GLUCOSE-BASED FEEDING STRATEGY DEVELOPMENT IN RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *Pichia pastoris* FOR SEMI-BATCH BIOREACTOR OPERATION

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

BY

ABDULLAH KESKİN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMICAL ENGINEERING

AUGUST 2014

Approval of the thesis:

GLUCOSE-BASED FEEDING STRATEGY DEVELOPMENT IN RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *Pichia pastoris* FOR SEMI-BATCH BIOREACTOR OPERATION

submitted by **ABDULLAH KESKİN** in partial fulfillment of the requirements for the degree of **Master of Science in Chemical Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

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

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

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

Examining Committee Members:

Prof. Dr. Halil Kalıpçılar
Chemical Engineering Dept., METU _____

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

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

Asst. Prof. Dr. Erhan Bat
Chemical Engineering Dept., METU _____

Dr. Robert W. Murdoch
Environmental Engineering Dept., METU _____

Date: 26.08.2014

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: Abdulah Keskin

Signature :

ABSTRACT

GLUCOSE-BASED FEEDING STRATEGY DEVELOPMENT
IN RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION
BY *Pichia pastoris*
FOR SEMI-BATCH BIOREACTOR OPERATION

Keskin, Abdullah

M.Sc., Department of Chemical Engineering

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

August 2014, 128 Pages

The aim of this study was to develop an effective feeding strategy to enhance recombinant human growth hormone (rhGH) production by *Pichia pastoris* Mut+ strain harboring pGAPZαA::*hGH* which is integrated to *glyceraldehyde-3-phosphate dehydrogenase* gene (*GAP)* locus of *P. pastoris*. In this context, eight semi-batch bioreactor experiments were conducted at pilot-scale; their batch phase was same in which glycerol was used as the sole carbon source. For semi-batch operation phase, eight feeding strategies were designed using exponential substrate feeding, constant substrate feeding, specific substrate consumption rate based substrate feeding, and combination of these feeding modes. The feeding strategies were designed by using three different pre-determined specific growth rates, μ_0=0.10, 0.15, and 0.175 h^{-1}, two constant volumetric flow rates of glucose, Q(t≥15 h) =12 ml h^{-1} or Q(t≥11 h) =24 ml h^{-1}, and one constant specific glucose consumption rate, q_S=0.05 g g^{-1} h^{-1}. Further,

to investigate the effects of carbon sources, glucose and pre-treated molasses were used. The best feeding strategy was the one conducted based on exponential glucose feeding with the pre-determined specific growth rate of μ_0= 0.15 h^{-1} wherein the highest rhGH was attained as C_{rhGH} = 508 mg L^{-1} at t=19 h and the cell concentration was C_X =65.4 g L^{-1}. The overall product and cell yield on total substrate were obtained as $Y'_{P/S}$ = 1.9 mg g^{-1} and $Y'_{X/S}$=0.22 g g^{-1}, respectively.

Keywords: Recombinant human growth hormone, *P. pastoris*, *GAP* promoter, semi-batch, feeding strategy.

ÖZ

Pichia pastoris ile
REKOMBİNANT İNSAN BÜYÜME HORMONU ÜRETİMİNDE
YARI-KESİKLİ BİYOREAKTÖR İŞLETİMİ İÇİN
GLUKOZ TEMELLİ BESLEME STRATEJİSİ GELİŞTİRİLMESİ

Keskin, Abdullah

Yüksek Lisans, Kimya Mühendisliği Bölümü

Tez Yöneticisi: Prof. Dr. Pınar Çalık

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

Ağustos 2014, 128 sayfa

Bu çalışmada amaç *Pichia pastoris* Mut+ hücrelerinin *gliseraldehit-3-fosfat dehidrogenaz* geni (*GAP*) bölgesine entegre edilmiş pGAPZαA::*hGH* taşıyan konak hücre ile rekombinant insan büyüme hormonu (rhGH) üretimini artıracak yarı-kesikli biyoreaktör işletimi için besleme stratejisi geliştirilmesidir. Sekiz yarı-kesikli biyoreaktör işletim stratejisi tasarlanmıştır. Tasarlanan tüm stratejilerin kesikli fazı aynıdır; gliserol tek karbon kaynağı olarak kullanılmıştır. Proseslerin yarı-kesikli fazlarında belirlenen spesifik çoğalma hızları için önceden-hesaplanan üstel- ve sabit- debide seçilen substratların sürekli beslendiği besleme stratejileriyle, özgül substrat tüketim hızı temelli besleme stratejileri tasarlanmış; ve ek olarak da bu stratejilerin kombinasyonlarından oluşan bir strateji tasarlanmıştır. Besleme stratejilerinin tasarımında üç farklı ön-görülmüş özgül çoğalma hızı, μ_0=0.10, 0.15, ve 0.175 st^{-1}; iki farklı sabit akış hızı, Q(t≥15 st) =12 ml st^{-1} veya Q(t≥11 st) = 24 ml st^{-1}, ve özgül subtrat tüketim hızı, q_S=0.05 g g^{-1} st^{-1}, kullanılmıştır. Bunlara ek

olarak, karbon kaynağı etkisini incelemek için glukoz ve ön-işlem görmüş melas kullanılmıştır. En yüksek rhGH derişimi t=19 st'te C_{rhGH} = 508 mg L^{-1} olarak μ_0=0.15 st^{-1} için önceden-hesaplanan üstel beslemeli yarı-kesikli işletim stratejisinde elde edilmiştir; t=19 st'te hücre derişimi C_X =65.4 g L^{-1} değerinde toplam substrat tüketimi üzerinden ürün ve hücre verimi $Y'_{P/S}$ = 1.9 mg g^{-1} and $Y'_{X/S}$=0.22 g g^{-1} olarak bulunmuştur.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, *P. pastoris*, *GAP* promoter, yarı-kesikli, besleme stratejisi.

To my family

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Dr. Pınar Çalık for her guidance, knowledge, support and help throughout my M. Sc. study in all the possible way. I also thank to my co-supervisor Prof. Dr. Tunçer H. Özdamar for his worthy critics and advices.

My deepest thanks are to my family for loving, encouraging, supporting and motivating me in all the possible way throughout my life.

I appreciate to my labmates in our research group at Industrial Biotechnology and Metabolic Engineering Laboratory: H. Gül Zerze, Erdem Boy, Özge Ata Akyol, Burcu Akdağ, Burcu Gökbudak, Sibel Öztürk, Hande Güneş, Aslan Massahi and Sena Yaman for their friendship, support, help and contribution in the experiments. I also want to thank to research group of Prof. Dr. Ufuk Bölükbaşı for their cooperation and friendship.

I wish to acknowledge the contributions and teaching from the faculty members of Chemical Engineering Department, METU, by lectures, seminars and laboratories during my undergraduate and graduate program. I would like to thank also to administrative and technical staff of Department of Chemical Engineering, METU for their help.

Abdullah Keskin

26.08.2014

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

NOMENCLATURE

C	Concentration	$g\ L^{-1}$
DO	Dissolved oxygen	%
N	Agitation rate	min^{-1}
Q	Volumetric flow rate	$L\ h^{-1}$
q	Specific formation or consumption rate	$g\ g^{-1}\ h^{-1}$ or $mg\ g^{-1}\ h^{-1}$
r	Formation or consumption rate	$g\ L^{-1}\ h^{-1}$
t	Cultivation (residence) time	h
T	Medium temperature	^{o}C
U	One unit of an enzymatic activity	
V	Volume of fermentation medium	L
Y	Yield	$g\ g^{-1}$ or $mg\ g^{-1}$
Y'	Overall yield	$g\ g^{-1}$ or $mg\ g^{-1}$
OUR	Oxygen uptake rate	$mol\ m^{-3}\ sec^{-1}$
OTR	Oxygen transfer rate	$mol\ m^{-3}\ sec^{-1}$

Greek Letters

ρ	Density	$g\ L^{-1}$
μ	Specific growth rate	h^{-1}
μ_0	Pre-determined specific growth rate	h^{-1}

Subscripts

0	Initial condition
O	Oxygen
p	Product
S	Substrate
X	Cell
G	Glucose
F	Fructose
pro	Protease

Abbreviations

*AOX*1	Alcohol oxidase 1
BMGY	Buffered glycerol-complex medium
BSM	Basal salt medium
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
EDTA	Ethylenedinitrilotetraacetic acid
*FLD*1	*Formaldehyde dehydrogenase 1*
GAP	*Glyceraldehyde-3-phosphate dehydrogenase*
GB	Glycerol batch phase
GFB	Glucose semi-batch phase
hGH	Human growth hormone
hGM	Human granulocyte-macrophage colony stimulating factor

HIV	Human immune deficiency virus
HPLC	High performance liquid chromatography
ICL1	*Isocitrate lyase 1*
OD	Optical density
PCR	Polymerase chain reaction
*PGK*1	*Phosphoglycerate kinase*
rhGH	Recombinant human growth hormone
r-protein	Recombinant protein
PMSF	Sufonyl fluoride
PTM1	Pichia trace minerals
tPA	Tissue plasminogen activator
YPD	Yeast Extract Peptone Dextrose

CHAPTER 1

INTRODUCTION

Microorganisms were used since the ancient times for the production of bread, cheese, and yoghurt etc. About 150 years ago Louis Pasteur noticed that microorganisms play a role in these biochemical processes. After then some important chemicals were produced by using microorganisms. This was continued by finding several bacteria with high productivity in Japan and a new bioprocess, named as "L-glutamic acid fermentation", was developed in "applied microbiology" field. This research field is then called as "microbial biotechnology" which can be described as "industrial biotechnology" at the industrial scale or when producing industrial products. Using chemical engineering background as well as biology and chemistry, biochemical engineering was born. In 1982 OECD defined biotechnology as "the application of scientific and engineering principles to the processing materials to provide goods and services". With the discovery of the DNA molecule in 1953, by R.Franklin, M. Wilkins, J. Watson, and F. Crick in England, genetic engineering has been developed and incorporated into industrial biotechnology. Production of compounds in living organisms was made possible by genetic manipulation of organisms which is done with cloning of the target gene to the genome of the organism by recombinant DNA technology (Nielsen *et al.*, 2003). Hence industrial biotechnology made it possible to produce complex compounds like hormones and enzymes in larger scale together with producing crucial biomaterials.

Food and Drug Administration (FDA) and European Medicines Agency (EMEA) have approved 165 recombinant proteins (r-proteins) for pharmaceutical use and in the following five years, it is predicted to reach 200 r-proteins (Corchero

et al. 2013). Therefore industrial biotechnology get significant attention since the biopharmaceutical industry is expected to reach a market of $182.5 billion by 2015 (www.researchandmarkets.com). The market of some biotech mammalian polypeptides are $13.1 billion for erythropoietin (EPO), $6 billion for interferon-α, $5.6 billion for human insulin, $1.8 billion for human growth hormone (hGH), $640 million for tissue plasminogen activator (tPA) (Demain, 2007).

Recombinant hGH (rhGH) is used for treating burn, injury, bleeding ulcer, bone fracture, hypopituitary dwarfism, Turner syndrome, growth hormone deficiency, chronic renal failure and human immunodeficiency virus (HIV) syndrome (Baulieu and Kelly, 1990; Binkley 1994; Trevino *et al*,. 2000). Before the production of rhGH, hGH was obtained from the pituitary glands of the cadavers restrictedly. In 1979, Goeddel *et al*. produced rhGH by *Escherichia coli* intracellularly. In the coming years rhGH was also produced by the other microorganisms *i.e. Bacillus subtilis* (Özdamar *et al*., 2009), *Saccharomyces cerevisiae* (Tokunaga *et al*., 1985) and *Pichia pastoris*; (Trevino *et al*. 2000; Çalık *et al*., 2008)

The r-proteins are mostly produced by mammalian cell hosts with a percentage of 45% and it is followed by the bacterium *E.coli* with 39% and *S.cerevisiae* with 15% (Sanchez and Demain, 2012). However in recent years, *Pichia pastoris* has turned out to be a popular and appropriate host microorganism for r-protein expression. It get interest because it can grow on a cheap, defined medium to high cell densities, secrete the products to extracellular medium which enhances separation and purification (Çelik and Çalık 2012), produce active form of the proteins which structurally and functionally overlaps with the native form (Cereghino and Cregg 2000). Several strong inducible and constitutive promoters have been identified, providing excellent tools for producing r-proteins in *P. pastoris* at high levels. *GAP* promoter is a constitutive promoter which can express r-proteins at high levels (Waterham *et al*., 1997). It is a good alternative to the strong, inducible *AOX1* promoter since it overcomes the imperfections of the inducible promoters. In *GAP*-driven expression systems there is no need for two different carbon sources and phases. Moreover, continuous operation is possible since carbon sources do not repress or depress the cell growth or r-protein production. Methanol use in the *AOX1*

promoter is also problematic due to its storage and transportation costs. Since *GAP* promoter is induced by glucose and methanol is not used in the process, it is favored over the *AOX1* promoter. Furthermore *GAP* is also a strong promoter whose performance is comparable with *AOX1* promoter (Cos *et al*., 2006).

In our research group, for producing rhGH production a novel expression system was developed in which *P.pastoris* was used as a host microorganism and *AOX1* was used as the promoter (Çalık *et al*., 2008). By using this expression system Orman *et al*. (2009) obtained 110 mg L^{-1} rhGH concentration. Açık (2009) attained 301 mg L^{-1}, İnankur (2010) 290 mg L^{-1}, Bozkurt (2012) 640mg L^{-1} and Zerze (2012) 1200 mg L^{-1} rhGH by using co-carbon sources and developing feeding strategies. In 2012, Zerze also constructed a *P. pastoris* expression system in which rhGH was produced under glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter. A 200 mg L^{-1} rhGH was reached in pilot-scale bioreactor experiments where glucose was fed exponentially with a pre-determined specific growth rate of μ_0=0.2 h^{-1} within t=0-3 h and then glucose was fed with constant flow rate of 6 g L^{-1}.

In this study, the objective was to develop a feeding strategy to produce rhGH in a *GAP*-driven *P.pastoris* expression system. To increase rhGH production, the effects of feeding strategies based on the pre-determined specific growth rates (μ_0) at μ_0=0.10, 0.15 and 0.175 h^{-1} were investigated. Moreover, a two-step feeding strategy in which at t =0- 6 h μ_0 =0.175 h^{-1} and at t ≥ 6 h μ_0 =0.15 h^{-1} was applied. Two other feeding strategies were designed in which glucose was fed first with an exponentially increased continuous volumetric flow rate based on a selected μ_0 value than constantly at Q(t≥15 h) =12 ml h^{-1} or Q(t≥11 h) =24 ml h^{-1}. Yield coefficients, consumption and production rates were calculated by using the data obtained in all the designed semi-batch bioreactor experiments to clarify the fermentation characteristics of *P. pastoris* and responses of *P. pastoris* to the designed semi-batch bioreactor continuous feeding strategies.

CHAPTER 2

LITERATURE SURVEY

For recombinant protein production, properties and structure of the protein, characteristics of the host microorganism with the selected promoter, optimum bioprocess operation parameters and bioprocess operation modes should be studied in detail. Moreover, to understand the efficiency of the bioprocess in terms of growth of the microorganism, protein and by-product formation, substrate utilization, the yield coefficients and specific rates of growth, specific production rate of the r-protein and specific consumption rate of the substrate should be defined. In this context, literature on the target protein human growth hormone (hGH), host microorganism *Pichia pastoris*, *glyceraldehyde-3-phosphate dehydrogenase (GAP)* promoter, expression mechanism of the yeast *Pichia pastoris*, bioprocess operation parameters and modes and fermentation characteristics are summarized and analyzed.

2.1 Product: Human Growth Hormone

Hormones are synthesized by endocrine glands and act as chemical messengers carrying signals to the target cells. They have many influences on human body including regulation of the metabolism, stimulation of growth, activation of the immune system and initiation for an incoming activity or stage of life (Baulieu and Kelly, 1990; Binkley, 1994).

There are some general characteristics of the hormones. They are not stored after the secretion for the need of the body. The secretion of the hormones is

regulated by a negative feedback system with neurotransmitters and other hormones. Transportation of hormones is carried out by blood stream with small transmission rate while their duration is generally long-lasting. Furthermore a hormone can behave antagonistically or synergistically to another hormone (Schwyzer, 1964).

Human growth hormone (hGH) is one of the crucial hormones for human body secreted by the somatotroph cells in pituitary gland. This hormone was first described in 1921 (Evans and Long, 1921). It was first isolated from human pituitaries in 1956. Initially the only way of obtaining hGH was extracting it from pituitaries of cadavers (Krysiak *et al.*, 2007). In 1972, biochemical structure of the hormone was found. In 1979, recombinant hGH (rhGH) was produced by using *Escherichia coli* by genetic engineering techniques for the first time (Goeddel *et al*., 1979). The native form of the molecule whose amino acid sequence and conformation was identical to the hGH in the body was produced from *E. coli* in 1985 (Gray *et al*., 1985). The clinical use of recombinant hGH was approved in the same year (Kopchick *et al*., 2003).

The effects of human growth hormone on human body is tabulated in Table 2.1

Table 2.1 Effects of hGH in human body (Chawla *et al*. 1983)

Metabolic	Stimulates amino acid transport
	Stimulates protein synthesis in most cell types
	Stimulates DNA/RNA synthesis in most cell types
	Stimulates polyamine synthesis
	Stimulates lipolysis
	Inhibits insulin action on glucose metabolism
Physiological	Increases renal blood flow, glomerular filtration rate and tubular reabsorption of PO_4
	Increases basal metabolic rate
	Stimulates new bone formation
	Stimulates erythropoiesis
	Expands extracellular fluid space
Anatomic	Accelerates linear growth
	Reduces adipose mass and enlarges lean body mass (muscle, liver, kidney, heart, GI tract, pancreas, skeleton, connective tissue)

As the effects of hGH is summarized in Table 2.1 the recombinant hGH can also be used for these purposes. It is used for growth hormone deficiency in children and adults, treatment of hypopituitary dwarfism, Turner's syndrome, human immune deficiency virus (HIV) syndrome and chronic renal failure. It is also used clinically in the treatment of bone fractures, injuries, burns, and bleeding ulcers (Tritos, 1998).

2.1.1 Properties and Structure of hGH

Human growth hormone is a non-glycosylated 191 aminoacid polypeptide hormone. It has a molecular weight of 22 kDa and its empirical formula is $C_{990}H_{1529}N_{262}O_{300}S_7$ (Kasimova *et al*., 2002). The isoelelectric point of hGH is in the range of 4.9 to 5.1 and at pH 7.0 pI, is 4.9 (Binkley, 1994).

The genes encoding hGH are located in chromosome 17 in the q22-24 region (http://www.ncbi.nlm.nih.gov). The 191 amino acids fold into a four-helix bundle structure with two di-sulfide bonds (De Vos *et al.*, 1992). The two disulfide bonds, resulted by four cysteine biomolecules that are found on 35th, 165th, 182nd, and 189th positions of the hGH chain, enable it to form a tertiary structure, which is the active form of the protein, while the helices are necessary for the interaction with the hGH receptors (Binkley, 1994). Nucleotide sequence and tertiary structure are given in Figures 2.1 and 2.2, respectively.

```
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
```

Figure 2.1 Nucleotide sequence of hGH (Baulieu and Kelly, 1990; Binkley, 1994)

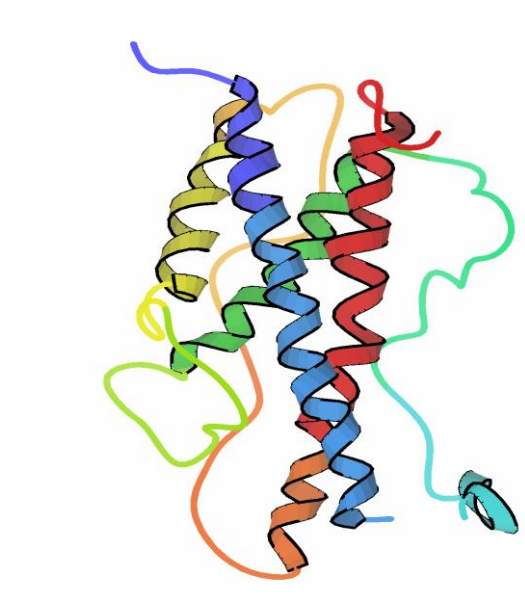


Figure 2.2 Tertiary structure of hGH (http://www.ncbi.nlm.nih.gov)

In Figure 2.3 primary structure, amino acid sequence, secondary structure, disulfide bridges and alpha helices are shown for hGH with accession number 1HGU.

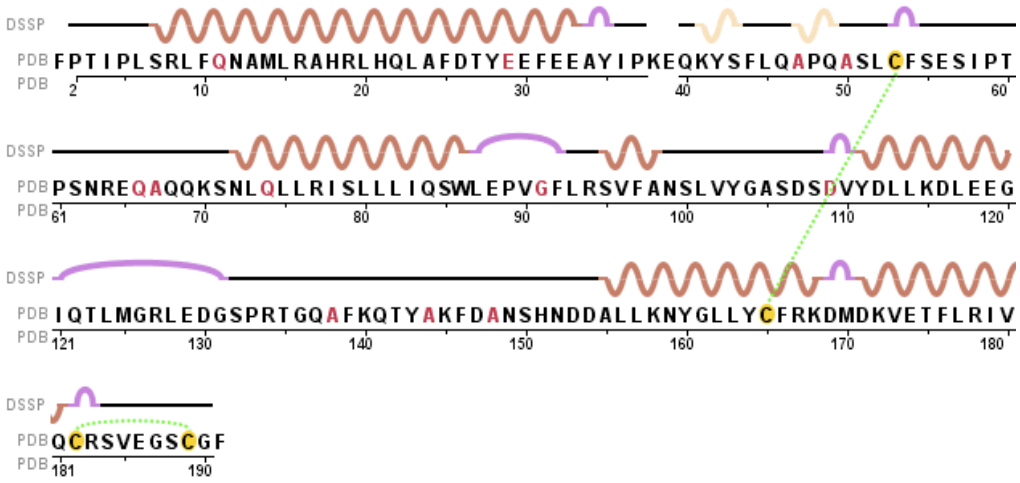


Figure 2.3 Primary and secondary structure of hGH; purple and brown curves: major α-helices; pink curves: shorter connective α-helices; dashed green lines: disulfide bridges between cystein residues. (http://www.pdb.org/pdb)

2.2 Selection of Microorganism for rhGH Production

As microorganisms act as micro bioreactors in recombinant protein production, the selection of appropriate microorganism was the crucial part of the work. Potential hosts should produce and secrete high amount of proteins, should have high yields and they have to be suitable for industrial fermentations; should not need expensive and harmful substances in the production medium, be safe and not give any undesirable by products (Kirk and Othmer, 1994). *E. coli* and *Saccharomyces cerevisiae* are best characterized and studied microorganisms in recombinant protein production although they have some disadvantages. *E. coli* cannot perform many post-translational modifications like glycosylation while *S. cerevisiae* hyper-glycosylates proteins which give rise to decrease of secretion rates and alters protein functionality (Walker, 1998).

For the production of hGH the first and most widely used microorganism is *E. coli* (Goeddel *et al*., 1979; Gray *et al*., 1985; Hsiung *et al*., 1986; Kato *et al*., 1987; Shin *et al*., 1998; Patra *et al*., 2000; Tabandeh *et al*., 2004; Singh *et al*., 2009). The *E. coli* based fermentations are preferred because it can grow and express proteins in cheap media and it has fast growth rate which shortens the process. However it is hard to purify rhGH in these bioprocesses since the hGH production takes place in inclusion bodies.

Another prokaryote microorganism for rhGH production is *Bacillus subtilis* (Nakayama *et al*., 1988; Franchi *et al*., 1991; Özdamar *et al*., 2009). High protease production rate hence high proteolytic activity is the major drawback of using this microorganism. For reducing the proteolytic degradation of recombinant protein, protease inhibitors should be added to the medium (Özdamar *et al*., 2009) or protease deficient strains should be used.

Yeasts are one of the eukaryotic cells that can be used in producing recombinant proteins. They are capable of performing post-translational modifications; they can reach to a high cell densities in cheap and defined media, express proteins extracellularly. For these purposes higher fermentation efficiencies can be obtained by using yeasts in expression systems. *Pichia pastoris* is one of the promising yeast species for high-level protein production (Cregg *et al*., 1993; Romanos, 1992; Sreekrishna *et al*., 1997). Recombinant hGH was first produced from *P.pastoris* by Trevino *et al*. (2000), who obtained 11 mg L^{-1} in shake tubes having 3 ml of defined medium. They also produced 49 mg L^{-1} rhGH in a 2-L bioreactor. Then, Eurwilaichitr *et al.* (2002) studied the production of hGH from *P. pastoris* and attained 190 mg L^{-1} rhGH concentration. In 2008 a new expression system in which host microorganism was *P. pastoris* was designed for rhGH production and purification under control of *AOX1* promoter and obtained 110 mg L^{-1} rhGH production (Orman *et al*., 2008). Çalık *et al.* (2013) studied the effects of semi-batch methanol feeding without any other carbon source, semi-batch methanol feeding with pulse sorbitol feeding and semi-batch methanol feeding with semi-batch sorbitol feeding on rhGH production by using *P. pastoris* and *AOX1* promoter. The highest rhGH concentration was found as 0.64 g L^{-1} at t=42 h with the feeding strategy where exponential methanol feeding with a pre-determined specific growth

rate of μ_0=0.03 h^{-1} was performed and sorbitol concentration was maintained at 50 g L^{-1} (Çalık *et al*., 2013). Zerze (2012) obtained 1.2 g L^{-1} where exponential methanol feeding was performed with μ_0=0.03 h^{-1} within t=0-19.5 h and mannitol was fed to keep at 50 g L^{-1} within t= 0-6 h, then a second methanol transition phase was applied for half an hour which was followed by dynamic methanol feeding to maintain μ=0.03 h^{-1}. Zerze (2012) also designed and constructed an rhGH expression system under the control of *glyceraldehyde-3-phosphate dehydrogenase* (*GAP*) which is a constitutive promoter and obtained a 200 mg L^{-1} rhGH concentration (Zerze, 2012).

2.2.1 Expression Host: *Pichia pastoris*

Some yeast species have been found to have the ability of utilizing methanol as sole carbon and energy source (Ogata *et al*., 1969). These species are identified as methylotrophic yeasts, and up to now four genera are identified to utilize methanol which are *Hansenula, Pichia, Candida and Torulopsis* (Faber *et al*., 1995). *P.pastoris* from *Pichia* genera is also a methylotropic yeast whose media and protocols were first developed by Phillips Petroleum Company during 1970s. After that time, the relevance on *P. pastoris* has increased significantly.

Over 400 recombinant proteins are reported to be produced by *P. pastoris* (Cereghino *et al*., 2000). Besides being methylotropic it is also a mesophilic yeast that can live at temperatures around 25-35 ^{o}C (Macauley-Patrick *et al*., 2005). It can grow in media with a wide pH range of 3 to 7 (Cregg *et al*., 2000). Like other yeast cells *P.pastoris* is unicellular, has a diameter of 1-5 μm wide and 5-30 μm long, have typical eukaryotic cell structure with a polysaccharide cell wall and they are facultative anaerobes. Taxonomically *P. pastoris* is placed under the Kingdom Fungi, Division Eumycota, Subdivision Ascomycotina, Class Hemoascomycetes, Order Endomycetales, Family Saccharomycetaceae and Genus Pichia (http://www.ncbi.nlm.nih.gov).

P. pastoris can be a good expression host because of its advantages. First of all, the manipulations of its genetics are simple and protocols are presented as well as it can perform many post-translational modifications (Cereghino *et al*., 2002). Furthermore, fermentation protocols were developed which yield high cell densities on simple, defined and inexpensive media (Cos *et al*., 2006). Moreover, it is a non-

pathogenic microorganism (Daly and Hearn, 2005) which can produce proteins intracellularly or extracellularly at high levels which eases the separation and purification of the products (Macauley-Patrick *et al*., 2005, Ragon *et al*., 2007). The other advantage of *P. pastoris* is that it has strongly regulated promoters as *alcohol oxidase* 1 (*AOX1*) promoter and *GAP* promoter (Cereghino and Cregg, 2000, Cos *et al*., 2006).

There are also some disadvantages of using *P. pastoris* as an host microorganism besides all these advantages. Firstly, compared to prokaryotic cells its growth rate is slower; hence the cultivation time is longer compared to that of prokaryotic ones. Secondly it secretes protease enzymes so it shows high proteolytic activity which can be overcame by addition of protease inhibitor (Kobayashi *et al*., 2000, Sinha *et al*., 2004) or using strains which are protease deficient (Sreekrishna *et al*., 1997). Another disadvantage should be taken into account when using *AOX1* promoter. Because methanol is used to induce the *AOX1* promoter and it is a hazardous compound. The transportation and storage of methanol is difficult in industrial scale production (Zhang et *al*., 2009). On the other hand, there are other promoters like *glyceraldehyde-3-phosphate dehydrogenase* (*GAP*) (Waterham *et al*., 1997), *formaldehyde dehydrogenase 1* (*FLD1*) (Shen *et al*., 1998); *isocitrate lyase* (*ICL1*) (Menendez *et al*., 2003) and *3-phosphoglycerate kinase* (*PGK1*) (Almeida *et al*., 2005) for *P. pastoris* that avoid the necessity of methanol usage. The advantages and disadvantages of using *P. pastoris* as host organism are summarized in Table 2.2.

