

FEEDING STRATEGY DEVELOPMENT FOR HUMAN GROWTH
HORMONE PRODUCTION BY *Pichia pastoris*

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

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JULY 2012

Approval of the thesis:

**FEEDING STRATEGY DEVELOPMENT FOR HUMAN GROWTH
HORMONE PRODUCTION BY *Pichia pastoris***

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ABSTRACT

FEEDING STRATEGY DEVELOPMENT FOR HUMAN GROWTH HORMONE PRODUCTION BY *Pichia pastoris*

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JULY 2012, 140 pages

In this study, recombinant human growth hormone (rhGH) production by *Pichia pastoris-Mut⁺* strain was improved by designing feeding strategies which were applied in the production phase of the bioreactor operations. During the bioreactor experiments the cell growth, sorbitol and methanol consumptions, recombinant hGH production, alcohol oxidase (AOX) activity, the by-products protease and organic acid concentrations were followed and analyzed. In this context, in the first part of the study, three bioreactor operations were designed and performed. In general, the designed strategies are fundamentally based on simultaneous feeding of the two substrates starting at $t=0$ h of the production phase, i.e., batch-wise 50 gL⁻¹sorbitol feeding, together with fed-batch methanol feeding with a specific growth rate of $\mu_0=0.03$ h⁻¹ or $\mu_0=0.04$ h⁻¹, and fed-batch sorbitol

feeding with a specific growth rate of $\mu_0=0.025\text{h}^{-1}$ which was calculated based on the specific consumption rate $q_s=0.152\text{ g g}^{-1}\text{h}^{-1}$ of sorbitol. Consequently, sorbitol concentration was kept constant at 50 gL^{-1} within $t=0\text{-}15\text{h}$ of the production phase; where, sorbitol feeding was terminated at $t=15\text{h}$. Amongst, in the first strategy (SSM1), methanol was fed to the system with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$, and the H^+ concentration (pH) in the bioreactor was kept constant at $\text{pH}=5.0$. In the second strategy (SSM2), pH was kept constant at 5.5 until $t=24\text{h}$ of the induction phase (production phase), thereafter, was reduced to $\text{pH}=5.0$; where methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$. In the third strategy (SSM3), methanol was fed with the specific growth rate of $\mu_0=0.04\text{ h}^{-1}$, and the pH in the bioreactor was kept constant at $\text{pH} 5.0$. The highest rhGH production and cell concentration were achieved in the first strategy SSM1 as $C_{\text{rhGH}}=640\text{ mg L}^{-1}$ and $C_X=105.3\text{ g L}^{-1}$, and the overall cell and product yields on total substrate were calculated as $Y_{X/S}=0.21\text{ g g}^{-1}$ and $Y_{C_{\text{rhGH}}/S}=1.83\text{ mg g}^{-1}$.

In the second part of this study the two-substrates sorbitol and methanol were fed simultaneously in a solution compose of 1.37 mol sorbitol and 6.21 mol methanol in 13.88 mol water, which is named as SM. In this strategy (SM), the two-substrate solution was fed to the medium with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$ on sorbitol until $t=30\text{h}$; thereafter, only methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$. The highest cell and rhGH concentrations obtained in SM were, respectively, $C_X=104.7\text{ g L}^{-1}$ and $C_{\text{rhGH}}=124\text{ mg L}^{-1}$; and the overall cell and product yields on the total substrate were calculated as $Y_{X/S}=0.21\text{ g g}^{-1}$ and $Y_{C_{\text{rhGH}}/S}=0.39\text{ mg g}^{-1}$. Although the highest cell concentration obtained at SM is close to that of the SSM1, the rhGH concentration obtained at SM is 5.2-fold lower than that of the strategy SSM1.

Keywords: Recombinant human growth hormone, *Pichia pastoris*, feeding strategy, sorbitol, mixed feed

ÖZ

Pichia pastoris İLE İNSAN BÜYÜME HORMONU ÜRETİMİ İÇİN BESLEME STRATEJİLERİNİN GELİŞTİRİLMESİ

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TEMMUZ 2012, 140 sayfa

Bu çalışmada, yüksek derişimde rekombinant insan büyüme hormonu (rhGH) üretmek amacı ile ikinci karbon kaynağı sorbitolun varlığında farklı üretim stratejilerinin rhGH üretimi üzerine etkisi araştırılmıştır. İnsan büyüme hormonu geni taşıyan rekombinant *Pichia pastoris*-Mut⁺ suşu kullanılmış; proses süresince hücre, sorbitol, metanol, rhGH, AOX ve yan-ürünler proteaz ve organik asit derişimleri ölçülerek izlenmiştir. Belirtilen amaç doğrultusunda çalışmanın ilk bölümünde üç biyoreyaktör işletim stratejisi tasarlanmıştır. Tasarlanan üç işletim stratejisi temel olarak üretim fazına geçiş anı t=0 st'ta başlamak üzere çift-substrat metanol ile sorbitolun eşanlı olarak: i) kesikli-işletimle 50 g L⁻¹ sorbitol beslemesi; ii) yarı-kesikli işletimle özgül çoğalma hızı $\mu_0=0.03 \text{ st}^{-1}$ veya $\mu_0=0.04 \text{ st}^{-1}$ olacak şekilde metanol beslemesi ve; iii) sorbitol özgül tüketim hızı $q_s=0.152 \text{ g g}^{-1}\text{st}^{-1}$ kullanılarak hesaplanan özgül çoğalma hızı $\mu_0=0.025 \text{ st}^{-1}$ olacak şekilde

yarı-kesikli sorbitol beslemesi ile oluşturulmuştur. Böylece, üretim fazında t=0–15 st arasında biyoreaktörde sorbitol derişimi 50 gL^{-1} deęerinde sabit tutulmuş, ve sorbitol beslemesi t=15 st'ta kesilmiştir. Tasarlanan ilk stratejide (SSM1) metanol $\mu_0=0.03 \text{ h}^{-1}$ çoęalma hızı ile yarı-kesikli olarak beslenmiş ve üretim ortamı H^+ iyonu derişimi pH=5.0 olacak şekilde ayarlanmıştır. Uygulanan ikinci stratejide (SSM2) üretimde, yarı-kesikli metanol beslemesi $\mu_0=0.03 \text{ h}^{-1}$ çoęalma hızı ile gerçekleştirilmiştir; dięer taraftan biyoreaktörde H^+ iyonu derişimi t=0 st'da pH=5.5'de başlanmış ve t=0-24 st arasında bu deęerde sabit tutulmuş, ancak t=24 st sonunda pH deęeri 5.0'e düşürülerek proses sürdürülmüştür. Üçüncü stratejide (SSM3), metanol beslemesi $\mu_0=0.04 \text{ st}^{-1}$ çoęalma hızı ile gerçekleştirilmiştir; dięer parametre pH=5.0'te üretim fazı süresince sabit tutulmuştur. En yüksek rhGH üretimi ve hücre derişimine birinci strateji SSM1'de $C_{\text{rhGH}}=640 \text{ mg L}^{-1}$ ve $C_X=105.3 \text{ g L}^{-1}$ derişimleri ile ulaşılmış; çift substratlı üretim sisteminde, toplam substrat temel alınarak en yüksek ürün ve hücre verimleri, sırasıyla, $Y_{C_{\text{rhGH}}/S}=1.83 \text{ mg g}^{-1}$ ve $Y_{X/S}=0.21 \text{ g g}^{-1}$ olarak hesaplanmıştır.

Çalışmanın ikinci kısmında iki substrat, sorbitol ve metanol birlikte suda çözelti olarak biyoreaktöre yarı-kesikli olarak beslenmiştir. Besleme çözeltisinde 13.88 mol suda, 1.37 mol sorbitol ve 6.21 mol metanol olacak şekilde ayarlanmıştır ve sorbitol üzerinden $\mu_0=0.03 \text{ st}^{-1}$ çoęalma hızı ile üretim ortamına 30 st süreyle beslenmiştir; t=30 st'ten sonra ise yalnız metanol beslenmiştir. En yüksek hücre ve rhGH derişimleri sırası ile $C_X=104.7 \text{ g L}^{-1}$ ve $C_{\text{rhGH}}=124 \text{ mg L}^{-1}$ olarak hesaplanmıştır. Toplam substrat temel alınarak elde edilen en yüksek hücre ve ürün verimleri ise sırasıyla $Y_{X/S}=0.21 \text{ g g}^{-1}$ ve $C_{\text{rhGH}/S}=0.39 \text{ mg g}^{-1}$ olarak bulunmuştur. SM için bulunan en yüksek hücre derişiminin SSM1 için bulunan en yüksek hücre derişimine yakın olduęu; ancak, SM stratejisi ile en yüksek rhGH derişiminin SSM1 stratejisinden 5.2-kat daha düşük olduęu belirlenmiştir.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, *Pichia pastoris*, besleme stratejisi, karışık besleme

To my family,

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my supervisor Prof. Dr. Pınar Çalık for her support, guidance and help, in all the possible way, throughout this study.

I am thankful to my co-supervisor Prof. Dr. Tunçer Özdamar for his valuable advices and critics.

My special thanks are to Ph.D. student Erdem Boy, for sharing his experience, his support to my experimental studies; moreover, during the bioreactor experiments to his tolerance to my late night calls which enabled me to solve the operational problems. I am grateful to my friends, the graduate students in our research group: Gül Özer Zerze, Melda Eskitoros, Burcu Akdağ, Özge Ata, and Burcu Gökbudak, and also to Hasan Zerze; without their great friendship, the encouragement and cooperation even in sleepless nights, I could not have carried out my studies with success. And also I am thankful to research group of Prof. Dr. Ufuk Bölükbaşı for their friendship and cooperation during the laboratory studies.

I should like to acknowledge with gratitude the contributions, through the graduate lectures and seminars, I have received from the Faculty Members of Chemical Engineering and Biotechnology Departments during my studies at METU.

I also like to acknowledge the financial supports of the Middle East Technical University Research Fund Project, and The Scientific and Technological Research Council of Turkey (TÜBİTAK- Project 109R015).

Last but not least, my warmest thanks to my family for loving, supporting and encouraging me all through my life.

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LIST OF SYMBOLS AND ABBREVIATIONS

C	Concentration in the medium	g L^{-1} or mol m^{-3}
C_{O^*}	Saturated dissolved oxygen concentration	Mol m^{-3}
Da	Damköhler number (=OD/OTR _{max} ; Maximum possible oxygen utilization rate per mass transfer rate)	
DO	Dissolved oxygen	%
E	Enhancement factor (=K _L a/K _L a ₀)	
K _L a	Overall liquid phase mass transfer coefficient	s^{-1}
K _L a ₀	Physical overall liquid phase mass transfer coefficient	s^{-1}
N	Agitation rate	min^{-1}
OUR	Oxygen uptake rate	$\text{mol m}^{-3} \text{sec}^{-1}$
OTR	Oxygen transfer rate	$\text{mol m}^{-3} \text{sec}^{-1}$
OD	Oxygen demand	$\text{mol m}^{-3} \text{sec}^{-1}$
Q	Feed inlet rate	L h^{-1}
q	Specific formation or consumption rate	$\text{g g}^{-1} \text{h}^{-1}$
r	Formation or consumption rate	$\text{g g}^{-1} \text{h}^{-1}$
t	Cultivation time	h
T	Bioreaction medium temperature	°C
U	One unit of an enzyme	
V	Volume of the bioreactor	L
Y	Yield (overall)	g g^{-1}

Greek Letters

ρ	Density	g L^{-1}
η	Effectiveness factor (=OUR/OD)	
μ_0	Desired specific growth rate	h^{-1}
$\mu_{s,\text{max}}$	Maximum specific growth rate on sorbitol	h^{-1}
μ_t	Total specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Refers to initial condition
AOX	Refers to alcohol oxidase
G	Refers to glycerol
M	Refers to methanol
O	Refers to oxygen
p	Refers to protein
pro	Refers to protease
R	Refers to bioreaction medium
rp	Refers to recombinant protein
S	Refers to sorbitol or substrate
St	Refers to total substrate
X	Refers to cell

Abbreviations

AOX	Alcohol oxidase
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
FLD	Glutathione-dependent formaldehyde dehydrogenase
GAP	Glyceraldehyde-3-phosphate dehydrogenase

hGH	Human growth hormone
HPCE	High pressure capillary electrophoresis
HPLC	High pressure liquid chromatography
MSS-0.03	Batch-wise sorbitol addition at t=0 h and t=9 h and methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$
MSSS-0.03	Batch-wise sorbitol addition at t=0 h, t=14 h and t=31 h and methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$
rhGH	Recombinant human growth hormone
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SM	A mixed substrate solution with 1.37 mol sorbitol and 6.21 mol methanol in 13.88 mol water with $\mu_0=0.03 \text{ h}^{-1}$ (t=0-30h); methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$ (t=30-42h); pH 5.0 50 gL^{-1} batch-wise sorbitol pulse feeding (t=0 h) and keeping sorbitol concentration at 50 gL^{-1} at t=0-15 h; methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$; pH 5.0
SSM1	50 gL^{-1} batch-wise sorbitol pulse feeding (t=0 h) and keeping sorbitol concentration at 50 gL^{-1} at t=0-15 h; methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$; pH 5.0
SSM2	50 gL^{-1} batch-wise sorbitol pulse feeding (t=0 h) and keeping sorbitol concentration at 50 gL^{-1} at t=0-15 h; methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$; pH 5.5 (t=0-24h), pH 5.0 (t=24-48h)
SSM3	50 gL^{-1} batch-wise sorbitol pulse feeding (t=0 h) and keeping sorbitol concentration at 50 gL^{-1} at t=0-15 h; methanol feeding with $\mu_0=0.04 \text{ h}^{-1}$; pH 5.0
TCA	Tricarboxylic acid
TCP	Total cell protein

CHAPTER 1

INTRODUCTION

Although biotechnological products (e.g., wine, bread, cheese, yoghurt, vinegar, and beer) have been produced since thousands of years, it was only a little more than 150 years that Louis Pasteur pointed out the role that living organisms play in these processes. In the years that followed, a number of commercially important biochemicals (e.g., penicillin, ethanol, acetone-butanol) were produced by the utilization of the activities of various organisms. In 1957 several novel bacteria with high productivity of L-glutamic acid were found, and a new fermentation method called “L-glutamic acid fermentation” commenced in the field of “applied microbiology” in Japan, which is presently named as “Microbial Biotechnology” or in a wider context as “Industrial Biotechnology”. The scientists of the field, i.e., microbiologists, biochemists and chemical engineers, had required an engineering approach to the field, dealing with the industrial utilization of microorganisms; and this was achieved in 1964 by S. Aiba in Tokyo University and the book “Biochemical Engineering” was published. Besides, although the double-helix structure of DNA molecule was discovered in 1953 by the four scientists (R. Franklin, M. Wilkins, J. Watson, and F. Crick) in England, their discovery was awarded with the Nobel Prize in 1962, to the living latter three scientists. Over the past few decades, the scientists have introduced molecular biology and genetic engineering to the field of industrial biotechnology; and with the invention of the polymerase chain reaction (PCR) in 1988 and the design of the “thermal cycler” equipment, the frontier in the genetic engineering applications was lifted. Since almost two decades, every year an increasing number of

new biomolecules are introduced into the world markets through innovative metabolic engineering designs of recombinant systems which are constructed by the application of molecular genetic methods.

Today more than 200 recombinant pharmaceutical proteins and peptides are approved by Food and Drug Administration (FDA) (Demain and Vaishnav 2009). Thus, the biotechnological industry gained an important role in the business world. Biopharmaceuticals is projected to reach \$182.5 billion by 2015 (www.researchandmarkets.com). The most well-known biotech products are mammalian polypeptides, e.g., erythropoietin (EPO) with a \$13.1 billion market; interferon- α , \$6 billion; human growth hormone (HGH) a \$1.8 billion; human insulin, \$5.6 billion; tissue plasminogen activator (tPA), \$640 million (Demain 2007). Among these proteins rhGH is widely used for the treatment of several diseases such as burns, injuries, bleeding ulcers, bone fractures, hypopituitary dwarfism, and moreover it supplies benefits for girls with Turner syndrome, adults with growth hormone deficiency, children with chronic renal failure and human immunodeficiency virus (HIV) syndrome (Baulieu *et al.*, 1990; Binkley 1994; Trevino *et al.*, 2000).

Before taking the advantage of metabolic engineering, hGH used for the treatment of the diseases, was extracted initially from the pituitaries of cadavers; but caused a disease. In 1979, Goddel *et al.* (1979) reported intracellular production rhGH in recombinant *Escherichia coli*. The recombinant rhGH whose amino acid sequence and conformation was identical to the native molecule, was produced by using *E.coli* in 1985 by Gray *et al.* (1985). However, since *E.coli* is a prokaryote, type of the foreign protein to be expressed is limited due to the incorrect folding and inefficient posttranslational modifications. Also the reducing environment of the cytosol inhibits the disulfide bond formation and therefore proteins, especially ones that contain disulfide bridges in their native structure, aggregate as inclusion bodies. Hence, alternative host microorganisms have been used for the production of rhGH which are *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Pseudomonas* and *Pichia pastoris* (Gray *et al.* 1985; Tokunaga *et al.*

1985; Treviño *et al.* 2000; Özdamar *et al.* 2009; Çalık *et al.*, 2008, Orman *et al.*, 2009, Çalık *et al.*, 2010a, Çalık *et al.*, 2010b).

In the last decade, *Pichia pastoris* have become a popular and widely used expression system owing to its advantages over the other host microorganisms. What make *P.pastoris* the most preferred host microorganism are; besides being an eukaryote, the ease of genetic manipulation; ability of performing posttranslational modifications, e.g., glycosylation, correct disulfide bond formation and proteolytic processing; comprising the strongest and tightly regulated alcohol oxidase 1 (AOX1) promoter in its structure (Cereghino and Cregg 1999; 2000). The first study used *P.pastoris* for rhGH production was performed by Trevino *et al.* (2000). In their study by using 2-L bioreactor, 49 mg L⁻¹ rhGH was produced in high cell density cultures. In 2002, Eurwilaichitr *et al.* reported that glutamic acid-alanine spacer was not necessary for the removal of MF α -1 signal sequence. The same group investigated the optimal condition for high level expression of rhGH. As a result, by induction with C_{MeOH}=3% (v/v) throughout 3 days of operation, they achieved 190 mg L⁻¹ rhGH production in a complex medium.

In our research group, Çalık *et al.* (2008) constructed an expression system using pPICZ α A vector carrying hGH gene under the control of AOX1 promoter. Thereafter, they investigated the carbon source effects on rhGH production by using two *P. pastoris* strains; Mut⁺ and Mut^S. For this purpose, the defined medium containing 30 g L⁻¹ glycerol with 1% (v/v) methanol was used. Consequently, 110 mg L⁻¹ rhGH was produced by using Mut⁺ strain (Orman *et al.*, 2009). Açık (2009) developed a strategy abbreviated as MSS-0.03 for rhGH production by *P. pastoris* Mut⁺ strain. In this strategy methanol was fed with the specific growth rate of $\mu=0.03$ h⁻¹ simultaneously with pulse sorbitol feeding at t=0 h and at t=9 h in order to provide 50 g L⁻¹ sorbitol in the fermentation medium. The highest rhGH was produced at t=30h as C_{rhGH}=301 mg L⁻¹. Thereafter, Inankur (2010) applied a strategy where sorbitol was fed to the bioreactor in three pulses at t=0 h, t=14 h, and t=31 h, in order to provide 50 g L⁻¹ sorbitol in the medium simultaneously together with the methanol feeding at the specific growth

rate of $\mu_0=0.03 \text{ h}^{-1}$, thereupon, 290 mg L^{-1} rhGH was obtained at $t=39 \text{ h}$ of the process. Çalık *et al.* (2010a) studied the influences of methanol feeding rates on bioprocess characteristics in the presence of sorbitol. In this context, rhGH productivity was investigated under three different specific growth rates (μ) on methanol which are; $\mu_0=0.02 \text{ h}^{-1}$, $\mu_0=0.03 \text{ h}^{-1}$, and $\mu_0=0.04 \text{ h}^{-1}$. In Çalık *et al.* (2010a), the highest rhGH production was obtained during feeding methanol at the predetermined growth rate of $\mu_0=0.03 \text{ h}^{-1}$ as $C_{\text{rhGH}}=270 \text{ mg L}^{-1}$. Also Çalık *et al.* (2010b) studied the influences of pH operation conditions within pH 4.0-6.0; and their effects on rhGH production by *P. pastoris* in the presence of sorbitol simultaneously together with methanol feeding at the predetermined growth rate of $\mu_0=0.03 \text{ h}^{-1}$. Among these pH values, although the highest cell concentration was obtained at pH=6.0, the most favorable operation condition was found at pH 5.0 in terms of the rhGH production, protease formation and AOX activity. The highest rhGH concentration obtained at pH 5.0 as $C_{\text{rhGH}}=270 \text{ mg L}^{-1}$.

In this M.Sc. thesis the aim is to develop a feeding strategy in order to enhance rhGH production by recombinant *Pichia pastoris*. In this context, sorbitol is used as the co-substrate besides methanol. In the first part of this study, the common characteristic of the original designed strategies is, keeping sorbitol concentration constant within $t=0-15 \text{ h}$ of the induction phase at 50 g L^{-1} . Therefore, 50 g L^{-1} sorbitol was fed batch-wise at $t=0 \text{ h}$, and methanol was fed semi-batch simultaneously with the fed-batch feeding in addition to methanol. In the second part of the study, a feeding strategy reported by Jungo *et al.* (2007) used with a feed solution containing a mixture of sorbitol and methanol in water in a defined ratio; and the results were compared with the results of the original strategies applied in the first part of the M.Sc. The effects of the feeding strategies on the bioprocess and rhGH production were investigated, analyzed and compared with the literature.

CHAPTER 2

LITERATURE SURVEY

2.1 Target Product: Human Growth Hormone

Hormones are signaling chemicals released into the blood stream in small amounts to reveal a typical physiological response in other cells named as target cells. These chemical messengers help the body to carry out vital functions such as growth, development, reproduction, regulating blood glucose level and blood pressure (Rushton 2009).

Hormones are classified into two groups: steroid hormones and non-steroid hormones which include proteins such as insulin and growth hormone. Human growth hormone (hGH) is secreted by somatotroph cells within the anterior pituitary gland in a pulsatile fashion and regulated by the two hormones, i.e., growth hormone releasing hormone which stimulates the secretion of hGH and somatostatin which inhibits the secretion by back regulation (Saugy *et al.* 2006). It exerts its biological activity by binding to specific receptors on target cells (Kelly *et al.* 1991). Growth hormone can be considered as an anabolic hormone and affects the body directly or indirectly. The biological effects associated with hGH on human body are summarized in Table 2.1.

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

Metabolic	Stimulates amino acid transport Stimulates protein synthesis in most cell types Stimulates DNA/RNA synthesis in most cell types Stimulates polyamine synthesis Stimulates lipolysis Inhibits insulin action on glucose metabolism
Physiological	Increases renal blood flow, glomerular filtration rate and tubular reabsorption of PO ₄ Increases basal metabolic rate Stimulates new bone formation Stimulates erythropoiesis Expands extracellular fluid space
Anatomic	Accelerates linear growth Reduces adipose mass and enlarges lean body mass (muscle, liver, kidney, heart, GI tract, pancreas, skeleton, connective tissue)

2.1.1 History

The history of human growth hormone (hGH) has its origin from 1920s. It was described by Evans and Long (1921) and isolated from the human pituitaries in 1956 (Krysiak *et al.* 2007). The first use of growth hormone (GH) as a treatment for GH deficiency was reported in 1958 (Raben 1958). For a long time the only way of obtaining GH was by its extraction from the cadaver pituitaries and the clinical application was limited with the children who suffers from growth hormone deficiency disease (Cazares-Delgado *et al.* 2011). Cadaver-GH was

used until 1985 and ceased when the Creutzfeld-Jacob disease was diagnosed in the individuals who received GH treatment (Hintz 1995).

The developments in molecular biology enabled the first recombinant human growth hormone (rhGH; somatropin) expression by *E. coli* in 1979 (Goeddel *et al.*, 1979). In 1985, Gray *et al.* (1985) achieved to produce rhGH in a native form by using the host *E. coli* (Gray *et al.*, 1985); consequently, in 1985 US Food and Drug Administration (FDA) approved Genentech Inc. (San Francisco, CA) to market recombinantly produced human GH (rhGH) (Flodh 1986). Although until 1985, GH treatment was limited to the children with growth hormone deficiency, up to today several indications were approved by FDA, the indications are given in Table 2.1 (Kemp and Frindik 2011).

Table 2.2. Approved indications for GH use by FDA

Indication	Year of approval
GH-deficiency state	
Childhood growth- hormone deficiency	1985
Adult growth-hormone deficiency	1996
Pubertal dosing	2000
Non-GH-deficiency states	
Chronic kidney disease	1993
Turner syndrome	1996
AIDS wasting	1996
Prader-Willi syndrome	2000
Small for gestational age	2001
Idiopathic short stature	2003
Small bowel syndrome	2004
SHOX deletion	2006
Noonan syndrome	2007

2.1.2 Structure and Isoforms

Human growth hormone, also known as somatotropin, is non-glycosylated single-chain polypeptide, is synthesized, stored and secreted by somatotroph cells within the lateral section of the anterior pituitary (Cazares-Delgadillo, Ganem-Rondero and Kalia 2011). hGH is a heterogeneous protein consisting of several molecular isoforms (Baumann 1991). The human genome carries two genes encoding for GH (Hirt *et al.*, 1987) where the genes are located in q22-24 region of the chromosome 17. The products of this two distinct genes on chromosome 17 are named as *GH-1* and *GH-2* and they are expressed principally in somatotroph cells of pituitary gland and in the syncytiotrophoblast of placenta, respectively (Popii and Baumann 2004).

The largest intact molecule encoded by GH-1 gene is what is typically referred to as hGH and is the most predominant hormone of anterior pituitary. It has a molecular weight of 22 kDa and consist of 191 amino acid residues (Havel *et al.*, 1989) which folds into four helical twisted bundle structure comprising two disulfide bridges; one connecting distant parts of the molecule involving amino acid residues 53 and 165 forming a large loop, another between residues 182 and 189 forming a small loop (Figure 2.1 and Figure 2.2) (Patra *et al.*, 2000). For the small loop of hGH, it is reported that the integrity of the loop is nonessential for secretion (Chen *et al.*, 1992) and biological activity of the hormone (Graf *et al.*, 1976; Graf *et al.*, 1975).

The α -helices run up-up-down-down topology where the aminoacid residues from 35 to 71 links helix 1 to helix 2 and from 129 to 154 links the helix 3 and helix 4. In addition to these there are two short helices (residues K38-N47 and R64-k70) in the crossover connection of helix 1 and helix 2 which are involved in contact of hormone and receptor and one (residue R-94-S100) in the short linker between helix 2 and helix 3 (de Vos *et al.* 1992; Abdel-Meguid *et al.* 1987). The core of hGH mostly made up of hydrophobic side chains and the pI ranges of hGH is between 4.9-5.1 and the net charge at pH 7.0 is -4.9 (Binkley 1994).

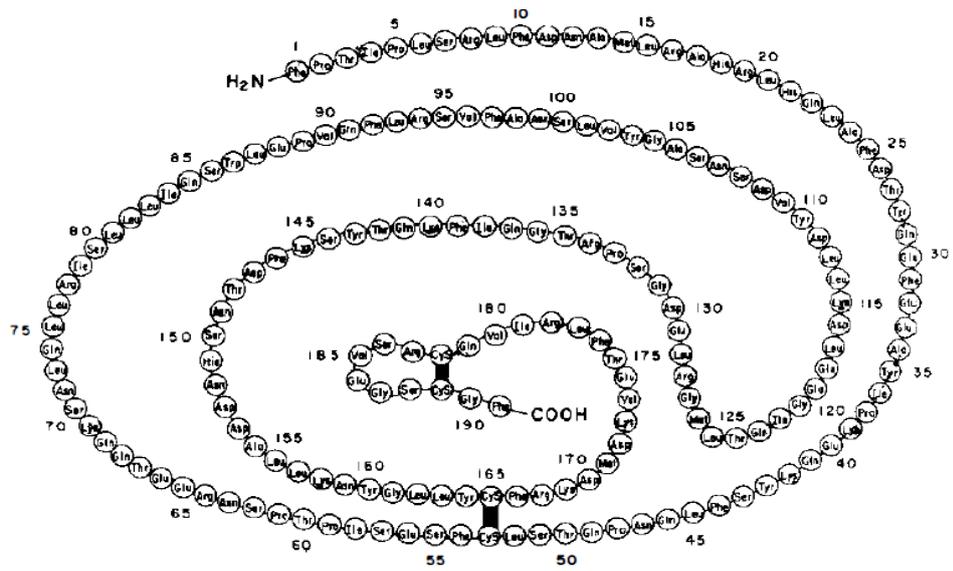


Figure 2.1 Covalent structure of hGH (Chawla *et al.*, 1983)

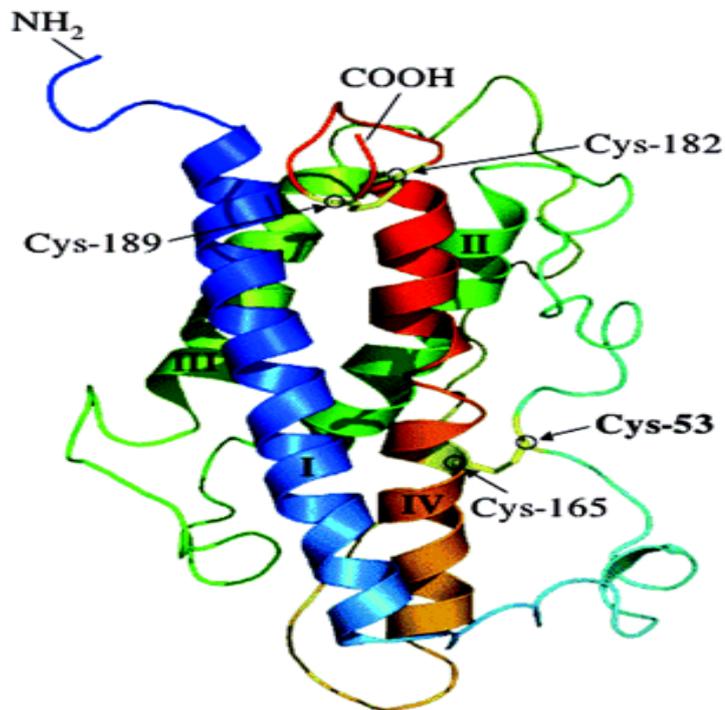


Figure 2.2 Three dimensional model of hGH: The four α -helices are shown, numbered I-IV; the four cysteines are shown with the disulfide bonds they form (Besson *et al.*, 2005).

20 kDa isoform of GH is yielded by the alternative splicing of GH1 gene and it differs from 22 kDa hGH with deleted 15 aminoacids (residues 32-46) at the internal sequence. This variant accounts for 5-10% of the total growth hormone production. It has generally less biological activity and diminished metabolic activity, binds to GH receptors with lower affinity when compared with 22 kDa GH (Popii and Baumann, 2004).

Other isoforms include glycosylated hGHs with molecular weights of 12 kDa and 24 kDa (Haro *et al* 1996), oligomeric hGHs, a 35 kDa hGH, and cleaved hGHs with molecular weights of 5 kDa and 17 kDa (Baumann 1991).

2.2 Yeast Expression Systems

Microbial organisms and eukaryotic cells have been used increasingly for the expression of heterologous proteins for medical or industrial use (Idiris *et al.*, 2010). The choice of the expression system depends on the characteristics and the applications of the compound to be expressed which are determined by the economical basis for the production of enzymes or by safety criteria for the production of pharmaceutical proteins (Strasser and Gellissen 1998). From this point, yeasts offer advantages over other expression systems. Due to the ease of genetic manipulation; sharing molecular, genetic and biochemical characteristics with higher eukaryotes; ability of posttranslational modifications; rapid growth on inexpensive media and not comprising pyrogens and pathogens, yeasts are suitable organisms for the production of recombinant proteins (Gellissen, 2000).

Several proteins originated from human, animal, plant, bacteria and virus have been successfully expressed by the yeast expression systems over 20 years (Demain and Vaishnav, 2009). In the literature there are several examples demonstrating the ability of yeasts to secrete mature human proteins having the expected biological properties, e.g., single chain antibody fragments (Damasceno *et al.*, 2004; Hackel *et al.*, 2006; Braren *et al.*, 2007), interferon α -2b (Shi *et al.*, 2007) and neuromedin U subtype II receptor (Shukla *et al.*, 2007).

The baker's yeast *Saccharomyces cerevisiae* was the first yeast system applied for the production of heterologous proteins and was successfully used in the production of several FDA-approved pharmaceuticals such as insulin and HBsAg (Gellissen *et al.*, 2005). In the early 1990 it obtained GRAS status (Generally Recognized as Safe) (Goffeau *et al.*, 1996). However the limitations such as tendency to hyperglycosylate the recombinant proteins and allergenic mannosylation led to characterization of alternative yeast expression systems (Böer *et al.*, 2007). After identification of methylotrophic yeasts, *Pichia pastoris* gained an important role in heterologous protein production.

2.3 Microbial Organisms for rhGH Production

Escherichia coli has been the first and most reported host microorganism for the production of rhGH (Goeddel *et al.*, 1979; Gray *et al.*, 1985; Becker and Hsiung, 1986; Kato *et al.*, 1987). However, the high level expressions of rhGH results in inclusion body formation (Mukhija *et al.*, 1983). Therefore, requirement of additional steps such as solubilization and purification where high concentrations of chaotropic reagents are used, make the process by r-*E.coli* more expensive and complex; whereupon, lowers the bioactivity of rhGH (Shin *et al.*, 1998). Besides, disulfide bridge formation is inhibited because of the reducing environment of bacterial cytosol, and initiation of desired products with formylmethionine (fMet) may play role in antibody formation in patients treated with the product. As an alternative to *Escherichia coli*, *Bacillus subtilis* has also been used for rhGH production (Nakayama *et al.*, 1988; Franchi *et al.*, 1991; Özdamar *et al.*, 2009). There are some advantages using *Bacillus* species as the host microorganism such as ability to secrete functional proteins directly to the medium, being lack of pathogenicity and the absence of endotoxins from the cell wall (Simonen and Palva, 1993). However high protease production is the major problem which reduces recombinant protein production efficiency. To overcome this problem either protease inhibitors are added (Özdamar *et al.*, 2009) or protease deficient strains are used.

The first study in the production of recombinant human growth hormone (rhGH) by *Pichia pastoris* was performed by Trevino *et al.*, (2000). They achieved to produce mature and active rhGH with a concentration of 49 mg L⁻¹ in a 2L bioreactor system. In the study reported by Eurwilaichitr *et al.*, (2002), rhGH was produced by using three different vectors and moreover the optimum medium condition was determined as complex medium with 3% (v/v) methanol concentration. Consequently, 190 mg L⁻¹ rhGH was obtained after 3 days of induction. Çalık *et al.*, (2008) constructed a plasmid carrying human growth hormone gene; *pPICZαA::hGH*. The plasmid was integrated to the genome of two different strains of *Pichia pastoris*; Mut⁺ and Mut^s. The comparison of the two strains was studied in batch cultivations and the highest concentration 110 mg L⁻¹ rhGH was obtained by Mut⁺ strain in a defined medium consisting of 1% (v/v) methanol with 30 g L⁻¹ glycerol (Orman *et al.*, 2009). Açık (2009) and Inankur (2010) used *P.pastoris*-Mut⁺ strain for rhGH production and developed strategies which were abbreviated as MSS-0.03 and MSSS-0.003, respectively. At MSS-0.03, two pulses of sorbitol was fed to the bioreactor at t=0 h and t=9 h and at MSSS-0.03 three pulses of sorbitol was fed to the bioreactor at t=0h, t=14 h and t=31 h of induction phase in order to provide 50 g L⁻¹ sorbitol in the medium. In addition to sorbitol pulse feeding, methanol was fed to the sytem with the predetermined growth rate of $\mu_0=0.03$ h⁻¹ at both strategies. As a result, the highest rhGH production was achieved at t=30 h as C_{rhGH}=301 mg L⁻¹ at MSS-0.03 and at t=39h as C_{rhGH}=290 mg L⁻¹ at MSSS-0.03. Çalık *et al.*, (2010a; 2010b) investigated the effects of different methanol feeding rates and pH on rhGH production by using *P.pastoris* Mut⁺ strain. In those studies, since sorbitol was found as the non-inhibitory co-substrate to use in methanol fed-batch experiments, it was fed to the bioreactor batch-wise in order to provide 50 g L⁻¹ sorbitol. Among the different specific growth rates, feeding methanol at $\mu_0=0.03$ h⁻¹ was found as the optimal condition where the highest rhGH concentration was achieved as 270 mg L⁻¹. In addition to this, among the different pH values the

optimal pH was found as 5.0 for the production of rhGH where the highest rhGH concentration was obtained as 271 mg L⁻¹.

2.3.1 Host Microorganism: *Pichia pastoris*

2.3.1.1 Background

Methylotrophic yeasts are first described by Ogata *et al.*, in 1969. Taxonomic studies carried out afterwards, characterised the four genera; *Candida*, *Hansenula*, *Pichia* and *Torulopsis* (Lee and Komagata 1980) . The yeast species belong to these genera have the ability to utilize methanol as sole energy and carbon source. *P. pastoris* was first discovered about four decades ago during the search of sources for single-cell protein (SCP) production which was abandoned; the media and the protocols for the growth was developed by Philips Petroleum Company (Cereghino and Cregg, 2000).

In 1980s, Philips Petroleum Company and Salk Institute of Biotechnology/ Industrial Associates Inc. (SIBIA, La Jolla, CA) started to study *P. pastoris* as a system for heterologous protein production. Vectors, strains and protocols were developed by SIBIA. Since then it has become an important biological tool in recombinant protein production and a model eukaryote system used in cell biology research. Up to now with the developments in metabolic engineering techniques over 500 recombinant proteins were expressed in *P. pastoris* (Plantz *et al.*, 2006). Today the current patent holder of *Pichia* system is still Research Corporation Technologies (Tucson, AZ) since 1993. The recent important advance is the approval of the first *P.pastoris* produced therapeutic, KALBITOR[®] (ecalantide) by Food and Drug Administration (Walsh, 2010).

