DEFINED AND COMPLEX MEDIUM BASED FEEDING STRATEGY DEVELOPMENT FOR RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *P. pastoris* UNDER GAP PROMOTER

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DEFINED AND COMPLEX MEDIUM BASED FEEDING STRATEGY DEVELOPMENT FOR RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *P. pastoris* UNDER GAP PROMOTER

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ABSTRACT

DEFINED AND COMPLEX MEDIUM BASED FEEDING STRATEGY DEVELOPMENT FOR RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *P. pastoris* UNDER GAP PROMOTER

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The objective of this study is to investigate different feeding strategies leading to higher recombinant human growth hormone (rhGH) production by *Pichia pastoris*, driven under constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene. rhGH production took place in a pilot bioreactor where glucose and molasses were examined as carbon sources. For batch phase, which is the first production phase of the reactor, all experiments share the same characteristics where glycerol was utilized as the carbon source. For semi-batch phase, exponential and consecutive strategies, were designed by using predetermined specific growth rates as μ_o =0.07, 0.10, and 0.15 h^{-1}. Consecutive feeding consisted of designed cyclic repetitions of 1.5h exponential feeding as semi-batch operation followed by a 0.5h of interruption period where bioreactor operates batch-wise. The cells were more responsive to feeding + starvation strategies, when glucose was utilized as carbon source by obtaining cell concentrations up to 135 g L^{-1}. For consecutive feeding strategy with glucose based continuous feed stream designed with μ_o = 0.10 h^{-1}, the maximum C_{rhGH} was obtained as 611 mg L^{-1} and rhGH production increased by 1.37-fold compared to continuous exponential feeding profile with the same μ_o = 0.10 h^{-1}. On the other hand, molasses

was a more effective carbon source, when was exponentially fed to the production medium with μ_o =0.07 h^{-1} and resulted in higher production yields on the cell and substrate. Among all the strategies, this strategy produced the highest rhGH concentration as C_{rhGH} = 650 mg L^{-1} at t =17 h where the cell concentration was C_X =71.2 g L^{-1}. The overall product and cell yield on total substrate were obtained as $Y'_{P/S}$ = 10.7 mg g^{-1} and $Y'_{X/S}$=0.434 g g^{-1}, respectively.

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

ÖZ

Pichia pastoris ile

REKOMBİNANT İNSAN BÜYÜME HORMONU ÜRETİMİ İÇİN TANIMLI VE KOMPLEKS ORTAM TEMELLİ BESLEME STRATEJİSİ GELİŞTİRİLMESİ

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Bu çalışmada *Pichia pastoris* ile rekombinant insan büyüme hormonu (rhGH) üretimini arttırmaya yönelik farklı besleme stratejilerinin araştırılması amaçlanmaktadır. Bu amaç için, ilk olarak hücre-dışı insan büyüme hormonu üretebilen yapısal başlatıcı *gliseraldehit-3-fosfat dehidrogenaz* geni ile yönlendirilmiş rekombinant *Pichia pastoris* geliştirilmiştir. Geliştirilen r-*Pichia pastoris* tanımlı ve kompleks üretim ortamların etkilerinin araştırıldığı pilot ölçek biyoreaktörde rhGH üretiminde kullanılmıştır. Gliserolün karbon kaynağı olarak kullanıldığı reaktörün ilk üretim fazında (kesikli faz) tüm deneylerin üretim koşulları aynıdır. İkinci faz olan yarı-kesikli faz için, öngörülmüş özgül çoğalma hızı, μ_o = 0.07, 0.10, and 0.15 st^{-1} değerleri kullanılarak farklı besleme stratejileri tasarlanmıştır. Karbon tüketimi ve hücre metabolik aktivitesini arttırmak için, üstel beslemenin yanı sıra, üstel besleme profilinin ardışık beslemesiz aralıklarla birleştirildiği besleme stratejisi tasarımları yapılmıştır. Her 1.5 saatlik beslemeden sonra ardışık 0.5 saatlik bekleme uygulanmıştır. Glukoz (tanımlı ortam) ve sukrozun glukoz ve fruktoza hidrolizi ile elde edilen indirgenmiş melas (kompleks ortam) karbon kaynağı olarak kullanılmış ve

karşılaştırılmıştır. Tasarlanan stratejiler arasında, glukoz ile uygulanan ikili besleme stratejisi ile daha yüksek rhGH derişimi ve özgül üretim hızı elde edilmiştir. Önceden belirlenmiş özgül çoğalma hızı μ_o = 0.07 st^{-1} olan üretim ortamında üstel olarak beslendiğinde melasın daha etkin karbon kaynağı olduğu bulunmuştur. En yüksek rhGH derişimi t = 17 st'te, C_{rhGH} = 650 mg L^{-1} ve hücre derişiminin C_X = 71.2 g L^{-1} değerlerinde elde edilmiştir. Toplam substratta tüm ürün ve hücre verimi $Y'_{P/S}$ = 10.7 mg g^{-1} ve $Y'_{X/S}$ = 0.434 g g^{-1} olarak bulunmuştur.

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

To my family

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B. Hoxha

29.08.2016

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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

AOX1	Alcohol oxidase 1
BMGY	Buffered glycerol-complex medium
BSM	Basal salt medium
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
EDTA	Ethylenedinitrilotetraacetic acid
FLD1	*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

HPLC	High performance liquid chromatography
OD	Optical density
PCR	Polymerase chain reaction
PGK1	*Phosphoglycerate kinase*
rhGH	Recombinant human growth hormone
r-protein	Recombinant protein
PTM1	Pichia trace minerals
YPD	Yeast Extract Peptone Dextrose

CHAPTER 1

INTRODUCTION

The earliest innovative steps of biotechnology date back to ancient times where microorganisms were used for food and fermentation processes. The detection of microbial metabolic activities in the 19th century marked the beginning of modern biotechnology where important chemicals were produced by using microorganisms. The discovery of the DNA molecule in 1953 by F. Crick, J. Watson, M. Wilkins, and R. Franklin, broadened the application of genetic engineering which further expanded the sophistication, scope and applicability of industrial biotechnology. In 1982 OECD defined biotechnology as "the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services" (http://www.oecd.org). The main driving force that focused industry on biotechnology applications was the increasing demand for medical, agricultural and food products.

Till 1980s, microorganism expression systems have been developed and utilised for basic research and industrial biotechnology applications. Biotechnology is used for traditional fermentations (e.g. beer, wine, sake, soy sauce), for production of food and feed ingredients (e.g. enzymes, flavours, pigments, amino acids, organic acids), biofuels (e.g. bioethanol, biodiesel, farmesene, amorphadiene), biocatalysts (e.g. pharmaceuticals, chiral, chemical intermediates, biotransformation), heterologous protein production (e.g. protein pharmaceuticals, enzymes, hormones, vaccines, toxins), fundamental biological research (e.g. molecular and cellular biology, genomics, pathway engineering, system biology mechanisms), biomedical research

(e.g. drug discovery, drug resistance and metabolism elucidation), biocontrol (e.g. crop protection, food and feed safety, probiotics), environmental biotechnology (e.g. bioremediation, pollutant degradation, removal of heavy metals from waste waters).

Biopharmaceuticals, approved by regulatory authorities for therapeutic applications, are mostly proteins that have been produced by recombinant DNA technology and they represent the most invested segment of pharmaceutical market. Biopharmaceuticals can be further classified as: anticoagulants, blood factors, enzymes, growth factors, interferons and interleukins, hormones, monoclonal antibodies, thrombolytics and vaccines. The selling prices for recombinant proteins per gram are: $375 for human insulin, $23,000 for tissue Plasminogen Activator (tPA), $35,000 for human growth hormone, $384,000 for Granulocyte macrophage colony Stimulating factor (GM-CSF), $450,000 for G-CSF and $840,000 for Erythropoietin (EPO) compared to yeast's general selling price of $0.003 per gram (Demain *et al*. 2009). In late 2011 statistics, out of 211 biopharmaceuticals, 31 % were produced in *Escherichia coli*, 15 % in yeast and 43 % in mammalian cells (Berlec *et al*. 2013).

Hormones are chemical substances that act as messenger molecules in the body by travelling in the blood to the particular target cell. They control and regulate the activity of certain cells or organs (Norman, 1997). The human growth hormone, also known as somatotropin, is an anionic, non-glycosylated, four helix-bundle protein that contains 191 amino acid residues displaying a molecular mass of 22 kDa. It is secreted by the somatotropic cells in pituitary gland and provides the growth of bone, muscle and cartilage cells. Due to gene mutation or damage in hypothalamus/pituitary gland, disorders in hGH secretion levels lead to idiopathic short stature, dwarfism, bone fractures, bleeding ulcers, skin burns, wounds, cardiovascular diseases, and chronic renal insufficiency. These diseases can be treated by exogenous hGH administration (Walsh *et al*. 2007).

Before the application of recombinant technology, the only way to obtain hGH was from pituitary glands of cadavers. The first rhGH intracellular production by recombinant technology was performed by using *E. coli* as a host microorganism in 1979 (Goeddel *et al*. 1979). Since then rhGH is being produced by recombinant DNA

technology in the context of metabolic engineering by mainly using the host microorganism *Escherichia coli* and *Pichia pastoris.* Companies of recombinant human growth hormone (rhGH) production and the respective drug name are given in Table 1.1.

Table 1.1. Companies and respective drugs of recombinant human growth hormone (rhGH)

Companies	**Drug Product**	**U.S FDA Approval Date**
Eli Lilly	Humatrope	1987
Biotechnology General	BioTropin	1991
Genentech	Nutropin	1994
Pharmacia and UpJohn	Genotropin	1995
Ferring Pharmaceuticals	Tev-Tropin	1995
EMD Serono	Saizen & Serostim	1996
Novo Nordisk Inc.	Norditropin	2000
Pharmacia	Somavert	2003
Sandoz	Omnitrope	2006
Biopartner	Valtropin	2007

There are about 1500 yeast species and only around 70-80 species, have been potentially useful for biotechnology purposes. Compared to mammalian and microbial cell lines, yeasts lead to higher cell yields, higher productivity and shorter fermentation cycles. Furthermore yeast have both non-pathogenic and non-pyrogenic posttranslational modification pathways (Demain *et al*. 2009). The passage of proteins through these secretory pathways provide proteolytic maturation, glycosylation and disulphide bond formation. However before 1980s, yeasts were not capable of producing human proteins containing N-linked carbohydrates applied as injectable pharmaceutical drugs, because their sugars consisted of a high mannose type which is very different from the human's sugar composition and configuration (Cregg *et al*. 2009). The unrecognizable glycoproteins would further be rejected from the human

immune system which lead to the patient's death. These obstacles were studied and overcome, resulting in yeasts being potential expression systems. The utilization of yeasts for production of heterologous proteins that are unavailable from traditional sources, has had a great impact on biotechnological processes which was marked with the cloning, expression, processing, and secretion of human proinsulin in *S. cerevisiae* (Branduardi and Porro, 2012). Some of the commonly used yeast species investigated for recombinant protein (r-protein) production are *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe, Yarrowia lipolytica, Arxula adeninivorans* (Türker *et al*. 2014).

The methylotrophic yeast *Pichia pastoris* is a very preferable host microorganism, which provides both extracellular and intracellular protein production in high expression levels (Sreekrishna et al., 1997; Cregg, 1999). Compared to higher eukaryotes, it can grow in minimal media and can reach high cell densities which makes the production conditions easier, faster and less expensive (Cregg *et al.* 2000). *P. pastoris*, being a eukaryotic unicellular microorganism, is capable of disulfide bond formation, folding, proteolytic processing, and glycosylation. In order to produce heterologous proteins by *P. pastoris*, a variety of promoters, different types of process operations and feeding strategies have been examined. *P. pastoris* has strong promoters such as alcohol oxidase 1 (AOX1) promoter and the glyceraldehyde-3-phosphate dehydrogenase gene (GAP) promoter that lead to high expression levels (Cereghino and Cregg, 2000, Cos *et al*., 2006). However, the usage of methanol to induce AOX1 promoter is disadvantageous since methanol is a hazardous compound and its storage for industrial scale production is difficult. Most important, for the production of human proteins using methanol can be toxic as cannot be separated or purified totally. Furthermore its usage for food products is considered unsuitable because methanol is derived from petrochemical sources (Macauley-Patrick *et al*. 2005). Using constitutive promoters such as glycolytic GAP promoter avoids the usage of methanol and simplifies the production process.

Under a constitutive promoter the r-protein concentration increases proportionally with the cell growth and there is no need for carbon source shifts during the bioprocess (Kottmeier *et al.* 2012). Despite the differences in their regulation P_{GAP} and P_{AOX1} are

both strong promoters and reach similar expression levels (Waterham *et al*. 1997). The GAP promoter has been recently used in a wide range for production of several proteins and is the most frequently used alternative to P_{AOX1}. Furthermore using P_{GAP} instead of P_{AOX1} results in cheaper and shorter cultivation processes because of the elimination of carbons source shifts (Vogl and Glieder, 2013).

P. pastoris can utilize a high variety of carbons sources for energy production such as through fermentation of glucose, fructose, galactose, maltose, sugar alcohols, sorbitol and mannitol, or through oxidation of a variety of fermentation products, i.e., glycerol, ethanol, and lactate (Çalık *et al*. 2014). Furthermore, carbon source selection for cell growth is an important step since it affects the strength of the GAP promoter (Waterham *et al.* 1997). In this study both glucose and molasses were investigated as potential carbon sources. Molasses is a by-product of sucrose production from sugar beets and is seen as a potential carbon source because of its rich composition with organic non-sugars, vitamins, and essential materials which are effective at cell growth. It is easily obtainable with a low cost (Baratti *et al*. 1991).

Semi-batch operation consists of a substrate input stream whose flow rate and feeding strategy are determined in a way that improves the productivity of the desired recombinant protein. The most effective feeding strategy applied for semi-batch operation has been the exponential feeding strategy (Rebnegger *et al.* 2014, Garcia-Ortega *et al.* 2013). Furthermore, to enhance the productivity of a particular recombinant protein by *Pichia pastoris* under GAP promoter, Garcia-Ortega *et al.* (2016) for the semi-batch phase have examined a combination of exponential feeding with starvation intervals which resulted in higher specific production rates and product yields on substrate. It was proposed that higher secretion rates of proteins under regulation of GAP promoter were obtained during the starvation periods because of the ability of yeast to adapt to environmental stress. 1.5 h feeding followed by 0.5 h starvation resulted as the best optimization method when the specific predetermined growth rate was $\mu_o = 0.15 h^{-1}$ (Garcia-Ortega *et al.* 2016).

In this study, to shift the carbon source utilization from *P. pastoris* primary metabolism regulation to rhGH production, the designed strategies based on exponential feeding

followed by starvation period were investigated for both glucose and molasses. In these strategies glucose or molasses was fed to the system with predetermined specific growth rate of μ_o= 0.07 h^{-1}, 0.10 h^{-1}, 0.125 h^{-1}, 0.15 h^{-1} respectively, each interrupted 1.5 h by 0.5 h of no feeding period. These strategies were compared with the standard exponential feeding with μ_o = 0.07 h^{-1}, μ_o = 0.10 h^{-1}. By using the fermentation data for each designed semi-batch bioreactor experiment, yield coefficients, substrate consumption rates, and productivities were calculated.

CHAPTER 2

LITERATURE SURVEY

2.1 Hormones

In the endocrine system, there are different glands that are grouped according to their anatomical and physiological functions. Each gland consists of specialized cells that synthesize, store and secrete hormones (Fox *et al*. 2015). Hormones, as chemical messengers, are transported through the bloodstream to a particular organ to regulate its physiological processes (Baxter *et al*. 1997). Some of the general functions of different hormones are growth stimulation or inhibition, metabolism regulation, immune system activation or suppression and promotion of sleep or wakefulness. The main common role of hormones is keeping the human body in a state of physiological equilibrium by providing a balance between body's interior environment and alterations of the outer environment (Fox *et al*. 2015). Additionally hormones provide non-endocrine functions; paracrine function (can affect the cell neighboring their released organ), aurocrine function (affect the origin cell that they are released from) and exocrine function (the chemicals that are released by the sweat glands in the environment have an effect on the other being of the same species around them) (Neave, 2007) .

2.1.1 Hormonal signaling pathway (Fox et al. 2015)

In the hormonal signaling pathway many steps are involved starting with hormone synthesis which is carried out by cells in the endocrine gland. Then peptide and amine hormones different from lipid hormones are stored to be released from the gland into

the bloodstream when required. During the transport to the target organ the hormone travels independently or by means of transport proteins. The next step is receptor binding where the hormone attaches to the receptor molecule that can be found either in the membrane of the cells or inside the cells. When the hormone binds to the cell membrane it alters the cell metabolism. However hormones that bind to nuclear or cytoplasmic receptors inside the cell affect the expression of genes in the cell. The signal is received when the signal molecule binds to a specific receptor protein in or on the cell which has a specific shape to be recognized by the signal's receptors (Campbell and Reece, 2005). Finally the hormones are released from the cells of the target organ.

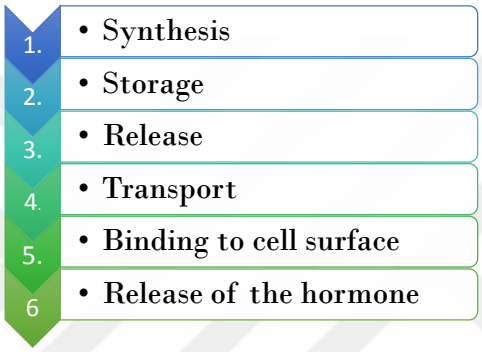


Figure 2.1 Hormonal signaling pathway (Fox et al. 2015)

2.1.2 Classification of hormones

Hormones are classified according to their chemical properties, and effects. According to the chemical structure hormones are categorized as peptides, amine and lipids (Fox *et al*. 2015)**:**

1. **Peptides-Peptide hormones:** The hormones in this class are chains of amino acids (polypeptides). These chains range in length from 3 to 100 Å. They are categorized according to their size into small peptides, large peptides and polypeptides.
2. **Amines-Amine hormones** are derived from aromatic amino acids such as tryptophan, phenylalanine and tyrosine.
3. **Lipids** (mainly steroids): Hormones of this group are derivatives of cholesterol and classified either as ketones or alcohols. Their chemical structure consists

of three six-carbon rings plus one five-carbon ring. All steroids are fat soluble and pass easily through cell membranes (Neave, 2007)

According to their affecting way at their target cells hormones are classified in two groups (Neave, 2007):

1. Hormones of this group cannot penetrate the cell, so they bind to receptors at the surface of the cell and affect the cell metabolism or gene expression indirectly by binding to other proteins responsible for these actions (Neave, 2007). All polypeptide hormones and monoamines are classified in this group (Lazar, 2002).
2. Hormones capable of entering the cells, attach to intracellular receptors to structure a 'hormone-receptor complex' which enters the cell nucleus and attaches to 'accceptor sites' on the chromosome (McEwen, 1976).

2.1.3 Human Growth Hormone (hGH)

The human Growth hormone (hGH) is a peptide hormone named as somatotropin and is mainly responsible for growth stimulation, cell reproduction and cell regeneration (Norman, 1997).

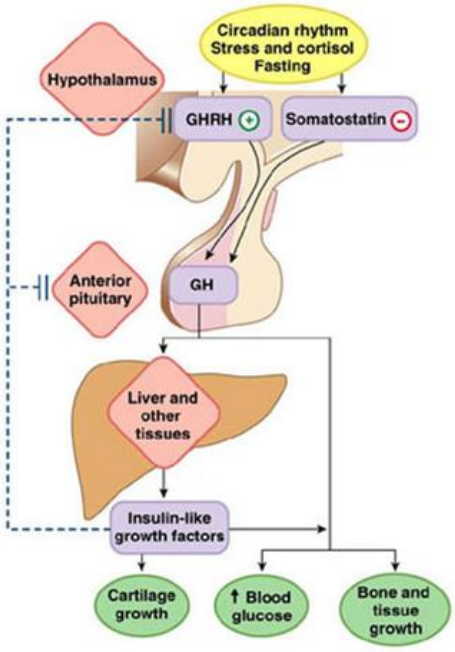


Figure 2.1. hGH Release Control (http://schoolworkhelper.net)

As illustrated by Figure 2.2, hGH is secreted from the pituitary gland and its regulation is managed by neurosecretory nuclei of hypothalamus which release somatocrinin (GH-releasing hormone) and somatostatin (GH-inhibiting hormone). The balance of these two molecules' secretion, determines whether hGH is released or not in the pituitary (Bartholomew *et al* 2009). Additionally the insulin-like growth factor 1 (IGF-1) produced by the liver affects the hGH activities when the hormone travels to the liver to aid cartilage growth.

2.1.4 Gene of hGH

The human GH gene is found on the long arm of human chromosome 17 accompanied with four other genes that are structurally and functionally similar. There are two hGH genes; hGH-N (normal) and hGH-V (variant) and three placental lactogen (PL) genes (Henry and Norman, 2001). These genes have an identical molecular structure that consists of four small introns that divide the transcriptional units at the same locations. Furthermore hGH-N is the predominant gene which produces HGH22k with a molecular weight of 22kDa (Chawla *et al*. 1983) and provides the modulation of carbohydrate, lipid, nitrogen and mineral metabolism (Barrera-Saldaña, 2012). HGH20k is an isoform of hGH that is also produced by hGH-N gene from another pathway of the primary transcript by means of a second mRNA generation where mRNA lacks the first 45 nucleotides of the third exon (Barrera-Saldaña, 2012).

2.1.5 Physical and Chemical Properties

Human GH is a nonglycosylated polypeptide composed of 191 amino acids with a molecular weight of 22 kDa (Figure 2.3), that contains two disulfide bonds and four helices in its tertiary structure (Figure 2.4) that help in interacting with the GH receptor (Norman, 1997). Its isoelectric point (pI) is 5.1 (Goeddel *et al*. 1979) and the empiric formula is $C_{990}H_{1529}N_{262}O_{300}S_7$. Isoform of hGH has 176 amino acids with a molecular mass of 20,274 Da (Baumann, 2009) because it lacks the amino acids of positions 32-46 of the native hormone (Barrera-Saldaña, 2012). hGH is synthesized as a pro-hormone including a signal peptide at the amino-terminus which is removed when the hormone is secreted from the cell and has a half-life of 25 min (Henry and Norman, 2001).

Figure 2.3. hGH Amino acid Sequence (Li *et al*. 1969)

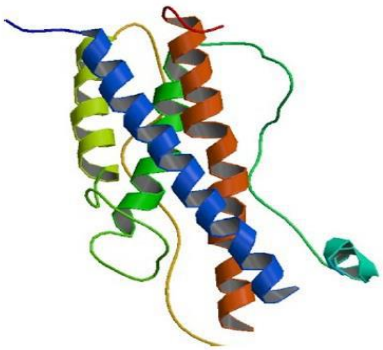


Figure 2.4. Tertiary structure of hGH, (Norman, 1997)

2.1.6 Biological Effects

Beside the pituitary gland, hGH is also synthesized within other tissues throughout the body on which it implements both tropic and nontropic effects (Campbell and Reece, 2005). Human growth hormone is well known for its ability to stimulate the growth of muscle, bone and cartilage. To perform such function, first hGH signals the liver to release IGFs which are directly responsible for growth. Recently its effect on immune cell function such as ulcer treatments, resistance to common disease is studied (Chappel *et al.* 2000). Additionally it has a significant impact on metabolic effect to raise glucose in blood which is the reverse action of insulin (Campbell and Reece, 2005). In table 2.1 the biological effects of hGH are summarized.

Table 2.1 Biological effects of hGH

Metabolic	Amino acid transport
	Protein synthesis in most cell types
	Polyamine synthesis
	Insulin action inhibition
Physiologic	Increase of blood flow and basal metabolic rate
	Stimulation of new bone formation
	Stimulation of erythropoiesis
	Extracellular fluid space expansion
Anatomic	Accelerates linear growth
	Reduces adipose mass and enlarges lean body mass

2.1.7 Therapeutic Uses of hGH

Different disorders occur because of hGH hypersecretion or hyposecretion in different time periods of human life. If hypersecretion happens during childhood, it might lead to gigantism where the individual can reach heights as much as 2.4m. However this abnormality in secretion does not affect the body proportions (Campbell and Reece, 2005). On the other hand if hypersecretion occurs during adulthood it causes acromegaly which stimulates the bony growth in the face, hands, and the feet (Campbell and Reece, 2005). Growth hormone deficiency caused by hyposecretion results in pituitary dwarfism which can be treated effectively with hGH if it is detected before puberty.

There are athletes who use hGH to ameliorate their athletic shape, however for adults with no deficiency or abnormalities, the impact of the hormone on the muscle mass and strength is insignificant (Campbell and Reece, 2005). Some other conditions accompanied by short stature that can be cured by hGH are chronic renal insufficiency, Turner syndrome, Prader-Willi syndrome, and idiopathic short stature (Hintz, 2004). Additionally human growth hormone is used to improve the conditions of dosages used for HIV-associated visceral fat accumulation (Burgess *et al.* 2005) and has shown a significant role on treating HIV-associated wasting disease (Gelato et al. 2007). In table 2.2 hGH therapeutic uses in children and adults are listed.

Table 2.2. hGH therapeutic uses **(Tritos, 2009)**

Growth failure in children with:	Adults with:
Growth hormone deficiency (idiopathic or as a result of pituitary disease	Growth hormone deficiency as a result of pituitary disease or childhood-onset GHD
Chronic renal failure	HIV infection-associated cachexia
Intraurerine growth retardation	Short bowel syndrome
Idiopathic short stature	
Turner syndrome	
Prader Willi syndrome	
Noonan syndrome	

2.1.8 Recombinant human growth hormone (rhGH) production

Until the middle of the 20th century, pituitary gland was the only hGH supplier since it was not possible to obtain hGH from any artificial source. In 1985 scientists were able to produce biosynthetic form of human growth hormone by using the techniques of genetic engineering and recombinant DNA technology (Gray *et al*. 1985). The most commonly used host microorganism for rhGH production is *E. coli*. However, due to undesirable modifications such as incorrect folding and being contaminated with highly pyrogenic substances, recovery of the recombinant protein requires too much effort. Consequently, to avoid such obstacles other expressions systems are used such as *Bacillus subtilis, Saccharomyces cerevisiae* and *Pichia pastoris*. Especially in yeast expression systems which are capable of complex posttranslational modifications, genetic manipulation is easier and is accompanied with rapid growth on inexpensive medium that leads to high cell densities (Gellissen *et al*. 2005).

Recombinant biological products, secreted by yeast are free of endotoxins and oncogenic and viral DNA which lowers the toxicity in the culture medium (Damasceno *et al*. 2011). For these purposes higher fermentation efficiencies can be obtained by using yeasts in expression systems. The most popular yeast species that are generally used are *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha and Kluyveromyces lactis*. In *P. pastoris*, glycosylation of proteins is less extensive than in *S. cerevisiae* which makes *P. pastoris* potentially more suitable for production of heterologous enzymes (Varnai *et al.* 2014). The following table is a summary of the studies performed by *Pichia pastoris* between 1985 and 2016.

In Table 2.3 the studies performed for production of rhGH by *Pichia pastoris* are listed. To enhance the productivity of the recombinant protein, the mentioned studies have developed the expression system of the host microorganism and studied the effect of different feeding regimes for a variety of carbon sources.

Table 2.3. rhGH production studies performed by *Pichia pastoris* from 1985-2016

Researcher	Year	Microorganism	Approach of the study	r-protein
Trevino *et al*.	2000	*Pichia Pastoris*	To produce hGH by the expression system of alcohol oxidase (AOX1) promoter	49 mg L^{-1}
Eurwilaichitr *et al.*	2002	*Pichia Pastoris*		190 mg L^{-1}
Çalık *et al*.	2008	*Pichia Pastoris*	To construct a novel expression system as a host microorganism to produce recombinant human growth hormone.	350 mg L^{-1}
Orman *et al.*	2009	*Pichia pastoris*	To investigate the impacts of carbon sources on the production of rhGH in batch processes.	110 mg L^{-1}
Çalık *et al*.	2010	*Pichia pastoris*	To investigate the impacts of carbon sources on the production of rhGH in batch processes.	270 mg L^{-1}
Çalık *et al*.	2011	*Pichia pastoris*	To investigate the effect of feeding rate of methanol on the intracellular reaction rates.	270 mg L^{-1}

Table 2.3 continued.

Researcher	Year	Microorganism	Approach of the study	r-protein
Zerze	2012	*Pichia pastoris*	Exponential methanol and mannitol feeding GAP promoter	1200 mg L^{-1} 220 mg L^{-1}
Güneş *et al*.	2014	*Pichia pastoris*	To investigate the effect of designed methanol and co-substrate feeding strategies on fed-batch rhGH production	1300 mg L^{-1}
Keskin	2014	*Pichia pastoris*	To obtain an enhancement on rhGH production by developing a glucose-based feeding strategy under P_{GAP} expression.	508 mg L^{-1}
Lee *et al*.	2015	*Pichia pastoris*	To construct a recombinant *P. pastoris* expressing a human growth hormone (hGH) fusion protein	790 mg L^{-1}

2.2 Host Microorganism

Among the limited number of yeast species that are able to utilize methanol as sole carbon and energy source, *Pichia pastoris* is unique. It is classified under the Kingdom Fungi, Division Eumycota Subdivision Ascomycotina, Class Hemoascomycetes, Order Endomycetales, Family Saccharomycetaceae, and Subfamily Saccharomycetoideae (Damasceno *et al*. 2011). As a yeast, *Pichia pastoris* is a unicellular microorganism, which has a polysaccharide cell wall and an oval shape with diameter 1-5 μm wide and 5-30 μm long. It is a facultative aerobe mesophilic yeast that is accommodated in a temperature range of 25-35^{0}C (Macauley-Patrick *et al*. 2005) and pH range of 3 to 7 (Cregg *et al*. 2000).

Pichia pastoris was first considered as a host microorganism in late 1960s because of its efficient utilization of methanol, when the cost of methanol synthesis from natural methane was low. However in 1970s during the oil crisis, the methane price increased and the production of r-proteins in this yeast became economically inconvenient (Cregg *et al.* 2000). In the 1980s *P. pastoris*-based systems for expression of r-proteins were developed. Additionally vectors and strains were constructed for the genetic manipulation of *P. pastoris* based on its use of methanol as carbon and energy source. Since then more than 1000 proteins have been expressed and produced by this host microorganism (Damasceno *et al*. 2011).

P. pastoris as a eukaryote offers advantages like efficient secretion and post-translational modifications such as proteolytic processing, glycosylation and disulfide bond formation. It is easily and rapidly cultivated on inexpensive salt-based media where the contamination probability with viral DNAs is significantly low (Romanos *et al*. 1992). Another advantageous aspect of *P. pastoris* is its ability to reach high cell densities up to 13 g L^{-1} (Xiong *et al*. 2006) by demanding small amount of energy during fermentation (Jahic *et al*. 2002). Extracellular recombinant protein secretion provides the direct capture of active product from the cultivation medium, lowering the cost and avoiding the low-yield cell disruption or refolding processes (Damasceno *et al*. 2011).

However there are some drawbacks that are faced when using *P.pastoris* as host microorganism such as slow growth rate compared to prokaryotic cells, leading to longer cultivation times. Additionally it has a high proteolytic activity that secretes protease enzymes that degrade the secreted recombinant protein. To avoid this drawback, protease deficient strains are designed (Sreekrishna *et al*. 1997). Furthermore N- and O-linked oligosaccharide structures are distinguished from the ones of mammalian cells because of heterogeneous high-mannose content. When attached to recombinant glycoproteins they are washed away from the human bloodstream and can further lead to immunogenic reactions. To circumvent this obstacle GlycoFi's glycoengineering technology provides generation of yeast strains that are able to replicate the most important steps of the N-glycosylation pathway found in mammals (Valero, 2013).

2.2.1 Promoters of Pichia pastoris

One of the reasons that *Pichia pastoris* has become the most favorable yeast for the production of recombinant proteins is having tightly regulated promoters (Cregg *et al*. 2009). To regulate transcription, promoters provide specific DNA-binding sites for RNA polymerase and transcription factors which repress or activate the transcription (Vogl and Glieder, 2013). Since the initial transcription of the particular gene is one of the crucial steps in heterologous protein production, picking a strong and controllable promoter is very important to optimize production of the protein. There are several promoters that are used for r-protein production in *P. pastoris* where the most preferable ones are the inducible alcohol oxidase 1 (AOX1) (Cregg et al. 2000) and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) (Zhang *et al*. 2009).

As a methylotrophic yeast, *P. pastoris* has been preferred for its strong methanol inducible promoters AOX1 and AOX2 which control the expression of alcohol oxidase (AOX) in the methanol utilization pathway. The expression of these promoters is inhibited by ethanol, glucose and glycerol and induced by methanol (Cregg *et al*. 2000). By using one of the repressive carbon sources initially a significant cell growth is reached and then by inducing methanol gene product is expressed (Macauley-Patrick *et al*. 2005) so the expression of r-proteins toxic to the cells can be controlled. However

the storage of methanol can be dangerous for large scale production since it a fire hazard. On the other hand GAP is a constitutive promoter which avoids the usage of methanol by making the cultivation process easier where its expression strength relies on the carbon source (Várnai *et al*. 2014).

