EFFECT OF pH ON ERYTHROPOIETIN PRODUCTION BY RECOMBINANT *Pichia pastoris* IN FED-BATCH OPERATION

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

BY

ELİF ŞÜKRAN SOYASLAN

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

AUGUST 2010

Approval of the thesis:

EFFECT OF pH ON ERYTHROPOIETIN PRODUCTION BY RECOMBINANT *Pichia pastoris* IN FED-BATCH OPERATION

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

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

Prof.Dr. Gürkan Karakaş _____
Head of Department, **Chemical Engineering**

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

Examining Committee Members:

Prof.Dr. Tunçer Özdamar _____
Chemical Engineering Dept., Ankara Universıty

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

Prof. Dr. Ufuk Bakır _____
Chemical Engineering Dept., METU

Prof.Dr. Semra Şahin _____
Food Engineering Dept., METU

Dr. Remziye Yılmaz _____
Central Laboratory, METU

Date: 31. 08. 2010

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

Name, Last name: ELİF ŞÜKRAN SOYASLAN

Signature :

ABSTRACT

EFFECT OF pH ON ERYTHROPOIETIN PRODUCTION BY RECOMBINANT *Pichia pastoris* FED-BATCH OPERATION

Soyaslan, Elif Şükran

M.Sc., Department of Chemical Engineering

Supervisor : Prof.Dr. Pınar Çalık

August 2010, 124 pages

In this study, the effects of pH on therapeutically important protein, recombinant human erythropoietin (rhuEPO), production by *Pichia pastoris* was investigated at pH=4.0, 4.5, 5.0, 5.5 and 6.0. rHuEPO production was started by methanol induction in fed-batch mode. The highest cell concentration was obtained at pH=4.5 as 81.4 g L^{-1}. The co-substrate substrate sorbitol, which was added batch-wise, was consumed at t=15 h of the operations at pH=4.0, 4.5 and 5.0. However as the pH increases above pH=5.0 the sorbitol consumption rate decreases. The highest rHuEPO concentration was achieved at pH=4.5 as 0.158 g L^{-1} which was 1.43-, 1.24-, 1.95- and 1.23-fold higher than those obtained at pH=4.0, 5.0, 5.5, and 6.0, respectively. Also at pH=4.5 overall cell yield on substrate was 0.51 g g^{-1} and overall rHuEPO yield on substrate was 1.45 mg g^{-1}.

rHuEPO concentration was decreased in the last 3-6 hour of the operation due to proteolysis. Therefore extracellular protease concentrations in the medium were determined. As expected, since the investigated pH range was acidic, the amount of acidic proteases was found to be higher than neutral and basic proteases. Furthermore the total protease concentration increased linearly in the fermentation broth, having close values at different pH values. Thus, pH did not have a significant effect on extracellular protease activity.

Alcohol oxidase (AOX) activities showed similar behavior at different pH. The highest specific AOX activity was attained at pH=4.5, at which the highest rHuEPO concentration was achieved, as 110.1 U g^{-1} CDW.

Keywords: Recombinant human erythropoietin, *Pichia pastoris*, optimization of pH, alcohol oxidase activity

ÖZ

REKOMBİNANT *Pichia pastoris* İLE ERİTROPOİETİN ÜRETİMİNDE pH ETKİSİNİN YARI KESİKLİ İŞLETİM İLE ARAŞTIRILMASI

Soyaslan, Elif Şükran
Yüksek Lisans, Kimya Mühendisliği Bölümü
Tez Yöneticisi : Prof.Dr. Pınar Çalık

Ağustos 2010, 124 sayfa

Bu çalışmada, pH'ın *Pichia pastoris* ile rekombinant insan eritropoetini (rHuEPO) üretimi üzerine etkisi pH=4.0, 4.5, 5.0, 5.5 ve 6.0 işletim koşullarında araştırılmıştır. rHuEPO üretimi yarı-kesikli metanol indüksiyonuyla başlatılmıştır. En yüksek hücre derişimi pH=4.5' de 81.4 g L^{-1} olarak elde edilmiştir. İkinci karbon kaynağı olan sorbitol kesikli olarak biyoreaktöre eklenmiştir, pH=4.0, 4.5 ve 5.0'de yapılan işletimlerde sorbitol t=15 st'te tükenmiştir. Ancak pH=5.0'in üzerindeki işletimlerde sorbitol tüketim hızının artan pH ile azaldığı gözlemlenmiştir. Elde edilen en yüksek rHuEPO derişimi 0.158 g L^{-1} olarak pH=4.5'te elde edilmiştir. Elde edilen bu değer pH=4.0, 5.0, 5.5 ve 6.0 da elde edilen derişimlerden sırasıyla 1.43-, 1.24-, 1.95- and 1.23-kat daha yüksektir. Ayrıca pH=4.5'te substrate üzerinden hücre ve rHuEPO verim katsayıları sırasıyla 0.521 g g^{-1} ve 1.45 mg g^{-1} olarak bulunmuştur.

rHuEPO derişimlerinde işletimlerin son fazlarında proteoliz nedeniyle düşme göstermiştir. Araştırılan pH aralığı asitik olduğundan, ortamdaki asitik proteazların nötral ve bazik proteazlardan daha fazla olduğu bulunmuştur. Buna ek olarak toplam proteaz derişiminin zamanla doğrusal olarak arttığı ve farklı pH'larda yakın değerlere ulaştığı gözlemlenmiştir. Bu nedenle üretim

ortamı pH'ı hücre dışı proteaz aktivitesi üzerinde belirgin bir etkiye sahip değildir. Alkol oksidaz (AOX) aktiviteleri farklı pH'larda benzer davranışlar göstermiştir. Elde edilen en yüksek spesifik AOX aktivitesi 110.1 U g^{-1} CDW olup, en yüksek rHuEPO derişiminin elde edildiği pH olan pH=4.5 de elde edilmiştir.

Anahtar Kelimeler: Rekombinant insan eritropoetini, *Pichia pastoris*, pH optimizasyonu, alkol oksidaz aktivitesi

To my beloved family

ACKNOWLEDGEMENTS

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

I would like to express my special thanks to Prof. Dr. Tunçer H. Özdamar for giving me the opportunity to use Ankara University, Biochemical Reaction Engineering Laboratories.

I am also thankful my friends Hatice Taşpınar, Merve Şahin, Bahar İnankur and Erdem Boy who became a lifelong friends, for their great friendship and continuous support for two years even in sleepless days and nights and also Dr. Eda Çelik Akdur for her continuous support, help, and friendship throughout my graduate program.

I am grateful to my friends in our research group in Industrial Biotechnology Laboratory, Eda Açık, Eda Baraktar, Vahideh Anghardi, Bade Kavurt, and also my friends in Biochemical Reaction Engineering Laboratory Department of Chemical Engineering (Ankara University); Özge Yılmaz, Pınar Kocabaş and to research group of Prof. Dr Ufuk Bakır for their support, friendship, advice and encouragement throughout my graduate program.

Financial support of Scientific and Technical Research Council of the TURKEY (TÜBİTAK) through the project MAG-107M285 and METU-Research Fund are gratefully acknowledged.

Above all, I would like to thank to my family for loving, supporting and encouraging me all through my life.

TABLE OF CONTENT

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

NOMENCLATURE

C_O^*	Saturated dissolved oxygen concentration	mol m^{-3}
Da	Damköhler number (=OD / OTR_{max} ; Maximum possible oxygen utilization rate per maximum mass transfer rate)	
DO	Dissolved oxygen	%
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient	
K_La	Overall liquid phase mass transfer coefficient	s^{-1}
K_La_0	Physical overall liquid phase mass transfer coef.	s^{-1}
N	Agitation rate	min^{-1}
OUR	Oxygen uptake rate	mol m^{-3} sec^{-1}
OTR	Oxygen transfer rate	mol m^{-3} sec^{-1}
OD	Oxygen demand	mol m^{-3} sec^{-1}
Q	Feed inlet rate	L h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
r	Reaction rate	g L^{-1} h^{-1}
t	Cultivation time	h
T	Bioreaction medium temperature,	°C
U	One unit of an enzyme	
V	Volume of the bioreactor	L
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}
μ_{max}	Maximum specific growth rate	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" or "product"
pro	Refers to "protease"
R	Refers to "bioreaction medium"
rHuEPO	Refers to "recombinant human erythropoietin"
rp	Refers to "recombinant protein"
S	Refers to "sorbitol" or "substrate"
X	Refers to "cell"

Abbreviations

AOX	Alcohol oxidase
BHK	Baby Hamster Kidney
BSM	Basal salt medium
CDW	Cell dry weight
CHO	Chinese Hamster Ovary
DNA	Deoxyribonucleic acid
EPO	Human erythropoietin hormone
GB	Glycerol batch
GFB	Glycerol fed-batch
HPCE	High pressure capillary electrophoresis
HPLC	High pressure liquid chromatography
MFB	Methanol fed-batch
MT	Methanol transition
PTM1	Trace salt solution
rHuEPO	Recombinant human erythropoietin
rhGH	Recombinant Human growth hormone
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid

CHAPTER 1

INTRODUCTION

In the 20th century biological sciences and different engineering disciplines was combined in biotechnology to improve human health and environment. Health care, environment, crop production and agriculture, non food uses of crops and other products (biodegradable plastics, vegetable oil, biofuels) are the industrial areas in which applications of this interdisciplinary filed is widely used (Nielsen et al., 2003). Industrial biotechnology is one of the fastest growing area of science enabling the production and optimization of the production of industrially important bio-molecules, thus the demand can be met.

Different bio-molecules such as hormones, enzymes, antibodies are being used as a therapeutic drugs. However derivation of these drugs from human or animal sources fails to meet the demand. Recent advances in recombinant DNA technology helps to overcome the need to obtain the bio-molecules from its original source, by cloning the targeted gene responsible for the production of the desired bio-molecules (Nielsen, 2003). Recombinant human erythropoietin (rHuEPO) is one of these bio-molecules that is widely used for treatment of anemia caused by renal failure or cancer (Jelkmann, 1992). Erythropoietin (EPO, which is a glycoprotein hormone, is synthesized in kidneys during adult life and in liver during fetal life (Jacobson et al., 1957; Zanjani et al., 1977). In human body the function of EPO is to stimulate red blood cell formation in bone marrows, and also to initiate hemoglobin production. Therefore decrease in EPO production due to kidney failure results in anemia. Red blood cell formation is regulated by accelerating or reducing the EPO production in cases of hypoxia or hyperoxia (Krantz, 1991).

Recombinant human erythropoietin (rHuEPO) is a U.S. Food and Drug Administration approved therapeutic drug which is produced in Chinese hamster ovary cell lines (Egrie et al., 1986). However animal cell lines have disadvantages such as low efficiency and high cost, thus an alternative host for more efficient production of rHuEPO is needed. Several different microorganisms were tested as hosts to produce rHuEPO. RHuEPO was expressed in bacterial hosts such as *E. coli* (Lee-Huang, 1984) and *B. brevis* (Sasaki et al., 1997). EPO is a glycoprotein hormone that needs to be glycosylated to attain biological activity (Delorme et al., 1992); since being prokaryote bacteria do not utilize the ability of glycosylation and therefore fail to produce an active rHuEPO. An eukaryote, that was used for production of EPO, was *S. cerevisiae* and yet again the product is not biologically active because of the hyperglycosylation of the proteins by this microorganism (Bretthauer et al., 1999).

Pichia pastoris, that can be regarded as an alternative to *S. cerevisiae*, has become a popular host and a useful system for expression of proteins both in research studies and industrial applications. *P. pastoris* is a methylotrophic yeast and has a strong promoter (AOX1) which is induced by methanol. Furthermore being a eukaryote *P. pastoris* can perform post translational modifications, which is an ability that bacteria lack and it needs less time and effort with higher yields when compared to animal cell lines. Recently Çelik et al. (2007) has developed a *P. pastoris* strain that produces rHuEPO extracellularly and investigated the effects of different production media and specific growth rate in fed-batch fermentations. Furthermore in a more recent study it was found that using this strain a method for fed-batch methanol feeding strategy with sorbitol feeding in batch mode was developed (Çelik et al., 2009). In the study it was found that the specific growth rate has an important effect on rHuEPO production, and the optimum value was determined to be 0.03 h^{-1}. Also the operation pH of the bioreactor experiment was pH=5.0 which was determined by a shake flask experiment in which pH was adjusted to the desired value at the beginning but remained uncontrolled during the production.

For the development of a fermentation process, after the construction of the strain and choosing the appropriate medium, optimum process

conditions should be determined (Nielsen et al., 2003). A microorganism functions as a micro-bioreactor by interacting with its micro-environment in the bioreactor (Çalık et al., 2003). Thus the operation conditions of the bioreactor have an important effect on the metabolic reactions occurring in the microorganism and thus help increasing the yield (Çalık et al., 1999).

Different bioreactor operation parameters such as pH, temperature and oxygen transfer were investigated for the production of different recombinant proteins in *P. pastoris*. Amongst those pH is an important factor affecting the productivity of the cells as well as protein stability.

In this context, the aim of this study is to improve the production of rHuEPO in *P. pastoris* by optimizing the operation pH. For this purpose a four phase fermentation process was employed at the pH values of pH=4.0, 4.5, 5.0, 5.5 and 6.0 to obtain high cell densities and consequently high rHuEPO concentrations. Furthermore to obtain a better insight of the process the effects of pH on cell growth, substrate consumption, rHuEPO production, alcohol oxidase and protease production were investigated.

CHAPTER 2

LITERATURE SURVEY

A large variety of proteins are being produced using microorganisms by means of recombinant DNA technology. The development of a process for production of these biomolecules, begins with the selection of a host microorganism considering the properties of the product. Later, effects of medium composition and bioreactor operation conditions should be examined since they have crucial effects on quality and quantity of the product. Furthermore, for better evaluation bioprocess characteristics, which are mass transfer coefficient, oxygen uptake and transfer rates, yields and productivity should be calculated; these tools enables a robust investigation of different conditions. In this study, the literature on the product human erythropoietin, the host microorganism *Pichia pastoris*, medium design and bioreactor operation conditions are reviewed in addition to bioprocess characteristics.

2.1 Product: Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone that is synthesized in kidneys during adult life and in liver during fetal life (Jacobson et al., 1957; Zanjani et al., 1977). Its function in human body is to stimulate red blood cell formation in bone marrows, and also to initiate hemoglobin production. In case of hypoxia or hyperoxia EPO production is accelerated or reduced respectively, to regulate red blood cell formation (Krantz, 1991). It is used for therapeutic purposes for treatment of anemia caused by renal failure, cancer, etc. (Jelkman, 1992). In 1997, Miyake et al. purified EPO from human urine and showed that there are two forms of EPO, namely EPO α and EPO β, having the same activity *in vivo*.

Using recombinant DNA technology; human EPO cDNA was successfully expressed first in *E. coli* in 1984 (Lee-Huang, 1984). EPO was also produced extracellulary, using a secretion signal with different microorganisms like *S. cerevisiae* or *B. brevis* (Elliott et al., 1989; Sasaki et al., 1997). Prior to these studies human EPO gene was isolated and expressed in Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells (Lin et al., 1985; Jacobs et al., 1985). EPO produced in these studies, using mammalian cell lines, were biologically active both *in-vivo* and *in-vitro*. Furthermore recombinant human erythropoietin (rHuEPO), which was approved by U.S. Food and Drug Administration (FDA), is therapeutically used worldwide. Recently rHuEPO was successfully expressed in *Pichia pastoris* extracellularly (Çelik et al., 2007). Also to be able to purify rHuEPO easily a polyhistidine tag was fused to the amino terminal end of the protein.

2.1.1 Biological and Genetic Structure of EPO

Human urinary EPO was analyzed and primary structure of it was determined to have 165 amino acids having a molecular weight about 18 kDa (Lai et al., 1986). Human EPO gene, having a single copy in a 5.4 kb region, is located on chromosome 7 q11-q12 and includes five exons and four introns for 193 amino acids (Law et al., 1986; Jacobs et al., 1985; Lai et al,. 1986; Lin et al., 1985). The first 27 amino acids from the amino-terminal is cleaved prior to secretion of the hormone leaving 166 amino acids, one of which -carboxy terminal arginine- is also cut off by an intracellular carboxypeptidase (Lai et al., 1986; Recny et al., 1987).

The molecular weight studies showed that EPO has a molecular weight of 34-38.5 kDa, 40 % of which consists of carbohydrates including N-acetylglucoseamine, mannose and sialic acid (Dordal et al., 1985). Three *N*-glycosylation sites for carbohydrate moieties were reported at asparagines 24, 38, 83 and one *O*-glycosylation site at serine 126 (Lai et al., 1986; Goldwasser et al., 1990; Egrie et al., 1986). Human EPO also has two disulfide bond formations between cysteines 7-161 and 29-33 (Figure 2.1), these bonds are needed to attain the tertiary structure (Figure 2.2) and so the biological activity (Wang et al., 1985).

Carbohydrates have important role in attaining biological activity of erythropoietin. It was proved that *N*-glycosylation is essential to have *in vivo* activity while *O*-glycosylation is not known to have such effect (Delorme et al., 1992). These sugar chains also enable the stabilization of EPO by covering up the hydrophobic regions and correct folding (Narhi et al., 1991). Furthermore the form of the carbohydrate not only ensures the biological activity, but also prevents clearance from circulation by avoiding the detection and removal by galactose binding receptors of hepatocytes (Fukuda et al., 1989; Elliott et al., 2004).

2.2 Host Microorganism: *Pichia pastoris*

Since the techniques for DNA manipulation was discovered, various host microorganisms were used for recombinant protein production. In 1970s an interest for methanol usage as sole carbon source has arisen, and four different genera namely *Hansenula, Candida, Turolopsis* and *Pichia,* were found to have the ability of methanol utilization *(*Hazeu et al., 1987); amongst, a specific strain, *Pichia pastoris* gained more attention and citation since it was found to be a useful system for expression of proteins both in research studies and industrial applications. Being a eukaryote it can perform post translational modifications, which is an ability that bacteria lack. Also *P. pastoris* needs less time and effort with higher yields when compared to animal cell lines.

The first growth media and protocols for growing *P. pastoris* at high cell densities were developed by Phillips Petroleum Company in 1970s. Since that time, *P. pastoris* was used for expression of more than 500 recombinant proteins (Cregg, 1999).

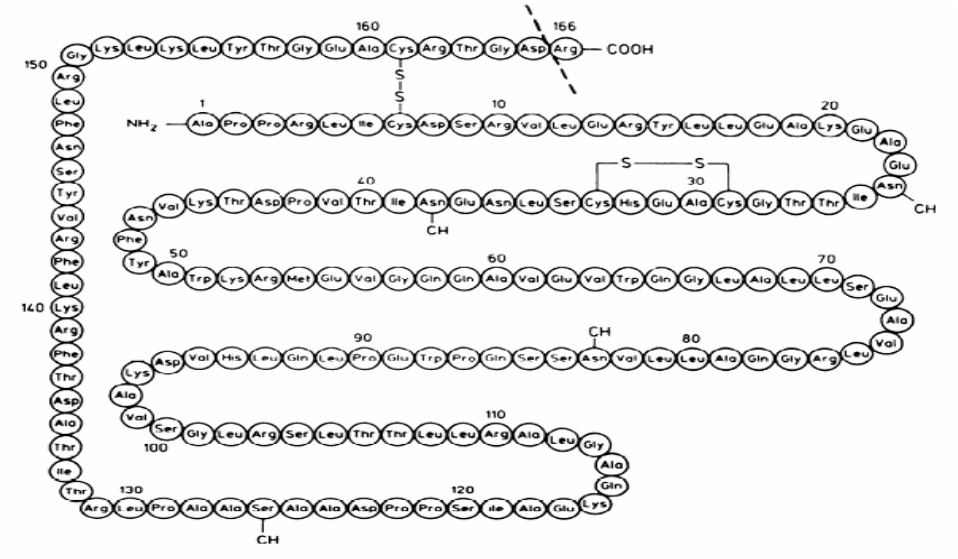

Figure 2.1 Primary structure of rHuEPO (Jelkmann, 1992)

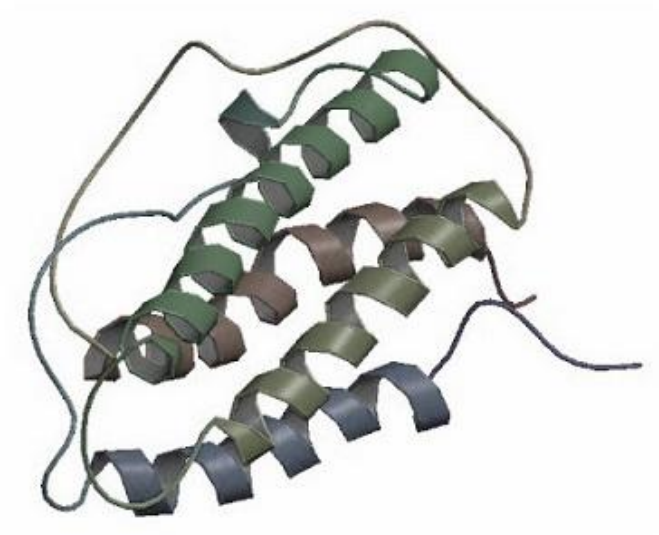

Figure 2.2 Tertiary structure of rHuEPO. (http://www.rcsb.org/pdb/explore/explore.do?structureId=1BUY, Last accessed: September 2008).

2.2.1 General Characteristics

Pichia pastoris is classified under the kingdom *Fungi*, Division *Eumycota*, Subdivision *Ascomycotina*, Class *Hemoascomycetes*, Order *Endomycetales*, Family *Saccharomycetaceae* and Genus *Pichia*. It is a mesophilic and methylotrophic yeast and like other yeast species it is a unicellular fungi having oval cells 1-5 µm wide by 5-30 µm long with a typical eukaryotic cell structure and thick polysaccharide cell wall. Besides *P. pastoris* is a facultative anaerobe and reproduce sexually (Cregg, 1999).

The advantage of using *P. pastoris* as an expression system stems from being a eukaryote and the simplicity of being unicellular. Some advantages and disadvantages of *P. pastoris* have been reported (Cregg, 1999; Daly and Hearn, 2005; Macauley-Patrick et al., 2005). First advantage is that one can obtain high product yield and productivity using a simple chemically defined medium with an inexpensive formulation. Being a eukaryote it can perform many post-translational modifications like disulfide-bond formation and glycosylation. Although sugar chains still differ from than that of human, affecting biological activity, hyper-glycosylation is not as much as it is in *S. cerevisiae*. By-products like ethanol or acetate caused by fermentative mode of growth, creates problems in *S. cerevisiae* fermentations. Another advantage over *S. cerevisiae* is that *P. pastoris* prefers respiratory mode of growth rather than fermentative and therefore by-product formation is decreased. Yet another and may be the most important advantage of *P. pastoris* is Alcohol Oxidase (AOX1) promoter of it. It is a very strong promoter and enables initiating foreign protein production just by induction of methanol. However there are some disadvantages of using methanol; monitoring during the process is difficult for AOX induction, dangerous since having fire hazard risk and might be unsuitable for use in food industry being a petrochemical. But methanol is easily accessible making it an advantageous chemical to use. Production of extracellular proteins using a proper secretion signal can be considered another advantage of *P. pastoris.* Besides being non-pathogenic *P. pastoris* have no endotoxin problem and has a broad growing pH being between 3-7.

2.2.2 Glycerol, Methanol and Sorbitol Mechanisms

Although *P. pastoris* is a methylotrophic yeast, in standard growth procedures at first glycerol is preferred as a carbon source since growth on glycerol has higher biomass yield and specific maximum growth than those of methanol. The reason for not using glycerol for protein production steps is that it represses protein expression. However, glycerol is still preferable over glucose, because when glucose is used as carbon source; high amounts of byproduct, ethanol, formation is observed (Macauley-Patrick et al., 2005). It was proved that ethanol represses AOX promoter even at low concentrations; 10-50 mg L^{-1} (Inan and Meagher, 2001-a).

The utilization pathway of glycerol (Figure 2.3) begins with phosphorylation of glycerol into glycerol-3-phosphate (G3P) by a glycerol kinase. G3P is further oxidized by a FAD-dependant glycerol-3-phosphate dehydrogenase, which is located on the outer surface of the mitochondrial inner membrane. As a result of this reaction dihydroxyacetone phosphate, which then enters to glycolytic pathway, is formed (Nevoigt and Stahl, 1997). The product of glycolysis, pyruvate, is then oxidized to acetyl-CoA via pyruvate dehydrogenase. Later acetyl-CoA enters tricarboxylic (TCA) cycle which yields many metabolites to be used in the synthesis of cellular components such as nucleic acids, amino acids and also cell wall constituents. Moreover energy need for cell growth and maintenance is obtained from TCA cycle in the form of ATP and NADH. Also a small portion of biomass is assumed to come from G3P (Ren et al., 2003).

Methylotrophic yeasts have some essential enzymes and a specialized organelle for utilization of methanol. Methanol utilization pathway (Figure 2.4) begins with oxidation of methanol to hydrogen peroxide and formaldehyde (Form) and this first step is catalyzed by a specific enzyme alcohol oxidase (AOX). When cells are grown on methanol, AOX can account for up to 30% of the total cell proteins. While the enzyme is induced by methanol; glucose, glycerol and ethanol represses the enzyme production (Walker, 1998). AOX is isolated in a specialized organelles, *peroxisomes*, to avoid toxicity of hydrogen peroxide produced in the reaction catalyzed by it. Catalase that degrades hydrogen peroxide to oxygen and H_2O, is also sequestered in *peroxisome*

(Creghino and Cregg, 2000). This organelle can occupy 80% of the cell volume when cells are grown on methanol (Veenhuis et al., 1983; Sreekrishna and Kropp, 1996).

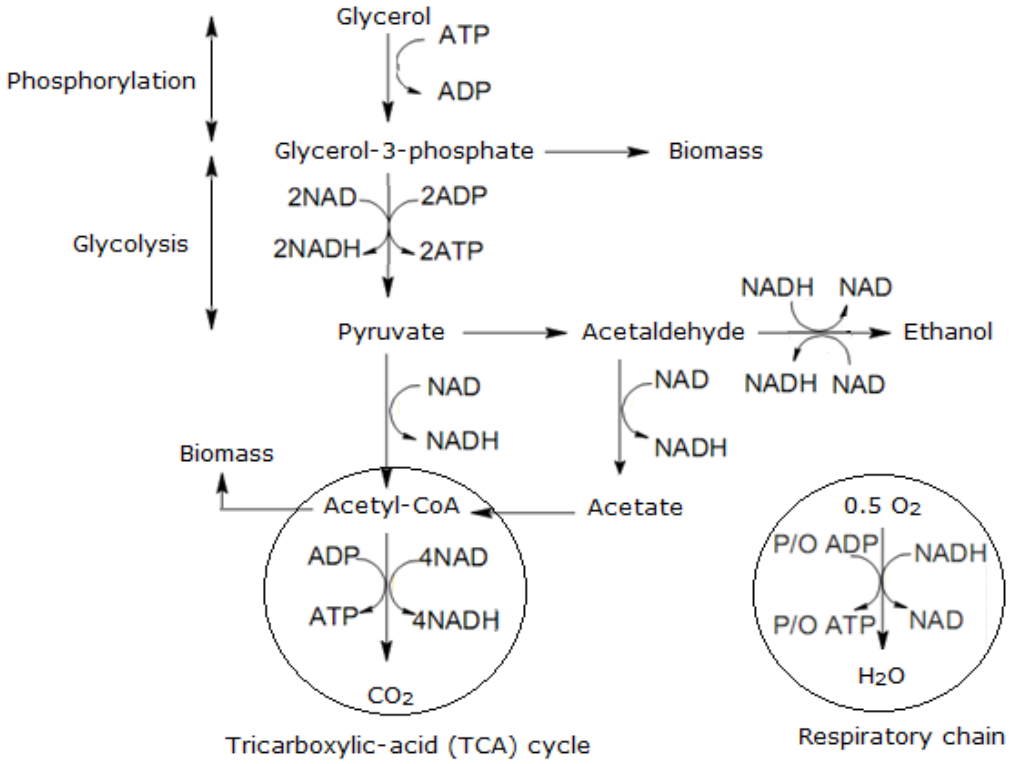

Figure 2.3 Metabolic pathways of glycerol in *Pichia pastoris* (Ren et al, 2003)

Formaldehyde yielded from methanol via AOX can enter either in dissimilatory or assimilatory pathways. A portion of formaldehyde leaves the peroxisome to enter dissimilatory pathway. Here Form is oxidized to formate by formaldehyde dehydrogenase (FLD) and formate is further oxidized by formate dehydrogenase (FDH) to carbon dioxide. These two reactions

generate energy in the form of NADH and enable detoxification of formaldehyde in methylotrophic yeasts (Lee et al., 2002). The remaining formaldehyde is assimilated by reactions in cytosol. In the *peroxisome* condensation of formate with xylulose-5-phosphate is catalyzed by the third and the last peroxisomal enzyme dihydroxyacetone synthase (DHAS). This reaction yields dihydroxyacetone (DHA) and glyceraldehyde-3-phosptahe (GAP), and these C_3 compounds leave the peroxisome afterwards. GAP enters the TCA cycle and yields further energy in the form of ATP and NADH. DHA enters xylulose monophosphate cycle yielding xylulose-5-phosphate and biomass.