2.3.1.2 Properties

Pichia pastoris (Figure 2.3) is a mesophilic and methylotrophic yeast that has an ability to live at temperatures around 25-35°C and metabolize methanol as sole energy and carbon source (Macauley-Patrick *et al.*, 2005). The culture of this microorganism can be grown at a broad range of pH 3 to 7 (Cregg *et al.*, 2000). It

has the general yeast characteristics, i.e., being unicellular, having diameter of 1-5 μm wide and 5-30 μm long and having typical eukaryotic cell structure with a thick polysaccharide cell wall. In addition to these instead of fermentative it prefers respiratory mode of growth thus by-products such as ethanol and acetic acid does not create such problems as seen in *S. cerevisiae*. The taxonomy of the *Pichia pastoris* is shown in Figure 2.4.

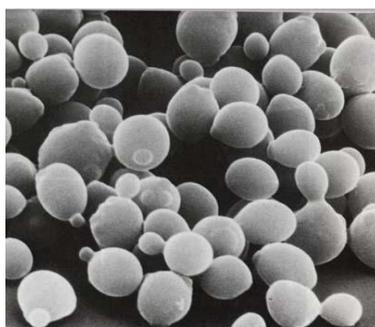


Figure 2.3 Microscopic view of *Pichia pastoris*

P. pastoris has become a very popular and widely used yeast host due to its several advantages over other microorganisms. The factors that make this system popular can be specified as; it does not require complex growth medium (Macauley-Patrick *et al.*, 2005) which makes it less expensive when compared to mammalian cells and can be reached to high cell densities by using minimal media (Wegner, 1990); as it is a single-celled eukaryote, it enables the genetic manipulations and has the ability to perform post-translational modifications such as disulfide bond formation, folding, proteolytic processing, and glycosylation thereby, proteins produced as inclusion bodies in the bacterial systems can be produced biologically active form in *Pichia* system (Cereghino *et al.*, 2002; Jahic *et al.*, 2006), Foreign proteins, even with high molecular weight, expressed in this system can be directed to extracellular media through signal peptides (Cereghino and Cregg, 2000) which then simplifies the downstream processing.

The most important fact about *P. pastoris* is, it comprises an alcohol oxidase (AOX1) promoter which is known as the strongest and tightly regulated eukaryotic promoter (Cereghino and Cregg, 1999). The AOX1 promoter is induced in the presence of methanol as sole carbon source and repressed in the presence of repressing carbon source, such as glucose in the medium.

Although it is an effective system, since the flammable and explosive properties, storage of methanol may presents challenges for the large-scale operations and health risks (Paulová *et al.*, 2012).

Fungi (Kingdom)
Eumycota (Division)
Ascomycotina (Subdivision)
Hemoascomycetes (Class)
Endomycetales (Order)
Saccharomycetaceae (Family)
Saccharomycetoideae (Sub-family)
Pichia pastoris

Figure 2.4 Taxonomy of the *Pichia pastoris* (Gellissen *et al.* 2005)

Besides methanol having a fire hazard characteristic and difficulty in monitoring during a process, there are other disadvantages of *Pichia pastoris* system such as long cultivation time compared to bacteria, non-native glycosylation and potentiality of proteolysis.

2.3.1.3 AOX1 and Alternative Promoters

The use of yeast promoters was indicated as essential for an efficient transcription of a foreign gene (Hitzeman *et al.*, 1981). The popularity and widely use of *Pichia pastoris* expression system stems from its success in production of heterologous protein production which is directly related with strongly and tightly regulated promoter alcohol oxidase I (AOX1).

Pichia pastoris genome contains two genes that encode alcohol oxidase: *AOX1* and *AOX2* (Cereghino and Cregg, 2000). *AOX1* was isolated by Ellis *et al.*, (1985) and *AOX2* gene was discovered by Cregg and Madden (1987); thereafter its isolation and physical characterization was studied by Cregg *et al.*, (1989). In the study of Koutz *et al.*, (1989) it was found out that the protein coding portions of *AOX1* and *AOX2* are greater than 90% homologous at both DNA and predicted amino acid sequence, whereas the difference in sequence located at 5' of the protein coding portion of the two genes resulted in AOX activity differences. The *AOX1* promoter is responsible for 85% of alcohol oxidase activity in the cell while *AOX2* is less active (Cregg *et al.*, 1989). *AOX1* gene is regulated in the transcriptional level and repressed by glucose, glycerol and ethanol, whereas totally induced when the culture is shifted to the fermentation period where only methanol was fed as carbon source (Cregg *et al.*, 2000). With the benefit of this characteristic, *AOX1* promoter can be switched off and selecting non-expressing mutants/contaminants can be minimized during biomass formation (Daly and Hearn, 2005).

In methanol-grown cells the portion of the *AOX1* enzyme can reach 30% of total cellular protein (TCP) content which is possibly due to the low affinity of *AOX1* enzyme for oxygen and so compensation of cells by producing large amount of the enzyme (Gellissen, 2000; Cregg *et al.*, 1993). Besides the favorable characteristics of *AOX1* promoter system, some disadvantages were reported in the literature which are originated from methanol usage. Since the methanol is a petrochemical product, the usage of methanol is not suitable for the food industry as well as storing the large quantities is undesirable (Macauley-Patrick *et al.*, 2005).

With regard to methanol-utilizing capacity, there are three phenotypes of *Pichia pastoris* expression strains (Stratton, Chiruvolu, and Meagher, 1998) which are summarized below:

- **Methanol utilization plus (Mut⁺) phenotype:** grow at the wild-type rate on methanol, requires large amount of methanol but very

sensitive to the high concentrations. Both *AOX1* and *AOX2* genes are active but most of the AOX activity relies on *AOX1* promoter. The maximum specific growth rate for Mut^+ strain is found as $0.14\ h^{-1}$ (Jungo *et al.*, 2006).

- **Methanol utilization slow (Mut^s) phenotype:** contains non-functional *AOX1* gene and relies on *AOX2* gene. *AOX2* and *AOX1* have the same activity however *AOX2* has a lower expression level and consume methanol slowly. In some cases Mut^s strain is found as advantageous due to the alleviation of oxygen limitations and difficulties of high cell density cultivation of rapid growth strains (Cos *et al.*, 2005). The maximum specific growth rate of Mut^s on methanol is found to be $0.04\ h^{-1}$ (Jungo *et al.*, 2006).
- **Methanol utilization minus (Mut^-) phenotype:** defective in both *AOX1* and *AOX2* and unable to grow on methanol (Chiruvolu, Gregg, and Meagher, 1997). The maximum specific growth rate for Mut^- strain is $0.0\ h^{-1}$ (Jungo *et al.*, 2006).

Alternative promoters that are not induced by methanol have been also taking attention. Waterham *et al.* (1997) isolated the glyceraldehyde-3-phosphate dehydrogenase (GAP) gene. GAP promoter enables the cell to grow without the need of methanol induction, and there is no need to switch the cultures from one carbon source to another (Daly and Hearn 2005; Cereghino and Cregg, 2000). The other alternative promoter is the glutathione-dependent formaldehyde dehydrogenase (FLD1) which involves in methanol dissimilatory pathway and can be induced with either methanol or methylamine (Cereghino and Cregg, 2000). Moreover, glycerol or glucose can be used not only as the carbon source but also for the induction instead of methanol (Resina *et al.*, 2004). The other two promoters are *PEX8* encodes for a peroxisomal matrix protein and *YPT1* encodes for GTPase.

2.3.1.4 Glycerol, Methanol and Sorbitol Metabolism

In the glycerol utilization pathway the first reaction is the phosphorylation of glycerol to glycerol-3-phosphate (G3P) under the control of glycerol kinase. In the pathway, G3P is oxidized to dihydroxyacetone phosphate, which enters to the glycolytic pathway, by FDA-dependent glycerol-3-phosphate dehydrogenase. The product of glycolysis, pyruvate, is further oxidized to acetyl-CoA by the enzyme pyruvate dehydrogenase. As the acetyl-CoA enters to the citric acid (the TCA or Krebs) cycle, many metabolites are produced for the formation of cellular and cell wall components (Ren *et al.*, 2003). Most of the energy needed by the cells for maintenance and growth is also yielded in the TCA (Ratledge, 2001). Utilization of glycerol and the pathways is given in Figure 2.5.

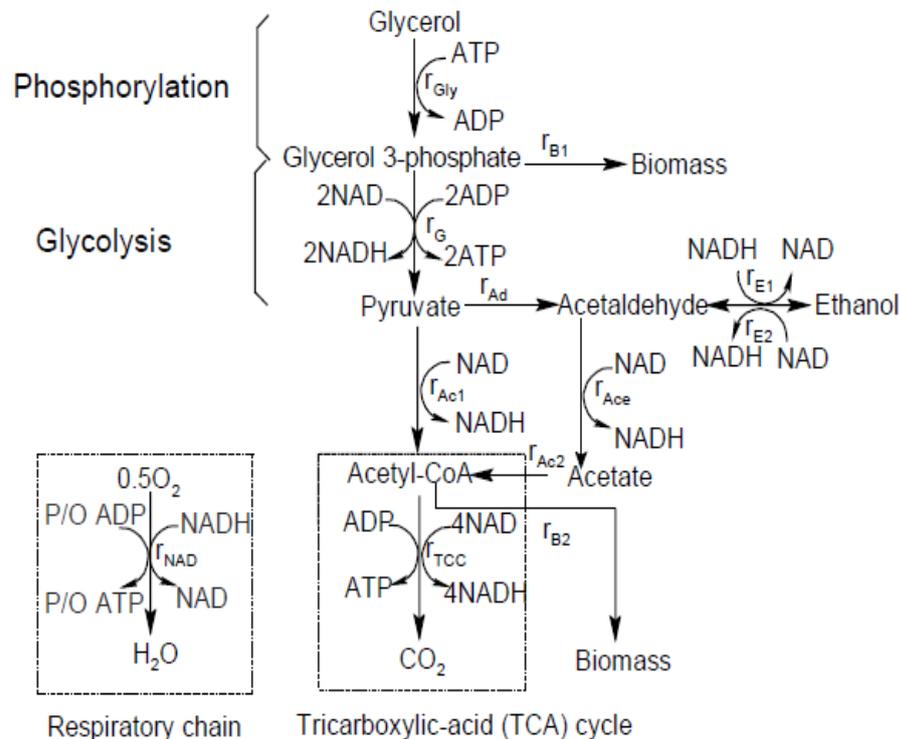


Figure 2.5 Metabolic pathways of glycerol in *Pichia pastoris* (Ren *et al.*, 2003)

Pichia pastoris has a compartmentalized methanol-metabolic pathway as shown in Figure 2.6. The key enzymes of this metabolism; AOX (alcohol oxidase), FMD (formate dehydrogenase) and DHAS (dihydroxyacetone synthase), are present in high amounts when the cells grown on methanol (Gellissen *et al.*, 1992). Methanol utilization starts with oxidation of methanol to formaldehyde and hydrogen peroxide. This reaction is catalyzed by the enzyme alcohol oxidase (AOX) which is sequestered in peroxisome in order to avoid toxicity of hydrogen peroxide.

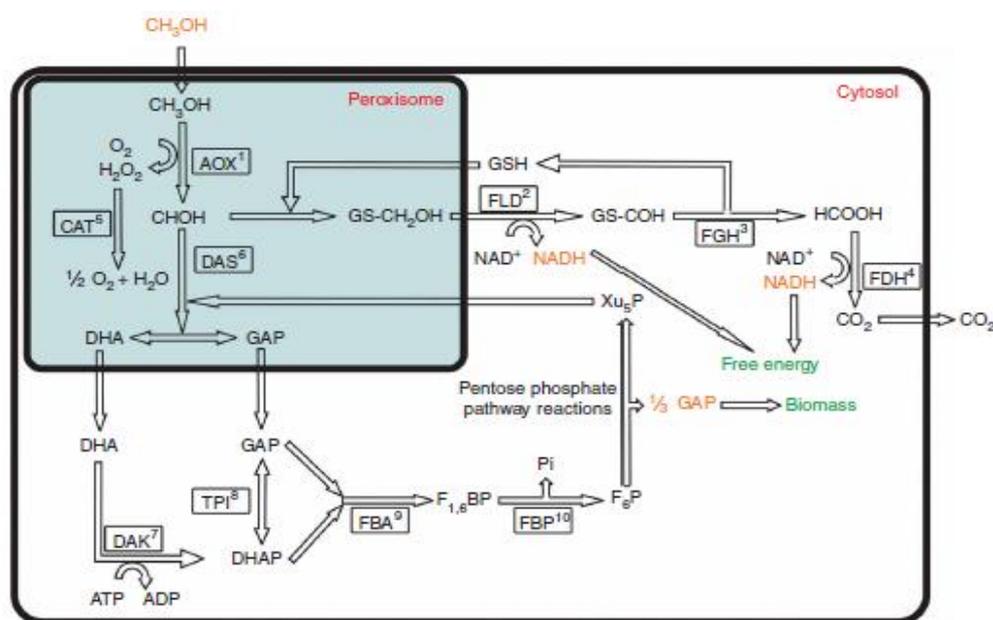


Figure 2.6 Methanol utilization pathway in *Pichia pastoris*. ¹AOX, alcohol oxidase; ²FLD, formaldehyde dehydrogenase; ³FGH, S-ormylglutathione hydrolase; ⁴FDH, formate dehydrogenase; ⁵CAT, catalase; ⁶DAS, dihydroxyacetone synthase; ⁷DAK, dihydroxyacetone kinase; ⁸TPI, triosephosphate isomerase; ⁹FBA, fructose-1,6-bisphosphate aldolase; ¹⁰FBP, fructose-1,6-bisphosphatase; DHA, dihydroxyacetone; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; Pi, phosphate; Xu_5P , xylulose-5-phosphate; GSH, glutathione (De Schutter *et al.* 2009).

Formation of formaldehyde from methanol is reported as a rate-limiting step and the regulation is achieved with the increasing amount of AOX enzyme which is demonstrated in the study of Couderc *et al.*, (1980) (Couderc and Baratti, 1980). The hydrogen peroxide which is the by-product of AOX activity is degraded to oxygen and water by peroxisome catalase (Gellissen, 2000). A portion of the formaldehyde is further oxidized either yielding energy by the dissimilatory pathway or generating the cell by the assimilatory pathway (Cereghino and Cregg, 2000). The assimilatory pathway starts with the activation of DHAS which condensates formaldehyde with xylulose-5-monophosphate ending up with the formation of glyceraldehydes-3-phosphate (GAP) and dihydroxyacetone (DHA). And then, GAP enters the TCA cycle, yielding further energy in the form of ATP and NADH; and on the other side, DHA enters xylulose monophosphate cycle yielding xylulose 5-phosphosphate and the cell. In the dissimilatory pathway, enzymes play role in energy production and detoxification of formaldehyde and formate. Formaldehyde firstly oxidized to formate by NAD⁺-dependent formaldehyde dehydrogenase (FLD) then formate is oxidized by NAD⁺-dependent formate dehydrogenase (FDH) to carbon dioxide. In both reactions NADH is generated and used in energy production for the growth on methanol (Hartner and Glieder, 2006).

In order to improve of foreign protein production by *P. pastoris*, the use of multicarbon substrate besides methanol has been reported many times in the literature (Cregg *et al.* 1993; Loewen *et al.*, 1997; McGrew *et al.*, 1997; Katakura *et al.*,1998; Zhang *et al.*,2003). Due to the inhibition effect of excess glycerol, other carbon sources rather than glycerol took the attention. For instance, as the non-repressing carbon source, sorbitol has been used to improve the fed-batch strategy with recombinant *Pichia pastoris* strains (Thorpe *et al.*,1999; Jungo *et al.*,2007; Wang *et al.*,2009).

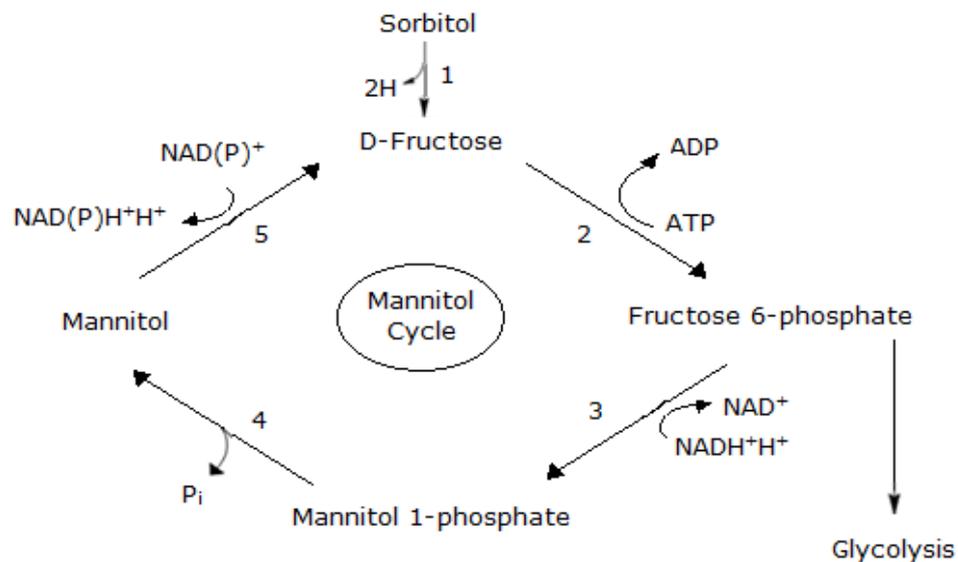


Figure 2.7 Metabolic pathway of sorbitol and mannitol in yeasts. 1, D-glucitol dehydrogenase; 2, fructokinase; 3, mannitol-phosphate dehydrogenase; 4, phosphatase; 5, mannitol dehydrogenase (Walker 1998).

In this study, sorbitol was used as co-substrate to enhance the production whose utilization begins with the oxidation to fructose by sorbitol dehydrogenase; thereafter enters the mannitol cycle (Figure 2.7). Fructose is phosphorylated to fructose-6-phosphate via fructokinase and enters glycolysis pathway (Walker, 1998).

2.3.1.5 Posttranslational Modifications and Protein Secretion

When compared to bacterial expression systems, *Pichia pastoris* exhibits major advantages in performing many post-translational modifications as; disulfide bridge formation, correct folding, *O*- and *N*-linked glycosylation and signal sequence processing (Cregg *et al.*, 2000; Macauley-Patrick *et al.*, 2005).

Secretion Signals

One of the important consideration in recombinant protein production is the choice of the extracellular secretion or intracellular expression which depends

on the characteristic of the interested protein. Intracellular expression can be alternative to secretion in the case of the interested protein is not secreted in its native system however extracellular production of foreign proteins occur to be more favorable to simplify the downstream porcess (Daly and Hearn, 2005). For the secretion of proteins into the culture medium, a specific signal sequence is required. This signal sequence can be the protein's own native secretion signal, the acid phosphatase signal sequence (PHO), the invertase signal sequence (SUC2) or *S.cerevisiae* alpha-mating factor pre-pro leader sequence (α -MF) (Li *et al.*,2001). The most widely used and highly successful signal sequence is *S.cerevisiae* α -MF which presents in many of the *P.pastoris* expression systems (Sreekrishna *et al.*, 1997) and can lead to higher amount of foreign protein secretion than using the native signal peptide while the product secreted by its native signal was found as more biologically active (Daly and Hearn, 2005). *S.cerevisiae* α -MF consist of a pre-sequence signal peptide of 19 amino acids and a pro-region of 60 hydrophilic amino acid (Julius *et al.*,1984; Brake *et al.*, 1984). During the translocation process within the protein secretion pathway, the signal peptide sequence is cleaved by signal peptidase and the pro-region is cleaved by the dibasic aminopeptidase, kex2 protease resulting in releasing the mature protein (Daly and Hearn, 2005).

Protein Folding and Disulfide Bond Formation

Disulfide bond formation and proper folding are important steps in post-translational modifications and moreover in some cases identified as rate-limiting step for foreign protein production (Hohenblum *et al.* 2004). In protein folding, firstly secondary structures are being formed (Hlodan and Hartl, 1994) and afterwards, in endoplasmic reticulum (ER) a rapid generation of disulfide bonds occurs (Holst *et al.*,1996).

Protein folding process in ER is under the strict quality control (QC) and only properly folded proteins are exported from ER to Golgi apparatus (Klausner 1989). When mis-folded or improperly processed proteins are acumulated in ER, the ER-associated degradation is activated in order to reduce the stress and

mis-folded proteins are eliminated (Wu and Kaufman, 2006). When there is no stress, nascent peptides are maintained in a soluble form by the ER-resident chaperons, e.g. immunoglobulin binding protein (BiP), and the immature proteins are stabilized and prevented by these chaperons not to form aggregates with unfolded/misfolded proteins (Damasceno *et al.*, 2011). Since protein folding in ER is reported as the major bottleneck due to the strict QC system several strategies such as overexpression of protein disulfide isomerases (PDIs), multiple chaperons and other folding helpers have been applied to increase secretion of proteins (Idiris *et al.* 2010).

N- and O-Linked Glycosylation

Protein glycosylation is the major posttranslational modification performed by *P.pastoris*. Glycosylation is important for structural stability, protein folding, specific signal transduction, oligomer assembly, recognition and secretion processes (Wright and Morrison, 1997; Jenkins *et al.*, 1996).

Although *P.pastoris* is capable of performing *O*- and *N*-linked glycosylation, there are many differences when compared to mammalian cells. In mammals *O*-linked oligosaccharides are mainly composed of galactose, sialic acid, and *N*-acetylgalactosamine whereas *P.pastoris* adds only mannose residues linked α -1,2 in arrangement (Cereghino and Cregg, 2000; Duman *et al.*, 1998). It is known that the *O*-linked glycans are added to the hydroxy-amino acids such as serine and threonine and initiated in ER by protein-*O*-mannosyltransferases (Schutter *et al.*, 2009) yet, mainly occur in Golgi apparatus (Dennis *et al.*, 1999).

N-linked oligosaccharide pathway is conserved in yeast and most higher eukaryotes. The glycosylation pathway in human and yeast is shown in Figure 2.8. After protein translation, glycosylation takes place in the lumen of endoplasmic reticulum where glycan portion of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ -PP-dolichol lipid-linked precursor is transferred to suitable asparagine residue at the recognition site Asn-X-Ser/Thr on nascent polypeptide (Gemmill and Trimble, 1999). Subsequently three glucose residues are removed by glycosidase I and II. $\text{Man}_8\text{GlcNAc}_2$ is formed after removal of α -1-2-linked mannose residue by α -1-

2-mannosidase (Daly and Hearn, 2005). These steps are conserved between yeast and mammalian cells however glycosylation patterns begin to differ when the glycosylated protein is transported to Golgi for further processing.

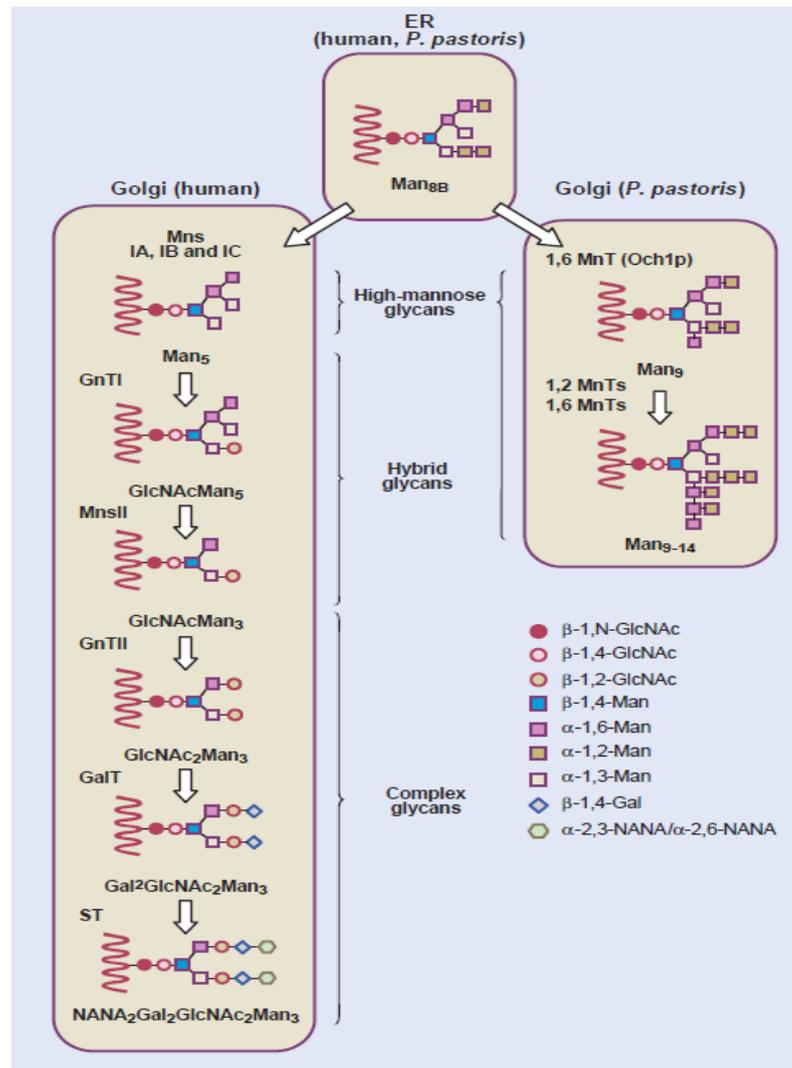


Figure 2.8 Glycosylation pathways in human and yeast. Mns, α -1,2-mannosidase; MnsII, mannosidase II; GnTI, β -1,2-*N*-acetylglucosaminyltransferase I; GnTII, β -1,2-*N*-acetylglucosaminyltransferase II; GalT, β -1,4-galactosyltransferase; SY, sialyltransferase; MnT, mannosyl-transferase (Gerngross 2004).

Mammalian cells rely on additional α -1-2-mannosidase to trim mannose residues (Tulsiani serine and threonine and initiated in ER by protein-*O*-mannosyltransferases (Schutter *et al.*,2009) yet, mainly occur in Golgi apparatus (Dennis *et al.*,1999).1982), while yeasts initiate a series of mannosyltransferase reactions yielding hypermannosylated glycan structures (Gerngross, 2004). For this reason, therapeutic glycoprotein production in yeasts is hampered. Yeast *N*-glycosylation confers a short in vivo half-life to the protein and reveals an immunogenic response to the foreign carbohydrate moiety (Helenius and Aebi, 2001). Among the yeasts, *P.pastoris*, is more favorable host system since the oligosaccharide chains are shorter than that of *S. cerevisiae*, and unlike *S. cerevisiae*, glycoproteins do not comprise of terminal α -1,3-linked mannose residues which increases the antigenic activity (Li serine and threonine and initiated in ER by protein-*O*-mannosyltransferases (Schutter *et al.*,2009) yet, mainly occur in Golgi apparatus (Dennis *et al.*,1999).2007). Moreover, with the developments in glycoengineering, *P.pastoris* strains were engineered with fully human *N*-glycosylation (Jacobs *et al.*, 2009). In regard to *S.cerevisiae* glycosylation pathway, Vervecken *et al.*, (2004) achieved to humanize the N glycosylation of *P.pastoris*. Moreover, Gerngross research group was able to produce fully functional recombinant human erythropoietin by glycoengineered *P.pastoris* (Damasceno *et al.*, 2011).

2.3.1.6 Proteolytic Degradation

One of the major drawback of *P.pastoris* is the post-secretory degradation of recombinant proteins which paralyse the efficient secretion and purification (Idiris *et al.* 2010). The stress conditions such as change of carbon sources, starvation, toxic chemicals or heat and pH changes can lead to proteolytic response (Hilt and Wolf 1992). It is reported that in the cell cultures grown on methanol, the extracellular and intracellular protease levels are higher than those on glycerol owing to the oxidative stress arising from high oxygen level requirement of methanol metabolism and production of hydrogen peroxide as the

by-product (Sinha *et al.* 2005; Potvin *et al.* 2010). Especially, in high cell density fermentation, the main factor in protein degradation is vacuolar proteases together with the cell lysis. There are four types of vacuolar proteases; PrA, PrB, CpY and aminopeptidase which are usually detected in the supernatants of *P.pastoris* cultures (Shen *et al.* 1998).

Proteolytic degradation can lead to reduction of product yield, loss of biological activity, contamination of the product by degradation intermediates in downstream processing (Jahic 2003). Several approaches such as controlling cultivation conditions (e.g., temperature, pH); addition of protease inhibitors, peptone, casamino acids or specific amino acids; changing medium composition (e.g., carbon and nitrogen sources) were studied in order to overcome the problems mentioned above (Enfors 1992; Gonzalez-Lopez *et al.* 2002; Jones 1991). However, most of these approaches are protein or host specific and limitedly effective and therefore more effective strains are being constructed with genetic manipulations. Several protease-deficient strains; SMD1168 (*his4 pep4*), SMD1165 (*his4 prb1*) and SMD1163 (*his4 pep4 prb1*) have been effectively used in heterologous protein production (Brierley 1998; White *et al.* 1995).

The effect of culture conditions has been shown in several studies. In the study of Li *et al.* (2001) besides the cell viability, the concentration of herring antifreeze protein was increased from 5.3 mg/L to 18 mg/L by decreasing temperature from 30°C to 23°C. A similar study was carried out by Dragosits *et al.* (2009) where the specific productivity of 3H6 Fab fragment was 3-fold higher in the chemostat culture performed at 20°C than that of at 30°C. Besides the temperature, another critical parameter is pH since protein formation, cell growth and protein stability also depends on the optimum pH. Related with this matter, Jahic *et al.* (2003) reported that expression of CBM (cellulose-binding module)-CALB (cellulose 6A and lipase B) in *P. pastoris* increased from 40 to 90% when pH of the bioreactor culture decreased from 5.0 to 4.0.

2.4 Fermentation Process Parameters

Fermentation processes are important for the production of secreted proteins since the concentration of the secreted protein in the bioreactor medium is roughly correlated with the cell density, whereas, in shake-bioreactor experiments high yield expression of heterologous protein is difficult owing to the limited volume, oxygen transfer limitation, substrate addition and inability for efficient monitoring of these factors (Macauley-Patrick *et al.*, 2005). Several factors at both genetic and cultivation level (Potvin *et al.*, 2010), e.g., properties of the desired protein, the host strain and its cultivation conditions, vector system, promoter choice, codon usage, processing, folding and secretion (Niebauer and Robinson, 2005) affect the expression of foreign proteins. Although the general protocols for *P. pastoris* fermentation are provided by Invitrogen, in many studies it is indicated that the optimization should be done according to the individual processes with regard to established principles.

2.4.1 Medium Composition

The composition of the medium is important in recombinant protein production since it affects the cell growth and viability (Kang *et al.*, 2000; Chen *et al.*, 2000).

Basal salt medium (BSM), provides higher concentrations of basic elements and commonly used for the high cell density fermentation of *P. pastoris*. Moreover it provides micronutrients such as Fe, Mn, Cu and biotin supplied with trace salt solution PTM1 which is proposed by Invitrogen (Invitrogen Co, 2002). Related with the medium composition, the study of O'Callaghan *et al.*, (2002) showed that, at least 200 μM presence of copper was necessary for the optimum laccase activity in *Pichia* culture. Similarly, Boze and coworkers (2001) demonstrated that for the improvement of porcine follicle-stimulating hormone production, supplementation of seven vitamins and two trace elements within a basal medium increased the product formation and cell growth in *P. pastoris*.

As a nitrogen source generally ammonium hydroxide, which also applied for adjusting pH to the desired level, is used. It is reported that the cell growth could be inhibited due to the prolonged lag phase in the case of increased ammonium concentration of 0.6 M and above within the medium (Yang *et al.*, 2004). On the other hand, the lack of nitrogen directly leads to increase in the proteolytic activity and protein degradation (Kobayashi *et al.*, 2000).

2.4.2. Temperature

For *P.pastoris* cultures optimum growth and production temperature is determined as 30°C and for the cultures above 32°C, a quick decrease in the cell growth and cessation in protein expression are reported (Cos *et al.*, 2006). On the other hand, some studies showed that when the induction temperature was reduced to 20°C or even to lower levels, heterologous protein production was improved and moreover cell lysis and proteolytic activity was reduced (Hong *et al.*, 2002; Dragosits *et al.*, 2009) besides the increase in AOX activity (Jahic *et al.*, 2003). In the work of Jin *et al.*, (2011) for the production of porcine interferon- α (pIFN- α), the induction temperature of the bioprocess was reduced to 20°C and performed under the same operation conditions with the process performed previously at 30°C. They reported that, the adaptation period of the cell was reduced from 6h to 1h; moreover, pIFN- α concentration, specific methanol consumption rate, specific activity of alcohol oxidase was enhanced by decreasing the induction temperature.

2.4.3 pH

pH is one of the crucial parameter in the production of recombinant proteins in genetically engineered yeasts. pH of the cell culture highly affects the cell growth (Chiruvolu *et al.*, 1999), specific expression rate (Roubin *et al.*, 1992), secretion efficiency (Bae *et al.*, 1999), and proteolytic degradation (Clare *et al.*, 1991; Song and Chung, 1999). Optimum pH shows differences between hosts

as well as proteins since proteins have different characteristics and proteases require different pH values for the optimum activity (Moon *et al.*, 2002).

Pichia pastoris cultures can grow within a wide range of pH values (pH = 3 to 7). It is indicated that this range does not affect the cell growth significantly and provides freedom in adjusting the pH (Wagner *et al.*, 1983; Inan *et al.*, 1999); on the other hand, pH values greater than 5.0 were pointed out to cause precipitation and operational problems such as starvation of nutrients and difficulty in OD measurement in the case of BSM use (Cos *et al.*, 2006). Sreekrishna *et al.* (1990) reported that the production of HSA was improved significantly by increasing the pH of the medium from 5.2 to 6.0. In the study of Soyaslan and Çalık, (2011), the highest production of rEPO by *P.pastoris* Mut⁺ strain was obtained when cultivation was performed at pH=5.0 in the glycerol-batch and glycerol fed-batch phases and at pH=4.5 in the induction phase; whereas, in a recent work of Çalık *et al.* (2010b) the highest rhGH was obtained when the bioreactor system was operated at pH 5.0.

2.4.4 Process Control Strategies

2.4.4.1. Fed-Batch Cultivation

In order to enhance recombinant protein production by *P. pastoris*, development of novel fermentation strategies have been vital research issues for bioreactor design (Loewen *et al.*, 1997; Cregg and Higgins, 1995). In general, fed-batch operation is applied since it helps to overcome several effects such as substrate inhibition, product inhibition, glucose effects, catabolite repression and auxotrophic mutations (Agrawal *et al.*, 1989; Yang, 1992).

A fed-batch bioprocess for *P. pastoris* under the control of AOX promoter involves three stages: the glycerol batch and fed-batch stages, and the methanol induction phase. In the first two phases *P. pastoris* formation is aimed until depletion of glycerol, and induction phase begins by feeding methanol to the bioreactor in order to induce the gene expression. Between the cell formation and methanol induction phases, an optional transition phase can be applied for the adaptation of

the cells to the second carbon source. During transition phase repressing factors of inductions; residual glycerol and metabolic waste, are removed which results in improvement of protein expression (Potvin *et al.*, 2010). Chen *et al.* (2004) reported that when transition phase was applied, recombinant phytase activity was increased 260% in high density fermentation of *P.pastoris*.

In *P.pastoris* processes methanol acts as carbon and energy source; however, excess amount of methanol inhibits the gene expression, contrarily presence of methanol in sufficient quantities is important to fully induce foreign protein expression without inhibiting product formation and the cell growth (d'Anjou and Daugulis, 2000). To keep methanol concentration within the optimal limits and to maximize protein production, bioreactor operation strategies have been applied, i.e., constant specific growth rate feeding, constant DO feeding, constant methanol concentration feeding, temperature limited fed-batch, and oxygen-limited fed-batch.

2.4.4.2. DO-Stat Control

Dissolved oxygen (DO) which indicates the relative percent of oxygen in the medium, is one of the important parameters for the cell growth and recombinant protein production by *P.pastoris*. The importance of oxygen is related with the methanol utilization pathway where methanol is oxidized to formaldehyhde and finally product formation is ocurred (Sibirny *et al.*, 1990). DO can be controlled by the agitation rate, and the air or O₂ flow rate (Çelik and Çalık, 2011). Typically DO levels for *P. pastoris* cultures are kept between 15 and 20% of saturation.

Lim *et al.*, (2003) applied DO-stat control strategy for production of rGuamerin. In this study, partial pressure of pure O₂ in the inlet air and the feed rate of methanol were controlled; consequently, comparing with the fed-batch process with manual methanol feeding control, the cell concentration was increased to high values more than 140 gL⁻¹ and 40% higher rGuamerin expression was achieved. DO-stat control strategy have been applied successfully

in several studies, however the influence of methanol concentration and specific growth rate on production can not be determined accurately since these parameters are not constant in this strategy (Cos *et al.*, 2006; Inan and Meagher, 2001-a; Chung, 2000; Lee *et al.*, 2003)

2.4.4.3 μ -Stat Control

μ -stat control strategy is based on the feeding rate profile derived from mass balance equations and maintaining the specific growth rate (μ) constant (Cos *et al.*, 2006). This strategy takes into account the simple cell growth models and moreover does not require on-line monitoring of the process parameters (d'Anjou and Daugulis, 2001; Sinha *et al.*, 2003; Trinh *et al.*, 2003; Zhang *et al.*, 2000b).

In the study of Sinha *et al.*, (2003) the cell growth on methanol was modeled by using the substrate-feed equation which was used for controlling the process; where, the key parameter for controlled protein production and protease production was the methanol feeding strategy. Trinh *et al.*, (2003) used three methanol feeding strategies which are based on, respectively, methanol consumption, DO concentration, μ -stat control under limited methanol feeding with predetermined exponential feeding rate of $\mu=0.02 \text{ h}^{-1}$. For all the three strategies total protein production was found similar, but the production of recombinant protein per unit biomass per unit methanol was 2-fold higher in the μ -stat culture than the other two strategies.

2.4.5 Oxygen Limited Fed-Batch Process (OLFB)

As *P. pastoris* prefers aerobic fermentation, generally oxygen limitation is the undesired condition during the induction phase since it is detrimental for foreign protein synthesis. However, it is also showed that recombinant protein production in oxygen limited cultivations resulted in higher protein yields rather than methanol limited processes. For the production of the Thai Rosewood β -glucosidase, OLFB process was applied and methanol concentration controlled at

350 mg L⁻¹ during the induction phase. The oxygen uptake rate was found 35% higher in OLFB than methanol limited fed batch strategy, which increases methanol consumption rate and cell productivity. However, due to the high maintenance demand, biomass yield per methanol decreased (Charoenrat *et al.*, 2005). Comparative studies showed that in OLFB processes the increase in the oxygen transfer results in approximately 40% higher oxygen transfer and methanol consumption rates compared to DO-stat control processes (Khatri and Hoffmann, 2006b) which increase the cell density and protein production (Khatri and Hoffmann, 2006a; Khatri and Hoffmann, 2006b).

