EFFECTS OF CO-CARBON SOURCES IN RECOMBINANT HUMAN ERYTHROPOIETIN PRODUCTION BY PICHIA PASTORIS

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

BY

ŞÜKRAN MELDA ESKİTOROS

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

JANUARY 2013

Approval of the thesis:

EFFECTS OF CO-CARBON SOURCES IN RECOMBINANT HUMAN ERYTHROPOIETIN PRODUCTION BY PICHIA PASTORIS

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ABSTRACT

EFFECTS OF CO-CARBON SOURCES IN RECOMBINANT HUMAN ERYTHROPOIETIN PRODUCTION BY PICHIA PASTORIS

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January 2013, 89 pages

In this study, it was aimed to investigate the effects of different co-carbon sources on therapeutically important glycoprotein, recombinant human erythropoietin (rHuEPO) production by *Pichia pastoris* by designing feeding strategies which were applied in the production phase of the bioprocess. During the experiments, the cell growth, sorbitol, mannitol, and methanol consumptions, recombinant human EPO production, alcohol oxidase activity, total protease concentrations and the by-products organic acid concentrations were analyzed.

In this context, firstly, laboratory scale air filtered shake bioreactor experiments were performed by *P. pastoris* Mut^+ strain to investigate the effects of mannitol and sorbitol. 50 gL^{-1} initial concentration of co-substrates was found more affordable and appropriate for cell concentration and recombinant protein production. Thereafter, six pilot scale bioreactor operations were designed and performed. In the first designed strategy (named as SSM strategy), batch-wise 50 g L^{-1} sorbitol was fed at t=0 h of the production phase and then sorbitol concentration was kept constant at 50 g L^{-1} by fed-batch feeding with a pre-determined specific growth rate of μ_{Srb0}=0.025 h^{-1} within t=0-15 h of the production phase together with fed-batch methanol feeding with a pre-determined specific growth rate of μ_{M0}=0.03 h^{-1}. In the following bioreactor experiments co-substrate mannitol was fed to the system with different feeding strategies together with fed-batch methanol feeding with a pre-determined specific growth rate of μ_{M0}=0.03 h^{-1}. In the second strategy (MM), only 40 g L^{-1} mannitol was added to the system at t=0 h of the production phase. In the third strategy (MMM), after adding 50 g L^{-1} mannitol at t=0 h, mannitol concentration was kept constant at 50 g L^{-1} by fed-batch feeding with a pre-determined specific growth rate of μ_{Man0}=0.11 h^{-1} within t=0-9 h of the production phase when the same cell concentration was attained in SSM strategy. In the fourth one (MLM), limiting amount of mannitol, 3 g L^{-1}, was added at t=0 h and then mannitol concentration was kept constant at 3 g L^{-1} by fed-batch feeding with a pre-determined specific growth rate of μ_{Man0}=0.005 h^{-1} within t=0-10 h of the production phase. After these strategies, several pulses, batch-wise, mannitol feeding strategies were performed. In the fifth strategy (MPM), besides 50 g L^{-1} initial mannitol feeding at t=0 h, adding second batch-wise mannitol at t=6 h, and third one at t=12 h were applied. In the last strategy (MPMG), four 50 g L^{-1} pulse feeding of mannitol were performed at t=0 h, 7 h, 14 h, and 24 h, containing glycerol, with an initial concentration in the fermentation medium being 8 g L^{-1}. The highest extracellular rHuEPO production was achieved in the fifth strategy MPM as C_{rHuEPO}=645 mg L^{-1} at t=9 h while the highest cell concentration was achieved in the first strategy SSM as C_x=109 gL^{-1} at t=48 h. The overall cell and product yields on total substrate were calculated as $Y_{X/St}$=0.22 g g^{-1} and $Y_{P/St}$=2.23 mg g^{-1} in the highest rHuEPO production case.

Keywords: Recombinant human erythropoietin, *Pichia pastoris*, co-carbon sources, feeding strategy

ÖZ

PICHIA PASTORIS İLE REKOMBİNANT İNSAN ERİTROPOİETİN ÜRETİMİNDE İKİNCİ KARBON KAYNAKLARININ ETKİLERİ

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Ocak 2013, 89 sayfa

Bu yüksek lisans tezinde, farklı ikinci karbon kaynaklarının değişik üretim stratejileri kullanılarak, *Pichia pastoris* ile terapatik proteinlerden glikoprotein yapısındaki rekombinant insan eritropoietin (rHuEPO) üretimi üzerine etkilerinin araştırılması amaçlanmıştır. Proses süresince hücre, sorbitol, mannitol, metanol, rHuEPO, AOX, proteaz ve yan ürünler olan organic asit derişimleri ölçülerek izlenmiştir.

Belirtilen amaç doğrultusunda ilk olarak, mannitol ve sorbitolün ikinci karbon kaynağı olarak etkileri *P. pastoris* Mut^+ suşu kullanılarak laboratuvar ölçekli biyoreaktörlerde incelenmiştir. Kullanılan farklı başlangıç konsantrasyonları arasından, 50 g L^{-1} ikinci karbon kaynağı konsantrasyonun hücre derişimi ve rekombinant protein üretimi için daha ekonomik ve uygun olduğu saptanmıştır. Sonraki aşamada, altı çeşit biyoreaktör işletim stratejisi tasarlanmıştır. Tasarlanan ilk stratejide (SSM) kesikli işletimle 50 g L^{-1} sorbitol, üretim fazına geçiş anı t=0 st'te beslenmiş ve ardından proses boyunca yarı-kesikli işletimle önceden belirlenen özgül çoğalma hızı μ_{M0}=0.03 st^{-1} olacak şekilde metanol beslemesiyle eşzamanlı olarak, üretim fazının t=0-15 st arasında biyoreaktördeki sorbitol derişimi 50 g L^{-1} değerinde, özgül çoğalma hızı μ_{Srb0}=0.025 st^{-1} olacak şekilde yarı-kesikli beslemeyle sabit tutulmuştur. Bunu takip eden biyoreaktör deneylerinde ikinci karbon kaynağı olan mannitol kullanılmış, her birinde metanolde özgül çoğalma hızı μ_{M0}=0.03 st^{-1} olacak şekilde yarı-kesikli beslenmiştir. Uygulanan ikinci stratejide, (MM) yalnızca üretim fazına geçiş anı t=0 st'te 40 g L^{-1} mannitol eklenmiştir. Tasarlanan üçüncü stratejide (MMM), üretim fazı başında 50 g L^{-1} mannitolün ortama eklenmesinden sonra üretim fazında, ilk tasarlanan strateji (SSM) ile aynı hücre konsantrasyonuna gelene kadar, t=0-9 st arasında, özgül çoğalma hızı μ_{Man0}=0.11 st^{-1} olacak şekilde mannitol konsantrasyonu 50 g L^{-1} de sabit tutulmuştur. Dördüncü stratejide (MLM), limit miktar olan 3 g L^{-1} mannitol, üretim fazına geçiş anında kesikli olarak eklenmiş, devamında üretim süresince t=0-10 st arasında özgül çoğalma hızı μ_{Man0}=0.005 st^{-1} olacak şekilde mannitol konsantrasyonu 3 g L^{-1} de sabit tutulmuştur. Tasarlanan bu stratejilerden sonra, birden fazla kesikli mannitol eklemelerinin yapıldığı yeni besleme stratejileri geliştirilmiştir. Tasarlanan beşinci stratejide (MPM), üretim fazının t=0, 6 ve 12 st de 50 g L^{-1} mannitol kesikli olarak eklenmiştir. En son tasarlanan stratejide (MPMG) ise, t=0, 7, 14, ve 24 st'de dört defa kesikli olarak, 50 g L^{-1} mannitol ile fermentasyon ortamında 8 g L^{-1} olacak şekilde gliserol beslenmiştir. En yüksek rekombinant protein derişimine 645 mg L^{-1} olarak beşinci stratejinin (MPM) t=9 st'te elde edilirken, en yüksek hücre derişimi olan 109 g L^{-1} ye ilk strateji (SSM) ile t=48 st de ulaşılmıştır. En yüksek üretimin elde edildiği üretim koşulunda, toplam substrat üzerinden elde edilen en yüksek hücre ve ürün verimleri, sırasıyla $Y_{X/St}$=0.22 g g^{-1} ve $Y_{P/St}$=2.23 mg g^{-1} olarak bulunmuştur.

Anahtar Kelimeler: Rekombinant insan eritropoetini, *Pichia pastoris*, karbon kaynakları, besleme stratejileri

ACKNOWLEDGMENTS

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

I am also thankful to my co-supervisor Dr. Eda Çelik-Akdur for her valuable advices and critics.

I am also thankful to my friends in Industrial Biotechnology and Metabolic Engineering Research Group for their help and advice. I would like to especially thank to Burcu Gökbudak, Bahar Bozkurt, Burcu Akdağ, Özge Ata, Burcu Gündüz, Gül Zerze, Erdem Boy and Ali Toğay for their great friendship and support for two years in sleepless days and nights. And also I am thankful to Research Group of Prof. Dr. Ufuk Bölükbaşı for their friendship and cooperation during the laboratory studies.

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

I also like to acknowledge the financial supports of The Scientific and Technological Research Council of Turkey (TUBITAK-BIDEB 2210) and the Middle East Technical University Research Fund Project.

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

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NOMENCLATURE

C	Concentration	g L^{-1} or mol m^{-3}
EPO	Erythropoietin	
N	Agitation rate	min^{-1}
Q	Volumetric flow rate	L h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
r	Formation or consumption rate	g g^{-1} h^{-1}
t	Cultivation time	h
T	Bioreaction liquid medium temperature	°C
U	One unit of an enzyme	U
V	Volume of the bioreactor medium	L
Y	Yield (overall)	g g^{-1}

Greek Letters

ρ	Density	g L^{-1}
μ	Specific growth rate	h^{-1}
μ_t	Total specific growth rate	h^{-1}
μ_{M0}	Pre-determined specific growth rate on methanol	h^{-1}
μ_{Srb0}	Pre-determined specific growth rate on sorbitol	h^{-1}
μ_{Man0}	Pre-determined specific growth rate on mannitol	h^{-1}
μ_{Gly0}	Pre-determined specific growth rate on glycerol	h^{-1}

Subscripts

0	Refers to initial condition and stock concentration
AOX	Refers to alcohol oxidase
Gly	Refers to glycerol
M	Refers to methanol

Man	Refers to mannitol
O	Refers to oxygen
p	Refers to protein
Pro	Refers to protease
R	Refers to bioreaction medium
rHuEPO	Refers to recombinant human erythropoietin
rp	Refers to recombinant protein
Srb	Refers to sorbitol
X	Refers to cell

Abbreviations

AOX1	Alcohol oxidase I
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
FLD	Glutathione-dependent formaldeyhde dehydrogenase
GAP	Glyceraldehyde-3-phosphate dehydrogenase
EPO	Erythropoietin
HPLC	High pressure liquid chromatography
MS	50 g L^{-1} batch-wise sorbitol pulse feeding (t=0 h); methanol feeding with μ_{M0}=0.03 h^{-1}
MM	40 g L^{-1} batch-wise mannitol pulse feeding (t=0 h); methanol feeding with μ_{M0}=0.03 h^{-1}
MMM	50 g L^{-1} batch-wise mannitol pulse feeding (t=0 h) and keeping mannitol concentration at 50 g L^{-1} at t=0-9 h; methanol feeding with μ_{M0}=0.03 h^{-1}
MLM	3 g L^{-1} batch-wise mannitol pulse feeding (t=0 h) and feeding mannitol with pre-determined μ_{Man0}=0.005 h^{-1} (t=0-10 h); methanol feeding with μ_{M0}=0.03h^{-1}
MPM	50 g L^{-1} batch-wise mannitol pulse feeding at t = 0, 6, 12 h; methanol feeding with μ_{M0}=0.03 h^{-1}
MPMG	50 g L^{-1} batch-wise mannitol pulse feeding at t = 0, 7, 14 and 24 h with containing glycerol; methanol feeding with μ_{M0}=0.03 h^{-1}
rHuEPO	Recombinant human erythropoietin

SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SSM	50 g L^{-1} batch-wise sorbitol pulse feeding (t=0 h) and keeping sorbitol concentration at 50 g L^{-1} at t=0-15 h; methanol feeding with μ_{M0}=0.03 h^{-1}
TCA	Tricarboxylic acid
TCP	Total cell protein

CHAPTER 1

INTRODUCTION

The word of “biotechnology” refers the use of microorganisms in order to create useful chemical compounds. This explanation shows that biotechnology has a very long historical background because people have been producing useful commodities (e.g., bread, wine, cheese, yogurt, and daily products) by the use of living microorganisms from the prehistoric period until today. Moreover, this new era of technology has rapidly grown and expanded with the increase in the population from the production of antibiotics such as penicillin in the mid-forties to the discovering of the role of DNA as the carrier of genetic information in the early 1950s (Nielsen *et al*., 2003). Thereafter, industrial or “white” biotechnology has been developed in order to produce chemicals, pharmaceuticals and a wide range of products for food, pulp, and textile industries. Many scientists including chemical engineers, microbiologists and biochemists have been dealing with this area to develop novel commodities. By considering these developments, “white” biotechnology depends on environmental friendly products i.e. chemicals, pharmaceuticals and bio-energy. Additionally, industrial biotechnology provides several industries such as energy, chemical and food industry with the opportunity to reduce energy consumption and also diminish the costs. With the advancement in this technology, firstly different methods have been developed in order to produce biomolecules such as hormones, enzymes and antibodies industrially. Then the selected bioprocess has been optimized. Industrial biotechnology that is growing area composes of some bioprocess stages. Firstly, the properties of target protein are examined in detail. Therefore, the applications of the product are searched whether it has a large scale use in the sector. In other words, economic feasibility can be performed. Then, the characteristics of a proper host cell are determined. Host microorganisms well suited for production of the target compound are isolated naturally (Otero and Nielsen, 2010). Furthermore, under controlled environments, the growth and production characteristics of the cells should be obtained (Çelik, 2008). Thus, fermentation strategies are developed in order to obtain efficient bioprocess development and recombinant protein production. Fermentation parameters that are optimum medium composition, pH and temperature are firstly determined in laboratory scale shake bioreactor experiments. Analyses of product are carried out due to different co-substrates in these experiments. After the optimum production conditions are determined, pilot scale bioreactor experiments are performed with adjusting and measuring pH, temperature, stirrer speed etc. Rather than shake bioreactor experiments, better control of fermentation parameters is performed with pilot scale bioreactor (Çelik and Çalık, 2012). During the fermentation, growth, oxygen requirements and foaming are analyzed. Moreover, for investigating effect of different parameters on the production; cell growth, by-product formation, the specific production rates and the yield coefficients should be defined. The best operation mode as batch, fed-batch or continuous should be decided.

The utilization of one of the well-known recombinant therapeutic proteins, erythropoietin (EPO) as a therapeutic drug for low blood level in the human body caused by kidney failure, prematurity, cancer and human immunodeficiency virus infection has been accepted by the U.S. FDA (Jelkmann, 1992), with global sales exceeding $13.1 billion in 2007. EPO is produced in kidneys for adult life and in liver during fetal life (Zanjani *et al*., 1977). Moreover, anemia can occur in the absence of EPO due to kidney failure.

Many hormones used in the clinical applications needs to be produced in mammalian cells. Therefore, EPO was firstly taken from human urine in 1977 by Miyake *et al*. Thereafter some groups

such as Lin *et al.* (1985) expressed human EPO cDNA in mammalian cells that were Chinese Hamster Ovary cells (CHO) and Baby Hamster Kidney cells (BHK) by using recombinant DNA technology. However, using mammalian cell cultures cause significant disadvantages that are low efficiency and high cost. This forced the scientists to acquire new methods. So, Lee-Huang (1984), Elliott *et al*. (1989), and Nagao *et al.* (1997) studied with bacterial and eukaryotic hosts to obtain an alternative host for the production of EPO.

Glycosylation that is one of the post-translational modifications is important for EPO to be the biologically active protein. Kim *et al.* (2005) investigated the *N*-glycan structures of rHuEPO during employing *Drosophila melanogaster* Schneider-2 cells as a simpler eukaryotic expression system. Whereas, prokaryotes cannot glycosylate the proteins, some eukaryotes such as *Saccharomyces cerevisiae* forms hyperglycosyl-type proteins. However, hyper-glycosylated proteins are not biologically active (Bretthauer *et al.*, 1999).

Pichia pastoris that is an alternative to *S. cerevisiae* is a well-known host microorganism for r-protein production. *P. pastoris* expression system has a widespread utilization area on account of its capability to increase recombinant protein production, its ability in performing post-translational modifications, i.e., disulfide bond formation, glycosylation; and the availability of the strong and tightly regulated AOX1 promoter (Cereghino and Cregg 1999). Firstly Çelik *et al*. (2007) cloned and expressed a *P. pastoris* strain and produced rHuEPO extracellularly under the control of AOX promoter. Furthermore, they studied to find a new strategy that is the continuous methanol feeding throughout the bioprocess with batch sorbitol feeding at t=0 h (Çelik *et al*., 2009). The optimum specific growth rate was determined as 0.03 h^{-1} on rHuEPO production and 50g L^{-1} sorbitol as the non-inhibiting concentration limit for production of rHuEPO in the mentioned study. The last study was performed by Soyaslan and Çalık (2011). In the study, different pH values were applied at pH= 4.0, 4.5, 5.0, 5.5 and 6.0 for rHuEPO production by *Pichia pastoris*. Finally, 0.158 g L^{-1} protein was produced at pH=4.5 (Soyaslan and Çalık, 2011).

In this study, the aim is to enhance the production of rHuEPO in *P. pastoris* by using different feeding strategies with different co-substrates. For this purpose, sorbitol and mannitol were used as co-substrates together with the primary carbon source methanol. In this context, the effects of co-substrates were initially determined in the shake bioreactor experiments. In the pilot scale bioreactor experiments the bioprocess was started batch-wise with 50 g L^{-1} sorbitol, and then semi-batch operation was used by continuous sorbitol feeding during the recombinant production phase. Further, the effects of feeding strategies using an alternative co-substrate, i.e., mannitol on cell growth, rHuEPO production and by-product formation were investigated in pilot scale bioreactor.

CHAPTER 2

LITERATURE SURVEY

2.1 Product: Erythropoietin (EPO)

2.1.1 Properties of Erythropoietin

Erythropoietin (EPO) that is a glycoprotein hormone adjusts and controls red blood cell level in bone marrows. EPO is produced in the kidney during adult life and in the liver during fetal life (Zanjani *et al.,* 1977). Firstly, Miyake *et al*. took erythropoietin gene from urine in 1977. Afterwards, human EPO cDNA was expressed in mammalian cells that were Chinese Hamster Ovary cells (CHO) and Baby Hamster Kidney cells (BHK) by using recombinant DNA technology (Lin *et al.,* 1985). However, low efficiency and high cost of mammalian cell cultures has been the driving force to obtain different host microorganisms for EPO formation (Fernandez and Hoeffler, 1999). For that reason, different microorganisms has been tried to produce EPO. For instance, the production of EPO was studied for *E.coli* (Lee-Huang 1984) and for *B. brevis* (Nagao *et al.,* 1997) as a bacterial host and *S. cerevisiae* as an eukaryotic host (Elliott *et al*., 1989). In 2005, Kim *et al.* employed *Drosophila melanogaster* Schneider-2 cells as a simpler eukaryotic expression system and the *N*-glycan structures of rHuEPO were investigated during the production. On the other hand, prokaryotes cannot glycosylate the proteins, although *Saccharomyces cerevisiae* forms hyperglycosyl-type proteins. So these systems cannot be the alternative to CHO cells.

Anemia occurs from the lower EPO level in the blood because of renal failure. EPO entered clinical trials in 1985 and then U.S. FDA has confirmed recombinant human erythropoietin (rHuEPO) as a drug created in Chinese hamster ovary (CHO) cell (Egrie *et al*., 1986). It has been used for the treatment of anemia because of kidney failure, prematurity, cancer, chronic inflammatory disease and human immunodeficiency virus infection (Jelkmann *et al*., 1992).

2.1.2 Structure of Erythropoietin

Studies have revealed that human EPO gene has a single copy that is placed on chromosome 7 in q11-q12 region and it can extend over 5.4 kb region that composes of 193 amino acids (Law *et al.,* 1986; Jacobs *et al*., 1985; Lin *et al*., 1985). However, 165 amino acids form in the mature form of EPO which has a molecular weight about 18 kDa (Lai *et al*., 1986).

EPO is a heavily glycosylated protein having a molecular weight 34-38.5 kDa that has 40% of its total mass composed of carbohydrates (Dordal *et al*., 1985). Residues of EPO and glycan structures have an important effect on its function, structure and stability (Higuchi *at al*. 1992). There are three *N*-glycans that locate at asparagine (Asn) at 24, 38 and 83 and has a *O*-glycan that locate at Ser-126 (Egrie *et al*., 1986; Goldwasser *et al*., 1990). Also human EPO has two disulfide bonds between 7 and 161, and between 29 and 33 (Wang *et al*., 1985) (Figure 2.1).

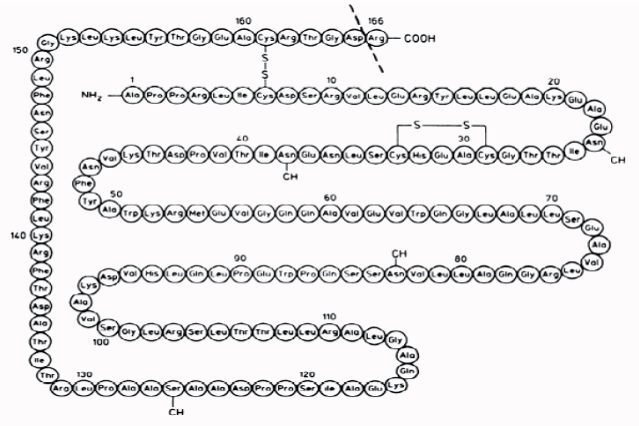

Figure 2.1 The structure of recombinant human erythropoietin (Jelkmann, 1992)

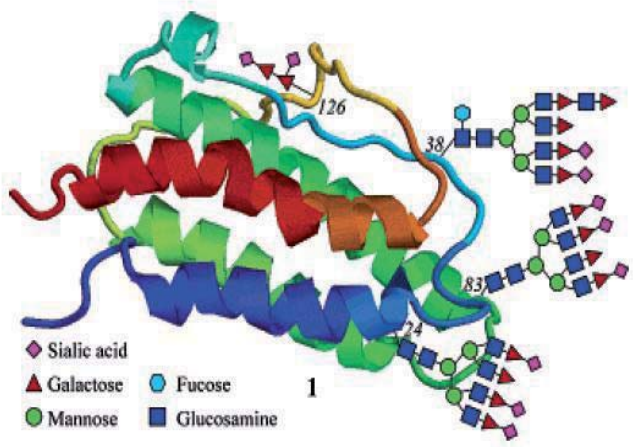

Figure 2.2 Tertiary structure of recombinant human erythropoietin (Warren *et al*., 2004)

2.2 Advantages and Disadvantages of Host Microorganism

The host microorganism selection is the second stage of the development of an industrial bioprocess. In order to select the appropriate microorganism, some parameters should be considered such as having efficient recombinant protein expression and secretion capabilities, producing product with adequate yield and minimum by-products, growing on economical medium and moderate process conditions. Additionally, potential hosts should grow to large cell mass per volume in a reasonable time and be suitable for easy scale-up. Microorganism also should not produce detrimental substances (Kirk and Othmer, 1994; Soetaert and Vandamme, 2010). Microorganisms are more suitable for recombinant protein production rather than plant and animal cells because of simpler cultivation mediums, higher growing rates and easier product purification.

Expression of foreign proteins in yeast is really important for the pharmaceutical industry in order to synthesize the drugs. Yeast that can produce foreign proteins has been used for several therapeutic applications with different properties. The species *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis, Yarrowia lipolytica* and *Arxula adeninivorans* are the most important yeast expression hosts (Çelik and Çalık, 2012). There are some advantages and disadvantages of using yeast for expression systems:

Advantages of using yeasts for expression;

- Economical
- Provide higher yields
- Need less time and effort
- Easy microbial growth
- Manipulation of gene
- Not contain pyrogens, pathogens or viral inclusions
- Ability to accomplish some eukaryotic post-translational modifications

Disadvantages of using yeasts for expression;

- Inability to accomplish complex post-translational modifications: prolyl hydroxylation, amidation, some phosphorylation and hyper-glycosylation.

By the beginning of recombinant production processes, *E. coli* and *S. cerevisiae* started to be used in industry. After some research, it was found out that *E. coli* and *S. cerevisiae* have some disadvantages. Although *E. coli* may not perform post-translational modifications*, S. cerevisiae* has the hyper-glycosylation and changes the protein functionality. On the other hand, *P. pastoris* has become more popular in recent times and has more advantages for recombinant protein production (Çelik and Çalık, 2012). Therefore, since 1984 this culture system has been used to express approximately 300 recombinant proteins. The utilizabilities and limitations of *P. pastoris* system are itemized below:

Factors for this system utilizability (Cereghino and Cregg, 1999; Macauley-Patrick *et al*., 2005; Çelik and Çalık, 2012):

- Higher yield and productivity can be obtained.
- *AOX1* promoter that is the mostly regulated one is used.
- Purification cost is lower.
- It has a eukaryotic post-translational modification as glycosylation and disulfide-bond formation.
- *P. pastoris* does not have as much hyper-glycosylation as *in S. cerevisiae.*
- Expression plasmids can be stably combined to specific sites in the genome.
- Strains can be cultivated in the bioreactors.
- A kit is found from Invitrogen Co.
- It does not have any pathogenic effect and cause any endotoxin problems.
- This microorganism has a broad range of pH between 3 and 7 for growing.
- Methanol can be used as a carbon source.
- *P. pastoris* chooses respiratory mode of growth rather than fermentative causing lower by-product production.

Limitations to this system utilizability:

- Methanol has a risk of firing.
- *P. pastoris* does not have promoters that are moderately expressed. High level expression that is occurred with *AOX1, FLD1* and *GAB* are harmful.
- There are not a lot of selectable markers for transformation such as *HIS4, ARG4* and *Sh ble.*
- Cell cultivation of *P. pastoris* is longer than bacteria.
- It has a proteolytic activity.

2.2.1 Host Microorganism: *Pichia pastoris*

Phillips Petroleum Company developed the *Pichia pastoris* growing culture system on methanol in 1970s. During that time, researches on *Pichia pastoris* were not improved due to the increase in the price of methanol with Oil crisis (Cos *et al.,* 2006). But one decade later, the developments on *P. pastoris* were accelerated for academic and industrial purposes. Then Invitrogen Corporation was licensed by Phillips Petroleum Company in order to sell *P. pastoris* expression system components. Today, lots of heterologous proteins have been produced by the help of *P. pastoris* culture system (Cregg, 2004; Macauley-Patrick *et al*., 2005)

2.2.1.1 General Characteristics

Pichia pastoris that is methylotrophic yeast has been studied to express lots of different biological products (Sreekrishna, 1997; Chiruvolu and Cregg, 1997). The assortment of *Pichia pastoris* can be defined as the kingdom *Fungi*, Division *Eumycota*, Subdivision *Ascomycotina*, Class *Hemoascomycetes*, Order *Endomycetales*, Family *Saccharomycetaceae* and Genus *Pichia* (Cregg, 1999). *P. pastoris* is a unicellular fungus. Its eukaryotic cells are 1-5 μm wide by 5-30 μm long with a cell wall. Since *P. pastoris* chooses a respiratory mode for growth, fermentation products do not build up quickly (Cereghino *et al*., 2002). Primarily, it has been started to be used expression microorganisms of several proteins that has drawn attention to industrial and academic fields (Cereghino and Cregg, 1999, 2000).