Table 2.2 Advantages and disadvantages of using *P.pastoris* as host organism

Advantages	Disadvantages
High yield and productivity	Proteolytic degradation
Strong promoters (*AOX1*,*GAP*)	Non-native glycosylation
Simple, cheap and defined media	Long cultivation time
Able to process the products like mammalian cells	In *AOX1* based expression systems methanol use is problematic due to the transportation and storage. Methanol used systems are also unsuitable for food industry.
Low purification cost	
High protein expression levels intracellularly and extracellularly	
Perform post-translational modifications	
No endotoxin problem	
Non-pathogenic	
Wide pH-range: 3-7	
Prefer aerobic respiration rather than anaerobic: an advantage over *S.cerevisia*	
Crab-tree negative	
Hyper-glycosylation is not a problem like in *S.cerevisia*	

2.2.2 Expression with GAP promoter

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is homotetrameric protein with 333 amino acid residues which is an enzyme in glycolysis and gluconeogenesis pathways as seen in Figure 2.4. Its molecular weight is 35.4 kDa (Waterham *et al.* 1997). GAP promoter is a constitutive promoter that expresses genes at high levels (Waterham *et al*. 1997) using gene dosage(Wu *et al*., 2003a; Wu

et al., 2003b;Cos *et al*., 2006; Zhang *et al*., 2009; Ferreira *et al*., 2012; Potvin *et al*. 2012) and through precisely designed feeding strategies (Çalık *et al*., 2014). Moreover, this promoter can be a good alternative that overcomes the imperfections in the usage of inducible promoters (Pal *et al*. 2006). In the expression of GAP promoter carbon source selection is crucial since it modulates the strength of expression and recombinant protein production (Waterham *et al*. 1997). Generally, glucose expressed systems have the highest expression levels while glycerol follows it as a carbon source in *GAP*-driven expressions (Waterham *et al*., 1997; Pal *et al*., 2006;.Fei *et al*., 2009; Garcia-Ortega *et al*., 2013)

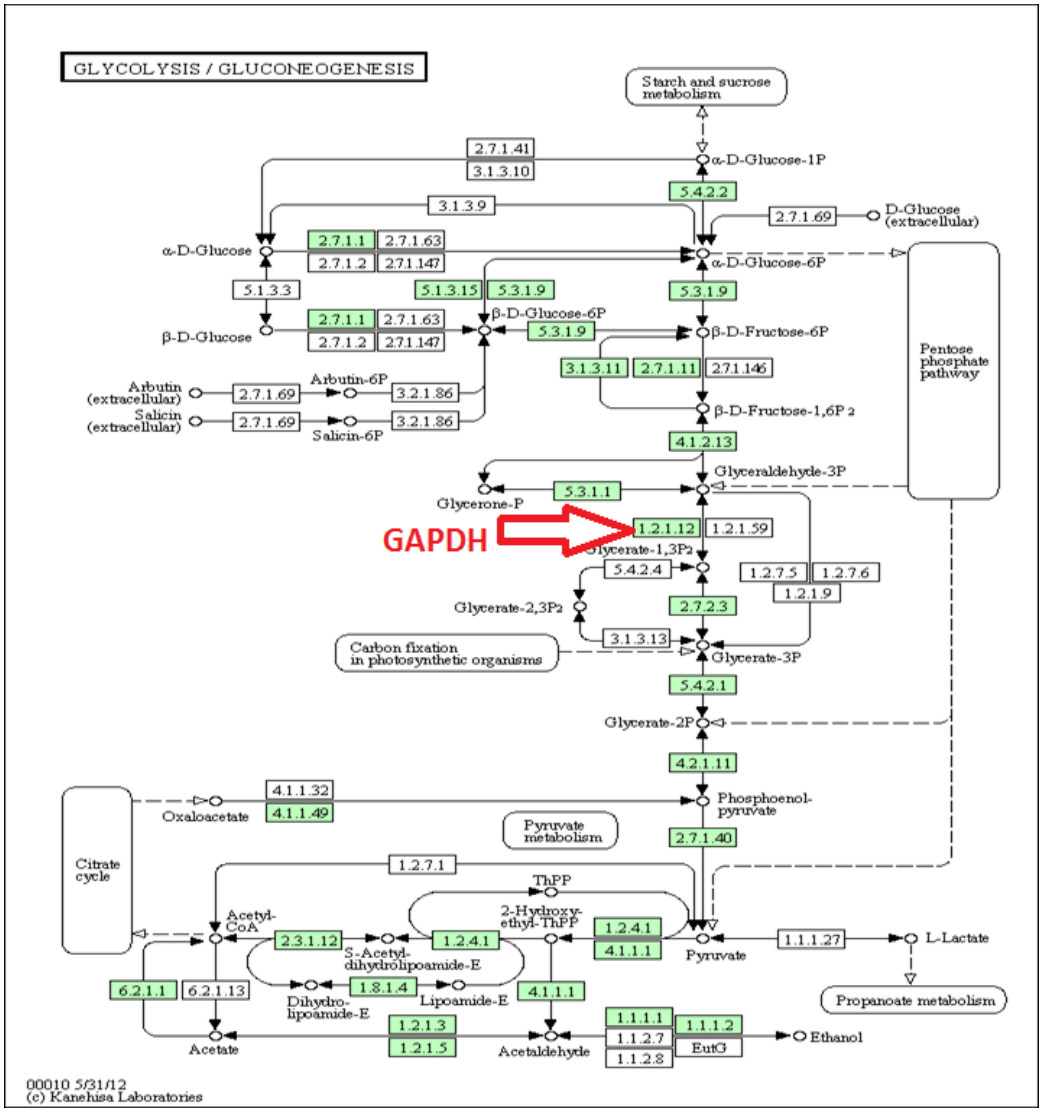


Figure 2.4 Glycolysis Pathway indicating GAPDH enzyme (http://www.genome.jp/kegg/pathway/map/map00010.html)

2.2.2.1 Comparison of AOX1 and GAP promoters

In *P. pastoris* expression systems the most used promoters are the inducible *AOX1* promoter and constitutive *GAP* promoter (Potvin *et al*. 2012). Among all the promoters used in *P. pastoris* expressions the most known and studied is the *AOX1* promoter which is a strong and tightly regulated promoter. *P. pastoris* has the ability of producing recombinant proteins at high levels and fermentations under the control of *AOX1* promoter which have an established protocol. This promoter is a repressible/depressible one which is repressed by glycerol and depressed in the presence of methanol. Furthermore, there have to be two main phases since the specific growth rate of *P. pastoris* cells on methanol is low and glycerol, in which cells have a high specific growth rate, represses the expression of recombinant protein. Therefore, the cells are grown to a certain cell density with the carbon source glycerol and then the production is induced with the feeding of methanol. Methanol usage in the *AOX1* driven systems is another disadvantage since the transportation and storage of methanol is problematic because it is a hazardous and flammable substance. And as it is a toxic material, its use in food and drug industry is questionable. Moreover, in the *AOX1* driven systems because of the methanol metabolism some by-products like formaldehyde and hydrogen peroxide accumulate (Zhao *et al*. 2008). These by-products can be a reason of the raise in the concentration of contaminating proteins which are the release of endogenous cellular proteins resulting from cell lysis (Wang *et al*. 2012).

GAP promoter based expression system overcame the explained disadvantages. First it does not require methanol to induce the production so it eliminates the cost of transportation and storage of methanol. Next as the expression system is a constitutive one, the cells can produce the protein while they are growing which gets rid of different phases and it does not require carbon source shifts in the process (Kottmeier *et al*. 2012). Moreover, one single carbon source, glucose or glycerol, is sufficient in the fermentations (Zhang *et al*., 2009) and hence the continuous operation is possible in the production of recombinant proteins which is preferable in large scale operations.

There are also some other constitutive promoters used in recombinant protein production in *P. pastoris* such as *AOD, ENO1, GPM1, HSP82, ILV5, KAR2, YPT1* and *SDH* (Çalık *et al*., 2014). However their expression levels, except *SDH*, are not comparable with *GAP* promoter since their expression levels are very low compared to *GAP* promoter. Furthermore there are studies that are done by using inducible *DAS, AOX2, FLD1* promoters (Çalık *et al*., 2014).

The production and yields can be different depending on the produced protein for *GAP* and *AOX1* expression systems. Some of the studies were done to compare these two promoter systems. In some of them *AOX*1 system shows higher expression levels (Sears *et al*., 1998; Vassileva *et al*., 2001; Kim *et al*., 2009) while in some other cases *GAP* promoter based systems expresses higher amount of protein (Döring *et al*., 1998; Delroisse *et al*., 2005). Cos *et al.* (2006) tabulated this comparison in their review paper as shown in the Table 2.3.

Table 2.3 The comparison of *GAP* and *AOX1* promoters' productions (Cos *et al.* 2006)

Protein	Promoter		Substrate	Total protein (mg/L)[1] (U/mL)[2]	Productivity (mg/L h)[1] (U/L h)[2]	Operational mode	Bioreactor
Insect esterase	*GAP*	Intracellular	Glucose	7[1]	--	Batch	Shake flasks 50 mL
		Extracellular		80[1]	--		
	AOX1	Extracellular	Methanol	40[1]	--		
Fructose exo-levanase	*GAP*	Extracellular	Glycerol	26.6[2]	682[2]	Fed-batch	Bioreactor 7.5 L
	AOX1	Extracellular	Methanol	21.1[2]	220[2]	Fed-batch	
Human trysinogen	*GAP*	Extracellular	Glycerol	--	--	Fed-batch	Bioreactor 2 L
	AOX1	Extracellular	Methanol	--	--	Fed-batch	
Vitellogenin	*GAP*	Intracellular	Glucose	12[2]	--	Fed-batch	Bioreactor 2 L
hGM-CSF	*GAP*	Extracellular	Glucose	90[1]	1.2[1]	Batch	Shake flasks 50 mL
	GAP+AOX1	Extracellular	Methanol	180[1]	2.4[1]	Batch	
Aqualysin I	*GAP*	Extracellular	Glucose	1000[1]		Batch	Shake flasks
h-Chitinase	*GAP*	Extracellular	Glucose	450[1]	2.8[1]	Fed-batch	Bioreactor 3 L
				--	15[1]	Continuous	
	AOX	Extracellular	Methanol	350[1]	2.9[1]	Fed-batch	
HBsAg	GAP	Extracellular	Glucose	--	--	Fed-batch	Shake flasks 500 mL
	GAP multicopy	Extracellular		--	--	Fed-batch	
				--	--	Cyclic batch	
	AOX1	Extracellular	Methanol	--	--	Fed-batch	
Cellobiohydrolase	GAP	Extracellular	Glycerol	--	--	Fed-batch	Bioreactor 7.5 L
	AOX1	Extracellular	Methanol	--	--	Fed-batch	

2.2.3 Proteolytic Degradation in *Pichia Pastoris*

Pichia pastoris can reach to high cell densities which is an advantage for higher expression levels. However, high cell density cultures bring higher proteolytic activity (Van Den Hazel *et al*., 1996; Kobayashi *et al*., 2000; Gimenez *et al*., 2000; Sinha *et al*., 2004). Other than the degradation of the produced protein, proteolysis also gives rise to decrease of product yield and contaminates the medium which makes downstream processing difficult (Kobayashi *et al*., 2000).

To overcome proteolytic degradation, some strategies are developed. Addition of casamino acid and peptone to the medium is one of the developed strategies that prevent proteins from proteolytic degradation. These amino acid rich components reduce proteolytic degradation because they can eliminate nitrogen limitation (Clare *et al*., 1991; Goodrick *et al*., 2001; Sinha *et al*., 2004; Pal *et al*., 2006; Khasa *et al*., 2007).

The other strategy is using the inhibitors for proteases. Generally aspartic, cystine, and serine type proteases are present in the production medium (Shi *et al*., 2003); thus protease inhibitors like antipain, chymostatin, diisopropyl fluorophosphate, elastatinal or phenyl methyl sufonyl fluoride (PMSF) and ethylenedinitrilotetraacetic acid (EDTA) have been investigated. Adding EDTA or PMSF result a decrease in the protease activity by 45% and 78%, respectively (Sinha *et al*., 2004) and an increase in the recombinant protein concentration is seen when the protease inhibitors are added to the medium (Kobayashi *et al*., 2000).

Using protease-deficient strains is also a way to prevent proteolysis. In this strategy the genes that encodes proteases is disrupted. SMD1163 (his4 pep4 prb1), SMD1165 (his4 pep4), and SMD1168 (his4 pep4) are the strains with a disruption in the genes encoding proteinase A and proteinase B (Sreekrishna *et al*., 1997; Cereghino and Cregg, 2000). Proteinase A and B are encoded by pep4 and prb1 genes, respectively (Jahic *et al*., 2006). So the use pep4 and prb1 mutants in the production of proteins results a reduction or elimination in the proteolytic activity (Higgins and Cregg, 1998).

For reducing the protease activity temperature and pH should be adjusted to a proper value. Optimum temperature for *P.pastoris* fermentations is found to be 30 ^{o}C (Wegner, 1983) and higher temperatures, above 32 ^{o}C, can cause cell lysis and higher protease activity which damages expression of the proteins (Invitrogen, 2002; Inan *et al*., 1999). Due to kinetics of proteases low temperatures results a decrease in the proteolytic activity (Li *et al*., 2001; Hong *et al*., 2002; Macauley-Patrick *et al*., 2005). Choosing a pH value that is not optimal for protease activation can also reduce the proteolytic degradation of proteins (Jahic *et al*., 2002). *P. pastoris* cells can live in a wide pH range 3.0 to 7.0. It was found that pH has little effect on growth rate which is an advantage for finding a proper pH value that is not appropriate for protease activity (Wegner, 1983; Inan *et al*., 1999; Sreekrishna *et al*., 1997).

2.3 Bioreactor Operation Parameters and Medium Design

Cells are the micro-bioreactor that produces the recombinant protein or other molecules via intracellular biochemical reactions. Therefore cell growth and the expression of the proteins are highly related with the production and feeding medium design as well as the bioreactor operation parameters such as temperature, pH and oxygen transfer. Intracellular metabolic pathways and fluxes are affected by these parameters. Thus for an industrial production optimum conditions should be found and applied (Çalık *et al*. 1999).

2.3.1 Medium Design

The design of the fermentation medium is a key factor in bioprocesses as the cell growth and expression of proteins is the result of uptake and using the nutrients in the medium in the intracellular reactions. Therefore the fermentation medium should contain carbon, nitrogen and energy sources as well as essential minerals required for the cells and expression (Nielsen *et al.*, 2003). It should be cheap enough, easily found and its quality should be sufficient for the operation. Moreover it should not result difficulty in the downstream processing like separation and purification (Nielsen *et al.*, 2003).

Nutrients needed for the fermentation can be classified into two groups according to their amount in the medium, namely macronutrients and micronutrients. Macronutrients needed in higher amounts, above 10^{-4} M and includes carbon, oxygen, nitrogen, hydrogen, sulfur, phosphorus, magnesium and potassium. Micronutrients are required below 10^{-4} M and they are trace elements like Ca, Cu, Fe, Na, Mn, Mo, Zn, and vitamins (Fiecher *et al*., 1984).

In bioprocesses, complex medium, containing different amino acids, vitamins and minerals, and defined medium, whose composition is known, are used. Although complex medium performs better in most of the processes, defined medium is preferable in industry since it is necessary to standardize and validate the process, medium and product as well as to eliminate the consequent downstream operations (Nielsen *et al.*, 2003; Macauley-Patrick *et al*., 2005).

Basal salt medium (BSM), which contains main macronutrients, is the most preferred medium with the carbon source(s) and Pichia trace salt solution (PTM), which contains micronutrients (Zhang *et al*., 2000; Jungo *et al*., 2006; Dietzsch *et al*., 2011). As a nitrogen source mostly ammonium hydroxide was added to the medium which also adjust pH of the fermentation broth (Wang *et al*., 2009; Soyaslan and Çalık, 2011; Gao *et al*., 2012).

Carbon source selection is an important factor on growth and expression in GAP driven systems. Glucose, glycerol, and oleic acid were used as sole carbon sources in *GAP* promoter driven expression systems (Zhang *et al*., 2009). Fructose, sorbitol, mannitol, and trehalose were also used as alternative carbon sources since they can be utilized in carbon metabolism (Çalık *et al*., 2014).

Glucose and glycerol were compared as a carbon source in recombinant protein production in *GAP*-driven systems. Some of the studies showed that glucose-based systems resulted in high recombinant protein concentrations (Fei *et al*., 2009; Pal *et al*., 2006; Waterham *et al*., 1997). On the other hand, Goodrick *et al*. (2001) reported that the concentration of human chitinase was higher in glycerol-based medium whereas the cell density, the specific and volumetric productivity was greater in glucose based system. In a recent study a fermentation strategy was developed with two stages, batch and semi-batch. In the batch phase glycerol was

described as the superior carbon source, while in the semi-batch phase glucose was the preferred one due to its lower heat generation and oxygen yield values (Garcia-Ortega *et al*., 2013). To conclude the carbon source choice is dependent on the bioreactor operation mode, batch, semi-batch or combinations of them, fermentation strategy which can be different such as producing r-proteins with high yields or expressing the r-protein at high levels together with high cell density (Çalık *et al*., 2014).

The effect of casamino acids which are found in the complex media were also studied as they had been found to prevent proteolytic degradation (Goodrick *et al*., 2001; Pal *et al*., 2006; Khasa *et al*., 2007). In one of the study it was found that addition of 10 g L^{-1} to the medium stabilizes the yield of recombinant protein (Goodrick *et al*., 2001). Another study revealed a 1.4- fold increase in r-protein production and 1.3- fold increase in cell concentration was obtained by adding casamino acids. The concentration of casamino acids was investigated and 20 g L^{-1} was found as the best concentration for the maximum cell and r-protein concentration (Khasa *et al*., 2007). To avoid using complex medium, casamino acids, some of the precursor amino acids, specifically glutamic acid, cysteine, and glycine, were added all at 20 mmol L^{-1} to the production medium and 4.6- fold higher glutathione yield and with a 2.5- fold higher productivity were found while the cell concentration remained constant (Fei *et al*., 2009). Therefore addition of amino acids frees metabolic network to increase the cell density and r-protein expression (Heyland *et al*., 2011).

2.3.1.1 Molasses

Molasses is formed during the production of sucrose. Beet molasses is comprised as a by-product besides sucrose that is produced from sugar beet (Curtin, 1983). Ingredients of the beet molasses are introduced in Table 2.4 in detail (Olbrich, 1963). In this study sugar beet molasses was taken from Ankara Sugar Factory and the components are tabulated in Table 2.5.

Since molasses is a cheap by-product which is a rich medium that can be found in large amount, it is a good alternative as substrate for host microorganisms. As it contains organic non-sugars, vitamins and essential minerals, it enhances cell growth (Park and Baratti, 1991; Kirk and Othmer, 1994). It is used as a substrate for the host microorganisms like *Zymomonas mobilis, Saccharomyces cerevisiae, Mycobacteriurn phlei, E.coli* (Park and Baratti, 1991; Diwany *et al.,* 1992; Ghozlan, 1994; Agarwal *et al,*. 2006; Çalık and Levent, 2009). If molasses is treated so that the sucrose is converted to glucose and fructose; it can also be used as a feed for *GAP* driven *P. pastoris*. In this study pre-treated molasses in which sucrose was converted to glucose and fructose was used in one of the experiments.

Table 2.4 Composition of molasses obtained from European sugar beet samples (Olbrich, 1963)

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

Table 2.5 Composition of sugar beet molasses obtained from Ankara Sugar Factory

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

2.3.2 Bioreactor Operation Conditions: Oxygen Transfer, pH and Temperature

2.3.2.1Oxygen Transfer

Pichia pastoris is a facultative anaerobic microorganism which prefers aerobic respiration in the presence of oxygen but can switch to anaerobic respiration due to the lack of oxygen in the medium. The oxygen transfer (OTR) and oxygen uptake (OUR) rates which affects the metabolic pathways and metabolic fluxes are two important fermentation characteristics, where DO indicates the accumulation of oxygen in the bioreactor production medium (Çalık *et al*., 1999 and 2000). Air or oxygen enriched air is supplied for oxygen requirement of the *P.pastoris* cells. The OTR is manipulated by either changing the inlet air rate or the agitation rate (Lee *et al*., 2003; Çelik, 2008).

In biotechnological processes where *P. pastoris* is used, dissolved oxygen is generally kept around 20-30 % to achieve fully aerobic fermentation. As *P. pastoris* cells can reach high cell concentrations, oxygen requirement increases. This can be overcome by feeding oxygen enriched air (Gasser *et al*., 2006; Zheng *et al*., 2006; Zhang *et al*., 2007; Fei *et al*., 2009;).

There are also studies with *P. pastoris* on "hypoxic cultivations" which sometimes is used for limited oxygen transfer conditions. Baumann *et al*. (2008)

reported in the specific productivity was increased 2.5- fold at limited oxygen transfer conditions. They designed a strategy on oxidofermentative process which resulted 3- to 6- fold increase in specific productivity with a 3-fold decrease in the cultivation time. However Garcia-Ortega *et al*. (2013) claimed that keeping DO higher, *e.g.*, 50%, was better since fermentative by-products were found in even 35% DO level, who did not mention the OTR or OUR. As there are conflicting arguments based on the DO concentration, the DO concentration changes need to be investigated within oxygen transfer effects together with the oxygen uptake and oxygen transfer rates (Çalık *et al*., 2014).

2.3.2.2 pH

Hydrogen ion concentration, *i.e.*, pH, is one of the crucial bioreactor operation parameter that affects the reactions in the cell which influences the growth, enzyme activities, and transport through cell membrane. Although microorganisms can keep their pH constant in the cell, their energy requirement increases with the pH difference across the cell membrane (Nielsen *et al*., 2003). Yeasts are able to grow in a wide pH range from 4.5 to 6.5 (Walker, 1998); specifically, *P. pastoris* can grow in a wider range between pH values from 3 to 7, and Macauley-Patrick *et al*. (2005) reported that changing pH does not cause a difference in the growth considerably.

In *GAP* promoter *P. pastoris* expression systems optimum pH is found to be 6.0 for obtaining high product concentrations (Zheng *et al*., 2006; Hu *et al*., 2008; Wang *et al*., 2012). Zhao *et al*. (2008) designed a strategy where they changed pH and temperature of the medium at a certain time; where they grew the cells at a pH of 5.5 and temperature of 29 ^{o}C, and then shifted the pH to 6.5 and temperature to 26 ^{o}C for higher r-protein production.

2.3.2.3 Temperature

As the microorganisms are not able to regulate their internal temperature it is very crucial to maintain the temperature at the optimum value for their metabolic activity. Higher temperatures will give rise to the cell death while lower temperatures decrease the rate of reactions taking place in the cell (Donati, 2007). Moreover, nutritional requirements, cell growth rate, r-protein expression and yield coefficients are also influenced by medium temperature. The optimum temperature for growth and r-protein production may be also different values which can be challenging.

Contrary to pH, *P. pastoris* fermentation systems have a narrow temperature range as 25-30^{o}C. In most of *GAP* promoter based *P. pastoris* fermentation studies temperature was set to 30 ^{o}C (Waterham *et al*.,1997; Sears *et al*., 1998; Wu *et al*. 2003a; Menéndez *et al*., 2004; Zheng *et al*., 2006; Rupa *et al*., 2007; Fei *et al*., 2009) while some used 25 ^{o}C (Gasser *et al*., 2006) and 28 ^{o}C (Qiao *et al*., 2010). Although decreasing the temperature results an increase in the solubility of oxygen which increases the oxygen transfer rate, however causes a reduction in the growth and reaction rates. The effect of temperature, from 20 ^{o}C to 30 ^{o}C was studied and a 3-fold increase in the specific productivity for r-protein production was attained at lower temperatures and it was mentioned that folding stress was reduced at lower temperatures which allows more efficient protein secretion (Dragosits *et al*., 2010). As stated in section 2.3.2.2, Zhao *et al*. (2008) changed the temperature from 29 ^{o}C to 26 ^{o}C for increasing r-protein production.

2.4 Bioreactors and Operation Modes

Reactors in industry based on the hydrodynamics in the reactor and the operation mode, are divided into two main groups: ***i)*** "mixed-flow reactors" which can be operated as batch-, semi-batch- or backmixed-flow reactors; and ***ii)*** laminar-flow, turbulent-flow, and plug flow- continuous reactors which are operated at steady-state. In aerobic fermentation processes using microorganisms, as air or oxygen causes axial dispersion, in a "gas (air)- liquid (aqueous phase)- solid (microorganism)" reaction system, continuous operation under plug-flow or the laminar- or turbulent-flow is impossible. On the other hand, as microorganism acts as a semi-batch bioreactor system and ages during the process, steady-state microbial

production under back-mixed flow conditions is meaningless, except chemo-stat experiments. Therefore, in biotechnological processes, batch- and semi-batch bioreactors under completely-stirred or well-mixed hydrodynamic conditions are used.

The advantage of using stirred batch- and semi-batch reactors in aerobic fermentations is to contact the well mixed liquid phase with the sparged-gas (air or oxygen) phase that passes through the liquid medium which contributes to high OTR and OUR (Nielsen *et al*., 2003).

2.4.1 Batch Operation

There are five main phases in the microbial growth. The first phase is the lag phase which is the adaptation time of the microorganisms to the medium. Growth of the cells is very low during this phase; however the cells produce the enzymes for utilizing the nutrients in the cultivation medium. The second stage is the exponential (logarithmic) growth phase in which the cells grows rapidly and thus the cells reach maximum growth rate. In this phase cells synthesizes the metabolites which are needed. After the exponential phase cells show a decrease in the growth rate which is called deceleration phase. The next phase is the stationary phase in which the growth and the death of the cells balanced. In the last phase, death phase, the rate of cell degradation is dominant to the growth of the cells due to the diminution of the nutrients and substrates as well as there can be some by-products which may be toxic to the cells (Liu, 2013).

In *GAP* promoter based *P.pastoris* fermentations, batch mode is not commonly used for protein production as it has lower production yields, there are few studies in the literature (Çalık *et al*., 2014). Amongst, Chen *et al*. (2007) produced and purified goat lactoferrin with a yield of 2.0 mg L^{-1} in a five day experiment; Li *et al*. (2010) produced 0.78 mg ml^{-1} endo-β-1,4-xylanase in a three day batch process; and, *Rhizopus oryzae* α-amylase is also expressed as 1.9 U ml^{-1} α-amylase activity in a 140 h batch fermentation process (Li *et al*. 2011).

2.4.2 Semi-batch (Fed-batch) Operation

Semi-batch operations are generally designed as two stages which are batch and semi-batch phase. The bioprocess is started as a batch and cells grow to a high concentration by utilizing the initial substrate. At a particular time, usually when the initial substrate consumed totally, the feeding that contains the inducer which switches the metabolic pathways for the desired production is initiated. During the semi-batch operation no product is withdrawn from the bioreactor (Nielsen *et al*., 2003).

Semi-batch operations are favorable in following situations: substrate inhibition, high glucose effect, catabolite repression, to reach high cell density, to prolong production period, to add the water which is lost with evaporation and to reduce the fermentation medium viscosity (Yamanè and Shimizu, 1984). In bioprocesses that use *P. pastoris*, semi-batch operation is preferred to increase the r-protein production by extending the cultivation time by feeding the appropriate substrate(s) after the batch phase.

In *GAP* promoter driven fermentations, semi-batch is also designed as two stages. Batch phase is the first stage in which cells is grown to a certain concentration or until the substrate is consumed totally from an initial carbon source concentration from 10 to 50 g L^{-1}, generally 40 g L^{-1}. Cell proliferation is the major objective in the batch phase. After the batch phase, semi-batch phase is initiated by feeding the feed stream(s) which contain carbon and nitrogen sources together with the essential minerals and trace elements (Çalık *et al*., 2014).

There are experimental studies that used direct and indirect feedback control. Since monitoring the substrate concentration is difficult, there are few experiments reported with direct feed control (Kottmeier *et al*. 2012; Zhao *et al*. 2008). The concentration of the glucose is monitored and three pulses were fed when the glucose is consumed totally (Kottmeier *et al*., 2012).

In indirect feedback controls, DO, respiratory quotient, pH and the exit gas composition was used. Zhao *et al*. (2008) performed experiments using substrate-stat, pH-stat, μ-stat and the combination of pH- and μ-stat feedback controls for

production of lipase. They found the combination of pH- and μ-stat strategy performed best in which they produced the highest amount of lipase. Nevertheless, pH-stat feedback controls are not favored since the agent that adjusts pH is also the nitrogen source. DO-stat indirect feedback control was also used in some studies (Baumann *et al*., 2008; Hu *et al*. 2008; Pepeliaev *et al*., 2011; Ferreira *et al*., 2012). Baumann *et al*. (2008) perform DO-stat experiments using different DO levels. Experiments followed by a pre-determined exponential feeding until the DO level decreased to 0% and ethanol concentration in the exit gas to 1% (v/v). Thereafter substrate was fed to keep ethanol concentration at 1% (v/v). They stated that with this strategy they obtained an increase in volumetric and specific productivity. In a recent study, DO was maintained between 25-40% by regulating the substrate feeding and highest cell and r-protein concentrations were obtained as 75 g L^{-1} and 250 mg L^{-1}, respectively (Guan *et al.,* 2013).