2.4.6 Temperature Limited Fed Batch Process (TLFB)

In TLFB, temperature is selected as the limiting parameter for avoiding oxygen limitation (Jahic *et al.*, 2003). While methanol concentration is maintained at constant values, culture temperature is lowered that limits the cell growth. Jahic *et al.*, (2006) applied TLFB technique for the production of a proteolysis-sensitive fusion protein composed of a cellulose-binding module (CBM) from *Neocallimastix patriciarum cellulase* 6A and lipase B from *Candida antarctica* (CALB). They reported lower fraction of dead cells, higher cell density, lower proteolytic degradation of recombinant protein and twice higher concentration of the product compared to methanol limited fed batch process. The increase in AOX activity also reported to contribute to the high yield of production.

2.4.7 Mixed Feed Fed Batch Process

Use of a multi-carbon substrate in addition to methanol has been used in order to improve heterologous protein production and reduce the process time (Files *et al.*, 2001). This strategy generally applied for Mut^S strains since their induction time is longer because of the slow assimilation of methanol (Zhang *et al.*, 2005b). In the literature several carbon sources such as methanol, sorbitol, glycerol, glucose, trehalose, mannitol, lactic acid, acetate and ethanol have been

used for cultivation of *P. pastoris* (Brierly *et al.*, 1990; Sreekrishna *et al.*, 1997; Thorpe *et al.*, 1999; Inan and Meagher, 2001-a, 2001-b; Xie *et al.*, 2005).

Glycerol is used generally as the co-substrate for a successful cell growth and expression of heterologous protein (Thorpe *et al.*, 1999); however excess concentrations repress the AOX1 promoter, consequently, lowers the specific recombinant protein productivity (Xie *et al.*, 2005). For Mut⁺ strains of *P.pastoris* several studies where glycerol-methanol mixed feeding strategy is used, exhibited successful results. McGrew *et al.* (1997) employed mixed feed of glycerol: methanol with a ratio of 1:1 and as a result they doubled the expression of CD40 ligand compared to feeding methanol alone. Katakura *et al.*, 1998) improved product formation by simultaneous feeding of glycerol at 5 ml L⁻¹ h⁻¹ while maintaining the residual methanol concentration at 0.55% (v/v).

Sorbitol is another carbon source which is non-repressive for *P. pastoris*. Mixed feed of methanol and sorbitol exhibit several advantages over mixed feed of glycerol and methanol, since recombinant protein production does not affected by sorbitol accumulation (Thorpe *et al.*, 1999); thus, residual sorbitol concentration is less critical than the glycerol concentration in the the mixed feed of glycerol and methanol. Moreover, the growth on sorbitol provides lower oxygen consumption rate and lower heat production rate than the growth on glycerol or methanol which is very advantageous in high cell density cultures (Jungo *et al.*, 2007). Wang *et al.*, (2009) used sorbitol as a methanol co-substrate to enhance the cell viability and production of polygalacturonate lyase (PGL). In the induction phase, 50% (w/v) sorbitol solution was fed to bioreactor with a constant flow rate of 3.6 g h⁻¹ L⁻¹ in addition to methanol. Therefore, PGL activity was enhanced 1.85-fold compared to control (only methanol), due to sorbitol co-feeding which decreased the cell mortality to 8.8% and reduced proteolytic degradation of lyase enzyme.

2.4.8 Continuous Cultivation

Although traditional *P. pastoris* fermentations are performed by fed-batch operation, Schilling *et al.*, (2001) asserted that continuous cultivation may offer several advantages such as higher volumetric productivity, product uniformity, product quality as well as reduced oxidation or inactivation.

For pAOX1-regulated systems continuous cultivations are divided into three phases: glycerol batch and fed-batch phases as in the fed-batch cultivations, and the continuous feeding phase (Potvin *et al.*, 2010). For continuous cultures dilution rate (D) is the critical operation which influences protein production. In d'Anjou and Daugulis, a set of back-mixed-flow bioreactor (CSTR) experiments were performed with a dilution rate within $D = 0.01 \text{ h}^{-1}$ and 0.09 h^{-1} , using mixed feed of glycerol and methanol. The maximum production was reported at the lowest dilution rate as expected; while the maximum specific production rate was reported at the highest dilution rate; moreover, claimed that higher productivity was obtained with the CSTR compared to fed-batch operation. Paulová *et al.* (2012) operated a continuous cultivation of *P.pastoris* Mut⁺ strain for the production of recombinant trypsinogen with the use of a mixed feed of glucose and methanol. The sub-limit of the repressive effect of glucose was found at $D = 0.15 \text{ h}^{-1}$ and the cell yield was found 1.4-fold higher than the yield obtained by methanol fed operation; and the maximum production was reported at $D = 0.07 \text{ h}^{-1}$; where glucose was asserted as a feasible partial substitute for methanol in continuous cultures of *P.pastoris*.

2.5 Cell Growth Kinetics

2.5.1 Yield Coefficients and Specific Rates

Overall and Instantaneous Yield Coefficients

Yield coefficient can be defined as the ratio of the amount of a substance generated to the amount of a substance consumed for any reactant or product. The

yield coefficients are presented in Table 2.3. The general definition of a yield coefficient is given in equation 2.1:

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \quad (2.1)$$

S and P are, respectively, substrate and product, $Y_{P/S}$ is the overall yield coefficient, ΔP and ΔS represent the mass or moles of P produced and the mass or moles of S consumed, respectively. The overall yield coefficient represents the average value for whole process. Due to the variations in metabolic functions and growth rate of the microorganism the overall yield coefficient may vary during the process. Instantaneous yield is calculated as follows:

$$Y'_{P/S} = -\frac{dP/dt}{dS/dt} = -\frac{dP}{dS} \quad (2.2)$$

Specific Growth Rate

Specific growth (μ) rate defines the microbial cell growth. In a batch or a fed-batch bioprocess, for the component “the cell (C_x)”, the bioprocess is batch; therefore, it is derived by applying the general mass balance for a batch bioprocess as follows:

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

According to the equation 2.3, the cells are in “batch operation”, therefore should not be lost through sampling. The biomass formation rate (r_X) is defined as:

$$r_X = \mu C_X \quad (2.4)$$

where, μ is specific growth rate and C_X is the cell concentration.

Table 2.3 Definition of yield coefficients

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

If one combines equation (2.3) and (2.4):

$$\frac{d(C_X V)}{dt} = \mu C_X V \quad (2.5)$$

In the fed-batch operation volume change is due to substrate feed, e.g., methanol and sorbitol. By applying the continuity equation to the semi-batch (fed-batch) bioreactor, assuming the density of the reaction system is constant (the second assumption), and knowing that there is no exit stream ($Q_{\text{out}}=0$), the continuity equation is reduced to the form as follows:

$$\frac{dV}{dt} = Q \quad (2.6)$$

By inserting the equation (2.6) into equation (2.5):

$$\frac{dC_X}{dt} = \left(\mu - \frac{Q}{V}\right) C_X \quad (2.7)$$

and then by rearranging equation (2.7):

$$\mu = \frac{dC_X}{dt} \frac{1}{C_X} + \frac{Q}{V} \quad (2.8)$$

Mass balance equation for a substrate in a semi-batch (fed-batch) bioreactor is as follows:

$$QC_S - 0 + r_S V = \frac{d(C_S V)}{dt} \quad (2.9)$$

where, Q is volumetric flow rate, and V is bioreactor reaction volume.

In this M.Sc. thesis semi-batch (fed-batch) operation were used:

1. For the first substrate methanol until the end of the bioprocess ($t \geq 0$ h);
2. For the second substrate sorbitol within $t=0$ - 15h of the bioprocess;

therefore, equation (2.9) is used for the two substrates, during the cultivation times as mentioned above. For each substrate an equation in the form of equation 2.9 is written.

Therefore, substrate consumption rate (r_S) can be defined as;

$$r_S = q_S C_X \quad (2.10)$$

where, q_S is specific substrate consumption rate. By inserting the equation (2.10) into equation (2.9):

$$QC_S + q_S C_X V = V \frac{dC_S}{dt} + C_S \frac{dV}{dt} \quad (2.11)$$

As the substrates in this work (methanol and sorbitol) fed to bioreactor in a predetermined feeding rate and did not create an accumulation, the bioreactor is assumed to be operated at quasi-steady state; therefore:

$$C_S \frac{dV}{dt} \sim 0 \quad (2.12)$$

Dividing by V, the final form of equation (2.11) is:

$$\frac{dC_S}{dt} = \frac{Q}{V} C_S + q_S C_X \quad (2.13)$$

Rearranging the equation (2.13) specific consumption rate of substrate (methanol or sorbitol) (q_S) can be obtained;

$$q_S = - \left(\frac{Q}{V} \frac{C_{S0}}{C_X} - \frac{1}{C_X} \frac{dC_S}{dt} \right) \quad (2.14)$$

For the designed strategies SSM1, SSM2, and SSM3 until $t=15$ h, the bio-process is semi-batch for sorbitol as mention before; thereafter, the bioprocess is batch. Therefore, in the period $t \geq 15$ h, the mass balance equation for the component sorbitol is:

$$r_S V = \frac{d(C_S V)}{dt} \quad (2.15)$$

Sorbitol consumption rate r_S after $t > 15$ h, is:

$$r_S = q_S C_X \quad (2.16)$$

where, q_S is the specific sorbitol consumption rate. By inserting equation (2.16) into equation (2.15):

$$\frac{dC_S}{dt} = - \frac{Q}{V} C_S + q_S C_X \quad (2.17)$$

the specific sorbitol consumption rate (q_S) is obtained by rearranging the equation (2.17) as follows;

$$q_S = \left(\frac{dC_S}{dt} + \frac{Q}{V} C_S \right) \frac{1}{C_X} \quad (2.18)$$

As the bioreactor is batch for the recombinant protein rhGH, by constructing the mass balance equation, the recombinant protein production is achieved according to the following equation:

$$\frac{dC_{rp}}{dt} = q_{rp}C_x - \left(\frac{Q}{V}\right)C_{rp} \quad (2.19)$$

Consequently, the specific recombinant protein production rate is defined as:

$$q_{rp} = \left(\frac{dC_{rp}}{dt} + \frac{Q}{V}C_{rp}\right) \frac{1}{C_x} \quad (2.20)$$

thus, the specific rates μ , q_s , q_M , and q_{rp} can be calculated from experimental data using equations 2.8, 2.14, 2.18 and 2.20.

2.5.2 Oxygen Transfer Characteristics

Oxygen is the key substrate in aerobic fermentation processes and it strongly affects the productivity of cell and bioproducts (Liang and Yuan 2007). In high cell density fermentations oxygen transfer is limiting factor due to the given cell density and unable capacity of the bioreactor to sustain the oxygen metabolic demand (Oliveira *et al.*, 2005). When the cells do not form aggregates and dispersed well in the fermentation medium, the resistance to oxygen transfer between the liquid and air bubble gains a significant importance. The oxygen transfer rate (ORT) from gas to liquid can be defined as;

$$OTR = K_L a (C_0^* - C_0) \quad (2.21)$$

where, a is the gas-liquid interfacial area, C_0 and C_0^* are saturated dissolved oxygen concentration and actual dissolved oxygen concentration in the fermentation broth, respectively.

The liquid mass transfer resistance is the dominant resistance due to the low solubility of oxygen in aqueous solutions. Overall liquid mass transfer coefficient $K_L a$, is nearly equal to liquid phase mass transfer coefficient, $k_L a$ (Villad-

sen and Nielsen, 1990). The maximum possible mass transfer rate is defined as in equation 2.22:

$$OTR = K_L a C_0^* \quad (2.22)$$

The next oxygen transfer characteristics, the oxygen uptake rate (OUR: , - r_0) per unit volume of medium is:

$$OUR = -r_0 = q_0 C_X \quad (2.23)$$

where, q_0 is the specific oxygen consumption rate.

To calculate $K_L a$ values the widely used method is the dynamic method (Bandyopadhyay and Humprey, 1967) which should be applied during the fermentation process. It is based on the material balance for oxygen;

$$\frac{dC_0}{dt} = OTR - OUR = K_L a (C_0^* - C_0) + r_0 \quad (2.24)$$

In the dynamic method, firstly de-oxygenation step is applied by stopping the air flow and reducing the agitation rate to a minimal level to avoid surface aeration. During this period, there is no oxygen transfer since microorganisms consume the dissolved oxygen (C_0) within the broth which causes a drop in C_0 . Thus the equation (2.24) becomes;

$$\frac{dC_0}{dt} = r_0 \quad (2.25)$$

Oxygen uptake rate $-r_0$, can be calculated using equation (2.25) in region II of Figure 2.9

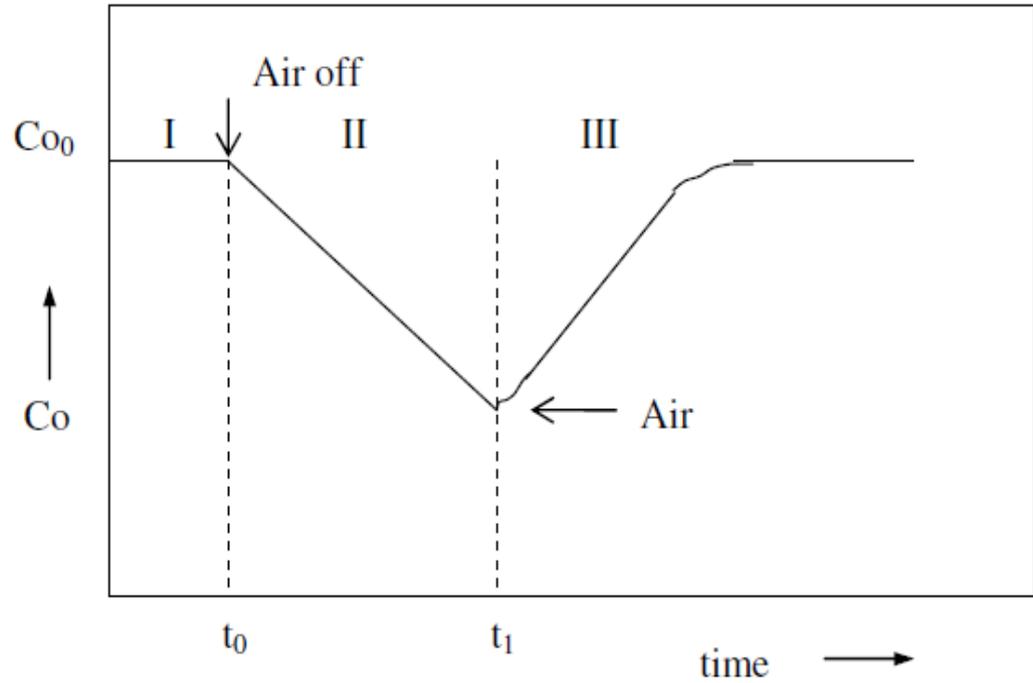


Figure 2.9 Variation of dissolved oxygen concentration with time in dynamic measurement of $K_L a$.

And then, air inlet is turned back “on” and the increase in C_0 is monitored as a function of time (Figure 2.9 Region III). r_0 which was calculated in region II was assumed to be the same for region III. In order to obtain $k_L a$ equation (2.24) is rearranged and equation (2.26) is obtained.

$$C_0 = -\frac{1}{(K_L a)} \left(\frac{dC_0}{dt} - r_0 \right) + C_0^* \quad (2.26)$$

By plotting C_0 versus $(dC_0 / dt - r_0)$ $K_L a$ can be determined from the slope (Figure 2.10).

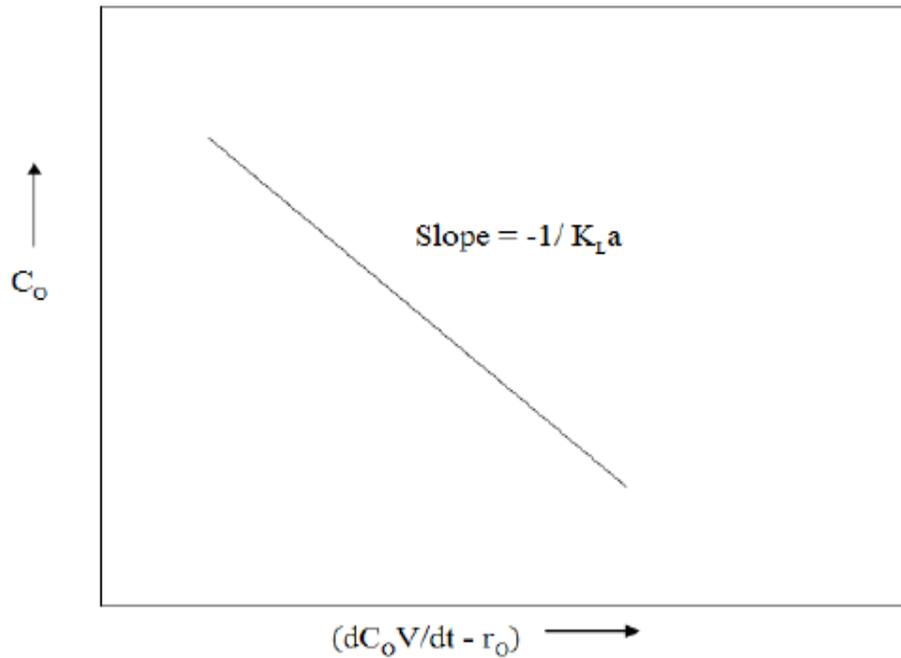


Figure 2.10 Evaluation of $K_L a$ using the Dynamic Method

To understand the effect of additional mass transfer resistance in the presence of microorganism, $k_L a$ can be calculated when there is no microorganism in the same fermentation broth. This term is symbolized as $k_{L a_0}$. For the calculation of $k_{L a_0}$ an experiment is required. Therefore, the medium in the absence of microorganism is de-oxygenated by sparging nitrogen into the bioreactor. Then the air inlet is turned on and the increase in C_0 versus time is recorded. Since $r_0=0$, equation (2.26) can be simplified as;

$$C_0 = -\frac{1}{K_L a} \frac{dC_0}{dt} + C_0^* \quad (2.27)$$

therefore, C_0 is plotted versus $d(C_0V/dt)$, and the slope gives the physical mass transfer coefficient, $K_L a_0$.

In order to evaluate the influences of the oxygen transfer further as described by Çalık *et al.* (2000), the maximum possible oxygen utilization rate, i.e. oxygen demand (OD) is calculated using the equations given below:

$$OTR_{max} = K_L a C_0^* \quad (2.28)$$

$$OD = \frac{\mu_{max} C_0^*}{Y_{X/S}} \quad (2.29)$$

Damköhler number (Da) which is defined as the maximum possible oxygen utilization rate per maximum mass transfer rate; and effectiveness factor (η) that is the oxygen uptake rate per maximum possible oxygen utilization rate, are formulated as (Çalık *et al.*, 2000):

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

$$\eta = \frac{OUR}{OD} \quad (2.31)$$

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals and solvents were analytical grade and they are supplied by Sigma Ltd., Fluka Ltd., Merck Ltd., Roche .

3.2 Buffers and Stock Solutions

All stock solutions and buffers listed in Appendix A were prepared with ultra purified water and sterilized either by autoclaving at 121 °C for 20 min or by filter sterilization through 0.20 µm filters (Sartorius AG, Gottingen, Germany) and stored at +4 °C or at room temperature.

3.3 Microorganism

The rhGH production was achieved by using *Pichia pastoris* pPICZαA::hGH::Mut⁺ strain constructed by Orman *et al.* (2008) (Figure 3.1). The Human GH gene was fused with polyhistidine tag to enable affinity purification and a target site for the factor Xa protease recognition in order to produce native hGH after protease cleavage. The fused gene was cloned to the vector pPICZαA that carries α-factor signal peptide, AOX1 promoter and Zeocin resistance gene. The recombinant microorganisms are stored in microbanks (PRO-LAB), by inoculating young colonial growth into cryopreservative fluid present in the vial. Firstly, adsorption of microorganisms into the porous beads was performed afterwards excess cryopreservative was aspirated and inoculated into cryovial stored at -55 °C.

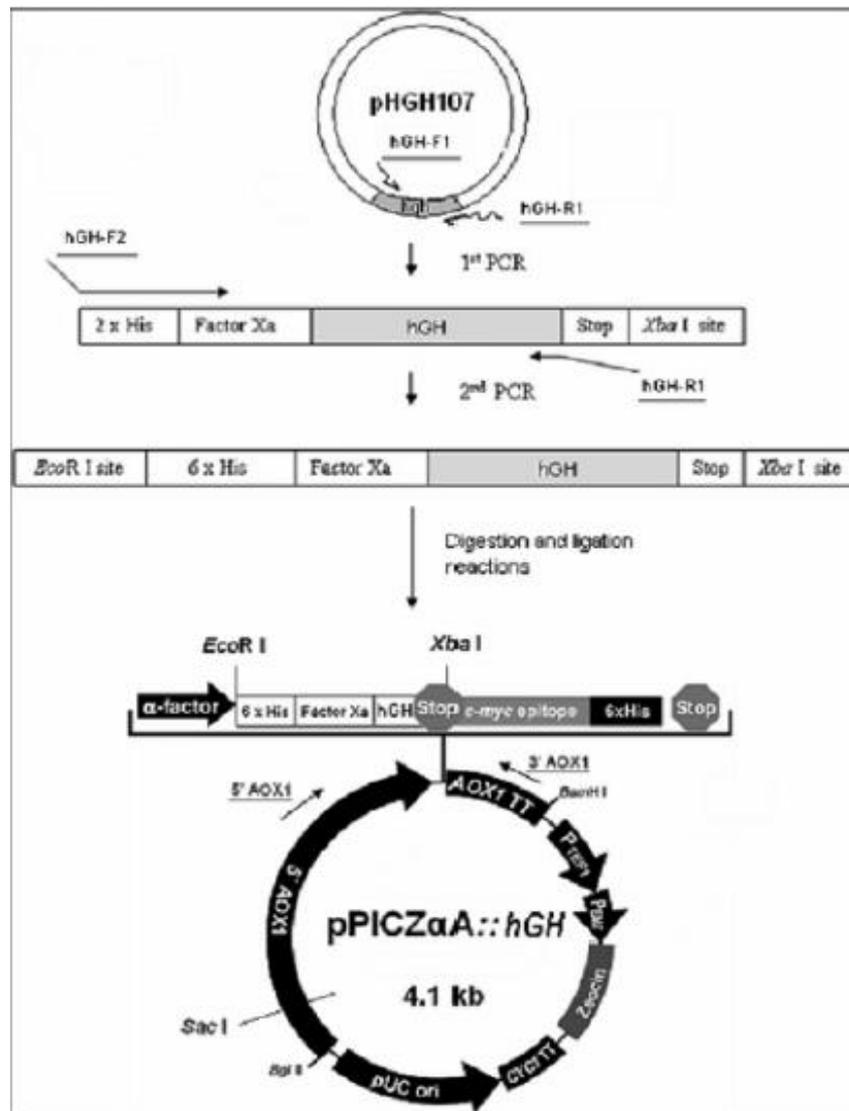


Figure 3.1 Schematic representation of *hGH* amplification, integration of specific recognition sites by two step PCR, and construction of the *PPICZαA::hGH* plasmid. Shown are the *Eco*RI and *Xba*I sites used in ligation of the insert to the vector, the *Sac*I site used to linearize the plasmid before transformation, and the primers used for sequencing. There are 976 nucleotides between the 5' AOX1 primer and *hGH-R1* primer, and 793 nucleotides between the *hGH-F2* primer and 3' AOX1 primer (Çalık *et al.* 2008).

3.4 Growth Medium

3.4.1 Solid Medium

Recombinant *Pichia pastoris* strain cells stored in microbanks at -55 °C were cultivated in solid medium by inoculation onto YPD agar, containing 0.1 g L⁻¹ Zeocin as a selective antibiotic. The agars were incubated at 30 °C for 48-60 hour and stored at +4 °C, afterwards. The composition of YPD agar is listed in Table 3.1.

Table 3.1 The composition of the YPD, solid medium

Compound	Concentration, g L ⁻¹
Yeast extract	10
Peptone	20
Glucose	20
Agar	20
Zeocin	0.1

3.4.2 Precultivation Medium and Stock Preparation

Recombinant *Pichia pastoris* Mut⁺ strain was inoculated into precultivation YPD liquid medium (Table 3.2) followed by growth on YPD agar. In order to prepare glycerol stock, harvested cells from YPD were resuspended in glycerol stock solution (Table 3.3) and stored at -55°C or to continue with the production single colonies from solid media were directly inoculated into BMGY (Buffered Glycerol Complex Medium), second precultivation medium whose composition was given in Table 3.4. Precultivation mediums were sterilized and right after the selective antibiotics, zeocin or chloramphenicol was added in the stated amounts in Table 3.2 and 3.4.

Table 3.2 The composition of YPD, the first precultivation medium

Compound	Concentration, g/L
Yeast extract	10
Peptone	20
Glucose	20
Zeocin	1 ml

Table 3.3 The composition of the glycerol stock solution (Jungo *et al.* 2007)

Compound	Concentration, g/L
NaCl	9
Glycerol	20

Table 3.4 The composition of BMGY, second precultivation medium

Compound	Concentration, g/L
Yeast extract	10.0
Peptone	20.0
Potassium phosphate buffer pH6.0	0.1 M
YNB	13.4
Biotin	4×10^{-5}
Glycerol (mL)	10.0
Chloramphenicol* (mL)	1

* Chloramphenicol is prepared as 34 mg/ml stock in pure ethanol, kept in sterile dark bottle at -20 °C.

3.4.3 Production Medium

After inoculation to second pre-cultivation medium, BMGY, recombinant *P. pastoris* cells were harvested by centrifugation and transferred to production medium. The defined production medium containing sorbitol together with methanol, basal salts solution and nitrogen sources whose composition was reported by Jungo *et al.* (2006), was used with modification in nitrogen source for the production of recombinant protein in air filtered shake bioreactor experiments. Instead of ammonium chloride, ammonium sulfate was used with the ratio of Carbon/Nitrogen and Methanol/Nitrogen as 4.57 and 2.19 (Jungo *et al.* 2006). The composition of the medium is listed in Table 3.5. In a pilot scale bioreactor, Basal Salt Medium (BSM) whose composition was given in Table 3.6 was used as a recombinant protein production medium. After sterilization all of medium components at 121 °C for 20 min, 0.1% chloramphenicol, 0.1% antifoam, sterile filtered trace salts (PTM1) having the listed composition in Table 3.7 was added and to adjust and control the pH at the desired value, 25% ammonium hydroxide (NH₃OH) solution was used.

Table 3.5 The composition of the defined production medium (Jungo *et al.*, 2006)

Compound	Composition, g/L
Methanol	1 mL
Sorbitol	30
Ammonium sulfate	21.75
Potassium phosphate buffer pH=6	0.1 M
MgSO ₄ .7H ₂ O	14.9
CaSO ₄ .2H ₂ O	1.17
Chloroamphenicol	1 mL
PTM1	4.35 mL

Table 3.6 The composition of Basal Salt Medium (BSM)

Compound	Concentration, g/L
85% H ₃ PO ₄	26.7
CaSO ₄ .2H ₂ O	1.17
MgSO ₄ .7H ₂ O	14.9
KOH	4.13
Glycerol	40.0
Chloramphenicol (mL from stock)	1
10% Antifoam (mL)	1
PTM1 (mL)	4.35

Table 3.7 The composition of PTM1 (Sibirny *et al.*, 1987)

Compound	Concentration, g/mL
CuSO ₄ .5H ₂ O	0.6
NaI	0.008
MnSO ₄ .H ₂ O	0.3
Na ₂ MoO ₄ .2H ₂ O	0.02
H ₃ BO ₃	0.002
ZnCl ₂	2
FeSO ₄ .7H ₂ O	6.5
CoCl ₂ .6H ₂ O	0.09
H ₂ SO ₄	0.5
Biotin*	0.02

*Biotin was prepared as 0.2g/L stock and stored in dark bottle at +4 °C.

3.5 Recombinant Human Growth Hormone Production

The production of rhGH was performed in a pilot scale bioreactor for the investigation of different feeding strategies.

3.5.1 Precultivation

P. pastoris Mut⁺ strains carrying human growth hormone gene, was inoculated onto solid medium whose composition was given in Table 3.1 and incubated for t=48-60 h at 30°C. After that two or more colonies were resuspended in BMGY precultivation medium. In this stage air filtered shake bioreactors were used. Precultivation medium inoculated with the cells harvested from YPD was divided equally into flasks of 150 mL in size and having a working volume of 50 mL. Precultivation was carried out about 20-24 h until the cells reach to OD₆₀₀=2-6, in an orbital shaker (B.Braun, Cermotat BS-1) where a agitation and heat rate controlled at 30°C and at N=225 min⁻¹. After the OD₆₀₀ reached to the desired values the medium was centrifuged at 4000 rpm for 10 min at +4°C and then the cell pellets were resuspended in 40 mL sterile water and transferred into bioreactor containing the production medium.

3.5.2 rhGH Production in the Pilot Scale Bioreactor

For the pilot scale bioreactor experiments, Braun CT2-2 having a working volume of V_R=0.5-2.0 L bioreactor system, where the temperature, foam, stirring rate, feed inlet rate and dissolved oxygen (DO) was controlled by the system itself, was used. The temperature control and sterilization was achieved by using a jacket around the bioreactor, an external cooler and a steam generator. The bioreactor consisting of four baffles and a sparger was stirred with two four-bladed Rushton turbines to manage a homogenous mixture and oxygen transfer within the bioreactor. For the air supply a compressor was used and a pure oxygen adjusted by the mass flow was employed where the air supplied by the compressor was not sufficient. Feed solutions placed on balances were transferred through

inlet ports by using peristaltic pumps. The schematic presentation of the bioreactor and cultivation steps for production were given in figure 3.2

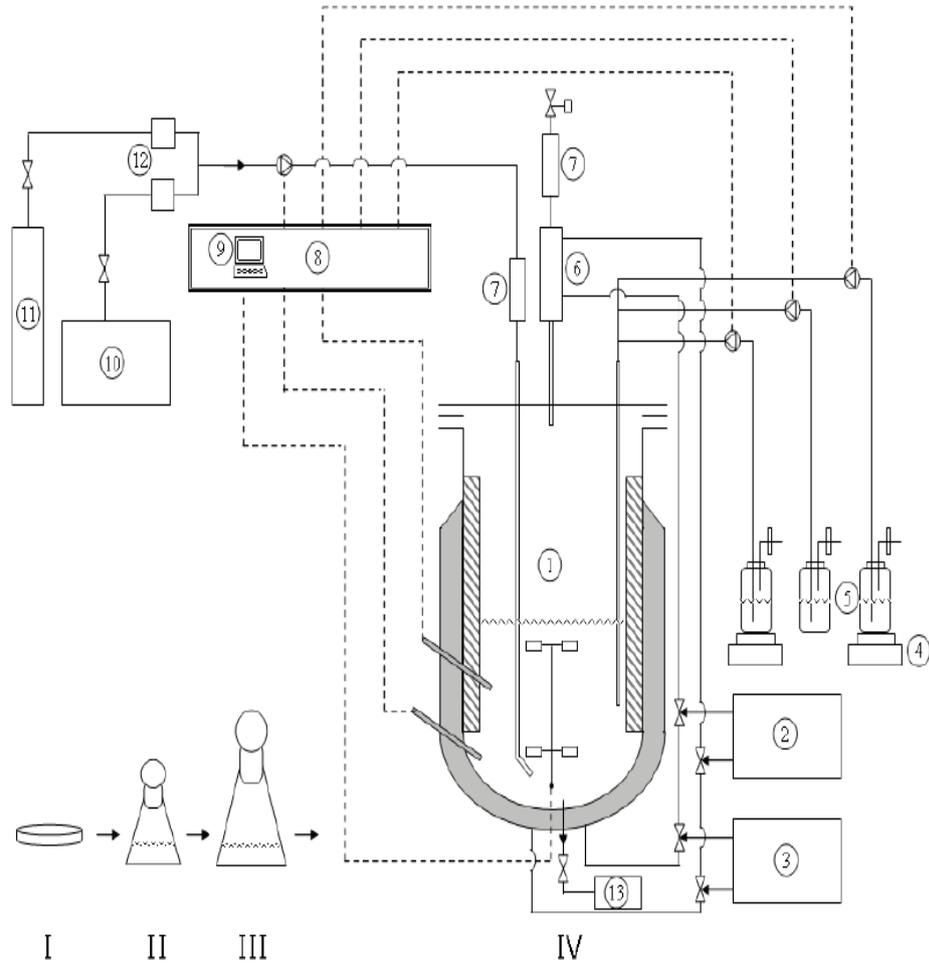


Figure 3.2 Scale up steps and pilot scale bioreactor system. I: Solid medium inoculated from stock culture; II: 1st Precultivation medium, V= 10 mL; III: 2nd Precultivation medium, V=50 mL; IV: Pilot scale bioreactor system, V₀= 1L, which is composed of (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O₂ tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik 2008).

3.5.2.1 Bioreactor Operation Parameters

PI controller system of the reactor was used to control the temperature constant at 30°C throughout the process with the help of the external cooler and steam generator. The pH was adjusted at desired values by addition of 25 % ammonia solution to the fermentation medium (Çelik *et al.* 2009). The pH of the medium was controlled by using the PI controller of the bioreactor system with the parameters of $X_p=30\%$, $T_I=30s$ and 10% opened base-pump-valve. The agitation rate was kept constant at $N=900$ rpm to manage homogenous mixture and better oxygen transfer within the bioreactor.

Pichia pastoris is an aerobic microorganism and has high oxygen consumption rate due to the oxygen requirement of methanol utilization pathway. Thus as reported in the literature (Çelik *et al.* 2009, Horstkotte *et al.* 2008) dissolved oxygen (DO) in the medium is an extremely important parameter to maintain at the desired levels to prevent oxygen limitations. DO was maintained above 20% of air saturation by an inlet air and when the oxygen in the air was not sufficient pure oxygen was fed to the medium under the control of mass flow controller.

In order to prevent foaming which is an important issue to overcome and mostly seen at the production phase, antifoam solution (10% (v/v)) was added firstly to the initial medium and when required manually added in a very small amounts during the process.

3.5.2.2. Precultivation, Sorbitol Addition and Sorbitol and Methanol Feeding Rate in Fed-Batch Pilot Scale Bioreactor Operations

For the expression of rhGH in *Pichia pastoris* under the control of AOX1 promoter, a standard protocol (Stratton, Chiruvolu, and Meagher 1998) modified by Çelik *et al.* (2009) was applied. First the cells harvested from the precultivation medium were transferred and resuspended in production medium, BSM, present in the bioreactor such that initial $OD_{600}=1$ which corresponds to a cell

concentration of 0.275 g L^{-1} . Afterwards, the four-phased protocol was applied, which involves;

- **Glycerol batch phase (GB):** The phase was initiated with the re-suspension of the cells in the production medium containing 40 g L^{-1} glycerol which was reported as the non-inhibitory limit for cell growth (Cos *et al.* 2006). The main purpose of this phase was to provide biomass accumulation which lasted approximately 15-18h until $\text{OD}_{600}=30$ which corresponds to the cell concentration of $C_X=10 \text{ g L}^{-1}$. Rather than methanol, glycerol was used as the growth substrate since cells grown on glycerol have higher specific growth rate than the cells grown on methanol.

- **Glycerol fed-batch phase (GFB):** In this phase 50 % glycerol containing 12 mL L^{-1} PTM1 was fed with the pre-determined exponential feeding profile in order to prevent glycerol accumulation and provide biomass formation. Another purpose of this phase was to prepare the cells to another carbon source which was methanol. This phase was continued until the cell concentration reached to $\text{OD}_{600}=80$ which corresponds to $C_X=20\text{-}23 \text{ g L}^{-1}$. During the first two phases no recombinant proteins were produced.

- **Methanol Transition Phase (MT):** This phase was applied to aid the cellular adaptation to methanol. Methanol (100% methanol containing 12 mL L^{-1} PTM1) was fed to the bioreactor based on the suggested feeding profile of Invitrogen *Pichia* Fermentation Process Guideline (Invitrogen 2002) such that 3.6 ml/hr per liter initial fermentation volume within 4 hours.

- **Production Phase :** The production phase applied in the first part of this study consisted of two sub-phases which were performed simultaneously;

Methanol fed-batch phase (MFB): MFB mode was started at $t=0\text{h}$ of the production phase by 100% methanol (containing 12 mL L^{-1} PTM1) feeding with the predetermined exponential feeding profile in order to induce recombinant protein production.

Sorbitol fed-batch: At $t=0\text{h}$ of the production phase, sorbitol (500 g L^{-1} sterile solution) was added batch-wise to the bioreactor, such that $C_{\text{so}}=50 \text{ g}$

L⁻¹ was obtained and simultaneously, 500 g L⁻¹ sorbitol containing 12 mL L⁻¹ PTM1 was fed to the system with the predetermined feeding profile in order to keep sorbitol concentration constant at 50 g L⁻¹ within t=0-15h of production phase.

In the second part of this study, production phase was performed by simultaneous feeding of sorbitol and methanol mixture within a solution with the predetermined feeding profile.

