was carefully flicked 4-5 times to mix the cells and DNA.
11. Mixture was stuck in ice for 30 minutes.
12. Heat-shock was applied at 42°C in water bath for exactly 90 seconds.
13. The tube was plunged into ice for 5 minutes.
14. Mixture was transferred to a 50 mL sterile falcon.
15. 900 µl LB-Broth stored at room temperature was added into the mixture and incubated at 37°C and 200 rpm for 1 hour.

16. Inoculated cells were thoroughly mixed by flicking and 25 to 200 µl of the cells were put upon the LSLB plates comprising appropriate antibiotics and was instantly spread over. Also10 fold dilution was performed with Super Optimal broth with Catabolite repression (SOC) Medium (Table 3.9) and 50 to 100 µl mixture was spread over the LSLB plates.
17. Cells are incubated at 37°C for 16-20 hours.

3.5.10 DNA Sequencing

Subsequent to transformation into *E. coli*, inserted plasmid sequence was confirmed by Applied Biosystems Hitachi 3500 Genetic Analyzer (Tokyo, Japan). Sequence of pPDC3.5Kα*::hGH* plasmid and result of the sequencing was given in Appendix C.

Table 3.9 Composition of SOC Medium.

Compound	**Concentration (g L^{-1})**
Tryptone	20
Yeast Extract	5
NaCl (10 mM)	0.584
KCl (2.5 mM)	0.186
$MgCl_2$ (10 mM)	0.952
$MgSO_4$ (10 mM)	1.2
Glucose	3.6
dH_2O	to 1 L

3.5.11 Plasmid Linearization

In the transfection of *P. pastoris* linearized (single-digested) plasmids were used. After the sequence was verified, the plasmids were isolated and restriction digestion was performed with the appropriate restriction enzyme. Linearization reaction mixture of vector was given in Table 3.10.

Table 3.10 Reaction mixture used for plasmid linearization.

Component	Amount
10X Buffer O	2 µL
SalI	2 µL
DNA (0.5-1 µg μL^{-1})	1 µL
Nuclease-free water	15 µL
Total volume	20 µL

Plasmids were digested by using the SalI restriction enzyme and by incubating the plasmids at 37°C for 3 hours. The enzyme was thermally inactivated by incubating the mixture at 65°C for 20 minutes and the reaction was terminated. Single digestion was confirmed by running 1 µL reaction mixture on agarose gel and concentration of plasmid was arranged as 80 ng μL^{-1}. The mixture was purified by GeneJET PCR Purification Kit and final product was eluted in 50 µL pure water and stored at -20°C to be used in the transfection of *P. pastoris*.

3.5.12 Transfection into *Pichia pastoris*

Transfection into *P. pastoris* pGAPZαA*::hGH*/X-33 was performed by the chemical LiCl method provided by the manufacturer (Invitrogen, 2010). Requisite solutions used in the procedure were given in Appendix A and the protocol as follows:

1. *P. pastoris* pGAPZαA*::hGH*/X-33 cells were incubated on YPD agar containing Zeocin at 30°C for 48 hours.
2. Single colony was inoculated in a 250 mL baffled erlenmeyer flask in 50 mL of YPD Broth at 30°C and 200 rpm for 12-15 hours.
3. When OD_{600} reached 0.8 to 1.0, mixture was transferred to a 50 mL sterile falcon and cells were harvested by centrifugation at 4°C and 4000 rcf for 5 minutes and the supernatant was discarded.
4. Cells were washed with 25 mL autoclaved water.

5. Mixture was centrifuged for 10 minutes at 1500 rcf at room temperature and supernatant was removed.
6. In the meantime, 30 µl of 2 mg mL^{-1} Salmon sperm DNA (single-stranded DNA) was boiled at 95°C for 5 minutes and instantly submerged on ice.
7. Pellet was resuspended in 1 mL 100 mM LiCl (filter-sterilized) solution and the mixture was transferred to a 1.5 mL sterile Eppendorf tube.
8. Mixture was centrifuged at maximum speed for 15 seconds in a microcentrifuge and LiCl was removed with a pipette.
9. Cells were resuspended in 400 µL 100 mM LiCl.
10. For each transfection, 50 µl of cells were distributed into 1.5 mL Eppendorf tubes and quickly centrifuged for 15 seconds at maximum speed.
11. LiCl was removed with a pipette.

During the transfection of the cells the following substances were added quite slowly, heedfully and in the given order:

- 240 µL of 50% PEG,
- 36 µLof 1 M LiCl,
- 25 µLof 2 mg mL^{-1} Salmon sperm DNA,
- 2.5 µg linearized plasmid DNA in 50 µl sterile water

12. The tube was vortexed vigorously for approximately 1 minute and incubated at 30°C for 30 min without shaking.
13. Heat-shock was applied at 42°C in water bath for 20-25 minutes.
14. The tube was centrifuged at 4000 rcf for 15 seconds.
15. Solution was removed by a pipette and the cells were resuspended in 1 mL YPD Broth and the medium transferred to a 50 mL sterile falcon.
16. Cells recovery was performed at 30°C with shaking at 200 rpm for 4 hours.
17. 25-200 µL of the medium was spread over the MD plates (Table 3.11) and incubated for 3 days in order to select the His^+ transformants.

18. At the end of 3 days selected colonies were inoculated on the YPD plates containing 0.25 g L^{-1} G-418.

Table 3.11 Composition of solid MD medium.

Compound	**Concentration (g L^{-1})**
Yeast Nitrogen Base (YNB)	13.4
Dextrose	20
Biotin	0.0004
Agar	20

Table 3.12 Composition of solid YPD medium used for recombinant *P. pastoris* GAP-PDC*::hGH* strain.

Compound	**Concentration (g L^{-1})**
Yeast extract	10
Peptone	20
Glucose	20
G-418	0.25
Agar	20

3.5.13 Isolation of Genomic DNA from *P. pastoris*

P. pastoris genomic DNA isolation was performed by the method described by Burke et al., (2000) with slight modifications. Followed by the transfection, genomic DNA isolation was applied to the selected colonies both to verify the colonies whether they transfected or not and to use the genomic DNA of the confirmed colonies in the RT-qPRC for copy number determination. The method was listed below:

1. Selected colonies were proliferated on YPD (Table 3.4) agar plates at 30°C for 48 to 60 hours. Cells were scraped, transferred to sterile 2 mL Eppendorf tube and were used directly for the genomic DNA isolation.
2. Cells were resuspended in 200 µL yeast lysis solution (filter-sterilized) the tubes were mixed by flicking to make sure of cell lysis.
3. 200 µL phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube in the fume hood and approximately 0.3 g glass beads (acid-washed) were added.
4. Parafilm was used to wrap the tubes and they were vortexed for 4 minutes.
5. 200 µL Tris-EDTA (TE) buffer solution with pH of 8.0 was added and for 2 minutes tubes were centrifuged at 16 000 rcf in a microcentrifuge.
6. Approximately 200 µL of aqueous layer was taken and transferred to a new sterile 2 mL Eppendorf tube.
7. 1 mL of 100 % EtOH was put upon the solution and they were mixed by inversion.
8. The pellet was harvested by centrifugation at 16 000 rcf for 2 minutes and supernatant was removed and the pellet was air-dried.
9. The pellet was dissolved in 400 µL TE buffer and 4 µL of 10 mg mL-1 RNase A was added.
10. The mixture was incubated at 37 °C for 15 minutes.
11. Followed by incubation 14 µL of 3 M sodium acetate and 1 mL of 100 % EtOH was put upon the solution and they were mixed by inversion.

12. The pellet (genomic DNA) was separated by centrifugation at 16 000 rcf for 2 minutes and supernatant was discarded.
13. Air-dried pellet was dissolved in 50 µL of TE Buffer or sterile water.

3.5.14 Determination of Gene Copy Number

Both PCR and the Quantitative RT-PCR (qRT-PCR) share the same purpose which is the amplification of the DNA fragment. Besides, qRT-PCR is also highly accurate, sensitive and precise, reproducible, and rapid method and has a broad quantification range (Heid et al. 1996) allowing the determination of the initial copy number of the target sequence. It utilizes the linear DNA amplification and quantifies the absolute or relative DNA sequence. In qRT-PCR two types of fluorescent reporter molecules, an intercalating dye which binds the double-stranded DNA or a dye- labeled probe can be used and progression of the reaction is monitored during the thermal cycling. Amplified product amount is proportional to the accumulated fluorescence signal due to binding of amplicons to a fluorescence dye. Reaction-limiting situations are least during the exponential phase of the reaction; therefore, fluorescence intensity is monitored during the amplification (throughout the 30 to 45 cycles) and measured in the exponential phase. If the primary number of DNA molecules higher in a sample the fluorescence is perceived in earlier cycles. For a fluorescent signal, the number of cycles desired to intercept the threshold is defined as cycle threshold (C_t) and quantitation cycle (C_q) is described as the cycle in which fluorescence signal detected. Higher C_q values means lower initial gene copy number.

In this research both absolute and relative quantification was performed. As a housekeeping gene argininosuccinate lyase, *ARG4*, (PAS_chr1-1_0389, XM_002490002.1) was utilized because the *ARG4* gene was found as single copy in the haploid host, thus it was appropriate as an endogenous control (Abad et al., 2010). Also, the strain pGAPZαA:*:hGH*/X-33 which was constructed Massahi and Çalık, (2016) was determined as a single copy in the genome of *P. pastoris* and it was utilized for relative quantification during qRT-PCR experiments. In order to

improve the qRT-PCR by lowering the nonspecific amplification and limit of detection nested PCR technique was used (Haft, 1994). List of external and internal primers was given in Table 3.13 (Massahi and Çalık, 2016). While constitute standard curves external primers of *ARG4* and *hGH* was used and samples with known concentrations were procured and the standard curves covering the copy quantity ranges for *ARG4* and *hGH* which was primed by serial dilutions, was presented in Table 3.14.

Concentrations were converted to the copy quantities (number of copies) by the following equation:

$$Copy\ quantity = \frac{amount\ of\ sample\ (ng) \cdot 6.022 \cdot 10^{23}}{length\ of\ DNA\ (bp) \cdot 10^{9} \cdot 650}$$

where, $6.022 \cdot 10^{23}$ is the Avagadro number and the average weight of a single DNA base pair (bp) is 650 Daltons (g mol^{-1}).

Table 3.13 The external and internal primers used in qPCR experiments.

Name of the Primer	**Nucleotide Sequence (5'-3')**	**Amplicon Size (bp)**
External Primers		
GAP forward	GTCCCTATTTCAATCAATTGA	540
AOX reverse	GCAAATGGCATTCTGACATC	
ARG4-Std-F	CTTGAACATTGATGCCGAAA	330
ARG4-Std-R	GACTCTAGCTTTTCATTCAGC	
Internal Primers		
hGH-F	ACGTCTATGACCTCCTAAAGG	202
hGH-R	AATGTCTCGACCTTGTCCATG	
ARG-F	TCCATTGACTCCCGTTTTGAG	84
ARG-R	TCCTCCGGTGGCAGTTCTT	

Table 3.14 The copy quantity ranges for *ARG4* and *hGH*.

ARG4		***hGH***	
Sample	**Copy µL^{-1}**	**Sample**	**Copy µL^{-1}**
Stock	$3.41 \cdot 10^8$	Stock	$8.91 \cdot 10^8$
Std-ARG1	$3.41 \cdot 10^6$	Std-hGH1	$8.91 \cdot 10^6$
Std-ARG2	$3.41 \cdot 10^5$	Std-hGH2	$8.91 \cdot 10^5$
Std-ARG3	$3.41 \cdot 10^4$	Std-hGH3	$8.91 \cdot 10^4$
Std-ARG4	$3.41 \cdot 10^3$	Std-hGH4	$8.91 \cdot 10^3$

In order to determine the copy number of the unknown samples a couple of standard curves, both for *ARG4* and *hGH* were generated. Reaction efficiency, E, was quite significant, the accepted range for qPCR is 90-105%, and it can be calculated by the equation given below:

$$E = (10^{-1/slope} - 1) \cdot 100$$

In that study iTaq™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA) was used as intercalating fluorescent dye. Merely when the dye binds to double-stranded DNA, fluorescence is observed and the fluorescence increases with each cycle all along the PCR amplification reaction. While conducting the qRT-PCR experiments CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA) was used to determine and quantify the nucleic acids in the samples. Genomic DNAs of the unknown samples, serially diluted standards and nuclease-free water for non-template control (NTC) were used as DNA templates during qRT-PCR experiments. Operation conditions of qPCR reactions and compositions of the qPCR reaction mixtures were presented in Table 3.15 and 3.16, respectively.

Table 3.15 qPCR reaction operation parameters.

Number of Cycles	Temperature	Time
1 cycle	95°C	3 min
35 cycles	95°C	1 min
	55°C	1 min
	72°C	3 min

When the standard curves were constructed copy quantity of the unknown sample can be determined by using the C_q values obtained from the qPCR amplification. Expected C_q values should be within the range of $12 < C_q < 26$. Once the copy quantities of the target gene (*hGH*) and the housekeeping gene (*ARG4*) were determined absolute quantification, independent of the precise amount of the input genomic DNA, was performed by normalization to calculate the copy number of the unknown sample by the following equation:

$$\text{Copy number of target gene} = \frac{\text{Copy quantity of target gene } (hGH)}{\text{Copy quantity of housekeeping gene } (ARG4)}$$

Table 3.16 qPCR reaction mixture composition.

Component	Amount	Final Concentration
SYBR Green Supermix (2X)	5.0 µL	1X
Forward Primer (5 µM)	0.8 µL	400 nM
Reverse Primer (5 µM)	0.8 µL	400 nM
DNA Template	2.0 µL	50 ng - 5 pg
Nuclease-free water	1.4 µL	-
Total volume	10 µL	-

Melt Curve Analysis

Melt curve analysis is routinely applied in qPCR experiments when double-stranded DNA-binding fluorescent dyes are utilized due to the probability of binding onto primer-dimers or other non-specific amplification products. In order to eliminate the overestimation of the target copy quantity or to evaluate the specificity by the help of NTC analysis, melt curve assay is followed by validating the results. Melt curves were created subsequent to each qPCR run. The temperature was increased from 60°C to 99°C and 0.5°C increment for each step and hold for 2 seconds. A proper melt curve analysis has expected to have only one clear peak yet extra peaks imply either primer-dimers or non-specific PCR products.

3.6 Production of rhGH

In this research, recombinant human growth hormone production was performed by genetically engineered *P. pastoris* strain carrying two vectors with two different constitutive promoters by conducting both air filtered shake bioreactor experiments and pilot scale bioreactor experiments.

In *P. pastoris* productions of recombinant proteins are depend on two-stage cultivation. The first stage is the precultivation which brings cells in the same growth phase in a low initial medium and the other step is the cultivation which the expression of recombinant proteins is observed.

3.6.1 Precultivation

Precultivation is a technique which was applied to the microorganisms that show overflow metabolism (Pham et al., 1999, Mattanovich et al., 2009). The medium includes all the required nutrients with low carbon source concentration in a little medium volume. In this study, buffered glycerol complex medium, BMGY was used as precultivation medium and its composition was given in Table 3.17. Biotin and chloramphenicol were added afterwards the sterilization and subsequent cooling to approximately 50°C.

Table 3.17 Composition of BMGY, precultivation medium.

Compound	**Composition (g L^{-1})**
Yeast extract	10
Peptone	20
Potassium phosphate buffer (pH=6.0)	0.1 M
Yeast Nitrogen Base (YNB) (w/o amino acids)	3.4
Ammonium Sulfate	10
Glycerol	10
Biotin	4×10^{-5}
Chloramphenicol (34 mg mL^{-1})	1 mL L^{-1}
dH_2O	to 1 L

P. pastoris GAP-PDC*::hGH* strain from its microbank was inoculated on YPD solid medium (Table 3.4) for 48 hours at 30°C. A single colony was picked and cultivated in BMGY with shaking at 200 rpm at 30°C for approximately 16 to 18 hours until the OD_{600} reach to its value between 2-6. Subsequently, cells were collected by centrifugation at 4°C for at 1500 rcf 10 minutes and they were resuspended in sterile water and added to the production medium, either in shake flasks or pilot scale bioreactor, by adjusting the initial production OD_{600} as 1.

3.6.2 Production of rhGH in Air Filtered Shake Bioreactors

Followed by precultivation, *P. pastoris* GAP-PDC*::hGH* was cultivated in the production medium to produce rhGH. During the production 250 mL air filtered, baffled shake flasks were used with a working volume of 50 mL. Composition of the defined production medium developed by Jungo et al. (2006) was given in Table 3.18 with a slight difference. As nitrogen source ammonium sulfate was added rather than ammonium chloride. Pichia Trace Metals, PTM1 was prepared as filter-sterilized. It developed by Sibirny et al. (1987) and its composition was presented in Table 3.19.

Table 3.18 Composition of production medium.

Compound	**Composition (g L^{-1})**
Glucose	20
Ammonium Sulfate	9.54
Potassium phosphate buffer (pH=6.0)	0.1 M
$MgSO_4 \cdot 7H_2O$	14.9
$CaSO_4 \cdot 2H_2O$	1.17
Chloramphenicol (34 mg mL^{-1})	1 mL
PTM1	4.35 mL

Table 3.19 Composition of PTM1 solution.

Compound	**Amount per liter**
Cupric sulfate-pentahydrate ($CuSO_4.5H_2O$)	6.0 g
Sodium iodide (NaI)	0.08 g
Manganese sulfate monohydrate ($MnSO_4.H_2O$)	3.0 g
Sodium molybdate dehydrate ($Na_2MoO_4.2H_2O$)	0.2 g
Boric Acid (H_3BO_3)	0.02 g
Cobalt chloride ($CoCl_2$)	0.5 g
Zinc chloride ($ZnCl_2$)	20.0 g
Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	65.0 g
Biotin	0.2 g
Sulfuric Acid (H_2SO_4)	5.0 mL
dH_2O	to1 L

3.6.3 rhGH Production in Pilot Scale Bioreactor

Pilot scale 7 L bioreactor (Sartorius BIOSTAT® Cplus, Germany) with a minimum working volume of 1.6 L was used. During fermentation Temperature, Dissolved Oxygen (DO), pH, agitation rate, foam and level can be measured and controlled. By PID cascade multi-stage controller was applied to control the gas filling controller (air, O_2 or N_2), gas flow controller, agitation rate controller and substrate supply controllers. The agitator with double mechanical seal with 6-blade disk agitators was used for stirring. In order to distribute the oxygen homogeneously bioreactor includes four baffles and a sparger. The fermenter has an external cooler, an electrical heater and a jacket to insulate and to maintain isothermal bioreactor operation condition as well as to sterilize the bioreactor. Oxygen and pH are perpetually measured by probes (Hamilton VisiFerm DO 120 probe, Switzerland). A compressor (Larfon Top Silent 1.5, Italy) is used for the air supply into the bioreactor. Besides, when the DO concentration decreases below the set point value a cascade system is activated to enrich the oxygen either by changing the inlet gas flow rate (Q/V) or changing the agitation rate (N) or supplying pure oxygen from an oxygen tube. A peristaltic pump is used to transfer the media into the culture vessel.

Subsequent to precultivation, when the OD_{600} reached to 4 on average, the fed-batch fermentation in the bioreactor proceeded with two phases namely, glycerol batch phase and glucose fed-batch phase. In the beginning of the glycerol batch phase OD_{600} was adjusted as 1, temperature was kept constant at 30°C and basal salt medium; BSM (Table 3.20) including 18 g L^{-1} glycerol was used where working volume of the bioreactor was set as 1.8 L. In order to maintain homogeneous oxygen and substrate transport agitation rate was set as 600 rpm.

Table 3.20 Composition of BSM.

Basal Salts Medium	**Amount per liter**
Phosphoric Acid (85%)	26.7 mL
Calcium Sulfate Dihydrate	1.17 g
Potassium Sulfate	18.2 g
Magnesium Sulfate heptahydrate	14.9 g
Potassium Hydroxide	4.13 g
Glycerol (100%)	18 g
Antifoam 30%	1 mL
PTM1	4.35 mL
Chloramphenicol	1 mL

When the glycerol was depleted glucose fed-batch phase was initiated. Within the 14 to 16 h, OD_{600}=41-45 corresponding to C_X=10.0-10.8 was determined (Equation 3) where the cell yield on the glycerol $Y_{X/S}$ (g g^{-1}) was determined as 0.56. pH was kept at 5.0 by suppling 25% ammonium hydroxide (NH_4OH) base as well as being a nitrogen source (Çelik et al., 2009). DO concentration was kept at either 5% or 15% of air saturation value, by feeding air or oxygen enriched air at a volumetric oxygen flow rate of Q_0=10 L min^{-1} and by cascading agitation rate (N=600-900 min^{-1}). 500 g L^{-1} glucose solution containing 12 ml L^{-1} PTM1 was commenced to feed into the bioreactor with a pre-determined specific growth rate $\mu_0 = 0.15$ h^{-1}. Pre-determined exponential feeding rate was calculated with the following equation derived in section 2.15 (Çalık et al. 2016):

$$Q_S = \frac{\mu_0 \cdot C_{X_0} \cdot V_0}{Y_{X/S} \cdot (C_S^0 - C_S)} \exp(\mu_0 \cdot t) \qquad (3.2)$$

where, Q_S (L h^{-1}) is the feed volumetric flow rate, μ_0 (h^{-1}) is the pre-determined specific growth rate, C_{X_0}(g L^{-1}) is the initial cell concentration at the inception of glucose fed-batch phase, V_0 (L) is the initial volume of the culture vessel at the inception of glucose fed-batch phase, $Y_{X/S}$ (g g^{-1}) is the cell yield on substrate, C_S^0 (g L^{-1}) is the feed substrate concentration, C_S is the substrate concentration in the bioreactor. However, C_S was taken as zero due to accumulation of glucose was not allowed in the bioreactor; therefore, Equation 2 is reduced to (Çalık et al. 2016):

$$Q_S = \frac{\mu_0 \cdot C_{X_0} \cdot V_0}{Y_{X/S} \cdot C_S^0} \exp(\mu_0 \cdot t) \qquad (3.3)$$

During the glucose fed-batch phase while determining the value of Q_S, the values of other parameters are; $\mu_0 = 0.15$ h^{-1}, $C_{X_0} = 10$ g L^{-1}, $V_0 = 1.9$ L, $Y_{X/S} = 0.48$g g^{-1} for glucose, and $C_S^0 = 500$ g L^{-1}.

3.7 Analysis

During the fed-batch fermentation samples were taken for every 3 hours centrifuged at 1500 rcf and 4°C for 10 minutes. Supernatants were taken to new tubes and pellets and supernatants were stored at -80°C. Supernatants were used to measure the protein concentration, glucose concentration, by-product, ethanol, concentration and organic acid concentration. Pellets were used to determine the intracellular protein concentration.

3.7.1 Cell concentration determination by optical density (OD_{600})

OD measurements of the cultures were performed at 600 nm in a UV-Vis Spectrophotometer (Spectroquant, Merck, Germany). Biomass concentration (C_X) can be proportionately correlated to the optical density (OD_{600}) by applying the Beer-Lambert law. Samples should be within the range 0.1 and 0.8 otherwise

samples giving absorbance value higher than 0.8 have to be diluted with dH_2O. A calibration curve was generated to associate the absorbance with dry weight. Depending on the calibration curve C_X (g L^{-1}) was determined by the equation:

$$C_X = 0.244 \times OD_{600} \times Dilution\ Factor \quad (3.3)$$

3.7.2 Extracellular rhGH Concentration

In order to measure the extracellular rhGH concentration Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis was performed developed by Laemmli (1970) with a few modifications. Gels were prepared by TGX Stain-Free FastCast Acrylamide Kit, 12% (Bio-Rad, CA, USA) according to manufacturer's instructions and they were stained by silver nitrate.

Samples which were loaded onto gel were prepared according to Table 3.21. Preparation of loading buffer was presented in Appendix A. Loading buffer and samples or hGH Standard with a concentration of 10 mg mL^{-1} were added in 1:4 ratio including 1 M DL-Dithiothreitol (DTT) and boiled for 5 minutes in a water bath. 3 µL PageRuler Prestained Protein Ladder and 15 µL of mixtures were loaded into the wells of the SDS gel. Gels were run in 1X SDS running buffer (Appendix A) at constant 40 mA and in the sequel silver staining protocol of SDS-PAGE gels was applied.

Table 3.21 Composition of sample mixture loaded onto SDS-PAGE.

Compound	**Amount**
1M DTT	2 µL
Sample or hGH Standard (10 mg mL^{-1})	13 µL
4X Loading Buffer	5 µL
Total volume	20 µL

Silver Staining Procedure:

Silver staining procedure of proteins was given as Table 3.22. All of the solutions were freshly prepared and their compositions were supplied in Appendix A. The incubations were performed at room temperature with shaking. The basic steps of silver staining protocol include: 1) fixation to dispose the interfering compounds, 2) rinses to enhance the contrast and the susceptibility of the staining, 3) impregnation with silver nitrate solution, 4) rinses and development to create the silver image, 5) rinse and stop to terminate the development to prevent the formation of redundant background and further processing.

Table 3.22 Silver staining of SDS-PAGEs.

Treatment	**Solution**	**Duration**
Fixing	Fixer	1 to 16 hours
Washing	50% EtOH	3 times for exactly 20 seconds each
Rinsing	dH_2O	3 times for exactly 20 seconds each
Pretreatment	Pretreatment Solution	1 minute
Rinsing	dH_2O	3 times for exactly 20 seconds each
Impregnate	Silver Nitrate Solution	20 minutes
Rinsing	dH_2O	2 times for exactly 20 seconds each
Developing	Developing Solution	About 5 minutes
Stop	Stop solution	More than 5 minutes

3.7.6 Glucose Concentration

Glucose concentration in the medium was measured by using Glucose Analysis Kit (Biasis, Turkey) by the method of glucose oxidation. In the assay, D-glucose is oxidized by the enzyme glucose oxidase in the presence of H_2O to form gluconate and hydrogen peroxide (H_2O_2). Nascent H_2O_2 reacts with 4-aminoantipyrine and phenol and the reaction is catalyzed by peroxidase yielding

red iminoquinone dye. The colored complex is proportional to concentration of glucose and is measured by UV/Vis Spetrophotometer at 505 nm. The sequence of the enzymatic reactions applied in the assay is as follows:

$$D - glucose + O_2 + H_2O \rightarrow gluconate + H_2O_2$$

$$H_2O_2 + 4 - aminoantipyrine + phenol \rightarrow iminoquinone + H_2O$$

By applying serial dilutions glucose standard calibration curve was obtained (Appendix E). Samples were diluted with distilled water if they include more than 2 g L^{-1} glucose. During quantifications 2.05 mL distilled water, 0.40 mL glucose analysis buffer, 0.05 mL glucose analysis reactive and 0.05 mL sample was mixed and incubated at 37°C for 20 minutes. The absorbance was measured at 505 nm with UV/Vis Spectrophotometer.

3.7.7 Ethanol Concentration

In order to measure the ethanol concentration in the cultivation media Ethanol analysis kit (Megazyme, Ireland)is used. The assay depends on two enzymatic reactions. The first reaction is the oxidation of ethanol to acetaldehyde in the presence of nicotinamide-adenine dinucleotide (NAD^+) catalyzed by the enzyme alcohol dehydrogenase (ADH). Yet the equilibrium reaction favors the left side. Therefore, by the quantitative oxidation acetaldehyde and NAD^+ reacts by the enzyme aldehyde dehydrogenase (Al-DH) to get acetic acid.

$$Ethanol + NAD^+ \rightarrow acetaldehyde + NADH + H^+$$

$$Acetaldehyde + NAD^+ + H_2O \rightarrow acetic\ acid + NADH + H^+$$

Ethanol standard calibration curve was constructed by performing serial dilutions to ethanol standard solution with a concentration of 5 mg mL^{-1}. Also, dilutions were applied to samples if their concentration were greater than 0.12 mg mL^{-1}. For measurements 2.0 mL distilled water, 0.1 mL sample, 0.2 mL buffer, 0.2 mL NAD^+ solution, 0.05 mL aldehyde dehydrogenase solution and 0.02 mL alcohol dehydrogenase suspension were mixed and incubated for 5 minutes. The absorbance was read at 340 nm with UV/Vis Spectrophotometer which was the NADH. The amount of NADH generated stoichiometrically is twice of the amount of ethanol. The protocol for ethanol concentration measurements were summarized in Table 3.23 and the equations used in the calculations were given below:

$$\Delta A = A_2 - A_1$$

$$\Delta A_{EtOH} = \Delta A_{sample} - \Delta A_{Blank}$$

$$C = 0.09397 \times \Delta A_{EtOH}$$

where, A_1 is the firts absorbance value of the samples after alcohol dehydrogenase was added, A_2 is the second absorbance value after the addition of aldehyde dehydrogenase, C is the ethanol concentration (g L^{-1}).

Table 3.23 Components and procedure of the ethanol assay kit.

Component	**Blank**	**Sample**
dH_2O	2.10 mL	2.00 mL
Sample	-	100 µL
Buffer solution	200 µL	200 µL
Solution 2 (NAD^+)	200 µL	200 µL
Solution 3 (Al-DH)	50 µL	50 µL
Mix and read the absorbance at 340 nm (A_1) after waiting for 2 min.		
Suspension 4 (ADH)	20 µL	20 µL
Mix and read the absorbance at 340 nm (A_2) after waiting for 5min		

3.7.8 Concentrations of Organic Acids

Concentrations of secreted organic acids in the production medium were measured by High Pressure Liquid Chromatography, HPLC (Waters, Alliance 2695, Milford, MA). Standard solutions of organic acids were utilized to obtain calibration curves of HPLC chromatograms and they were given in Appendix E. Samples were filtered by using 0.45 μm porous filters (Sartorius, AG) and they were diluted with the mobile phase with various ratios according to the requirement of the samples. For injections 200 μL of samples were put inside the vials. All solutions and distilled water were degassed in an ultrasonic water bath before the analysis. The operational conditions and the applied protocol were given in Table 3.24:

Table 3.24 The operational conditions for HPLC system for organic acid determination (Ileri and Calik, 2006).

Column	Capital Optimal ODS, 5 μm
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Detector	Waters 2487-Dual absorbance, 210 nm
Detector temperature	30°C
Mobile phase	0.312% (w/v) NaH_2PO_4 and 0.062% (v/v) H_3PO_4
Flow rate of mobile phase	0.8 mL/min
Column temperature	30°C
Injection volume	5 μL
Analysis period	15 minutes
Space time	5 minutes

3.8 Metabolic Flux Analysis

In order to investigate the metabolic network of the biochemical reactions of the r-*P. pastoris* strains metabolic flux analysis (MFA) was performed. The biochemical reactions which were already applied to r-*P. pastoris* strains expressing rhGH by Çalık et al. (2011) were utilized with slight modifications. Reactions were supplied in Appendix F including 102 metabolites with 147 reactions. During the analysis, the program GAMS 2.25 (General Algebraic Modeling System, GAMS Development Corp., Washington DC) was utilized to determine objective function Z, which was used to designate the distribution of intracellular fluxes by minimizing the rhGH accumulation within the cell (Çalık and Özdamar 2002). The variables of the model which were the fluxes of the metabolic reactions stated in mmol g_{DW}^{-1} h^{-1} and and flux through biomass was expressed in h^{-1}.

CHAPTER 4

RESULTS AND DISCUSSION

The scope of the M.Sc. thesis is to construct a *Pichia pastoris* strain containing double promoter expression cassettes and to investigate the expression level of recombinant human growth hormone (rhGH); and then to compare the novel recombinant strain with two previously constructed recombinant *P.pastoris* strains (Massahi, 2017) carrying hGH gene as single copy in the genome of the host by expressing the gene hGH under two constitutive promoters, separately. In the first part of the research, the cloning vector, pPIC3.5K, was manipulated by transforming the DNA fragment containing the constitutive pyruvate decarboxylase (PDC) promoter region, α-mating factor (MFα-1) secretion signal and the hGH gene. Afterwards, the newly synthesized plasmids were propagated, linearized and transfected into the genome of the recombinant *P.pastoris* strain which was already constructed to express *hGH* under GAP promoter. In order to determine the gene copy number, qRT-PCR studies were performed and copy number of the integrated expression cassette was determined. In the second part of the work, batch shaker-bioreactor experiments and pilot-scale bioreactor experiments were conducted to produce extracellular rhGH. Results were evaluated by SDS-PAGE analyses and the fermentation characteristics of the three strains were compared according to cell growth rates, rhGH production rates, and substrate consumption rates.