Another approach for feeding is the exponential feeding strategy in which specific growth rate can be controlled. This can provide effective metabolic regulation and increase r-protein expression. In this strategy the amount of substrate(s) necessary for the cells to attain the desired cell concentration is calculated by using pre-determined specific growth rate. The calculated amount of substrate is fed continuously to the bioreactor (Çalık *et al*., 2014). As a substrate, glucose and glycerol is used and they were fed to the system according to the equation- 2.18 as derived from mass balances in Section 2.5.2 (Çalık *et al*. 2013; Yamanè and Shimizu 1984):

In *GAP* promoter driven *P.pastoris* expression systems, Yx/s is taken to be 0.50 and 0.47 for glycerol and glucose, respectively (Baumann *et al*., 2010; Cos *et al*., 2005). But for glucose this value can reduce to 0.35 g cell g^{-1} glucose due to the formation of by-products even under fully aerobic fermentations (Tang *et al.,* 2010). On the other hand when glycerol is used as substrate, by-products do not form because glycerol is a less fermentative substrate under totally aerobic conditions. The concentrations of glycerol and glucose in the feed medium are commonly 630 and 500-600g L^{-1}, respectively (Çalık *et al*., 2014).

Zhao *et al*. (2008) studied the effect of glucose feeding rate for lipase production using pre-determined specific growth rates, μ_0= 0.10, 0.15, 0.20, and 0.25 h^{-1}. They obtained the highest product yield on the cell at μ = 0.15 h^{-1} where the r-protein production did not alter at μ = 0.15 h^{-1}. Baumann *et al*. (2008) fed glucose at μ = 0.2 h^{-1} and obtained the mean specific growth rates as μ = 0.0675 h^{-1} with the highest activity of 22.34 U L^{-1} for r-human trypsinogen, and μ = 0.0428 h^{-1} highest activity of 86.95 U L^{-1} for r-porcine trypsinogen. Ragon *et al*. (2008) compared the effect of two pre-determined specific growth rates at μ_0= 0.01 and 0.04 h^{-1} using glucose and they obtained the same amount of r-protein; however they also stated that increasing the pre-determined specific growth rate,μ_0,decreases specific activity 1.5- fold. Recently Garcia-Ortega *et al*. (2013) conducted experiments where glucose was fed at, μ_0= 0.05, 0.10, and 0.15 h^{-1} specific growth rates. It was represented that r-protein production and overall yields and total productivity in the case of μ_0 = 0.15 h^{-1} is 20% higher than those of μ_0 = 0.10 h^{-1} although the final cell densities are similar.

Constant feeding of the substrate is another approach in the feeding strategies. Goodrick *et al*. (2001) fed glucose and glycerol at a mass flow rate of 4.8 g h^{-1} for the production of human chitinase and found better results in glycerol feeding. In another study, Gasser *et al*. (2006) fed 4.1 g h^{-1} glucose and obtained 41 mg L^{-1} 2F5 Fab and 160 g L^{-1} cell concentration. Baumann *et al*. (2008) also constantly fed glucose with a quite high flow rate of 162 g h^{-1}. They obtained a cell density of 112 g L^{-1} and r-protein concentration of 47 mg L^{-1}. In 2009 Fei *et al*. conducted experiment in which glucose was fed constantly with 2.9 ml L^{-1} h^{-1} flow rate and they got 4.15 g L^{-1} r-protein and 83 mg L^{-1} h^{-1} productivity. Different from the glucose Menendez *et al*. (2013) fed cane sugar with a mass flow rate of 8 ml L^{-1} and resulted 135 U ml^{-1} β-fructosidase and 115 g L^{-1} cell concentration.

2.5 Fermentation Characteristics

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

The performance of a bioprocess is evaluated by calculating the yield coefficients and specific rates. Specific rate of production or consumption can be defined as the variation of the quantity of the molecule or cell per unit time, either per unit volume of fermentation broth, or per unit cell concentration. Specific growth rate, μ, is a key parameter which characterizes microbial growth in fermentations. As there is no inlet of the cells to the bioreactor, the material balance for the cell in semi-batch can be written as it is in batch processes, as follows:

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

where r_x is the cell formation rate in [g L^{-1} h^{-1}], V is bioprocess working volume in [L], C_X is cell concentration [g L^{-1}]. The relation between cell density and the cell formation rate can be defined with a first order kinetic equation where the kinetic constant μ is the specific growth rate, as follows:

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

In the cell balance equation it is assumed that volume, V(t), does not change due to the sampling if the total volume of samples are kept negligible ($V_{Total\text{-}samples} << V(t)$). Equation- 2.2 is substituted into equation-2.1:

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

In semi-batch systems bioreactor volume change due to the feeding stream; by taking the density of the system as constant the continuity equation is written, as follows:

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

Since there is no outlet stream in the semi-batch bioreactor, Q_{out} is zero, therefore, $Q_{in} = Q_s > 0$, where Q_s is the feed volumetric flow rate [$m^3\ h^{-1}$]; equation-2.4 becomes:

$$\frac{dV}{dt} = Q_s \tag{2.5}$$

If equation- 2.5 is combined with equation-2.3, one can derive:

$$C_x \frac{dV}{dt} + V \frac{dC_x}{dt} = \mu C_x V \tag{2.6}$$

Therefore, the specific growth rate for semi-batch systems can be expressed by rearranging the equation (2.9), as follows:

$$\mu = \frac{1}{C_x}\frac{dC_x}{dt} + \frac{1}{V}\frac{dV}{dt} \tag{2.7}$$

2.5.2 Mass Balance Equation for Substrate in Semi-Batch Reactor

In the semi-batch systems selected substrate(s) is (are) continuously fed the bioreactor; therefore the material balance equation is written as follows:

$$\frac{d(C_s V)}{dt} = Q_s C_{s_0} + r_s V \tag{2.8}$$

where r_S is the rate of consumption of glucose, and Q_S is the feed stream flow rate. The rate of consumption of a substrate can be expressed with a first-order kinetic equation with respect to the cell concentration as follows:

$$-r_s = q_s C_x \tag{2.9}$$

where q_s is the specific substrate consumption rate. As the glucose is not accumulated in the bioreactor ($(dC_s/dt) = 0$), and the volume change is negligible, the semi-batch fermentation system can be assumed quasi-steady state for glucose, and the material balance for glucose may be modified as:

$$\frac{Q_s}{V} C_s - q_s C_x = 0 \qquad (2.10)$$

Hence the specific glucose consumption rate q_s is derived as:

$$q_s = \left(\frac{Q_s}{V}\frac{C_{s_o}}{C_x}\right) \qquad (2.11)$$

If the substrate is accumulated in the medium then: $C_s \neq 0$ and $dC_s/dt \neq 0$. Combining equation- 2.8 and -2.9 to express q_s:

$$q_s = \left(\frac{Q_s(C_s - C_{s_o})}{C_x V}\right) + \left(\frac{1}{C_x}\frac{dC_s}{dt}\right) \qquad (2.12)$$

Substrate consumption rate, r_s, can be expressed in terms of yield of cell on substrate, $Y_{X/S}$:

$$-r_s = (r_x/Y_{x/s}) \qquad (2.13)$$

Assuming substrate does not accumulate in the bioreactor ($dC_S/dt = 0$), if equation- 2.13 is inserted into equation- 2.8 the equation becomes:

$$Q_s C_{s_0} - \frac{r_x V}{Y_{x/s}} = C_s \frac{dV}{dt} \qquad (2.14)$$

Equations-2.2 and -2.5 are inserted into equation-2.14, and:

$$Q_s C_{s_0} - \frac{\mu C_x V}{Y_{x/s}} = C_s Q_s \qquad (2.15)$$

As the solution of the equation- 2.1 using the initial conditions is:

$$C_x V = C_{x_0} V_0 e^{\mu t} \qquad (2.16)$$

by combining the equations- 2.15 and -2.16, volumetric rate of the feed stream Q_S is derived as:

$$Q_s = \frac{\mu C_{x_0} V_0}{Y_{x/s}(C_{s_0} - C_s)} e^{\mu t} \quad (2.17)$$

If the substrate is not accumulated in the bioreactor production medium (C_S = 0), or if $C_{SO} >> C_S$ is assumed, equation-2.17 is reduced to:

$$Q_s = \frac{\mu C_{x_0} V_0}{Y_{x/s} C_{s_0}} e^{\mu t} \quad (2.18)$$

2.5.3 Mass Balance Equation for Product in Semi-Batch Reactor

The product is a batch- component which is synthesized in the cells and secreted to the bioreactor aqueous production medium; therefore, the mass balance equation for the product (rhGH) is as follows:

$$\frac{d(C_{rhGH} V)}{dt} = r_{rhGH} V \quad (2.19)$$

where r_{rhGH} is the recombinant human growth hormone formation rate which can be defined in the production region with a first order kinetic equation as follows:

$$r_{rhGH} = q_{rhGH} C_x \quad (2.20)$$

where, q_{rhGH} is the specific rhGH formation rate. Equation-2.19 is inserted into equation-2.18, and q_{rhGH} is derived as follows:

$$q_{rhGH} = \frac{1}{C_x}\left(\frac{dC_{rhGH}}{dt} + \frac{C_{rhGH}}{V}\frac{dV}{dt}\right) \quad (2.21)$$

2.5.4 Overall and Instantaneous Yield Coefficients

Yield coefficients that are the selectivity values in bioprocesses can be defined as the ratio of the rate of formation of molecules to the rate of consumption of the molecules. It can be mathematically expressed as the following equation:

$$Y_{x/s} = \frac{r_x}{-r_s} = \left(\frac{dC_x/dt}{-dC_s/dt}\right) \tag{2.22}$$

$$Y_{P/s} = \frac{r_x}{-r_s} = \left(\frac{dC_P/dt}{-dC_s/dt}\right) \tag{2.23}$$

Other than the instantaneous yield coefficient, overall yield coefficients are calculated for a period of time, which can be determined as:

$$Y'_{X/S} = \frac{r_x}{-r_s} = \frac{\Delta C_x/\Delta t}{-\Delta C_s/\Delta t} \tag{2.24}$$

$$Y'_{P/S} = \frac{r_P}{-r_s} = \frac{\Delta C_P/\Delta t}{-\Delta C_s/\Delta t} \tag{2.25}$$

Yield coefficients that are usually used in evaluating the performance of the bioprocess are tabulated, in the following Table 2.6:

Table 2.6 Yield coefficients and definitions

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

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

The chemicals that are used in this study were analytical grade and acquired from Sigma, Fluka and Merck.

3.2 Buffers and Stock Solutions

Buffers and stock solutions and their formulation and preparation are presented in Appendix A. Depending on the solution, sterilization was made by either autoclaving at 121 ^{o}C for 20 minutes or filtering through filters having 0.25 or 0.45 μm pore size (Millipore Corporation Bedford, MA, USA).

3.3 Microorganism

The microorganism used in this study, *P. pastoris* Mut+ strain, was constructed by Zerze (2012) after the amplification of hGH gene from *Escherichia coli* TOP10 that carries pPICZαA::*hGH* plasmid with zeocin resistance gene. *Pichia pastoris* wild type strain X-33 and the shuttle vector, pGAPZαA, which encloses zeocin resistance gene were obtained from Invitrogen. pGAPZαA vector is propagated by using *E. coli* DH5α and pGAPZαA::*hGH* plasmid was also constructed and amplified in *E. coli* DH5α. After that *P. pastoris* Mut+ strain that contains *GAP* promoter was developed. Schematic illustration is presented in Appendix B. The

microorganisms were kept in microbanks (Prolab Diagnostics) or 15% glycerol containing glycerol stocks at -55°C.

3.4 Growth Media

All growth media were sterilized by autoclaving 20 minutes at 121^{o}C and after cooling to about 50 ^{o}C, suitable antibiotics were added.

3.4.1 Solid Media

P. pastoris Mut+ cells that were stored in the microbanks at -55^{o}C were inoculated on YPD agar containing yeast extract, peptone, glucose and the antibiotic Zeocin. Cells were incubated at 30 ^{o}C for about 48 hours and kept at +4 ^{o}C. The composition is in the following table:

Table 3.1 Composition of YPD agar solid medium.

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

*Glucose was sterilized by filtering 0.20 μm and added to the medium after autoclave.

3.4.2 Precultivation Media

Pichia pastoris cells taken from the solid medium were inoculated to precultivation medium, buffered glycerol complex medium (BMGY). It contains the carbon source as glycerol and the antibiotic chloramphenicol. The cells were precultivated in 50 ml of BMGY in 250 ml baffled air-filtered shake bioreactors with

an agitation rate of 200 rpm at 30 ^{o}C temperature. The contents and their compositions are given in the Table 3.2:

Table 3.2 Composition of precultivation medium, BMGY

Compound	**Concentration (g L^{-1})**
Yeast extract	10
Peptone	20
Potassium phosphate buffer (pH=6.0)	0.1 M
Yeast nitrogen base (YNB)	3.4
$(NH_4)_2SO_4$	10.0
Biotin *	4 x 10^{-4}
Glycerol	10
Chloramphenicol (34 mg ml^{-1})*	1ml L^{-1}

*Biotin and chloramphenicol were added to the medium after autoclave.

3.4.3 Production Media

The production medium that were used in laboratory scale air filtered shake bioreactor and pilot-scale bioreactor is the basal salt medium (BSM). It contains calcium, potassium, magnesium, phosphor, sulphur and the carbon source. It also contains the Pichia trace minerals (PTM1). The ingredients and the compositions of BSM and PTM1 are in the Table 3.3 and Table 3.4.

Table 3.3 Production medium, Basal salt medium (BSM), for baffled air-filtered shake bioreactor

Compound	Concentration (g L^{-1})
Carbon source*	20**
$(NH_4)_2SO_4$	C/N =4.57
Potassium phosphate buffer pH=6	0.1 M
$MgSO_4.7H_2O$	14.9
$CaSO_4.2H_2O$	1.17
Chloramphenicol	1 mL
PTM1	4.35 mL

*Carbon source may be glucose, glycerol, fructose, sucrose, molasses, sorbitol, or mannitol

** For molasses the concentration was 40 g L^{-1}

Table 3.4 Production medium, Basal salt medium (BSM), for pilot-scale bioreactor

Compound	Concentration (g L^{-1})
85% H_3PO_4	26.7 ml
$CaSO_4.2H_2O$	1.17
$MgSO_4.7H_2O$	14.9
KOH	4.13
K_2SO_4	18.2
Glycerol	40.0
Chloramphenicol*	1 mL
20% antifoam*	1 ml
PTM1*	4.35 mL

* Chloramphenicol, antifoam and PTM1 were added after autoclaving.

Table 3.5 Composition of PTM1 trace salt solution (Sibirny *et al*., 1990)

Compound	Concentration (g L^{-1})
$CuSO_4.5H_2O$	0.6
NaI	0.008
$MnSO_4.H_2O$	0.3
$Na_2MoO_4.2H_2O$	0.02
H_3BO_3	0.002
$ZnCl_2$	2
$FeSO_4.7H_2O$	6.5
$CoCl_2.6H_2O$	0.09
H_2SO_4	0.5
Biotin	0.02

3.4.4 Feed Stream Composition

In the semi-batch phase of the fermentation, nitrogen and carbon sources were fed to the bioreactor. Nitrogen source was 25% ammonium hydroxide which also adjusted pH of the medium. Carbon source was glucose which induced the constitutive *GAP* promoter to produce rhGH. PTM1 was also fed together with the glucose solution. The concentration is 500 g glucose L^{-1} and 12 ml PTM1 L^{-1}. In one of the experiments pre-treated molasses was fed to the bioreactor. Its concentration was 440 g L^{-1}. Half of the molasses is sucrose and as it was inverted to glucose and fructose by acid hydrolysis. The concentration of the glucose and fructose in the feed stream was 110 g L^{-1}. In this experiment PTM1 was not added to the feed.

3.4.4.1 Acid Hydrolysis of Molasses

Molasses that was used in this study was taken from Ankara Beet Sugar Factory (Ankara, Turkey).

P. pastoris cannot utilize sucrose due to the lack of invertase enzyme (Stambuk *et al.,* 2000). Therefore it was pre-treated by acid hydrolysis to obtain 1:1 mixture of glucose and fructose. Acid hydrolysis was made with the following steps:

1. 440 g molasses was dissolved in 1 L ultra pure water.
2. The solution was centrifuged at 6000 g at +4 ^{o}C to get rid of the impurities and precipitates.
3. pH of the solution was reduced to 1.8 with 37% HCl at room temperature.
4. Acid hydrolysis pretreatment was performed for 3 hours in 90 ^{o}C water bath. In that stage sucrose was inverted to glucose and fructose.
5. After hydrolysis, pH was set to 5.0 by adding 5 M KOH solution since the pH of fermentation medium was also set to 5.0.

3.5 Recombinant Protein Production

Recombinant human growth hormone is produced by metabolically engineered *P.pastoris* carrying pGAPZαA::*hGH* using pilot-scale bioreactor.

3.5.1 Precultivation

The *P. pastoris* Mut+ strain cells were plated onto YPD agar solid medium (Table 3.1) and incubated at 30 ^{o}C. After 48-60 hours, a single colony was taken and inoculated into the precultivation medium which is buffered glycerol complex medium (BMGY) (Table 3.2). Using laboratory scale baffled air-filtered shake flasks, the precultivation was made at an agitation rate of 200 rpm and temperature of 30 ^{o}C for about 15-18 hours. When the cells reached OD_{600} of 2-6, which reveals that they were in the exponential phase, they were harvested by centrifuging 10 minutes at 1500g and 4 ^{o}C. The precipitated cells were collected and resuspended in

water to feed them into the production medium. The cells were fed to the bioreactor such that the initial OD_{600} = 1 was obtained which corresponded to C_{X0}= 0.275 g L^{-1}.

3.5.2 Recombinant Human Growth Hormone Production in Pilot-scale Bioreactor

In this study, a 3 L pilot-scale bioreactor (Braun CT2-2) was used that had a working volume of 1-2 L. Dissolved oxygen, pH, temperature, agitation rate, foam, and feed inlet rate can be controlled. The bioreactor is represented in Figure 3.1. For sensing dissolved oxygen it had an oxygen probe (Hamilton, Switzerland). Air was used as the main oxygen source and it supplied by a compressor. If the oxygen in the air was not sufficient, pure oxygen was fed through a mass flow rate controller. In the bioreactor there were four baffles and a sparger for distributing the oxygen homogenously in the fermentation medium. pH was also sensed by a pH probe (Hamilton, Switzerland) and for keeping it constant either base or acid solutions can be fed by peristaltic pumps. To keep the temperature, which was measured by a thermocouple, at the desired value, an external cooler and a steam generator were used. Steam generator was also the heat source for sterilization. Agitation was carried out by an electrical motor with two four-blade Rushton turbines. The substrates, acid and base solutions were fed through the inlet port by peristaltic pumps for semi-batch operations.

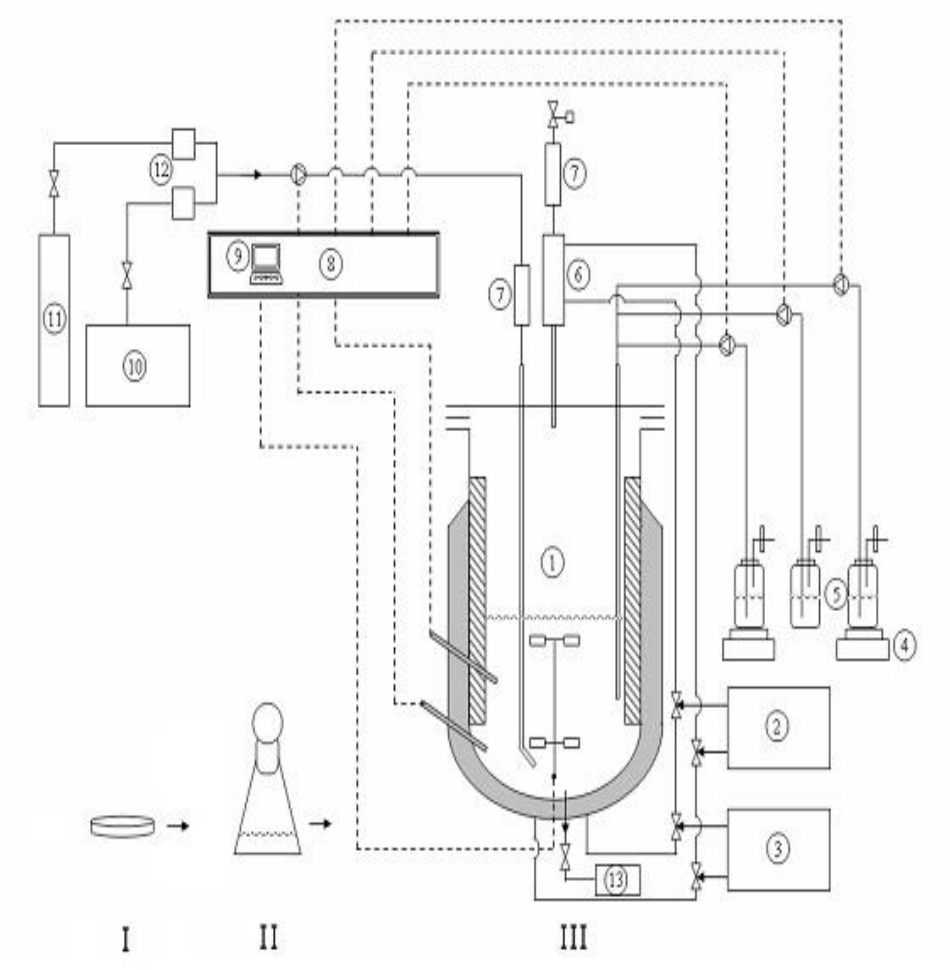


Figure 3.1 Precultivation steps and pilot-scale bioreactor system set up I. Solid medium containing inoculated cells from stock culture. II. Precultivation medium III. Pilot-scale bioreactor system. (1) Bio-reaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2 tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008)

3.5.2.1 Bioreactor Operation Parameters for Pilot-scale Bioreactor Operation

The fermentation was carried out at 30 ^{o}C which was kept constant by using external cooler and steam generator. pH value was adjusted to 5.0 by feeding 25% ammonium hydroxide solution that was also the nitrogen source for the cells. For homogenous and effective mass transfer, agitation rate was set to 900 rpm. Dissolved oxygen was 20% to avoid anaerobic fermentation. If the air feeding was not enough for keeping DO at 20%, pure oxygen was fed together with the air. 20% antifoam was used to avoid excess foaming. The bioprocesses were followed by sampling every 2 or 3 hours. The collected samples were centrifuged at +4 ^{o}C and 4000 rpm for 10 minutes for obtaining the supernatants, filtrates and pellets. These samples were stored at -55 ^{o}C.

3.5.2.2 Production Phases in Semi-batch Pilot-scale Bioreactor Operation

The cells grown in the precultivation medium were harvested and resuspended in the BSM so that they had an OD_{600} of 1 which represents 0.275 g L^{-1} cell density. This was the beginning of the first phase which is the glycerol batch phase. The production phases are explained as follows:

- **Glycerol Batch Phase (GB):** Inoculation of the cells was the starting of the glycerol batch phase. In this phase, glycerol was the carbon source since the purpose of this phase was to increase the cells population in the fermentation broth and glycerol was represented as a better substrate for the cell growth. There was no inlet or outlet stream during this stage of the bioprocess. GB was continued about 12-15 hours until the cells grew to a concentration about 10 g L^{-1} which corresponds the optical density of OD_{600} = 30 – 35.
- **Glucose Semi-batch Phase (GFB):** The feed stream containing 50% (w/v) glucose which was the substrate for inducing the production of rhGH and containing 12 ml L^{-1} PTM1, was fed continuously with a feeding strategy either pre-calculated exponential feeding or constant feeding. The objective of the glucose fed batch phase is to produce rhGH together with growing the *P. pastoris* cells since *GAP* promoter

is a constitutive promoter. Glucose accumulation should be avoided because high glucose concentrations may inhibit the expression of the r-protein and growth of the cells.

Using the pre-determined exponential growth rate, the feeding rate of the glucose solution was calculated with the following equation derived in Section 2.5.2:

$$Q_S(t) = \frac{\mu_0 C_{x_0} V_0}{Y_{X/S} C_{S_0}} \exp(\mu_0 t) \qquad (2.18)$$

In the equation- 2.18 μ_0 is the pre-determined growth rate (h^{-1}), V_0 is the initial volume of the fermentation medium (L), C_{X0} is the initial cell density (g L^{-1}), C_{S0} is the feed substrate (glucose) concentration (g L^{-1}), and $Y_{X/S}$ is the yield of cell on substrate (g g^{-1}). The pre-determined growth rate, μ_0, was altered in different experiments from 0.1 to 0.175 h^{-1}. The glucose concentration in the feed stream, C_{S0}, was 500 g L^{-1}. And the cell yield on glucose was taken as 0.5 g g^{-1} in the calculation of the amount of the feed.

3.6 Analyses

During the fermentation, samples were collected every 2 or 3 hours. From the sample itself the cell concentration was measured. Then the supernatants were obtained by centrifuging the samples and precipitating the pellets which included the cells at 4^{o}C and 4000 rpm for 10 minutes. This supernatant was used for determining the r-protein, glucose and protease concentration. The supernatants were filtered by 20 μm to get filtrates that were used in the HPLC to obtain the concentrations of the organic acids.

3.6.1 Cell Concentration

Pichia pastoris cell concentration was obtained by using UV-Vis Spectrophotometer (Thermo Spectronic, Hελιosα) at 600 nm, immediately after the sample was taken. Most of the time, the sample should be diluted since the reading range of the spectrophotometer is 0.1 to 0.9. For the calculation of the cell

concentration with the absorbance values the following equation was used (Orman, 2007):

$$Cx = OD_{600} x\ 2.75 x Dilution\ Factor \quad (3.1)$$

3.6.2 rhGH Concentration

The concentration of the product of the bioprocess, rhGH, was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) described by Laemmli (1970). 20 µl of supernantant and 10 µl sample loading buffer were mixed and by a thermocycler it was treated at 95 ^{o}C for 5 minutes and then kept on ice for 5 minutes. After that 15 µl of the samples and 3 µl of prestained protein molecular weight marker (Appendix C) were loaded to the wells of SDS-PAGE gels. The gels were run for about 45 minutes at 40 mA constant current. Then by silver staining of the gels, proteins and their compositions can be detected.

The SDS-PAGE analysis has three major parts which are preparation of the gel, gel electrophoresis and staining of the gels. The buffers and solutions required for SDS-PAGE analysis are presented in Appendix A. The analyses were done as described in the following part:

Preparation of SDS polyacrylamide gel:

1. The two glass plates which were used for the preparing the SDS gels were cleaned and assembled as described in the manufacturer's manuals.
2. In erlenmayer flasks 12% resolving gel and 5% resolving gel were prepared without NNN'N'- Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulphate (APS) solutions. The percent of the resolving gel changes according to the molecular weight of the analyzed protein. For analyzing rhGH 12% resolving gel is suitable which can be used 10-200 kDa range. The gel ingredients are given in Appendix A.
3. After the addition of APS and TEMED solutions to the resolving gel, the mixture was shaken vigorously. Then the solution was immediately poured by micropipette into the gel cast between the two glass plates.

Bubble formation and polymerization should be disallowed. There should be sufficient space for the stacking gel in the gel cast.

4. Isopropanol was added onto the resolving gel to flatten the gel surface.
5. The gel was left in a vertical position for polymerization for about 45 minutes. Isopropanol was removed and the space gap between the glasses was washed with distilled water and the water was removed using filter paper.
6. The preparation of the stacking gel was completed with the addition of APS and TEMED solutions. And the contents were shaken strongly and poured the space between the glasses left for the stacking gel. This should be made as soon as possible. The comb which formed the wells was also placed instantaneously and carefully to avoid polymerization and bubble formation. Stacking gel was generally polymerized in 25 minutes.
7. The comb was removed and the wells were washed with distilled water after the stacking gel polymerized. After drying with filter paper the SDS gels can be used instantly or can be kept at +4 ^{o}C with wrapping up wetted paper towel.

Gel Electrophoresis:

1. 10 µl loading buffer and 20 µl sample were mixed and treated at 95 ^{o}C for 5 minutes and then kept on ice for 5 minutes.
2. The setup for electrophoresis was prepared by placing the gels in the apparatus. And 1X SDS running buffer was put in the reservoir.
3. 3 µl of prestained protein molecular weight marker and 15 µl of the mixture of supernatant and sample loading buffer were loaded into the wells. At constant current of 40 mA, the gels were run for approximately 45 minutes.

Staining the SDS Gels (Blum *et al.*, 1987):

The final part of the SDS-PAGE analyses is the staining the gels. The required solutions and their compositions are given in Appendix A. The instructions are tabulated as:

Table 3.6 SDS-PAGE Silver staining instructions (Blum *et al.*, 1987)

	Treatment	Solution	Time	Comments
1	Fixing	Fixer	≥ 1 hour	Overnight incubation can be done
2	Washing	50% Ethanol	3X20 min	Should be fresh
3	Pretreatment	Pretreatment Solution	1 min	Should be fresh
4	Rinse	Distilled Water	3X20 sec	Time should be exact
5	Impregnate	Silver nitrate Solution	20 min	
6	Rinse	Distilled water	2X20 sec	Time should be exact
7	Developing	Developing Solution	~5 min	After the colors occurred water should be added to slow down the reaction.
8	Stop	Stop solution	>10 min	Gels can be kept in this solution.

3.6.3 Organic Acid Concentrations

Reverse phase high pressure liquid chromatography (HPLC) (Waters HPLC, Alliance 2695, Milford, MA) was used to measure organic acid concentrations. Capital Optimal ODS- 5μm column (Capital HPLC, West Lothian, UK) was used. Calibration curves, given in Appendix E, are obtained from the standard organic acid chromatograms. The organic acid concentrations were determined by calculating the areas under the corresponding peaks on the chromatogram. These calculated areas were then converted to concentrations by using calibration curves. The mobile phase was composed of 3.12% (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4. The samples that were analyzed and the mobile phases were filtered using 45 μm filters

(ACRODISC CR PTFE) and if necessary the samples were diluted with the mobile phase. For loading the samples to the systems vials are filled with 100-200 µl sample. The dissolved gas in the buffers and ultra-pure water was removed by degassing for 15 minutes with ultrasonic water bath. The system and the conditions are revealed in Table 3.7.