The predetermined exponential feeding profiles for glycerol, methanol and sorbitol were determined using equation 3.1, calculated for a constant specific growth rate.

$$F(t) = \frac{\mu_0 V_0 C_{X0}}{C_{S0} Y_{X/S}} \exp(\mu_0 t) \quad (3.1)$$

In the equation 3.1, μ_0 is the desired growth rate (h⁻¹), V_0 is the initial volume (L), C_{X0} is the initial cell concentration (g L⁻¹), $Y_{X/S}$ is the cell yield on the substrate (g g⁻¹) and C_{S0} is the feed substrate concentration (gL⁻¹). Parameters of equation 3.1 for glycerol, methanol, sorbitol and mixed feed are given in Table 3.8

Table 3.8 Parameters of Equation 3.1 for Glycerol, Methanol and Sorbitol Fed-Batch Feeding (Çelik *et al.*, 2009)

Parameter	Glycerol	Methanol	Sorbitol	Mixed Feed
	Fed-Batch Feeding	Fed-Batch Feeding	Fed-Batch Feeding	Fed-Batch Feeding
μ_0 (h ⁻¹)	0.18	0.03-0.04	0.025	0.03
$Y_{X/S}$ (g/g)	0.5*	0.42**	0.15	0.23
C_{S0} (g L ⁻¹)	1130	790	500	1136

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

In order to calculate the yield coefficient for sorbitol given in Table 3.8 the study of Çelik *et al.* (2009) was taken into account. In this study, for the production of recombinant protein several strategies were applied with and without sorbitol by using *P.pastoris* Mut⁺ strain. As a result, in the strategy where only methanol was fed to the medium with a specific growth rate as $\mu_0=0.03 \text{ h}^{-1}$, the overall cell yield on total substrate was found as $Y_{X/S}=0.30 \text{ (g g}^{-1}\text{)}$ and in the other strategy where 50 g L^{-1} sorbitol was added to the system as a batch-wise at $t=0\text{h}$ of the production phase in addition to methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$, the overall cell yield on total substrate was found as $Y_{X/S}=0.45 \text{ (g g}^{-1}\text{)}$. Based on this two strategies, subtraction of this two yield coefficient values yielded cell yield on sorbitol which was calculated as $Y_{X/S}=0.15 \text{ (g g}^{-1}\text{)}$. Furthermore, the specific growth rate of sorbitol was calculated from the sorbitol consumption rates data of the previous studies. The calculation of specific growth rate on sorbitol was given in Appendix H. Using the parameters given in Table 3.8, the predetermined feeding profiles for glycerol, methanol and sorbitol are plotted in Figure 3.3, Figure 3.4 and Figure 3.5.

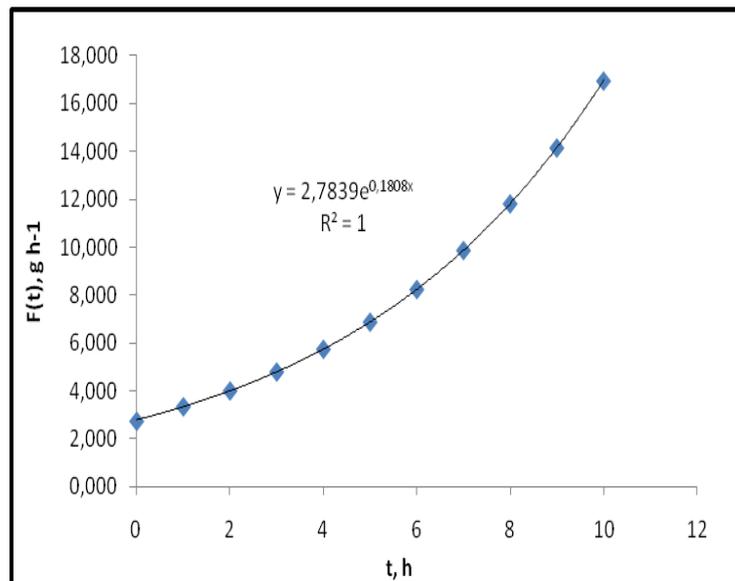


Figure 3.3 the predetermined feeding profile for glycerol, calculated for specific growth rate (μ_0) of 0.18 h^{-1} .

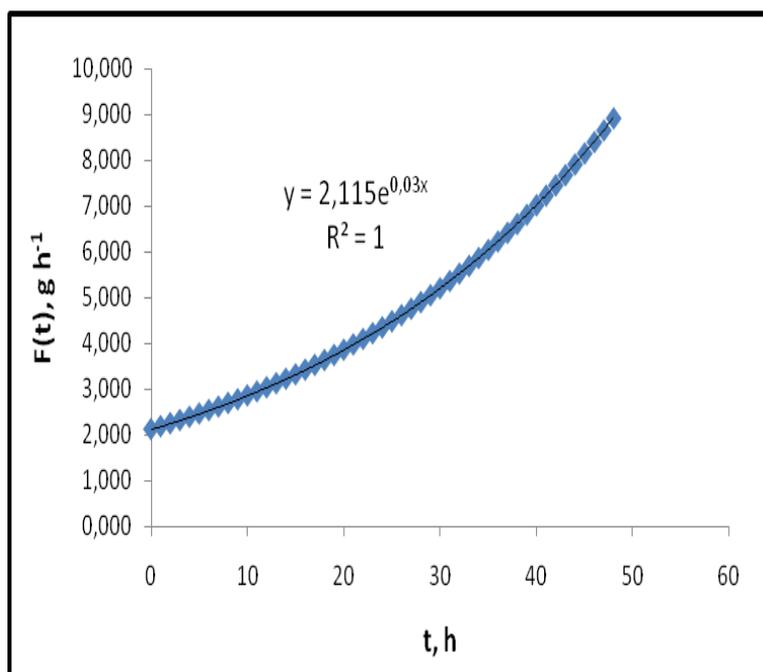


Figure 3.4 The predetermined feeding profile for methanol, calculated for specific growth rate (μ_0) of 0.03 h^{-1} .

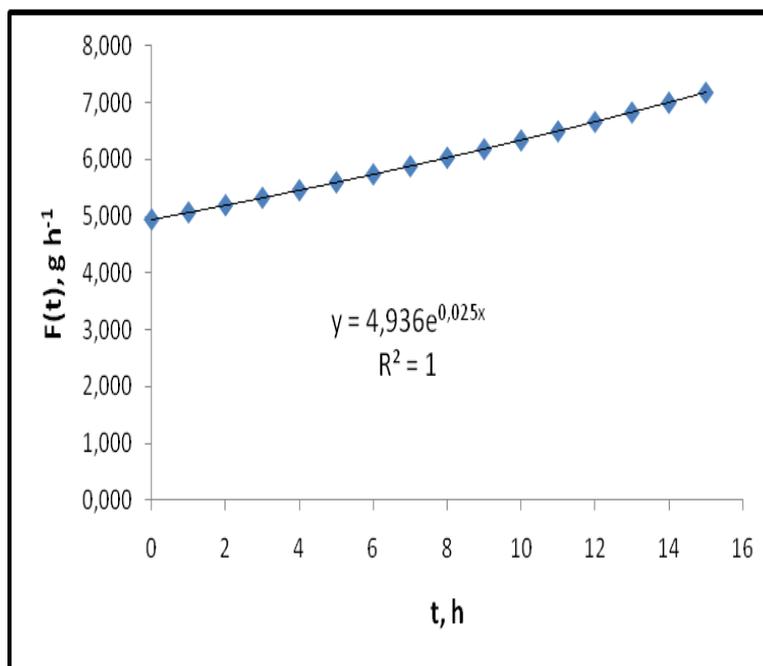


Figure 3.5 The predetermined feeding profile for sorbitol, calculated for specific growth rate (μ_0) of 0.025 h^{-1} .

In the last part of this study, the feeding strategy developed by Jungo et al. (2007) was applied for production of rhGH with some adjustments. In the study of Jungo et al. (2007) mix feed of 1.37 mol sorbitol and 6.21 mol methanol in 13.88 mol water at a specific growth rate of 0.03 h^{-1} was fed to the fermentation medium in order to improve the production of recombinant avidin. This optimal ratio which yielded the highest recombinant avidin production was obtained from the transient nutrient gradient continuous cultivation performed by the same group. Consequently, this strategy resulted in 1.3-fold higher avidin productivity and at the rate of 38% lowers oxygen consumption and heat production rate than with a culture on methanol. Therefore, this suggested optimal sorbitol/methanol ratio was applied in the induction phase of the last strategy in this study. The mixed feed was prepared as described in the study of Jungo et al. (2007) and fed to the fermentation medium at the predetermined feeding rate of $\mu_{0=} 0.03 \text{ h}^{-1}$ within $t=0-30 \text{ h}$; thereafter, only methanol was fed with the predetermined growth rate of $\mu_{0=} 0.03 \text{ h}^{-1}$. The parameters for predetermined feeding profile are given in Table 3.8. The residual sorbitol and methanol concentration was monitored and analyzed with HPLC simultaneously. The overall cell yield on total substrate was calculated as the sum of the yield coefficients for each substrate multiplied by the percentage of each substrate in the medium. Figure 3.6 shows the predetermined feeding profile of the mixed feed of methanol and sorbitol.

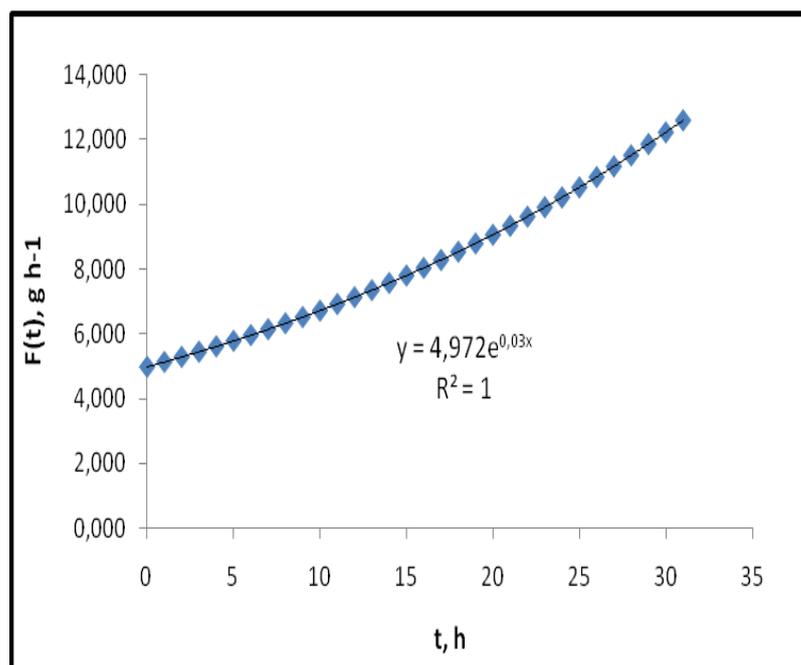


Figure 3.6 The predetermined feeding profile for the mixed substrate solution with 1.37 mol sorbitol and 6.21 mol methanol in 13.88 mol water, calculated for specific growth rate (μ_0) of 0.03 h^{-1} .

3.6 Analysis

Throughout the production phase, samples were collected at every 3h for the first 24h; thereafter at every 6h of the process. Firstly, the cell concentration was determined and afterwards the collected medium was centrifuged at 13200 g for 10 min at $+4^\circ\text{C}$. Cells, supernatants and filtrates were stored at -55°C separately for further analysis. Supernatants were used for the determination of total protein, protease concentration and SDS-Gel electrophoresis. Filtrates were used for determination of sorbitol, methanol, organic acid and hGH concentrations. To determine AOX activity the harvested cells were used.

3.6.1 Cell Concentration

Cell concentration was measured by using a UV-Vis Spectrophotometer (Thermo Spectronic, Heliossa) at 600 nm. To keep the sample between the range of $OD_{600}=0.1-0.9$ dilution with distilled water (dH_2O) was performed. Equation 3.2 was used to convert the absorbance to the cell concentration, C_X ($g L^{-1}$) (Çelik 2008).

$$C_X = 0.275 * OD_{600} * \text{Dilution rate} \quad (3.2)$$

3.6.2 Total Protein Concentration

Bradford assay (Bradford 1976) which is a spectrophotometric method was used to determine the total protein concentration. The protocol was taken from the instruction manual of BioRad (Bio-Rad 2012). 20 μL of sample was mixed with 1 mL of Bradford reagent (Bio-Rad) and incubated in a dark place at room temperature for 5-15 min. The absorbance was read at 595 nm by UV-spectrophotometer. The calibration curve was obtained by using BSA in the concentration range between 0-2 $mg mL^{-1}$ (Appendix B).

3.6.3 rhGH Concentration

rhGH concentrations were measured by using a high performance capillary electrophoresis (Agilent CE). Samples were filtered with 0.45 μm cellulose acetate filters (Milipore) and analyzed at 12 kV and 15°C with a positive power supply using 75 cm x 75 μm silica capillary. As a separation buffer 50 mM borate buffer at $pH=10$ containing zwitter ion (Z1-Methyl reagent, Waters), with the aim of preventing protein adsorption to the capillary column, was used. Proteins were detected by UV absorbance at 214 nm, as mentioned elsewhere (Çalık 1998). A sample electropherogram which belongs to a hGH Standard can be seen in Appendix E.

3.6.4 Ultrafiltration

The aim of performing ultrafiltration was to remove the salts and concentrate the supernatants obtained by centrifugation of samples. For this purpose 400 mL stirred cells (Amicon) with 10 kDa cut-off regenerated cellulose ultrafiltration membranes (Milipore) were used. The ultrafiltration process was carried out at $T=4-8^{\circ}\text{C}$ in a cold room using N_2 gas at a pressure of 3.5 bars until at least 10-fold concentrated medium was obtained. Desalting was done by adding filter sterilized ultra pure water.

3.6.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed as described by Laemmli (1970). The sample buffer and protein samples were mixed (1:3 volume) and heated at 95°C for 4 min and after that samples were taken onto the ice immediately. $3\mu\text{L}$ of a dual color prestained protein MW marker (Appendix G) and $15\mu\text{L}$ of the samples were loaded to gel and run simultaneously at constant current of 40 mA around 40-45 min. The buffers used in SDS-PAGE are given in Appendix A. The protocol of SDS-PAGE is given below;

1. Clean the glasses with ethanol and assemble the glass plates according to the manufacturer's instructions.
2. In an Erlenmeyer flask, prepare resolving and stacking solutions using the values given in Appendix A. To start polymerization add NNN'N'-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) into the solutions afterwards.
3. Swirl the mixture rapidly and immediately pour the solution into the gap between the glass plates by micropipette in order to prevent polymerization and bubble formation. Leave sufficient space for the stacking gel.
4. Pour a thin layer of isopropanol onto the gel to obtain a smooth gel surface. Leave the gel polymerize at least 45 minutes.

5. After polymerization pour the isopropanol and wash the space between the glasses with dH₂O. Dry the excess water with filter papers.
6. Prepare stacking gel and add TEMED and APS. Gently mix the solution and pour into the gel cast. Place the comb immediately by tilting and avoid bubble formation.
7. Leave the gel to polymerize at least 20 minutes and then wrap the gel into a tissue soaked with dH₂O and store in +4°C for up to two weeks.

Preparation of Samples and Running the Gel:

1. Mix the sample loading buffer and samples (1:2), heat at 95°C for 4 minutes and store in ice for 5 minutes, centrifuge for a short time and vortex.
2. Remove the comb between the glasses wash the wells with dH₂O and assemble the glass into the electrophoresis unit. Fill the apparatus with 1 X SDS-PAGE running buffer until the gels are covered.
3. Load 15 µL of each samples and 3 µL prestained protein MW marker into the wells and run the gel at constant current of 40 mA for 40-45 minutes.

3.6.6 Staining of SDS-PAGE Gels

After running the gels, the glasses were separated from each other and the stacking gel was taken out. The gels were stained with silver salts using the procedure (Blum *et al.* 1987) given in Table 3.9. The solutions are given in Appendix A.

Table 3.9 Procedure for silver staining of SDS-PAGE

Step	Solution	Time of Treatment	Comments
1.Fixing	Fixer	≥ 1 h	Can be incubated overnight
2.Washing	50% Ethanol	3x20 min	Should be fresh
3. Pre-treatment	Pretreatment Solution	1 min	Should be fresh
4.Rinse	Distilled Water	3x20 sec	Time should be exact
5.Impregnate	Silver Nitrate Solution	20 min	
6.Rinse	Distilled Water	2x20 sec	Time should be exact
7.Developing	Developing Solution	~5 min	Time is determined according to the color development
8.Wash	Distilled Water	2x2 min	
9.Stop	Stop Solution	≥ 10 min	Gels can be kept overnight in this solution

3.6.7 Methanol, Sorbitol and Organic Acid Concentrations

Methanol, sorbitol and organic acid concentrations were measured with reversed phase HPLC (Waters HPLC, Alliance 2695, Milford, MA) on Capital Optimal ODS-5 μ m column (Capital HPLC, West Lothian, UK) (Çelik *et al.* 2009). The concentration were calculated from the chromatogram, based on the chromatogram of the standard solutions. Samples were filtered with 45 μ m filters (ACRODISC CR PTFE) and 150 μ L of the samples were loaded to the analysis system. For the dilution of samples mobile phase was used. 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.5 mL min⁻¹, and refractive index detector (Waters 2414) at 30°C were used to determine methanol and sorbitol concentrations. The calibration curves for methanol and sorbitol are given in Appendix C. All the buffers used in the HPLC system should be degassed before

their use. The specific condition for methanol and sorbitol concentration determination are given in Table 3.10.

For the organic acid concentration analysis, 3.12% (w/v) NaH_2PO_4 and $0.62 \times 10^{-3}\%$ (v/v) H_3PO_4 (İleri and Çalık 2006) as a mobile phase at a flow rate of 0.8 mL min^{-1} , and Dual absorbance detector (Waters 2487) were used. The calibration curves are given in Appendix D. The specific conditions for the analysis are given in Table 3.11.

Table 3.10 Conditions for HPLC system for methanol and sorbitol analyses

Column	Capital Optimal ODS, $5 \mu\text{m}$
Column dimensions	4.6x250 mm
System	Reversed phase chromatography
Mobile phase	5 mM H_2SO_4
Mobile phase flow rate	0.5 mL/min
Column temperature	30°C
Detector type and wavelength	Waters 2414 Refractive Index detector, 410 nm
Detector temperature	30°C
Injection volume	$5 \mu\text{L}$
Analysis period	10 min
Space time	5 min

Table 3.11 Conditions for HPLC system for organic acid analysis (İleri and Çalık 2006)

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

3.6.8 Protease Activity Assay

Proteolytic activity was determined by hydrolysis of casein. Three different buffer solutions were used to determine the acidic, basic and neutral protease activities. Hammerstein casein solution was prepared in either 0.05 M borate buffer (pH 10), 0.05 M sodium acetate buffer (pH 5) and 0.05 M sodium phosphate buffer (pH 7). 2 mL of the casein solution were mixed with 1 mL of diluted supernatant sample and hydrolyzed at 30°C for 20 min. To cease the reaction 10% (w/v) trichloroacetic acid (TCA) was added to the mixture and kept in ice for 20 min. After termination of the reaction the samples were centrifugated at 10500 rpm for 10 min at +4°C and then kept at room temperature for 5 min. The absorbance of supernatant was measured at 275 nm in UV-Vis spectrophotometer in quartz cuvettes. In the case of need, dilutions were made such that the absorbance was between 0.2-0.6.

It is defined that one unit protease activity is the activity that releases 4 nmole tyrosine per minute (Moon and Parulekar 1991). The calibration equation for converting absorbance to protease activity ($U\text{ cm}^{-3}$) is given in the equation 3.2 (Çalık 1998).

$$A = \left(\frac{\text{Absorbance}}{0.8 \times 10^{-3} / \mu\text{mol} \cdot \text{cm}^{-3}} \right) \left(\frac{1U}{4 \text{nmolmin}} \right) \left(\frac{1}{20 \text{min}} \right) \left(\frac{1000 \text{nmol}}{1 \mu\text{mol}} \right) \left(\frac{\text{Dilution}}{\text{Ratio}} \right) \quad (3.2)$$

3.6.9 Determination of AOX Activity

3.6.9.1 Yeast Lysis to Obtain Intracellular Medium

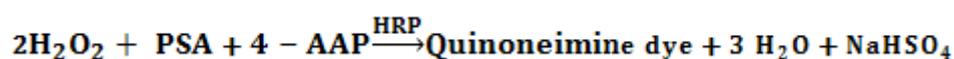
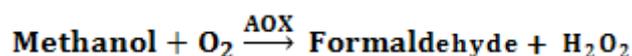
Since AOX is the first enzyme of methanol metabolism of *Pichia pastoris*, the specific activity of the AOX has to be determined. For that purpose, first of all the intracellular medium of cells should be extracted because of being AOX an intracellular enzyme. 1 mL of fermentation medium was centrifuged (10 min, 12500 rpm, +4°C) and supernatant was removed. For extraction of intracellular medium yeast lysis buffer (Appendix A) was added on the cells such that final volume was 500 μL , then mixed up for 20 sec and kept on ice for 30 sec which was done three times in series. Afterwards the same procedure was applied with addition of a spoon of glass beads.

When this process was finished cells were centrifugated at 3000 rpm for 2 min at +4°C and the supernatans were taken away from the cells and centrifugated again at 12500 rpm for 5 min at +4°C. The supernatants obtained from the second centrifugation were used for determination of AOX activity.

3.6.9.2 AOX Activity

With the aim of monitoring the oxidation of methanol to formaldehyde by AOX, a bi-enzymatic assay combining alcohol oxidase (AOX) and horseradish peroxidase (HRP) was used. This colorimetric system based on the enzymatic reaction of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) and

measurement the concentration of liberated H₂O₂ with activity of AOX. The first step of the reaction series starts with the oxidation of methanol to H₂O₂ and formaldehyde which is catalyzed by AOX. Secondly, two moles of H₂O₂ reacts with the one mole of PSA and one mole of 4-AAP, yielding one mole of quinoneimine dye one mole of sodium hydrogensulfate and three moles of water.



The quinoneimine dye has a characteristic magenta color with a maximum absorption around 500 nm. The AOX activity was determined with the monitoring the increase in the absorbance at 500 nm which is proportional to the production rate of H₂O₂ and rate of methanol consumption as well. One unit of AOX activity (U) was defined as the number of μmol of H₂O₂ produced per minute at 25°C (Azevedo *et al.* 2004). The reaction mixture containing 3 mL standard assay reaction mixture, 30 μL HRP, 375 μL methanol and 75 μL sample was mixed in a cuvette and the increase in the absorbance at 500 nm was monitored for four minutes and recorded in every 30 sec intervals.

To convert absorbance to specific AOX activity the equation 3.3 was formed using calibration curve given in Appendix F.

$$C_{AOX} \left(\frac{U}{gCDW} \right) = 15.67 \left(\frac{UmL^{-1}}{Absorbance} \right) * OD_{500} * \frac{1}{C_X} \quad (3.3)$$

3.6.10 Oxygen Uptake Rate and Liquid Phase Mass Transfer Coefficient

In rhGH production process to determine the liquid phase mass transfer coefficient and oxygen uptake rate the dynamic method (Bandyopadhyay and Humpre 1967) was used. The method was explained in 2.4.2.

Before the inoculation of the microorganism to the production medium in the bioreactor the physical mass transfer coefficient (K_{La_0}) was determined. During the production phase, at a certain cultivation times the dynamic oxygen transfer experiments were performed. To minimize the effect of low oxygen levels on the microorganisms, the experiments were carried out in a short period of time.

CHAPTER 4

RESULTS AND DISCUSSION

The aim of this M.Sc. thesis is, to design a feeding strategy for the substrates, i.e., methanol and sorbitol, of the semi-batch bioprocess for recombinant human growth hormone (rhGH) production, by *P.pastoris Mut⁺* strain carrying *hGH* gene (Çalık *et al.*, 2008; Orman *et al.*, 2009). In this context, in the first part of the study three feeding strategies were designed and used in the pilot scale bioreactor system. In the second part, a feeding strategy based on a study in literature was tested to compare the rhGH productivity with the designed strategies applied in the first part. To be able to make a proper comparison and to investigate the effects of the strategies on the cell growth, substrate consumption and rhGH production rates, alcohol oxidase (AOX) and protease activities were followed by taking samples. Moreover, the organic acid profiles, yield coefficients, specific rates, and the oxygen transfer characteristics were investigated in order to understand the effects of the designed strategies on rhGH production.

4.1 Strategy Design for Production of Recombinant Human Growth Hormone by *Pichia pastoirs*

The designed strategies are fundamentally based on simultaneous feeding of the two substrates starting at $t=0$ h of the production phase, i.e., batch-wise 50 gL^{-1} sorbitol feeding, together with fed-batch methanol feeding with a specific growth rate of $\mu_0=0.03 \text{ h}^{-1}$ or $\mu_0=0.04 \text{ h}^{-1}$, and fed-batch sorbitol feeding with a specific growth rate of $\mu_0=0.025\text{h}^{-1}$ which was calculated based on the specific consumption rate $q_s=0.152 \text{ g g}^{-1}\text{h}^{-1}$ of sorbitol. Consequently, sorbitol concentra-

tion was kept constant at 50 gL^{-1} within $t=0-15\text{h}$ of the production phase; where, sorbitol feeding was terminated at $t=15\text{h}$.

The descriptions of the designed strategies are presented in Table 4.1. In the first strategy (SSM1), methanol was fed to the system with the specific growth rate of $\mu_0=0.03 \text{ h}^{-1}$, and the H^+ concentration (pH) in the bioreactor was kept constant at $\text{pH}=5.0$. In the second strategy (SSM2), pH was kept constant at 5.5 until $t=24\text{h}$ of the induction phase (production phase), thereafter, was reduced to $\text{pH}= 5.0$; where methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03 \text{ h}^{-1}$. In the third strategy (SSM3), methanol was fed with the specific growth rate of $\mu_0=0.04 \text{ h}^{-1}$, and the pH in the bioreactor was kept constant at $\text{pH}=5$.

Table 4.1 The abbreviations used for experiments

Strategy	Strategy Definition
Name	
SSM1	50 gL^{-1} batch-wise sorbitol pulse feeding ($t=0 \text{ h}$) and keeping sorbitol concentration at 50 gL^{-1} at $t=0-15 \text{ h}$; methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$; $\text{pH} 5.0$
SSM2	50 gL^{-1} batch-wise sorbitol pulse feeding ($t=0 \text{ h}$) and keeping sorbitol concentration at 50 gL^{-1} at $t=0-15 \text{ h}$; methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$; $\text{pH} 5.5$ ($t=0-24\text{h}$), $\text{pH} 5.0$ ($t=24-48\text{h}$)
SSM3	50 gL^{-1} batch-wise sorbitol pulse feeding ($t=0 \text{ h}$) and keeping sorbitol concentration at 50 gL^{-1} at $t=0-15 \text{ h}$; methanol feeding with $\mu_0=0.04 \text{ h}^{-1}$; $\text{pH} 5.0$
SM	Mixed feed of 1.37 mol sorbitol and 6.21 mol methanol in 13.88 mol water with $\mu_0=0.03 \text{ h}^{-1}$ ($t=0-30\text{h}$); methanol feeding with $\mu_0=0.03 \text{ h}^{-1}$ ($t=30-42\text{h}$); $\text{pH} 5.0$

Before the production phase, the process proceeds through the first three phases, i.e., glycerol batch-phase (GB), glycerol fed-batch phase (GFB), and methanol transition-phase (MT), in which, glycerol batch-phase is the starting instant of the bioprocess, following glycerol addition whose concentration in the bioreactor medium is 40 g L^{-1} , *P. pastoris* is inoculated. Glycerol fed-batch (GFB) phase is carried out with glycerol feeding according to the predetermined feeding profile (Figure 3.3) whose parameters are given in Table 3.8. The third consecutive phase that is before the production phase, is methanol transition (MT) phase performed by fed-batch feeding of 100% methanol with the feed rate of 3.6 mL/h per initial fermentation volume within 4 hours.

The isothermal temperature in the bioreactor was set to 30°C which was found as the optimum temperature for the production of rhGH (Inankur 2010), and the temperature was kept constant at $30 (\pm 0.1)^\circ\text{C}$ by the PI controller of the bioreactor system. H^+ concentration in the bioreactor was kept constant at $\text{pH}=5.0$ or at $\text{pH}=5.5$, within the defined time periods of the designed fed-batch strategies.

In the induction (production) phase of the four bioreactor operations, sorbitol was used as the co-substrate which is known as the non-inhibitory carbon source for AOX promoter. Batch-wise feeding of sorbitol at $t=0\text{h}$ of the production phase, was suggested to eliminate the long lag-phase as well as proliferation of the cell growth (Çelik *et al.*, 2009, Açık, 2009, Inankur, 2010); and, 50 g L^{-1} sorbitol was indicated as the non-inhibitory limit for Mut^+ strain producing rhGH (Açık, 2009). Based on these knowledge, besides the predetermined methanol feeding, batch-wise 50 g L^{-1} sorbitol feeding at $t=0 \text{ h}$ of the production phase proceed by fed-batch sorbitol feeding until $t=15\text{h}$, to keep sorbitol concentration constant at 50 g L^{-1} within $t=0-15\text{h}$ of the strategies SSM1, SSM2 and SSM3. The parameters for the predetermined methanol and sorbitol feeding are given in Table 3.8.

pH was kept constant at $\text{pH}=5.0$ throughout of the bioprocess in the designed SSM1 and SSM3, and in the tested operation condition SM. As Çalık *et al.* (2010b) reported that the rhGH production was higher until $t=15 \text{ h}$ of the process

operated at pH 5.5 than the process operated at pH 5.0; in SSM2, pH was set to 5.5 until $t=24\text{h}$ of the induction phase, thereafter reduced to 5.0.

Related with the other parameter, i.e., methanol feed rate, as Çalık *et al.* (2010a) reported that the rhGH production was higher within $t=0-15\text{h}$ of the induction phase when methanol was fed with the predetermined feed rate calculated for the specific growth rate of $\mu_0=0.04\text{ h}^{-1}$ than that of the rate for $\mu_0=0.03\text{ h}^{-1}$, in SSM3 methanol was fed to the bioreactor with the predetermined growth rate calculated for $\mu_0=0.04\text{ h}^{-1}$; while in SSM1 and SSM2, the predetermined feed rates were calculated for the specific growth rates of $\mu_0=0.03\text{ h}^{-1}$.

The strategy SM, was derived from the study of Jungo *et al.* (2007) and tested in order to compare the results obtained with those of SSM1, SSM2 and SSM3. A mixed substrate solution was prepared with 1.37 mol sorbitol and 6.21 mol methanol in 13.88 mol water. The solution was fed to the bioreactor until $t=30\text{h}$ of the induction phase with the predetermined growth rate of $\mu_0=0.03\text{ h}^{-1}$; thereafter only methanol was fed to the bioreactor with the growth rate of $\mu_0=0.03\text{ h}^{-1}$.

For an in-depth analyses of the results produced from the designed strategies and the tested strategy (SM) on the rhGH production, comparisons are also made with the former strategies used in our laboratory, i.e., MSS-0.03 (Açık, 2009) and MSSS-0.03 (Inankur, 2010). The descriptions of these strategies are given in Table 4.2.

Table 4.2 Definitions for MSS-0.03 and MSSS-0.03 strategies

Strategy Name	Strategy Definition
MSS-0.03	Batch-wise sorbitol addition at $t=0\text{ h}$ and $t=9\text{ h}$ and methanol feeding with $\mu_0=0.03\text{ h}^{-1}$
MSSS-0.03	Batch-wise sorbitol addition at $t=0\text{ h}$, $t=14\text{ h}$ and $t=31\text{ h}$ and methanol feeding with $\mu_0=0.03\text{ h}^{-1}$

4.1.1 Sorbitol Consumption

Sorbitol concentration was kept constant at 50 g L^{-1} until $t=15\text{h}$ of the induction phase where predetermined feeding strategies for methanol and sorbitol are applied (Figure 4.1). As shown in Table 4.3, until $t=15\text{h}$ of the designed processes, sorbitol consumption rates, q_s , were higher at $t=0\text{h}$, i.e., $0.144 \text{ g g}^{-1} \text{ h}^{-1}$ at SSM1, $0.167 \text{ g g}^{-1} \text{ h}^{-1}$ both at SSM2 and SSM3, than the subsequent consumption rates and the processes proceeded at almost constant values. After $t=15\text{h}$, with the termination of fed-batch sorbitol feeding, sorbitol consumption rates began to decrease (Table 4.3) owing to the increase in cell concentration and decrease in sorbitol concentration and it is totally consumed at $t=24\text{h}$ at SSM1, at $t=36\text{h}$ at SSM2 and at $t=25\text{h}$ at SSM3 showing a linear variation with the cultivation time.

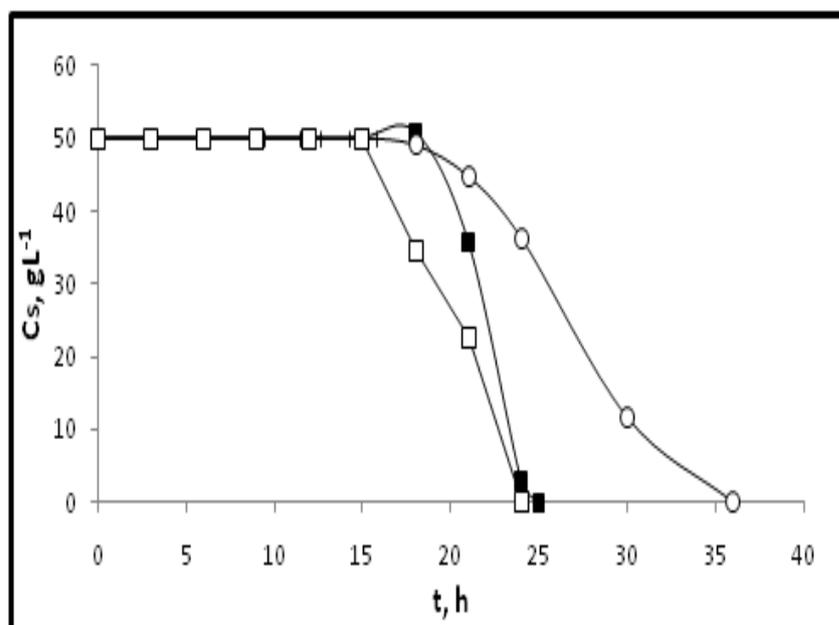


Figure 4.1 Variation in sorbitol concentration with the cultivation time in the production phase: SSM1 (■), SSM2 (○) and SSM3 (□).

Related with sorbitol consumption, Aık (2009) and Inankur (2010) found that depletion of sorbitol lasted 15 h in the strategies MSS-0.03 and MSSS-0.03 (Table 4.2) in the production of rhGH by the same recombinant *P.pastoris*. In SSM1 and SSM3, sorbitol is totally consumed after the $t=15\text{h}$ long $C_S=50\text{ g L}^{-1}$ constant-sorbitol concentration period within proceeding 10h, until $t=25\text{h}$ (Figure 4.1); where, the cell concentrations were, respectively, 2.4- and 2.5-fold, and 2.6- and 2.8-fold higher than that of the MSS-0.03 and MSSS-0.03. Nevertheless in SSM2, where the process pH was constant at pH 5.5 until $t=24\text{h}$, sorbitol is totally consumed after the $C_S=50\text{ g L}^{-1}$ constant-sorbitol concentration period within proceeding 22h, until $t=37\text{h}$. The analyses of these results reveal that the cell concentrations were lower throughout SSM2 than that of SSM1 and SSM3, whereupon the sorbitol consumption rate was lower than that of SSM1 and SSM3, whereas the cells consumed nearly the same amount of methanol in SSM2 (188.2 g L^{-1}) as in SSM1 (190.5 g L^{-1}). The influences are, both AOX activity and rhGH production rate are higher in SSM2 than that of SSM1 until $t=18\text{h}$, and than that of SSM3 for the whole process. Moreover, methanol is the preferred substrate by the cells than sorbitol in SSM2 which is operated at $\text{pH}=5.5$ until $t=24\text{h}$; thus, sorbitol consumed within a lengthened cultivation time, more than 10h in SSM3 compared to SSM1. The specific consumption rates calculated for the three designed strategies are slightly lower than those obtained in the strategies MSS-0.03 (Aık 2009), MSSS-0.03 (Inankur 2010), due to higher cell concentrations obtained in SSM2 and SSM1.

Methanol and sorbitol concentrations were followed by HPLC. Methanol was not detected in the fermentation media, and the specific methanol consumption rate, q_M , remained constant throughout the fermentations (Table 4.3). Similar with the findings of Jungo *et al.* (2007), elik *et al.* (2009), and alık *et al.* (2010a), methanol and sorbitol consumed simultaneously, which can be seen from the specific consumption rates. In SSM3, related with methanol consumption, as methanol was fed to the bioreactor with the feeding rate of $\mu_0=0.04\text{h}^{-1}$, methanol consumption rates were higher than those of SSM1 and SSM2 strate-

gies as expected, which is in agreement with the results of Çalık *et al.* (2010a) . In SSM1 and SSM2 , the specific methanol consumption rates were close to each other, as methanol was fed with the predetermined growth rate of $\mu_0=0.03 \text{ h}^{-1}$.

4.1.2 The Cell Growth

In order to understand the effects of the simultaneous feeding of sorbitol and methanol, the analysis of the cell growth rates are indeed important. The consecutive three phases of the bioprocess before the production phase, i.e., GB, GFB, and MT, were performed identically in SSM1, SSM2 and SSM3. The cell concentration profiles in the three consecutive phases are presented in Figure 4.2a, which shows that the process starts and proceeds following the same course of the cell concentration without any significant difference through the first two phases, i.e., GB and GFB, for all the designed strategies. Nevertheless, in MT phase of SSM2, the cell concentration is slightly lower than the others, as in SSM2 the bioreactor was operated at pH=5.5.

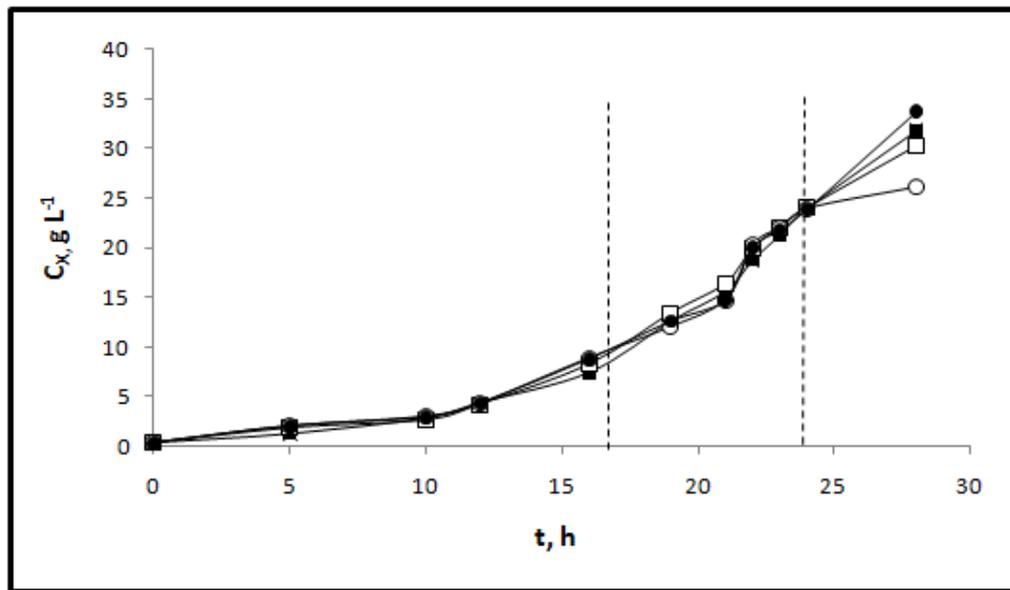


Figure 4.2a Variation in the cell concentration with the cultivation time at glycerol batch (GB), glycerol fed-batch (GFB) and methanol transition (MT) phase of each strategy; SSM1 (■), SSM2 (○), SSM3 (□) and SM (●).