2.2.1.2 *Pichia pastoris* Expression system

Pichia pastoris is the most preferable microorganism since it has a strong, tightly regulated promoter, *AOX1* which is affected from methanol (Cereghino and Cregg, 2002; Çelik and Çalık, 2012). Many host strains of *Pichia pastoris* grow on methanol easily (Cereghino and Cregg, 2000). Alcohol oxidase that has two genes (*AOX1*, *AOX2*) is initially used in the methanol consumption pathway. *AOX1* promoter is highly affected in methanol existence whereas the *AOX2* is slightly induced (Cregg, 1999).

The methanol utilization ability is shown for *P. pastoris* phenotypes (Stratton *et al*., 1998; Jungo *et al*., 2006):

- **Methanol utilization plus (Mut^+) phenotype:** grown on methanol; presence of functional *AOX1* and *AOX2* genes; and more sensitivity to excess methanol. The highest μon methanol is obtained as 0.14 h^{-1}.

- **Methanol utilization slow (Mut^S) phenotype:** corruption of *AOX1* gene; depending on the transcriptional weaker AOX2 gene; and slowly grown on methanol. The highest μ on methanol is obtained as 0.04 h^{-1}.

- **Methanol utilization minus (Mut^-) phenotype:** corruption of *AOX1* and *AOX2* genes; not to utilize methanol at all; and the requirement of another carbon source. It has μ=0.03 h^{-1} on methanol.

Pichia pastoris has several alternative promoters as *GAP, FLD1, PEX8* and *YPT1* to *AOX1* promoter. For using of *GAP*, methanol is not needed for induction but in order for production of toxic proteins for the yeast it is not sufficient during expression of *GAP* promoter. Methanol as a sole carbon source or methylamine as a sole nitrogen source is used for induction of the *FLD1* promoter. The *YPT1* is expressed in glucose, methanol or mannitol as carbon sources. The *PEX8* gene is needed for peroxisomal biogenesis (Cereghino and Cregg, 2000).

2.2.1.3 Metabolism of *Pichia pastoris*

Glycerol, methanol and also sorbitol and mannitol metabolisms and their pathways should be examined, so the host microorganism expression system can be comprehended under the control of *AOX1* promoter.

Glycerol metabolism:

General growth mechanism of *P. pastoris* is started with glycerol as a carbon source because biomass yield and maximum specific growth rate of glycerol are higher than those of methanol. However glycerol represses the protein expression during the growth. If glycerol is compared with glucose, glycerol will be preferred, since higher amounts of ethanol formation that are by-product are observed on glucose using as a carbon source (Macauley-Patrick *et al*., 2005). Besides lower ethanol concentrations repress the alcohol oxidase promoter at levels of 10-50 mg L^{-1} (Inan and Meagher, 2001).

The glycerol metabolic pathway is schematically showed in Figure 2.3. Firstly, glycerol is converted to G3P by phosphorylation reaction with glycerol kinase. Then G3P is turned to dihydroxyacetone phosphate (DHAP) by oxidation with glycerol-3-phosphate dehydrogenase (Nevoigt and Stahl, 1997). Later, oxidation of pyruvate that is the product of the glycolysis occurs. As a result of oxidation, acetyl-CoA is formed. Moreover acetyl-CoA goes into TCA cycle to produce several cellular constituents, i.e., amino acids, nucleic acids, cell wall components. Also TCA cycle provides energy for biomass and maintenance as as form of ATP and NADH. At the same time, acetaldehyde is formed from pyruvate by pyruvate decarboxylase and then ethanol is formed by the oxidation of acetaldehyde by alcohol dehydrogenase in the fermentative bypass (Inan and Meagher, 2001). After the oxidation of ethanol to acetaldehyde, acetate is formed by acetaldehyde dehydrogenase, and it is turned into acetyl–CoA by acetyl–CoA synthetase (Pronk *et al*., 1996; Vanrolleghem *et al*., 1996). Whereas a small part of biomass comes from G3P, the biggest one is from acetyl–CoA (Ren *et al*., 2003).

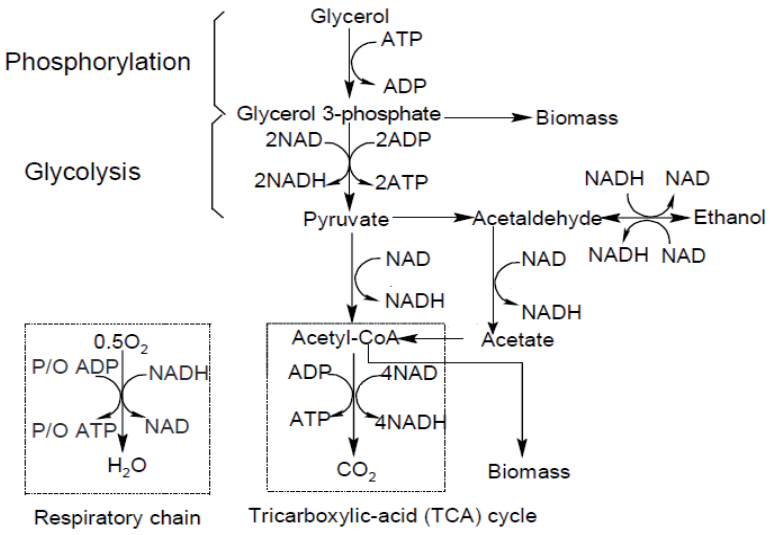

Figure 2.3 Metabolic pathway of glycerol in *Pichia pastoris* (Ren *et al*., 2003).

Methanol Metabolism:

Metabolic pathway including different enzymes is followed during the methanol utilization as shown in Fig. 2.4. Methanol utilization pathway starts with the oxidation reaction. Methanol was converted to formaldehyde and hydrogen peroxide by alcohol oxidase (AOX). When methanol was found in the production medium, AOX can be responsible to form approximately 35% of the total

proteins but it is repressed by the carbon sources as glucose, glycerol and ethanol (Walker, 1998). *Peroxisomes* are specialized organelles that isolate AOX to avoid toxicity of hydrogen peroxide produced in the reaction. In the peroxisome, degradation of hydrogen peroxide to oxygen and water occurs with catalase. After leaving of a portion of formaldehyde from peroxisome, it enters the dissimilatory pathway and the oxidation of formaldehyde to formate and carbon dioxide is proceeded with formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH), respectively, providing reducing power in the form of NADH (Cereghino and Cregg, 2000).

The rest of formaldehyde goes on cytosol. Formaldehyde is turned to xylulose-5-phosphate by dihydroxyacetone synthase (DHAS) to glyceraldehyde-3-phosphate (GAP), which enters the TCA cycle to yield energy and dihydroxyacetone (DHA). DHA enters xylulose monophosphate cycle and forms xylulose-5-phosphate. The biomass formation comes from GAP and acetyl–CoA (Cereghino and Cregg, 2000).

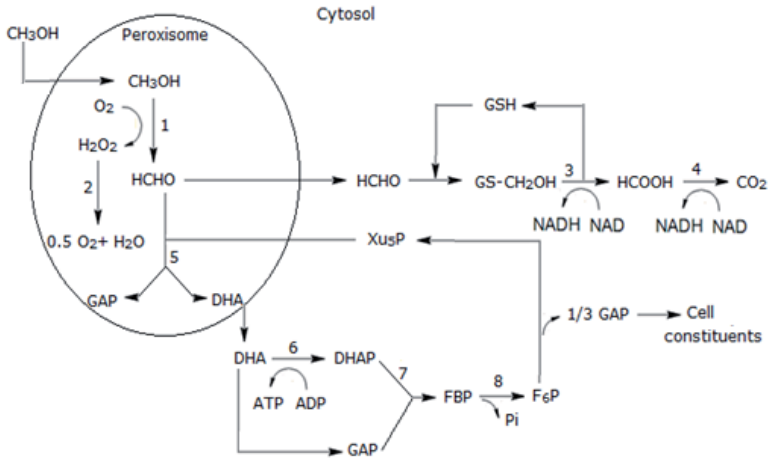

Figure 2.4 Methanol utilization pathway in *Pichia pastoris.* 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, di-hydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase (Cereghino and Cregg,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

Sorbitol and Mannitol Metabolism:

In order to improve r-protein expression by host microorganism, addition of multi-carbon source besides methanol has been investigated in the literature (Zhang *et al.*, 2003).

In this study, mannitol and sorbitol that are less repressive to methanol utilization pathway are used as co-substrates in order to enhance the productivity of rHuEPO (Sreekrishna *et al.*, 1997).

Mannitol and sorbitol enter to the glycolysis pathway. Before entering they converted to other metabolites. Sorbitol is firstly converted to fructose by D-glucitol dehydrogenase and then enters the mannitol cycle. It is turned to fructose-6-phosphate by fructokinase (Figure 2.5). However, metabolism of mannitol involves specific hexitol phosphate dehydrogenase activity (Figure 2.5) (Walker, 1998) and it can be used two different ways. One of them is the same as sorbitol, which is oxidized to fructose and then turned to F6P. The other one is that mannitol is converted to mannitol-1-phosphate by mannitol kinase and then turned into fructose-6-phosphate by mannitol-1-phosphate dehydrogenase.

Mannitol dehydrogenases have been studied in several organisms and two forms were described, one NAD^+-dependent and other $NADP^+$-dependent. D-mannitol and NADPH are oxidized by mannitol dehydrogenase predominantly, and it reduces D-Fructose and $NADP^+$, but some of the organisms oxidizes at low rate also D-glucitol (sorbitol) and may use also NAD^+ and NADH as cofactors. Apparently the enzyme activity is dependent on the carbon source used for growth (Quain and Boulton, 1987). In the case of mannitol grown cells, mannitol may be also utilized through the fructose pathway. This is probably the case, since hexokinase has the highest activity values in mannitol grown cell.

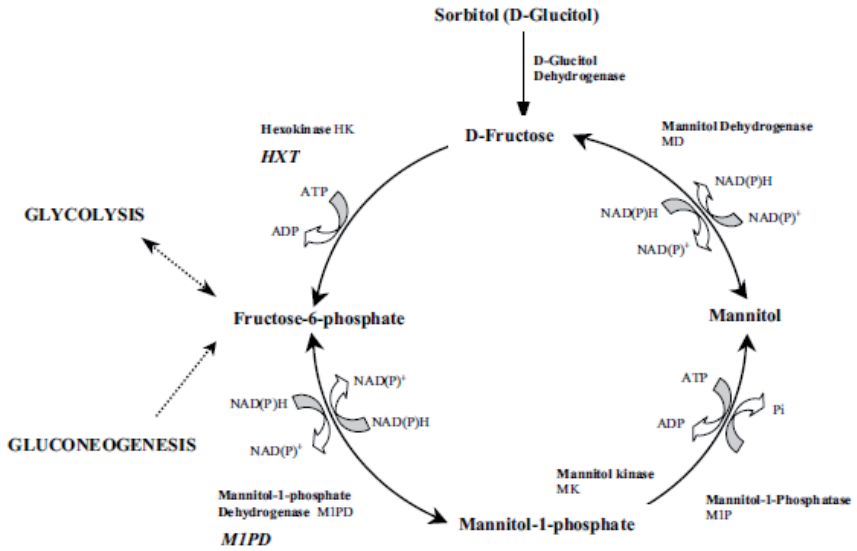

Figure 2.5 Metabolic pathways of sorbitol and mannitol in yeasts. 1, D-glucitol dehydrogenase; 2, D-fructokinase; 3, mannitol-phosphate dehydrogenase; 4, mannitol-1-phosphatase; 5, mannitol dehydrogenase (Walker, 1998).

2.2.1.4 Post-translational modification of secreted proteins

Glycosylation has an important role on the protein modification that is found in bacteria, archaea and eukarya (Varki *et al.*, 2009). Moreover it has a critical role on the protein stability against proteolysis, solubility, rigidity, immune response, cellular signaling and adhesion and intracellular localization (Larkin and Imperiali, 2011). However, glycosylation can be different for every host and also clone (Werner, 2007).

Several host cells can be utilized as host cells for glycoprotein productions. Moreover, glycosylation varies the molecular masses of rHuEPO that was produced and secreted such as ~35 kDa in CHO cells (Lin *et al.*, 1985), ~31 kDa in tobacco cells (Matsumoto *et al.*, 1995), ~25 kDa in *Drosophila* S2 cells (Kim *et al.*, 2005), higher than 29 kDa in *S. cerevisiae* (Elliott *et al.*, 1989) and ~30 kDa in *Physcomitrella patens* (Weise *et al.*, 2007), while human urinary EPO (or native EPO) is reportedly ~34 kDa (Dordal *et al.*, 1985). Çelik *et al.* (2007) notified that the molecular mass of rHuEPO expressed in *P. pastoris* was 30 kDa.

P. pastoris can perform *N*- and *O*- linked glycosylation by linkage of carbohydrate moieties in order to secrete proteins (Cereghino *et al.*, 2002). *N*-linked glycosylation includes the transfer of an oligosaccharide onto the side chain amide nitrogen of asparagine residues within the acceptor proteins. Therefore, when Asn-X-Thr/Ser sequences, where X is any amino acid except proline, is introduced to the precursor transferring enzyme *N*-linked glycosylation is started (Werner, 2007). *O*-linked

glycosylation adds the monosaccharides in a sequential manner onto the side chain hydroxyl oxygen atom of either serine or threonine residues.

2.2.1.4.1 *N*-Linked Glycosylation

- ***N*-Linked Glycosylation in ER:**

N-linked glycosylation is firstly performed at the membrane of the endoplasmic reticulum (Helenius and Aebi, 2001), in eukaryotes such as humans, other high mammalians and yeasts (Wildt *et al.*, 2005). Firstly, UDP-GlcNAc and GDP-Man that are high concentration of nucleotide sugar donors are found as substrates for glycosyltransferases in the glycan assembly. After the production of heptasaccharide is finished, $Man_5GlcNAc_2$-PP-Dol intermediate transferred from cytoplasmic side to inside part of ER but the process can not be known exactly. After that point, further elongation continues to form at the inside part of ER. Finally, a tetradecasaccharide ($Glc_3Man_9GlcNAc_2$) is transferred from a dolichyldiphosphate carrier onto the amide side chain nitrogen of an acceptor protein. All eukaryotes that include from yeast to humans occur this process and it is conserved in all of them (Larkin and Imperiali, 2011). The *N*-linked glycosylation pathway at the ER membrane for *S. cerevisiae* is shown in Figure 2.6.

After $Glc_3Man_9GlcNAc_2$-PP-Dol has been formed, it is bound to asparagine in the N-X-S/T consensus by a multimeric oligosaccharyl transferase (OT) complex. After it is transferred, the glucosidases GLS-I and GLS-II trim the glucose residues. Then one of the mannose residue is removed by mannosidase-I (Man-I). From now on, this glycoprotein that is $Man_8GlcNAc_2$ can go out from the ER. Therefore, it is transported to the Golgi apparatus (Callewaert *et al.*, 2010).

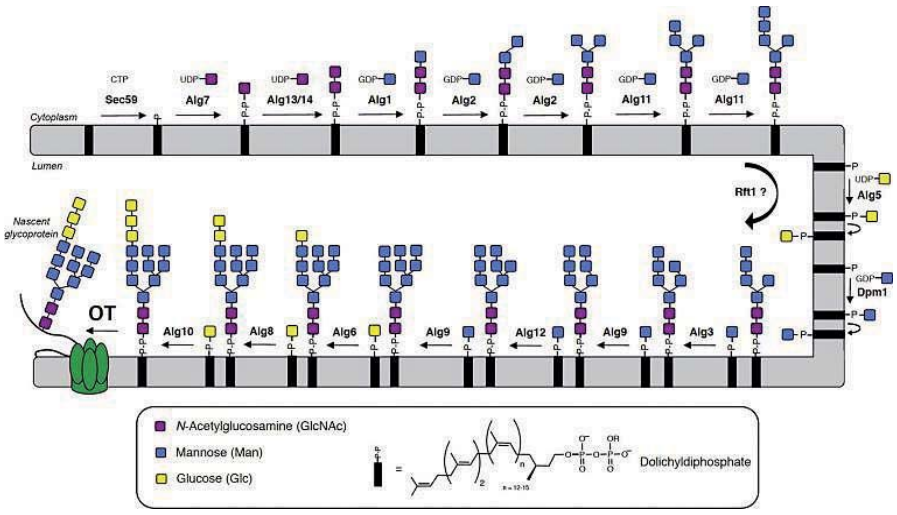

Figure 2.6 Pathway of *N*-linked glycosylation at the ER membrane (Larkin *et al.*, 2011).

- ***N*-Linked Glycosylation in Golgi:**

N-linked glycan has a different processing in the Golgi (Wildt *et al.*, 2005). Further trimming of mannoses occurs after the moving to the Golgi apparatus. Moreover during the terminal glycosylation new sugars that include GlcNAc, galactose, sialic acid and fucose are added in order to produce complex *N*-linked glycans in some cases (Helenius and Aebi, 2001). The *N*-Linked glycosylation pathway in Golgi apparatus is shown in Figure 2.7.

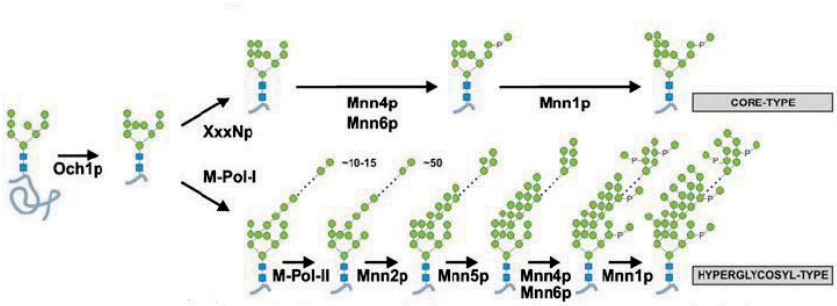

Figure 2.7 Pathway of *N*-linked glycosylation in Golgi apparatus (Callewaert *et al.*, 2010).

P. pastoris is more preferable host microorganism due to its short polysaccharide chains with the deficiency of α-1,3-mannosylation part (Bretthauer *et al.*, 1999; Macauley-Patrick *et al.*, 2005). Çelik *et al.* (2007) notified that the molecular mass of rHuEPO was 30 kDa and that the major glycan attached to all three *N*-linked glycosylation sites was $Man_{17}(GlcNAc)_2$.

2.2.1.5 Proteolytic Degradation in *Pichia pastoris*

Proteolytic degradation of heterologous protein in *Pichia pastoris* fermentations is an important problem to overcome. It causes the deficiency of biological activity of protein. Therefore, it reduces the product yield (Kobayashi *et al.*, 2000).

Vacuolar and extracellular proteases are present in *P. pastoris*. However, the secretion of extracellular proteases is lower (Jahic *et al.*, 2006). Therefore the vacuolar proteases cause the large part of the proteolytic degradation.

If cells are kept viable, vacuolar proteases secreted into the production medium can be prevented. Addition of protease inhibitors (Kobayashi *et al.*, 2000) helps to deal with the problem caused by the yeast lysis from the cultivation parameters, starvation and some harmful substances (Hilt and Wolf, 1992). The use of protease deficient strains such as SMD1163 (*his4 pep4 prb1*), and SMD1168 (*his4 pep4*) is the way rather than changing operation conditions; pH, temperature and medium compositions; carbon, nitrogen sources (Cereghino and Cregg, 2000).

2.3 Medium Design and Bioreactor Operation Parameters

Design of the optimal production system has depended on fermentation parameters, growth conditions, host cell physiology, protease activity, translation start codon context, secretion signals, expression cassette copy number, and mode of chromosomal integration of the expression cassette (Sreekrishna *et al.*, 1997).

Moreover, maximizing efficiency, production level of proteins, and also obtaining a recurrent protein expression under controlled and automated conditions are important for industrial purposes (Cos *et al.*, 2006). The best conditions that are medium, pH, temperature should be different with respect to strain and the foreign protein expressed (Sinha *et al.*, 2003). In order to bioreactor design there are some important steps shown below:

1. Medium design
2. Fermentation parameters design
    - pH
    - Temperature

- Oxygen transfer rate

2.3.1 Medium Composition Design

An organism can grow and produce metabolites with the help of the interaction between intercellular and extracellular effectors. Therefore, determination of necessary components and their concentrations in the medium is the most essential thing for the design of the medium (Scragg, 1988).

A fermentation medium should include carbon, nitrogen, energy sources, mineral sources for cell growth; all necessary growth factors for high product yield (Nielsen *et al*., 2003).

- **Water:** It is the most important component for all media.
- **Nutrients:** They can be divided into two groups.

1. **Macronutrients:** They are needed in the medium at concentrations higher than 10^{-4} M. Carbon, nitrogen, oxygen, magnesium, potassium, sulphur are the major macronutrients.

2. Micronutrients: They are needed in concentrations of less than 10^{-4} M such as trace elements; Ca^{2+}, Cu^{2+}, Fe^{2+}, Na^{2+}, Mn^{2+}, Mo^{2+}, Zn^{2+}, and vitamins, hormones etc. (Shuler and Kari, 2002).

- **Trace Elements**: Less than 0.1% in the medium
- **Buffers**: Control the pH of bioreactor liquid media.
- **Antifoams:** Surface active agents

There are two basic types of growth media; defined and complex medium. Defined medium contains the exact amounts of chemical compounds whereas complex medium contains unknown amounts of chemical. However, complex medium that includes yeast extract, peptones, molasses can increase cell concentration than defined medium. However, defined medium has better fermentation control, easier and cheaper recovery and purification of the protein (Shuler and Kargi, 2002).

In order to obtain high cell concentrations with r-protein production in fermentation processes, basal salt medium (BSM) with trace salt medium (PTM1) is mostly selected fermentation medium (Çelik and Çalık, 2012) proposed by Invitrogen (Invitrogen, 2000). PTM1 contains micronutrients like Fe, Mn, Cu and biotin, etc. Boze *et al*. (2001) reported that using vitamins and trace salts in BSM medium is more effective on the production than using BSM and PTM1. This medium is accepted as a standard but it cannot be optimum (Cos *et al*., 2006). For that reason, alternative media like d'Anjou that was proposed by d'Anjou *et al*. (2000) or FM22 that was formulated by Stratton *et al*. (1998) have been described for high cell densities in fed batch cultures. There are some similarities and also differences between them. The BSM medium has the basic elements that are higher than others.

Nitrogen source is other important nutrient in *P. pastoris* fermentations. Ammonium hydroxide is added into the BSM and FM22 as a nitrogen source for controlling pH. However, all nitrogen is supplied at the initially in the d'Anjou medium (Cos *et al*., 2006). The important thing for nitrogen source is to avoid the accumulation of it because it inhibits the growth and enlarges the lag phase (Yang *et al*., 2004).

Carbon source plays an important role on recombinant protein production and cell growth. *P. pastoris* mostly select methanol, glycerol, sorbitol, mannitol, trehalose, and acetate as a carbon source (Brierley *et al*., 1990; Sreekrishna *et al*., 1997; Thorpe *et al*., 1999; Inan and Meagher, 2001; Xie *et*

al., 2005). Methanol is the most crucial primary substrate since it induces the expression of recombinant protein in Mut^+ and Mut^s strains of *P. pastoris* and also it is utilized as carbon and energy source. However, biomass is inhibited by methanol above certain levels that is the toxic limit of 4 g L^{-1} (Zhang *et al*., 2000-b). Therefore, fed-batch fermentation preferred generally during methanol utilization.

2.3.2 Bioreactor Operation Parameters

There are some parameters that affect the bioprocesses such as pH, T, DO, agitation rate and composition of the medium in the bioreactor. By modifying bioreactor operation conditions, cell and product yield can be increased by the help of changing metabolic fluxes and influencing metabolic pathways (Çalık *et al*., 1999).

2.3.2.1 pH

Hydrogen ion concentration (pH) has an important role on the enzyme activities, cell growth rate, gene expression, protein secretion and proteolytic degradation in the fermentation processes (Macauley-Patrick *et al*., 2005).

P. pastoris has a wide pH range, containing from 3.0 to 7.0 (Cregg *et al*., 1993). However, the stability and nature of recombinant protein that is produced by *P. pastoris* change the optimum pH value for growth (Inan *et al*., 1999).

There are several studies to find optimum pH values. The production pH of insulin-like growth factor-I, was found 3.0 (Brierly *et al*., 1994). pH = 4.0 was found optimum to produce high-level of enterkinase (Zhang *et al*., 2009). The production of hGH was highly increased at pH = 5.0 (Çalık *et al*., 2010). The most suitable pH values for mouse epidermal factor (Clare *et al*., 1991) and α-amylase (Choi and Park, 2006) was determined as pH 6.0, whereas pH is 6.3 for mini-proinsulin (Pais-Chanfrau *et al*., 2004). EPO production with *P. pastoris* was performed at pH 5.0 (Çelik *et al*., 2009). Soyaslan and Çalık (2011) considered to use pH values as 4.0, 4.5, 5.0, 5.5, and 6.0 for the growth of *P. pastoris.* The highest rHuEPO production, and the highest specific AOX activity was achieved at pH=5.0 in precultivation phases and at pH=4.5 in the production phase. Therefore, in this study, the pH value has been used at 5.0 at the first three phases and at 4.5 in the induction phase to improve the product stability and decrease the proteolytic activity.

2.3.2.2 Temperature

Another important physical parameter for bioprocess is temperature. An optimum temperature is needed for metabolic activity of yeast. Cell and environmental temperatures are equal to each other because microorganisms cannot change the temperature when it is not at the optimum value. Growth rate of microorganisms and formation of protein can be affected from temperature changes. Increasing temperature from the optimum value begins the denaturation of protein.

The optimum temperature is 30 ºC in *P. pastoris* since protein expression stops and cell growth rapidly decreases when the temperature increases above 32 ºC (Invitrogen, 2000). Lowering the temperature below 30 °C might not enchance the production of r-protein expressed by *P. pastoris* (Inan *et al*., 1999; Kupcsulik and Sevella, 2005).

There are some studies for investigating temperature effects on the production of heterogeneous biological products. Lie *et al*. (2001) changed the culture temperature to 23 ºC. Decreasing temperature improved the yields of proteins (Chen *et al*., 2000; Whittaker and Whittaker, 2000; Sarramegna *et al*., 2002). Moreover, protease activity and cellular lysis are decreased during induction phase with a decreasing temperature profile by Jahic *et al*. (2003-a). Also the temperature-lowering strategy performed to improve the expression efficiency of an alkali β-mannanase from *Bacillus sp.* N16-5 in *Pichia pastoris* (Zhu *et al*., 2011). They lowered temperature and added sorbitol at the production step. Therefore Zhu *et al*. (2011) increased the β-mannanase production level. In this study, rHuEPO production by *P. pastoris* was carried out at the optimal temperature value stated as 30°C.

2.3.2.3 Oxygen Transfer Rate

Oxygen affects the formation of products in aerobic fermentation processes due to the effects of metabolic pathways and also changes of metabolic fluxes (Çalık *et al*., 1999).

P. pastoris is obligate aerobe by growing on methanol. Therefore, *P. pastoris* needs more oxygen and also requires high oxygen transfer rates for its methanol metabolism. Operating the stirrer more rapidly and enhancing the aeration rate increase the oxygen transfer coefficient (K_La) and also the oxygen transfer rate (OTR) in *P. pastoris* (Charoenrat *et al*., 2006).

Dissolved oxygen level is employed above 20% by Çelik *et al*. (2008). This level is found suitable for *P. pastoris* and in this study dissolved oxygen level was kept above 20% as well.

2.4 Bioreactor Operation Processes

P. pastoris is the essential and versatile microorganism in the production of various products. Depending on its *AOX1* promoter, its capacity for secretion of foreign proteins, its facility to apply post-translational modifications, the capability to use defined media and its preference for respiratory, *P. pastoris* expression system has been preferred for industrial applications (Potvin *et al*., 2010). Development of fermentation strategy is the most important issue in order to enhance recombinant protein production.