Table 3.7 Operation conditions of HPLC for organic acids concentration analyses

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

3.6.4 Protease Activity Assay

Proteolytic activity was evaluated by hydrolysis of casein. Although there are alkali, neutral and acidic protease activities, only the acidic activity was performed since the pH of the fermentation broth was 5.0. As the product was secreted to the extracellular medium, supernatants were used in this analysis. Hammerstein casein solution (0.5% w/v) was prepared in 0.05 M sodium acetate buffer (pH=5.0) for acidic proteolytic activity. 2 ml of this solution was added to 1 ml of the sample which was diluted sufficiently. Thereafter hydrolyzation was made for 20 minutes at

37 ^{o}C. 10% trichloroacetic acid (TCA) was added to stop the hydrolyzation reaction. The reaction mixture was put on ice for 20 minutes. After that it was centrifuged at +4 ^{o}C and 10500 g for 10 minutes to separate the layers of the mixture. This separated mixture was incubated at room temperature for 5 minutes. The absorbance of the supernatant of the mixture was read at 275 nm in UV-Vis spectrophotometer. The formation of 4 nmole tyrosine per minute corresponds to one unit of protease activity (Moon and Parulekar, 1991). The equation relating the absorbance to activity is as follows:

$$A = \left(\frac{Absorbance}{0.8 \times 1/\mu mol.\, cm^{-3}}\right)\left(\frac{1U}{4\ nmol/min}\right)\left(\frac{1}{20\ min}\right)\left(\frac{1000\ nmol}{1\mu mol}\right)\left(\frac{Dilution}{Factor}\right) \quad (3.2)$$

3.6.5 Glucose Concentration

The concentration of glucose was determined by using glucose analysis kit (Biasis, Ankara) based on the glucose oxidation method (Boyacı, 2005). Glucose oxidase converts D-glucose to gluconate and H_2O_2 occurs. This H_2O_2 reacts with 4-aminoantiprine and phenol to produce iminoquinone dye which can change the color of the solution. It can also be read in the UV/Vis Spectrophotometer at 505 nm. The amount of the iminoquinone dye is proportional to the glucose concentration which can be easily seen in the following reaction:

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

$$H_2O_2 + 4 - aminoantiprine + Phenol \xrightarrow{Peroxidase} iminoquinone + H_2O$$

Glucose analysis kit involves a glucose analysis reactive and buffer. The reactive consists of the enzymes glucose oxidase and peroxidase; and 4-aminoantiprine while the buffer consists of potassium dihydrogen phosphate and phenol. The samples which contain more than 2 g L^{-1} need to be diluted. 2.0 ml distilled water, 0.4 ml buffer, 0.05 ml reactive, and 0.05 ml sample were mixed and the mixture was kept at 37 ^{o}C for 20 minutes. After 20 minutes, absorbance of the mixture was read at 505 nm in UV/Vis Spectrophotometer. Calibration curve which

was plotted by using the standard glucose solutions was used to define the glucose concentration in the sample. The calibration curve is given in Appendix F.

3.6.6 Fructose Concentration

The fructose concentration in the fermentation medium was measured by cysteine-carbazol-sulfuric acid method as defined by Dische and Borenfreund (1951). The supernatant sample was added to 0.1 M HCl. Thereafter the prepared 1.8 ml of 70% (v/v) sulfuric acid, 60 µl of 1.5% cysteine in 37% HCl, and 60 µl of 0.12% (w/v) carbazol in 95% ethanol was introduced to the mixture in the given order. The fructose concentration was detected by UV-Vis spectrophotometer at 560 nm. Calibration curve for fructose analysis is given in Appendix G.

CHAPTER 4

RESULTS AND DISCUSSION

The objective of this study was to develop an effective feeding strategy to improve recombinant human growth hormone (rhGH) production by *Pichia pastoris* Mut+ strain carrying pGAPZαA::*hGH*. For this purpose, eight semi-batch bioreactor operation strategies were designed in order to investigate the effects of carbon sources and feeding profiles on the cell growth and r-protein production. As the carbon source either glucose or molasses were used. The feeding strategies were designed using equation 2.18 for pre-calculation of the feed flow rate as a function of the cultivation time by using: ***i)*** three different pre-determined specific growth rates of μ_0=0.10, 0.15, and 0.175 h^{-1}; ***ii)*** two constant flow rates of glucose, Q(t≥15 h) =12 ml h^{-1} or Q(t≥11 h) =24 ml h^{-1}; ***iii)*** and, specific glucose consumption rate, q_S=0.05 g g^{-1} h^{-1}. Throughout the experiments the substrate, cell, rhGH, organic acid concentrations, protease activities were measured and the fermentation characteristics such as specific rates and yield coefficients were calculated in order to determine the efficiency of the designed feeding strategies.

4.1 Strategy Design

The abbreviations and explanations of eight semi-batch operation strategies designed were given in Table 4.1 and continuous feed profiles were demonstrated in Figure 4.1, Figure 4.2a and 2b, and Figure 4.3. In all bioreactor experiments, except BR-8, a feed stream containing 50% (w/v) glucose and 12 ml L^{-1} PTM1 was used. In BR-8 pre-treated molasses solution, with a concentration of 440 g L^{-1}, was used as the substrate containing 110 g L^{-1} glucose and 110 g L^{-1} fructose. The DO oxygen level was fixed to 20% of air saturation except the experiment of BR-2. In BR-2, DO

concentration was kept at 40-50 %. The other bioreactor operation conditions were T=30^{o}C, pH=5.0 and N=900 rpm for all the experiments.

In the first feeding strategy (BR-1), glucose stock solution was fed to the bioreactor (equation-2.18) with the pre-determined specific growth rate of μ_0 = 0.3 h^{-1}, within t=0-6 h and then with the pre-calculated specific glucose consumption rate (equation-2.11) of q_G=0.05 g g^{-1} h^{-1}, within t=6-18 h according to the result of Zerze (2012), as higher specific rhGH formation rates were obtained when specific glucose consumption rate was around q_G=0.05 g g^{-1} h^{-1}.

Second semi-batch bioreactor strategy (BR-2) was designed to decrease the cultivation time required to achieve the highest rhGH concentration. In this context, glucose was fed with a pre-calculated Q(t) based on the pre-determined specific growth rate of μ_0 = 0.15 h^{-1}, within t=0-15 h. After the cell concentration reached to C_X=55 g L^{-1}, glucose was started to feed constantly with the volumetric flow rate of Q(t≥15 h) =12 ml h^{-1}. In BR-3, the pre-determined specific growth rate based exponential feeding with μ_0 = 0.15 h^{-1} within t=0-11 h was followed by constant feeding with the volumetric flow rate of Q(t≥11 h) =24 ml h^{-1},within t=11-18 h in order to investigate the effect of 2-fold higher constant flow rate on the cell growth and r-protein production.

The feeding profiles of first three experiments, BR-1, BR-2, and BR-3 were illustrated in Figure 4.1 and the corresponding feeding profiles in terms of mass flow rates of glucose is represented in Appendix I.

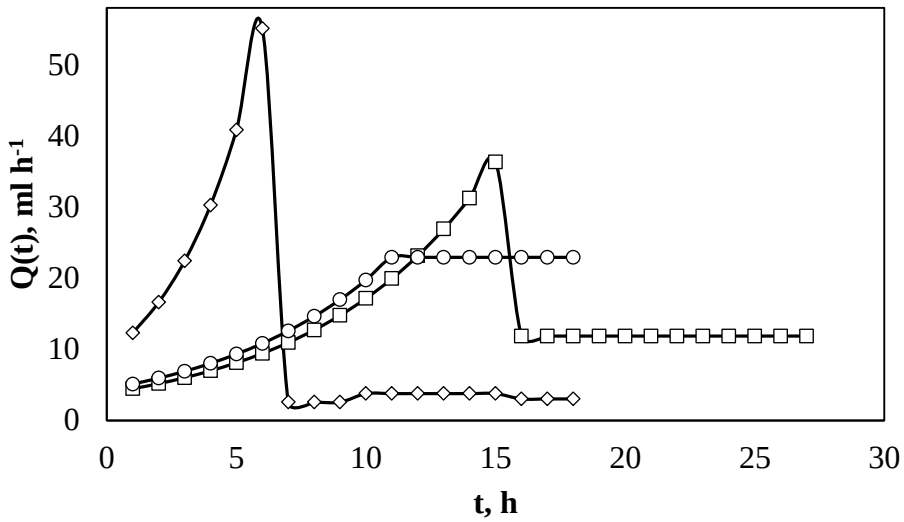


Figure 4.1 Feeding profiles of glucose in BR-1 (◊), BR-2(□), and BR-3 (○)

Four following semi-batch bioreactor operation strategies, BR-4, BR-5, BR-6, and BR-7, were performed to investigate the effects of pre-determined specific growth rates on semi-batch bioreactor operation conditions design, and to test the results reported on r-protein production by using glucose fed *GAP* driven *P. pastoris* expression systems (Zhao et al., 2008; Baumann *et al*., 2008; Ragon *et al*., 2008, Garcia-Ortega *et al*., 2013). Therefore exponential feeding was conducted throughout the semi-batch operation at the pre-determined specific growth rates of μ_0=0.10, 0.15, and 0.175 h^{-1} in BR-4, BR-5, and BR-6, respectively. On the other hand in BR-7, the pre-determined specific growth rate was μ_0 =0.175 h^{-1} within $0 \leq t < 6$ h, thereafter glucose was fed to bioreactor with a calculated Q(t) based on μ_0 =0.15 h^{-1} within $6 \leq t < 19$ in order to achieve continuous increase in r-protein concentration through the operation and decrease operation time. Figure 4.2a demonstrates the feeding profiles of BR-4, BR-5, BR-6, and BR-7.

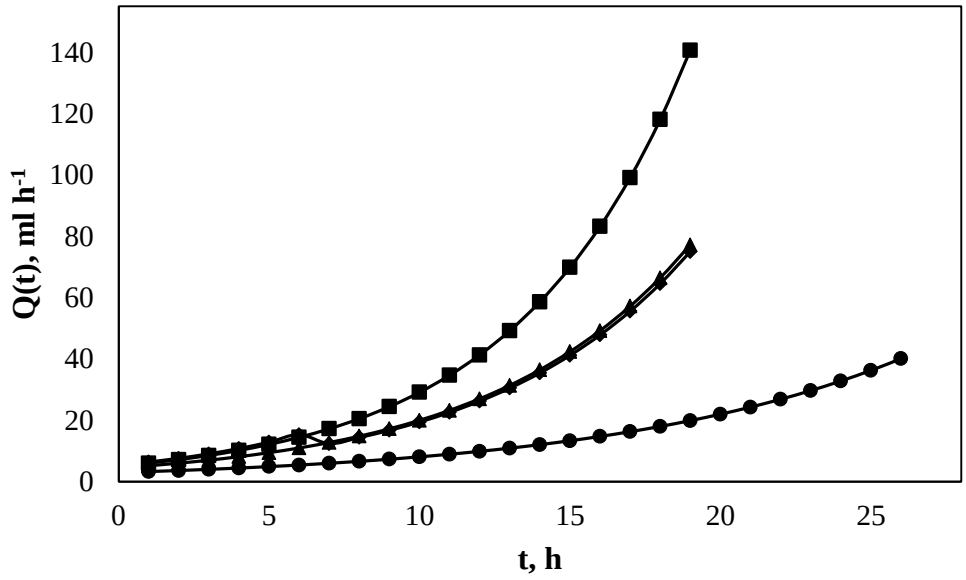


Figure 4.2a Feeding profiles of glucose in BR-4 (●), BR-5(▲), BR-6 (■) and BR-7 (♦)

The corresponding feeding profiles in terms of mass flow rate of glucose, F(t) is presented in Figure 4.2b as:

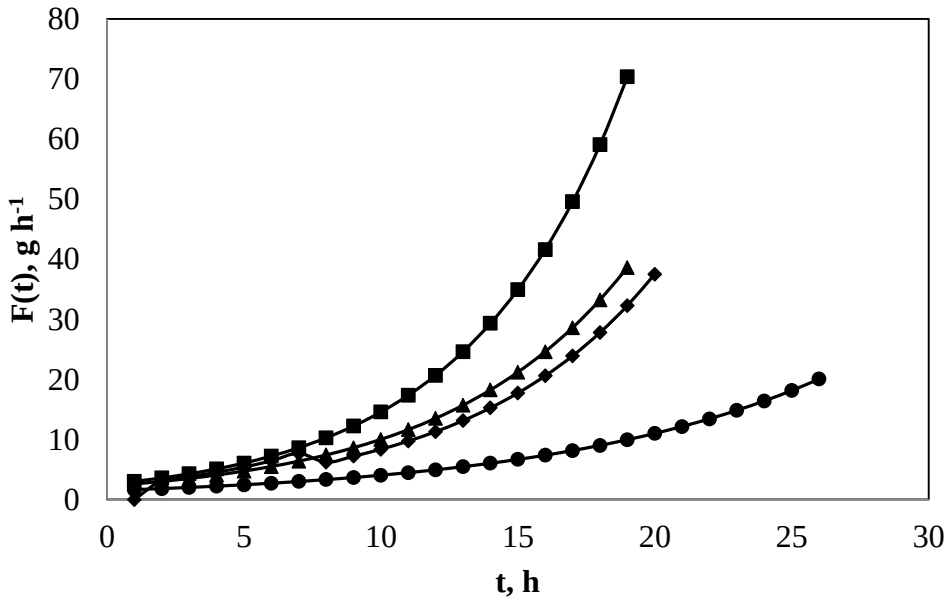


Figure 4.2b Feeding profiles of glucose in terms of mass flow rate of glucose in BR-4 (●), BR-5(▲), BR-6 (■) and BR-7 (♦)

In the last feeding strategy (BR-8), pre-treated molasses was used as the carbon and energy source and fed to the bioreactor with the pre-determined specific growth rate, μ_0 =0.15 h^{-1} as in BR-5. In the calculation of required substrate amount, both glucose and fructose were considered as the carbon sources utilized by *P. pastoris*. Feeding profile of molasses with the cultivation time was shown in Figure 4.3 and the corresponding feeding profile in terms of mass flow rate of molasses is given in Appendix I.

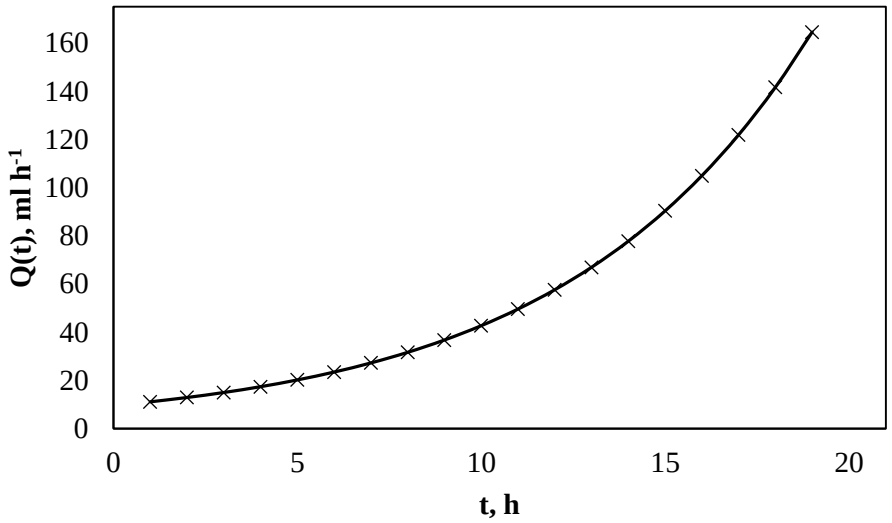


Figure 4.3 Feeding profile of pre-treated molasses in BR-8

The summary of the designed semi-batch feeding strategies is presented in Table 4.1.

Table 4.1 Summary of the designed semi-batch bioreactor feeding strategies

Strategy Name	Semi-batch Feeding Strategy Definition
BR-1	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.3 h^{-1} within 0≤ t <6 h , and then constant feeding based on q_G=0.05 g g^{-1} h^{-1} within 6≤ t <18 h
BR-2	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.15 h^{-1} within 0≤ t <15 h, and then constant feeding at a flow rate of 12 ml h^{-1} within 15≤ t <27 where DO level was kept 40-50 %
BR-3	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.15 h^{-1} within 0≤ t <11 h, and then constant feeding at a flow rate of 24 ml h^{-1} within 11≤ t <18 h
BR-4	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.10 h^{-1} within 0≤ t <26 h
BR-5	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.15 h^{-1} within 0≤ t <19 h
BR-6	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.175 h^{-1} within 0≤ t <19
BR-7	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.175 h^{-1} within 0≤ t <6 h , shifted to second exponential glucose feeding based on μ_0 = 0.15 h^{-1} within 6≤ t <19 h
BR-8	Pre-calculated exponential glucose feed flow rate using μ_0 = 0.15 h^{-1} within 0≤ t <19 h, using pre-treated molasses that contains equimolar glucose and fructose

4.2 Substrate (Glucose) Consumption

Glucose was not detected in the fermentation broth throughout the processes except BR-7. Thus, all the glucose fed to the bioreactor was immediately utilized by *P.pastoris* cells for the growth and r-protein production. Contrariwise, in BR-7, glucose concentration reached to C_G=29 g L^{-1} at t = 19 h. Although glucose concentration reached to a high glucose concentration due to high feeding rate, where half of glucose fed to the bioreactor was consumed by the cells.

Variations in the specific glucose consumption rates (q_G) with the cultivation time are given in Table 4.2. The highest q_G value was attained as q_G =1.042 g g^{-1} h^{-1} at t = 6 h in BR-1 in which glucose was fed at μ_0 = 0.3 h^{-1}; and then in BR-6 strategy with a value of q_G =0.729 g g^{-1} h^{-1} at t = 17 h; contrariwise, the lowest value was obtained as q_G = 0.039 g g^{-1} h^{-1} in BR-1 in which the feeding was made with a q_G of 0.05 g $g^{-1}h^{-1}$ which is 1.28-fold lower than the pre-calculated q_G value. These results shows that *P. pastoris* can utilize glucose with a specific glucose consumption rate of q_G=1.062 g g^{-1} h^{-1}. The trend of the specific glucose consumption rates was dependent on the feeding strategy as expected; however, after the upper limit value of μ_0 = 0.175 h^{-1} the cells were not be able to metabolize high amounts of glucose fed to the bioreactor after a certain time (t=17 h).

4.3 Fructose Concentration

As molasses contains 50% glucose and 50% fructose, variations in glucose and fructose concentration with the cultivation time were followed through the bioprocess, in BR-8. Glucose was not detected in the fermentation broth; however, fructose accumulated from the beginning of the semi-batch phase which is represented in Figure 4.4. As seen in Figure 4.4 fructose concentration was increased linearly within t=0-12 h, after t=12 h fructose concentration was increased with a higher slope within t=12-19 h. Although most of the fructose fed was utilized by the cells, it was accumulated during the process which implies that fructose was not utilized as much as glucose. At the end of the fermentation, t=19 h, almost 50% of

the fructose fed was accumulated in the medium. This accumulation may be because of glucose inhibition since glucose inhibits the utilization of fructose.

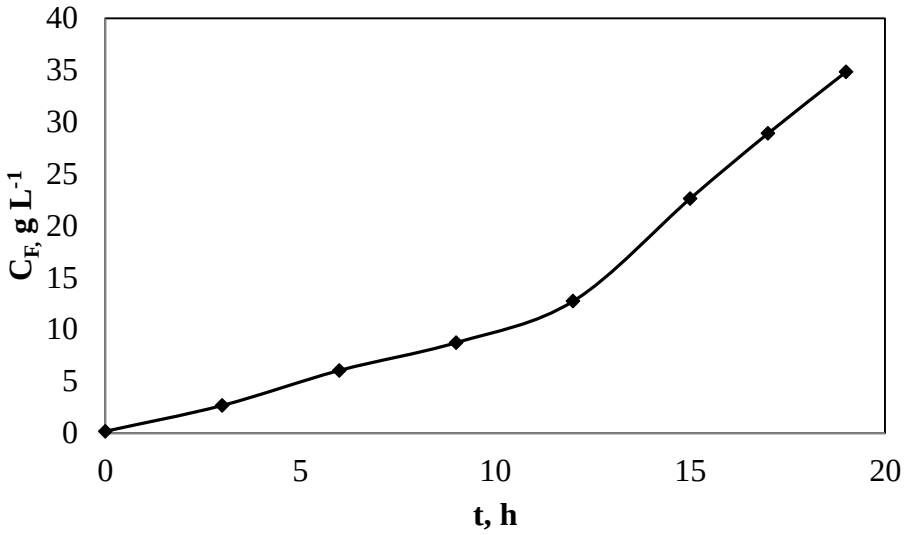


Figure 4.4 Concentration profile of fructose in BR-8 bioreactor experiment

Table 4.2 Variations of the specific rates of growth, glucose consumption and rhGH production with the cultivation time

Experiment	t h	μ h^{-1}	q_G $g\ g^{-1}h^{-1}$	q_{rhGH} $mg\ g^{-1}\ h^{-1}$
BR-1	1	0.183	0.609	0.149
	3	0.117	0.618	0.187
	6	0.194	1.042	0.104
	9	0.018	0.033	-0.046
	12	0	0.046	-0.035
	15	0	0.047	-0.038
	18	0	0.039	-0.037
BR-2	1	0.239	0.250	-0.063
	3	0.133	0.200	-0.068
	6	0.086	0.222	-0.031
	9	0.117	0.273	-0.038
	12	0.156	0.311	-0.002
	15	0.108	0.321	0.005
	18	0.008	0.082	-0.003
	21	0.012	0.080	0.000
	24	0.020	0.077	-0.001
	27	0.019	0.073	-0.001
BR-3	1	0.149	0.282	1.282
	3	0.114	0.263	0.376
	6	0.205	0.304	0.789
	9	0.074	0.290	0.684
	12	0.088	0.312	0.921
	15	0.075	0.244	0.382
	18	0.065	0.199	0.331

Table 4.2 Variations of the specific rates of growth, glucose consumption and rhGH production with the cultivation time (continued)

Experiment	t h	μ h^{-1}	q_G $g\ g^{-1}\ h^{-1}$	q_{rhGH} $mg\ g^{-1}\ h^{-1}$
BR-4	1	0.093	0.180	0.940
	3	0.095	0.159	0.469
	6	0.088	0.167	0.753
	9	0.079	0.178	0.148
	12	0.062	0.193	0.187
	15	0.058	0.217	0.549
	18	0.093	0.246	0.255
	21	0.047	0.255	0.448
	24	0.057	0.293	0.277
	26	0.073	0.308	0.349
BR-5	1	0.158	0.278	0.734
	3	0.120	0.256	0.745
	6	0.225	0.292	1.524
	9	0.124	0.268	0.558
	12	0.117	0.297	0.798
	15	0.115	0.330	1.404
	17	0.024	0.360	0.718
	19	0.035	0.451	0.853
BR-6	1	0.180	0.317	1.976
	3	0.112	0.284	0.487
	6	0.164	0.356	1.418
	9	0.067	0.389	1.073
	12	0.106	0.527	0.629
	15	0.102	0.626	-0.524
	17	0.040	0.729	0.076
	19	0.054	0.577	0.128

Table 4.2 Variations of the specific rates of growth, glucose consumption and rhGH production with the cultivation time (continued)

Experiment	t h	μ h-1	q_G g $g^{-1}h^{-1}$	q_{rhGH} mg g^{-1} h^{-1}
BR-7	1	0.162	0.331	1.895
	3	0.125	0.312	0.504
	6	0.138	0.378	0.931
	9	0.083	0.289	1.243
	12	0.110	0.352	0.257
	15	0.099	0.397	0.589
	17	0.066	0.447	-0.564
	19	0.073	0.519	-0.526
BR-8	1	0.164	0.145	0.894
	3	0.120	0.131	0.938
	6	0.088	0.146	0.699
	9	0.111	0.174	0.277
	12	0.026	0.192	0.741
	15	0.005	0.264	1.582
	17	0.025	0.377	-0.602
	19	0.042	0.464	-0.581

4.4 Cell Concentration Profiles

The variations in cell concentration with the cultivation time in designed semi-batch bioreactor operation strategies were presented in Figure 4.5. The highest cell concentration was obtained as C_X = 76 g L^{-1} in BR-2 at t = 27 h. In BR-1 and BR-3 strategies in which glucose was fed with q_G=0.05 g g^{-1} h^{-1} and constant feeding rate to the bioreactor with Q(t≥11 h) =24 ml h^{-1}, respectively, the highest cell

concentrations were obtained as 42 g L^{-1} at t = 18 h and 54 g L^{-1} at t = 18 h, respectively. The cell concentration profile did not change for 7 hours of constant feeding in BR-3, t=11-18 h. The reason for that may be the glucose flow rate was higher, 2-fold of the BR-2, so that growth was not affected by glucose limitation like in the case of BR-2. In bioprocesses with μ_0 based feeding strategies, the highest cell concentrations were C_X = 60, 67, 51, and 57 g L^{-1} in BR-4, BR-5, BR-6 and BR-7, respectively. This values were attained at the last hours in BR-3, at t = 26 h, and BR-7, at t = 19 h. However the highest cell concentrations were determined at t = 17 h in BR-5 and BR-6 strategies. The cell concentration profile obtained at BR-7 was not affected from the step decrease from μ_0 = 0.175 h^{-1} to μ_0=0.15 h^{-1}. The reason for the decrease in the cell density in BR-5 and BR-6 may be due to the cells reached to deceleration or stationary phase or the increase in the volume by feeding stream since the concentration is inversely proportional to volume. In BR-5 and BR-6, exponential feeding with μ_0=0.15 $h^{-1}$and μ_0=0.175 h^{-1}, the amount of feed that was fed into the bioreactor in the last hours of the process significantly affected the volume of fermentation medium. Lastly in BR-8, the cell concentration did not reach to high levels, where molasses was used as substrate, as much as BR-5 in which the feeding was performed with the same pre-determined specific growth rate of μ_0=0.15 h^{-1}. The maximum concentration was attained as C_X = 29 g L^{-1} at t = 12 h. The reasons may be the same reason for the cases for BR-5 and BR-6, increase in the volume of fermentation medium was considerably high. Moreover, it seems that fructose was not utilized as much as glucose by the cells for growth since fructose was not effectively utilized like glucose and it was accumulated in the bioreactor up to a concentration of 35 g L^{-1} at t=19 h. Further, the absence of PTM1in the feed stream can also lead to lower cell growth.

The specific cell growth rates for the designed strategies were given in Table 4.2. For all the experiments, the specific growth rates were lower than the pre-determined growth rates. The highest specific growth rate for *P.pastoris* Mut+ strain cells that are producing rhGH under the control of *GAP* promoter attained in this study was μ_{max}=0.225 h^{-1} at t=6 h in BR-5. The specific growth rates obtained in BR-1, BR-2 and BR-3 after glucose was fed at a constant flow rate (t ≥6 h for BR-1, t ≥15 h for BR-2 t ≥11 h for BR-3) were lower than the specific growth rates

obtained in the other strategies where glucose was fed exponentially the with pre-determined specific growth rates(μ_0). Comparing the BR-2 and BR-3, as the constant flow rate of the feed was 2-fold in BR-3, the specific growth rates were obtained about 5-fold higher. The pre-determined specific growth rates were not attained in BR-4, BR-5, BR-6, BR-7 and BR-8. But with the increase of the pre-determined specific growth rates, the experimentally obtained specific growth rates also increased in the early stages of the processes. However at the later stages a sharp decrease in specific growth rates was observed in BR-5 and BR-8. In BR-4 and BR-6 it was seen that the growth was sustained during the process with a lower decrease in specific growth rate.

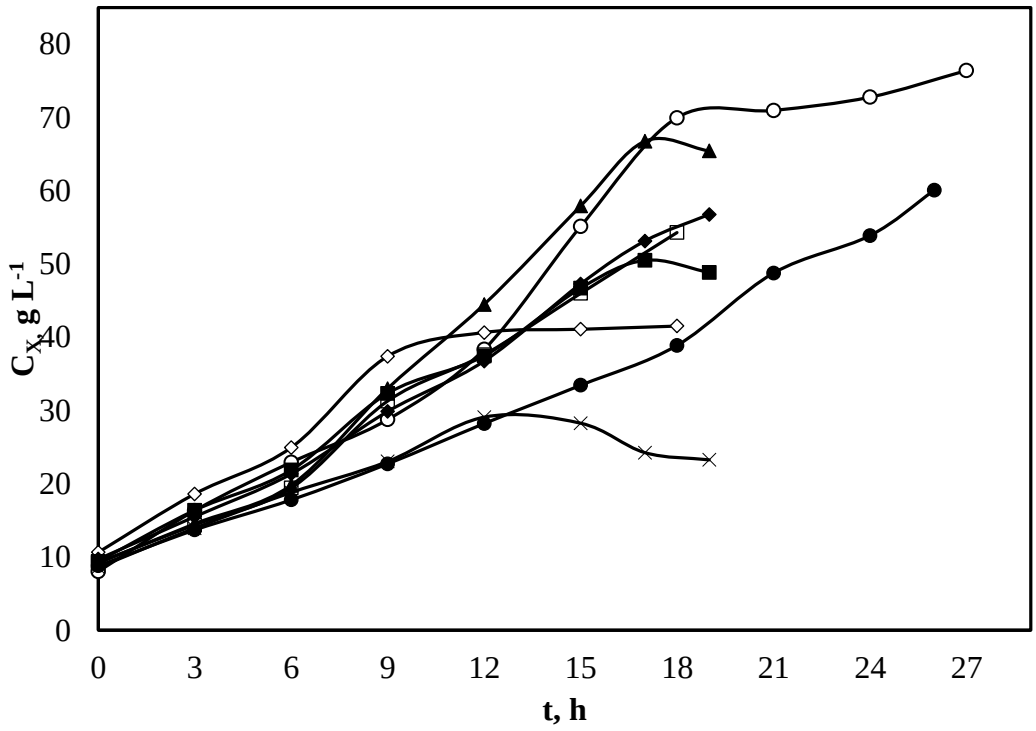


Figure 4.5 Cell concentration profiles in bioreactor experiments, BR-1 (◊), BR-2 (○), BR-3(□), BR-4 (●), BR-5(▲), BR-6(■), BR-7 (♦), BR-8 (x).