The cell concentration profile obtained in the production phase of SSM1, SSM2 and SSM3 are presented in Figure 4.2b. The $C_S=50 \text{ g L}^{-1}$ constant-sorbitol concentration period prevented the long lag phase ($t=0-9\text{h}$) which was observed only in the bioreactor operations with sole substrate methanol feed. The cells started to proliferate immediately, similar to the findings of Aık (2009) and Bayraktar (2009). The highest cell concentrations obtained in SSM1, SSM2, and SSM3 are, respectively, 105.325 g L^{-1} , 99.3 g L^{-1} , and 92.4 g L^{-1} at $t=48\text{h}$, which are 1.8-, 1.6-, and 1.5-fold higher than the cell concentrations obtained at $t=48\text{h}$ in MSSS-0.03 (İnankur, 2010), and 1.9-, 1.8-, and 1.7-fold higher than the cell concentration obtained at $t=30\text{h}$ in MSS-0.03 (Aık, 2009); whereas at $t=30 \text{ h}$, the cell concentrations in SSM1, SSM2, and SSM3 are also 1.7-, 1.4-, 1.5-fold higher than the cell concentration obtained at $t=30\text{h}$ in MSS-0.03. Therefore, the $C_S=50 \text{ g L}^{-1}$ constant-sorbitol concentration period within the first 15h of the process provides more *P. pastoris* cells instead of two or three pulse sorbitol feeding.

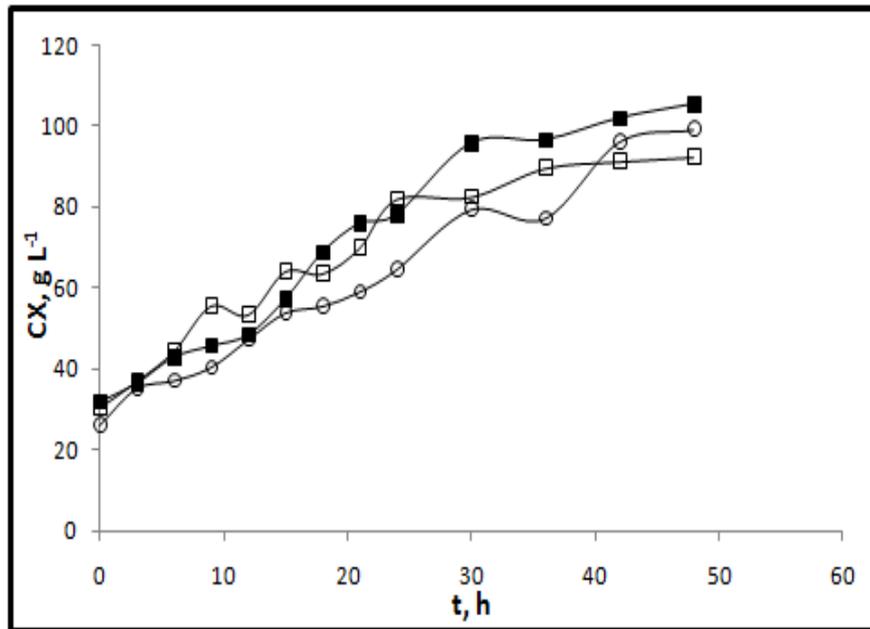


Figure 4.2b Variation in cell concentrations with cultivation time in the production phase of bioprocesses with different feeding profiles: SSM1 (■), SSM2 (○) and SSM3 (□).

In general, the cell growth rates in the three designed strategies are close to each other (Table 4.3); however, are higher than that of MSSS-0.03 (İnankur, 2010) and MSS-0.03 Aık, 2009). The highest specific growth rates obtained in SSM1, SSM2 and SSM3 are, respectively, 0.067, 0.123 and 0.081 h⁻¹. After sorbitol was consumed, in SSM1 and SSM3 at t=25h, and in SSM2 at t=36h, the bioprocess turns to single-substrate fermentation, where the cell growth rates decrease as the cells could find only the other substrate methanol for the growth, which were also observed in MSSS-0.03 (İnankur, 2010) and MSS-0.03 (Aık, 2009). Besides, the decrease in the cell concentrations in MSSS-0.03 (İnankur, 2010) is also because of sorbitol pulse-feeding that creates drastic sorbitol-concentration changes in the micro-environment of *P. pastoris* cells, which was prevented by fed-batch sorbitol feeding by creating the C_S=50 gL⁻¹ constant-sorbitol concentration period in SSM1, SSM2, and SSM3.

Table 4.3 Variations in specific rates throughout the fermentation bioprocess

Exp. Name	t h	μ_t h ⁻¹	q_s g g ⁻¹ h ⁻¹	q_M g g ⁻¹ h ⁻¹	$q_{rp} * 1000$ g g ⁻¹ h ⁻¹
	0	0.056	0.144	0.071	0.130
	3	0.058	0.123	0.067	0.073
	6	0.043	0.103	0.061	0.076
	9	0.028	0.095	0.061	0.100
SSM1	12	0.048	0.088	0.061	0.098
	15	0.067	0.072	0.055	0.320
	18	0.050	0.031	0.049	0.385
	21	0.025	0.103	0.048	0.386
	24	0.033	0.051	0.051	0.480
	30	0.022		0.048	0.261
	36	0.012		0.055	0.115
	42	0.015		0.060	0.042
	48	0.014		0.065	-0.028

Table 4.3 Continued

Exp. Name	t h	μ_t h ⁻¹	q_s g g ⁻¹ h ⁻¹	q_M g g ⁻¹ h ⁻¹	$q_{rp} * 1000$ g g ⁻¹ h ⁻¹
SSM2	0	0.123	0.167	0.071	0.105
	3	0.059	0.130	0.057	0.220
	6	0.030	0.131	0.058	0.201
	9	0.049	0.127	0.057	0.050
	12	0.055	0.114	0.052	0.267
	15	0.034	0.105	0.049	0.274
	18	0.019	0.050	0.051	0.163
	21	0.029	0.033	0.052	0.209
	24	0.039	0.055	0.052	0.224
	30	0.018	0.051	0.049	0.203
	36	0.024	0.025	0.058	0.228
	42	0.026		0.054	0.159
	48	0.013		0.060	0.118
SSM3	0	0.123	0.167	0.071	0.105
	3	0.059	0.130	0.057	0.220
	6	0.030	0.131	0.058	0.201
	9	0.037	0.106	0.063	0.027
	12	0.037	0.115	0.070	0.030
	15	0.037	0.098	0.062	0.052
	18	0.021	0.064	0.067	0.054
	21	0.049	0.080	0.066	0.034
	24	0.023	0.031	0.060	0.020
	30	0.015		0.069	0.027
	36	0.016		0.072	0.033
	42	0.012		0.081	0.066
	48	-0.233		0.092	0.117

4.1.3 rhGH Production

The influences of the designed substrate feeding strategies on the rhGH production were analyzed by using the data obtained by SDS-PAGE analyses of the samples collected from the fermentation broth, besides the quantitative analyses of the samples by HPCE (Figure 4.3). The highest rhGH concentration was obtained in SSM1 as $C_{rhGH}=640 \text{ mg L}^{-1}$ at $t=42\text{h}$ (Figure 4.3), which is 84% of the

total proteins secreted in SSM1. The increase in rhGH concentration with the cultivation time can be seen from the SDS-PAGE results of SSM1 (Figure 4.4).

In the previous strategies designed in our laboratory, the highest rhGH value reached in MSS-0.03 (Açık, 2009) was $C_{rhGH} = 301 \text{ mg L}^{-1}$ at $t = 30 \text{ h}$ of the induction phase, which is higher than that of MSSS-0.03 (Inankur, 2010) where the highest rhGH concentration was obtained as $C_{rhGH} = 290 \text{ mg L}^{-1}$ at $t = 39 \text{ h}$. These values are compared with the designed three strategies of this work; amongst, in SSM1 the highest rhGH concentration is, respectively, 2.1- and 2.2-fold higher than that obtained in MSS-0.03 and MSSS-0.03 (Figure 4.3). As the cultivation time of MSS-0.03 (Açık, 2009) fermentation is shorter as $t = 30 \text{ h}$, based on the same cultivation time, rhGH concentration obtained in SSM1 is 1.8-fold higher than that obtained in MSS-0.03.

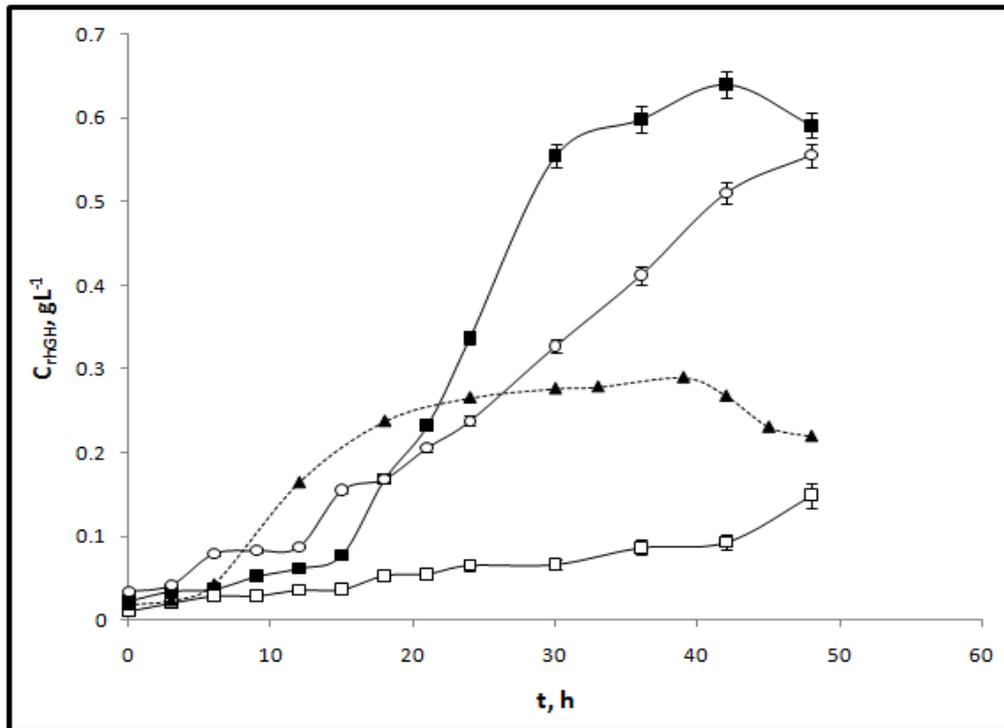


Figure 4.3 Variations in rhGH concentration with the cultivation time: SSM1 (■), SSM2 (○) and SSM3 (□), MSSS-0.03 (▲) (error ratio standard deviation is calculated as : 2.5%).

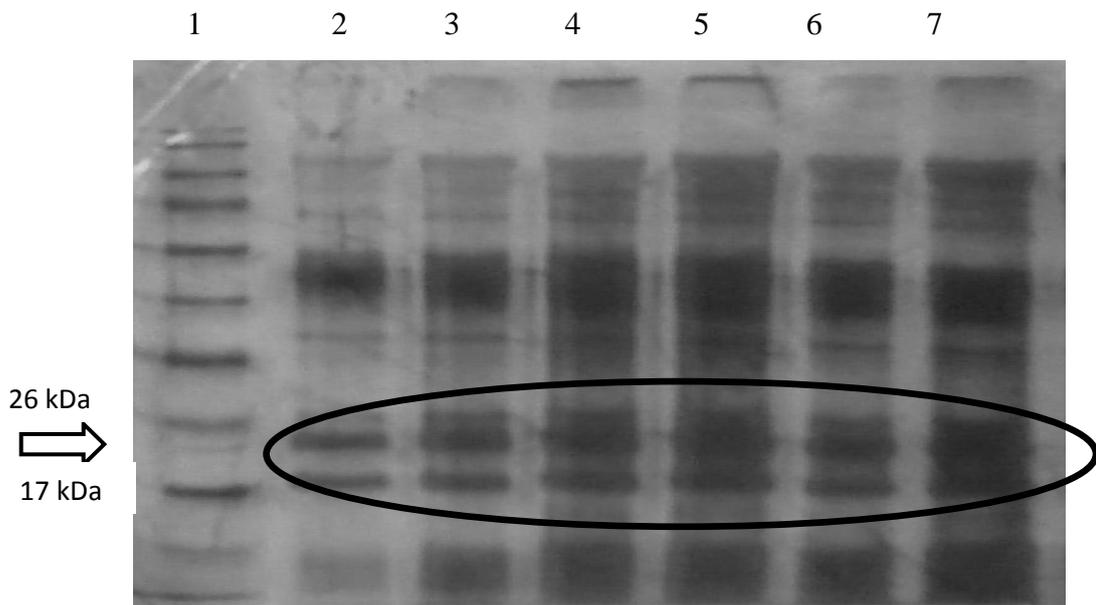


Figure 4.4 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in SSM1: Lane-1: protein marker, lane-2: t=21h, lane-3: t=24h, lane-4: t=30h, lane-5: t=36h, lane-6: t=42h, lane-7: t=48h.

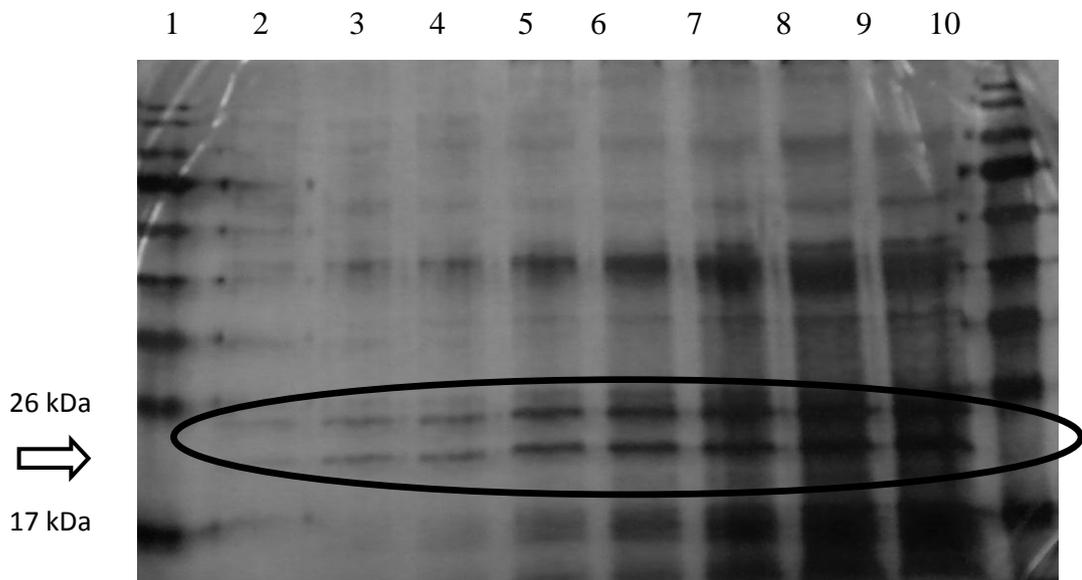


Figure 4.5 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in SSM2: Lanes-1 and 10: protein marker, lane-2: t=9h, lane-3: t=12h, lane-4: t=15h, lane-5: t=21h, lane-6: t=30h, lane-7: t=30h, lane-8: t=36h, lane-9: t=48h.

The specific recombinant production rate in SSM1 increased with the cultivation time and reached to the highest value as $0.480 \text{ g g}^{-1} \text{ h}^{-1}$ at $t=24\text{h}$, the cultivation time which corresponds to sorbitol depletion (Figure 4.1), is indeed noteworthy. Whereas in MSS-0.03 strategy (Açık, 2009), the highest specific rhGH production rate was $q_{rp}= 0.380 \text{ g g}^{-1} \text{ h}^{-1}$ at $t=9\text{h}$ which is 1.3-fold lower than that of the SSM1.

In SSM2, the highest rhGH concentration corresponds 71% of the proteins secreted into the fermentation broth, obtained at $t=48\text{h}$ as $\text{Crp}=556 \text{ mg L}^{-1}$ which is 0.9-fold lower than that obtained in SSM1; however, 1.8- and 1.9-fold higher than that obtained, respectively, in MSS-0.03 (Açık, 2009) and MSSS-0.03 (Inankur, 2010). In SSM2 until $t=18\text{h}$, the specific rhGH production, specific growth, and specific sorbitol consumption rates were higher than those obtained in SSM1 until the pH change. After pH was decreased to 5.0 in the bioreactor, an increase in the specific rhGH production, growth, and sorbitol consumption rates were observed, which were continued until the depletion of sorbitol at $t=36\text{h}$. Although the rhGH concentration obtained in SSM2 is lower than that of the SSM1, by lengthening the cultivation time up to $t=48\text{h}$ than that of SSM1, but with a lower slope, is increased to a high value ($\text{Crp}=556 \text{ mg L}^{-1}$) (Figure 4.3 and 4.5). The difference between the recombinant rhGH production performances and fermentation characteristics of *P. pastoris* in SSM1 and SSM2 should primarily be due to higher initial pH ($\text{pH}=5.5$) and pH operation condition until $t=24\text{h}$ in SSM2, which influenced the sorbitol consumption.

Among the three designed strategies, the lowest rhGH production was obtained in SSM3, where the predetermined feeding rate for methanol was calculated for the higher specific growth rate of $\mu_0=0.04 \text{ h}^{-1}$. Although the highest sorbitol and methanol consumption rates $q_s=0.167 \text{ g g}^{-1} \text{ h}^{-1}$, $q_m=0.095 \text{ g g}^{-1} \text{ h}^{-1}$ were obtained in SSM3, the influences on the AOX activity resulted in lower rhGH production. The highest rhGH concentration obtained in SSM3 was $\text{Crp}=149 \text{ mg L}^{-1}$ which is 4.3-, 3.7-, and 1.9-fold lower than those obtained, respectively, in the designed strategies SSM1 and SSM2 of this work, and MSSS-0.03 (Inankur,

2010). Çalık *et al.* (2010a) reported the effects of methanol feeding on rhGH production and concluded that higher rhGH concentration in the fermentation process is obtained when methanol is fed with the predetermined growth rate calculated for $\mu_0=0.03 \text{ h}^{-1}$ rather than the growth rate for $\mu_0=0.04 \text{ h}^{-1}$, similar to the findings of this work. The recombinant protein rhGH is ca. 71% of the total proteins secreted in SSM3. The SDS-PAGE analyses of the three strategies are presented in Figure 4.6 for comparison.

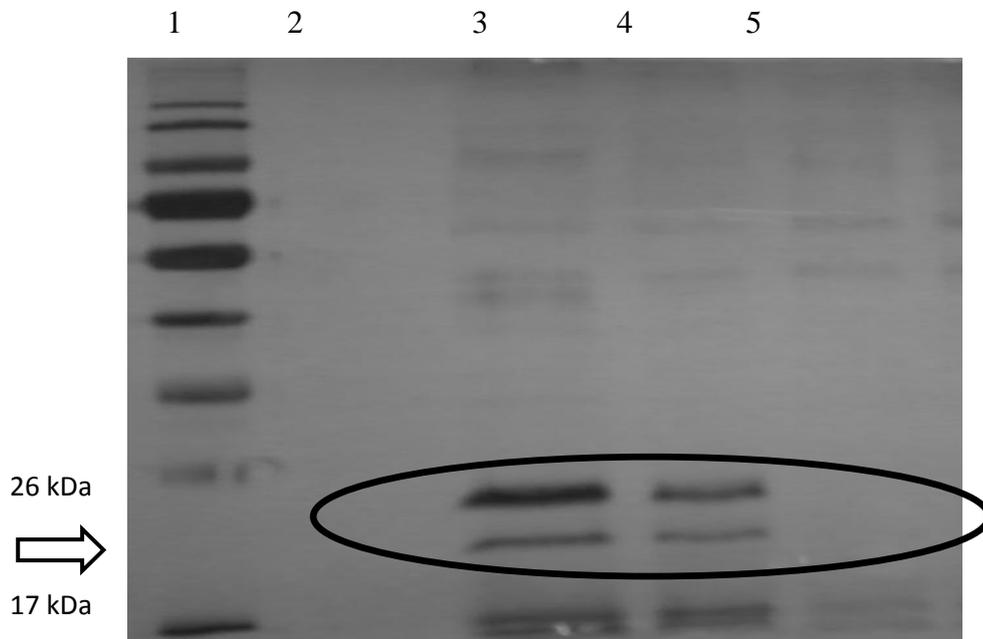


Figure 4.6 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in pilot scale bioreactor to observe effect of SSM1, SSM2 and SSM3 operations on rhGH production. 1. well: protein marker, 3. well: t=42 for SSM1, 4. well: t=48h for SSM2, 5.well: t=48h for SSM3 (Dilution ratio of the samples=1/2 (v/v)).

4.1.4 Influences on Alcohol Oxidase Activity in *P. pastoris*

Alcohol oxidase (AOX) is the first enzyme in the methanol utilization pathway in *P. pastoris*. As induction of rhGH production is under the control of AOX promoter, AOX activities were determined and presented in Figure 4.7 for SSM1, SSM2 and SSM3.

In the three designed strategies, the variations in AOX activities with the cultivation time show oscillatory changes, started with a maxima at the beginning of the induction phase, which continue with decaying oscillations, lasted in SSM1 with a peak at ca. $t=37\text{h}$; in SSM2 significantly at ca. $t=25\text{h}$, further at ca. $t=48\text{h}$ with a very low AOX activity and impact on rhGH production; and in SSM3 lasted at ca. $t=8\text{h}$, further with an almost decayed peak at ca. $t=42\text{h}$.

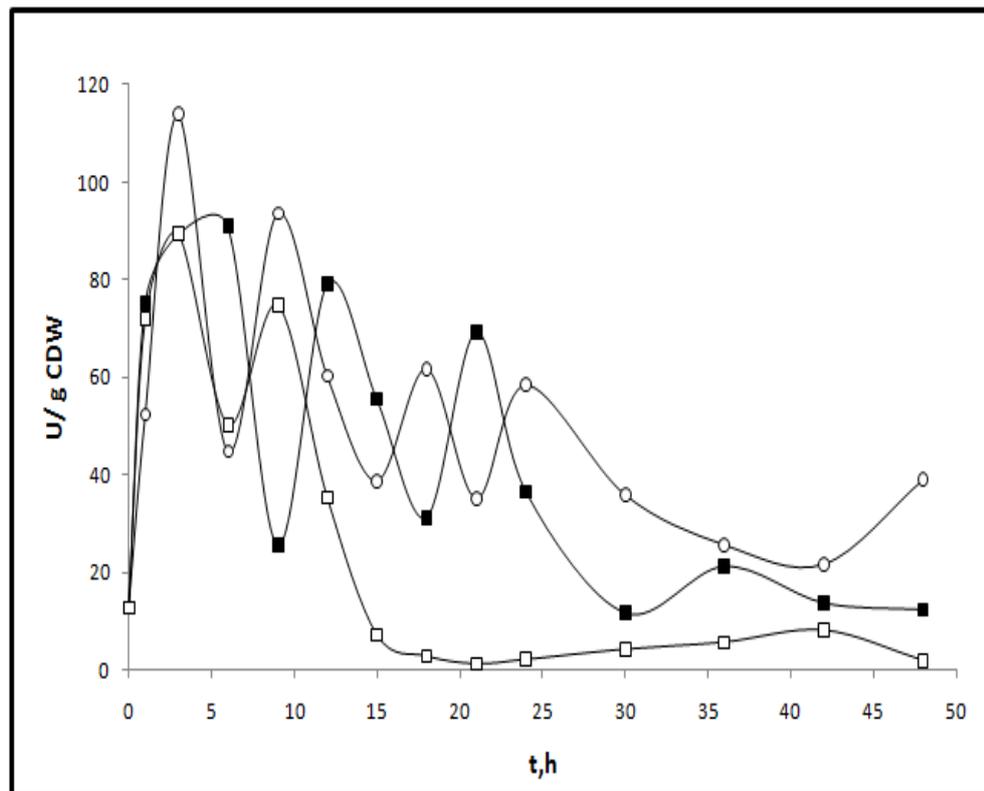


Figure 4.7 Variations in AOX activity with cultivation time: SSM1 (■), SSM2 (○) and SSM3 (□).

It is clear that the differences are due to the changed parameters in designing the three strategies, one of which is the pH operation used in SSM2 which has a lowering impact on AOX activity after ca. $t=10\text{h}$, whereupon the rhGH production than that of SSM1; secondly, the predetermined feeding rate of methanol calculated using the higher specific growth rate, i.e., $\mu_0=0.04\text{ h}^{-1}$ in SSM3, which has more detrimental effect on AOX activity, whereupon on the rhGH production than that of SSM1.

Sorbitol and its feeding strategy within the designed strategies is appeared as a vital common part of the strategies; in the very beginning of the common $t=15\text{h}$ long $C_S=50\text{ gL}^{-1}$ constant-sorbitol concentration period, within one hour sharp increase in AOX activities were observed, and in less than one hour reached to 5.9-, 4.1-, 5.6-fold higher values obtained than that of, respectively, at $t=0\text{h}$ of SSM1, SSM2 and SSM3. Until $t=9\text{h}$ AOX activity in SSM2 was higher than that of at SSM1 which resulted in higher rhGH production until $t=18\text{h}$ (Figure 4.3); nevertheless, after $t=9\text{h}$, AOX activity decreased.

When considering the previous studies in our laboratory, the production phase started with approximately 4.2- and 6.2-fold higher AOX activities (Figure 4.7) than that of at MSS-0.03 (Açık, 2009) and MSSS-0.03 (Inankur, 2010) due to the condition changed in methanol transition (MT) phase, before the induction phase. On the other hand, the non-repressing carbon source feeding provides higher AOX activities in a shorter time, besides it shortens the lag phase of the cell growth, as also reported by Açık (2009) and Inankur (2010).

The highest AOX activity was obtained as 113.9 U/g CDW at $t=3\text{h}$ at SSM2 which is 3.3-fold and 2.9-fold higher than the highest AOX activity obtained at MSS-0.03 (Açık, 2009) and MSSS-0.03 (Inankur, 2010), respectively.

4.1.5 Influences on Protease Activity

Total protease activity which includes neutral, acidic and basic proteases within the extracellular medium was determined and converted to protease concentration. The total protease concentrations determined for SSM1, SSM2 and SSM3 strategies are given in Figure 4.8. At all the three strategies protease formation begins with lower levels and increases with respect to time (Figure 4.8). The highest protease concentration was obtained as 0.160 g L^{-1} at $t=48\text{h}$ at SSM1 strategy where the highest rhGH concentration was obtained.

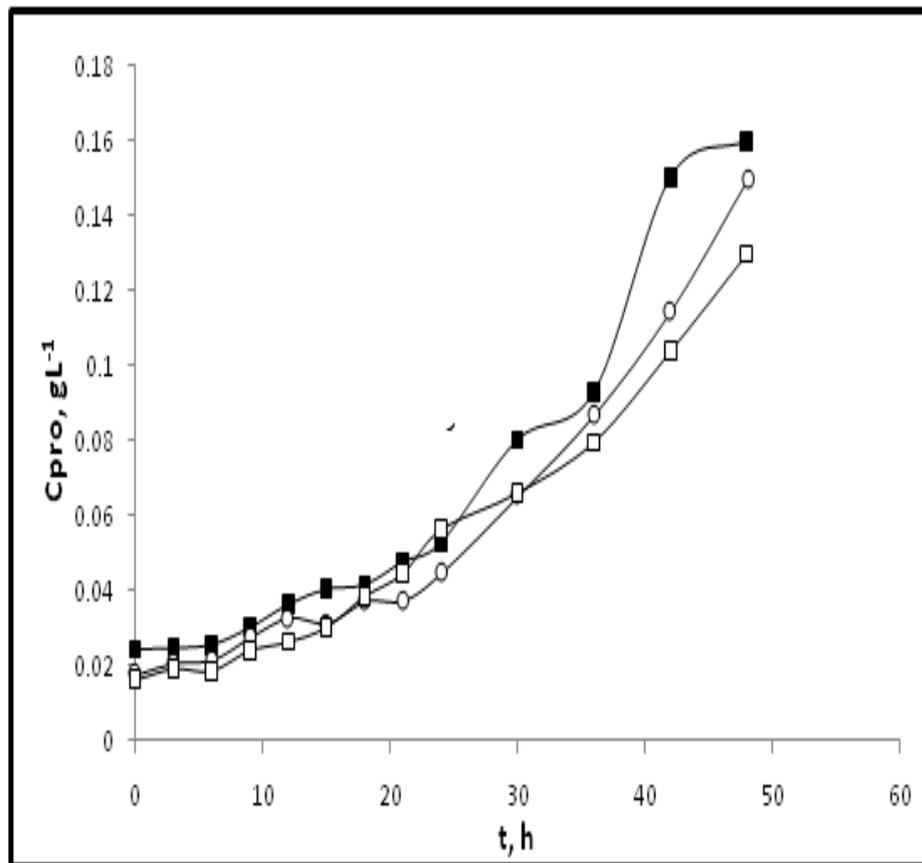


Figure 4.8 Variations in protease concentration with the cultivation time: SSM1 (■), SSM2 (○) and SSM3 (□).

When considering the MSSS-0.03 protease concentration profile, within the 24h of the induction phase, the total protease concentration was higher than that of the SSM1 but afterwards with the depletion of sorbitol at t=24h in SSM1 which yielded an increase in protease concentration, the last total protease concentration reached to closer value with the that of at MSSS-0.03. At MSS-0.03 strategy, protease concentration was 1.7 fold higher than that obtained at SSM1 at t=24h. Thereby the reason of production of higher rhGH at SSM1 became clearer when considering together with the cell and protease concentration together with AOX activity.

As Çalık *et al* (2010b) reported that the protease concentration obtained at the strategy operated at pH=5.5 was higher than the that of pH=5.0, identically in this study protease concentration obtained at SSM2 was lower than the that of at SSM1 since the operation pH of SSM2 was kept constant at 5.5 until t=24h. After pH was reduced to 5.0 at t=24h a sharp increase in protease formation was observed (Figure 4.8). As a result, lower total protease concentration together with higher AOX activity until t=18h at SSM2 than those of the SSM1 resulted in higher rhGH concentration. When considering the MSS-0.03 strategy, the highest protease concentration was 1.4-fold higher than SSM2, together with lower cell concentration and AOX activity resulted in lower rhGH production than that of SSM2.

The lowest last protease concentration was obtained as 0.129 g L^{-1} at t=48h for SSM3 which is 1.2-fold and 1.15-fold lower than SSM1 and SSM2 although until t=24h it was close to that of SSM1.

4.1.6 Influences on Organic Acid Profiles

Organic acids are regulated by the intracellular reaction networks of the cells. Determining the concentration of these metabolites in the medium can give insight about the supply and demand of the metabolites. Variations in organic acid concentrations are given in Table 4.4.

Table 4.4 Variations in organic acid concentrations with respect to time for different feeding strategies in g L⁻¹

	Strategy t(h)	3	12	24	36	42	48
SSM1	Formic acid	-	-	0.1046	0.1280	0.0733	0.1304
	Acetic acid	-	-	0.0158	0.0282	0.0246	0.0427
	Succinic acid			0.0087	0.0342	0.0339	0.0594
	Citric acid	-	-	0.0137	0.0832	0.0810	0.1655
	Pyruvic acid			0.0115	0.0522	0.0268	0.0455
	Oxalic acid	0.0019	0.0016	0.0274	0.0392	0.0543	0.0658
	Fumaric acid	-	-	0.0015	0.0042	0.0036	0.0058
	Maleic acid	-	-	0.0002	0.0005	0.0006	0.0009
	Strategy t(h)	3	12	24	36	42	48
SSM2	Formic acid	0.0682	0.0696	0.0756	0.0839	0.1105	0.1605
	Acetic acid	-	-	0.0219	0.0164	0.0325	0.0612
	Succinic acid	0.0262	0.0301	0.0609	0.1413	0.1614	0.2148
	Citric acid	0.0106	0.0049	0.0181	0.0942	0.1333	0.1962
	Pyruvic acid	0.0023	0.0013	0.0009	0.0010	0.0012	0.0033
	Oxalic acid	0.0042	0.0067	0.0107	0.0417	0.0554	0.0768
	Fumaric acid	0.0010	0.0010	0.0014	0.0032	0.0044	0.0058
	Maleic acid	0.0002	0.0001	0.0000	0.0003	0.0005	0.0008
	Strategy t(h)	3	12	24	36	42	48
SSM3	Formic acid	0.0425	0.0821	0.1227	0.1358	0.1461	0.1850
	Acetic acid	-	-	-	0.0112	0.0426	0.0469
	Succinic acid	0.0304	0.0346	0.0286	0.0615	0.0507	0.0705
	Citric acid	-	-	0.0310	0.0795	0.1092	0.1288
	Pyruvic acid	0.0044	0.0030	0.0092	0.0010	0.0098	0.0168
	Oxalic acid	0.0054	0.0045	0.0182	0.0324	0.0747	0.0669
	Fumaric acid	0.0004	0.0005	0.0007	0.0020	0.0034	0.0040
	Maleic acid	-	0.0001	0.0000	0.0003	0.0007	0.0009

At all the three cases succinic acid, citric acid, pyruvic acid, oxalic acid, fumaric acid, maleic acid, formic acid and acetic acid were detected in the bioreactor medium. On the other hand, lactic acid was not detected in the bioreactor medium of SSM1 and SSM2 operations but detected in the bioreactor medium of SSM3 even in small amounts. Lactic acid is mainly produced in the case of oxygen limited fermentations where the TCA cycle can not take place efficiently. Thus, it was an expected result for SSM3 strategy since air enriched with oxygen was never sufficient throughout the process. The highest lactic acid concentration determined at SSM3 was 0.49 g L^{-1} at $t=48\text{h}$.

In the existence of imbalance between the initial oxidation of methanol and formaldehyde consuming reactions, the concentration of formaldehyde in the cell elevates during the cell growth and product formation (Charoenrat *et al.* 2006). Normally, formaldehyde is oxidized to formic acid or goes into assimilatory pathway and enters the glycolysis. It is indicated that formic acid concentration increases with respect to time with increasing methanol feeding rate and in the absence of co-substrate its concentration is much higher in the extracellular medium since it is dissimilated rather than entering the glycolysis pathway. In this study instead of formaldehyde formic acid was detected in the bioreactor medium of the three strategies and its concentration increased with respect to time (Table 4.4). After the depletion of sorbitol, the cell growth slowed down and a remarkable amount of increase was observed in formic acid concentration since formaldehyde was most probably converted to formate instead of forming cell constituents. The highest formic acid concentration was obtained at SSM3 as 0.185 g L^{-1} at $t=48\text{h}$ due to higher methanol feeding rate of $\mu_0=0.04 \text{ h}^{-1}$ than that of the SSM1 and SSM2.

Other organic acids detected in the extracellular medium were mainly the TCA cycle metabolites such as oxalic acid, pyruvic acid, acetic acid, citric acid, fumaric acid, succinic acid, maleic acid. The concentration of these organic acids increased with respect to time especially after the depletion of sorbitol. The highest obtained organic acid concentrations at each strategy are, namely; pyruvic

acid as 0.045 g L⁻¹ at t=48h at SSM1, succinic acid as 0.21 g L⁻¹ at t=48h for SSM2, lactic acid as 0.49 g L⁻¹ at t=48h at SSM3.

In SSM1 until t=24h which is the time when sorbitol was totally depleted, most of the organic acids were undetectable (Table 4.4) which may be explained such that the metabolites are consumed efficiently in TCA and addition of insufficient organic acids externally to the bioreactor medium within 24h would improve the process.

4.1.7 Yield Coefficients

In order to better understand and evaluate the bioprocess it is necessary to determine the overall yield coefficients. The overall yield of cell generated per mass of total substrate consumed ($Y_{X/St}$), the overall yield of product formed per mass of cells generated ($Y_{P/X}$), the overall yield of product per mass of total substrate ($Y_{P/St}$) were calculated for rhGH production processes under different feeding strategies (Table 4.5).

Table 4.5 Overall Yield Coefficients

Strategy	Y_{X/s_t} g/g	Y_{p/s_t} mg/g	$Y_{p/x}$ mg/g
SSM1	0.21	1.83	8.81
SSM2	0.38	1.73	7.12
SSM3	0.15	0.36	2.28
SM	0.22	0.39	1.75

The total amount of carbon sources consumed at SSM2 (302.1 g total substrate) was lower than that of the SSM1 (336.4 g total substrate) and SSM3 (412.5 g total substrate) and moreover, SSM2 was operated at pH 5.5 within t=0-24h of the production phase, consequently, the overall cell yield on total substrate, $Y_{X/St}$, calculated for SSM2 was higher than that of the SSM3 and SSM1

which were operated at pH 5.0. The highest $Y_{X/St}$ was calculated as 0.38 g g^{-1} at SSM2 which gets along with the study operated at pH5.5 of Inankur (2010) where $Y_{X/St}$ was found 1.2-fold higher than the strategy carried out at pH 5.0. Although $Y_{X/St}$ was highest at SSM2, when considering the overall product yield on cell, the calculated $Y_{P/X}$ was 1.2-fold and 3.9-fold higher at SSM1 (Table 4.5) than that of the SSM2 and SSM3, respectively. As a result it can be concluded that as rhGH production is somehow related with the cell concentration since the highest rhGH production was obtained at SSM1.

When considering the overall product yield on total substrate, the highest $Y_{P/St}$ was calculated as 1.83 mg g^{-1} for SSM1 which is close to that of at SSM2 and 5-fold higher than the $Y_{P/St}$ calculated for SSM3 (Table 4.5). All the overall yields calculated for SSM3 are the lowest which was an expected result since rhGH production could not be enhanced with this operation.