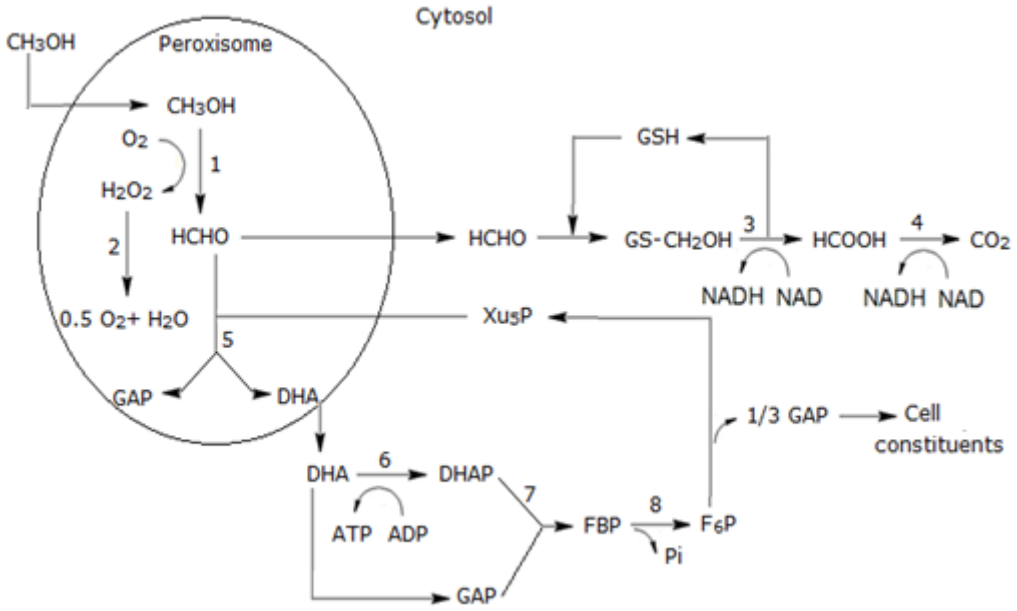

Figure 2.4 Metabolic pathway of methanol in *Pichia pastoris*. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, di-hydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase (Cereghino and Cregg, 2000)

As mentioned above although glycerol and glucose enables achieving high cell growth rates, they repress recombinant protein production by repressing the AOX promoter. Alternative carbon sources, by which the promoter is not repressed and meanwhile high cell growth rates are achieved, was investigated. Sorbitol, which has been the most abundantly used alternative carbon source, was a strong candidate for this purpose. In sorbitol utilization pathway (Figure 2.5), sorbitol enters mannitol cycle after being oxidized to fructose by sorbitol dehydrogenase. Via fructokinase, fructose is phosphorylated and the produced fructose-6-phosphate enters glycolysis (Walker, 1998).

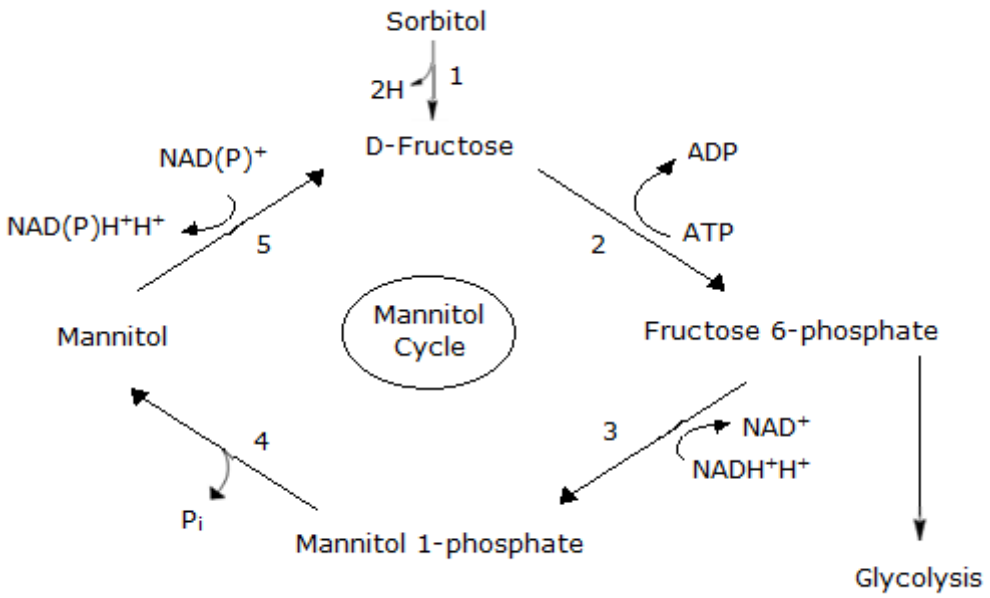

Figure 2.5 Metabolic pathway of sorbitol and mannitol in yeasts. 1, D-glucitol dehydrogenase; 2, fructokinase; 3, mannitol-phosphate dehydrogenase; 4, phosphatase; 5, mannitol dehydrogenase (Walker, 1998)

2.2.3 Expression System of *Pichia pastoris*

Expression of recombinant proteins in *Pichia pastoris* depends on the fundamental enzyme that is needed for methanol utilization. This enzyme, alcohol oxidase, enables a strong, tightly regulated and methanol induced expression system with its promoter, AOX1 (Cereghino et al., 2002; Jahic et al., 2006). Expression of this gene depends on presence of methanol however the promoter is highly repressed in cells grown on glucose or glycerol. Therefore recombinant protein production in *P. pastoris* designs are based on this fact, and it has been the most successful and widely used system reported for *P. pastoris* (Cereghino et al., 2001).

The *AOX1* gene from *Pichia pastoris* was isolated and characterized (Ellis et al., 1985). Besides *AOX1* gene, *P. pastoris* has a second alcohol oxidase gene; *AOX2* (Koutz et al., 1989). Hence the AOX1 deficient strains grow slowly on methanol, it is thought that AOX1 is responsible for about 90% of the enzyme activity (Cregg et al., 1989; Cos et al., 2006). Since the product of the reaction catalyzed by AOX is toxic to the cell this enzyme is isolated in a specialized organelle, peroxisome. This organelle might occupy up to 80% of the cell volume, and AOX can account up to 30% of the total cell proteins, when cells are grown on methanol (Walker, 1998).

Some advantages and disadvantages of AOX1 promoter was reported (Macauley-Patrick et al., 2005). The transcription is easily induced just by addition of methanol. Production of recombinant protein is tightly regulated and controlled by a repression/derepression mechanism. Repression of the promoter by carbon sources other than methanol, becomes an advantage in the production of foreign proteins that are toxic to the cell enables high level production of these proteins (Cereghino et al., 2002). Using a carbon source other than methanol before the expression of recombinant protein, ensures a good cell growth without overexpressing the gene product. However this situation creates a need for usage of two different carbon sources with switching from one to another with a precise timing. Another disadvantage of using AOX1 promoter is using methanol. Methanol is not only a petrochemical that might be unsuitable for usage in production of food products or additives, but it also has fire hazard risk so that storage of large quantities is undesired.

Lastly methanol monitoring during the process is difficult. On-line and off-line monitoring is possible; but off-line monitoring methods are complicated and on-line methods are unreliable.

To avoid methanol usage different promoters were developed for *Pichia pastoris*. Glyceraldehyde-3-phosphate dehydrogenase (GAP) is a constitutive promoter which has not been widely used since constitutive production of recombinant proteins might have cytotoxic effects (Waterham et al., 1997; Menedez et al., 2004). Other alternative promoters are formaldehyde dehydrogenase (FLD1) (Shen et al., 1998), isocitrate lyase (ICL1) (Menedez et al., 2003), 3-phosphoglycerate kinase (PGK1) (de Almeida et al., 2005), YPT1 and PEX8 (Cereghino and Cregg, 2000).

2.2.4 Methanol Utilization Phenotypes of *Pichia pastoris*

There are three different phenotypes of *P. pastoris* host strains according to methanol utilization ability (Stratton et al., 1998). The first phenotype, which is methanol utilization plus (Mut^+), can grow at wild type rate on methanol. In this phenotype both *AOX1* and *AOX2* genes are functional. The second phenotype, which has a disruption in *AOX1* gene, is methanol utilization slow (Mut^S) phenotype. The cell growth and methanol utilization is slower than those of Mut^+ strain since the cells have to rely on the weaker *AOX2* gene for methanol metabolism. For production of some recombinant proteins like hepatitis B surface antigen, Mut^S strains were found to be advantageous over Mut^+ strain. The third and the last phenotype is methanol utilization minus (Mut^-). This phenotype has disruption in both *AOX1* and *AOX2* genes and therefore cannot grow on methanol which is necessary for induction of recombinant protein production. Low growth rate of Mut^- phenotype is sometimes preferable for production of some proteins (Cregg et al., 1987). The reported maximum specific growth rates (μ) on methanol are; 0.14 h^{-1}, 0.04 h^{-1} and 0.0 h^{-1} for Mut^+, Mut^S and Mut^- *Pichia pastoris* strains respectively (Jungo et al., 2006; Stratton et al., 1998).

2.2.5 Post Translational Modifications and Secretion of Proteins

Being a eukaryote *Pichia pastoris* is able to perform many post-translational modifications. This property makes it advantageous over bacterial expression systems. The post-translational modifications that are performed in higher eukaryotes are *O*- and *N*-linked glycosylation, correct folding, disulphide bond formation, processing of secretion signals and certain types of lipid addition (Macauley-Patrick et al., 2005; Cereghino and Cregg, 2000).

Foreign proteins can be expressed either extracellularly or intracellularly in *P. pastoris*. By addition of a proper secretion signals to the protein of interest, it can be exported out of the cell. Producing the recombinant protein enables easier purification i.e. eliminates the extraction of the protein from cell (Cereghino et al., 2002; Macauley-Patrick et al., 2005). Foreign proteins, that are normally secreted by their native hosts, are preferred to be produced extracellularly because of the protein stability and folding requirements. There are two secretion signals that are used widely in *P. pastoris*, namely *S. cerevisiae* α-factor prepro peptide and *P. pastoris* acid phoshphatase (*PHO1*) signal. Although there are several other secretion signal sequences can be used, *S. cerevisiae* α-factor prepro peptide has been the most widely and successfully used signal peptide. It sonsists of 19-amino acid signal (pre) sequence that are followed by a 66-residue (pro) sequence containing three consensus *N*-linked glycosylation sites and a dibasic Kex2 endopeptidase processing site (Kurjan and Herskowitz, 1982). This sequence is processed in three steps; removal of the pre signal by signal peptidase in the endoplasmic reticulum, cleavage between Arg-Lys of the pro leader sequence by Kex2 endopeptidase, and lastly cleavage of Glu-Ala repeats by the Ste13 protein (Cereghino and Cregg, 2000). When the most frequently used secretion signals, *S. cerevisiae* α-factor prepro peptide or *PHO1*, does not work; other signal sequences should be examined, and to find the proper secretion signal trial and error experiments should be conducted for a specific protein (Macauley-Patrick et al., 2005).

Not only secretion but also folding and disulphide bond formation of foreign protein is an important step in terms of productivity in *P. pastoris*; and

in some cases it is defined as the rate-limiting step. (Hohenblum et al., 2004). Disulphide bonds, which are formed in endoplasmic reticulum after the formation of secondary structure, are essential for the mature protein to attain its active form (Hlodan et al., 1994; Holst et al., 1996). *P. pastoris* is able to produce highly disulphide bonded proteins successfully unlike prokaryotic systems in which proteins are sometimes misfolded due to reducing environment of the cytoplasm (White et al., 1994).

The other higher-eukaryotic post-translational modification that *P. pastoris* is able to perform is *O*- and *N*-linked glycosylation. Attachment of carbohydrate moieties is important for mammalian native proteins to attain biological activity. In this context, yeasts are a good model for glycoprotein production (Macauley-Patrick et al., 2005).

There are three types of *N*-linked glycans; high mannose, complex which includes several different sugars and hybrid which is the combination of hybrid and high mannose types. The glycosyl chains are attached to the protein in Endoplasmic Reticulum and then transferred to the Golgi for further processing of the protein and the glycans (Figure 2.6). Glycosylation patterns in Golgi apparatus differ in human and yeasts, and also among yeast species. Yeasts do not trim farther than $Man_8GlcNAc_2$ and do not add different type sugars like sialic acid unlike mammalian cells (Gemmill and Trimble, 1999). Yeasts are known to employ only high mannose type *N*-linked glycan addition. However unlike *S. cerevisiae*, *P. pastoris* does not hyper mannosylate the recombinant protein, i.e. the protein has a shorter glycosylation chains which makes *P. pastoris* an attractive host for recombinant glycoprotein production (Bretthauer et al., 1999; Macauley-Patrick et al., 2005). The reason why *P. pastoris* has shorter glycosylation chains is that it lacks terminal α-1,3-linked mannosylation, while *S. cerevisiae* do not, Therefore *N*-linked glycans of *S. cerevisiae,* include 50-150 mannose residues in length (Cereghino and Cregg, 2000; Bretthauer et al., 1999).

Presence of sialic acids in the oligosaccharide chains ensures the biological activity. Since *P. pastoris* glycosylate proteins with high-mannose type *N*-glycans, half-life of the protein produced is reduced and the therapeutic function is conceded.

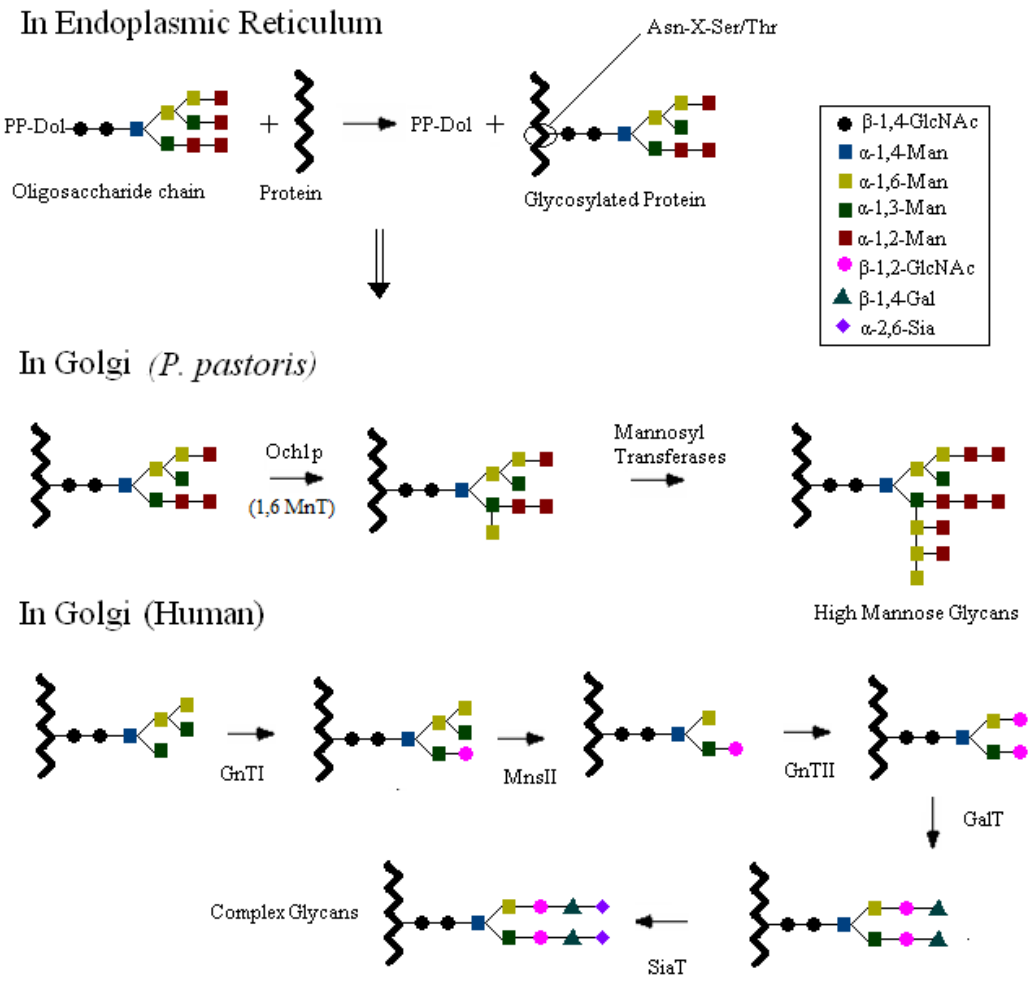

Figure 2.6 Yeast and mammalian N-linked glycosylation pathway. Och1p (1,6 MnT):α-1,6-mannosyltransferase, GnTI: β-1,2-*N*-acetylglucoaminyltransferase I, MnsII: mannosidase II, GnTII: β-1,2-*N*-acetylglucoaminyltransferase II, GalT: β-1,4-galactosyltransferase, SiaT: α-2,6-sialyltransferase (Adapted from Hamilton et al., 2006)

To obtain a similar *N*-glycan pattern humanizing the yeast glycosylation pathway was attempted by several researchers (Callewaert et al., 2001; Choi et al., 2003; Bobrowicz et al., 2004). Glycosylation of the protein that takes place in Endoplasmic Reticulum is identical in human and yeasts. Although the glycan structure of the protein, transferred from the Endoplasmic reticulum to Golgi, is identical; because of the difference in further trimming and extension processes in human and yeast, the final form of these glycans differ from each other (Hamilton and Gerngross, 2007). Therefore *N*-glycan engineering studies are focused on this part to obtain hybrid type *N*-glycans in *P. pastoris*; by deleting *OCH1* gene, increasing the expression of α-1,2-mannosidase, and expression and localization of *N*-acetylglucosaminyl-transferase (GnTI) I and β-1,4-galactosyltransferase (GalT) (Callewaert et al., 2001; Choi et al., 2003; Vervecken et al., 2004;Hamilton et al., 2006).

There is a little information about *O*-linked glycans concerning the mechanism of addition and specificity, in the literature. Eukaryotes attach *O*-linked oligosaccharide onto the hydroxyl group of serine or threonine. However the sugars included, varies between higher and lower eukaryotes. Higher eukaryotic (like mammalian) *O*-linked oligosaccharides consist of different sugars like galactose and sialic acid while lower eukaryotes like *P. pastoris* have *O*-linked glycans including only mannose residues. Furthermore it is known that *P. pastoris* sometimes may not glycosylate the same serine and threonine residues as it is in the native host of the recombinant protein. (Cereghino and Cregg et al., 2000).

2.2.6 Proteolytic Activity in *Pichia pastoris*

Yeasts have different vacuolar proteases like serine, metallo and aspartyl proteases causing degradation of the recombinant protein produced (van Den Hazel et al., 1996; Gimenez 2000). Proteolytic degradation of the recombinant protein is an important problem also in *Pichia pastoris* fermentations. The proteolytic activity might be caused by extracellular proteases, cell-bound proteases and intracellular proteases from dead cells (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005). The extracellular

proteases are not well studied in *P. pastoris* and yet their existence remains as a possibility, however induction of secretion of vacuolar proteases are thought to be increased in case of overexpression (Kobayashi et al., 2000-b; Stevens et al., 1986). Furthermore, it is reported that the proteases secreted form dead cells also contribute to the degradation of the recombinant protein extracellularly as well as intracellular proteolytic activity (Jahic et al., 2003-a; Hong et al., 2002). Proteolysis introduces some problems during and after production. For instance, since the product is degraded the yield decreases and/or biological activity might be lost. Moreover having similar characteristics with the target protein, the degradation products might interfere in downstream processes (Macauley-Patrick et al., 2005).

Different factors may affect and accelerate cell viability and proteolytic activity during fermentations. Hansen et al. (1977) showed that protease amount increases 2-3 fold if the yeasts grow on poor nitrogen. To overcome nitrogen limitation problem, addition of casamino acids or peptone was applied and was successful to reduce proteolytic activity (Clare et al., 1991; Brankamp et al., 1995; Goodrick et al., 2001; Ohya et al., 2002). However casamino acid addition of was not found to be an ideal option, being animal originated. Also when scale up and downstream processes were taken into consideration; peptone, having a high cost, also becomes a unideal candidate for preventing proteolytic degradation (Goodrick et al., 2001; Jahic et al., 2006). When casamino acids were not successful decreasing proteolysis, L-arginine and ammonium sulphate is added to the medium which enabled inhibition of proteolytic activity (Tsujikawa et al., 1996). Kobayashi et al. (2000-b) revealed that ammonium ion amount has an important effect on protease activity. When ammonia concentration was below 0.3 mg/L, protease activity and recombinant protein degradation were observed.

Nevertheless in some cases nitrogen limitation is not the factor affecting proteolytic activity and medium pH was also shown to be an important factor (Sinha et al., 2004). Buffering the medium pH at 6.0 was found to be optimal to prevent proteolysis in some studies (Clare et al., 1991; Brankamp et al., 1995; Sreekrishna et al., 1997). Consistent with these studies; with an increase in medium pH from 5.0 to 6.0, Sinha et al. (2004) observed a significant decrease in proteolytic activity. Shi et al. (2003) and

Kobayashi et al. (2000-b) also showed that as the at higher medium pH, proteases activity decreases resulting in increased protein expression. On the contrary in some studies, minimization of proteolytic degradation of the product was achieved by maintaining low pH when methanol is induced since alkaline and neutral proteases are generally responsible for damage to recombinant proteins in the culture broth (Scorer et al., 1993; Werten et al., 1999).

Since as the amount of viable cells decreases, proteolytic activity increases; cultivation time might affect proteolysis (Daly and Hearn, 2005). Longer cultivation times cause increase in protease amount in time and it is reported that replacing the media with fresh media is an effective solution to this problem (Ogunjimi et al., 1999; Daly and Hearn, 2005; Jahic et al., 2006). It is also known that since oxidation of methanol by alcohol oxidase yields formaldehyde and hydrogen peroxide; accumulation of these molecules causes *P. pastoris* cells to die when methanol is fed as a sole carbon source. Therefore proteolytic activity might increase due to proteases secreted from dead cells (Shi et al., 2003).

Not only feeding methanol as the only carbon source, but also elevated temperatures lead to cell lysis and death causing high protease activity in fermentation medium (Inan et al., 1999). *Pichia pastoris* has an optimum temperature of 30^{O}C for growth, and temperatures higher than 32^{O}C leads to cell death (Wegner et al., 1983; Invitrogen, 2002). Low temperature and temperature limited fed-batch operations were shown to yield less proteolytic activity and higher recombinant protein concentrations were achieved (Hong et al., 2002; Jahic et al., 2003-b). The low temperature operation improves protein folding and cell viability. Furthermore it enables stable cell membrane thus the protease amount released form dead cells is decreased (Li et al., 2001).

Other than changing temperature or pH, and addition of a nitrogen source; usage of protease deficient strains is an alternative method to prevent proteolytic degradation of the recombinant protein. Three different strains, namely SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*) and SMD1168 (*his4 pep4*), having disruption in genes encoding protease A (*pep4*) and/or

protease B (*prb1*) were investigated. However these strains have lower viability and show slow growth; they are recommended to be used when other methods to prevent proteolysis fail (Sreekrishna et al., 1997; Cereghino and Cregg, 2000).

2.3 Medium Design and Bioreactor Operation Parameters

An industrial microorganism can be thought as a micro-bioreactor, functioning by interacting with its micro-environment in the bioreactor (Çalık et al., 2003). After the identification of the product and construction of the strain, the next step in development of a fermentation process is to choose the correct fermentation medium and the process conditions that are optimum (Nielsen et al., 2003). Furthermore The metabolic reactions are strongly related to bioreactor operation parameters which also serves as a fine-tuning agent for bioreactor for increasing the yield (Çalık et al., 1999). In *P. pastoris* fermentations most frequently investigated bioreactor operation parameters are pH, temperature and oxygen.

2.3.1 Medium Design

Cells remove the desirable compounds from their extracellular environment and converting these nutrients, they produce other compounds (Shuler and Kargı, 2002). Therefore selection of fermentation medium becomes an important aspect for the development of the processes. There are some criteria that should be fulfilled by the fermentation medium so that the nutritional requirements of cells are met for optimization of cell growth and product formation. According to Nielsen et al., (2003), a fermentation medium should easily available throughout the year and include;

- Carbon, nitrogen and energy sources
- All essential minerals that are necessary for cell growth
- To obtain rapid growth and high product yield, all necessary growth factors

as well as causing minimum problems with;

- The downstream processing
- Some other factors affecting the fermentation process, such as mass transfer.

According to their required amounts in the medium, nutrients are classified into two groups. The first group, namely macro-nutrients, are the ones that are needed in concentrations larger than 10^{-4} M. The fundamental macro-nutrients are; carbon, nitrogen, oxygen, hydrogen, sulphur, phosphorus, Mg^{2+}, K^{+}. The latter, micro-nutrients, are needed in concentrations lower than 10^{-4} M. Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Na^{2+}, and vitamins are the nutrients that are needed in trace amounts (Shuler and Kargı, 2002).

The growth media can be classified into three major types; defined, semi-defined and complex medium. Specific amounts of pure chemical compounds, having known chemical compositions, are included in defined media; while complex media contain natural compounds whose compositions are not known exactly. Complex media might contain; yeast extract, peptones, molasses or corn steep liquor. These components enable higher cell yields when compared to defined medium, by supplying hormones and vitamins. Although complex media are less expensive and enable high cell yields; both seasonal and storage caused changes in the composition makes it disadvantageous. Furthermore defined media has other advantages like more reproducible results and better control of fermentation as well as easy recovery and purification of the target protein (Shuler and Kargı, 2002; Nielsen et al., 2003). Since the use of yeast extract or peptones may lead to variations between operations, such complex components are desired to be eliminated from the medium.

Basal Salt Medium (BSM) is the most common fermentation medium, used for high cell density fermentations of *P. pastoris* (Macauley-Patrick et al., 2005). Also addition of vitamins and trace elements was found to be useful to achieve high product formation (Boze et al., 2001). BSM along with trace salt medium (PTM1) is considered to be a standard medium, however it may not be optimum and reveal some problems such as unbalanced composition, precipitates and ionic strength (Cereghino et al., 2002; Cos et al., 2006).

Alternative media like FM22 were proposed and formulated in a few study (d'Anjou et al., 2000; Stratton et al., 1998; Zhang et al., 2007). Also researchers have made some modifications to overcome the problems coming from BSM (Brady et al., 2001; Thope et al., 1999; Jungo et al., 2006; Zhao et al., 2008).

In *P. pastoris* fermentations the second important nutrient, is the nitrogen source. For both media, BSM or FM22, this component is supplied by addition of ammonium hydroxide which also serves as a tool for adjusting the pH (Cos et al., 2006). Increase of ammonia concentration in the medium causes longer lag phase and so inhibits cell growth and product formation, while nitrogen starvation increases proteolysis (Kobayashi et al., 2000-b; Xie et al., 2003; Yang et al., 2004). Addition of ammonia as nitrogen source not only overcomes these problems but also enables pH adjustment.

The carbon source plays the most crucial role in bioprocesses, both for cell growth and recombinant protein production. Several different carbon sources; methanol, glycerol, sorbitol, glucose, mannitol, glucose ,trehalose, lactic acid, ethanol and acetate, have been used for *P. pastoris* fermentations (Brierly et al., 1990; Sreekrishna et al., 1997; Thorpe et al., 1999; Inan and Meagher, 2001-a, 2001-b; Xie et al., 2005). Methanol is the irrevocable carbon source since it is necessary for induction of recombinant protein production and besides it can be used as sole carbon source. Certain levels of methanol was found to be growth inhibitory (Zhang et al., 2000; Kobayashi et al., 2000-a). Therefore fed-batch mode of methanol feed is preferred. The most commonly applied fed-batch fermentation protocol consists of three stages. It begins with a batch in which a repressive carbon source is used to obtain high levels of biomass rapidly before the production. Since the maximum specific growth rate of wild type *P. pastoris* growing on glycerol is higher (0.18 h^{-1}) than many other carbon sources, glycerol batch (GB) operation is preferred in the first step. After the glycerol is totally consumed, second stage in which glycerol is fed to the system as sole carbon source at growth-limited levels, is started for further biomass increase (Cos et al., 2006). Exponential feeding in the glycerol fed-batch (GFB) stage has been applied in some studies (Zhang et al., 2000). GFB phase can be considered as a transition phase which enables derepression of the enzymes in methanol

utilization pathway and also adaptation of cells to growth on methanol (Chiruvolu et al., 1997). Also a 3-6 hour transition step can be conducted after GFB to shorten the time required for cells to adapt methanol consumption (Zhang et al., 2000; Çelik et al., 2009). Once the desired biomass level is obtained the last stage is started by methanol induction in fed-batch mode and recombinant protein production begins.

The carbon source feeding strategy becomes an important aspect, to obtain high cell densities and productivities, as well as shorter induction times. Several different strategies have been developed by researchers for this purpose. A typical feeding strategy is using an additional carbon source with methanol (Files et al., 2001). Egli et al. (1982), proved that mixed feeds of glucose and methanol enables higher productivities with methylotrophic yeasts. Mixed feeds of glycerol and methanol were the first approach applied by several researchers (Brierley et al., 1990). Later following this strategy, some other groups reported higher cell densities when compared to sole methanol feeding (Cregg et al., 1993; Loewen et al., 1997; McGrew et al., 1997; Katakura et al., 1998; Zhang et al., 2003; Jungo et al., 2007-a).

Although glycerol/methanol mixed feeding enables high cell densities, lower specific productivities are obtained since glycerol AOX1 promoter (Sreekrishna et al., 1997; Xie et al., 2005). Therefore a non-repressing carbon sources for AOX1 promoter have been searched. Sorbitol was proven to be a non-repressing carbon source. Mixed feed of sorbitol and methanol was first used in cultivation of Mut^S phenotype of *P. pastoris* (Thorpe et al., 1999; Boze et al., 2001; Inan and Meagher, 2001; Xie et al., 2005). Although using methanol/sorbitol mixed feeding, lower cell yield on substrate are obtained when compared to that of methanol/glycerol mixed feed; the specific product formation rate appears to be higher. This result can be explained by residual sorbitol in the medium being non-repressive for the AOX1 promoter (Thorpe et al., 1999). Jungo et al. (2007-b) studied effect of sorbitol/methanol co-feeding on production of recombinant avidin with *P. pastoris* Mut^+ strain at constant specific growth rate. Productivity of recombinant protein was increased using this co-feeding strategy. Furthermore this strategy revealed other advantages such as lower heat production rate and oxygen consumption rate when compared to

methanol/glycerol mixed feeding. Also sorbitol accumulation was found to be ineffective on recombinant protein production. Recently Çelik et al. (2009) investigated effect of sorbitol on growth of *P. pastoris* Mut^+ phenotype expressing recombinant erythropoietin and productivity. Consistent with Jungo et al. (2007-b) residual sorbitol was proven to be non-inhibitory and 50 g L^{-1} sorbitol to be optimum for both recombinant protein production and cell growth. Consequently instead of methanol/sorbitol mixed feeding, addition of sorbitol batch wise was proven to be applicable (Çelik et al., 2009).

Since methanol inhibits cell growth at certain levels, the feeding strategy becomes important. Methanol-limited feeding by exponential increase, enables controlling the specific growth rate, which is kept below maximum (μ_{max}), at desired values. This exponential feeding strategy allows better growth and production (Zhang et al., 2000). Moreover studies revealed that specific growth rate is an important parameter that affects cell growth rate and recombinant protein production (Kobayashi et al., 2000-a; Cunha et al., 2004; Zhang et al., 2005). Çelik et al. (2009) studied effect of specific growth rate, using a predetermined methanol exponential feeding, on recombinant erythropoietin production. Among the investigated specific growth rate values, $\mu=0.03h^{-1}$ was found to be optimum for obtaining the highest recombinant protein concentration, while the highest specific yield was obtained at $\mu=0.02h^{-1}$.