However selecting a strongly regulated promoter such as P_{AOX1} and P_{GAP} is not always the best choice for r-protein production (Table 2.4). There are conditions where the correct folding of the protein or processing in the secretory pathway restricts high concentrations of active product. For example, P_{PEX8} and P_{YPT1} are weak promoters that are considered for such cases, however their application has been very rare (Vogl and Glieder, 2013). Other alternative *P. pastoris* derived promoters, constitutive or inducible are P_{FLD1}, P_{DAS}, P_{TEF1}, P_{PGK1}, P_{ICL1}, and P_{GK} (Hartner et al. 2008).

2.2.2 GAP promoter

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a tetrameric NAD-binding enzyme along the glycolysis and gluconeogenesis pathways illustrated in Figure 2.4. It catalyzes an important energy-yielding step (the sixth step of glycolysis) in carbohydrate metabolism, which is reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD) (NCBI, 2016). The GAPDH protein composed of 333 amino acid residues, has a molecular weight of 35.4 kDa and is expressed constitutively reaching high amounts of protein (Waterham *et al.* 1997). Cregg *et al,* (1985) used colony hybridization to isolate the GAP gene from a *P. pastoris* genomic DNA library.

The GAP promoter has been recently used in a wide range for production of several proteins by being the most frequently used alternative to P_{AOX1}. Despite the differences in their regulation, P_{GAP} and P_{AOX1} are both strong promoters and reach similar expression levels (Waterham *et al*. 1997). However, using P_{GAP} instead of P_{AOX1} reduces the cultivation time and expenses because there is no need for a carbon source shift for the constitutive promoter leading to higher space/time yield (Vogl and Glieder, 2013).

Table 2.4. Inducible and constitutive promoters of *Pichia pastoris*

Inducible Promoters		**Constitutive Promoters**	
Gene	**Gene Product**	**Gene**	**Gene Product**
AOX1	Alcohol oxidase 1	*GAP*	Glyceraldehyde3-phosphate dehydrogenase
AOX2	Alcohol oxidase 2	*ENO1*	Enolase
DAS	Dihydroxyacetone synthase	*GPM1*	Phosphoglycerate mutase
FLD1	Formaldehyde dehydrogenase	*HSP82*	Cytoplasmic chaperone
ICL1	Isocitrate lyase	*ILV5*	Acetohydroxy acid isomeroreductase
PEX8	Peroxisomal matrix protein	*KAR2*	ER resident chaperone
PHO89 or NSP	Sodium-coupled phosphate symporter	*KEX2*	Endopeptidase involved in the processing of secreted proteins
		PET9	ADP/ATP carrier of the inner mitochondrial membrane
		PGK1	Phosphoglycerate kinase
		SSA4	Heat shock protein
		TEF1	Translation elongation factor 1 alpha
		THI11	Protein involved in thiame biosynthesis
		TPI1	Triose phostphate isomerase
		YPT1	GTPase involved in secretion

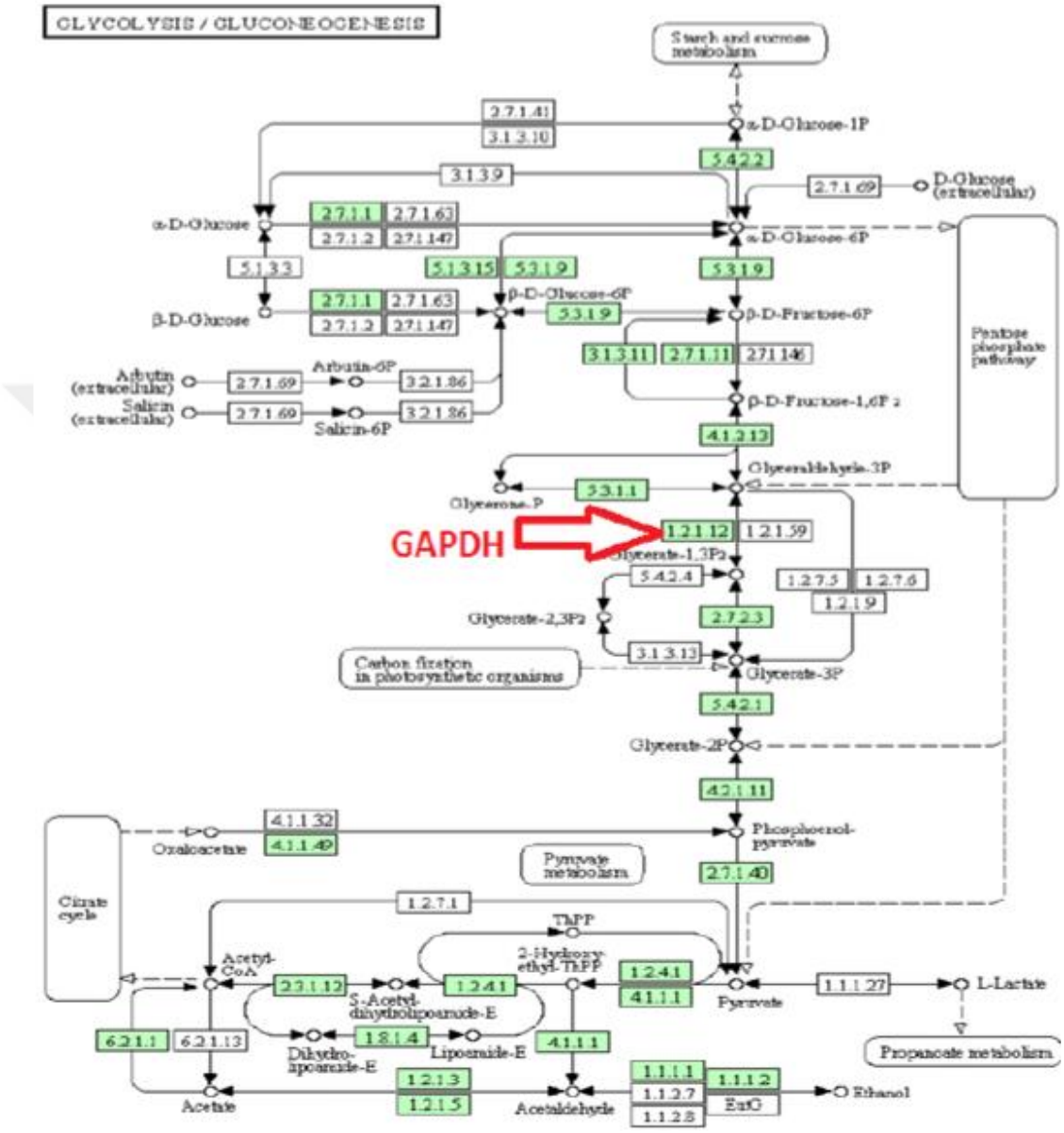


Figure 2.5 Glycolysis Pathway indicating GAPDH enzyme (http://www. genome.jp/ kegg /pathway/map/map00010.html)

Even though GAP is a constitutive promoter, its carbon source affinity differs from one source to another. The highest GAP mRNA levels are achieved in cultivation media where glucose is sole carbon source. The expression level reduces by 30% for glycerol, 50% for oleic acid and 65% for methanol (Waterham *et al*. 1997). Furthermore, Kern *et al*. observed that protein secretion under P_{GAP} control increased significantly when glucose in the production medium was entirely consumed by creating a starvation condition (Kern *et al.* 2007).

Another important parameter that affects the P_{GAP} driven heterologous protein production is the oxygen supply. It was observed that hypoxic conditions enhanced the specific productivity of particular proteins by three- to six-fold and the volumetric productivity by approximately two-fold. On the other hand, for low dissolved oxygen concentrations the cultivation time was shorter (Baumann *et al*. 2008).

2.2.3 Carbon Source Utilization of Pichia pastoris

The intracellular network of *P. pastoris* is a complex system which is very responsive to external nutrients and signaling molecules and provides a better understanding of the cell's developmental and growth decisions (Wang *et al*. 2004). For energy generation *P. pastoris* makes use of fermentation of fructose and glucose or sugar alcohols such as mannitol and sorbitol and is capable of utilizing fermentation products; glycerol, ethanol and lactate by oxidation. However it cannot utilize xylose and galactose lacking the uptake specific genes and sucrose because invertase gene is absent in *Pichia pastoris* (Çalık *et al*. 2015). Furthermore, fermentable carbon sources are consumed more efficiently which results in a more rapid growth and a greater capacity for mass accumulation. The uptake rate of the above mentioned substrates determines the growth kinetics and the characteristics of biotechnological processes (Mattanovich *et al*. 2009). In Figure 2.5 the metabolic pathways of *P. pastoris* are represented schematically.

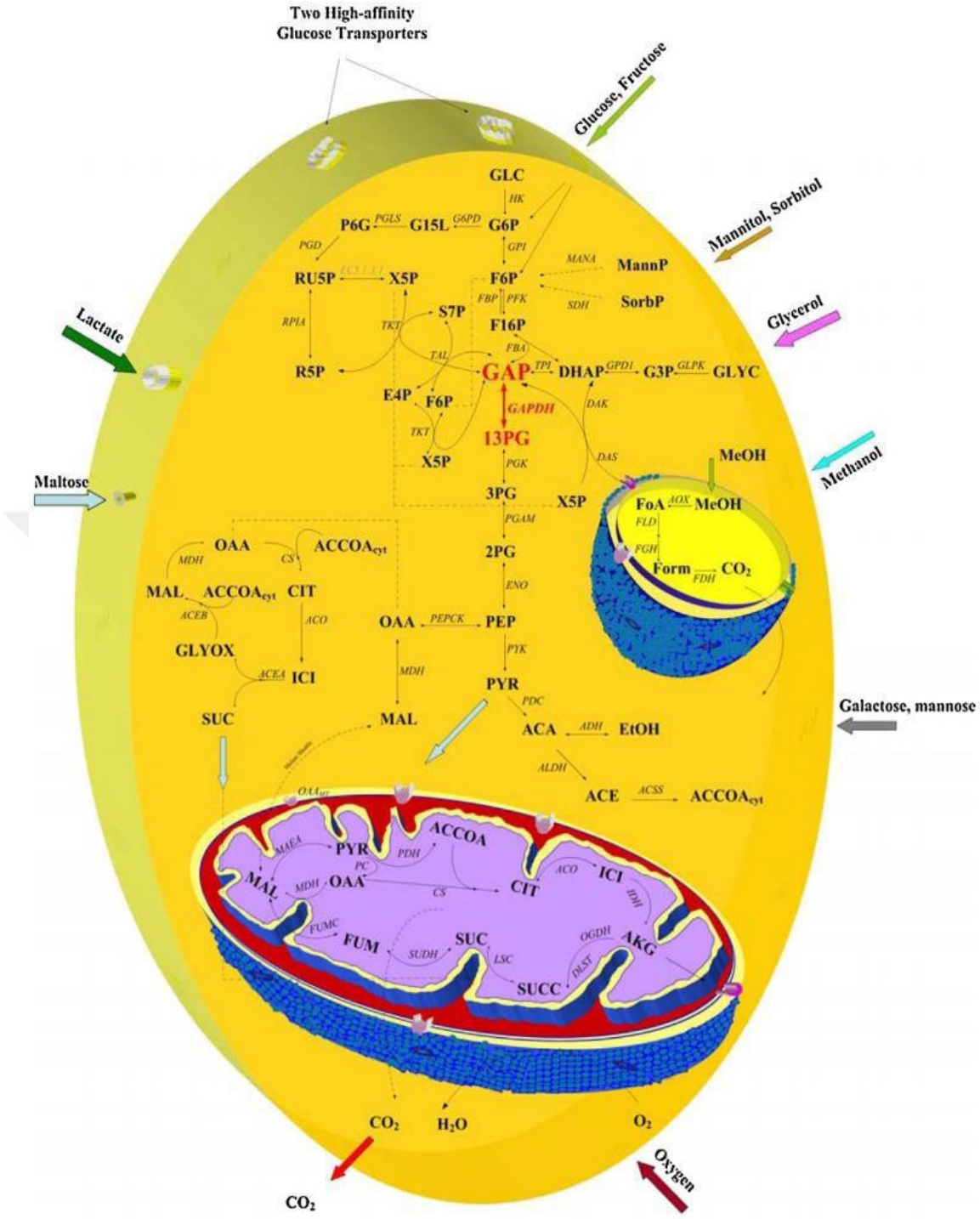


Figure 2.6. Central Carbon Metabolism of *Pichia pastoris* (Çalık *et al*. 2015).

Carbon sources are taken up according to a certain order with irregular lag phases (Klein *et al*, 1998). The uptake of these carbohydrates is mediated by both high and low affinity transport systems. Low affinity transport is provided by constitutive diffusion and the high affinity transport system is assisted by the activity of phosphorylating enzymes (D'Amore *et al.* 1989). Genes responsible for transporters are strongly regulated by the glucose concentration in the medium. Consequently, the glucose presence in the medium represses genes which are concerned with the use of alternative carbon sources by implicating a negative effect in the metabolism of other sugars (Wang *et al*. 2004).

The glucose role on hexose transporters is assumed to be the major reason for facilitated growth and high cell densities. However, unlike the fermentative yeast *S. cerevisiae*, respiratory yeast *P. pastoris* contains a fewer number of hexose transporter genes which encode energy dependent symporters and limit the glucose uptake. To increase energy supply and compensate for the limited resources, the Crabtree-negative yeast redirects the intracellular fluxes and prevents a metabolism overflow. As a result, yeast reduces cell yield and causes the recombinant protein formation (Çalık *et al*. 2015). At maximum growth rate $\mu_{max} = 0.193\ h^{-1}$, the glucose uptake rate is limited to $q_{Smax} = 0.35\ g\ g^{-1}$ for chemostat cultivations (Mattanovich *et al*. 2009).

A study carried out from Bauman *et al*, (2008) concluded that hypoxic cultivations result in higher glycolytic flux and assist in upregulation of glycolytic genes like GAP. Furthermore, it is speculated that also the heterologous genes controlled by GAP promoter may be upregulated in low dissolved oxygen concentration. For such hypoxic condition, oxygen limitation is responsible for adjusting the metabolism and heterologous protein productivity while cell growth is controlled by carbon limitation (Baumann et al. 2008).

2.2.4 Proteolytic Activity of Pichia pastoris

Alterations in cultivation conditions such as change of carbon source, heat shock, pH value variations, and accumulation of toxic chemicals cause a stress on the yeast cells which give a proteolytic response. In high cell density fermentation process, concentration of proteases excreted by *P. pastoris* in the production medium increases

and leads to degradation of the extracellular protein product. Furthermore, proteolytic degradation reduces the product yield and biological activity of the recombinant protein. Degradation intermediates created as a result of proteolysis have a high resemblance in physicochemical and affinity characteristics with the recombinant product which leads to contamination during downstream processing (Macauley-Patrick *et al*. 2005). In order to overcome the aforementioned problems, several strategies are applied to control proteolysis.

At the cell level, genetic modifications are applied to obtain protease deficient host strains (Gleeson *et al*. 1998). Since the proteases of yeast are of vacuolar origin, cells were mutated by introducing a nonvacuolar set which prevented the complete proteolytic process (Jones, 1991). Compared to the wild-type strain the r-protein secretion was delayed, however the product yield was higher (Li *et al*. 2001).

Particular amino acid chains in the r-protein may be recognized by the proteases of the host microorganism making them more vulnerable to degradation. In cases when these amino acid sequences were not crucial for the r-protein function, they were deleted to increase the protein resistance over proteases (Gustavsson *et al*. 2001). Another way of controlling proteolytic activity is by adding inhibitors of proteases to the medium. Enzymes used for this purpose are classified in four groups; namely, serine, cysteine, aspartic, and metallo (Holmquist *et al*. 1997; Shi *et al*. 2003).

It has been observed that activity of proteases diminishes when the cultivation pH is lowered (Jahic *et al*. 2002). The pH range for *P. pastoris* growth is between 3.0- 7.0 and is possible to adjust a value favorable to lower proteolytic activity since pH does not affect the growth radically. Decreasing the pH value from 5.0 to 4.0 elicited an increase on the final product concentration (Jahic *et al*. 2003). Another process parameter that is examined to reduce proteolysis is temperature, for which the optimum value for *P. pastoris* is 30°C. Higher cultivation temperature leads to cell death which results in cell lysis consequently higher protease activity. On the other hand low temperature values provide higher stability of the recombinant protein and a lower proteolysis rate because of thermodynamic reasons (Jahic *et al*. 2003).

Ammonium presence in the cultivation medium decreases the protease activity which is caused by the nitrogen starvation (Kobayashi *et al*. 2000). To provide nitrogen in the culture medium casamino acids or peptone is added which at the same time act as a second substrate (Clare *et al*. 1991a). Furthermore, the production stability and concentration was enhanced by adding amino-acid supplements.

Another strategy to control proteolysis is by controlling the specific growth rate. A lower growth rate results in lower protease secretion in the medium (Zhang *et al*. 2002).

2.3 Medium Design

To design an effective medium, the microorganism's metabolism and the type of the promoter for the regulation of the recombinant protein should be considered. For cell growth, nutrition elements can be grouped into three main categories, which are sugars, amino acids and minerals. Medium design is a very important factor for the productivity of the fermentation process because it directly affects cell growth behavior. In accordance with Pichia fermentation process guidelines proposed by Invitrogen (USA), Basal salt medium (BSM) is the most commonly used medium to maintain high cell density fermentation of *P. pastoris*. Although this is the used standard medium, it may not be the optimum for every heterologous protein produced by *P. pastoris* and has some drawbacks such as high ionic strength, precipitation and unbalanced composition (Cos *et al.* 2006). To ameliorate the effect of BSM Zhang et al added 2% peptone and 1% yeast extract in its composition (Zhang *et al*. 2009).

According to the chemical composition, cultivation media are classified as defined, semi-defined and undefined/complex. In the defined medium the mixed compounds are in known ratio, whereas in the undefined media the components are in their natural form and do not have defined composition ratios. Generally complex media consist of different amino acids, minerals and vitamins leading to higher cell densities and r-protein concentration. However, when considered for application in pharmaceutical products they are not very favorable because of the required validation of the process and difficulties in the downstream operations (Macauley-Patrick *et al*. 2005).

2.3.1 Carbon Source

For the P_{GAP}-driven expression system the most effective and applied carbon sources are glucose, glycerol, methanol and oleic acid (Zhang *et al*. 2009). In addition, considering the carbon metabolism in glycolysis pathway, fructose, mannitol, trehalose, and sorbitol are potential carbon sources (Çalık *et al*. 2015). In a study made by Garcia-Ortega and co-workers the combination of glycerol and glucose as sole carbon sources for batch and fed batch phases respectively was found as the most effective arrangement. Different from glucose utilization during batch phase, there were no by-products or cell aggregates detectable in the bioprocess while using glycerol as the carbon source. After calculating the lower heat yield and oxygen to biomass yield, glucose was found as the best alternative for industrial scale (Garcia-Ortega *et al*. 2013).

2.3.2 Nitrogen source

Yeast specific genes and pathways determine the yeast's capacity to assimilate and metabolize nitrogen (Kingsbury *et al*. 2006). Nitrogen along with carbon utilization is important for the transcription of several yeast genes. *P. pastoris* is capable of utilizing a variety of nitrogen containing compounds, where glutamine, glutamic acid and ammonium are the most preferable sources. There have been developed regulation mechanisms specifically for utilization of these compounds (Magasanik, 1992). In BSM medium, ammonium hydroxide is used as the nitrogen source which is added throughout the process to control pH. When the cell reaches a biomass of 50 g/L nitrogen starvation occurs (Cos *et al.* 2005) which induces the proteolysis activity which may become a serious problem when reaching high cell concentrations. For this reason, Ghosalkar *et al*. integrated to the initial formulation of the production medium, ammonium sulfate as nitrogen source (Ghosalkar *et al.* 2008).

2.3.3 The Source of Trace and Essential Elements

Minerals are essential for cells during the cultivation. Iron, zinc, manganese, molybdenum, cobalt, copper, and calcium are the trace elements required in small amounts by providing functions like vitamin synthesis. Manganese acts mainly on the production of enzymes. In Pichia pastoris, PTM1 trace salts rich with these micronutrients is added to the production medium in small amounts (Cos *et al*. 2006).

2.3.4 Amino Acids

When glucose was used as sole carbon source, a limitation on recombinant protein production was observed. The reason for such restriction in the product yield is not related with transcription or translation machinery, but has to do with the operation of cellular network. To provide the required resources for recombinant protein synthesis, *P.pastoris,* reduces the formation of by-products and increases energy generation by TCA cycle. Addition of amino acids and its simultaneous utilization with glucose, facilitate the cellular metabolism obstacles by increasing the energy generation required for cell growth and recombinant protein synthesis (Heyland *et al.* 2011). Heyland *et al* examined four different additional amino acid media: glutamine, TCA cycle derived amino acids, energetically expensive amino acids like histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine and addition of highly incorporated amino acids (Heyland *et al.* 2011).

Casamino Acids are also added to the culture medium as supplementary substrates and have resulted in a better cell growth and in a constant specific product yield with a 1.75-fold higher value (Pal *et al.* 2006). When added to *P. pastoris* cultures casamino acids have shown a positive effect in reducing the proteolytic degradation (Clare *et al.,* 1991a and Goodrick *et al.*, 2001).

2.3.5 Molasses

Molasses is the noncrystallizable byproduct of sucrose production from sugar beets (Curtin *et al.* 1983). Although sole carbon sources such as glucose and glycerol are favored, rich complex medium molasses is an attractive alternative to increase cell growth and r-protein production. As a rich medium, it contains organic non-sugars, vitamins, and essential minerals which are very effective at enhancing cell growth (Curtin *et al.* 1983; Baratti *et al.,* 1991). What makes it a good alternative as a substrate for microorganisms, is being inexpensive and easily obtainable (Baratti *et al.* 1991).

Molasses is generally used for alcohol production; mainly ethanol, and has been described as an optimal carbon source for the microorganism metabolism (Jimènez *et al.* 2004). The detailed composition of the beet molasses is presented in Table 2.5.

Table 2.5. Molasses Composition from European sugar beet industry **(Olbrich, 1963)**

Constituents	Average Composition
Sucrose	51.0
Organic non-sugars	19.0
Water	16.5
Glucose and Fructose	1.00
Raffinose	1.00
Ash Components:	11.5
K_2O	3.90
CO_3	3.5
Na_2O	1.30
Cl	1.60
SiO_2	0.10
CaO	0.26
MgO	0.16
P_2O_5	0.06
Fe_2O_3	0.02
Al_2O_3	0.07
Sulfates as SO_3	0.55
Vitamins (mg/100g)	
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

For this research, Ankara Sugar Factory was the main distributor for sugar beet molasse and the constituents are presented in Table 2.6.

Table 2.6. Composition of sugar beet molasses obtained from Ankara Sugar Factory

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

In previous studies molasses feeding was examined under host microorganisms such as *Zymomonas mobilis*, *Saccharomyces cerevisiae*, *E. coli (*Park and Baratti, 1991, Agarwal *et al.* 2006, Çalık and Levent, 2009). Half of the molasses medium composition consists of sucrose. However, *P. pastoris* is not capable of utilizing sucrose because of the lack of invertase enzyme (Pınar Çalık *et al.* 2014). Therefore, the molasses is pretreated by hydrolysis to convert sucrose to glucose and fructose mixture.

2.4 Bioreactor Operation Parameters

Bioreactor and operation conditions design are the most crucial part of the bioprocesses. Before, the microorganism need to be selected or developed and the fermentation medium is designed. In the bioreactor the pH conditions and temperature values are defined. Also the aeration and agitation rates and oxygen supply need to be investigated. Some current experimental studies have emphasized the significant influence of culture conditions like specific growth rate, pH, temperature and oxygen for proper post-translational protein modification. (Looser *et al.* 2015)

2.4.1 Temperature

Biochemical activity is directly affected by temperature. *P. pastoris* has a specific temperature range of 25–30°C where it grows optimally. For values higher than 32°C the yeast is incapable of protein expression and the growth rate slows down (Cos *et al.* 2006). It is very important for the process to keep the temperature constant at the required value. The designed operation temperature also affects the consumption of the carbon and energy sources, yield of the product, stress response, and protein folding (Dragosits *et al.* 2008).

For the recombinant protein production under GAP promoter, Rebnegger *et al*., Maurer *et al*., and Buchetics *et al*. selected 25°C as the operation temperature. It has been observed that working at low temperatures ameliorates the heterologous protein production (Li *et al.* 2001). As Jahic *et al*, (2003) concluded, working with a low temperature value reduces the protease activity and cellular lysis. However, depending on the composition of culture medium, working with cultivation temperatures lower

than 30 °C, has a negative impact on cell growth rate (Looser *et al*. 2015). Waterham *et al*., Zheng *et al*., Khasa *et al*., Wu *et al*., and Fei *et al*. worked with T= 30°C.

2.4.2 pH

The metabolic activity of the cell affects the hydrogen ion concentration. Microorganisms keep their intracellular pH value constant while the extracellular value changes because of external factors. This pH value difference between the inner and outer sides of the cell leads to a proton gradient which is the driving force for mass and energy transfer (Nielsen *et al.* 2003). Medium pH has a significant effect on the cell growth rate, the activity of enzymes, specific expression rates, secretion efficiency and proteolytic degradation (Çalık *et al.* 2010). *P. pastoris* is capable of growing in a wide pH range between 3 and 7 (Cregg *et al.* 1993). However, for the research studies pH is selected a value between 5 and 6. When *P. pastoris* grows in BSM medium, for pH greater than 5.0, operational problems like lack of nutrients and OD measurement interference occur (Cos *et al.* 2006).

Birijlall *et al*. concluded that optimum controlled-pH parameter is dependent on the r-protein physical properties. In order to avoid precipitation, pH medium should not be close to isoelectric point (pI) of the protein. For rhGH protein production under AOX promoter, Çalık *et al*, (2010) investigated the pH effect, where pH = 5.0 resulted in the highest rhGH concentration. Former studies from Trevino *et al*., Eurwilaichitr *et al*., and Orman *et al*. for rhGH production were carried out at pH = 6.0.

2.4.3 Oxygen Transfer

Pichia pastoris is a Crabtree-negative yeast which adapts its oxygen necessity according to the dissolved oxygen availability. It carries out aerobic respiration in the presence of oxygen and is capable of switching to anaerobic respiration in the absence of oxygen in the medium (Baumann *et al.* 2008). The oxygen supply in the cultivation medium is an important issue in bioreactor operations. Beside its crucial impact on cell growth and protein production, oxygen also effects the cellular redox reactions which are interconnected with protein folding reactions within the cell. Furthermore, it is a critical factor on the economy of aerobic biosynthesis systems.

2.4.3.1 Oxygen Transfer Characteristics

For aerobic fermentation, oxygen is an essential requirement. In 1 m^3 aqueous culture medium about 7-8.10^{-3} kg dissolved oxygen is found. For a concentrated microorganism population, this amount of oxygen is exhausted only in a few seconds. For *P. pastoris* cells, air or pure oxygen is supplied into the production medium. Since mixing is helpful for aeration, stirred-tank and bubble column bioreactors are favored types of bioreactors in cultivation processes.

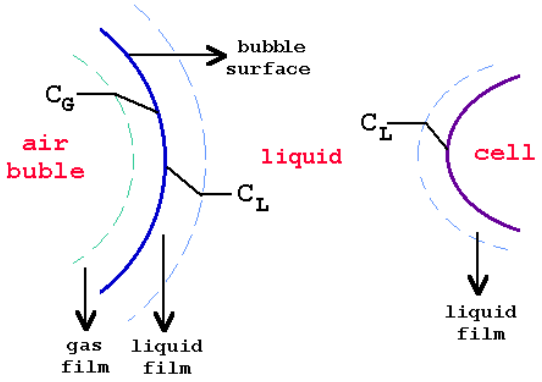


Figure 2.7. Mass Transfer in Aerobic Bioprocesses

Oxygen mass transfer, unlike chemical processes, is more complex due to polyphasic composition of the broths which generally consist of three phases (gas–liquid–solid) or four phases (gas–liquid–liquid–solid) as presented in Figure 2.7. Oxygen solubility and diffusion rate into the medium, define the aeration efficiency (Caşcaval *et al.* 2011).

Oxygen transfer (OTR) and oxygen uptake (OUR) rates are two important fermentation characteristics and affect the metabolic pathways and metabolic fluxes. (Çalık *et al*. 1999 and 2000). Cell growth and metabolic pathway analysis are good indicators for deciding on working at high oxygen transfer rate conditions or controlled oxygen transfer rates which regulate the oxygen uptake rates (Çalık *et al.* 1998). Oxygen uptake rate is balanced with oxygen transfer rate and is dependent on the cell metabolism. Oxygen transfer rate can be calculated by the following equation:

$$OTR = k_L \times a \times (C^*_{DO} - C_{DO}) \qquad (2.1)$$

Where C_{DO}^{*} is the saturation concentration of dissolved oxygen at phase boundary, effected by temperature and C_{DO} is the concentration of dissolved oxygen in liquid. k_L is the mass transfer coefficient, a is the specific exchange surface. However, measurement of these parameters separately is very difficult. Consequently, the k_La parameter (volumetric mass transfer coefficient) is measured instead. This parameter plays a significant role in the design of mixing/sparging equipment for the bioreactor. Geometrical and operational characteristics of the vessel, medium composition, and microorganism concentration affect the k_La values (Caşcaval *et al.* 2011).

The oxygen transfer coefficient proportionally increases with agitation rate while oxygen consumption rate increases linearly with cell concentration (Çalık *et al.* 1998). Biomass accumulation affects the rheological characteristic of the broth, specifically the viscosity. The increase of viscosity encourages the reduction of turbulence and agitation of bubble dispersion which result in a lower oxygen solubility and a blocking effect where cells are adsorbed to air bubble surface. However the adsorbed solid particles can provide a surface generation which has a positive effect on oxygen mass transfer (Caşcaval *et al*. 2011).

2.4.3.2 Dissolved Oxygen Concentration (C_{DO})

The oxygen demand depends on cell concentration, as for low cell densities air is an adequate oxygen supplier leading to fully aerobic conditions; whereas for high cell densities is not sufficient and results in limited oxygen conditions (Çalık *et al.* 2015). In *P. pastoris* fermentation processes the dissolved oxygen concentration is commonly kept in the range of 20–30% air saturation by supplying the production medium with air or oxygen in a cascade system (Gasser *et al.* 2006; Zheng *et al.* 2006; Zhang *et al.* 2007; Fei *et al.* 2009; Çalık *et al.* 2015).

Furthermore, taking into consideration that the carbon utilization pathway affects the oxygen requirement of *P. pastoris*, for different carbon sources, different dissolved oxygen concentrations are examined. In P_{AOX1}-driven expression systems where methanol is the carbon source, the methanol oxidation with molecular oxygen is the first step of carbon consumption and growth on methanol is an aerobic process. An excess quantity of oxygen may result in accumulation of formaldehyde (Baumann *et al.* 2008). On the other hand, low oxygen concentration limits the cell growth and

maintains a higher methanol concentration which further contributes in induction of AOX1 promoter for heterologous gene expression (Charoenrat *et al.* 2005).

On the other hand, when glucose is the carbon source, under limited oxygen conditions *P. pastoris* behaves as anaerobic and does not limit the substrate consumption. Moreover, it shifts to an alternative metabolic pathway where ethanol is produced as by-product and its fermentation takes place (Baumann *et al.* 2008). Since lower oxygen concentrations on glucose do not affect respiratory activity negatively, hypoxic conditions were further studied. Low dissolved oxygen concentration leads to a higher specific growth rate (Hu *et al.* 2008), an increase of the specific productivity and lower biomass (Baumann *et al.* 2008), and higher productivity.

As the metabolic flux through glycolytic pathway is higher in hypoxic bioprocesses, it should be expected that glycolytic genes like GAP will be upregulated. Güneş *et al.*, (2016) investigated the effect of oxygen transfer on the r-protein production under GAP promoter and concluded that for fed-batch phase of the bioprocess where glucose is the carbon source, C_{DO}=15 % was the optimum dissolved oxygen concentration.

2.5 Bioreactors and Operation Modes

The main aim of selecting a proper bioreactor operation mode is to provide a controlled environment for cell growth and r-protein secretion. According to hydrodynamics and the distinctions of volume of the fermentation medium with respect to time, the operation modes are classified as batch reactors with mixed-flow which can be operated as batch-, semi-batch- or backmixed-flow reactors, and continuous reactors operated at steady-state with laminar-flow, turbulent-flow, or plug flow. However, for aerobic fermentations, turbulent flow and plug flow fail to provide a steady state operation because the entering oxygen source causes axial dispersion, furthermore making impossible the "gas (oxygen source)- liquid (production medium)- solid (microorganism)" reaction system.