Although methanol is a sole carbon and energy source in *P. pastoris* bioprocesses, excess amount of methanol causes the inhibition on expression of proteins, formation of products and cell growth (Zhang *et al*., 2000). Mostly used feeding strategies are shown below developed for protein production by *P. pastoris*:

2.4.1 Fed-Batch Cultivation Process

Fed-batch operation is the most privileged cultivation system to achieve high cell densities with easy control. Fed-batch operation in *P. pastoris* culture system is composed of the GB and GFB phases, and the MFB phase.

Cell growth phases are continued until whole glycerol is consumed. Glycerol used as the growth substrate has a μ as 0.18 h^{-1}. This value is higher than the highest μ of wild type *P. pastoris* growing on methanol that is 0.14 h^{-1}. After the whole glycerol is depleted and the dissolved oxygen (DO) sharply increases, induction phase starts with feeding of methanol to the fermentation media. The last stage that is induction phase depends on process parameters such as the temperature, pH, culture medium, phenotype and specific characteristics of the protein (Cos *et al*., 2006). In this stage, recombinant protein production is excited with methanol added continuously to the bioreactor medium. Moreover, methanol transition phase can be performed among the precultivation phases and production phase to adapt the cells to another carbon source, methanol (Potvin *et al*., 2010). In this phase, during methanol is fed to the system, remaining glycerol in the reactor gradually decreases.

Several fed-batch feeding strategies for addition of methanol have been developed (Zhang *et al*., 2000-b). Ohya *et al*. (2002) and Zhang *et al*. (2005) and Çelik *et al*. (2009) revealed that protein expression is affected from the specific growth rate. Jungo *et al*. (2007-a) pointed out that specific product productivity was affected by μ which is under 0.08 h^{-1} by using pre-determined feeding profile. It was observed that specific productivity increased with μ for higher than 0.02 h^{-1}. However, specific productivities are decreased below 0.02 h^{-1}. Zhang *et al*. (2000-a) and Çelik *et al*. (2009) have been conducted transition step between 3 and 6 hours to adapt the cell to growth on methanol. In this study, transition phase has been performed as *Invitrogen* that has indicated to feed methanol to 3.6 ml hr^{-1} per liter initial fermentation volume (Invitrogen, 2000).

2.4.2 Oxygen Limited Fed-Batch Process (OLFB)

Though *P. pastoris* prefers aerobic fermentation and oxygen limitation can cause harmful results for protein production in the induction phase, higher foreign protein is yielded in oxygen limited cultivations rather than methanol limited processes.

Trentmann (2004) explained that supplementation of low amount of oxygen increased the yield of purified scFv and also reduced the cell lysis. Additionally, Charoenrat *et al*. (2005) investigate the differences between OLFB process and MLFB process. They applied OLFB process to produce the Thai Rosewood β-glucosidase and controlled primary carbon-source concentration at 350 mg L^{-1} in the induction phase. They investigated the oxygen consumption rate was greater 35% in OLFB than those MLFB process. In addition, higher specific activity and productivity were observed with OLFB strategy.

2.4.3 Temperature Limited Fed-Batch Process (TLFB)

In these processes, rather than methanol limitation, temperature is limited to prevent oxygen shortage at higher cell concentrations (Jahic *et al*., 2003). While the methanol concentration is carried on constant in the medium, the culture temperature is decreased to keep DO at a specific set value. Therefore, temperature limits cell growth (Potvin *et al*., 2010). Notably, TLFB process is essential for Mut^+ strains because cell death and oxygen limitation can be occurred with non-limiting methanol concentrations (Surribas *et al*., 2007).

For the production of fusion protein, TLFB process was applied. Jahic and co-workers (2006) resulted in higher cell and protein concentration, lower dead cells and lower proteolytic degradation of r-protein to MLFB process (Jahic *et al.*, 2006).

2.4.4 Mixed Feed Fed-Batch Process

To enhance biomass or process producibility and also reduce the induction time, another fermentation strategy, which is the using mixed feed fed-batch processes with methanol, has been developed.

Egli *et al*. (1982) firstly conducted the methanol/glucose mixed feed strategy and they found out that mixed feed fermentation has higher productivity than methanol as a sole carbon source. Thereafter, multi-carbon source with methanol strategy has been applied for Mut^- and Mut^s strains.

Generally, glycerol has been used as the co-carbon source for a successful increment of cell concentration and expression of proteins (Thorpe *et al.*, 1999). But excess concentrations of glycerol repress the activity of *AOX* and lower the productivity (Xie *et al.*, 2005). Brierley *et al*. (1990) firstly conducted the fed-batch feeding strategy with mixing glycerol/methanol for Mut^- strain of *P. pastoris.* However, protein expression did not reach to optimal level because glycerol was suppressed the *AOX1* promoter (Brierley *et al*., 1990). Files *et al*. (2001) added glycerol during MFB phase in the semi-batch bioreactor with a constant rate of methanol and different glycerol feeding rate with Mut^s phenotype. They reported that the productivity of product was increased. However, they found out feeding glycerol at higher rates decreased the concentration of heterologous protein (Files *et al*., 2001). Moreover, this strategy is studied in *P. pastoris* Mut^+ phenotype. Katakura *et al*. (1998) stated that glycerol enhanced the specific growth rate and the productivity more than feeding only methanol at a constant C_M=5.5 g L^{-1}. Besides, Hellwig *et al*. (2001) proved that the lowest glycerol feeding rate was helped to reach the highest level of recombinant protein. Zhang *et al*. (2003) fed methanol at a certain feeding rate as μ_M of 0.015 h^{-1} while a feeding strategy with glycerol growth rate ratios varying from 1 to 4. They reported that glycerol that was supplied with $\mu_{Gly} \leq 0.06$ h^{-1} increased the overall growth. Therefore, using a mixed feed without growth inhibition by glycerol is valid and logical (Zhang *et al*., 2003). Other groups were also followed this strategy to enhance the cell concentration, volumetric productivity and energy supply (Cregg *et al*., 1993). Although glycerol/methanol mixed feeding strategy increases the cell densities, high concentrations of glycerol inhibits the growth. Therefore, new mixed substrate strategy has begun to be researched to change glycerol with other co-substrates that does not affect AOX1 promoter.

Another co-carbon source, i.e., sorbitol has been used in the bioprocesses. Sreekrishna *et al*. (1997) found out sorbitol does not repress the *AOX1* promoter as much as glycerol and also support the growth. Then Thorpe *et al*. (1999) compared the mixed-feed strategies that are methanol/glycerol and methanol/sorbitol with a Mut^S phenotype of *P. pastoris*. They explained that excess sorbitol is less repressive to *AOX1* promoter and high specific production rate are observed with using sorbitol whereas cell yields are lower. Thereafter, Jungo *et al*. (2007-d) investigated whether methanol/sorbitol ratio affected the recombinant glycosylated avidin production by *P. pastoris*. Sorbitol reported as a non-repressing co-substrate to *AOX1* (Xie *et al*., 2005) can improve the productivity. Another advantageous for achieving biomass concentrations with mixing of methanol and sorbitol feeding is that oxygen depletion and heat production rates were reduced upto 38% with methanol at μ of 0.03 h^{-1} against to using only methanol for growth (Jungo *et al*., 2007d). Addition of sorbitol as a co-substrate for rHuEPO production by *P. pastoris* was investigated by Çelik *et al*. (2009). They reported that 50g/L sorbitol was the non-inhibiting concentration limit in the rHuEPO production. Also feeding sorbitol in a batch mode at the beginning of the induction phase was more preferable than mixed feeding of methanol and sorbitol (Çelik *et al*., 2009). Wang *et al*. (2010) compared the sorbitol co-feeding strategy with the traditional strategy that addition of methanol as a primary carbon source in the semi-batch cultivation to increase the production of an alkaline polygalacturonate lyase. When sorbitol was fed at a rate of r_{Srb} = 3.6 g L^{-1} h^{-1} into the production phase, they achieved 1.85-fold increase after 100 h of induction, compared to sole methanol feeding (Wang *et al*., 2010). Moreover, Gao *et al*. (2012) investigated the feeding strategy of sorbitol as a co-substrate with methanol for pIFN-α production at 30 °C. They reported that the production of product by *Pichia pastoris* was increased with this feeding strategy (Goa *et al*., 2012). Also formaldehyde accumulation was repressed.

In addition to previous studies, different carbon sources are also compared that can be used as a co-substrate with methanol. Inan and Meagher (2001) tried different carbon sources to enhance the growth and expression of β-Gal in *P. pastoris* Mut^- strain in shake flasks studies. They found out that Mut^- strain growing in media containing mannitol, sorbitol with methanol increased the amounts of β-Gal more than a methanol utilization positive phenotype. Moreover, glycerol, sorbitol, acetate and lactic acid were fed beside methanol in order to increase recombinant *P. pastoris* cell concentrations with a methanol utilization slow phenotype and the production of angiostatin (Xie *et al*., 2005). The highest production of angiostatin was obtained with lactic acid-methanol combination as 191 mg/l. Some amount of lactic acid accumulated throughout the production phase but it did not affect the production of angiostatin badly. Therefore, these results showed that alanine, mannitol, trehalose, lactic acid and sorbitol were appeared as non-repressing carbon sources. In this study, sorbitol and mannitol were used as alternative co-substrates in the production medium.

2.5 Bioprocess Characteristics

2.5.1 Yield Coefficients and Specific Rates

To investigate the fermentation process and also process efficiency, calculations of substrate consumption, product formation, specific growth rates and yield coefficients are important.

2.5.1.1 Overall and Instantaneous Yield Coefficients

To evaluate the bioprocess correctly, specific rates and yield coefficients are the most important terms. The yield coefficients are given in Table 2.1. The yield coefficient, $Y_{P/S}$, is described, as the ratio of the mass or concentration of the product formed per the amount of the selected substrate consumed.

The overall product yield on substrate, $Y_{P/S}$, which is defined within a finite the cultivation time interval (Δt), is formulated as follows:

$$Y_{P/S} = -\left(\frac{\Delta C_P}{\Delta t}\right) \Big/ \left(\frac{\Delta C_S}{\Delta t}\right)$$
$$Y_{P/S} = -\frac{\Delta C_P}{\Delta C_S} \quad (2.1)$$

The instantaneous yield on substrate, $Y_{P/S}$, which is defined at the cultivation time interval (Δt), is formulated as follows:

$$Y_{P/S} = -\left(\frac{dC_P}{dt}\right) \Big/ \left(\frac{dC_S}{dt}\right)$$
$$Y_{P/S} = -\frac{dC_P}{dC_S} \quad (2.2)$$

Table 2.1 Definition of overall yield coefficients.

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

2.5.1.2 Specific Growth Rates

Specific growth rate (μ) that explains the microbial cell growth is an important process variable (Çelik *et al*., 2009). The batch mass balance on biomass in the bioreactor is represented below; as follow:

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

In which r_X is the biomass production rate.

The biomass rate can be stated as the function of C_X, where μ is the specific biomass formation rate:

$$r_x = \mu C_x \quad (2.4)$$

The main assumption for the equations (2.3) and (2.4) is that C_X (t) and V (t) are not affected by sampling, confirming that small sample volumes should be taken. By combining equations (2.3) and (2.4),

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

In this work the operation mode is semi-batch (fed-batch), depending on continuous feeding of substrates, so the fermentation volume (liquid phase) changes throughout the process. Volume variation is expressed with the assumption of constant density. The continuity equation for the fed-batch operated bioreactor with feeding input having the flow rate of Q_t is shown as follows:

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

By inserting equation (2.6) into equation (2.5),

$$\begin{aligned} C_X \frac{d(V)}{dt} + V \frac{d(C_X)}{dt} &= \mu C_X V \\ C_X Q_t + V \frac{d(C_X)}{dt} &= \mu C_X V \end{aligned} \tag{2.7}$$

Therefore, specific biomass formation rate depending on continuous substrates addition with Q_t can be got easily, as follows:

$$\mu = \frac{d(C_X)}{dt}\frac{1}{C_X} + \frac{Q_t}{V} \tag{2.8}$$

2.5.1.3 Methanol Consumption Rate

The first substrate, methanol was fed to the system with semi-batch bioreactor operation throughout the process. Therefore, the material balance for the continuously fed substrate in semi-batch process can be written as follows:

$$\frac{d(C_M V)}{dt} = Q_M C_{M_o} - 0 + r_M V \tag{2.9}$$

in which r_M is the methanol utilization rate. The methanol utilization rate can be stated as the function of C_X, where q_M is the specific methanol utilization rate:

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

By inserting the equation (2.10) into equation (2.9), the equation (2.11) is obtained as follows:

$$V \frac{dC_M}{dt} + C_M \frac{dV}{dt} = Q_M C_{M_o} - q_M C_X V \tag{2.11}$$

In the semi-batch process, methanol fed with pre-determined feeding rate of Q_M was consumed totally, so methanol did not accumulate in the bioreactor; thus:

$$\frac{dC_M}{dt} = 0 \tag{2.12}$$

Moreover, the bioreactor liquid volume can be assumed not to be changed:

$$\frac{dV}{dt} \approx 0 \tag{2.13}$$

So, the bioreactor system is assumed in quasi-steady state condition, as follows:

$$\frac{Q_M}{V} C_{M_o} - q_M C_X = 0 \tag{2.14}$$

By rearranging equation (2.14), the specific consumption rate of methanol (q_M) can be defined as follows:

$$q_M = \left(\frac{Q_M}{V} \frac{C_{M_o}}{C_X} \right) \tag{2.15}$$

2.5.1.4 Sorbitol Consumption Rate

In this study one of the co-carbon sources, sorbitol was fed to the bioreactor continuously besides methanol with semi-batch operation mode to keep the sorbitol concentration at 50 g L^{-1} in the fermentation medium within t=0-15 h of the bioprocess in SSM strategy. In the continuous methanol and sorbitol fed semi-batch bioreactor experiment (SSM), the total flow rate Q_t is equal to the summation of the volumetric flow rates of methanol and sorbitol streams ($Q_t = Q_M + Q_{Srb}$). Therefore, the material balance equation for sorbitol for fed-batch bioreactor is; as follows:

$$Q_{Srb} C_{Srb_o} + r_{Srb} V = \frac{d(C_{Srb} V)}{dt} = C_{Srb} \frac{dV}{dt} + V \frac{dC_{Srb}}{dt} \tag{2.16}$$

Since sorbitol concentration was kept as a constant within the continuous methanol feeding period, the change in sorbitol concentration is as follows:

$$\frac{dC_{Srb}}{dt} = 0 \tag{2.17}$$

The sorbitol consumption rate can be defined with the function of C_X, where q_{Srb} is the specific sorbitol consumption rate of the cells:

$$-r_{Srb} = q_{Srb} C_x \tag{2.18}$$

By combining the equation (2.18) and equation (2.16) as follows:

$$Q_{Srb} C_{Srb_o} - q_{Srb} C_X V = C_{Srb} \frac{dV}{dt} \tag{2.19}$$

where,

$$\frac{dV}{dt} = Q_{Srb} + Q_M = Q_t \tag{2.20}$$

By rearranging equation (2.19) the specific sorbitol consumption rate (q_{Srb}) can be defined as follows:

$$q_{Srb} = \frac{Q_{Srb}}{V}\frac{C_{Srb_o}}{C_X} - \frac{C_{Srb}}{C_X}\frac{Q_t}{V} \tag{2.21}$$

After t=15 h sorbitol was not fed to the bioreactor; therefore, the bioprocess is batch. In the period of t ≥ 15 h, the material balance equation for sorbitol is as follows:

$$r_{Srb}V = \frac{d(C_{Srb}V)}{dt} \tag{2.22}$$

where r_{Srb} is the sorbitol consumption rate. The sorbitol consumption rate can be defined with the function of C_X, where q_{Srb} is the specific sorbitol consumption rate of the cells:

$$-r_{Srb} = q_{Srb}C_X \tag{2.23}$$

By combining equation (2.23) and equation (2.22) gives:

$$-q_{Srb}C_X V = V\frac{d(C_{Srb})}{dt} + C_{Srb}\frac{d(V)}{dt} \tag{2.24}$$

Therefore, the specific sorbitol consumption rate (q_{Srb}) can be defined by rearranging equation (2.24) as follows:

$$q_{Srb} = -\frac{1}{C_X}\left(\frac{dC_{Srb}}{dt} + \frac{Q_t}{V}C_{Srb}\right) \tag{2.25}$$

where Q_t is assumed equal to Q_M.

2.5.1.5 Mannitol Consumption Rate

The alternative co-substrate, i.e., mannitol was fed to the bioreactor with two different feeding strategies:

1. In MM strategy, mannitol was fed to the bioreactor batch-wise at t=0 h of production; however, in MPM and MPMG strategies, pulse mannitol was fed to the system. Therefore, the mannitol mass balance equation is as follows:

$$r_{Man}V = \frac{d(C_{Man}V)}{dt} \tag{2.26}$$

where r_{Man} is the mannitol utilization rate. The mannitol utilization rate can be defined with the function of C_X, where q_{Man} is the specific mannitol utilization rate of the cells:

$$-r_{Man} = q_{Man} C_X \tag{2.27}$$

By combining equation (2.26) and equation (2.27) gives:

$$-q_{Man} C_X V = V \frac{d(C_{Man})}{dt} + C_{Man} \frac{d(V)}{dt} \tag{2.28}$$

Therefore, the specific mannitol utilization rate (q_{Man}) can be defined by rearranging the equation (2.28) as follows:

$$q_{Man} = -\frac{1}{C_X}\left(\frac{dC_{Man}}{dt} + \frac{Q_t}{V} C_{Man}\right) \tag{2.29}$$

where Q_t is assumed equal to Q_M.

2. In MMM and MLM strategies, fed-batch operation were used with mannitol within t=0-9 h and t=0-10 h, respectively. This is analog to SSM strategy where sorbitol was fed to the bioreactor with semi-batch operation.

In the fed-batch methanol and mannitol experiments the total flow rate Q_t is equal to the summation of the volumetric flow rates of methanol and mannitol streams ($Q_t = Q_M + Q_{Man}$). Therefore, the material balance equation for mannitol for the semi-batch bioreactor system is as follows:

$$Q_{Man} C_{Man_o} + r_{Man} V = \frac{d(C_{Man} V)}{dt} = C_{Man} \frac{dV}{dt} + V \frac{dC_{Man}}{dt} \tag{2.30}$$

Since mannitol concentration was kept as a constant within the continuous methanol feeding period, the change in mannitol concentration is as follows:

$$\frac{dC_{Man}}{dt} = 0 \tag{2.31}$$

The mannitol utilization rate can be defined with the function of C_X, where q_{Man} is the specific mannitol utilization rate of the cells:

$$-r_{Man} = q_{Man} C_x \tag{2.32}$$

By combining the equation (2.32) and equation (2.30) as follows:

$$Q_{Man} C_{Man_o} - q_{Man} C_X V = C_{Man} \frac{dV}{dt} \tag{2.33}$$

where,

$$\frac{dV}{dt} = Q_{Man} + Q_M = Q_t \tag{2.34}$$

By rearranging equation (2.33) the specific mannitol utilization rate (q_{Man}) can be defined as follows:

$$q_{Man} = \frac{Q_{Man}}{V}\frac{C_{Man_o}}{C_X} - \frac{C_{Man}}{C_X}\frac{Q_t}{V} \tag{2.35}$$

After t=9 h for MMM strategy and t=10 h for MLM strategy mannitol was not fed to the bioreactor; therefore, the bioprocess is batch. In this period, the material balance equation for mannitol is as follows:

$$r_{Man}V = \frac{d(C_{Man}V)}{dt} \tag{2.36}$$

where r_{Man} is the mannitol consumption rate. The mannitol consumption rate can be defined with the function of C_X, where q_{Man} is the specific mannitol consumption rate of the cells:

$$-r_{Man} = q_{Man}C_X \tag{2.37}$$

By inserting equation (2.37) into equation (2.36):

$$-q_{Man}C_XV = V\frac{d(C_{Man})}{dt} + C_{Man}\frac{d(V)}{dt} \tag{2.38}$$

Therefore, the specific mannitol consumption rate (q_{Man}) can be defined by rearranging equation (2.38) as follows:

3. $$q_{Man} = -\frac{1}{C_X}\left(\frac{dC_{Man}}{dt} + \frac{Q_t}{V}C_{Man}\right) \tag{2.39}$$

where Q_t is assumed equal to Q_M.

2.5.1.6 Recombinant Protein Production Rate

The mass balance for rHuEPO which is found as batch-wise in the bioreactor is defined as follows:

$$r_{rp}V = \frac{d(C_{rp}V)}{dt} \tag{2.40}$$

The recombinant protein formation rate (r_{rp}) can be defined with the function of C_X, where q_{rp} is the specific r-protein formation rate:

$$r_{rp} = q_{rp} C_X \tag{2.41}$$

By combining equation (2.41) and equation (2.40) the specific r-protein formation rate can be determined as follows:

$$q_{rp} = \frac{1}{C_X}\left(\frac{dC_{rp}}{dt} + \frac{Q_t}{V} C_{rp}\right) \tag{2.42}$$

Consequently, the specific rates μ, q_M, q_{Srb}, q_{Man} and q_{rp} in semi-batch fermentations can be calculated from experimental data using equations (2.8), (2.15), (2.25), (2.29), (2.39), and (2.42), respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

The chemical substances and prepared solutions used in the experiments were analytical grade, and taken from Sigma Aldrich Co., Fluka Co., Merck & Co. Inc., and Roche.

3.2 Buffers and Stock Solutions

Buffers or solutions were prepared with distilled water disinfected with exposing high temperature and pressure at 121 ^{o}C or percolated through filters (Sartorius AG, Gottingen, Germany). After these processing, they were got into +4 ^{o}C or room temperature. They are given in Appendix A.

3.3 Microorganisms, Plasmids and Storage

As a microorganism, *Pichia pastoris*-E17 Mut^{+} carrying EPO cDNA was used in the experiments (Çelik *et al*., 2007). Into the gene, poly-histidine tag and also factor Xa protease recognition site were put together. This fused gene was cloned to the vector pPICZαA that carries α-factor signal peptide, *AOX1* promoter and Zeocin resistance gene (Çelik *et al*., 2007).

3.4 Cell Growth Medium

3.4.1 Solid Medium

P. pastoris cells stored in micro banks at -55 ^{o}C were transferred into the solid medium, including 0.1 g L^{-1} Zeocin as antibiotic. The solid mediums were kept approximately 48 h at 30 °C. The solid medium components and their concentrations are shown in Table 3.1.

Table 3.1 The composition of the YPD, solid medium

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

3.4.2 Precultivation Medium and Glycerol Stock Solution

Preparation of glycerol stock was achieved with transferring a single colony grown on YPD agar into YPG medium (Table 3.2) after incubation. The medium was centrifuged at 1500xg for 4 minutes at the 24th hour of the cultivation. The cell pellets were taken into a saline glycerol solution (Table 3.3), and kept at -55^{o}C until they were analyzed. Necessary amount of glycerol stock was directly transferred to BMGY-Buffered Glycerol Complex Medium (Table 3.4). The selective antibiotic, chloramphenicol, was taken into the media after the removal of contaminants.

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*., 2008).

Compound	**Concentration (g L^{-1})**
NaCl	9
Glycerol	20

Table 3.4 The composition of BMGY, precultivation medium.

Compound	**Concentration (g L^{-1})**
Yeast extract	10
Peptone	20
Potassium phosphate buffer pH=6.0	0.1 M
YNB	13.4
Ammonium sulphate	10
Biotin	4 x 10^{-5}
Glycerol (mL)	20
Chloramphenicol (mL)	1

3.4.3 Production Medium

In the production phase, cells taken from BMGY were transferred into production medium when the cells reached the optimum growth. There are some differences in the production method between the laboratory scale air filtered shake bioreactor experiments and the pilot scale bioreactor

experiments. In the shake bioreactor experiments, a defined production medium (Table 3.6) containing sorbitol or mannitol and methanol, basal salts solution and nitrogen sources was used that was stated by Jungo *et al*. (2006). However, ammonium sulfate was chosen instead of ammonium chloride with the amount as carbon to nitrogen ratio and methanol to nitrogen ratio are 4.57 and 2.19, respectively in this study (Jungo *et al*., 2006). Also a different trace salt solution (PTM1) that is listed in Table 3.5 was used. On the other hand, in the pilot scale experiments, Basal Salt Medium (BSM) was used for the production of rHuEPO. After the sterilization of BSM (Table 3.7), 0.1 % antifoam, PTM1 and 0.1 % chloramphenicol were put into the medium.

Table 3.5 The composition of the trace salt solutions PTM1 (Sibirny *et al*., 1990).

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

Table 3.6 The composition of the defined production medium (Jungo *et al*., 2006)

Compound	**Concentration (g L^{-1})**
Methanol (mL)	1
Sorbitol/Mannitol	30-70
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
Chloramphenicol (mL)	1
PTM1 (mL)	4.35

Table 3.7 The composition of Basal Salt Medium (BSM) (Sibirny *et al*., 1990).

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

3.5 Recombinant Erythropoietin Production

rHuEPO was produced by using two different experiments, i.e., laboratory scale air filtered shake bioreactor experiments and pilot scale bioreactor experiments.

3.5.1 Precultivation

P. pastoris cells taken from the stock were directly got into precultivation medium. The cells were precultivated at 30°C and shaken at N=225 rpm for 20-24 h in an orbital shaker (B.Braun, Certomat BS-1) containing air-filtered 150 mL bioreactors with V=50 mL. When the cells reach to C_X=1.8-2.4 g L^{-1}, precultivation medium was centrifuged at 6000xg for 10 min. Although precultivation method was the same, the production method in the laboratory scale shake bioreactor experiments differs than that in the pilot scale bioreactor experiments. They were explained, as follows:

3.5.2 rHuEPO Production in Laboratory Scale Air Filtered Shake Bioreactors

In the laboratory scale air filtered shake bioreactor experiments, 250 mL shake bioreactors with V=50 mL containing the defined medium, and other co-substrates, i.e., sorbitol and mannitol, were utilized. After the cells were taken from precultivation medium, they were got into the defined production medium having the composition listed in Table 3.6. Batch fermentations were performed within 49 h at T=30°C and N=225 min^{-1}. 1% (v/v) methanol was induced to the medium in every 24 h to induce rHuEPO production.

3.5.3 rHuEPO Production in the Pilot Scale Bioreactor System

In the pilot scale bioreactor experiments, protein generation was performed in V=3.0 L bioreactor (Braun CT2-2), with V=1.0-2.0 L. Controlling the bioprocess operation parameters is really important. Temperature, pH, foam, stirring rate, feed inlet rate and dissolved oxygen control systems are taken place in the bioreactor. The representation of the bioreactor and phases for the production is represented in Figure 3.1. The details of controlling the bioprocess operation parameters were explained below:

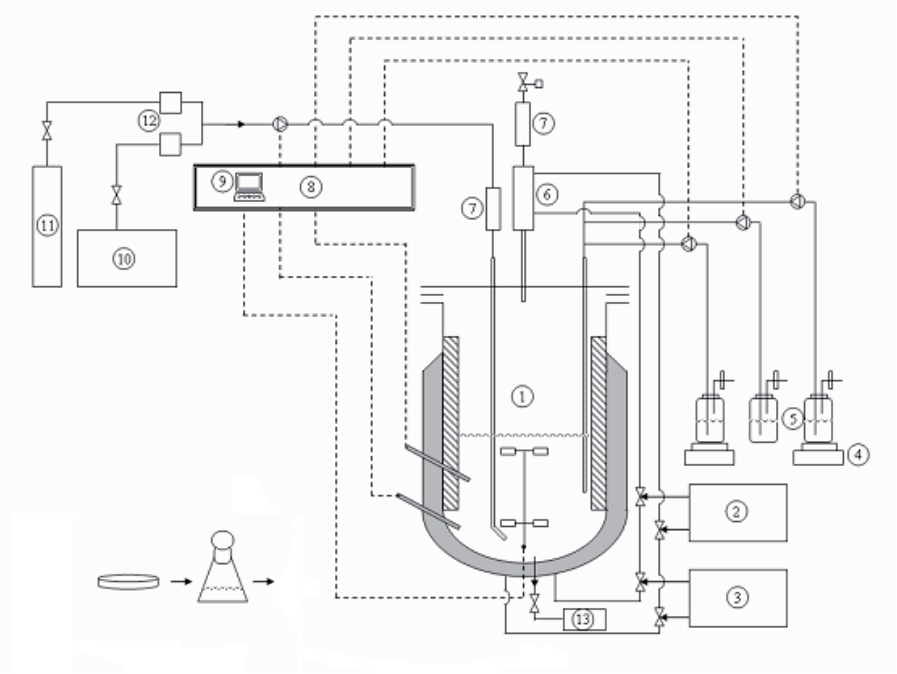

Figure 3.1 The pilot scale bioreactor system. I: Solid medium; II: Precultivation medium, V = 50 mL; III: Pilot scale bioreactor system, composed of (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2 tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008).