4.1 Development of the Strain

In order to develop a recombinant *P. pastoris* strain producing hGH under two constitutive promoters namely, P_{GAP} and P_{PDC}, four main steps were administered and summarized in Figure 4.1. As the first step DNA fragment including the P_{PDC}, MFα-1, and *hGH* gene was amplified by PCR with the designed primers from the previously constructed plasmid pPDCZα-A*::hGH* (Massahi, 2017) and, also pPIC3.5K plasmids were propagated and isolated. Both the DNA fragment and the expression vector were cut by the same restriction enzymes, then ligated and transformed into *E.coli* DH5α strain. Plasmids carrying the DNA fragment were detected after the restriction digestion of the plasmids and PCR amplification. Besides selected transformant was also verified by DNA sequencing. In the third step recombianant *P.pastoris* strain, previously transfected with pGAPZα-A*::hGH* (Massahi, 2017), was transfected with the newly constructed recombinant plasmid once again and transformants were selected in two consequtive steps. The His^+ transformants were selected on minus histidine plates then they were transferred and selected under Geneticin resistance. r-*P. pastoris* strain that express *hGH* under both P_{PDC} and P_{GAP} was constructed and named as GAP-PDC*::hGH*. Lastly, copy number of the selected colony was detected by qRT-PCR.

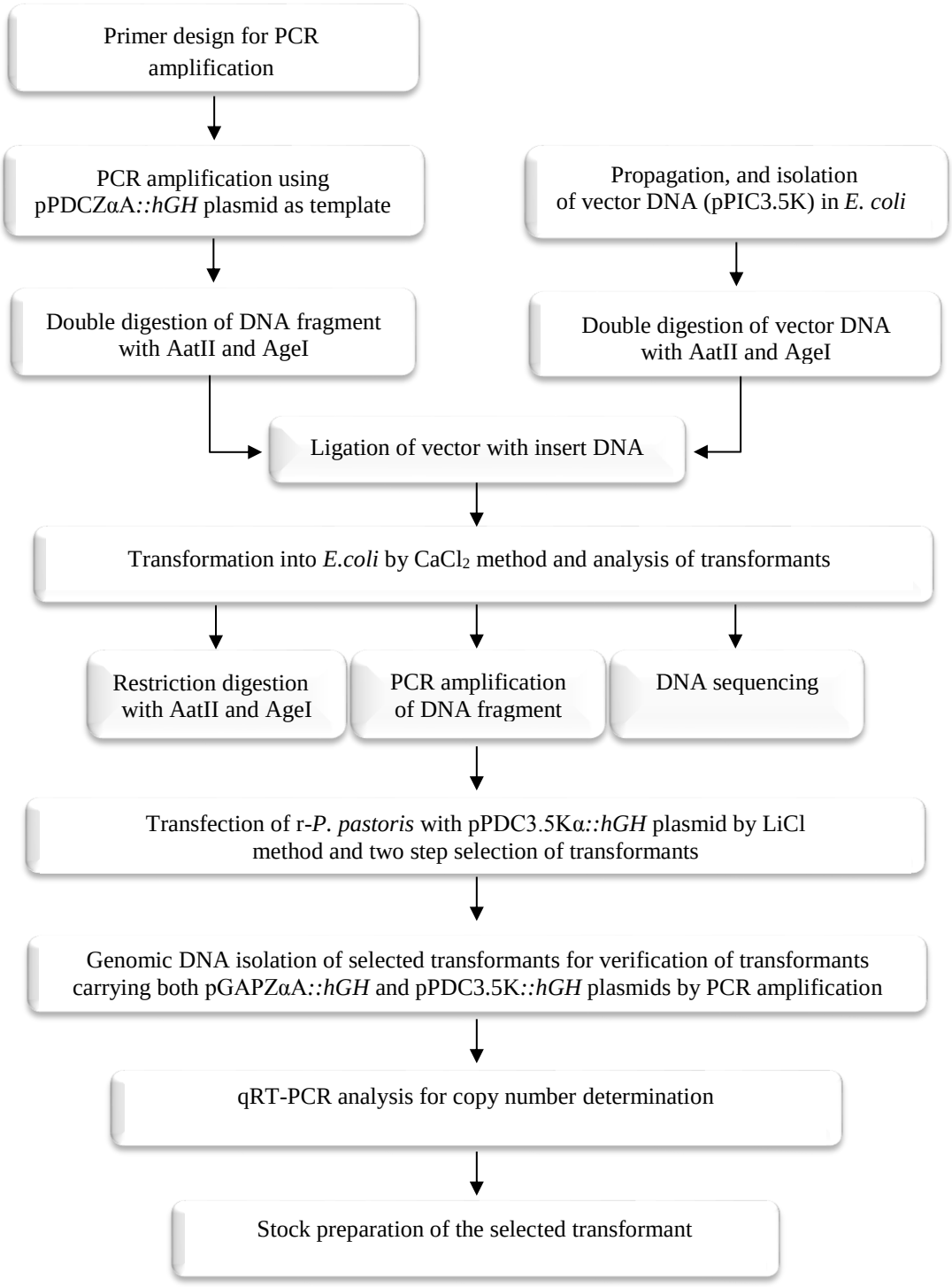


Figure 4.1 Flowchart of double promoter expression system construction in *P.pastoris*.

4.1.1 Development of the plasmid

For constructution of the plasmid pPDC3.5K*::hGH*, pPDCZα-A*::hGH* plasmid was utilized as a parent plasmid. The pPIC3.5K vector was transformed into *E.coli*. A sigle colony was selected, microbanks were provided for propagation of the plasmids and stored at -80°C.

Once the microbanks were prepared wild-type *E.coli* cells were inoculated on LSLB plates and colonies were further incubated on LB broth. Plasmid isolation was performed with GeneJet Plasmid Miniprep Kit according to the instructions Isolated circular plasmids were given in Figure 4.2.

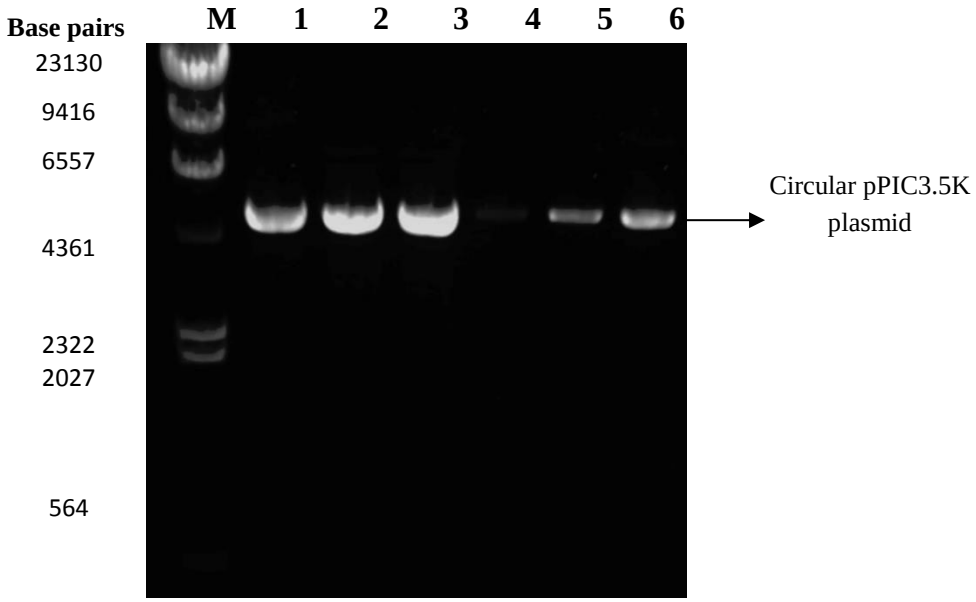


Figure 4.2 Gel electrophoresis image of the plasmids of the transformed *E.coli* colonies. M: Lambda DNA/HindIII Marker, whereas 1 to 6 shows the selected *E.coli* transformants.

In order to verify the size of the plasmids a single digestion with the restriction enzyme AatII was carried out. Circular plasmids run through the gel matrix faster than the linearized plasmids due to surfacing less space and linearized (single digested) and circular plasmids were compared in Figure 4.3.

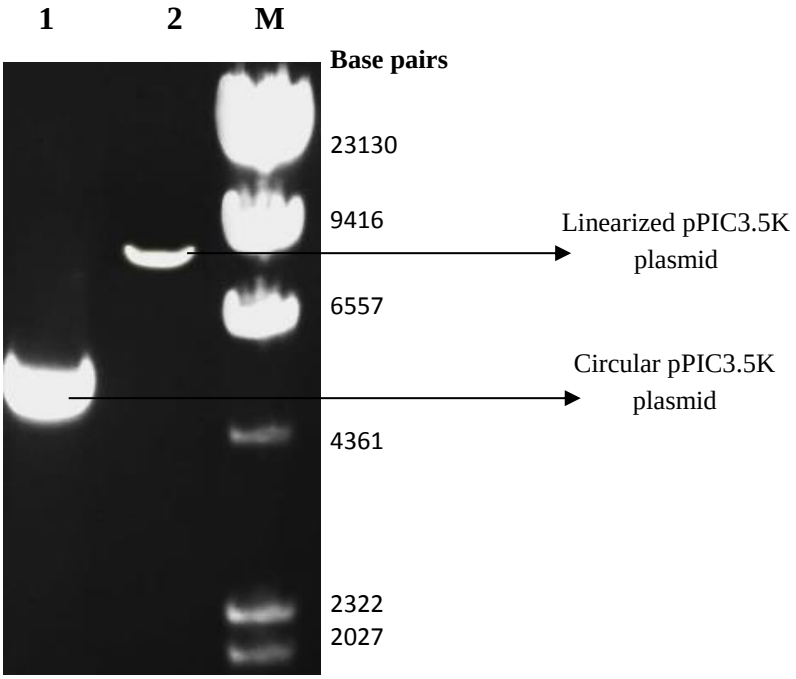


Figure 4.3 Gel electrophoresis output of pPIC3.5K vector. 1: circular pPIC3.5K, 2: linear pPIC3.5K, M: Lambda DNA/HindIII Marker.

Locus_tag of the pyruvate decarboxylase enzyme was defined as PAS_chr3_0188 in *P. pastoris* GS115 strain and the promoter region of the enzyme was chosen as a DNA segment interval beginning from the upstream of the PAS_chr3_0188 to the end of PAS_chr3_0189 having a length of 546 nucleotides (Figure 4.4).

5'…**GACGT^**CGGGACAAGCACACGATTACCCAATCACTTGATATGCACCAATTTGTTC
CGTTGTTTATGCCATATTTACCGAATTTTCTTCCCAGGTTTTTCCGAATGGACATCTGT
AGTCCACTTTTTGGTTATCATAATCGTCCCACAAGTCGTGGATTTAACCAGAACCTAG
TAATTTTAAGTTCGCTATTAATCACTCAGAATGGTCTCACCTTGCTATTGGCCAAGTCT
GGAGTCGCCAGCTACCACCTCAGAGGCTACATAGACCTCCCAATGTCATCTCCTCAGT
GCGCTCTTCAATCTCGTGTCTTTTCCGTTAAAACTCCGTTCGTTTCACCCTATACTGCC
CCTGGTTGTGCAGCTCTTACCACTTCGCGCCGCTACTATCCGTAGTGGTCGAGCCGCA
TCAATATCACGTTGAAATAGAATAACTCCCTACAAAAGCCGCACGCAACCATCAAAT
CTATATAAGGAACCTCAAATATCTAGCAACATCTTTTCAATTTACTACAACATATTCGT
TAATCATCAATCAATTAGCTAGTACACAACA… **3'**

Figure 4.4 Promoter region of the pyruvate decarboxylase gene used in that study. Bold six letters given at the 5' end of the sequence are restriction site equaled to AatII.

While the mature *hGH* gene comprising 573 nucleotides, constituted and utilized *hGH* gene was attached with restriction sites at both ends a polyhistidinetag (6xHis tag) and a Factor Xa cleavage site in order to provide proper DNA insertion into demanded plasmid, to enable affinity purification and to cleave non-native sequences to obtain a mature rhGH. 612 bp nucleotide sequence of the *hGH* gene along with its annexes were given in Figure 4.5.

5'… **CACCATCACCATCACCAT** **ATTGAAGGGAGA** ttc cca act ata cca cta tct cgt cta ttc gat aac gct atg ctt cgt gct cat cgt ctt cat cag ctg gcc ttt gac acc tac cag gag ttt gaa gaa gcc tat atc cca aag gaa cag aag tat tca ttc ctg cag aac ccc cag acc tcc ctc tgt ttc tca gag tct att ccg aca ccc tcc aac agg gag gaa aca caa cag aaa tcc aac cta gag ctg ctc cgc atc tcc ctg ctg ctc atc cag tcg tgg ctg gag ccc gtg cag ttc ctc agg agt gtc ttc gcc aac agc ctg gtg tac ggc gcc tct gac agc aac gtc tat gac ctc cta aag gac cta gag gaa ggc atc caa acg ctg atg ggg agg ctg gaa gat ggc agc ccc cgg act ggg cag atc ttc aag cag acc tac agc aag ttc gac aca aac tca cac aac gat gac gca cta ctc aag aac tac ggg ctg ctc tac tgc ttc agg aag gac atg gac aag gtc gag aca ttc ctg cgc atc gtg cag tgc cgc tct gtg gag ggc agc tgt ggc ttc **TAG** **A^CCGGT**… **3'**

Figure 4.5 Nucleotide sequence of the *hGH* gene**.** Small letters represents the mature *hGH*. Black bold letters at the 3' end represents the restriction site of AgeI (BshTI). Red letters represents the 6xHis tag. Green letters are the Factor Xa cleavage site. Blue letters are the stop codon.

4.1.2 Propagation of pPDCZαA*::hGH* Plasmids

E.coli DH5α cells were utilized for the propagation of the plasmid pPDCZαA*::hGH*. Cells stored in the microbanks at -80°C were incubated on LSLB plates containing 25 µg mL^{-1} Zeocin, overnight at 37°C. A single colony was selected and transferred to LSLB broth including the same concentration of antibiotic and grown for 16 to 20 hours with shaking. Thereafter plasmid isolation was performed according to the manufacturers instructions. Plasmids were run on agarose gel and the results were presented in Figure 4.6.

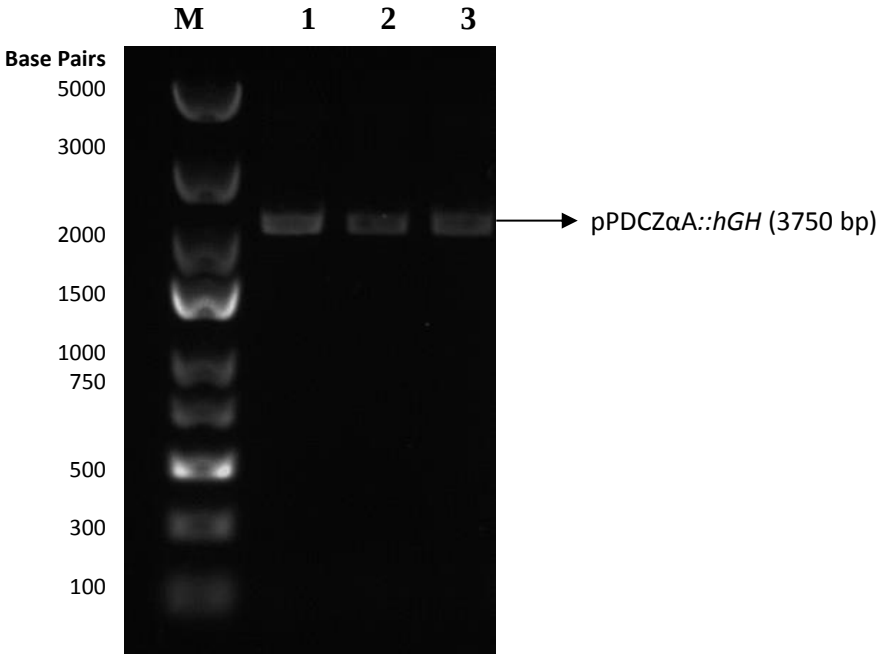


Figure 4.6 Agarose gel image of pPDCZα-A*::hGH* plasmid. M: GeneRuler Express DNA Ladder, besides the other lanes exhibit the isolated pPDCZα-A*::hGH* plasmids.

4.1.2 Primer Design for Amplification of the DNA Fragment

Primers used in the amplification of the DNA fragment including, PDC promoter region, the signal peptide and *hGH* gene were designed by using the restriction sites mentioned in section 3.4.3. The two primers were abbreviated as PDC-Fw and hGH-Rv and were presented in Table 4.1. Forward primer was

designed with AatII recognition site (6 bp) at the 5' end of the PDC-Fw primer and reverse primer was designed with AgeI (BshTI) recognition site (6 bp) at the 3' end of the hGH-Rv primer. During the design of the primers GC contents, melting temperatures (T_M), thermodynamic properties, hairpin, self- and hetero-dimer properties were evaluated by OligoAnalyzer program. Features of the primers were given in Appendix B.

Table 4.1 Primers utilized in the PCR amplification.Underlined letters are the recognition sites of the restriction endonucleases.

Abbreviation	**Sequence (5' → 3')**	**Length (bp)**
PDC-Fw	AGACGTCGGGACAAGCACACGATTA	25
hGH-Rv	CATACCGGTCTAGAAGCCACAGCTG	25

4.1.3 Amplification of the DNA Fragment

After the design of forward and reverse primers with the proper restriction sites, annealing temperature was determined as 58°C for the first cycle to enhance the primer annealing and 61°C for the remaining 29 cycles to avoid non-specific primer binding. Extension time, based on the utilized DNA polymerase and length of the DNA fragment is another crucial parameter in PCR and was optimized as 3 minutes at 72°C in order to achieve complete target replication according to the manuel of *Pfu* DNA Polymerase recommending the extension step as 2 min/kb at 72°C. Concentration of the salt, $MgSO_4$, was also optimized as 3mM increasing the primer binding efficiency while preventing the non-specific primer binding. Results of the amplified products performed in regard to optimized PCR conditions with three different $MgSO_4$ concentrations were given in Figure 4.7.

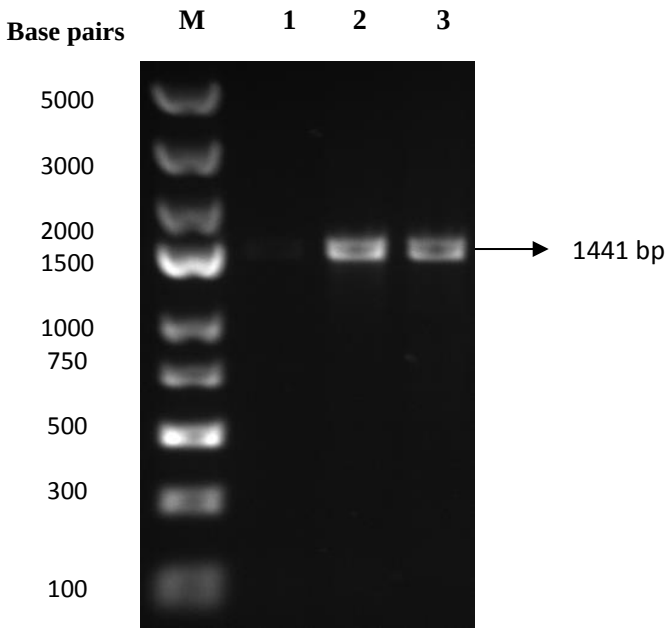


Figure 4.7 Agarose gel electrophoresis display of amplified DNA fragment with an expected size of 1441 base pairs. M: GeneRuler Express DNA Ladder, 1: 2 mM MgSO4, 2: 3 mM MgSO4,3: 4 mM MgSO4.

In order to amplify the DNA fragment including pyruvate decarboxylase promoter region, the signal peptide, alpha mating factor (αMF-1), and *hGH* gene the designed primers (Table 4.1), were utilized in the PCR according to the optimized PCR conditions. 1 µL of pPDCZα-A*::hGH* plasmid was added into the reaction mixture and utilized as template for the PCR. Thermal-cycler operation parameters and reaction mixture composition were presented in Table 3.4 and Table 3.5, respectively. The amplified PCR products were run on 0.8% agarose gel and represented in Figure 4.8.

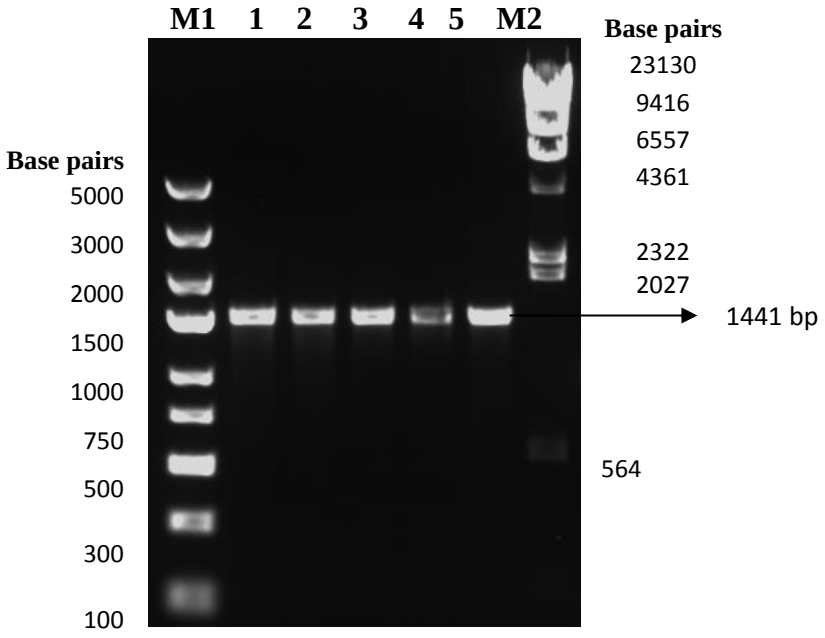


Figure 4.8 Agarose gel electrophoresis display of amplified 1441 base pair DNA fragments. M1: GeneRuler Express DNA Ladder, M2: Lambda DNA/HindIII Marker, other wells include amplified DNA region.

4.1.4 Double Digestion of pPIC3.5K vector and the PCR Products

After the selection of the restriction enzymes, AatII and AgeI (BshTI), using the Restriction Mapper of USA Molecular Biology Resources (www.restrictionmapper.org), in order to obtain sticky and complementary ends in both sides of the plasmids and the amplified DNA region, subsequent double digestion with the restriction enzymes AatII and AgeI (BshTI) was performed. Reaction mixture used in the restriction digestion of PCR products was supplied in Table 3.6 and the reaction mixture utilized in the restriction digestion of pPIC3.5K vector was presented in Table 3.7. The results of the single digestion by AatII and double digestion by AatII and AgeI (BshTI) of the vector were presented in Figure 4.9. After the double digestion of the amplified region only a few nucleotides were cleaved. Therefore, the difference between the pre-and post- digestions on the agarose gel was not observed.

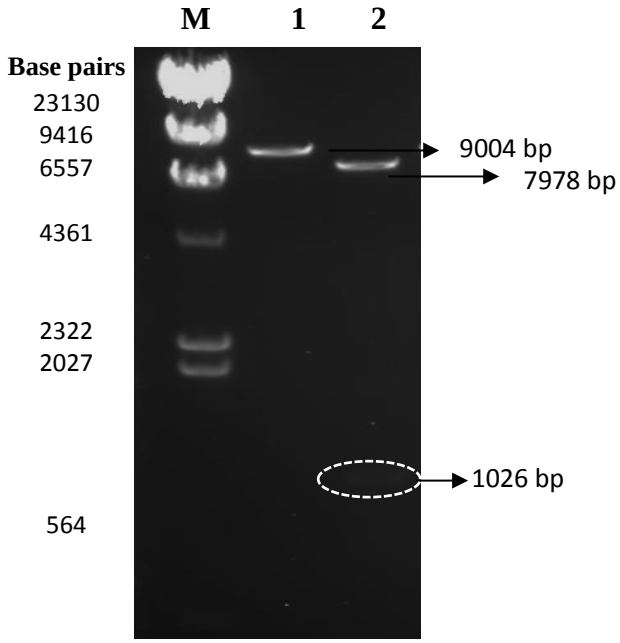


Figure 4.9 Gel electrophoresis image of single digested (with AatII) and double digested (with AatII and AgeI) pPIC3.5K plasmid. M: Lambda DNA/HindIII Marker, 1: single digested pPIC3.5K, 2: double digested pPIC3.5K.

After the double digestion reactions of the plasmids and PCR products were were terminated for the purifications of the cleaved plasmids GeneJET Gel Extraction Kit was utilized and for the purifications of the cleaved amplicons GeneJET PCR Purification Kit was used. Agarose gel electrophoresis results of the purified plasmids and amplicons were presented in Figure 4.10.

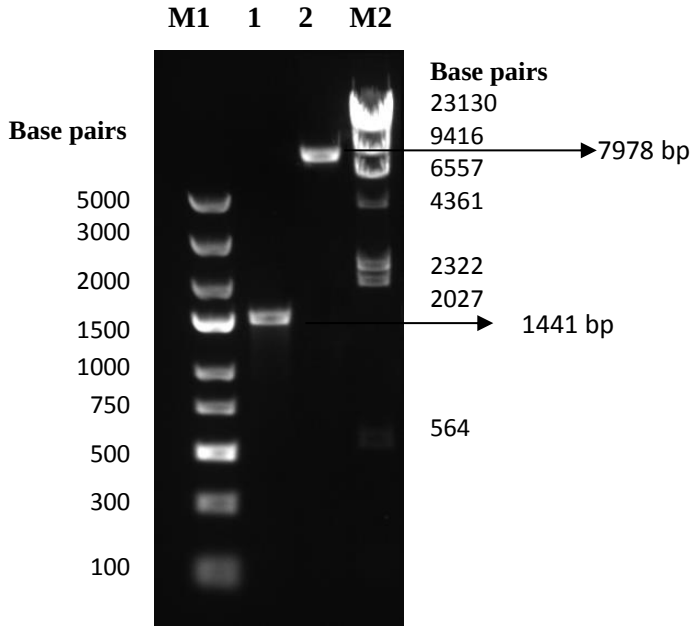


Figure 4.10 Control of digested pPIC3.5K plasmids and amplified DNA fragments after purifications.1: amplified DNA fragment, 2: double digested pPIC3.5K plasmids, M: Lambda DNA/HindIII Marker.

4.1.5 Ligation Reaction

After purified plasmids and amplicons were attained, ligation reaction was performed to attach compatible ends using the T4 DNA ligase enzyme elucidated as in 3.5.8. The molar ratio between the insert and vector was set as 3:1 and composition of the reaction mixture was presented in Table 4.2. Schematic representation of the constructed plasmid was named as pPDC3.5Kα*::hGH* and represented in Figure 4.11.

Table 4.2 Ligation reaction mixture composition.

Component	**Amount**
10X T4 DNA ligase buffer	2 µL
Linear vector DNA	100 ng
Insert DNA	54 ng
T4 DNA Ligase	1 µL
Up to	20 µL

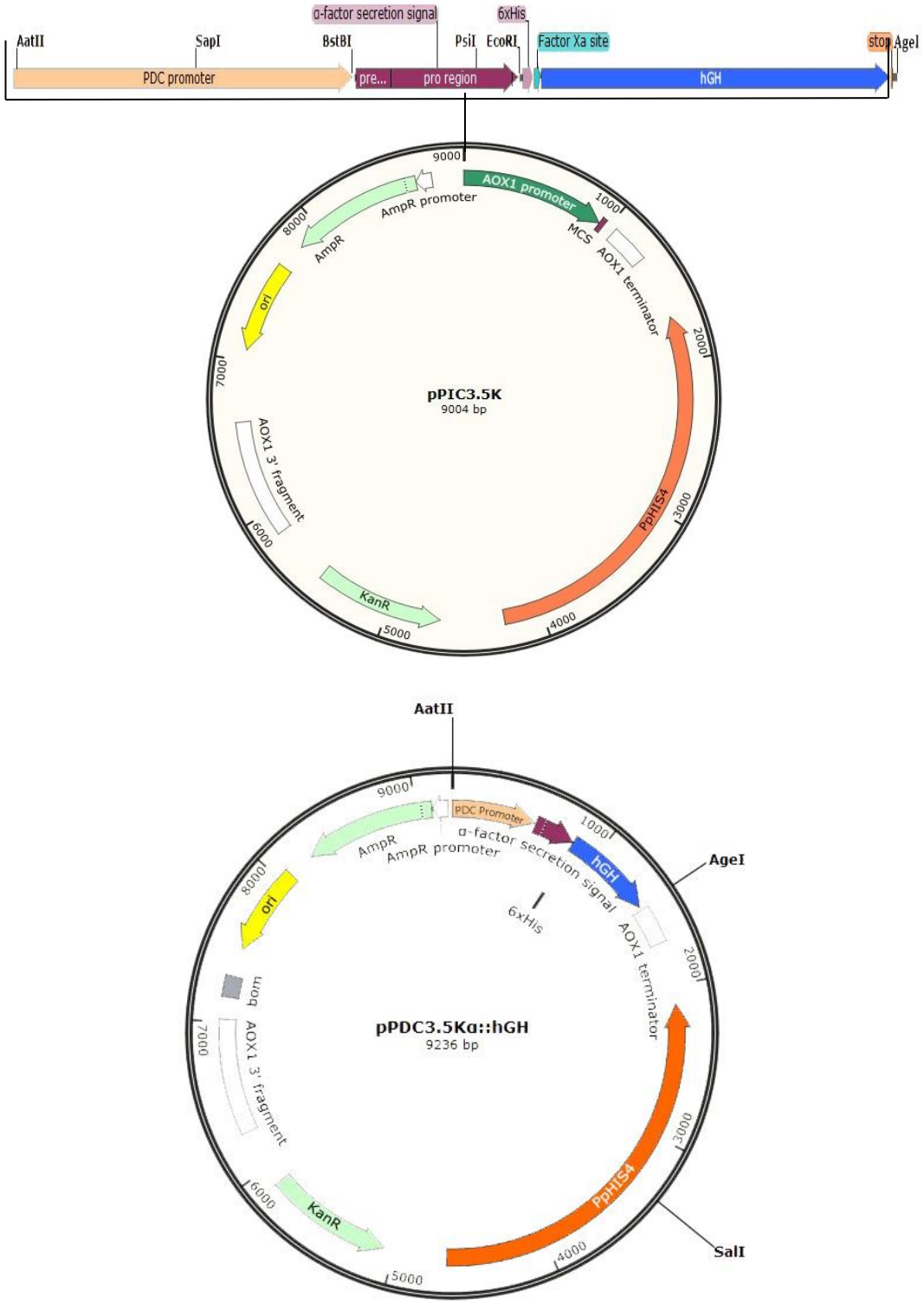


Figure 4.11 Schematic representation of the construction of pPDC3.5K*α::hGH* plasmid.

4.1.6 Transformation of pPDC3.5Kα*::hGH* into *E.coli* cells

Ligated amplicons and plasmid backbone were transformed into *E.coli* DH5α strain by $CaCl_2$ method as elucidated in 3.5.9. After the protocol was applied the cells were spread over the LSLB plates containing 0.1 g L^{-1} ampicillin. After 16 hours transformants came out, selected and transferred to new LSLB plates. Utilizing GeneJet Plasmid Miniprep Kit, the plasmids of the eight selected colonies were isolated as described in 3.5.1. As it could be seen from the Figure 4.12 six of the plasmids of the colonies have the expected size; however, two of them were false positives. Besides, pPIC3.5K were loaded to on the far right of the agarose gel as a positive control yet the size of the six colonies cannot be separated from positive control because, both plasmids are too large in size and the number of nucleotides between the two constructs was too small to be distinguished. Therefore, four colonies were selected and their plasmids were used in the further analyses.