2.5.1 Batch Operation

In batch fermentation, substrate and yeast culture are added to the bioreactor along with the production medium. Since batch fermentation process is an isolated process without output and input streams all cell growth phases as presented in Figure 2.9 can

be observed. The first phase is the lag phase where there is no division of cells. In this phase, cells are only developing but not dividing because they are adapting in the new medium. For utilization of the nutrients in the cultivation medium the cells produce some particular enzymes. To keep this phase short, the microorganisms must have been in former medium that is similar to the production medium from its volume and component composition to prevent the shock and the adaption takes less time. Dilution rate is also important for cell growth. If the cultivation process is started with a small amount of cells lower than 1OD in 1L solution the lag phase would last for hours or days. After cells are adapted, they enter the second phase and start to divide by creating so an exponential growth. Under these conditions the cell mass increases with a constant specific growth rate. This phase is called the logarithmic phase, and the required metabolites are synthesized. After the log phase, a decline in the specific growth rate is observed defining this period as the deceleration phase.

Log phase $\rightarrow \frac{dN}{dt} = \mu \times N \qquad \rightarrow N = N_0 \times e^{\mu(t-t_{lag})}$

The next phase is the stationary phase where the nutrients are totally consumed and there are no more substrate, carbon and nitrogen sources. Also waste products which can be potentially toxic, are accumulated in the bioreactor. For such reasons cells stop increasing. The last phase is the death phase where the durability of the cells decreases and they start to die gradually. Most biotechnological operations are carried on the log phase or on the stationary phase (Liu, 2013)

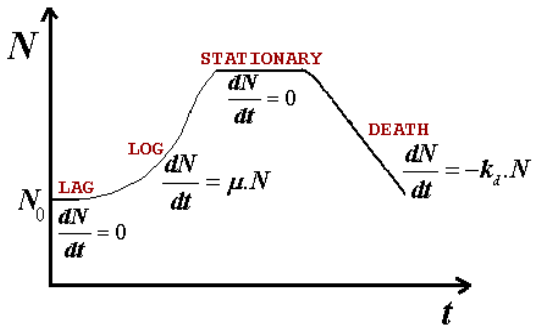


Figure 2.8 The phases of cell growth in batch reactor.

Batch operation is easily controlled and sterilization of the system is more obtainable because there are no input flows during the fermentation. Even though investment

cost for this operation is relatively lower than other processes, the cultivation time is a great deal longer compared to fed-batch or continuous processes and it may last for days (Li *et al.* 2010). During batch process after a particular time the nutrient exhaustion may occur and furthermore results in a limited cell growth in the cultivation culture (Radford *et al.* 1997). Liu et al studied the effect of fermentation conditions in *Pichia pastoris* and observed that batch operation showed a significantly lower performance compared with continuous fermentation (Liu *et al.* 2005).

2.5.2 Continuous Operation

In continuous operations, there is a continuous medium flow through the reactor. Incoming stream (feed) contains the substrate and leaving stream (effluent) contains the product. Two main assumptions of continuous operations are constant total number of cells and constant total volume of bioreactor.

Continuous fermentation process is efficient by avoiding the down-time and it is highly productive. Product is obtained with uniform characteristics. Quality of the product is almost the same from time to time. Another important advantage of continuous bioreactor is that there is no accumulation of inhibitory products. Physiological state of the biomass is uniform. One of the risks during continuous bioprocess is the contamination because the maintenance of the sterile conditions is very difficult. Degradation of the recombinant plasmids because of long time performance is possible by decreasing the yield of production.

$$\frac{dC_x}{dt} = \mu C_x - DC_x \qquad (2.2)$$

For steady state condition the time dependent term of the equation becomes zero. This leads to $D = \mu$ which means that by knowing the dilution ratio the specific growth rate can be controlled.

P_{GAP} favors the application of continuous production of r-proteins, by avoiding the induction phase that is required for r-protein expression under P_{AOX1} promoter. These continuous fermentations are preceded by a batch-wise phase where the substrate is included in the fermentation medium to maintain the cell growth. Goodrick *et al.* (2001) investigated rhuman chitinase production under P_{GAP}-driven expression, and

observed a reduction of the proteolytic activity which stabilized the r-protein secretion. Beside these advantages the process lasted for 26 days and resulted in lower volumetric and specific production rates.

2.5.3 Semi-Batch (Fed-Batch) Operation

Semi-Batch operation is a combination of batch and continuous operations. More than one nutrient enters the bioreactor continuously but there is no output stream until the end of the process. Carbon-energy sources like glucose are generally rapidly metabolized by causing a high intracellular concentration of ATP which may further repress the enzyme biosynthesis. By changing the feed rate it is possible to control the concentration of the substrates fed into the production medium by either avoiding the inhibitory effects or supplying a higher substrate concentration for high cell cultivation. In bioprocesses with extended cultivation periods, semi-batch operation provides replacement of water loss and dilution of the medium to decrease the viscosity. Otherwise high biomass concentrations would increase the viscosity of the medium significantly leading to lower oxygen transfer efficiency. By controlling the aforementioned obstacles of the bioprocess, the semi-batch operation mode provides a longer cultivation period (Yamane *et al.* 1984).

Semi-batch operation for r-protein production under the constitutive GAP promoter, consists of two-phases where in the first phase, the system operates in batch mode. The main scope of this phase is the cell proliferation where the recombinant strain is adapted to the production medium and it reaches a certain C_X. The end of batch phase is determined by the total consumption of the initial substrate and its duration varies between t = 17–22 h. Carbon source exhaustion is accompanied by a significant increase of the dissolved oxygen. Accordingly, an elevated oxygen concentration is used as an indicator to shift to semi-batch operation where substrate can be introduced to the medium by different feeding profiles.

Based on the controlling method of the semi-batch process, it is possible to classify them as bioreactors with feed-forward control and feedback control as illustrated in Figure 2.9

a) Operations without feedback control

For operations with feed-forward control, the substrate flow rate is pre-calculated and added to the cultivation culture independent of the response of the system. According to feeding profile semi-batch operations with feed-forward control are further subdivided as intermittent, constant, exponential and optimized feeding. For intermittent addition, substrate is fed to the medium by repetitive pulses in certain hours of the process which can affect the cultivation environment's stability. Substrate pulses may disturb the steady growing pattern of cells and depending on the cell's requirement, a sudden increase in the substrate concentration can slow the cell metabolism (Cheng *et al.* 2002).

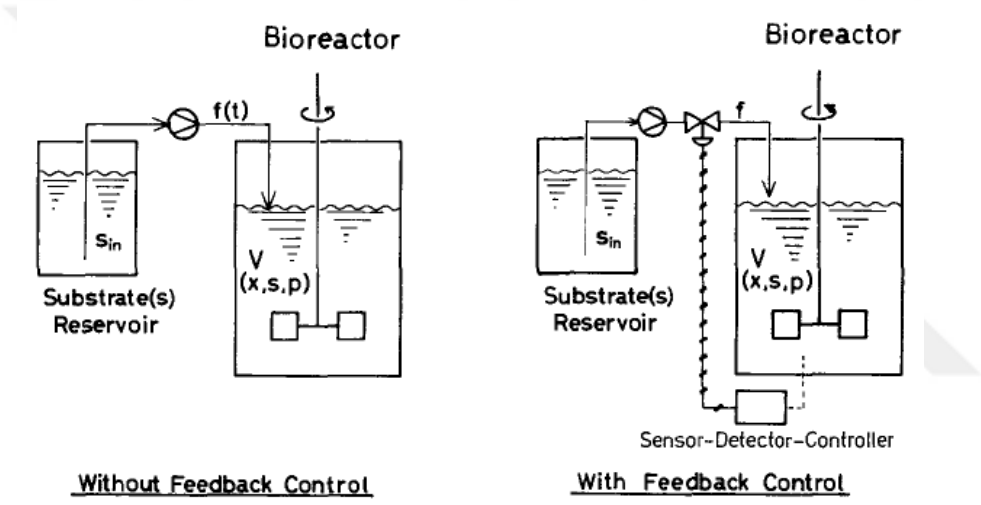


Figure 2.9. Schematic diagram of fed-batch operation in a microbial process (Yamane *et al.* 1984)

Constant feeding is introduced to the system with a constant nutrient pre-determined rate, which is not very effective for providing a constant specific growth and high cell concentrations. Glucose and glycerol were investigated as carbon sources for the production of human chitinase where they entered the system with a constant mass flow rate of 4.8 g h^{-1}. This strategy was more effective for glucose by achieving a higher biomass and higher production rate (Goodrick *et al.* 2001). Following this study, glucose was fed to the bioreactor with a certain mass flow rate, to maintain the

production of other r-protein production (Gasser *et al*. 2006, Baumann *et al*. 2008, Fei *et al*. 2009)

To keep a constant specific growth rate, exponential feeding strategy is the best alternative since, the feeding rate increases exponentially along with the cell proliferation. In fed-batch cultivation a pre-programmed exponential feeding profile is calculated and introduced to the system to maintain the desired specific growth rate. For the systems where expression is driven by GAP promoter specific growth rate plays an important role on the bioprocess productivity. Furthermore, with increasing specific growth rate, the genes responsible for protein folding and secretion are upregulated leading to higher specific protein secretion rates (Rebnegger *et al.* 2014). Another study carried by Garcia-Ortega et al., (2013) compared three different specific growth rates: μ_0 = 0.05 h^{-1}, 0.10 h^{-1}, and 0.15 h^{-1} for glucose feeding in the bioreactor and concluded that μ_0 = 0.15 h^{-1} obtained the highest r-protein production and overall yields and total productivity.

Even though exponential feeding is very effective and successful, after a certain time cells enter their stationary phase and the exponentially fed nutrients may accumulate in the medium to further repress the metabolic activity of the cells. In this case optimized feeding strategies are designed where exponential and linear feeding intervals are combined with each other to enhance the bioprocess performance. In order to enhance the volumetric productivity, Maurer *et al*. (2006) designed an optimum feed profile which included an exponential feed at μ_{max} = 0.2 h^{-1}, followed by linearly increasing feed rate, and a feeding with steadily decreasing specific growth rate μ = 0.05 h^{-1}. This feeding combination significantly increased the volumetric productivity by 2.2-fold compared to standard fed batch where glucose was fed exponentially with μ= 0.05 h^{-1}.

Recently Garcia-Ortega *et al.* (2016) designed a new feeding strategy where exponential feeding is combined with carbon-starving intervals. The combinations investigated were 3 h exponential feeding followed by 1 h stop interval, 3 h exponential feeding followed by 0.5 h stop interval, and 1.5 h exponential feeding followed by 0.5 h stop interval. All the investigated combinations were carried out with the same specific growth rate 0.15 h^{-1} which is calculated from the derivation of

mass balance equations. The additional starvation periods were combined with the exponential feeding to study the effect of carbon-starving conditions. It was observed that at the end of each stop interval the specific production rate was significantly increased. Furthermore overall yields and r-protein production were enhanced by 50% where the best combination arranged was exponential feeding for 1.5 h and carbon-starving interval for 0.5 h. It was concluded that for short non-fed intervals r-protein secretion by GAP promoter, is still carried on by the yeast metabolism beside the glucose depletion (Garcia-Ortega *et al*. 2016).

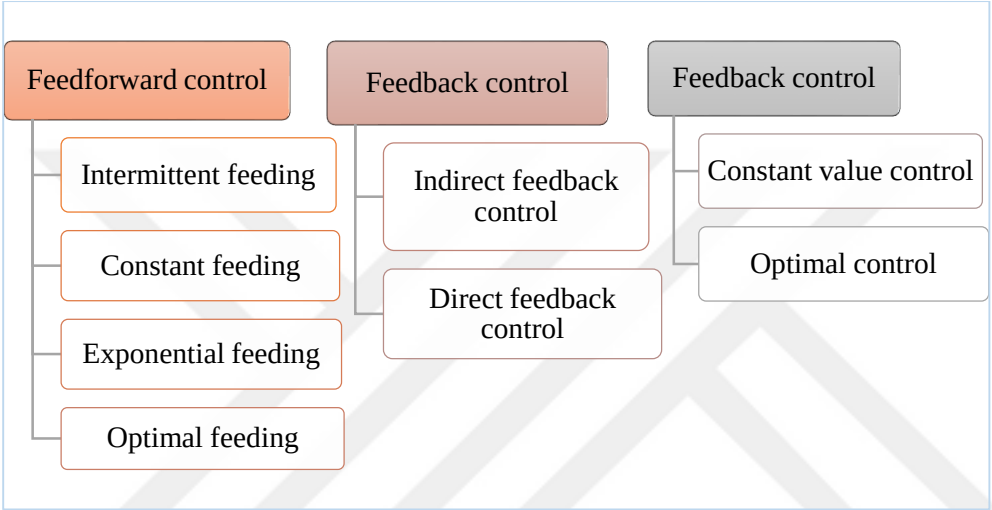


Figure 2.10 Classification of semi-batch operations.

b) Operation with feedback control

Systems of bioreactors with feedback control, take into consideration the output values of the parameters such as pH, C_{DO}, temperature, and substrate concentration and adjust the desired output response. In semi-batch operation, feedback control is enabled either directly where the substrate concentration decides the amount of further addition rate of substrate or indirectly where other feedback parameters such as C_{DO}, pH determine the input flow rate of the substrate. For example, in cases where C_{DO} is significantly higher than its set point, it shows that substrate in the medium is totally consumed. To sustain the desired limit, substrate is added to the medium. By using DO-stat control, Hu *et al.* (2008) investigated the glycerol feeding strategies where limited feeding was interrelated with dissolved oxygen (DO) values determined as 0 %, 25 % and 50%. In another study, glucose feeding rate was controlled by dissolved oxygen tension which

is an indicator of the cells' state (Pepeliaev *et al.* 2011). For both control systems, substrate is either added to the medium by a continuous constant feed or by intermittent intervals. To increase the lipase activity and production under GAP promoter, different feedback control strategies like μ-stat, pH stat, substrate-stat and combination of these strategies were investigated. The best feeding strategy was the combined control of μ-stat and pH-stat (Zhao *et al.* 2008). The following diagram is an illustration of fed-batch operations classification.

2.6 Bioprocess Kinetics in Semi-Batch Operation

The performance and maintenance of the bioprocess are defined by specific growth rate of the cell, consumption rate of the substrate, and specific production rate. To compare different semi-batch operation strategies with each other, the absolute values of specific rates and yields are calculated. The mathematical models applied for these calculations are derived from the material balances of cell, substrate and product.

2.6.1 Material Balance Equation for the Cell

The mass balance equation is written by assuming that there is no cell loss and the number of microorganisms is always increasing. Considering that the cells are added to the production medium at the beginning of the bioprocess, there is no input and output streams:

$$r_x V = \frac{d(C_x V)}{dt} \tag{2.3}$$

Where r_x is the cell growth rate, V is the working volume of the bioreactor, Cx is the cell concentration and t is cultivation time. To introduce the specific growth rate μ in this equation r_x can be represented as

$$r_x = \mu \times C_x \tag{2.4}$$

When equation 2.4 is inserted in equation 2.3 the equation is further written as:

$$\mu C_x V = \frac{d(C_x V)}{dt} \tag{2.5}$$

Which is can be also written as:

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

In semi-batch operation substrate is continuously added to the system by causing an increase in the reactor volume with time. Assuming that there is a uniform density in the production medium the volume change is calculated as:

$$Q_{in} - Q_{out} = \frac{dV}{dt} \tag{2.7}$$

Where Q_{in} and Q_{out} are the input and output volumetric flow rates respectively. Since there is no output for semi-batch process, equation 2.6 is modified as:

$$Q_{in} = \frac{dV}{dt} \tag{2.8}$$

When Equation 2.8 is inserted in Equation 2.6 the derived equation is further simplified as:

$$\frac{dC_x}{dt} = \left(\mu - \frac{Q}{V}\right) C_x \tag{2.9}$$

Arranging equation 2.9, the specific growth rate for semi-batch bioreactor is presented as follows:

$$\mu = \frac{dC_x}{dt} \frac{1}{C_x} + \frac{Q}{V} \tag{2.10}$$

2.6.2 Material Balance Equation for the Substrate

In semi-batch operation, substrate is fed into the bioreactor without an output stream and if the microorganism is not capable of utilizing it rapidly beside the consumption rate an accumulation rate is also included in the mass balance equation:

$$Q_s C_{s_0} + r_s V = \frac{d(C_s V)}{dt} \tag{2.11}$$

where Qs is the volumetric feed rate of the substrate, C_{S0} is the concentration of the substrate in the feed medium and C_S is the concentration of the substrate in the

bioreactor. Substrate utilization rate is defined by a first order kinetic equation which is a function cell concentration:

$$-r_s = q_s C_x \tag{2.12}$$

where q_s is the specific substrate utilization rate. To create a direct relation between C_X and C_S, equation 2.12 is inserted in equation 2.11 and is re-written as:

$$Q_s C_{s_0} - q_s C_x V = C_s \frac{dV}{dt} + V \frac{dC_s}{dt} \tag{2.13}$$

For q_s the equation 2.13 is rearranged as:

$$q_s = \frac{1}{C_x}\left(\frac{Q_s}{V} C_{s_0} - \frac{C_s}{V}\frac{dV}{dt} - \frac{dC_s}{dt}\right) \tag{2.14}$$

The operation of semi-batch concerning the concentration of substrate in the cultivation medium of the bioreactor, provides a quasi-steady state condition which means that:$dC_s/dt = 0$. In such condition, consumption of substrate is only associated with cell formation. To create a correlation between cell formation rate and substrate utilization rate, cell yield on the substrate parameter is introduced:

$$-r_S = (r_x/Y_{X/S}) \tag{2.15}$$

Furthermore, when Equation 2.15 is inserted into Equation 2.11 the derived equation is written as:

$$Q_s C_{s_0} - \frac{r_x V}{Y_{x/s}} = C_s \frac{dV}{dt} \tag{2.16}$$

Additionally, to include μ term in Equation 2.16, r_x is replaced with μCx to give:

$$Q_s C_{s_0} - \frac{\mu C_x V}{Y_{x/s}} = C_s Q_s \tag{2.17}$$

The differential Equation 2.3 can be written as a linear equation by using Equation 2.4 and the obtained expression is:

$$C_x V = C_{x_0} V_0 e^{\mu t} \tag{2.18}$$

Equation 2.18 is further inserted to Equation 2.17 which is arranged to present the volumetric feed rate of the substrate as function of time:

$$Q_s = \frac{\mu C_{x_0} V_0}{Y_{X/S} C_0} \exp(\mu t) \tag{2.19}$$

2.6.3 Material Balance Equation for the Product (rhGH)

In semi-batch operation, mass balance of product is only represented by the production rate and the accumulation rate in the medium since there is no input or output streams involving product.

$$r_p V = \frac{d(C_p V)}{dt} \tag{2.20}$$

Where C_p is the product concentration in the cultivation medium, and r_p is the product formation rate which is written as a function of cell concentration by means of a first order kinetic equation

$$r_p = q_p C_x \tag{2.21}$$

Where q_P is the specific formation rate of the product ($g_{product}\ g_{cell}^{-1}\ h^{-1}$). Specific formation rate of the product is obtained by rearranging the above equations:

$$q_p = \frac{1}{C_x}\left(\frac{C_p}{V} Q + \frac{dC_p}{dt}\right) \tag{2.22}$$

2.6.4 Yield Coefficients

Yield coefficients represent the mass of cell or product formed per unit mass of substrate:

$$Y_{X/S} = \frac{r_X}{-r_s} = \frac{dC_x/dt}{-dC_s/dt} \tag{2.23}$$

$$Y_{P/S} = \frac{r_P}{-r_s} = \frac{dC_P/dt}{-dC_s/dt} \tag{2.24}$$

To obtain the overall yield coefficients for the entire bioprocess, Equation 2.23 and 2.24 are presented as linear equations for finite period of time

$$\bar{Y}_{X/S} = \frac{\Delta C_x/\Delta t}{-\Delta C_s/\Delta t} \quad (2.25)$$

$$\bar{Y}_{P/S} = \frac{\Delta C_P/\Delta t}{-\Delta C_s/\Delta t} \quad (2.26)$$

In aerobic processes, microorganisms consume oxygen intensively to carry out their oxidative reactions and to synthesise the recombinant protein. For the consumed oxygen, the cell and product yields on oxygen are also calculated:

$$Y_{X/O} = \frac{r_X}{-r_O} \quad (2.27)$$

$$Y_{P/O} = \frac{r_P}{-r_O} \quad (2.28)$$

The following table summarizes the most used yield coefficients with their symbols and the particular definitions:

Table 2.7. Definition of commonly calculated yield (selectivity) coefficients

Symbol	***Definition***
$Y_{X/S}$	Cell production rate per unit substrate consumption rate
$Y_{X/O}$	Cell production rate per unit of oxygen consumption rate
$Y_{S/O}$	Substrate consumption rate per unit oxygen consumption rate
$Y_{P/X}$	Product production rate per unit of cell production rate
$Y_{P/S}$	Product production rate per unit of substrate consumption rate
$Y_{P/O}$	Product production rate per unit mass of oxygen consumption rate

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Chemicals used in this study were provided from the following chemical companies and laboratories: Sigma Aldrich Co., Merck & Co. Inc., and Fluka Co. Each chemical was accompanied by its analytical grade definition.

3.2 Buffers and Stock Solutions

Prior to their usage, buffers and stock solutions were sterilized by using either autoclaving for 20 minutes at 121°C or filtering through filters with pore sizes of 0.25 or 0.45 μm (Millipore Corporation Bedford, MA, USA). In APPENDIX A, the formulation and preparation of the buffers and stock solutions are presented in detail.

3.3 The Microorganism

The host microorganism used in human growth hormone production was recombinant *Pichia pastoris* strain constructed by Massahi and Çalık, (2016). The pGAPZαA*::hGH* plasmid was constructed and amplified in *E. coli* DH5α and beside the amplified hGH gene it consists of a zeocin resistance gene and a his-tag gene sequence. pGAPZα-A is the shuttle vector concerned with zeocin resistance gene enclosing and it is also propagated in *E. coli* DH5α cells. Plasmid is isolated and further linearized by single digestion linearization. Transfection of *P. pastoris* wild type X-33 host strain is done by lithium chloride transformation method. Both the shuttle vector pGAPZα-A and *Pichia pastoris* wild type strain X-33 were obtained from Invitrogen. After the transformation the recombinant strains are inoculated and grown for 48-60 h and then stored in microbanks.

3.3.1 Microbank

The purpose of Microbank™ construction is to store bacterial and yeast systems. It is a convenient, ready-to-use system that is used for storage of yeast cultures. Compared to repetitive subculture, which has some serious drawbacks such as lost organisms or contaminated cultures, this method is more reliable. It consists of porous beads that provide adhesion and a preservative solution (www.pro-lab.com). After inoculation, the beads are washed to provide absorption and then stored at -80°C. For each culture requirement one of the beads can be received used for inoculation.

3.4 Growth Media for rhGH by *P. pastoris*

For sterilization of growth media, the prepared compositions were autoclaved for 20 minutes at 121 °C. After the sterilization it was waited for the temperature to drop under 50 °C and then the proper antibiotics were added.

3.4.1 Solid Medium

After the YPD agar was autoclaved, filtered glucose and the antibiotic zeocin were added in the medium. In every petri dish 30 mL of the sterilized medium was spread and waited for 10 minutes for the solution to cool off and solidify. Then the microorganisms stored in Microbank™ were picked out and inoculated to the YPD Agar plates. Inoculation was carried out for 48 hours at 30 °C. The compounds of solid medium with their specific concentrations for recombinant *Pichia pastoris* for rhGH production is given in Table 3.1.

Table 3.1. The Composition of the solid medium for recombinant *P. pastoris*

Component	**Concentration (g/L)**
Yeast Extract	10
Peptone	20
Agar	20
Glucose*	20
Zeocin™	1 ml
dH_2O	to 1 L

*Instead of autoclaving the glucose is sterilized by a 0.2 μm pore diameter filter.

3.4.2 Precultivation Medium

At the end of 48 h of inoculation the recombinant strain of *P. pastoris* was transferred to precultivation medium, buffered glycerol complex medium (BMGY) whose composition is presented in Table 3.2 (Invitrogen). After sterilizing the BMGY medium, chloramphenicol and Biotin were added to the composition and 50 ml of medium were placed in 250 ml baffled air-filtered shake bioreactors. After inoculation, the air-filtered bioreactors were placed in an orbital shaker (Sartorius, Germany) which worked with an agitation speed of 200 rpm and temperature of 30°C. Precultivation phase generally lasted for 15-20 h, which was the required time for the cells to reach the desired concentration at a range of OD_{600} = 4-8.

Table 3.2. Composition of precultivation medium, BMGY

Component	**Concentration (g/L)**
Yeast Extract	10
Peptone	20
Potassium phosphate buffer (pH = 6.0)	0.1 M
Yeast nitrogen base (YNB)	13.4
Ammonium sulfate $(NH_4)_2SO_4$	10
Glycerol	10
Chloramphenicol	1ml L^{-1}
Biotin	4 x 10^{-5}
dH_2O	to 1 L

3.4.3 Production Medium

After the required cell concentration was obtained at precultivation phase, the cells were prepared to be inoculated in the production medium in the bioreactor. To separate the cells from the precultivation medium, the cell containing BMGY medium, was centrifuged at 1500 g and 4°C for 10 minutes. The precipitated cells also known as pellet, after being detached from the supernatant, were later dissolved in water and fed to the bioreactor. The concentration of the cells in the water was arranged in a way that

the production phase started with OD_{600} = 2. For production of rhGH with *P. pastoris* under P_{GAP}, production phase was carried out in two phases. In the first phase cells were inoculated in basal salt medium (BSM), where glycerol was the sole carbon source. For the second phase glucose or molasses were utilized as the carbon source. To maintain a healthy cell growth in production media for both phases Pichia trace minerals (PTM) were added. The composition of PTM is shown in Table 3.4.
Components of the production medium and their particular concentrations for rhGH production are presented in Table 3.3:

Table 3.3. Composition of production medium

Components	**Concentration (g/L)**
Glycerol (86 %)	50 ml
85% H_3PO_4	26.7ml
K_2SO_4	18.2
$MgSO_4.7\ h_2O$	14.9
KOH	4.13
$CaSO_4.2\ h_2O$	1.17
Chloromphenicol*	1mL
PTM1 *	4.35mL
10% antifoam*	1 mL
dH_2O	to 1 L

*Added after autoclaving

3.4.4 Feed Stream Composition

At the depletion of glycerol, the second phase of the production also known as fed-batch started where nitrogen and carbon sources enter in the bioreactor continuously. The nitrogen source used in this study was 25% ammonium hydroxide which at the same time served for pH adjustment in the production medium. Glucose was added to the bioreactor as sole carbon source for one part of the experiments where its stock solution concentration was 500 g L^{-1}. To investigate the effect of complex medium on yeast *Pichia pastoris* metabolism, molasses was also used as carbon source where the solution's stock concentration was 440 g L^{-1}. Sucrose, being 50% of the entire

composition, is the basic carbon source of molasses. To convert sucrose to glucose and fructose, acid hydrolysis method was applied where 110 g L^{-1} of glucose and fructose respectively were obtained and fed to the bioreactor.

Table 3.4 Composition of *Pichia* trace salts, PTM1

Components	Concentration g/L
$CuSO_4.5\ h_2O$	6
H_3BO_3	0.02
NaI	0.08
$Na_2MoO_4.2\ h_2O$	0.2
MnSO4.H2O	3
$ZnCl_2$	20
$FeSO_4.7\ h_2O$	65
$CoCl.6\ h_2O$	0.916
H_2SO_4	5 mL
Biotin	0.2 mL
dH_2O	to 1 L

3.4.4.1 Acid Hydrolysis of Molasses

When molasses was pre-treated by acid hydrolysis, sucrose was converted to 1:1 mixture of glucose and fructose. The steps followed for acid hydrolysis were:

1) **Molasses Feeding**: 440 g molasses was dissolved in 1 L ultra-pure water. The added dH_2O in this step did not complete exactly 1L since further chemicals were added to the molasses
2) **Centrifugation**: To remove the probable impurities that may have accumulated with molasses, the mixture was centrifuged at 6000 g at +4°C for 20 minutes
3) **Dropping the pH value**: To reduce the pH of the solution, 37% HCl was added slowly while continuously checking the pH. Approximately 30 ml of acid were added to lower adjust pH = 1.8

4) **Hydrolysis:** During this step sucrose was converted to glucose and fructose. For the sucrose to be entirely inverted, the solution was placed in water bath at 90°C to be pretreated for 3 hours.
5) **pH adjustment:** After 3 hours, base KOH was added to the solution to rearrange the pH value as 5.0 since this was the fermentation pH value.

3.5 rhGH production in Pilot-scale Bioreactor

The pilot-scale bioreactor with a 3 L volume (Braun CT2-2) used for rhGH production, was put to work with a volume of 1-1.5 L. Detailed properties of the bioreactor are presented in Table 3.5.

During the fermentation process beside the substrate feeding rate other important parameters for the bioreactor are essential to control the bioprocess. Temperature, pH, oxygen supply, antifoam, mixing and aeration conditions were investigated during the fermentation. To control these parameters, the set points data for each parameter were inserted in the control system.

Table 3.5. Geometric properties of the bioreactor

Property	Size
Tank diameter	T = 12 cm
Impeller diameter	D = T/2.4
Impeller height	h = T/12
Off-bottom clearance	C = T/6
Average height of working volume	H= 0.667 T

3.5.1 Bioreactor Operation parameters

The temperature of the medium was kept at the constant value of 30°C by the PI controller of the system. According to its deviation the temperature value was either adjusted by an external cooler or steam generator and a jacket. The pH value of the medium at 5.0 was controlled by a pH probe and the control unit. For every necessary

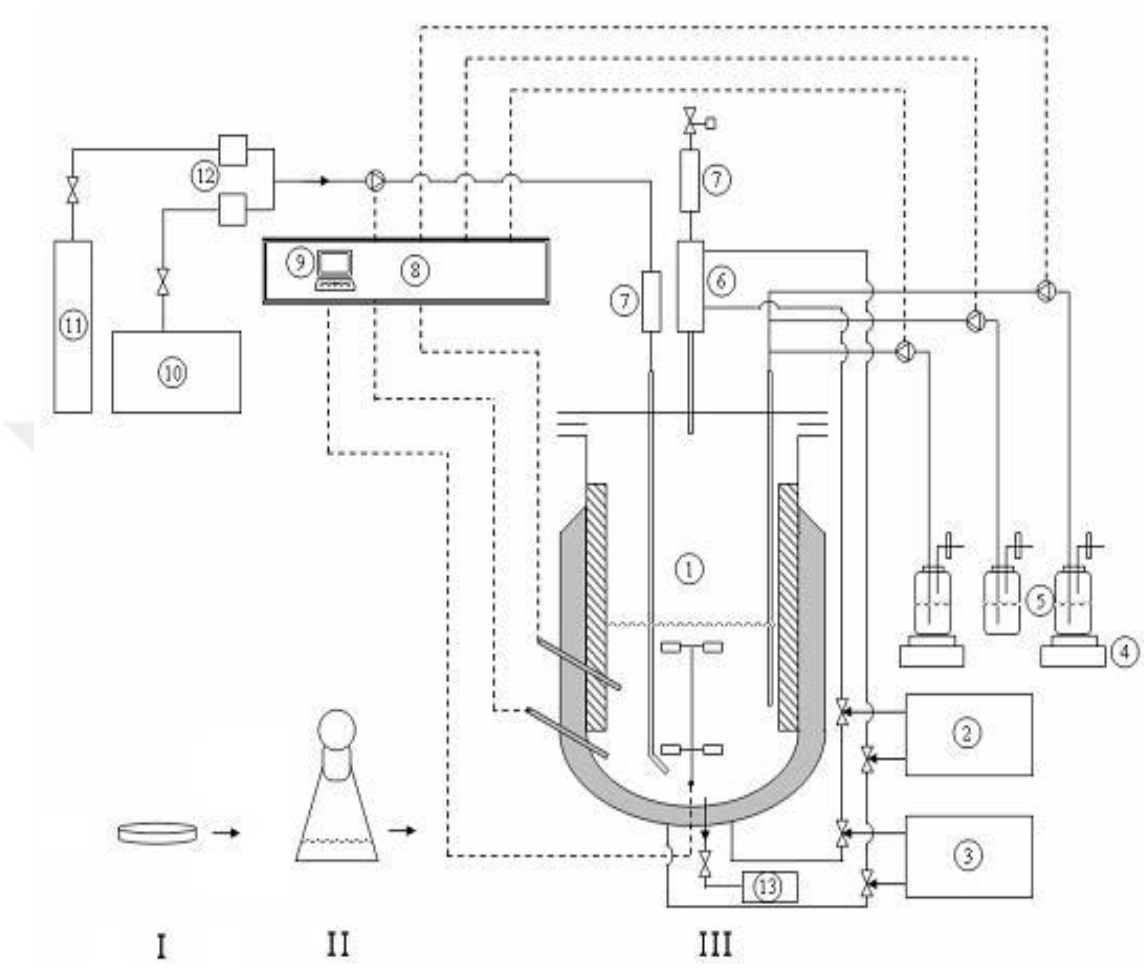


Figure 3.1 Precultivation steps and pilot-scale bioreactor system set up. I. Solid medium containing inoculated cells from stock culture. II. Precultivation medium III. Pilot-scale bioreactor system. (1) Bio-reaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, 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)

pH elevation, 25% ammonium hydroxide was fed to the bioreactor automatically. To provide the necessary oxygen transfer, agitation was arranged by six-bladed Rushton turbines and the dissolved oxygen concentration in the medium was determined by the oxygen probe. Air or pure oxygen were supplied to the bioreactor to keep the oxygen concentration at a specific value. Foaming is one the obstacles of the bioprocess and it should be kept in control to prevent blockage of outlet filter and problems in oxygen mass transfer. In order to keep a low level of foam, at the beginning of batch phase, 1 mL of antifoam solution (Y-30 emulsion, Sigma) was added to BSM medium. Additionally, small amounts of antifoam are added during the fed-batch phase. Peristaltic pumps correlated with PI controller provided the supplement of base, antifoam and substrate through the inlet port of bioreactor. All the steps required for rhGH production under P. pastoris are illustrated in Figure 3.1.