2.3.2 pH

Since pH affects the microbial growth rate, the activity of enzymes, transport mechanisms and other extracellular and intracellular events, hydrogen ion concentration (pH) is considered as one of the most important bioreactor operation parameters. Microbial cells are able to maintain a constant intracellular pH even if the extracellular pH shows large fluctuations. However this effort costs an increase in the maintenance energy demand, because more Gibbs free energy is being used to be able to maintain the proton gradient across the cell membrane (Nielsen and Villadsen, 1994).

Pichia pastoris is able to grow at a wide range of pH (3.0-7.0). Since pH values between 3.5 to 5.5, have almost no effect on cell growth, this pH range provides freedom in adjusting the pH (Wegner et al., 1983; Inan et al., 1999). The operation pH should be optimized in order to reduce proteolytic activity and to obtain higher recombinant protein production and stability (Sreekrishna et al., 1997; Macauley-Patrick et al., 2005).

The optimum pH for growth of *P. pastoris* Mut^+ strain was shown to be 5.0. Also if the fermentation was conducted without pH control, pH of the medium decreases, and the growth lasts until the pH is 2.5. No cell growth was observed at pH 2.2 (Chiruvolu et al., 1998). Therefore to keep the pH constant addition of a base is needed and the most frequently used source is ammonium hydroxide that also acts as a nitrogen source (Cos et al., 2006). Although there is an optimum pH for growth, in literature different pH values were found to be optimum for different recombinant proteins produced by *P. pastoris*; since it changes due to the nature and stability of the recombinant protein. For example for some recombinant proteins low pH values were found to be optimum. pH 3.0 was the optimum value for production of insulin-like growth factor-I, cytokine growth-blocking peptide and Fv antibody fragment (Brierly et al., 1994; Koganesawa et al., 2002; Damasceno et al., 2004). The recombinant protein degradation was decreased sharply at pH 3.0 and no production was observed at pH 6.0 (Damasceno et al., 2004). Zhang et al. (2009) preferred to use potassium hydroxide to maintain a constant pH, and achieved high-level production of enterokinase at pH 4.0. For the production of mouse epidermal factor (Clare et al., 1991) and α-amylase (Choi and Park , 2006) optimum pH was determined as pH 6.0; while for mini-proinsulin it is 6.3 (Pais-Chanfrau et al., 2004). Two studies have revealed unconventionally high optimum pH values for recombinant protein production in *P. pastoris*. Although the cell growth was very similar to each other at every pH value examined, highest recombinant production was achieved at pH 6.8, and no product was obtained at pH values lower than 5.0 (Wang et al., 2005). The highest optimum pH value obtained for *P. pastoris,* was in production of human μ-opioid receptor (Sarramegna et al., 2002). The study revealed that operations at pH values 7.0, 8.0, 9.0 and 10.0 the production was higher when compared to lower pH values.

Bioreactor operation pH effects product formation also by influencing the protease activity. Different results were obtained regarding the effect of pH on proteolysis; in some studies, increasing the pH reduces proteolytic activity while in others it does not. Recombinant human serum albumin (rHSA) was highly degraded at pH 4.3 because of proteolytic activity, however by increasing the pH to pH 5.6, degradation of the product was prevented. Also at pH 4.3 rHSA was degraded at a rate of 660 mg rHSA L^{-1} h^{-1} (Kobayashi et al., 2000-b). Sinha et al. (2004) also observed increased proteolytic activity at lower pH values, and recombinant protein degradation was decreased at pH 6.0. Lastly a fusion protein was cleaved the linker between the proteins at pH 5.0. On the other hand when the pH was decreased to 4.0 the fraction of the full length fusion protein was increased from 40 to 90%, as well as lipase activity which is a constituent of the fusion protein (Jahic et al., 2003-a). Laborde et al. (2004) also showed that proteolysis decreases at pH 3.2 and the recombinant protein yield increases, achieving the maximum. Production of recombinant erythropoietin using *P. pastoris* in bioreactor was conducted at pH 5.0 (Çelik et al., 2009). In literature, there is no study related with optimization of pH for production of rHuEPO by *P. pastoris*. Also there is no study that investigates the effects of pH on bioprocess characteristics. In this context; this study is focused on effects of pH on production of rHuEPO and other bioprocess characteristics.

2.3.3 Temperature

Microorganisms are not able to control their intracellular temperature. Therefore, cell temperature is always equal to environmental temperature and all of the biochemical reactions, that take place in the cells, depend directly on the external temperature. In addition to the biochemical reactions, also metabolic regulation, nutritional requirements, biomass composition and product formation will be affected by temperature. Thus, temperature is an important bioreactor operation parameter whose optimum value can differ for growth and recombinant protein production. Differentiation of temperature from the optimum value causes increase in maintenance energy (Nielsen and Villadsen, 1994).

Since cell growth is the overall result of a series of enzymatic reactions, temperature affects the maximum specific growth rate in a similar manner that it affects the activity of an enzyme. Increase is observed with increasing temperature until the protein degradation begins, and after this point a rapid decrease is obtained. Maximum specific growth rate can be explained by Arrhenius equation, since it increases much the same way as a normal chemical rate constant; but only at temperatures below the point where degradation starts (Nielsen et al., 2003).

Pichia pastoris has an optimum temperature of 30^{O}C for growth (Wegner et al., 1983). Temperatures above 32^{O} C was determined to be harmful for protein formation and also may lead to cell death (Invitrogen, 2002). Inan et al. (1999) showed that temperatures above 30^{O}C was not appropriate for recombinant protein production, since higher temperatures cause cell lysis and increase in proteolytic activity in fermentation medium.

Due to high proteolysis possibility at higher temperatures, operation at lower temperatures were conducted, and decreasing the cultivation temperature was found to be useful to increase final recombinant protein production (Hong et al., 2002; Jahic et al., 2003-a; Pais-Chanfrau et al., 2004). At low temperatures cell are more viable when compared with the same cell concentrations obtained at higher temperatures. This increased viability might be due to the cell membrane being more stable at low temperatures; leading to reduced amounts of proteases released from the dead cells and reduced proteolytic degradation of the recombinant protein (Li et al., 2001). Also in E. coli cultivations it is observed that lower temperature enables better protein folding and improves the solubility of proteins (Georgiou and Valax, 1996). Furthermore at low temperatures the ability of the cells to synthesize proteins is reduced and so more time for folding of the proteins properly is provided (Li et al.,2001). The lowest operation temperature examined for *P. pastoris* was 15^{O}C. It was proved that *P. pastoris* can be cultivated even at this temperature and due to reduced protease amounts recombinant protein production was improved at 15^{O}C. The study also revealed that, when cultivated at 15^{O}C cells preserve their ability to accumulate recombinant protein. However this ability was decreased rapidly when cultivation is conducted at 30^{O}C (Shi et al., 2003).

However it should be noted that decreasing the temperature below 30^{O}C might not improve the recombinant protein expression by *P. pastoris*, in some cases (Inan et al., 1999; Kupcsulik and Sevella, 2005). In this study temperature was kept at 30^{O}C, which is optimum for *P. pastoris* growth, during the fermentation.

2.3.4 Oxygen Transfer Rate

Aerobic fermentation processes are affected by oxygen that influence metabolic pathways and changes metabolic fluxes (Çalık et al., 1999 and 2000). Gaseous oxygen is supplied to the medium either by spraying air or surface aeration. Also oxygen transfer rate can be kept at desired levels by adjusting the air inlet rate, agitation rate and/or oxygen supplementation (Lee et al, 2003; Çelik, 2008). Oxygen requirement of the cells are determined by not only the metabolic pathway characteristics of the microorganism, but also the carbon source and other nutrition used.

P. pastoris is an obligately aerobic organism and prefers respiratory mode of growth instead of fermentative, thus byproducts like ethanol and acetate is not produced (Cereghino et al., 2002). This property is advantageous though high oxygen transfer rates are needed when it grows on methanol since methanol metabolism utilizes oxygen at high rate (Cereghino and Cregg, 2000). Molecular oxygen is used for both the respiration and formation of formaldehyde by oxidation of methanol. Thus oxygen limitation might have detrimental effects on cell growth (Couderc et al., 1998). However in some studies oxygen limited fed-batch (OLFB) process is applied and when compared to methanol limited fed-batch operations increased growth and recombinant protein production were observed (Trentmann et al., 2004; Charoenrat et al., 2005). Oxygen limited fed-batch operations require higher maintenance than that of methanol-limited fed-batch processes (Charoenrat et al., 2006). Thus to improve production of the recombinant protein higher methanol concentrations should be present in the medium when compared to oxygen-sufficient operations (Khatri and Hoffmann, 2006). In literature, predominantly, keeping the dissolved oxygen level above 20-30% was preferred in *P. pastoris* fermentations during the methanol induction phase

(Jahic et al., 2006). In a more recent study Çelik (2008) employed above 20% dissolved oxygen level, and observed that this value was sufficient for *P. pastoris* utilizing both sorbitol and methanol. Dissolved oxygen level was kept above 20% in this study as well.

2.4 Computation of Bioprocess Characteristics

2.4.1 Yield Coefficients and Specific Rates

For further and better evaluation of a bioprocess certain tools are being used. These tools, which are stoichiometrically related properties, are yield coefficients. A yield coefficient can be defined as in equation 2.1. In this equation P and S are product and substrate respectively. ΔP is the mass or moles of P produced, ΔS is the mass or moles of S consumed and $Y_{P/S}$ represents the overall yield coefficient.

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \tag{2.1}$$

This definition provides an average value for the whole process. On the other hand during the batch or fed-batch processes, the yield coefficients might vary during the operation since variations in growth rate and metabolic functions are obrserved. Thus, evaluation of the instantaneous yield at a certain time of the process becomes necessary. Instantaneous yield can be calculated as below:

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

Other yield coefficients that presenting the efficiency of conversions between substrate and oxygen or product or biomass are listed in Table 2.1.

Table 2.1 Definition of yield coefficients.

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

Cellular growth can be considered as an autocatalytic event in which cells synthesize more cells (Nielsen et., 2003). When cells are introduced to cultivation, a characteristic path is followed for cell growth, called cell growth cycle. In the first phase, namely lag phase, cells get used to their environment, and only a slight growth is observed. Later in an accelartion phase growth rate increases until a point where the maximum value is ttained in growth phase. In the next phase which is stationary phase, not net growth is obtained until the nutrients are totally consumed and finally cell death starts (Atkinson and Mavituna, 1991; Shuler and Kargı, 2002).

Specific growth rate (μ) is a demonstration of microbial cell growth and also an important process variable (Jungo et al., 2007-b; Çelik et al., 2009). Fo the fed-batch operations it is derived from the general mass balance for biomass, presented below:

$$r_X V = \frac{d(C_X V)}{dt} \tag{2.3}$$

In equation 2.3, the main assumption is that cells are batch-wise and not lost trough sampling. Also another assumption is that density of the medium remains constant throughout the process.

The biomass formation rate (r_X) is the product of specific cell growth rate (μ) and cell concentration (C_X), as formulated below:

$$r_X = \mu C_X \tag{2.4}$$

Inserting equation 2.4 into equation 2.3 yields:

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

Volume changes due to the substrate, which is methanol in this case, feeding in fed-batch operations during the process. This variation of the volume can be derived from the general mass balance, with the assumption of constant density for simplicity.

$$\frac{dV}{dt} = Q \tag{2.6}$$

Inserting the equation 2.6 into equation 2.5 yields:

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

To obtain a definition for specific growth rate (μ), equation 2.7 is rearranged:

$$\mu = \frac{dC_X}{dt}\frac{1}{C_X} + \frac{Q}{V} \tag{2.8}$$

In this study methanol, which is the substrate introduced to the system in a fed-batch manner, was added with an exponential volumetric flow rate of Q to the sytem of a reaction volume of V. Thus the mass balance for methanol can be written as below:

$$QC_{M0} - 0 + r_M V = \frac{d(C_M V)}{dt} \tag{2.9}$$

Also as is it in equation 2.4, the substrate consumption rate (r_M) can be written as the product of specific substrate consumption rate (q_M) and cell concentration (C_X):

$$r_M = q_M C_X \tag{2.10}$$

Inserting the equation 2.10 into the equation 2.9 yields:

$$QC_{M0} + q_M C_X V = V\frac{dC_M}{dt} + C_M\frac{dV}{dt} \tag{2.11}$$

There is no substrate accumulation since nutrient consumption rate is almost equal to its feed rate. This is a property of fed-batch systems which are assumed to be operating at quasi-steady state (Shuler and Kargı, 2002). Therefore the last term in equation 2.11 can be neglected:

$$C_M\frac{dV}{dt} \sim 0 \tag{2.12}$$

The final form of the equation 2.11, dividing by V, is:

$$\frac{dC_M}{dt} = \frac{Q}{V}C_{M0} + q_M C_X \tag{2.13}$$

To obtain a definition for specific methanol consumption rate (q_M), equation 2.13 is rearranged:

$$q_M = \frac{Q}{V}\frac{C_{M0}}{C_X} - \frac{1}{C_X}\frac{dC_M}{dt} \tag{2.14}$$

The second substrate of this study, sorbitol, is added to the system batch-wise and C_{S0}=0. Therefore mass balance for sorbitol can be written as:

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

The substrate consumption rate (r_S) can be written as the product of the specific substrate consumption rate (q_S) and cell concentration (C_X):

$$r_S = q_S C_X \tag{2.16}$$

Inserting the equation 2.16 into the equation 2.15 and dividing by V yields:

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

To obtain a definition for the specific sorbitol consumption rate (q_S), equation 2.17 is rearranged:

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

Finally, using the same approach that is used for sorbitol, a definition for the specific recombinant protein production rate (q_{rp}) can be obtained:

$$q_{rp} = \left(\frac{dC_{rp}}{dt} + \frac{Q}{V}C_{rp}\right)\frac{1}{C_X} \tag{2.19}$$

Consequently, using the experimental data the specific rates; μ, q_M, q_S and q_{rp} can be determined by the equations 2.8, 2.14, 2.18 and 2.19 respectively.

2.4.2 Oxygen Transfer Characteristics

In fermentations of *P. pastoris* oxygen is not only needed for respiration but also for methanol metabolism, thus oxygen requirement is much more higher than that of a regular yeast (Cereghino and Cregg, 2000). In this context oxygen transfer characteristics becomes important to meet the needs of cells.

Several steps are involved in the oxygen transfer from the medium to the microorganism (Bailey and Ollis, 1986). If the cells in the medium are dispersed well and the fermentation broth is thoroughly mixed, the oxygen transfer resistance in the liquid film surrounding the air bubbles, attains significant importance.

Oxygen transfer rate (OTR) from gas to liquid can be defined as:

$$OTR = K_L a(C_O^* - C_O) \tag{2.20}$$

In equation 2.20, a is the gas-liquid interfacial area, C_O and C_O* are saturated dissolved oxygen concentration and actual dissolved oxygen concentration in the fermentation broth, respectively.

Solubility of oxygen in aqeous solutions is very low, therefore the dominant resistance becomes the liquid phase mass trasfer resistance. Also the overall liquid phase mass transfer coefficient, K_La, is almost equal to liquid phase mass transfer coefficient, k_La (Shuler and Kargı, 2002). Definition of the maximum possible mass trasfer rate is defined as in equation 2.21.

$$OTR = K_L a C_O^* \tag{2.21}$$

The oxygen uptake rate (OUR), $-r_O$, per unit volume of medium is defined as below:

$$OUR = -r_O = q_O C_X \tag{2.22}$$

In equation 2.22, q_O is the specific oxygen consumption rate and C_X is the cell concentration (Shuler and Kargı, 2002).

There are several different methods were developed to calculate the K_La values. Dynamic method is the most frequently used one. This method should be applied during the process and its basis is the material balance for oxygen:

$$\frac{dC_O}{dt} = OTR - OUR = K_L a(C_O^* - C_O) + r_O \tag{2.23}$$

In dynamic method, at first the air flow is stopped and the agitation speed is lowered to a minimum level to avoid surface aeration. During this process since the oxygen is being consumed by the microorganism present in the medium, dissolved oxygen concentration (C_O) drops. Thus, there is no oxygen transfer, equation 2.23 becomes as follows:

$$\frac{dC_O}{dt} = r_O \tag{2.24}$$

If C_O versus time was plotted using equation 2.24, as shown in the Figure 2.7; using the region-II, oxygen uptake rate ($-r_O$) can be calculated.

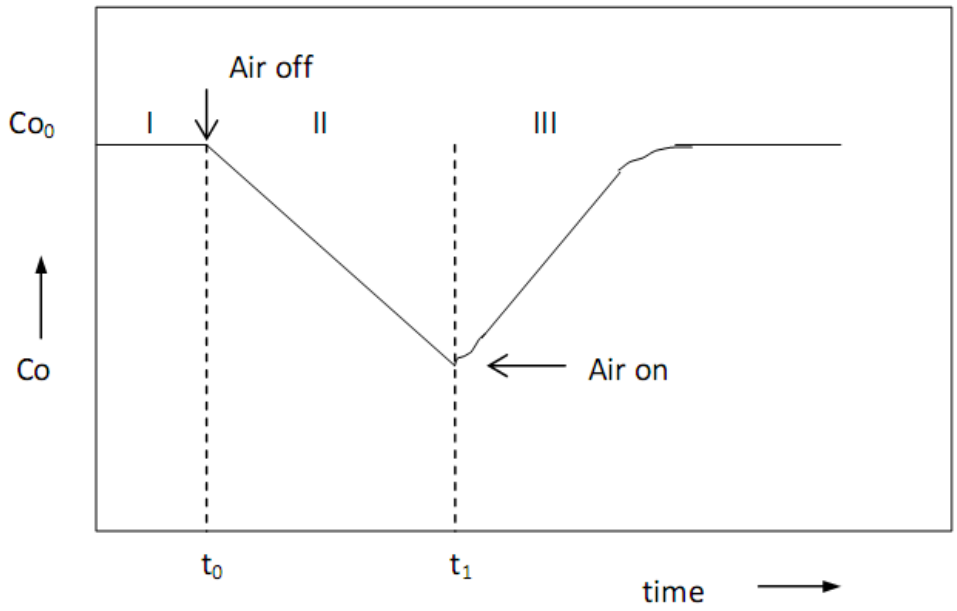

Figure 2.7 Variation of dissolved oxygen concentration with time in dynamic determination of $K_L a$.

Later dissolved oxygen concentration is increased by opening the air flow, and it is recorded versus time. The equation 2.23 is rearranged to obtain the below equation:

$$C_O = \frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_O\right) + C_O^* \qquad (2.25)$$

In equation 2.25, r_O is known and $K_L a$ can be calculated from the slope of the plot of C_O versus (dC_O/dt-r_O).

The dynamic method is also applied when there is no reaction, i.e. $r_O=0$ (Nielsen et al., 2003). To apply this method, the medium is de-oxygenated by spraying nitrogen to the reactor. Later to be able to increase the C_O, air inlet is turned on, and dissolved oxygen concentration is recorded versus time. Since $r_O=0$, the equation 2.25 can be written as below:

$$C_O = -\frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \tag{2.26}$$

If C_O versus $d(C_O V)/dt$ is plotted, the physical mass transfer coefficient, using the slope, $K_L a_0$ can be obtained.

Comparison between the relative rates of maximum oxygen transfer and biochemical reactions and finding the rate limiting step of the process is needed for further investigation of the process. For this purpose, the maximum possible oxygen utilization rate, i.e. oxygen demand (OD), is calculated as below:

$$OD = \frac{\mu_{max} C_X}{Y_{X/O}} \tag{2.27}$$

Furthermore to determine the oxygen limitation theoretically; effectiveness factor (η) which is defined as the oxygen uptake rate per maximum possible oxygen utilization rate, and Damköhler number (Da) which defined as maximum possible oxygen utilization rate per maximum mass transfer rate. As defined by Çalık et al. (2000), η and Da are formulated as below:

$$\eta = \frac{OUR}{OD} \tag{2.28}$$

$$Da = \frac{OD}{OTR_{max}} \tag{2.29}$$

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals and solutions were analytical grade unless otherwise stated, and obtained from Sigma Ltd., Difco Laboratories, Fluka Ltd., Merck Ltd. and Roche.

3.2 Buffers and Stock Solutions

All buffers and stock solutions were prepared with distilled water and they are sterilized either by autoclaving at 121^{O}C for 20 minutes or by filtering with 0.20 or 0.45 μm filters (Sartorius AG, Gottingen, Germany) After sterilization they are stored at +4^{O}C or room temperature. A list of all buffers and stock solutions used is presented in Appendix A.

3.3 The Microorganism: Strain and Storage

Pichia pastoris-E17 Mut^{+} strain carrying pPICZαA::epo plasmid, was used for rHuEPO production (Çelik et al., 2007). The Human EPO gene was fused with polyhistidine tag and a target site for the factor Xa protease recognition and the fused gene was cloned to the vector pPICZαA that carries α-factor signal peptide, *AOX1* promoter and Zeocin resistance gene. The recombinant microorganisms, that were stored in the microbanks (PRO-LAB), were first cultivated to prepare a cell stock. After the cultivation, cells are preserved in saline glycerol solution at -55^{O}C.

3.4 Growth Medium

3.4.1 Solid Medium

Recombinant *P. pastoris* strains from cell stock, were cultivated in solid medium. The cells are inoculated on YPD agar, containing 0.1 g L^{-1} Zeocin, the selective antibiotic. The composition of YPD agar is listed in Table 3.1. After an incubation of 36-48 hour at 30^{O}C, the agars containing *P. pastoris* colonies were stored at +4^{O}C.

Table 3.1 The composition of YPD agar.

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

3.4.2 Stock Preparation and Precultivation Medium

After incubation of 36-48 hours a single colony from YPG agar was transferred into a YPG medium. At the 24th hour of the cultivation the medium was collected and centrifuged at 1500 g for 4 minutes. The cell pellets obtained from 100 mL of YPG medium were suspended in a saline glycerol solution, and divided into 1.5 mL Eppendorff tubes. The tubes were preserved at -55^{O}C for storage. Table 3.2 and Table 3.3 list the composition of YPG medium and saline glycerol solution. A certain amount of saline glycerol stock was directly inoculated into BMGY which is the precultivation medium with the composition listed in Table 3.4. The selective antibiotics, zeocin or chloramphenicol, were added to the mediums after sterilization.

Table 3.2 The composition of YPG, cultivation medium for stock preparation.

Compound	Concentration, g L^{-1}
Yeast extract	6
Peptone	5
Glycerol	20

Table 3.3 The composition of saline glycerol solution (Schenk et al., 2007).

Compound	Concentration, g L^{-1}
NaCl	9
Glycerol	20

Table 3.4 The composition of BMGY, precultivation medium.

Compound	Composition, g L^{-1}
Yeast extract	10
Peptone	20
Potassium phosphate buffer pH=6.0,	0.1 M
YNB	13.4
Ammonium sulphate	10
Biotin	$4x10^{-5}$
Glycerol	10
Chloramphenicol	1 mL L^{-1}

3.4.3 Production Medium

After inoculation in precultivation medium, cells were harvested by centrifugation and the cells pellets of recombinant *P. pastoris* were immediately transferred into production medium. This medium, which is a defined medium reported in a previous study (Jungo et al., 2006), contains sorbitol together with methanol, basal salts solution and nitrogen was used for air filtered shake bioreactors. However some modifications were done in this medium. Instead of ammonium chloride, ammonium sulfate was used, also a different trace salt solution (PTM1) was added to the medium after sterilization, the composition of the medium is listed in Table 3.5. For production of rHuEPO in pilot scale bioreactor Basal Salt Medium (BSM), which is the most commonly used medium *for P. pastoris* fermentations, was used. After the sterilization of BSM having the listed composition (Table 3.6), 0.1 % antifoam, PTM1 having the listed composition (Table 3.7), and 0.1 % chloramphenicol was added to the medium. 25% ammonium hydroxide (NH_3OH) solution was used to adjust and control the pH constant at the desired value.

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

Compound	**Composition, g L^{-1}**
Methanol	1 mL
Sorbitol	30
Ammonium sulphate	4.35
Potassium phosphate buffer pH=6.0	0.1 M
$MgSO_4.7H_2O$	14.9
$CaSO_4.2H_2O$	1.17
Chloraphenicol	1 mL
PTM1	4.35 mL

Table 3.6 The composition of Basal Salt Medium (BSM) (Sibirny et al., 1987).

Compound	Composition, g L^{-1}
85 % H_3PO_4	26.7 mL
$CaSO_4.2H_2O$	1.17
$MgSO_4.7H_2O$	14.9
KOH	4.13
K_2SO_4	18.2
Glycerol	40.0

Table 3.7 The composition of the trace salt solution (PTM1) (Sibirny et al., 1987).

Compound	Composition, g L^{-1}
$CuSO_4.5H_2O$	6
NaI	0.08
$MnSO_4.H_2O$	3
$Na_2MoO_4.\ 2H_2O$	0.2
H_3BO_3	0.02
$ZnCl_2$	20
$FeSO_4.7H_2O$	65
$CoCl_2$	0.5
H_2SO_4	5
Biotin	0.2

3.5. Recombinant Erythropoietin Production and Purification

Recombinant human erythropoietin was produced in laboratory scale air filtered bioreactors and a pilot scale bioreactor for the investigation of the optimum pH for production.

3.5.1 Precultivation

A certain amount of *P. pastoris* Mut^+ strain was transferred from the glycerol stock, directly to BMGY precultivation medium. Air filtered shake bioreactors were used in this stage. The medium was maintained at 30^O and shaken at N=225 min^{-1} in an orbital shaker which is agitation and heating rate controlled (B.Braun, Certomat BS-1). The cells were precultivated in flasks of 150 mL in size and having a working volume of 50 mL for 20-24 h, until the cells reach to OD_{600}=6-8, i.e. concentration of 1.8-2.4 g L^{-1}. Once the desired cell concentration was achieved the medium was centrifuged at 4000 rpm for 10 min and then the cell pellets were immediately resuspended in 10 mL sterile water and transferred into the bioreactor including the production medium.

3.5.2 RHuEPO Production in Laboratory Scale Air Filtered Shake Bioreactor

Baffled and air filtered Erlenmeyer flasks of 250 mL in size was used for laboratory scale air filtered shake bioreactor experiments. The cells, harvested from the precultivation medium, were transferred into the 50 mL of defined production medium having the composition listed in Table 3.5. Methanol was induced every 24 h in order to induce recombinant protein production. The process was performed as batch and continued for 50 h.

3.5.3 RHuEPO Production in the Pilot Scale Bioreactor

The production in a pilot scale bioreactor was conducted in 3 L bioreactor having a working volume of 1 L (Braun CT2-2). The bioreactor system is shown in Figure 3.1. Temperature, foam level, stirring rate, feed

inlet rate and dissolved oxygen (DO) was controlled by the reactor. pH was kept constant throughout the process and controlled manually by addition of 25 % ammonia solution. Temperature and stirring were kept constant at 30^{O}C and N=900 rpm respectively. DO concentration was maintained above 20% of air saturation by an inlet air, and when needed the inlet was supplied with pure oxygen passing from a mass flow controller.

The standard protocol was applied for the production of a recombinant protein by *P. pastoris* under the control of AOX1 promoter (Stratton et al., 1998), with some modifications (Çelik et al., 2009). This strategy includes four steps; glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and methanol fed-batch (MFB) or production phases. The cells that were harvested from precultivation were transferred and resuspended in the production medium present in the bioreactor, so that the initial OD_{600}=1, i.e. initial cell concentration being 0.3 g L^{-1}, and the glycerol batch phase was started. The purpose of this stage is to achieve high cell concentration using a AOX1 repressing carbon source enabling high specific growth rate, prior to production. GB phase lasted about 17 h, until the glycerol was totally consumed. At the end of this step OD_{600}=26-30, i.e. cell concentration was 8-9 g L^{-1}. Later to obtain further increase in biomass, GFB was started with feeding of 50% glycerol including 12 mL L^{-1} PTM1. A predetermined exponential feed rate was determined using equation 3.1, calculated for a constant specific growth rate.

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

In the above equation μ_0 is the desired specific growth rate, V_0 is the initial volume, C_{X0} is the initial cell concentration, C_{S0} is the feed substrate concentration and $Y_{X/S}$ is the cell yield on the substrate. In the GFB phase, the predetermined parameters were μ_0, $Y_{X/S}$ and C_{S0} being 0.18 h^{-1}, 0.5 and 790 g L^{-1} respectively (Çelik et al., 2009). Once OD_{600}= 70-80, i.e. cell concentration reached to 21-24 g L^{-1}, a 6 h a methanol pre-induction was conducted for cells to get used to methanol utilization.

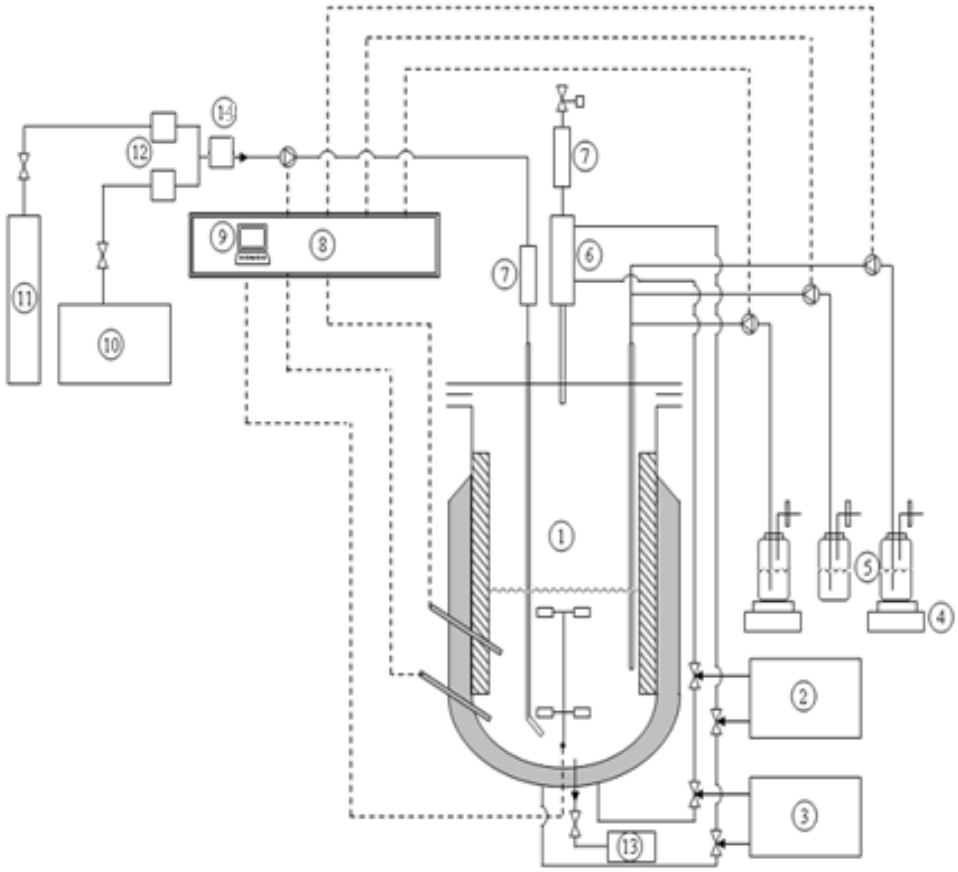

Figure 3.1 The pilot scale bioreactor system, consisting of (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2 tank (12) Digital mass flow controllers (13) Sampling bottle (14) Regulator (Çelik, 2008).