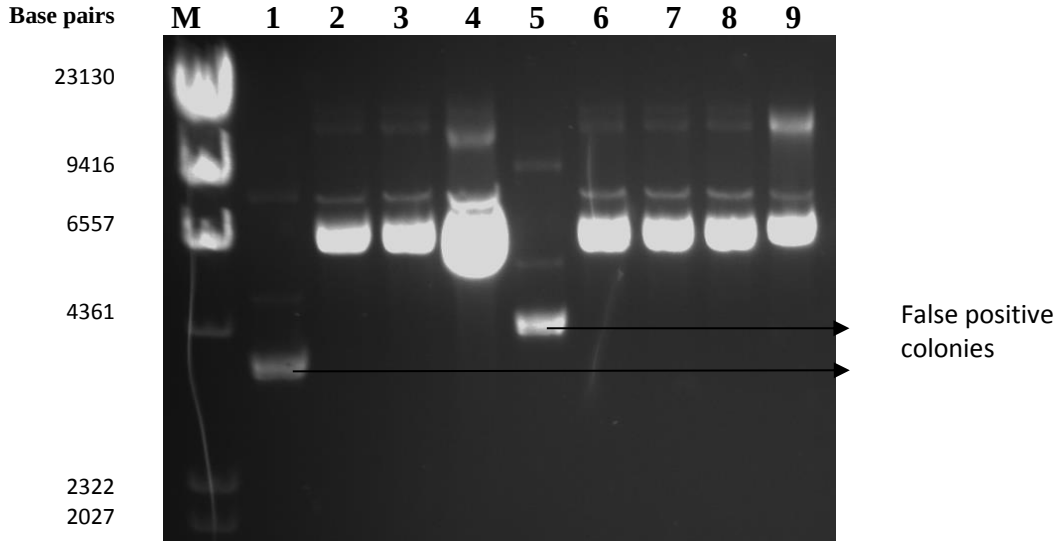


Figure 4.12 Gel electrophoresis display of transformed recombinant pPDC3.5Kα*::hGH* plasmid. M: Lambda DNA/HindIII Marker, 1,5: false positive colonies 2,3,4,6,7,8 are the circular recombinant pPDC3.5Kα*::hGH* vectors, 9: circular and empty pPIC3.5K vector.

Next, four colonies namely,2, 3, 4, 6 were chosen and their plasmids were utilized as templates for futher analysis in PCR using the designed primers in order to verify that the plasmids carry the DNA fragment. The results were presented in Figure 4.13.

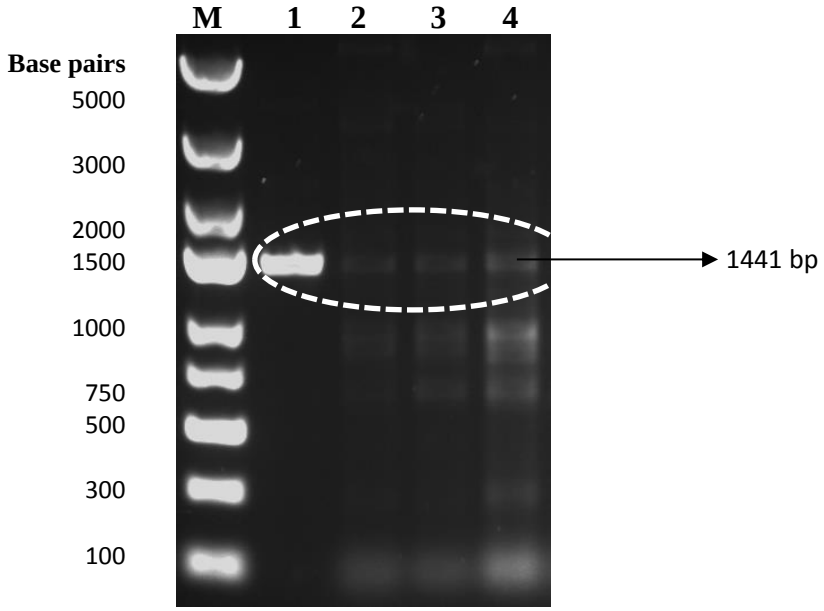


Figure 4.13 Gel electrophoresis display of amplified DNA fragments. M: GeneRuler Express DNA Ladder, 1-4: amplified DNA region including P_{PDC}, α-MF and *hGH*.

From the image it could be clearly understood that the plasmid of the colony 2 carries the 1441 bp amplicon. Subsequently the plasmid of the transformant carrying the DNA fragment was single and double digested with the restriction enzymes and the results were presented in Figure 4.14. Besides, the bands appeared after double digestion also verifed that the plasmid carries the DNA fragment; consequently the construction of the recombinant plasmid was accomplished.

As a final control DNA sequence of the insert was analyzed and sequenced by DNA sequencers (Applied Biosystems Hitachi 3500 Genetic Analyzer). The DNA nucleotide sequences were analyzed by National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) and the sequence of the insert was verified (Appendix C).

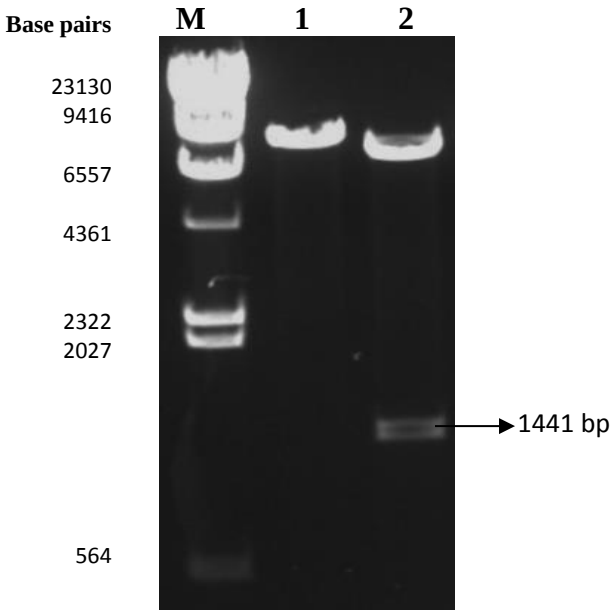


Figure 4.14 Single and double digestion of the pPDC3.5Kα::hGH plasmids of the selected colony. M: Lambda DNA/HindIII Marker, 1: Linearized pPDC3.5Kα*::hGH* plasmid by AatII restriction enzyme, 2: Double digested pPDC3.5Kα*::hGH* plasmid by the enzymes, AatII and AgeI.

4.1.7 Transfection of *P.pastoris* cells with pPDC3.5Kα*::hGH* plasmids

In order to insert the pPDC3.5Kα*::hGH* plasmid into r-*P. pastoris* strain which was previously transformed by the pGAPZαA*::hGH* plasmid, the pPDC3.5Kα*::hGH* vector was primarily linearized and thereby homologous recombination event could take place at HIS4 locus of the gene of the r-*P. pastoris* while preventing the homologous recombination at alpha mating factor secretion signal, the *hGH* gene or *AOX1* transcription termination region which were common in both plasmids. To that end, the restriction enzyme, SalI, which divides the *HIS4* gene located in the plasmid, was selected. The applied protocol for the linearization process was given in section 3.5.11 and linearized plasmids were verified by agarose gel electrophoresis. The plasmids were further purified by GeneJET PCR Purification Kit and were eluted in 50 µL dH_2O. Their concentration was arranged to be between 2.5 to 3 ng μL^{-1} in order to achieve single copy integration.

Concentrations of the circular pPDC3.5Kα*::hGH* vector and linearized, purified pPDC3.5Kα*::hGH* vector were compared in Figure 4.15. For transfection into r-*P.pastoris* LiCl method was applied as explained in 3.5.12. Schematic diagram representing the gene insertion event at HIS4 locus of the r-*P.pastoris* genome was introduced in Figure 4.16. Due to inefficacy in the direct selection of geneticin (G-418) in *P.pastoris*, initial selection of His^+ transformants and subsequent G-418 resistant clone determination was conducted. For that purpose, transfected r-*P.pastoris* cells were spread over the minimal dextrose (MD) plates and were incubated for approximately 3 days until they express adequate amount of resistance factor. Then the grown colonies were transferred to YPD plates including 0.25 g L^{-1} G-418 and were incubated for 3 more days. The genome of the colony was isolated as described in section 3.5.13 and used in PCR as template for the verification of the proper integration inside the genome (Figure 4.18).

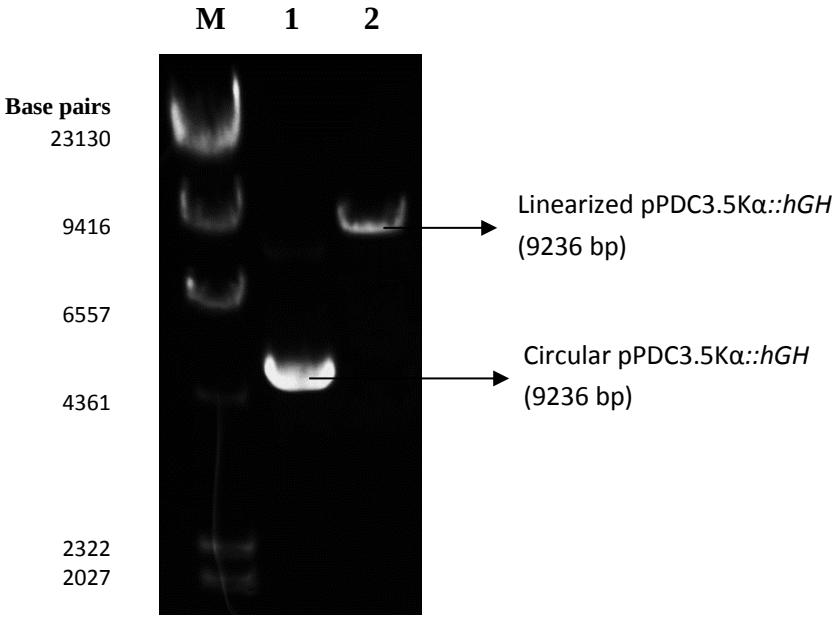


Figure 4.15 Gel electrophoresis results of circular and linear pPDC3.5Kα*::hGH* plasmids. M: Lambda DNA/HindIII Marker, 1: Circular pPDC3.5Kα*::hGH* plasmid before linearization and purification, 2: Linearized pPDC3.5Kα*::hGH* plasmid after purification.

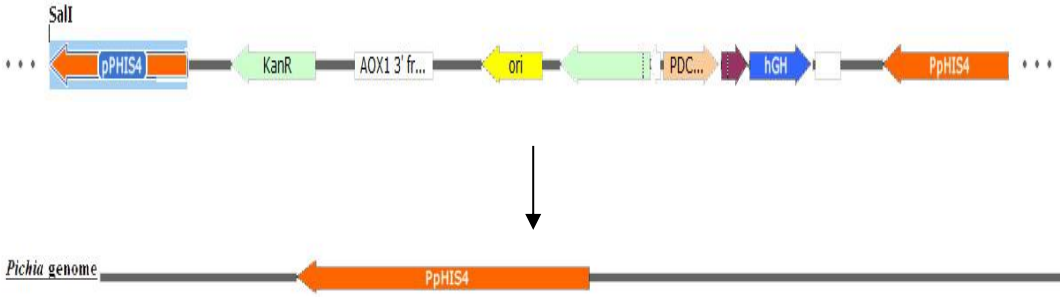


Figure 4.16 Schematic diagram that represents insertion event of pPDC3.5Kα*::hGH* plasmids at HIS4 locus.

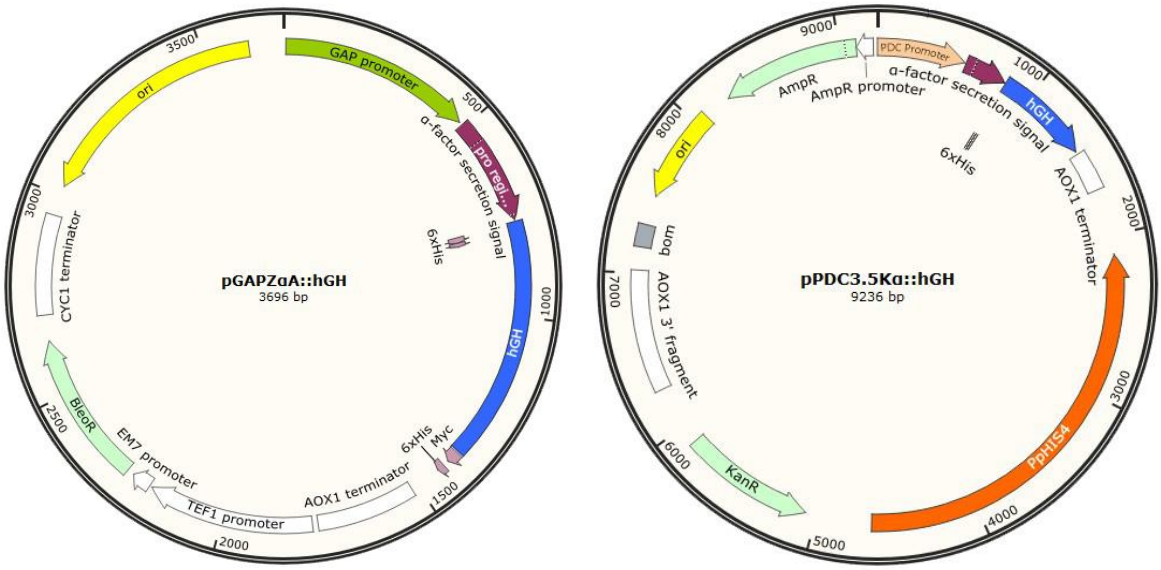


Figure 4.17 Schematic diagrams of the inserted vectors into the genome of *P. pastoris*.

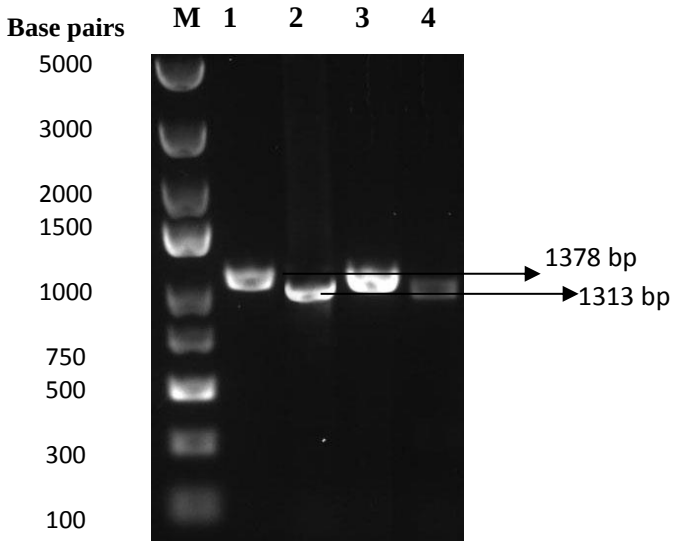


Figure 4.18 Gel electrophoresis display of amplified DNA fragments. M: GeneRuler Express DNA Ladder, 1-3: amplified DNA region including P_{PDC}, α-MF and *hGH*, 2-4: amplified DNA region including P_{GAP}, α-MF and *hGH*.

4.2 Determination of the Gene Copy Number

In order to determine the copy number of the integrated *hGH* gene into r-*P.pastoris* genome, qRT-PCR experiments were conducted and performed as elucidated in detail in section 3.5.14. In the preparation of the unknown samples, isolated genomic DNA of the colonies were used as templates after their concentration was determined at A_{260} by NanoDrop® 2000 (Thermoscientific). To implement relative quantification, internal primers namely, *ARG4* and *hGH* was utilized and copy number of the inserted *hGH* gene was determined by dividing copy quantity of the *hGH* gene to copy quantity of the housekeeping gene *ARG4*.

Throughout the experiments fluorescence emission which is directly proportional the accumulation of the amplicons in each cycle, were detected via the specialized qRT-PCR instrument. Serial dilutions (Table 3.14) were performed to readily-prepared standards and the standard curve was constituted by plotting log starting quantity with respect to C_q (quantification cycle) then efficiency of the reaction was determined. The threshold value was arranged automatically by the

software of the system when the amplification fluorescence surpass the background fluorescence.

In order to obtain precise hGH gene copy number evaluation for double promoter expression system, in each qRT-PCR run a single copy integrant which was previously proven by Massahi, 2017 was used as a control. Both serially diluted standards and unknown samples were prepared and analyzed as duplicates in each experiment. Besides, the biological replicates of the experiments were also performed in duplicates. Plot of amplification curve of one of the relative fluorescence units (RFU) against cycles of the qRT-PCR run was given in Figure 4.19. Results of the qRT-PCR experiments were summarized in Table 4.3.

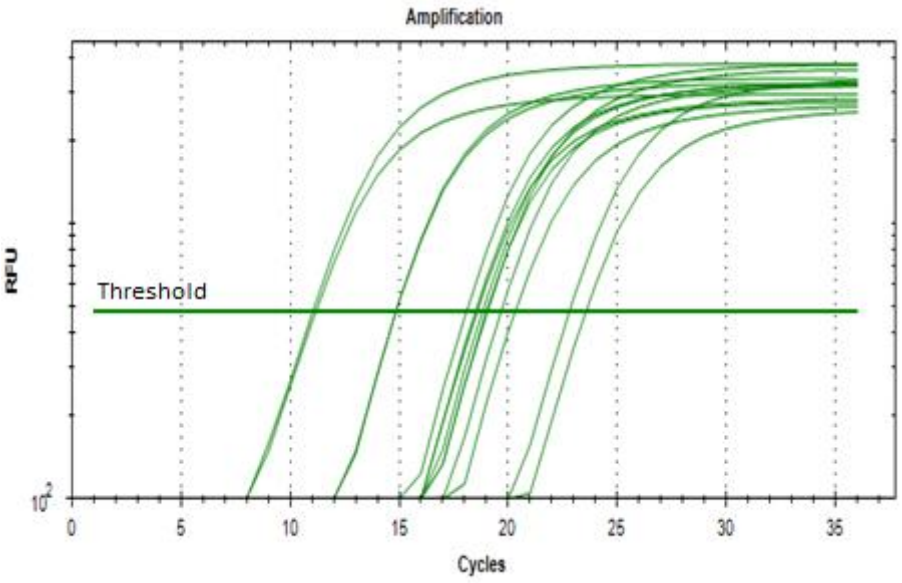


Figure 4.19 Amplification curve attained utilizing the serial dilutions of standards and unknown samples with threshold line in logarithmic scale for the y axis.

Subsequent to amplification of the DNA fragments melt curve analysis were performed at the end of each PRC run in order to verify specificity of annealing. Starting from 60°C temperature was increased to 99°C for 0.5°C increment and separated peaks of *ARG4* and *hGH* were represented in Figure 4.20.

Table 4.3 Copy number of the two colonies obtained by duplicated qRT-PCR experiments.

Strain		Efficiency (%)	R^2-Value		Copy number
First qRT-PCR Run					
pGAPZαA::*hGH* and GAP-PDC::*hGH*	***ARG***	96.0794	***ARG***	0.9901	0.682 and 1.286
	hGH	91.306	***hGH***	0.99936	
Second qRT-PCR Run					
pGAPZαA::*hGH* and GAP-PDC::*hGH*	***ARG***	97.345	***ARG***	0.99885	0.733 and 1.298
	hGH	91.57	***hGH***	0.99771	

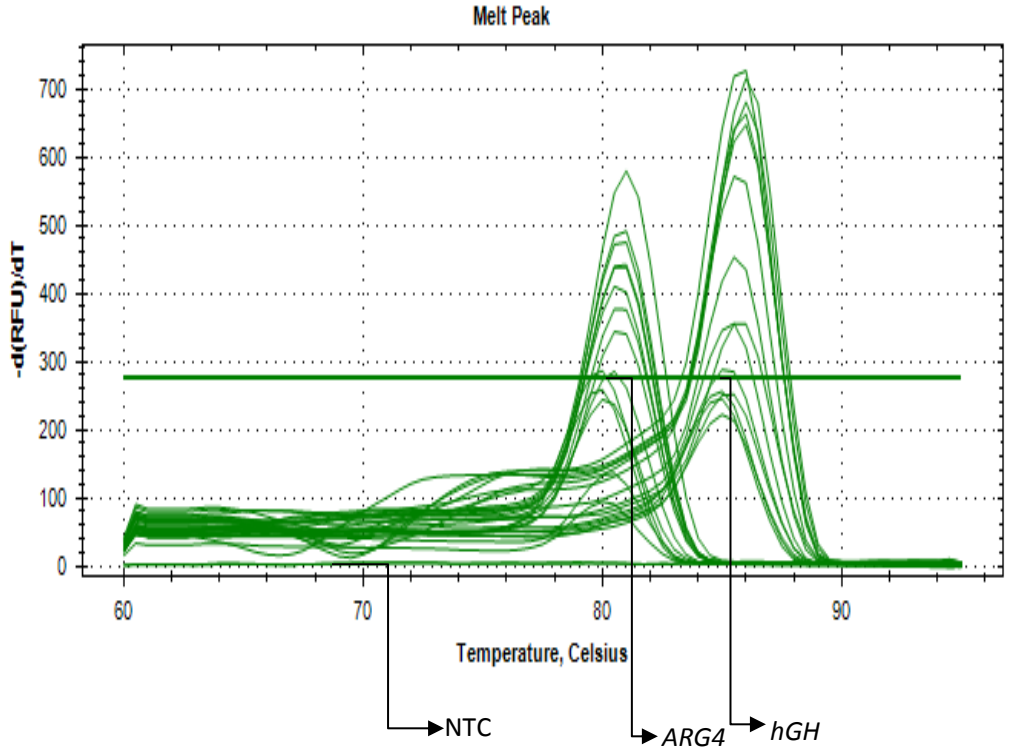


Figure 4.20 Melt curve plot of *ARG4* and *hGH* genes which was plotted as the derivative of relative fluorescence unit with respect to temperature.

4.3 Recombinant Human Growth Hormone Production

After the r-*P.pastoris* strain that carry *hGH* gene under two different promoters as single-copy integrant of each, productivity of the double promoter expression system was investigated primarily in air-filtered shake bioreactors and then by pilot scale bioreactor experiments.

4.3.1 rhGH Production in Air-Filtered Shake Bioreactors

After pPDC3.5Kα*::hGH* plasmid insertion the genome of r-*P. pastoris*, the colony that express hGH gene under one copy of P_{GAP} promoter and one copy of P_{PDC} promoter was selected, in order to investigate rhGH expression level under double constitutive promoters. To distinguish the difference between the double promoter expression system and single copy expression system, strains GAP-PDC*::hGH* and pGAPZα*::hGH*/X-33 were incubated on solid YPD medium (Table 3.3). After 48 hours, the cells were inoculated into BMGY precultivation medium (Table 3.17) at 200 rpm at 30°C for 15-18 hours inside air-filtered and baffled 250 mL Erlenmeyer flasks as shake bioreactors including 50 mL of precultivation medium. Once the OD_{600} value of the cells reached to a value between 2 to 6 they were harvested in centrifuge at 1500 g at 4ºC for 10 minutes. Later on, the initial OD_{600} value was adjusted to be as 1 in 50 mL production medium (Table 3.18) and r-*P. patoris* strains were incubated at 30ºC at 200 rpm for 30 hours in 250 mL air filtered shake bioreactors. Starting from the 21 hours of production samples were taken in every 3 hours period until the 30 hours of production.

The cell growth of the two strains were controlled and compared during the shaker experiments and the results were shown in Figure 4.21 Starting from the same initial cell concentration it was observed that the cell growth of GAP-PDC*::hGH* strain was faster. The maximum cell concentrations were obtained at the 27 hour of experiment as 9.78 and 11.1 g L^{-1} for pGAPZα*::hGH*/X-33 and GAP-PDC*::hGH*, respectively.

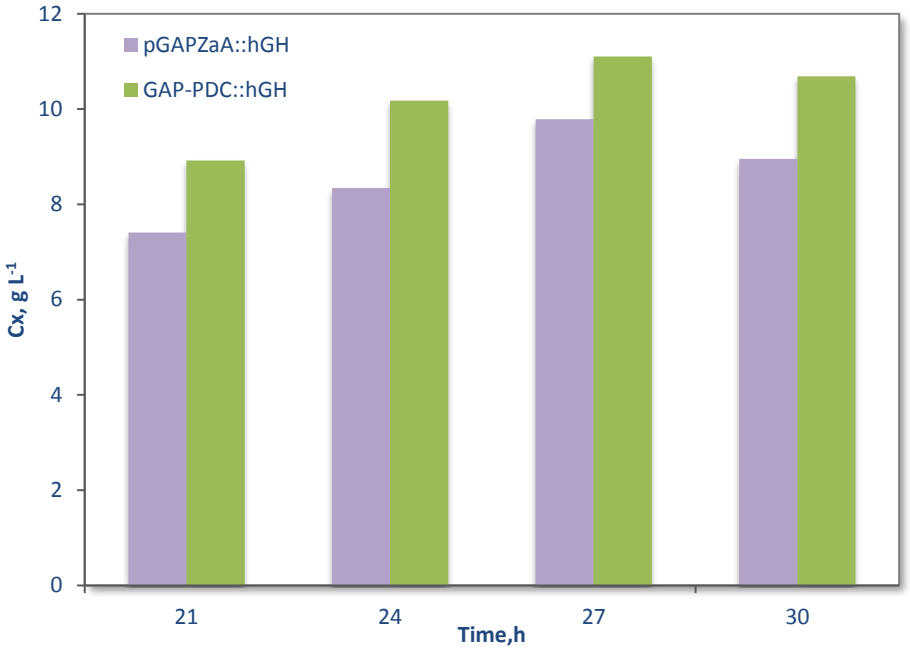


Figure 4.21 The cell concentrations of the r-P. pastoris pGAPZα*::hGH*/X-33 and GAP-PDC*::hGH* strains at different cultivation times of production in air-filtered shake bioreactor experiments.

In order to compare the rhGH production levels of the two strains SDS-PAGE analyses was conducted. The samples collected throughout the experiment were centrifuged at 4°C for at 1500 rcf for 10 minutes and supernatants were separated. 15 µL of supernatants were utilized in the SDS-PAGE analysis according to the protocol that was explained in section 3.7.2. Figure 4.22 demostrates the rhGH concentration levels of the air-filtered shake bioreactor experiment of the two strain.

As can be seen in the figure below there were two bands that belongs to rhGH one on the top of the other. The upper band was the rhGH secreted to the production medium with polyhistidinetag (6xHis) and Factor Xa cleavage site and the lower band was the rhGH secreted to the production medium without 6xHis tag due to its removal by the machinery of the cell.

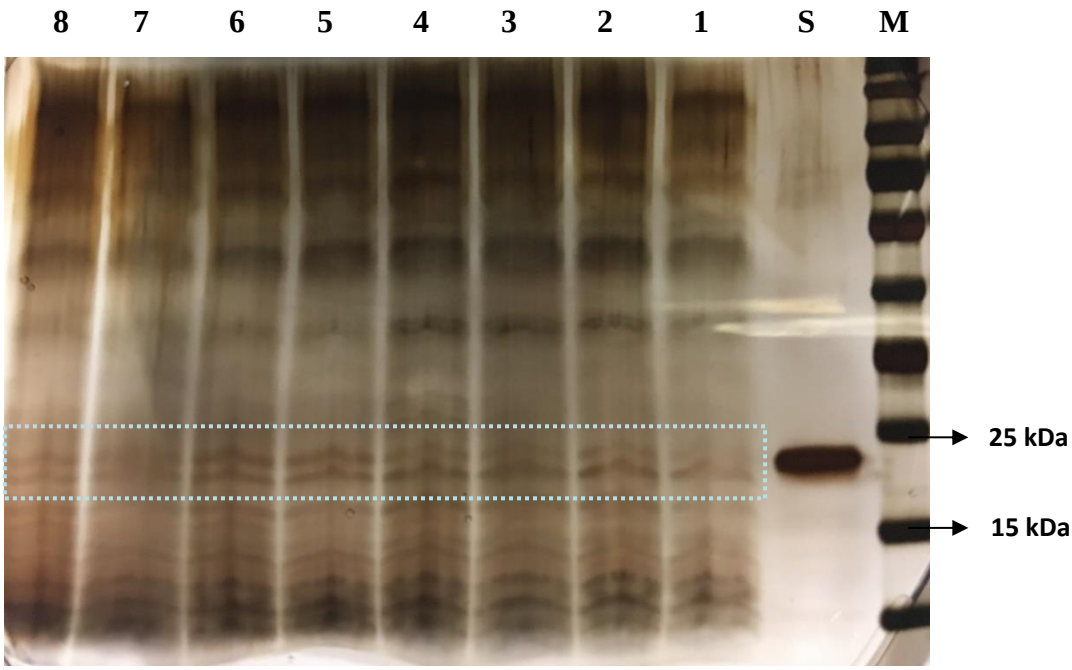


Figure 4.22 SDS-PAGE analysis results of the shaker experiment at t=21 h,24 h, 27 h, and 30 h of production. M: protein marker. S: hGH standard 0.05 g L^{-1}, 1: supernatant of pGAPZα*::hGH*/X-33 at 21 h, 2: supernatant of pGAPZα*::hGH*/X-33 at 24 h, 3: supernatant of pGAPZα*::hGH*/X-33 at 27 h, 4: supernatant of pGAPZα*::hGH*/X-33 at 30 h, 5: supernatant of GAP-PDC*::hGH* at 21 h, 6: supernatant of GAP-PDC*::hGH* at 24 h, 7: supernatant of GAP-PDC*::hGH* at 27 h, 8: supernatant of GAP-PDC*::hGH* at 30 h.

According to the results of air-filtered shake bioreactor experiment, it was observed that higher amount of rhGH production was achieved in the earlier hours (i.e., 21 h and 24 h) of the experiment by the double promoter expression system with more rhGH nonsegregated form including the polyhistidinetag (6xHis) and Factor Xa cleavage site. However, rhGH expressions of the two strains diminished after twenty-seventh hour of the experiment.

4.3.2 rhGH Production in Pilot Scale Bioreactor

Subsequent to the air-filtered shake bioreactor experiment expression levels of single-copy pPDCZαA*::hGH*, pGAPZαA*::hGH* strains, and the r-*P. pastoris* strain that express rhGH under P_{GAP} and P_{PDC} were investigated in pilot scale bioreactor in a controlled system as detailly elucidated in section 3.6.3. The pilot

scale bioreactor experiment which was conducted to cultivate pPDCZαA*::hGH* strain was named as BR-1, the second pilot scale bioreactor experiment performed to cultivate pGAPZαA*::hGH* strain was called as BR-2, and the next pilot scale bioreactor experiment implemented for GAP-PDC*::hGH* strain was named as BR-3. As explained in section 3.6.3 at the end of glycerol batch phase, after glycerol depletion 10 g L^{-1} cell concentration was obtained and the process shifted to glucose fed-batch phase with glucose. In the all pilot scale bioreactor experiments pre-determined specific growth rate used in the calculation of continuous feed stream Q_s (Eqn 2.25) was selected as $\mu_0 = 0.15\ h^{-1}$. Concentration of the stock solution of glucose (C_S^0) including 12 ml L^{-1} PTM1 was 500 g L^{-1} and the solution was fed to the bioreactor according to the equation 3.3 in order to maintain the constant specific growth rate at 0.15 h^{-1}. Dissolved oxygen concentration, C_{DO}, was kept at 5% during the experiments and the three recombinant strains were compared at the condition reported by Baumann et al. (2010) asserted that using the term hypoxic conditions instead of oxygen-limitation, yields stronger transcriptional regulation, regulates protein folding and reduces protein trafficking of *P.pastoris* (Baumann et al., 2010). However, being on the fermentative pathway it was expected that r-protein expression under P_{PDC} lead to higher amount of rhGH. Moreover, it was also reported that the recombinant protein production under P_{GAP} could be enhanced when the C_{DO} was increased to 15% (Güneş and Çalık, 2016). Therefore, another pilot scale bioreactor experiment was conducted to analyze the expression level of GAP-PDC*::hGH* strain at the same pre-determined specific growth rate with same initial cell concentration at a higher C_{DO} level by increasing the C_{DO} to 15%, so called BR-4.

Taking the samples in 3-hour time intervals beginning from the glucose fed-batch phase, the cell concentrations, secreted rhGH concentrations, accumulated glucose and ethanol concentrations in the production mediums were determined and compared for each pilot scale bioreactor experiment.

The highest cell concentration was obtained in BR-4 as 103.9 g L^{-1} at t= 18 h then a stationary phase was observed at t=21 h. However, among BR-1, BR-2, BR-3 slightly higher cell concentrations were attained in BR-3 as 76.9 g L^{-1} at t= 18 h, in BR-1 as 63.6 g L^{-1} at t= 15 h, and in BR-2 as 63.2 g L^{-1} at t= 18 h. Figure 4.23 shows the variations in the cell concentrations throughout the bioreactor experiments. As can be seen from the figure increasing the constant C_{DO} from 5% (BR-3) to 15% (BR-4) enhances the cell concentration by conserving the growth behavior of the strain GAP-PDC*::hGH*. Besides, growth of pGAPZαA*::hGH* strain displayed a similar behavior. However, growth behavior of the pPDCZαA*::hGH* strain was different after t>15h from the other two strains although the metabolic load implemented by the foreign protein production was nearly the same in the two strains namely, pGAPZαA*::hGH* and pPDCZαA*::hGH*.