3.5.3.1 Control of Bioreactor Operation Parameters

The bioreactor has some operation parameters. In this study temperature was kept at 30±0.1°C with PI controller of the system by means of the external cooler and steam generator throughout the process, which is the mostly known as an optimum value for growth and production in the previous experiments.

The optimum pH value for rHuEPO production by *Pichia pastoris* was reported by Soyaslan and Çalık (2011). In this previous study five different pH values 4.0, 4.5, 5.0, 5.5, and 6.0 was considered. Therefore, the highest protein production was obtained at pH= 4.5. In this study, the pH value has been kept at pH=5.0 at the first three phases and then lowered to pH=4.5 in the production phase. For the adjustment of pH 25% NH_3OH solution was added to the medium to control manually (Çelik, 2008). Additionally, there is an automatic PI controller with parameters as Xp=30% and TI= 30 s in order to retain the base-pump-valve open at 10%. It controlled pH with ±0.1 sensitivity. In this study, sometimes manual and sometimes automatic control system was used to adjust the pH value in the fermentation process.

Another important parameter, dissolved oxygen level, has been maintained above 20% saturation to prevent oxygen limitation. *P. pastoris* that is an aerobic microorganism consumes oxygen at high rates. Therefore, control of DO is difficult. In early phases of the fermentation air was fed to the system to control dissolved oxygen. However, cells needed much more air to grow while the glycerol batch phase was being finished. For that reason, pure oxygen was used to enrich the air by controlling digital mass flow rate controller.

In order to prevent the formation of foam, negligible amounts of 10% antifoam solution (1 or 2 mL) was added to the fermenter broth manually drop-wise in the experiments. The agitation rate was maintained constant at N=900 rpm while it was fixed at N=225 rpm in the laboratory scale shake

bioreactor experiments. N=900 rpm was found as an optimum because higher agitation rates causes shear damage to the cells and also increase in temperature and foaming (Çelik, 2008).

3.5.3.2 Fed-Batch Pilot Scale Bioreactor Operations

The general procedure used for the r-protein expression by *P. pastoris* through *AOX1* promoter (Stratton *et al*., 1998) is performed in four steps (Çelik *et al*., 2009); glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and production or methanol fed-batch (MFB) phases. When biomass concentration reached 0.3 g L^{-1} as initial OD_{600} =1, they were taken from the precultivation medium and transferred into the production medium and, the first phase, GB began, as follows:

- **Glycerol Batch Phase (GB)**:

GB phase started when the cells are transferred into the fermentation medium containing 40 g L^{-1} glycerol that is used batch-wise. Higher amount of glycerol should not be used because it inhibits the growth of cells (Cos *et al.,* 2006). The purpose of GB is to raise biomass concentration. This phase is performed until all glycerol are consumed (15 - 17 h) with OD_{600}=26-30 corresponding C_X=8-9 g L^{-1} of cell concentration.

- **Glycerol Fed-Batch Phase (GFB):**

In glycerol fed-batch step, glycerol solution including 12 mL L^{-1} PTM1 is fed continuously to the bioreactor, with a pre-calculated exponential feeding rate. This phase is proceeded until the cell concentration reaches to 21- 24 g L^{-1} (OD_{600}=80-90). In this phase, there is no recombinant protein synthesized in the cells since glycerol represses *AOX1* promoter. The purpose of GFB is to enhance the cell growth while de-repressing the AOX enzyme and preventing the accumulation of glycerol.

- **Methanol Transition Phase (MT)**:

In this phase, another carbon source, methanol solution including 12 mL L^{-1} PTM1 was added to the bioreactor, with a process given that 2.8 g hr^{-1} per liter of initial fermentation volume during 4 h (Invitrogen, 2002). The aim in this step is to adapt the cells to methanol.

- **Methanol Fed-Batch Phase (MFB):**

Recombinant protein production phase, where 100% methanol including 12 mL L^{-1} PTM1 was added continuously to the semi-batch operated bioreactor, with the pre-determined exponential feeding profile with μ_{M0}=0.03 h^{-1}, $Y_{X/St}$= 0.42 and C_{S0}= 630 g L^{-1} (Çelik *et al*., 2009). Furthermore, alternative co-substrates, mannitol and sorbitol, were used based on the findings of the shake-bioreactor experiments.

In SSM strategy, sorbitol was fed continuously (500 g L^{-1} solution) to retain its concentration at 50 g L^{-1} in the semi-batch operated bioreactor. In the second (MM) experiment, mannitol was used batch-wise; however, in the third (MMM) experiment, mannitol was fed continuously (250 g L^{-1} solution in BSM) to keep its concentration at 50 g L^{-1} in the semi-batch operated bioreactor. Moreover, in the fourth (MLM) strategy, mannitol was added (220 g L^{-1} solution in BSM) to keep its concentration at 3 g L^{-1} in the semi-batch operated bioreactor. In the fifth (MPM) strategy, three-pulse mannitol was used batch-wise at t=0 h, 6 h, and 12 h. In the last (MPMG) experiment, four-pulse mannitol was used batch-wise at t=0 h, 7 h, 14 h, and 24 h containing glycerol, with the initial concentration in the bioreactor being 8 g L^{-1}.

The pre-calculated exponential feed rate was determined as a constant specific growth rate in SSM, MMM and MLM strategies as an equation 3.1:

$$Q(t) = \frac{\mu_o V_o C_{Xo}}{C_{So} Y_{X/S}} \exp(\mu_o t) \qquad (3.1)$$

where, Q is volumetric flow rate, 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.

3.6 Analysis

During the production process, samples were collected at every 3 h. Then the medium was centrifuged 10 min at 6000xg and +4°C. Cells, supernatants and filtrates were kept at -55°C for analysis. By using the supernatant, total protein, protease concentration and SDS-Gel electrophoresis were determined. Filtrates were used to determine mannitol, sorbitol, methanol, organic acid concentrations while AOX activity was determined from the cells.

3.6.1 Cell Concentration

Cell concentration was determined by a UV-Vis Spectrophotometer (Thermo Spectronic, Hελios-α). Cells were mixed with water to obtain correct range in the spectrophotometer at 600 nm that is 0.1-0.9. By using equation 3.2 the absorbance was converted to cell concentration, C_X (g/L) (Çelik *et al*., 2009)

$$C_X = 0.3 \times OD_{600} \times DilutionRatio \tag{3.2}$$

3.6.2 Protein Analysis

3.6.2.1 Total Protein Concentration

Total protein concentration was determined by a spectrophotometric method at 595 nm with Bradford assay (Bradford, 1976). After the mixture which contains 20 µL sample and 1 mL Bradford reagent (Bio-Rad) was kept at room temperature for 10 min in dark, the absorbance was determined. A calibration curve which obtained with bovine serum albumin (BSA) was given in Appendix B.

3.6.2.2 RHuEPO Concentration

RHuEPO concentrations were calculated by using SDS-PAGE analysis. Samples were analyzed by the comparison of marker, standard and samples by using LABWORKS Image Acquisition and Analysis Software Program (Ver. 4.6, Cambridge, UK). The SDS-PAGE protocol is given in section 3.6.2.3. The silver stained SDS-PAGE gel view of extracellular proteins produced by *P. pastoris* for all strategies with respect to time are illustrated in Appendix G.

3.6.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the way of Laemmli (1970). Buffer was added to the samples with 1:3 volume ratio. The mixture was denatured 4 min at 95 °C and put into ice. In order to analyze 15 µL of the sample and 3 µL of a dual color prestained protein MW marker (Appendix F) were loaded to gel and the analyze was run at constant current of 40 mA. The buffers used are given in Appendix A. The SDS-PAGE protocol is given below:

Preparation steps of SDS-PAGE gels

1. The glasses were cleaned with ethanol and they were assembled plates according to the manufacturer's instructions. Then the gel cast was set-up.

2. The resolving and stacking gels were prepared 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 pipetted into the gel cast quickly to avoid polymerization and bubble formation. The gel cast was filled 0.5 cm space below the comb-till the bottom of green line.

4. A thin layer of isopropanol was poured onto the gel to smooth the gel surface.

5. After at least 45 minutes, isopropanol was poured away and the glass 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 inserted to prevent bubble formation under the comb.

7. The gel was left at least 20 minutes to polymerize. The gel was wrapped into a tissue that was soaked in dH_2O and stored in $+4^{O}C$ for up to two weeks.

Preparation of Samples and Gel electrophoresis steps

1. Sample loading buffer was added to samples (1:3) and the mixture was heated at 95°C for 4 minutes, stored in ice for 5 minutes, centrifuged and vortexed.

2. The comb between the glasses was removed slowly and the wells were washed with dH2O and assembled into the electrophoresis unit.

3. The apparatus was filled with 1XSDS-PAGE running buffer up to a point where the bottom of the gels was covered. The electrodes do not wet.

4. 15 µL of the prepared samples and 3 µL of prestained protein MW marker were loaded in the wells with Pasteur pipette quickly.

5. The lid of the apparatus was closed by attaching the correct red/black power leads.

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

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

Staining the SDS-PAGE Gels

After the gel electrophoresis, 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.

Table 3.8 Procedure for silver staining of SDS-PAGE gels

Step	Solution	Time of Treatment
1. Fixing	Fixer	≥ 1 h
2. Washing	50% Ethanol	3 x 20 min
3.Pre-Treatment	Pretreatment Solution	1 min
4. Rinse	Distilled Water	3 x 20 sec
5.Impregnate	Silver Nitrate Solution	20 min
6. Rinse	Distilled Water	2 x 20 sec
7. Developing	Developing Solution	~5 min
8. Wash	Distilled Water	2 x 2 min
9. Stop	Stop Solution	≥ 10 min

3.6.3 Glycerol, Methanol, Sorbitol and Mannitol Concentrations

Glycerol, methanol, sorbitol, and mannitol concentrations were determined and analyzed by reversed phase HPLC (Waters HPLC, Alliance 2695, Milford, MA) (Helik *et al*., 2009). Determination analysis depends on the chromatogram of the standard where solutions enable to calculate the concentrations from the chromatogram. 150 µL samples filtered through 45 µm filters (ACRODISC CR PTFE) were taken to use in the system. In order to determine methanol, mannitol and sorbitol concentrations, 5 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1} was used as a mobile phase and refractive index detector (Waters-2414) were used at 30°C. Also the calibration curves can be seen in Appendix C. The methanol, mannitol and sorbitol concentration analyzed under the specified conditions are listed in Table 3.9.

Table 3.9 Conditions for HPLC system for methanol, mannitol and sorbitol analyses

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

3.6.4 Organic Acids Concentrations

Organic acid concentrations were determined by reversed phase HPLC (Waters, HPLC, Alliance 2695). 150 μL samples filtered through 45 μm filters (ACRODISC CR PTFE) were used into the analysis system. 3.12 % (w/v) NaH_2PO_4 and $0.62x10^{-3}$ % (v/v) H_3PO_4 were used as the mobile phase. Organic acid concentration analyzed under the specified conditions are listed in Table 3.10 (Çelik *et al*, 2009). The organic acids calibration curves are shown in Appendix D.

Table 3.10 Conditions for HPLC system for organic acids analysis (Çelik *et al*, 2009)

Column	Capital Optimal ODS, 5μm
Column dimensions	4.6×250 mm
System	Reversed phase chromatography
Mobile phase	3.12% (w/v) NaH_2PO_4 and $0.62x\ 10^{-3}$ % (v/v) H_3PO_4
Mobile phase flow rate	0.8 mL min^{-1}
Column temperature	30°C
Detector-wavelength	Waters 2487 Dual absorbance detector, 210 nm
Injection volume	5 μL
Analysis period	15 min
Space time	5min

3.6.5 Protease Activity Assay

Proteolytic activity of samples was determined by hydrolysis of casein. In order for acidic, neutral and basic protease activities, casein dissolved in either borate buffer (0.05 M, pH 10), sodium acetate buffer (0.05 M, pH 5) or sodium phosphate buffer (0.05 M, pH 7) with mixing for 20 min with one mL of diluted medium at 30°C. 10% (w/v) trichloroacetic acid was added and hydrolysis was stopped and kept within ice for 20 min. Before the absorbance at 275 nm was measured in UV-Vis spectrophotometer in quartz cuvettes, medium was centrifugated at 19000xg for 10 min at +4°C.

One unit protease activity was defined as the activity that releases 4 nmole tyrosine per minute (Moon and Parulekar, 1991). The equation 3.3 shown below was used for turning into absorbance to protease activity (Ucm^{-3}) (Çalık, 1998).

$$A = \left(\frac{Absorbance}{0.8x1/\mu mol.cm^{-3}}\right)\left(\frac{1U}{4nmol/\min}\right)\left(\frac{1}{20\min}\right)\left(\frac{1000nmol}{1\mu mol}\right)\left(\begin{matrix}Dilution\\ ratio\end{matrix}\right) \quad (3.3)$$

3.6.6 Alcohol Oxidase Activity

3.6.6.1 Yeast Lysis to Obtain Intracellular Medium

Since AOX is an intracellular enzyme, by using yeast lysis buffer intracellular medium was got out of the cells (Appendix A). After 500 µL yeast lysis buffer was added into 1 mL of cell sample, they were shaked three times for 20 sec and kept on ice for 30 sec for each cycle. After that a spoon of glass beads were added to the mixture and the same procedure was applied. When the process was completed, mixtures are centrifuged at 3000xg for 2 min at +4 °C and the supernatant was centrifuged again at 12000xg for 5 min at +4 °C. The second centrifugation supernatant was used in the analysis method.

3.6.6.2 AOX Activity Assay

In order to determine the oxidation of methanol to formaldehyde by AOX enzyme alcohol oxidase (AOX) and horseradish peroxidase (HRP) were utilized in the assay. UV-Vis spectrophotometer obtains the concentration of H_2O_2 produced by AOX with addition of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP). Firstly methanol oxidizes to formaldehyde and H_2O_2 by the help of AOX. Then 2 moles of H_2O_2 reacts with 1 mole of PSA and 4-AAP to form 1 mole of quinoneimine dye, 1 mole of sodium hydrogen sulfate and 3 moles of water.

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

$$2\,H_2O_2 + \text{PSA} + \text{4-AAP} \xrightarrow{\text{HPR}} \text{Quinoneimine dye} + 3\,H_2O + NaHSO_4$$

Individual magenta color found in the dye was absorbed at around 500 nm. The standard assay reaction mixture composed of 0.4mM 4-AAP, 25mM PSA, and 2U mL^{-1} HRP in 0.1M phosphate buffer with pH 7.5 was used at 25°C. 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 was performed with addition of 3 mL standard reaction mixture, 30 µL HRP, 375 µL methanol and 75 µL sample. Increment in absorbance at 500 nm was monitored for 4 minutes with 30 sec intervals. The calibration curve is given in Appendix E. The AOX concentration (mg mL^{-1}) was obtained by using equation 3.4.

$$C_{AOX}\left(\frac{U}{gCDW}\right) = 21.1\frac{U/mL}{absorbance}\,xOD_{500}x\frac{1}{C_X} \quad (3.4)$$

CHAPTER 4

RESULTS AND DISCUSSION

In this study, two co-carbon sources (mannitol and sorbitol) and different feeding strategies were investigated for recombinant human erythropoietin production by *Pichia pastoris.* In the first part of the study, effects of co-carbon sources on recombinant human erythropoietin production were investigated in laboratory scale shake bioreactor experiments. In the second part, depending on the findings of the shake flask experiments, pilot-scale bioreactor experiments were designed and conducted. Therefore, the effects of feeding strategies on the cell growth, substrate consumption, rHuEPO production, alcohol oxidase (AOX), and protease activities were analyzed. Furthermore, yield coefficients, specific rates, and organic acid profiles were investigated in order to determine the effects of these feeding strategies on rHuEPO production.

4.1 Production of Recombinant Human Erythropoietin by *Pichia pastoris* using Two Alternative Co-Carbon Sources in Laboratory Scale Air Filtered Shake Bioreactors

In this study, by using *P. pastoris* Mut^+ strain carrying EPO gene (Çelik *et al*., 2007) the effects of co-carbon sources were investigated further in the laboratory-scale bioreactor experiments. However, in order to design feeding strategies, laboratory-scale air filtered shake-bioreactor experiments were firstly conducted.

4.1.1 Effect of Co-Carbon Sources on Cell Concentration in the Production of rHuEPO

The concentrations of the co-carbon sources in the production phase were determined according to the previous studies and adjusted to be found in the growth medium at equivalent carbon atoms. Each different production media was induced with 1% (v/v) methanol in every 24 h. After the cells were precultivated in BMGY medium, they were transferred to production medium (BSM) with the initial cell concentration at C_x=0.3 g L^{-1}. The processes lasted for t =49 h and the effects of co-carbons at different concentrations were analyzed and compared.

Firstly, sorbitol was used in the production medium. Concentrations of sorbitol were adjusted by taking the optimized amount that was reported as the single co-substrate by Çelik *et al.* (2009) as 50 g L^{-1}. In order to make comparisons, 30 g L^{-1} sorbitol and to investigate the dual co-substrate effect of mannitol and sorbitol, at C_{Man0}=C_{Srb0}=15 g L^{-1}, were experimented. Then, mannitol tested as the alternative co-carbon source at different concentrations of C_{Man0} = 30, 40, 50, 60, 70 g L^{-1} at an initial methanol concentration of 1% (v/v), in order to enhance the production of rHuEPO in *P. pastoris*. Variations in the cell concentrations for each media at t= 24 h and t=49 h can be seen in Figure 4.1.

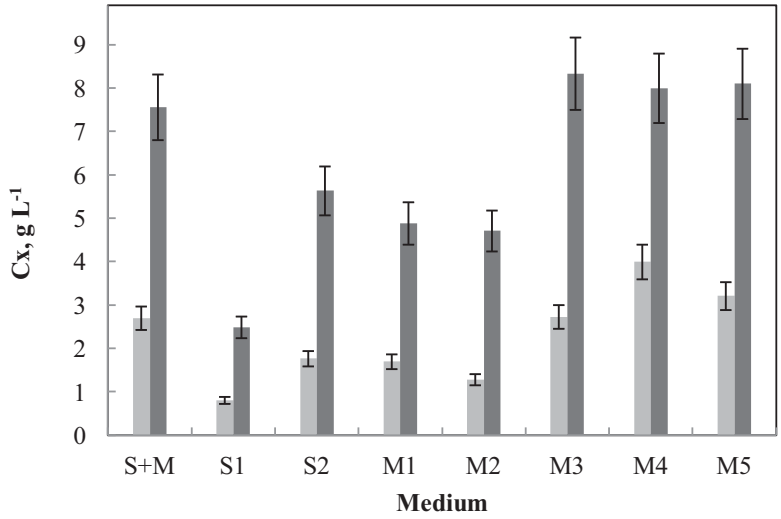

Figure 4.1 Variation in the cell concentration with the cultivation time at t=24 h and t=49 h and co-carbon sources with 1% (v/v) methanol. S+M: 15 g L^{-1} sorbitol and 15 gL^{-1} mannitol, S1: 30 g L^{-1} sorbitol, S2: 50 g L^{-1} sorbitol, M1: 30 g L^{-1} mannitol, M2: 40 g L^{-1} mannitol, M3: 50 g L^{-1} mannitol, M4: 60 g L^{-1} mannitol, M5: 70 g L^{-1} mannitol; at t= 24 h (■), t=49 h (■).

The cell concentration profiles show that the lowest cell concentration (C_x=0.8 g L^{-1} at t=24 h, and C_x=2.5 g L^{-1} at t=49 h) was acquired with C_{Srb0}=30 g L^{-1}. Although C_{Srb0}=50 g L^{-1} (C_x=5.6 g L^{-1}) was found to be the optimum value for the production of rHuEPO by *P. pastoris* (Çelik *et al*., 2009), the cell concentration was not as high as the simultaneous sorbitol and mannitol use at C_{Srb0}=C_{Man0}=15 g L^{-1} (C_x=7.7 g L^{-1}). It probably occurs from the increasing effect of mannitol on the cell growth. The cell concentration profiles were close to each other above C_{Man0}= 50 g L^{-1} at t=49 h. However, Çelik *et al*. (2009) reported that C_{Srb0}=50 g L^{-1} was the non-inhibitory limit for the Mut^+ phenotype of *P. pastoris* to express recombinant human erythropoietin (rHuEPO). As a conclusion, C_{Man0}=50 g L^{-1} (C_X=8.3 g L^{-1}) was obtained as economic and also suitable for rHuEPO production by *P. pastoris* on the basis of inhibition effect and process economy.

4.1.2 Effect of Co-Carbon Sources on rHuEPO Production

To investigate the optimum co-carbon source for increasing rHuEPO expression, the highest r-protein concentrations at t=49 h were compared using SDS-PAGE (Figure 4.2). In the production medium containing C_{Srb0}=30 g L^{-1} together with 1% (v/v) methanol, the lowest rHuEPO concentration was obtained as well as the lowest cell concentration at t=49 h (Figure 4.1). When the recombinant protein concentrations produced in the medium containing C_{Srb0}=50 g L^{-1} (Line 5) and C_{Man0}=50 g L^{-1} (Line 6) are compared, the highest amount of rHuEPO is obtained as 70 mg L^{-1} in medium containing C_{Man0}=50 g L^{-1} containing 1% (v/v) methanol, that is 5-fold higher than that containing C_{Srb0}=50 gL^{-1}. Although the rHuEPO production with the simultaneous mannitol and sorbitol use at C_{Srb0}=C_{Man0}=15 g L^{-1} are 4-fold higher than production with using sorbitol as a sole co-carbon source in the production medium, it does not reach the higher value as the production at C_{Man0}=50 g L^{-1}. Besides the comparison of the amount of final proteins at initial mannitol concentrations of C_{Man0}=30, 40, 50, 60, 70 g L^{-1} (Fig. 4.2. Line 3, Line 4, Line 6, Line 7, Line 8, respectively), the maximum production was achieved when the initial mannitol concentration was selected as C_{Man0}=50 g L^{-1} that was 5-, 5.5-, 1.3-, and 1.4- fold higher, respectively. It was shown that above 50 g L^{-1} initial mannitol concentrations has the inhibitory effect for the Mut^+ phenotype of *P. pastoris* producing rHuEPO. As a result, C_{Man0}=50 g L^{-1} mannitol concentration was found to result in optimum growth rate and rHuEPO production. Therefore, mannitol was chosen as the best alternative to sorbitol.

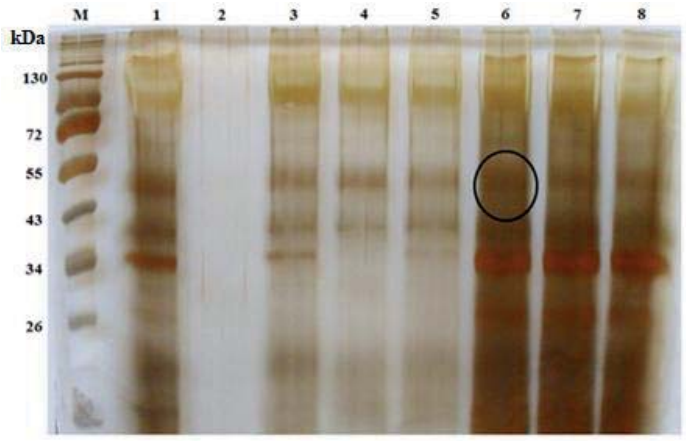

Figure 4.2 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale shake bioreactors to observe the difference in rHuEPO production between different initial sorbitol and mannitol concentrations at t=49 h. M: protein marker, 1. well: 15 g L^{-1} sorbitol and 15 g L^{-1} mannitol, 2. well: 30 g L^{-1} sorbitol, 3. well: 30 g L^{-1} mannitol, 4. well: 40 g L^{-1} mannitol, 5. well: 50 g L^{-1} sorbitol, 6. well: 50 g L^{-1} mannitol, 7. well: 60 g L^{-1} mannitol, 8. well: 70 g L^{-1} mannitol.

4.2 Production of Recombinant Human Erythropoietin by *Pichia pastoris* using Two Alternative Co-Carbon Sources in Pilot Scale Bioreactor System

Due to the findings of the experiments performed in laboratory scale air filtered shake bioreactors, the effects of sorbitol and mannitol on rHuEPO production were investigated separately by the pilot-scale bioreactor experiments. By using the pilot-scale bioreactor system having a working volume 1.0-2.0 L with pH, foam, temperature, agitation rate and dissolved oxygen controller, higher cell growth and product formation can be achieved. Furthermore, fed-batch feeding that provides better cell growth and production can be performed in the pilot-scale bioreactor system. Therefore, in this part of the study, six pilot scale experiments were designed and conducted with different feeding strategies in order to enhance the production of rHuEPO with *P. pastoris*.

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

One of the important parameters in fed-batch fermentations is the specific growth rate that is utilized to calculate the exponential feed inlet rate of the limiting substrates with the equation 4.1, as follows:

$$Q(t) = \frac{\mu_O V_O C_{XO}}{C_{SO} Y_{X/S}} \exp(\mu_O t) \tag{3.1}$$

where Q is volumetric flow rate, (L h^{-1}), μ_O is the desired specific growth rate (h^{-1}), V_O is the initial fermentation volume (L), C_{XO} (g L^{-1}) is the initial cell concentration at t=0 h, $Y_{X/S}$ (g g^{-1}) is the cell yield on the given substrate and C_{SO} (g L^{-1}) is feed substrate concentration.

The variations of glycerol, methanol, sorbitol and mannitol used in equation 3.1 are represented in Table 4.1.

Table 4.1 Parameters of Equation 3.1

Parameter	Glycerol Fed-Batch Feeding	Methanol Fed-Batch Feeding	Sorbitol Fed-Batch Feeding	Mannitol Fed-Batch Feeding in MMM	Mannitol Fed-Batch Feeding in MLM
μ_0 (h^{-1})	0.18	0.03	0.025	0.11	0.005
$Y_{X/S}$ (g/g)	0.5*	0.42**	0.30***	0.4	0.128
C_{SO} (g L^{-1})	630	790	500	250	220

*Cos *et al*., 2006, **Jungo *et al*., 2006, ***Çalık, 2013

In order to calculate the parameters for sorbitol in SSM strategy given in Table 4.1, previously published findings were used. First, Çelik *et al.* (2009) investigated the expression of rHuEPO by using *P. pastoris* Mut^+ strain with two feeding strategies, with and without sorbitol. In these studies, firstly, primary carbon source was only added with a pre-determined specific growth rate of μ_{M0}=0.03 h^{-1} and the overall cell yield on total substrate was determined as $Y_{X/S}$=0.30 (g g^{-1}). Further experiments were carried out with feeding 50 g L^{-1} batch-wise sorbitol at t=0 h beside methanol feeding rate with μ_{M0}=0.03 h^{-1} and the overall cell yield on total substrate was determined as $Y_{X/S}$=0.45 (g g^{-1}). By using these studies, Çalık (2013) calculated the cell yield on sorbitol as $Y_{X/S}$=0.30 (g g^{-1}) which is the subtraction of these two experiments' yield coefficient values. Furthermore, the pre-determined specific rate on sorbitol was calculated as μ_{Srb0}=0.025 h^{-1} from the consumption rates data of sorbitol that were taken from the study of Inankur *et al*. (2010).