In MT a pulse feed of methanol, so that initial concentration was 1.5 g L^{-1}, was given to the medium by feeding 100 % methanol including 12 mL L^{-1} PTM1. After MT phase is completed, sorbitol was fed to the bioreactor in batch mode such that its initial concentration was 50 g L^{-1}. Then immediately rHuEPO production was induced by a fed-batch feed of methanol at limiting rates. To determine the feed rate equation 3.1 was used with predetermined parameters being; were μ_0=0.03 h^{-1} (optimum specific growth rate for rHuEPO production in *P. pastoris*), $Y_{X/S}$= 0.42 and C_{S0}= 1130 g L^{-1} (Çelik et al., 2009).

3.5.4 Ultrafiltration

At the end of the production the medium was centrifuged and the supernatant was concentrated and desalted by ultrafiltration. The process was carried out in a 400 mL stirred cells (Amicon) and 10 kDa cut-off regenerated cellulose ultrafiltration membranes (Millipore). To prevent the degeneration of the protein, ultrafiltration was performed in a cold room (2-6^{O}C), using N_2 gas pressure of maximum 55 psi (3.8 bar). Ultrafiltration lasted until at least 10 fold concentration of the medium was obtained. Also for desalting purposes dH_2O was added to the ultrafiltration cell in 4-5 fold volume of the medium.

3.5.5 Polyhistidine-Tag Affinity Purification of RHuEPO

Using its polyhistidine tag, rHuEPO was purified with cobalt-based metal affinity resins (BD Talon). 1 mL of resin was used for 10 mL of concentrated medium. A detailed procedure is represented below.

1. The BD TALON Resin was thoroughly resuspended.
2. The required amount of resin was immediately transferred into a sterile tube which will accommodate 10-20 times the resin bed volume.
3. The mixture was centrifuged at 5000 rpm for 7 min in order to pellet the resin, later it was put in ices for 2 min.
4. The supernatant was removed and discarded.

5. 10 bed volumes of 1X Equilibration/Wash Buffer was added onto the resin and mixed briefly to pre-equilibrate the resin.
6. The mixture was centrifuged at 5000 rpm for 10 min, and put in ice for 5 min.
7. Steps 5 and 6 was repeated.
8. The concentrated sample was mixed with the resin.
9. The mixture of resin and sample was gently agitated at room temperature for 20 min on a platform shaker to allow the polyhistidine-tagged protein to bind the resin.
10. The mixture was centrifuged at 5000 rpm for 10 min, and put in ice for 5 min.
11. Without disturbing the resin pellet supernatant was carefully removed as much as possible, and it was stored.
12. The resin was washed by adding 10-20 bed volumes of 1X Equilibration/Wash Buffer and gently agitating the suspension at room temperature for 10 min on a platform shaker to promote thorough washing.
13. The mixture was centrifuged at 5000 rpm for 10 min, and put in ice for 5 min.
14. The supernatant was remove and discarded.
15. Steps 12-14 was repeated.
16. One bed volume of 1X Equilibration/Wash Buffer was added onto the resin and resuspended by vortexing.
17. The mixture was transferred to a 2-ml gravity-flow column (Sartorius) with an end-cap in place, and put in ice for 10 min to allow the resin to settle out of suspension.
18. The end-cap was removed, and the buffer was allowed to drain until it reaches the top of the resin bed, making sure no air bubbles are trapped in the resin bed.
19. The column was washed once with 5 bed volumes of 1X Equilibration/Wash Buffer.
20. The column was washed once with 5 bed volumes of Pre-elution Buffer.

21. The polyhistidine-tagged protein was eluted by adding 5 bed volumes of Elution Buffer to the column, and the eluate was collected in 500 µL fractions.

Note: Under most conditions, the majority of the polyhistidine-tagged protein will be recovered in the first two bed volumes.

22. To determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein, spectrophotometric and SDS-PAGE analyses were conducted.

23. To clean the resins and remove imidazole the resin was first washed with MES Buffer and then with dH_2O. The regenerated resin was stored at 4^OC in 20% ethanol.

3.6 Analysis

During the production, samples were collected at every 3 h. After determination of cell concentration, the collected medium was centrifuged at 13200 rpm for 10 min. The supernatant and the cells obtained were stored in -55^OC for analyses.

3.6.1 Cell Concentration

Cell concentration was measured by a UV-Vis Spectrophotometer (Thermo Spectronic, Heλiosα) at 600 nm. The range to read OD_{600} was between 0.1 and 0.9. Thus, the samples taken from the sample was diluted with distilled water (dH_2O). Equation 3.2 was used to convert the absorbance to the cell concentration, C_X (g L^{-1}) (Çelik, 2008).

$$C_X = 0.3 * OD_{600} * \text{Dilution rate} \quad (3.2)$$

3.6.2 Total Protein Concentration

A spectrophotometric method, Bradford assay (Bradford, 1976), was used to determine total protein concentration . 50 µL sample was mixed with 1.5 mL Bradford reagent (BioRad). The mixture was incubated in dark and at

room temperature for 10-13 min. Later the absorbance was read at 595 nm by a UV-spectrophotometer. Also a calibration curve was obtained using bovine serum albumin (BSA) as a standard, in the concentration range of 0-1 g L^{-1} (Appendix B).

3.6.3 RhuEPO Concentration

rHuEPO concentrations were measured using a high-performance capillary electrophoresis (Capillary Electrophoresis System, Agilent). Samples were filtered using a 0.45 μm cellulose acetate filters prior to analysis. The filtered samples were analyzed at 12 kV and 15^{O}C with a positive power supply using 75 cm x 75 μm silica capillary and 50 mM borate buffer (pH=10) containing zwitter ion (Z1-Methyl reagent, Waters) as the separation buffer. The column was washed with 0.1 M NaOH for 5 min at the beginning. The purpose of using the zwitter ion is to prevent protein adsorption to the capillary column. Proteins were detected by UV absorbance at 214 nm, as mentioned elsewhere (Çalık et al., 1998). A sample electropherogram belonging to a rHuEPO standard and the calibration curve are presented in Appendix C.

3.6.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970). The sample buffer and protein samples were mixed (1:2 volume) and kept at 95^{O}C water batch for 4 min. 3 μL of a dual color prestained protein MW marker (Appendix D) and 15 μL of the sample were loaded to gel and were run simultaneously at constant current of 40 mA . The buffers used can be seen in Appendix A.

Preparation steps of SDS-PAGE gels are given below:

1. The glasses were cleaned with ethanol and they were assembled plates according to the manufacturer's instructions.

2. The resolving and stacking gels were prepared accordingly in an Erlenmeyer flask, without the addition of NNN'N'-

Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS).

3. TEMED and APS were added to the resolving gel. After 2-3 times gently mixing the solution, the gel was poured into the gel cast, as quickly as possible to prevent polymerization and avoiding bubble formation. The gel cast was filled so that sufficient place for stacking gel was left.

4. To obtain a smooth gel surface, a thin layer of isopropanol was poured onto the gel.

5. After polymerization of at least 45 minutes, isopropanol was poured out and the space between the glasses was washed with dH_2O. Excess water is shaken out and dried using filter paper.

6. TEMED and APS were added to the stacking gel. After 2-3 times gently mixing the solution, it was poured into the gel cast. The comb was immediately placed by tilting it so that its teeth were at a slight angle, to prevent bubble formation under the comb.

7. After polymerization of at least 20 minutes, the gel was wrapped into a tissue that was soaked in dH2O and stored in $+4^{O}C$ for up to two weeks.

Sample preparation and gel electrophoresis steps are listed below:

1. Sample loading buffer and samples, taken from medium supernatant, were mixed (1:2). Tthe mixture was heated at 95°C for 4 minutes, stored in ice for 5 minutes, centrifuged for a short time and vortexed.

2. In the meantime the comb between the glasses was removed and the wells were washed with dH_2O and assembled into the electrophoresis unit. The apparatus was filled with 1XSDS-PAGE running buffer up to a point where the bottom of the gels were covered.

3. 15 µL of the prepared samples and 3 µL of prestained protein MW marker were poured in the wells in a predetermined order.

4. The lid of the apparatus was closed thoroughly.

5. The gels were run simultaneously at constant current of 40 mA.

6. The gels were run for 45-50 minutes, until the lanes of the protein marker was separated visibly.

3.6.5 Staining of SDS-PAGE Gels

After running the gels, the glasses were separated from each other and the stacking gel was cut off. The gels were silver stained according to Blum et al. (1987). The procedure can be seen in Table 3.8. The solutions in Table 3.8 are given in Appendix A.

3.6.6 Methanol and Sorbitol Concentrations

Methanol and sorbitol concentrations were measured with reversed phase HPLC (Waters HPLC, Alliance 2695, Milford, MA) on Capital Optimal ODS-5µm column (Capital HPLC, West Lothian, UK) (Çelik et al., 2009). The method enables to calculate the concentrations from the chromatogram, based on the chromatogram of the standard solutions. Calibration curves for methanol and sorbitol can be seen in Appendix E. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and 100 µL of them were loaded to the analysis system. 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 specified conditions were used for analysis are listed in Table 3.9.

Table 3.8 Procedure for silver staining of SDS-PAGE gels

Step	Solution	Time of Treatment	Comments
1. Fixing	Fixer	≥ 1 h	Can be incubated overnight
2. Washing	50% Ethanol	3 x 20 min	Should be fresh
3. Pre-treatment	Pretreatment Solutior	1 min	Should be fresh
4. Rinse	Distilled Water	3 x 20 sec	Time should be exact
5.Impregnate	Silver Nitrate Solutior	20 min	
6. Rinse	Distilled Water	2 x 20 sec	Time should be exact
7. Developing	Developing Solution	~5 min	To slow down the reaction distilled
8. Wash	Distilled Water	2 x 2 min	
9. Stop	Stop Solution	≥ 10 min	In this solution gels can be kept overnight

Table 3.9 Conditions for HPLC system for methanol and sorbitol analysis

Column	Capital Optimal ODS, 5µm
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Mobile phase and flow rate	5 mM H_2SO_4, 0.5 mL min^{-1}
Column temperature	30^OC
Detector	Waters 2414 Refractive Index Detector
Detector temperature	30^OC
Detector wavelength	410 nm
Injection volume	5 µL
Analysis period	10 min

3.6.7 Amino Acids Concentrations

Amino acid concentrations were measured with an amino acid analysis system (Waters, HPLC), using the Pico Tag method (Cohen, 1983). In this method, which is based on reversed phase HPLC, using a pre-column derivation technique with a gradient program was developed for amino acids (Çalık et al., 1999). The amino acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard amino acids solution. 6.0% (v/v) acetonitrile as a mobile phase was used. The specified conditions were used for analysis are listed in Table 3.10.

3.6.8 Organic Acids Concentrations

An organic acid analysis system (Waters, HPLC, Alliance 2695) was used for organic acid concentration measurement. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and 100 µL of them were loaded to the analysis system. 3.12 % (w/v) NaH_2PO_4 and $0.62x10^{-3}$ (v/v) H_3PO_4 (Ileri and Çalık) was used as the mobile phase The specified conditions were used for

analysis are listed in Table 3.11. Calibration curves for organic acids are given in Appendix F.

Table 3.10 Conditions for HPLC system for amino acids analysis

Column	Amino acid analysis (Nova-Pak C18,
Column dimensions	3.9 mm x 30 cm
System	Reversed phase chromatography
Mobile phase and flow rate	1 mL min^{-1}
Column temperature	38^{O}C
Detector and wavelength	UV//VIS, 254 nm
Injection volume	4 µL
Analysis period	20 min

Table 3.11 Conditions for HPLC system for organic acids analysis

Column	Capital Optimal ODS, 5µm
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Mobile phase flow rate	0.8 mL min^{-1}
Column temperature	30^{O}C
Detector	Waters 2487 Dual absorbance
Detector temperature and	30^{O}C, 210 nm
Injection volume	5 µL
Analysis period	15 min

3.6.9 Protease Activity Assay

Proteolytic activity was determined by hydrolysis of casein. Three different buffer solutions were used to evaluate three different types of proteins. Hammerstein casein was dissolved in either 0.05 M borate buffer (pH 10), 0.05 M sodium acetate buffer (pH 5) or 0.05 M sodium phosphate buffer (pH 7), so that its concentration is 5 g L^{-1}. Later 2 mL of the casein solutions were mixed with 1 mL of diluted medium supernatant and hydrolyzed at 30^{O}C for 20 min. Later to cease the reaction 10% (w/v) trichloroacetic acid (TCA) was added to the mixture. Then it was stored in ice for 20 min. After centrifugation at 10500 rpm for 10 min at 4^{O}C, and afterwards waiting at room temperature for 5 min, the absorbance was measured at 275 nm in UV-Vis spectrophotometer. One unit protease activity was defined as the activity that releases 4 nmole tyrosine per minute (Moon and Parulekar, 1991). The equation 3.3 represents the calibration equation used for converting absorbance to protease activity (U cm^{-3}) (Çalık, 1998).

$$A = \left(\frac{Absorbance}{0.8x\ 1/\mu mol.cm^{-3}}\right)\left(\frac{1U}{4\ nmolmin}\right)\left(\frac{1}{20\ min}\right)\left(\frac{1000nmol}{1\mu mol}\right)\left(\frac{Dilution}{Ratio}\right) \quad (3.3)$$

3.6.10 Determination of Alcohol Oxidase Activity

3.6.10.1 Yeast Lysis to Obtain Intracellular Medium

To determine the alcohol oxidase (AOX) activity, since AOX is an intracellular enzyme, at first the intracellular medium of the cells should be extracted. Yeast lysis buffer (Appendix A) was used for this purpose. The volume of the cell samples were completed to 500 µL by yeast lysis buffer and mixed thoroughly. Then a spoon of glass beads was added to this mixture. Later by centrifugation at 3000 g for 2 min was applied, and the supernatant was again centrifuged at 12000g for 5 min to get rid of the remaining cells. After the second centrifugation the intracellular medium obtained was used for AOX assay.

3.6.10.2 AOX Activity Assay

To monitor the oxidation of methanol to formaldehyde by AOX an assay combining two enzymes, AOX and horseradish peroxidase (HRP), was used. This colorimetric system measures the concentration of H_2O_2 liberated by AOX (Çelik, 2008), whose basis is the enzymatic reaction of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP). The series of the reactions starts with AOX oxidizing methanol to form formaldehyde and H_2O_2. Later two moles of H_2O_2 reacts with one mole of PSA and one mole of 4-AAP, yielding one mole of quinoneimine dye. one mole of sodium hydrogenosulfate and three moles of water.

$$\text{Methanol} + O_2 \xrightarrow{\text{AOX}} \text{Formaldehyde} + H_2O_2$$

$$2\ H_2O_2 + \text{PSA} + \text{4-AAP} \xrightarrow{\text{HRP}} \text{Quinoneimine dye} + 3\ H_2O + NaHNO_3$$

Quinoneimine dye has a characteristic magenta color with maximum absorption around 500 nm. To determine the AOX activity the increase in absorbance at 500 nm was measured with UV-Vis spectrophotometer. The increase is proportional to the production rate of H_2O_2 and eventually the consumption rate of methanol. The assay was conducted at 25^{O}C using a standard assay reaction mixture, including 0.4mM 4-AAP, 25mM PSA, and 2 U mL^{-1} HRP in 0.1M phosphate buffer, pH 7.5. One unit of AOX activity (U) was defined as the number of µmol of H_2O_2 produced per minute at 25°C (Azevedo et al., 2004). The reaction mixture consists of; 3 mL standard assay reaction mixture, 30 µL HRP, 375 µL methanol and 75 µL sample was mixed in a cuvette. Later the increase in absorbance at 500 nm was monitored for four minutes with 30 sec intervals. The obtained absorbance was converted to AOX concentration (mg mL^{-1}) using equation 3.4 and the calibration curve is given in Appendix G.

$$C_{AOX}(U/g\ CDW) = 21.1\ \frac{U/mL}{absorbance} \times OD_{500} \times \frac{1}{C_X} \qquad (3.4)$$

3.6.11 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

A dynamic method (Bandyopadhyay and Humprey, 1967) was used to determine the liquid phase mass transfer coefficient and oxygen uptake rate in the rHuEPO production process. The method was explained in Section 2.4.2.

The physical mass transfer coefficient (K_La_0) was determined before inoculation of the microorganism to the production medium in the bioreactor. The dynamic oxygen transfer experiments were performed at certain cultivation times during production phase. To minimize the effect of low level oxygen on the microorganisms, the experiments were carried out in a short period of time.

CHAPTER 4

RESULTS AND DISCUSSION

In this study, the aim is to investigate the effects of operation pH on recombinant human erythropoietin (rHuEPO) production by *Pichia pastoris*. To obtain information as how cells respond to different operation pH values; cell growth, substrate consumption, rHuEPO production, alcohol oxidase and protease production were analyzed. Amino and organic acid profiles were obtained for better understanding of intracellular reaction networks. Furthermore bioprocess characteristics, yield coefficients and the specific rates were investigated as well as oxygen transfer characteristics to determine the effects of these properties on rHuEPO production.

4.1 Expression of Recombinant Human Erythropoietin by *P. pastoris* in Pilot Scale Bioreactors

Recently Çelik (2008) developed a recombinant *P. pastoris* strain producing rHuEPO and analyzed the effects of medium components and the specific growth rate on production. Furthermore the cell growth and rHuEPO production was investigated in air filtered laboratory-scale bioreactor experiments. Laboratory-scale air-filtered bioreactor experiments conducted by Çelik (2008) showed that for the cell growth the favored pH was 5.0-6.0 while for production it was 4.0-5.0. However it was observed that the buffering capacity of citric acid-phosphate buffer was insufficient for long-term productions. Although the pH was adjusted to different values for each flask and the pH at end of the production was about 2.2 in all flasks, which is inappropriate for cell growth (Chiruvolu et al., 1998). For more realistic analysis of optimum operation pH; a pilot-scale bioreactor having 1 L working volume; with temperature, foam, stirring rate, feed inlet rate and dissolve

oxygen control was used in this study. In this context, five different sets of fed-batch bioreactor experiments were performed to investigate the effects of operation pH on rHuEPO production.

4.2 Control of Bioreactor Operation Parameters in Pilot-Scale Bioreactor

The bioreactor operation parameters, i.e., temperature, agitation rate, dissolved oxygen was controlled by the bioreactor system as precisely as possible while pH was controlled manually to be able to keep it as constant as possible.

In this study the investigated parameter, pH, was kept constant manually throughout the process. For this purpose, 25 % ammonia was added to the medium, and pH of the medium was controlled at the desired value with ±0.1 sensitivity. The PI controller of the bioreactor system was not preferred to keep pH constant because it provides rather high changes (±0.3) in pH when compared to manual control.

The temperature was controlled by the PI controller of the bioreactor system at 30 ±0.1 ^{O}C. An antifoam solution was added to the production medium (0.1% v/v) prior to inoculation to prevent foaming. During the process negligible amounts of antifoam (maximum 1-2 mL) was added to the medium in all the experiments. Agitation rate was fixed at N=900 rpm which is a high value as high oxygen consumption rates are observed in fermentations by *P. pastoris*. Since higher agitation rates might cause not only shear damage to the cells but also increase in temperature and foaming, N=900 rpm was found to be appropriate to meet the oxygen need of *P. pastoris* (Çelik, 2008).

Control of dissolved oxygen (DO) was difficult since *P. pastoris* consumes oxygen at very high rates. It is important to keep the DO level above 20% saturation to prevent oxygen limitation. Air was used to control DO at the beginning of the fermentation, but it was not sufficient after the 12th hour of the glycerol batch phase. Therefore the air inlet was supplied with pure oxygen to be able to keep DO above 20% saturation. A mass flow rate

controller was used to adjust the oxygen amount enriching the air before feeding to the bioreactor.

4.3 Glycerol, Sorbitol and Methanol Feeding Rates in Fed-Batch Pilot Scale Bioreactor Operations

In fed-batch processes, constant specific growth rate is achieved by using predetermined exponential feeding profile. Feeding rate, F(t), in g h^{-1} is varied according to equation 4.1 (Weigand et al., 1979).

$$F(t) = \frac{\mu_0 V_0 C_{X0}}{Y_{X/S}} \exp(\mu_0 t) \quad (4.1)$$

In equation 4.1; μ_0 is the specific growth rate (h^{-1}), V_0 is the initial volume (L), C_{X0} is the initial cell concentration (g L^{-1}) and $Y_{X/S}$ is the cell yield on substrate (g g^{-1}).

In glycerol fed-batch (GFB) and methanol fed-batch (MFB) phases substrates were fed to the system according to equation 4.1. In GFB phase specific growth rate was kept at 0.18 h^{-1}, which is the maximum specific growth rate of *P. pastoris* grown on glycerol. Also the cell yield on glycerol ($Y_{X/G}$) is 0.5 g g^{-1} (Cos et al., 2005). The feeding profile changes from experiment to experiment since the initial volume and cell concentrations differ. A sample predetermined exponential glycerol feeding profile can be seen in Figure 4.1.

Before beginning the methanol fed-batch phase co-substrate, sorbitol, was added to the medium such that its initial concentration (C_{S0}) was 50 g L^{-1}. The maximum specific growth rate (μ_{max}) of *P. pastoris* on methanol is 0.14 h^{-1}, while it is 0.032 h^{-1} on sorbitol (Jungo et al., 2007). Since *P. pastoris* needs high oxygen transfer rates when grown on methanol, specific growth rate cannot be kept at the maximum level. Furthermore Çelik (2008) investigated effect of specific growth rate (μ) on rHuEPO production and found that μ=0.03 h^{-1} was optimum value to achieve high production. Therefore in this study in methanol fed-batch phase to calculate the predetermined exponential feeding

rate, μ=0.03 h^{-1} was used. A sample predetermined exponential methanol feeding profile can be seen in Figure 4.2.

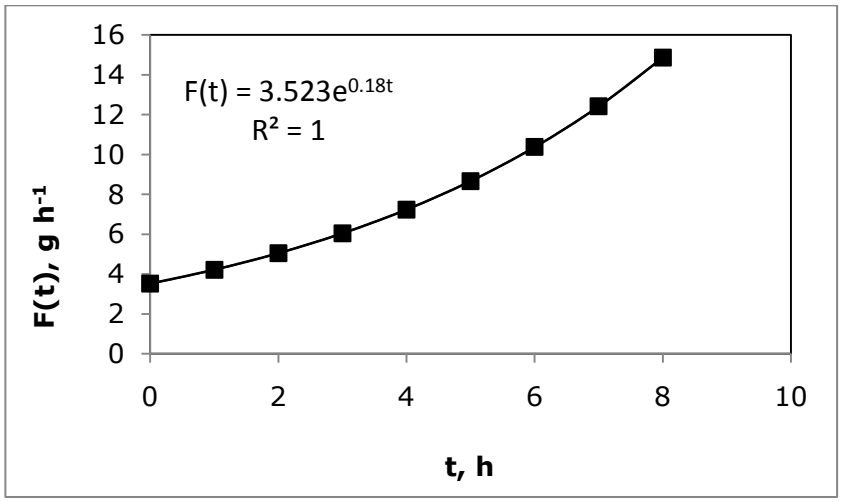

Figure 4.1 A predetermined feeding profile for glycerol fed-batch phase

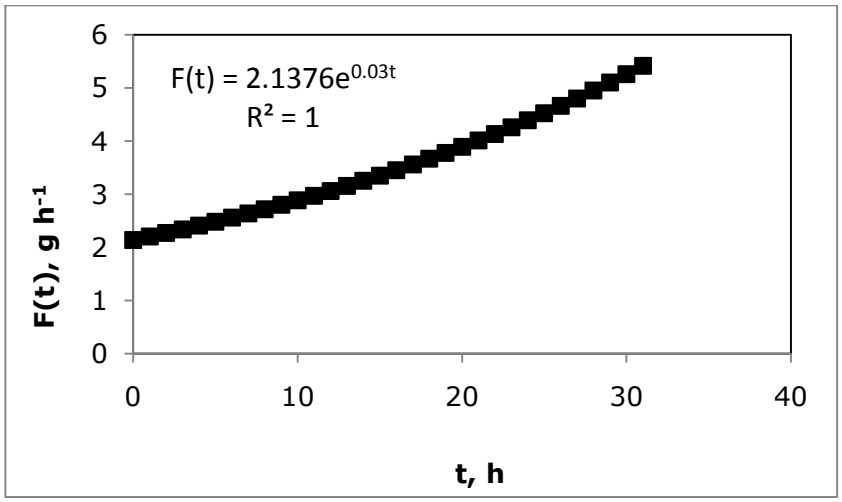

Figure 4.2 A predetermined feeding profile for methanol fed-batch phase

4.4 Effect of pH on Cell Growth

The results of the shake flask experiment conducted by Çelik (2008), showed that the cell growth and rHuEPO production was high between pH 4.0 and pH 6.0. Considering this fact five pilot scale bioreactor experiments were conducted in this study to investigate the effect of pH on rHuEPO production with pH values between 4.0 and 6.0 that changes by 0.5 increments. For the first three phases, glycerol batch (GB), glycerol fed-batch (GFB) and methanol transition (MT), the growth profiles were almost the same for pH=5.0, 5.5 and 6.0. However, in operations at pH below 5.0 the growth rate was so low that 4 hours of additional cultivation time was needed in GB phase to reach the cell concentration (C_X) obtained at higher pH values, at the end of the phase (Figure 4.3a). The purpose of the GB and GFB phases was to obtain high cell concentration in as soon as possible before production. Since the specific growth rate was low at pH values below 5.0, in these operations GB and GFB phases were conducted at pH 5.0. Later in MT phase pH was allowed to decrease until the desired value was obtained and then kept constant in the last phase which is methanol fed-batch (MFB).

The cell growth profiles in GB, GFB and MT phases (Figure 4.3b) were not changed with change in operation pH. This result also suggests that at pH=5.0, 5.5 and 6.0 there was no nitrogen limitation caused by feeding of ammonia, which is used for pH adjustment, as the only nitrogen source.

At t=30h the methanol fed-batch (MFB) was started to induce rHuEPO production. For simplicity this cultivation time was designated as t=0, that is the beginning of the production phase (Figure 4.4). In all the experiments the initial cell concentration (C_{X0}) was almost the same, ca. 28 g L^{-1}. The highest C_X was obtained at pH=4.5 as 81.4 g L^{-1} which is 10% higher than that of the second highest cell concentration obtained. The final cell concentration achieved at different pH, tended to increase with increasing pH, with a contradictory value observed at pH 4.5. The difference between the cell growth profiles can be attributed to pH since neither nitrogen nor oxygen limitation exists.

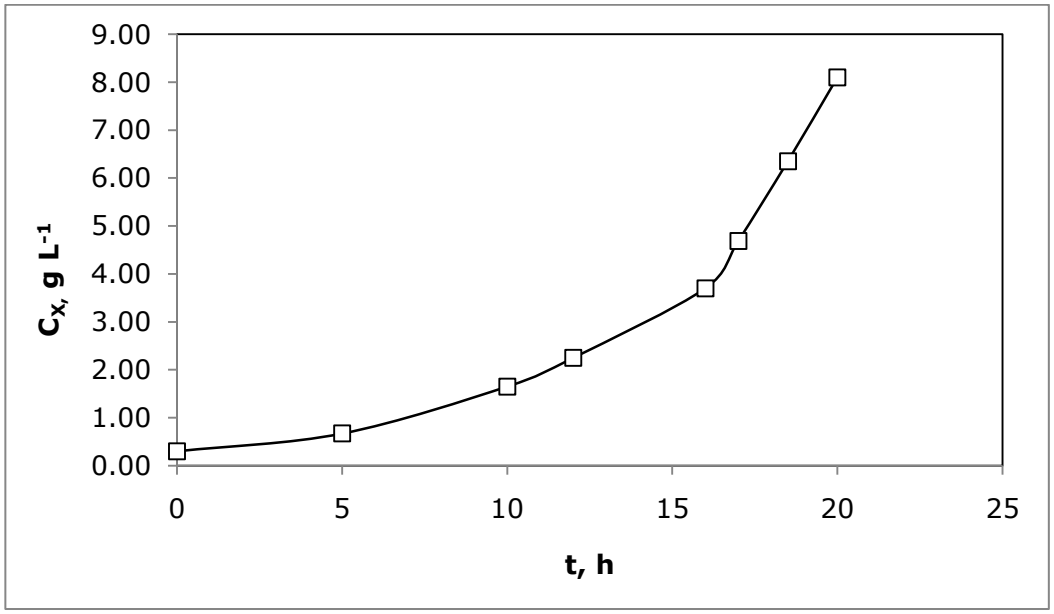

Figure 4.3a Variation in the cell concentration with the cultivation time and at pH=4.5 in glycerol batch (GB) phase.