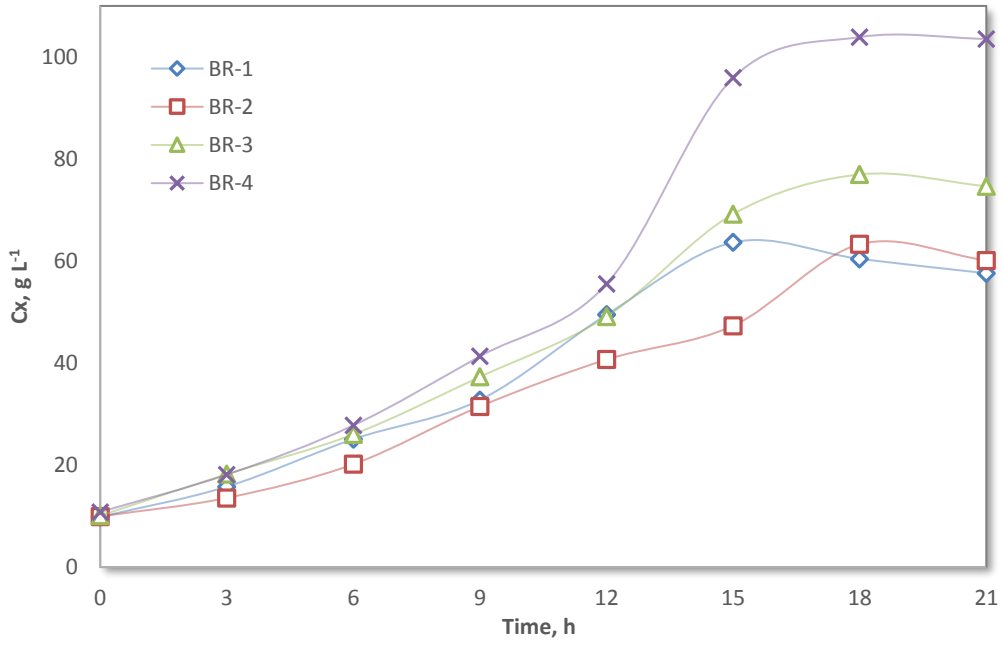


Figure 4.23 Variations in cell concentrations during glucose fed-batch phase in pilot scale bioreactor experiments of the strains pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%.

In order to measure the extracellular rhGH concentrations supernatants of the samples were primarily filtrated by 0.45 μm filters and were used in the SDS-PAGE analysis of the fermentations. Results of the bioreactor experiments were presented in Figure 4.24, Figure 4.25, Figure 4.26, and in Figure 4.27 for BR-1, BR-2, BR-3, and for BR-4, respectively.

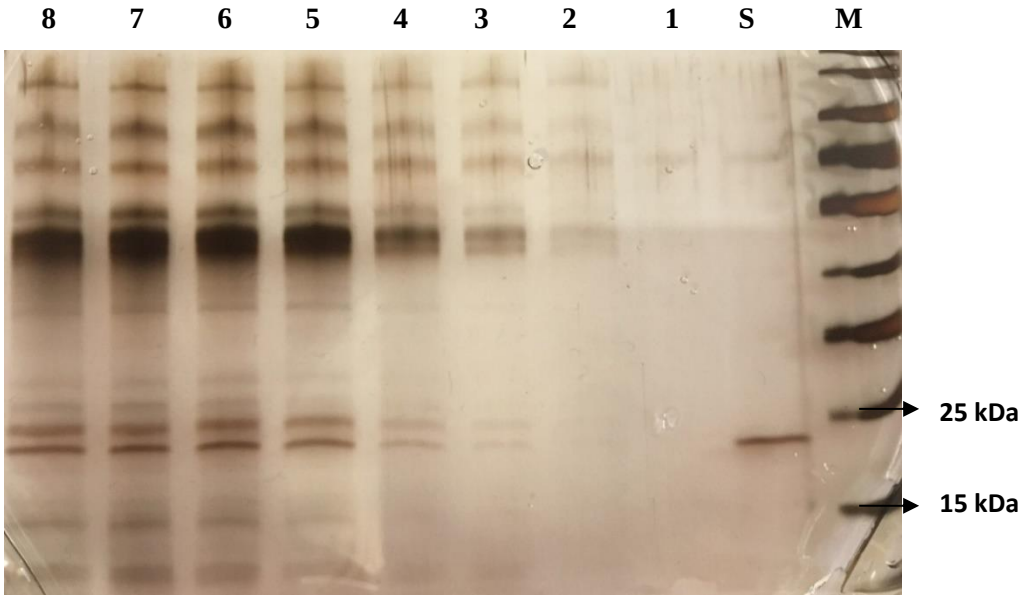


Figure 4.24 SDS-PAGE analysis result of the strain r-*P. pastoris* pPDCZαA*::hGH* (BR-1) M: protein marker, S: hGH standard 0.01 g L^{-1}, 1-8: corresponds the samples for t= 0, 3, 6, 9, 12, 15, 18, and 21 h during the fed-batch pilot scale bioreactor experiment with 1:5 dilution at C_{DO}= 5% with μ_0=0.15 h^{-1}, respectively.

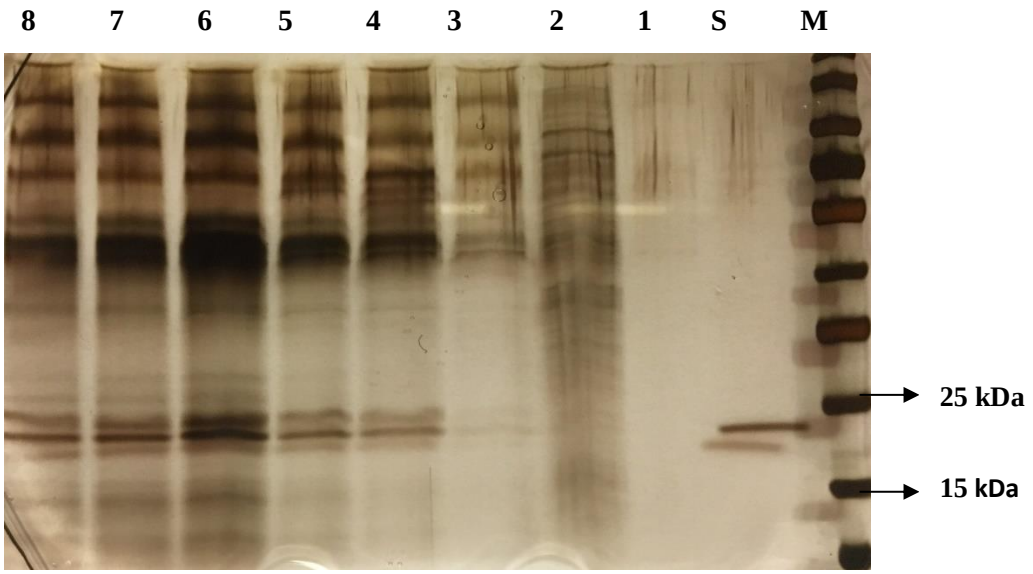


Figure 4.25 SDS-PAGE analysis result of the strain r-*P. pastoris* pGAPZαA*::hGH (*BR-2) M: protein marker, S: hGH standard 0.01 g L^{-1}, 1-8: corresponds the samples for t= 0, 3, 6, 9, 12, 15, 18, and 21 h during the fed-batch pilot scale bioreactor experiment with 1:5 dilution at C_{DO}= 5% with μ_0=0.15 h^{-1}, respectively.

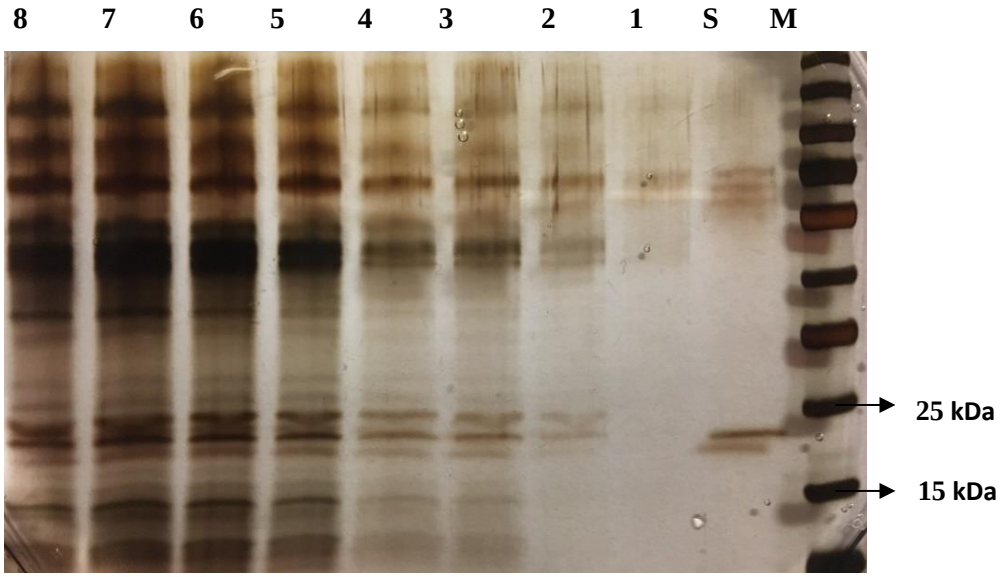


Figure 4.26 SDS-PAGE analysis result of the strain r-*P. pastoris* GAP-PDC*::hGH* (BR-3) M: protein marker, S: hGH standard 0.01 g L^{-1}, 1-8: corresponds the samples for t= 0, 3, 6, 9, 12, 15, 18, and 21 h during the fed-batch pilot scale bioreactor experiment with 1:5 dilution at C_{DO}= 5% with μ_0=0.15 h^{-1}, respectively.

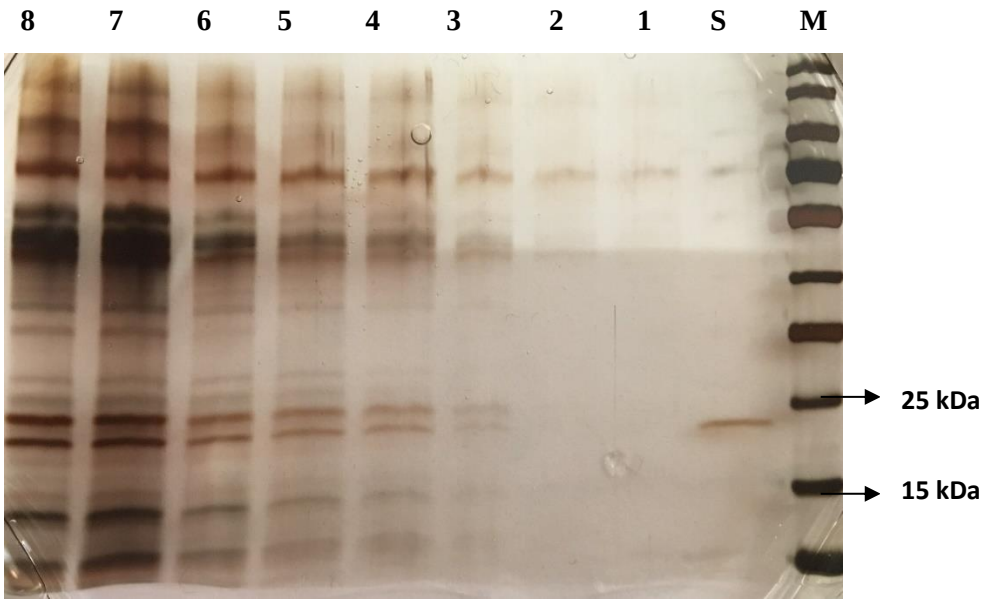


Figure 4.27 SDS-PAGE analysis result of the strain r-*P. pastoris* GAP-PDC*::hGH* (BR-4) M: protein marker, S: hGH standard 0.01 g L^{-1}, 1-8: corresponds the samples for t= 0, 3, 6, 9, 12, 15, 18, and 21 h during the fed-batch pilot scale bioreactor experiment with 1:5 dilution at C_{DO}= 15% with μ_0=0.15 h^{-1}, respectively.

In addition to conducted four SDS-PAGE analysis which were performed separately for each pilot scale bioreactor experiment, another SDS-PAGE analysis was applied to the samples which exhibited the highest amount of extracellular rhGH during the processes in order to determine the concentrations of secreted rhGH synchronically in a SDS-PAGE with a more precise measurement method, and the results were given in Figure 4.28.

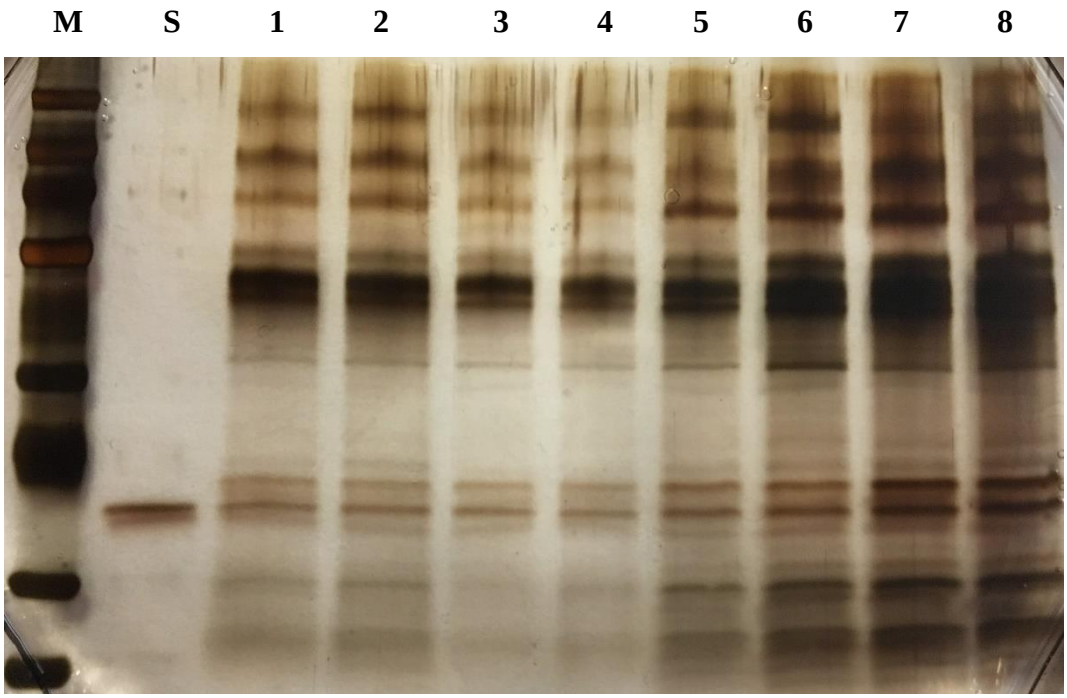


Figure 4.28 SDS-PAGE analysis result of the most extracellular rhGH produced hours of the four pilot scale bioreactor experiments. M: protein marker, S: hGH standard 0.01 g L^{-1}, 1 and 2: r-*P. pastoris* pPDCZαA*::hGH* (BR-1) at t=12 h and t=15h, 3 and 4: r-*P. pastoris* pPDCZαA*::hGH* (BR-2) at t=15 h and t=18 h, 5 and 6: r-*P. pastoris* GAP-PDC*::hGH* (BR-3) at t=15 h and t=18 h, 7 and 8: r-P. pastoris GAP-PDC*::hGH* (BR-4) at t=15 h and t=18 h with 1:5 dilution.

As can be seen in Figure 4.29 the amount of rhGH secreted to the production medium were enhanced with the increase in the cell concentration due to recombinant foreign protein production under the constitutive promoters. Extracellular rhGH production by the strains pPDCZαA*::hGH* and pPDCZαA*::hGH* showed a similar trend during the experiments. Nevertheless, although the growth of the strain GAP-PDC*::hGH* followed the same trend with the oxygen transfer conditions designed with C_{DO} set values of 5% and 15%, rhGH secretion during C_{DO}=15% (BR-4) performed a slightly different profile especially after t=9 h. When the C_{DO} was kept at 5% the highest extracellular rhGH concentration was obtained in BR-3 as 179 mg L^{-1} at t=18 h. Besides, in single-copy integrants of hGH gene under P_{PDC} and P_{GAP} expressions the highest extracellular rhGH concentration was obtained at t=15h of the fermentations as 80.6 and 107.8

mg L^{-1}, respectively. Under the double promoter (BR-3), the total amount of rhGH produced in BR-1 and BR-2 with the single promoters were produced. Furthermore, by increasing the dissolved oxygen concentration set point C_{DO} from 5% to 15% by increasing the oxygen transfer enhances the rhGH production approximately 2.4-fold. Secreted rhGH concentration (C_{hGH}) was measured as 429 mg L^{-1} at t=18 h in BR-4. In all the pilot scale bioreactor experiments a decline was observed after the C_{hGH} reached to a maximum, because of degradation of the protein by proteases.

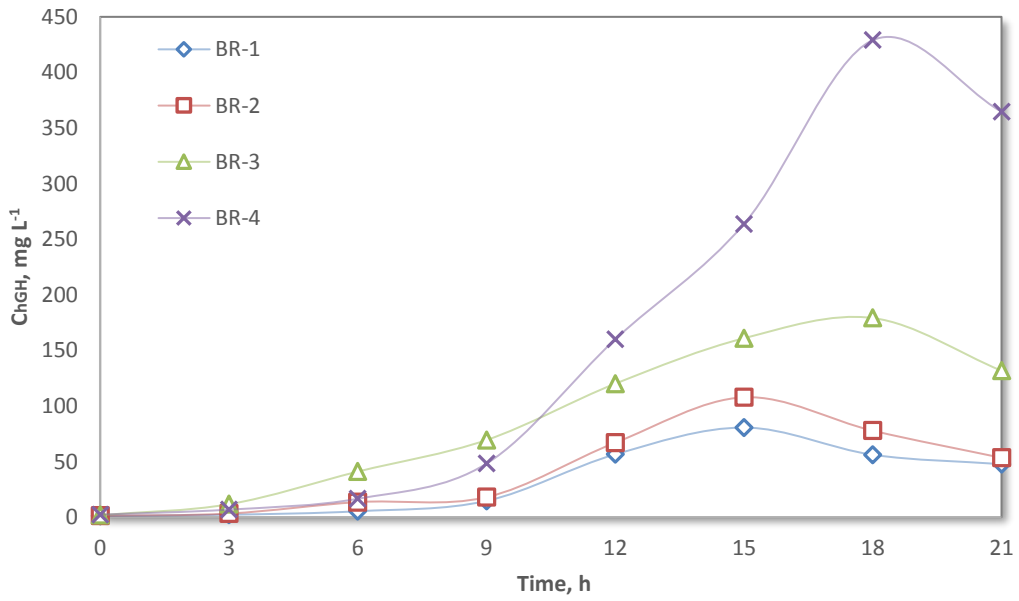


Figure 4.29 Variations in rhGH concentrations during glucose fed-batch phase in pilot scale bioreactor experiments with the strains pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%.

Instantaneous specific rhGH formation rates, q_{rhGH} (g g^{-1} h^{-1}), and rhGH formation rates, r_{rhGH} (g L^{-1} h^{-1}), were presented in Table 4.4. The highest q_{rhGH} was calculated as 1.25 g g^{-1} h^{-1} at t=18 h in BR-4, and the second highest q_{rhGH} was calculated as 0.518 g g^{-1} h^{-1} at t=15 h in BR-3. Those results indicated that when the strain with double promoter GAP-PDC*::hGH* was used the specific rhGH formation rate enhanced with the increase in C_{DO} with the increase in oxygen transfer. Moreover, the overall product yield on the cell $Y'_{P/X}$ was calculated as

1.42 (at t=15 h), 2.67 (at t=15 h), 2.51 (at t=18 h), and 4.38 (at t=18 h) mg g^{-1} at the maximum C_{rhGH} measured in BR-1, BR-2, BR-3, and BR-4, respectively.

Table 4.4 The specific rhGH formation rates and rhGH formation rates of the four bioreactor experiments.

Cultivation Time (h)	q_{rhGH} (g g^{-1} h^{-1})				r_{rhGH} (g L^{-1} h^{-1})			
	BR-1	**BR-2**	**BR-3**	**BR-4**	**BR-1**	**BR-2**	**BR-3**	**BR-4**
0	-	-	-	-	-	-	-	-
3	0.024	0.045	0.099	0.099	0.38	0.707	3.37	1.56
6	0.045	0.14	0.111	0.138	1.12	3.61	6.33	3.47
9	0.10	0.26	0.231	0.270	3.39	5.83	10.9	11.5
12	0.31	0.37	0.412	0.503	15.6	18.2	20.4	41.7
15	0.18	0.28	0.518	0.706	11.3	17.9	23.2	44.9
18	-0.09	-0.10	0.246	1.25	-5.44	-6.28	14.9	75.3

Residual glucose concentrations during the glucose fed-batch phase in the pilot scale bioreactor experiments were measured as explained in section 3.7.6. Glucose accumulations during the bioprocesses were observed to be compatible with the cell concentration profiles of the experiments. In other words, glucose in the production medium was detected in all the experiments when the cell concentrations started to drop as represented in Figure 4.30. Due to its maximum C_X value at t=15 h of the experiment, residual glucose concentration of BR-1 was more than the other experiments at the end of the bioprocess. At t=21 h, glucose concentrations were measured as 35.3, 9.7, 9.0 and 3.3 g L^{-1} for BR-1, BR-2, BR-3, and BR-4, respectively.

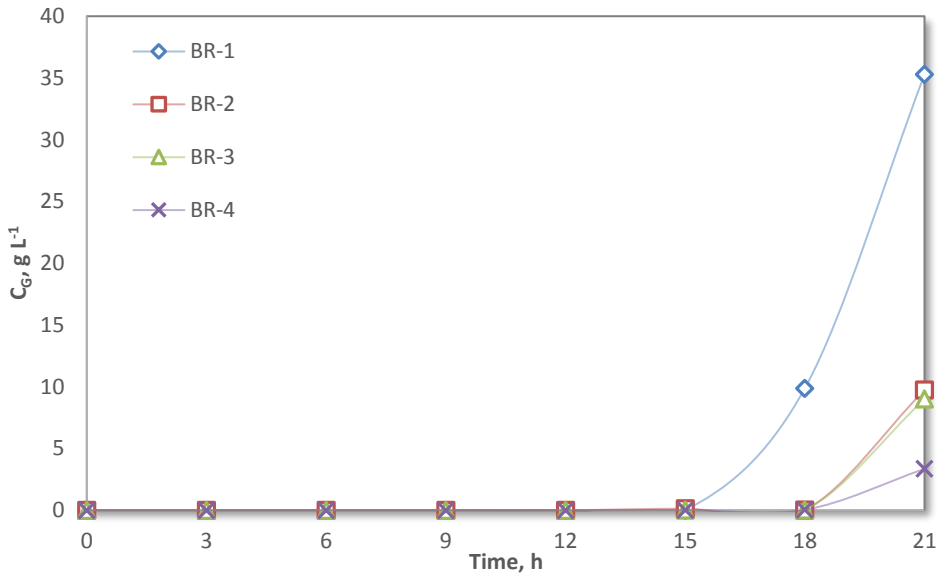


Figure 4.30 Accumulated glucose concentration during glucose fed-batch phase in pilot scale bioreactor experiments of the strains pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%.

Instantaneous specific glucose consumption rates, q_G (g g^{-1} h^{-1}), and glucose consumption rates, r_G (g L^{-1} h^{-1}), were presented in Table 4.5. The overall cell yield on substrate $Y'_{X/S}$ was calculated as 0.38, 0.47, 0.36, and 0.48 g g^{-1}. Also, the overall product yield on substrate $Y'_{P/S}$ was calculated as 0.54, 0.73, 0.92, 2.12 for BR-1, BR-2, BR-3, and BR-4, respectively.

In the first three pilot scale bioreactor experiments the effect of reduced dissolved oxygen concentration (limited-oxygen transfer condition at C_{DO}=5%), and in BR-4 impact of the higher dissolved oxygen concentration, C_{DO}=15%, on the cell yields and excreted the by-product, ethanol, were investigated to clarify the compatibility between the respiratory and the fermentative metabolism. Concentrations of the secreted ethanol was measured as explained in section 3.7.7 and the results were represented in Figure 4.31.

Table 4.5 The specific glucose consumption rates and glucose consumption rates of the four bioreactor experiments.

Cultivation Time (h)	q_G (g g^{-1} h^{-1})				r_G (g L^{-1} h^{-1})			
	BR-1	BR-2	BR-3	BR-4	BR-1	BR-2	BR-3	BR-4
0	-	-	-	-	-	-	-	-
3	0.27	0.32	0.24	0.24	4.29	4.28	4.28	4.27
6	0.26	0.33	0.25	0.23	6.59	6.57	6.60	6.51
9	0.30	0.31	0.27	0.24	9.92	9.90	9.97	9.75
12	0.29	0.35	0.29	0.25	14.29	14.27	14.48	14.04
15	0.32	0.42	0.30	0.20	20.16	20.10	20.55	19.62
18	0.45	0.38	0.32	0.22	26.88	23.86	24.58	23.14
21	0.18	0.10	0.07	0.04	10.31	5.72	4.96	4.58

In the all pilot scale bioreactor experiments ethanol was not detected until t=6 h. Ethanol concentrations started to increase at t=9 h of BR-1, BR-2, and BR-3. Although the cell concentration in BR-3 was higher compared to the other two bioreactor experiments, the lowest ethanol concentration was measured in double promoter expression system; whereas, the highest ethanol was detected with P_{GAP} driven expression system. Besides, due to increased oxygen transfer, ethanol concentration in BR-4 started to increase after t=12 h. The maximum ethanol concentrations were detected at t=21 h in all experiments as 12.9, 16.5, 10.9, and 1.92 g L^{-1} in BR-1, BR-2, BR-3, and BR-4, respectively. Being a 'Crabtree negative' yeast, *P. pastoris*, oxygen limitation shifted the metabolism to fermentative pathway from the respiratory pathway and caused cells to utilize their energy to produce ethanol rather than to use cell growth. Therefore, while the increase in cell concentrations ceased at the end of the processes, ethanol concentrations continued to enhance.

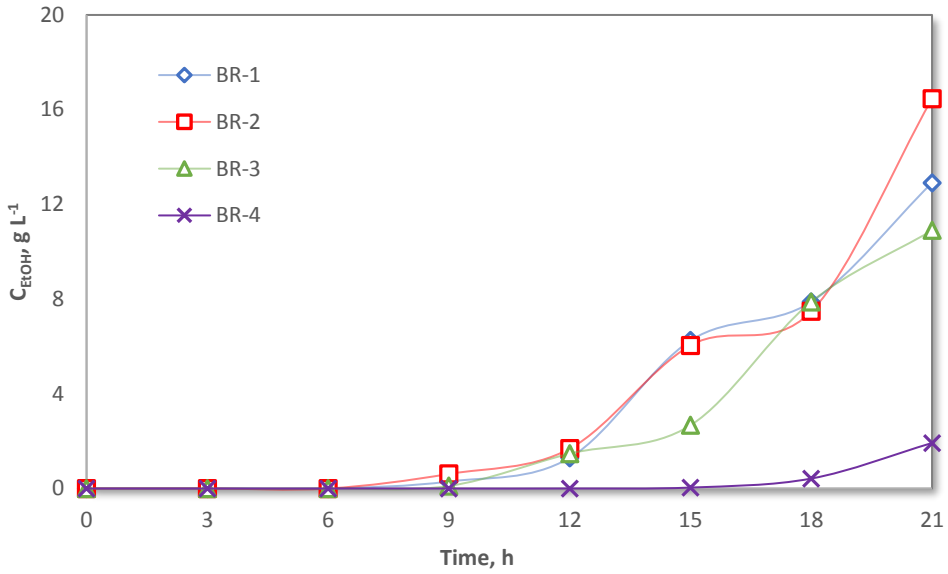


Figure 4.31 Variation of produced ethanol concentration during glucose fed-batch phase in pilot scale bioreactor experiments of the strains pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%.

Organic acids are produced as endo-metabolomes in the cell which are excreted towards its environment throughout the growth in order to keep the intracellular pH as constant. Therefore, determination of the concentrations of organic acids during fermentation give an idea about the functioning of the intracellular network of the cells. Organic acids that were present in the extracellular medium was measured as elucidated in section 3.7.8 and results were given in Table 4.6.

The results of the analysis with HPLC verified that by keeping the dissolved oxygen concentration at lower levels caused increase in the extracellular organic acid concentrations which were produced via the TCA cycle. Among the constituents of the TCA cycle, namely fumaric acid, citric acid, oxalic acid and succinic acid, and malic acid, larger amount of oxalic acid were measured in all the cultivations. The concentrations of those acids were generally increased as the

cultivation time increases. Besides, the concentrations of the organic acids in the broth were found in proportionally lower levels. When the dissolved oxygen concentration was increased to 15%, detected organic acids of the TCA cycle were considerably lower due to the interaction between the respiratory pathway and the TCA cycle. At pyruvate node, pyruvic acid concentration increased with increase in the cultivation time. In addition, accumulation of acetic acid was significantly higher at the late growth phases of BR-2, BR-3, and of BR-4. Nevertheless, lactic acid and formic acid accumulations were lower in all the experiments.

4.4 Metabolic Flux Analysis

Metabolic flux analysis (MFA) is the analysis of intracellular reaction rates by reaction engineering principles which is used to comprehend the physiology of the cell, to estimate its metabolic capacity in response to variations in its environment or to genetic modifications (Zamboni and Sauer, 2009, Lee et al., 2011). Therefore, stoichiometry based MFA assuming pseudo-steady state in the cell for the endo-metabolomes enables to understand the intracellular reaction network of the three different r-*P. pastoris* strains in-depth, consequently, to optimize the bioprocess parameters for heterologous protein production (Çelik et al., 2010). For the two different *P. pastoris* strains expressing rhGH under different promoters and the third *P. pastoris* strain that express rhGH with the combination of those two promoters, the calculated fluxes were compared at limited-oxygen transfer conditions. Furthermore, effect of different oxygen transfer conditions on double promoter expression systems were investigated. The intracellular biochemical reaction pathway map of r-*P. pastoris* expressing rhGH was presented in Figure 4.32.

In order to construct the intracellular metabolic flux model the measured rhGH and organic acid production rates, glucose uptake rates, and biomass formation rates were used. By analysing the cell growth profiles of the pilot scale bioreactor experiments, the periods for the analyses were determined. Period I was between $0 < t \leq 9$ h of the cultivations where early exponential growth phase was observed and the analysis was carried out at t=5 h. Likewise, the cultivation time

interval of $9 < t \leq 15$ h was designated as period II where exponential growth phase occurred and the analysis was carried out for t=12 h of the fermentations. Lastly, period III was defined for BR-3 and BR-4 within $15 < t \leq 18$ h where the cell growth rate decreased whereas rhGH formation rates augmented; and the analysis was carried out with the data at t=16 h. Results of the normalized flux values with respect to glucose uptake rates (R6) for the all the bioreactor experiments were presented in Table 4.7.

Primarily, each analysis was evaluated separately, then the results were compared to reveal the effects of the three rhGH expression systems and the effects of the oxygen trasnfer, in other words dissolved oxygen concentration in the bioreactor on r-protein production. Metabolic flux distributions at the principle nodes, Pyr and G3P were analyzed, and data was given in percentage. The fluxes towards intracellular amino acids were also calculated and total ATP production was determined and data was normalized with respect to glucose uptake rates of the periods.