4.5 rhGH Concentration Profiles

The variations in rhGH concentration with the cultivation time in semi-batch bioreactor operation strategies designed were presented in Figure 4.6. In BR-1 strategy, the highest rhGH concentration was obtained as C_{rhGH} = 26 mg L^{-1} at t = 9 h. Although the cell growth was continued throughout the process, rhGH was not expressed at high levels showing that glucose was not sufficient for the production of rhGH. And after t =9 h, the rhGH concentration decreased as the protease enzymes formed in the bioprocess hydrolyze rhGH. In BR-2, glucose was fed at μ_0 = 0.15 h^{-1} at t=0-15 h and then at t=15-27 h it was fed at a flow rate of 12 ml h^{-1}. Although this strategy should be appropriate for the cell growth and r-protein production, rhGH was not expressed by the cells because of the inhibition effect of high oxygen concentrations r-protein production. The highest rhGH concentration was obtained at C_{rhGH} = 12 mg L^{-1} at t = 0 h which was produced in the batch mode of the bioprocess in which glycerol was used as the substrate. In BR-3, the highest rhGH concentration was obtained as C_{rhGH} = 337 mg L^{-1} at t = 18 h. Increase in the cell and r-protein concentrations were continued until the end of the process implying that glucose limitation was not observed in the constant glucose feeding, with Q(t≥11 h) =24 ml h^{-1}.

The highest rhGH concentration was achieved as C_{rhGH} = 508 mg L^{-1} at t = 19 h in BR-5 in which μ_0 = 0.15 h^{-1} throughout the bioprocess. On the other hand, the highest rhGH concentrations were obtained as C_{rhGH} = 313, 381, and 357 mg L^{-1} in BR-4 at t = 26 h, BR-6 at t = 15 h and BR-7 at t = 17 h, respectively. Among the designed strategies, for sustainable and high rhGH production BR-5 in which glucose was fed with a pre-determined specific growth rate of μ_0 = 0.15 h^{-1} was found as the best strategy. However, in BR-6, as the highest rhGH concentration was reached at an earlier cultivation time but with a cost of 1.3-fold decrease in C_{rhGH}.

As the fructose was not utilized effectively in BR-8, the expression level of rhGH was low similar to the cell growth profile. Although a complex medium, molasses, was used, the highest attained rhGH concentration in BR-8 was 239 mg L^{-1} at t = 17 h. The significant rise in the volume and the lack of PTM1 in the feed

stream also enhanced this low concentration of rhGH compared to the BR-5where glucose was fed with $\mu_0 = 0.15\ h^{-1}$.

The specific rhGH production rates (q_{rhGH}) for the designed strategies were given in the Table 4.2. q_{rhGH} values were relatively high at the beginning of the bioprocess and then decreased. The specific production rates, q_{rhGH}, in BR-1 and BR-2 were very small since rhGH production was low. Specifically after the feeding regimes were changed, the q_{rhGH} became significantly less than the previous amount. In BR-3 the alteration of the feeding at t =11 h q_{rhGH} was decreased about 2- fold. Therefore the objective of the change of the feeding regime, which was the shifting the cells to production rather than the cell growth, did not succeed. The cells continued to grow and production was not favored. The maximum q_{rhGH} attained in this study was 1.976 mg g^{-1} h^{-1} in BR-6 at the beginning of the process. After BR-6, higher q_{rhGH} values were obtained in BR-7 as 1.895 mg g^{-1} h^{-1}, BR-5 as 1.524 mg g^{-1} h^{-1} and BR-4 as 0.940 mg g^{-1} h^{-1}. Specific production rate values generally reduced throughout the processes which indicated that cells in the exponential phase can produce more r-proteins than the cells in the deceleration or stationary phase.

To conclude, the highest concentration was achieved in BR-5 as 508 mg L^{-1} at t =19 h and the process, in which exponential feeding was performed with $\mu_0 = 0.15$ h^{-1}, is the best strategy for rhGH production. However, 381 mg h^{-1} rhGH was produced at t=15 h in BR-6, exponential feeding with μ_0 =0.175 h^{-1}, and shorter process time makes this process favorable. Applying first exponential feeding μ_0 = 0.15 h^{-1} for t=0-6 h then with μ_0 0.175 h^{-1} for the rest t=6-19 h did not produce the expected amount of rhGH in BR-7.

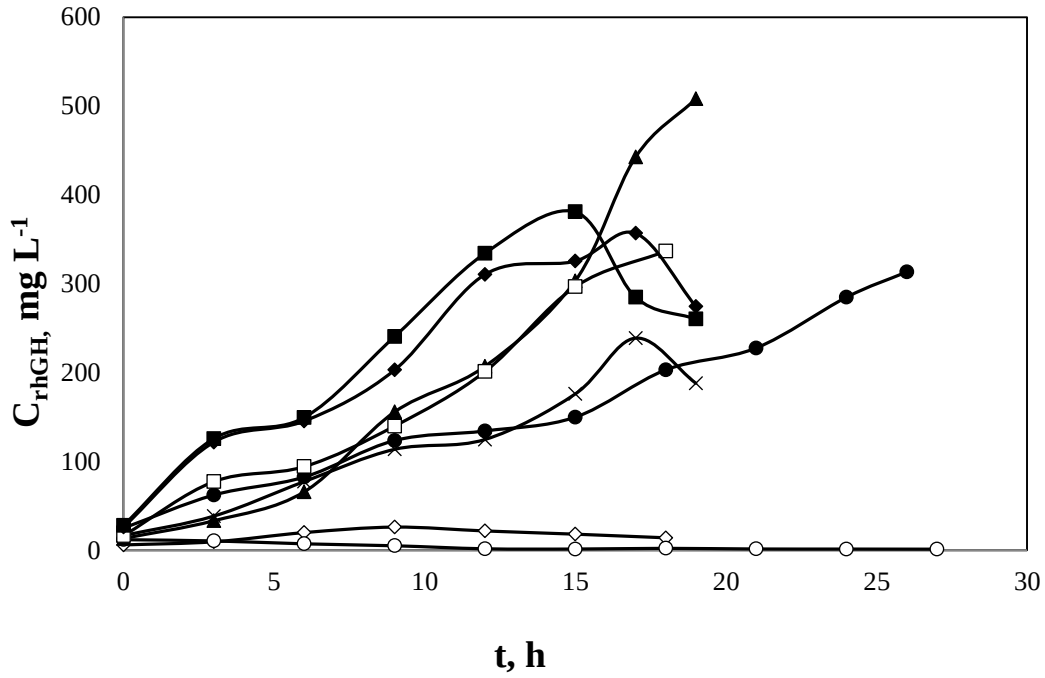


Figure 4.6 Concentration profiles of rhGH in bioreactor experiments, BR-1 (◊), BR-2 (○), BR-3(□), BR-4 (●), BR-5(▲), BR-6(■), BR-7(♦), and BR-8 (x).

4.6 Protease Activity Assay

The variations in protease concentrations with the cultivation time in semi-batch bioreactor operation strategies applied were given in Figure 4.7, except BR8. The highest protease concentration was determined in BR-3 as 8.5 mg L^{-1}. In BR-4 and BR-6 strategies, 8.0 mg L^{-1} protease was produced. On the other hand, the smallest protease concentration value was attained in BR-2 as C_{pro}=3.7 mg L^{-1}. Comparing the same hours (t=3, 9, 15 and 19 h) of BR-4, BR-5 and BR-6, exponential glucose feeding with μ_0=0.10, 0.15 and 0.175 h^{-1}, respectively, protease concentration was increased with the increase in predetermined specific growth rate, μ_0. On the other hand the highest concentrations of proteases were measured in the last hours of the bioprocesses (t=18h for BR-1 and BR-3; t=21 for BR-2; t=26 for BR-4; and t=19 for BR-5, BR-6, and BR-7). Moreover, as the processes extended, the protease concentration increased exponentially.

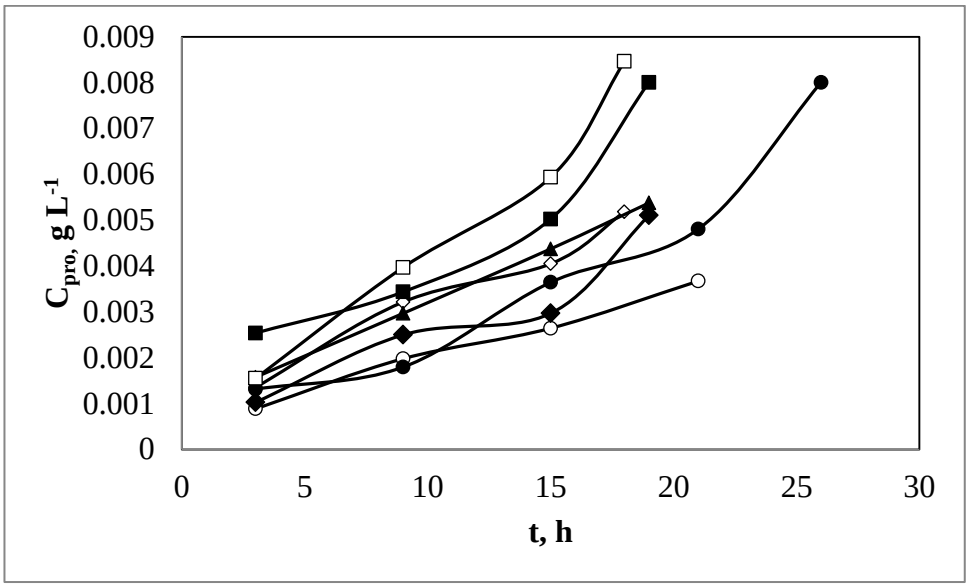


Figure 4.7 Concentration of protease enzymes for BR-1 (◊), BR-2 (○), BR-3(□), BR-4 (●), BR-5(▲), BR-6(■), and BR-7(♦).

4.7 Organic Acid Concentration Profiles

The variations in organic acid concentrations with the cultivation time in semi-batch bioreactor operation strategies applied were given in Table 4.3, except BR8. The organic acids detected in all of the experiments were gluconic acid, formic acid, malic acid, lactic acid, acetic acid, maleic acid, fumaric acid, succinic acid, pyruvic acid and oxalic acid. Citric acid was detected in BR-2, BR-, BR-4 and BR-8.

Gluconic acid is produced from the oxidation of gluconate which is formed by the oxidation of glucose, in the biochemical reaction network of *P. pastoris*. Gluconic acid concentrations were higher in BR-1, BR-2, BR-3, BR-4 and BR-5 indicating that some of the glucose did not enter the glycolysis pathway, instead oxidized and converted toe gluconic acid. Glucose induces r-protein production under *GAP* promoter when it enters the glycolysis pathway. The yield of substrate on rhGH values in BR-1, BR-2, BR-3, BR-4 and BR-5 strategies, are lower since glucose was converted to gluconate rather than entering the glycolysis pathway. The concentration of gluconic acid was measured between 0 to 10.8 g L^{-1}, and the maximum concentration was in BR-5 where glucose was fed with μ_0=0.15 h^{-1}.

Lactic acid concentrations were between 0.3 to 11 g L^{-1} in the experiments except BR-5. In BR-5, in which the highest rhGH concentration was obtained, lactic acid concentration was only detected in t=19 as 0.29 g L^{-1}. As lactic acid is mostly produced in oxygen limited bioprocesses where TCA cycle cannot occur effectively, it can be concluded that TCA cycle in BR-5 took place more efficiently than the other strategies.

Formic acid concentrations were higher at the beginning of the process (t=3h); thereafter decreased showing that formic acid formed due to glycerol metabolism in the batch phase of the fermentation processes utilized in the semi-batch operation mode for all strategies. However, in BR-4, BR-5, BR-6 and BR-7 strategies, formic acid concentrations were also increased with the cultivation time. In BR-1, BR-2 and BR-3 strategies formic acid concentration did not increase with the cultivation time.

Other organic acids detected in the medium were oxalic acid, pyruvic acid, acetic acid, citric acid, succinic acid and maleic acid. These organic acids are TCA cycle metabolites. Their concentrations were lower than the that of gluconic acid, formic acid, and lactic acid (Table 4.3).

Table 4.3 Variations in organic acid concentrations with the cultivation time for different feeding strategies in g L^{-1}

	Strategy, t(h)	3 h	9h	15 h	18 h
BR-1	**Gluconic acid**	2.0624	2.6017	1.9737	4.3861
	Formic acid	0.8268	-	-	0.4910
	Malic acid	0.8141	0.1356	0.1559	0.7838
	Lactic acid	3.3705	0.7633	0.7353	1.2826
	Acetic acid	0.1048	0.2673	0.5458	0.9478
	Maleic acid	0.0010	0.0011	0.0011	0.0008
	Citric acid	-	-	-	-
	Fumaric acid	0.0024	0.0028	0.0025	0.0038
	Succinic acid	-	0.2405	0.2948	0.6448
	Pyruvic acid	0.1245	0.0366	0.0401	0.0629
	Oxalic acid	0.1243	0.1721	0.1905	0.2541

	Strategy, t(h)	3 h	9h	15 h	21 h
BR-2	**Gluconic acid**	2.1139	3.0786	1.3762	2.6678
	Formic acid	5.0094	0.2051	0.2957	0.4173
	Malic acid	1.5348	0.1725	0.1855	0.1996
	Lactic acid	0.3353	0.4200	0.3978	0.5312
	Acetic acid	0.0680	0.1869	0.1098	0.1133
	Maleic acid	0.0117	-	0.0006	0.0004
	Citric acid	-	-	-	0.3526
	Fumaric acid	0.0008	0.0014	0.0008	0.0011
	Succinic acid	-	0.1739	0.2685	0.4498
	Pyruvic acid	-	0.0492	0.0311	0.0221
	Oxalic acid	0.0878	0.1279	0.1143	0.1427

Table 4.3 Variations in organic acid concentrations with the cultivation time for different feeding strategies in g L^{-1} (continued).

	Strategy, t(h)	3 h	9h	15 h	18 h
BR-3	**Gluconic acid**	0.6385	5.5089	0.5136	5.7903
	Formic acid	7.0485	-	0.2136	0.2882
	Malic acid	2.2359	0.6058	1.2766	0.9433
	Lactic acid	0.2437	0.3660	0.6413	0.7146
	Acetic acid	0.2522	0.1876	0.3562	0.6477
	Maleic acid	0.0154	-	0.0012	0.0046
	Citric acid	-	-	0.3241	0.1158
	Fumaric acid	0.0018	0.0027	0.0026	0.0021
	Succinic acid	-	0.2545	0.5393	0.7451
	Pyruvic acid	-	0.0472	0.0995	0.0735
	Oxalic acid	0.1794	0.1896	0.2765	0.1837

	Strategy, t(h)	3 h	9h	15 h	21 h	26 h
BR-4	**Gluconic acid**	-	0.3272	4.6724	2.4607	1.9108
	Formic acid	9.4408	0.3292	0.1940	2.0010	7.6366
	Malic acid	3.2630	-	-	-	-
	Lactic acid	0.3407	0.3411	0.4017	0.6639	0.7914
	Acetic acid	0.2757	0.1664	0.1643	0.4576	0.6453
	Maleic acid	0.0232	-	0.0006	0.0014	0.0058
	Citric acid	-	-	0.2619	0.1508	0.2290
	Fumaric acid	0.0023	0.0026	0.0025	0.0036	0.0040
	Succinic acid	-	-	0.2652	0.4352	0.5384
	Pyruvic acid	-	0.0410	0.0613	0.1186	0.1285
	Oxalic acid	0.2315	0.2669	0.1829	0.2044	0.3080

Table 4.3 Variations in organic acid concentrations with the cultivation time for different feeding strategies in g L^{-1} (continued).

	Strategy, t(h)	3 h	9h	15 h	19 h
BR-5	**Gluconic acid**	-	5.0069	-	10.8100
	Formic acid	4.4000	0.2721	4.9489	11.8432
	Malic acid	1.3023	0.2601	0.3156	5.8538
	Lactic acid	-	-	-	0.2900
	Acetic acid	0.0858	-	0.2470	0.7463
	Maleic acid	0.0057	-	0.0028	0.0960
	Citric acid	-	-	-	-
	Fumaric acid	0.0009	0.0024	0.0034	0.0194
	Succinic acid	-	0.2944	0.3375	0.4898
	Pyruvic acid	-	0.0507	-	-
	Oxalic acid	0.1154	0.1576	0.2469	0.8673

	Strategy, t(h)	3 h	9h	15 h	19 h
BR-6	**Gluconic acid**	1.9820	-	-	-
	Formic acid	0.4379	1.9470	11.0245	11.9162
	Malic acid	-	1.9422	4.2934	6.7629
	Lactic acid	0.2243	0.3550	1.0396	1.7193
	Acetic acid	0.0874	0.0862	0.4570	0.5353
	Maleic acid	-	0.0137	0.1270	0.1558
	Citric acid	-	-	-	-
	Fumaric acid	0.0013	0.0013	0.0167	0.0314
	Succinic acid	-	0.1866	0.4806	0.2827
	Pyruvic acid	0.0264	0.0794	-	-
	Oxalic acid	0.0863	0.1840	0.9714	1.0482

Table 4.3 Variations in organic acid concentrations with the cultivation time for different feeding strategies in g L^{-1} (continued).

	Strategy, t(h)	3 h	9h	15 h	19 h
BR-7	**Gluconic acid**	3.3023	-	7.1217	-
	Formic acid	0.7407	1.8459	11.0245	11.9162
	Malic acid	-	0.12	0.2298	6.3689
	Lactic acid	0.2728	0.6244	0.3507	0.7003
	Acetic acid	-	0.6170	0.0698	0.2236
	Maleic acid	0.0045	0.2116	0.0064	0.0465
	Fumaric acid	0.0011	0.0282	0.0012	0.0048
	Succinic acid	-	0.2299	0.2781	0.1211
	Pyruvic acid	0.0358	-	0.0595	-
	Oxalic acid	0.1399	0.1488	0.1587	0.4789

4.8 Yield Coefficients

Yield coefficients for certain intervals and overall yield coefficients were calculated for all of the strategies and are given in Table 4.4 and Table 4.5. Except BR-8, glucose was used as the substrate; however; in BR-8, pre-treated molasses containing both glucose and fructose was fed to the bioreactor; thus in the calculations of yield coefficients in BR-8 both glucose and fructose were considered as substrates.

The highest yields coefficients for cell generation per substrate consumed, $Y_{X/S}$, were obtained as 3.236, 1.064, 0.569, 0.894, 0.588, 0.642, 0.502 and 0.825 g g^{-1} for BR-1, BR-2, BR-3, BR-4, BR-5, BR-6, BR-7, and BR-8, respectively. Generally, the highest $Y_{X/S}$ values were attained in the early hours of the process where the glucose was fed exponentially with μ_0= 0.10, 0.15 and 0.175 h^{-1} in BR-4, BR-5, BR-6 and BR-7, respectively. Moreover when the substrate feeding rate increased, the $Y_{X/S}$ also raised. On the other hand, the highest $Y_{P/S}$ values were 1.606, 6.770, 6.798, 4.010, 8.980, 8.294, and 3.581 mg g^{-1} in BR-1, BR-3, BR-4, BR-5, BR-6, BR-7, and BR-8, respectively. The strategy BR-6, where glucose was fed exponentially with μ_0 =0.175 h^{-1} throughout the operation, has the highest yield

coefficients, $Y_{P/S}$ and it is followed by BR-7 where glucose was fed exponentially with μ_0 =0.175 h^{-1} at t=0-6 h and then with μ_0 =0.15 h^{-1} at t=6-19 h. Furthermore, uppermost $Y_{P/X}$ values were found as 1.638, 11.889, 11.173, 15.777, 18.319, 16.531 and 8.583mg g^{-1} for BR-1, BR-3, BR-4, BR-5, BR-6, BR-7, and BR-8, respectively. The $Y_{P/X}$ value in BR-8 at t=19 h was not taken into consideration since the cell and rhGH concentration decreased in this hour.

Overall yield coefficients were determined based at the cultivation time where the highest rhGH concentration was obtained, at BR-1, BR-3, BR-4, BR-5, BR-6, BR-7 and BR8 at t=9h, 18h, 26h, 19h, 15h, 17h and 17h, respectively. Overall yield coefficients were not evaluated for BR-2, since rhGH was not produced by the cells during semi-batch phase of the operation. The highest $Y'_{X/S}$, $Y'_{P/S}$, and $Y'_{P/X}$ were calculated as 0.307 mg g^{-1} in BR-3, 2.182 mg g^{-1} in BR-3 and 14.172 mg g^{-1} in BR-8. The highest overall yields, $Y'_{X/S}$, and $Y'_{P/S}$ were obtained as 0.307 g g^{-1} and 2.182 mg g^{-1}, respectively, in BR-6 strategy which is consisted of exponential feeding with μ_0 =0.15 h^{-1} within t=0-11 h and constant flow rate Q=24 ml h^{-1} within t=11-18 h. Although total glucose amount fed to the bioreactor in BR-3 was less than that of BR-4, BR-5, BR-6 and BR-7 strategies, glucose utilization for cell growth and rhGH production were higher. The complex feed, molasses, in BR-8 gave rise to the maximum $Y'_{P/X}$ value as 14.172 mg g^{-1} in BR-8. The overall yields of the strategy in which the highest rhGH, 508 mg L^{-1}, was produced was also high even though the glucose was fed exponentially with pre-determined specific growth rate of μ_0 =0.15 h^{-1}.

Table 4.4 Yield coefficients for the designed strategies

Strategy	t h	Yx/s g g^{-1}	Yp/s mg g^{-1}	Yp/x mg g^{-1}
BR-1	3	0.311	0.137	0.441
	6	0.100	0.164	1.638
	9	3.236	1.606	0.496
	12	0.574	-0.770	-1.340
	15	0.078	-0.641	-8.235
	18	0.097	-0.904	-9.296
BR-2	3	1.064	-0.176	-0.166
	6	0.539	-0.269	-0.498
	9	0.304	-0.113	-0.373
	12	0.318	-0.113	-0.355
	15	0.355	-0.006	-0.017
	18	0.835	0.044	0.053
	21	0.056	-0.041	-0.732
	24	0.102	-0.004	-0.040
	27	0.204	-0.008	-0.040
BR-3	3	0.569	6.770	11.889
	6	0.357	1.193	3.340
	9	0.538	2.066	3.841
	12	0.193	1.870	9.695
	15	0.243	2.781	11.438
	18	0.241	1.162	4.812
BR-4	3	0.894	6.798	7.602
	6	0.556	2.734	4.922
	9	0.493	4.126	8.374
	12	0.408	0.799	1.956
	15	0.287	0.854	2.970
	18	0.222	2.168	9.771
	21	0.298	0.742	2.490
	24	0.114	1.278	11.173
	26	0.162	0.746	4.603

Table 4.4 Yield coefficients for the designed strategies (continued)

Strategy	t h	Yx/s g g^{-1}	Yp/s mg g^{-1}	Yp/x mg g^{-1}
BR-5	3	0.588	2.185	3.713
	6	0.367	2.277	6.198
	9	0.589	4.010	6.811
	12	0.326	1.457	4.468
	15	0.244	1.746	7.158
	17	0.166	2.616	15.777
	19	-0.018	0.913	-49.694
BR-6	3	0.642	8.980	13.996
	6	0.297	1.287	4.329
	9	0.337	2.929	8.696
	12	0.097	1.782	18.319
	15	0.104	0.525	5.048
	17	0.042	-1.053	-25.299
	19	-0.020	-0.293	14.885
BR-7	3	0.502	8.294	16.531
	6	0.299	1.214	4.063
	9	0.391	2.639	6.741
	12	0.201	3.149	15.641
	15	0.197	0.282	1.432
	17	0.113	0.605	5.340
	19	0.052	-1.179	-22.669
BR-8	3	0.825	3.365	4.076
	6	0.449	3.581	7.970
	9	0.219	1.877	8.583
	12	0.201	0.367	1.824
	15	-0.020	1.260	-62.209
	17	-0.093	1.458	-15.640
	19	-0.016	-0.836	51.273

Table 4.5 Overall yield coefficients

Strategy	Y'x/s g g^{-1}	Y'p/s mg g^{-1}	Y'p/x mg g^{-1}
BR-1	0.289	0.217	0.750
BR-3	0.307	2.182	7.112
BR-4	0.263	1.478	5.623
BR-5	0.215	1.894	8.797
BR-6	0.185	1.748	9.464
BR-7	0.226	1.721	7.620
BR-8	0.104	1.475	14.172

CHAPTER 5

CONCLUSIONS

In this M. Sc. study, in order to enhance rhGH production by *P. pastoris* under *GAP* promoter, different feeding strategies were designed. In the experiments glucose was used to induce *GAP* promoter to express rhGH. Additionally in BR-8 pre-treated molasses, in which sucrose was inverted to glucose and fructose, was used as the substrate since fructose can also induces *GAP* promoter.

In BR-2 and BR-3, exponential feeding $\mu_0 = 0.15\ h^{-1}$ was followed by constant feeding, with volumetric flow rates of $Q(t \geq 15\ h) = 12\ ml\ h^{-1}$ and $Q(t \geq 11\ h) = 24\ ml\ h^{-1}$, respectively. The transition cultivation time from exponential feeding to constant feeding was at t = 15 h in BR-2, when the cells reached to a concentration of 55 g L^{-1}, and at t= 11 h in BR-3 when the flow rate reached to 24 ml h^{-1} feed. In BR-1, glucose was fed with $\mu_0 = 0.3\ h^{-1}$ within t=0-6 h and after that within t=6-18 h it was fed with $q_G = 0.05\ g\ g^{-1}\ h^{-1}$. In BR-4, BR-5 and BR-6, glucose was fed exponentially throughout the process based on pre-determined specific growth rates of 0.10 h^{-1}, 0.15 h^{-1} and 0.175 h^{-1}, respectively. In BR-7, two consecutive pre-determined specific growth rates were used to feed glucose μ_0=0.175 h^{-1} within t=0-6 h and μ_0=0.15 h^{-1} within t=6-19 h. BR-8 was the only experiment in which pre-treated molasses was used as the feed and it was fed with $\mu_0 = 0.15\ h^{-1}$ for the whole process, t=0-19 h, considering both glucose and fructose concentrations in molasses.

Among the designed strategies, the highest rhGH concentration was obtained in BR-5 where glucose was exponentially fed to the bioreactor with the pre-determined specific growth rate of μ_0=0.15 h^{-1}. The highest rhGH concentration was attained as 508 mg L^{-1} at t=19 h of BR-5 where the cell concentration was 65 g L^{-1}.

At t=19 h, the specific glucose consumption rate, q_G and specific rhGH production rate, q_{rhGH} were calculated as 0.451 g $g^{-1}h^{-1}$, and 0.853 mg $g^{-1}h^{-1}$, respectively. The overall yields, $Y'_{X/S}$, $Y'_{P/S}$, and $Y'_{P/X}$ for BR-5 were 0.215 g g^{-1}, 1.894 mg g^{-1}, 8.797 mg g^{-1}, respectively.

The following major results are:

- The highest cell concentration was obtained in BR-2 as C_X=76 g L^{-1} where rhGH was not produced. It was followed by BR-5 as C_X=66.7 g L^{-1}. In BR-8, where pre-treated molasses was used as feed without PTM1, and BR-1, the maximum cell concentrations obtained were lower being, C_X=29.1 and 41.5 g L^{-1}, respectively. Therefore, cell concentration is dependent on the carbon source used, feeding strategy of the carbon source and trace minerals which exists in PTM1.
- Pre-determined μ_0 values were not obtained experimentally. The maximum specific growth rates were attained in the early hours of the processes. Generally specific growth rates were decreased as time passed because the cells entered the stationary phase and the volume of the fermentation medium increased with time. Moreover for obtaining the pre-determined specific growth rate the variation of $Y_{X/S}$ value should be taken into consideration in the calculation of the feed amount.
- Glucose was consumed totally in all experiments except BR-7 in which 29 g L^{-1} glucose was accumulated at t = 19 h. In BR-8, fructose was also detected in all samples of the experiment which implies fructose was not utilized by the cells as much as glucose.
- Maximum q_G value was 1.062 g g^{-1} h^{-1} in BR-1. Therefore the maximum amount of glucose that can be utilized by 1 g of *P. pastoris* cells in one hour was 1.062 g.
- In BR-6 and BR-7, 381 and 357 mg L^{-1} were produced being 1.33- and 1.42-fold lower than that produced in BR-5, respectively.
- Maximum q_{rhGH} values were obtained as 1.976 mg g^{-1} $h^{-}1$ and 1.895 mg g^{-1} h^{-1} in BR-6 and BR-7, respectively, at the beginning of the processes. Thus the rate of the production was the highest in μ_0 = 0.175 h^{-1}.