On the other side, in order to calculate the parameter for mannitol in MMM strategy given in Table 4.1, the previous experiment data that is MM strategy was utilized. In MM strategy, $Y_{X/S}$ was determined as 0.40 (g g^{-1}). Additionally, for calculation of mannitol specific growth rate, the highest specific utilization rates of mannitol which is the first six hours of the production phase in MM strategy was used. From the specific consumption rate, q_{Man}, the volumetric consumption rate, r_{Man}, of mannitol was calculated. Thereafter, based on the pre-determined feeding profile calculated with volumetric mannitol consumption rate, the pre-determined specific growth rate of mannitol was found as μ_{Man0}=0.11 h^{-1}.

Other parameters in Table 4.1 for mannitol in MLM strategy, calculations were carried out with the experimental data in MMM strategy. For the first three hours in MMM strategy, the pre-determined specific growth rate of mannitol was calculated as μ_{Man0}=0.005 h^{-1}. $Y_{X/S}$ in MMM was determined as 0.128 (g g^{-1}). Therefore, by using these values, the feeding strategies of sorbitol and also mannitol were conducted in each experiment. The pre-determined feeding profiles for glycerol, methanol, sorbitol in SSM strategy and mannitol in MMM and MLM strategies are plotted in Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.6 and Figure 4.7.

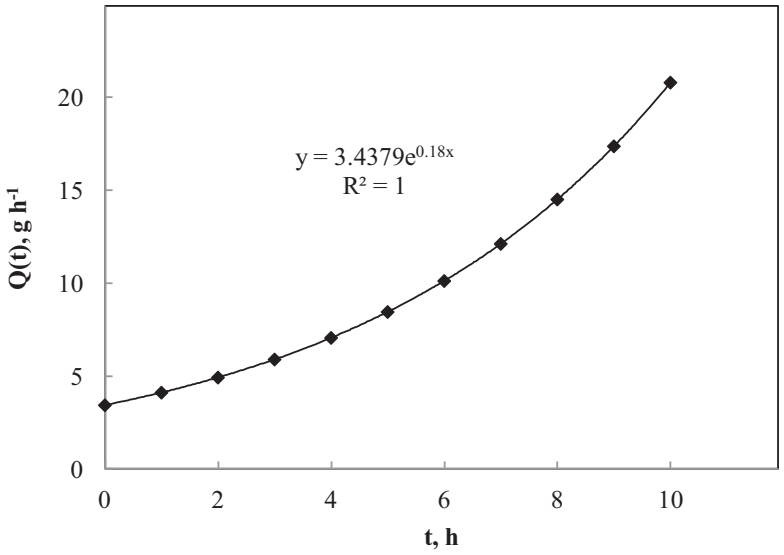

Figure 4.3 The pre-determined feeding profile for glycerol in all strategies, calculated for specific growth rate (μ_{Gly0}) of 0.18 h^{-1}.

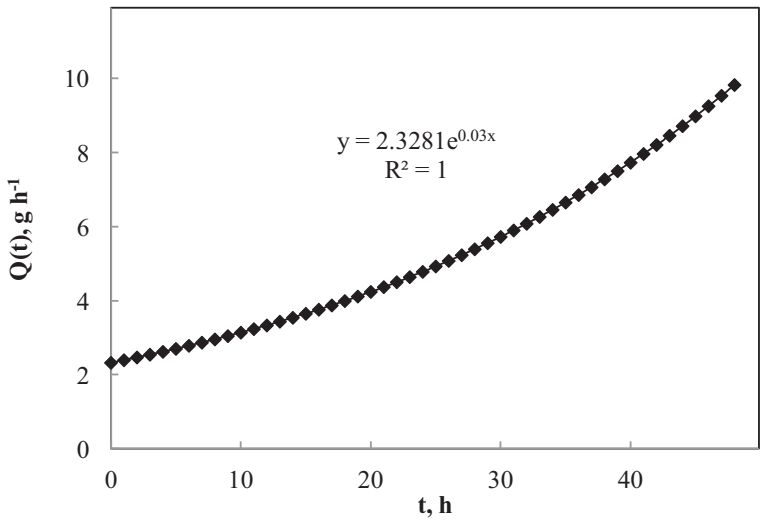

Figure 4.4 The pre-determined feeding profile for methanol in all strategies, calculated for specific growth rate (μ_{M0}) of 0.03 h^{-1}.

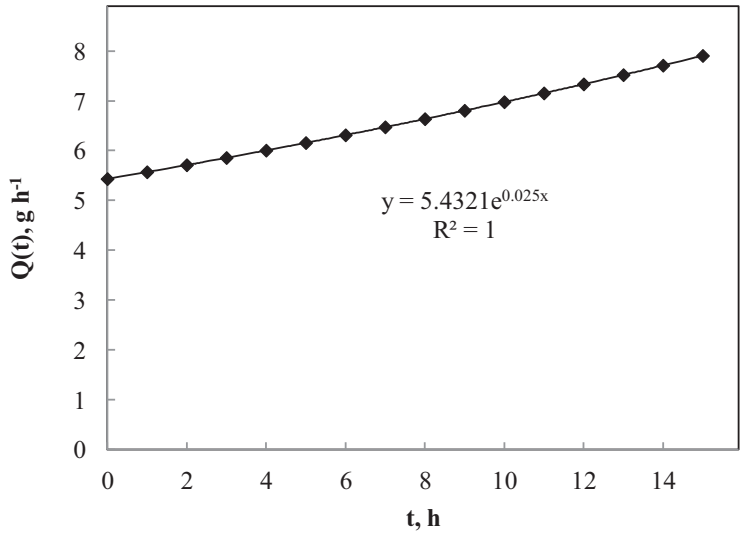

Figure 4.5 The pre-determined feeding profile for sorbitol in SSM strategy, calculated for specific growth rate (μ_{Srb0}) of 0.025 h^{-1}.

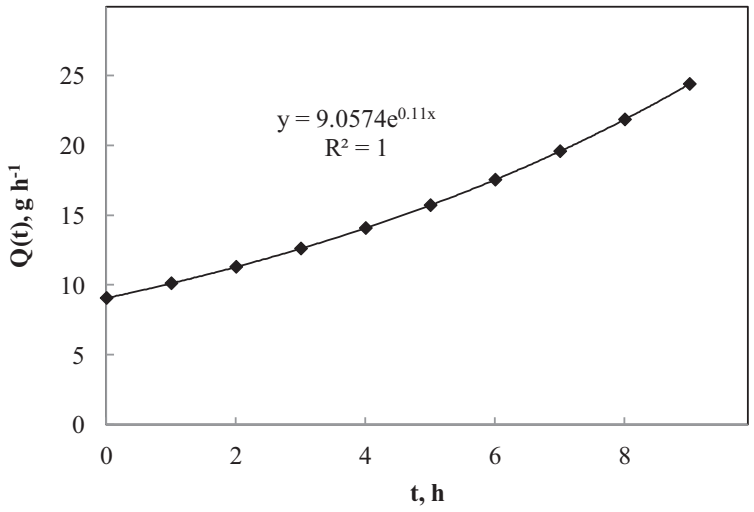

Figure 4.6 The pre-determined feeding profile for mannitol in MMM strategy, calculated for specific growth rate (μ_{Man0}) of 0.11 h^{-1}.

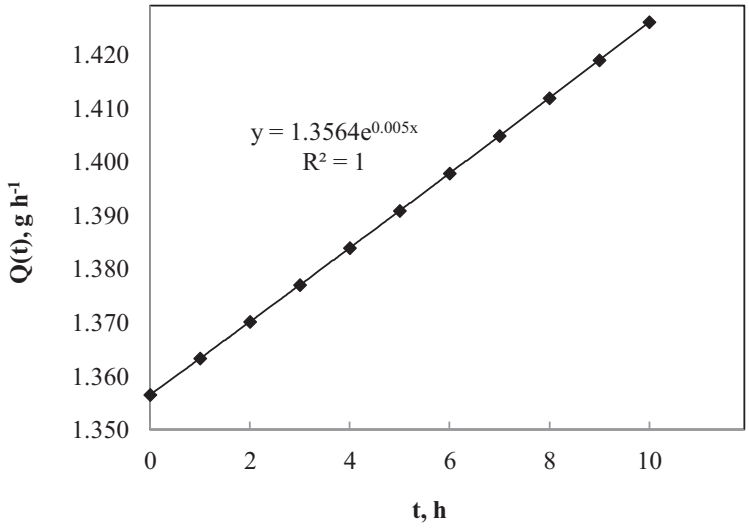

Figure 4.7 The pre-determined feeding profile for mannitol in MLM strategy, calculated for specific growth rate (μ_{Man0}) of 0.005 h^{-1}.

4.2.2 Feeding Strategy Development for Production of Recombinant Human Erythropoietin by *Pichia pastoris*

In all experiments, bioreactor operation parameters were set to values as explained in section 3.5.3.1. Operation phases were carried out as explained in section 3.5.3.2. Before production phase, the same three consecutive phases were applied. Firstly, glycerol batch (GB) phase was conducted with 40 g L^{-1} glycerol. Secondly, glycerol fed-batch (GFB) phase was performed by fed-batch feeding of glycerol with the pre-determined specific growth rate of μ_{Gly0}=0.18 h^{-1}. Thirdly, methanol transition (MT) phase was accomplished with feeding 100% methanol containing 12ml L^{-1} PTM1 solution to the medium with 3.6 ml/hr per liter of initial fermentation volume within 4 hours. Lastly, the production phase was conducted in the semi-batch operated bioreactor with different feeding strategies of the co-substrates together with continuous methanol feeding according to the pre-determined specific growth rate of μ_{M0}=0.03 h^{-1}.

In the first feeding strategy abbreviated as SSM, the strategy designed by Bozkurt (2012), was conducted and examined for rHuEPO production by *P. pastoris*. In this experiment, 50 g L^{-1} sorbitol was fed to the semi-batch operated bioreactor medium as a batch-wise mode at t=0 h. After that two substrates, methanol and sorbitol, were simultaneously added to the bioreactor with a pre-determined specific growth rate of μ_{M0}=0.03 h^{-1} and μ_{Srb0}=0.025 h^{-1}. While methanol was continued to be fed until the end of the experiment, sorbitol concentration was kept at 50 g L^{-1} as a constant within t=0-15 h of the production phase (Table 4.2.)

In the second designed experiment for the production of rHuEPO in bioreactor abbreviated as MM, the co-substrate was mannitol with 40 g L^{-1} initial concentration. It was added batch-wise to the medium with feeding methanol according to the pre-calculated specific growth rate of μ_{M0}=0.03 h^{-1} within t=0-30 h.

The third strategy; MMM was performed similar to the first strategy; SSM. While sorbitol was used in SSM, 50 g L^{-1} mannitol was fed to the bioreactor as a batch-wise mode starting the production phase at t=0 h. Thereafter, while methanol was fed to the medium with the pre-determined specific growth rate of μ_{M0}=0.03 h^{-1}, mannitol was simultaneously fed to the system with the pre-determined

specific growth rate of μ_{Man0}=0.11 h^{-1} within t=0-9 h, when the cell concentration at t=9 h in MMM was the same as that obtained at t=15 h in SSM, in order to keep the mannitol concentration constant at C_{Man0}=50 g L^{-1}. In order to develop MMM strategy, the previous experimental data in MM strategy was utilized.

In the fourth strategy; MLM, initially 3 g L^{-1} mannitol added at t=0 h and then mannitol was fed to the bioreactor with the pre-determined specific growth rate of μ_{Man0}=0.005 h^{-1} within t=0-10 h which was calculated from MM and MMM strategies as a limiting value besides fed-batch methanol feeding with μ_{M0}=0.03 h^{-1} to the end of the process.

In the fifth experiment; MPM, mannitol was fed to the bioreactor for three times at t=0 h, t=6 h and t= 12 h of the production phase to reach 50 g L^{-1} mannitol concentration in the semi-batch operated bioreactor. In addition, methanol was fed according to the pre-determined specific growth rate of μ_{M0}=0.03 h^{-1} until the bioprocess was stopped.

In the last production strategy; MPMG, pulse mannitol feeding was performed at t=0 h, t=7 h, t=14, and t= 24 h of production to attain 50 g L^{-1} mannitol concentration. This strategy was the same as MPM strategy. However, in order to examine the strategies, glycerol with the initial concentration in the fermentation medium being 8 g L^{-1} was fed in MPMG strategy.

The data for MS strategy developed and performed by Soyaslan (2010) was also used, in order to thoroughly evaluate the effects of the feeding strategy. The descriptions of the designed strategies are summarized in Table 4.2.

Table 4.2 The abbreviations used for experiments

Experiment Name	Strategy Definition
MS*	50 g L^{-1} batch-wise sorbitol pulse feeding (t=0 h); methanol feeding with μ_{M0}=0.03 h^{-1}
SSM	50 g L^{-1} batch-wise sorbitol pulse feeding (t=0 h) and keeping sorbitol concentration at 50 g L^{-1} at t=0-15 h; methanol feeding with μ_{M0}=0.03 h^{-1}
MM	40 g L^{-1} batch-wise mannitol pulse feeding (t=0 h); methanol feeding with μ_{M0}=0.03 h^{-1}
MMM	50 g L^{-1} batch-wise mannitol pulse feeding (t=0 h) and keeping mannitol concentration at 50 g L^{-1} at t=0-9 h; methanol feeding with μ_{M0}=0.03 h^{-1}
MLM	3 g L^{-1} batch-wise mannitol pulse feeding (t=0 h) and feeding mannitol with pre-determined μ_{Man0}=0.005 h^{-1} (t=0-10 h); methanol feeding with μ_{M0}=0.03 h^{-1}
MPM	50 g L^{-1} batch-wise mannitol pulse feeding at t = 0, 6, 12 h; methanol feeding with μ_{M0}=0.03 h^{-1}
MPMG	50 g L^{-1} batch-wise mannitol pulse feeding at t = 0, 7, 14, and 24 h containing glycerol; methanol feeding with μ_{M0}=0.03 h^{-1}

*Soyaslan and Çalık, 2011

4.2.3 Effect of Feeding Strategies on Co-Substrates Consumption Profiles

The sorbitol and mannitol consumption profiles of the experiments are illustrated in Figure 4.8 and Figure 4.9. Methanol was not detected in the fermentation media, confirming all methanol was consumed instantly. The cells expended all methanol fed with pre-determined exponential specific growth rate calculated by using equation 4.1. Moreover, the specific methanol consumption rates, q_M, did not change significantly throughout the bioprocesses (Table 4.5 and Table 4.6). Additionally, in MLM strategy mannitol was also not detected in the medium, since limiting amount of mannitol was fed continuously together with methanol, and mannitol and methanol were metabolized simultaneously.

Sorbitol consumption profile was investigated in the first feeding strategy, SSM where sorbitol concentration was kept constant at 50 g L^{-1} until t=15 h of the production phase and the pre-determined feeding strategies were used for methanol and sorbitol. Moreover, the designed strategy, MS, where 50 g L^{-1} sorbitol was only added initially, also showed the sorbitol consumption profile (Figure 4.8). Sorbitol consumption began at t=0 h in both feeding strategies, SSM and MS. In SSM and MS strategies, sorbitol was totally utilized at t=30 and 15 h, respectively (Figure 4.8). In SSM strategy, until t=15 h, sorbitol consumption rates, q_{srb}, were found nearly constant between 0.149-0.086 g g^{-1} h^{-1} (Table 4.5). After t=15 h, sorbitol consumption rates began to decrease and the whole sorbitol was consumed 15 hours at t=30 h. In addition, in MS strategy, Soyaslan (2010) found that consumption of sorbitol started at t=0 h and its depletion lasted 15 h in the production of rHuEPO by the same recombinant *P. pastoris.* Therefore, it was seen that all sorbitol consumed 15 h for both strategies, SSM and MS. However, after t=15 h the cell concentrations of SSM strategy began to be higher than those of MS strategy. At t= 30 h the cell concentration of SSM was 1.2- fold higher than that of MS strategy. It was shown that methanol and sorbitol were consumed simultaneously, which was similar with the results of Jungo *et al*. (2007) and Çelik *et al*. (2009).

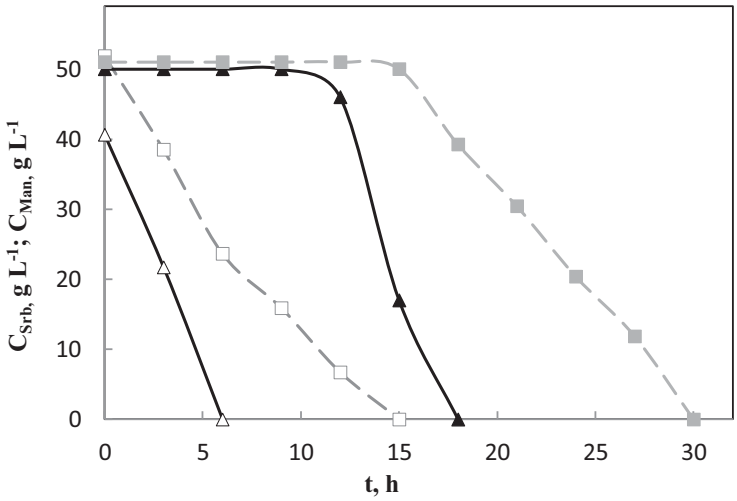

Figure 4.8 Variations in sorbitol and mannitol concentrations with the cultivation time: Black curves: MM (Δ), MMM (▲), Dotted and gray curves: MS (□), SSM (■).

Moreover, mannitol concentration profiles were investigated in MM, MMM, MPM and MPMG strategies. In MM strategy, where 40 g L^{-1} mannitol was added to the bioreactor at t=0 h of the production phase, mannitol was totally consumed within 6 hours (Figure 4.8). The specific mannitol consumption rate, q_{Man}, values were found between 0.183-0.179 g g^{-1} h^{-1} and it decreased with respect to time until t=6 h (Table 4.6). In MMM strategy, mannitol concentration was kept as a constant at 50 g L^{-1} until t=9 h when the cell concentration of MMM strategy at t=9 h was reached the same value as C_x= 70 g L^{-1} to that of SSM strategy at t=15 h. While the mannitol consumption rate, q_{Man}, values in MMM were constant until t=9 h, values were dramatically decreased after t=9 h and lasted to be

depleted within 9 hours (Table 4.6). Due to the mannitol and sorbitol consumption profiles between SSM and MMM, and also between MM and MS, it can be clearly seen that mannitol was depleted significantly much faster than sorbitol. The reason for that should be explained by the consumption pathway of these co-carbon sources and also the enzyme activities used in this pathway. It is known that both of these substrates enter to the glycolysis pathway. Before entering, they are converted to other metabolites. Sorbitol is firstly converted to D-fructose by D-glucitol dehydrogenase and then turned to fructose-6-phosphate by fructokinase (Figure 2.5). However, mannitol can be used two different ways in order to enter the glycolysis pathway. One of them is the same as sorbitol, which is oxidized to D-fructose and then phosphorylated to fructose-6-phosphate. The other one is that mannitol phosphorylated to mannitol-1-phosphate by mannitol kinase and then oxidized to fructose-6-phosphate by mannitol-1-phosphate dehydrogenase (Figure 2.5). However the phosphorylative mannitol utilization pathway, which involves firstly the action of mannitol kinase and followed by an oxidative step catalyzed by a mannitol-1-phosphate dehydrogenase, was not explained in detail to be used in fungi (Graça, 2004). It can be understood that whichever ways mannitol selects, there are two possibilities for the activity of enzymes used in the consumption pathway of both co-carbon sources. One is that the activity of mannitol dehydrogenase should be much higher than that of D-glucitol dehydrogenase. Quain and Boulton (1987) and Perfect *et al*. (1996) explained that in the presence of oxygen, a NAD^+-dependent mannitol dehydrogenase was highly found out in *S. cerevisiae*. They also pointed out oxygen used biomass formation is absolutely important for mannitol metabolism (Quain and Boulton, 1987; Perfect *et al*., 1996). Other possibility is that the expression level of mannitol dehydrogenase can be much lower than that of mannitol kinase. Therefore, cells can metabolize mannitol much faster than sorbitol by the help of enzymes in this pathway (Figure 2.5). Additionally, it can be explained by the specific consumption rates of sorbitol and mannitol. Whereas the highest sorbitol uptake rate, q_{srb}, was 0.149 g g^{-1} h^{-1} at t=3 h in SSM and 0.148 g g^{-1} h^{-1} at t=3 h in MS (Soyaslan, 2010) (Table 4.5), the highest mannitol uptake rate, q_{Man}, was found 0.183 g g^{-1} h^{-1} at t=3 h in MM and 0.249 g g^{-1} h^{-1} at t=3 h in MMM (Table 4.6). Thus, mannitol consumption rate was higher than sorbitol consumption rate.

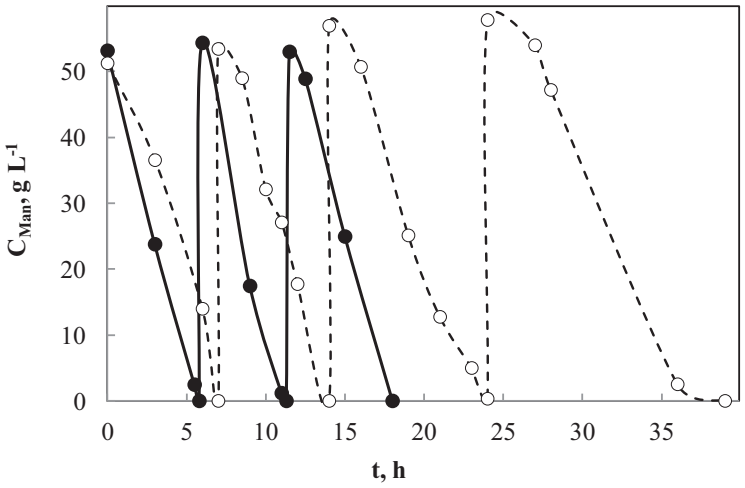

Figure 4.9 Variations in mannitol concentration with the cultivation time: MPM (●), MPMG (○).

In the last two experiments, MPM and MPMG, several pulse of mannitol was fed to the fermentation media in order to attain the mannitol concentration to 50 g L^{-1} after the previous pulse mannitol was consumed. In MPM strategy, mannitol pulses were consumed within 6 h, 5.5 h and 6.5 h for the first, second and third pulses, respectively. However, in MPMG, first pulse mannitol was consumed within 7 h at t=7 h, second one was also 7 h at t=14 h, third one was 10 h at t=24h and the last pulse was consumed within 15 h at t=39 h (Figure 4.9). It shows that mannitol consumption was taken approximately 6 hours. Nevertheless, the time for consumption of mannitol in MPMG was higher than time in MP since glycerol found in MPMG strategy decreased the specific mannitol consumption rates in this strategy. When the specific consumption rates are considered, it is explained that glycerol, with an initial concentration in the fermentation medium being 8 g L^{-1}, was metabolized with mannitol and methanol. While the highest mannitol consumption rate, q_{Man} in MPM was 0.272 g g^{-1} h^{-1} at t=3 h, 0.209 g g^{-1} h^{-1} at t=6 h was the maximum value in MPMG (Table 4.6).

4.2.4 Effect of Feeding Strategies on the Cell Growth

Cell growth is the most important parameter to be examined the effects of feeding strategies and different co-carbon sources. Before the production phase, the precultivation phases of the bioprocess, i.e., GB, GFB, and MT, were the same. The cell growth profiles in three phases are represented in Figure 4.10. In order to simplify the calculations the production phase began at t=0 h although this phase starts at around t=30 h after MT phase is completed. At t=0 h, co-carbon source was fed to the fermentation media batch-wise or in a fed- batch mode.

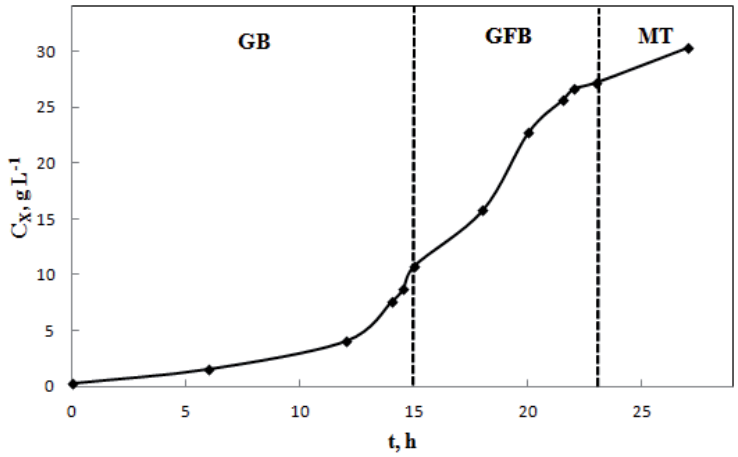

Figure 4.10 Variations in the cell concentration with the cultivation time in the precultivation phases: glycerol batch phase (GB), glycerol fed-batch phase (GFB) and methanol transition phase (MT).

The cell concentration profiles in the production phase of all feeding strategies are represented in Figure 4.11a. Addition of co-substrate to the medium, sorbitol or mannitol, decreased the long lag phase for the cells (Çelik, 2008). Therefore, the cells began to proliferate immediately.

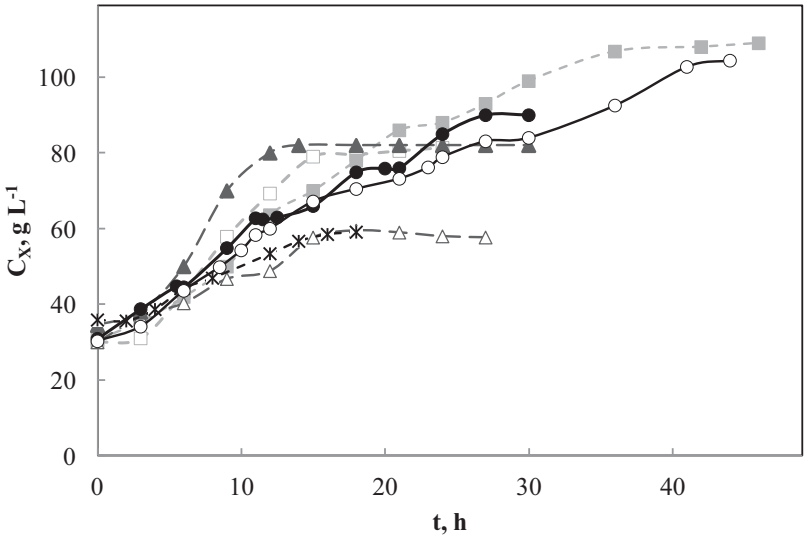

Figure 4.11a Variations in the cell concentration with the cultivation time in the production phase: MPM (●), MPMG (○), Dotted and black curves: MM (Δ), MMM (▲), MLM (*), Dotted and gray curves: MS (□), SSM (■).

The highest cell concentrations obtained in SSM and MS (Soyaslan, 2010) are 109 g L^{-1} at t=48 h and 81.4 g L^{-1} at t=24 h, respectively. The process time in SSM was longer than MS strategy since cells were attained the stationary phase barely. In addition, the biomass obtained at t=24 h in SSM was 1.2-fold higher than the cell concentration at t=24 h in MS. Therefore, fed-batch feeding of sorbitol beside methanol and keeping constant of sorbitol concentration at C_{Srb0}=50 g L^{-1} within the first 15 hours of the process is better than only 50 g L^{-1} sorbitol feeding batch-wise at t=0 h. Additionally, SSM strategy not only helped the cells to enter the stationary phase slowly, but it also provided more *P. pastoris* cells. The cell growth rates of both experiments were close to each other (Table 4.5). However, after sorbitol was consumed in SSM at t=30 h and in MS at t=15 h, bioprocess begin to only methanol fermentation and the cell growth rates decrease, which was also observed Çelik (2007).