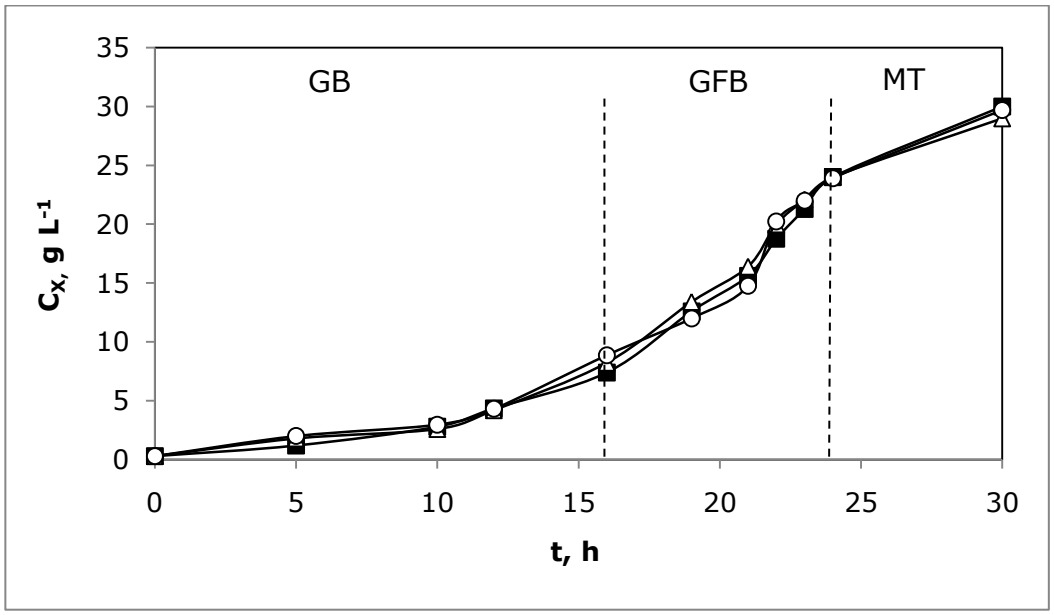

Figure 4.3b Variation in the cell concentration with the cultivation time and pH in glycerol batch (GB), glycerol fed-batch (GFB) and methanol transition (MT) phases. pH 5.0(■), pH 5.5 (Δ), pH 6.0 (○).

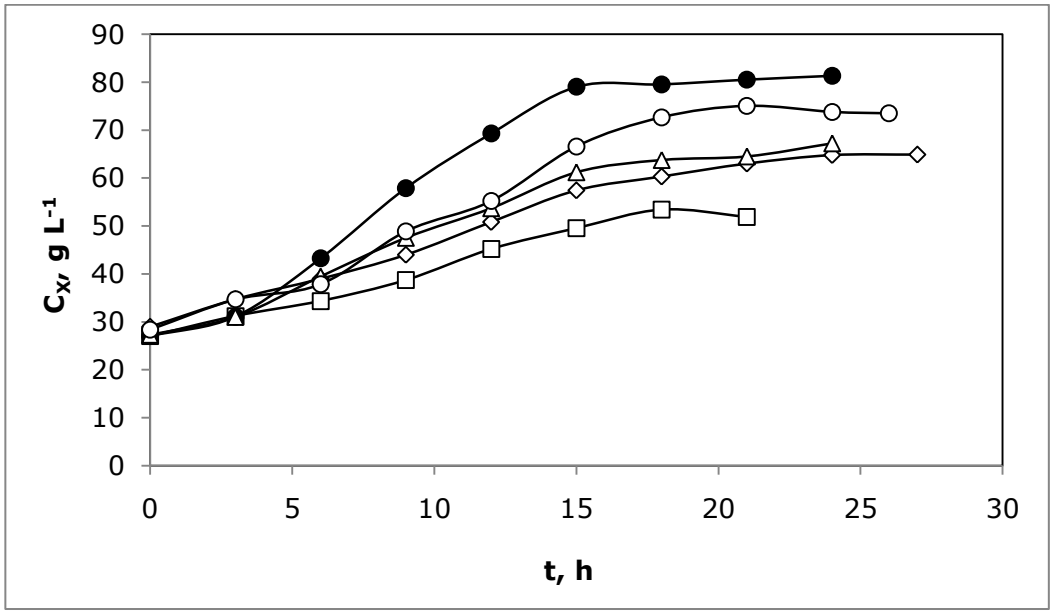

Figure 4.4 Variation in the cell concentration with the cultivation time and pH in methanol fed-batch phase. pH 4.0 (□), pH 4.5 (●) pH 5.0 (◊), pH 5.5 (Δ), pH 6.0 (○).

4.5 Effect of pH on Sorbitol and Methanol Consumption

Methanol was fed to the system in limited amounts by an exponential feeding strategy calculated using equation 4.1. Therefore, methanol was not detected in the production medium showing that methanol fed to system was immediately consumed by the cells. Both for eliminating the lag phase and enhancing the growth, sorbitol was added to the medium at the beginning of the production phase such that its initial concentration (C_{S0}) was 50 g L^{-1}, in all experiments. Sorbitol consumption began at t=0h, and the concentration decreased linearly with cultivation time (Figure 4.5). The sorbitol consumption rate was not influenced by pH at low pH values, thus it was depleted at the 15^{th} hour at pH=4.0, 4.5 and 5.0. On the other hand sorbitol consumption rate decreased with increasing pH at between 5.0 and 6.0, it was depleted at t=18 and 21 in operations at pH=5.5 and 6.0, respectively.

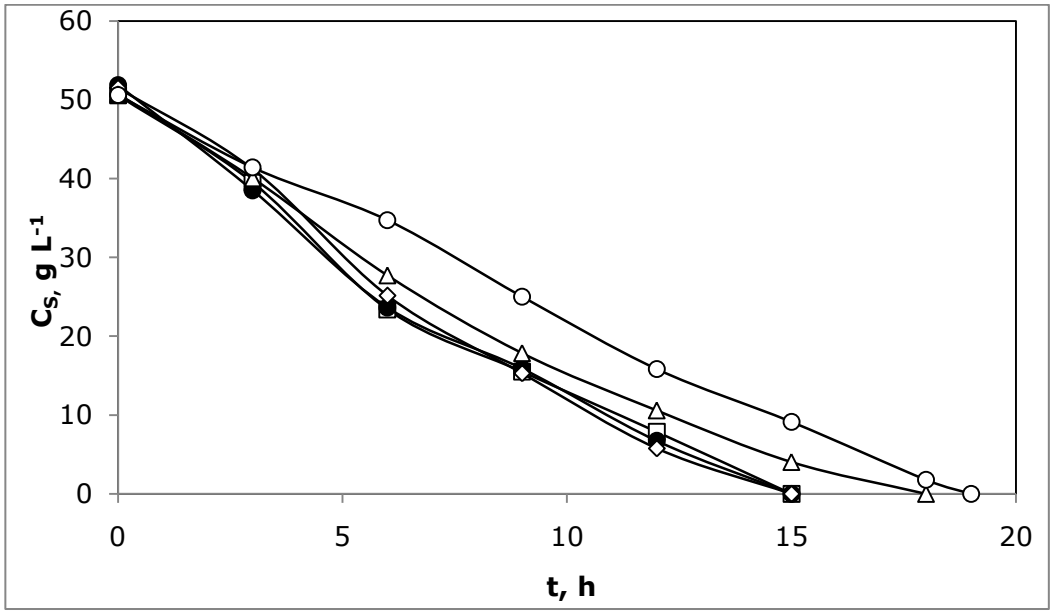

Figure 4.5 Variation in sorbitol concentrations (C_S) with the cultivation time and pH. pH 4.0 (□), pH 4.5 (●) pH 5.0 (◊), pH 5.5 (Δ), pH 6.0 (○).

Changes in the sorbitol consumption rate can be attributed to pH, since no relation between the cell growth and sorbitol consumption was observed. For instance although the cell growth at pH=4.5 was higher than that of pH=4.0 the sorbitol was totally consumed at t=15h in both operations. The results confirmed Çelik's (2008) results in which it was revealed that *P. pastoris* was able to consume sorbitol and methanol simultaneously.

On the other hand it was also observed that presence of sorbitol enhances the cell growth rate. The cell concentration was increased exponentially until the sorbitol was totally consumed. After C_S=0, cells entered to the stationary phase or the cell growth rate decreased drastically For example at pH=6.0, since the sorbitol was depleted at the late hours of the production; cells entered the stationary phase later than other pH values, which resulted in higher cell concentrations at pH=6.0.

4.6 Effect of pH on rHuEPO production

The aim of this study was to investigate the optimum pH for high rHuEPO production. In the experiments the recombinant product concentration increased with the cultivation time, the highest rHuEPO concentrations were achieved at t=15, 21, 18, 21, 18 h for pH=4.0, 4.5, 5.0, 5.5, 6.0 respectively, and the highest rHuEPO concentrations were compared using SDS-PAGE (Figure 4.6).

To determine the rHuEPO concentrations (C_{rHuEPO}) samples were analyzed by HPCE (Figure 4.7). The highest rHuEPO concentration was achieved at pH=4.5 as 0.158 g L^{-1}. This value was 1.43-, 1.24-, 1.95- and 1.23-fold higher than those obtained at pH=4.0, 5.0, 5.5, and 6.0 respectively. After the highest concentration was obtained C_{rHuEPO} was decreased drastically in all the operations. This might be due to presence of proteases in the fermentation medium. Also it can be resulted that C_{rHuEPO} profiles were almost the same for pH=4.0, 5.0 and 6.0 for the first 10 hours of the operations. Although the production rate was low at pH=4.5, after t=6h the rate increases and the highest C_{rHuEPO} was obtained at this pH. Finally the lowest C_{rHuEPO} were obtained at pH=5.5, however until t=21h it increased linearly.

The highest C_{rHuEPO} was obtained at pH=4.5 where the highest cell concentration was achieved. At this pH, the increase in the cell concentration enhanced the recombinant protein production. On the other hand, although the highest C_X attained at pH=6.0 was 1.14-fold higher than that of pH=5.0, the highest C_{rHuEPO} achieved at pH=6.0 was almost the same as that of pH=5.0.

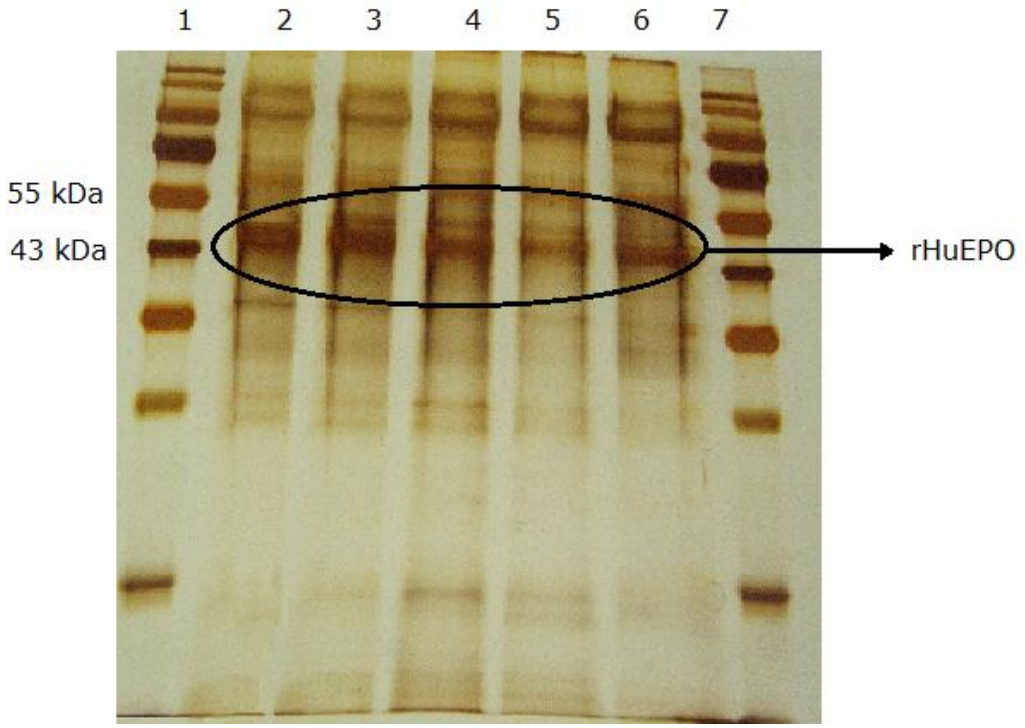

Figure 4.6 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in pilot scale bioreactor to observe effects of pH on rHuEPO production. 1. and 7. well: protein marker (PageRuller^{TM} prestained protein ladder, Fermentas) , 2. well: t=15h of pH 4.0, 3. well: t=21h of pH 4.5, 4. well: t=18h of pH 5.0, 5. well: t=21 of pH 5.5, 6. well t=18 of pH 6.0 (equal volume of samples were loaded to each well).

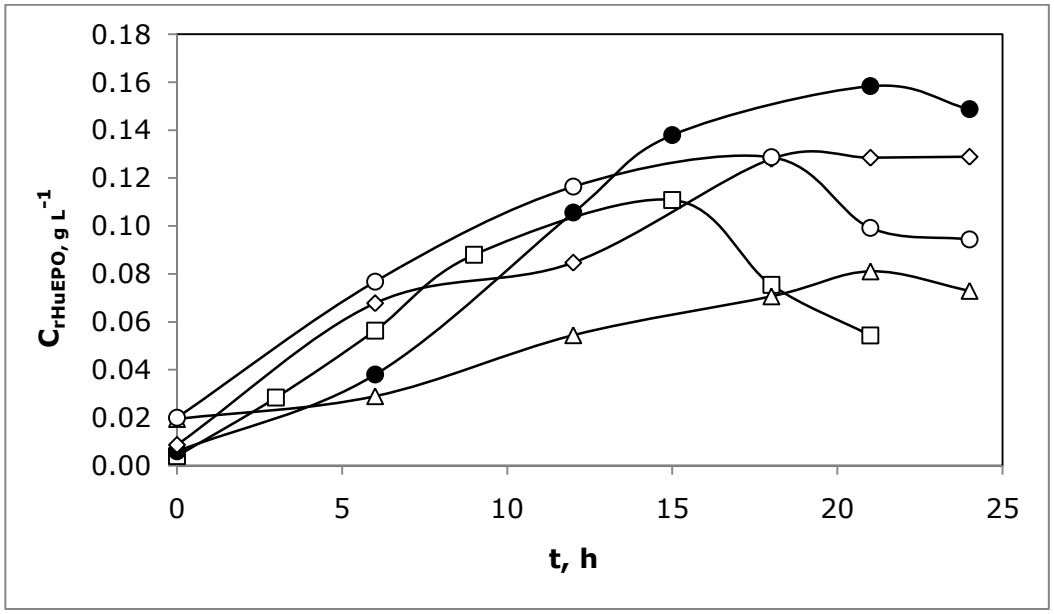

Figure 4.7 Variation in recombinant human erythropoietin concentration (C_{rHuEPO}) with the cultivation time and pH. pH 4.0 (□), pH 4.5 (●) pH 5.0 (◊), pH 5.5 (Δ), pH 6.0 (○).

It was also observed that the highest rHuEPO concentrations were attained prior to or just after the cells entered the stationary growth phase. This might be due to increased maintenance energy during this phase as well as proteolytic activity. In this study highest rHuEPO concentration achieved at pH=5.0 was 0.128 g L^{-1} which is very close to the C_{rHuEPO} obtained in Çelik's study (2008), which was 0.130 g L^{-1} , with the same conditions. This was the highest C_{rHuEPO} achieved for *P. pastoris*. When compared to Çelik's results, 1.22-fold higher rHuEPO concentration was achieved in this study by operation at pH=4.5.

4.7 Effect of pH on Alcohol Oxidase Activity

Specific alcohol oxidase (AOX) activity profiles showed dependence on pH. At low pH values, pH=4.0 and 4.5 the highest activities were obtained at t=3-6h which is later than those obtained at higher pH values (Figure 4.8). Since the highest rHuEPO was obtained at pH=4.5 , as expected the highest specific AOX activity was attained at this pH as 110.1 U g^{-1} CDW. The rHuEPO produced at pH=5.0 and 6.0 was very close to each other. Consistently the maximum specific AOX activity obtained at pH=5.0 was 72.9 U g^{-1} CDW which was 1.32-fold higher than that obtained at pH=6.0. However the rHuEPO concentration achieved at pH=6.0 was higher when compared to that obtained at pH=5.0. This might be due to higher AOX activities obtained at pH=6.0 throughout the fermentation, that allowed higher rHuEPO production.

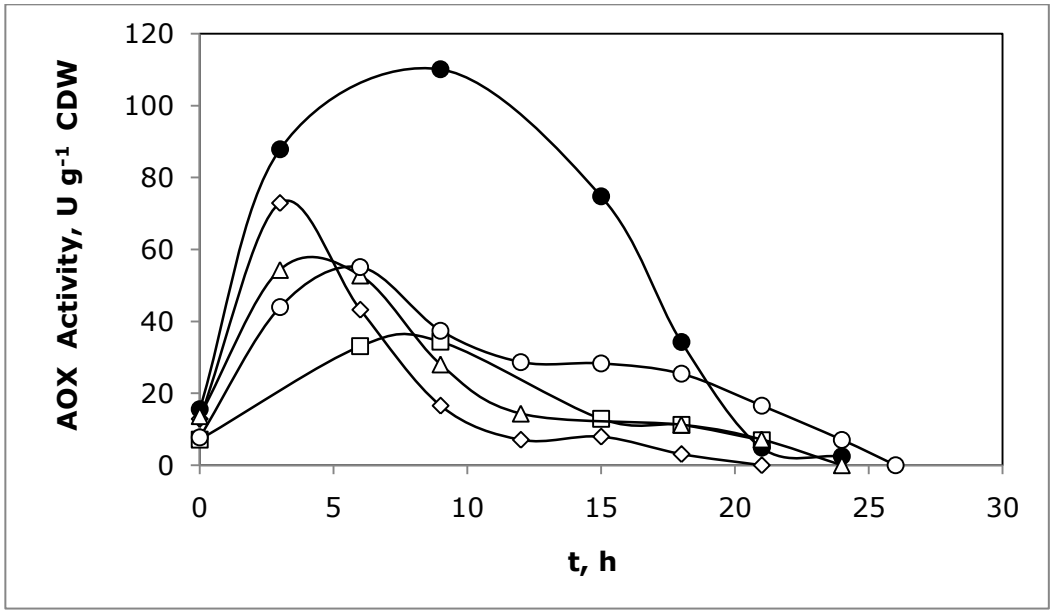

Figure 4.8 Variation in specific alcohol oxidase activity with the cultivation time and pH. pH 4.0 (□), pH 4.5 (●) pH 5.0 (◊), pH 5.5 (Δ), pH 6.0 (○).

The AOX activity indicates the respiratory status of the cells. In all of the experiments there was no methanol accumulation in the fermentation broth showing that the methanol fed to the system was consumed efficiently and thus there was no problem in AOX synthesis.

4.8 Effect of pH on Total Protease Concentration

Activities of acidic, neutral and alkali proteases were measured and the total activity obtained was converted to total protease concentration. The protease concentration profiles were very close to each other for all pH values. C_{pro} concentrations were almost constant until t=6h, for all operations (Figure 4.9). Later C_{pro} increases linearly throughout the fermentations. However the concentrations decreased after giving a maximum at t=21h for all experiments.

The highest C_{pro} achieved at pH= 4.5, 5.0 and 6.0 are only about 15% higher than those of pH=4.0 and 5.5. This result suggests that extracellular protease activity was not affected by pH significantly. Total protease concentrations converged to the same value for pH=4.0 and 5.5 at t=21h being 0.0609 g L^{-1}. The highest C_{pro} achieved at pH=4.5 was 0.0704 g L^{-1} which is slightly over those obtained at pH=5.0 and 6.0.

Extracellular protease concentration is known to affect the recombinant protein production in *P. pastoris* (Macauley-Patrick et al., 2005). Also pH is an important factor in terms of proteolytic activity (Sinha et al., 2004). Therefore to minimize the product degradation, extracellular protease concentration (C_{pro}) was investigated. However, it was observed that change in medium pH did not affect the proteolytic activity significantly. On the other hand rHuEPO concentrations decreased between t=18-21 for all experiments, when the protease concentrations reached the highest value.

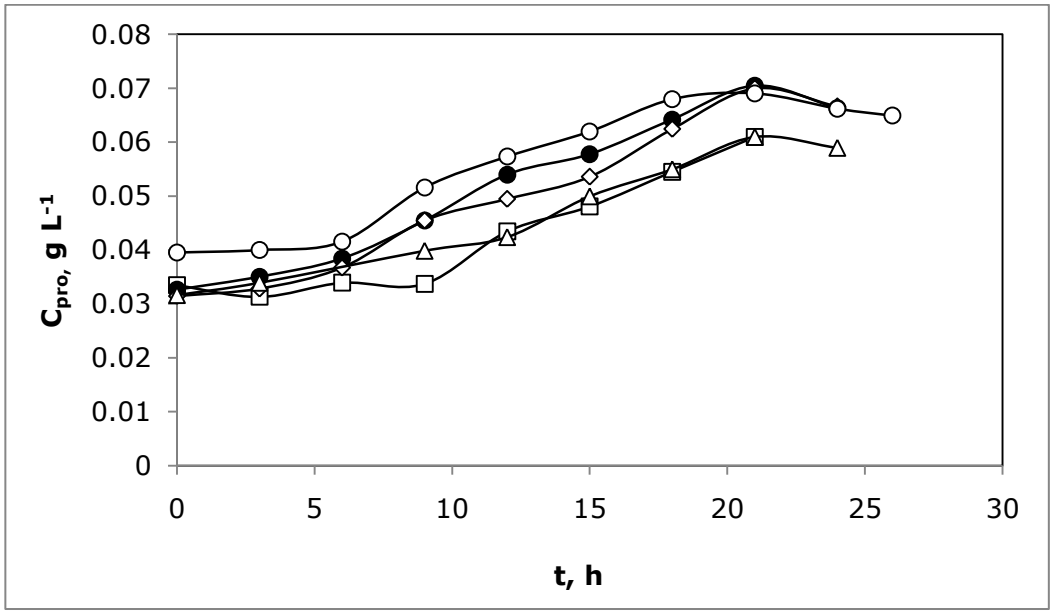

Figure 4.9 Variation in total protease concentration (C_{pro}) with the cultivation time and pH. pH 4.0 (□), pH 4.5 (●) pH 5.0 (◊), pH 5.5 (Δ), pH 6.0 (○).

Since the investigated pH range was acidic, as expected acidic proteases were higher than neutral and alkali proteases. For example at pH=5.0, neutral and alkali protease concentrations were 0.0212 and 0.0214 g L^{-1}, respectively, while acidic protease concentration was 0.027 g L^{-1} at t=21h.

4.9 Amino and Organic Acid Concentration Profiles

Intracellular reaction network regulates the demand and supply for amino and organic acids, thus the concentration profiles of these metabolites provide an insight. Microorganisms are able to synthesize the essential amino acids needed for synthesis of proteins using carbon and nitrogen sources. Depending on the demand and supply of the amino acids in the cells they can be transferred in or out of the microorganism causing fluctuation of these metabolites in the fermentation medium. Also degradation of the proteins in

the medium by extracellular proteases contributes to the amino acids concentrations in the fermentation medium. The variations in amino acid concentrations were represented in Table 4.1.

At all pH values operated; glutamic acid (Glu), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), methionine (Met), arginine (Arg), valine (Val) and tyrosine (Tyr) were not detected in the fermentation broth. Since rHuEPO includes high Ala and Leu, which are 13 and 11% respectively, absence of these amino acids were expected. Amongst the amino acids that were present in the medium, Lysine (Lys) attained the highest concentration in all experiments except pH=4.0, at which the highest amino acid concentrations achieved were serine (Ser) and Tryptophan (Trp). Furthermore the highest total amino acid concentration was obtained at pH= 6.0 as 0.513 g L^{-1} at t=0h. Also total amino acid concentrations at pH=4.5, 5.0 and 6.0 were remained at high levels throughout the processes.

High methanol concentration is harmful for *P. pastoris* since the accumulation of methanol oxidation products, hydrogen peroxide and formaldehyde, are toxic to the cells. After being produced formaldehyde either is oxidized to formic acid or enters the assimilatory pathway. Therefore formaldehyde was not detected in the fermentation broth. Unlike formaldehyde, formic acid was present in the medium at high pH values which are pH=5.5 and 6.0 (Table 4.2). Presence of formic acid suggests that formaldehyde entered in dissimilatory pathway at high pH. The maximum formic acid concentrations obtained at pH=5.5 and 6.0 were 0.0786 g L^{-1} and 0.0653 g L^{-1}, respectively.

Table 4.1 Variation in amino acid concentration in the fermentation broth with the cultivation time and pH

	pH 4.0				pH 4.5			
Time(h)	**3**	**6**	**12**	**18**	**3**	**6**	**12**	**18**
Asn	0.009	0.018	0.010	0.004	0.091	0.069	0.003	0.007
Asp	0.001	0.007	0.003	0.001	0.000	0.011	0.011	0.000
Cys	0.011	0.008	0.009	0.000	0.015	0.016	0.013	0.008
Gln	0.020	0.019	0.023	0.016	0.029	0.008	0.013	0.018
Gly	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.004
His	0.000	0.003	0.023	0.011	0.014	0.026	0.010	0.003
Lys	0.008	0.088	0.022	0.023	0.209	0.198	0.073	0.101
Pro	0.002	0.002	0.000	0.000	0.000	0.006	0.004	0.002
Ser	0.058	0.059	0.069	0.048	0.086	0.025	0.039	0.053
Thr	0.003	0.002	0.000	0.000	0.001	0.003	0.001	0.000
Trp	0.041	0.040	0.055	0.057	0.010	0.000	0.004	0.006
Total	0.144	0.228	0.204	0.158	0.364	0.293	0.168	0.195
	pH 5.0				**pH 5.5**			
Time(h)	**3**	**6**	**12**	**18**	**3**	**6**	**12**	**18**
Asn	0.008	0.007	0.000	0.000	0.018	0.014	0.017	0.014
Asp	0.066	0.029	0.142	0.056	0.004	0.003	0.006	0.002
Cys	0.000	0.018	0.019	0.000	0.012	0.007	0.000	0.000
Gln	0.005	0.000	0.000	0.000	0.002	0.002	0.000	0.000
Gly	0.013	0.011	0.015	0.019	0.005	0.004	0.005	0.003
His	0.004	0.013	0.027	0.012	0.008	0.010	0.012	0.005
Lys	0.184	0.148	0.148	0.141	0.127	0.131	0.124	0.122
Pro	0.007	0.003	0.001	0.000	0.000	0.000	0.000	0.000
Ser	0.015	0.000	0.000	0.000	0.007	0.007	0.000	0.000
Thr	0.001	0.005	0.003	0.004	0.004	0.002	0.005	0.000
Trp	0.015	0.004	0.004	0.004	0.004	0.004	0.005	0.003
Total	0.310	0.231	0.359	0.236	0.173	0.170	0.157	0.135

Table 4.1 Variation in amino acid concentration in the fermentation broth with the cultivation time and pH (continued)

	pH 6.0			
Time(h)	**3**	**6**	**12**	**18**
Asn	0.004	0.005	0.004	0.002
Asp	0.041	0.028	0.019	0.069
Cys	0.099	0.017	0.018	0.021
Gln	0.000	0.000	0.000	0.016
Gly	0.008	0.000	0.000	0.000
His	0.009	0.017	0.034	0.011
Lys	0.340	0.308	0.209	0.208
Pro	0.005	0.006	0.003	0.001
Ser	0.000	0.000	0.000	0.047
Thr	0.002	0.005	0.002	0.001
Trp	0.009	0.003	0.002	0.000
Total	0.513	0.384	0.287	0.374

Other organic acids detected were mostly the TCA cycle metabolites; which are oxalic acid, gluconic acid, pyruvic acid, acetic acid, citric acid, fumaric acid, succinic acid, malic acid and maleic acid. Oxalic and gluconic acids were detected at all pH values and increased with time. Pyruvic acid was also detected at all pH values other than pH=4.5, having higher values at pH=5.0 when compared to other pH. Furthermore acetic acid was present in the medium at later hours of the operation. At low pH values, pH=4.0 and 4.5, succinic acid was not detected in the medium showing that the operations were not oxygen limited. However this result does not necessarily reveals that operations at pH=5.0, 5.5 and 6.0 were oxygen limited, since lactic acid is the main metabolite that indicates whether the oxygen was sufficient in the medium or not. Lactic acid was not detected in any of the

operations showing that TCA cycle worked efficiently, and it was not related to operation pH. Lastly total organic acid concentration in the fermentation medium was increased with pH until pH=5.0, and then it was decreased (Figure 4.10).

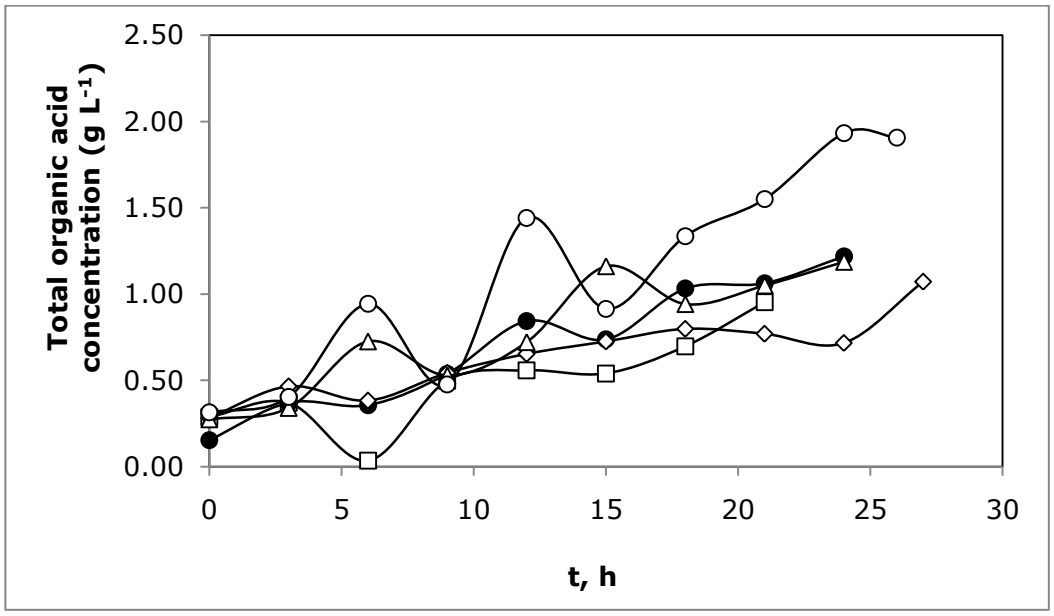

Figure 4.10 Variation in total organic acid concentration in the fermentation broth with cultivation time and pH. pH 4.0 (□), pH 4.5 (●) pH 5.0 (◊), pH 5.5 (Δ), pH 6.0 (○).