3.5.2 Bioreactor Arrangements and Preparations

For every experiment, prior to the cell cultivation, some crucial steps such as sterilization were carried out. The system was turned on approximately 4 hours before the cultivation for polarization of the oxygen probe. Oxygen and pH probes were calibrated for every fermentation process investigated to avoid any error in reading of the probes and for the maintenance of the system.

3.5.2.1 pH calibration

To calibrate the pH measurement, two standard buffer solutions (Mettler Toledo) at pH = 4.0 and pH = 7.0 respectively were used. The pH probe was inserted in both of the solutions and from the control unit monitor it was waited for the values on the screen to stabilize at 4 and 7. After calibration probe was plugged in the bioreactor.

3.5.2.2 Sterilization

Sterilization of the bioreactor vessel was performed before loading the production medium by the superheated steam. Bioreactor vessel was filled with distilled water up to the baffle level. Protective jacket of bioreactor made by stainless steel was attached to the outer glass surface. The position of the inlet air filter was adjusted from fermentation to sterilization position. The sterilization procedure was commanded from the control unit and it was carried out for 20 minutes at 121^{o}C. At the end of

sterilization the temperature of the vessel was adjusted as 30°C, the water was discharged, and the sterilized BSM production medium was added to the bioreactor by a sterilized silicon hose. BSM medium has a pH value around 2. To increase the pH to 5 ammonium hydroxide was pumped to the medium manually.

3.5.2.3 Oxygen calibration

The air inlet filter was rearranged to fermentation position. To adjust pO_2 = 0%, instead of oxygen, pure nıtrogen was fed to the reactor and the medium was deoxygenated completely. Subsequently the oxygen input stream was open to oxygenate the medium totally until it reached the maximum pO_2 which was further set as pO_2 = 100%. The theoretic zero and slope values obtained for a normalized calibration are 0 nA and 60 nA respectively. If the obtained values after the calibration were very different, pO_2 calibration was repeated.

3.5.3 Production phases in Semi-batch operation

For semi batch operation in *P. pastoris* under GAP promoter two production phases were required to optimize the r-protein production. When the cells were inoculated in the BSM medium into the reactor they marked the beginning of the batch phase. The initial OD_{600}=2 coincides with a cell concentration of 0.55 g L^{-1}.

1. Glycerol Batch Phase

The aim of this phase was to increase the cell concentration and glycerol was found as the most suitable carbon source for optimizing the cell growth. There was no input stream to supply the production medium with other nutrients. Consequently, the depletion of glycerol was the indicator of the end of the batch phase. Since the yield coefficient of glycerol on cell is 0.5 g g^{-1}, when starting with 45 g glycerol, at the end of this phase cell concentration varied from 25–27 g L^{-1} corresponding to an OD_{600} = 100.

2. Semi-batch Phase

In this phase the purpose of selection of the carbon source was to increase the rhGH production along with the cell growth. In this study both glucose and molasses were investigated as potential carbon sources where they were fed to the bioreactor with

different feeding strategies as explained in Table 3.6. To calculate the feeding profile according to the predetermined specific growth rate Equation 2.19 was used:

$$Q_s = \frac{\mu C_{x_0} V_0}{Y_{X/S} C_{S0}} \exp(\mu t) \qquad (2.19)$$

Where μ_o is the predetermined specific growth rate (h^{-1}), C_{X_o} is the initial cell concentration (g L^{-1}) , V_o is the initial volume of the bioreactor medium at the beginning of the fed-batch phase (L), $Y_{X/S}$ is cell yield on substrate (g g^{-1}), C_S^o is feed substrate concentration (g L^{-1}).

Table 3.6. Parameter values for the feed profile represented by equation 2.19

Parameter	Value adjusted
C_{x0}	27 g L^{-1}
V_0	0.94 L
C_{S0} (glucose)	500 g L^{-1}
C_{S0} (molasses)	440 g L^{-1}
Yx/s	0.48 g g^{-1}

3.5.3.1 Exponential and combined feeding strategies studied

The aim of this study was to increase the rhGH productivity of *P. pastoris* under GAP promoter by investigating two critical parameters of semi-batch operation; the selection of the carbon source and the design of feeding profile. Under consideration of the yeast metabolism and central carbon pathways of *P. pastoris*, glucose and molasses were selected as the potential substrates.

There were two experiment sets that were carried out in the pilot scale bioreactor by using semi-batch operation where the substrates; glucose or molasses were introduced to the system with an exponential profile or combined feeding profile. Exponential feeding strategies were named as G1 (μ_o = 0.10 h^{-1}) and G2 (μ_o = 0.15 h^{-1}) for glucose, whereas M1 (μ_o = 0.07 h^{-1}) and M2 (μ_o = 0.10 h^{-1}), for molasses. Combined feeding strategies consisted of 1.5 h exponential feeding with a particular μ_o followed by 0.5 h non-feeding intervals in a repetitive cycle. GS1, GS2 and GS3 strategies were designed

for glucose with μ_o = 0.07 h^{-1}, 0.10 h^{-1} and 0.125 h^{-1} and MS1, MS2, and MS3 strategies were designed for molasses with μ_o = 0.07 h^{-1}, 0.10 h^{-1} and 0.15 h^{-1}

Table 3.7. Explanation of applied feeding strategies for rhGH production

Strategy	Fed-batch Feeding Strategy Description
G1	Exponential glucose feed flow rate defined with μ_o = 0.10 h^{-1} within 0≤ t <17 h
G2	Exponential glucose feed flow rate defined with μ_o = 0.15 h^{-1} within 0≤ t <15 h
GS1	Exponential glucose feed flow rate defined with μ_o = 0.07 h^{-1} for 1.5 h accompanied with a starvation period of 0.5 h
GS2	Exponential glucose feed flow rate defined with μ_o = 0.10 h^{-1} for 1.5 h accompanied with a starvation period of 0.5 h
GS3	Exponential glucose feed flow rate pre-calculated using μ_o = 0.125 h^{-1} for 1.5 h accompanied with a starvation period of 0.5 h
M1	Exponential molasses feed flow rate defined with μ_o = 0.07 h^{-1} within 0≤ t <17 h
M2	Exponential molasses feed flow rate defined with μ_o = 0.10 h^{-1} within 0≤ t <17 h
MS1	Exponential molasses feed flow rate defined with μ_o = 0.07 h^{-1} within 0≤ t <17 h in intervals of 1.5 hours accompanied with a gap interval of 0.5 h.
MS2	Exponential molasses feed flow rate defined with μ_o = 0.10 h^{-1} within 0≤ t <17 h in intervals of 1.5 hours accompanied with a gap interval of 0.5 h.
MS3	Exponential molasses feed flow rate defined with μ_o = 0.15 h^{-1} within 0≤ t <17 h in intervals of 1.5 hours accompanied with a gap interval of 0.5 h.

3.6 Analysis

To follow the cell growth, r-protein production, the medium maintenance, and the proteolytic activity, medium samples were taken from the bioreactor in specific intervals; every 1.5 or 3 hours. The cell concentration was measured from these samples. Since the recombinant strain of *P. pastoris* produced, rhGH extracellularly the medium was further centrifuged to separate pellets from the supernatant. Centrifugation took place at 1500 g and 4^{o}C for 10 minutes. Furthermore supernatants were passed through filters with a pore diameter of 0.2 μm and were used to determine the organic acid concentration in HPLC.

3.6.1 Cell Concentration

Optical density of the cells was measured by using UV-Vis spectrophotometer at a wavelength of 600nm. For an accurate reading the reading of UV-Vis spectrophotometer should vary in the range of (0.1-0.8 Å). As mentioned before, cells reach densities up to OD = 100 or higher so the samples were diluted before using the apparatus. To convert the optical density to dry cell weight in g L^{-1} for *P. pastoris,* a calibration curve was drawn to correlate the absorbance value with cell concentration:

$$C_x = 0.244 \times OD_{600} \times DF \qquad (3.1)$$

Where C_X is the dry cell weight per liter, OD_{600} is the optical density obtained at 600 nm and DF is the dilution factor.

3.6.2 rhGH Concentration

For the measurement of the r-protein concentration of hGH Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). For the sample preparation 20 μL of supernatant was mixed with 10 μL of loading buffer from which 15 μL of the mixture, were loaded to the wells of SDS-PAGE gels. On the other hand for the prestained protein molecular weight marker only 3 μL solution were loaded. Marker (Appendix C) is composed of buffers with different molecular weights. After loading the samples in the gel, a constant current of 40mA was run through the gels for 45 minutes. Furthermore, silver staining of the gels took place and at the final step of this procedure, proteins were able to be detected.

To cast the SDS polyacrylamide gel these steps were followed:

1. For each gel preparation two Bio-Rad glass plates were required so that the solution of the gel was dropped into the gap between two glasses. The glasses were cleaned and positioned in a stationary posture.
2. Solutions required for the gel were prepared by TGX Stain-Free™ and FastCast™ acrylamide kits. Two different solutions named Resolver and Stacker solutions were prepared separately. First Resolver solution was prepared by mixing 3mL of Resolver A and B respectively with 30 µL APS (10% ammonium persulphate) and 3 µL TEMED (NNN'N'-Tetramethylethylenediamine). To create a uniform mixture, the obtained solution was shaken.
3. The gap between the two glass plates was filled with Resolver solution until 0.5-1 cm below bottom of comb teeth.
4. Then Stacker solution was prepared by mixing 1mL of Stacker A and B respectively with 10 µL APS (10% ammonium persulphate) and 2 µL TEMED (NNN'N'- Tetramethylethylenediamine).
5. Stacker solution was added above the Resolver solution to fill the gap entirely.
6. Comb was inserted and then it was waited for 30-45 minutes for polymerization to take place.
7. After the polymerization the wells were washed with distilled water to get rid of any gel parts and then were totally dried.

For Gel Electrophoresis the following steps were taken:

1. The glass plates were put in the apparatus's cell in such a way that the shorter glass was placed in the inner side of the system. Running buffer solution was added between the two gels until it covers the whole space and the gel wells.
2. Then the 3 µL of prestained protein molecular weight marker and 15 µL of the prepared samples were loaded in the gel wells.
3. The current of the system was adjusted at 40 mA for 45 approximately minutes. To understand the position of the running samples, marker was observed and waited for its noticeable lines to widen from each other.

4. After samples finished running the system was turned off and the gels were delicately separated from the glass plates.

Silver Staining procedure was followed according to Blum *et al.* (1987) and is the last stage of SDS-PAGE. The solutions used for this procedure are given in Appendix A. Figure 3.2 illustrates the steps followed:

Figure 3.2 Silver Staining Procedure steps

3.6.3 Glucose Concentration

To measure glucose concentration, glucose oxidation method was performed by the help of a Glucose Analysis Kit (Biasis Ankara). In the presence of glucose oxidase catalyst, two subsequent reactions occur in series for conversion of glucose to gluconate and hydrogen peroxide. The second reaction's product, iminoquinone has the same concentration with glucose converted and its production is accompanied with a pink colour.

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

$$H_2O_2 + 4 - aminantripine + Phenol \rightarrow iminoquinone + H_2O$$

For this reaction to occur, using the glucose analysis kit 0.05 ml glucose analysis reactive, 0.4 ml glucose analysis buffer, 0.05 ml sample consisted of supernatant and 2.0 ml distilled water were mixed together. When the glucose concentration in the sample exceeded 2 g L^{-1}, the sample was diluted with DF = 10. These mixtures were incubated for 20 minutes at 37°C to complete the glucose oxidation. In the end of this reaction, the OD_{505} of the iminoquinone was measured by UV-Vis Spectrophotometer at a wavelength of 505 nm. To calculate the concentration of glucose, calibration curve was obtained to write concentration as a function of absorbance. To draw the calibration curve given in APPENDIX B, OD_{505} of standard glucose solutions with known concentrations was measured.

3.6.4 Fructose Concentration

To measure the fructose concentration, a method determined by Dische and Borenfreund (1951) known as cysteine- carbazol- sulfuric acid method. In a glass tube, 540 µL of 0.1 M HCl was mixed with 60 µL medium sample, 60 µL of 1.5% cysteine in 37% HCl, 60 µL of 0.12% (w/v) carbazol in 95% ethanol, and 1.8 mL of 70% (v/v) sulfuric acid. The solutions were added in this order and the reaction started when carbazol was added to the mixture. To provide a better mixing of the chemicals, each tube was vortexed and then were waited at room temperature for 30 minutes. Fructose was absorbed at a wavelength of 560 nm by UV-Vis spectrophotometer. The calibration curve of fructose is presented in APPENDIX B.

3.6.5 Organic Acid Concentrations

Organic acid concentrations were measured by using Reverse phase high pressure liquid chromatography (HPLC) (Waters HPLC, Alliance 2695, Milford, MA). Organic acids were separated, identified and quantified on the basis of their molecular structure; the molecules that display stronger interaction with the stationary phase, move more slowly, resulting to later retention times compared to the organic acids

with weaker interactions. The stationary phase consisted of the column which is filled with an adsorbent material and the mobile phase was composed of 3.12% (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4. Mobile phase flowed through column and carried the samples made of filtered supernatants. Samples, in cases of high concentration of organic acids, were diluted and further loaded to the system's vials with a required volume of 100-200 μL.

Table 3.8. HPLC operation characteristic and condition for identifying and quantifying the organic acids

Column	Capital Optimal ODS
Column dimensions	4.6 mm x 250 mm x 5 m
System	Reversed phase chromatography
Mobile phase	3.12% (w/v) NaH_2PO_4, 0.62·10-3 (v/v) H_3PO_4
Flow rate of mobile phase	0.8 ml min^{-1}
Column temperature	30°C
Detector type and wavelength	Waters 2487 Dual Absorbance Detector, 210 nm
Detector temperature	30°C
Injection volume	5 l
Analysis period	15 min
Delay time	5 min

Before loading the samples in HPLC, first, solution standards for each organic acid were read in the system and the respective retention times were determined to identify the organic acids. Area of the peaks displayed presented the concentration of the components. Calibration curves given in APPENDIX E, provide the relation of peak areas with concentration of the organic acids.

3.6.6 Protease Activity Assay

There are three different protease activities named as alkali, neutral and acidic. Since pH of the fermentation medium was 5.0, only the acidic activity of the proteases was examined. The extracellular acidic activity was determined by hydrolysis of casein by using the supernatants of the medium samples. Hammerstein casein solution (0.5% w/v) was prepared in 0.05 M sodium acetate buffer (pH=5.0). 2 ml of this solution was added to 1 ml of the sample which was diluted sufficiently. Hydrolyzation took place at 37°C for 20 minutes. To terminate this reaction 10% trichloroacetic acid (TCA) was added. The reaction mixture was further centrifuged at +4°C and 10500 g for 10 minutes to separate the layers of the mixture. Further it was required for the mixture to be incubated at room temperature for 5 minutes. By using UV-Vis spectrophotometer the solution's supernatant was absorbed at a wavelength of 275nm. The formation of 4 nmol tyrosine per minute corresponds to one unit of protease activity. The equation where activity is presented as a function of absorbance is as follows:

$$A(UL^{-1}) = \left(\frac{Abs(275nm)}{0.8}\right)\left(\frac{1U}{4nmol\ min^{-1}}\right)\left(\frac{1}{20\text{min}}\right)\left(\frac{1000nmol}{1\mu mol}\right)(DF) \qquad (3.2)$$

CHAPTER 4

RESULTS AND DISCUSSION

The aim of this study was to increase the recombinant human growth hormone (rhGH) production by *Pichia pastoris* strain carrying pGAPZαA*::hGH* plasmid, focusing on the effect of semi-batch feeding strategies where two different feeding media; glucose and molasses were investigated. Feeding strategies were designed for each of the carbon sources, glucose and molasses respectively in a pilot-scale bioreactor. The abbreviations and description of each strategy was presented in Table 3.7. The glycerol batch phase of the bioprocess was carried out under the same conditions presented in 3.5.1 for all the experiments and approximately lasted for 17~18 hours. The semi-batch bioreactor operation strategies were designed according to Equation 2.19 where the feed flow rate was pre-calculated as a function of the cultivation time. The initiation time of the semi-batch process was considered as t = 0 h for a simpler presentation and description of the results. For the first set of experiments where glucose was utilized as the carbon source, two exponential feeding strategies with predetermined specific growth rate μ_o = 0.10 h^{-1} and 0.15 h^{-1} were examined. Furthermore, three combined feeding strategies were carried out where glucose was fed with an exponential profile for 1.5 h with μ_o = 0.07 h^{-1}, 0.10 h^{-1}, 0.125 h^{-1} and then the feeding was stopped and the bioreactor was operated batch-wise for 0.5 h, and feeding restarted by following the designed strategy constitutively in a repetitive cycle. In the second set of experiments, molasses was utilized as the carbon source with an exponential feeding similar to glucose based feeding strategies with μ_o = 0.07 h^{-1} and 0.10 h^{-1}, and with consecutive exponential feeding with μ_o= 0.07 h^{-1}, 0.10 h^{-1}, 0.15 h^{-1} followed by 0.5 h

of batchwise operation of bioreactor. To determine the fermentation characteristics concentrations of cell, rhGH, substrate, organic acids, and protease activities were measured for each experiment. Furthermore, fermentation characteristics such as yield coefficients and specific rates were calculated. The two experiment set results were further compared with each other in a quantitative and qualitative perspective.

4.1 Exponential Feeding strategies with glucose as the carbon source

In G1 and G2 strategies, glucose was introduced to the bioreactor with an exponential profile; the volumetric flow rate Q (t), of glucose was presented in Figure 4.1.

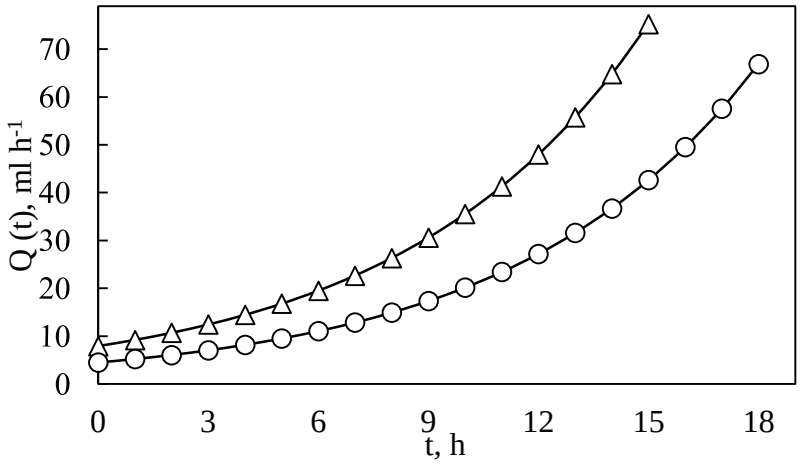


Figure 4.2. Exponential feeding profiles of glucose in G1 (○), G2 (Δ)

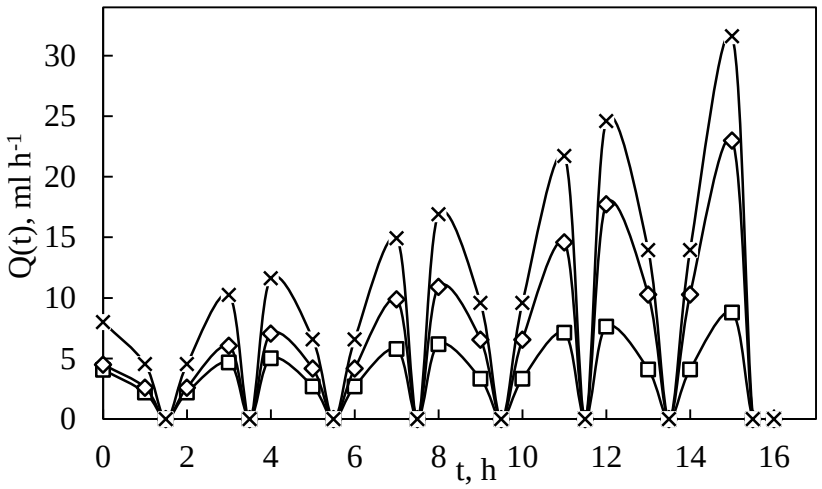


Figure 4.3. Consecutive feeding strategy profiles of glucose in GS1 (□), GS2 (◊), and GS3 (×)

The exponential glucose feed followed by 0.5 h batchwise operation in GS1, GS2, and GS3 strategies were presented in Figure 4.2 As it was depicted in Figure 4.2 glucose feeding performed an exponential curve. However, the locus of maximum in each consecutive strategy has an exponential character.

4.1.1 Glucose Concentration and Specific Glucose Consumption Rate

Glucose concentration in the cultivation medium was measured throughout the bioprocess to determine if substrate was being accumulated or depleted during the process. In this experiment set there was no glucose accumulation in the cultivation except for G1 and G2 operation strategies where the accumulated concentration of glucose was measured as 9 g L^{-1} at t = 17 h and 20 g L^{-1} at t = 15 h, respectively. This shows that the entire glucose supplied in the cultivation medium was immediately utilized by *P. pastoris* cells for the growth and r-protein production. The accumulation of the glucose in strategies G1 and G2 were accompanied with an increase in dissolved oxygen concentration in the medium showing that the cells were not consuming any more the oxygen and glucose and they have entered the stationary phase.

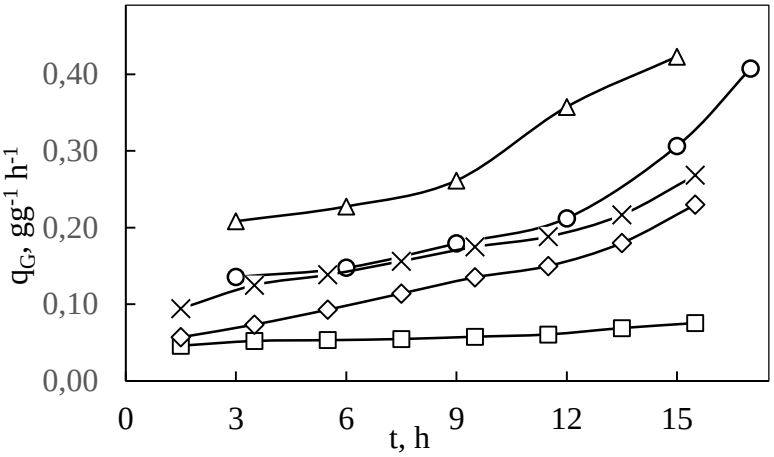


Figure 4.3. Variations in the specific substrate (glucose) consumption rates with the cultivation time G1 (○), G2 (Δ), GS1 (□), GS2 (◊), GS3 (×).

Variations in the specific glucose consumption rate (q_G) with the cultivation time were presented in Figure 4.3. Since glucose was entirely utilized during the whole process for all the glucose fed-batch feeding strategies the consumption rate values increased along the cultivation time. As expected, q_G values increased by increasing μ_o. The highest specific glucose consumption rate was obtained as 0.41 g g^{-1} h^{-1} at t = 15 h for

G2 strategy where glucose was fed with $\mu_o = 0.15\ h^{-1}$. The lowest specific consumption rate was obtained in GS-1 strategy where q_G values calculated were around 0.07 g g^{-1} h^{-1}.

4.1.2 Cell concentration and Specific Cell Growth Rate

For the designed semi-batch bioreactor operation strategies, the variations in cell concentrations with the cultivation time were presented in Figure 4.4. The cell concentration profiles were similar with each other for the first 9 hours of the semi-batch operation. For strategies G1 and G2, the cell concentrations reduced after this time. A reason for such behavior can be the accumulated glucose in the medium after t = 12 h which has a repressive effect on the cell growth. In strategy G2 where the cells were supposed to grow with a specific growth rate of 0.15 h^{-1} the lowest cell concentration was obtained as cells could not utilize glucose effectively resulting in the accumulating in the medium. Furthermore, the limited cell growth in the bioprocess may be related with physical restrictions such as mass transfer of oxygen, as oxygen limitation in aerobic processes, becomes an obstacle for maintaining the desired specific growth rate.

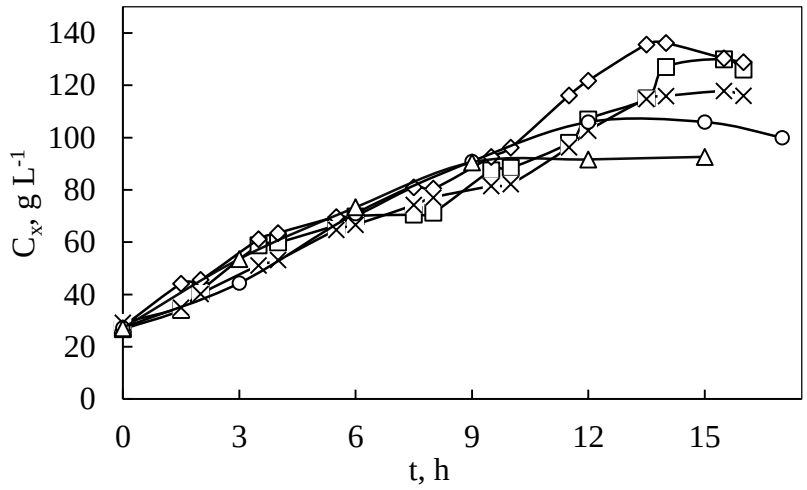


Figure 4.4. Variation in cell concentration with the cultivation time G1 (○), G2 (Δ), GS1 (□), GS2 (◊), GS3 (×).

For strategies GS1, GS2, and GS3 higher cell concentrations were obtained in later hours of the bioprocess at t =13.5 h ~ 14 h, even though less glucose fed into the fermentation media designed with 0.5 h feed interruption periods. When continuous

glucose feeding was interrupted, it results to a lower flux through the glycolysis since glucose is the main carbon source of the glycolytic pathway. However, the cells can adapt their metabolism to short starving intervals, where they reserve their capacity for rapid restart growth in case there will be another glucose supply in the media (Garcia-Ortega *et al*. 2016). As it was shown in the Figure 4.4 during the feed interruption periods there were no significant increase in the cell concentration which supports the results of Garcia-Ortega *et al*. (2016) where the cell was not affected during the non-feeding intervals. The highest cell concentration was obtained at GS2 strategy as C_X =136 g L^{-1} at t = 14 h. The maximum cell densities reached in the other semi-batch strategies were: 106 g L^{-1} at t = 15 h for G1, 92.7 g L^{-1} at t = 12 h for G2, 130 g L^{-1} at t = 15.5 h for GS1, and 118 g L^{-1} at t = 15.5 h for GS3. The highest cell concentration reached in GS2 was 1.29-, 1.46-, 1.05-, and 1.15-fold higher than G1, G2, GS1, and GS3 respectively. In both G1 and GS2 strategies, the exponential feeding of glucose was calculated with a predetermined specific growth rate of 0.10 h^{-1}. It was observed that including the batchwise intervals the entire cell concentration of the bioprocess enhanced by 1.29-fold.

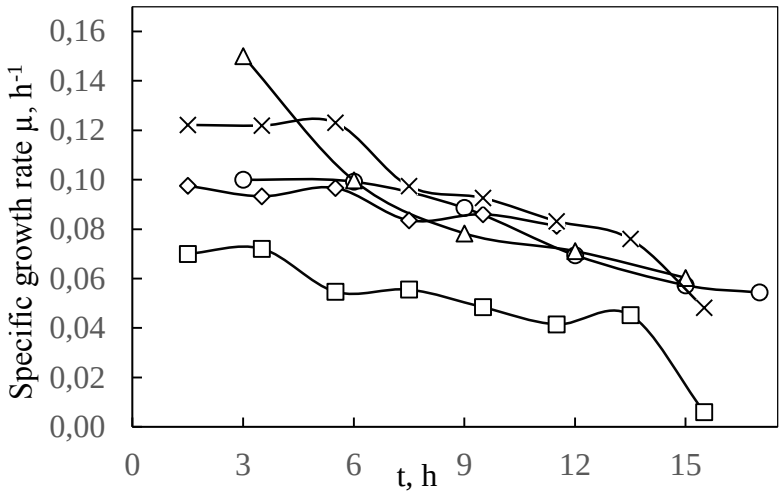


Figure 4.5. Variations in the specific growth rate with the cultivation time G1 (○), G2 (Δ), GS1 (□), GS2 (◊), GS3 (×).

The variations in the specific growth rate were presented in Figure 4.5 where it was shown that during the first hours of the semi-batch operation 0 h < t < 6 h the cell formation continued with an approximate constant growth rate and after that the

specific growth rate reduced gradually. The highest specific growth rate attained in this study was μ_{max} =0.15 h^{-1} at t=3 h in G2. Theoretically for G1 and G2 strategies cells were presumed to grow with the constant predetermined growth rate as μ =0.10 h^{-1} and 0.15 h^{-1}. However the deviation from the expected value increased throughout the cultivation. For GS1, GS2 and GS3 strategies, a deviation from μ_o was expectable since the feeding of glucose followed a delayed exponential profile.

4.1.3 Recombinant hGH Concentration Profiles

The variations in rhGH concentration with the cultivation time in the designed semi-batch bioreactor operation strategies were presented in Figure 4.6. The r-protein concentration increased proportionally with the cell growth which was expected for rhGH expression under constitutive GAP promoter. However, for G1 and G2 feeding strategies after t = 9 h and t = 6 h, respectively, the rhGH concentration started to decrease significantly because of the hydrolysis by synthesized proteases. In these specific hours the maximum rhGH was obtained as 446 mg L^{-1} and 278 mg L^{-1}, respectively, as presented in Table 4.1.

Table 4.1 Characteristics and performances of glucose designed feeding strategies

Semi-Batch Operation	**Overall values at t_{max}**				
	t_{max}, h	Cell concentration, g L^{-1}	rhGH concentration, mg L^{-1}	Volume, L	Total substrate consumed, g
G1	9	90.58	446	0.977	21
G2	6	73.34	278	1.013	23
GS1	8	77.71	380	0.83	8
GS2	8	77.12	611	0.81	13
GS3	10	82.25	598	0.84	22

In a previous study, where glucose was used as the carbon source the exponential feeding with μ_o = 0.15 h^{-1} was found as the optimal feeding strategy for rhGH production where the highest C_{rhGH} achieved was 508 g L^{-1} at t = 19 h (Keskin, 2014).

However, in that study initial cell concentration at the beginning of the semi-batch operation was 10 g L^{-1}. Whereas, in the current work initial cell concentration at the beginning of semi-batch operation was 27 g L^{-1}. This difference affected the amount of glucose fed to the medium and shortened the semi-batch operation time. In G1 feeding strategy with μ_o = 0.10 h^{-1} the r-protein concentration obtained was only 1.13-fold lower than the best value obtained by Keskin. (2014), but the cultivation time to achieve this maximum amount was shortened two times. However, the product to cell yield and product to substrate yield, for G1 and G2 strategies, were relatively lower which shows that glucose was not utilized very effectively. To increase the product yield on substrate and enhance the utilization of glucose for rhGH production the combined feeding strategies GS1, GS2, and GS3 were designed. Furthermore, consumption of high concentrations of glucose or other repressing carbon sources facilitates the r-protein production with a negative effect on transcription. It leads to an improper high recombinant protein secretion which is suppressed by glucose supply and results in unfolded or misfolded proteins which may cause the saturation of the secretory pathways (Inan *et al*. 2005). By introducing 30 minutes starvation intervals the stress on the glycolytic pathway was reduced. The gradual increase of rhGH levels along the carbon-starving periods of the semi-batch cultivation performed applying strategies GS1, GS2, and GS3 were also presented in Figure 4.6. The slightly increase of rhGH concentration in the end of each starvation period, means that the cellular stress in ER (endoplasmic reticulum) and Golgi was lessened.