In MM the highest cell concentration is 59.5 g L^{-1} at t=18 h, which is 1.4-fold lower than the maximum biomass obtained in MS since mannitol was metabolized efficiently within 6 h of the process and the time for consuming sorbitol was 2.5-fold higher than the time for mannitol (Figure 4.11b). After all mannitol was consumed, the cell growth was slowed down. However, while the cells using sorbitol as a co-carbon source were entered to the lag phase, the cells using mannitol began to proliferate without entering lag phase in early hours of the production phase. The highest cell growth rate of MM was found as 0.049 h^{-1} at the beginning of the bioprocess (Table 4.6). However, the maximum cell concentration obtained in MMM is 85 g L^{-1} at t=12 h, which is 1.1-, and 1.4-fold higher than the cells found in MS (Soyaslan, 2010) at t=24 h and MM at t=18 h (Figure 4.11b). It can be stated that feeding mannitol as a co-substrate and using fed-batch feeding strategy for co-carbon source, mannitol is the best way in order to increase cell concentration. Mannitol was consumed immediately within 6 h of the process; however, the cell concentration at t=48 h in SSM is 1.8-fold higher than that obtained at t=18 h in MMM. The reason is that cell production in MMM was much faster than that in SSM, so the time for achieving maximum cell concentration in MMM is obtained in early hours of the production.

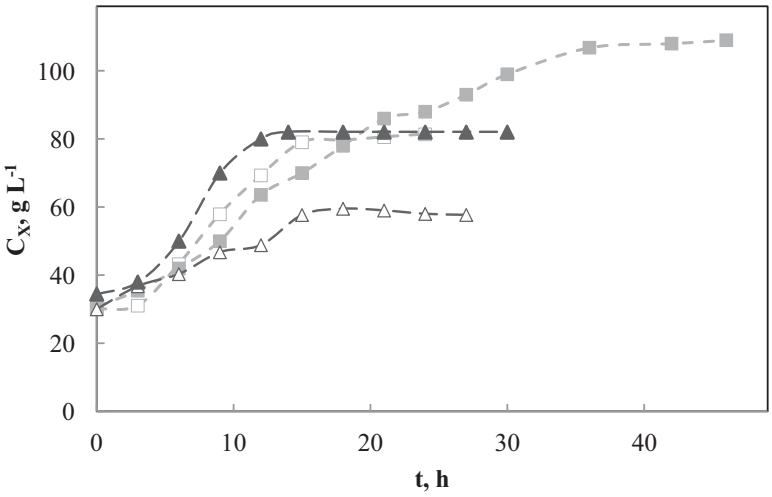

Figure 4.11b Variations in the cell concentration with the cultivation time in the production phase: Dotted and black curves: MM (Δ), MMM (▲) and Dotted and gray curves: MS (□), SSM (■).

The highest cell concentration obtained in MPM and MPMG strategies are 90 g L^{-1} at t=30 h and 104.4 g L^{-1} at t=44 h (Figure 4.11c). Although the cell growth profiles in MPM and MPMG strategies show nearly same trend, the time for entering the stationary phase for MPMG takes much more than the time for MPM. It can be occurred from longer time between mannitol pulses into the medium in MPMG. Furthermore, MLM and MM strategies have the same cell growth profiles and they attain the same highest cell concentration at the same time as C_X=59.5 g L^{-1} at t=18 h. That should be occurred from the calculations of mannitol consumption profiles that were used the data from taken MM strategy. When considering the cell growth rates, the highest values obtained in MMM, MPM and MPMG are 0.145, 0.075, and 0.093 h^{-1}, respectively (Table 4.6). After consumption of mannitol into the medium, the specific growth rates decreases since cells can only metabolize methanol for the growth.

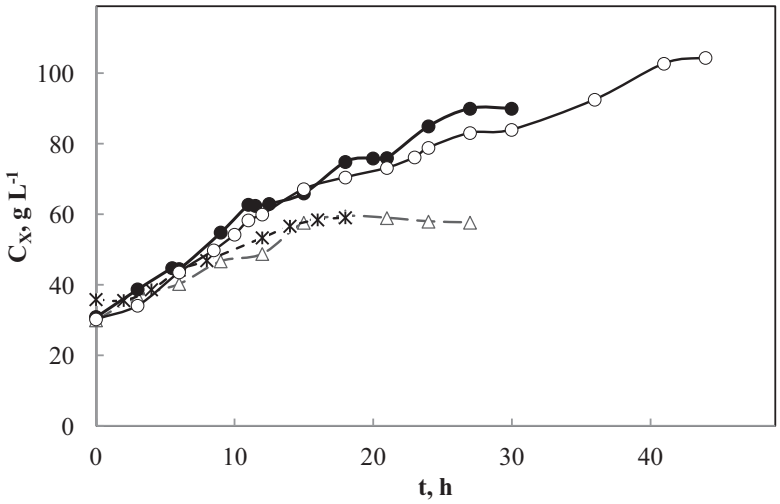

Figure 4.11c Variations in the cell concentration with the cultivation time in the production phase: MPM (●), MPMG (○), Dotted and black curves: MM (Δ), MLM (*).

4.2.5 Effect of Feeding Strategies on the Recombinant EPO Production

The aim of the bioprocess experiments is to enhance the recombinant protein production. The effects of the co-substrates and feeding strategies designed on the rHuEPO production were determined by SDS-PAGE analyses of the samples. The variation in rHuEPO concentrations with the cultivation time with different feeding strategies is represented in Figure 4.13a. The highest rHuEPO concentrations were compared using SDS-PAGE in Figure 4.12. Additionally, SDS-PAGE analyses of all experiments with the cultivation time are illustrated in Appendix G.

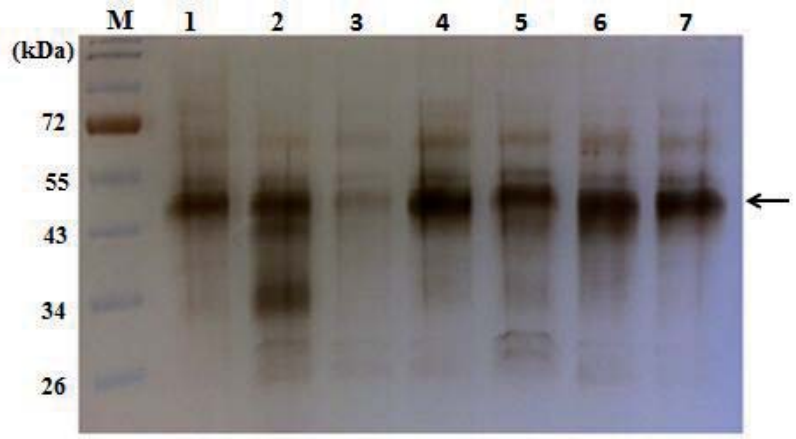

Figure 4.12 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in pilot scale bioreactors: M: protein marker, 1. well: t=21 h for MS, 2. well: t=18 h for SSM, 3. well: t=6 h for MM, 4. well: t=9 h for MMM, 5. well: t=10 h for MLM, 6. well: t=9 h for MPM, 7. well: t=15 h for MPMG.

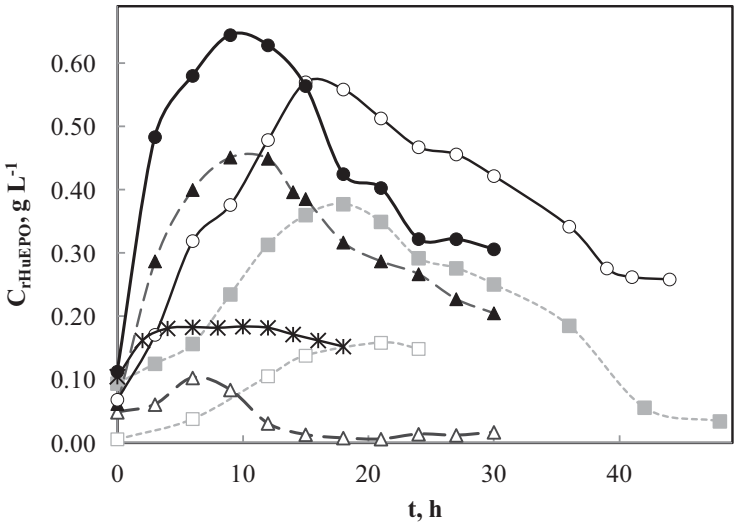

Figure 4.13a Variations in rHuEPO concentrations with the cultivation time: MPM (●), MPMG (○), Dotted and black curves: MM (Δ), MMM (▲), MLM (*), Dotted and gray curves: MS (□), SSM (■).

In SSM strategy the highest recombinant protein was determined as C_{rHuEPO}=377 mg L^{-1} at t=18 h, which is 75% of the total proteins secreted. It is 2.4-fold higher than the highest rHuEPO concentration at t=21 h in MS strategy (Soyaslan, 2010). The specific recombinant protein production rate in SSM strategy enhanced throughout the cultivation time and the maximum rate reached was found as 0.590 g g^{-1} L^{-1} at t=9 h (Table 4.5) whereas in MS strategy (Soyaslan, 2010), the highest specific rHuEPO production was q_{rHuEPO}= 0.198 g g^{-1} L^{-1} at t=9 h which is 2.9-fold lower than that of SSM strategy. Therefore, fed-batch sorbitol feeding is more preferable strategy than sorbitol feeding only at the beginning of the production phase.

The lowest rHuEPO production was obtained in MM; however, the maximum rHuEPO concentration obtained in this strategy at t=6 h as C_{rHuEPO}= 83.9 mg L^{-1} which is 35% of the proteins secreted to the broth (Figure 4.13b). Moreover, the highest specific protein production rate in MM was q_{rHuEPO}= 0.423 g g^{-1} L^{-1} at t=3 h which is 2.1- fold higher than that obtained in MS. Although mannitol concentration that was 40 g L^{-1} in the fermentation broth was metabolized immediately within 6 h, the cell growth and also the recombinant protein production was not as high as other feeding strategies.

For another strategy, MMM, the highest recombinant protein production that is 89% of the total protein secreted was found out as C_{rHuEPO}= 451 mg L^{-1} at t=9 h (Figure 4.13b). It is 5.4-, and 1.2-fold higher than that obtained in MM and SSM, respectively. Furthermore, in MMM, the highest specific rHuEPO production rate was obtained q_{rHuEPO}= 1.8 g g^{-1} L^{-1} at t=3 h. It is clearly explained that feeding mannitol as a co-substrate is the best option among strategies. In MLM strategy, there is nearly constant rHuEPO production. The highest rHuEPO production corresponds 58% of the secreted proteins, attained at t=10 h as C_{rHuEPO}= 184 mg L^{-1}. Its highest specific rHuEPO production rate was found as q_{rHuEPO}= 0.58 g g^{-1} L^{-1} at t=3 h which is 1.4- fold higher than that obtained in MM; however, 3.0- fold lower than that obtained in MMM (Figure 4.13b). The reason is that limiting amount of mannitol is not sufficient in order to produce much more protein and also increase the cell growth.

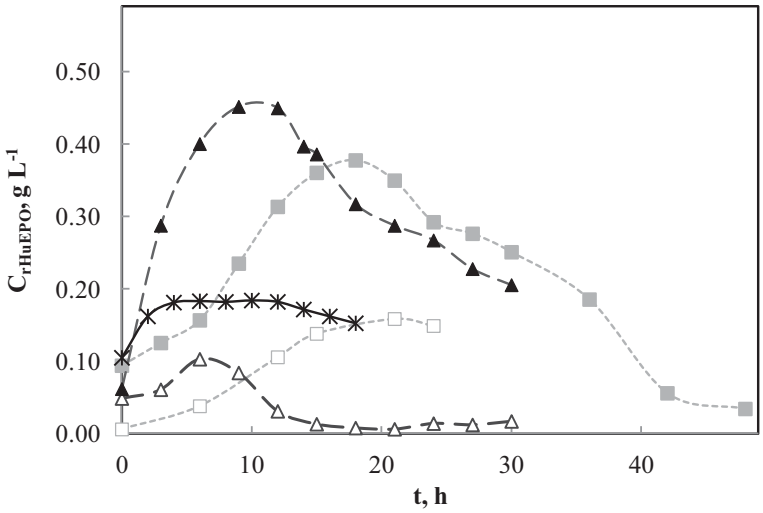

Figure 4.13b Variations in rHuEPO concentration with the cultivation time: Dotted and black curves: MM (Δ), MMM (▲), MLM (*), Dotted and gray curves: MS (□), SSM (■).

The highest C_{rHuEPO} was obtained in MPM as C_{rHuEPO}= 645 mg L^{-1} at t=9 h, which is 83% of the total secreted proteins. It is 1.2-, and 1.4-fold higher than that in MPMG at t= 15 h and MMM at t= 9 h, respectively (Figure 4.13c). Moreover, the specific recombinant production rate in MPM enhanced with the cultivation time and the maximum value is found as q_{rHuEPO}= 2.1 g g^{-1} L^{-1} at t=3 h which is 1.7-, and 1.2- fold higher than that acquired in MPMG and MMM, respectively. The highest rHuEPO production was attained in early hours of the strategy MPM. Therefore, by using mannitol at C_{Man0}= 50 g L^{-1} with pulse feeding strategy the process time can be lowered and in early hours higher cell and protein concentrations were obtained.

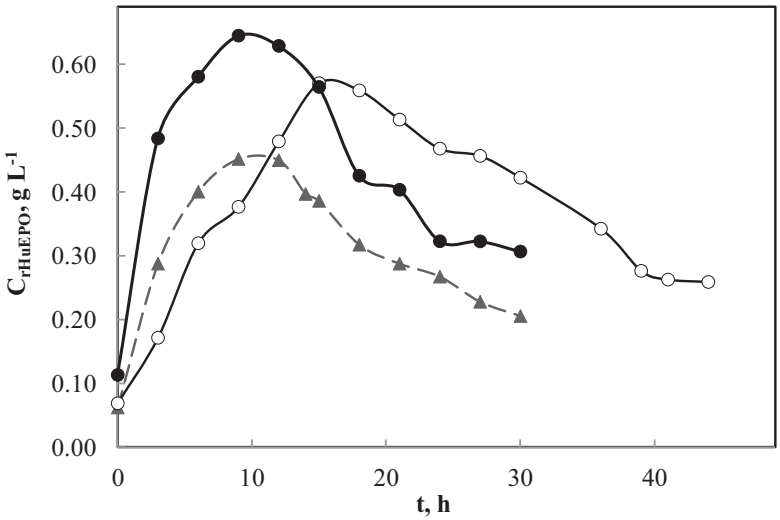

Figure 4.13c Variations in rHuEPO concentration with the cultivation time for the best three strategies: MPM (●), MPMG (○), Dotted and black curves: MMM (▲).

4.2.6 Effect of Feeding Strategies on Alcohol Oxidase Activity

Alcohol oxidase (AOX) is the key enzyme in the methanol metabolism of *P. pastoris*. AOX activities were represented in Figure 4.14 for all strategies since rHuEPO production began with the induction of AOX promoter.

In all experiments except MLM and MS strategies (Soyaslan, 2010), AOX activities achieved maximum values at the beginning of the experiments and then they diminished to lower values. It is known that the highest rHuEPO production rates were also obtained in the early hours of the processes for MM, MMM, MPM, MPMG and SSM. For that reason, the highest AOX activities have the same profiles as the highest recombinant protein production.

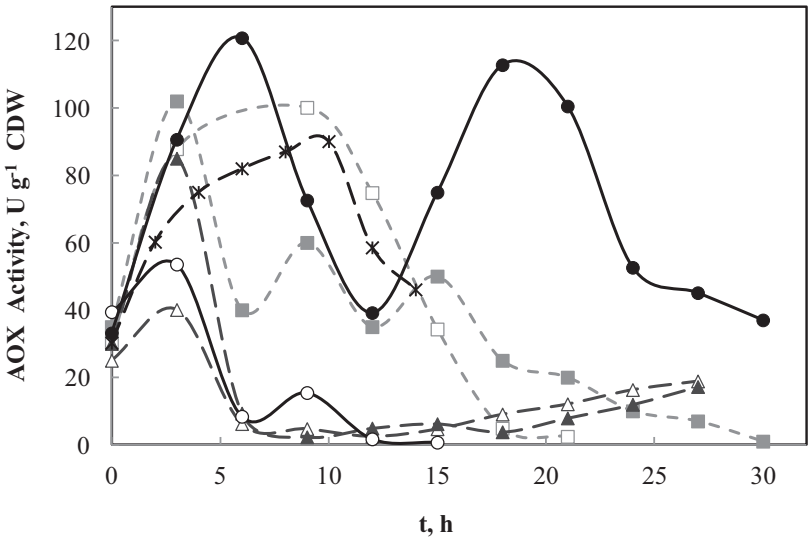

Figure 4.14 Variations in alcohol oxidase activity with the cultivation time: MPM (●), MPMG (○), Dotted and black curves: MM (Δ), MMM (▲), MLM (*), Dotted and gray curves: MS (□), SSM (■).

In the presence of sorbitol for SSM and MS strategies, AOX activities show an increasing trend at the beginning and then continue to decrease until the total depletion of sorbitol. The highest AOX activity in SSM was found at t=3 h as 102 U g^{-1} CDW, which was 1.1-fold higher than the highest AOX activity in MS obtained at t=9 h. The reason is that methanol transition phase in this study was applied as feeding methanol at a rate of 3.6 mL L^{-1} h^{-1} within 4 hours in place of a pulse methanol feeding, C_M=1.5 g L^{-1} for 6 hours (Soyaslan, 2010). Therefore, in early hours of the production phase higher AOX activities were achieved.

In MMM, the highest AOX activity was also achieved at t=3 h as 85 U g^{-1} CDW, which was 2.1-fold higher than that obtained in MM; however, it was 1.2-fold lower than that obtained in SSM. In MLM strategy, the highest AOX activity was found as 100 U g^{-1} CDW at t=12 h.

For several-pulse feeding strategies applied in MPM and MPMG the highest AOX activities was again measured at the early hours of the production phase as 120 U g^{-1} CDW at t=6 h and 53.6 U g^{-1} CDW at t=3 h, respectively. The reason why their AOX activities were different from each other is that glycerol with the initial concentration in the fermentation medium being 8 g L^{-1} might be repressing the AOX promoter. In MPM strategy, the highest activity value is 1.4-fold higher than that obtained in MMM. This might be a result of stress on cells due to the feeding mannitol within t=0-9 h

instead of a pulse feeding of mannitol. Therefore, while addition of mannitol and sorbitol help to shorten the lag-phase, mannitol provides to reach high AOX activities in shorter times.

4.2.7 Effect of Feeding Strategies on Total Protease Activity

The activities of acidic, neutral and alkali proteases in the extracellular medium were determined separately and converted to total protease concentration. The total protease concentrations of all strategies are illustrated on Figure 4.15a. In all feeding strategies total protease concentrations begin with lower values and then increased with respect to the cultivation time as given in Figure 4.15a.

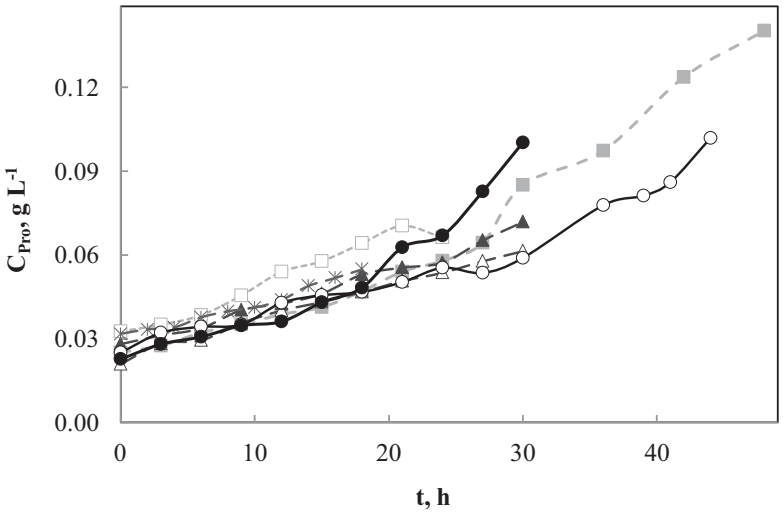

Figure 4.15a Variations in total protease concentration with the cultivation time: MPM (●), MPMG (○), Dotted and black curves: MM (Δ), MMM (▲), MLM (*), Dotted and gray curves: MS (□), SSM (■).

When considering protease concentration profile in SSM strategy, it was lower than that of the MS (Soyaslan, 2010) within the 21 h of the production phase. Afterwards protease concentration of SSM strategy began to increase much higher than before due to the depletion of sorbitol at t=30 h. Whereas the maximum protease concentration in SSM was achieved at t=48 h as C_{Pro}=0.140 g L^{-1}, in MS strategy it was observed at t=24 h and 2-fold lower than that obtained in SSM (Figure 4.15b).

In MMM strategy, the total protease concentration profile shows the same linear increasing trend as MM and also MLM (Figure 4.15b). The reason of the production of higher rHuEPO at MM, MMM and MLM in early hours of the processes became clear with cell and protease concentrations and also AOX activities. Moreover, it was seen that there is no effect of mannitol on protease concentration in these strategies so that after the depletion of mannitol, the total protease concentration was not changed significantly.

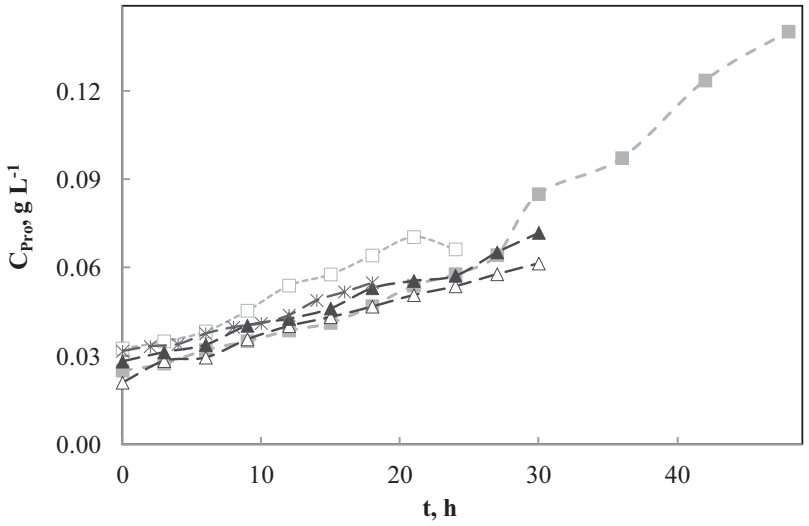

Figure 4.15b Variations in total protease concentration with the cultivation time: Dotted and black curves: MM (Δ), MMM (▲), MLM (*), Dotted and gray curves: MS (□), SSM (■).

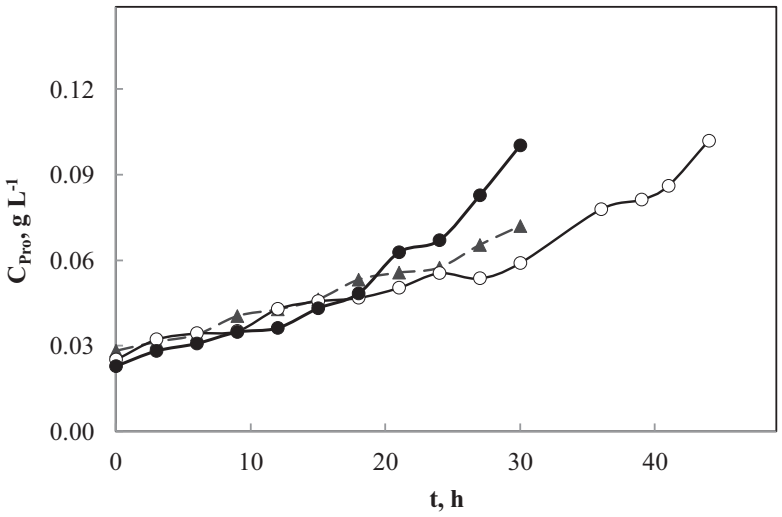

Figure 4.15c Variations in total protease concentration with the cultivation for the best three strategies: MPM (●), MPMG (○), and Dotted and black curves: MMM (▲).

4.2.8 Effect of Feeding Strategies on Organic Acid Concentration Profiles

Organic acid profiles throughout the process help to understand the intracellular reaction networks of recombinant *Pichia pastoris* more deeply. Therefore, organic acid profiles of different fermentation media were investigated and variations in organic acid concentrations are shown in Table 4.3.

For all strategies, oxalic acid, gluconic acid, pyruvic acid, formic acid, malic acid, acetic acid, citric acid, and succinic acid which are mostly involved in TCA cycle reactions were found out in the fermentation medium. However, lactic acid was only detected in SSM and MLM as the metabolic byproduct. Lactic acid is formed when the oxygen in the medium is not sufficient. Besides the TCA

cycle need oxygen to take place efficiently. Therefore, air that was enriched with oxygen was not sufficient throughout the processes in SSM and MLM strategies. The highest lactic acid concentrations found at SSM and MLM as 1.33 g L^{-1} at t=42 h and 0.39 g L^{-1} at t=14 h, respectively.

Methanol is converted to hydrogen peroxide and formaldehyde by the help of AOX enzyme in methanol utilization pathway. Additionally, it is known that hydrogen peroxide and formaldehyde are toxic (Zhang *et al*., 2000). When there is instability between methanol oxidation and formaldehyde consumption, formaldehyde concentration increases throughout the cell growth and product formation (Charoenrat *et al*., 2006). Formaldehyde is normally oxidized to formic acid or goes into assimilatory pathway and then it enters the glycolysis. While formic acid concentrations in SSM, MM, MMM and MLM strategies increased with respect to time, at the beginning of MPM and MPMG bioprocesses it was high but then there is no any formic acid detected in the production medium. Because it may have been used in anabolic reactions and so it was not detected.