Table 4.6 Variences in organic acid concentrations during pilot scale bioreactor experiments

	Cultivation time, h	Oxalic Acid	Formic Acid	Pyruvic Acid	Malic Acid	Lactic Acid	Acetic Acid	Maleic Acid	Citric Acid	Fumaric Acid	Succinic Acid	Total
BR-1	0	0.1679	0.0067	0.1600	-	-	0.3037	-	-	-	-	0.6382
	3	0.2306	-	0.0870	-	-	-	-	-	-	-	0.3177
	6	0.0105	0.0098	0.2158	-	0.0519	0.3032	0.0008	-	0.0010	-	0.5929
	9	0.0082	0.0195	0.2998	-	0.0434	1.0009	0.0014	0.0063	0.0061	0.0323	1.4180
	12	0.0157	0.0147	0.3238	-	0.0778	0.4547	0.0012	-	0.0015	-	0.8893
	15	0.3035	0.0684	0.7033	0.0116	0.0847	2.3963	0.0017	0.0331	0.0106	-	3.6132
	18	0.2478	0.3691	1.4113	0.1067	-	-	0.0015	0.0968	0.0344	-	2.2677
	21	0.0299	0.4854	2.0478	0.0541	-	-	0.0012	0.1860	0.0405	-	2.8449
BR-2	0	0.2027	-	0.1308	-	0.0332	-	-	-	0.0002	-	0.3669
	3	0.1601	0.0023	0.2840	-	-	0.3641	0.0002	-	0.0003	-	0.8110
	6	0.2590	-	0.3217	0.0214	-	0.3489	0.0009	-	0.0005	0.0316	0.9840
	9	0.5689	-	0.4236	-	0.0322	0.1187	0.0003	-	-	0.0328	2.1765
	12	0.1721	-	0.5629	-	0.1103	0.3349	0.0011	-	0.0007	-	1.1820
	15	0.0241	0.1821	1.2335	-	0.0676	1.4816	0.0045	-	0.0060	0.1006	3.1000
	18	0.5129	0.7378	1.9939	0.0766	-	3.9370	0.0015	-	0.0263	-	7.2860
	21	0.4411	0.4424	1.2387	0.0492	-	2.3373	0.0006	0.0533	0.0183	-	4.5809
BR-3	0	0.1954	-	0.0221	-	-	0.0344	-	-	0.0004	-	0.2523
	3	0.0180	-	0.0890	-	0.0371	0.2892	0.0001	-	0.0037	-	0.4370
	6	0.2786	-	0.1507	-	-	0.1097	0.0000	-	0.0002	0.0489	0.5882
	9	0.1417	0.0105	0.1842	-	0.0401	0.1266	0.0005	-	0.0002	0.0379	0.5416
	12	0.1603	-	0.2236	-	-	0.2045	0.0007	-	0.0008	0.0656	0.6555
	15	0.2122	-	0.4543	-	0.0682	0.3019	0.0014	-	0.0074	0.1605	1.2060
	18	0.9288	0.0920	0.7926	-	0.0101	1.8301	0.0014	-	0.0002	-	3.6553
	21	0.1482	0.4000	1.7479	0.2169	0.1915	3.1922	0.0025	-	0.0003	0.3454	6.2450
BR-4	0	0.0475	-	0.0297	-	-	0.0323	-	-	0.0001	-	0.1095
	3	0.0499	0.0052	0.0163	-	-	0.0333	-	-	0.0001	-	0.1047
	6	0.0749	-	0.0153	-	-	0.0452	-	-	-	-	0.1354
	9	0.0197	0.0587	0.0196	-	-	0.0982	0.0002	0.0137	-	0.0265	0.2367
	12	0.0567	0.0065	0.0146	-	-	0.1120	-	-	0.0003	-	0.1901
	15	0.0733	0.2217	0.0533	-	-	1.7563	0.0003	0.0806	0.0139	-	2.1994
	18	0.0129	0.0155	0.1889	0.0512	0.0278	3.6339	-	0.0112	0.0239	-	3.9654
	21	0.1800	0.0608	0.4360	0.1203	0.0506	10.0191	-	-	0.0716	0.1967	11.135

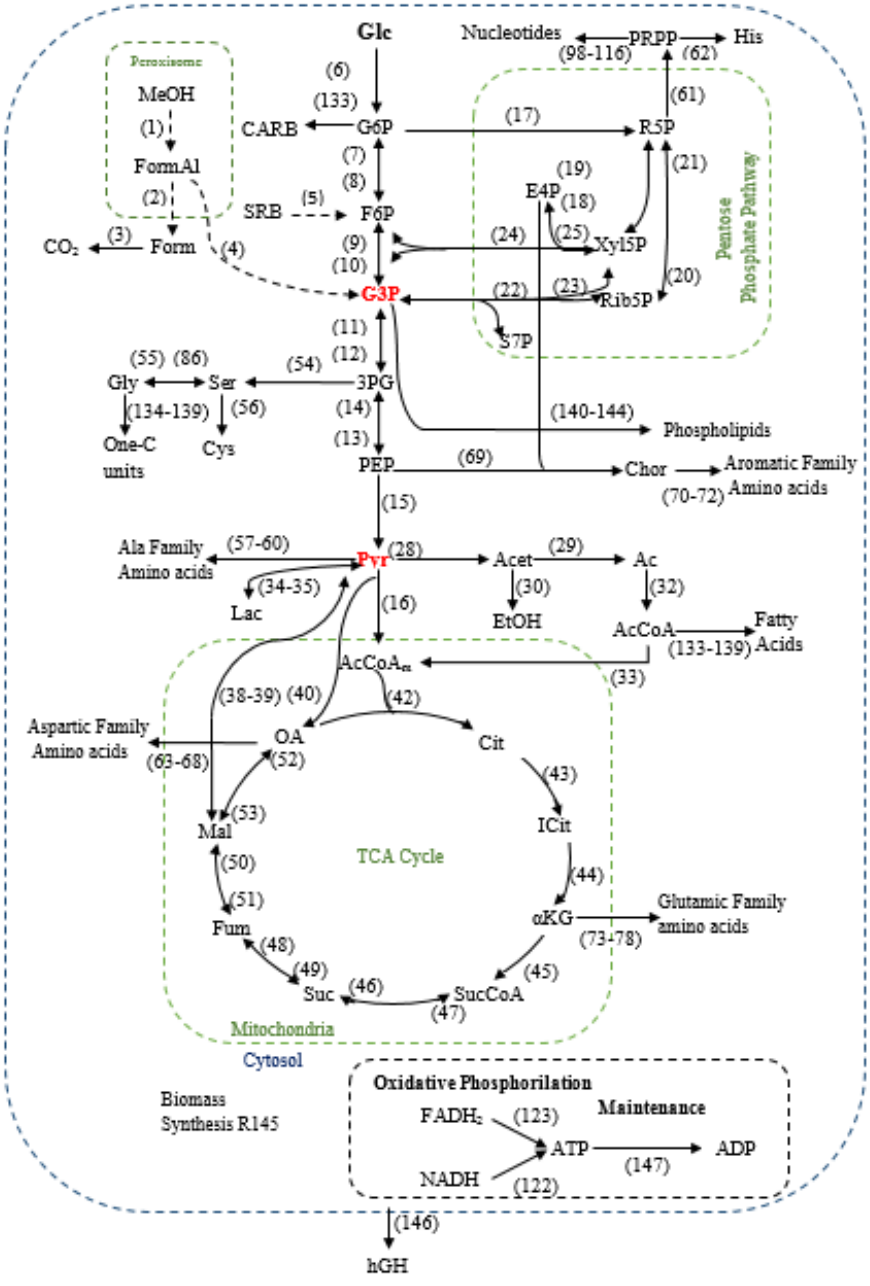


Figure 4.32 The metabolic pathway map of r-*P. pastoris* producing rhGH.

Table 4.8 Normalized flux distributions (with respect to glucose uptake rate) of rhGH production by r-*P. pastoris*.

R #	BR-1		BR-2		BR-3			BR-4		
	t=5 h	t= 12h	t=5 h	t=12h	t=5 h	t=12h	t=16h	t=5 h	t=12h	t=16h
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
3	0.00	0.00	17.79	0.00	8.59	0.00	0.00	24.93	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
7	8.29	0.00	240.0	0.00	0.00	153.8	0.00	0.00	56.07	0.00
8	0.00	0.00	0.00	66.15	0.00	0.00	99.05	64.39	0.00	0.00
9	42.36	27.83	0.00	68.02	45.19	0.00	87.91	64.39	32.07	52.37
10	0.00	0.00	17.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11	111.1	75.22	64.01	136.5	111.9	75.92	170.0	127.8	107.6	130.5
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13	103.9	32.93	49.53	92.45	83.85	61.21	145.3	81.01	105.9	99.92
14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15	89.07	2.87	45.81	90.63	79.41	56.94	143.9	76.35	102.1	98.55
16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17	104.0	96.94	337.0	32.40	96.52	250.5	0.00	31.82	153.6	99.16

Table 4.7 Normalized flux distributions of rhGH production by r-*P. pastoris* (Continued).

18	50.64	27.83	217.9	1.82	45.19	153.8	0.00	0.00	88.13	52.37
19	0.00	0.00	0.00	0.00	0.00	0.00	11.14	0.00	0.00	0.00
20	29.07	21.40	109.9	1.35	23.70	77.96	0.00	1.15	45.00	26.49
21	0.00	0.00	0.00	0.00	0.00	0.00	5.25	0.00	0.00	0.00
22	29.07	21.40	109.9	1.35	23.70	77.96	0.00	1.15	45.00	26.49
23	0.00	0.00	0.00	0.00	0.00	0.00	5.25	0.00	0.00	0.00
24	21.57	6.37	108.0	0.47	21.48	75.86	0.00	0.00	43.13	25.80
25	0.00	0.00	0.00	0.00	0.00	0.00	5.89	1.15	0.00	0.00
26	29.07	21.40	109.9	1.35	23.70	77.96	0.00	1.15	45.00	26.49
27	0.00	0.00	0.00	0.00	0.00	0.00	5.25	0.00	0.00	0.00
28	0.06	5.16	0.00	1.98	2.89	13.38	99.05	0.20	0.07	44.27
29	0.06	4.71	0.00	1.51	2.89	0.57	65.51	0.20	0.07	22.98
30	0.00	0.45	0.00	0.47	0.00	12.80	33.54	0.00	0.00	21.30
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	61.77	0.00	0.00	0.00
33	51.93	11.21	10.35	46.61	18.22	12.36	51.01	13.92	46.00	24.66
34	0.64	0.03	0.00	0.36	0.44	0.06	0.13	0.00	0.00	0.08
35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
36	0.00	0.00	17.79	0.00	9.26	0.00	0.57	25.07	0.00	0.00
37	4.64	12.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.15
38	67.07	0.00	0.00	57.14	0.00	0.00	0.00	77.91	0.00	0.00

Table 4.7 Normalized flux distributions of rhGH production by r-P. pastoris (Continued).

39	0.00	0.00	15.41	0.00	0.00	0.00	11.77	0.00	0.00	0.00
40	144.2	0.00	0.00	72.08	50.74	11.53	0.00	80.74	91.47	11.07
41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
42	43.93	0.03	7.09	11.72	14.00	0.00	49.94	9.86	26.73	23.51
43	43.93	0.00	7.09	11.72	14.00	0.00	49.94	9.86	26.67	23.21
44	43.93	0.00	7.09	11.72	14.00	0.00	49.94	9.86	26.67	23.21
45	31.50	0.00	0.00	0.00	0.00	0.00	43.16	0.00	0.00	15.04
46	31.50	14.65	0.00	0.00	0.00	0.00	43.16	16.35	0.93	54.66
47	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
48	31.50	14.65	0.00	0.00	0.00	0.00	42.85	16.35	0.93	54.58
49	0.00	0.00	1.10	0.10	0.44	0.13	0.00	0.00	0.00	0.00
50	67.07	73.76	15.06	57.14	52.07	26.43	74.81	74.73	56.00	94.27
51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
52	0.00	73.76	30.29	0.00	52.07	26.43	86.52	272.6	56.00	94.20
53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
54	7.14	42.29	14.42	44.06	28.07	14.78	24.75	46.69	1.73	30.69
55	0.00	27.52	12.79	43.28	26.15	12.87	24.18	44.66	0.00	30.08
56	0.07	0.05	0.05	0.02	0.06	0.06	0.02	0.06	0.05	0.03
57	0.00	0.00	3.20	1.56	3.78	3.63	1.08	4.05	2.27	1.07
58	9.07	12.68	3.90	35.26	5.11	13.18	1.33	21.35	3.27	1.37
59	2.71	10.57	1.86	0.89	2.22	2.10	0.63	18.72	1.53	0.61

Table 4.7 Normalized flux distributions of rhGH production by r-P. pastoris (Continued).

60	6.36	2.17	2.09	34.38	2.89	11.08	0.70	2.64	1.73	0.76
61	24.29	47.71	9.24	29.22	27.63	18.66	16.39	30.68	20.53	20.23
62	0.71	16.05	0.47	0.21	0.52	8.66	0.13	0.61	0.40	0.15
63	101.4	72.29	23.08	60.63	88.81	37.90	34.24	342.9	120.6	81.83
64	1.07	0.76	0.70	59.48	0.89	0.83	0.25	0.88	0.60	0.31
65	60.00	9.17	3.02	1.46	31.70	6.94	1.01	3.85	62.33	40.69
66	59.50	8.79	2.67	1.30	31.26	6.50	0.89	3.38	62.07	40.61
67	2.00	7.39	1.34	0.68	1.63	1.53	0.44	1.69	2.07	40.08
68	0.71	0.51	0.47	0.26	0.59	0.57	0.19	0.61	0.40	0.15
69	7.43	15.03	1.86	0.89	2.22	2.10	0.63	2.36	1.93	0.69
70	1.36	0.96	0.93	0.47	1.11	1.08	0.32	1.22	0.80	0.61
71	1.07	0.76	0.70	0.36	0.81	0.83	0.25	0.88	0.60	0.00
72	5.00	13.31	0.17	0.10	0.22	0.19	0.06	0.27	0.53	0.06
73	84.86	131.0	58.31	114.8	141.6	76.43	65.95	140.2	155.9	119.6
74	45.79	76.05	19.83	118.2	56.81	30.76	33.16	62.97	58.47	41.07
75	1.71	1.21	292.9	0.57	1.33	196.5	0.38	1.49	0.93	0.38
76	3.29	10.19	2.21	1.09	2.59	2.55	0.76	2.84	18.40	0.69
77	1.64	9.04	1.10	0.57	1.33	1.27	0.38	1.42	17.53	0.38
78	4.93	2.36	1.98	9.22	7.93	2.29	5.06	3.38	5.80	6.41
79	0.00	9.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
80	0.00	7.90	0.00	0.00	0.00	0.00	0.00	0.00	16.60	0.00

Table 4.7 Normalized flux distributions of rhGH production by r-P. pastoris (Continued).

81	0.00	0.00	0.00	59.11	0.00	0.00	0.00	0.00	0.00	0.00
82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	275.7	0.00	0.00
83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
85	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
86	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
87	0.00	15.54	0.00	0.00	0.00	8.15	0.00	0.00	0.00	0.00
88	0.00	5.99	0.00	0.00	0.00	0.00	0.00	0.00	0.93	39.62
89	3.29	0.00	0.00	33.33	0.44	8.73	0.00	0.00	0.00	0.00
90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23
91	0.00	0.00	291.8	0.00	0.00	195.2	0.00	0.00	0.00	0.00
92	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
93	55.50	0.00	0.00	0.00	28.07	3.44	0.00	0.00	58.87	0.00
94	4.71	13.12	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00
95	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	8.60	0.00	0.00	0.00	0.00	0.00	16.35	0.00	0.00
97	1.93	0.25	0.00	8.23	5.56	0.00	4.43	0.81	4.20	5.80
98	17.00	17.26	7.50	28.85	25.56	8.60	15.76	28.45	18.73	19.69
99	17.64	33.31	7.97	28.59	26.15	17.20	15.95	29.05	19.13	19.85
100	0.71	0.51	0.47	0.21	0.52	0.51	0.13	0.61	0.40	0.15
101	0.14	0.13	0.12	0.05	0.15	0.13	0.04	0.14	0.07	0.03

Table 4.7 Normalized flux distributions of rhGH production by r-P. pastoris (Continued).

102	17.00	32.80	7.50	28.33	25.56	16.69	15.82	28.45	18.80	19.77
103	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
104	0.21	0.19	0.17	0.10	0.22	0.19	0.05	0.20	0.13	0.05
105	0.14	0.13	0.12	0.05	0.15	0.13	0.04	0.14	0.07	0.03
106	17.00	32.80	7.50	28.33	25.56	16.69	15.82	28.45	18.80	19.77
107	43.07	81.85	18.02	117.3	54.67	36.82	32.59	60.68	40.40	40.46
108	1.64	1.15	1.10	0.52	1.26	1.21	0.38	1.42	0.87	0.31
109	0.93	0.64	0.64	0.31	0.74	0.70	0.19	0.81	0.53	0.15
110	0.79	0.51	0.52	0.26	0.59	0.57	0.19	0.68	0.40	0.15
111	0.79	0.51	0.52	0.26	0.59	0.57	0.19	0.68	0.40	0.15
112	0.57	0.38	0.41	0.21	0.44	0.45	0.13	0.47	0.33	0.15
113	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
114	0.36	0.25	0.23	0.10	0.30	0.25	0.06	0.27	0.20	0.07
115	0.21	0.19	0.17	0.10	0.22	0.19	0.05	0.20	0.13	0.05
116	0.14	0.13	0.12	0.05	0.15	0.13	0.04	0.14	0.07	0.03
117	0.14	0.13	0.12	0.05	0.15	0.13	0.04	0.14	0.07	0.03
118	35.57	8.15	3.26	13.96	26.22	5.48	7.72	13.58	38.40	9.69
119	0.71	0.51	0.47	0.26	0.59	0.57	0.19	0.61	0.40	0.15
120	34.64	35.03	15.47	56.93	51.70	17.64	31.71	57.50	37.93	39.54
121	34.64	35.03	15.47	56.93	51.70	17.64	31.71	57.50	37.93	39.54
122	318.9	304.0	437.4	301.0	296.5	352.0	364.7	282.4	311.7	381.1

Table 4.7 Normalized flux distributions of rhGH production by r-P. pastoris (Continued).

123	39.50	42.36	290.7	33.23	0.00	203.8	42.85	32.77	2.27	94.20
124	155.7	159.5	352.4	104.8	95.93	278.2	184.6	96.42	128.1	254.9
125	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
126	138.7	147.6	74.01	159.5	171.2	92.61	91.08	188.6	140.8	110.9
127	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
128	0.57	0.45	0.41	0.21	0.52	0.45	0.13	0.54	0.33	0.15
129	27.50	65.54	11.40	89.38	30.22	29.30	17.09	33.45	22.40	21.07
130	194.9	51.53	22.33	157.0	127.3	37.01	47.28	165.8	147.6	70.15
131	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
132	243.5	230.3	400.1	289.3	230.4	325.6	185.1	0.00	229.1	248.6
133	4.29	2.99	2.91	1.41	3.41	3.31	0.95	3.72	2.33	0.84
134	6.64	4.65	4.42	2.19	5.26	5.10	1.46	5.68	3.67	1.30
135	0.64	0.45	0.41	0.21	0.52	0.45	0.13	0.54	0.33	0.15
136	0.50	0.32	0.29	0.16	0.37	0.38	0.13	0.41	0.27	0.08
137	0.29	0.19	0.17	0.10	0.22	0.19	0.06	0.27	0.13	0.06
138	0.21	0.13	0.12	0.05	0.15	0.13	0.04	0.14	0.07	0.04
139	0.05	0.03	0.03	0.01	0.04	0.03	0.01	0.04	0.02	0.01
140	0.21	0.13	0.12	0.05	0.15	0.13	0.04	0.14	0.13	0.04
141	0.14	0.06	0.12	0.04	0.07	0.13	0.03	0.14	0.07	0.03
142	0.14	0.06	0.06	0.04	0.07	0.06	0.03	0.07	0.06	0.02
143	0.06	0.04	0.04	0.02	0.05	0.05	0.01	0.05	0.03	0.01

Table 4.7 Normalized flux distributions of rhGH production by r-P. pastoris (Continued).

144	0.05	0.04	0.03	0.02	0.04	0.04	0.01	0.04	0.03	0.01
145	10.29	7.20	6.92	3.39	8.22	7.90	2.28	8.85	5.67	2.06
146	0.0001	0.0008	0.0009	0.0003	0.0011	0.001	0.0006	0.0001	0.0020	0.0046
147	0.00	0.00	596.6	0.00	7.56	342.1	576.4	0.00	0.00	613.2

***** From left to right fluxes were normalized by 1.4, 1.57, 1.72, 1.92, 1.35, 1.57, 1.58, 1.48, 1.50, 1.31 mmol g_{DW}^{-1} h^{-1}, respectively.

4.4.1 Metabolic Flux Analysis for rhGH production under P_{PDC}

When rhGH expression under P_{PDC} was performed, it was seen that the glucose uptake rate enhanced from 1.40 to 1.57 mmol g_{DW}^{-1} h^{-1} in period I to period II and all fluxes normalized with respect to the corresponding glucose uptake rates. Considering the fluxes of R17 majority of G6P entered the PPP with an increase from period I to period II. Causing an interruption in the glycolysis pathway flux through G6P to F6P (R8) was calculated as zero in both periods and maintenance of the pathway, F6P formation, was provided via the reactions in the PPP. Regarding the gluconeogenesis pathway flux from F6P to G6P was observed only in period I leading an increase in the fluxes of R24 and R26. Due to decrease in R15 pyruvate generation by utilizing Pi reduced. $AcCoA_m$ formation from PYR by R16 was found as zero and it was revealed that all $AcCoA_m$ was produced from AcCoA via R33. As *P. pastoris* is a Crabtree-negative yeast, when oxygen was kept at lower levels, efficiency of the TCA cycle reduced by inducing the metabolism to shift to fermentative pathway to produce the by-products.

As a crucial branch point when Pyr was taken into consideration, the flux through pyruvate to formate (R36) was found as zero whereas the reaction R34 was active therefore lactate production was determined in both periods with decreasing fluxes from 0.64 to 0.03 in period I to period II, respectively. Although the anaplerotic reaction R40 that produces OA from Pyr was highly active in period I,

the reaction that was catalyzed by the enzyme pyruvate decarboxylase which converts Pyr to Acet was also active and the flux through Pyr to Acet (R28) enhanced almost 100-fold from period I to period II when the Pyr accumulation increased (Agarwal et al., 2013) due to reduction in Pyr participation in the TCA cycle. Thus, Pyr was directed to Acet. Besides, detection of ethanol in the production medium verified the shift towards the fermentative pathway. On the other hand, the other anaplerotic reaction R38 (Pyr replenishment reaction) was active in period I and the flux reduced to zero in period II. Another anaplerotic reaction that produces Mal from Pyr (R39) was found as inactive in both periods leading Mal production mainly from Fum with ever-increasing fluxes (R50).

When the reactions of the TCA cycle were analyzed two distinctive attitudes were observed. In period I, the TCA cycle was not completed as R52 was inactive. One of the key intermediates, OA, was supplied through the anaplerotic reaction R40 from pyruvate; also αKG, the other key intermediate in the TCA cycle was produced via the reaction R44 to form glutamic family amino acids. In period II however, fluxes through the reactions R43, R44, R45, and R49 were zero indicating that the incomplete TCA cycle, and the glutamic family amino acids were generated from different amino acid biosynthesis reactions. The increase in the flux of R62 could be the possible source for αKG production. Contrarily, the reactions R46, R48, R50, R52 were active to form OA and to further generate aspartic family amino acids from OA. In addition, reduction in the flux of R132 which provides transport of NADH from cytoplasm to mitochondrion causes ineffective TCA cycle operation.

When ATP generation fluxes were taken into consideration a decrease was observed from period I to period II mainly due to reduction in the flux of R15 from 9.8 % to 0.39 %. The highest contribution to ATP generation supplied from the oxidative phosphorylation of $NADH_m$ with an increase in fluxes from 70.1 % to 81.6 % between period I and period II, respectively. Also, oxidative phosphorylation of $FADH_2$ (R123) enhanced from 4.3 % to 5.7 % from period I to period II. However, total ATP generation decreased almost 10 %.

Due to reduction in total ATP generation fluxes the biomass synthesis flux (R145) was decreased approximately 30 % from 0.144 mmol g_{DW}^{-1} h^{-1} in period I to 0.113 mmol g_{DW}^{-1} h^{-1} in period II (normalized as 10.3 to 7.2) which was an inevitable consequence of diminished ATP formation. Moreover, flux through rhGH synthesis (R146) enhanced 683 % from 0.00012 in period I to 0.00082 in period II. Likewise, the reaction R28 which was catalyzed by pyruvate decarboxylase was enhanced 52-fold from period I to period II. Because rhGH was expressed under the promoter of the gene of that enzyme expected and compatible results were achieved.

4.4.2 Metabolic Flux Analysis for rhGH production under P_{GAP}

During rhGH production under P_{GAP} it was revealed that glucose uptake rate increased to 1.72 to 1.92 mmol g_{DW}^{-1} h^{-1} and intracellular fluxes were normalized with respect to those glucose uptake rates for period I and period II, respectively. Different from the previous analysis R3 was active in period I to generate NADH and CO_2 from For. In addition, in period I most of the G6P attended into the PPP (R17) while the fluxes towards G6P to F6P (R8) and F6P to G3P (R8) were zero causing an interruption in the glycolysis pathway and leading G3P production via reactions of PPP (R22 and R24). Moreover, R7 and R10 in the gluconeogenesis pathway were active. On the other hand, in period II the fluxes through the PPP decreased, which caused an active glycolysis pathway and inactive gluconeogenesis pathway. Due to increase in the glycolysis pathway fluxes between the two periods, the flux towards the Pyr from PEP enhanced from 45.8 in period I to 90.6 in period II leading increased formation of Pyr and ATP. The flux through $AcCoA_m$ from Pyr (R16) was zero in period I and period II and $AcCoA_m$ was supplied by the conversion of AcCoA via the R33. Due to low dissolved oxygen concentrations, efficiency of the TCA cycle was low and the metabolism of the yeast preferred the fermentative pathway yielding ethanol as by-product in period II.

At Pyr branch point Lac metabolism (R34 and R35) was found to be inactive in period I; however, R34 was active in period II to produce lactate from pyruvate.

Besides, in period I, R36 was active to form formate from pyruvate yet the flux of R36 was zero in period II. The reactions that convert Pyr to Acet (R28), Acet to Ac (R29), and Acet to EtOH (R30) were inactive in period I; whereas, in period II the reactions were inactive.

Regarding the anaplerotic reactions R38 (flux through Mal to Pyr) and R40 (flux through Pyr to OA) the two reactions were inactive, whereas R39 (flux through Pyr to Mal) was active in period I. Contrarily, in period II the anaplerotic reactions R38 and R40 became active and R39 was inactive.

The fluxes in TCA cycle resulted in an incomplete cycle in both periods. In both periods the reactions R42, R43, R44, and R49 were active and αKG provided by TCA cycle with the formation of glutamic family amino acids via R44, with increase in fluxes being 7.1 in period I and 11.7 in period II. Besides, R45, R46, R47, R48, R51, and R53 were inactive in both periods. Flux through Fum to Mal (R50) enhanced from 15.1 to 57.1 while Fum was probably produced via R106 which increased from 7.5 in period I to 28.3 in period II. Surprisingly, while in period I Mal was transformed to OA (R52), in period II the flux through R52 was zero and by the help of the anaplerotic reaction R38 generated Mal was transformed to Pyr and OA was produced via the anaplerotic reaction R40 to form aspartic family amino acids.

Total ATP generation was reduced from 12.3 in period I to 7.72 in period II (normalized with respect to glucose uptake rate) mostly because of the decrease in oxidative phosphorylation reactions. R122 was diminished 23.2 % from period I to period II while R123 decreased 87.2 % from period I to period II. On the other hand, owing to substrate-level oxidations (ATP_{SP}) in R11 ATP production enhanced from 5.2 % in period I to 17.7 % in period II. Consequently, total ATP generation was decreased for 30 % between period I to period II.

Because of the 39.2 % decrease in ATP generation flux via oxidative phosphorylation reactions leaded 50.7 % reduction in biomass synthesis fluxes (R145) as 0.119 mmol g_{DW}^{-1} h^{-1} in period I to 0.065 mmol g_{DW}^{-1} h^{-1} in period II

(normalized values were 6.9 in period I and 3.4 in period II, respectively). Additionally, flux towards rhGH synthesis (R146) also decreased 64.8 % from 0.00089 in period I to 0.00031 in period II which contradicted to the 2.4-fold increased flux of R11, catalyzed by gleceraldehyde-3-phosphate dehydrogenase enzyme, from 1.101 in period I to 2.621 in period II. Although it was reported that the glycolytic genes were upregulated under limited-oxygen availability conditions and result were parallel to the literature, reduction in R146 were considered to be unexpected and affected from other parameters.

4.4.3 Metabolic Flux Analysis for rhGH Production via Double Promoter Expression System under Limited-Oxygen Conditions

The glucose uptake rates increased during experiment from 1.35 mmol g_{DW}^{-1} h^{-1} in period I to 1.57 mmol g_{DW}^{-1} h^{-1} in period II and slightly increased to 1.58 mmol g_{DW}^{-1} h^{-1} in period III. In period I, at the entrance point to glycolysis pathway most of the G6P directed to PPP (R17) and the flux towards G6P to F6P (R8) was zero therefore glycolysis pathway was disrupted at that point. It was revealed that F6P was supplied via the PPP reactions (R24 and R26) to resume the pathway. Besides, with the contribution of another PPP reaction R22 flux from F6P to G3P increased and glycolysis pathway sustained until the ATP generated Pyr production (R15). Gluconeogenesis pathway on the other hand was totally inactive in period I. Regarding the period II, flux from G6P to PPP (R17) increased dramatically while the reactions of glycolysis pathway (R8 and R9) were zero meaning that the G6P was provided to glycolysis pathway only by the help of PPP reactions namely, R22, and R24. However, due to decrease in the flux of R11 flux towards Pyr from PEP reduced to 56.9 in period II from 79.4 in period I leading a reduced level of pyruvate production. In period III, totally opposite behavior was observed. The flux of G6P to PPP (R17) was zero and completely active glycolysis pathway and totally inactive gluconeogenesis pathway was achieved. On account of high fluxes in the upstream the flux from PEP to Pyr (R15) increased to ATP enhanced Pyr production. Yet, owing to low-oxygen condition the flux that form AcCoAm from

Pyr was zero and the metabolism of the Crabtree-negative yeast was directed to fermentative pathway to produce by-products.

At Pyr branch point, the reaction R34, that produce lactate from pyruvate was observed to be active in all periods whereas, the reverse reaction (R35) was inactive. In the meantime, the reaction catalyzed by pyruvate decarboxylase enzyme, which converted Pyr to Acet (R28), had ever-increasing fluxes either to produce by-product (R30), ethanol, or to transformed to Ac (R29). Due to allosterical regulation of pyruvate decarboxylase enzyme and its stimulation from its substrate, Pyr, might cause the increase in fluxes of R28 (Agarwal et al., 2013). Being inactive in period I and period II, Ac to AcCoA (R32) was active in period III. AcCoA was probably produced via R89 and R93 in period I and period II, and the reaction AcCoA to $AcCoA_m$ (R33) was active in all periods.

When anaplerotic reactions considered, because the unemployed pyruvate was present in production medium as pyruvic acid, the transformation of Mal to Pyr (R38) was inactive in the three periods. The reaction that converts Pyr to OA (R40) was found to be active in the first two periods and it was inactive in period III. On the contrary, Pyr to Mal (R39) was in the first two periods and the flux enhanced to 11.8 in period III to form Mal.

The TCA cycle was found to be incomplete in period I and period II whereas the cycle completed in period III. In period I, the cycle resumed until αKG, via reactions R42, R43, and R44, which was an important precursor in the production of glutamic family amino acids. Further, R45, R46, R47, R48, R51, and R53 were inactive in period I. Flux towards Fum to Mal (R50) was calculated as 52.1 while although R49 was active Fum presumably mainly generated via R98 and R106. Mal was converted to OA (R52) which was a crucial intermediate in the production of several amino acids. The TCA cycle reactions R42, R43, R44, R45, R46, R47, R48, R51, and R53 were inactive in period II, because of the low-oxygen concentration in the medium. The substantial intermediate αKG, which was utilized in the production of glutamic family amino acids could not be formed via TCA cycle

reactions therefore αKG must be produced via other reactions; enhanced flux of R60 from 2.9 in period I to 11.1 in period II might be explanation of αKG formation. In period II it was revealed that the majority of Fum was obtained via R106. Fluxes through Mal from Fum reduced to 26.4 in period II from 52.1 in period I. All Mal was converted to OA via R52 in order to maintain vital reactions. As previously mentioned, complete TCA cycle behavior was observed in period III.

Total ATP generation regarding the periods, followed an increasing trend, changed from 7.85 in period I to 10.4 in period II to 11.3 in period III (normalized with respect to glucose uptake rate). When the first two periods compared the reason in the enhancement of ATP generation was mainly due to 1.38-fold increase of the oxidative phosphorylation flux of R122 and 3.2-fold increase of the oxidative phosphorylation flux of R123. In addition, substrate-level oxidation (ATP_{SP}) in R11 contributed to increase in ATP generation. Moreover, if period II and period III were compared, it was understood that the increase in ATP generation was mainly caused by substrate-level oxidations (ATP_{SP}) of R11 and R15 due to augmentations in the fluxes by 2.25 and 2.54-fold, respectively. Oxidative phosphorylation flux R122 increased approximately 105% and R123 on the other hand reduced 78.8% in period III.