The observed increase in the secreted rhGH during the non-fed intervals, shows that yeast *P. pastoris*, could still produce recombinant proteins under expression of GAP promoter for short starvation periods. Even though the fluxes of glycolytic pathway decrease due to no glucose entrance into the pathway, the adaptive capacity of the yeast provides the upregulation of the transcription and translation of the glycolytic genes (Garcia-Ortega *et al*., 2016). Since rhGH was expressed under the glycolytic GAP promoter, its productivity could be upregulated in the carbon-starving conditions.

The maximum rhGH concentrations were achieved with GS1, GS2, and GS3 at t = 8 h as 380 mg L^{-1}, t = 8 h as 611 mg L^{-1}, t = 10 h as 598 mg L^{-1}, respectively, as presented in Table 4.1. By using this consecutive feeding and starvation periods, the production

increased by 1.37-fold compared to G1 strategy designed with exponential feeding with $\mu_o = 0.10\ h^{-1}$ throughout the process.

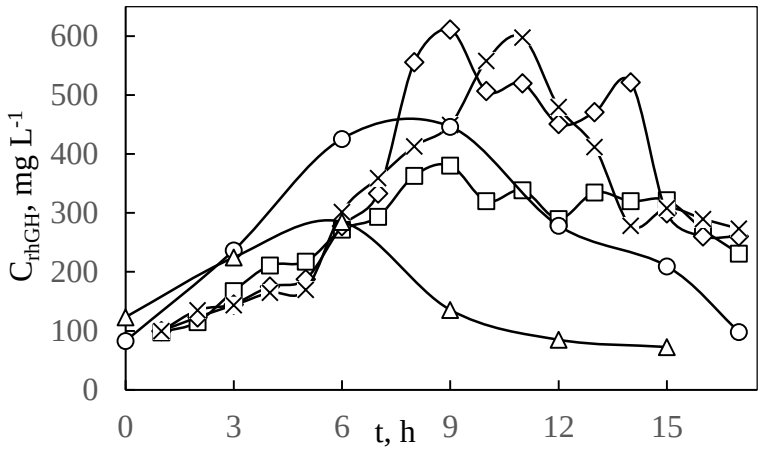


Figure 4.6. Variations in rhGH concentration with the cultivation time G1 (○), G2 (Δ), GS1 (□), GS2 (◊), GS3 (×).

The specific rhGH production rates (q_{rhGH}) for the designed strategies were presented in Figure 4.7. For G1 and G2 strategies, q_{rhGH} values were higher during the initial times of the cultivation and then decreased. As shown in the Figure 4.7, the q_{rhGH} values for G1 and G2 strategies were lower compared to GS1, GS2 and GS3 operation strategies, since the concentrations of rhGH were low. The highest specific production rates were calculated at t = 3 h as 1.22 mg g^{-1} h^{-1} and 0.72 mg g^{-1} h^{-1}, respectively, for G1 and G2. For the consecutive feeding strategies GS1, GS2 and GS3, the specific production rates increased between $0 < t < 8$ h and then decreased significantly. At GS2 strategy the highest q_{rhGH} was calculated as 1.92 mg g^{-1} h^{-1}. In GS1 and GS3 the highest q_{rhGH} values were calculated at t = 8 h as 0.68 mg g^{-1} h^{-1} and 1.49 mg g^{-1} h^{-1}, respectively. Thereafter the cell concentration continued to increase but the rhGH synthesis stopped, meaning that the consumed glucose was utilized for the cell growth. While the cells were in their exponential phase, rhGH production was favored compared to the rhGH secretion levels during the stationary phase.

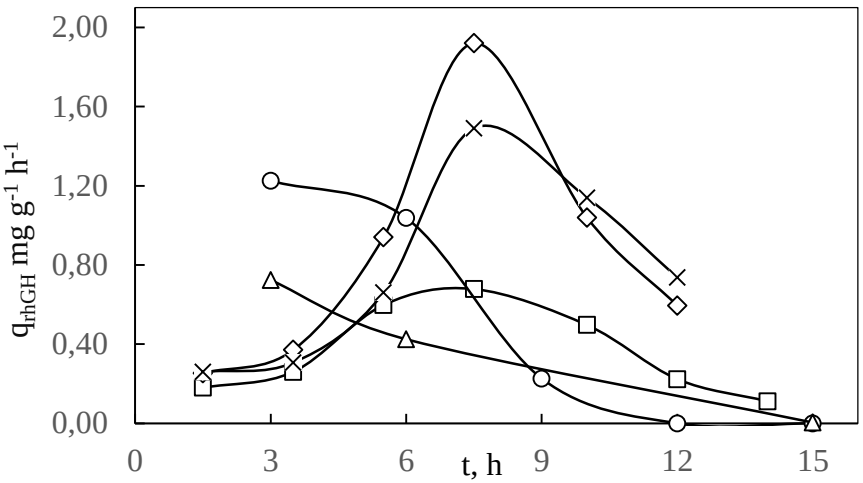


Figure 4.7. Variations in the specific rhGH, production rates with the cultivation time. G1 (○), G2 (Δ), GS1 (□), GS2 (◊), GS3 (×).

4.1.4 Yield coefficients

Along with the specific growth, consumption, and production rates, yield coefficients were calculated throughout the semi batch process for each designed feeding strategy. The respective values obtained for $Y_{X/S}$, $Y_{P/S}$, and $Y_{P/X}$ were presented in Table 4.2. Yield coefficient values were higher during the initial cultivation times and then decreased. The reason that the process was not able to maintain constant yield values was the fact that the cell growth did not follow a smooth exponential growth profile which was also observed when the specific growth rate values were presented in Figure 4.5. The highest cell yield values on substrate were calculated both for G1 and G2 as 0.5 g g^{-1} at t = 3 h, both for GS1 and GS3 as 0.5 g g^{-1} until t = 5.5 h, and for GS2 as 0.43 g g^{-1} until t = 7.5 h. The theoretical cell yield on glucose is 0.48 g g^{-1} (Cos *et al*. 2005) which shows that calculated values were reciprocal with the theoretical values for the initial cultivation times. Considering the overall yield coefficients for G2 and GS1 strategies maintained the highest $Y'_{X/S}$ values as 0.5 g g^{-1}. For G2 strategy it was observed that $Y_{P/X}$ values were relatively lower than G1 and the consecutive feeding strategies GS1, GS2 and GS3 showing that substrate (glucose) was mostly utilized for the cell growth. In GS1 strategy an overall high cell yield on substrate was obtained because the cell growth was high despite the fact that the lowest amount of glucose fed into the bioreactor with $\mu_o = 0.07$ h^{-1}.

On the other hand, the highest product yield on substrate was calculated for GS2 as 11.53 mg g^{-1} at t = 7.5 h, where the highest rhGH concentration was obtained at t = 8 h. For GS2, the overall $Y_{X/S}$ was 0.37 g g^{-1} which was lower than 0.5 g g^{-1} calculated for the other strategies showing that higher amount of glucose directed to the r-protein production. Furthermore, the overall $Y_{P/S}$ calculated for GS2 strategy was 4.79 mg g^{-1} which was 1.36-, 1.98-, 1.07-, and 1.77- higher than G1, G2, GS1, and GS3, respectively. Product yield on the cell was also calculated and the maximum value was obtained for GS2 strategy as 26.85 mg g^{-1} and at t = 7.5 h. The $Y_{P/X}$ values were the lowest for G2 where the rhGH production level was the lowest and the cell concentration was relatively high leading to a low product yield on the cell.

Table 4.2. Yield Coefficients for glucose feeding strategies

Strategy	**t** **h**	**$Y_{X/S}$** **g g^{-1}**	**$Y_{P/S}$** **mg g^{-1}**	**$Y_{P/X}$** **mg g^{-1}**
G1	3	0.50	6.52	11.37
	6	0.48	7.16	15.01
	9	0.40	1.34	3.34
	Overall	0.17	3.52	7.99
G2	3	0.50	2.77	5.54
	6	0.39	1.77	4.50
	Overall	0.50	2.42	4.79
GS1	5.5	0.50	5.15	10.30
	7.5	0.45	6.77	15.10
	Overall	0.50	4.47	8.98
GS2	5.5	0.43	6.46	15.11
	7.5	0.43	11.53	26.85
	13.5	0.20	1.80	8.83
	Overall	0.37	4.79	13.01
GS3	5.5	0.50	1.61	2.24
	7.5	0.26	6.09	12.14
	9.5	0.22	2.55	9.91
	Overall	0.17	2.70	15.97

By the introduction of 30 minute non-feeding intervals in GS1 and GS2 strategies, $Y_{X/S}$, $Y_{P/S}$, and $Y_{P/X}$ increased significantly leading to more effective feeding strategies. When compared with the G1 and GS2 strategies, where glucose was fed with $\mu_o = 0.10$ h^{-1} the overall $Y_{X/S}$ increased by 2.17-fold, $Y_{P/S}$ by 1.36-fold, and $Y_{P/X}$ enhanced by 1.62-fold.

4.1.5 Proteolytic Activity

In Figure 4.8 protease activities were represented for the time when maximum rhGH was obtained (t_{max}) and at the final hour of continuous feeding t_{final}**.** For this study, only acidic protease activity was measured since the pH values varied around 5.0. The protease activity increased gradually along with the cultivation time and reached its peak at the last hour of the continuous feeding. As presented in Figure 4.8, the highest proteolytic activity was detected in strategy GS3 at t = 16 h as 164 U mL^{-1} and the lowest activity was 37.34 U mL^{-1} obtained in GS2 at t_{max} = 8 h. For G1 semi-batch operation strategy, t_{max} = 9 h and t_{final} = 17 h, whereas in G2 t_{max} = 6 h and t_{final} = 15 h meaning that in G2 strategy a higher concentration of glucose utilized for higher protease production. The same behavior was observed for GS1, GS2 and GS3 strategies where the protease activity was higher for the higher predetermined μ values. However, for the consecutive feeding and starvation strategies, a lower proteolytic activity was measured compared to exponential feeding.

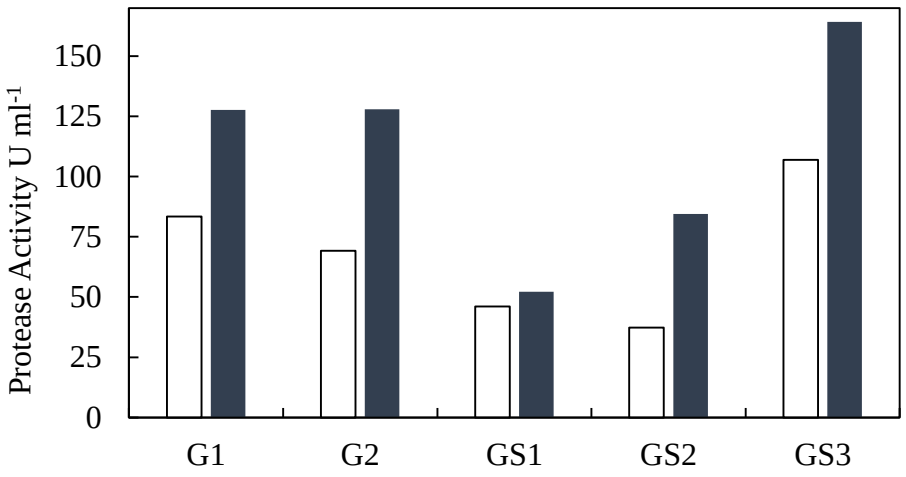
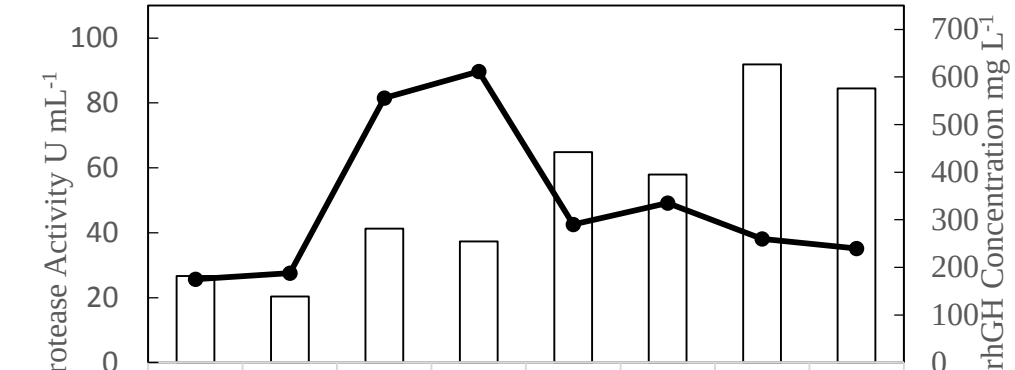


Figure 4.8. Variation in protease activity for each of the designed strategies G1, G2, GS1, GS2, and GS3 at t_{max} (□) and t_{final} (■).

In Figure 4.9 the variations in protease activity along with the cultivation time for GS2 semi-batch operation strategy was presented. By measuring the protease activity before and after the specific 0.5 h starvation intervals, the effect of these intervals were also investigated. It was observed that after each interval the proteolytic activity reduced by 10%. The reason for this phenomenon can be the decrease in the cell growth during these periods showing that the cells were forced to regulate their metabolic activity which reduces the protease secretion also. Furthermore, proteolytic activity is one of the main factors that affect the decrease of rhGH concentration in the medium. To illustrate the correlation between r-protein secretion and proteolytic activity, in Figure 4.9 rhGH concentrations also are presented. After t_{max} = 8 h, proteolytic activity was significantly higher by and diminished the positive effect that interruption periods had on r-protein secretion.



	3.5 h	4 h	7.5 h	8 h	11.5 h	12 h	15.5 h	16 h
U	27	20	41	37	65	58	92	85
rhGH	175	188	556	612	290	335	260	240

Figure 4.9. Variations in protease activity for GS2 strategy along the cultivation time

4.1.6 Organic Acid Concentrations

The chain of reactions that starts with the oxidation of the carbon source through the glycolytic pathway is followed by the tricarboxylic acid (TCA) cycle which is fully functional during the aerobic cultivation. The organic acids that are produced in the TCA cycle or through the glycolysis and pentose phosphate pathways need to be kept under control. A significant accumulation of these organic acids can result in a down-regulation of the metabolic pathways of *P. pastoris*. Determination of the concentration of the organic acids in the cultivation medium gives a better

understanding of the bioprocess performance, whether the metabolic pathways are active and the carbon source is used effectively to produce the required recombinant protein.

Variations in the organic acid concentrations measured for each strategy throughout the bioprocess were presented in Figures 4.10 – 4.14 and tabulated at Table 4.3. The generally detected organic acids in the semi-batch bioprocess operations where glucose was the sole carbon source were, gluconic-, malic-, pyruvic-, lactic-, acetic-, succinic- and fumaric acids.

In the biochemical reaction network of *P. pastoris*, gluconic acid is the product of gluconate oxidation which is formed by the oxidation of glucose. Gluconic acid is further used by the pentose phosphate pathway (PPP) when the dissolved oxygen in the cultivation medium is moderate or high. Therefore under hypoxic conditions, gluconic acid starts accumulating in the medium. On the other hand, when glucose concentration in the medium is very low, the amount of gluconic acid produced through oxidation of glucose is not sufficient to enter the pentose phosphate pathway and consequently starts accumulating. Gluconic acid was present in all the 5 semi-batch operation strategies where the highest concentration was detected in G1 and G2 as 3.5 g L^{-1} at the end of the cultivation. During the last hours of the cultivation, concentration of dissolved oxygen started to decrease and was difficult to keep constant at 15% (APPENDIX F) which created by a hypoxic condition. On the other hand, for GS1, GS2, and GS3 consecutive feeding strategies, accumulation of gluconic acid was a result of incomplete glucose oxidation since glucose was supplied in smaller amounts. It was observed that for GS3 the amount of gluconic acid was lower than GS1 and GS2 since glucose was fed into the bioreactor in higher amounts.

The pyruvate node is the place where glycolytic reactions are connected to TCA cycle enzymes. In presence of oxygen accompanied with a complete oxidation of carbon source, pyruvic acid is further involved in metabolic reactions of the TCA cycle. However, in the absence of oxygen, yeast minimizes the contribution to the TCA cycle and pyruvic acid starts to accumulate or being reduced to lactic acid or ethanol. In this study pyruvic acid was detected in the last hours of the cultivation 9 h < t < 17 h where it was accumulated up to 3 g L^{-1}. In GS1 and GS2 strategy pyruvic acid was hardly detected showing that pyruvic acid was further used in the TCA cycle.

Lactic acid in conditions of limited oxygen is produced from pyruvate where, *P. pastoris* shifts from aerobic to anaerobic respiration. Lactic acid concentration in the production medium was found in relatively low amounts showing that the metabolic fluxes of the yeast worked mostly aerobically and the TCA cycle functioned smoothly. The highest lactic acid concentration accumulated was up to 3.2 g L^{-1} in the last hour of cultivation t = 15.5– 16 h in GS2 feeding strategy showing that oxygen was not sufficient for the production medium which was accompanied with the cells entering the stationary phase (APPENDIX F).

Acetic acid and maleic acid were detected in the medium in very small amounts. The other organic acids detected were malic-, succinic- and fumaric acids which are part of the TCA cycle metabolites. Malic acid was the organic acid involved in TCA cycle that was accumulated mostly in the cultivation medium in the exponential feeding strategies G1 and G2 where the TCA cycle fluxes were down-regulated by high fluxes of glucose mostly in the last hours of the continuous feeding. On the other hand, in consecutive strategies GS1, GS2, and GS3, organic acids produced in the TCA cycle were not accumulated in the bioreactor, showing that Krebs cycle worked efficiently accompanied with the energy generation for the yeast's metabolic needs.

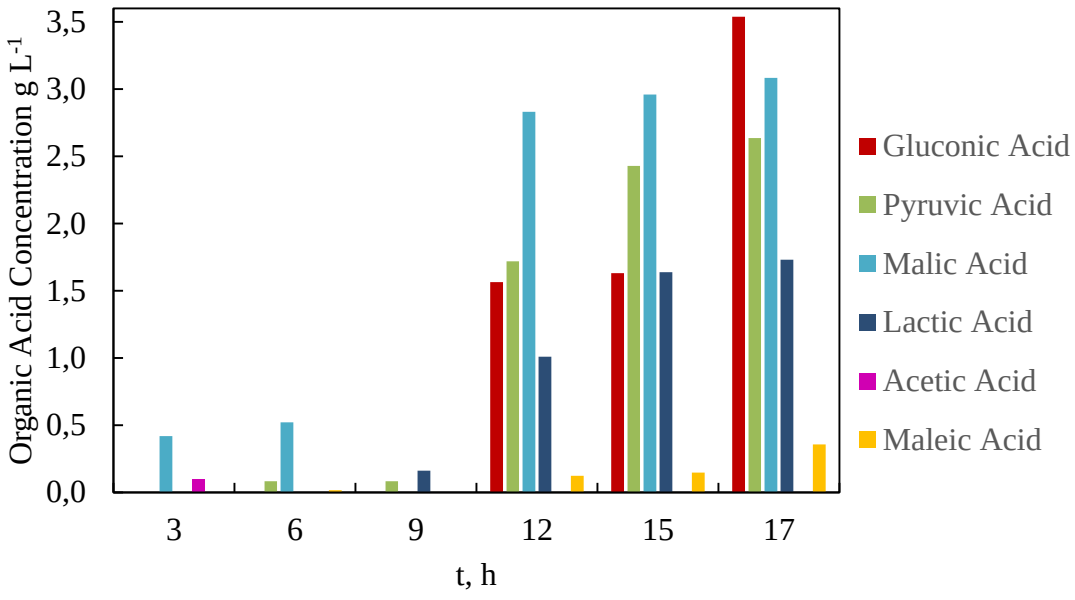


Figure 4.10. Variations in organic acid concentrations with the cultivation time for G1 strategy

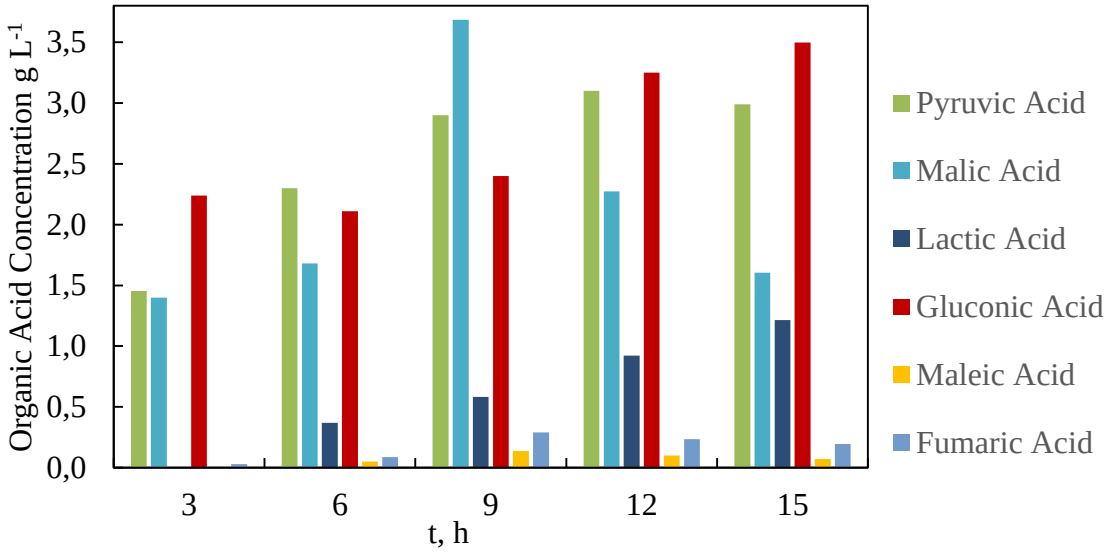


Figure 4.11. Variations in organic acid concentrations with the cultivation time for G2 strategy

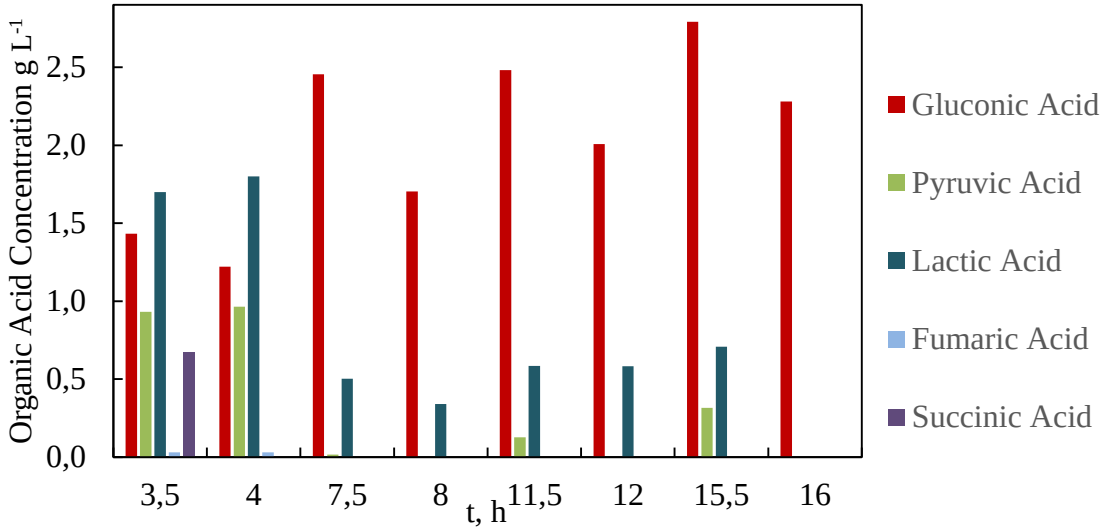


Figure 4.12. Variations in organic acid concentrations with the cultivation time for GS1 strategy

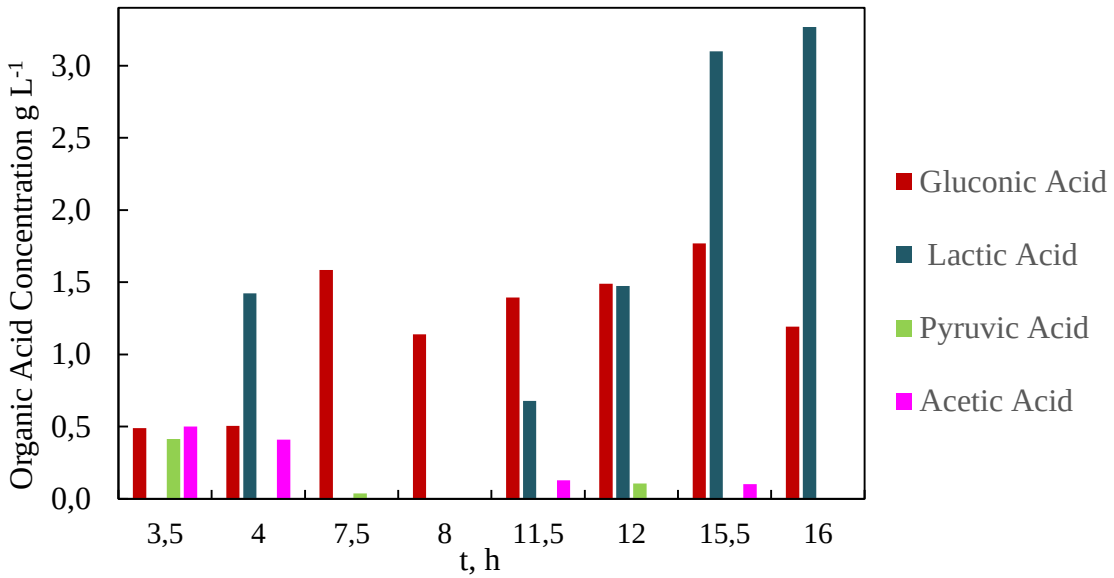


Figure 4.13. Variations in organic acid concentrations with the cultivation time for GS2 strategy

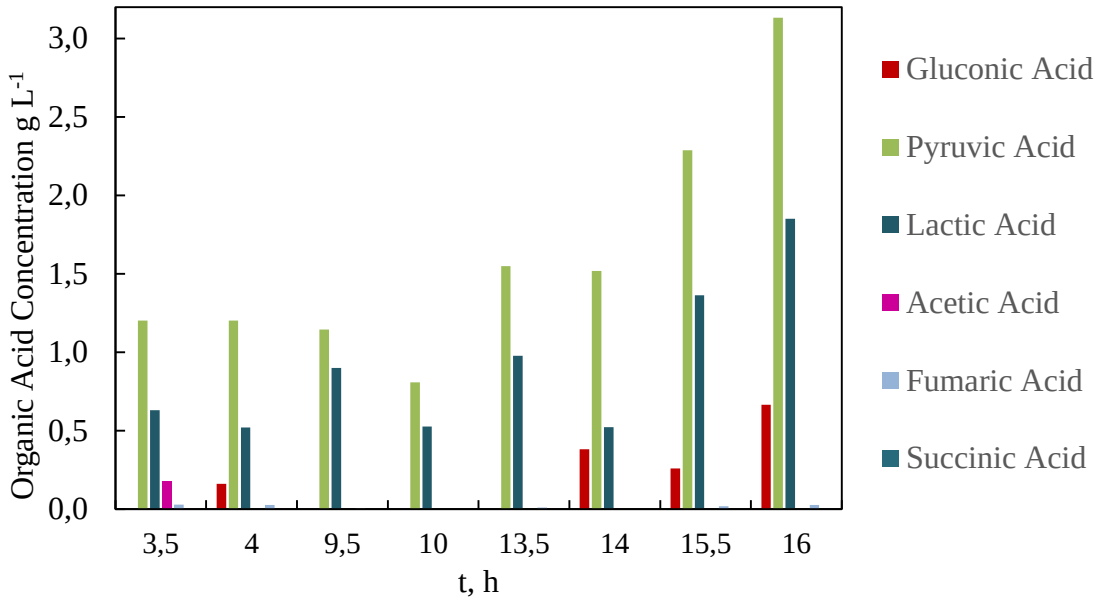


Figure 4.14. Variations in organic acid concentrations with the cultivation time for GS3 strategy

Table 4.3 Variations in the total excreted organic acid concentrations with the cultivation time

Semi-batch	**C_{OA}, g L^{-1}**					
operation	**3 h**	**6 h**	**9 h**	**12 h**	**15 h**	**17 h**
G1	0.52	0.62	1.24	7.24	8.81	11.35
G2	6.72	8.51	12.30	12.38	12.5	-
	3.5 h	**4 h**	**7.5 h**	**8 h**	**15.5**	**16 h**
GS1	4.76	4.01	2.97	2.04	3.81	2.27
GS2	9.31	2.33	1.62	1.14	1.86	1.19
	3.5 h	**4 h**	**9.5 h**	**10 h**	**15.5**	**16 h**
GS3	2.03	1.91	2.05	1.33	3.93	7.76

4.2 Exponential Feeding strategies with Molasses as the carbon source

In M1 and M2 semi-batch operation strategies, molasses was introduced into the bioreactor with an exponential profile and the volumetric flowrates of glucose and fructose solution were presented in Figure 4.15.

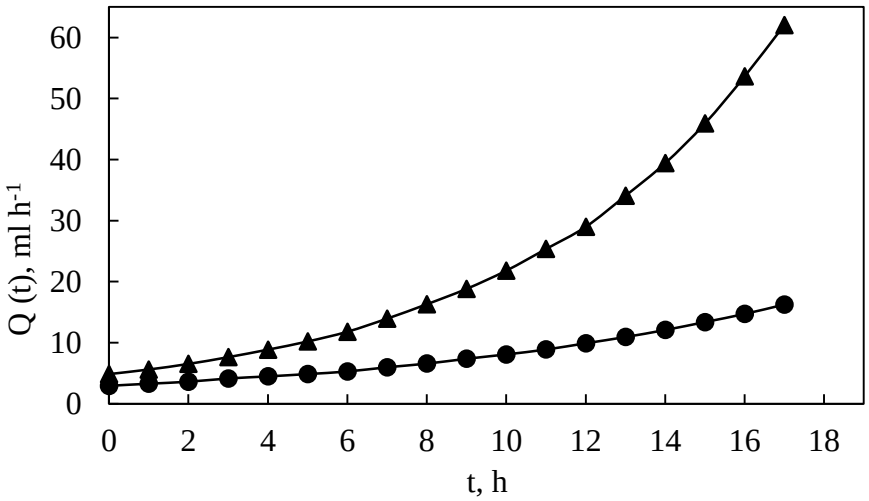


Figure 4.15. Exponential feeding profiles of glucose:fructose mixture in M1 (●), M2 (▲)

The volumetric flow rate Q (t) of glucose and fructose in molasses solution in MS1, MS2, and MS3 strategies was presented in Figure 4.16.

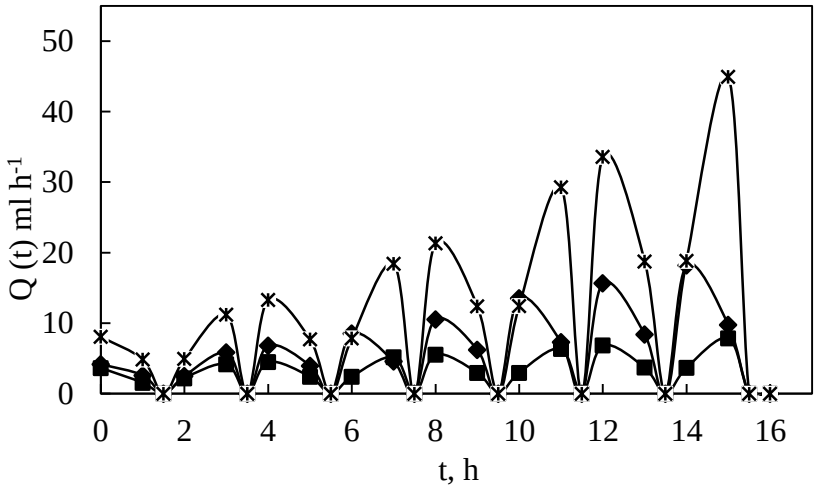


Figure 4.16. Consecutive feeding strategy profiles of glucose and fructose in MS1 (■), MS2 (♦), and MS3 (×)

4.2.1 Glucose Concentrations and Specific Glucose Consumption Rates

Every sample taken from the cultivation medium was analyzed and there was no glucose detected throughout the whole process. The concentration of glucose in the stock solution of molasses composition fed into the bioreactor was 110 g L^{-1} representing 50% of the carbon sources. Since yeasts including *P. pastoris*, have higher affinity for glucose than fructose it was expected for glucose to be consumed faster.

Variations in the specific glucose consumption rates (q_G) with the cultivation time were presented in Figure 4.17. Among the molasses semi-batch operation strategies, the highest glucose feed flow rate was supplied into the bioreactor production medium in M2 strategy. The consumption of the carbon sources for this strategy resulted in lower cell concentrations C_{Xmax} = 67 g L^{-1} showing that the consumption of glucose did not influence the cell growth compared to M1 strategy, where C_{Xmax} was obtained as 75 g L^{-1} even though the specific consumption rate of glucose q_G = 0.07 g g^{-1} h^{-1} was 2-fold lower than the average glucose consumption rate of M2 strategy. A reason for lower cell densities in M2 strategy may be the negative effect on the cell of the

accumulation of fructose in the medium which will be further discussed later in the next page. The highest q_G value was calculated as 0.24 g g^{-1} h^{-1} in M2 strategy. For the consecutive feeding strategies q_G values were relatively lower since less glucose was supplied into the bioreactor.