Table 4.2 Variation in organic acid concentration (g L^{-1}) profiles with cultivation time at different pH

	pH 4.0							
t	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**
Oxalic acid	0.0089	0.0109	0.0082	0.0156	0.0174	0.0178	0.0194	0.0359
Gluconic acid	0.2387	0.3099	0.0184	0.4411	0.4995	0.5026	0.6054	0.8238
Pyruvic acid	0.0088	0.0107	0.0066	0.0146	0.0178	0.0187	0.0266	0.0359
Acetic acid	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0446	0.0557
Citric acid	0.0308	0.0379	0.0000	0.0271	0.0223	0.0000	0.0000	0.0000
Fumaric acid	0.0005	0.0006	0.0009	0.0008	0.0009	0.0009	0.0011	0.0012

	pH 4.5								
t	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Oxalic acid	0.0072	0.0096	0.0109	0.0156	0.0227	0.0222	0.0394	0.0423	0.0503
Gluconic acid	0.1205	0.2956	0.2834	0.4874	0.7774	0.6826	0.8836	0.8473	1.0528
Acetic acid	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0676	0.1306	0.0647
Citric acid	0.0240	0.0628	0.0602	0.0329	0.0408	0.0303	0.0389	0.0401	0.0479
Fumaric acid	0.0009	0.0011	0.0011	0.0016	0.0022	0.0019	0.0028	0.0026	0.0029

	pH 5.0									
t	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**
Oxalic acid	0.0063	0.0260	0.0174	0.0181	0.0180	0.0170	0.0169	0.0138	0.0196	0.0207
Gluconic acid	0.1567	0.2785	0.2148	0.3154	0.4063	0.4236	0.4488	0.4244	0.3996	0.5017
Pyruvic acid	0.0347	0.0596	0.0450	0.0800	0.0755	0.0797	0.1255	0.1106	0.0928	0.1335
Acetic acid	0.0000	0.0000	0.0000	0.0000	0.0391	0.0328	0.0366	0.0370	0.0371	0.1918
Citric acid	0.0105	0.0176	0.0152	0.0000	0.0000	0.0199	0.0000	0.0000	0.0000	0.0000
Succinic acid	0.0753	0.0838	0.0619	0.0873	0.1019	0.0906	0.1167	0.1101	0.1019	0.1389
Malic acid	0.0000	0.0000	0.0288	0.0424	0.0126	0.0619	0.0539	0.0742	0.0657	0.0854

Table 4.2 Variation in organic acid concentration (g L^{-1}) profiles with cultivation time at different pH (continued)

	pH 5.5								
t	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Oxalic acid	0.0089	0.0117	0.0162	0.0123	0.0148	0.0303	0.0277	0.0376	0.0388
Gluconic acid	0.1836	0.1643	0.4512	0.3462	0.4924	0.7881	0.6398	0.6869	0.8455
Pyruvic acid	0.0176	0.0213	0.0339	0.0164	0.0279	0.0425	0.0351	0.0693	0.0589
Acetic acid	0.0000	0.0507	0.0945	0.0642	0.0767	0.1180	0.1029	0.0648	0.0659
Citric acid	0.0255	0.0406	0.0570	0.0271	0.0339	0.0447	0.0347	0.0622	0.0811
Succinic acid	0.0378	0.0508	0.0613	0.0379	0.0480	0.0592	0.0441	0.0728	0.0538
Formic acid	0.0000	0.0000	0.0113	0.0236	0.0275	0.0786	0.0576	0.0562	0.0431

	pH 6.0									
t	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**
Oxalic acid	0.0125	0.0171	0.0227	0.0131	0.0274	0.0198	0.0248	0.0256	0.0328	0.0397
Gluconic acid	0.0386	0.1293	0.2405	0.2635	0.7366	0.5190	0.6733	0.7369	0.8158	0.9749
Pyruvic acid	0.0169	0.0156	0.0146	0.0000	0.0142	0.0000	0.0164	0.0284	0.0452	0.0373
Acetic acid	0.0830	0.0000	0.0000	0.0000	0.2039	0.0375	0.1869	0.0434	0.1332	0.1123
Citric acid	0.0436	0.0512	0.1990	0.0334	0.1158	0.0391	0.0369	0.0658	0.0939	0.0738
Fumaric acid	0.0000	0.0004	0.0003	0.0003	0.0000	0.0000	0.0000	0.0008	0.0009	0.0011
Succinic acid	0.0861	0.1451	0.3751	0.1161	0.2385	0.2055	0.2779	0.4817	0.6262	0.5297
Malic acid	0.0336	0.0444	0.0791	0.0331	0.0767	0.0712	0.0787	0.1019	0.1493	0.1113
Formic acid	0.0000	0.0000	0.0116	0.0176	0.0279	0.0216	0.0407	0.0653	0.0350	0.0267
Maleic acid	0.0000	0.0000	0.0003	0.0000	0.0003	0.0000	0.0002	0.0003	0.0003	0.0003

4.10 Yield Coefficients and Specific Rates of the Bioprocess

Efficiency and profitability of a bioprocess can be evaluated by using the overall yield coefficients. To have a better insight, the overall yield of cell generated per mass of substrate consumed ($Y_{X/S}$), the overall yield of rHuEPO formed per mass of cells generated ($Y_{rHuEPO/X}$) and the overall yield of rHuEPO formed per mass of substrate consumed ($Y_{rHuEPO/S}$) were calculated for the production processes conducted at different pH values. For the calculation of the overall yield coefficients related to substrate, both methanol and sorbitol was taken into consideration. Table 4.3.

Table 4.3 Overall yield coefficients at different pH

Operation pH	$Y_{X/S}$ g g^{-1}	$Y_{P/X}$ mg g^{-1}	$Y_{P/S}$ mg g^{-1}
4.0	0.27	4.75	1.26
4.5	0.51	2.84	1.45
5.0	0.31	3.81	1.19
5.5	0.35	1.67	0.58
6.0	0.46	2.45	1.12

The highest overall cell yield on substrate was obtained at pH=4.5 as 0.51 g g^{-1}. Although the initial cell concentrations were almost the same for all pH values, $Y_{X/S}$ increased with increasing pH and achieved a maximum value at pH=4.5. The $Y_{X/S}$ values at different pH values are within or close the range, which is 0.4-0.8 for most fungi and yeast species, stated in the literature (Bailey and Ollis, 1986). The highest overall rHuEPO yield on cell was obtained at pH=4.0 as 4.75 mg g^{-1} which was 1.67-, 1.25-, 2.84- and 1.94-fold higher than those obtained at pH=4.5, 5.0, 5.5 and 6.0. However the overall rHuEPO yield on substrate was the highest at pH=4.5. $Y_{P/S}$ values obtained at pH=4.0, 5.0 and 6.0 were close to 1 mg g^{-1}. $Y_{X/S}$ at pH=5.5 was 0.35 g g^{-1} which was close that obtained at pH=4.0 and 5.0. On the other

hand at pH=5.5 $Y_{P/S}$ was 2-fold lower when compared to those of pH=4.0 and 5.0.

The overall yields are important parameters in evaluating the bioprocess efficiency and profitability. However it should be noted that the final amount of the product is more crucial for proteins with high commercial values. Therefore operation at pH=4.5 is more profitable since the final rHuEPO concentration achieved was the highest. Also it can be seen that at pH=4.5 $Y_{P/S}$ was the highest amongst the operated pH values. This result shows that with low substrate expenses, achievement of high rHuEPO concentrations would be enabled.

Total specific growth rate (μ_t), the specific sorbitol consumption rate (q_S), the specific methanol consumption rate (q_M), the specific rHuEPO formation rate (q_{rHuEPO}), the specific oxygen uptake rate (q_O) and the cell yields on oxygen ($Y_{X/O}$) were also calculated for the processes at different pH, and tabulated in Table 4.4.

Since the represented value of the specific growth rate was related to both carbon sources, it was expected that μ_t would exceed the specific growth rate on methanol which is 0.03 h^{-1}. The maximum total specific growth rate was obtained at pH=4.5, and for most of the process it remained higher when compared to other pH values; thus the highest cell concentration was attained at this pH. Similarly total specific growth rates obtained for pH=4.0 was lower than those of other pH values throughout the process and the lowest cell concentration was obtained.

The highest specific sorbitol consumption rates were achieved at the beginning of the processes (t=0-3h) and then decreased. Among the different operation pH values, the highest q_S was obtained at pH=4.5 as 0.16 g g^{-1} h^{-1} at t=0h. At low pH, pH=4.0 and 4.5, q_S was higher when compared to pH=5.0, 5.5 and 6.0 since the growth rate was low at the beginning of the processes at low pH values.

The initial cell concentrations were very close to each other, thus methanol feeding profiles, which were formed by using equation 4.1, were nearly the same in all operations. This was resulted in very close specific

methanol consumption rates for all pH values. Also no significant change in q_M was observed throughout the processes. Furthermore since the cells enter to the stationary phase and the methanol fed to the bioreactor increases at the end of the process, q_M increased slightly.

The highest specific rHuEPO formation rate was achieved at pH=4.0 as 0.425 mg g^{-1} h^{-1} at t=0h, while low q_{rHuEPO} values was obtained at pH=5.5 throughout the process. It was also observed that although the highest rHuEPO concentration was obtained at pH=4.5, q_{rHuEPO} was lower than those of pH=4.0, 5.0 and 6.0. Therefore it can be thought that, the ability of the cells to produce the recombinant protein was not improved and the high rHuEPO concentration was achieved by the increased cell concentration at pH=4.5.

Specific oxygen uptake rates remained higher at pH=4.0 when compared to other pH values. Also mostly q_O tends to increase at the beginning of the process and later decreases due to increase in cell concentration.

The highest cell yield on oxygen was obtained at pH=4.0 as 1.44. When compared with other pH, cell yield on oxygen values at pH=6.0 remained at higher values while they remained at lower values at pH=5.0. Furthermore for all ph values operated, a sudden decrease in $Y_{X/O}$ was observed since the cells entered the stationary phase and the oxygen demand increased in the late hours of the operations.

Since the cell growth terminates in the last hours of processes, the variation in cell concentration was negative. Also rHuEPO concentration decreased in those hour too. Therefore some total specific growth rate values, specific rHuEPO formation rate and cell yields on oxygen cannot be calculated.

Table 4.4 Variation in the specific rates with cultivation time at different pH

Time	C_X	μ_t	q_S	q_M	q_{rHuEPO}	q_O	$Y_{X/O}$
h	g L^{-1}	h^{-1}	g g^{-1} h^{-1}	g g^{-1} h^{-1}	mg g^{-1} h^{-1}	g g^{-1} h^{-1}	
				pH 4.0			
0	27.05	0.054	0.132	0.069	0.425	0.108	0.48
3	31.19	0.042	0.142	0.065	0.264	0.104	0.38
6	34.34	0.039	0.115	0.064	0.245	0.069	0.53
9	38.70	0.049	0.066	0.062	0.180	0.062	0.76
12	45.18	0.043	0.057	0.058	0.091	0.028	1.44
15	49.52	0.031	0.053	0.057		0.027	1.01
18	53.40	0.011		0.057		0.068	0.11
21	51.90			0.065		0.062	
				pH 4.5			
0	27.12	0.053	0.160	0.071	0.157	0.086	0.59
3	31.07	0.046	0.148	0.067	0.173	0.041	1.08
6	43.25	0.097	0.086	0.053	0.159	0.103	0.92
9	57.90	0.078	0.048	0.043	0.198	0.089	0.95
12	69.30	0.054	0.038	0.039	0.194	0.068	0.75
15	79.05	0.025	0.028	0.037	0.089	0.066	0.33
18	79.56	0.007		0.039	0.049	0.016	0.19
21	80.55	0.008		0.042	0.016	0.013	0.28
24	81.36	0.007		0.045		0.005	0.67
				pH 5.0			
0	28.75	0.069	0.114	0.075	0.273	0.062	1.06
3	34.74	0.051	0.123	0.068	0.287	0.053	0.90
6	39.05	0.043	0.109	0.066	0.179	0.105	0.38
9	44.00	0.048	0.072	0.063	0.109	0.048	0.93
12	50.82	0.048	0.049	0.059	0.093	0.041	1.08
15	57.42	0.032	0.033	0.056	0.082	0.037	0.75
18	60.30	0.019		0.058	0.069	0.018	0.86
21	63.35	0.017		0.060		0.019	0.61
24	64.77	0.010		0.063			
27	64.87	0.006		0.064		0.006	0.09

Table 4.4 Variation in the specific rates with cultivation time at different pH (continued)

Time	C_X	μ_t	q_S	q_M	q_{rHuEPO}	q_O	$Y_{X/O}$
h	g L^{-1}	h^{-1}	g g^{-1} h^{-1}	g g^{-1} h^{-1}	mg g^{-1} h^{-1}	g g^{-1} h^{-1}	
pH 5.5							
0	27.30	0.048	0.126	0.071	0.064	0.052	0.87
3	31.02	0.068	0.120	0.068	0.053	0.080	0.82
6	39.46	0.073	0.092	0.058	0.042	0.077	0.90
9	47.52	0.053	0.059	0.052	0.092	0.098	0.51
12	53.70	0.046	0.042	0.050	0.089	0.053	0.80
15	61.20	0.031	0.029	0.048	0.048	0.056	0.49
18	63.75	0.011	0.021	0.049	0.055	0.049	0.15
21	64.04	0.013		0.053	0.011	0.016	0.55
24	67.23	0.021		0.055		0.036	0.44
pH 6.0							
0	28.40	0.076	0.103	0.071	0.260	0.102	0.72
3	34.68	0.048	0.073	0.063	0.276	0.050	0.92
6	37.86	0.066	0.069	0.063	0.261	0.061	1.03
9	48.90	0.062	0.063	0.053	0.142	0.062	0.95
12	55.20	0.057	0.047	0.051	0.077	0.049	1.08
15	66.60	0.048	0.035	0.046	0.038	0.036	1.21
18	72.67	0.024	0.021	0.045		0.020	0.95
21	75.06	0.007	0.008	0.047		0.011	0.22
24	73.80			0.052		0.011	
26	73.50			0.057			

4.11 Oxygen Transfer Characteristics of Bioprocess

The Dynamic Method was applied to determine the oxygen transfer parameters, which are the oxygen uptake rate (OUR), oxygen transfer rate (OTR) and oxygen transfer coefficient, K_La, throughout the processes for all operations. Also other oxygen transfer characteristics, the enhancement factor E (K_La/K_La_0), maximum possible oxygen utilization rate or oxygen demand (OD), Damköhler number (Da) and effectiveness factor (η) throughout the bioprocesses were calculated and presented in Table 4.5 with variations in OUR, OTR and K_La. The physical oxygen transfer coefficient, K_La_0 was measured at t<0h, before the cells were transferred into the medium.

Agitation rate, temperature, rheological properties of the fermentation medium are known to be affecting the K_La as well as fine particles that are present in the medium. Since the agitation rate and temperature was kept constant in the experiments, changes in the K_La can be attributed to rheological properties of the medium and the fine particles in it. Despite the fact that K_La was observed to be oscillating during the fermentation, it had a tendency to increase at the beginning of the fermentation. The highest K_La was obtained at pH=4.5 as 0.109 s^{-1} at t=12h and when compared to other experiments higher K_La values were achieved. Secretion of proteins and other metabolites into the medium creates a resistance zone for mass transfer by limiting the contact area of cells with the gas bubbles and causes a decrease in K_La. Although the highest rHuEPO concentration was achieved at pH=4.5, since the cell concentrations attained at this pH was high throughout the fermentation, higher values of OUR was achieved. Therefore obtaining higher K_La values at pH=4.5 was an expected result since high OUR creates a driving force. The maximum K_La values attained at pH=4.0, 5.0, 5.5 and 6.0 were 0.092, 0.103, 0.089 and 0.089 s^{-1}, respectively.

The OUR, being affected from the cell growth and substrate consumption rates, tends to increase at the beginning of the fermentation due to high values of these rates. The highest OUR was obtained at pH=4.5 as 45.1 mol m^{-3} s^{-1} at t=15h, since the highest total specific growth and sorbitol consumption rates were achieved at this pH increasing the oxygen demand. The maximum values of OUR was mostly obtained at t=3-9h of the processes

other than pH=4.5, reaching to 28.2, 35.7, 40.5 and 26.5 mol m^{-3} s^{-1}, respectively, for pH=4.0, 5.0, 5.5 and 6.0. Furthermore throughout the fermentations higher OUR values were obtained before sorbitol was totally consumed. This was an expected result since utilization of both methanol and sorbitol needs oxygen, causing an increase in oxygen demand of the cells, when compared to feeding of methanol as sole carbon source.

The OTR has a similar behavior as the OUR since the OUR creates a driving force by decreasing the dissolved oxygen concentration in the medium. The OTR attained slightly higher but close values with OUR showing that cells effectively consumed most of the oxygen fed to the bioreactor. Consistent with the OUR results, the highest OTR amongst the pH values operated was achieved at pH=4.5 as 47.7 mol m^{-3} s^{-1}. Respectively higher values of OTR and OUR at pH=4.5 can be explained by, not only the high specific growth rate and substrate consumption, but also high rHuEPO production causing an increase in the oxygen demand. Therefore, high values of the maximum oxygen utilization rate (OD= $\mu_{max}C_X / Y_{X/O}$) was obtained at pH=4.5. Also OD values increases with the cultivation time due to higher cell growth rates.

OD and maximum possible mass transfer rate ($OTR_{max}=K_La C_o^*$) was calculated to be able to find the rate limiting step of the fermentation process. The modified Damköhler number ($Da=OD/OTR_{max}$) represents the relation between these parameters (Çalık et al., 2000). For all the experiments Da was higher than 1, showing that the mass transfer resistance was significant and dominated the biochemical reaction resistance. Since the high oxygen demand cannot be met because of the very low OTR_{max} available, the highest Da was obtained at pH=6.0 as 27.9 which was very close to the maximum Da value obtained at pH=4.5. It was observed that Da and effectiveness factor (η), which is the oxygen uptake rate per maximum possible oxygen utilization rate in the absence of mass transfer resistance (Çalık et al., 2000), had an inverse relation with each other. While Da increased within time, η decreased. Effectiveness factor, η, was always below 1 for all operations. Low η values indicates that cells might be consuming lower oxygen than the oxygen demand. Highest η value was achieved at pH=4.5 as 0.67 and it remained at high values until a sharp decrease was observed at t=18h. This results

reveals that at pH=4.5 maximum possible oxygen utilization (OD) values was approached only at this pH. Da and OD are theoretical values calculated assuming absence of resistances and at the highest specific growth rate (μ_{max}) which is 0.14 h^{-1} for *Pichia pastoris*. In this study the aimed and calculated specific growth rates are much lower than the μ_{max}. Therefore it was an expected result that oxygen uptake rate could not reach the maximum possible oxygen demand (OD). Also since the OTR_{max} is directly proportional with K_La, Da«1 was not expected. Thus the values of OD and Da calculated do not show that the processes were oxygen limited.

During the determination of K_La_0, properties of the medium was different from that includes the microorganism. Therefore defining of an enhancement factor, E (K_La/K_La_0), becomes necessary. The highest E was 9.91 which was obtained at pH=4.5 at t=12h.

Table 4.5 Variation in the oxygen transfer parameters with the cultivation time at different pH

t	K_La	E	$OTRx10^3$	$OTR_{max}x10^3$	$OURx10^3$	$ODx10^3$	Da	η
h	s^{-1}	K_La/ K_La_0	mol m^{-3} s^{-1}	mol m^{-3} s^{-1}	mol m^{-3} s^{-1}	mol m^{-3} s^{-1}		
				pH 4.0				
0	0.092	8.35	31.2	39.0	25.3	68.4	1.8	0.37
3	0.090	8.20	33.8	42.2	28.2	100.8	2.4	0.28
6	0.070	6.36	22.0	27.5	20.5	78.9	2.9	0.26
9	0.071	6.44	24.3	30.4	20.8	62.3	2.0	0.33
12	0.047	4.25	15.4	19.2	10.8	38.0	2.0	0.29
15	0.040	3.67	14.6	18.2	11.8	59.7	3.3	0.20
18	0.095	8.66	34.2	42.8	31.4	592.7	13.9	0.05
21	0.092	8.39	32.9	41.2	28.0			
				pH 4.5				
0	0.071	6.48	24.2	30.3	20.0	55.9	1.8	0.36
3	0.046	4.16	15.6	19.4	10.9	35.1	1.8	0.31
6	0.100	9.10	42.0	52.5	38.5	57.4	1.1	0.67
9	0.089	8.09	43.5	54.4	39.6	74.0	1.4	0.54
12	0.109	9.91	45.9	57.4	40.8	112.3	2.0	0.36
15	0.081	7.36	47.7	59.7	45.1	291.8	4.9	0.15
18	0.035	3.14	14.7	18.3	11.4	506.9	27.7	0.02
21	0.033	3.03	19.0	23.8	9.2			
24	0.018	1.67	6.9	8.6	3.5			
				pH 5.0				
0	0.059	5.37	18.1	22.6	15.6	33.2	1.5	0.47
3	0.065	5.95	20.9	26.2	16.1	47.0	1.8	0.34
6	0.103	9.33	37.6	47.0	35.7	126.3	2.7	0.28
9	0.062	5.68	19.4	24.2	18.3	57.2	2.4	0.32
12	0.050	4.52	19.2	24.0	18.0	57.2	2.4	0.31
15	0.058	5.23	19.4	24.3	18.3	92.9	3.8	0.20
18	0.033	2.97	11.3	14.1	9.4	85.2	6.0	0.11
21	0.035	3.16	11.9	14.9	10.6	125.1	8.4	0.08
27	0.018	1.60	6.0	7.6	3.5			

Table 4.5 Variation in the oxygen transfer parameters with the cultivation time at different pH (continued)

t	K_La	E	$OTRx10^3$	$OTR_{max}x10^3$	$OURx10^3$	$ODx10^3$	Da	η
h	s^{-1}	K_La/K_La_0	mol m^{-3} s^{-1}	mol m^{-3} s^{-1}	mol m^{-3} s^{-1}	mol m^{-3} s^{-1}		
				pH 5.5				
0	0.051	4.67	14.6	18.3	12.4	38.2	2.1	0.32
3	0.063	5.70	26.4	33.0	21.5	46.0	1.4	0.47
6	0.075	6.81	29.5	36.9	26.5	53.2	1.4	0.50
9	0.089	8.10	42.5	53.1	40.5	113.6	2.1	0.36
12	0.061	5.56	26.1	32.7	24.7	81.4	2.5	0.30
15	0.049	4.42	32.1	40.1	29.5	150.9	3.8	0.20
18	0.068	6.20	28.9	36.2	27.0	509.7	14.1	0.05
21	0.024	2.19	9.8	12.2	9.1	140.5	11.5	0.06
24	0.054	4.95	24.2	30.3	21.1	186.4	6.2	0.11
				pH 6.0				
0	0.073	6.67	28.7	35.8	25.2	48.0	1.3	0.53
3	0.060	5.42	15.8	19.8	15.0	46.1	2.3	0.32
6	0.065	5.93	20.5	25.6	19.9	44.5	1.7	0.45
9	0.087	7.92	27.3	34.2	26.5	62.7	1.8	0.42
12	0.089	8.09	24.6	30.7	23.7	62.1	2.0	0.38
15	0.082	7.41	23.3	29.1	20.9	66.9	2.3	0.31
18	0.051	4.61	14.6	18.2	12.9	93.3	5.1	0.14
21	0.048	4.38	12.0	14.9	7.4	417.4	27.9	0.02
24	0.034	3.07	8.4	10.5	7.2			
26	0.012	1.10	3.1	3.9				

CHAPTER 5

CONCLUSIONS

In this study, the aim is to investigate effects of pH on recombinant human erythropoietin (rHuEPO) production by *Pichia pastoris.* Five different laboratory scale bioreactor experiments were conducted between pH=4.0 and pH=6.0. The process consists of four phases which are glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and methanol fed-batch (MFB). The first two steps were conducted to obtain the cell concentration as high as possible prior to the recombinant protein production. The third step was conducted to enable the cells to get used to methanol utilization. The cell growth profiles at the first three steps of the process were very similar and at the end of the MT phase almost the same cell concentrations were achieved. However at pH values below pH=5.0 the cell growth rate was so low that additional 4 hours of operation was needed to obtain a similar cell growth profile with pH=5.0, 5.5 and 6.0. Therefore, the first three steps of the processes at pH=4.0 and 4.5 were conducted at pH=5.0 and the desired cell growth profiles were obtained at GB, GFB, and MT phases. Consequently the initial cell concentration in the MFB phase was almost the same for all experiments. The results obtained for the production of rHuEPO are summarized below.

- The highest cell concentration was obtained at pH=4.5 as 81.4 g L^{-1} while the lowest value was 53.4 g L^{-1} which was obtained at pH=4.0.
- Methanol was fed to the system at limiting amounts, thus no methanol was detected in the fermentation medium. The secondary substrate, sorbitol, was consumed at t=15h of the production at pH=4.0, 4.5 and 5.0. On the other hand at pH values above pH=5.0 the sorbitol consumption rate was decreased. Sorbitol was depleted at t=18h and t=21h at pH=5.5 and pH=6.0, respectively.

- The highest rHuEPO was achieved at pH=4.5 as 0.158 g L^{-1}. This value was 1.43-, 1.24-, 1.95- and 1.23-fold higher than those obtained at pH=4.0, 5.0, 5.5, and 6.0 respectively. Another result is that the highest rHuEPO concentrations were attained prior to or just after the cells entered the stationary growth phase which might be due to increased maintenance energy during this phase as well as proteolytic activity. Furthermore recently Bayraktar (2009) showed that the optimum pH for production of recombinant human growth hormone (rhGH) by *P. pastoris* was pH=5.0. Thus, the highest recombinant protein concentration was achieved for production of a polypeptide hormone, which is rhGH, at pH=5.0 while it was pH=4.5 for production of a glycoprotein hormone, which is rHuEPO. Finally the highest rHuEPO concentration achieved in this study is 1.2-fold higher than that obtained in a previous study (Çelik, 2008).
- The highest specific AOX activity was attained at pH=4.5, at which the highest rHuEPO concentration was achieved, as 110.1 U g^{-1} CDW.
- Extracellular protease concentration was almost constant for the first six hours of the operations and then increased linearly. The highest C_{pro} achieved at pH=4.5 was 0.0704 g $^{L-1}$ which is slightly over those obtained at pH=5.0 and 6.0. When the rHuEPO concentration profiles are considered, it can be said that proteases were effective in extracellular protein degradation between 0.04-0.07 g L^{-1}.
- Formic acid was found in the medium at high pH values which are pH=5.5 and 6.0. Presence of formic acid suggests that formaldehyde entered in dissimilatory pathway at high pH. The highest formic acid concentrations obtained at pH=5.5 and 6.0 were 0.0786 g L^{-1} and 0.0653 g L^{-1}, respectively.
- The highest cell yield on substrate, $Y_{X/S}$, was achieved at pH=4.5 as 0.51 g g^{-1} . The highest overall rHuEPO yield on cell was obtained at pH=4.0 as 4.75 mg g^{-1} which was 1.67-, 1.25-, 2.84- and 1.94-fold higher than those obtained at pH=4.5, 5.0, 5.5 and 6.0. The overall rHuEPO yield on substrate was the highest at pH=4.5. $Y_{X/S}$ at pH=5.5 was 0.35 g g^{-1} which was close that obtained at pH=4.0 and 5.0. On the other hand at pH=5.5. $Y_{P/S}$ was 2-fold lower when compared to those of pH=4.0 and 5.0.

- Although the highest rHuEPO concentration was obtained at pH=4.5, q_{rHuEPO} was lower than those of pH=4.0, 5.0 and 6.0 showing that, the ability of the cells to produce the recombinant protein was not improved and the high rHuEPO concentration was achieved by the increased cell concentration at pH=4.5.
- Higher mass transfer coefficient values were obtained at pH=4.5 throughout the process when compared to other pH values due to high cell concentrations in the medium. The highest K_La was obtained at this pH as 0.109 s^{-1} at t=12h. The maximum K_La values attained at pH=4.0, 5.0, 5.5 and 6.0 were 0.092, 0.103, 0.089 and 0.089 s^{-1} respectively. Also the highest OUR was obtained at pH=4.5 as 45.1 mol m^{-3} s^{-1} at t=15h, since the highest total specific growth and sorbitol consumption rates were achieved at this pH increasing the oxygen demand.

REFERENCES

Atkinson, B., Mavituna, F., 1991. Biochemical Engineering and Biotechnology Handbook, 2nd ed., Macmillan Publishers Ltd., England.

Azevedo, A.M., Cabral, J.M.S., Prazeres, D.M.F., Gibson, T.D., Fonseca. L.P., 2004. Thermal and operational stabilities of Hansenula polymorpha alcohol oxidase. Journal of Molecular Catalysis B: Enzymatic, 27: 37-45.

Bailey, E. J., Ollis, F. D., 1986. Biochemical Engineering Fundamentals, 2nd ed., McGraw-Hill, Inc., Singapore.

Bandyopashyay, B. and Humprey, A.E., 1967. Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. Biotechnology and Bioengineering, 9:533-544.

Bayraktar, E., 2009. Effects of pH on human growth hormone production by *Pichia pastoris* considering the expression levels of regulatory genes. Thesis Master of Science, Middle East Technical University, Ankara.

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

Bobrowicz, P., Davidson, R.C., Li, H.J., Potgieter, T.I., Nett, J.H.,Hamilton, S.R., Stadheim, T.A., Miele, R.G., Bobrowicz, B., Mitchell, T., Rausch, S., Renfer, E., Wildt, S., 2004. Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose. Glycobiology, 14(9): 757-766.

Boze, H., Laborde, C., Chemardin, P., Fabien, R., Venturin, C., Combarnous, Y., Moulin, G., 2001. High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*. Process Biochemistry 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.

Brady C.P., Shimp R.L., Miles A.P., Whitmore M., Stowers A.W., 2001. High-level production and purification of P30P2MSP119 an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expression and Purification, 23, 468-475.

Brankamp, R.G., Sreekrishna, K., Smith, P.L., Blankenship, D.T. and Cardin, A.D., 1995. Expression of a synthetic gene encoding the anticoagulan antimetastati protein ghilanten by the methylotrophic yeast *Pichia pastoris*. Protein Expression and Purification 6: 813-820.

Bretthauer, R.K., Castellino, F.J., 1999. Glycosylation of *Pichia pastoris*-derived proteins. Biotechology and Applied Biochemistry, 30:193-200.

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

Brierley, R.A., Davis, R.G., Holtz, C.G., 1994. Production of insulin-like growth factor-I in methylotrophic yeast cells. US Patent No.5, 324, 639.