- As a carbon source, pre-treated molasses did not perform as well as glucose. This can be because of the absence of the PTM1 in molasses or because of the glucose inhibition over fructose which leads to fructose accumulation in the medium.
- DO of 20% was found to be a better oxygen transfer condition, since rhGH was not produced in BR-2 where DO was kept at 40-50%.
- At μ_0 = 0.15 h^{-1}, the rhGH production and cell growth was the highest than the other designed strategies, whereas lowering μ_0 to 0.10 h^{-1} decreased both the cell and rhGH concentration. Increasing μ_0 to 0.175 h^{-1} did not increase the cell and rhGH concentrations; however, shortened process time.
- A very low feed rate of glucose was not suitable as in the cases BR-1 (q_G = 0.05 g g^{-1} h^{-1}), and BR-2 (Q(t≥11 h) =24 ml h^{-1}). A very high glucose rate did not also enhance r-protein effectively, as seen in BR-6. Accumulation of glucose to high concentrations inhibited cell growth and r-protein expression.
- Maximum cell yield on substrate, $Y_{X/S}$, were obtained as 3.236 g g^{-1} in BR-1at t=9h while highest product yield on substrate $Y_{P/S}$, were calculated as 8.980 mg g^{-1} in BR-6 at t=3 h. The product yield on cell, $Y_{P/X}$, was maximum as 18.319 mg g^{-1} in BR-6 at t=12h.
- The highest overall yield coefficients $Y'_{X/S}$, $Y'_{P/S}$, and $Y'_{P/X}$ were attained as 0.307 mg g^{-1} in BR-3, 2.182 mg g^{-1} in BR-3 and 14.172 mg g^{-1} in BR-8.

REFERENCES

About industrial biotechnology market. Retrieved 2014 from

(www.researchandmarkets.com).

About human growth hormone. Retrieved 2014 from

(http://www.ncbi.nlm.nih.gov).

About human growth hormone. Retrieved 2014 from

(http://www.pdb.org/pdb)

About glycolysis pathway. Retrieved 2014 from

(http://www.genome.jp/kegg/pathway/map/map00010.html)

Açık, E., 2009. "Effects of carbon sources and feeding strategies on recombinant human growth hormone production by metabolically engineered *Pichia pastoris*." Master of Science Thesis, Middle East Technical University, Ankara.

Agarwal, P.K., Uppada V., Noronha S.B., 2013. "Comparison of pyruvate decarboxylases from *Saccharomyces cerevisiae* and *Komagataella pastoris* (*Pichia pastoris*)." *Applied Microbiology Biotechnology*, 97:9439–9449.

Almeida, J.R.M., de Moraes, L.M.P., Torres F.A.G., 2005. "Molecular characterization of the 3- phosphoglycerate kinase gene (*PGK1*) from the methylotrophic yeast *Pichia pastoris*." *Yeast*, 22:7251–737.

Baulieu, E., Kelly, P. A., 1990. *HORMONES from molecules to disease*, Herman Press, New York.

Baumann, K., Maurer, M., Dragosits, M., Cos, O., Ferrer, P., Mattanovich, D., 2008. "Hypoxic semi-batch cultivation of *Pichia pastoris* increases specific and volumetric

productivity of recombinant proteins." *Biotechnology and Bioengineering*, 10(1):177–183.

Baumann, K., Carnicer, M., Dragosits, M., Graf, A.B., Stadlmann, J., Jouhten, P., Maaheimo, H., Gasser, B., Albiol, J., Mattanovich, D., Ferrer, P., 2010. "A multi-level study of recombinant *Pichia pastoris* in different oxygen conditions." *BMC Systems Biology*, 4:141.

Binkley, S. A., 1994. *Endocrinology*, Harper Collins College Publishers, New York.

Blum, H., Beier, H., Gross, H.J., 1987. "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels." *Electrophoresis*, 8:93-99.

Boyacı, İ.H., 2005. "A New approach for determination of enzyme kinetic constants using response surface methodology." *Biochemical Engineering Journal*, 25:p.55-62.

Bozkurt, B., 2012. "Feeding strategy development for human growth hormone production by *Pichia pastoris*." Master of Science Thesis, Middle East Technical University, Ankara.

Cereghino, J.L., Cregg, J.M., 2000. "Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*." *FEMS Microbiol Rev*, 24(1):45–66.

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002. "Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*." *Current Opinion in Biotechnology*, 13:329-332.

Chawla, R.K., Parks, J.S., Rudman, D., 1983. "Structural variants of human growth hormone: biochemical, genetic, and clinical aspects." *Ann. Rev. Med.*, 34:519-547.

Chen, G.H., Yin, L.J., Chiang I.H., Jiang, S.T., 2007. "Expression and purification of goat lactoferrin from *Pichia pastoris* expression system." *Journal of Food Science*, 72:67–71.

Clare, J.J., M.A. Romanos, F.B. Rayment, J.E. Rowedder, M.A. Smith, and M.M. Payne. 1991. "Production of mouse epidermal growth factor in yeast: high-level secreting using *Pichia pastoris* strains containing multiple gene copies." *Gene*, 105:205-212.

Corchero, J.L., Gasser, B., Resina, D., Smith, W., Parrilli, E., Vázquez, F., Abasolo, I., Giuliani, M., Jäntti, J., Ferrer, P., Saloheimo, M., Mattanovich, D., Schwartz, J.S., Tutino, M.L., Villaverde, A., 2013. "Unconventional microbial systems for the cost-

efficient production of high-quality protein therapeutics." *Biotechnology Advances*, 31(2):140–153.

Cos, O., Resina, D., Ferrer, P., Montesinos, J.L., Valero, F., 2005. "Heterologous Production of *Rhizopus oryzae* Lipase in *Pichia pastoris* Using the Alcohol Oxidase and Formaldehyde Dehydrogenase Promoters in Batch and Semi-batch Cultures." *Biochemical Engineering Journal*, 26:86–94.

Cos, O., Ramón, R., Montesinos, J.L., Valero, F., 2006. "Operational Strategies, Monitoring and Control of Heterologous Protein Production in the Methylotrophic Yeast *Pichia pastoris* Under Different Promoters: A Review." *Microbial Cell Factories*, 5:17.

Cregg, J.M., Vedvick, T.S. and Raschke, W.C., 1993. "Recent advances in the expression of foreign genes in *Pichia pastoris*." *BioTechnology* 11:905-910.

Cregg, J. M., J. L. Cereghino, J. Shi, and D. R. Higgins. 2000. "Recombinant protein expression in *Pichia pastoris*." *Mol. Biotechnol.* (16):23-52.

Curtin, L. V., 1983. Molasses-General Considerations. In Molasses in Animal Nutrition.

Çalık, P., Çalık, G., Takaç, S., Özdamar, T.H., 1999. "Metabolic flux analysis for serine alkaline protease fermentation by *Bacillus licheniformis* in a defined medium: Effects of the oxygen transfer rate." *Biotechnology and Bioengineering*, 64:151-167

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

Çalık, P., Orman M. A., Çelik E., Halloran S. M., Çalık G., and Özdamar T. H., 2008. "Expression system for biosynthesis and purification of recombinant human growth in *Pichia pastoris* and structural analysis by MALDI-ToF mass spectrometry." *Biotechnology Progress* 24(1):221-226.

Çalık, P., Bozkurt, B., Zerze, G.H., Inankur, B., Bayraktar, E., Boy, E., Orman, M.A., Açık, E., Özdamar, T.H., 2013. "Effect of co-substrate sorbitol different feeding strategy on human growth hormone production by recombinant *Pichia pastoris.*" *Journal of Chemical Technology and Biotechnology*, 88:1631-1640.

Çalık, P., Ata, Ö., Güneş, H., Massahi, A., Boy, E., Keskin, A., Öztürk, S., Zerze, G.H., Özdamar, T.H., submitted: 2014. "Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters."

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

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

Çelik, E., 2008. "Bioprocess development for therapeutical protein production."

Thesis Doctor of Philosophy, Middle East Technical University, Ankara.

Çelik, E., Çalık, P. 2012. "Production of recombinant proteins by yeast cells." *Biotechnol Adv* 30(5):1108–1118.

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

De Vos, A.M., Ultsch M., and Kossiakoff A.A.,1992. "Human growth hormone and extracellular domain of its receptor: crystal structure of the complex." *Science* 255:306-312.

Delroisse J.M., Dannau M., Gilsoul J.J., El Mejdoub T., Destain J., Portetelle D., Thonart P., Haubruge E., Vandenbol M., 2005. "Expression of a synthetic gene encoding a Tribolium castaneum carboxylesterase in *Pichia pastoris*." *Protein Expr. Purif* 42:286–294

Demain, A. L., 2007. "The business of biotechnology." *Industrial Biotechnology* 3 (3): 269-283.

Dietzsch C., Spadiut O., Herwig C., 2011. "A dynamic method based on the specific substrate uptake rate to set up a fedding strategy for *Pichia pastoris*." *Microbial Cell Factories* 10:14

Dische, Z. and E. Borenfreund., 1951. "A new spectrophotometric method for the detection and determination of keto sugars and trioses." *J. Biol. Chem* 192:583-587.

Diwany, A. I., Abyad, M. S., Refai, A. H., Sallam, L. A., Allam, R. F., 1992. Effect of some fermentation parameters on ethanol production from beet molasses by *Saccharomyces cerevisiae* Y-7. *Bioresource Technology*, 42, p.191-195.

Donati, E.R., Sand, W. (Eds). 2007, *Microbial Processing of Metal Sulfides*. Springer.

Doring F., Klapper M., Theis S., Daniel H., 1998. "Use of the glyceraldehyde-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast *Pichia pastoris*." *Biochem. Biophys. Res. Commun.* 250 (1998) 531–535.

Dragosits, M., Stadlmann, J., Graf, A., Gasser, B., Maurer, M., Sauer, M., Kreil, D.P., Altmann, F., Mattanovich, D., 2010. "The response to unfolded protein is involved in osmotolerance of *Pichia pastoris*." *BMC Genomics* 11:207.

Evans, H.M., Long, J.A., 1921. "The effect of the anterior lobe administered intraperitoneally upon growth, maturity and oestrus cycles of the rat." *Anat. Rec.* 21:62–63.

Eurwilaichitr, L., Roytroku, R., Suprosongsin,C., Manitchotpisit, P. and Panyim, S., 2002. "Glutamic acid and alanine spacer is not necassary for removal of MF_-1 signal sequence fused to the human growth hormone produced from *Pichia pastoris*." *World Journal of Microbiology and Biotechnology*, 18 : 493- 495.

Faber, K.N., Harder, W., Ab, G., Veenhuis, M., 1995. "Review: methylotrophic yeasts as factories for the production of foreign proteins." *Yeast* 11: 1331– 1344.

Fei, L., Wang, Y., Chen, S., 2009. "Improved glutathione production in *Pichia pastoris." Bioprocess Biosyst Eng* 32:729–735.

Ferreira, A.R., Ataíde, F., von Stosch, M., Dias, J.M.L., Clemente, J.J., Cunha, A.E., Oliveira, R., 2012. "Application of adaptive DO-stat feeding control to *Pichia pastoris* X33 cultures expressing a single chain antibody fragment (scFv)." *Bioprocess Biosyst Eng* 35(9):1603–1614.

Fischer, P.W.F., Giroux, A., L'Abbe, M.R. 1984. "Effect of zinc supplementation on copper excretion and retention in men." *Am J Clin Nutr*;40:743-6.

Franchi, E., Maisano F., Testori S.A., Galli G., Toma S., Parente L., Ferra F., and Grandi G., 1991. “A new human growth hormone production process using a recombinant Bacillus subtilis strain.” *Journal of Biotechnology* 18:41-54.

Gao, M.J., Li, Z., Yu, R.S., Wu, J.R., Zheng, Z.Y., Shi, Z.P., Zhan, X.B. Lin, C.C., 2012. “Methanol/sorbitol co-feeding induction enhanced porcine interferon-α production by *P. pastoris* associated with energy metabolism shift.” *Bioprocess Biosyst Eng.*

Garcia-Ortega, X., Ferrer, P., Montesinos, J.L., Valero, F., 2013. “Semi-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive *GAP* promoter.” *Biochem Eng J* 79:172–181.

Gasser, B., Maurer, M., Gach, J., Kunert, R., Mattanovich, D., 2006. “Engineering of *Pichia pastoris* for improved production of antibody fragments.” *Biotechnol Bioeng* 94(2):353–361.

Ghozlan, H. A., 1994. “Utilization of Beet Molasses for Riboflavin Production by *Mycobacteriurn phlei*.” *J. Basic Microbiol*., 34, 3, p.157-162.

Gimenez, J.A., Monkovic, D.D., Dekleva, M.L. 2000. “Identification and monitoring of protease activity in recombinant *Saccharomyces cerevisiae*.” *Biotechnol Bioeng* 67:245–251.

Goeddel, D.V., Heyneker, H.L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D.G., Ross, M.J., Miozzari, G., Crea, R., Seeburg, P.H., 1979. “Direct expression in *Escherichia coli* of a DNA sequence coding for growth hormone.” *Nature*, 281:544-548

Goodrick, J.C., Xu, M., Finnegan, R., Schilling, B.M., Schiavi, S., Hoppe, H., Wan, N.C., 2001. “High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system.” *Biotechnol Bioeng* 74(6):492–497.

Gray, G.L., Bladridge, J.S., McKeown, K.S., Heyneker, H.L., Chang, C.N., 1985. “Periplasmic production of correctly processed human growth hormone in *Escherichia coli* natural and bacterial signal sequences are interchangeable.” *Gene*. 29(2-3):247-254.

Guan, B., Chen, F., Lei, J., Li, Y., Duan, Z., Zhu, R., Chen, Y., Li, H., Jin, J., 2013. "Constitutive expression of a rhIL-2-HSA fusion protein in *Pichia pastoris* using glucose as carbon source." *Appl Biochem Biotechnol* 171(7):1792–1804.

Heyland, J., Fu, J., Blank, L.M., Schmid, A., 2011. "Carbon metabolism limits recombinant protein production in *Pichia pastoris*." *Biotechnol Bioeng* 108(8):1942-1953.

Higgins, D. R. and Cregg J. M., 1998. "Introduction to *Pichia pastoris*." Pp. 1-15 in *Pichia Protocols*. Totowa: Human Press Inc.

Hong, F., Meinander N.Q., Jonsson L.J., 2002. "Fermentation strategies for improved heterologous expression of laccase in Pichia pastoris." *Biotechnol Bioeng* 79(4):483-449.

Hsiung, H.M., Mayne, N.G., Becker, G.W., 1986. "High-level expression, efficient secretion and folding of human growth hormone in *Escherichia coli*." *Biotechnology*, 4:991-995.

Hu, X., Chu, J., Zhang, Z., Zhang, S., Zhuang, Y., Wang, Y., Guo, M.J., Chen, H.X., Yuan, Z.Y., 2008. "Effects of different glycerol feeding strategies on S-adenosyl-L-methionine biosynthesis by P_{GAP}-driven *Pichia pastoris* overexpressing methionine adenosyltransferase." *J Biotechnol* 137:44–49.

Inan M., Chiruvolu V., Eskridge K.M., Vlasuk G.P., Dickerson K., Brown S., Meagher M.M., 1999. "Optimization of temperature-glycerol-pH conditions for a fedbatch fermentation process for recombinant hookworm *(Ancylostoma caninum)* anticoagulant peptide (AcAP-5) production by *Pichia pastoris.*" *Enzyme and Microbial Technology* 24(7): 438-445.

Inankur, B., 2010. "Recombinant Human Growth Hormone Production by *Pichia pastoris* and Determination of its Interaction with Peptide Ligands." M.Sc. Thesis, Middle East Technical University, Ankara

Invitrogen, 2002. "Pichia Fermentation Process Guidelines." Retrieved 2014 (http://tools.invitrogen.com/content/sfs/manuals/pichiaferm_prot.pdf).

Jahic, M., Rotticci-Mulder, J.C., Martinelle, M., Hult, K., Enfors, S-O., 2002. "Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein." *Bioprocess and Biosystems Engineering*, 24: 385-393.

Jahic, M.A., Gustavsson A.K., Jansen M, and Enfors S.O., 2006. “Process technology for production and recovery of heterologous proteins with *Pichia pastoris*.” *Biotechnol Progress* 22:1465-1473.

Jungo C., Rerat C., Marison I.W., von Stockar U., 2006. “Quantitative characterization of the regulation of the synthesis of alcohol oxidase and of the expression of recombinant avidin in a *Pichia pastoris* Mut+ strain.” *Enzyme Microbial Technology*. 39 (4): 936–944.

Kasimova R.M.R., Kristensen S.M. Howe P.W.A., Christensen T., Matthiesen F.,

Petersen J., Sorensen H.H.M., Led J.J., 2002. “NMR studies of the backbone flexibility and structure of human growth hormone: a comparison of high and low pH conformations.” *Journal of Molecular Biology*. 318:679-695.

Kato, C., Kobayashi, T., Kudo, T., Frusato, T., Murakami, Y., Tanaka, T., Baba, H., Oishi, T., Ohtsuka, E., Ikehera, M.,Yanagida, T., Kato, H., Moriyama, S., Horikoshi, K., 1987. “Construction of an excretion vector and extracellular production of human growth hormone from *Escherichia coli*.” *Gene*, 54: 197-202

Khasa, Y.P., Khushoo, A., Srivastava, L., Mukherjee, K.J., 2007. “Kinetic studies of constitutive human granulocyte-macrophage colony stimulating factor (hGM-CSF) expression in continuous culture of *Pichia pastoris*.” *Biotechnol Lett* 29(12):1903–1908.

Kim, S.J., Lee J.A., Kim, Y.H., Song, B.K., 2009. “Optimization of the functional expression of Coprinus cinereus peroxidase in *Pichia pastoris* by varying the host and promoter.” *J. Microbiol. Biotechnol*. 19:966–971.

Kirk, R. E., Othmer, D. F., 2008. Food and Feed Technology.Wiley-Interscience, p.496-498.

Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Tomomitsu, K., 2000. “High level secretion of recombinant human serum albumin by fedbatch fermentation of the methylotrophic yeast, *Pichia pastoris*, based on optimal methanol feeding strategy.” *Journal of Bioscience and Bioengineering*. 90:280–288.

Kopchick, J.J., 2003. “History and Future of Growth Hormone Research. “*Hormone Resolutions.*” Vol: 60 (Suppl 3): 103-112.

Kottmeier, K., Günther, T.J., Weber, J., Kurtz, S., Ostermann, K., Rödel, G., Bley, T., 2012. "Constitutive expression of hydrophobin HFB1 from *Trichoderma reesei* in *Pichia pastoris* and its pre-purification by foam separation during cultivation." *Eng. Life Sci* 12(2):162–170.

Krysiak, R., Gdula-Dymek, A., Bernardska-Czerwinska, B.O., 2007. "Growth Hormone Therapy in Chilren and Adults", *Pharmacological Reports*, 59:500-516

Laemmli, U.K., 1970. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* 227 (5259): 680-685

Lee, C.Y., Nakano A., Shiomi N., Lee E.K., and Katoh S., 2003. "Effects of substrate feed rate on heterologous protein expression by *Pichia pastoris* in DOstat semi-batch fermentation." *Enzyme Microb Technol* 33:358-365.

Li, Z., Xiong F., Lin Q., D'Anjou M., Daugulis A.J., Yang D.S.C., and Hew C.L.. 2001. "Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*." *Protein Expression and Purification* 21:438-445.

Li, Y., Zhang, B., Chen, X., Xhen, Y., Cao, Y., 2010. "Improvement of *Aspergillus sulphureus* endo-β-1, 4-Xylanase expression in *Pichia pastoris* by codon optimization and analysis of the enzymic characterization." *Appl Biochem Biotechnol* 160(5):1321–1331.

Li, S., Sing, S., Wang, Z., 2011. "Improved expression of *Rhizopus oryzae* α-amylase in the methylotrophic yeast *Pichia pastoris*." *Protein Expr Purif* 79:142–148.

Liu, S., 2013. "Biopocess Engineering Kinetics, Biosystems, Sustainability and Reactor Design." p711-720

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M., 2005. "Heterologous protein production using the *Pichia pastoris* expression system." *Yeast* 22(4): 249–270.

Menendez, J., Valdes, I., Cabrera, N., 2003. "The ICL1 gene of *Pichia pastoris*, transcriptional regulation and use of its promoter." *Yeast*. 20:1097-1108

Menéndez, C., Hernández, L., Banguela, A., País, J., 2004. "Functional production and secretion of the *Gluconacetobacter diazotrophicus* fructose-releasing exo-levanase (LsdB) in *Pichia pastoris*." *Enzyme Microb Technol* 34:446–452.

Menéndez, C., Martínez, D., Trujillo, L.E., Mazola, Y., González, E., Pérez, E.R., Hernandez, L., 2013. "Constitutive high-level expression of a codon-optimized â-fructosidase gene from the hyperthermophile *Thermotoga maritima* in *Pichia pastoris.*" *Appl Microbiol Biotechnol* 97:1201–1212.

Moon, S. H. and Parulekar S. J., 1991. "A parametric study of protease production in batch and semi-batch cultures of Bacillus firmus." *Biotechnology and Bioengineering* 37:467-483.

Nakayama, A., Ando, K., Kawamura, K., Mita, I., Fukazawa, K., Hori, M., Honjo, M., Furutani, Y., 1988. "Efficient secretion of the authentic meture human growth hormone by *Bacillus subtilis.*" *Journal of Biotechnology*. 8: 123-134.

Nielsen, J., Villadsen, J., Liden, G., 2003. "*Bioreaction Engineering Principles*,"

Second Edition, Plenum Press, New York.

Ogata, K., Nishikawa, H., Ohsugi, M., 1969. "A Yeast Capable of Utilizing Methanol." *Agric. Biol. Chem*., 33, p.1519-1520.

Olbrich, H., 1963. The Molasses. Berlin: Fermentation Technologist Institut fur Zuckerindustrie.

Orman, M.A., 2007. "Extracellular Recombinant Human Growth Hormone Production by *Pichia pastoris*." Thesis of Master of Science, Middle East Technical University, Ankara

Orman, M.A, Çalık, P., Çalik, E., Halloran, S.M., Çalık, G., Özdamar, T.H., 2008. "Expression system for biosynthesis and purification of recombinant human growth in *Pichia pastoris* and structural analysis by MALDI-ToF mass spectrometry." *Biotechnology Progress*, 24 (1): 221-226

Orman M.A., Calık P., Ozdamar T.H., 2009. "The influence of carbon sources on recombinant-human-growth-hormone production by *Pichia pastoris* is dependent on phenotype: A comparison of MutS and Mut+." *Biotechnology and Applied Biochemistry*. 52 (3): 245-255

Özdamar, T.H., Senturk, B., Yilmaz, O.D., Calık, G., Celik, E., Calık P., 2009. "Expression system for recombinant human growth hormone production by *Bacillus subtilis.*" *Biotechnology Progress*. 25(1): 75-84.

Pal, Y., Khushoo, A., Mukherjee, K.J., 2006. “Process optimization of constitutive human granulocyte–macrophage colony-stimulating factor (hGM-CSF) expression in *Pichia pastoris* semi-batch culture.” *Appl Microbiol Biotechnol* 69(6):650–657.

Park, S. C. and Baratti, J., 1991. “Batch fermentation kinetics of sugar beet molasses by *Zymomonas mobilis*.” *Biotechnol Bioeng*.,38(3), p.304-13.

Patra, A.K., Mukhopadhyay, R., Mukhija, R., Krishnan, A., Garg, L.C. Garg, Panda, A.K., 2000. “Optimization of inclusion body solubilization and renaturation or recombinant human growth hormone from *Escherichia coli*.” *Protein Expression and Purification* 17: 182–192.

Pepeliaev, S., Krahulec, J., Černý, Z., Jílková, J., Tlustá, M., Dostálová, J., 2011. “High level expression of human enteropeptidase light chain in *Pichia pastoris.*” *J Biotechnol* 156(1):67–75.

Potvin, G., Ahmad, A., Zhang, Z., 2012. “Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review.” *Biochem Eng* J 64:91–105.

Qiao, J., Rao, Z., Dong, B., Cao, Y., 2010. “Expression of *Bacillus subtilis* MA139 β-mannanase in *Pichia pastoris* and the enzyme characterization.” *Appl Biochem Biotechnol* 160(5):1362–1370.

Ragon, M., Neugnot-Roux, V., Chemardin, P., Moulin, G., Boze, H., 2007. “Molecular gene cloning and overexpression of the phytase from *Debaryomyces castelii* CBS 2923.” *Protein Expression and Purification*. 58:275-283

Romanos, M.A., Scorer, C. A., Clare, J. J.,1992. “Foreign Gene-Expression in Yeast - a Review.” *Yeast*, 8, p.423-488.

Rupa, P., Nakamura, S., Mine, Y., 2007. “Genetically glycosylated ovomucoid third domain can modulate Immunoglobulin E antibody production and cytokine response in BALB/c mice.” *Clin Exp Allergy* 37(6):918–928.

Sanchez, S., Demain, A., 2012. “Special issue on the production of recombinant proteins.” *Biotechnol Adv* 30(5):1100–1111.

Schwyzer, R., 1964. “Chemistry and metabolic action of nonsteroid hormones.” *Annual Review of Biochemistry* 33: 259-286.

Sears, I.B., O'Connor, J., Rossanese, O.W., Glick, B.S., 1998. "A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*." *Yeast* 14:783–790.

Shen, S., Sulter, G., Jeffries, T.W., Cregg, J.M., 1998. "A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*." *Gene* 216: 93-102.

Shi, X., Karkut, T., Chamankhah, M., 2003. "Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*." *Prot Exp Pur*., 28,p.321–330.

Shin N.K., Kim D.Y., Shin C.S., Hong M.S., Lee J., Shin H.C., 1998. "High-level production of human growth hormone in *Escherichia coli* by a simple recombinant process." *Journal of Biotechnology*, 62: 143-151.

Sibirny, A.A., V.M. Ubiyvovk, M.V. Gonchar, V.I. Titorenko, A.Y. Voronovsky, and Y.G. Kapultsevich. 1990. "Reaction of direct formaldehyde oxidation to CO_2 are not essential for energy supply of yeast methylotrophic growth." *Arch Microbiol* 154:566-77.

Singh S.M., Sharma A., Pande A.K., 2009. "High throughput purification of recombinant human growth hormone using radial flow chromatography." *Protein Expression and Purification.* 68(1):54-59.

Sinha J., Plantz B.A, Inan M., Meagher, M.M., 2004. "Causes of proteolytic degredation of secreted recombinant proteins produced in methylotropic yeast *Pichia pastoris:* case study with recombinant ovine interferon-τ." *Biotechnology and Bioengineering.* 89:102-112.

Soyaslan, E.Ş., Çalık, P., 2011. Enhanced recombinant human erythropoietin production by *Pichia pastoris* in methanol semi-batch/sorbitol batch fermentation through pH optimization. *Biochem Eng J* 55:59–65.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A., Birkenberger, L.A. 1997. "Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*." *Gene* 190(1):55–62.

Stambuk, B.U., Batista, A.S., De Araujo, P.S., 2000. “Kinetics of active sucrose transport in *Saccharomyces cerevisiae*.” *J Biosci Bioeng* 89(2):212–214.

Tabandeh, F., Shojaosadeti, S.A., Zomorodipour, A., Khodabandeh, M., Sanati, M.H.,

Yakhchali, B., 2004. “Heat-induced production of human growth hormone by high cell density cultivation of recombinant *Escherichia coli*.” *Biotechnology Letters*, 26: 245-250.

Tang, S., Potvin, G., Raciche, A., Zhang, Z., 2010. “Modeling of phytase production by cultivation of *Pichia pastoris* under the control of the GAP promoter.” *Int J Chem React Eng* 8(1).

Trevino, L. L., J. M. Viader-Salvado, H. A. Barrera-Saldana, and M. Guerrero-Olazaran. 2000. “Biosynthesis and secretion of recombinant human growth hormone in *Pichia pastoris*.” *Biotechnology Letters* (22):109-114.

Tritos N.A., Mantzoros C.S., 1998.”Recombinant human growth hormone: old and novel uses”. *American Journal of Medicine*. 105(1):44-57.

Tokunaga, T., Iwai, S., Gomi, H., Kodama, K., Ohtsuka, E., Ikehara, M., Chisaka, O., Matsubara, K., 1985. “Expression of a synthetic human growth hormone gene in yeast.” *Gene*, 39: 117-120

Vassileva, A., Chugh, D.A., Swaminathan, S., Khanna, N., 2001. “Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the *GAP* promoter.” *J Biotechnol* 88:21–35.

Van den Hazel, H.B., Kielland-Brandt, M.C., Winther, J.R., 1996. “Review: biosynthesis and function of yeast vacuolar proteases.” *Yeast* 12: 1–16.

Walker, G.M. 1998. “*Yeast: Physiology and Biotechnology*.” John Wiley & Sons Inc., New York

Wang, Z., Wang Y., Zhang D., Li J., Hua Z., Du G., and Chen J., 2009. “Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation.” *Bioresource Technol* 101:1318-1323.

Wang, X., Sun, Y., Ke, F., Zhao, H., Liu, T., Xu, L., Liu, Y., Yan, Y., 2012. Constitutive expression of *Yarrowia lipolytica* lipase LIP2 in *Pichia pastoris* using *GAP* as promoter." *Appl Biochem Biotechnol* 166:1355–1367.

Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V., Cregg, J.M., 1997. "Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter." *Gene* 186:37–44.

Wegner, E. H, 1983.Biochemical conversion at yeast fermentations at high cell densities. US Patent 4414329

Wu, J.M., Lin, J.C., Chieng, L.L., Lee, C.K., Hsu, T.A., 2003a. "Combined use of *GAP* and *AOX1* promoter to enhance the expression of human granulocyte-macrophage colony-stimulating factor in *Pichia pastoris*." *Enzyme Microb Technol* 33(4):453–459.

Wu, J.M., Chieng, L.L., Hsu, T.A., Lee, C.K., 2003b. "Sequential expression of recombinant proteins and their separate recovery from a *Pichia pastoris* cultivation." *Biochem Eng J* 16:9–16.

Yamanè, T., Shimizu, S., 1984. "*Semi-batch Techniques in Microbial Processes. In: Fiechter, A. (ed), Biotechnology Bioprocess Parameters Control*" (Book Series: Advances in Biochemical Engineering), Vol. 30, Springer Berlin-Heidelberg, p.147-194.

Zerze, G.H., 2012. "Comparison of human growth hormone production performance of two different metabolically engineered *Pichia pastoris*." Thesis of Master of Science, Middle East Technical University, Ankara

Zhang, W., Inan, M. Meagher, M.M., 2000. "Fermentation strategies for recombinant protein expression in the methylotropic yeast *Pichia pastoris*." *Biotechnol. Bioprocess* Eng. 5: 275-287

Zhang, A.L., Zhang, T.Y., Luo, J.X., Chen, S.C., Guan, W.J., Fu, C.Y., Peng, S.Q., Li, H.L., 2007. "Constitutive expression of human angiostatin in *Pichia pastoris* by high-density cell culture." *J Ind Microbiol Biotechnol* 34:117–122.

Zhang, A.L., Luo, J.X., Zhang, T.Y., Pan, Y.W., Tan, Y.H., Fu, C.Y., Tu, F.Z., 2009. "Recent advances on the *GAP* promoter derived expression system of *Pichia pastoris*." *Mol Biol Rep* 36(6):1611–1619.

Zhao, W., Wang, .J, Deng, R., Wang, X., 2008. “Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the *GAP* promoter.” *J Ind Microbiol Biotechnol* 35(3):189–195.

Zheng, H., Wang, X., Chen, J., Zhu, K., Zhao, Y., Yang, Y., Yang, S., Jiang, W., 2006. “Expression, purification, and immobilization of His-tagged D-amino acid oxidase of *Trigonopsis variabilis* in *Pichia pastoris*.” *Appl Microbiol Biotechnol* 70(6):683–689.

APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Fermentation Medium

1 M potassium phosphate buffer, pH=6.0	56.48 g KH_2PO_4, 14.8 g K_2HPO_4 was dissolved in dH_2O and the volume completed to 500 mL. The pH was checked. The buffer was sterilized by autoclaving and stored at room temperature.
Antifoam	10 or 20% (v/v) antifoam solution was prepared with dH_2O and autoclaved.
Base	25% NH_3OH. No need to sterilize.

SDS-PAGE Solutions

10%(w/v) APS (Ammonium PerSulfate)	Add 0.1 g APS to 1 mL dH2O, freshly prepared.
1.5 M Tris-HCl, pH=8.8	36.3 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 8.8 with 6 N HCl. The buffer was completed to 200 mL with dH_2O. It was autoclaved and stored at 2-8 °C.
0.5 M Tris-HCl , pH=6.8	12.1 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 6.8 with 6N HCl. The buffer was completed to 200 mL with dH_2O. The buffer was autoclaved and stored at 2-8 °C.

Resolving Buffer (12%) (for 2 gels)	3.4mL dH_20, 4mL 30% Acrylamide-bis, 2.5 mL 1.5M Tris–HCl pH=8.8, 100µL 10%SDS, prior to gel preparation add 50µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine .
Stacking Buffer (5%) (for 2 gels)	2.8mL dH_20, 0.85mL 30% Acrylamide-bis, 1.25 mL 0.5M Tris –HCl pH=6.8, 50µL 10%SDS, prior to gel preparation add 25µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine
4 x Sample Loading Buffer for SDS-PAGE	200 mM Tris-HCl, pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue; 10% 2-mercaptoethanol. 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, 24 mL acetic acid, 100 Ml 37% formaldehyde and complete to 200 mL with dH_2O. This solution can be stored up to one month at room temperature.
Pretreatment Solution	Dissolve 0.05 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 250 mL distilled water by mixing with a glass rod. Take 2 mL and set aside for further use in developing solution preparation.

Silver Nitrate 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 dH_2O. Add 2 mL from pretreatment solution and 75 µL 37% formaldehyde.
Stop Solution	Mix 50 mL methanol, 12 mL acetic acid and complete to 100 mL with distilled water.

Protease Assay solutions

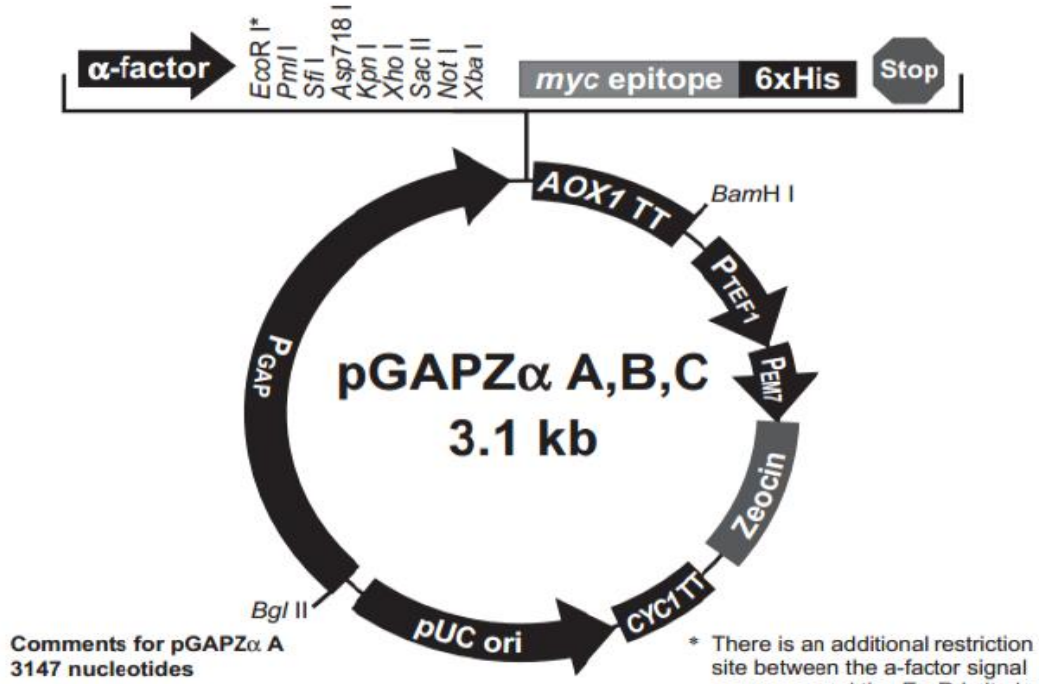
0.05 M Sodium Acetate buffer (for acidic proteases)	Dissolve 0.713 ml acetic acid in 25 ml total dH_2O. Dissolve 2.052 g sodium acetate in 50 ml dH_2O. Titrate sodium acetate solution with acetic acid solution to pH 5.0, and final V= 50 ml. Then dilute to 500 ml. Autoclave and store at +4 ^oC.

Fructose Analysis

70% (v/v) Sulfuric acid	70 ml sulfuric acid was gradually added to 30 ml dH_2O.
1.5% (w/v) Cysteine HCl	0.75 g cysteine was dissolved in 50ml dH_2O. Freshly prepared and used.
0.12%(w/v) carbazole	0.12 g carbazole was dissolved in 100ml dH_2O. Freshly prepared and used.

APPENDIX B

NUCLEOTIDE SEQUENCES AND PLASMIDS



Comments for pGAPZα A
3147 nucleotides

GAP promoter region: bases 1-483
α-factor signal sequence: bases 493-759
Multiple cloning site: bases 760-828
myc epitope tag: bases 827-856
Polyhistidine tag: bases 872-889
AOX1 transcription termination region: bases 893-1233
TEF1 promoter region: bases 1234-1644
EM7 promoter: bases 1645-1712
Sh ble ORF: bases 1713-2087
CYC1 transcription termination region: bases 2088-2405
pUC origin: bases 2416-3089

* There is an additional restriction site between the a-factor signal sequence and the *Eco*R I site in versions B and C of pGAPZa:

Pst I in pGAPZα B
Cla I in pGAPZα C

Figure B.1 Schematic representation of map of pGAPZαA vector supplied from Invitrogen

APPENDIX C

MOLECULAR WEIGHT MARKER

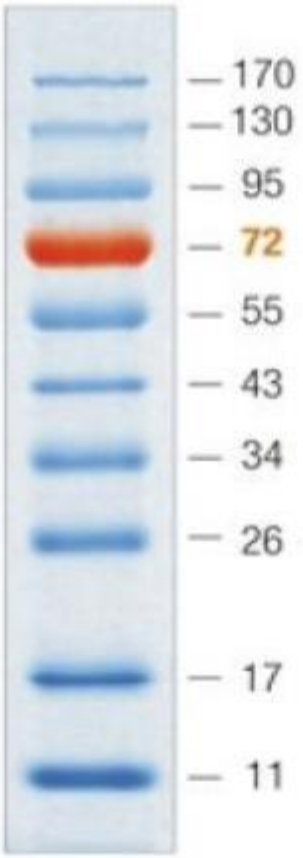


Figure C.1 PageRuler^{TM} Prestained Protein Ladder (Fermentas)

APPENDIX D

SDS-PAGE PROTEIN ANALYSES

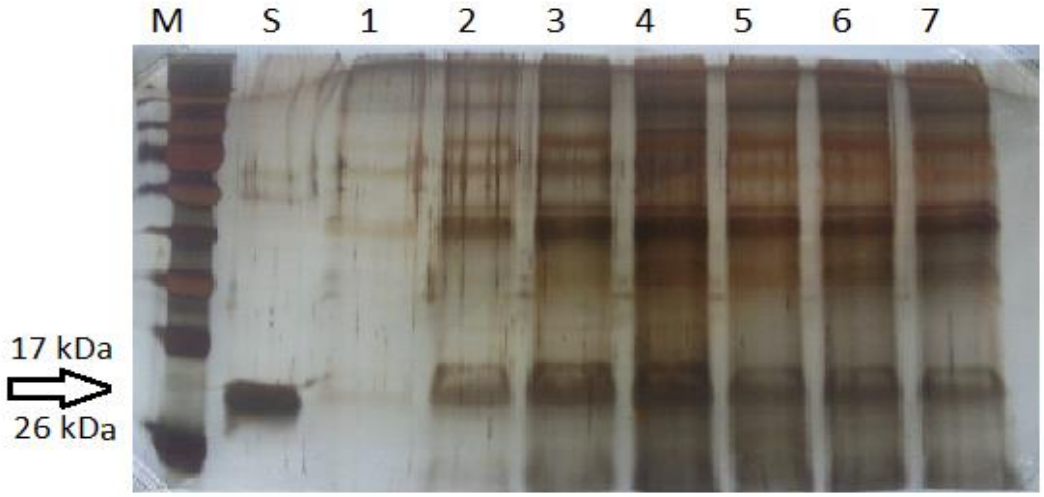


Figure D.1 Silver stained SDS-PAGE gel images of proteins produced in BR-1, 1:1 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18.

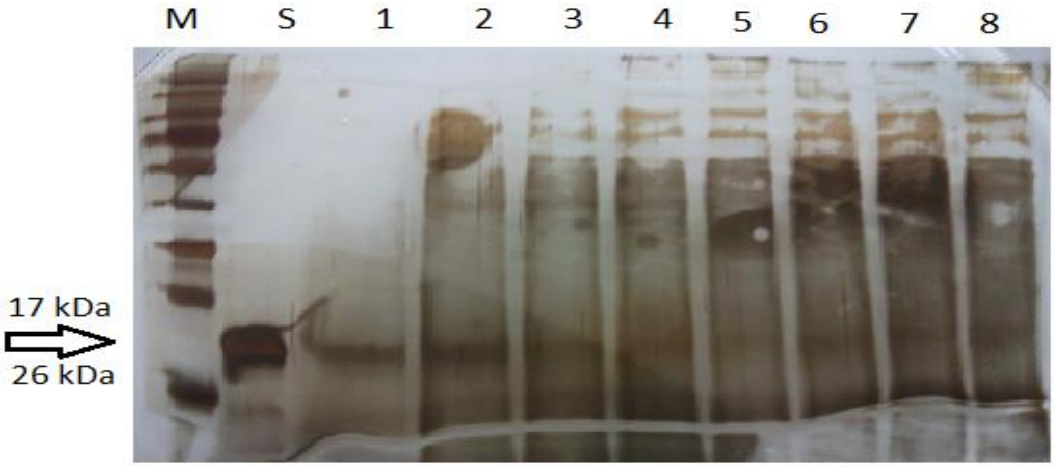


Figure D.2 Silver stained SDS-PAGE gel images of proteins produced in BR-2, 1:1 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=10.5 h, Lane 6: t=12 h, Lane 7: t=13.5 h, Lane 8: t=15 h.

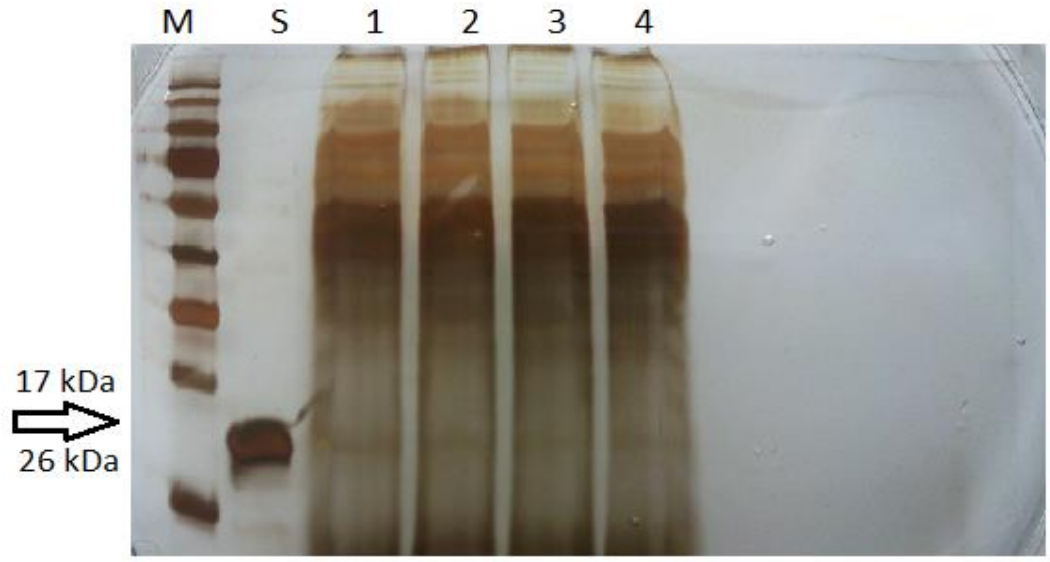


Figure D.3 Silver stained SDS-PAGE gel images of proteins produced in BR-2, 1:1 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: t=18 h, Lane 2: t=21 h, Lane 3: t=24 h, Lane 4: t=27 h.

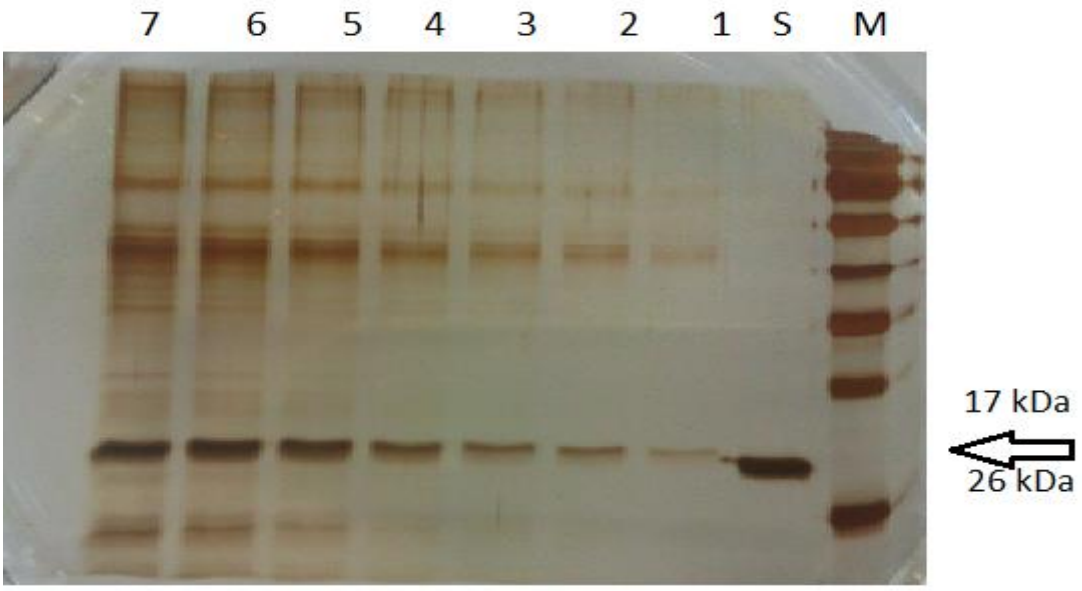


Figure D.4 Silver stained SDS-PAGE gel images of proteins produced in BR-3, 1:10 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18.

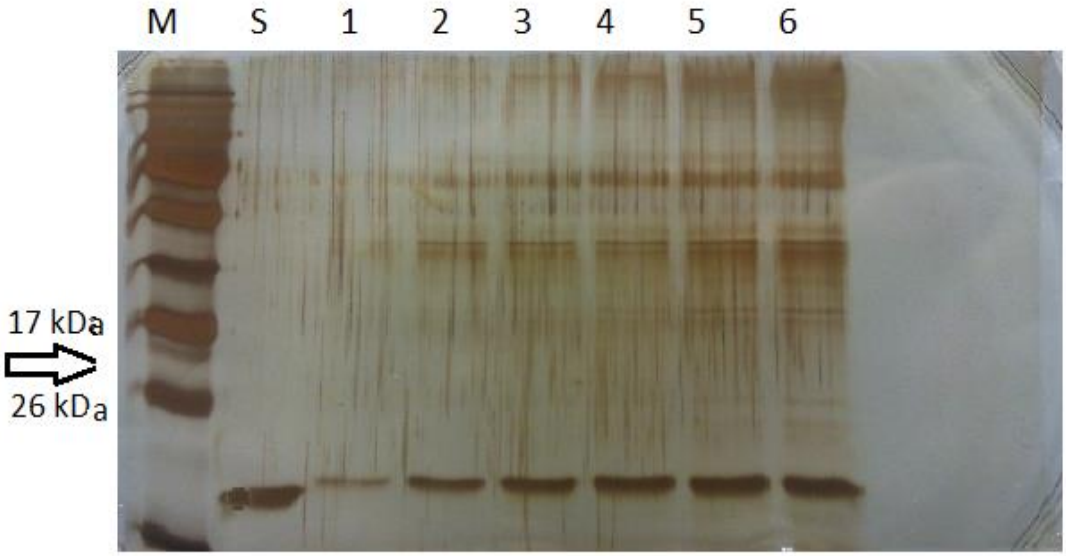


Figure D.5 Silver stained SDS-PAGE gel images of proteins produced in BR-4, 1:10 diluted samples. M: PageRuler protein ladder; S: 25 mg L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h.

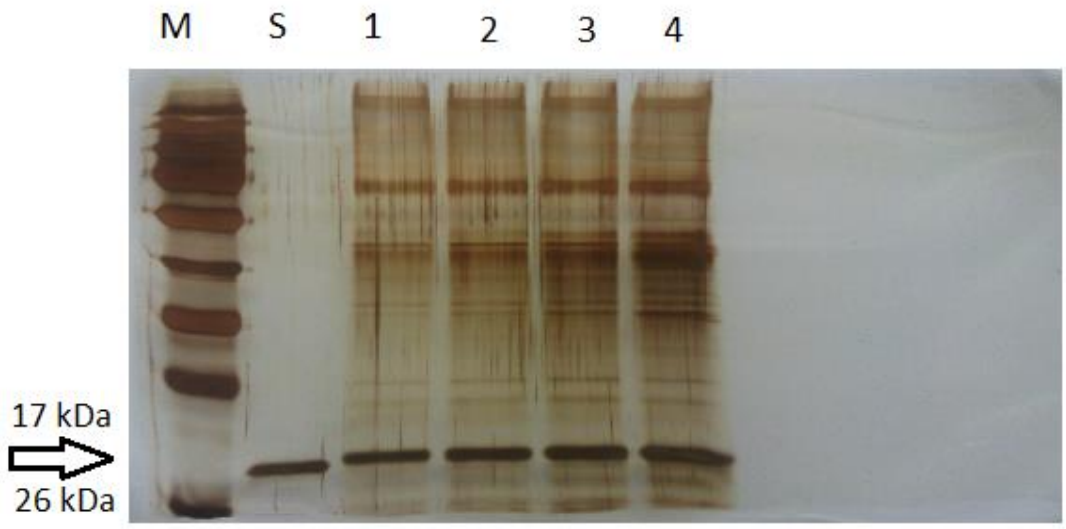


Figure D.6 Silver stained SDS-PAGE gel images of proteins produced in BR4, 1:10 diluted samples. M: PageRuler protein ladder; S: 25 mg L^{-1} hGH standard, Lane 1: t=18 h, Lane 2: t=21 h, Lane 3: t=24 h, Lane 4: t=26 h.

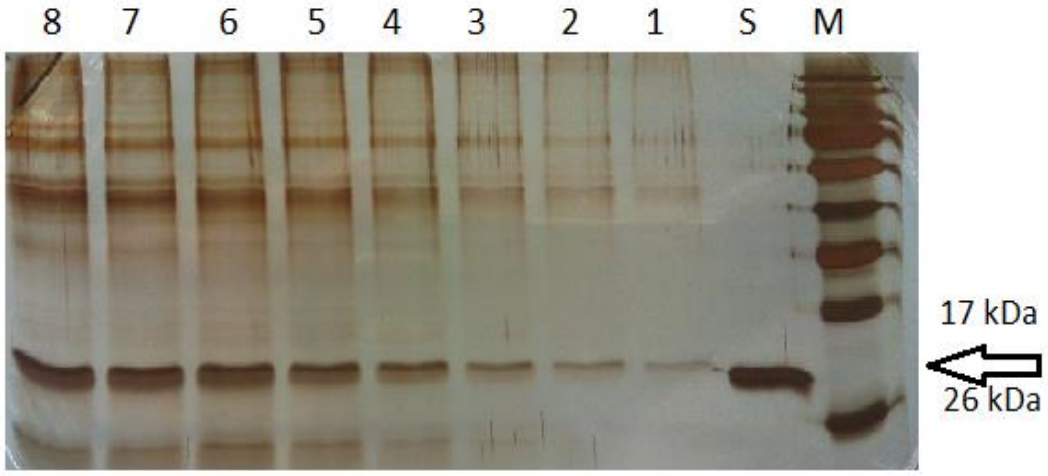


Figure D.7 Silver stained SDS-PAGE gel images of proteins produced in BR-5, 1:10 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=17, Lane 8: t=19h.

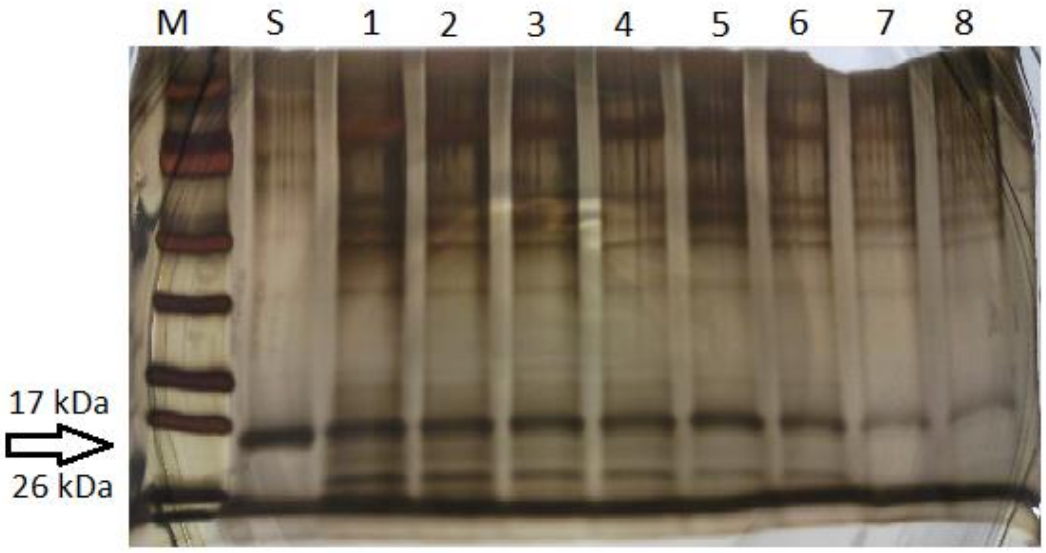


Figure D.8 Silver stained SDS-PAGE gel images of proteins produced in BR-6 and BR-7, 1:10 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: BR-7 t=9 h, Lane 2: BR-6 t=9 h, Lane 3: BR-7 t=6 h, Lane 4: BR-6 t=6 h, Lane 5: BR-7 t=3 h, Lane 6: BR-6 t=3 h, Lane 7: BR-7 t=0 h, Lane 8: BR-6 t=0h.

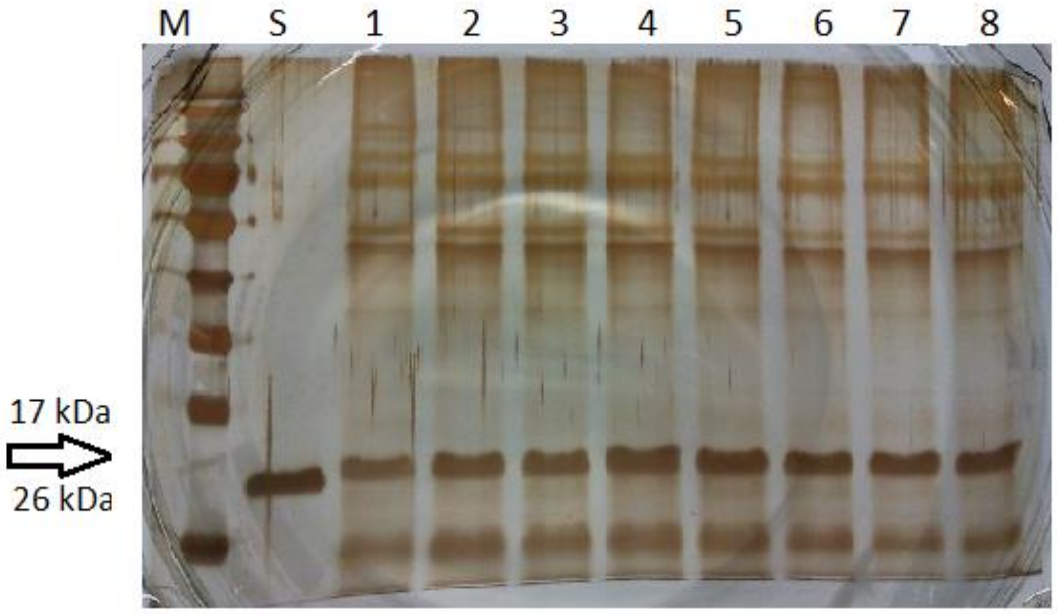


Figure D.9 Silver stained SDS-PAGE gel images of proteins produced in BR-6 and BR-7, 1:10 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: BR-7 t=19 h, Lane 2: BR-6 t=19 h, Lane 3: BR-7 t=17 h, Lane 4: BR-6 t=17 h, Lane 5: BR-7 t=15 h, Lane 6: BR-6 t=15 h, Lane 7: BR-7 t=12 h, Lane 8: BR-6 t=12 h.

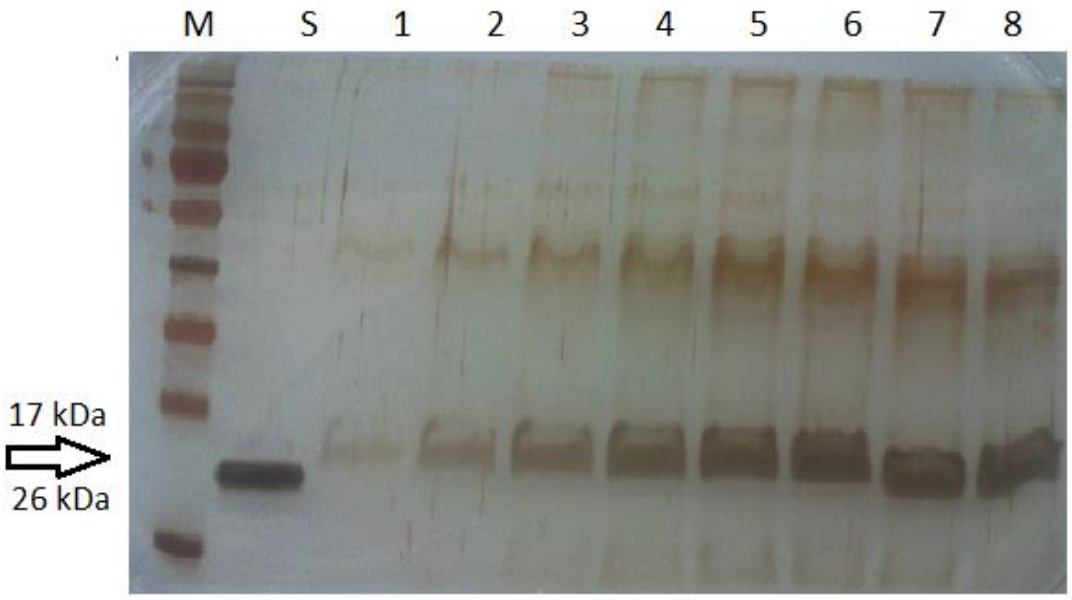


Figure D.10 Silver stained SDS-PAGE gel images of proteins produced in BR-8, 1:5 diluted samples. M: PageRuler protein ladder; S: 50 mg L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=17, Lane 8: t=19h.