When compared with MSS-0.03 strategy, the overall cell yield on substrate is 1.2-fold and 2.2-fold lower than those obtained at SSM1 and SSM3 strategies whereas close to that of the SSM3 which was an expected result when the cell growth profiles are examined. The overall product formation on cell at MSS-0.03 was 1.4- and 3.5-fold higher than those obtained at SSM2 and SSM3, respectively and close to that of the SSM1. The overall product yield on total substrate at MSS-0.03 was also close to that of the SSM1 and SSM2 since both the cell concentration and rhGH production were lower in MSS-0.03; however it was 1.9-fold higher than SSM3 owing to the low rhGH production and high cell concentration obtained in SSM3.

4.1.8 Oxygen Transfer Characteristics of the Bioprocess

As the oxygen affects the productivity of biomass and byproducts in the aerobic fermentation of *P. pastoris* it is important to determine the oxygen characteristics of the bioprocess. In order to find out the oxygen transfer parameters dynamic method was applied. The oxygen transfer parameters; the oxygen uptake rate (OUR), oxygen transfer rate (OTR), and oxygen transfer coefficient, K_{La} ; the

enhancement factor E (K_{La}/K_{La0}), oxygen utilization rate or oxygen demand (OD), Damköhler number (Da) and effectiveness factor (η) were calculated throughout the bioprocess which are listed in Table 4.5.

Concerning about the K_{La} , generally fluctuation was observed throughout the bioprocesses and the oxygen transfer coefficient did not show much differences among the three strategies. Since the agitation rate and temperature was kept constant from the beginning to the end of the process this fluctuation may be related with the rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. As the cells grew to high cell densities, the secreted metabolites and proteins which probably increased the resistance for oxygen transfer. In addition to this, at high cell densities, cells require high amount of oxygen for methanol oxidization thus while methanol is being fed to bioreactor oxygen requirement increases so does mass transfer coefficient, oppositely mass transfer coefficient decreases while methanol is not fed to the medium. The highest K_{La} values obtained as 0.105 s^{-1} at $t=21\text{h}$ at SSM1, 0.064 s^{-1} at $t=6\text{h}$ and $t=15\text{h}$ for SSM2 and SSM3, respectively.

When compared with the previous studies, generally, calculated K_{La} data at MSS-0.03 and MSSS-0.03 was higher than those at this study owing to the high cell concentration together with high amount of secreted metabolites resulted in oxygen transfer limitation at SSM1, SSM2 and SSM3. In MSSS-0.03 the highest K_{La} was calculated as 0.134 s^{-1} which is, 1.3- and 2.1-fold higher than that of the SSM1, SSM2 and SSM3, respectively. At MSS-0.03 the highest K_{La} value (0.162 s^{-1}) was 1.5- and 2.5-fold higher than that of SSM1, SSM2 and SSM3.

Table 4.6 Variations in oxygen transfer parameters with cultivation time in

Exp	t	$K_L a$	E	$OTR \times 10^3$	$OTR_{max} \times 10^3$	$OUR \times 10^3$	OD $\times 10^3$	Da	η	q_0	$Y_{x/o}$
	(h)	(s^{-1})	$K_L a / K_L a_0$	($mol\ m^{-3}\ s^{-1}$)	($mol\ m^{-3}\ s^{-1}$)	($mol\ m^{-3}\ s^{-1}$)	($mol\ m^{-3}\ s^{-1}$)			$g\ g^{-1}\ h^{-1}$	
	3	0.043	3.89	9.9	12.4	8.3	23.1	1.9	0.4	0.026	1.9
	6	0.024	2.17	5.0	6.3	3.2	12.6	2.0	0.3	0.009	4.1
SSM1	15	0.033	2.96	6.9	8.6	6.1	14.4	1.7	0.4	0.012	4.8
	18	0.061	5.55	12.8	16.0	9.9	30.6	1.9	0.3	0.017	2.7
	21	0.105	9.59	21.2	26.5	17.7	120.8	4.6	0.1	0.027	0.8
	24	0.023	2.13	4.9	6.1	4.3	21.4	3.5	0.2	0.006	4.4
	36	0.039	3.54	8.3	10.4	1.0	26.5	2.6	0.0	0.001	4.4
	48	0.023	2.05	4.8	6.0	0.4	9.5	1.6	0.0	0.0004	13.5

The oxygen uptake rate (OUR) depends on the metabolic functions and growth phase of the microorganism. It showed tendency to increase at the beginning of the production phase. OUR and oxygen transfer rate (OTR) values were close to each other which showed that the cells consumed nearly all oxygen transferred to the medium until $t=24\text{h}$ at SSM1 operation. Afterwards, with the depletion of sorbitol OUR decreased demonstrating that in the presence of co-substrate cells required high amount of oxygen and in addition to this the properties of fermentation medium affected OUR as in the case of K_{La} . This state was also supported by specific oxygen consumption rate (q_0) as it had a tendency to increase until $t=21\text{h}$ but decreased afterwards. At SSM1, the highest OUR was obtained at $t=21\text{h}$ as $17.7\text{ mol m}^{-3}\text{s}^{-1}$ which is 3.4-fold higher and 1.2-fold lower than the highest OUR values of SSM2 ($7.5\text{ mol m}^{-3}\text{s}^{-1}$) and SSM3 ($21.6\text{ mol m}^{-3}\text{s}^{-1}$), respectively. At SSM2, the highest value was obtained at $t=48\text{h}$ and showed oscillation during the process time similarly as in SSM3.

The highest OUR at MSS-0.03 ($61.5\text{ mol m}^{-3}\text{s}^{-1}$) was respectively, 3.5-, 8.2- and 2.8-fold higher than that of at SSM1, SSM2 and SSM3; and the highest OUR at MSSS-0.03 ($56.7\text{ mol m}^{-3}\text{s}^{-1}$) was 3.2-, 7.6- and 2.6-fold higher than that of at SSM1, SSM2 and SSM3. This result showed that, high cell concentration and the properties of the fermentation medium highly affected the oxygen transfer characteristics of the process.

Oxygen transfer rate (OTR) showed similar manner to OUR. However, since dissolved oxygen level was tried to keep $\text{DO}>20\%$ throughout the process, OTR values were higher than OUR values. OD and OTR_{max} which helps to find out the rate limiting step, calculated throughout the process. The relations between these parameters are defined as Damköhler number (Da) (Çalık *et al.* 2000). Da higher than 1 indicates that the mass transfer resistances dominate over the biochemical reactions and lower than 1 indicates the biochemical reaction limitation. As tabulated in Table 4.6 mostly Da was higher than 1 at SSM1 which shows that mass transfer resistances were effective most probably due to the high cell concentrations. SSM2 and SSM3 showed similarity however at the earlier

hours of SSM2 Da was lower than 1 where the mass transfer was biochemical reaction limited. The ideal condition for the effectiveness factor is $\eta=1$ and the effectiveness factor (η) has an inverse relation with Da. It was always lower than 1 for all conditions throughout the process time. Low η values mean that cells consume lower oxygen than the oxygen demand. The highest effectiveness factor was obtained as $\eta=0.8$ for SSM2 and $\eta=0.4$ for SSM1.

Owing to the high cell densities and low oxygen uptake rates, cell yield on oxygen was mostly high throughout the three processes. The highest cell yield on oxygen was calculated as $Y_{X/O}=6.42$ for SSM2 where the lowest OUR was observed. The prevalence case for the three operations is until the depletion of sorbitol, specific oxygen consumption rates showed an increasing trend in company with the OUR whereas the cell yield on oxygen was decreasing which then increased after total exhaustion of sorbitol due to the decrease in oxygen uptake rate.

4.1.9 Sorbitol/Methanol Mix-Feeding According to Stoichiometric Ratio

Jungo *et al.* (2007) used sorbitol as the co-substrate in order to investigate the advantages on production of recombinant avidin by *P. pastoris* Mut⁺ strain. They determined the optimum sorbitol/methanol stoichiometry within the mix-feed by performing transient nutrient gradient continuous cultivation with the dilution rate of 0.03 h^{-1} . With regard to that, in this study, the induction phase of the SM strategy was performed based upon the optimum sorbitol/methanol ratio found by Jungo *et al.* (2007). The aim of performing SM was to compare the operational responses with SSM1, SSM2 and SSM3 in terms of rhGH production. The strategy, SM, is defined in Table 4.1.

4.1.9.1 Effect on Cell Growth and Specific Rates

The cell growth profile of SM is given in Figure 4.9 with the comparison of SSM1, SSM2 and SSM3. It was observed that cell concentration of SM was close to the other three strategies and the highest cell concentration obtained at

SM was $C_X=104.8 \text{ g L}^{-1}$ at $t=48\text{h}$. On the other hand, at SM, cells entered a lag phase between $t=0\text{-}9\text{h}$ of the process which was seen in the cultures only fed with methanol which demonstrates that batch-wise sorbitol feeding to the medium at $t=0\text{h}$ of the production phase eliminates the lag phase and cells start to proliferate immediately as seen in SSM1, SSM2 and SSM3. Also, the specific growth rate (Table 4.7) calculated for SM also demonstrated that cell growth was low within $t=0\text{-}9 \text{ h}$ of the process and afterwards until $t=30\text{h}$ were almost constant with a slight oscillation. After the termination of mixed-substrate solution feeding at $t=30\text{h}$ cell growth rates began to decrease showing the same characteristic with SSM1, SSM2 and SSM3. The highest cell growth rate was calculated as 0.066 h^{-1} at $t=21\text{h}$

During the fermentation process, sorbitol and methanol concentrations in the bioreactor medium were analyzed by HPLC simultaneously with the operation. Sorbitol and methanol were not detected in the medium throughout the process which shows that both carbon sources were immediately and simultaneously consumed with the addition to the medium. The specific total substrate consumption rate increased until $t=12\text{h}$ and reached to its highest value as $0.142 \text{ g g}^{-1} \text{ h}^{-1}$ at $t=12\text{h}$ which mostly remained constant until $t=30\text{h}$ (Table 4.7). The slight decrease in substrate consumption rate until $t=30\text{h}$ can be due to high cell concentration which met the inadequate substrate concentration. At $t=30\text{h}$ a sharp decrease in substrate consumption rate was observed as a result of switching to only methanol feeding.

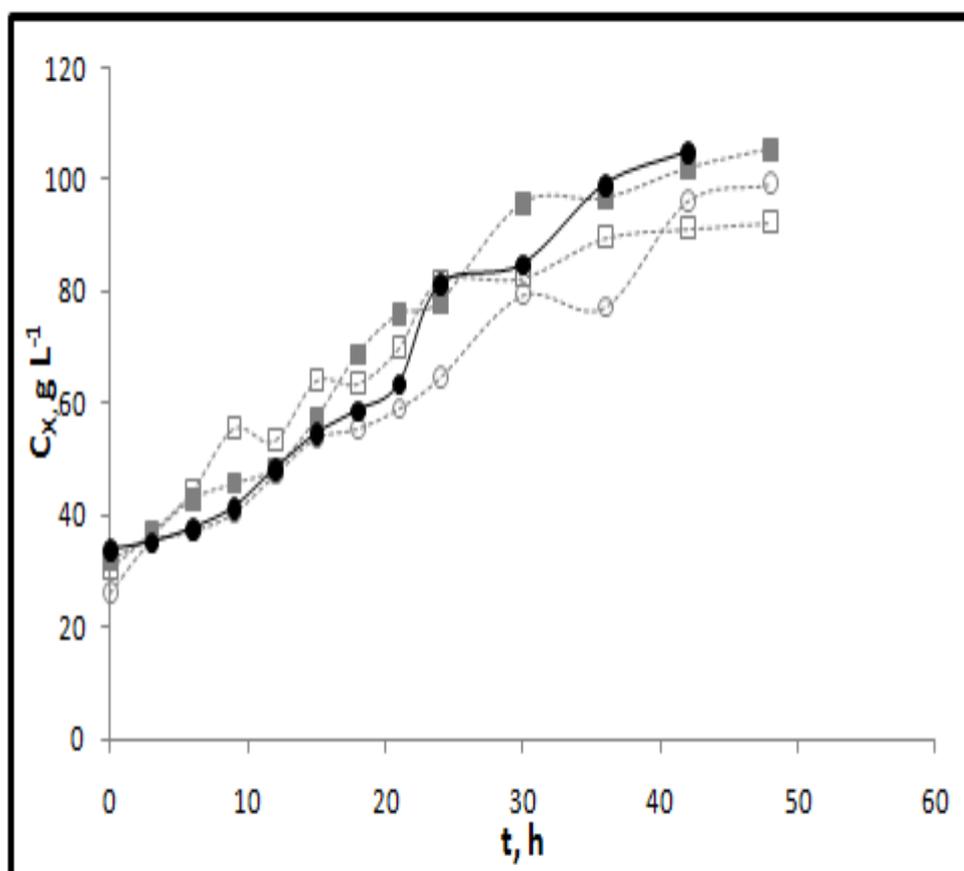


Figure 4.9 Variation in the cell concentration with the cultivation time in the production phase of SM; SM (●), Dotted and gray curves: SSM1 (■), SSM2 (○), SSM3 (□).

Considering the recombinant protein production rates until $t=9\text{h}$ rhGH was not produced since the cells entered the lag phase. The production of rhGH was started at $t=12\text{h}$ and the highest rhGH production rate was obtained as $108\text{ g g}^{-1}\text{ L}^{-1}$ at $t=15\text{h}$ which is 4.4- and 2.5-fold lower than obtained at SSM1, SSM2, respectively and close to that of at SSM3.

The overall yields were calculated as; $Y_{X/S}=0.22\text{ g g}^{-1}$, $Y_{P/S}=0.39\text{ mg g}^{-1}$ and $Y_{P/X}=1.75\text{ mg g}^{-1}$ (Table 4.5). Although yield of cells produced per amount of substrate was close to that of at SSM1, the overall yield of product produced per cell and substrate were, respectively, 5.0- and 4.7-fold lower than those of SSM1.

Table 4.7 Variations in specific rates throughout the bioprocess of SM

Exp. Name	T h	μ_t h ⁻¹	q_{st} g g ⁻¹ h ⁻¹	$q_{rp} \cdot 1000$ g g ⁻¹ h ⁻¹	q_o g g ⁻¹ h ⁻¹	$Y_{x/o}$
SM	0	0.018	0.150	0	0.024	1.11
	3	0.023	0.156	0		
	6	0.032	0.157	0	0.007	2.34
	9	0.048	0.154	0		
	12	0.051	0.142	0.083	0.034	0.95
	15	0.038	0.135	0.108	0.024	1.31
	18	0.032	0.135	0.101	0.014	1.79
	21	0.066	0.134	0.083		
	24	0.037	0.108	0.024	0.004	7.34
	30	0.025	0.065	0.034	0.002	11.16
	36	0.025	0.063	0.040	0.006	3.01
	42	0.018	0.067	0.027		

4.1.9.2 Effects on rhGH Production, AOX and Protease Profiles

The concentration profile of rhGH in SM was given Figure 4.10 which shows that within t=0-12h of the process, rhGH was not produced since the cells entered to the lag-phase but thereafter, the production began and the highest rhGH concentration was obtained t=42h as 0.124 g L⁻¹ which is 5.2-fold, 4.5-fold and 1.2-fold lower than the that of SSM1, SSM2 and SSM3, respectively. Although the cell concentration was close to that of the three strategies, it became clearer that the sorbitol/methanol ratio was insufficient for the production of rhGH when considering AOX activity and protease concentration. As a result, carbon sources may be mostly consumed for biomass formation and maintenance.

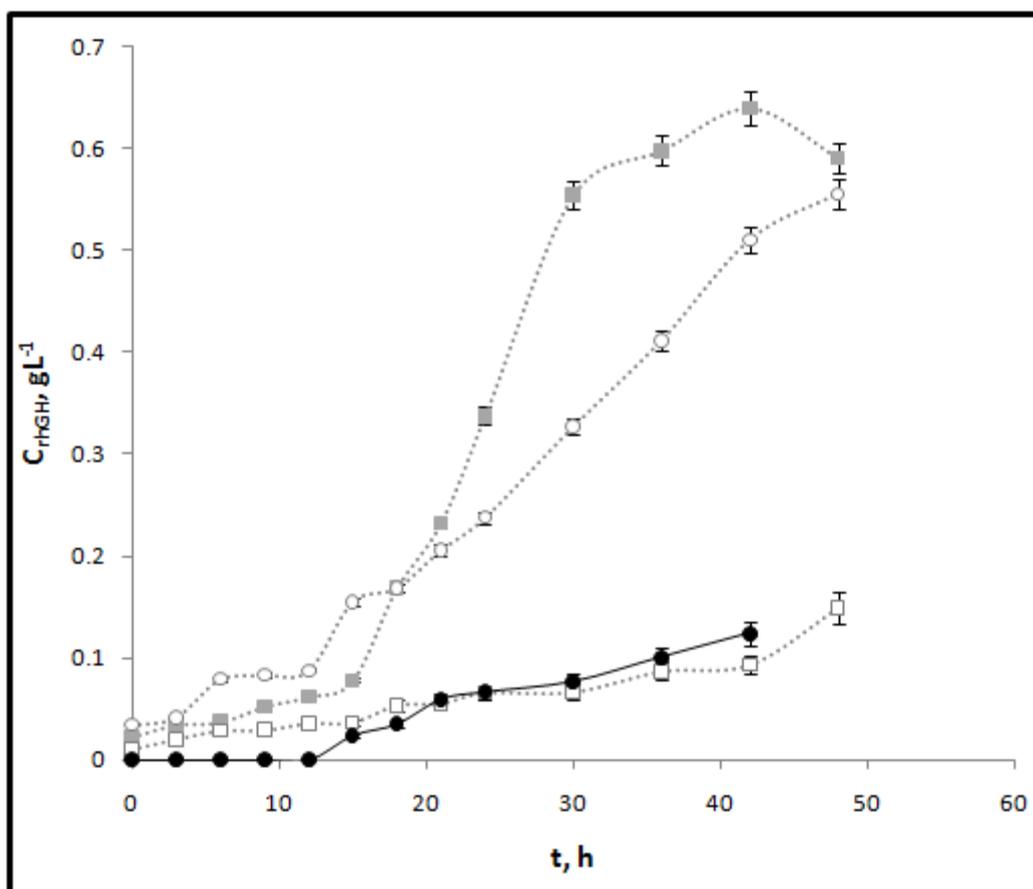


Figure 4.10 Variation in rhGH production with cultivation for SM; SM (●), Dotted and gray curves: SSM1 (■), SSM2 (○), SSM3 (□).

When AOX activity was analyzed, the reason of the lower rhGH production became clearer. Figure 4.11 shows the AOX activity profile for SM with the comparison of SSM1, SSM2 and SSM3. The highest AOX activity was obtained at t=15h as 37.9 U/ g CDW which is 2.4-fold, 3-fold and 2.4-fold lower than SSM1, SSM2 and SSM3, respectively. After termination of mixed substrate feeding at t=30h, a significant decrease in AOX activity was observed.

Although AOX activities at t=0h of all the four strategies were similar, after batch-wise addition of 50 gL⁻¹ sorbitol resulted in evaluated AOX activities in SSM1, SSM2 and SSM3 less than 1h (Figure 4.11). Consequently, the positive effect of batch-wise sorbitol addition at t=0h of the induction phase on AOX ac-

tivity became more significant. However, AOX activity level in SM was generally remained under the AOX activity levels of SSM1, SSM2 and SSM3 which can be due to the insufficient methanol and sorbitol concentration within the mixed substrate feed that met the high cell concentrations.

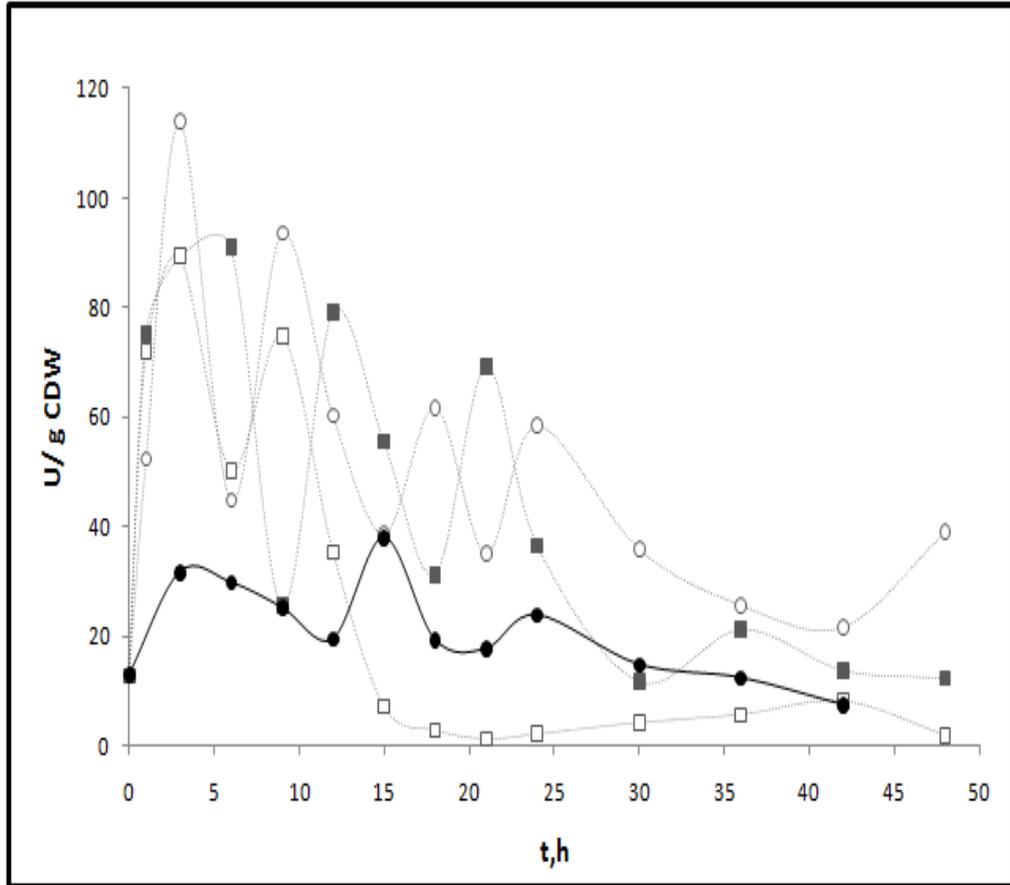


Figure 4.11 Variation in AOX activity with the cultivation time for SM; SM (●); Dotted and gray curves: SSM1 (■), SSM2 (○), SSM3 (□).

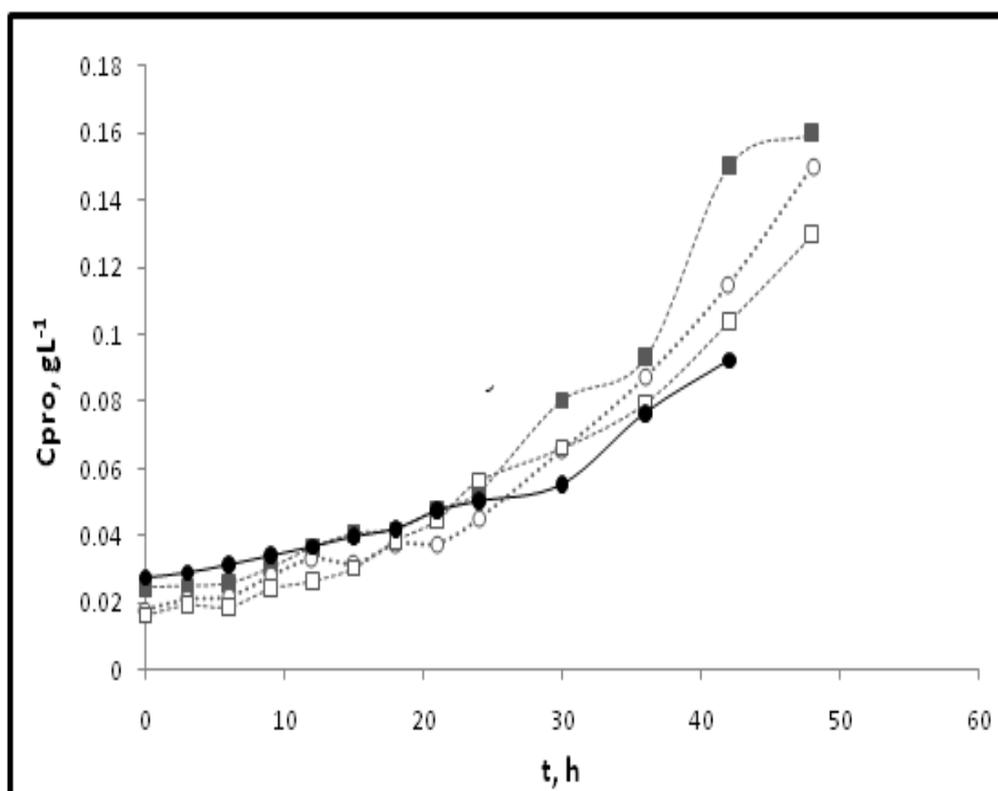


Figure 4.12 Variation in total protease concentration with the cultivation time for SM; SM (●); Dotted and gray curves: SSM1 (■), SSM2 (○), SSM3 (□).

Related with the total protease concentration of SM strategy, it was observed that until $t=24\text{h}$ the total protease concentration was higher than the total protease concentrations of the three strategies (Figure 4.12). However, afterwards, the total protease concentration remained at lower level than that of the SSM1, SSM2 and SSM3 since sorbitol was already depleted within the medium at SSM1 and SSM3 and pH was already set to 5.0 at SSM2 which resulted in a sharp increase of protease formation as mentioned elsewhere above. Moreover, similarly with the three strategies, after termination feeding of sorbitol containing mixed substrate feed at $t=30\text{h}$ a sharp increase also observed in this strategy. Consequently, the highest last protease concentration was obtained as 0.125 g L^{-1} at $t=42\text{h}$ which is 1.2-fold lower than the total protease concentration of SSM1 and SSM2 and close to that of the SSM3.

4.1.9.3 Effects on Organic Acid Concentrations

At SM operation the detected organic acids in the fermentation medium were formic acid, acetic acid, succinic acid, citric acid, pyruvic acid, oxalic acid, fumaric acid and maleic acid as tabulated in Table 4.8. Generally, the concentration of organic acids in the medium increased with the cultivation time and mostly lower than those obtained at SSM1, SSM2 and SSM3 owing to low rhGH production. Maleic acid concentration was the lowest of the other organic acids seen in the medium likewise in the medium of SSM1, SSM2 and SSM3. Lactic acid and formaldehyde were never detected in the medium as oxygen was never problematic throughout the fermentation process.

Table 4.8 Variations in organic acid concentrations for SM in g L⁻¹

Strategy t(h)		3	12	24	36	42
SM	Formic acid	0.0224	0.0246	0.0222	0.0825	0.1029
	Acetic acid	-	0.0056	0.0101	0.0148	0.0196
	Succinic acid	-	-	-	0.0232	0.0369
	Citric acid	0.0238	0.0057	0.0373	0.1005	0.1785
	Pyruvic acid	0.0038	0.0043	0.0115	0.0185	0.0316
	Oxalic acid	0.0031	0.0092	0.0167	0.0246	0.0518
	Fumaric acid	0.0021	0.0007	0.0012	0.0031	0.0036
	Maleic acid	0.0001	0.0001	0.0001	0.0002	0.0003

4.1.9.4 Oxygen Transfer Characteristics

The calculated oxygen transfer parameters; K_{La} , OUR, OTR, maximum possible oxygen transfer rate OTR_{max} , oxygen demand OD, K_{La_0} and η , Da and Ewere are tabulated in Table 4.9. In this operation oxygen mostly could be kept at 20% during the process. Thus, as expected OUR and OTR were mostly close to each other.

Table 4.9 Oxygen transfer characteristics of bioprocess SM

Exp	t	K_La	E	$OTR \times 10^3$	$OTR_{max} \times 10^3$	$OUR \times 10^3$	$OD \times 10^3$	Da	η
	(h)	(s^{-1})	K_{La}/K_{La0}	($mol\ m^{-3}\ s^{-1}$)	($mol\ m^{-3}\ s^{-1}$)	($mol\ m^{-3}\ s^{-1}$)	($mol\ m^{-3}\ s^{-1}$)		
SM	0	0.053	4.78	8.6	10.8	7.1	37.0	3.4	0.2
	6	0.023	2.06	4.0	5.0	2.2	19.5	3.9	0.1
	12	0.098	8.95	20.5	25.6	14.3	61.9	2.4	0.2
	15	0.096	8.72	20.1	25.1	11.6	50.6	2.0	0.2
	18	0.043	3.95	8.2	10.2	7.1	39.7	3.9	0.2
	24	0.019	1.77	4.1	5.1	2.8	13.4	2.6	0.2
	30	0.028	2.53	5.8	7.3	1.2	9.2	1.3	0.1
	36	0.033	3.01	9.1	11.4	4.8	39.9	3.5	0.1

OUR mostly depend on the substrate consumption rate. It has a positive effect on K_La as seen from the Table 4.8 increase in OUR resulted increase in K_La . Until $t=12h$ of the bioprocess oxygen uptake rate (OUR) and K_La was low due to the lag phase of the cells involved in. After exiting from the lag phase an increase in OUR and K_La was observed and the highest OUR value was obtained at $t=12h$ as $14.3\ mmol\ m^{-3}\ s^{-1}$ which is 1.2-, and 1.5-fold lower than that of SSM1 and SSM3, respectively; and 19-fold higher than that of SSM2. The highest K_La was calculated as $0,098\ s^{-1}$ which is close to that of SSM1 and 2-, and 1.5-fold higher than that of SSM2 and SSM3, respectively. With the increase in cell growth rate, after $t=15h$ of the process a decrease in OUR and K_La was observed. Depending on the OUR, OTR was increased to an extent of OUR.

Throughout the production phase it was observed that Da was always higher than 1 which means that the mass transfer resistance was dominant in the medium. Effectiveness factor, η , was lower than 0.2 during the process demonstrating that the SM operation was oxygen transfer limited; i.e. cells were consuming lower oxygen than the oxygen demand.

CHAPTER 5

CONCLUSION

In this M.Sc. thesis, to improve rhGH production by *Pichia pastoris* three original feeding strategies named as SSM1, SSM2, and SSM3 were designed for the production phase in semi-batch operation of bioreactor system. In all the bioreactor operations the temperature was kept constant at $T=30^{\circ}\text{C}$ and four-phased bioprocess was performed, i.e., glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and production (induction) phase. The cell growth profile in the first three phases were the same at each bioreactor operations since the designed feeding strategies were only applied in the production phase.

The production phase of the three bioreactor operations (SSM1, SSM2, SSM3) were performed in the presence of sorbitol which is known as the non-inhibitory carbon source for *P. pastoris*. Generally, these three bioreactor operations were based on simultaneous feeding of methanol and sorbitol starting at $t=0\text{h}$ of the production phase, i.e., batch-wise 50 gL^{-1} sorbitol feeding, together with methanol and sorbitol fed-batch feeding with the predetermined profiles. Sorbitol was fed to the bioreactor with the specific growth rate of $\mu_0=0.025\text{ h}^{-1}$ which was calculated based on the specific consumption rate $q_S=0.152\text{ g g}^{-1}\text{ h}^{-1}$. Consequently, sorbitol concentration was kept constant at 50 gL^{-1} within $t=0-15\text{h}$ of the induction phase where sorbitol feeding was terminated at $t=15\text{h}$. Methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$ or $\mu_0=0.04\text{ h}^{-1}$. In the first strategy, named as SSM1, methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$ and pH was kept constant at pH5.0 throughout the process. In the second strategy, SSM2, pH was kept constant at

5.5 until $t=24\text{h}$; thereafter, was reduced to 5.0 and methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03\text{ h}^{-1}$. In the third strategy, SSM3, methanol was fed to the medium with the specific growth rate of $\mu_0=0.04\text{ h}^{-1}$ and pH in the bioreactor was kept constant at pH 5.0. The results obtained for the three strategies are summarized below:

- The highest cell concentration was obtained in SSM1 as 105.3 gL^{-1} which is close to that obtained in SSM2 (99.3 gL^{-1}) and 1.13-fold higher than that obtained in SSM3 (92.4 gL^{-1}). In all the three strategies, cell growth rate decreased with the depletion of sorbitol.
- Methanol was not detected in the bioreactor, and sorbitol consumption began at $t=0\text{h}$ of the $t=15\text{h}$ long $C_S=50\text{ gL}^{-1}$ constant-sorbitol concentration period in the production phase; therefore, methanol and sorbitol was consumed simultaneously.
- Sorbitol consumption rates were higher within $t=0-15\text{h}$ of the bio-processes since the sorbitol concentration was kept constant at 50 gL^{-1} ; however, decreased by $t>15\text{h}$ as sorbitol fed-batch feeding was ended. Sorbitol was totally depleted at $t=24\text{h}$ in SSM1 and SSM2, and until $t=36\text{h}$ in SSM3. Since pH was kept constant at $\text{pH}=5.5$ until $t=24\text{h}$ of the process in SSM2, the consumption of sorbitol rate decreased and lengthened the sorbitol depletion time consequently the production of rhGH.
- Among SSM1, SSM2 and SSM3, the highest rhGH production was achieved in SSM1 as 640 mg L^{-1} which was 1.2-fold and 4.3-fold higher than that of SSM2 and SSM3, respectively. Therefore, the highest q_{rhGH} was calculated in SSM1 as $0.480\text{ gg}^{-1}\text{h}^{-1}$, although q_{rhGH} was higher in SSM2 until $t=15\text{h}$ of the production phase.
- The highest overall rhGH yields on the cell and on the total substrate consumed, were calculated for SSM1 as $Y_{p/x}=8.81\text{ mg g}^{-1}$, and $Y_{p/s_t}=1.83\text{ mg g}^{-1}$, respectively, while the highest overall cell

yield on the total substrate was calculated for SSM2 as $Y_{x/s_t}=0.38$ $g\ g^{-1}$.

- Although the highest rhGH concentration was achieved in SSM1, the highest AOX activity was obtained as 113.9 U/g CDW at $t=3h$ in SSM2 where q_{rhGH} was higher than that of the SSM1.
- Formic acid was detected in the production medium in all the three designed feeding strategies which suggests that a sum of formaldehyde entered into the dissimilatory pathway. Formic acid concentration increased with the cultivation time especially after the depletion of sorbitol; and the highest concentration was detected as $0.185\ g\ L^{-1}$ in SSM3. Lactic acid, which is formed when there is an oxygen limitation, was only detected in the production medium in SSM3 as oxygen was never sufficient in the bioreactor throughout the process.
- The total protease concentrations in the three strategies were remained at low concentrations during the presence of sorbitol in the medium. After depletion of sorbitol in SSM1 and SSM3, and the pH change in SSM2, a sharp increase was observed in protease formation. The highest total protease concentration was obtained as $0.160\ g\ L^{-1}$ in SSM1.
- In the three strategies, oscillations were observed in the oxygen transfer characteristics, i.e., K_{La} and OUR, as a result of the high cell concentration together with high amount of secreted metabolites which increased the resistance for oxygen transfer. Another reason of the oscillations can be explained as at high cell densities when the cells transfer methanol from the broth, the oxygen requirement, therefore the oxygen uptake rate (OUR) and mass transfer coefficient (K_{La}) increase; contrarily, in the absence of methanol oxygen requirement decreased and so the K_{La} and OUR. The highest K_{La} was obtained as $0.105s^{-1}$ in SSM1 where the high-

est rhGH was produced. Parallel with K_{La} , the highest OUR was calculated as $17.7 \text{ mol m}^{-3}\text{s}^{-1}$ at SSM1. In all the three strategies, until the depletion of sorbitol, the oxygen uptake rates and oxygen transfer rates were close to each other which indicate that the cells consumed nearly all the oxygen transferred into the medium.

- The positive effects of the alteration made in methanol transition phase were observed on the initial cell concentration and AOX activities in the production phases of all the three strategies.

Lastly, the feeding strategy named as SM was performed for the comparison with the three strategies which were mentioned above in terms of rhGH productivity. In this strategy methanol and sorbitol was simultaneously fed to the bioreactor in a solution containing 6.21 mol methanol and 1.37 mol sorbitol in 13.88 mol water. The solution was fed to the medium with the specific growth rate on sorbitol of $\mu_0=0.03 \text{ h}^{-1}$ until $t=30\text{h}$; thereafter only methanol was fed to the bioreactor with the specific growth rate of $\mu_0=0.03 \text{ h}^{-1}$. The results were summarized below:

- Cells entered to a lag-phase until $t=9\text{h}$ of the induction phase where the cell growth rates were low and there was no rhGH production. Afterwards, cell growth rate increased and the cell concentration reached its highest value as 104.8 gL^{-1} at $t=48\text{h}$ which was very close to that obtained in SSM1, SSM2 and SSM3.
- Sorbitol and methanol was never detected in the production medium throughout the process which demonstrated the both carbon sources were consumed simultaneously. Total substrate consumption rate, q_{st} , was remained at high values until $t=30\text{h}$ of the process where the highest q_{st} was obtained as $0.142 \text{ gg}^{-1}\text{h}^{-1}$ at $t=12\text{h}$.
- Since the cells were in the lag-phase until $t=9\text{h}$, rhGH was not produced within $t=0-9\text{h}$ of the process. Thereafter, with the termi-

nation of lag-phase rhGH production began and the highest concentration obtained at $t=42\text{h}$ as 0.124 g L^{-1} which was 5.2-, 4.5- and 1.2-fold lower than SSM1, SSM2 and SSM3, respectively.

- The overall cell yield on total substrate was calculated as $Y_{x/s}=0.21\text{ gg}^{-1}$ which was close to that of the SSM1. However overall product yield produced per cell ($Y_{p/x}=1.88\text{ gg}^{-1}$) was 5-fold lower than that of the SSM1 which demonstrated that carbon sources mostly consumed for biomass formation and maintenance at SM, rather than recombinant protein production.
- Related with protease concentration until $t=21\text{h}$, protease concentration was higher in SM than first three strategies. Thereafter, protease concentrations remained at lower values than that of SSM1, SSM2 and SSM3 since sorbitol was already depleted in those strategies at the time still presented in the medium of SM.
- The highest AOX activity was obtained at $t=15\text{h}$ as 37.9 U/ gCDW which is 2.4-fold, 3-fold and 2.4-fold lower than SSM1, SSM2 and SSM3 which clearly demonstrates the reason of low rhGH production. In addition, at $t=0\text{h}$ of the production phase although AOX activities were similar at the four strategies since sorbitol was not fed to the medium as batch-wise at $t=0\text{h}$ at SM, AOX activity remained at lower values than the other three strategies.