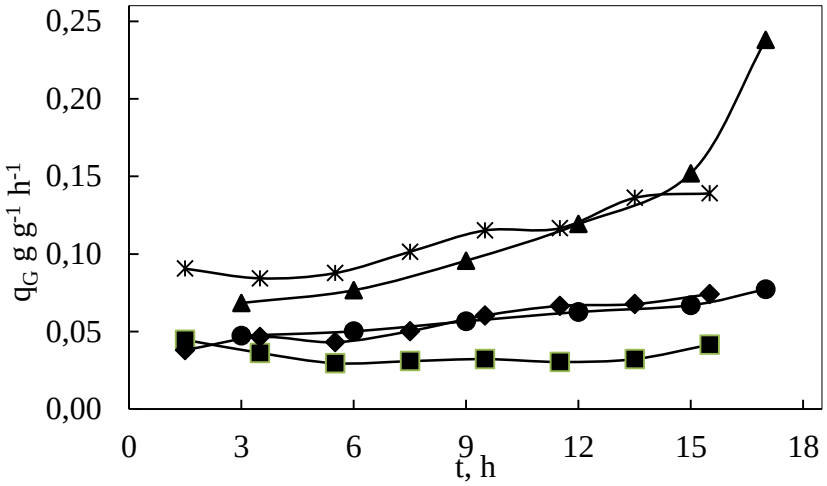


Figure 4.17. Variations in specific substrate (glucose) consumption rates with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×).

4.2.2 Fructose Concentrations and Specific Glucose Consumption Rates

While glucose was entirely consumed during the process in each strategy, fructose accumulation in the medium increased along with the cultivation. Both carbon sources glucose and fructose compete for the same membrane transporters where fructose was utilized with a lower uptake rate. A study carried out by D'Amore *et al.* (1988) concluded that when the two carbon sources glucose and fructose are fermented parallel in equal amounts, glucose utilization rate is about twice higher. The variations in fructose concentration along the cultivation times were presented in Figure 4.18. For strategy M1 and M2 where molasses was fed into the bioractor exponentially with μ_o = 0.07 h^{-1} and μ_o = 0.10 h^{-1} respectively, fructose concentration increased slightly within 0 < t < 12 h and then within 12 h < t < 17 h fructose accumulations were more significant.

To reduce the fructose accumulation, 1.5 hours of exponential feeding were followed by a batchwise 30-minute break to feeding where no substrate was introduced into the bioreactor, for strategies MS1, MS2, and MS3. Such modification of the feeding profile encouraged fructose consumption at MS1 and MS2.

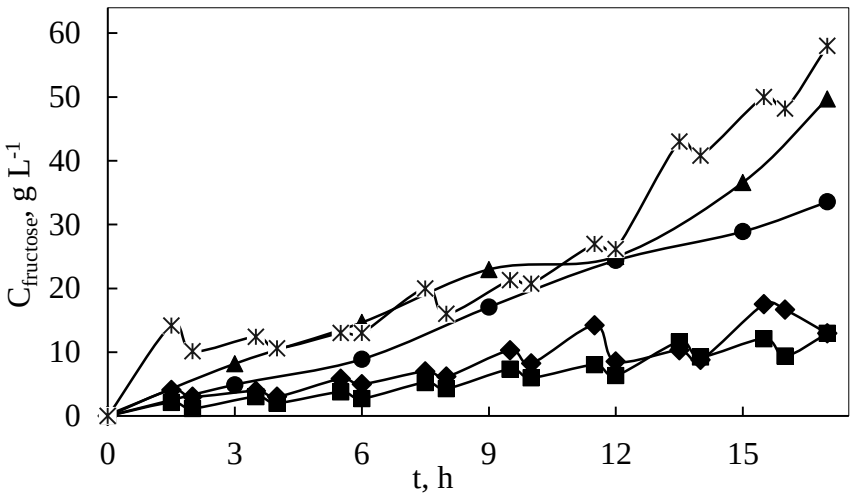


Figure 4.18. Variations in fructose concentration with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×).

As it was presented in Figure 4.18, in the end of each 30-minute interval, where the bioreactor was operated batchwise, the fructose concentration decreased considerably up to 2-fold. However, for MS3 where $\mu_o = 0.15\ h^{-1}$ there was no significant impact on fructose consumption. In MS3 semi-batch operation strategy, at t = 17 h almost all the fructose added to the cultivation medium was not utilized by the cells. A reason for that was the fact that the cells had already entered the stationary phase and might have slowed their metabolic activities. In table 4.4 fructose consumption ratios were tabulated for $t_{max,}$ where maximum rhGH concentrations were obtained, under each fed-batch operation.

Table 4.4 Fructose consumption at cultivation's t_{max}

Fed-batch Operation	**t_{max}, h**	**Total fructose fed until t_{max}, g**	**Total fructose consumed at $t=t_{max}$, g**	**Fructose consumption ratio %**
M1	17	82	60	73
M2	15	169	110	65
MS1	11.5	34	25	73
MS2	13.5	53	41	77
MS3	13.5	149	100	67

Variations in the fructose specific consumption rate for the investigated strategies were presented in Figure 4.19. It was observed that for M1 and M2 strategies the q_F values increased with the cultivation hours. However, the specific consumption rate of fructose was approximately 2-fold lower than that of glucose as expected. In M1 strategy the highest q_G calculated was 0.08 g g^{-1} h^{-1} whereas q_F was 0.049 g g^{-1} h^{-1} at t = 15 h and in M2 strategy the highest q_G was 0.24 g g^{-1} h^{-1} and the highest q_F was 0.11g g^{-1} h^{-1} at 15 h.

On the other hand, in the consecutive feeding strategies MS1, MS2, and MS3, the fructose consumption rate increased significantly compared to the glucose consumption rate. In MS1 strategy, the highest calculated q_G was 0.042 g g^{-1} h^{-1} and the reciprocal calculated q_F was 0.059 g g^{-1} h^{-1}, which was even higher than the glucose consumption rate showing that at low specific growth rates such as μ_o = 0.07 h^{-1} glucose was utilized in a shorter period and was not sufficient for the cell growth and r-protein production so the fructose consumption rate increased. In MS2 operation strategy the highest calculated q_G was 0.074 g g^{-1} h^{-1} at t = 15.5 h when q_F calculated value was 0.061 g g^{-1} h^{-1} only 1.21-fold lower than q_G. However, in MS3, fructose was consumed with a considerably slower rate compared to glucose showing that the 30 minutes no-feeding intervals were not very effective for feeding with high predetermined specific growth rates such as μ_o = 0.15 h^{-1}.

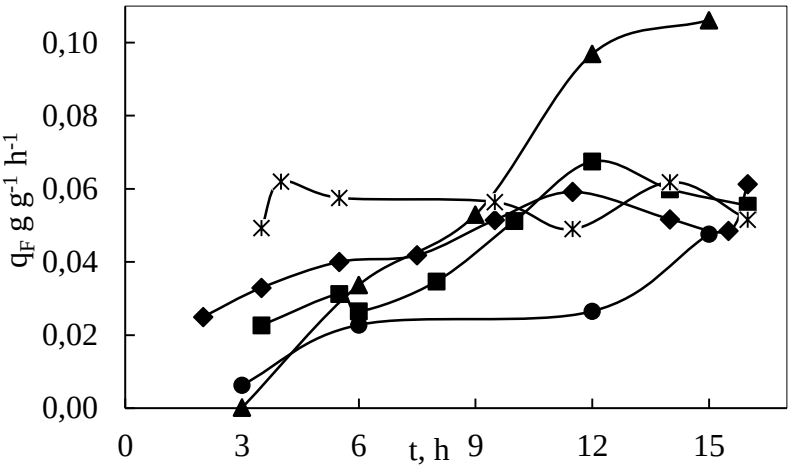


Figure 4.19. Variations in the fructose specific consumption rates with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×).

4.2.3 Cell Concentration and Specific Cell Growth Rate

The variations in the cell concentration variations along with the cultivation time for the designed semi-batch bioreactor operation strategies were presented in Figure 4.20. For M1 and M2, the highest cell concentrations were obtained as 74.8 g L^{-1} at t = 15 h and 67.5 g L^{-1} at t = 12 h respectively. When the exponential feeding strategy was proceeded, with batch-operations so called starvation intervals, the highest cell concentrations obtained as 91 g L^{-1} at t = 12 h , 91 g L^{-1} at t =14 h, and 76.5 g L^{-1} at t = 12 h in MS1, MS2, and MS3, respectively. By using the feeding + starvation strategy at MS1 and MS2 strategies the cell concentration obtained was 1.2-fold and 1.35-fold higher than M1 and M2, respectively showing that the introduced non-feeding batch-wise intervals assisted in regulation in the central carbon metabolism of *P. pastoris* which also lead to higher cell concentrations.

In M1 strategy the experimental specific growth rate was successfully achieved as the predetermined μ_o = 0.07 h^{-1} during the whole process; whereas at M2 experimental μ value was similar to that of predetermined value μ_o = 0.10 h^{-1} until t = 6 h, then it decreased till to 1.2-fold. This may be due to the specific substrate consumption rates which are higher at M2 strategy since the cell concentration reached lower values than the expected while the substrate was consumed in relatively high amounts.

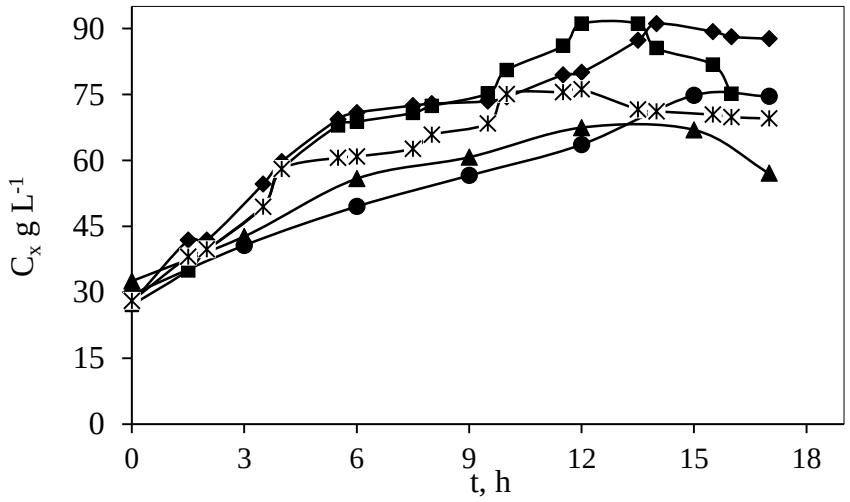


Figure 4.20. Variation in the cell concentration with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×)

For strategies MS1, MS2 and MS3 because of the 30 minutes intervals after each 1.5 h of the exponential feeding, the determined specific growth rate was lower than the predetermined μ_o, by diverging from the exponential growth profile.

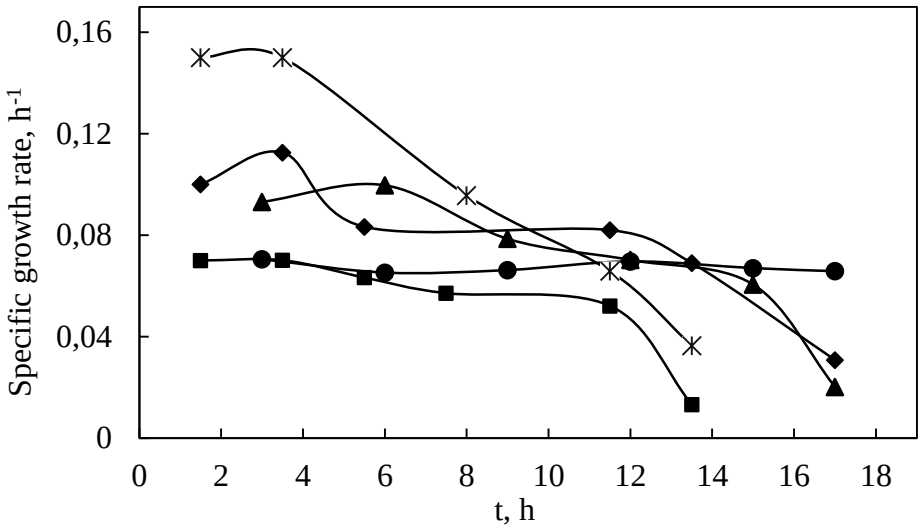


Figure 4.21. Variations in the specific growth rate with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×)

4.2.4 Recombinant hGH Concentrations and Specific production rates

The variations in rhGH concentrations with the cultivation time in semi-batch bioreactor operation strategies designed for molasses feeding were presented in Figure 4.22. For exponential feeding strategies M1 and M2, the highest rhGH obtained was 650 mg L^{-1} at t = 17 h and 544 mg L^{-1} at 15 h, respectively. The maximum rhGH concentration at M1 was 1.45-fold higher than G1 where glucose was fed exponentially based on predetermined μ_o = 0.1 h^{-1} showing that molasses was a more effective carbon source and its utilization was selected more in the rhGH production than the cell growth. Whereas, for the modified feeding strategies, the highest rhGH concentrations obtained were 487 mg L^{-1} at t= 11.5 h at MS1, 625 mg L^{-1} at t = 13.5 h at MS2 and 348 mg L^{-1} at t = 13.5 h at MS3 as presented in Table 4.5. M1 and MS1 were compared where the same specific growth rate μ_o = 0.07 h^{-1} was used for the design of continuous feed stream resulted in higher rhGH concentration. Therefore I conclude that at MS1 strategy, the cells were forced to consume only fructose for a

long cultivation period where the carbon source was not sufficient to provide rhGH production since the continuous feed flow rate was too low with the calculated Q (t) based on the predetermined $\mu_o = 0.07\ h^{-1}$. Also, at M1 it can be stated that accumulation of fructose up to 25 g L^{-1} did not inhibit the rhGH production; furthermore, the limited uptake rate of the second sugar fructose avoided an overflow in the metabolism.

Table 4.5. Characteristics and performances of molasses designed feeding strategies

Semi-Batch Operation	**Overall values at t_{max}**				
	t_{max}, h	Cell concentration, g L^{-1}	rhGH concentration, mg L^{-1}	Volume, L	Total sucrose consumed, g
M1	17	75	650	1.40	142
M2	15	67	544	1.66	279
MS1	11.5	79	487	0.89	59
MS2	13.5	87	625	1.03	94
MS3	13.5	72	348	1.33	249

On the other hand, MS2 compared to M2 lead to 1.2-fold higher rhGH concentration even though less molasses was introduced into the bioreactor by increasing the product yield on substrate. Fructose accumulation in M2 reached its maximum at t = 17 h as 49 g L^{-1}, whereas in MS2 the fructose consumption rate increased with the assistance of the 30 minute interruptions leading to a small amount of fructose accumulation up to 10 g L^{-1}. In MS2 the substrate was used more effectively and there were no excessive amounts of substrate to suppress the cell metabolism.

Under the pressure of glucose starvation, the cells were driven to consume fructose and the intracellular reaction network of *P. pastoris* was directed toward the synthesis of rhGH. In MS3 the same strategy was executed with $\mu_o = 0.15\ h^{-1}$; however, introducing 30 minutes of starvation periods to increase the fructose consumption did not have any significant effect and the rhGH concentration obtained was lower than the other strategies. At molasses feeding strategies, the highest r-protein concentrations

were obtained at relatively highest cell concentrations, showing that the GAP expression and r-protein secretion increased proportionally with the cell growth. Such behavior was expected since GAP is a constitutive promoter.

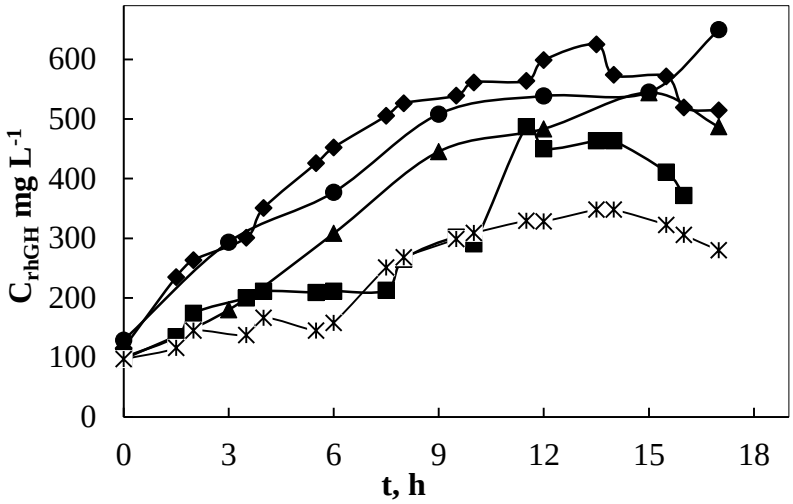


Figure 4.22. Variations in rhGH concentration with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×).

The reciprocal cell concentrations measured for the maximum rhGH, were 74.6 g L^{-1}, 67 g L^{-1}, 86 g L^{-1}, 87.3 g L^{-1} and 76.1 g L^{-1} respectively for M1, M2, MS1, MS2, and MS3. During exponential feeding increasing μ_o from 0.07 h^{-1} to 0.10 h^{-1} negatively affected the production rate of rhGH, however consecutive feeding + starvation positively influenced the production rate. However in MS3, although starvation period was introduced, the least rhGH was produced. This can be due to accumulation of feeding contents of molasses, including fructose which could be inhibitory after a critical value.

The variations in rhGH specific production rates, with the cultivation time in semi-batch bioreactor operation strategies designed for molasses feeding were presented in Figure 4.23. Productivity profile of rhGH for each applied strategy followed a declining slope where q_{rhGH} values were higher at the inital hours of the cultivation and then decreased gradually because the cell growth rate was greater than the rhGH production rate. The highest q_{rhGH} value was obtained at MS2 strategy as 1.99 mg g^{-1} h^{-1} in the first hours of the cultivation and then gradually decreased. A similar pattern was observed at M1, MS1, and MS3 where the highest q_{rhGH} were calculated as 1.47

mg g^{-1} h^{-1} at t =3 h in M1, 0.72 mg g^{-1} h^{-1} at t = 1.5 h in MS1, and 0.43 mg g^{-1} h^{-1} at t = 1.5 h in MS3. On the other hand, in strategy M2 where molasses was fed exponentially based on μ_o = 0.10 h^{-1}, the productivity values were close to each other throughout the process representing a stable productivity profile. The reason for this behavior may be the fact that the cell concentration profile for M2 was the lowest among the strategies and the cell growth did not follow the time course of the predetermined exponential growth.

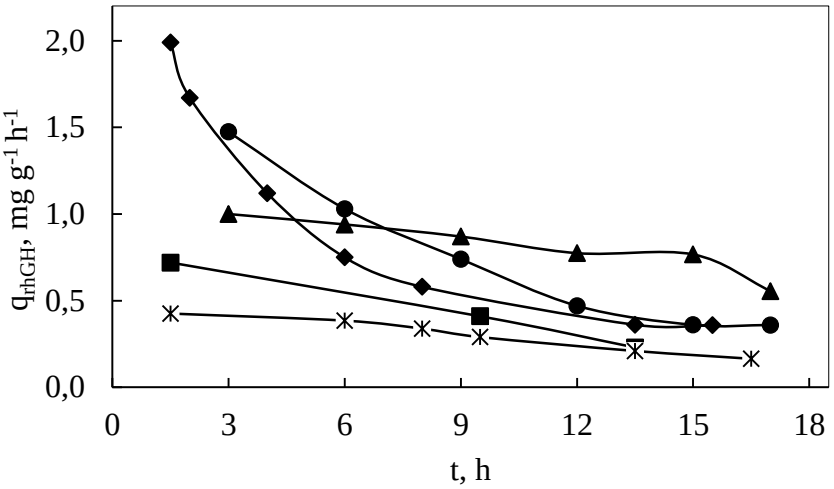


Figure 4.23 Variations in specific production rates with the cultivation time M1 (●), M2 (▲), MS1 (■), MS2 (♦), and MS3 (×).

4.2.5 Yield Coefficients

Variations in yield coefficients throughout the process and the respective overall yield coefficients of each strategy were presented in Table 4.6. The maximum cell yields on substrate were obtained at M1 and MS2 as $Y_{X/S}$ = 0.5 g g^{-1} at the beginning of the process until t = 9 h and t = 7.5 h respectively. In M1 and MS1 the cell yields on substrate approached high, constant values during the entire cultivation. However, in strategies M2, MS2 and MS3 the $Y_{X/S}$ values declined gradually along the process where the MS2 strategy enhanced the cell yield on substrate by 1.4-fold compared to M2.

Even though MS1 had the highest cell yield coefficients throughout the bioprocess, the product yield on substrate values were the lowest among the designed strategies. Compared to M1 strategy, carbon source in the medium was not sufficient for both

cell growth and r-protein production considering the fact that μ_o = 0.07 h^{-1} was accompanied further with non-feeding intervals that emphasized the carbon insufficiency. On the other hand, the product yield on substrate at MS2 was 6-fold higher than M2 concluding that substrate was used more efficiently when 30 minutes starvation periods with non-feeding were added to the regular exponential feeding. The highest $Y_{P/S}$ was obtained at MS2 as 20.84 mg g^{-1} at t = 12 h.

Table 4.6. Yield Coefficients for molasses feeding strategies

Strategy	**t, h**	**$Y_{X/S}$ g g^{-1}**	**$Y_{P/S}$ mg g^{-1}**	**$Y_{P/X}$ mg g-1**
M1	9	0.50	12.03	24.26
	12	0.46	5.51	11.95
	15	0.44	2.87	6.58
	Overall	0.434	10.70	24.6
M2	3	0.36	4.22	11.73
	6	0.23	3.69	16.19
	9	0.06	4.04	65.87
	12	0.10	2.91	29.55
	Overall	0.12	4.23	35.27
MS1	7.5	0.44	1.73	3.96
	12	0.46	-	-
	Overall	0.49	8.26	16.69
MS2	7.5	0.50	7.80	15.1
	12	0.36	20.84	58.51
	17	0.12	1.24	10.24
	Overall	0.29	6.25	21.22
MS3	2	0.42	7.22	17.22
	7.5	0.16	10.19	65.22
	9.5	0.15	3.54	24.32
	Overall	0.19	3.09	16.01

The highest $Y_{P/X}$ value was calculated for M2 strategy as 65.87 mg g^{-1} at t = 9 h, which was 1.12-fold higher than $Y_{P/X}$ in MS2. The product concentration in MS2 was higher than in M2, however the cell concentration obtained to reach the obtained r-protein production levels was higher.

4.2.6 Proteolytic Activity

Protease activities were presented in Figure 4.24 for two respective cultivation hours when the highest rhGH was reached symbolized as t_{max} and the final hour of the cultivation as t_{final}. Protease activity increased gradually with cultivation the time. Furthermore, it was observed that proteolytic activity was higher for the cultivations with higher predetermined specific growth rate values. At strategy MS3 where μ_o = 0.07 h^{-1} at t=16 h the highest protease activity was measured as 2400 U mL^{-1}.

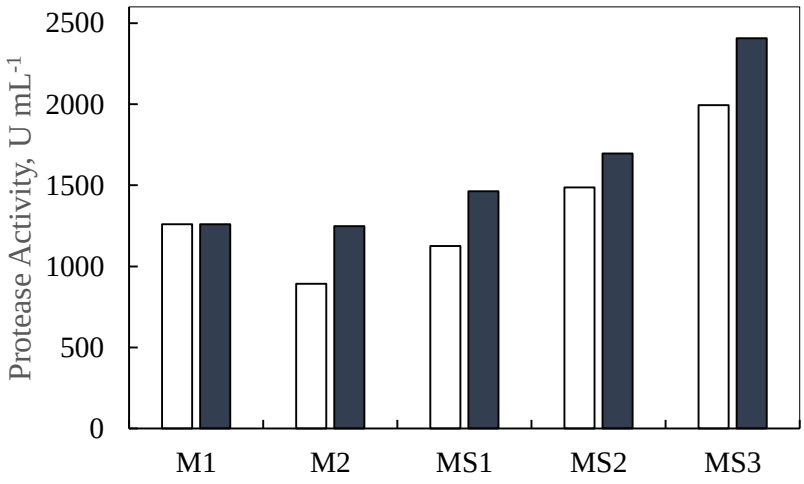


Figure 4.24. Variation in protease activity for each of the designed strategies M1, M2, MS1, MS2, and MS3 at t_{max} (□) and t_{final} (■).

4.2.7 Organic Acid Concentrations

As mentioned above, accumulation of organic acid in the production medium in small or low quantities is an important detector of yeast's metabolic pathways' performance. For molasses as the carbon source in the semi-batch operation strategies, concentration of the detected organic acids were shown in Figure 4.25 – 4.29. Gluconic acid was detected in M1 and M2 strategies up to a concentration of 2.45 g L^{-1} in the end of

continuous feeding: 12 h < t < 17 h. There was no trace of gluconic acid found in the MS1, MS2, and MS3 strategies showing that carbon source entered mostly glycolytic pathway instead of being oxidized to produce gluconic acid. The cell concentrations in M1 and M2 strategies were lower than in MS1, MS2, and MS3 feeding strategies, which may be related with carbon source, not used entirely for energy generation through the glycolytic pathway and the TCA cycle.

Pyruvic acid was detected in very small amounts up to 0.5 g L^{-1}. In MS1 feeding strategy, it was not detected in the production medium throughout the process showing that there was no accumulation of the pyruvic acid in pyruvate node and there was no barrier for the metabolic fluxes that cross through this node.

Malic acid is one of the organic acids produced and converted in the TCA cycle. Its presence in the production medium shows that malic acid was not able to be converted entirely to oxaloacetic acid. This affects the performance of the cycle by generating less energy than required and it can be a consequence of dissolved oxygen deficiency.

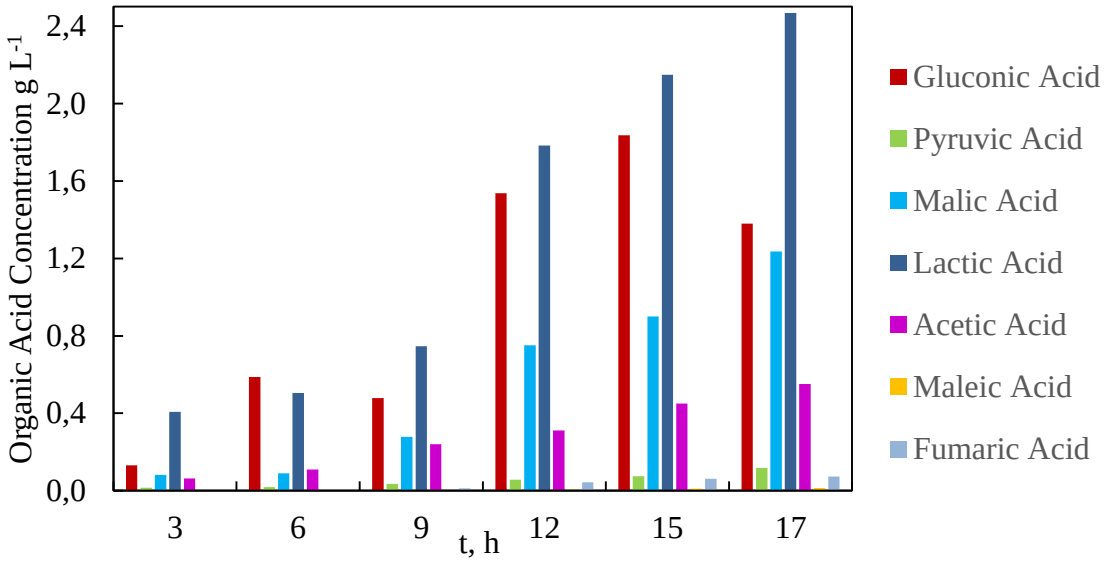


Figure 4.25. Variations in organic acid concentrations with the cultivation time for M1 strategy

The highest malic acid concentration accumulated in the production medium was measured as 3.43 g L^{-1} at t = 17 h at M2 strategy. Lactic acid was also present in the cultivation medium in significant amounts that increased with the cultivation time. In MS2 strategy the lowest lactic acid concentrations were measured showing that there

was no consequential oxygen deficiency (APPENDIX F) where C_{DO} was easier to be kept constant at 15%. Lactic acid accumulation furthermore inhibits the cell growth and in MS2 strategy where the lowest lactic acid concentration was detected, the highest cell concentration was measured at t = 14 h.

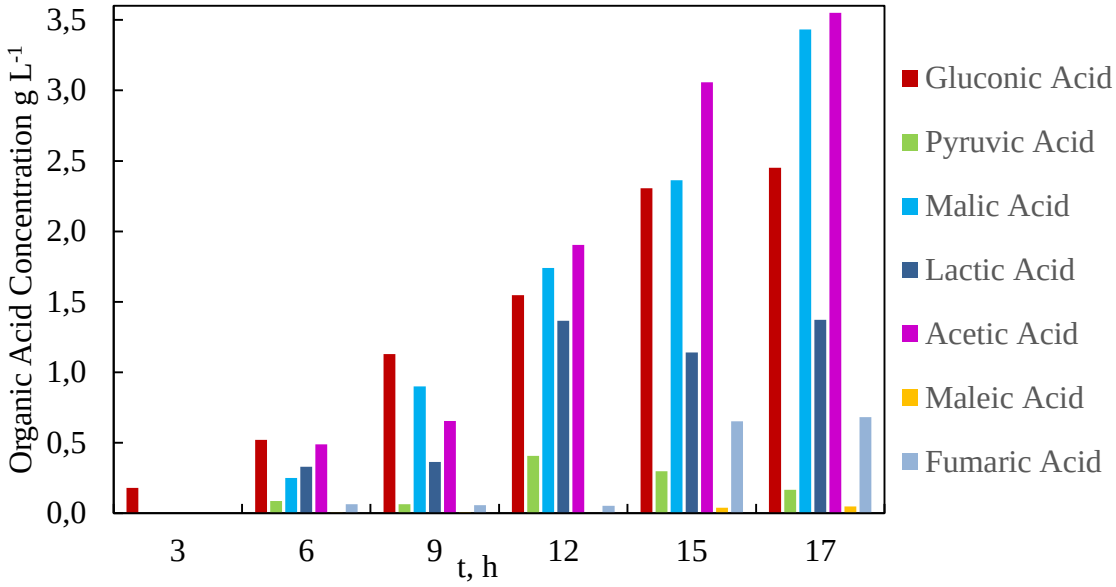


Figure 4.26. Variations in organic acid concentrations with the cultivation time for M2 strategy

In M2 strategy the highest acetic acid concentration was measured as 3.55 g L^{-1}. Acetic acid was always present in the cultivation medium in small quantities usually with an increasing concentration along the operation hours. Maleic acid and fumaric acid were barely detected in the cultivation media. The TCA cycle converts acetyl coenzyme A into citric acid which makes it the first organic acid of the cycle and for each full cycle of a citric acid molecule 38 molecules of ATP are generated (Lehninger, 1999). Citric acid was not detected in the cultivation media except in MS1 strategy where its concentration reached its maximum as 3.1 g L^{-1} at the final hour of the process. A reason for this considerable accumulation was the small amount of the carbon source that enters the glycolytic pathway resulting in low metabolic fluxes. Such accumulation affects negatively the TCA cycle to function smoothly and produced the necessary energy.