Callewaert, N., Laroy, W., Cadirgi, H., Geysens, S., Saelens, X., Jou, W.M., Contreras, R., 2001. Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1,2-K-D-mannosidase for *N*-glycan engineering in *Pichia pastoris*. FEBS Letters, 503: 173-178.

Cereghino, G.P.L. and Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiology Reviews, 24: 45-66.

Cereghino, G.P.L., Cereghino, J.L., Sunga, A.J., 2001. New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of *Pichia pastoris*. Gene, 263(1-2): 159-169.

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

Choi, B.K., Bobrowicz, P., Davidson, R.C., Hamilton, S.R., Kung, D.H., Huijuan, L., Miele, R.G., Nett, J.H., Wildt, S., Gerngross, T.U., 2003. Use of combinatorial genetic libraries to humanize *N*-linked glycosylation in the yeast *Pichia pastoris*. Proceedings of the National Academy of Sciences USA, 100(9): 5022-5027.

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

Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A., Enfors, S.E., 2006. Increased total air pressure versus oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. Biochemical Engineering Journal, 30: 205-211.

Chiruvolu, V., Cregg, J.M., Meagher, M.M., 1997. Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. Enzyme and Microbial Technology, 21(4): 277-283.

Chiruvolu, V., Eskridge, K., Cregg, J.M., Meagher, M., 1998. Effects of glycerol concentration and pH on growth of recombinant *Pichia pastoris* yeast. Applied Biochemistry and Biotechnology, 75: 163-173.

Choi, D.B., Park, E.Y., 2006. Enhanced production of mouse α-amylase by feeding combined nitrogen andcarbon sources in fed-batch culture of recombinant *Pichia pastoris*. Process Biochemistry, 41: 390-397.

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

Cohen, A., 1983. The PICO TAG System: a new method to analyze primary and secondary amino acids with one picomole sensitivity. Biotechiques, Sept/Oct: 273-275.

Cos, O., Resina, D., Ferrer, P., Montesinos, J.L., Valero, F., 2005. Heterologous production of Rhizopus oryzae lipase in *Pichia pastoris* using the alcohol oxidase and formaldeyhde dehydrogenase promoters in batch and fed-batch cultures. Bochemical Engineering Journal, 26: 86-94.

Cos, O., Ramon, R., Montesinos, J. L., Valero, F., 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microbial Cell Factories, 5:17.

Couderc, R., Baratti, J., 1998. Oxidation of methanol by the yeast *Pichia pastoris*: purification and properties of alcohol oxidase. Agricultural and Biological Chemistry, 44:2279–2289.

Cregg, J.M., Tschopp, J.F., Stillman, C., 1987. High-level expression and efficient assembly of hepatitis B surface antigen in *Pichia pastoris*. Biotechnology, 5: 479-485.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P., Stillman, C.A., 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., Vedvick, T.S., Raschke, W.C., 1993. Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*. Biotechnology, 11 (8): 905-910.

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

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

Çalık, P., Çalık, G., Takaç, S., Özdamar, T.H., 1999. Metabolic Flux Analysis for Serine Alkaline Protease Fermentation by *Bacillus licheniformis* in a

Defined Medium: Effects of the Oxygen Transfer Rate. Biotechnology and Bioengineering, 64:151-167.

Çalık, P., Çalık, G., Özdamar, T.H., 2000. Oxygen Transfer Strategy and its Regulation Effects in Serine Alkaline Protease Production by *Bacillus licheniformis*, Biotechnology and Bioengineering, 69: 301-311.

Çalık, P., Bilir, E., Çalık, G., Özdamar, T.H., 2003. Bioreactor operation parameters as tools for metabolic regulations in fermentation processes: influence of pH conditions. Chemical Engineering Science. 58: 759-766.

Çelik, E., Çalık, P., Halloran, S.M., Oliver, S.G., 2007. Production of recombinant human erythropoietin from *Pichia pastoris* and its structural analysis. Applied Microbiology, 103(6): 2084-2094.

Çelik, E., 2008. Bioprocess Development for Therapeutical Protein Production. Thesis Doctor of Philosophy University of Middle East Technical University, Ankara.

Çelik, E., Çalık, P., Oliver, S.G., 2009. Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. Yeast, 26(9): 473-484.

Daly, R. and Hearn, M.T.W., 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., Pla, I., Chang, H.J., Cohen, L., Ritter, G., Old, L.J., Batt, C.A., 2004. An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. Protein Expression and Purification, 37: 18-26.

d'Anjou, M.C., Daugulis, A.J., 2000. Mixed-feed exponential feeding for fed-batch culture of recombinant methylotrophic yeast. Biotechnology Letters, 22:341-346.

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

Delorme, E., Lorenzini, T., Giffin, J., Martin, F., Jacobsen, F., Boone, T., Elliott, S., 1992. Role of glycosylation on the secretion and biological activity of erythropoietin. Biochemistry, 31: 9871-9876.

Dordal, M.S., Wang, F.F., Goldwasser, E., 1985. The role of carbohydrate in erythropoietin action. Endocrinology, 116(6): 2293-2299.

Egli, T., Käppeli, O. and Fletcher, A., 1982. Mixed substrate growth of methylotrophic yeasts in chemostat culture: influence of the dilution rate on the utilisation of a mixture of glucose and methanol. Archives of Microbiology, 131: 8-13.

Egrie, J., Strickland, T.W., Lane, J., Aoki, K., Cohen, A.M., Smalling, R., Trial G., Lin, F.K., Browne, J.K., Hines, D.K., 1986. Characterization and biological effects of recombinant human erythropoietin. Immunobiology, 172:213-224.

Elliott, S., Giffin, J., Suggs, S., Lau, E.P.L., Banks, A.R., 1989. Secretion of glycosylated human erythropoietin from yeast directed by the α-factor leader region. Gene, 79: 167-180.

Elliott, S., Egrie, J., Browne, J., Lorenzini, T., Busse, L., Rogers, N., Ponting, I., 2004. Control of rHuEPO biological activity: the role of carbohydrate. Experimental Hematology, 32: 1146-1155.

Ellis, S.B., Brust, P.F., Koutz, P.J., Waters, A.F., Harpold, M.M., and Gingeras, T.R., 1985. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. Molecular and Cellular Biology, 5(5): 1111-1121.

Files, D., Ogawa, M., Scaman, C.H., Baldwin, S.A., 2001. A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-c. Enzyme and Microbiol Technology, 29:335-340.

Fukuda, M.N., Sasaki, H., Lopez, K., Fukuda, M., 1989. Survival of recombinant erythropoietin in the circulation the role of carbohydrates. Blood, 73: 84-89.

Georgiou, G., Valax, P., 1996. Expression of correctly folded proteins in *Escherichia coli*. Current Opinion in Biotechnology, 7(2): 190-197.

Gemmill, T.R., and Trimble, R.B., 1999. Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. Biochimica et Biophysica Acta, 1426: 227-237.

Gimenez, J.A., Monkovic, D.D., Dekleva, M.L., 2000. Identification and monitoring of protease activity in recombinant *Saccharomyces cerevisiae*. Biotechnology and Bioengineering, 67: 245-251.

Goldwasser, E., Beru, N., Smith, D., 1990. Erythropoietin: The primary regulator of red cell formation. Handbook of Experimental Pharmacology 95: 747-770.

Goodrick, J.C., Xu, M., Finnegan, R., Schilling, B.M., Schiavi, S., Hoppe. H,, Wan, N.C. 2001. High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. Biotechnolohy and Bioengineering, 76:492-497.

Hamilton, S.R., Davidson, R.C., Sethuraman, N., Nett, J.H., Jiang, Y.W., Rios, S., Bobrowicz, P., Stadheim, T.A., Li, H.J., Choi, B.K., Hopkins, D., Wischnewski, H., Roser, J., Mitchell, T., Strawbridge, R.R., Hoopes, J., Wildt, S., Gerngross, T.U., 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. Science, 313: 1441-1443.

Hamilton, S.R., and Gerngross, T.U., 2007. Glycosylation engineering in the yeast: the advent of fully humanized yeast. Current Opinion in Biotechnology, 18: 387-392.

Hazeu, W., de Bruyn, J.C., Bos, P., 1972. Methanol assimilation by yeasts. Archives of Microbiology, 87: 185-188.

Hlodan, R., Hartl, U.F., 1994. How the protein folds in the cell. In Mechanisms of Protein Folding, Pain RH (ed.). Oxford University Press: New York; 194–228.

Holst, B., Bruun, A.W., Kiellandbrandt, M.C., Winther, J.R., 1996. Competition between folding and glycosylation in the endoplasmic reticulum. EMBO J. 15: 3538–3546.

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

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

Inan, M., Meagher, M.M., 2001-a. The effect of ethanol and acetate on protein expression in *Pichia pastoris*. Journal of Bioscience and Bioengineering, 92: 337-341.

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

Invitrogen (2002).Pichia fermentation process guidelineswww.invitrogen.com. Last accessed: November 2008.

Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S.D., Kaufman, R.J., Mufson, A., Seehra, J., Jones, S.S., Hewick, R., Fritsch, E.F., 1985. Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature, 313 (6005): 806-810.

Jacobson, L.O., Goldwasser, E., Fried, E., 1957. Role of kidney in erythropoiesis. Nature (London), 179: 633-634.

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

Jahic, M., Gustavsson, M., Jansen, A. K., Martinelle, M., Enfors, S.O., 2003-a. Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. Journal of Biotechnology, 102: 45-53.

Jahic, M., Wallberg, F., Bollok, M., Garcia, P., Enfors, S.O., 2003-b. Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. Microbial Cell Factories, 2: 6.

Jahic, M., Viede, A., Charoenrat, T., Teeri, T., Enfors, S.O., 2006. Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. Biotechnology Progress, 22: 1465-1473.

Jeklmann, W., 1992. Erythropoietin: structure, control of production, and function. Physiological Reviews, 72: 449-489.

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

Jungo, C., Marison, I.W., von Stockar, U., 2007-a. Mixed feeds of glycerol and methanol con improve the performance of *Pichia pastoris* cultures: A quantitative study based on concentration gradients in transient continuous cultures. Journal of Biotechnology, 128 (4): 824-837

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

Katakura, Y., Zhang, W.H., Zhuang, G.Q., Omasa, T., Kishimoto, M., Goto, W., Suga, K.I., 1998. Effect of methanol concentration on the production of human beta(2)-glycoprotein I domain V by a recombinant *Pichia pastoris*: a simple system for the control of methanol concentration using a semiconductor gas sensor. Journal of Fermentation and Bioengineering, 86: 482–487.

Khatri, N.K. and Hoffmann, F., 2006. Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*. Biotechnology and Bioengineering, 93(5): 871-879.

Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Tomomitsu, K., 2000-a. High-level secretion of recombinant human serum albumin by fed-

batch fermentation of the methylotrophic yeast, *Pichia pastoris*, based on optimal methanol feeding strategy. Journal of Bioscience and Bioengineering, 90(3): 280-283.

Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Tomomitsu, K., 2000-b. High level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. Journal of Bioscience and Bioengineering, 89(1): 55-61.

Koganesawa, N., Aizawa, T., Shimojo, H., Miura, K., Ohnishi, A., Demura, M., Hayakawa, Y., Nitta, K., Kawano, K., 2002. Expression and purification of a small cytokine growth-blocking peptide from armyworm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris*. Protein Expression and Purification 25(3): 416–425.

Koutz, P., Davis, G.R., Stillman, C., Barringer, K., Cregg, J., Thill, G., 1989. Structural comparison of the *Pichia pastoris* alcohol oxidase genes. Yeast, 5 (3):167-177.

Krantz, S.B., 1991. Erythropoietin. Blood, 77: 419-434.

Kupcsulik, B. and Sevella, B., 2005. Optimization of specific product formation rate by statistical and formak kinetic model descriptions of an HSA producing *Pichia pastoris* Mut^S strain. Chemical and Biochemical Engineering Quarterly, 19(1): 99-108.

Kurjan, J., and Herskowitz, I., 1982. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. Cell, 30: 933-943.

Laborde, C., Chemardin, P., Bigey, G., Combamous, Y., Moulin, M., Boze, H., 2004. Overexpression of ovine leptin in *Pichia pastoris*: physiological yeast response to leptin production and characterization of the recombinant hormone. Yeast, 21: 249-263.

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

Lai, P.H., Everett, R., Wang, F.F., Arakawa, T., Goldwasser, E., 1986. Structural characterization of human erythropoietin. Journal of Biological Chemistry, 216: 3116-3121.

Law, M.L., Cai, G.Y., Lin, F.K., Wei, Q., Huang, S.Z., Hartz, J.H., Morse, H., Lin, C.H., Jones, C., Kao, F.T., 1986. Chromosomal assignment of the human erythropoietin gene and its DNA polymorphism. Proceedings of the National Academy of Sciences USA, 83(18): 6920-6924.

Lee, B., Yurimoto, H., Sakai, Y., and Kato, N., 2002. Physiological role of the glutathione-dependent formaldehyde dehydrogenase in the methylotrophic yeast *Candida boidinii*. Microbiology, 148: 2697-2704.

Lee, C.Y., Lee, S.J., Jung, K.H., Katoh, S., Lee, E.K., 2003. High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. Process Biochemistry, 38: 1147-1154.

Lee-Huang, S., 1984. Cloning and expression of human erythropoietin cDNA in *Escherichia coli*. Proceedings of the National Academy of Sciences USA-Biological Sciences 81(9): 2708-2712.

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

Lin, F.K., Suggs, S., Lin, C.H., Browne, J.K., Smalling, R., Egrie, J.C., Chen, K.K., Fox, G.M., Martin, F., Stabinsky, Z., Badrawi, S.M., Lai, P.H., Goldwasser, E., 1985. Cloning and expression of the human erythropoietin gene. Proceedings of the National Academy of Sciences USA, 82(22): 7580-7584.

Loewen, M.C., Liu, X., Davies, P.L. and Daugulis, A.J., 1997. Biosynthetic production of type II fish antifreeze protein: fermentation by *Pichia pastoris*. Applied Microbiology and Biotechnology, 48(4): 480-486.

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

McGrew, J.T., Leiske, D., Dell, B., Klinke, R., Krasts, D., Wee, S., Abbott, N., Armitage, R. and Harrington, K., 1997. Expression of trimeric CD41 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants. Gene, 187(2): 193-200.

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

Menedez, J., Hernandez, L., Banguela, A., Pais, J., 2004. Functional production and secretion of the *Gluconoacetobacter diazatrophicus* fructose-releasing exo-levanase (LsdB) in *Pichia pastoris*. Enzyme and Microbial Technology, 34(5): 446-452.

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

Narhi, L.O., Arakawa, T., Aoki, K.H., Elmore, R., Rohde, M.F., Boone, T., Strickland, T.W., 1991. The effect of carbohydrate on the structure and stability of erythropoeitin. Journal of Biological Chemistry, 34: 23022-23026.

Nevoigt, E. and Stahl, U., 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiology Reviews, 21: 231-241.

Nielsen, J., Villadsen, J., 1994. Bioreaction Engineering Principles, Second Edition, Plenum Press, New York.

Nielsen, J., Villadsen, J., Liden, G., 2003. Bioreaction Engineering Principles, Second Edition, Plenum Press, New York.

Ogunjiimi, A.A., Chandler, J.M., Gooding, C.M., Recinos III, A., Choudary, P.V., 1999. High-level secretory expression of immunologically active intact antibody from the yeast *Pichia pastoris*. Biotechnology Letters, 21: 561-567.

Ohya, T., Morita, M., Miura, M., Kuwae, S., and Kobayashi, K., 2002. High level production of Preurokinase-Annexin V Chimeras in the methylotrophic yeast *Pichia pastoris.* Journal of Bioscience and Bioengineering, 94 (5): 467-473.

Pais-Chanfrau, H.M., Garcia, Y., Licor, L., Besada, V., Castellanos-Serra, L., Cabello, C.L., Hernandez, L., Mansur, M., Plana, L., Hidalgo, A., Tambara, Y., del Abrahantes-Perez, M., del Toro, Y., Valdes, J., Martinez, E., 2004. Improving the expression of mini-proinsulin in *Pichia pastoris*. Biotechnology Letters, 26:1269-1272.

Recny, M.A., Scoble, H.A., Kim, Y., 1987. Structural characterization of natural urinary and recombinant DNA-derived erythropoietin. Journal of Biological Chemistry, 262: 17156-17163.

Ren, H. T., Yuan, J. Q., Bellgardt, K. H., 2003 Macrokinetic model for methylotrophic *Pichia pastoris* based on stoichiometric balance. Journal of Biotechnology, 106: 53-68.

Sasaki, H., Bothner, B., Dell, A., Fukuda, M., 1987. Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. Journal of Biological Chemistry, 262: 12059-12076.

Schenk, J., Balazs, K., Jungo, C., Urfer, J.,Wegmann, C., Zocchi, A., Marison, I.W., von Stockar, U., 2008. Influence of specific growth rate on specific productivity and glycosylation of a recombinant avidin produced by a *Pichia pastoris* Mut^+ strain. Biotechology and Bioengineering, 99(2): 368-376.

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

Shi, X., Karkut, T., Chamankhah, M., 2003. Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. Protein Expression and Purification, 28: 321–330.

Shuler, M.L., Kargi, F., 2002. Bioprocess Engineering: Basic Concepts, 2nd Ed., Prentice Hall Inc., USA.

Sibirny, A.A., Ubiyvovk, V.M., Gonchar, M.V., Titorenko, V.I., Voronovsky, A.Y., Kapultsevich, Y.G., Bliznik, K.M., 1990. Reaction of direct formaldehyde oxidation to CO2 are not essential for energy supply of yeast methylotrophic growth. Archives of Microbiology 154:566–575.

Sinha, J., Plantz, B.A., Inan, M., Meagher, M.M., 2004. Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-τ. Biotechnology and Bioengineering, 89(1): 102-112.

Sreekrishna, K., and Kroop, K.E., 1996. *Pichia pastoris*. Nonconventional yeasts in biotechnology. Wolf, K. Berlin, Springer, 203-253.

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

Stevens, T.H., Rothman, J.H., Payne, G.S., Schekman, R., 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. The Journal of Cell Biology, 102:1551–1557.

Stratton, J., Chiruvolu, V., Meagher, M., 1998. High cell-density fermentation. Methods in Molecular Biology. D.R. Higgins, J. M. C., Humana Press. Pichia Protocols, 107-120.

Thorpe, E.D., d'Anjou, M.C., Daugulis, A.J., 1999. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnology Letters, 21: 669–672.

Trentmann, O., Khatri, N.K., Hoffmann, F., 2004. Reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. Biotechnology Progress, 20:1766–1775.

Tsujikawa, M., Okabayashi, K., Morite, M., Tanabe, T., 1996. Secretion of a variant of human single-chain urokinase-type plasminogen activator without an *N*-gycosylatLon site in the methylotrophic yeast, *Pichia pastoris* and characterization of the secreted product. Yeast, 12:541-553.

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

Veenhuis, M., Vandijken, J.P., Harder, W., 1983. The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. Advances in Microbial Physiology, 24: 1-82.

Vervecken, W., Kaigorodov, V., Callewaert, N., Geysens, S., De Vusser, K., Contreras, R., 2004. In vivo synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. Applied and Environmental Microbiology, 70(5): 2639-2646.

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

Wang, F.F., Kung, C.K.H., Goldwasser, E., 1985. Some chemical properties of human erythropoietin. Endocrinology 116: 2286-2292.

Wang, J., Nguyen, V., Glen, J., Henderson, B., Saul, A., Miller, L.H., 2005. Improved yield of recombinant merozoite surface protein 3 (MP3) from *Pichia pastoris* using chemically defined media. Biotechnology and Bioengineering, 90 (7): 838-847.

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

Wegner, E.H., 1983. Biochemical conversions by yeast fermentation at high-cell densities. U.S. Patent 4, 414,329. US, Phillips Petroleum Company.

Weigand, W.A., Lim, H.C., Creagan, C.C., Mohlar, R.D., 1979. Optimization of repeated fed-batch reactor for maximum cell productivity. Biotechnology Bioengineering Symposium, 9:335-348.

Werten, M.W.T., van den Bosch, T.J., Wind, R.D., Mooibroek, H., de Wolf, F.A., 1999. High-yield secretion of recombinant gelatins by *Pichia pastoris*. Yeast, 15: 1087-1096.

White, C.E., Kempi, N.M., Komives, E.A. 1994. Expression of highly disulfide-bonded proteins in *Pichia pastoris*. Structure 2, (11): 1003–1005.

Yang, J., Zhou, X.S., Zhang, Y.X., 2004. Improvement of recombinant hirudin production by controlling NH4+ concentration in *Pichia pastoris* fermentation. Biotechnology Letters, 26:1013-1017.

Xie, J., Zhang, L., Ye, Q., Zhou, Q., Xin, L., Du, P., Gan, R., 2003. Angiostatin production in cultivation of *Pichia pastoris* fed with mixed carbon sources. Biotechnology Letters 25: 173–177.

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

Zanjani, E.D., Poster, J., Borlington, H., Mann, L.I., Wasserman, L.R., 1977. Liver as the primary site of erythropoietin formation in the fetus. Journal of Laboratory and Clinical Medicine, 89: 640-644.

Zhang, W., Bevins, M.A., Plantz, B.A., Smith, L.A., 2000. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of Botolinum Neurotoxin, Serotype A. Biotechnology and Bioengineering, 70 (1): 1-8.

Zhang, W.H., Potter, K.J.H., Plantz, B.A., Schlegel, V.L., Smith, L.A., Meagher, M.M., 2003. *Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement. Journal of Industrial Microbiology and Biotechnology, 30: 210-215.

Zhang, W., Sinha, J., Smith, L.A., Inan, M., Meagher, M.M., 2005. Maximization of production of secreted recombinant proteins in *Pichia pastoris* fed-batch fermentation. Biotechnology Progress, 21 (2): 386–393.

Zhang, W., Inan., M., Meagher, M.M., 2007. Rational Design and Optimization of Fed-Batch and Continuous Fermentations, *Pichia* Protocols (Ed. by Cregg, J.M.), Humana Press, Inc., Totowa-New Jersey.

Zhang, J.G., Wang, X.D., Moa, X.Z., Wei, D.Z., 2009. High-level production of bovine enterokinase light chain using fed-batches by recombinant *Pichia pastoris*. Chemical and Biochemical Engineering Quarterly, 23(2): 219-224.

Zhao, H.L., Xue, C., Wang, Y., Yao, X.Q., Liu, Z.M., 2008. Increasing the cell viability and heterologous protein expression of *Pichia pastoris* mutant deficient in *PMR1* gene by culture condition optimization. Biotechnological Products and Process Engineering, 81: 235-241.

APPENDIX A

BUFFERS AND STOCK SOLUTIONS

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 was dissolved in 80 mL dH_2O . NaOH was added until EDTA was dissolved. The final pH was further adjusted to pH 8.0 and the final volume was adjusted to 100 mL. The buffer was autoclaved and stored at room temperature.
1 M Tris-Cl, pH 8.0	12.1 g Tris base was dissolved in 80 mL dH_2O and the pH was adjusted to 8.0 by adding concentrated HCl. The volume was made up to 100 mL. The buffer was autoclaved and stored at room temperature.
Yeast Lysis Solution	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl-pH8.0, 1mM Na_2EDTA. The solution was autoclaved and stored at room temperature.
1.5 M Tris-HCl, pH 8.8	36.3 g Tris base was dissolved in 150 mL dH2O and pH was adjusted to 8.8 with 6N HCl. The buffer was made up to 200 mL with dH2O. The buffer was autoclaved and stored at 2-8°C.
0.5 M Tris-HCl, pH 6.8	12.1 g Tris base was dissolved in 150 mL dH2O and pH was adjusted to 6.8 with 6N HCl. The buffer was made up to 200 mL with dH2O. The buffer was autoclaved and stored at 2<8°C.

4 x Sample Loading Buffer for SDS-PAGE	200 mM Tris<HCl,pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue; 10% 2-mercaptoethanol. Distributed into microcentrifuge tubes and stored at -20°C.
5x SDS-PAGE Running Buffer	15 g Tris Base, 72 g glycine, 5 g SDS, dH_2O to1 liter. The buffer was stored at 2-8°C and diluted 1:5 with dH_2O prior to use.
1 M potassium phosphate, 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.
20x YNB Stock solution	17 g Yeast Nitrogen Base without amino acids, 50 g $(NH_4)_2SO_4$ was dissolved in dH_2O and the volume was made up to 500 mL. The solution was autoclaved, aliquoted into 50 mL Falcon® tubes and stored at room temperature in dark.
Fixer Solution	Mix 150 mL methanol + 36 mL acetic acid + 150 µL 37% formaldehyde and complete to 300 mL with distilled water. This solution can be used several times.
Pretreatment Solution	Dissolve 0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 400 mL distilled water by mixing with a glass rod. Take 8 mL and set aside for further use in developing solution preparation.
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
Antifoam	10 % (v/v) antifoam solution, prepared with dH_2O. Can be autoclaved once.
Base for Bioreactor	25 % NH_3OH (Sigma). No need to sterilize.
Borate buffer (for Alkali proteases)	2.381 g Boraks ($Na_2B_4O_7.10H_2O$) dissolved in 250 ml dH_2O. pH adjusted to 10 by 1 M NaOH (6-7 ml) and add dH_2O till 500 ml. Filter and store at $+4^OC$.
0.05 M Sodium Acetate buffer (For acidic proteases)	Dissolve 0.713 ml acetic acid in 25 ml total dH_2O. Dissolve 2.052 g sodium acetate in 50 ml dH_2O. Titrate sodium acetate solution with acetic acid solution to pH 5.0, and final V= 50 ml. Then dilute to 500 ml. Autoclave and store at $+4^OC$.
0.05 M Sodium Phosphate Buffer (for neutral proteases)	Dissolve 6.70 g $Na_2HPO_4.7H2O$ in 50 ml dH_2O. Dissolve 3.90 g $NaH_2PO_4.2H_2O$ in 50 ml dH_2O. Titrate till pH 7.0, and final V= 50 ml. Then dilute to 500 ml. Autoclave and store at room temperature.
1X Equilibration/wash buffer for His-Tag purification	50 mM sodium phosphate buffer, pH 7.0; 300 mM NaCl.
1X Elution buffer for His-Tag purification	50 mM sodium phosphate buffer, pH 7.0; 300 mM NaCl; 150 mM Imidazole
MES buffer for resin wash	20 mM MES buffer, pH 5.0; 0.1 M NaCl

APPENDIX B

CALIBRATION CURVE FOR BRADFORD ASSAY

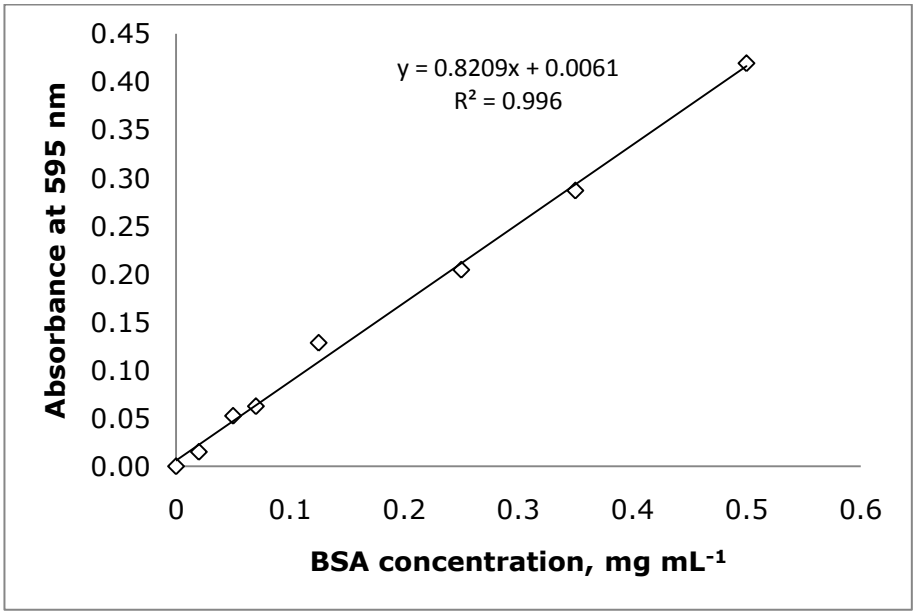

Figure B.1 Calibration curve for Bradford Assay

APPENDIX C

ELECTROPHEROGRAM AND CALIBRATION CURVE FOR rHuEPO STANDARD

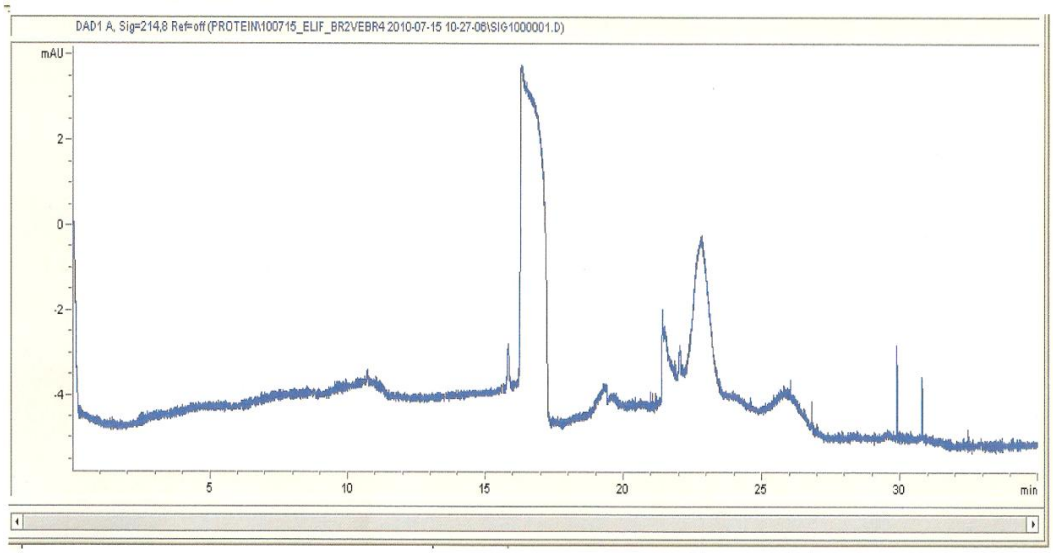

Figure C.1 Electropherogram of 0.05 g L^{-1} standard rHuEPO

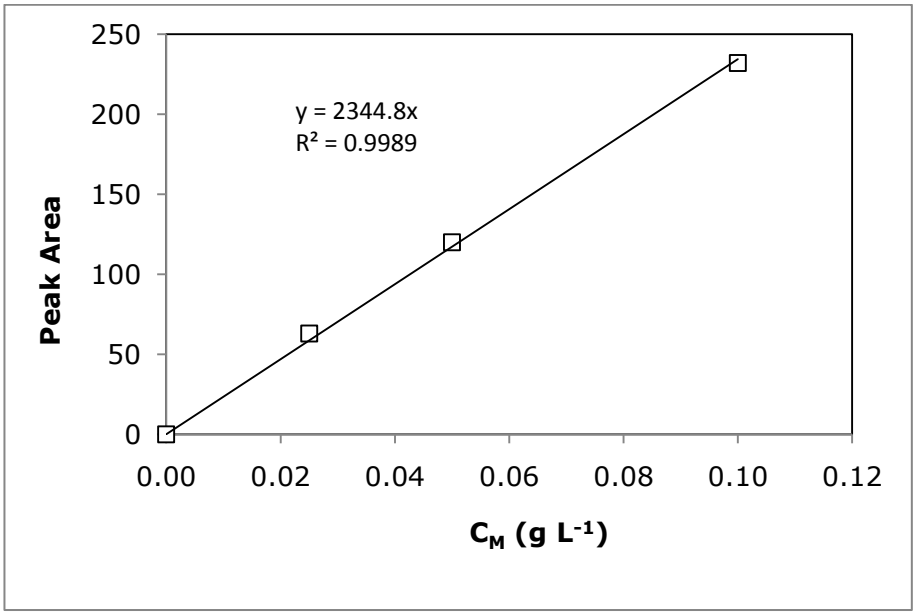

Figure C.2 Calibration curve for standard rHuEPO

APPENDIX D

MOLECULAR WEIGHT MARKER

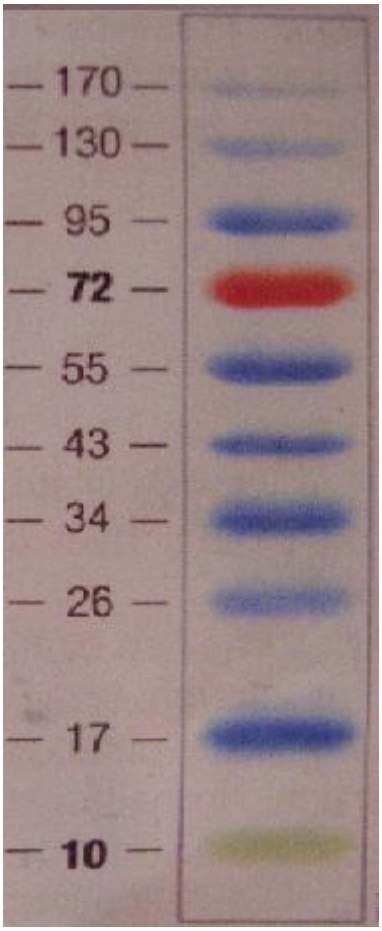

Figure D.1 PageRuler™ Prestained Protein Ladder (Fermentas)