REFERENCES

- Abdel-Meguid, S.S., H.S. Shieh, W.W. Smith, H.E. Dayringer, B.N. Violand, and L.A. Bentle. 1987. "Three-dimensional structure of a genetically engineered variant of porcine growth hormone." *Proc Natl Acad Sci* 84:6434-6437.
- Açık, E. 2009. "Effects of carbon sources and feeding strategies on human growth hormone production by metabolically engineered *Pichia pastoris*." MSc Thesis, Chemical Engineering, METU, Ankara.
- Agrawal, P., G. Koshy, and M. Ramseier. 1989. "An algorithm for operating a fed-batch fermentor at optimum specific growth rate." *Biotechnol and Bioeng* 33:115-125.
- Azevedo, A. M., J.M. S. Cabral, D.M. F. Prazeres, T. D. Gibson, and L. P. Fonseca. 2004. "Thermal and operational stabilities of *Hansenula polymorpha* alcohol oxidase." *Journal of Molecular Catalysis B: Enzymatic* 27:37-45.
- Bae, C.S., D.S. Yang, J. Lee, and Y.H. Park. 1999. "Improved process for production of recombinant yeast-derived monomeric human GCSF." *Appl Microbiol Biotechnol* 52:338-344.
- Bandyopadhyay, B. and A. E. Humpre. 1967. "Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems." *Biotechnology and Bioengineering* 9:533-544.
- Baumann, G. 1991. "Growth hormone heterogeneity: genes, isoforms, variants, and binding proteins." *Endocr. Rev.* (12):424-49.
- Bayraktar, E. 2009. "Effects of pH on human growth hormone production by *Pichia pastoris* considering the expression levels of regulatory genes." MSc Thesis, Chemical Engineering Department, METU, Ankara.
- Becker, G.W. and H.M. Hsiung. 1986. "Expression secretion and folding of human growth hormone in *Escherichia coli*." *FEBS Letters* 204:145-150.
- Besson, A., S. Salemi, J. Deladoey, J.M. Vuissoz, A. Eble, M. Bildingmaier, S. Bürgi, U. Honegger, C. Flück, and P.E. Mullis. 2005. "Short stature caused by a

biologically inactive mutant growth hormone (GH-C53S).” *The Jour of Clin Endoc & Metabol* 90(5):2493-2499.

Binkley, S.A. 1994. *Endocrinology*. New York: 1st Ed.Harper Collins Collage Publishers.

Bio-Rad. 2012. “Quick Start Bradford Protein Assay: Instruction Manual.” Bio-Rad.Retrieved2012(<http://www.biorad.com/webroot/web/pdf/lsr/literature/4110065A.pdf>).

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

Böer, E., G. Steinborn, G. Kunze, and G. Gellissen. 2007. “Yeast expression platforms.” *Appl Microbiol Biotechnol* (77):513-523.

Boze, H., C. Laborde, and P. e. a. Chemardin. 2001. “High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*.” *Process Biochem* 36:907-913.

Bradford, M. M. 1976. “A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding.” *Analytical Biochemistry* 72:248-254.

Brake, A.J., J.P. Merryweather, D.G. Coit, U.A. Heberlein, F.R. Masiarz, G.T. Mullenbach, M.S. Urdea, P. Valenzuela, and P.J. Barr. 1984. “Alpha-factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*.” *Proc Natl Acad of Sci* 81:4642-4646.

Braren, I., K. Greunke, O. Umland, S. Deckers, R. Bredehorst, and E. Spillner. 2007. “Comperative expression of different antibody formats in mammalian cells and *Pichia pastoris*.” *Biotechnol Appl Biochem* 47:205-214.

Brierley, R.A. 1998. “Secretion of recombinant human insulin-like growth factor (IGF-I).” *Methods in Mol Biol* 103:149-177.

Brierley, R.A., C. Bussineau, R. Kosson, A. Melton, and R.S. Siegel. 1990. “Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme.” *Annals New York Academy of Science* 589:350-362.

Çalık, P. 1998. “Bioprocess development for serine alkaline protease production.” PhD thesis, Ankara University, Ankara.

Çalık, P., G. Çalık, and T. H. Özdamar. 1998. "Oxygen transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis*." *Biotechnology Bioengineering* 69:301-311.

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

Çalık, P., B. İnankur, E.Ş. Soyaslan, M. Şahin, H. Taşpınar, E. Açık, and E. Bayraktar. 2010a. "Fermentation and oxygen transfer characteristics in recombinant human growth hormone production by *Pichia pastoris* in sorbitol batch and methanol fed-batch operation." *J Chem Technol Biotechnol* 85:226-233.

Çalık, P., E. Bayraktar, E.Ş. Soyaslan, M. Şahin, and H. e. a. Taşpınar. 2010b. "Influence of pH on recombinant human growth hormone production by *Pichia pastoris*." *J Chem Technol Biot* 85:1628-1635.

Cazares-Delgadillo, J., A. Ganem-Rondero, and Y. N. Kalia. 2011. "Human growth hormone: New delivery systems, alternative routes of administration, and their pharmacological relevance." *European Journal of Pharmaceutics and Biopharmaceutics* (78):278-288.

Çelik, E. 2008. "Bioprocess development for therapeutical protein production." Thesis Doctor of Philosophy, Middle East Technical University, Ankara.

Çelik, E. and P. Çalık. 2011. "Production of recombinant proteins by yeast cells." *Biotechnol Adv* In Press.

Çelik, E., P. Çalık, and S. G. Oliver. 2009. "Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol." *Yeast* 26:474-484.

Charoenrat, T., K.M. Cairns, H.S. Andersen, M. Jahic, and S.O. Enfors. 2005. "Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes." *Bioprocess Biosyst Eng* 27:399-406.

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

- Chen, X.Z., A.W. Shafer, J.S. Yun, Y.S. Li, T.E. Wagner, and J.J. Kopchick. 1992. "Conversion of bovine growth hormone cystein residues to serine affects secretion by cultured cells and growth rates in transgenic mice." *Mol Endocrinol* 6:598-606.
- Chen, D.C., B.D. Wang, P.Y. Chou, and T.T. Kuo. 2000. "Asparagine as nitrogen source for improving the secretion of mouse α -amylase in the *Saccharomyces cerevisiae* protease A deficient strains." *Yeast* 16:207-217.
- Chen, C.C., P.H. Wu, C.T. Huang, and K.J. Cheng. 2004. "*Pichia pastoris* fermentation strategy for enhancing the heterologous expression of *Escherichia coli* phytase." *Enzyme Microb Technol* 35:315-320.
- Chiruvolu, V., K. Eskridge, J. Cregg, and M. Meagher. 1999. "Effects of glycerol concentration and pH on growth of recombinant *Pichia pastoris*." *Yeast* 75:163-173.
- Chiruvolu, V., J.M. Gregg, and M.M. Meagher. 1997. "Recombinant protein production in an alcohol oxidase defective strain of *Pichia pastoris* in fedbatch fermentations." *Enzyme Microb. Technology* 21:277-283.
- Chung, J.D. 2000. "Design of metabolic feed controllers: application to high-density fermentations of *Pichia pastoris*." *Biotechnol Bioeng* 68:298-307.
- Clare, J.J., M.A. Romanos, F.B. Rayment, J.E. Rowedder, M.A. Smith, and M.M. Payne. 1991. "Production of mouse epidermal growth factor in yeast: high-level secreting using *Pichia pastoris* strains containing multiple gene copies." *Gene* 105:205-212.
- Cos, O., R. Ramon, J.L. Montesinos, and F. Valero. 2006. "Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review." *Microbial Cell Factories* 5:17.
- Cos, O., P. Resina, P. Ferrer, J.L. Montesinos, and F. Valero. 2005. "Heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fedbatch cultures." *Biochem.Eng.Journal* 26:86-94.
- Couderc, R. and J. Baratti. 1980. "Oxidation of methanol by the yeast, *Pichia pastoris* strain IFP206: purification and properties of the alcohol oxidase (EC 1.1.3.13)." *Agric. Biol. Chem.* 44:2279-2290.

- Cregg, J. M., J. L. Cereghino, J. Shi, and D. R. Higgins. 2000. "Recombinant protein expression in *Pichia pastoris*." *Mol. Biotechnol.* (16):23-52.
- Cregg, J.M. and D.R. Higgins. 1995. "Production of foreign proteins in the yeast *Pichia pastoris*." *Can J Bot* 73(Suppl.1):S891-S897.
- Cregg, J., P. Koutz, G.R. Davis, K. Barringer, and G. Thill. 1989. "Structural Comparison of the *Pichia pastoris* alcohol oxidase genes." *Yeast* 5:167-177.
- Cregg, J., K.R. Madden, K.J. Barringer, G.P. Thill, and C.A. Stillman. 1989. "Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*." *Molecular and Cellular Biology* 9(3):1316-1323.
- Cregg, J.M., T.S. Vedvick, and W.C. Raschke. 1993. "Recent advances in the expression of foreign genes in *Pichia pastoris*." *BioTechnology* 11:905-910.
- d'Anjou, M.C. and A.J. Daugulis. 1997. "A model-based feeding strategy for fed-batch fermentation of recombinant *Pichia pastoris*." *Biotechnol Technique* 11:865-868.
- d' Anjou, M.C. and A.J. Daugulis. 2000. "Mixed-feed exponential feeding for fed-batch culture of recombinant methylotrophic yeast." *Biotechnol Letts* 22:341-346.
- d' Anjou, M.C. and A.J. Daugulis. 2001. "A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation." *Biotechnol Bioeng* 72:1-11.
- Daly, R. and M.T.W. Hearn. 2005. "Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production." *Journal of Molecular Recognition* 18:119-138.
- Damasceno, L.M., C.Jr. Huang, and C.A. Batt. 2011. "Protein secretion in *Pichia pastoris* and advances in protein production." *Appl Microbiol Biotechnol* 93(1):31-39.
- Damasceno, L.M., I. Pla, H.J. Chang, L. Cohen, G. Ritter, L.J. Old, and C.A. Batt. 2004. "An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*." *Protein Expr Purif* 37:18-26.
- De Schutter, K., Y.C. Lin, P. Tiels, A. Van Hecke, S. Glinka, J. Weber-Lehmann, P. Rouze, Y. Van de Peer, and N. Callewaert. 2009. "Genome sequence of the

recombinant protein production host *Pichia pastoris*.” *Nature Biotechnol* 27(6):561-566.

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

Demain, A. L. 2000. “Small bugs, big business: the economic power of the microbe.” *Biotechnol Adv* 18:499-514.

Demain, A.L. and P. Vaishnav. 2009. “Production of recombinant proteins by microbes and higher organisms.” *Biotechnology Advances* 27:297-306.

Dennis, J.W., M. Granovsky, and C.E. Warren. 1999. “Protein glycosylation in development and disease.” *Bioessays* 21:412-421.

Dragosits, M., J. Stadlmann, J. Albiol, K. Baumann, M. Maurer, B. Gasser, M. Sauer, F. Altmann, P. Ferrer, and D. Mattanovich. 2009. “The effect of temperature on the proteome of recombinant *Pichia pastoris*.” *J Proteome Res* 8:1380-1392.

Duman, J.G., R.G. Miele, H. Liang, D.K. Grella, K.L. Sim, F.J. Castellino, and R.K. Bretthauer. 1998. “O-mannosylation of *Pichia pastoris* cellular and recombinant proteins.” *Biotechnol Appl Biochem* 28:39-45.

Enfors, S.O. 1992. “Control of in vivo proteolysis in the production of recombinant proteins.” *Trends Biotechnol* 10:310-315.

Flies, D., M. Ogawa, C.H. Scaman, and S.A. Baldwin. 2001. “A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-C.” *Enzyme and Microbiol Technol* 99:335-340.

Flodh, H. 1986. “Human growth hormone produced with recombinant DNA technology: development and production.” *Acta. Paediatr Scand. Suppl.* (325):1-9.

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

Gellissen, G. 2000. “Heterologous protein production in methylotrophic yeasts.” *Appl Microbiol Biotechnol* (54):741-750.

Gellissen, G., Z.A. Janowicz, U. Weydemann, K. Melber, A.W.M. Strasser, and C.P. Hollenberg. 1992. "High level expression of foreign genes in *Hansenula polymorpha*." *Biotechnol. Adv.* 10:179-189.

Gellissen, G., G. Kunze, C. Gaillardin, J. M. Cregg, E. Berardi, M. Veenhuis, and I. van der Klei. 2005. "New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica*-A comparison." *FEMS Yeast Research* (5):1079-1096.

Gemmill, T.R. and R.B. Trimble. 1999. "Overview of N- and O-linked oligosaccharide structures found in various yeast species." *BBA-General Subjects* 1426(2):227-237.

Gerngross, T.U. 2004. "Advances in the production of human therapeutic proteins in yeast and filamentous fungi." *Nature Biotechnol* 22(11):1409-1414.

Goeddel, D. V., H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. H. Seeburg. 1979. "Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone." *Nature* (281(5732)):544-548.

Goffeau, A., R. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Glibert, J. D. Hosheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver. 1996. "Life with 6000 genes." *Science* (274):563-567.

Gonzalez-Lopez, C.I., R. Szabo, S. Blanchin-Roland, and C. Gaillardin. 2002. "Genetic control of extracellular protease synthesis in the yeast *Yarrowia lipolytica*." *Genetics* 160:417-427.

Graf, L., J. Borvendeg, E. Barat, I. Hermann, and A. Patthy. 1976. "Reactivity and biological importance of the disulfide bonds in human growth hormone." *FEBS Lett* 66:233-237.

Graf, L., C.H. Li, and T.A. Bewley. 1975. "Selective reduction and alkylation of the COOH-terminal disulfide bridge in bovine growth hormone." *Int J Pept Protein Res* 7:467-473.

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

- Hackel, B.J, D. Huang, J.C. Bubolz, X.X. Wang, and E.V. Shusta. 2006. "Production of soluble and active transferrin receptor-targeting single-chain antibody using *Saccharomyces cerevisiae*." *Pharm Res* 23:79-797.
- Haro, L.S, U.J. Lewis, M. Garcia, J. Bustamante, A.O. Martinez, and N.C. Ling. 1996. "Glycosylated human growth hormone (hGH): a novel 24 kDa hGH-N variant." *Biochem and Biophys Research Commun* 228(2):549-556.
- Hartner, F.S. and A. Glieder. 2006. "Regulation of methanol utilisation pathway genes in yeasts." *Microbial Cell Factories* 5:39.
- Havel, H.A., R.S. Chao, R.J. Haskell, and T.J. Thamann. 1989. "Investigation of protein structure with optical spectroscopy: bovine growth hormone." *Anal Chem* 61:642-647.
- Helenius, A. and M. Aebi. 2001. "Intracellular functions of N-linked glycans." *Science* 291:2364-2369.
- Higgins, D. R. and J. M. Cregg 1998. "Introduction to *Pichia pastoris*." Pp. 1-15 in *Pichia Protocols*. Totowa: Human Press Inc.
- Hilt, W. and D.H. Wolf. 1992. "Stress-induced proteolysis in yeast." *Mol Microbiol* 6:2437-2442.
- Hintz, R. L. 1995. "The prismatic case of Creutzfeldt-Jacob disease associated with primary growth hormone treatment." *J. Clin. Endocrinol. Metab* (80(8)):2298-2301.
- Hitzeman, R.A., F.E. Hagie, H.L. Levine, D.V. Goseddel, G. Ammerer, and B.D. Hall. 1981. "Expression of a human gene for interferon in yeast." *Nature* 293:717-722.
- Hlodan, R. and U.F. Hartl 1994. "How the protein folds in the cell." Pp. 194-228 in *Mechanisms of Protein Folding*, edited by R.H. Pain. New York: Oxford University Press.
- Hohenblum, H., B. Gasser, M. Maurer, N. Borth, and D. Mattanovich. 2004. "Effects of gene dosage, promoters and substrates on unfolded protein stress of recombinant *Pichia pastoris*." *Biotechnol Bioeng* 85:367-375.
- Holst, B., A.W. Bruun, M.C. Kiellandbrandt, and J.R. Winther. 1996. "Competition between folding and glycosylation in the endoplasmic reticulum." *EMBO J* 15:3538-3546.

- Hong, F., N.Q. Meinander, and L.J. Jönsson. 2002. "Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*." *Biotechnol Bioeng* 79(4):483-449.
- Horstkotte, B., C. Arnau, V. Francisco, O. Elsholz, and V. Cerda. 2008. "Monitoring of sorbitol in *Pichia pastoris* cultivation applying sequential injection analysis." *Biochem Eng J* 42:77-83.
- Idiris, A., H. Tohda, H. Kumagai, and K. Takegawa. 2010. "Engineering of protein secretion in yeast: strategies and impact on protein production." *Appl. Microbiol. Biotechnol.* (86):403-417.
- İleri, N. and P. Çalık. 2006. "Effects oh pH strategy on endo- and exo-metabolome profiles and sodium potassium hydrogen ports of beta-lactamase producing *Bacillus licheniformis*." *Biotechnology Progress* 22(2):411-419.
- Inankur, B. 2010. "Recombinant human growth hormone production by *Pichia pastoris* and determination of its interaction with peptide ligands." MSc Thesis, Chemical Engineering Department, METU, Ankara.
- Inan, M. and M.M. Meagher. 2001-a. "The effect of ethanol and acetate on protein expression in *Pichia pastoris*." *J of Bioscience and Bioengineering* 92:337-341.
- Inan, M. and M.M. Meagher. 2001-b. "Non-repressing carbon sources for alcohol oxidase (AOZ1) promoter of *Pichia pastoris*." *Journal of Bioscience and Bioengineering* 92:585-589.
- Invitrogen. 2002. "Pichia Fermentation Process Guidelines." Retrieved 2012 (http://tools.invitrogen.com/content/sfs/manuals/pichiaferm_prot.pdf).
- Jacobs, J.J., S. Geysens, W. Verweken, R. Contreras, and N. Callewaert. 2009. "Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology." *Nat Protoc* 4:58-70.
- Jahic, M. 2003. "Process techniques for production of recombinant proteins with *Pichia pastoris*." PhD Thesis , Department of Biotechnology, Royal Institute of Technology, Stockholm, Sweden.
- Jahic, M., M. Gustavsson, A.K. Jansen, M. Martinelle, and S.O. Enfors. 2003. "Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes." *J Biotechnol* 102(1):45-53.

- Jahic, M., F. Wallberg, M. Bollok, P. Garcia, and S.O. Enfors. 2003. "Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures." *Microbiol Cell Fact* 2:6.
- Jenkins, N., R.B. Parekh, and D.C. James. 1996. "Getting the glycosylation right: implications for the biotechnology industry." *Nat Biotechnol* 14(8):975-981.
- Jin, H., G. Liu, K. Dai, H. Wang, Z. Li, and Z. Shi. 2011. "Improvement of porcine interferon- α production by recombinant *Pichia pastoris* via induction at low methanol concentration and low temperature." *Appl Biochem Biotechnol* 165(2):559-71.
- Jones, E.W. 1991. "Tackling the protease problem in *Saccharomyces cerevisiae*." *Methods Enzymol* 194:429-452.
- Julius, D., A. Brake, L. Blair, R. Kunisawa, and J. Thorner. 1984. "Isolation of the putative structural gene for the Lysine-Arginine-cleaving endopeptidase required for processing of the yeast prepro-alpha-factor." *Cell* 37:1075-1090.
- Jungo, C., C. Rerat, I.W. Marison, and U. von Stockar. 2006. "Quantative characterization of the regulation of the synthesis of alcohol oxidase and the expression of recombinant avidin in a *Pichia pastoris* Mut⁺ strain." *Enzyme and Microbial Technology* 39:936-944.
- Jungo, C., J. Schenk, M. Pasquier, I. W. Marison, and U. von Stockar. 2007. "A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*." *Journal of Biotechnolgy* 131:57-66.
- Kang, H.A., E.S. Choi, W.K. Hong, J.Y. Kim, S.M. Ko, J.H. Sohn, and S.K. Rhee. 2000. "Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*." *Appl Microbiol Biotechnol* 53:575-582.
- Katakura, Y., W. Zhang, G. Zhuang, T. Omasa, M. Kishimoto, Y. Goto, and K.-I. Suga. 1998. "Effect of methanol concentration on the production of human beta2-glycoprotein I domain V by a recombinant *Pichia pastoris*: a simple system for the control of methanol concentration using a semiconductor gas sensor." *J Ferment Bioeng* 86(5):482-487.
- Kato, C., T. Kobayashi, T. Kudo, T. Furusato, Y. Murakami, T. Tanaka, H. Baba, T. Oishi, E. Ohtuska, M. Ikehara, T. Yanagida, H. Kato, S. Moriyama, and K. Horikoshi. 1987. "Construction of an excretion vector and extracellular production of human growth hormone from *Escherichia coli*." *Gene* 54:197-202.

- Kelly, P.A., J. Djiane, and M.C. Postel-Vinay. 1991. "The prolactin/growth hormone receptor family." *Endocr Rev* 12:235-51.
- Kemp, S. F. and J. P. Frindik. 2011. "Emerging options in growth hormone therapy: an update." *Drug Design, Development and Therapy* (5):411-419.
- Khatri, N.K. and F. Hoffmann. 2006a. "Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*." *Biotechnol Bioeng* 93:871-879.
- Khatri, N.K. and F. Hoffmann. 2006b. "Oxygen-limited control of methanol uptake rate for improved production of a single-chain antibody fragment with recombinant *Pichia pastoris*." *Appl Microbiol Biotechnol* 72:492-498.
- Klausner, R.D. 1989. "Architectural editing: determining the fate of newly synthesized membrane proteins." *New Biol* 1:3-8.
- Kobayashi, K., S. Kuwae, T. Ohya, T. Ohda, M. Ohyama, H. Ohi, K. Tomomitsu, and T. Ohmura. 2000. "High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation." *J Bioscience Biotechnol* 89:55-61.
- Krysiak, R., A. Gdula-Dymek, A. Bednarska-Czerwinska, and B. Okopien. 2007. "Growth hormone therapy in children and adults." *Pharmacological Reports* (59):500-516.
- Kunkel, D. 2002-2012. "Yeast, *Pichia pastoris*." <http://www.corbisimages.com/>. Corbis Corporation. Retrieved May 6, 2012 (<http://www.corbisimages.com/stock-photo/rights-managed/42-23598844/yeast-pichia-pastoris>).
- Lee, C.Y., A. Nakano, N. Shiomi, E.K. Lee, and S. Katoh. 2003. "Effects of substrate feed rate on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation." *Enzyme Microb Technol* 33:358-365.
- Liang, J. and J. Yuan. 2007. "Oxygen transfer model in recombinant *Pichia pastoris* and its application in biomass estimation." *Biotechnol Lett* 29:27-35.
- Li, P., A. Anumanthan, X.G. Gao, K. Ilangovan, V.V. Suzara, N. Düzgüneş, and V. Renugopalakrishnan. 2007. "Expression of recombinant proteins in *Pichia pastoris*." *Appl Biochem Biotechnol* 142:105-124.

- Lim, H.K., S.J. Choi, K.Y. Kim, and K.H. Jung. 2003. "Dissolved-oxygen-stat controlling two variables for methanol induction of rGuamerin in *Pichia pastoris* and its application to repeated fed-batch." *Appl Microbiol Biotechnol* 62:342-348.
- Li, Z., F. Xiong, Q. Lin, M. D'Anjou, A.J. Daugulis, D.S.C. Yang, and C.L. Hew. 2001. "Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*." *Protein Expression and Purification* 21:438-445.
- Loewen, M.C., X. Liu, P.L. Davies, and A.J. Daugulis. 1997. "Biosynthetic production of type II fish antifreeze protein: fermentation by *Pichia pastoris*." *Appl Microbiol Biotechnol* 48(4):480-486.
- McGrew, J.T., D. Leiske, B. Dell, R. Klinke, D. Krasts, S.F. Wee, N. Abbott, R. Armitage, and K. Harrington. 1997. "Expression of trimeric CD40 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants." *Gene* 187:193-200.
- Moon, H., H. Kim, S.K. Rhee, E.S. Choi, I.H. Kim, and S.I. Hong. 2002. "Optimal strategy of pH control in the production of recombinant human epidermal growth factor by *Hansenula polymorpha*." *Process Biochem* 38:487-495.
- Moon, S. H. and S. J. Parulekar. 1991. "A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*." *Biotechnology and Bioengineering* 37:467-483.
- Mukhija, R., P. Rupa, D. Pillai, and L.C. Garg. 1983. "High level production of human growth hormone: Biochemical, genetic, and clinical aspects." *Annu.Rev.Med.* 34:519-547.
- Nakayama, A., K. Ando, K. Kawamura, I. Mita, K. Fukazawa, M. Hori, M. Honjo, and Y. Furutani. 1988. "Efficient secretion of the authentic mature human growth hormone by *Bacillus subtilis*." *Journal of Biotechnology* 8:123-134.
- Niebauer, R. T. and A. S. Robinson 2005. "Saccharomyces cerevisiae protein expression: from protein production to protein engineering." Pp. 253-296 in *Protein expression technologies*, edited by F. Baneyx. Norwich, Horizon.
- O'Callaghan, J., M.M. O'Brien, K. McClean, and A.D. Dobson. 2002. "Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*." *J of Indust Microbiol and Biotechnol* 29:55-59.

- Oliveira, R., J.J. Clemente, A.E. Cunha, and M.J.T. Carrondo. 2005. "Adaptive dissolved oxygen control through the glycerol feeding in a recombinant *Pichia pastoris* cultivation in conditions of oxygen transfer limitation." *Journ of Biotechnol* 116:35-50.
- Orman, M.A., P. Çalık, and T.H. Özdamar. 2009. "The influence of carbon sources on recombinant-human-growth-hormone production by *Pichia pastoris* is dependent on phenotype: A comparison of MutS and Mut+." *Biotechnology and Applied Biochemistry* 52(3):245-255.
- Özdamar, T.H., B. Senturk, O.D. Yilmaz, G. Çalık, E. Çelik, and P. Çalık. 2009. "Expression system for recombinant human growth hormone production by *Bacillus subtilis*." *Biotechnology Progress* 25(1):75-84.
- Patra, A.K., R. Mukhopadhyay, R. Mukhija, and A. Krishnan. 2000. "Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*." *Protein Expression and Purification* 18:182-192.
- Paulova, L., P. Hyka, B. Branska, K. Melzoch, and K. Kovar. 2012. "Use of a mixture of glucose and methanol as substrates for the production of recombinant trypsinogen in continuous cultures with *Pichia pastoris* Mut+." *J Biotechnol* 157:180-188.
- Paulová, L., P. Hyka, B. Branská, K. Melzoch, and K. Kovar. 2012. "Use of a mixture of glucose and methanol as substrates for the production of recombinant tyrpsinogen in continous cultures with *Pichia pastoris* Mut+." *Journal of Biotechnology* (157):180-188.
- Popii, V. and G. Baumann. 2004. "Laboratory measurement of growth hormone." *Clinica Chimica Acta* (350):1-16.
- Potvin, G., A. Ahmad, and Z. Zhang. 2010. "Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review." *Biochemical Engineering Journal*.
- Raben, M. S. 1958. "Treatment of pituitary dwarf with human growth hormone." *J. Clşn. Endocrinol. Metab.* (18):901-903.
- Ratledge, C. 2001. "Biochemistry and physiology of growth and metabolism." Pp. 17-45 in *Basic Biotechnology*, edited by C. Ratledge and B. Kristiansen. UK: Cambridge University Press.

- Ren, H.T., J.Q. Yuan, and K.H. Bellgardt. 2003. "Macrokinetic model for methylotrophic *Pichia pastoris* based on stoichiometric balance." *Journal of Biotechnology* 106:53-68.
- Resina, D., A. Serrano, F. Valero, and P. Ferrer. 2004. "Expression of a *Rhizopus oryzae* lipase in *Pichia pastoris* under the control of the nitrogen source-regulated formaldehyde dehydrogenase promoter." *J. Biotechnology* 109(1-2):103-113.
- Roubin, M.R., M.D. Cailas, S.H. Shen, and D. Groleau. 1992. "Influence of pH, nitrogen and phosphorous sources on the production of hepatitis B virus pre-S2 antigen by *Hansenula polymorpha*." *Appl Microbiol Biotechnol* 38:158-164.
- Rushton, L. 2009. *The Human Body How it Works; The Endocrine System*. New York: Chelsea House Publishers.
- Saugy, M., N. Robinson, C. Saudan, N. Baume, L. Avois, and P. Mangin. 2006. "Human growth hormone doping in sport." *Br J Sports Med* 40:i35-i39.
- Schilling, B.M., J.C. Goodrick, and N.C. Wan. 2001. "Scale-up of a high cell density continuous culture with *Pichia pastoris* X-33 for the constitutive expression of rh-chitinase." *Biotechnol Prog* 17:629-633.
- Shen, S., G. Sulter, T.W. Jeffries, and J.M. Cregg. 1998. "A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*." *Gene* 216:93-102.
- Shin, N.K., D.Y. Kim, C.S. Shin, M.S. Hong, J. Lee, and H.S. Shin. 1998. "High level production of human growth hormone in *Escherichia coli* by a simple recombinant process." *J. Biotechnology* 62:143-151.
- Shi, L., D. Wang, W. Chan, and L. Cheng. 2007. "Efficient expression and purification of human interferon alpha2b in the methylotrophic yeast, *Pichia pastoris*." *Protein Expr Purif* 54:220-226.
- Shukla, A.K., W. Haase, C. Reinhart, and H. Michel. 2007. "Heterologous expression and comparative characterization of the human neuromedin U subtype II receptor using the methylotrophic yeast *Pichia pastoris* and mammalian cells." *Int J Biochem Cell Biol* 39:931-942.
- Sibirny, A.A., V.M. Ubiyovk, M.V. Gonchar, V.I. Titorenko, A.Y. Voronovsky, and Y.G. Kapultsevich. 1990. "Reaction of direct formaldehyde oxidation to CO₂ are not essential for energy supply of yeast methylotrophic growth." *Arch Microbiol* 154:566-77.

- Simonen, M. and I. Palva. 1993. "Protein secretion in *Bacillus* species." *Microbial Rev.* 57:109-137.
- Sinha, J., B.A. Plantz, M. Inan, and M.M. Meagher. 2005. "Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: Case study with recombinant ovine interferon-t." *Biotechnol and Bioengineering* 89(1):102-112.
- Sinha, J., B.A. Plantz, W. Zhang, M. Gouthro, V.L. Schlegel, C-P. Liu, and M.M. Meagher. 2003. "Improved production of recombinant ovine interferon-t by Mut+ strain of *Pichia pastoris* using an optimized methanol feed profile." *Biotechnol Prog* 19:794-802.
- Song, G.Y. and B.H. Chung. 1999. "Use of high culture pH for the decreased proteolysis of human parathyroid hormone secreted by *Saccharomyces cerevisiae*." *Biotechnol Lett* 21:57-60.
- Soyaslan, E.Ş. and P. Çalık. 2011. "Enhanced recombinant human erythropoietin production by *Pichia pastoris* in methanol fed-batch/sorbitol batch fermentation through pH optimization." *Biochem Eng J* 55:59-65.
- Sreekishna, K., R.G. Brankamp, K.E. Kropp, D.T. Blankenship, J.T. Tsay, P.L. Smith, J.D. Wierschke, A. Subramaniam, and L.A. Birkenberger. 1997. "Strategies for optimal synthesis and secretion of heterologous proteins in methylotrophic yeast *Pichia pastoris*." *Gene* 190:55-62.
- Strasser, A.W. M. and G. Gellissen. 1998. "Yeast expression systems and their competitive environment.." *Pharm Manuf Int* (3):151-152.
- Stratton, J., V. M. Chiruvolu, and M. Meagher 1998. "High cell-density fermentation." Pp. 107-120 in *Pichia Protocols, Methods in Molecular Biology*, edited by D.R. Higgins and J.M.C. Human Press.
- Thorpe, E.D., M.C. d'Anjou, and A.J. Daugulis. 1999. "Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*." *Biotechnol Lett* 21:669-672.
- Trevino, L. L., J. M. Viader-Salvado, H. A. Barrera-Saldana, and M. Guerrero-Olazarán. 2000. "Biosynthesis and secretion of recombinant human growth hormone in *Pichia pastoris*." *Biotechnology Letters* (22):109-114.

- Trinh, L.B., J.N. Phue, and J. Shiloah. 2003. "Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin form *Pichia pastoris*." *Biotechnol Bioeng* 82:438-444.
- Tulsiani, D.R., S.C. Hubbard, P.W. Robbins, and O. Touster. 1982. "Alpha-D-mannosidases of rat liver Golgi membranes. Mannosidase II is the GlcNAcMan5-cleaving enzyme in glycoprotein biosynthesis and mannosidases IA and IB are the enzymes converting Man9 precursors to Man5 intermediates." *J Biol Chem* 257:3660-3668.
- Vervecken, W., V. Kaigorodov, N. Callewaert, S. Geysens, K. De Vusser, and R. Contreras. 2004. "In vivo synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*." *Appl Environ Microbiol* 70:2639-2646.
- Villadsen, J., Nielsen, J., 1990. " Modeling of fermentation kinetics, in Proceedings of European Congress Biotechnology", 259-266, Copenhagen.
- Walker, G.M. 1998. *Yeast: Physiology and Biotechnology*. New York: John Wiley & Sons Inc.
- Walsh, G. 2010. "Biopharmaceutical benchmarks." *Nat. Biotech.* 28:917-924.
- Wang, Z., Y. Wang, D. Zhang, J. Li, Z. Hua, G. Du, and J. Chen. 2009. "Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation." *Bioresource Technol* 101:1318-1323.
- Waterham, H.R., M.E. Digan, P.J. Koutz, S.V. Lair, and J.M. Cregg. 1997. "Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter." *Gene* 186:37-44.
- White, C.E., M.J. Hunter, D.P. Meininger, L.R. White, and E.A. Komives. 1995. "Large-scale expression, purification and characterization of small fragments of thrombomodulin: the roles of the sixth domain and methionine 388." *Protein Eng.* 8(11):1177-1187.
- Wright, A. and S.L. Morrison. 1997. "Effect of glycosylation on antibody function: implications for genetic engineering." *Trends in Biotechnology* 15(1):26-32.
- Wu, J. and R.J. Kaufman. 2006. "From acute ER stress to physiological roles of the unfolded protein response." *Cell Death Differ* 13:374-384.

www.researchandmarkets.com. 2010. "Biopharmaceuticals-a global market overview." Research and Markets. Retrieved June 2012 (www.researchandmarkets.com).

Xie, J., Q. Zhou, P. Du, R. Gan, and Q. Ye. 2005. "Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiostatin production." *Enzyme and Microbial Technology* 36:210-216.

Yang, X. 1992. "Optimization of a cultivation process for recombinant protein production by *Escherichia coli*." *J of Biotechnol* 23:271-289.

Yang, Z., Zhou X., and Y. Zhang. 2004. "Improvement of recombinant hirudin production by controlling NH₄⁺ concentration in *Pichia pastoris* fermentation." *Biotechnol Letts* 26:1013-1017.

Zhang, W., M.A. Bevins, B.A. Plantz, L.A. Smith, and M.M. Meagher. 2000b. "Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of Botulinum neurotoxin, Serotype A." *Biotechnol Bioeng* 70:1-8.

Zhang, L., K.J. Hywood Potter, B.A. Plantz, V.L. Schlegel, L.A. Smith, and M.M. Meagher. 2003. "*Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement." *J Ind Microbiol Biotechnol* 30:210-215.

Zhang, W.H., J. Sinha, L.A. Smith, M. Inan, and M.M. Meagher. 2005. "Maximization of production of secreted recombinant proteins in *Pichia pastoris* fed-batch fermentation." *Biotechnol Progress* 21:386-393.

APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Fermentation Medium

1 M potassium phosphate buffer, pH 6.0 56.48 g KH_2PO_4 , 14.8 g K_2HPO_4 was dissolved in dH_2O and the volume made up to 500 mL. The pH was controlled. The buffer was autoclaved and stored at room temperature.

Antifoam 10% (v/v) antifoam solution was prepared with dH_2O . Can be autoclaved once.

Base 25% NH_3OH (Sigma). No need to sterilize.

SDS-PAGE Solutions

10% (w/v) APS (Ammonium Persulfate) Add 0.1 g APS to 1 mL dH_2O , freshly prepared.

1.5 M Tris-HCl, pH 8.8 26.3 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 8.8 with 6 N HCl. The buffer was made up to 200 mL with dH_2O . The buffer was autoclaved and stored at 2-8°C.

0.5 M Tris-HCl, pH 6.8 12.2 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 6.8 with 6N HCl. The buffer was made up to 200 mL with dH_2O . The buffer was autoclaved and stored at 2-8°C.

Stacking Buffer (5%) (for 2 gels)	2.8mL dH ₂ O 0.85mL 30 Acrylamide-bis, 1.25mL 0.5M Tris-HCl pH6.8, 50µL 10% SDS, prior to gel preparation add 25µL APS and 5µL N,N,N',N'- Tetramethylethylenediamine.
Resolving Buffer (12%) (for 2 gels)	3.4 mL dH ₂ O, 4 mL 30% Acrylamide-bis, 2.5 mL 1.5 M Tris-HCl pH 8.8, 100 µL 10% SDS, prior to gel preparation add 50 µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine.
4 x Sample Loading Buf- fer for SDS-PAGE	200 mM Tris-HCl, pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue; 10% 2- mercaptoethanol. Distributed into microcentri- fuge tubes and stored at -20°C.
5 x SDS-PAGE Running Buffer	15 g Tris Base, 72 g glycine, 5g SDS, dH ₂ O to 1 liter. The buffer was stored at 2-8°C and di- luted 1:5 with dH ₂ O prior to use.
Fixer Solution	Mix 150 mL methanol + 36 mL acetic acid + 150 µL formaldehyde and complete to 300 mL with distilled water. This solution can be used several times.
Pretreatment Solution	Dissolve 0.08 g sodium thiosulphate (Na ₂ S ₂ O ₃ ·5H ₂ O) in 400 ml distilled water by mixing with glass rod. Take 8 mL and set aside for further use in developing solution prepara- tion.
Silver Nitrate Solution	Dissolve 0.8 g silver nitrate in 400 mL distilled water and add 300 µL 37% formaldehyde.
Developing Solution	Dissolve 9 g potassium carbonate in 400 mL distilled water. Add 8 mL from pretreatment solution and 300 µL 37% formaldehyde.
Stop Solution	Mix 200 mL methanol + 48 mL acetic acid and complete to 400 mL with distilled water.