APPENDIX E

CALIBRATION CURVES FOR ORGANIC ACIDS

- **Gluconic Acid**

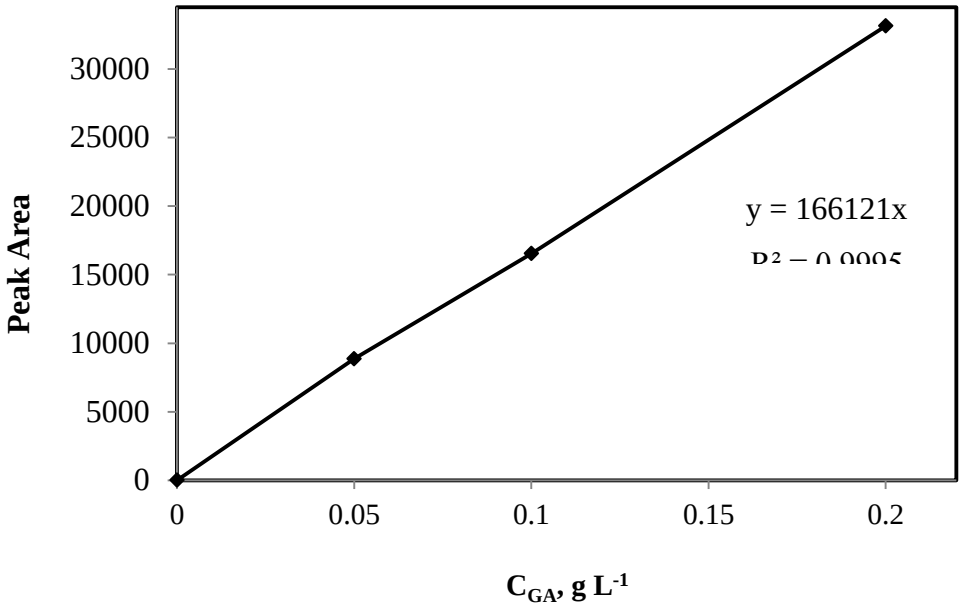


Figure E.1 Calibration curve for gluconic acid concentration

Based on the equation obtained from the calibration curve:

$$C_{GA} = \frac{Peak\ Area}{166121} \qquad (E.1)$$

- **Formic Acid**

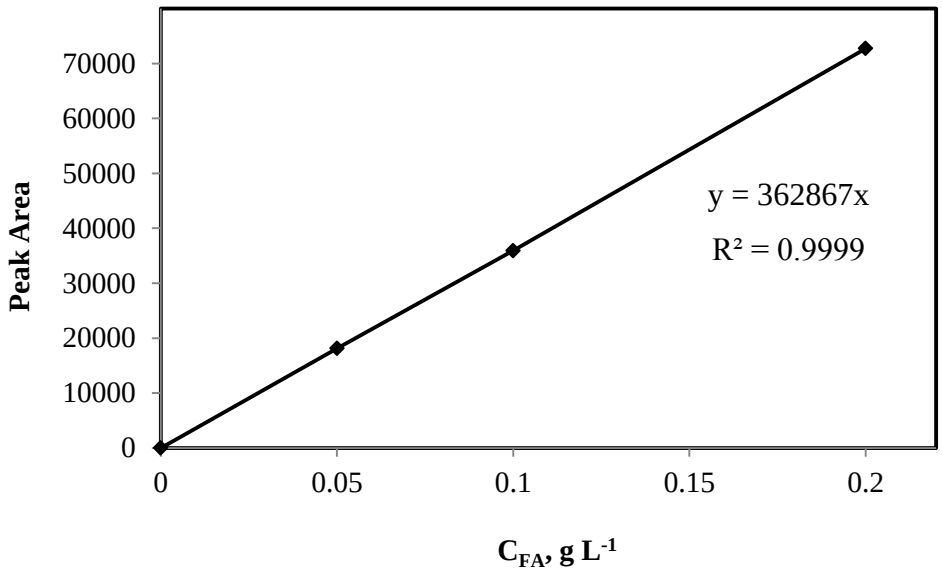


Figure E.2 Calibration curve for formic acid concentration

Based on the equation obtained from the calibration curve:

$$C_{FA} = \frac{Peak\ Area}{362867} \qquad (E.2)$$

- **Lactic Acid**

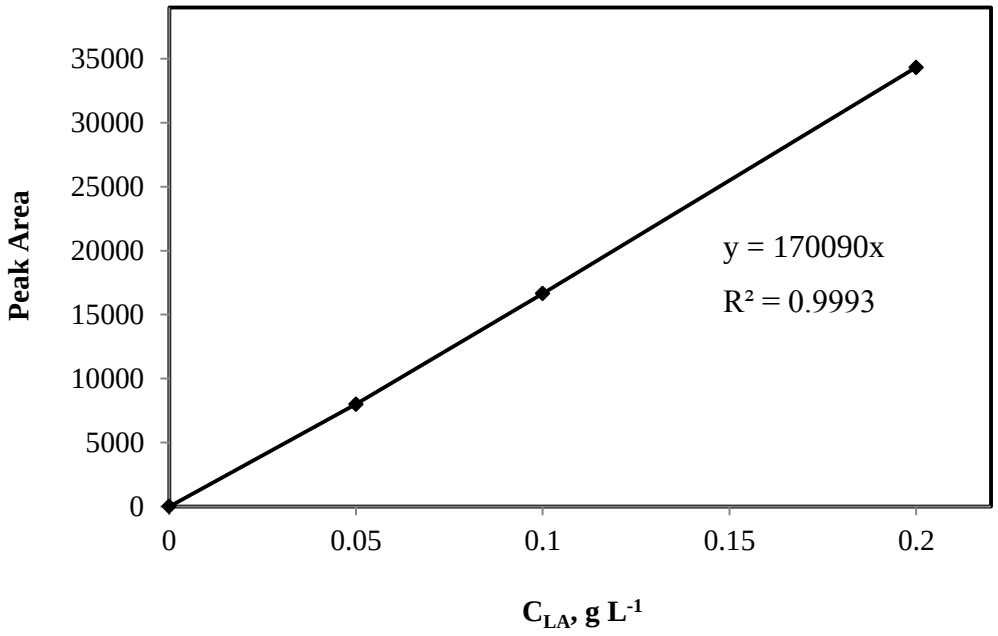


Figure E.3 Calibration curve for lactic acid concentration

Based on the equation obtained from calibration curve:

$$C_{LA} = \frac{Peak\ Area}{170090} \qquad (E.3)$$

- **Citric Acid**

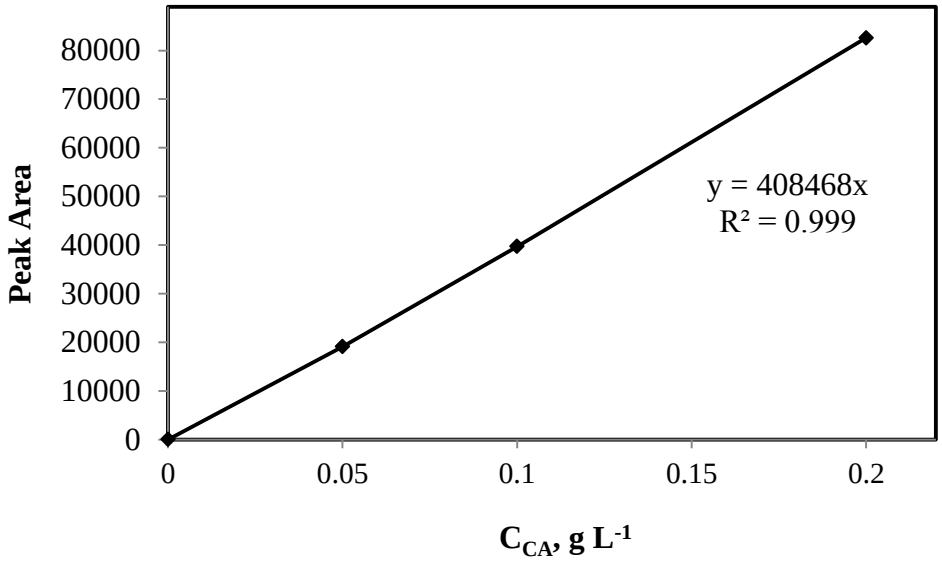


Figure E.4 Calibration curve for citric acid concentration

Based on the equation obtained from the calibration curve:

$$C_{CA} = \frac{Peak\ Area}{408468} \qquad (E.4)$$

- **Acetic acid**

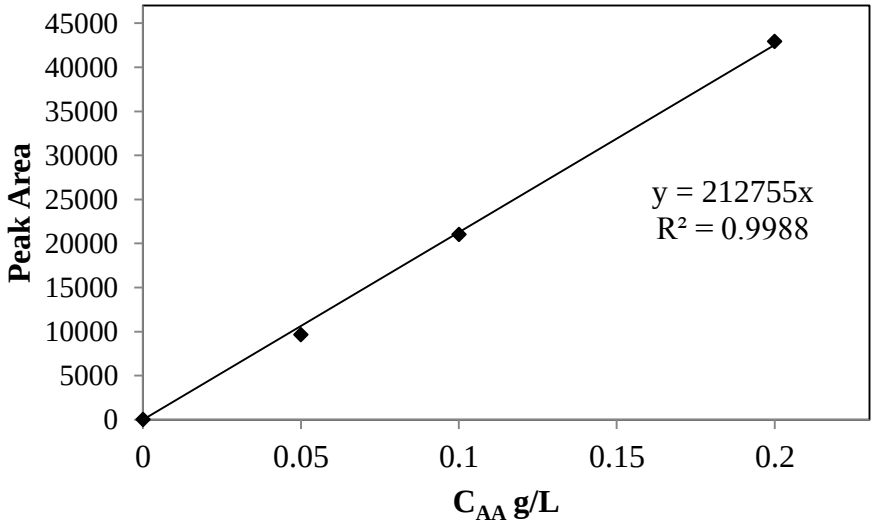


Figure E.5 Calibration curve for acetic acid concentration

Based on the equation obtained from the calibration curve:

$$C_{GA} = \frac{Peak\ Area}{212755} \qquad (E.5)$$

- **Maleic acid**

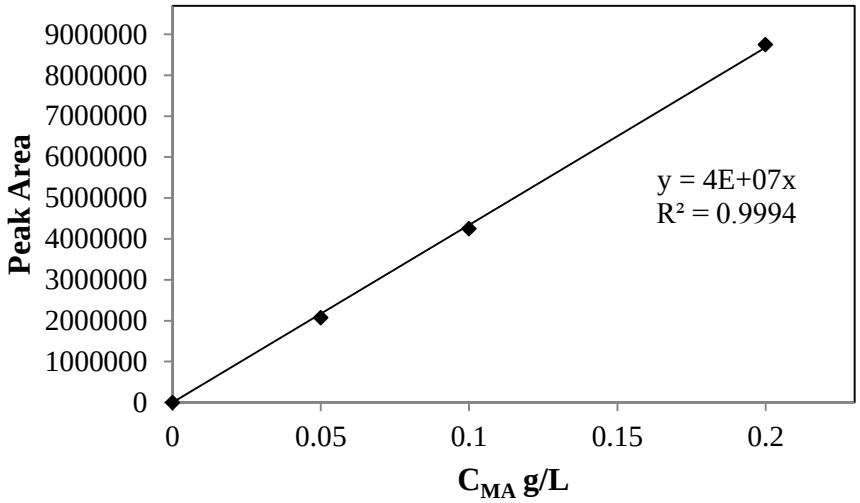


Figure E.6 Calibration curve for maleic acid concentration

Based on the equation obtained from calibration curve:

$$C_{GA} = \frac{Peak\ Area}{4 \times 10^7} \qquad (E.6)$$

- **Pyruvic acid**

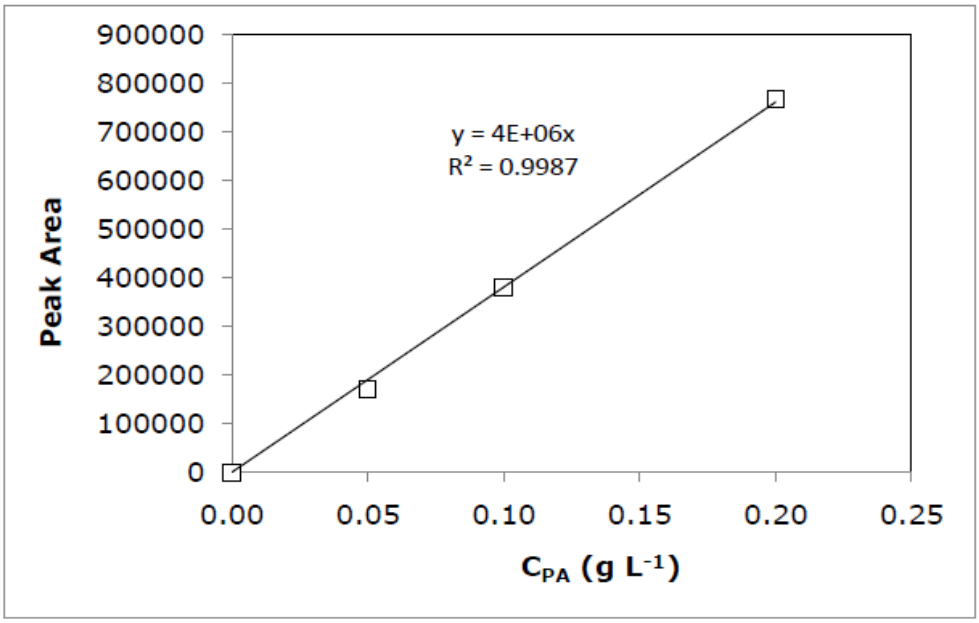


Figure E.7 Calibration curve for pyruvic acid concentration

Based on the equation obtained from calibration curve:

$$C_{GA} = \frac{Peak\ Area}{4 \times 10^6} \qquad (E.7)$$

- **Oxalic acid**

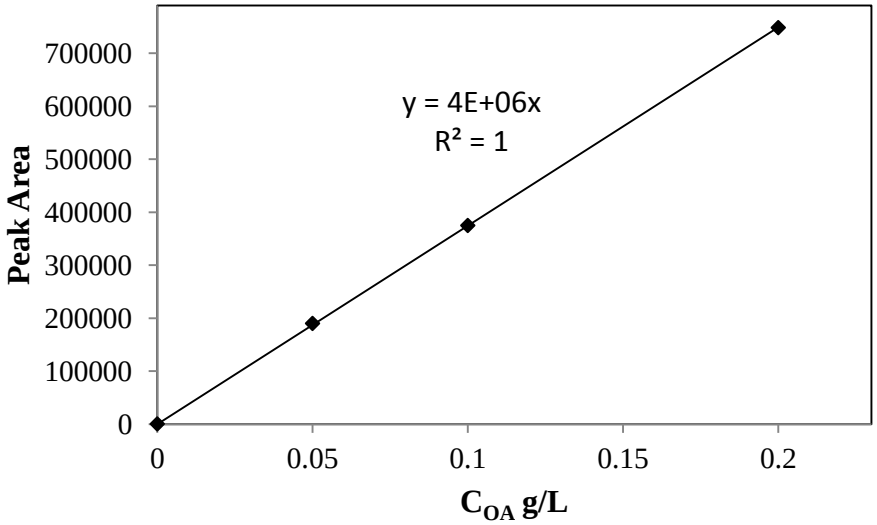


Figure E.8 Calibration curve for oxalic acid concentration

Based on the equation obtained from calibration curve:

$$C_{GA} = \frac{Peak\ Area}{4 \times 10^6} \qquad (E.8)$$

- **Succinic Acid**

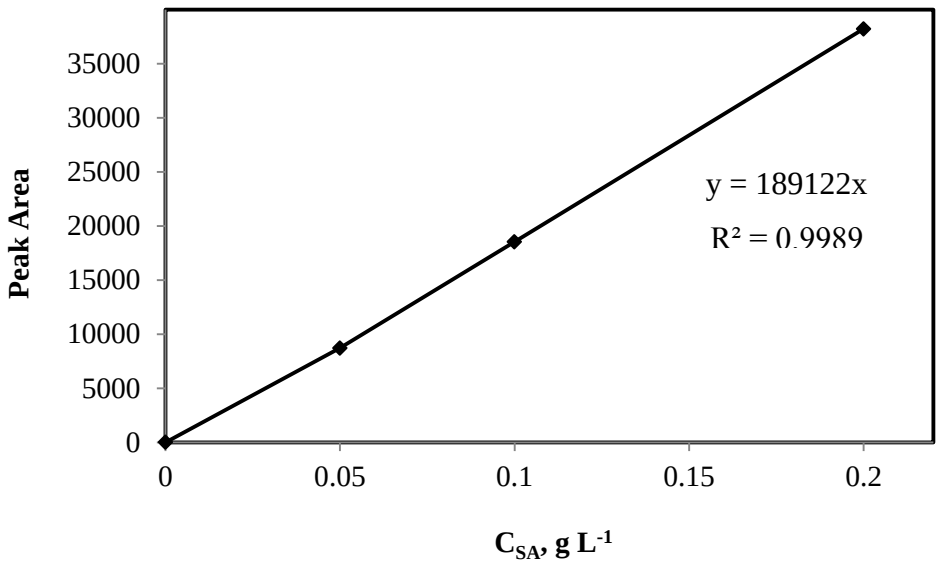


Figure E.9 Calibration curve for succinic acid concentration

Based on the equation obtained from calibration curve:

$$C_{SA} = \frac{Peak\ Area}{189122} \qquad (E.9)$$

- **Fumaric Acid**

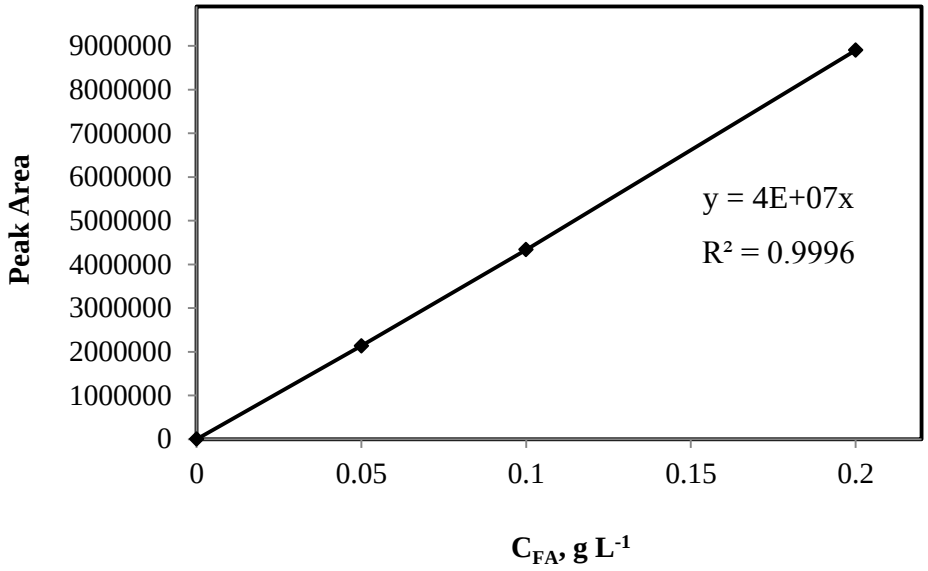


Figure E.10 Calibration curve for fumaric acid concentration

Based on the equation obtained from calibration curve:

$$C_{FA} = \frac{Peak\ Area}{4 \times 10^7} \qquad (E.10)$$

- **Malic Acid**

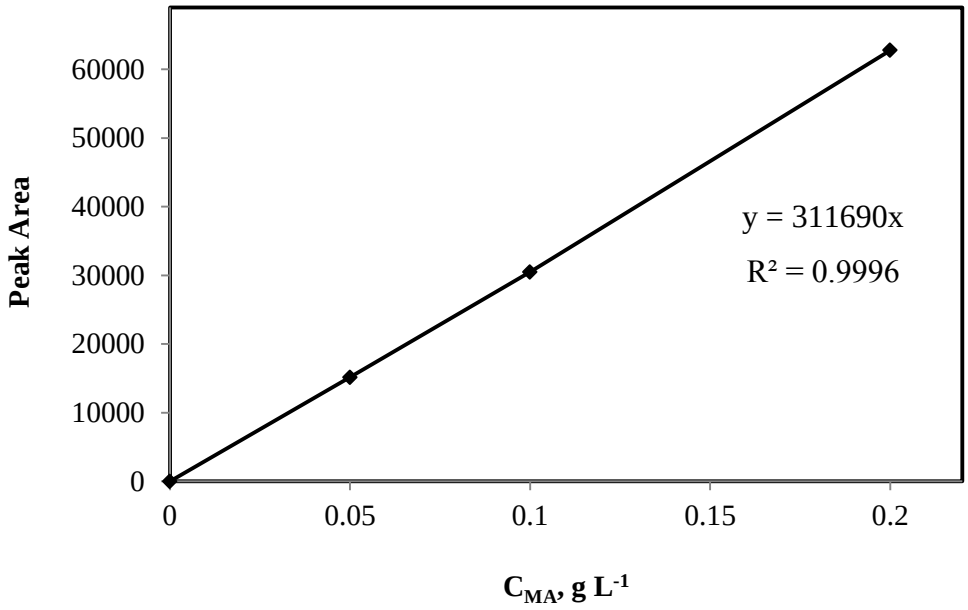


Figure E.11 Calibration curve for malic acid concentration

Based on the equation obtained from calibration curve:

$$C_{MA} = \frac{Peak\ Area}{311690} \qquad (E.11)$$

APPENDIX F

CALIBRATION CURVE FOR GLUCOSE CONCENTRATION

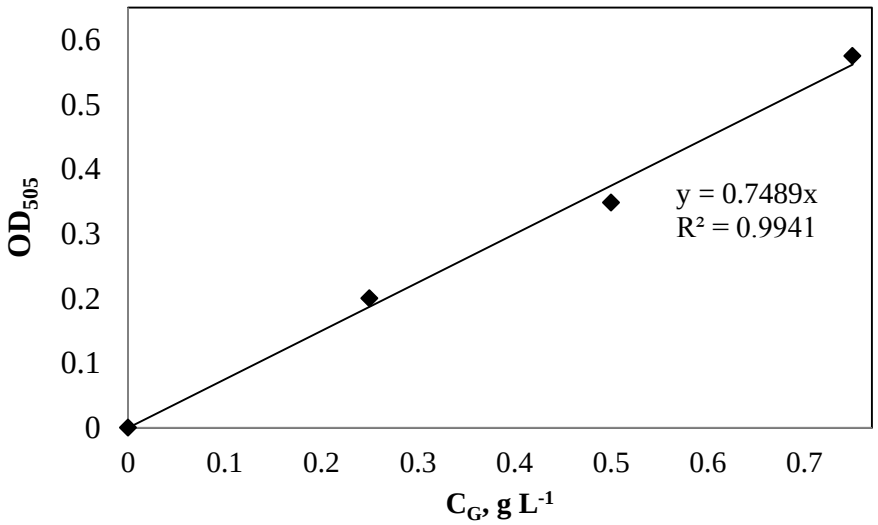


Figure F.1 Calibration curve for glucose concentration

Based on the equation obtained from calibration curve:

$$C_G = \frac{Absorbance}{0.7489} \times (Dilution\ ratio) \qquad (F.1)$$

APPENDIX G

CALIBRATION CURVE FOR FRUCTOSE CONCENTRATION

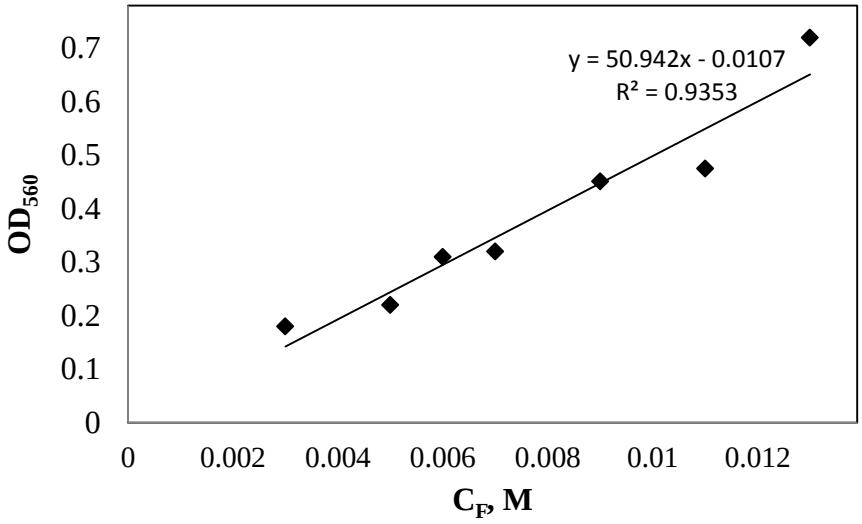


Figure G.1 Standard calibration curve for D-fructose concentration

Based on the equation obtained from calibration curve:

$$C_F(M) = \frac{(Absorbance - 0.010)}{50.942} \times (Dilution\ ratio) \qquad (G.1)$$

APPENDIX I

FEEDING PROFILES IN TERMS OF MASS FLOW RATES IN BR-1, BR-2, BR-3 AND BR-8

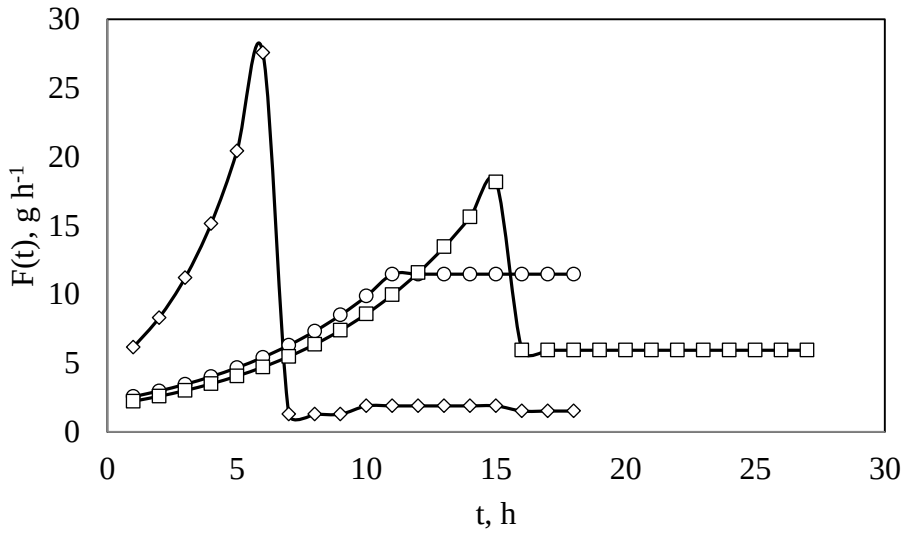


Figure I.1 Feeding profiles in terms of glucose mass flow rate in BR-1 (◊), BR-2(□), and BR-3 (○)

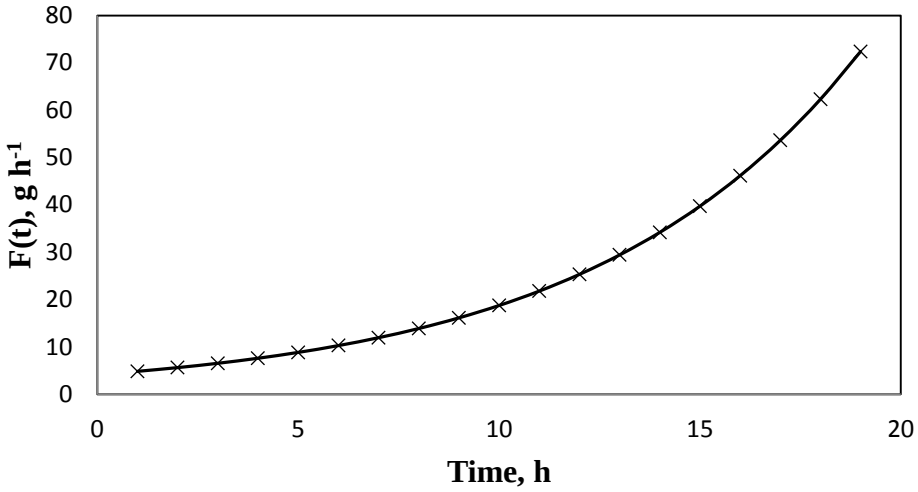


Figure I.2 Feeding profile in terms of molasses mass flow rate in BR-8