APPENDIX E

METHANOL AND SORBITOL CALIBRATION CURVES

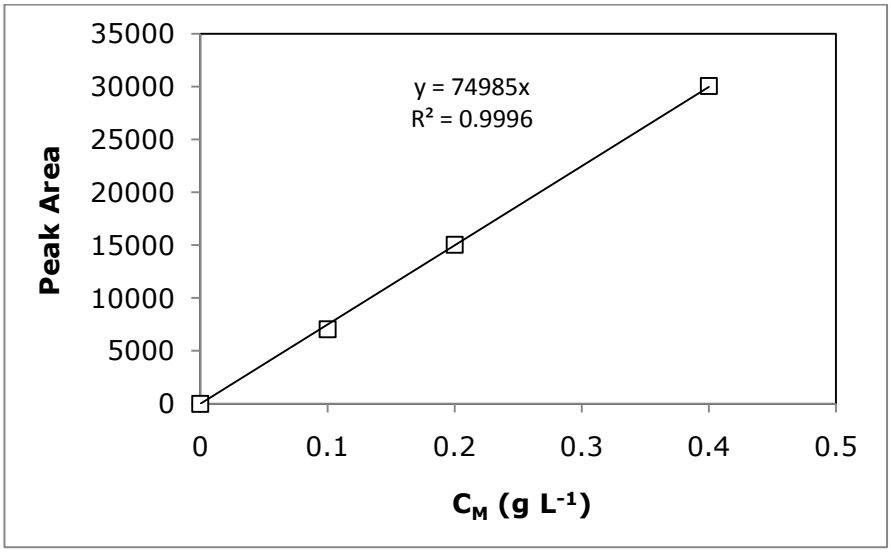

Figure E.1 Calibration curve obtained for methanol concentration; analysis was performed by HPLC.

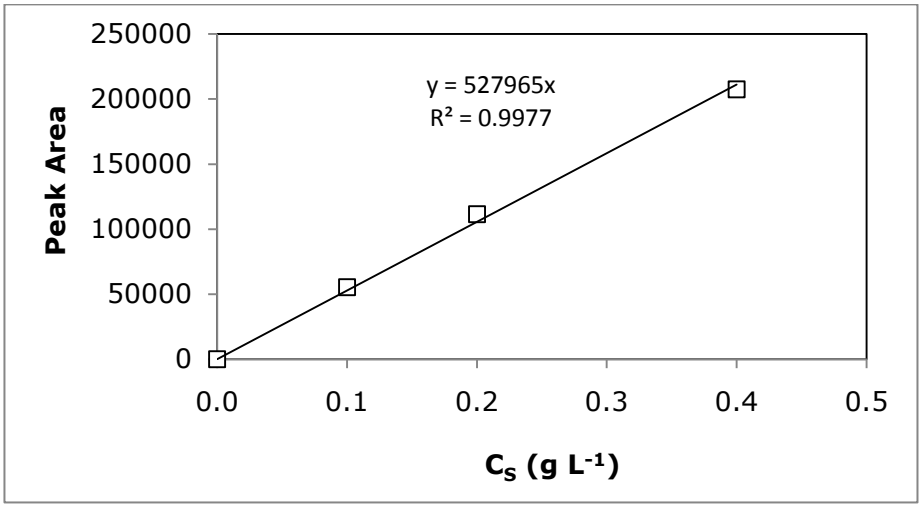

Figure E.2 Calibration curve obtained for sorbitol concentration; analysis was performed by HPLC.

APPENDIX F

ORGANIC ACIDS CALIBRATION CURVES

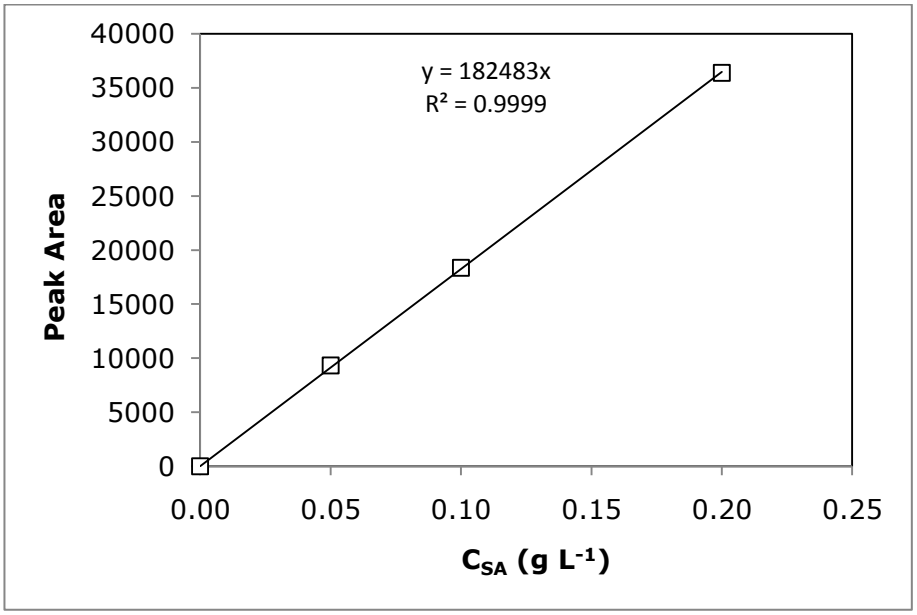

Figure F.1 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

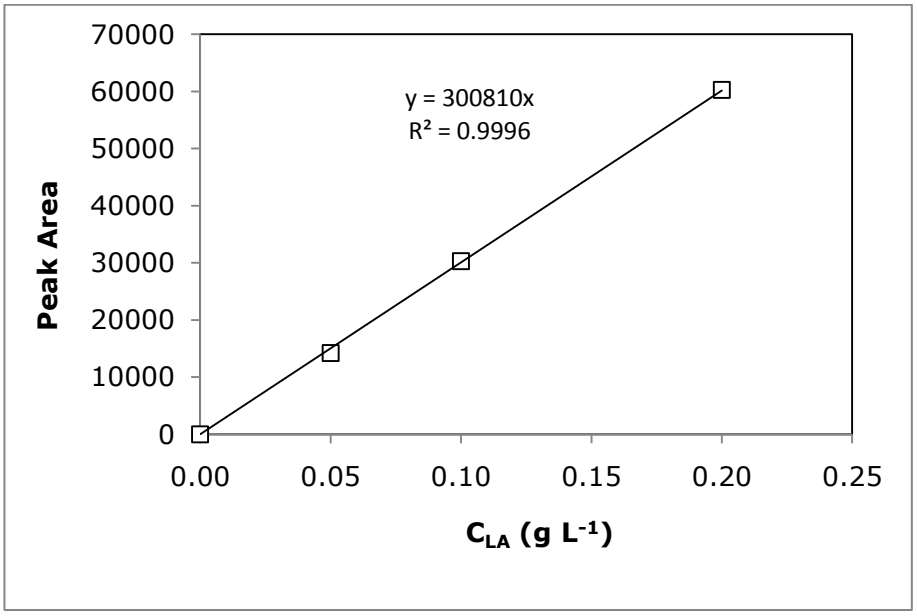

Figure F.2 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

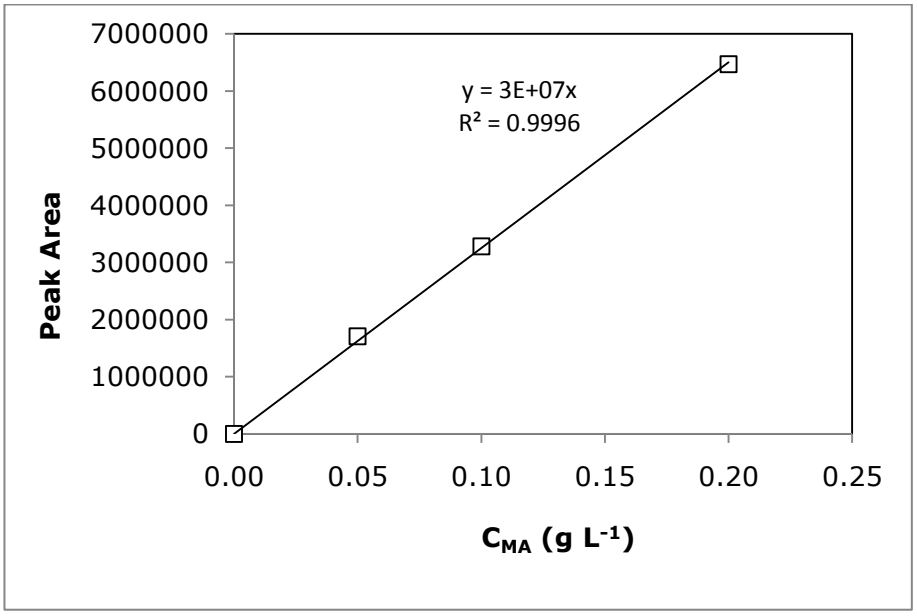

Figure F.3 Calibration curve obtained for maleic acid concentration; analysis was performed by HPLC.

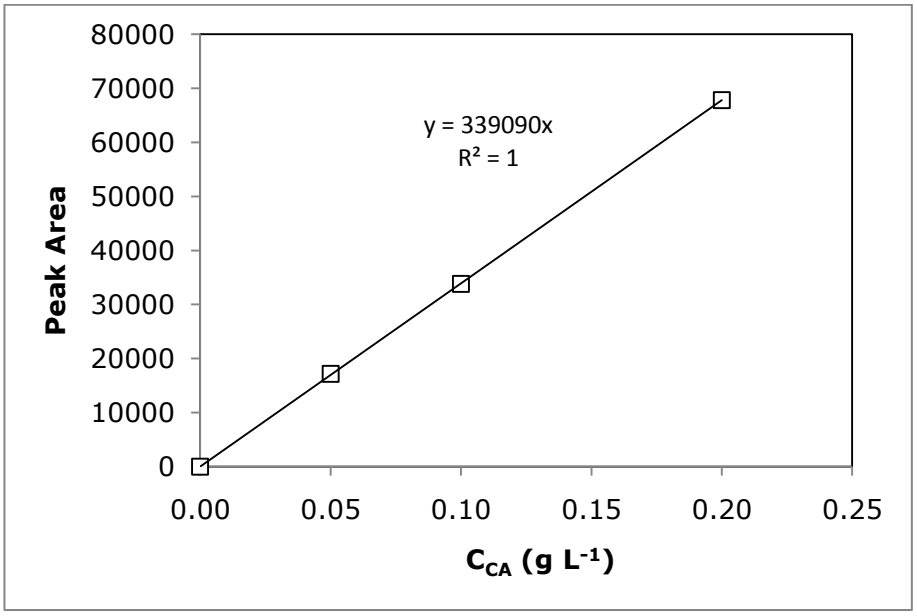

Figure F.4 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC.

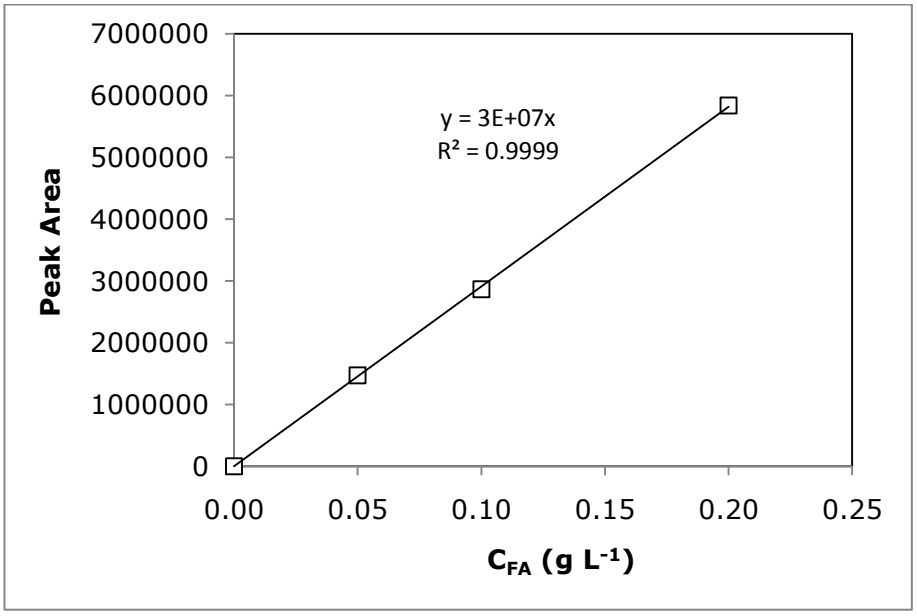

Figure F.5 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

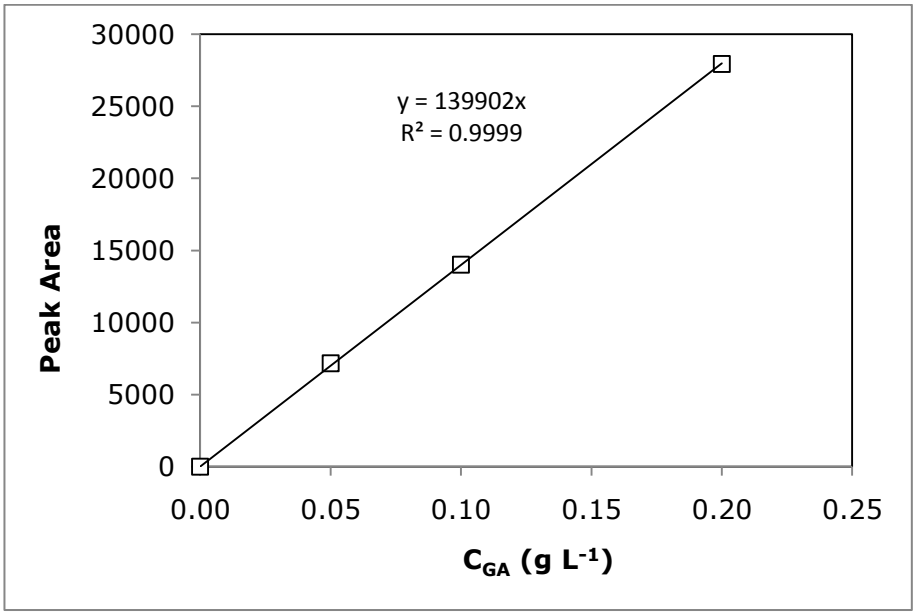

Figure F.6 Calibration curve obtained for gluconic acid concentration; analysis was performed by HPLC.

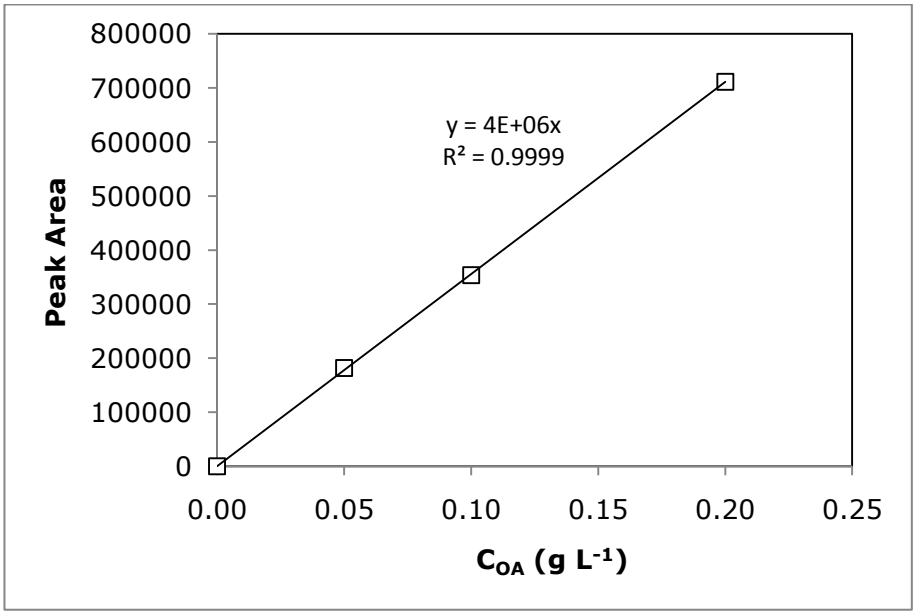

Figure F.7 Calibration curve obtained for oxalic acid concentration; analysis was performed by HPLC.

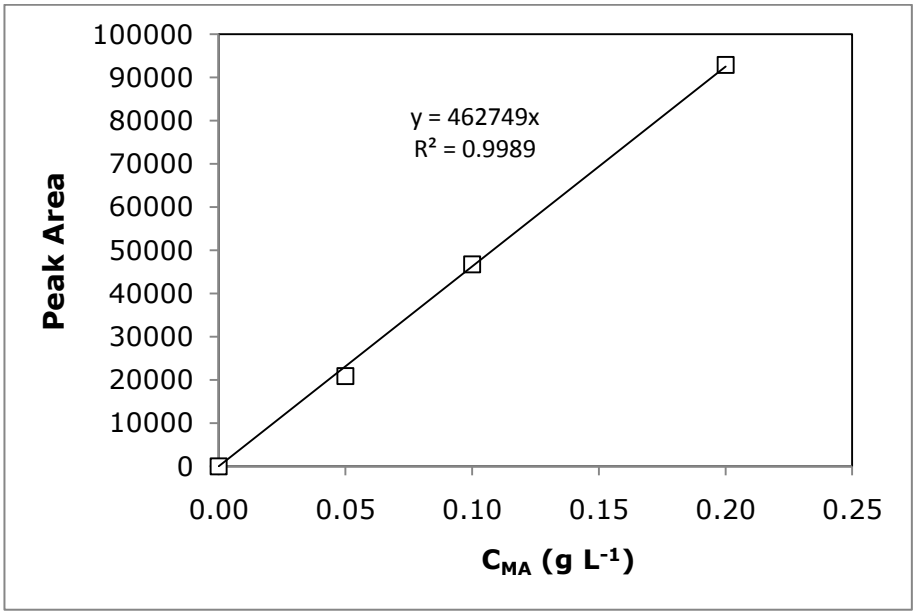

Figure F.8 Calibration curve obtained for malic acid concentration; analysis was performed by HPLC.

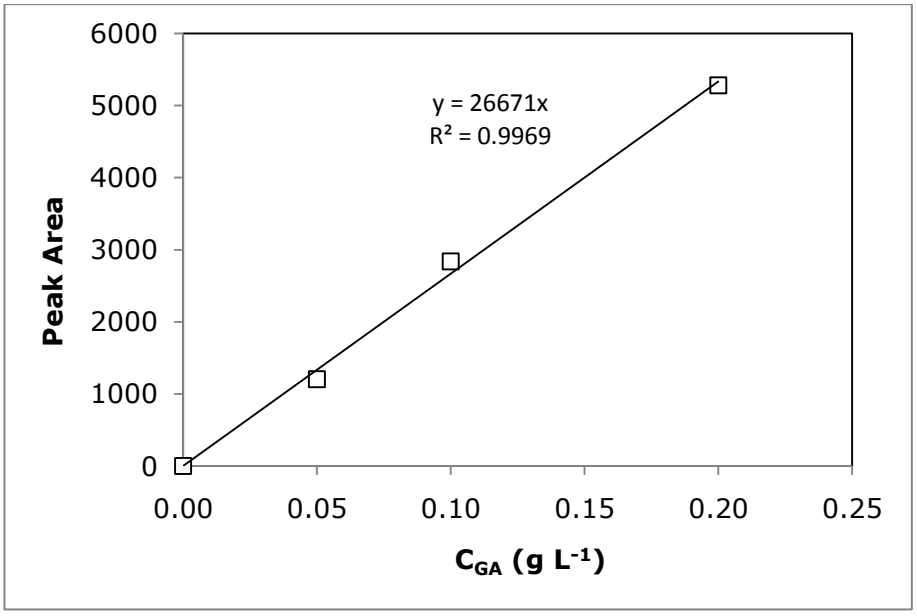

Figure F.9 Calibration curve obtained for glutaric acid concentration; analysis was performed by HPLC.

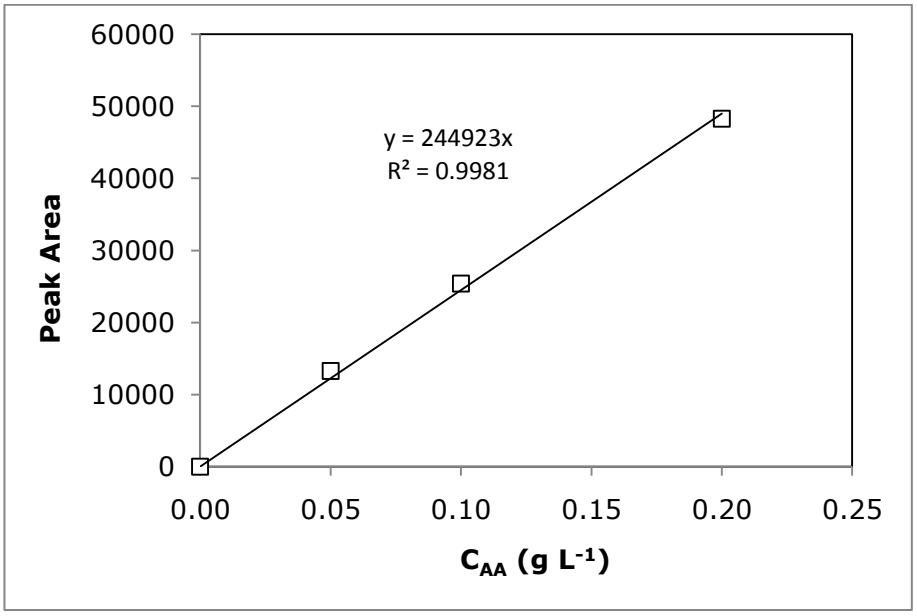

Figure F.10 Calibration curve obtained for acetic acid concentration; analysis was performed by HPLC.

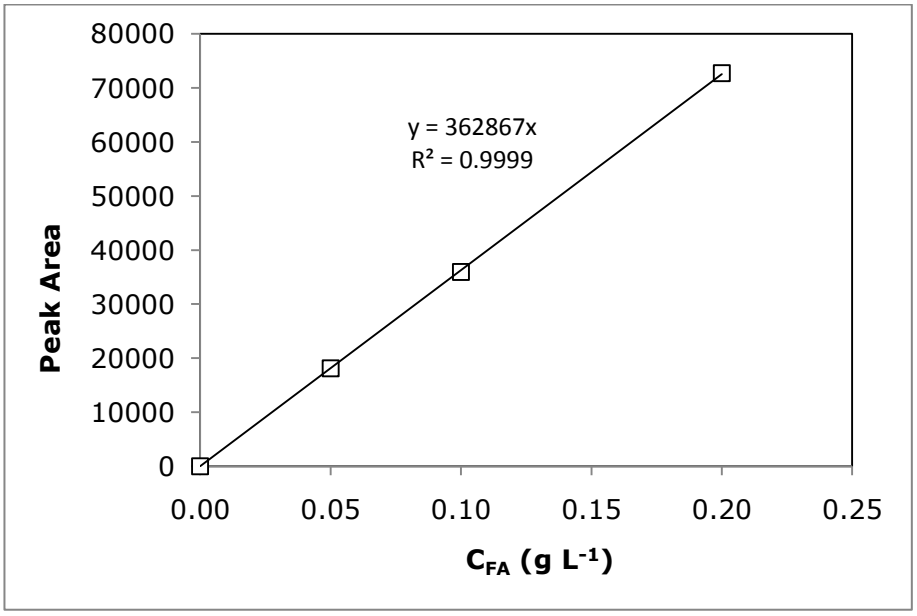

Figure F.11 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

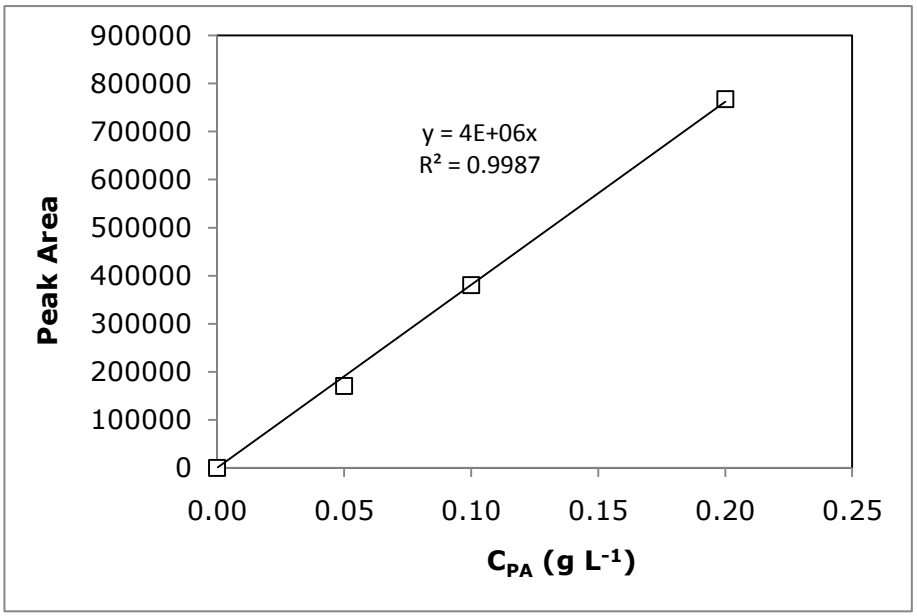

Figure F.12 Calibration curve obtained for pyruvic acid concentration; analysis was performed by HPLC.

APPENDIX G

CALIBRATION CURVE FOR AOX ACTIVITY

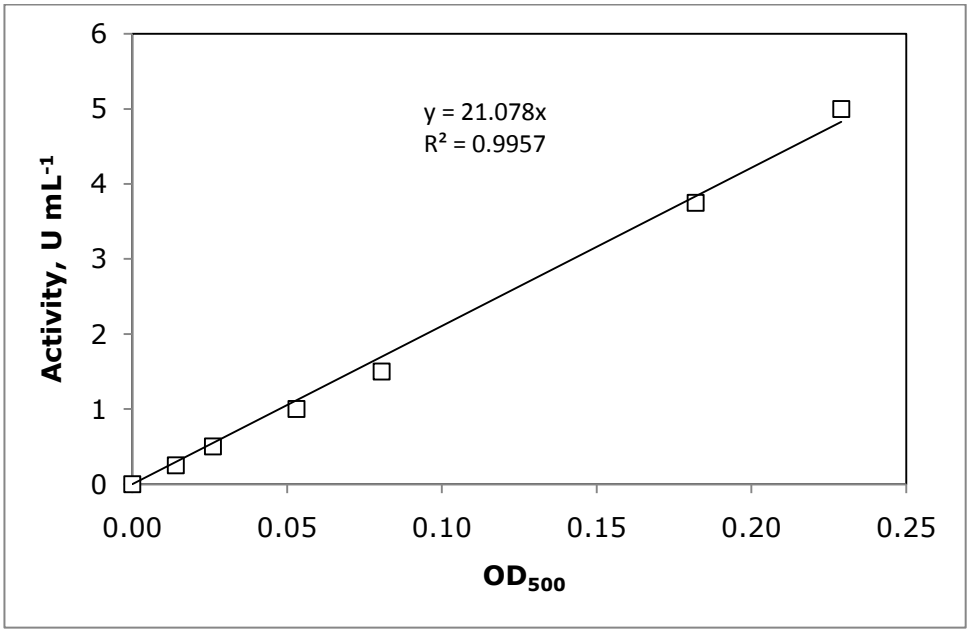

Figure G.1 Calibration curve obtained for AOX activity