Although ATP generation increasingly continue between the periods the biomass synthesis reaction (R145) increased from 0.111 to 0.124 mmol g_{DW}^{-1} h^{-1} from period I to period II and reduced from 0.124 to 0.036 mmol g_{DW}^{-1} h^{-1} from period II to period III. The unexpected result could be considered to be arise from the increase in maintenance energy (R147). It was revealed that the maintenance energy enhanced 45.3-fold in period I to period II and further increased 1.7-fold between period II and period III. In addition, rhGH secretion reaction flux (R146) decreased almost 6 % from 0.00106 in period I to 0.00100 in period II and almost 41% from 0.00100 in period II to 0.00059 in period III. Despite the increasing trend in the fluxes of R11 and R28 the enhanced gleceraldehyde-3-phosphate dehydrogenase, and pyruvate decarboxylase activities might be affected from their substrates and the downtrend in the fluxes of R146, the rhGH secretion reaction,

could be independent from the upregulation in the genes of the two enzymes in limited oxygen conditions.

4.4.4 Metabolic Flux Analysis for rhGH Production via Double Promoter Expression System under Low-oxygen Conditions

The glucose uptake rates fluctuated from 1.48 mmol g_{DW}^{-1} h^{-1} in period I to 1.50 mmol g_{DW}^{-1} h^{-1} in period II and decreased to 1.31 mmol g_{DW}^{-1} h^{-1} in period III. The fluxes were normalized with respect to those values. In period I glycolysis pathway was active. While almost two thirds of G6P was converted to F6P via R8, approximately one third directed to PPP via R17. Glycolysis pathway maintained until ATP generated Pyr production (R15). Nevertheless, gluconeogenesis pathway was totally inactive in period I. When period II was considered, incomplete glycolysis and gluconeogenesis pathways with enhanced PPP fluxes were determined. Unlike period I, F6P was totally produced via PPP reactions (R24 and R26) to sustain the glycolysis pathway because R8 was inactive. Similar to glucose uptake rates a decrease in the flux of R11 was detected. However, the flux of G3P to 3PG (R13), 3PG to PEP (R13), PEP to Pyr enhanced in period II causing an augmented level of ATP and Pyr production. Regarding gluconeogenesis pathway, the only active reaction was R8 in period II. Like in period II glycolysis pathway was disrupted at R8; flux through G6P to F6P was zero in period III. Flux came from glucose (R6) leaded to PPP (R17) and contributed in the generation of F6P and G3P via R22, R24, and R26. As of R9 glycolysis pathway was persued until Pyr production; fluxes however, reduced and Pyr to $AcCoA_m$ (R16) was found to be inactive in all periods leading metabolism the fermentative pathway.

Dealing with the Pyr branch point, it was revealed that the lactate metabolism was in active in the first two period. R34 that converts Pyr to Lac was active in period III though the flux value of the reverse reaction (R35) was again zero. Considering the flux through Pyr to Acet (R28) whose reaction was catalyzed via pyruvate decarboxylase enzyme anew fluctuated behavior was observed. Flux in period I was 0.2 and decreased to 0.1 in period II and increased to 44.3 in period

III and same trend followed by R29. Flux of Acet to EtOH (R30) was zero in the first two period and enhanced to 21.3 in period III. Ac to AcCoA (R32) was determined to be inactive in the three periods which lead to AcCoA production through alternative reactions. It was guessed that AcCoA was supplied via R36 in period I, R93 in period II, and R88 in period III. Meanwhile, AcCoA to AcCoAm (R33) was active in all periods.

The Pyr replenishment reaction, Mal to Pyr (R38), was active only in period I and the reverse reaction (R39) was inavtive in all periods. OA replenishment reaction (R40), on the other hand was active in all periods; Pyr to OA was 80.7 in period I, increased to 91.5 in period II and rapidly decreased to 11.1 in period III.

In period I and period II, TCA cycle could not be completed due to R45. In the first two periods the cycle sustained until αKG (R42, R43, and R44) which was utilized in the production of glutamic family amino acids. Starting from R46 TCA cycle resumed until the formation of OA; R46, R48, R50, and R52 were active. SucCoA was thought to be produced via R96. The increase in the flux of R52 was considered to be originated from the flux through R82. In period III, it was revealed that the TCA cycle was completed.

Total ATP production was undulated in the same manner; varied from 5.36 in period I to 5.25 in period II to 7.59 in period III (normalized with respect to glucose uptake rate). The downtrend from period I to perod II was mainly caused due to almost 14-fold decrease in the oxidative phosphorylation flux of R123. Besides, substrate-level oxidation (ATP_{SP}) in R11 contributed to decrease in ATP generation. Regarding period II and period III, the enhancement in ATP generation was majorly resulted from approximately 50-fold increase in the R46 and almost 36-fold enhancement of oxidative phosphorylation flux of R123.

On the contrary to the fluctuations in total energy current, biomass synthesis reaction (R145) decreased from 0.131 to 0.085 mmol g_{DW}^{-1} h^{-1} from period I to period II and diminished from 0.085 to 0.027 mmol g_{DW}^{-1} h^{-1} from period II to period III. The generated ATP was not squandered for maintenance (R147) in the

first two periods yet flux through maintenance increased to 613.2 in period III. Despite the fluctuations in the fluxes of the both gene namely, gleceraldehyde-3-phosphate dehydrogenase, and pyruvate decarboxylase, both of the fluxes (R10 and R28) leading the activity of the two enzymes upregulated in period III where the maximum amount of rhGH produced and rhGH secretion reaction flux (R146) enhanced 16.3-fold from 0.00013 in period I to 0.00203 in period II and 2.25-fold from 0.00203 in period II to 0.00457 in period III in low-oxygen conditions.

4.4.5 Comparison of the pilot scale bioreactor experiments in terms of ATP and amino acid generations

Following to the entrance of glucose into the central carbon metabolism and its phosphorylation to G6P (R6), when the flux through the PPP (R17) was taken into consideration an increase was determined in BR-1 and a sharp decrease was detected in BR-2 from period I to period II. Besides, immediately before the maximum hGH formation time or when the exponential growth rate occurred an enhancement was found and when cell growth rate decreased fluxes through PPP diminished in BR-3 and BR-4. Consequently, increment in the fluxes towards PPP yielded higher amount of NADPH which was utilized mostly in biosynthesis of amino acids and lipids, manintaing the anabolic reactions more effectively.

Total ATP generation fluxes (Table 4.8) reduced in BR-1 and BR-2 from period I to period II whereas, the total fluxes enhanced in BR-3 and BR-4 between the periods. Although incomplete TCA cycle situation both in BR-1 and BR-2, ATP generation via oxidative phosphorylation (R122 and R123) increased in BR-1 being less affected from reduced oxygen conditions. In BR-2 on the other hand, decline in the fluxes of oxidative phosphorylation reactions compensated with increased fluxes through glycolytic pathway correspondingly enhanced substrate-level phosphorylation (R11 and R15) to generate ATP. Moreover, in BR-3 increased trend was determined in the total ATP generation fluxes. Despite the partial TCA cycle fluxes, increase in oxidative phosphorilation reactions were found in perod II. However, it was revelaed that most of the energy was wasted by maintanence rather

than rhGH production. From period II to period III R122 slightly enhanced while R123 decreased and therefore, substrate-level phosphorylation increased and the almost half of the generated ATP was utilized for maintanence to conserve electrical potential across membranes, to regulate the pH gradient, or intracellular futile cycles, to synthesize macromolecular compounds for instance, enzymes and RNA, or to defense against O_2 stress (Nielsen et al., 2003; Bodegom, 2007). Regarding BR-4, unaccomplished TCA cycle caused reduced level of total ATP generation in period I and period II than in period III. Thus, in period III when the TCA cycle completed, increase in fluxes through oxidative phosphorilation reactions occurred. Nevertheless, almost 80 % of total ATP generation in period III consumed for maintenance and the total ATP flux was approximately half of total ATP generation of in BR-3 making the double promoter expression system better in lower oxygen availibility conditions in terms of energy.

Table 4.9 Variation in ATP generation during pilot scale bioreactor experiments (given in percentage).

R #	BR-1		BR-2		BR-3			BR-4		
	t=5 h	t=12h	t=5 h	t=12h	t=5 h	t=12h	t=16h	t=5 h	t=12h	t=16h
11	18.80	17.11	8.07	28.98	22.91	11.01	22.23	23.82	20.49	17.19
15	15.07	0.65	0.01	0.00	16.25	8.26	18.82	14.23	19.44	12.98
46	5.33	3.33	0.00	0.00	0.00	0.00	5.64	3.05	0.18	7.20
103	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
112	0.10	0.09	0.05	0.04	0.09	0.06	0.02	0.09	0.06	0.02
114	0.06	0.06	0.03	0.02	0.06	0.04	0.01	0.05	0.04	0.01
122	53.9	69.1	55.2	63.9	60.7	51.1	47.7	52.7	59.4	50.2
123	6.68	9.63	36.7	7.05	0.00	29.6	5.60	6.11	0.43	12.4
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Amino acids are crutial precursers for protein synthesis as well as they regulate the principle metabolic pathways (Carnicer et al., 2012). During high-level recombinant protein production demand for these precurserd increases. Glutamine (Glu) is one of the most significant amino acid due to its utilization for many other amino acids such as, Ser, Pro, Arg, Asp, Ala, Val, Leu, Ile, Phe, Tyr, Gln, and Lys (Çelik et al., 2010). Amino acid composition of *hGH* with N-terminus fusion tag was presented in Table 4.9 with number of amino acids and their relative percentages. When the synthesized amino acid families were considered, Glutamic acid familiy acids had the greatest share regarding the total amino acid fluxes in BR-1, BR-2, and in BR-3 (Table 4.10). Besides, the second majorly produced amino acid family was Aspartic acid family the lowest one was aromatic acid family amino acids in all bioreactor experiments.

Table 4. 10 Amino acid content of mature hGH including N-terminus fusion tag which was expressed in the experiments (Massahi, 2017).

Amino acid	Abbr.	Number (#)	Percent
Alanine	A	7	3.45
Arginine	R	12	5.91
Asparagine	N	9	4.43
Aspartic acid	D	11	5.42
Cysteine	C	4	1.97
Glutamic acid	E	16	7.88
Glutamine	Q	13	6.40
Glycine	G	9	4.43
Histidine	H	9	4.43
Isoleucine	I	9	4.43
Leucine	L	26	12.81
Lysine	K	9	4.43
Methionine	M	3	1.48
Phenylalanine	F	14	6.89
Proline	P	8	3.94
Serine	S	18	8.87
Threonine	T	10	4.93
Tryptophan	W	1	0.49
Tyrosine	Y	8	3.94
Valine	V	7	3.45

Table 4.11 Variance in amino acid fluxes during pilot scale bioreactor experiments. From top to bottom, pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%.

	Fluxes (mmol g^{-1} DW h^{-1})		
Amino acids	Period I	Period II	Period III
Serine Family amino acids	0.10	1.10	
Alanine Family amino acids	0.25	0.40	
Aspartic acid Family amino acids	3.15	1.55	
Aromatic acid Family amino acids	0.21	0.47	
Glutamic acid Family amino acids	1.99	3.61	
Histidine biosynthesis	0.35	1.00	
Total	6.05	8.13	
Serine Family amino acids	0.47	1.68	
Alanine Family amino acids	0.19	1.38	
Aspartic acid Family amino acids	0.54	2.38	
Aromatic acid Family amino acids	0.06	0.04	
Glutamic acid Family amino acids	6.47	4.69	
Histidine biosynthesis	0.17	0.57	
Total	7.90	10.73	
Serine Family amino acids	0.73	0.43	0.77
Alanine Family amino acids	0.19	0.47	0.06
Aspartic acid Family amino acids	2.09	0.85	0.59
Aromatic acid Family amino acids	0.06	0.07	0.02
Glutamic acid Family amino acids	2.86	4.86	1.67
Histidine biosynthesis	0.38	0.43	0.26
Total	6.31	7.12	3.37
Serine Family amino acids	1.35	0.03	0.80
Alanine Family amino acids	0.69	0.13	0.05
Aspartic acid Family amino acids	5.23	3.72	2.67
Aromatic acid Family amino acids	0.07	0.06	0.02
Glutamic acid Family amino acids	3.14	3.86	2.21
Histidine biosynthesis	0.46	0.31	0.27
Total	10.95	8.11	6.01

Total amino acid biosynthesis fluxes were determined to enhance from period I to period II in all experiments and decrease were determined in BR-3 and BR-4 when lower growth rates were observed (period III). Considering Table 4.9 the most common amino acids that contributes to the structure of hGH are Leu and Ser which were produced via R60 and R54, respectively. Therefore, the fluxes through them were substantial. Throughout the bioprocesses except from BR-2, flux through Leu and Ser reduced at the period where the maxiumum rhGH secretions were achieved. Thus, addition of Leu and Ser in period II of BR-1 and period III of BR-3 and BR-4 might enhance the rhGH production.

4.4.6 Comparison of the pilot scale bioreactor experiments in terms of rhGH Secretion Fluxes

Oxygen transfer affects the ATP generation, growth, and r-protein production. Under limited-oxygen conditions due to insufficiency of oxygen, TCA cycle was incomplete which resulted in reduced amount of ATP generation in both single copy hGH expression systems under P_{PDC} and P_{GAP}. In period II of the two experiments, where the maximum rhGH production was achieved, total ATP generation fluxes diminished, as the lack of oxygen, influenced the metabolism of the cells yielding energy deprivation and consequently decrease in biomass synthesis fluxes was led. Also, by causing a shift towards respiratory to fermentative pathway, by-product formation fluxes increased (i.e., R29 and R34). In the same manner, diminishment in ATP generation from period I to period II, conduced low rhGH synthesis fluxes when expressed under the control of the glycolytic glyceraldehyde-3-phosphate dehydrogenase promoter P_{GAP} whereas, when the promoter of pyruvate decarboxylase (P_{PDC}) whose gene involved in fermentation, was utilized rhGH synthesis fluxes increased under limited-oxygen conditions. Considering the expression system under the control of both promoters, the adaptation of cells to limited oxygen condition varied. Regarding the incomple TCA cycle fluxes in period I and period II and complete TCA cycle in period III, ATP generation fluxes together with biomass synthesis enhanced whereas rhGH secretion fluxes decreased from period I to period II. However, both biomass

synthesis and rhGH secretion fluxes reduced and by-product formation fluxes enhanced in period III.

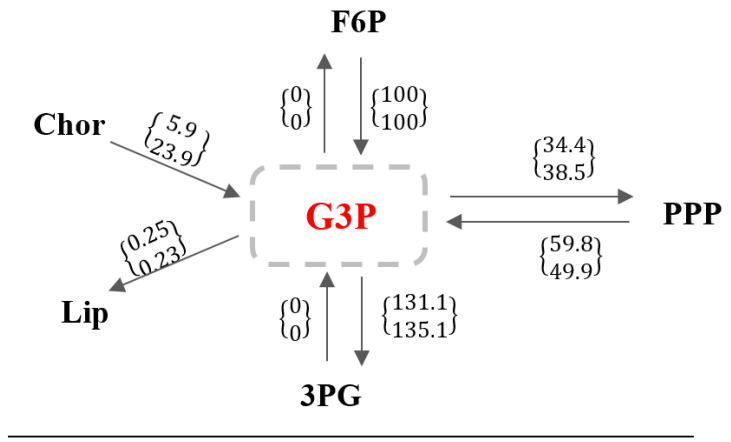
Considering the analysis of double promoter expression system under low-oxygen transfer conditions, it was revealed that when C_{DO} was enhanced cells adapted to C_{DO}=15% easier with complete TCA cycles in period II and in period III. Normalized ATP generation fluxes with respect to glucose uptake rates (Table 4.7) disclosed that ATP generation fluxes slightly enhanced from period I to period III and almost stable energy current was achived during fermentation. 4 ATP equivalents required to form a peptide bond (Çalık et al., 2011) and r-protein production is a multi-step process that requires energy (Baumann et al., 2010). Therefore, regarding the significance of ATP during r-protein synthesis, as expected rhGH secretion fluxes enhanced perpetually with the cultivation time whereas, biomass synthesis fluxes decresed continually from period I to period III. Besides, although a shift was determined from respiratory to fermentative pathway, fluxes towards by-product formation were lesser under low-oxygen conditions.

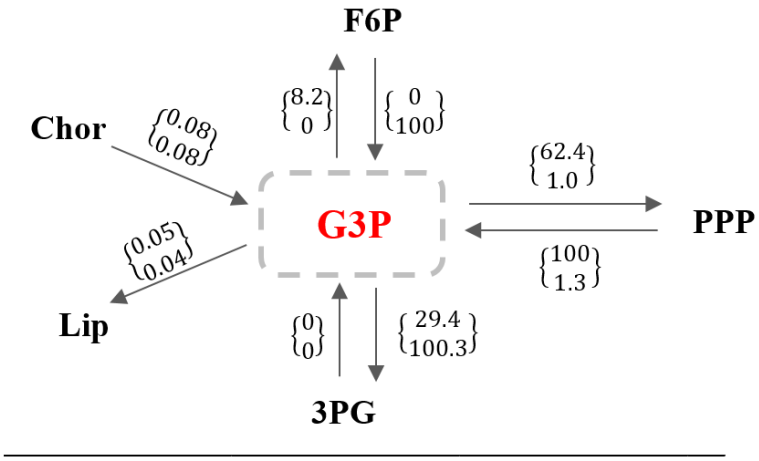
4.4.7 Metabolic Flux Distribution at Significant Nodes

Metabolic flux analysis (MFA) enables to ascertain the flux partitioning at the critical branch points (Çalık and Özdamar, 2002). The node, Pyr was selected due to being a crutial point with important flux distributions influencing the yield of the recombinant protein (Çelik et al., 2010). G3P on the other hand, was chosen because of its significance on glucose metabolism leading the yield of the r-protein.

In G3P node flux 100 was desinganated either R9 or summation of R22 and R24, due to complete flux shift to the PPP. It was revealed that the glycolysis pathway was steadyly operated only when the rhGH expression was performed under P_{PDC} (BR-1). Regarding the expression under P_{GAP} or double promoter system the fluxes through glycolysis pathway were unsteadyly changed between the periods. At reduced oxygen conditions it was understood that the PPP played a crucial role especially under P_{GAP} driven and double promoter expression systems. G3P generation was solely provided by the PPP reactions in period I of BR-2 and

in period II of BR-3. Fluctuations in the inlow and outflow through the PPP could because of the NADPH nucleotide demand of the metabolism in the cell. Besides, increase in dissolved oxygen concentration increased the glycolysis pathway fluxes in BR-4. Fluxes towards lipid metabolism and Chor can be regarded as insignificant when compared with the glycolysis and pentose phosphate pathways; still, the highest fluxes through the lipid metabolism and Chor were achieved in BR-1. Among the pilot scale bioreactor experiments, MFA analysis at the G3P revealed that the highest flux through the glycolysis pathway achieved in by BR-4 with the increase in oxygen transfer which created higher dissolved oxygen concentration set to C_{DO}=15%.





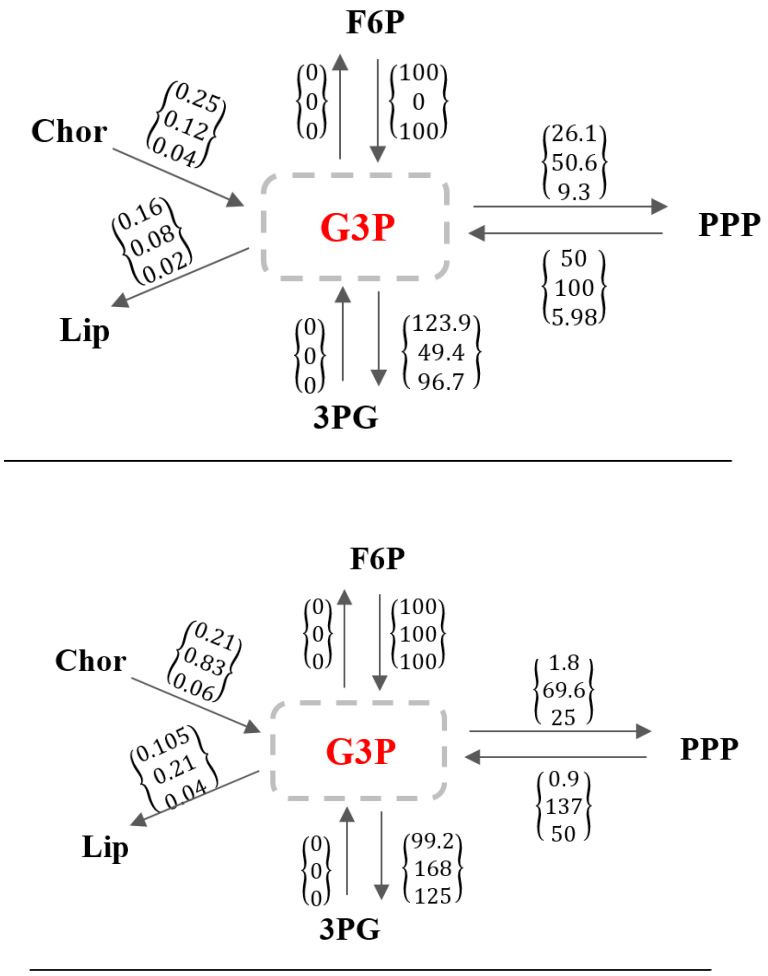
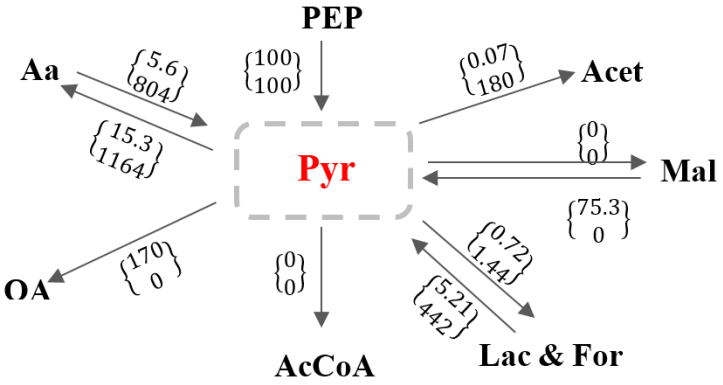


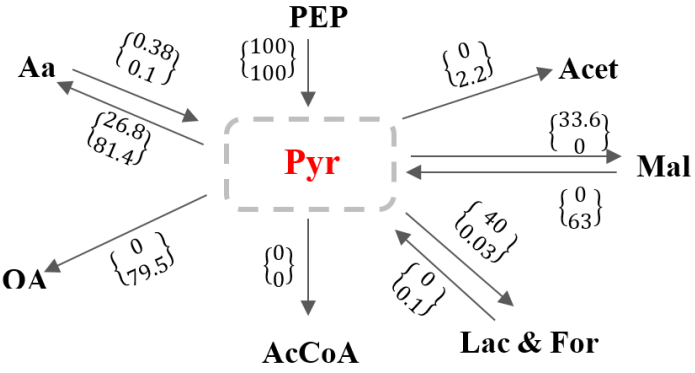
Figure 4.33 The normalized fluxes around G3P node throughout rhGH production in pilot scale bioreactor experiments of the strains pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%. From top to bottom, the numbers represent the period I, period II, and period III, respectively.

In the Pyr node flux 100 was designated for R15 in all analysis. The node was replenished via inlet flux towards Pyr from Mal mostly in BR-4. By-product, Lac, production was highest in BR-1 whereas the highest For formation was achieved in BR-4. Fluxes of OA replenishment reactions varied in lower oxygen availibity condition and enhanced at higher oxygen levels. When three expression systems were compared the lowest flux through acetaldehyde was achived during

production under P_{GAP} and due to the lowest by-product formation and desirable flux distribution, the favorable system was the double promoter expression system. Besides, when hGH productions under double promoter expression systems in different oxygen transfer conditions were compared, as expected lower flux towards acetaldehyde and higher flux through amino acids were determined in high oxygen transfer condition.

The analysis around two nodes, G3P and Pyr led to the conclusion that the two node were flexibile and adapted to flux alterations depending on the demand of the cell.





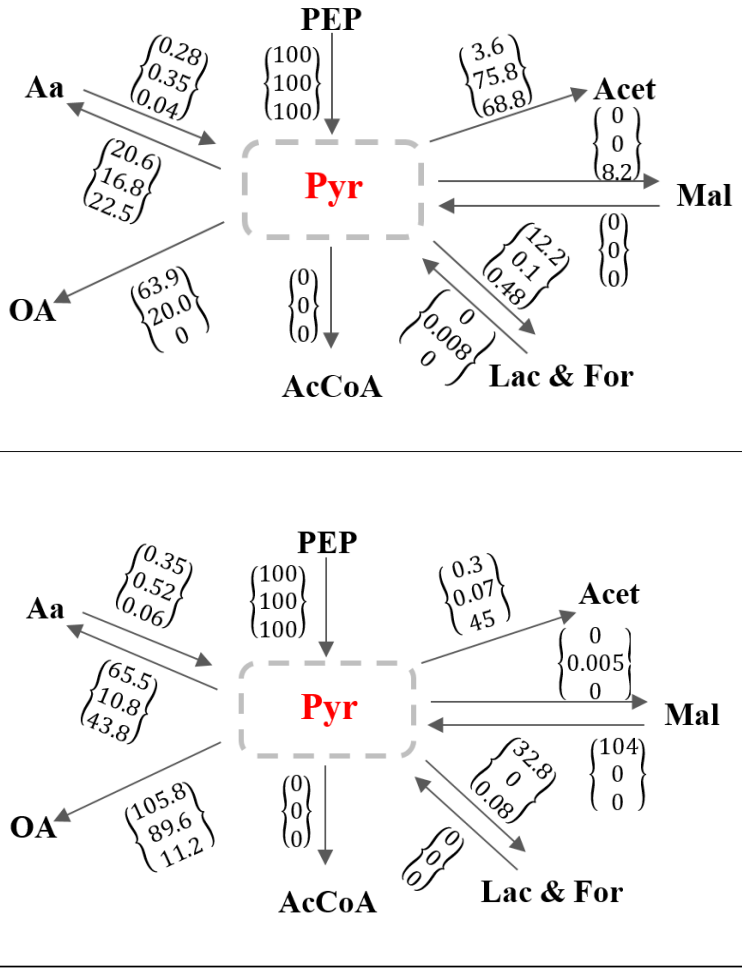


Figure 4.34 The normalized fluxes around Pyr node throughout rhGH production in pilot scale bioreactor experiments of the strains pPDCZαA*::hGH* (BR-1), pGAPZαA*::hGH* (BR-2), GAP-PDC*::hGH* (BR-3) at C_{DO}=5% and GAP-PDC*::hGH* (BR-4) at C_{DO}=15%. From top to bottom, the numbers represent the period I, period II, and period III, respectively.

CHAPTER 5

CONCLUSION

Within the scope of this M.Sc. thesis, simultaneous *hGH* expression under two different constitutive promoters namely, glyceraldehyde-3-phosphate dehydrogenase (GAP) and pyruvate decarboxylase (PDC), was performed to achieve higher rhGH production by the yeast *Pichia pastoris*.

For this purpose, the research was started with the amplification of DNA fragment containing P_{PDC} promoter region, α-MF1 secretion signal and *hGH* gene from the previously constructed pPDCZαA*::hGH* plasmid. By utilizing the metabolic engineering techniques, the DNA fragment was inserted into the shuttle vector pPIC3.5K, by deleting the DNA region including P_{AOX1} and multiple cloning site of the plasmid. The plasmid pPDC3.5Kα*::hGH* was subsequently transfected into the genome of r-*P. pastoris* which was priorly cloned by pGAPZαA*::hGH* plasmid involving single copy integrant of *hGH*. In order to select the single copy integrant of *hGH* which was to be expressed under P_{PDC}, qRT-PCR experiments were performed in duplicate and the chosen strain was named as GAP-PDC*::hGH*. Thereafter, the double rhGH expression of GAP-PDC*::hGH* was investigated and compared with the single copy expression by pGAPZαA*::hGH*/X-33 strain in air filtered shake bioreactors. By keeping pH, dissolved oxygen, and temperature constant with a pre-determined glucose feeding rate, rhGH production and cell growth were investigated for pPDCZαA*::hGH*/X-33, pGAPZαA*::hGH*/X-33, and GAP-PDC*::hGH* strains in limited-oxygen conditions in pilot scale bioreactor experiments. Then, effects of oxygen on rhGH production was studied for double

promoter expression system at oxygen-limited and at higher oxygen transfer conditions.

The findings attained with the thesis were listed below:

- pGAPZαA*::hGH*/X-33 was utilized as parent strain which includes single copy integrant of *hGH* gene and the constituted 9236 bp pPDC3.5Kα*::hGH* plasmid was linearized by SalI to transfect into HIS4 locus of that r-*P. pastoris* genome.
- In order to find single copy of hGH to be expressed under P_{PDC}, qRT-PCR experiments were performed by utilizing pGAPZαA*::hGH*/X-33 as control. During the experiments the colony that contain approximately two-fold higher copy number than pGAPZαA*::hGH*/X-33 was determined and the strain named as GAP-PDC*::hGH*.
- pGAPZαA*::hGH*/X-33 and GAP-PDC*::hGH* were compared in shake flask bioreactor experiment. Subsequently, pilot scale bioreactor experiments were performed to compare the rhGH productions at C_{DO}=5%.
- The highest cell concentration achieved during cultivations was at t=15 h as 64 g L^{-1}, at t=18 h 63 g L^{-1}, at =18 h as 77 g L^{-1} for the expressions under P_{PDC}, P_{GAP}, and double promoters, respectively.
- The maximum rhGH secretion was accomplished during fermentations was at t=15 h as 80 mg L^{-1}, at t=15 h 107 mg L^{-1}, at =18 h as 179 mg L^{-1} for the expressions under P_{PDC}, P_{GAP}, and double promoters, respectively.
- When C_{DO}=5% was enhanced to C_{DO}=15% cell concentration increased from 77 g L^{-1} to 104 g L^{-1} at t=18 h of production and in double promoter expression system.
- The highest rhGH expression was achieved as 429 mg L^{-1} at t=18 h of fermentation which was almost 5-fold higher expression than P_{PDC}, 4-fold higher expression than P_{GAP}, and approximately 2.4-fold higher expression than double promoters under limited-oxygen conditions.
- Due to reduced oxygen conditions ethanol concentrations were higher in the first three pilot scale bioreactor experiments and far fewer in enhanced

oxygen condition with the maximum amount of 16.5 g L^{-1} under P_{GAP} driven expression system.

In order to gain insight into physiology of the cells in low-oxygen conditions metabolic flux analysis (MFA) was performed:

- MFA revealed that both in limited- and low-oxygen conditions, a downtrend in biomass synthesis flux was observed with the cultivation time. Moreover, ATP generation fluxes attenuated from period I to peiod II under P_{PDC} and P_{GAP} driven systems whereas, ATP generation fluxes enhanced in double promoter expression systems in the process of time.
- Fluxes towards glycolysis pathway decreased from period I to period II indicating an adaptation to reduced oxygen conditions and correspondingly, conformance in TCA cycle fluxes. Therefore, most of the ATP was produced via oxidative phosphorylation reactions (in TCA cycle). Besides, almost stable fluxes towards glycolysis pathway in double promoter expression system at C_{DO}=15 % yielded approximately constant ATP generation throughout the cultivation.
- Although rhGH synthesis fluxes when produced under P_{PDC} in limited-oxygen conditions, and when produced under double promoters in low-oxygen conditions showed a similar trend with the fluxes towards the gene pyruvate decarboxylase and both of the genes glyceraldehyde-3-phosphate dehydrogenase and pyruvate decarboxylase, fluxes through rhGH synthesis reaction when expressed under P_{GAP} or double promoters under limited oxygen conditions had an opposite behavior. Therefore, when P_{PDC} was utilized for hGH expression keeping C_{DO} at 5%, and when double promoter expression system was used, enhancement of C_{DO} to 15% would be suitable to increase the rhGH production.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

1X TAE Agarose Gel Electrophoresis Buffer	40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
1 M Tris-Cl (pH 8.0)	Dissolve 12.1 g Tris base 80 mL dH2O and adjust the pH to 8.0 by adding concentrated HCl. Make up the volume up to 100 mL. Autoclave and store at room temperature.
0.5 M Ethylendiaminetetra acetic acit EDTA (pH:8.0)	Dissolve 18.61 g EDTA in 80 ml distilled water. Adjust pH to 8.0 and bring final volume up to 100 mL. Autoclave and store at room temperature.
TE Buffer	Mix 1 mL 1M Tris-HCl (pH 8.0) and 0.5 mL of 0.5M EDTA, and complete to 100mL with dH_2O.