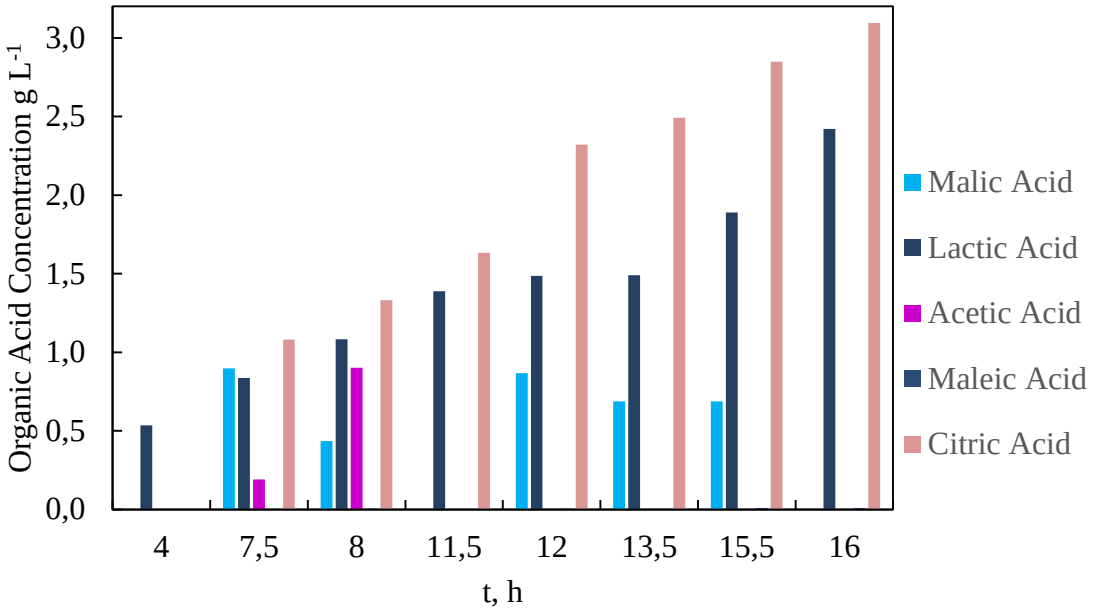


Figure 4.27 Variations in organic acid concentrations with the cultivation time for MS1 strategy

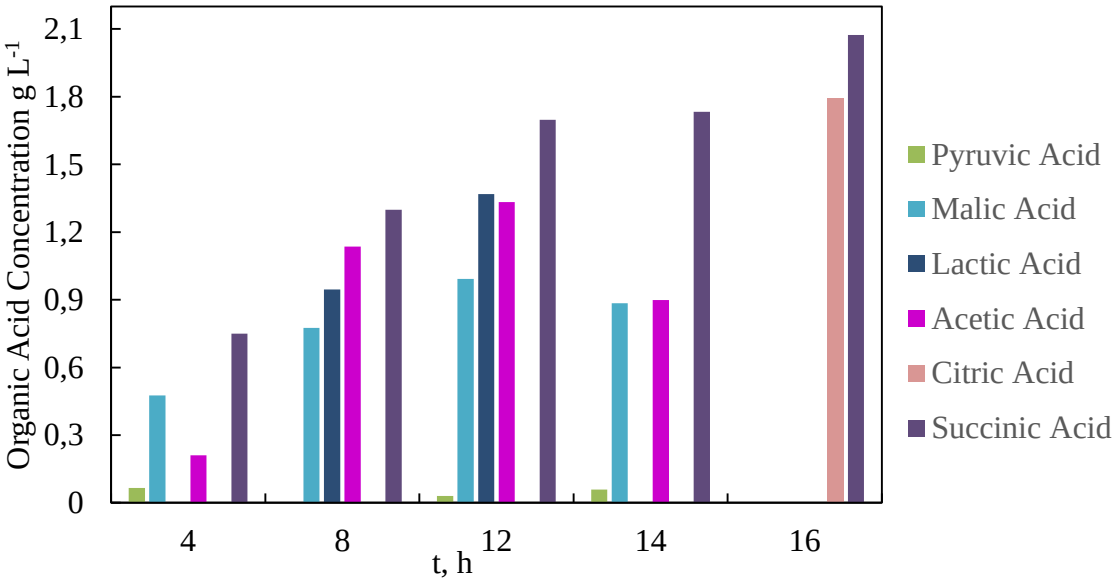


Figure 4.28. Variations in organic acid concentrations with the cultivation time for MS2 strategy

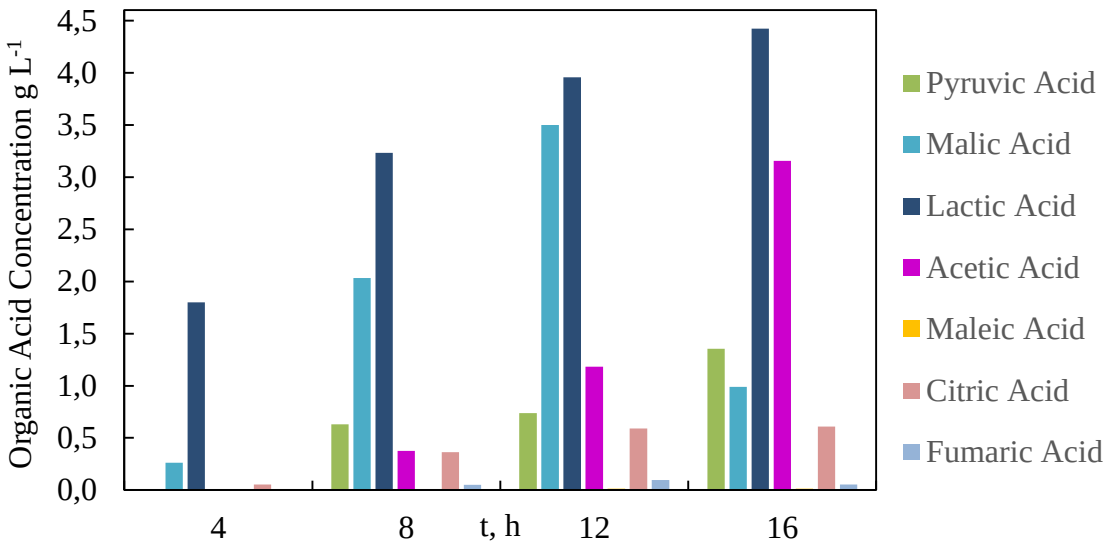


Figure 4.29. Variations in organic acid concentrations with the cultivation time for MS3 strategy

Table 4.7 Variations in the total excreted organic acid concentrations with the cultivation time

Semi-batch	**C_{OA}, g L^{-1}**					
operation	**3 h**	**6 h**	**9 h**	**12 h**	**15 h**	**17 h**
M1	0.70	1.31	1.79	4.49	5.48	6.26
M2	0.18	1.74	3.18	7.01	9.85	11.7
	3.5 h	**4 h**	**7.5 h**	**8 h**	**15.5 h**	**16 h**
MS1	0	0.53	3.00	3.75	5.43	5.52
	4 h	**8h**	**12 h**	**14 h**	**16 h**	
MS2	1.5	4.15	6.73	4.29	5.01	
MS3	2.12	6.69	10.07	10.1	10.599	

4.3 Comparison of glucose and molasses as carbon source in the designed semi-batch operation processes strategies

To increase the r-protein productivity in *P. pastoris*, the effects of two carbon sources were investigated. Glucose and molasses were selected as potential effective carbon sources to boost the performance of the central carbon pathways of *P. pastoris*. While glucose is determined as the most effective carbon source under GAP expression for semi-batch phase, pretreated beet molasses composed of two carbon sources has positively influenced in the yeast's metabolism. Furthermore, an advantageous aspect of molasses is its availability in considerable quantities under a very favorable cost representing a reasonable economical approach for low-cost production of rhGH.

The cell growth responded better in the presence of glucose making it more effective as a carbon source approaching 1.4- fold higher cell concentration compared to the dual carbon sources of molasses: fructose and glucose. For both carbon sources the highest cell concentration was obtained in the feeding strategy where 0.5 h non-feeding batchwise starvation intervals were proceeded after a continuous exponential feeding calculated with μ_o = 0.10 h^{-1}. The cell yield on substrate was higher for the glucose feeding strategies. In GS2, the overall cell yield on glucose was 1.24-fold higher than the cell yield on glucose and fructose mixture in MS2. The lower cell yields obtained in molasses feeding strategies show that fructose was not as effective as glucose for the cell growth. Since carbon sources are utilized in a certain selective order, glucose having a greater uptake rate was utilized first and then fructose. However, fructose was never consumed entirely but it was accumulated in the medium showing that cells are not able to use fructose with a high uptake rate.

For r-protein secretion driven by constitutive GAP promoter, high cell densities are desirable since recombinant protein is synthesized in the cell which acts as semibatch microbioreactor. Furthermore, it was observed that even though glucose feeding strategies provided higher cell growth, their rhGH productivity was lower than that approached by molasses feeding strategies. In G1 strategy the overall product yield on substrate was 1.2-fold lower than in M2 strategy which used the same exponential feeding profile with μ_o = 0.10 h^{-1}. Additionally, in MS1 and MS2 strategies the rhGH

yield on substrate was 1.8-fold and 1.3-fold higher than GS1 and GS2 strategies respectively. By comparing the product and cell yields on substrate for the two carbon sources it was observed that molasses utilization was more selective for the rhGH synthesis than the cell growth.

Since molasses medium is rich in vitamins and essential minerals, influenced the cell metabolism and glucose and fructose was not used mostly for the cell growth but it was utilized in the recombinant protein production. Additionally the maximum concentration of rhGH was obtained by molasses feeding with a value of 650 mg L^{-1} in M1 strategy where molasses was introduced to the process with μ_o = 0.07 h^{-1}. Exponential feeding strategies G1 and M2 with μ_o = 0.10 h^{-1} both resulted in low rhGH concentrations, of 445 mg L^{-1} and 544 mg L^{-1}, respectively; where molasses feeding resulted in 1.22-fold higher r-protein secretion. Low rhGH concentration can be a consequential result of high glucose and glucose and fructose fluxes in the metabolic pathways of *P. pastoris* which can impede the reaction steps of the glycolysis or TCA cycle.

When considering the consecutive exponential feeding strategies with starvation intervals it was observed that MS1 and MS2 strategies resulted in 1.27- fold and 1.023-fold higher rhGH concentration whereas MS3 strategy was 1.71-fold less successful than GS3 strategy. It was observed that introduction of non-feeding intervals was more effective in molasses compared to low predetermined specific growth rate glucose feeding strategies which produced relatively high specific growth rates. Interruption of exponential feeding for 0.5 h intervals influences the cell metabolism when glucose and or carbon sources of molasses were utilized. Non-feeding intervals which enable batchwise operations has a starvation effect on glucose feeding strategies which resulted in lower metabolic fluxes that lessened the stress on the secretory pathways and resulted in higher rhGH secretion at the end of each starvation period.

On the other hand, in semi-batch operation strategies where molasses was used as the carbon source, non-feeding intervals stimulated the utilization of fructose which increased its consumption rate and reduced fructose's accumulation in the cultivation medium. No starvation environment was simulated for molasses based operation strategies during the batch-wise periods, since cells carried on their metabolic activities

by consuming fructose during the 0.5 h interruption intervals. As shown in Figure 4.18, fructose was always present in the fermentation medium. Since cell affinity for fructose was lower than glucose, these interruption intervals were less effective for cell growth Consequently, for high predetermined specific growth rates, batchwise operations of 0.5 h were not very effective; thus significant amount of fructose was accumulated after t_{max} in strategy MS3 where $\mu_0 = 0.15\ h^{-1}$.

Furthermore, addition of starvation intervals in glucose feeding strategies has a significant effect on the proteolytic activity which was reduced after each starvation interval. This was related with the fact that the cell minimizes its metabolic activities in the absence of carbon source by reserving its energy sources which may result in a lower secretion of proteases. However, the same effect was not observed for the second set of experiments where molasses was fed to the system.

In addition the proteolytic activity was relatively high approximately 10-fold higher than the proteolytic activity in the production medium where glucose was present. A reason for high secretion of proteases is complex and rich medium of molasses with nutrients. Taking in consideration that protease hydrolysis the other proteins in the production medium, the reduction of its concentration, or suppression of its activity leads to higher rhGH concentrations.

While studying the organic acid concentrations it was observed that the most abundant organic acid in glucose feeding strategies present in the cultivation medium was gluconic acid which was related with the decrease in substrate concentration. However, in molasses feeding strategies gluconic acid concentration in the production media was found in low values, which can be related with the fact that the cells fed with molasses, were almost never left in starvation conditions.

Furthermore, lactic acid was accumulated in higher concentrations in the glucose feeding strategies showing that dissolved oxygen concentration was not entirely sufficient throughout the process which force the yeast to shift to anaerobic respiration and further produce lactic acid. The increased demand for oxygen in the operation strategies where glucose was the sole carbon source, was related with the high cell densities reached in the related experiment.

CHAPTER 5

CONCLUSIONS

In this work, two critical parameters of semi-batch operation namely the effect of carbon source and the design of continuous feed profile were investigated. Two groups of experiments were designed and carried out in the pilot scale bioreactor by using tailored semi-batch operations where the substrates glucose or molasses were introduced to the system with an exponential feeding profile or consecutive operation of semibatch and batch operation modes. Exponential feeding strategies were named as G1 (μ_o = 0.10 h^{-1}) and G2 (μ_o = 0.15 h^{-1}) for glucose, and M1 (μ_o = 0.07 h^{-1}) and M2 (μ_o = 0.10 h^{-1}), for molasses. Consecutive feeding strategies consisted of 1.5 h exponential feeding with a particular μ_o followed by 0.5 h batchwise non-feeding intervals in a repetitive cycle. GS1, GS2 and GS3 strategies were designed for glucose with μ_o = 0.07 h^{-1}, 0.10 h^{-1} and 0.125 h^{-1} and MS1, MS2, and MS3 strategies were designed for molasses with μ_o = 0.07 h^{-1}, 0.10 h^{-1} and 0.15 h^{-1}.

The most effective strategy for rhGH production was M1 where molasses was fed to the medium exponentially with μ_o = 0.07 h^{-1}. The maximum value of rhGH obtained was measured as 650 mg L^{-1} at t = 17 h when cell concentration was 75 g L^{-1}. Additionally, in this strategy, the highest overall product yield coefficients were obtained where $Y'_{P/S}$ and $Y'_{P/X}$ were calculated as 10.7 mg g^{-1} and 24 mg g^{-1} respectively. The highest specific production rate of rhGH calculated at M1 strategy was 1.48 g g^{-1} h^{-1} at t = 3 h and then q_{rhGH} decreased gradually.

The most important results achieved in this study are summarized as:

- The highest cell concentration, among the two experimental groups, was obtained in GS2 strategy as 136 g L^{-1} at t = 13.5 h. For molasses experimental set the highest C_X was obtained as 91 g L^{-1} at MS2 at t = 14 h showing that the consecutive feeding strategies has a positive effect on the cell growth and resulted in 1.35-fold higher cell growth than continuous exponential feeding.
- Glucose was more effective carbon source for the cell growth compared to molasses approaching higher cell densities up to 1.5-fold.
- The highest rhGH concentration obtained in glucose based experiment set was obtained in GS2 strategy at t = 8 h as 611 mg L^{-1}.
- Starting the semi-batch phase of the production with an initiated C_X = 27 g L^{-1} shortened the cultivation time by approaching the maximum cell and rhGH concentration in the earlier hours of the process.
- Molasses as well as glucose was mixed with PTM (*pichia* trace minerals) before being introduced to the medium. This increased molasses efficiency as a carbon source since PTM maintains an improved cell growth and metabolic activities.
- For the semi-batch operations, glucose was never detected in the production medium and the specific consumption rates of glucose increased proportionally with the cultivation time. The highest q_G calculated value was 0.41 g g^{-1} h^{-1} at t_{final} = 15 h in G2 strategy which was reciprocal to the highest glucose feeding rate among all the strategies.
- On the other hand, in molasses feeding strategies, fructose was always detected in the production medium and its concentration increased with the cultivation time. In combined feeding strategies MS1, MS2, and MS3 accumulation of fructose was reduced during the non-feeding intervals where cells were triggered to consume the accumulated sugar which further resulted in an enhanced consumption rate. Concentration of fructose accumulated in the media was measured up to 58 g L^{-1} in MS3 strategy at t = 16 h. The highest fructose consumption rate was calculated in M2 strategy as 0.11 g g^{-1} h^{-1} at t =15 h.

- Experimental specific growth rates were always lower than the pre-determined μ values. During the last hours of the cultivation, the cell enters the stationary phase by performing a greater deviation from the predetermined μ_o. M1 strategy where molasses was fed experimentally based on predetermined μ_o = 0.07 h^{-1}, was the only strategy where μ values were constant during the whole process. It can be concluded that it was easier to maintain the same experimental μ data for μ_o values. Furthermore, a deficiency in the dissolved oxygen concentrations, affects the maintenance of the specific growth rate negatively.
- For μ_o = 0.10 h^{-1} and μ_o = 0.15 h^{-1}, introduction of non-feeding intervals, improved the cell growth and increased recombinant hGH secretion significantly in both glucose and molasses feeding strategies. For the lower predetermined specific growth rate, μ_o = 0.07 h^{-1}, starvation period hindered the sugar utilization for rhGH secretion because carbon source was insufficient for both the cell growth and r-protein production.
- His-tagged protein expression facilitates the downstream process for purification of recombinant proteins and results in relatively pure protein when affinity chromatography is applied.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Fermentation Medium

1 M potassium phosphate buffer, pH=6.0	56.48 g KH_2PO_4 and 14.8 g $K_{2\ h}PO_4$ were 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% (v/v) antifoam solution was prepared by mixing 10-15 ml of antifoam solution with dH_2O up to 100-150ml and autoclaved.
Base	25% NH_3OH. Sterilization is not required

SDS-PAGE Solutions

10% (w/v) APS (Ammonium PerSulfate)	0.1 g APS is added to 1 mL dH_2O, freshly prepared.
Resolving gel	6 ml BioRad FastCast© resolving gel solution, 60 µl ammonium persulfate and 6 µl N,N,N',N'-tetramethylethylenediamine are mixed and poured into
Stacking gel	2 ml BioRad FastCast© stacking gel solution, 20 µl ammonium persulfate and 4 µl N,N,N',N'-tetramethylethylenediamine.

4X SDS-PAGE Sample loading buffer	200 mM Tris-HCl, pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue; 10% 2-mercaptoethanol. Store at -20°C.
5X SDS-PAGE Running buffer	15 g Tris Base, 72 g glycine, 5 g SDS, dH_2O to 1 L. Store at +4°C.

Silver Staining Solutions

Fixer	Mix 100 ml methanol, 24 ml acetic acid, 100 µL 37% formaldehyde. Add dH_2O up to 200 mL.
Pretreatment	Dissolve 0.05 g $Na_2S_2O_3.5\ h_2O$ in 250 ml distilled water by stirring with a glass rod. Take 2 ml and set aside for further use in developing solution preparation.
Silver nitrate	Dissolve 0.2 g silver nitrate in 100 ml dH_2O and add 75 µl 37% formaldehyde.
Developing	Dissolve 2.25 g potassium carbonate in 100 mL dH_2O. Add 2 ml from pretreatment solution and 75 µl 37% formaldehyde.
Stop	Mix 50 ml methanol, 12 ml acetic acid and dH_2O up to 100mL.

Protease Assay Solutions

0.05 M Sodium acetate buffer	Mix 0.713 mL acetic acid in 25 mL dH2O. Dissolve 2.052 g sodium acetate in 50 mL dH_2O. Titrate sodium acetate solution with acetic acid solution until pH = 5.0 and dilute to 500 ml. Autoclave and store at +4°C.

APPENDIX B

B.1. CALIBRATION CURVE FOR GLUCOSE CONCENTRATION

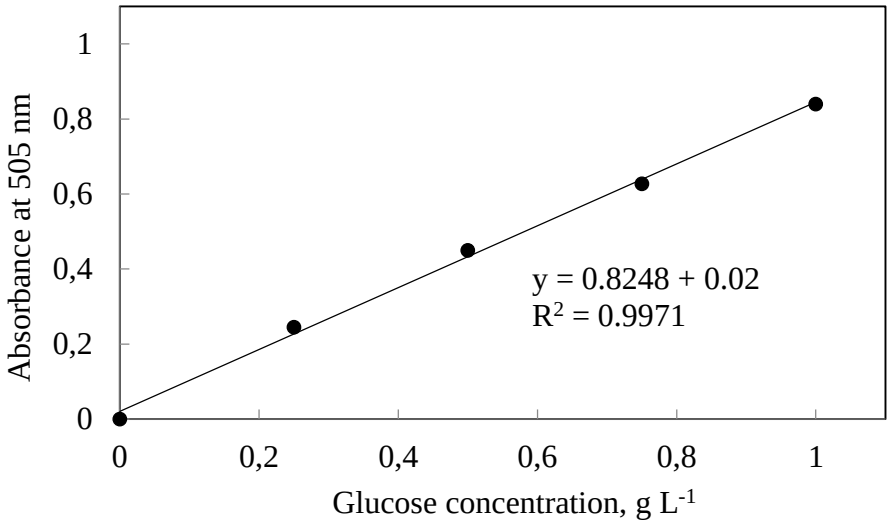

Figure B.1. Standard calibration curve for glucose concentration

B.2. CALIBRATION CURVE FOR FRUCTOSE CONCENTRATION

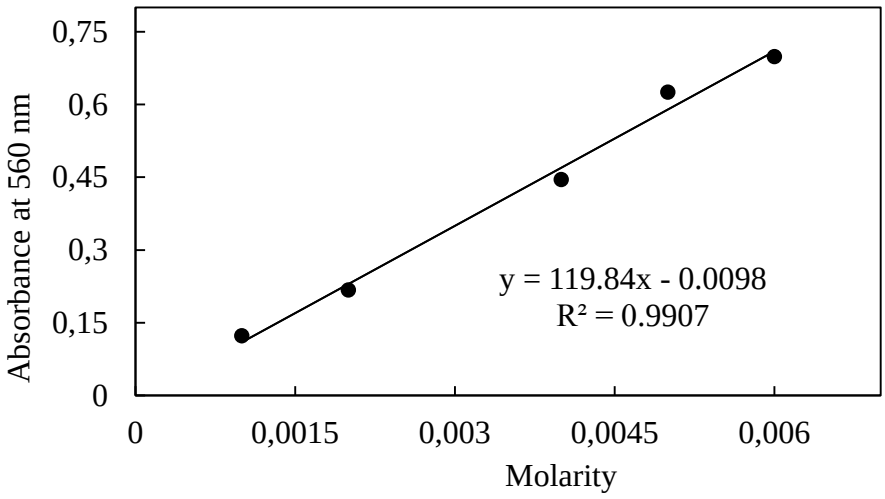

Figure B.1. Standard calibration curve for fructose concentration

APPENDIX C

MOLECULAR WEIGHT MARKER

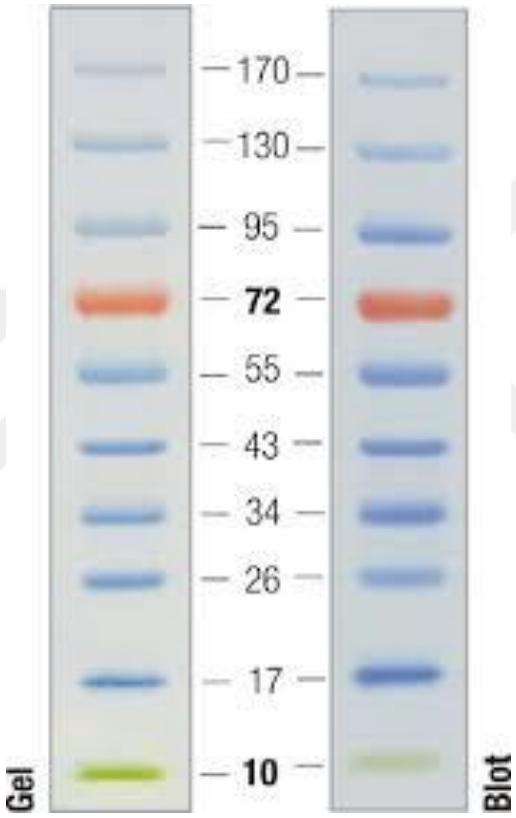

Figure C.1. PageRuler^{TM} Prestained Protein Ladder (Fermentas)

APPENDIX D

SDS-PAGE PROTEIN ANALYSES

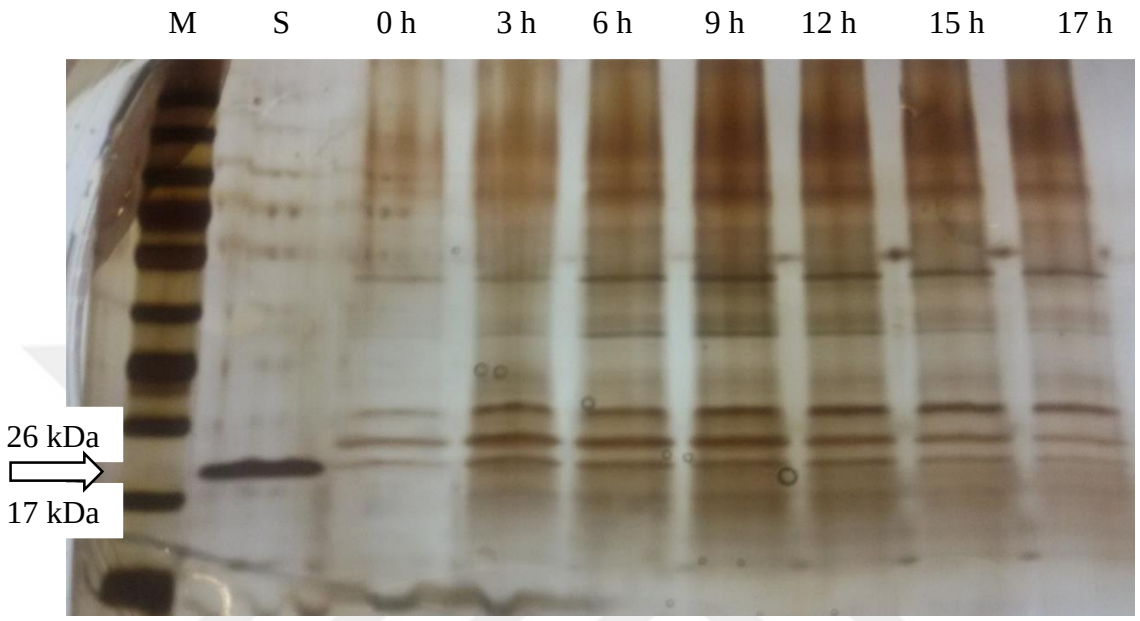

Figure D.1. Silver stained SDS-PAGE gel images of proteins produced in G1, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

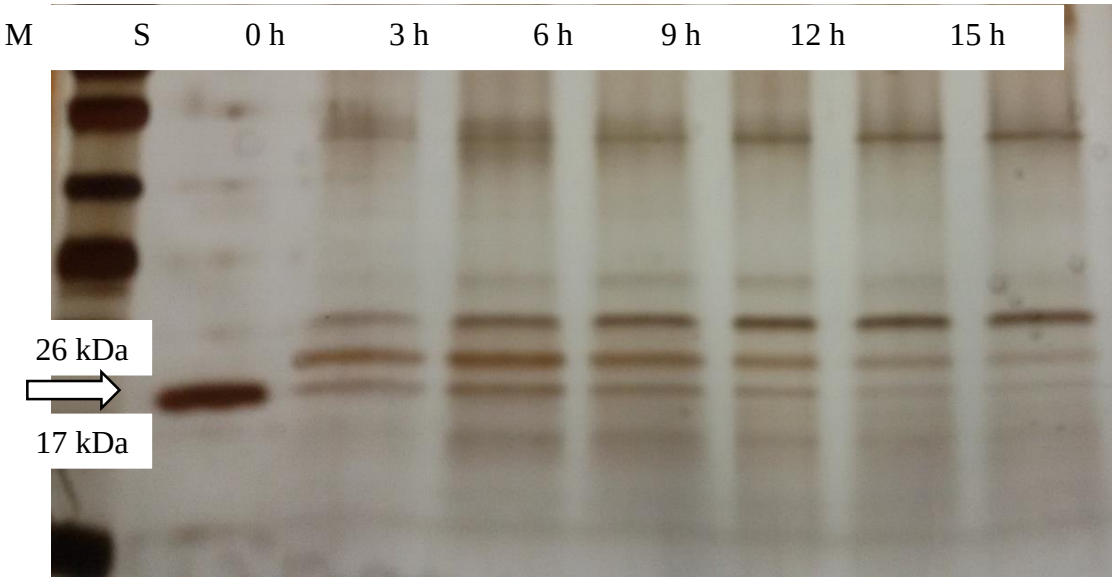

Figure D.2. Silver stained SDS-PAGE gel images of proteins produced in G2, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

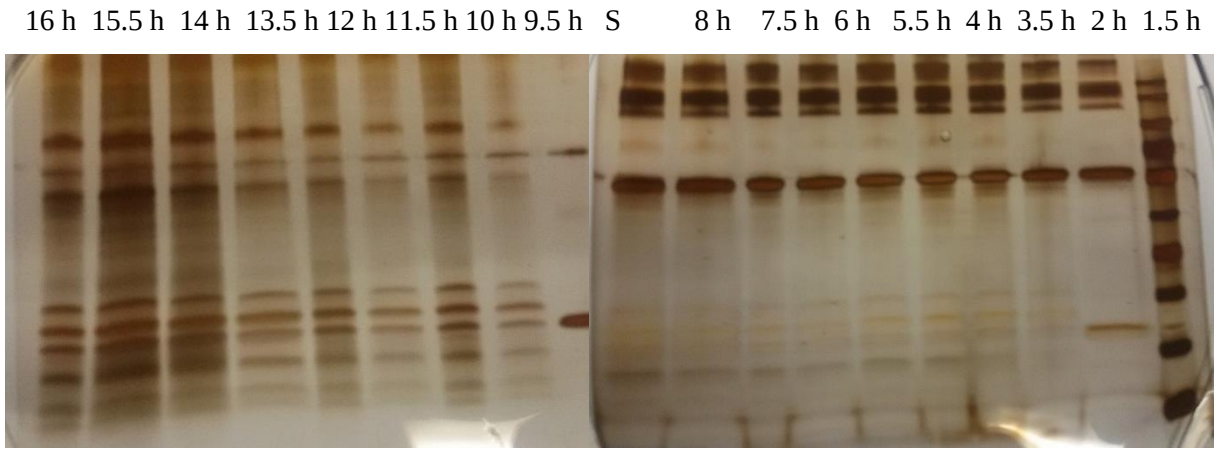

Figure D.3 Silver stained SDS-PAGE gel images of proteins produced in GS1, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

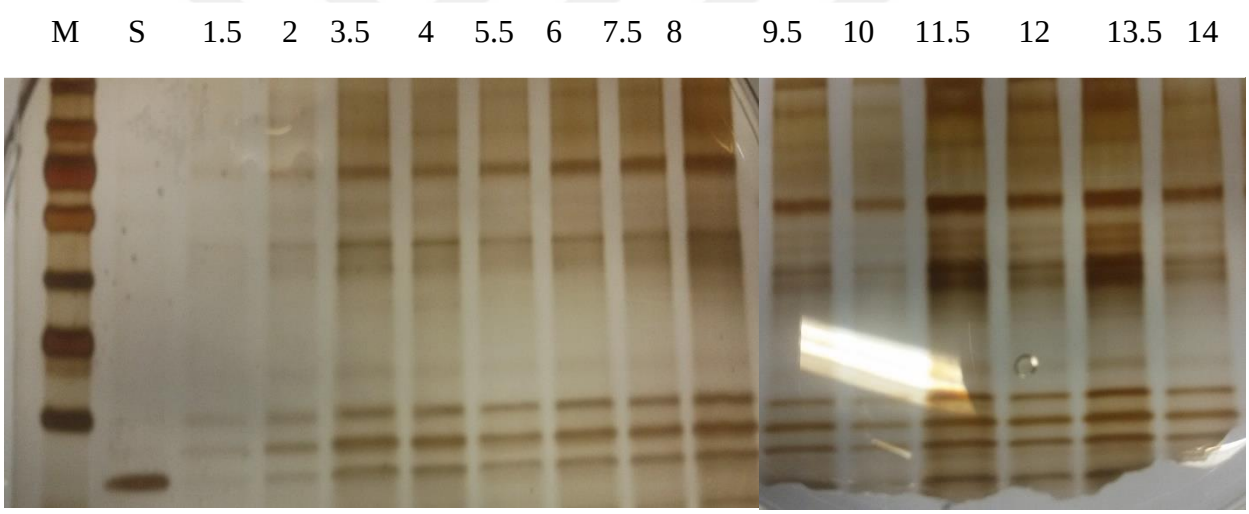

Figure D.4. Silver stained SDS-PAGE gel images of proteins produced in GS2, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

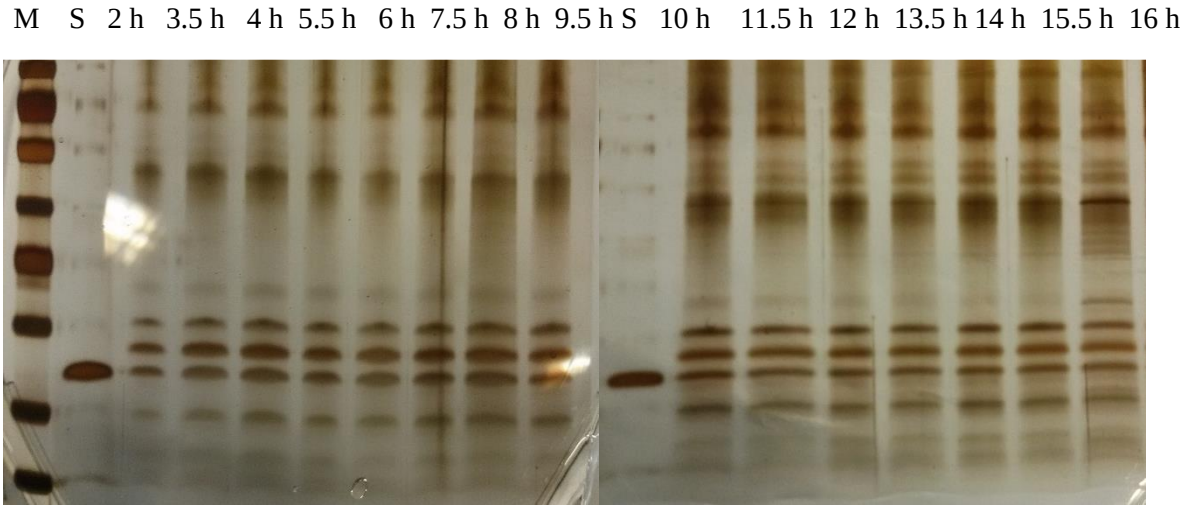

Figure D.5. Silver stained SDS-PAGE gel images of proteins produced in GS3, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