Table 4.3 Variation in organic acid concentrations with respect to time different feeding strategies in g L^{-1}

	SSM								
t (h)	**3**	**6**	**9**	**15**	**21**	**24**	**30**	**36**	**42**
Oxalic acid	0.028	0.038	0.030	0.032	0.046	0.037	0.037	0.055	0.073
Gluconic acid	0.103	0.344	0.339	0.476	0.620	0.609	0.763	1.226	1.365
Pyruvic acid	0.004	0.012	0.012	0.006	0.010	0.015	0.021	0.062	0.077
Formic acid	0.025	0.069	0.028	0.024	0.023	0.023	0.021	0.149	0.156
Malic acid	0.022	0.024	0.022	0.023	0.085	0.107	0.190	0.466	0.620
Lactic acid	0.232	0.232	0.229	0.221	0.347	0.418	0.671	1.062	1.327
Acetic acid	0.068	0.109	0.125	0.149	0.275	0.297	0.476	0.568	0.594
Citric acid	0.037	0.030	0.027	0.045	0.089	0.110	0.240	0.383	0.665
Succinic acid	0.047	0.036	0.037	0.043	0.061	0.043	0.076	0.077	0.115

	MM						
t (h)	**3**	**6**	**9**	**15**	**21**	**24**	**30**
Oxalic acid	0.032	0.022	0.016	0.028	0.081	0.021	0.043
Gluconic acid	0.114	0.254	0.368	0.612	0.784	0.787	1.044
Pyruvic acid	0.002	0.021	0.018	0.022	0.028	0.030	0.054
Formic acid	0.080	0.086	0.078	0.093	0.113	0.105	0.193
Malic acid	0.028	0.030	0.035	0.043	0.052	0.054	0.270
Acetic acid	0.053	0.079	0.077	0.242	0.199	0.269	0.516
Citric acid	0.059	0.080	0.074	0.094	0.147	0.129	0.192
Succinic acid	0.074	0.060	0.043	0.055	0.066	0.065	0.072

	MMM								
t (h)	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**
Oxalic acid	0.037	0.051	0.033	0.033	0.028	0.018	0.028	0.045	0.032
Gluconic acid	-	-	0.078	0.225	0.328	0.639	0.779	1.503	0.773
Pyruvic acid	0.008	0.013	0.011	0.012	0.013	0.015	0.018	0.043	0.047
Formic acid	0.023	0.069	0.069	0.032	0.028	0.099	0.110	0.214	0.153
Malic acid	0.020	0.021	0.031	0.043	0.051	0.063	0.076	0.423	0.390
Acetic acid	0.041	0.070	0.094	0.216	0.220	0.265	0.259	0.510	0.491
Citric acid	0.020	0.035	0.053	0.086	0.108	0.111	0.163	0.275	0.325
Succinic acid	0.028	0.037	0.051	0.056	0.059	0.068	0.071	0.124	0.097

Table 4.3 Continued

	MLM						
t (h)	**2**	**4**	**6**	**10**	**14**	**16**	**18**
Oxalic acid	0.024	0.030	0.029	0.036	0.039	0.034	0.036
Gluconic acid	0.280	0.203	0.167	0.482	0.620	0.527	0.466
Pyruvic acid	0.012	0.017	0.025	0.037	0.061	0.057	0.034
Formic acid	0.239	0.092	0.046	0.145	0.201	0.159	0.102
Malic acid	0.024	0.040	0.079	0.102	0.223	0.218	0.102
Lactic acid	0.262	0.387	0.373	0.359	0.393	0.371	0.284
Acetic acid	-	0.093	0.100	0.177	0.231	0.253	0.271
Citric acid	0.048	0.071	0.076	0.098	0.143	0.152	0.141
Succinic acid	0.098	0.092	0.053	0.069	0.074	0.079	0.073

	MPM									
t (h)	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
Oxalic acid	0.046	0.084	0.077	0.060	0.076	0.100	0.067	0.076	0.067	0.079
Gluconic acid	-	-	-	-	-	-	0.102	0.185	0.337	0.423
Pyruvic acid	0.008	0.022	0.228	0.280	0.381	0.420	0.099	0.046	0.072	0.037
Formic acid	0.017	0.018	-	-	-	-	-	-	-	-
Malic acid	0.036	0.043	0.095	0.112	0.212	0.253	0.118	0.124	0.610	0.477
Acetic acid	0.025	0.165	0.287	0.037	0.024	-	-	-	-	-
Citric acid	-	0.037	0.055	0.010	-	-	-	0.041	0.052	0.068
Succinic acid	0.030	0.026	0.029	0.031	0.029	0.020	0.040	0.063	0.036	0.068

	MPMG									
t (h)	**3**	**6**	**9**	**12**	**15**	**21**	**24**	**30**	**36**	**44**
Oxalic acid	0.035	0.043	0.051	0.046	0.037	0.072	0.087	0.079	0.075	0.118
Gluconic acid	-	-	-	-	-	-	-	0.092	0.132	0.116
Pyruvic acid	0.011	0.014	0.064	0.132	0.125	0.091	0.197	0.309	0.089	0.029
Formic acid	0.020	0.021	0.013	0.011	0.010	-	-	-	-	-
Malic acid	0.054	0.043	0.037	0.048	0.062	0.251	0.271	0.200	0.193	0.269
Acetic acid	-	-	-	-	-	-	-	0.064	0.211	0.501
Citric acid	-	-	0.014	0.022	0.014	0.022	0.020	0.010	0.026	0.046
Succinic acid	0.022	0.024	0.025	0.030	0.038	0.038	0.043	0.044	0.034	0.091

4.2.9 Yield Coefficients and Specific Rates

The overall yield coefficient should be determined in order to understand the efficiency and profitability of a bioprocess. The overall yield of biomassl generated per mass of total substrate consumed ($Y_{X/St}$), the overall yield of product formed per mass of total substrate consumed ($Y_{P/St}$), and the overall yield of product formed per mass of biomass generated ($Y_{P/X}$) were calculated for rHuEPO production processes for different feeding strategies (Table 4.4).

Table 4.4 Overall Yield Coefficients

Strategy	$Y_{X/St}$ (g/g)	$Y_{P/St}$ (mg/g)	$Y_{P/X}$ (mg/g)
MS*	0.51	1.45	2.85
SSM	0.17	1.35	7.18
MM	0.20	1.21	6.06
MMM	0.33	2.15	8.71
MLM	0.27	1.74	6.54
MPM	0.22	2.23	16.66
MPMG	0.15	2.08	13.59

*Soyaslan and Çalık, 2011

The cell yields on total substrates containing sorbitol and methanol in SSM and MS (Soyaslan, 2010) are different from each other. The total amount of carbon sources, sorbitol and methanol, consumed at SSM as 226.6 g was higher than that of MS strategy as 104.9 g. Moreover, the cell concentration of SSM at t=18 h, when the maximum production was obtained, was 1.2-fold lower than that of the MS at t=21 h. For that reason, the $Y_{X/St}$ value in SSM was 3-fold lower than that in MS. When considering the mannitol feeding strategies, the highest $Y_{X/St}$ value was calculated in MMM strategy. While the amount of carbon sources consumed at MMM was 1.6- and 2.7- fold higher than that of MM and MLM, respectively, it was 1.5- and 2.0-fold lower than that of the MPM and MPMG, respectively. Additionally, the cell concentration of MMM at t=6 h was 2.0-, 1.7-, 1.4- and 1.2-fold higher than that of MM, MLM, MPM and MPMG, respectively. Therefore, the cell concentrations and also used total carbon sources show that the highest cell yield on substrate containing mannitol and methanol was determined as 0.33 g g^{-1} at MMM.

The highest overall product yield on total substrate, $Y_{P/St}$ (sorbitol and methanol) was calculated as 1.45 mg g^{-1} for MS which is closed to that calculated for SSM (Table 4.4). However, considering the overall product yield on total substrate (mannitol and methanol), the highest value was found as 2.23 mg g^{-1} for MPM. This is an expected result since addition of mannitol into the medium as a pulse increased the product yield on total substrate.

The fed-batch addition of sorbitol into the SSM medium increased the product yield on cell as $Y_{P/X}$=7.18 mg g^{-1} and 2.5-fold from the MS which feeding sorbitol as batch-wise. However, feeding mannitol into the medium as fed-batch in MMM increased the product yield on cell much more than that of sorbitol as batch-wise in SSM about 1.2-fold. The highest $Y_{P/X}$ was obtained as 16.66 mg g^{-1} at MPM. Even though the overall yield coefficients are essential parameters to evaluate the process efficiency and better understand the process, it should be known that the products for having high commercial values, which is finally reached is more critical and important. Also, the aim of the study is to increase the final product, erythropoietin. Addition of three pulse mannitol in MPM was increased rHuEPO 1.2-fold than addition of four pulse mannitol in MPMG. Furthermore, by adding pulse mannitol as a co-substrate into the medium the process time was lowered and the highest rHuEPO was achieved in the early hours of the process in spite of the fact that the cost of mannitol was higher than that of sorbitol.

The variation in total specific growth rate (μ_t), specific sorbitol consumption rate (q_{Srb}), specific mannitol consumption rate (q_{Man}), specific methanol consumption rate (q_M), and specific recombinant product formation rate (q_{rHuEPO}) are shown in Table 4.5 and Table 4.6.

Though a specific growth rate of methanol (μ_M) was preferred as μ_{M0}=0.03 h^{-1} for all bioreactor experiments, it was not definitely constant at the desired value and it was achieved in average. Thereafter, adding sorbitol and mannitol batch-wise to the system in MS (Soyaslan, 2010) and in MM, MPM and MPMG, respectively without altering the feed rate of methanol, concluded higher specific growth rates as well as adding co-substrates in a fed-batch mode in SSM, MMM and MLM. The reason is that cells consume methanol (μ_M) and sorbitol (μ_{Srb}) or methanol and mannitol (μ_{Man}) simultaneously. Consequently, the total specific growth rate is assumed, $\mu_t = \mu_{M0} + \mu_{Srb}$ or $\mu_t = \mu_{M0} + \mu_{Man}$ where μ_{M0}=0.03=constant. For all processes, methanol consumption rates were nearly constant since they did not change notably (Table 4.5 and Table 4.6).

The specific sorbitol consumption rates (q_{Srb}) in SSM strategy were the highest at the beginning of the process and then lowered as well as those in MS (Soyaslan, 2010). The reason is that when cell concentration increases continually, sorbitol concentration in the medium decreases in MS. On the other hand, in SSM, fed-batch sorbitol feeding was stopped at t=15 h and the specific consumption rate values were lowered until it was consumed totally. When considering the mannitol consumption rates (q_{Man}), in MM the values were decreased with respect to time until t=6, when the all mannitol was metabolized as well as sorbitol (Table 4.6). In MMM, mannitol consumption rates were constant during the feeding time at t=0-9 h. After that, its consumption rate was diminished to q_{Man}=0.047 g g^{-1} h^{-1} within t=0-15 h. Due to the limiting amount of mannitol fed to the system, q_{Man} for MLM strategy was stayed constant within t=0-10 h. For other strategies, MPM and MPMG, mannitol consumption rate values were higher at the beginning of the process as 0.272 g g^{-1} h^{-1} for MPM and 0.209 g g^{-1} h^{-1} for MPMG. Then they decreased until the mannitol concentration was increased to C_{Man0}=50 g L^{-1}. After the final pulse of mannitol fed to the medium, q_{Man} decreased to 0.111 g g^{-1} h^{-1} and 0.056 g g^{-1} h^{-1} for MPM and MPMG, respectively (Table 4.6). Considering SSM and MS strategies, the highest recombinant production rate was obtained in SSM as q_{rHuEPO}=0.590 mg g^{-1} h^{-1} at t=9 h. On the other hand, when other strategies where mannitol was utilized as a co-substrate, the highest q_{rHuEPO} was obtained as 2.049 mg g^{-1} h^{-1} at the beginning of the process in MPM strategy which has the highest recombinant protein concentration and AOX activity.

Table 4.5 Variation in specific rates throughout the fermentation bioprocess with a co-substrate, sorbitol

Exp.	**t**	μ_t	q_{Srb}	q_M	q_{rHuEPO}
Name	h	h^{-1}	$g\ g^{-1}\ h^{-1}$	$g\ g^{-1}\ h^{-1}$	$mg\ g^{-1}\ h^{-1}$
	3	0.046	0.148	0.067	0.173
	6	0.097	0.086	0.053	0.159
	9	0.078	0.048	0.043	0.198
MS*	12	0.054	0.038	0.039	0.194
	15	0.025	0.028	0.037	0.089
	18	0.007	-	0.039	0.049
	21	0.008		0.042	0.016
	24	0.007		0.045	-
	3	0.067	0.149	0.065	0.342
	6	0.072	0.131	0.058	0.487
	9	0.087	0.113	0.051	0.590
SSM	12	0.067	0.092	0.042	0.403
	15	0.050	0.086	0.040	0.233
	18	0.038	0.039	0.039	-
	21	0.024	0.035	0.038	
	24	0.018	0.034	0.040	
	27	0.025	0.036	0.041	
	30	0.021	-	0.041	
	36	0.013		0.044	
	42	0.009		0.051	

*Soyaslan and Çalık, 2011

Table 4.6 Variation in specific rates throughout the fermentation bioprocess with a co-substrate, mannitol

Exp. Name	t h	μ_t h^{-1}	q_{Man} $g\ g^{-1}\ h^{-1}$	q_M $g\ g^{-1}\ h^{-1}$	q_{rHuEPO} $mg\ g^{-1}\ h^{-1}$
	3	0.049	0.183	0.053	0.423
	6	0.044	0.179	0.052	0.195
	9	0.033	0.000	0.049	-
MM	12	0.040	-	0.051	
	15	0.034		0.047	
	18	0.007		0.049	
	21	0.000		0.053	
	24	0.001		0.059	
	3	0.117	0.249	0.062	1.794
	6	0.145	0.232	0.044	0.942
	9	0.138	0.198	0.029	0.493
	12	0.028	0.067	0.028	0.240
MMM	15	0.009	0.047	0.029	-
	18	0.009	-	0.031	
	21	0.013		0.033	
	24	0.011		0.035	
	27	0.012		0.037	
	2	0.029	0.044	0.075	0.579
	4	0.065	0.040	0.072	0.181
	6	0.056	0.035	0.065	0.046
MLM	8	0.041	0.033	0.064	0.044
	10	0.042	0.030	0.063	0.037
	12	0.036	-	0.062	-
	14	0.027		0.061	
	16	0.015		0.063	

Table 4.6 Continued

Exp. Name	t h	μ_t h^{-1}	q_{Man} $g\ g^{-1}\ h^{-1}$	q_M $g\ g^{-1}\ h^{-1}$	q_{Gly} $mg\ g^{-1}\ h^{-1}$	q_{rHuEPO} $mg\ g^{-1}\ h^{-1}$
MPM	3	0.075	0.272	0.067	-	2.049
	5	0.046	0.227	0.061		
	6	0.060	0.102	0.063		0.649
	9	0.070	0.197	0.056		0.192
	11	0.044	0.189	0.051		
	12	0.006	0.103	0.053		0.173
	15	0.041	0.132	0.054		-
	18	0.031	0.111	0.051		
	21	0.012	-	0.054		
	24	0.014		0.057		
	27	0.021		0.060		
MPMG	3	0.068	0.179	0.069	0.723	1.239
	6	0.093	0.209	0.058		0.808
	7	0.067	0.000	0.052	0.544	
	9	0.045	0.202	0.053		0.514
	10	0.062	0.200	0.052		
	11	0.053	0.122	0.050		
	12	0.035	0.149	0.050		0.569
	14	0.032	0.000	0.049	0.500	
	19	0.022	0.087	0.051		
	21	0.021	0.171	0.051		0.174
	22	0.025	0.135	0.052		-
	23	0.037	0.081	0.052		
	24	0.029	0.000	0.051	0.527	
	28	0.015	0.022	0.054	-	
	30	0.020	0.073	0.056		
	36	0.029	0.056	0.059		
	39	0.023	0.000	0.059		
	41	0.022	-	0.059		

CHAPTER 5

CONCLUSION

In this M.Sc. thesis, it is aimed to investigate the effects of different co-carbon sources and feeding strategies in order to enhance therapeutically important glycoprotein, recombinant human erythropoietin (rHuEPO) production by *Pichia pastoris.* In this context, the research program was divided into two main sub-programs.

In the first part of the study, effects of two different co-carbon sources, mannitol and sorbitol, on cell growth and rHuEPO production were examined in laboratory scale air-filtered shake flask experiments. Depending on the process economy and based on the inhibitory effect, C_{Man0}=50 g L^{-1} was the most affordable and appropriate for the production of rHuEPO by *P. pastoris* because of the results explained below:

- The highest cell concentration was achieved as 8.3 g L^{-1} in the production medium containing C_{Man0}=50 g L^{-1} at the end of the process.

- The highest rHuEPO production was obtained as 70 mg L^{-1} in medium containing C_{Man0}=50 gL^{-1} with 1% (v/v) methanol, as well as the highest cell concentration.

In the second phase of the study, six different pilot-scale bioreactor experiments were performed to improve rHuEPO production by *P. pastoris*. The best feeding strategy for the production of rHuEPO was obtained as MPM strategy that included three-pulse mannitol feeding to the production medium with fed-batch feeding of methanol. Moreover, addition of mannitol to the fermentation medium helped to reach higher cell concentrations in early hours of the bioprocess compared to addition of sorbitol. However, the other key findings of the strategies are explained below:

- The highest cell concentration was obtained as C_X=109 g L^{-1} in SSM at t=48 h; however, cells reached the highest concentrations as C_X=85 g L^{-1} at t=12 h in MMM, whereas the same cell concentration was obtained at t=21 h in SSM. Therefore, cells using mannitol reached higher cell concentrations in early hours of the production phase. Moreover, based on the feeding strategy, fed-batch feeding of co-substrate was more advantageous on cell growth than batch-wise feeding of them. After consumption of mannitol and sorbitol in the medium, cell growth rates (μ_t) decreased in all strategies.

- Methanol was not detected in the medium since all methanol were consumed immediately. Moreover, methanol and co-substrates were consumed simultaneously during all processes.

- The depletion of sorbitol started at t=0 h and lasted within t=15-30 h when fed-batch sorbitol feeding was stopped. However, consumption of mannitol also begun at t=0 h and consumption time lasted for 6 or 9 h. Therefore, mannitol was metabolized much faster than sorbitol since activities of enzymes of the mannitol utilization pathway of yeasts should be much effective in the mannitol metabolism where aerobic respiration was important. During fed-batch feeding of co-substrates the consumption rates (q_{Srb} or q_{Man}) nearly remained constant; however, values decreased throughout the process time after their feeding stopped.

- The highest rHuEPO production was obtained in MPM as 645 mg L^{-1} at t=9 h in the early hours of the strategy. Also, the highest q_{rHuEPO} was obtained as 2.049 mg g^{-1} h^{-1} at the beginning of the process in MPM. Furthermore, the highest overall rHuEPO yields on cell and on the total substrate were obtained in MPM as 16.66 mg g^{-1} and as 2.23 mg g^{-1}, respectively while the highest cell yield on total substrate, $Y_{X/St}$, was achieved in MMM as 0.33gg^{-1}.

- In almost all experiments, the highest AOX activities achieved at the early hours of the production phase and lowered throughout the process time. The highest AOX activity was found as 120 U g^{-1} CDW at t=6 h in MPM, and the lowest one was obtained as 40 U g^{-1} CDW at t=3 h in MM.

- In all feeding strategies, an increasing total protease profile was observed throughout the process. Yet, after the sorbitol depletion, protease concentration began to increase highly. While mannitol found in the medium showed no effect on the protease concentrations of MM, MMM or MLM strategies, it affected the protease concentrations of MPM and MPMG so a sharp increment of C_{Pro} was observed. The maximum total protease concentration was found as 0.140 g L^{-1} at t=48 h in SSM, since more cells produce more proteases.

- In the organic acid profiles of feeding strategies, lactic acid was detected in SSM and MLM as 1.33 g L^{-1} at t=42 h and 0.39 g L^{-1} at t=14 h, respectively. This means that the oxygen was insufficient throughout the processes in SSM and MLM since lactic acid is the main by-product formed in the event of insufficient oxygen. Another organic acid, formic acid was found in SSM, MM, MMM and MLM increased with respect to time, and it was only found at the beginning of MPM and MPMG bioprocesses. Therefore, total formaldehyde entered into the dissimilatory pathway rather than entering the glycolysis pathway in SSM, MM, MMM and MLM. However, after t=9 h in MPM and t=21 h in MPMG, formaldehyde went into the assimilatory pathway and entered glycolysis. The maximum formic acid concentration was found as 0.24 g L^{-1} at t=2 h in MLM.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Fermentation Medium

Antifoam	10 % (v/v) antifoam solution, prepared with dH2O. Can be autoclaved once.
Base	25 % NH3OH (Sigma). No need to sterilize.
1 M potassium phosphate, pH=6.0	56.48 g KH_2PO_4, 14.8 g K_2HPO_4 was dissolved in dH20 and the volume made upto 500 mL. The pH was controlled. The buffer was autoclaved and stored at room temperature.

AOX Assay Solutions

Yeast Lysis Buffer	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl-pH8.0, 1mM Na2EDTA. The solution was autoclaved and stored at room temperature.
1 M potassium phosphate, pH=7.5	1M KH_2PO_4, 1M K_2HPO_4 was dissolved in dH20 and titer KH_2PO_4 with K_2HPO_4 while controlling pH. The buffer was autoclaved and stored at room temperature.

SDS-PAGE Solutions

10%(w/v) APS (Ammonium PerSulfate)	Add 0.1g APS to 1 mL dH_2O , freshly prepared.
1.5 M TrisHCl, 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 TrisHCl, 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 28°C.
Resolving Buffer (12%) (for 2 gels)	3.4mL dH20, 4mL 30% Acrylamide-bis, 2.5 mL 1.5M Tris–HCl pH=8.8, 100µL 10%SDS, prior to gel preparation add 50µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine.

Stacking Buffer (5%) (for 2 gels)	2.8mL dH20, 0.85mL 30% Acrylamide-bis, 1.25 mL 0.5M Tris – HCl pH=6.8, 50µL 10%SDS, prior to gel preparation add 25µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine.
4 x Sample Loading Buffer for SDSPAGE	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 SDSPAGE Running Buffer	15 g Tris Base, 72 g glycine, 5 g SDS, dH2O to 1 liter. The buffer was stored at 2-8°C and diluted 1:5 with dH2O prior to use.
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.

Protease Assay solutions

Borate buffer (for alkali proteases)	2.381 g Boraks (Na2B4O7.10 H2O) dissolved in 250 ml dH2O. pH is adjusted to 10 by 1 M NaOH (6-7 ml) and add dH2O till 500 ml. Filter and store at +4oC.
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 $Na_2HPO_4.7H_2O$ 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.

APPENDIX B

CALIBRATION CURVE FOR BRADFORD ASSAY

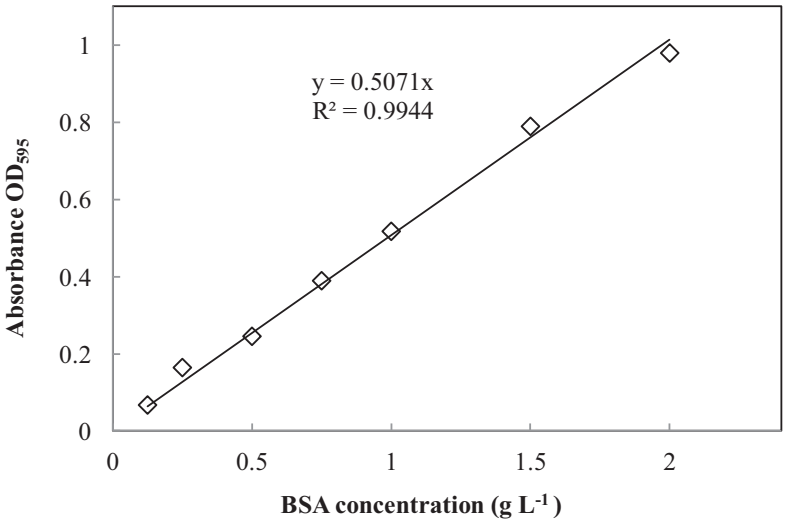

Figure B.1 Calibration curve for Bradford Assay

APPENDIX C

CALIBRATION CURVE FOR SORBITOL AND MANNITOL

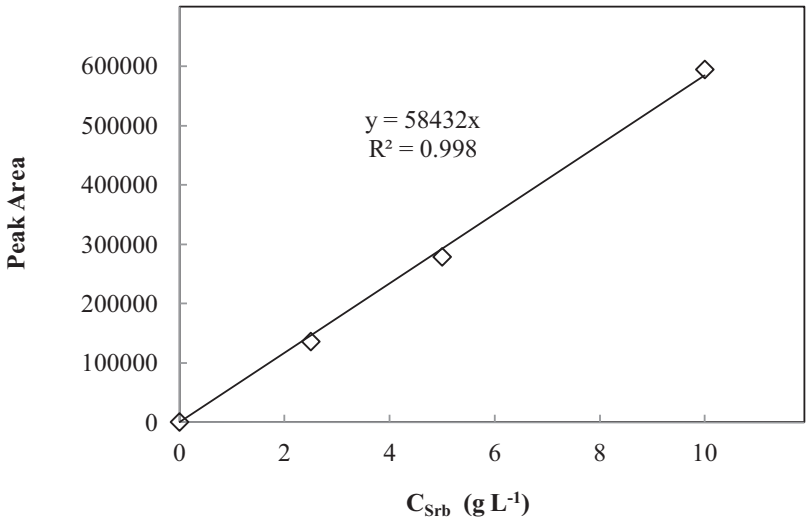

Figure C.1 Calibration curve for sorbitol concentration, analysis was performed by HPLC.

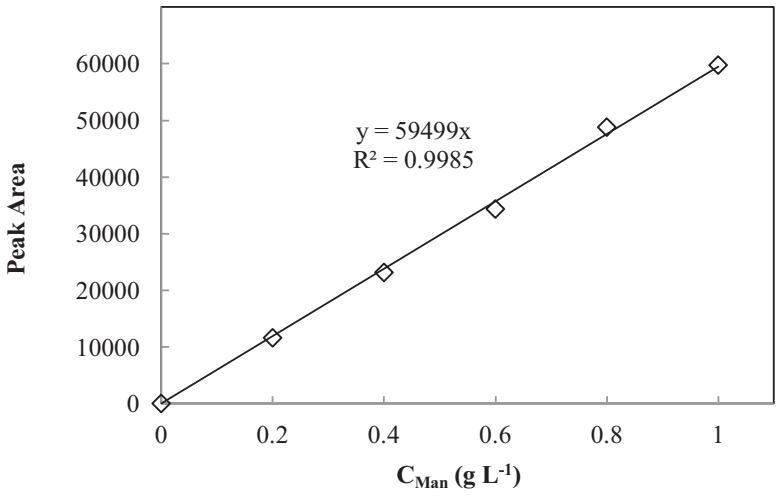

Figure C.2 Calibration curve for mannitol concentration, analysis was performed by HPLC.