Calcium chloride Transformation Solutions

$MgCl_2$-$CaCl_2$ Solution	Dissolve 8.13 g $MgCl_2.6H_2O$ with 1.1099 g $CaCl_2$ in 500 mL dH_2O. Filter-sterilize and store at +4°C.
0.1 M $CaCl_2$ Solution	Dissolve 11.1 g $CaCl_2$ in 1 L dH_2O. Filter-sterilize and store at +4°C.

Lithium Chloride Transfection Solutions

1M LiCl	Dissolve 4.24 g of LiCl in distilled water and filter sterilize. Dilute with sterile water when needed.
PEG	Dissolve 50 % polyethylene glycol (PEG-3350) in distilled water and filter sterilize. Store in tightly capped bottle.
Single-stranded DNA	2 mg/mL denaturated, fragmented salmon sperm DNA in TE (pH 8.0) buffer, store at -20°C.

Genomic DNA Isolation Solutions

Yeast Lysis Buffer for Genomic DNA Isolation	Dissolve 2 g of Triton-X 100, 1 g of SDS, 5.84 g of NaCl, 0.1 moles of Tris-Cl (pH=8.0) and 0.338 g of Na2EDTA in 1 L of dH_2O.

3 M Sodium Acetate	Add 24.6 g sodium acetate anhydrous (CH_3COONa) to 100 mL dH_2O store at room temperature.

SDS-PAGE Solutions

10%(w/v) APS (Ammonium PerSulfate)	Add 0.1g APS to 1 mL dH_2O, freshly prepared.
4X Sample Loading Buffer for SDS-PAGE	200 mM Tris-HCl, pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue. Distributed into microcentrifuge tubes and stored at -20°C.
5X SDS-PAGE Running Buffer	15 g Tris Base, 72 g glycine, 5 g SDS, dH_2O to 1 liter. The buffer can be stored at 2-8°C.
1X SDS-PAGE Running Buffer	Diluted from 5X buffer solution prior to use and can be used three times.
Fixer Solution	Mix 100 mL methanol and 24 mL acetic acid with100 µL 37% formaldehyde and complete to 250 mL with distilled water.

Pretreatment Solution	Dissolve 0.05 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 200 mL distilled water by mixing with a glass rod. Take 2 mL and set aside for further use in developing solution preparation.
Silver Nitrate Solution	Dissolve 0.2 g silver nitrate in 100 mL distilled water and add 75 µL 37% formaldehyde.
Developing Solution	Dissolve 2.25 g potassium carbonate in 100 mL distilled water. Add 2 mL from pretreatment solution and 75 µL 37% formaldehyde.
Stop Solution	Mix 50 mL methanol with 12 mL acetic acid and complete to 100 mL with distilled water.

APPENDIX B

THERMODYNAMIC PROPERTIES OF DESIGNED PRIMERS

OligoAnalyzer 3.1

Name :	PDC-Fw
Sequence	5'- AGA CGT CGG GAC AAG CAC ACG ATT A -3'
Complement	5'- TAA TCG TGT GCT TGT CCC GAC GTC T -3'
Length	25 bp
GC Content	52 %
Melt Temp	61.9 ºC
Molecular Weıght	7709.1 g/mole
Extınctıon Coefficıent	256600 L/(mole·cm)
nmole/OD_{260}:	3.9
µg/OD_{260}:	30.04

PDC-Fw Self-dimer

Dimer Sequence:

5'- AGACGTCGGGACAAGCACACGATTA -3'
Maximum Delta G: -48.5 kcal/mole

Delta G: -9.45 kcal/mole Base Pairs: 6
5' AGACGTCGGGACAAGCACACGATTA
||||||
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G: -5.19 kcal/mole Base Pairs: 3
5' AGACGTCGGGACAAGCACACGATTA
||| : : :::

```
3'   ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -4.95 kcal/mole  Base Pairs: 3
5'  AGACGTCGGGACAAGCACACGATTA
   :  |||  :     :  :::  :
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.61 kcal/mole  Base Pairs: 2
5'              AGACGTCGGGACAAGCACACGATTA
                 || ::
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.61 kcal/mole  Base Pairs: 2
5'           AGACGTCGGGACAAGCACACGATTA
                 ||
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.61 kcal/mole  Base Pairs: 2
5' AGACGTCGGGACAAGCACACGATTA
                :  ||  :
3'              ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.14 kcal/mole  Base Pairs: 2
5' AGACGTCGGGACAAGCACACGATTA
       :       ||       :
3'     ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -2.92 kcal/mole  Base Pairs: 3
5'         AGACGTCGGGACAAGCACACGATTA
             |||  :::
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.94 kcal/mole  Base Pairs: 2
5' AGACGTCGGGACAAGCACACGATTA
            || :    : ::
3'          ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.47 kcal/mole  Base Pairs: 2
5' AGACGTCGGGACAAGCACACGATTA
                   ||
3'                 ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.34 kcal/mole  Base Pairs: 2
5'    AGACGTCGGGACAAGCACACGATTA
         ||         ::
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -0.96 kcal/mole  Base Pairs: 2
5' AGACGTCGGGACAAGCACACGATTA
                    ||
3'                   ATTAGCACACGAACAGGGCTGCAGA
```

Name :	hGH-Rv
Sequence	5'- CAT ACC GGT CTA GAA GCC ACA GCT G -3'
Complement	5'- CAG CTG TGG CTT CTA GAC CGG TAT G -3'
Length	25 bp
GC Content	56 %
Melt Temp	60.8 ºC
Molecular Weight	7636 g/mole
Extinction Coefficient	240900 L/(mole·cm)
nmole/OD_{260}:	4.15
µg/OD_{260}:	31.7

hGH-Rv Self-dimer

```
Dimer Sequence:
5'- CATACCGGTCTAGAAGCCACAGCTG -3'
Maximum Delta G: -47.42 kcal/mole

Delta G:  -12.43 kcal/mole  Base Pairs: 6
5'            CATACCGGTCTAGAAGCCACAGCTG
              : |||||| :
3' GTCGACACCGAAGATCTGGCCATAC

Delta G:  -10.24 kcal/mole  Base Pairs: 6
5' CATACCGGTCTAGAAGCCACAGCTG
                ||||||
3'               GTCGACACCGAAGATCTGGCCATAC

Delta G:  -7.31 kcal/mole  Base Pairs: 6
5'   CATACCGGTCTAGAAGCCACAGCTG
    :      ||||||       :
3' GTCGACACCGAAGATCTGGCCATAC

Delta G:  -4.74 kcal/mole  Base Pairs: 3
5' CATACCGGTCTAGAAGCCACAGCTG
             |||   :::
3'           GTCGACACCGAAGATCTGGCCATAC

Delta G:  -3.14 kcal/mole  Base Pairs: 2
5' CATACCGGTCTAGAAGCCACAGCTG
           : ||  :
3'       GTCGACACCGAAGATCTGGCCATAC

Delta G:  -3.07 kcal/mole  Base Pairs: 2
```

```
5'  CATACCGGTCTAGAAGCCACAGCTG
        ||  :  :  ::
3' GTCGACACCGAAGATCTGGCCATAC

Delta G:  -1.95 kcal/mole  Base Pairs: 2
5' CATACCGGTCTAGAAGCCACAGCTG
  ||    : ::   :: :    ::
3' GTCGACACCGAAGATCTGGCCATAC

Delta G:  -1.95 kcal/mole  Base Pairs: 2
5' CATACCGGTCTAGAAGCCACAGCTG
               ||   ::
3'              GTCGACACCGAAGATCTGGCCATAC

Delta G:  -1.6 kcal/mole  Base Pairs: 2
5' CATACCGGTCTAGAAGCCACAGCTG
         ||       ::
3'      GTCGACACCGAAGATCTGGCCATAC

Delta G:  -1.6 kcal/mole  Base Pairs: 2
5' CATACCGGTCTAGAAGCCACAGCTG
           ||  :   :  ::
3'         GTCGACACCGAAGATCTGGCCATAC

Delta G:  -1.47 kcal/mole  Base Pairs: 2
5'                 CATACCGGTCTAGAAGCCACAGCTG
                   ||
3' GTCGACACCGAAGATCTGGCCATAC

Delta G:  -1.34 kcal/mole  Base Pairs: 2
5' CATACCGGTCTAGAAGCCACAGCTG
    : : ||       :: : :
3'   GTCGACACCGAAGATCTGGCCATAC

Delta G:  -0.96 kcal/mole  Base Pairs: 2
5'                 CATACCGGTCTAGAAGCCACAGCTG
                   ||
3' GTCGACACCGAAGATCTGGCCATAC

Delta G:  -0.96 kcal/mole  Base Pairs: 2
5'          CATACCGGTCTAGAAGCCACAGCTG
            ||     ::
3' GTCGACACCGAAGATCTGGCCATAC
```

PDC-Fw-hGH-Rv Hetero-Dimer

```
Primary Sequence:  5'- AGACGTCGGGACAAGCACACGATTA -3'

Secondary Sequence:  5'- CATACCGGTCTAGAAGCCACAGCTG -3'

Maximum Delta G:  -48.5 kcal/mole

Delta G:  -6.68 kcal/mole   Base Pairs3

5'           CATACCGGTCTAGAAGCCACAGCTG
              : |||   :
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -4.74 kcal/mole   Base Pairs3
5' CATACCGGTCTAGAAGCCACAGCTG
             :: :    |||
3'           ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -4.52 kcal/mole   Base Pairs4
5'            CATACCGGTCTAGAAGCCACAGCTG
                  ||||
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.61 kcal/mole   Base Pairs2
5'             CATACCGGTCTAGAAGCCACAGCTG
               :    ||
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.61 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
    : ||       :: :  :
3'  ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.14 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
              ||    : :
3'      ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.07 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
   : : ::      ||   :
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -3.07 kcal/mole   Base Pairs2
5'          CATACCGGTCTAGAAGCCACAGCTG
              || : :
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -2.92 kcal/mole   Base Pairs3
5'      CATACCGGTCTAGAAGCCACAGCTG
         : :  |||  :: : :
```

```
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.95 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
             :       ||
3'          ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.95 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
                :   ||
3'             ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.95 kcal/mole   Base Pairs2

5' CATACCGGTCTAGAAGCCACAGCTG
                :   ||
3'              ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.6 kcal/mole   Base Pairs2
5'  CATACCGGTCTAGAAGCCACAGCTG
    ::   :  || :   : ::  ::
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.57 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
         ||  :        :
3'     ATTAGCACACGAACAGGGCTGCAGA

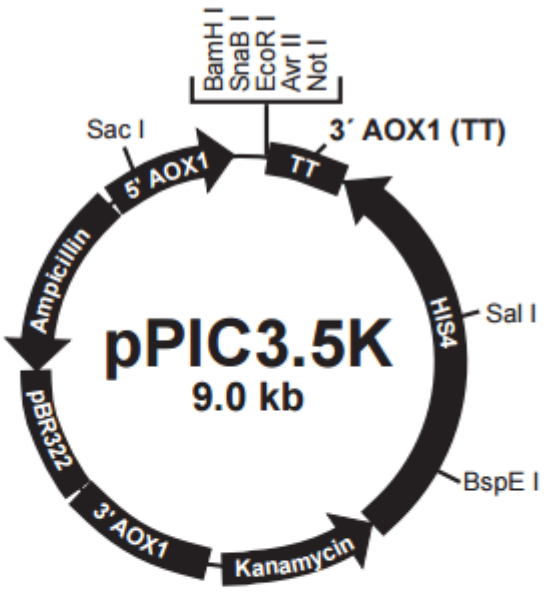
Delta G:  -1.34 kcal/mole   Base Pairs2
5'              CATACCGGTCTAGAAGCCACAGCTG
               :  ||   :
3' ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -1.34 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
    ::   || :    : : :  :
3'   ATTAGCACACGAACAGGGCTGCAGA

Delta G:  -0.96 kcal/mole   Base Pairs2
5' CATACCGGTCTAGAAGCCACAGCTG
           ||   :
3'          ATTAGCACACGAACAGGGCTGCAGA
```

APPENDIX C

NUCLEOTIDE SEQUENCES AND PLASMIDS



Comments for pPIC3.5K:
9004 nucleotides

5´ *AOX1* promoter fragment: bases 1-937
5´ *AOX1* primer site: bases 855-875
Multiple Cloning Site: bases 938-968
3´ *AOX1* primer site: bases 1055-1075
3´ *AOX1* transcription termination (TT):
bases 981-1314
HIS4 ORF: bases 4242-1708
Kanamycin resistance gene: bases 5471-4656
3´ *AOX1* fragment: bases 5850-6607
pBR322 origin: bases 7689-7016
Ampicillin resistance gene: bases 8694-7834

Figure C.1 Vector map of pPIC3.5K vector taken from Invitrogen.

Sequence of pPIC3.5K vector composed of 9004 nucleotides

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAG
GTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGT
TGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACC
AGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTA
ACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTA
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ACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGA
CAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAGGATCCTACGTAGAATTCCCTAG
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CGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAG
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CCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACGTTCGTTTGTGCAAGCTTATCGAT
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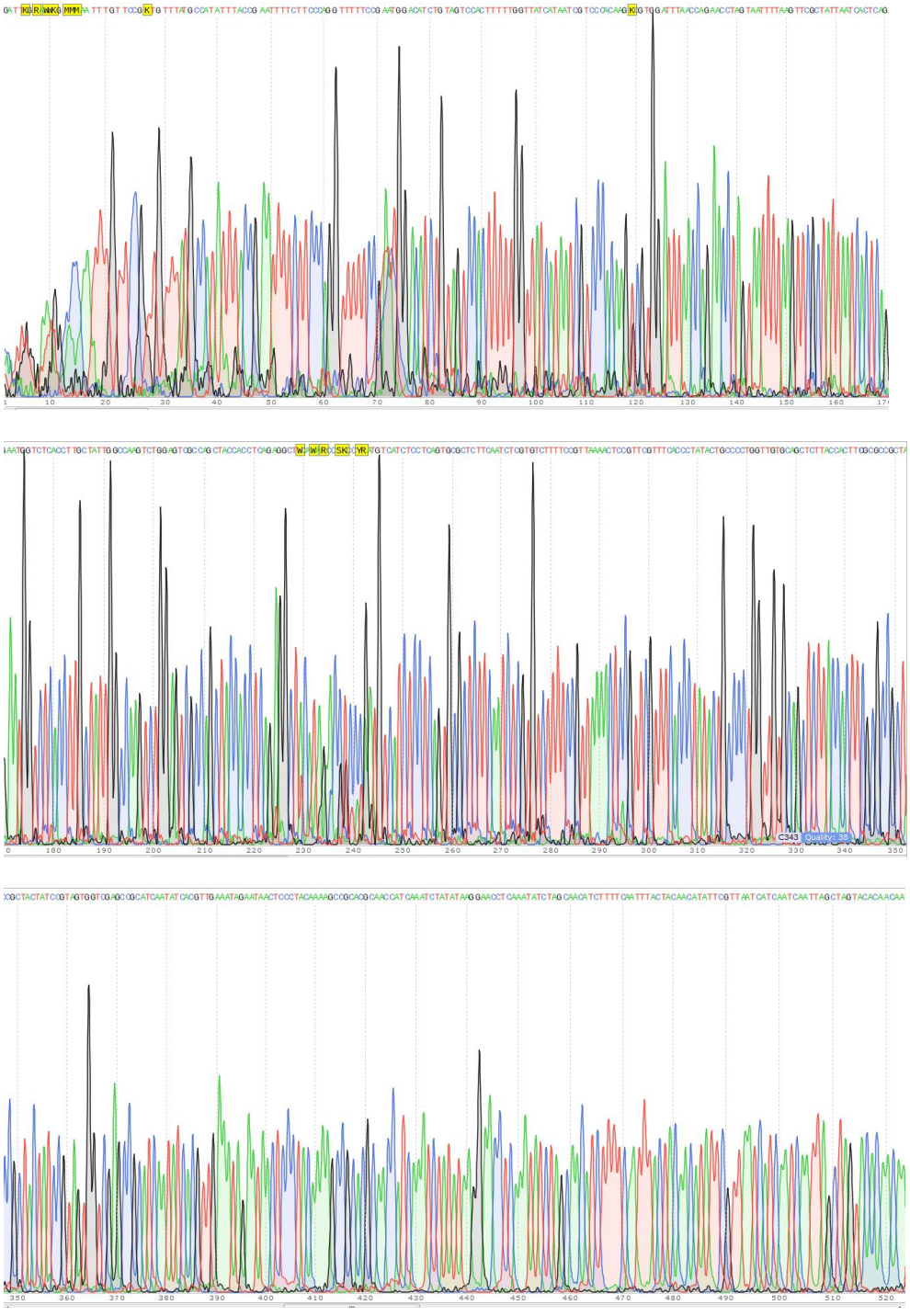
Figure C.2 Nucleotide sequence of pPIC3.5K vector.



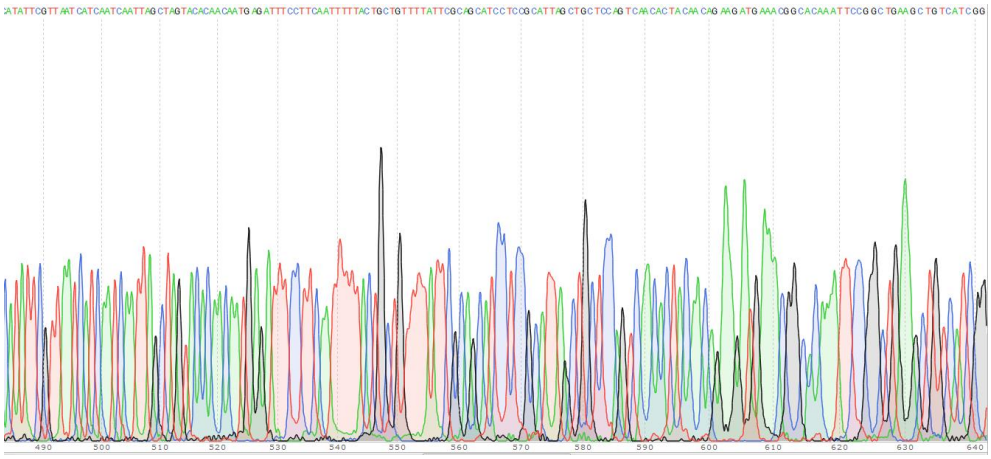


Figure C.3 Nucleotide sequence from the first forward reading.

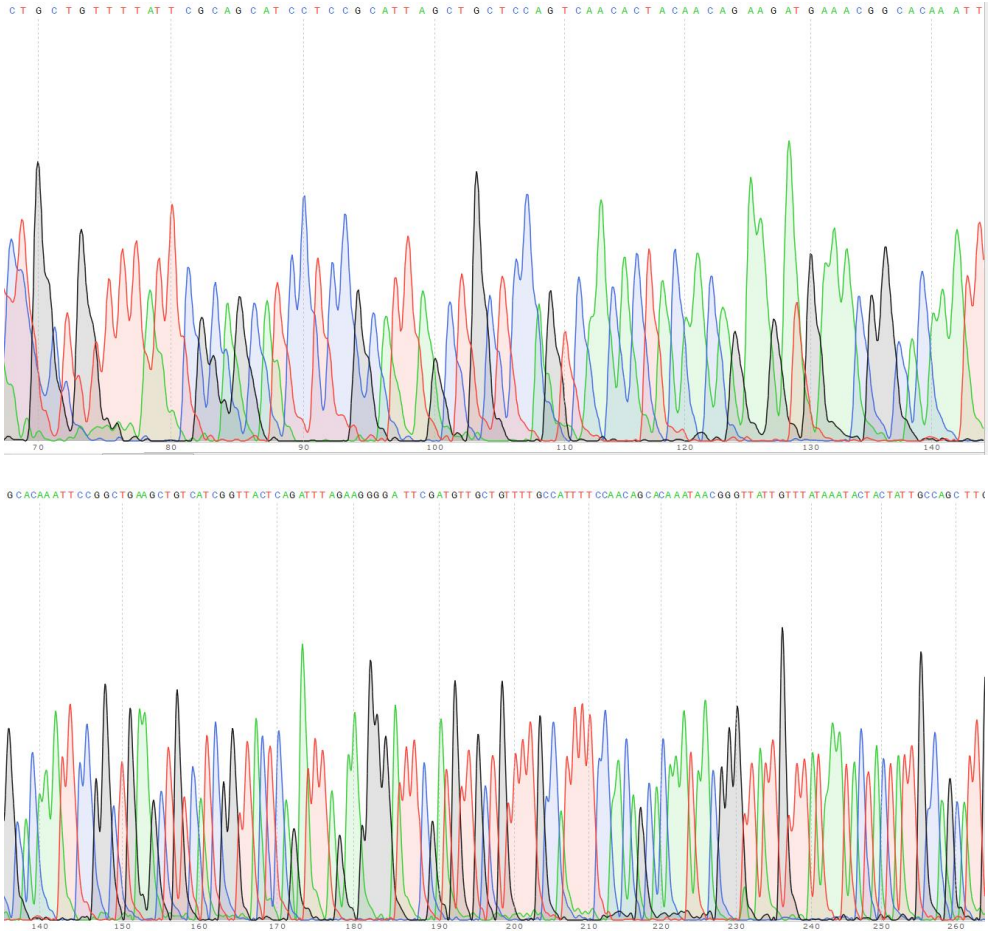


Figure C.4 Nucleotide sequence from the second forward reading

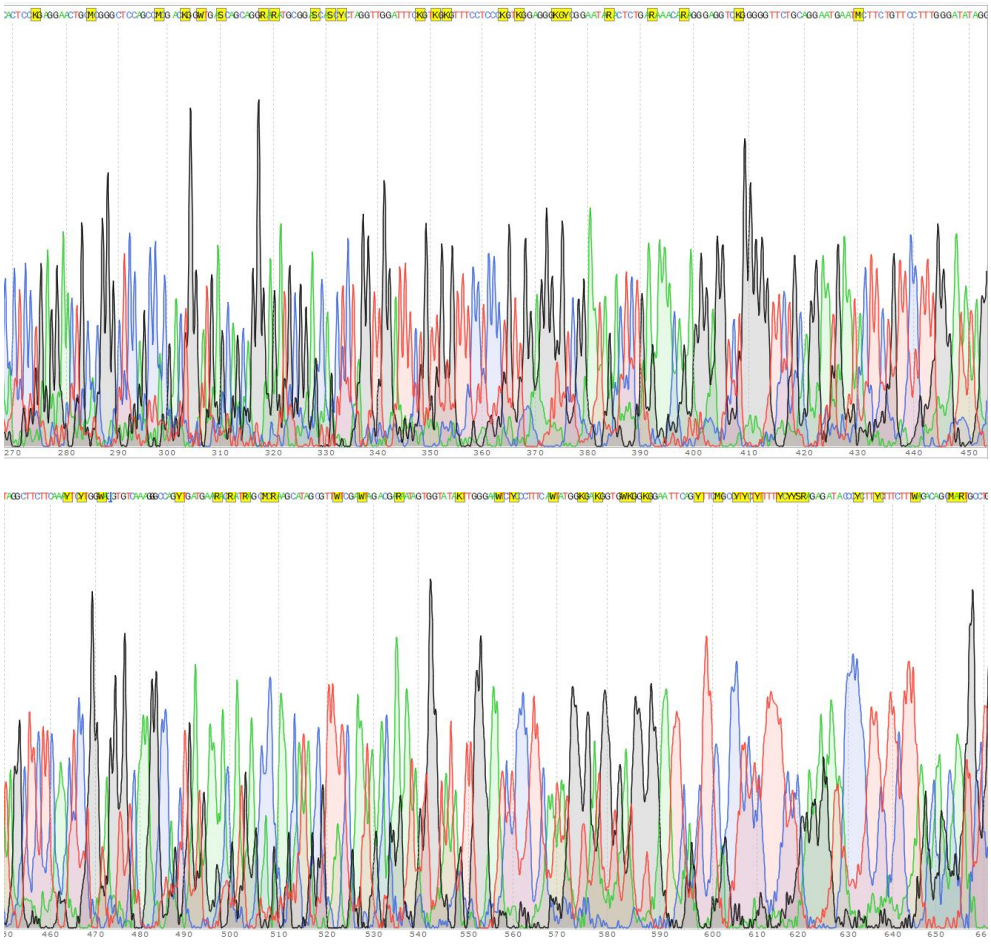


Figure C.5 Nucleotide sequence from reverse reading

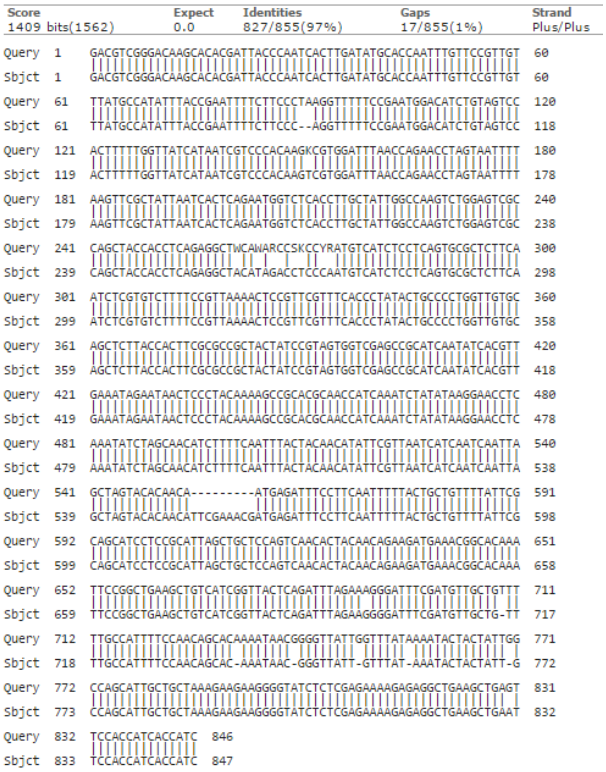


Figure C.6 Results of the BLAST for the first reading by NCBI (BLAST.NCBI.NLM.NIH.GOV/BLAST).

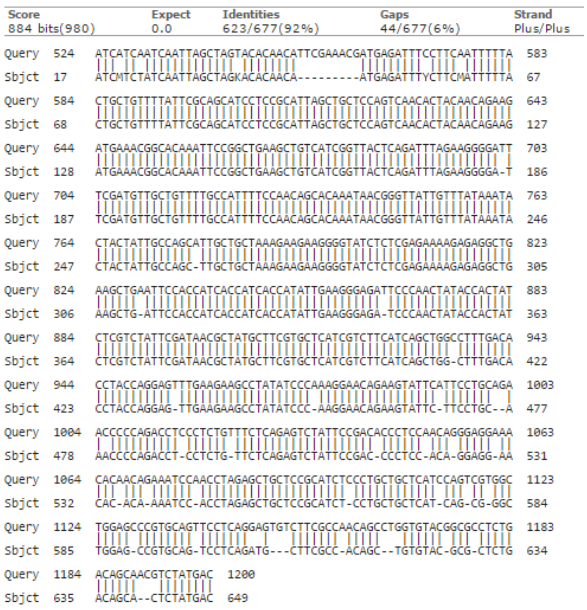


Figure C.7 Results of the BLAST for the second reading by NCBI (BLAST.NCBI.NLM.NIH.GOV/BLAST).

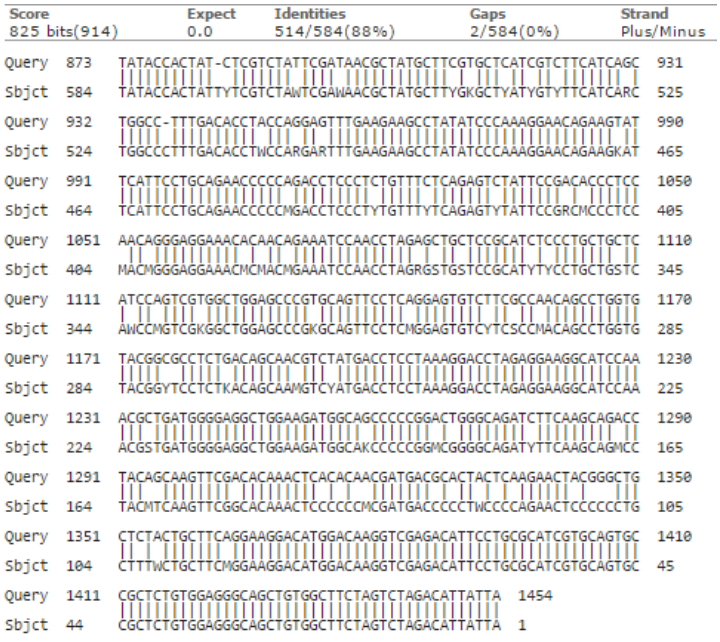


Figure C.8 Results of the BLAST for the reverse reading by NCBI (BLAST.NCBI.NLM.NIH.GOV/BLAST).

Sequence of pPDC3.5K*α::hGH* vector composed of 9236 nucleotides

*GACGTCGGGACAAGCACACGATTA*CCCAATCACTTGATATGCACCAATTTGTTCCGTTGT
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Figure C.9 Nucleotide sequence of constructed pPDC3.5Kα*::hGH* plasmid (9236 bp); nucleotides written in italics represent PDC forward and hGH reverse primers recognition sites, underlined lettets represent restriction enzyme recognition sites, nucleotides written in lower case letters represent mature hGH gene.

APPENDIX D

MOLECULAR WEIGHT MARKERS

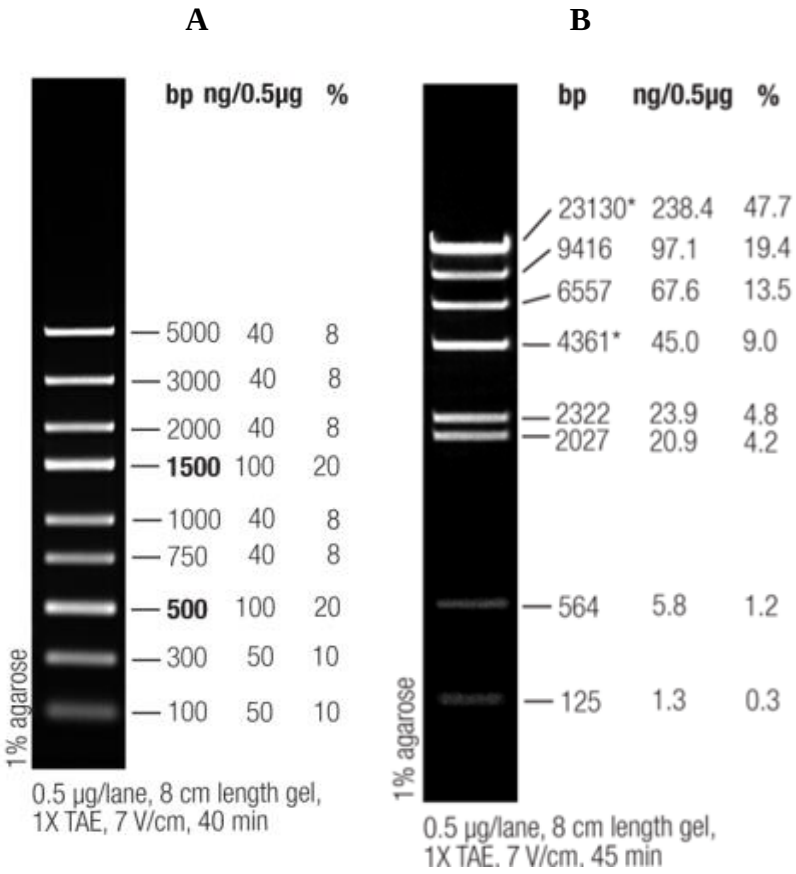

Figure D.1 Molecular weight markers utilized during agarose gel electrophoresis. A: GeneRuler^{TM} Express DNA Ladder, ready-to-use (Fermentas), B: Lambda DNA/HindIII Marker (Fermentas).

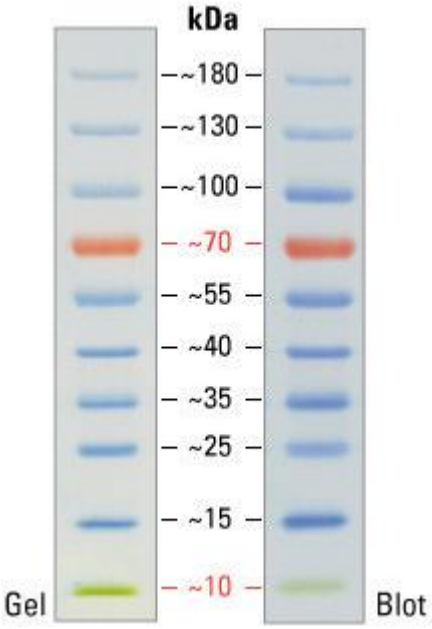

Figure D.2 SDS-PAGE band profile of the PageRulerTM Prestained Protein Ladder (Fermentas).