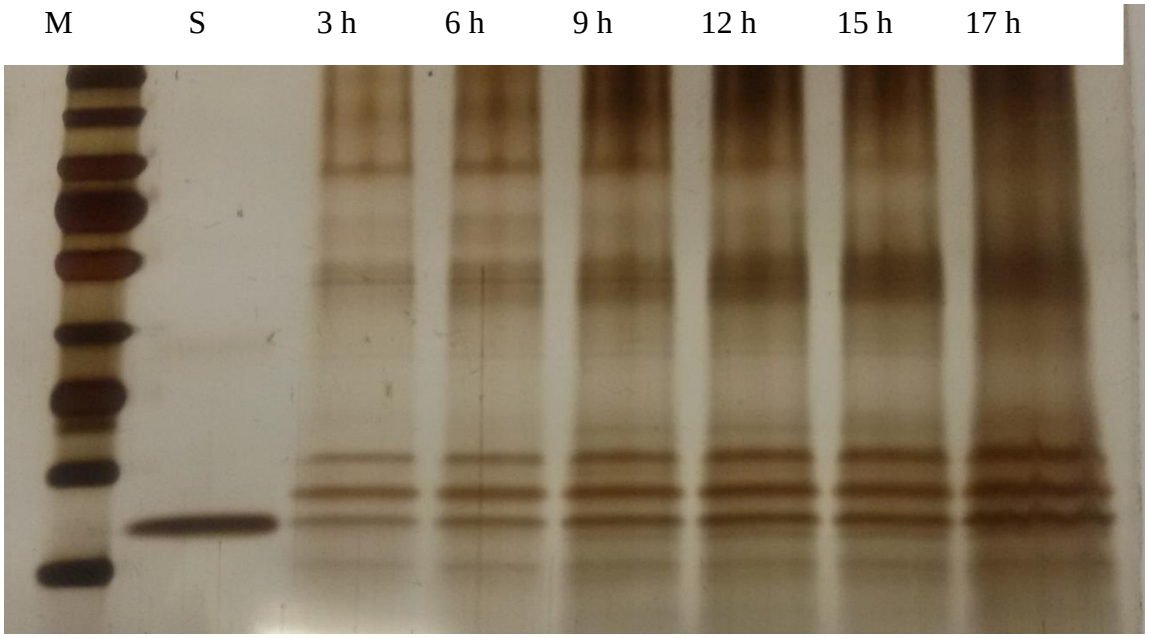

Figure D.6. Silver stained SDS-PAGE gel images of proteins produced in M1, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

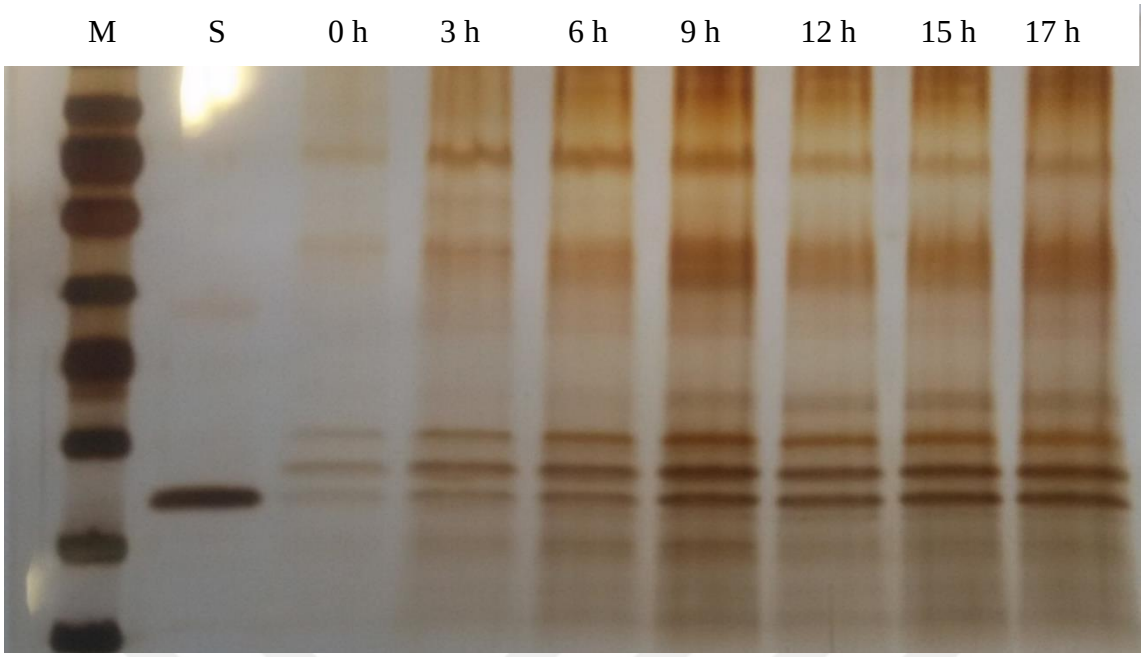

Figure D.7. Silver stained SDS-PAGE gel images of proteins produced in M2, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

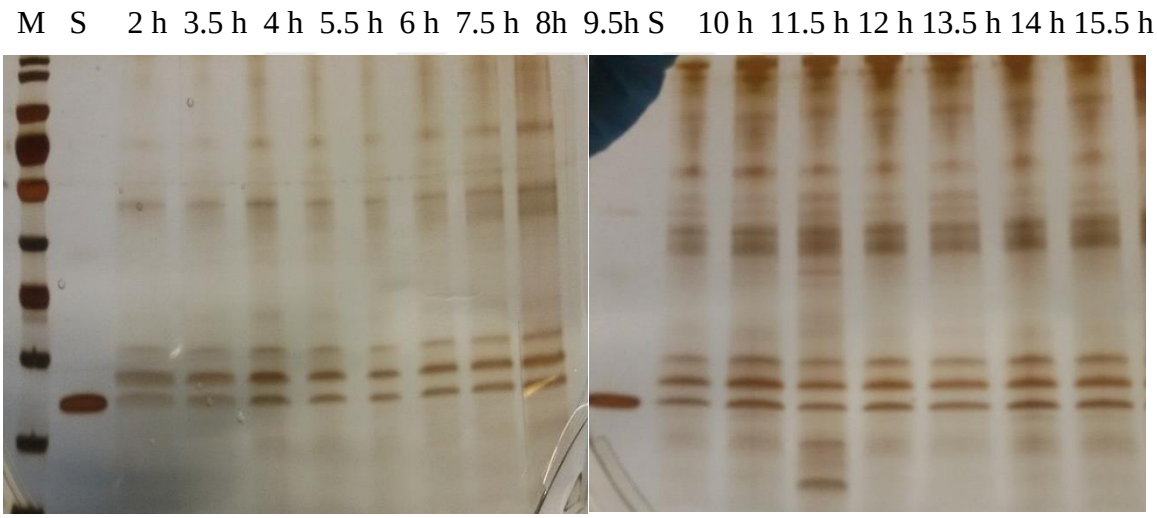

Figure D.8. Silver stained SDS-PAGE gel images of proteins produced in MS1, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

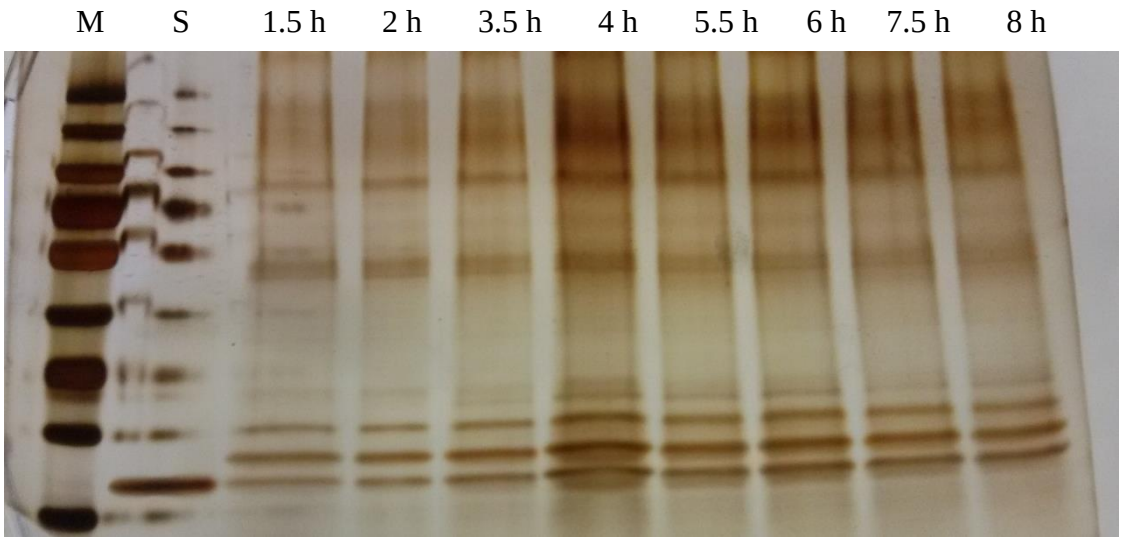

Figure D.9. Silver stained SDS-PAGE gel images of proteins produced in MS2-1, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

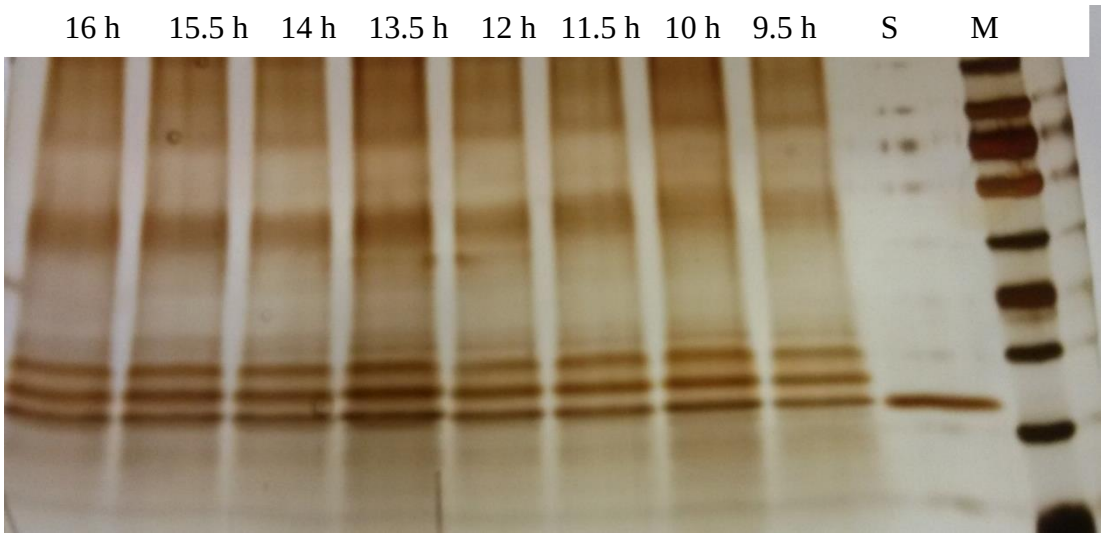

Figure D.10. Silver stained SDS-PAGE gel images of proteins produced in MS2-2, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

M S 1.5 h 2 h 3.5 h 4 h 5.5 h 6 h 7.5 h 8 h 9.5 h 10 h 11.5 h 12 h 13.5 h 14 h 15.5

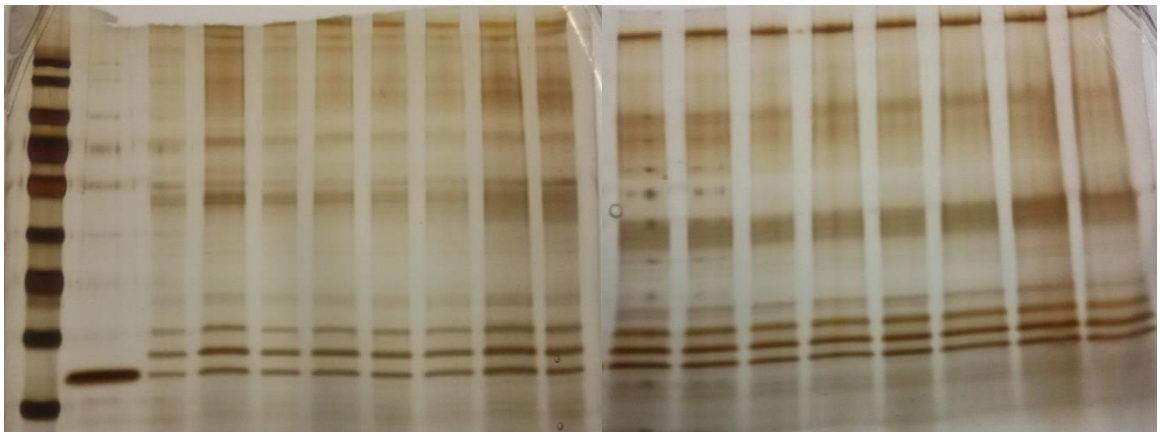

Figure D.11. Silver stained SDS-PAGE gel images of proteins produced in MS3, 1:10 diluted samples. M: Marker of PageRuler protein ladder; S: 50 mg L^{-1} hGH standard.

APPENDIX E

CALIBRATION CURVES FOR ORGANIC ACIDS

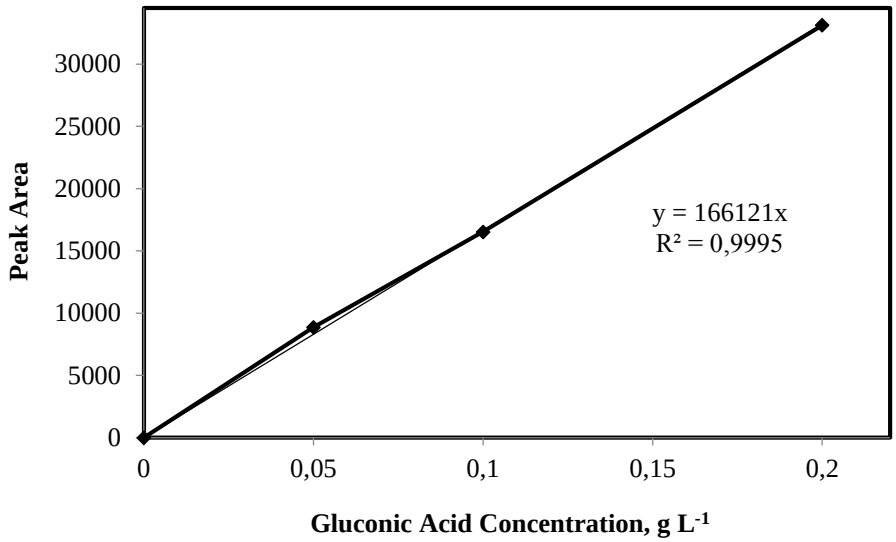

Figure E.1. Calibration curve for gluconic acid concentration determination by HPLC.

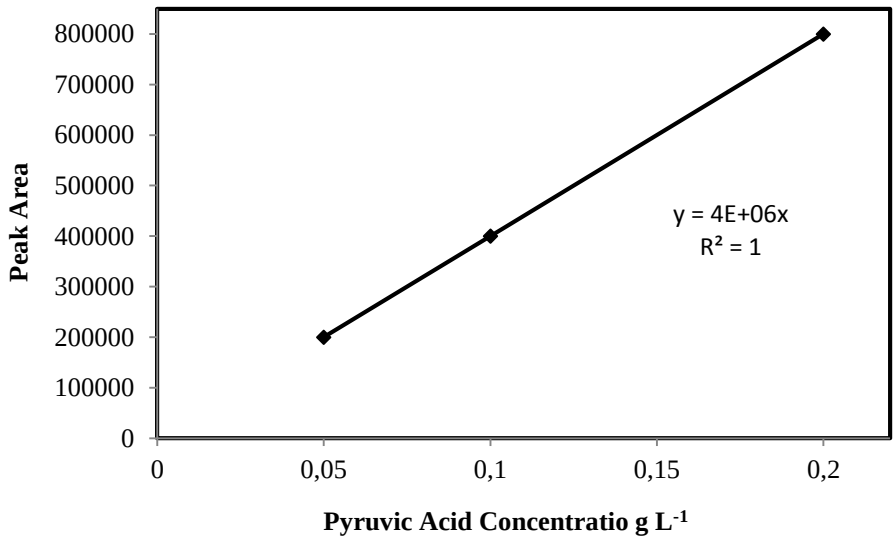

Figure E.2. Calibration curve for pyruvic acid concentration determination by HPLC.

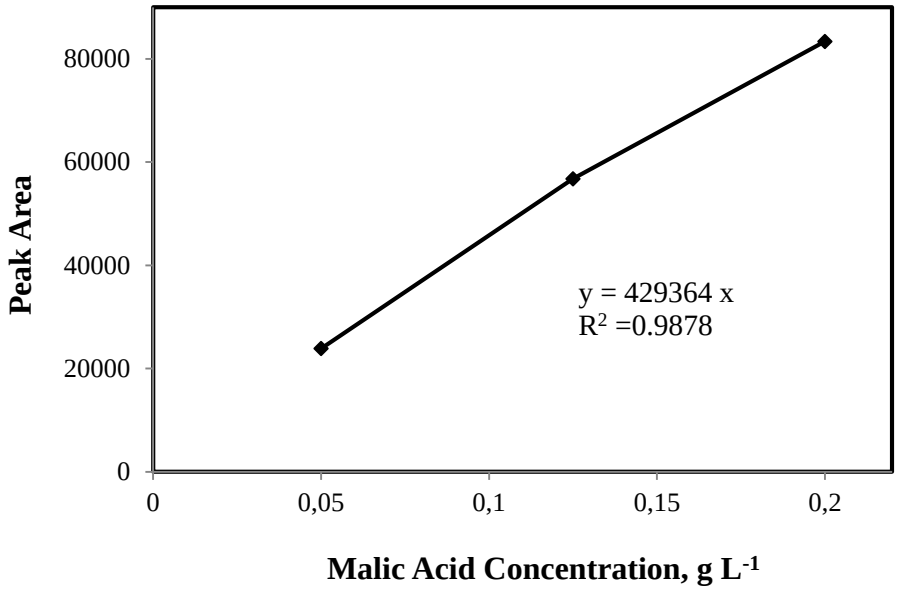

Figure E.3. Calibration curve for malic acid concentration determination by HPLC.

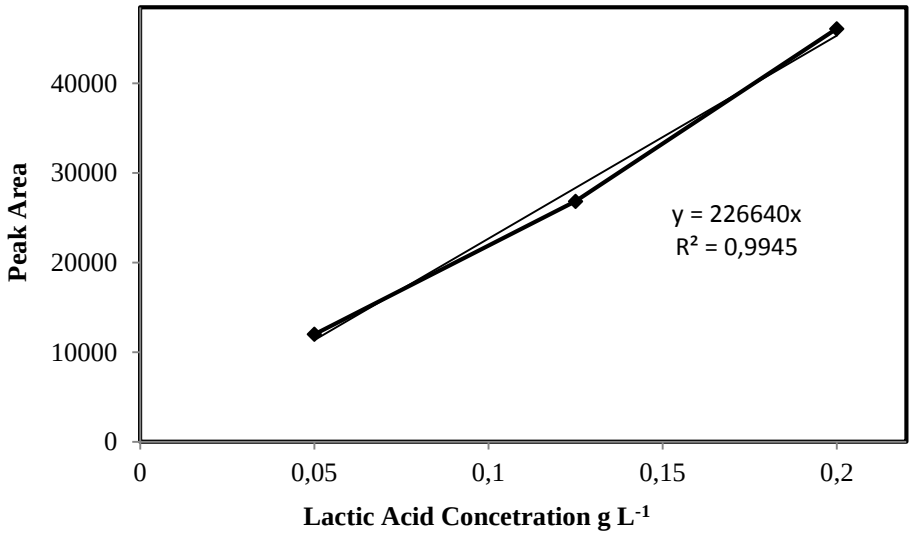

Figure E.4. Calibration curve for lactic acid concentration determination by HPLC**.**

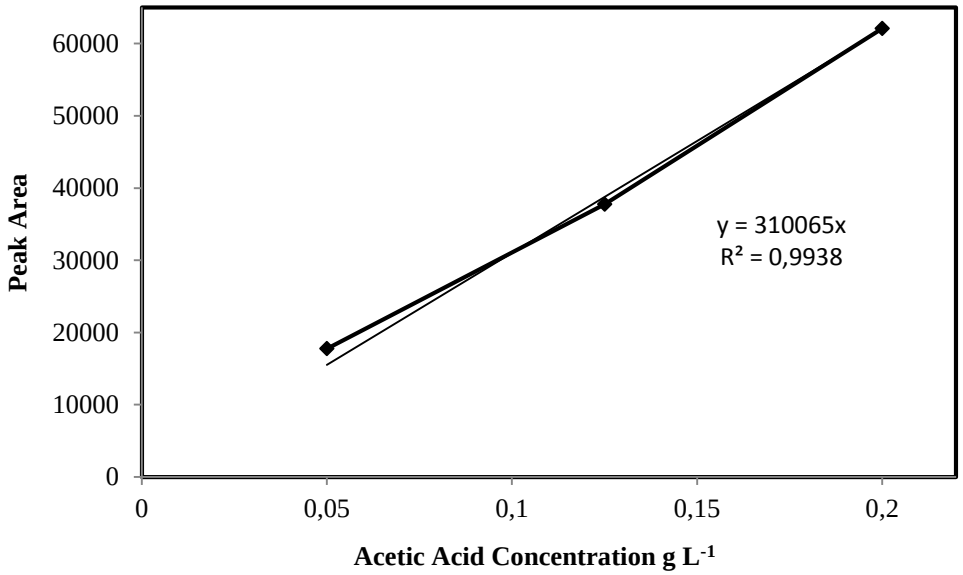

Figure E.5. Calibration curve for acetic acid concentration determination by HPLC.

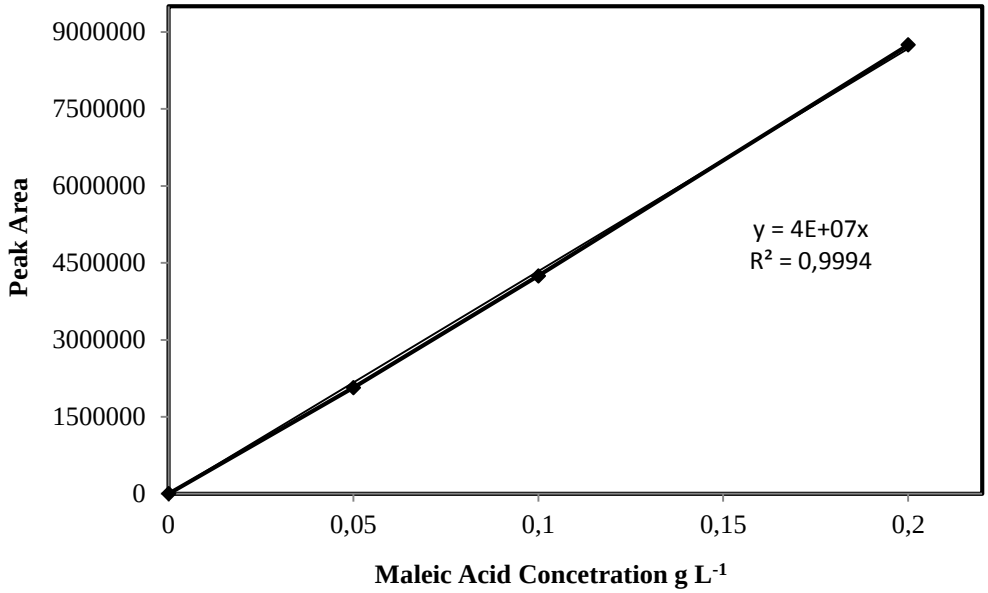

Figure E.6. Calibration curve for maleic acid concentration determination by HPLC.

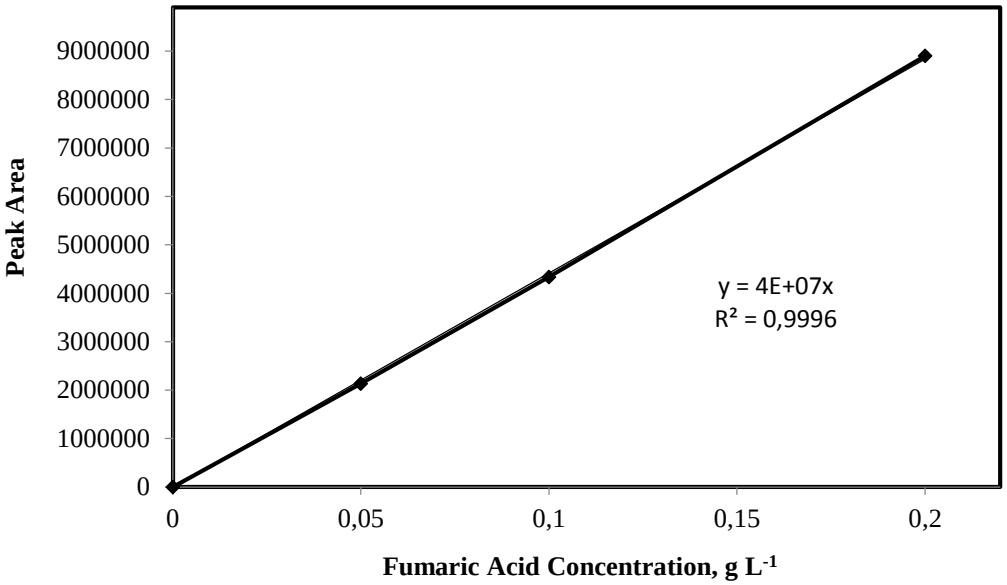

Figure E.7. Calibration curve for fumaric acid concentration determination by HPLC.

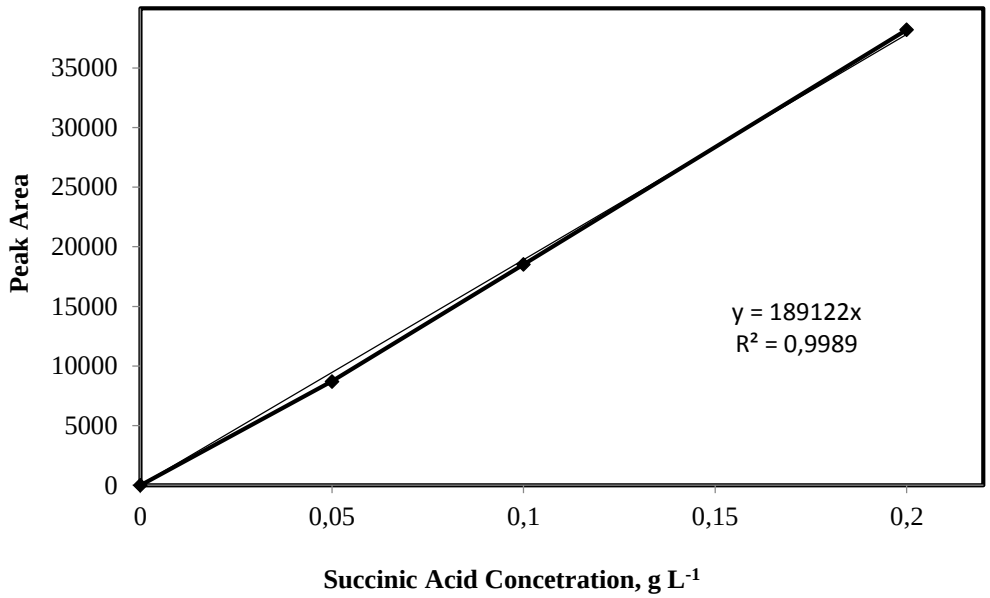

Figure E.8. Calibration curve for fumaric acid concentration determination by HPLC.

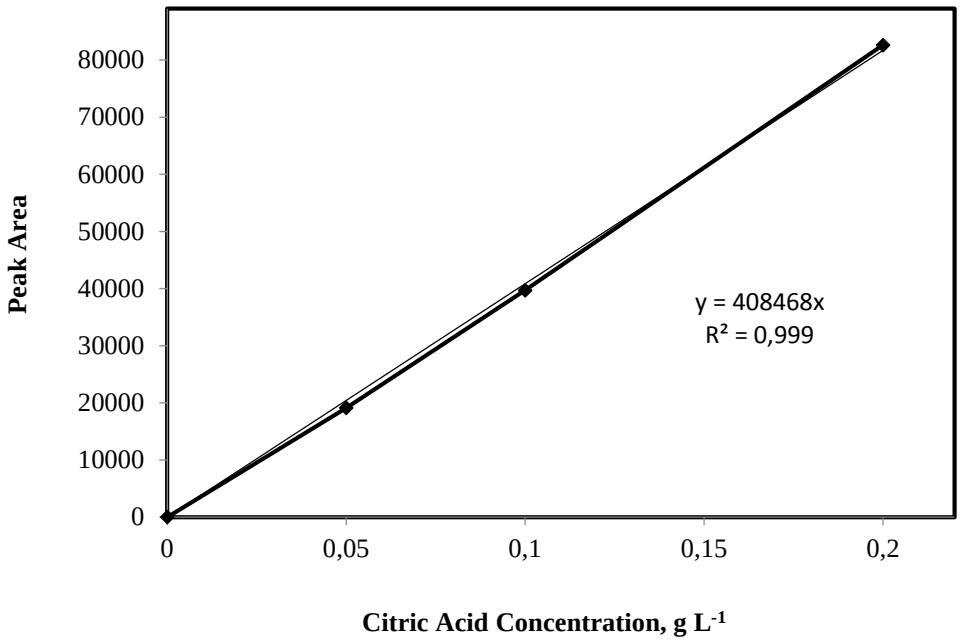

Figure E.9. Calibration curve for citric acid concentration determination by HPLC.

APPENDIX F

DISSOLVED OXYGEN CONCENTRATIONS

Table F.1 Dissolved Oxygen Concentration for glucose based feeding strategies

C_{DO} (%) for Glucose Based Feeding Strategies

t	**G1**	**G2**	**GS1**	**GS2**	**GS3**
0	14.8	14.9	14.5	15.4	13.9
1	16.4	13.1	13.6	14.9	15.3
2	15.1	13.2	13.6	13.9	14.7
3	14.8	12.5	13.3	14	15.8
4	15	10.3	12.2	21.9	16.7
5	16.6	7.9	11.0	15.9	11.8
6	16.3	8.5	11.3	17	15.4
7	8.4	6.7	10.4	18.3	10.7
8	12.4	4.6	9.3	32.3	10.5
9	7.7	3.3	8.7	12.3	10.4
10	2.8	2	8.0	10.5	10.3
11	2.3	2.1	8.1	15.2	10.2
12	12.3	2.2	8.1	23.9	10.2
13	2.3	3	8.5	1	5.2
14	0.4	3.1	8.6	1.3	0.2
15	0.6	3.8	8.9	0.4	0.3
16	0.8		12.2	0.4	0.3
17	0.7		13.8	0.5	0.3

Table F.2 Dissolved Oxygen Concentration for molasses based feeding strategies

C_{DO} *(%) for* **Molasses** *Based Feeding Strategies*					
t	**M1**	**M2**	**MS1**	**MS2**	**MS3**
0	14.8	19.8	15.6	12.8	15.5
1	16.9	21.4	22.3	15.5	13.2
2	15.1	20.1	21.5	15.3	12.7
3	14.8	19.8	20.2	15	12.2
4	15	20	43.7	17	15.8
5	16.6	21.6	3	15.2	11.2
6	16.3	21.3	21.9	12.5	20.4
7	8.4	8.4	22.4	13.5	10.8
8	12.4	12.4	44.8	22.9	11.1
9	17.7	7.7	23.2	14.9	10.8
10	12.8	2.8	47.5	20	10.8
11	11.1	0.2	20.1	12.6	12.6
12	18.1	0.3	48.1	13.1	19.1
13	2.3	0.3	20.3	11.5	15.4
14	6.4	0.4	11.8	20.2	21.4
15	4.3	0.6	20.7	5.7	0.7
16	0.8	0.3	50.7	6.7	0.6
17	0.7	3.4	10.7	1.9	0.5