AOX Assay Solutions

Yeast Lysis Buffer	100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10% glycerol. Add 1 protease inhibitor cocktail tablet per 50 mL buffer. Filter sterilize and store at +4°C for maximum 3 months.
1 M Tris-Cl, pH 8.0	12.1 g Tris base was dissolved in 80 mL dH ₂ O and the pH was adjusted to 8.0 by adding concentrated HCl. The volume was made up to 100 mL. The buffer was autoclaved and stored at room temperature.
0.125 M (or 0.5 M) EDTA, pH 8.0	4.65 g (or 18.61 g) Ethylenediaminetetra acetic acid disodium salt dihydrate was dissolved in 80 mL dH ₂ O. NaOH was added until EDTA was dissolved. The final pH was further adjusted to pH 8.0 and the final volume was adjusted to 100 mL. The buffer was autoclaved and stored at room temperature.
1 M potassium phosphate, pH 7.5	1 M KH ₂ PO ₄ , 1M K ₂ HPO ₄ was dissolved in dH ₂ O and titer KH ₂ PO ₄ with K ₂ HPO ₄ while controlling pH. The buffer was autoclaved and stored at room temperature.
0.05 M Sodium Acetate buffer (for acidic proteases)	Dissolve 0.713 mL acetic acid in 25 mL total dH ₂ O. Dissolve 2.052 g sodium acetate in 50 mL dH ₂ O. Titrate sodium acetate solution with acetic acid solution to pH 5.0 and final V=50 mL. Then dilute to 500 mL. Autoclave and store at +4°C.
0.05 M Sodium Phosphate Buffer (for neutral proteases)	Dissolve 6.70 g Na ₂ HPO ₄ ·7H ₂ O in 50 mL dH ₂ O. Dissolve 3.90 g NaH ₂ PO ₄ ·2H ₂ O in 50 mL dH ₂ O. Titrate till pH 7.0 and final V=50 mL. Then dilute to 500 mL. Autoclave and store at room temperature.

APPENDIX B

CALIBRATION CURVE FOR BRADFORD ASSAY

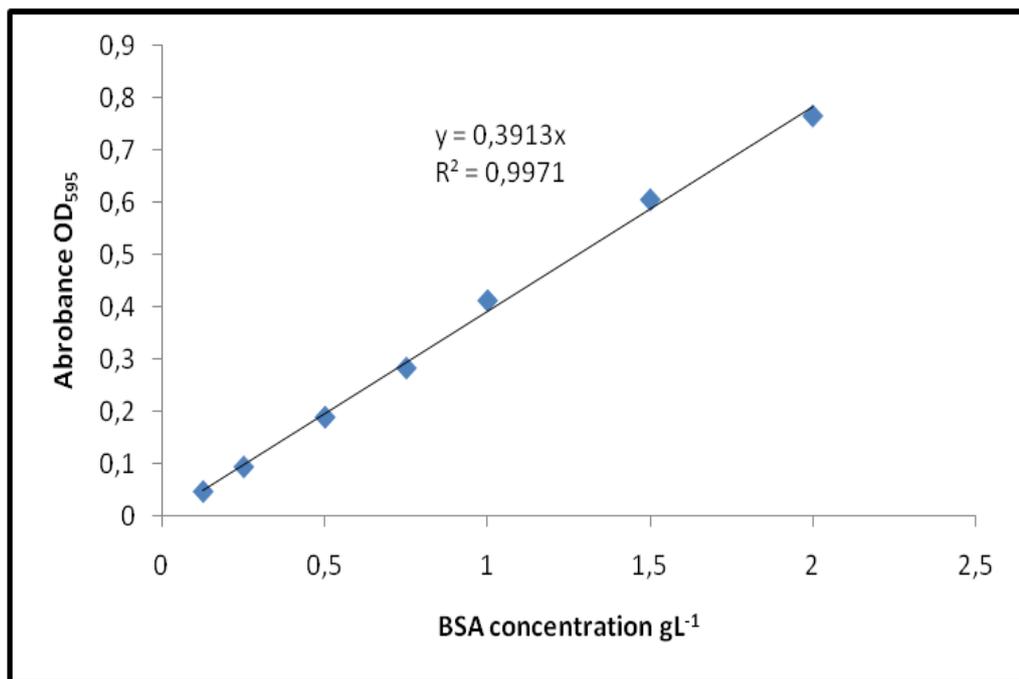


Figure B.1 Standard curve for Bradford Assay

APPENDIX C

CALIBRATION CURVES FOR METHANOL AND SORBITOL

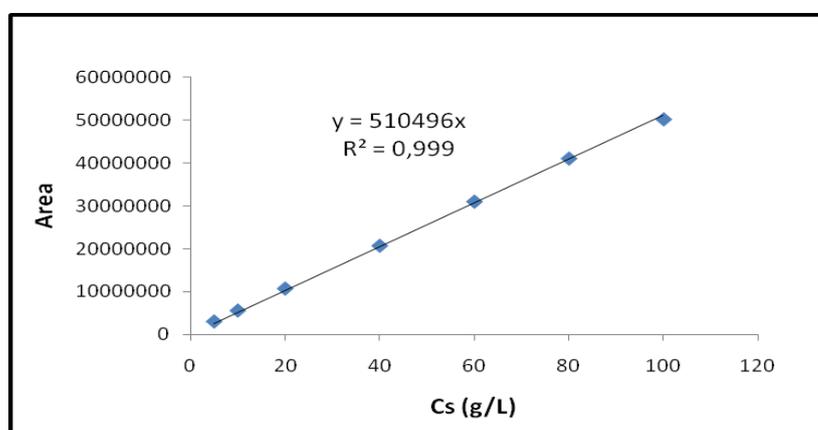


Figure C.1 HPLC Analysis: Calibration curve for sorbitol concentration

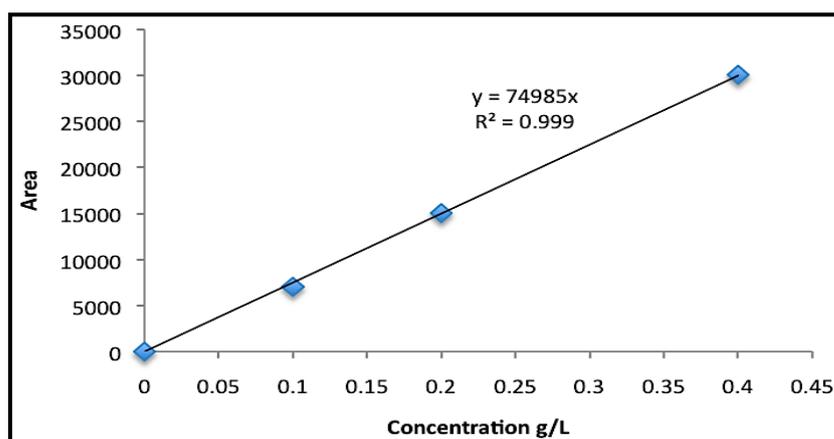


Figure C.2 HPLC Analysis: Calibration curve for methanol concentration

APPENDIX D

CALIBRATION CURVES FOR ORGANIC ACIDS

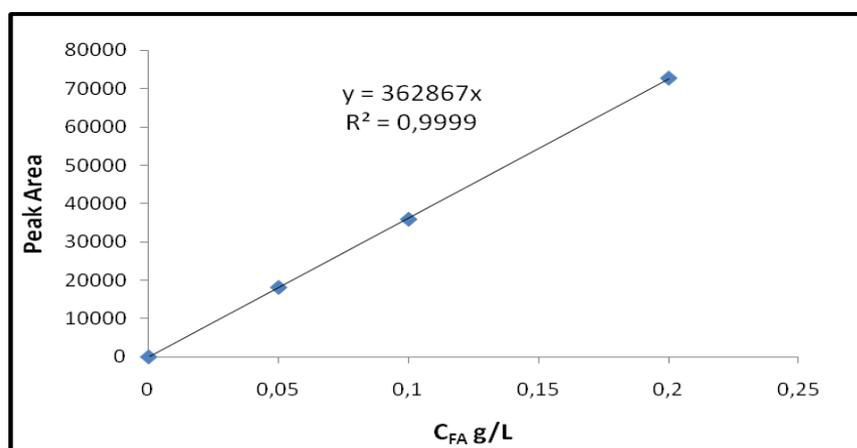


Figure D.1 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

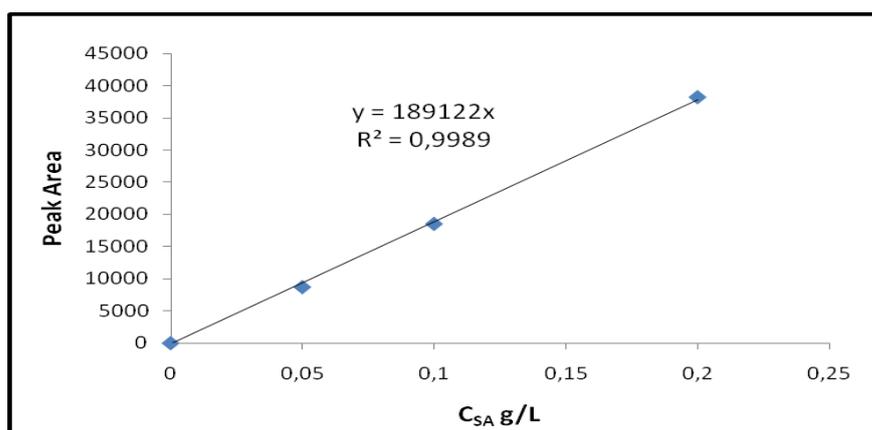


Figure D.2 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

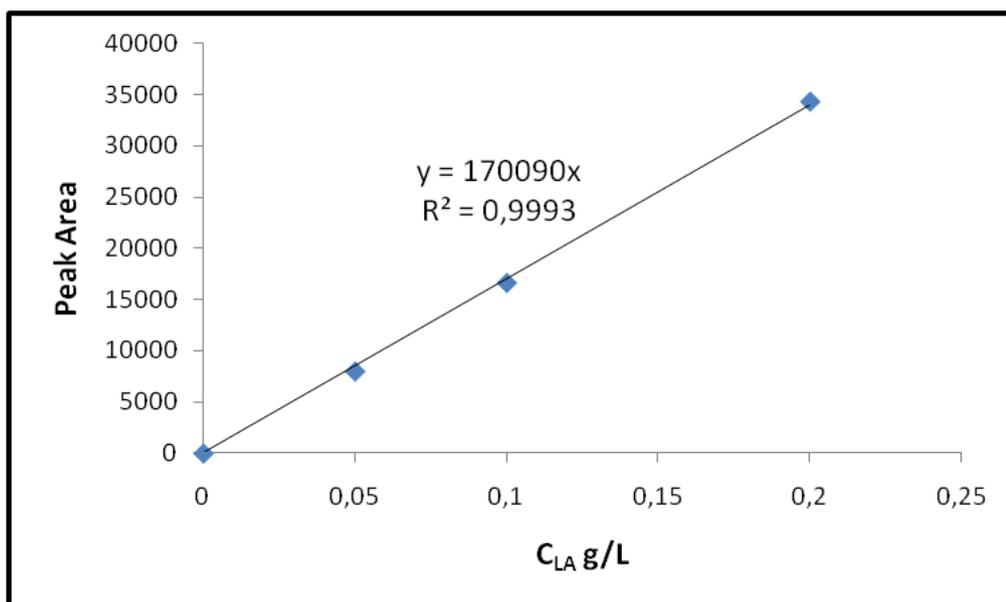


Figure D.3 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

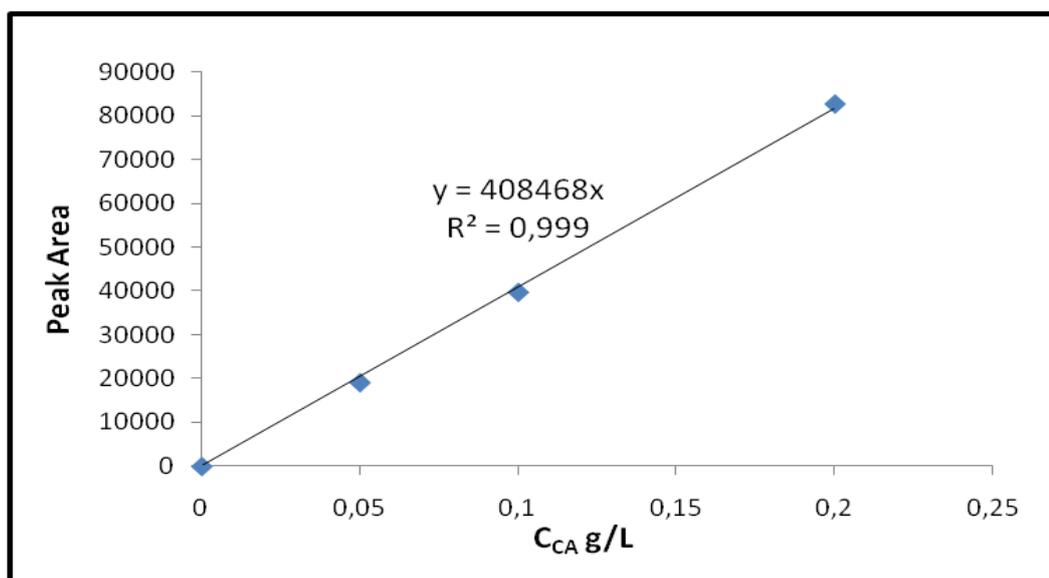


Figure D.4 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC

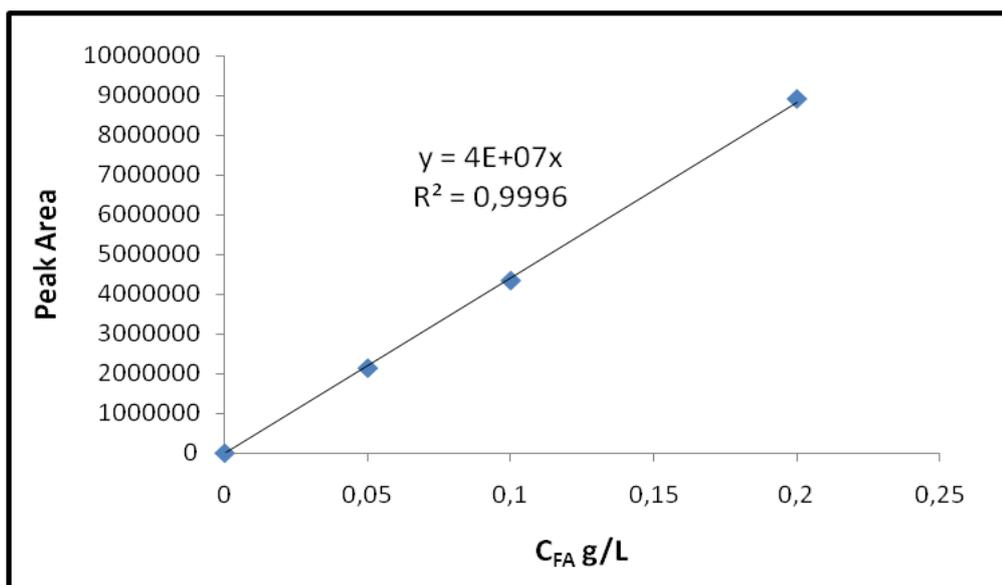


Figure D.5 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

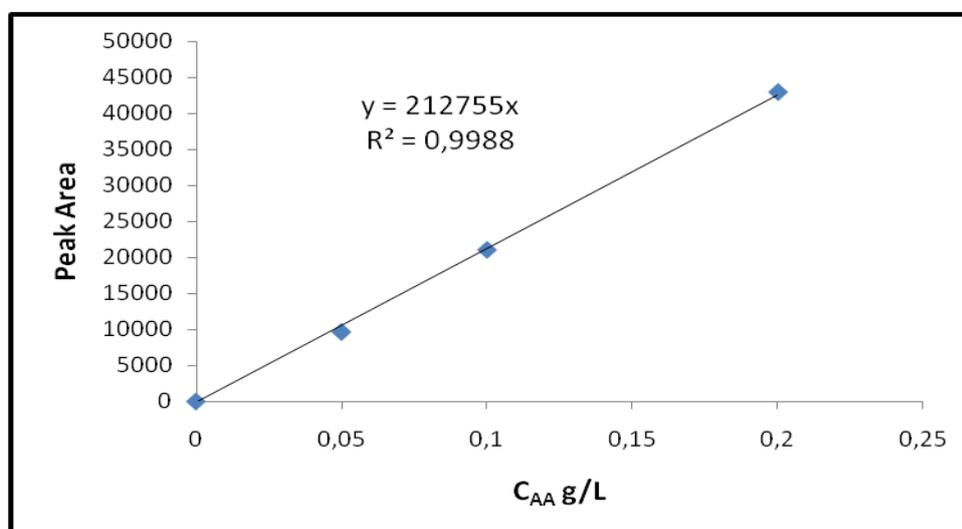


Figure D.6 Calibration curve obtained for acetic acid concentration; analysis was performed by HPLC.

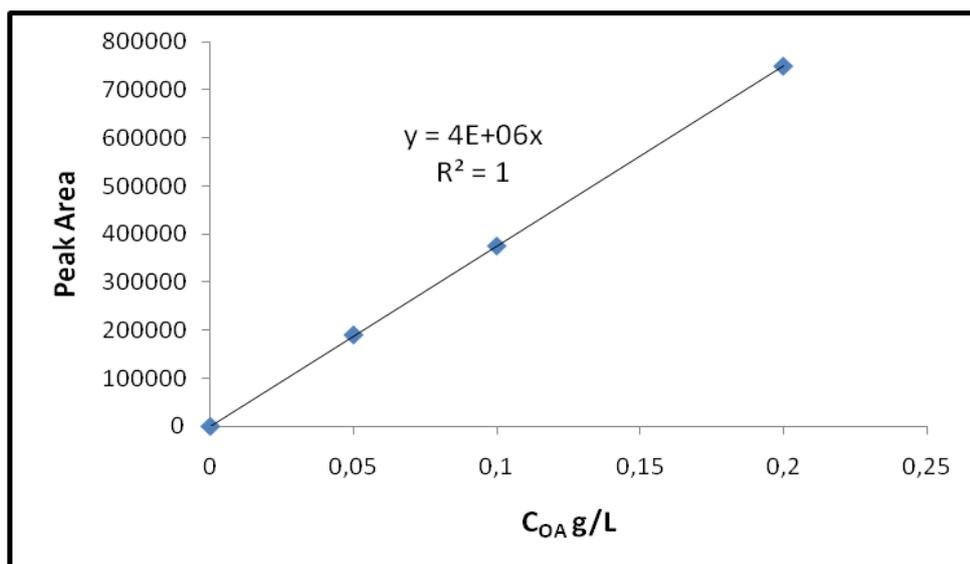


Figure D.7 Calibration curve obtained for oxalic acid concentration; analysis was performed by HPLC.

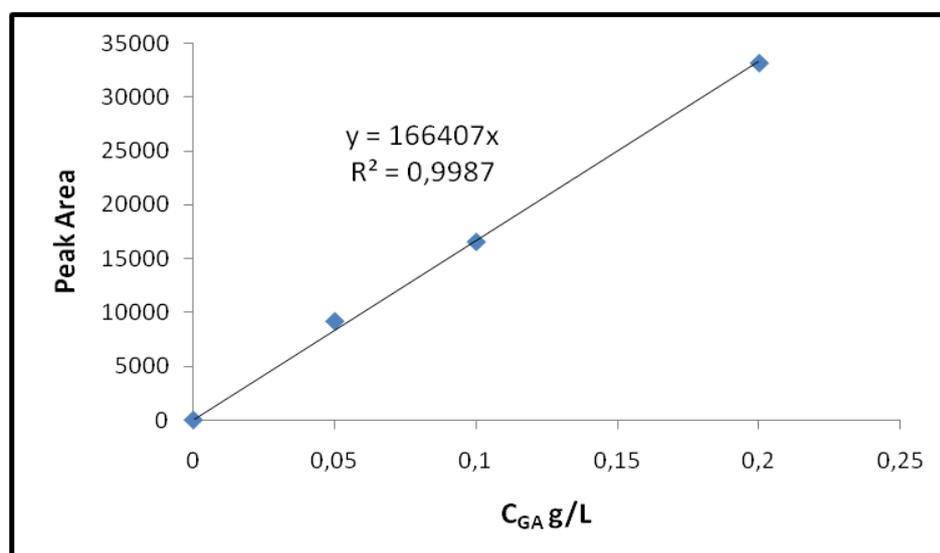


Figure D.8 Calibration curve obtained for gluconic acid concentration; analysis was performed by HPLC

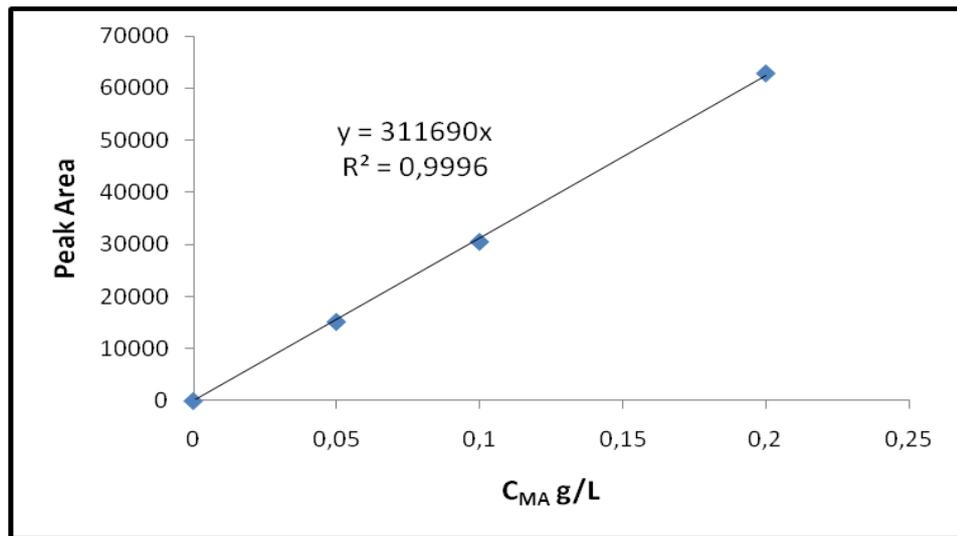


Figure D.9 Calibration curve obtained for malic acid concentration; analysis was performed by HPLC

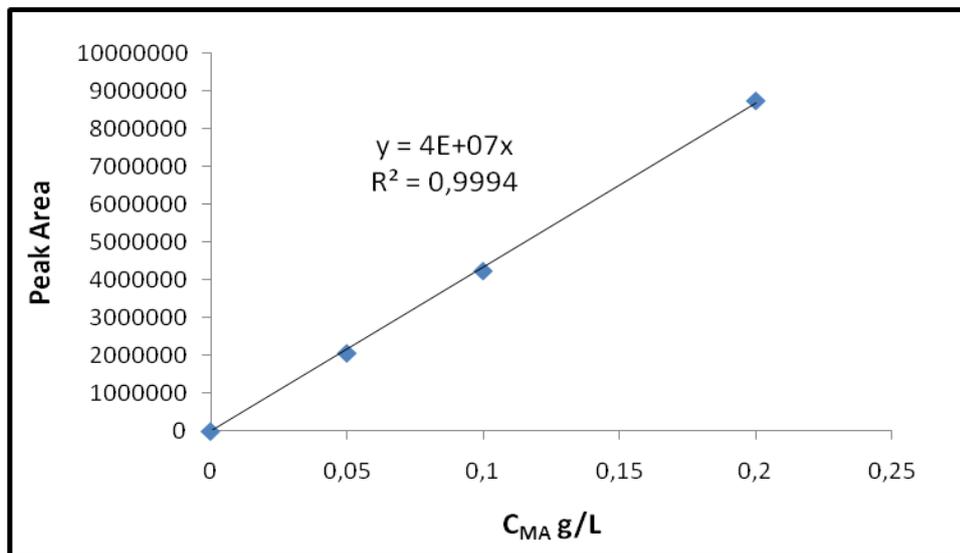


Figure D.10 Calibration curve obtained for maleic acid concentration; analysis was performed by HPLC.

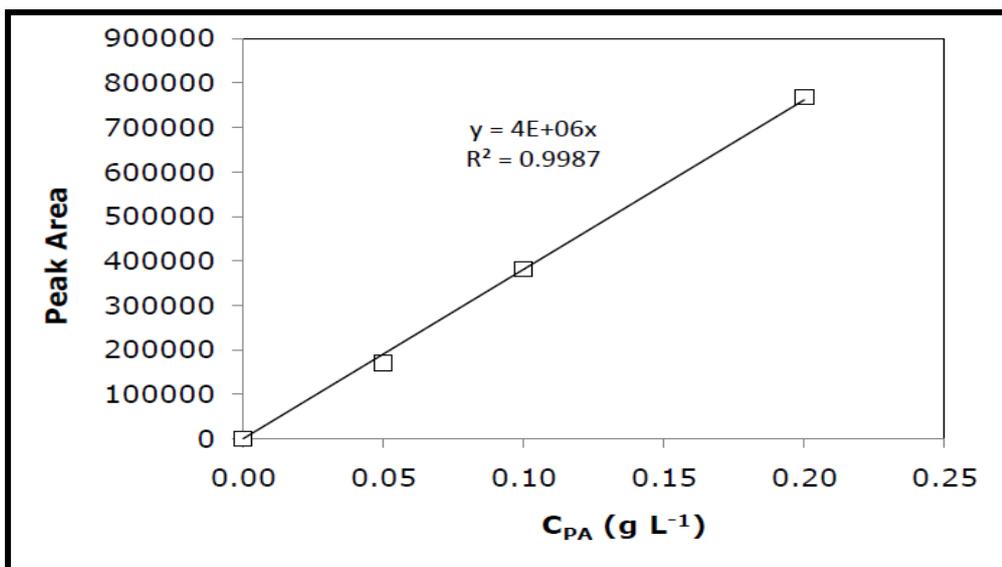


Figure D.11 Calibration curve obtained for pyruvic acid concentration; analysis was performed by HPLC

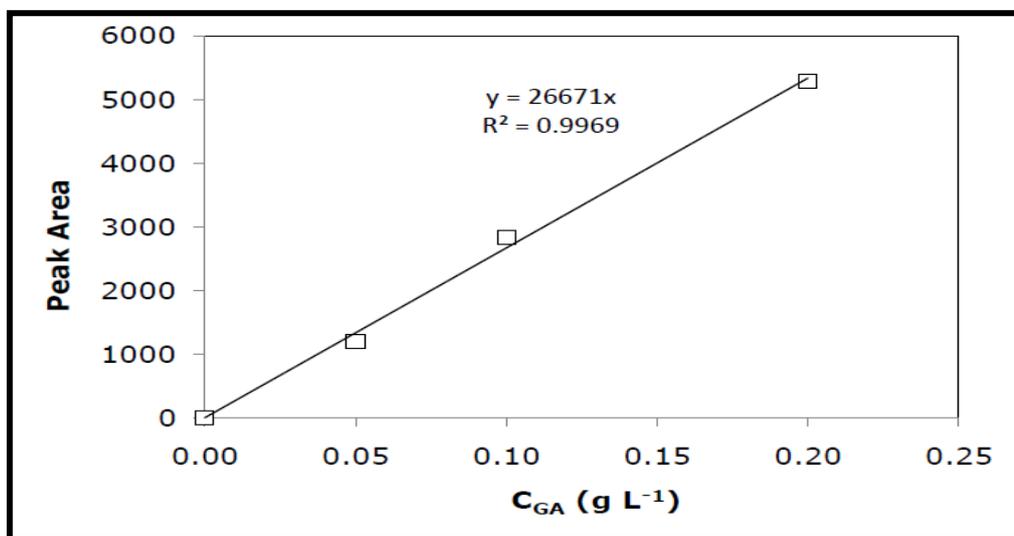


Figure D.12 Calibration curve obtained for glutaric acid concentration; analysis was performed by HPLC.

APPENDIX E

ELECTROPHEROGRAM AND CALIBRATION CURVE FOR rhGH STANDARD

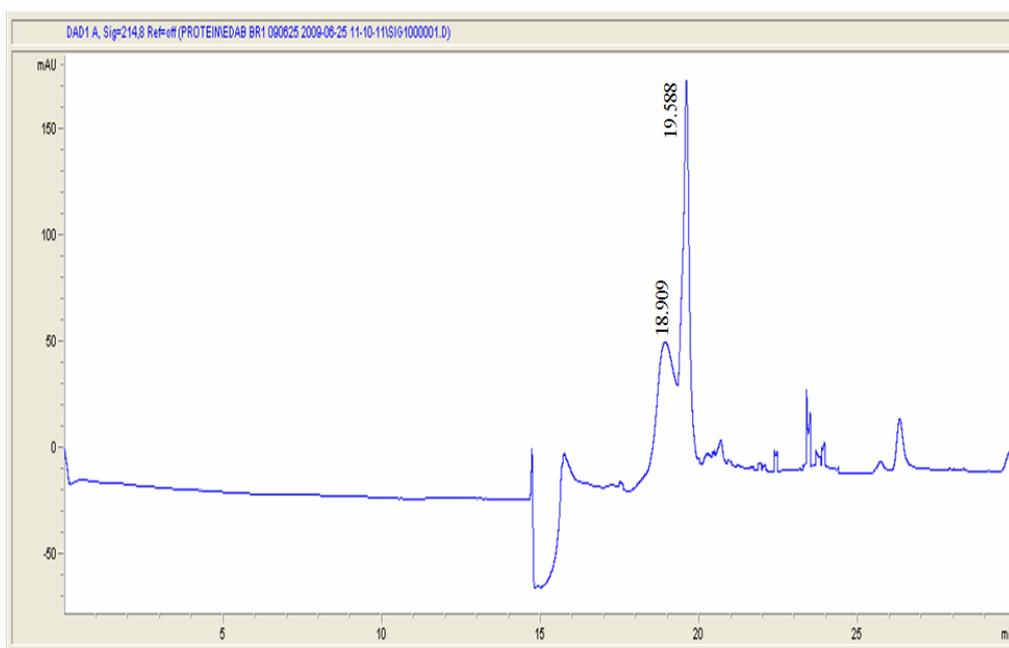


Figure E.1 Electropherogram of 0.05 g L⁻¹ hGH standard

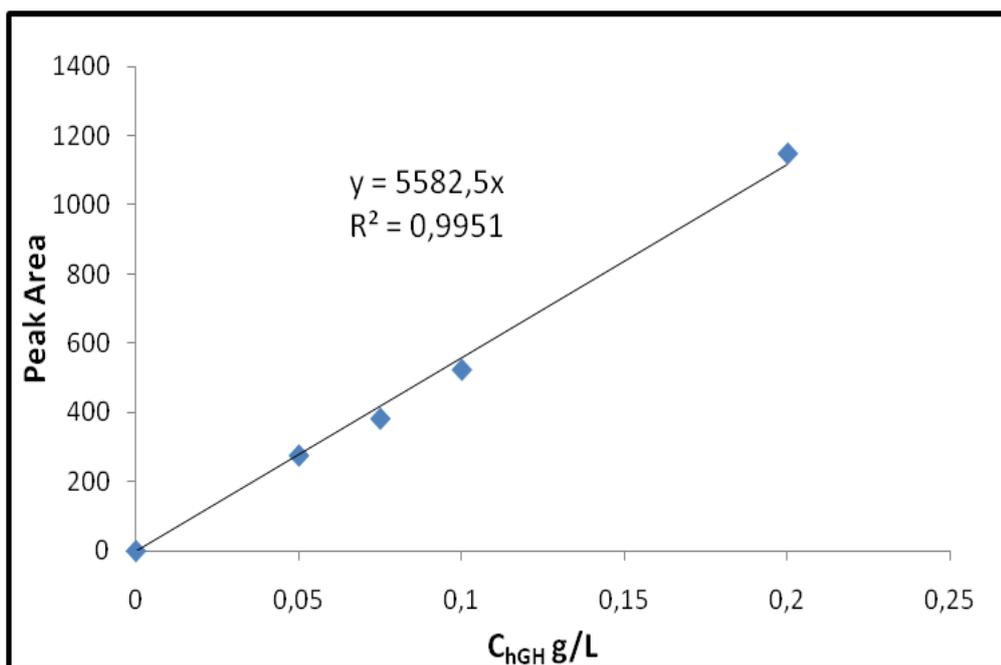


Figure E.2 Calibration curve for standard hGH

APPENDIX F

CALIBRATION CURVE FOR AOX ACTIVITY

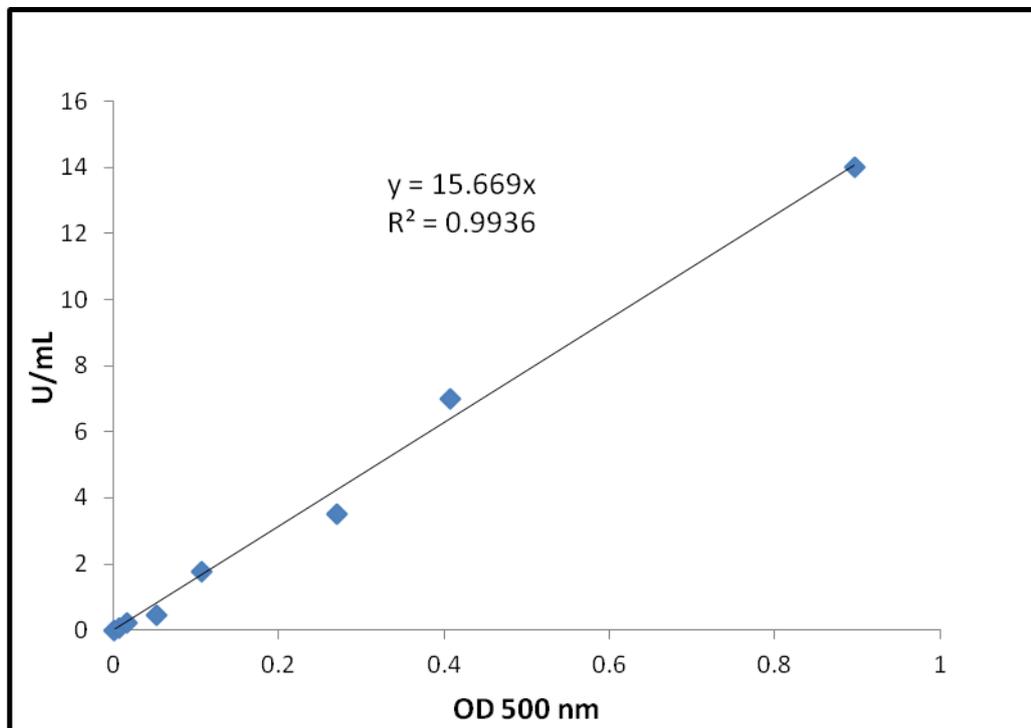


Figure F.1 Calibration curve obtained for AOX activity

APPENDIX G

MOLECULAR WEIGHT MARKER

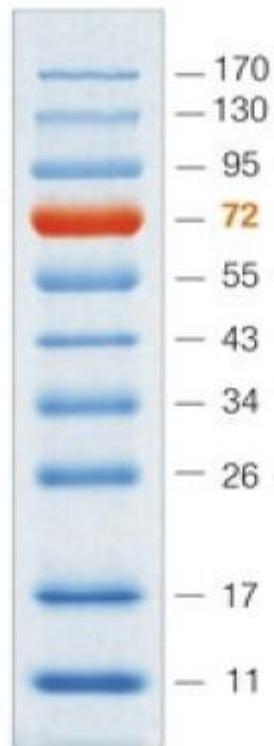


Figure G.1 PageRular™ Prestained Protein Ladder (Fermentas)

APPENDIX H

CALCULATION THE SPECIFIC GROWTH RATE OF SORBITOL

For the calculation of specific growth rate of sorbitol first six hours of the production phase where the highest specific consumption rates of sorbitol were observed, was taken into account. The datas (q_s and C_x) were taken from the study of Inankur (2010). Based on specific consumption rate, q_s , the volumetric consumption rate, r_s , of sorbitol was calculated (Table 4.9). The specific growth rate of sorbitol was determined by predetermined feeding profile calculated according to volumetric sorbitol consumption rate. The exponential value of the slope equation yielded the specific growth rate of sorbitol as 0.025 h^{-1} (Figure H.1).

Table 4.10 Sorbitol specific and volumetric consumption rates

t h	q_s g g ⁻¹ h ⁻¹	C_x g L ⁻¹	r_s g L ⁻¹ h ⁻¹
0	0.205	22.8	4.67
3	0.137	25.4	3.48
6	0.115	29.7	3.42
mean	0.152		3.86

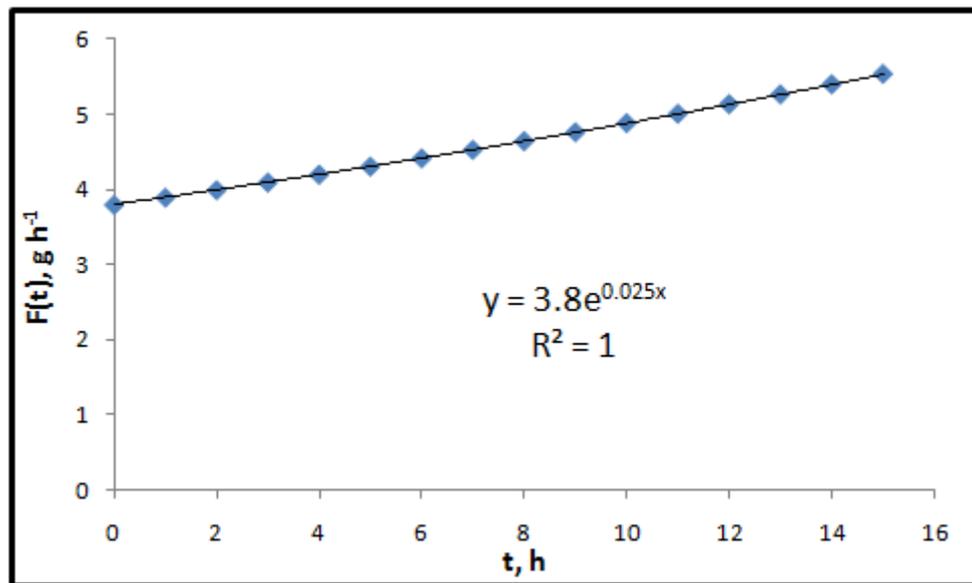


Figure H.1 Predetermined feeding profile for sorbitol calculated according to volumetric sorbitol consumption rate