APPENDIX E

CALIBRATION CURVES

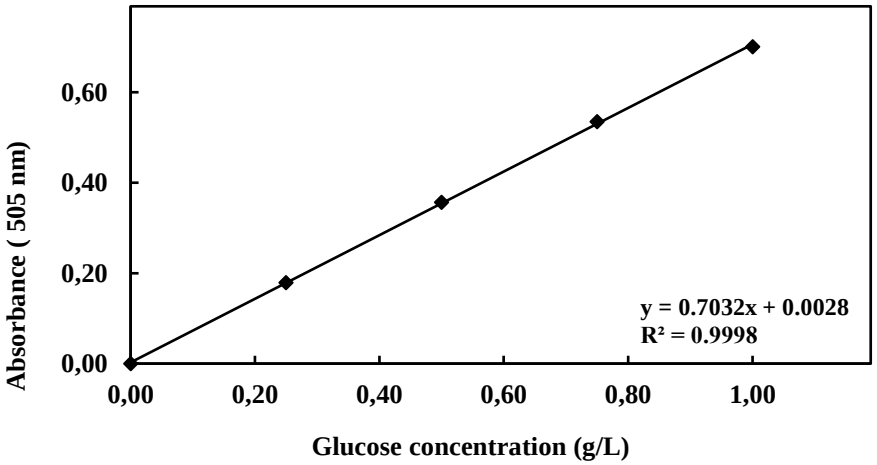

Figure E.1 Glucose standard curve used in residual glucose concentration measurement in pilot scale bioreactor experiments.

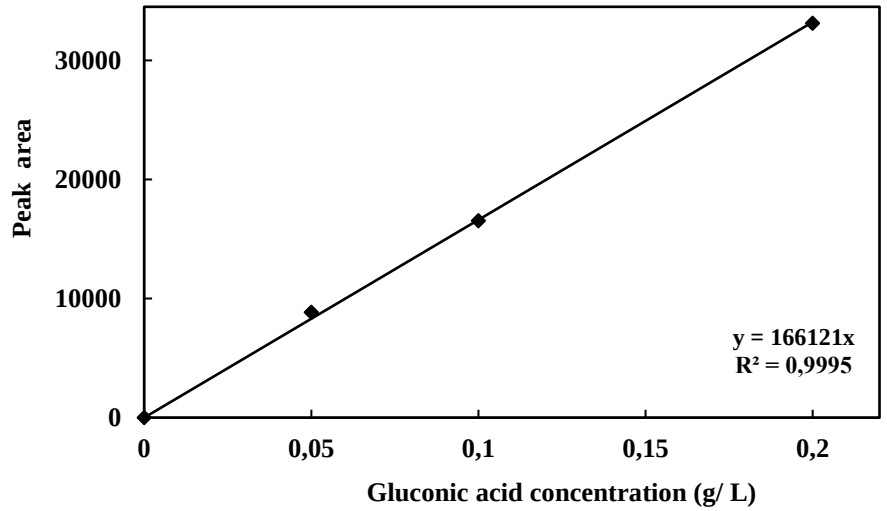

Figure E.2 Gluconic acid standard curve used in HPLC analysis.

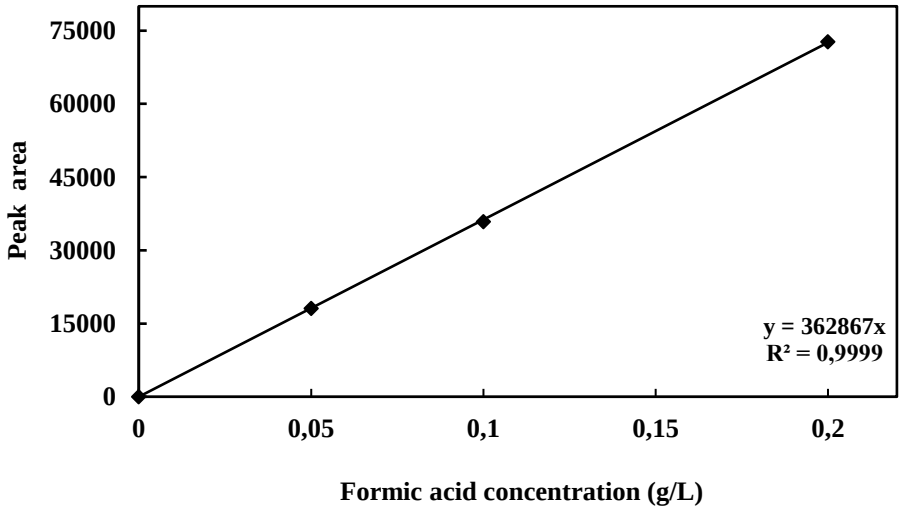

Figure E.3 Formic acid standard curve used in HPLC analysis.

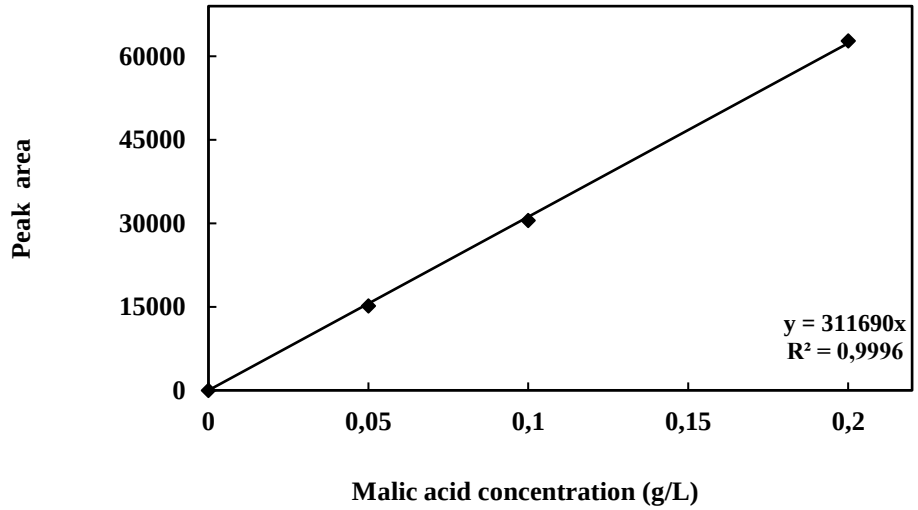

Figure E.4 Malic acid standard curve used in HPLC analysis.

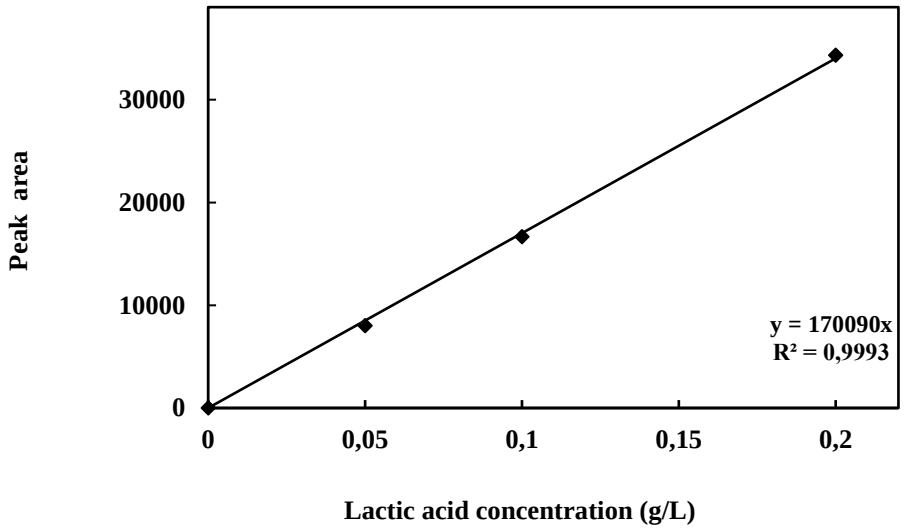

Figure E.5 Lactic acid standard curve used in HPLC analysis.

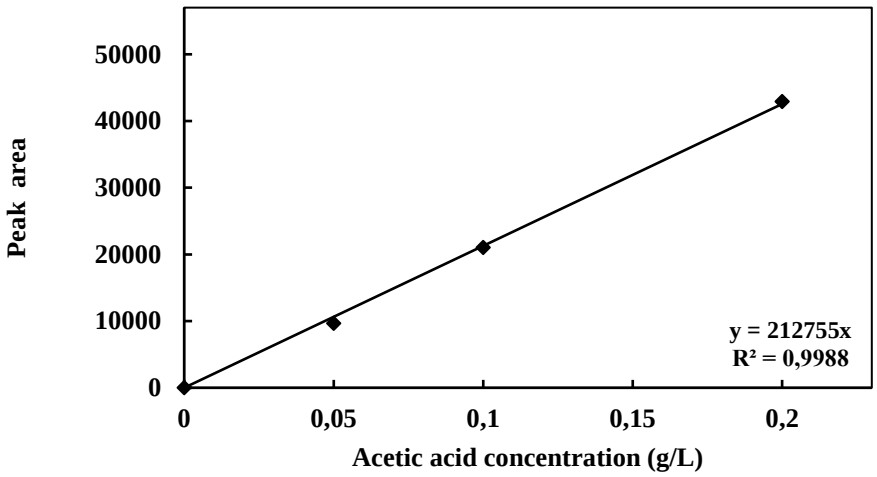

Figure E.6 Acetic acid standard curve used in HPLC analysis.

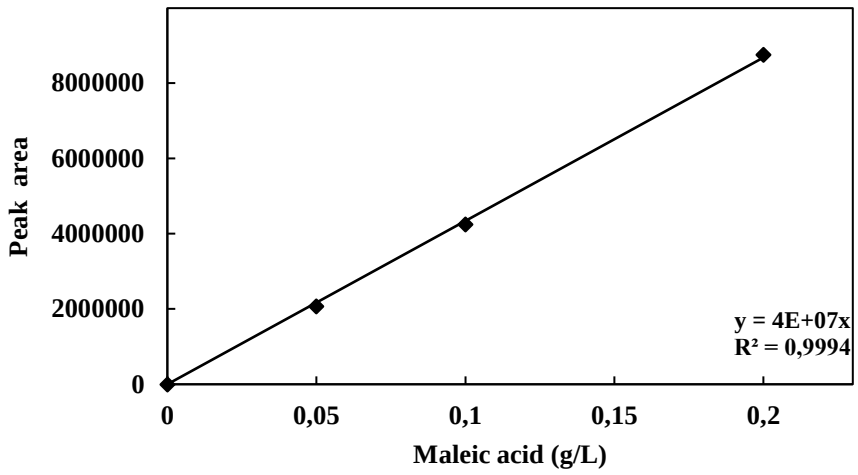

Figure E.7 Maleic acid standard curve used in HPLC analysis.

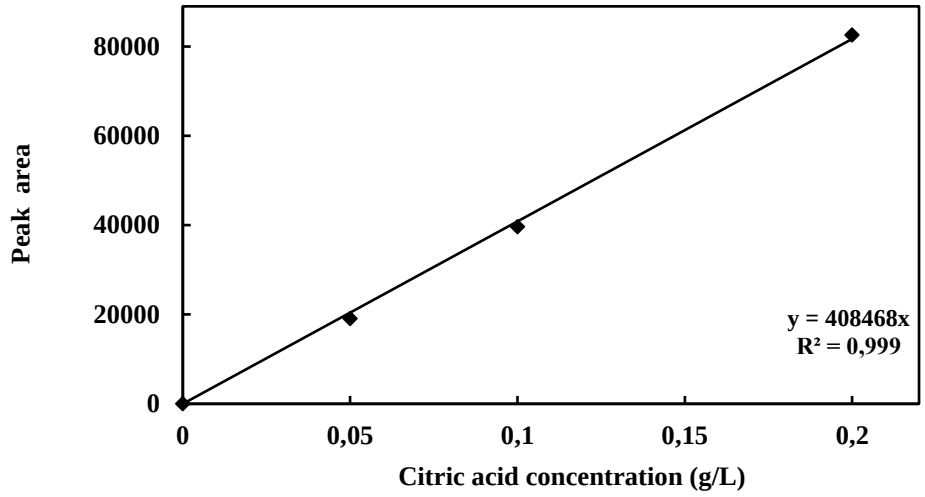

Figure E.8 Citric acid standard curve used in HPLC analysis.

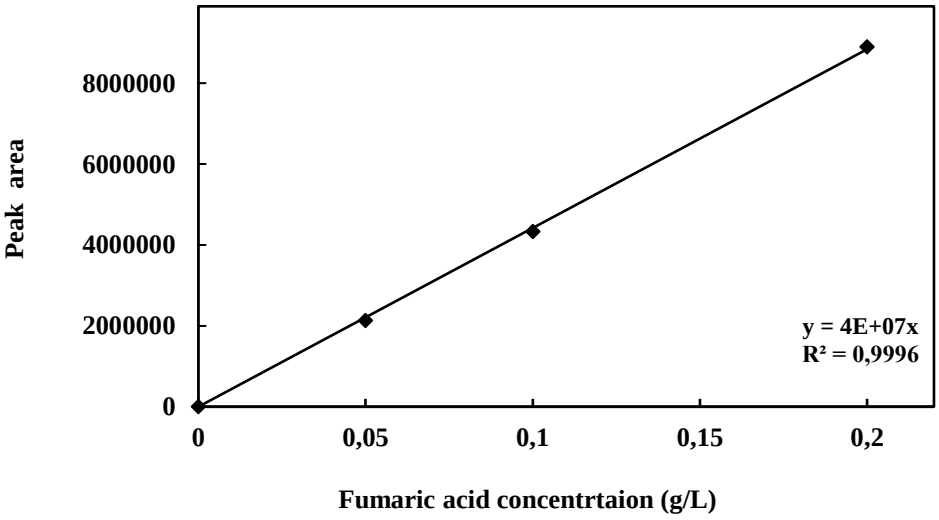

Figure E.9 Fumaric acid standard curve used in HPLC analysis.

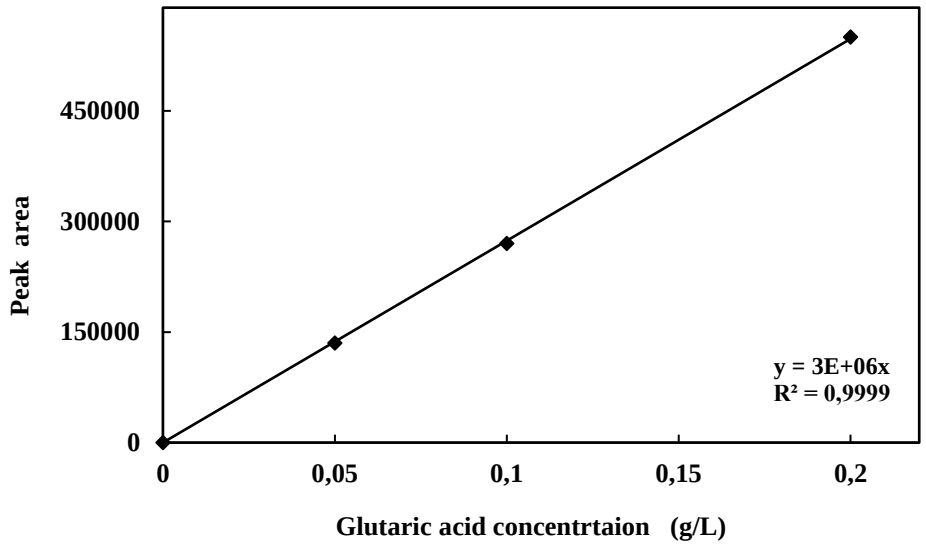

Figure E.10 Glutaric acid standard curve used in HPLC analysis.

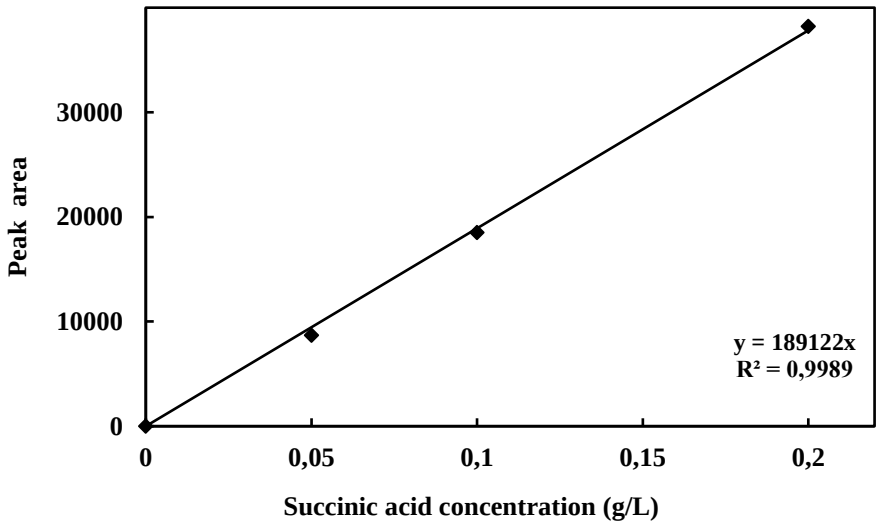

Figure E.11 Succinic acid standard curve used in HPLC analysis.

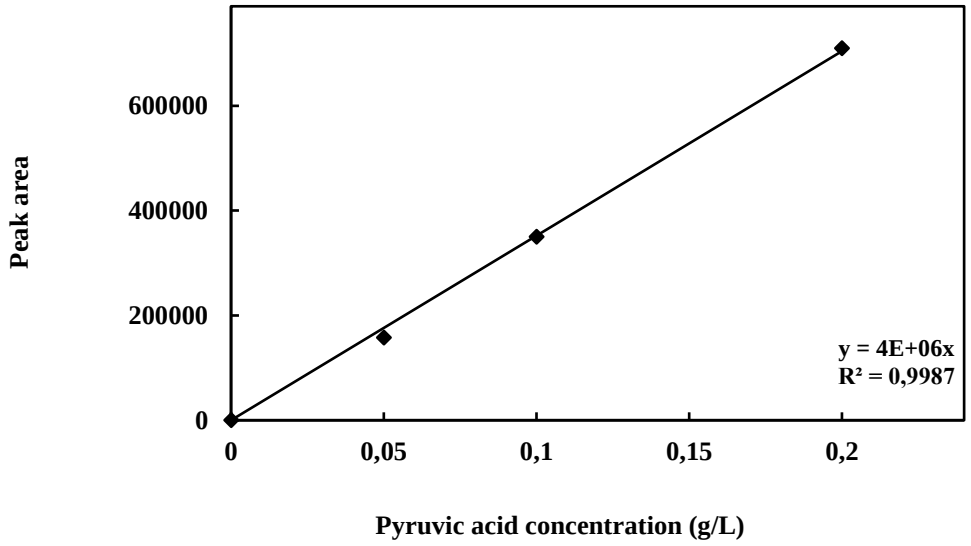

Figure E.12 Pyruvic acid standard curve used in HPLC analysis.

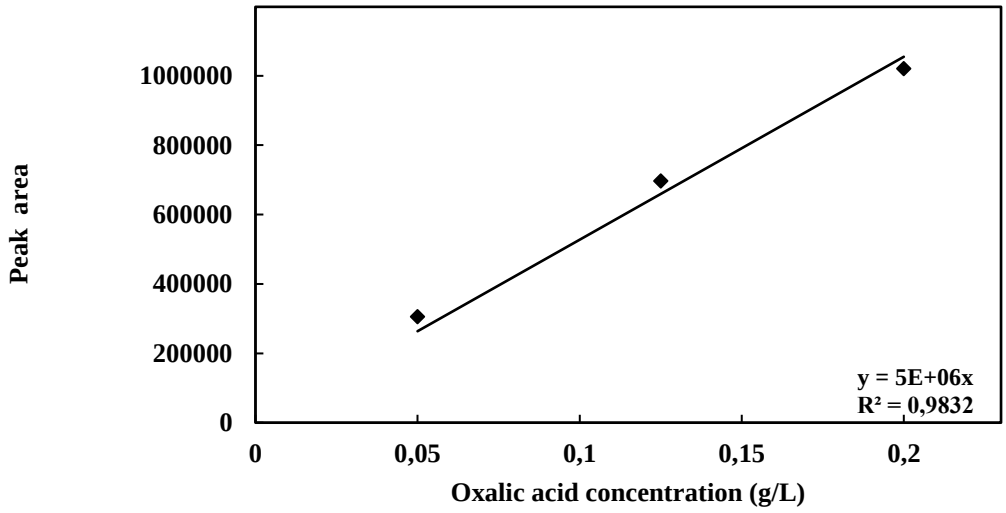

Figure E.13 Oxalic acid standard curve used in HPLC analysis.

APPENDIX F

Metabolic reactions for *P. pastoris* used in metabolic flux analysis

MeOH Metabolism

1. MeOH → FormAl
2. FormAl → For + NADH
3. For → NADH + CO_2
4. Xyl5P + FormAl + ATP → ADP + 2 G3P

Sorbitol Metabolism

5. Sorb + ATP – ADP + F6P +NADH

Glycolysis and Gluconeogenesis Pathway

6. Glc + ATP → G6P + ADP
7. F6P → G6P
8. G6P → F6P
9. F6P + ATP → 2 G3P + ADP
10. 2 G3P → F6P + Pi
11. G3P + ADP + Pi → 3PG + ATP + NADH

12. 3PG + ATP + NADH $\rightarrow$ G3P + ADP + Pi

13. 3PG $\rightarrow$ PEP

14. PEP $\rightarrow$ 3PG

15. PEP + ADP $\rightarrow$ Pyr + ATP

16. Pyr $\rightarrow$ $AcCoA_m + CO_2 + NADH_m$

Pentose Phosphate Pathway

17. G6P $\rightarrow$ R5P + 2 NADPH + CO_2

18. R5P $\rightarrow$ Xyl5P

19. Xyl5P $\rightarrow$ R5P

20. R5P $\rightarrow$ Rib5P

21. Rib5P $\rightarrow$ R5P

22. Xyl5P + Rib5P $\rightarrow$ S7P + G3P

23. S7P + G3P $\rightarrow$ Xyl5P + Rib5P

24. Xyl5P + E4P $\rightarrow$ F6P + G3P

25. F6P + G3P $\rightarrow$ Xyl5P + E4P

26. G3P +S7P $\rightarrow$ F6P + E4P

27. F6P + E4P $\rightarrow$ G3P +S7P

Branches from Glycolysis Pathway

28. Pyr $\rightarrow$ Acet + CO_2

29. Acet $\rightarrow$ Ac + NADPH

30. Acet + NADH $\rightarrow$ EtOH

31. EtOH $\rightarrow$ Acet + NADH

32. Ac + 2 ATP – AcCoA + 2 ADP + 2Pi

33. AcCoA $\rightarrow$ $AcCoA_m$

34. Pyr + NADH $\rightarrow$ Lac

35. Lac $\rightarrow$ NADH + Pyr

36. Pyr $\rightarrow$ For + AcCoA

37. For + AcCoA $\rightarrow$ Pyr

Anaplerotic Reactions

38. Mal $\rightarrow$ Pyr + CO_2 + NADPH

39. Pyr + CO_2 + NADPH $\rightarrow$ Mal

40. Pyr + CO_2 + ATP $\rightarrow$ OA + ADP

41. OA + ATP $\rightarrow$ PEP + ADP + CO_2

TCA cycle

42. $AcCoAm + OA \rightarrow Cit$

43. $Cit \rightarrow ICit$

44. $ICit \rightarrow \alpha KG + CO_2 + NADH_m$

45. $\alpha KG \rightarrow SucCoA + CO_2 + NADH_m$

46. $SucCoA + Pi + ADP \rightarrow Suc + ATP$

47. $Suc + ATP \rightarrow SucCoA + ADP + Pi$

48. $Suc \rightarrow Fum + FADH_2$

49. $Fum + FADH_2 \rightarrow Suc$

50. $Fum \rightarrow Mal$

51. $Mal \rightarrow Fum$

52. $Mal \rightarrow OA + NADH_m$

53. $NADH_m + OA \rightarrow Mal$

Biosynthesis of Serine Family Amino Acids

54. $3PG + Glu \rightarrow Ser + \alpha KG + NADH + Pi$

55. $Ser + THF \rightarrow Gly + MetTHF$

56. $Ser + AcCoA + H_2S \rightarrow Cys$

Biosynthesis of Alanine Family Amino Acids

57. Pyr + Glu $\rightarrow$ Ala + αKG

58. 2 Pyr + NADPH $\rightarrow$ Kval + CO_2

59. Kval + Glu $\rightarrow$ Val + αKG

60. Kval + $AcCoA_m$ + Glu $\rightarrow$ Leu + αKG + NADH + CO_2

Biosynthesis of Histidine

61. R5P + ATP $\rightarrow$ PRPP + AMP

62. PRPP + ATP + Gln $\rightarrow$ His + PRAIC + αKG + 2 PPi + 2 NADH + Pi

Biosynthesis of Aspartic Family Amino Acids

63. OA + Glu $\rightarrow$ Asp + αKG

64. Asp + Gln + ATP $\rightarrow$ Asn + Glu + AMP + PPi

65. Asp + ATP + 2 NADPH $\rightarrow$ Hser + ADP + Pi

66. HSer + ATP $\rightarrow$ Thr + ADP + Pi

67. Thr + NADPH + Glu + Pyr $\rightarrow$ Ile + αKG + NH_4 + CO_2

68. AcCoA + HSer + H_2S + MTHF $\rightarrow$ Met + THF

Biosynthesis of Aromatic Family Amino Acids

69. 2 PEP + E4P + ATP + NADPH → Chor + ADP + 4 Pi

70. Chor + Glu → Phe + αKG + CO_2

71. Chor + Glu → Tyr + αKG + NADH + CO_2

72. Chor + Gln + PRPP + Ser → Trp + Glu + Pyr + G3P + CO_2 + PPi

Biosynthesis of Glutamic Family Amino Acids

73. αKG + NH_4 + NADPH → Glu

74. Glu + ATP + NH_4 → Gln + ADP + Pi

75. Glu + ATP + 2 NADPH → Pro + ADP + Pi

76. Gln + CO_2 + 2 ATP → CaP + Glu + 2 ADP + Pi

77. 2 Glu + $AcCoA_m$ + 4ATP + NADPH + CaP + Asp → Arg + αKG + 4ADP + Fum + 5Pi

78. 2 Glu + AcCoA + 3 ATP + 2NADPH → 2 Glu + AcCoA + 3 ATP + 2 NADPH

Catabolism of Amino Acids

79. Ala + αKG → Pyr + Glu

80. Arg + αKG + NADPH → Glu + 2 NH_4 + CO_2

81. Asn → Asp + NH_4

82. Asp + αKG + NADH → Glu + Mal

83. Cys → Pyr + NH_4+ H_2S

84. Gln → Glu + NH_4

85. Glu → NH_4 + NADH + αKG

86. Gly + MetTHF → Ser + THF

87. His +THF → Glu + F10THF + NH_4

88. Ile + αKG → Glu + $FADH_2$ + 2 NADH + CO_2 + AcCoA + SucCoA

89. Leu + αKG + ATP → Glu + $FADH_2$ + NADH + 2 AcCoA + ADP + Pi

90. Phe + NADH → Tyr

91. Pro → Glu + NADH + $FADH_2$

92. Ser → Pyr + NH_4

93. Thr → Gly + NADH + AcCoA

94. Trp + NADPH → 2 AcCoA + Ala + CO_2 + NH_4 + For + 2NADH + $FADH_2$

95. Tyr + αKG → Glu + Fum + 2 AcCoA + CO_2

96. Val + αKG + ATP → Glu + $FADH_2$ + 3 NADH + CO_2 + SucCoA

97. Lys + AcCoA + 2 αKG → 2 Glu + NADH + CO_2

Biosynthesis of Nucleotides

98. PRPP + 2 Gln + Asp + CO_2 + Gly + 4 ATP + F10THF → 2 Glu + PPi + 4 ADP + 4 Pi + THF + PRAIC + Fum

99. PRAIC + F10THF → IMP +THF

100. IMP + Gln + ATP → NADH + GMP + Glu + AMP + PPi

101. GMP + ATP → GDP + ADP

102. GDP + ADP → ADP + GTP

103. ADP + GTP → ATP + GDP

104. NADPH + ATP → dATP

105. NADPH + GDP + ATP → dGTP + ADP

106. IMP + GTP + Asp → GDP + Pi + Fum + AMP

107. AMP + ATP → 2 ADP

108. PRPP + Asp + CaP → UMP + NADH + PPi + Pi + CO_2

109. UMP + ATP → UDP + ADP

110. UDP + ATP → ADP + UTP

111. UTP + NH_4 + ATP → CTP + ADP + Pi

112. CTP + ADP → CDP + ATP

113. CDP + ATP → CTP + ADP

114. CDP + ADP → CMP + ATP

115. ATP+ NADPH + CDP → dCTP + ADP

116. UDP + MetTHF + 3 ATP +NADPH → dTTP + DHF + 3 ADP + Ppi + Pi

Biosynthesis and Interconversion of One-carbon Units

117. DHF + NADPH → THF

118. Gly + THF → MetTHF + NH_4 + NADH + CO_2

119. MetTHF + NADH → MTHF

120. MetTHF → MeTHF + NADPH

121. MeTHF → F10THF

Oxidative Phosphorylation *(P/O = 2)*

122. $NADH_m$ + 2 ADP + 2 Pi → 2 ATP

123. $FADH_2$ + ADP + Pi → ATP

Transport Reactions

124. CO_2 → exp

125. imp → CO_2

126. imp → NH_4

127. NH_4 → exp

128. 2ATP + 4 NADPH → AMP + ADP + H_2S + PPi + Pi

129. PPi → 2Pi

130. imp → Pi

131. Pi → exp

132. NADH → $NADH_m$

Biosynthesis of Carbohydrate

133. ATP + G6P – ADP + 6 CARBH + 2 Pi

Biosynthesis of Lipids

134. AcCoA + ATP + CO_2 → ADP + MaCoA + Pi

135. AcCoA + 7 MaCoA + 14 NADPH → 7 CO_2 + PLM

136. NADPH + PLM + ATP → PLLM

137. AcCoA + 8 MaCoA + 16 NADPH → 8 CO_2 + STE

138. NADPH + STE + ATP → OLE

139. 1.7 OLE + 4.4 PLLM + 1.4 PLM +STE → 8.5 FA

140. 2 FA + G3P → PA

141. CTP + PA + Ser → 2 Pi + CMP + PS

142. PS → CO2 + PE

143. PE + 3ATP + 3 Met → PC + 3 AcCoA + 3 H_2S + 3 HSer + 9 Pi

144. PA + CTP + G6P → CMP + PINS

Biomass Synthesis

145. 0.459 Ala + 0.161 Arg + 0.102 Asn + 0.297 Asp + 0.007 Cys + 0.105 Gln + 0.302 Glu + 0.290 Gly + 0.066 His + 0.193 Ile + 0.296 Leu + 0.286 Lys + 0.051 Met + 0.134 Phe + 0.165 Pro + 0.185 Ser + 0.191 Thr + 0.028 Trp + 0.102 Tyr + 0.265 Val + 0.051 AMP + 0.051 GMP + 0.067 UMP + 0.05 CMP + 0.0024 dATP + 0.0016 dGTP + 0.0016 dTTP + 0.0024 dCTP + 0.0101 OLE + 0.0081 PLM +0.0263 PLLM + 0.0061 STE + 0.0006 PA + 0.005 PINS + 0.002 PS + 0.005 PE + 0.006 PC + 2.5 CARBH + 23.917 ATP → Biomass + 23.917 ADP + 23.946 Pi

Human Growth Hormone Synthesis

146. 7 Ala + 11 Arg + 9 Asn + 11 Asp + 4 Cys + 13 Gln + 14 Glu + 8 Gly + 3 His + 8 Ile + 26 Leu + 9 Lys + 3 Met + 13 Phe + 8 Pro + 18 Ser + 10 Thr + 1 Trp + 8 Tyr + 7 Val + 1168 ATP $\rightarrow$ rhGH + 1168 ADP + 1168 Pi

Maintenance Energy

147. ATP $\rightarrow$ ADP + Pi