APPENDIX D

CALIBRATION CURVES FOR ORGANIC ACID

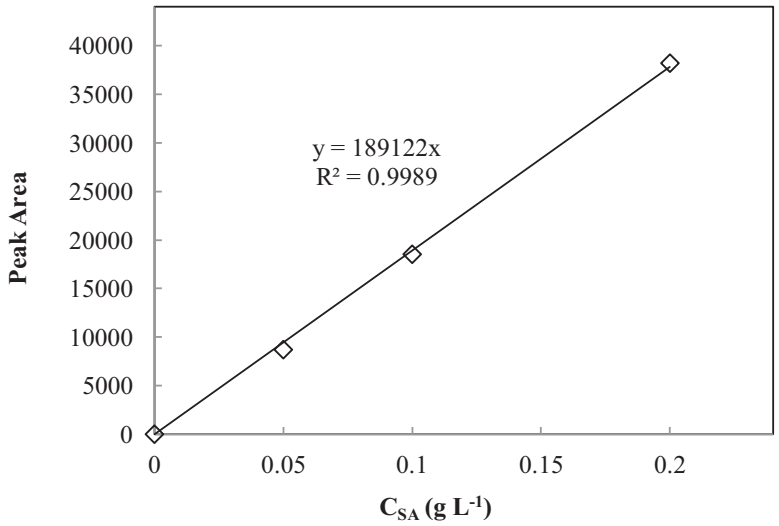

Figure D.1 Calibration curve obtained for succinic acid concentration, analysis was performed by HPLC

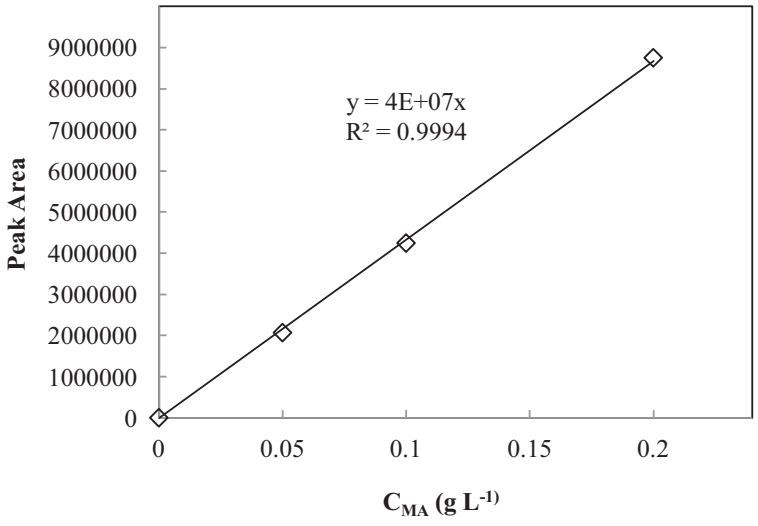

Figure D.2 Calibration curve obtained for maleic acid concentration, analysis was performed by HPLC

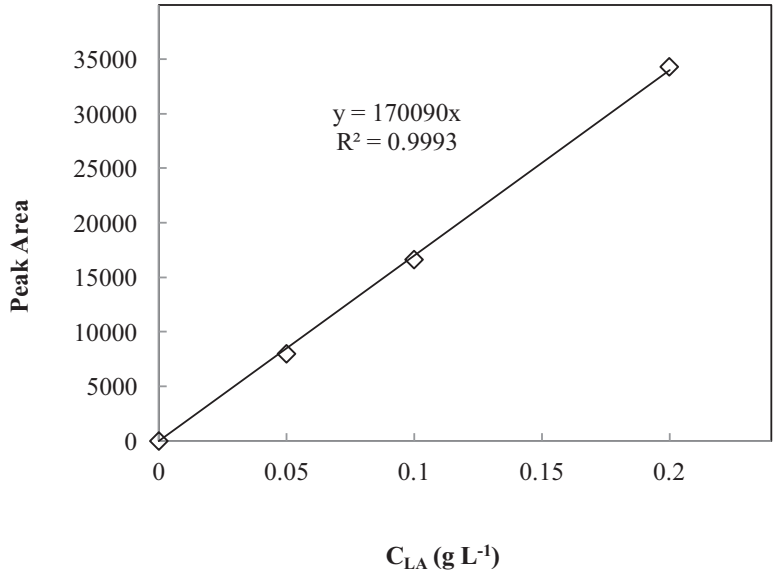

Figure D.3 Calibration curve obtained for lactic acid concentration, analysis was performed by HPLC

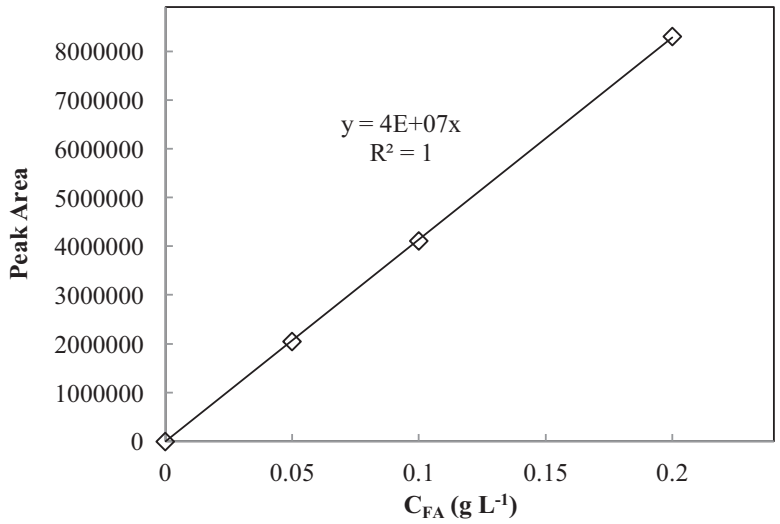

Figure D.4 Calibration curve obtained for formic acid concentration, analysis was performed by HPLC

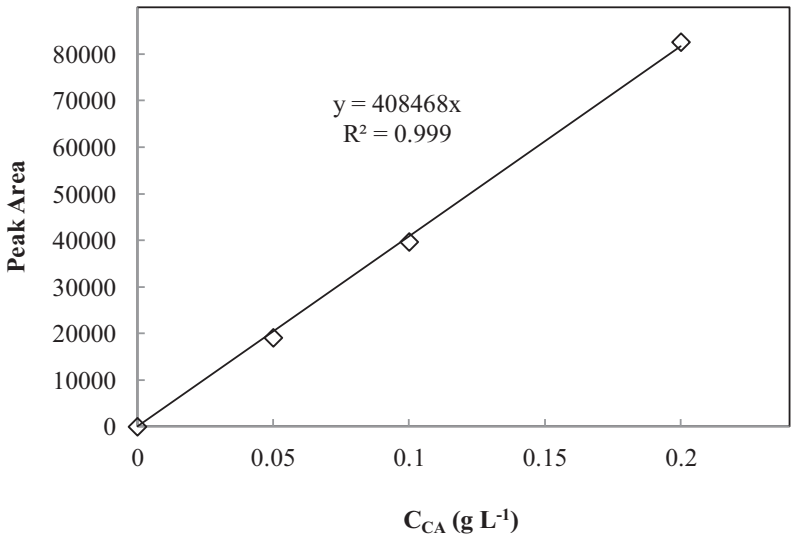

Figure D.5 Calibration curve obtained for citric acid concentration, analysis was performed by HPLC

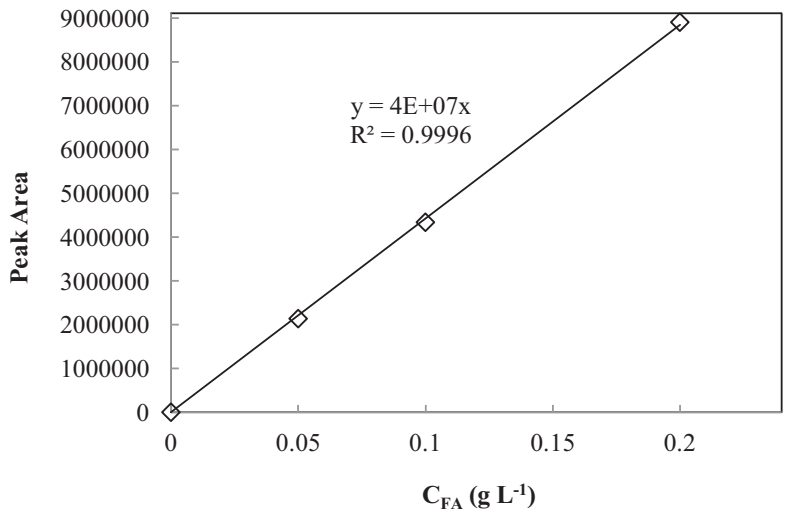

Figure D.6 Calibration curve obtained for fumaric acid concentration, analysis was performed by HPLC

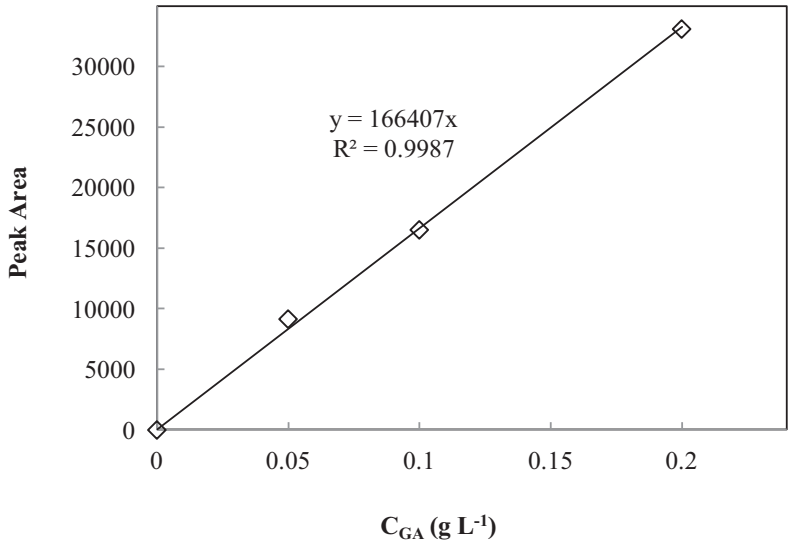

Figure D.7 Calibration curve obtained for gluconic acid concentration, analysis was performed by HPLC

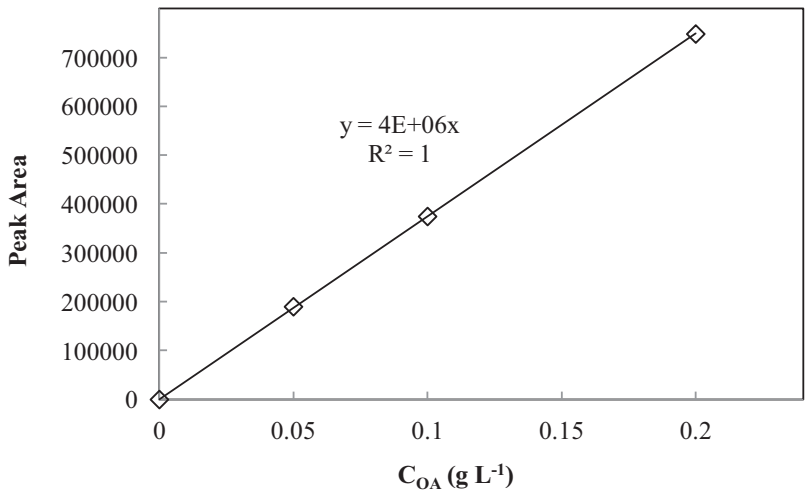

Figure D.8 Calibration curve obtained for oxalic acid concentration, analysis was performed by HPLC

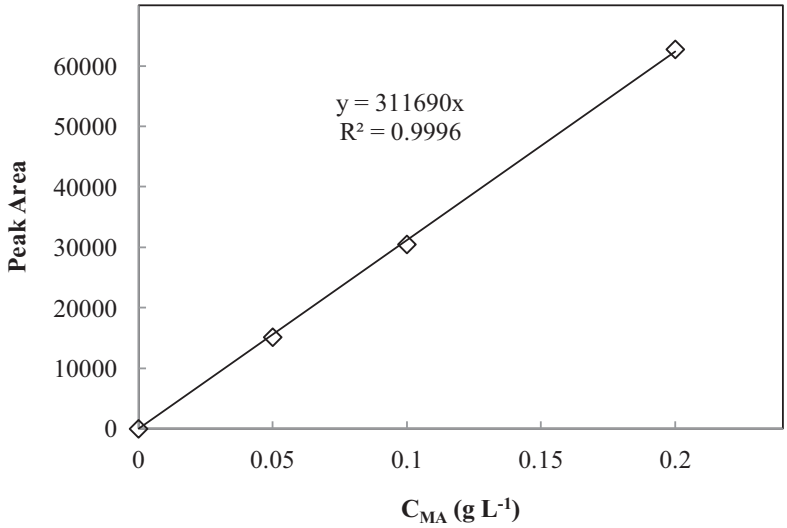

Figure D.9 Calibration curve obtained for malic acid concentration, analysis was performed by HPLC

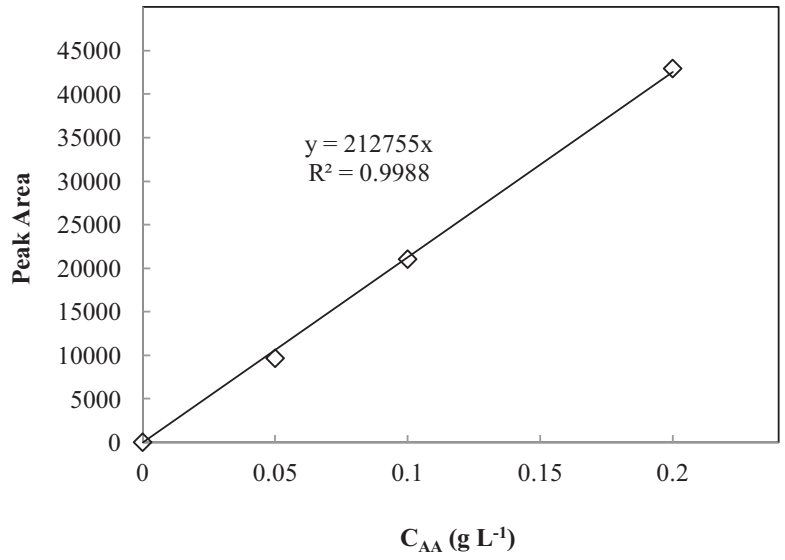

Figure D.10 Calibration curve obtained for acetic acid concentration, analysis was performed by HPLC

APPANDIX E

CALIBRATION CURVE FOR AOX ACTIVITY ASSAY

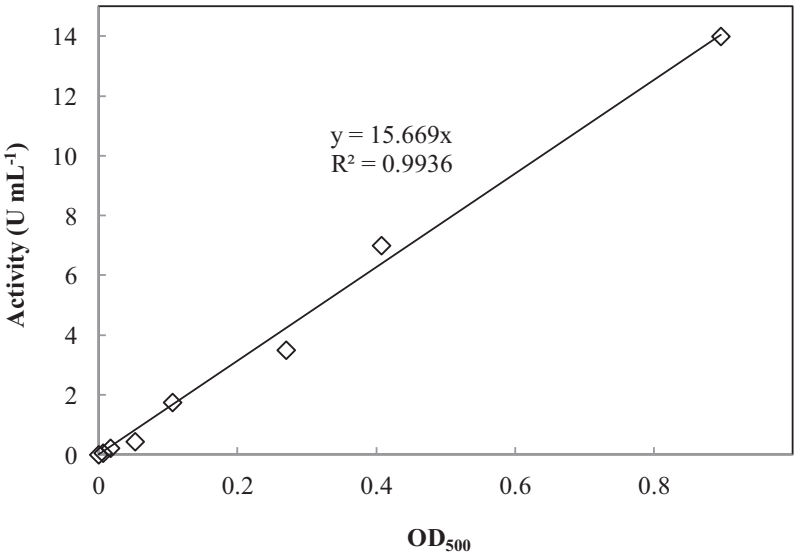

Figure E.1 Calibration curve for AOX activity assay

APPENDIX F

MOLECULAR WEIGHT MARKER

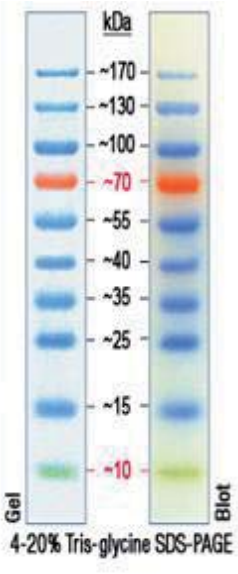

Figure F.1 PageRuler^{TM} Prestained Protein Ladder (Fermentas)

APPENDIX G

SDS-PAGE PROTEIN ANALYSIS

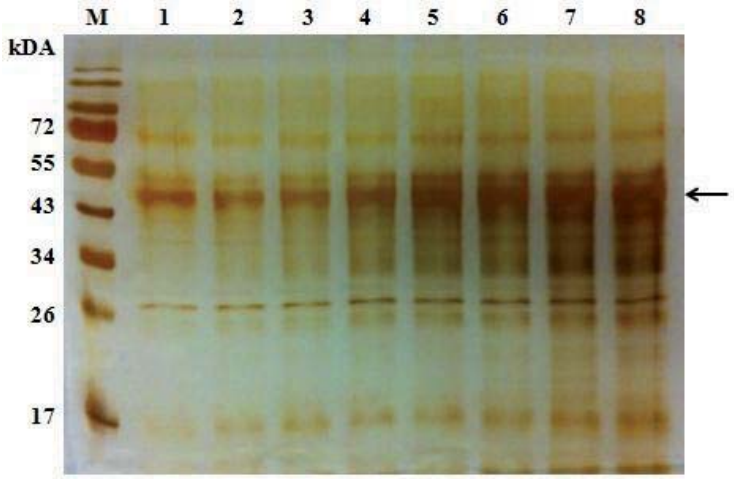

Figure G.1 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in SSM strategy. M: protein marker, 1. well: t=0 h, 2. well: t=3 h, 3. well: t=6 h, 4. well: t=9 h, 5. well: t=12 h, 6. well: t=15 h, 7. well: t=18 h, 8. well: t=21 h.

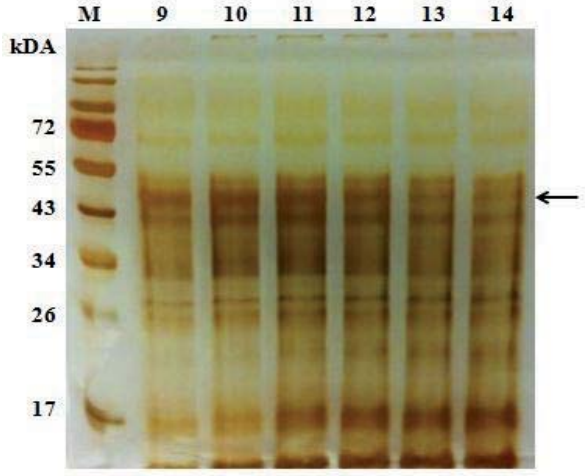

Figure G.2 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in SSM strategy. M: protein marker, 9. well: t=24 h, 10. well: t=27 h, 11. well: t=30 h, 12. well: t=36 h, 13. well: t=42 h, 14. well: t=48 h.

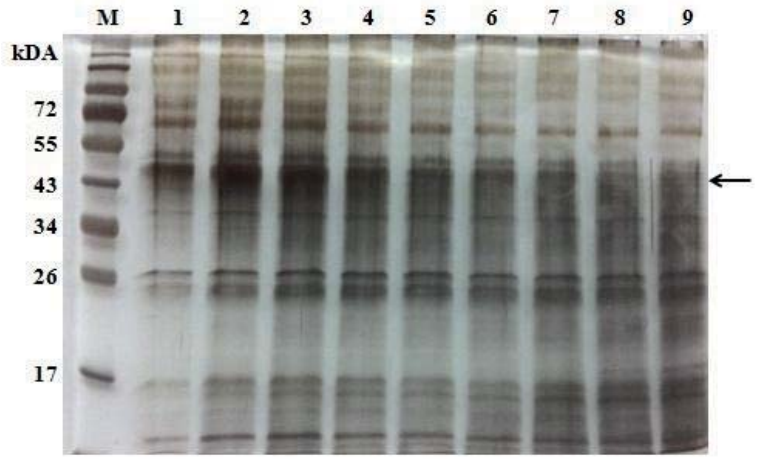

Figure G.3 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MM strategy. M: protein marker, 1. well: t=0 h, 2. well: t=6 h, 3. well: t=9 h, 4. well: t=12 h, 5. well: t=15 h, 6. well: t=18 h, 7. well: t=21 h, 8. well: t=27 h, 9. well: t=30 h.

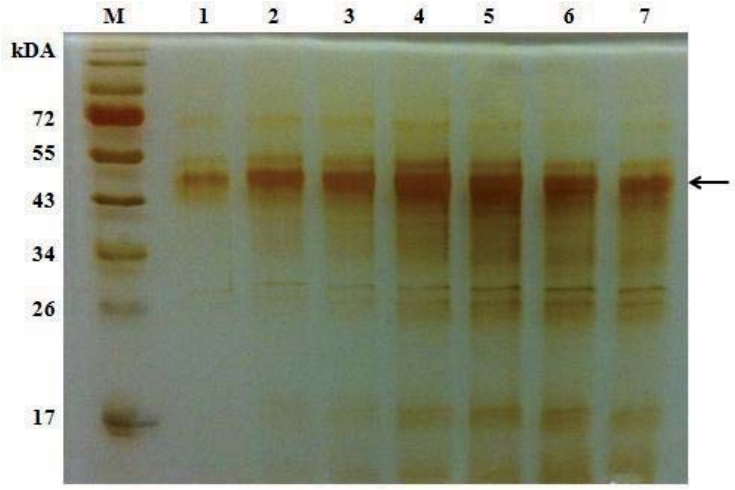

Figure G.4 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MMM strategy. M: protein marker, 1. well: t=0 h, 2. well: t=3 h, 3. well: t=6 h, 4. well: t=9 h, 5. well: t=12 h, 6. well: t=15 h, 7. well: t=18 h.

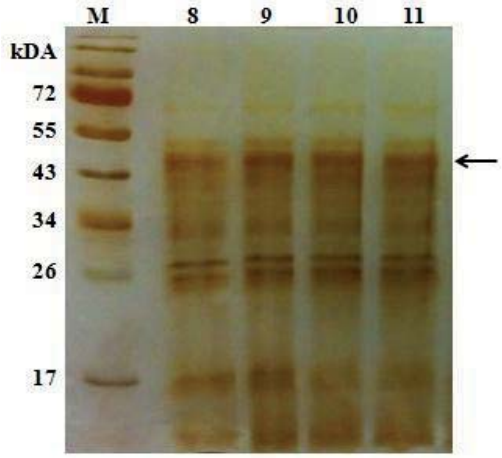

Figure G.5 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MMM strategy. M: protein marker, 8. well: t=21 h, 9. well: t=24 h, 10. well: t=27 h, 11. well: t=30 h.

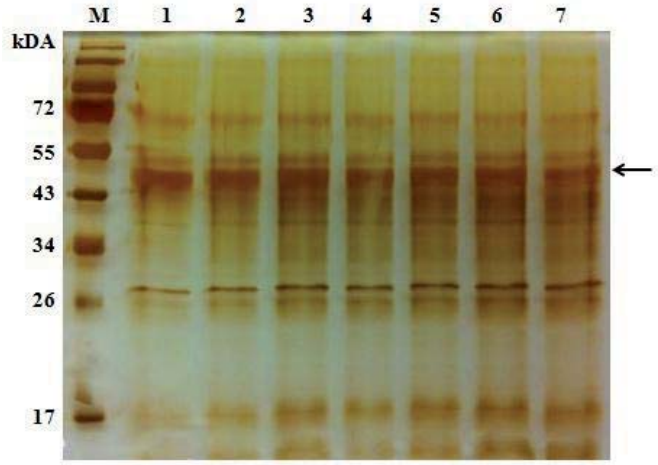

Figure G.6 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MLM strategy. M: protein marker, 1. well: t=0 h, 2. well: t=2 h, 3. well: t=4 h, 4. well: t=6 h, 5. well: t=8 h, 6. well: t=10 h, 7. well: t=12 h.

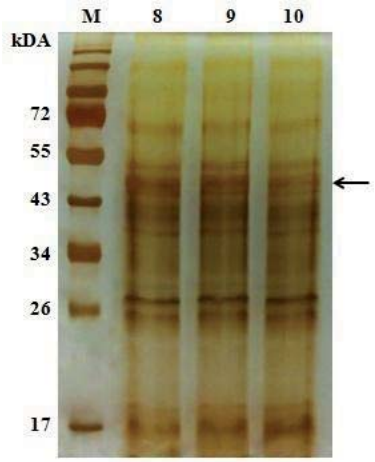

Figure G.7 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MLM strategy. M: protein marker, 8. well: t=14 h, 9. well: t=16 h, 10. well: t=18 h.

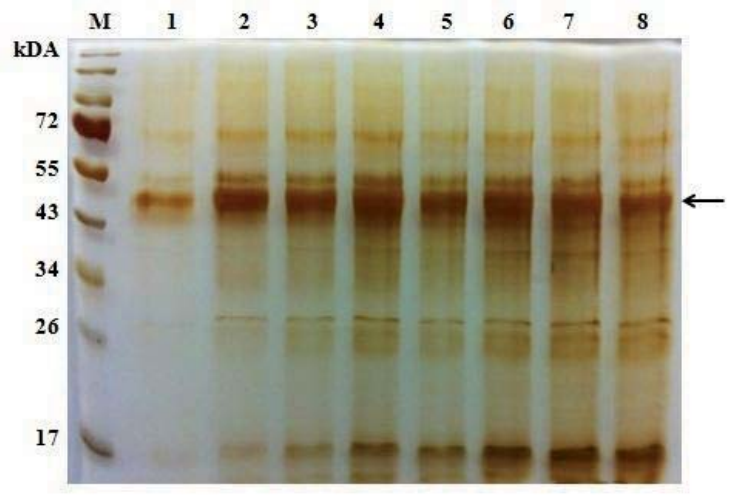

Figure G.8 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MPM strategy. M: protein marker, 1. well: t=0 h, 2. well: t=3 h, 3. well: t=6 h, 4. well: t=9 h, 5. well: t=12 h, 6. well: t=15 h, 7. well: t=18 h, 8. well: t=21 h

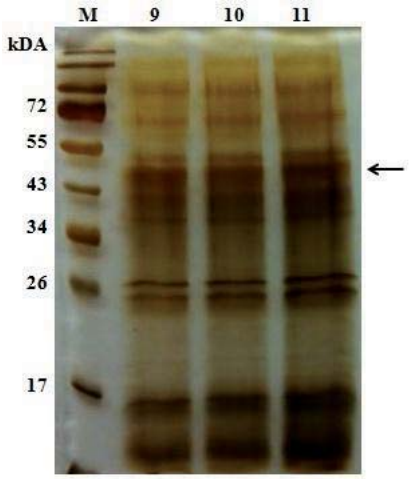

Figure G.9 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MPM strategy. M: protein marker, 9. well: t=24 h, 10. well: t=27 h, 11. well: t=30 h.

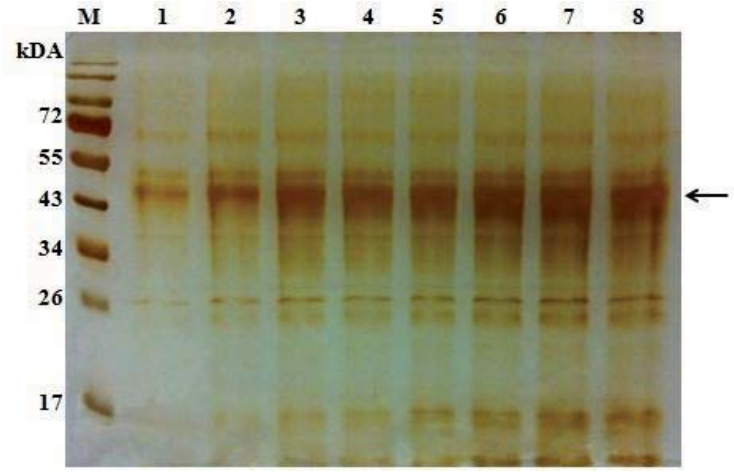

Figure G.10 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MPMG strategy. M: protein marker, 1. well: t=0 h, 2. well: t=3 h, 3. well: t=6 h, 4. well: t=9 h, 5. well: t=12 h, 6. well: t=15 h, 7. well: t=18 h, 8. well: t=21 h

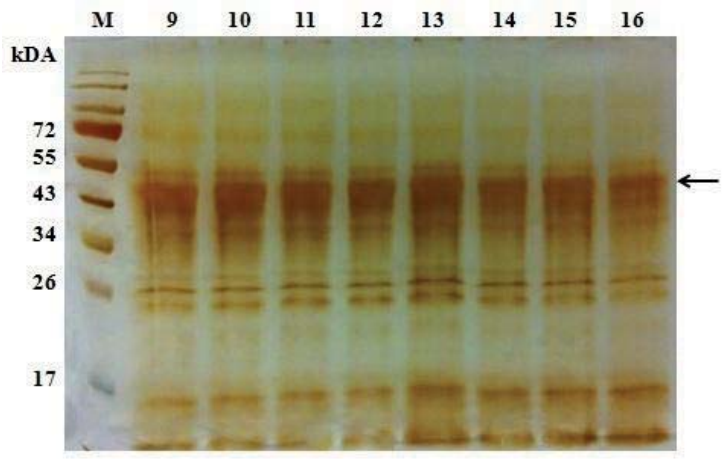

Figure G.11 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in MPMG strategy. M: protein marker, 9. well: t=24 h, 10. well: t=27 h, 11. well: t=30 h, 12. well: t=30 h, 13. well: t=36 h, 14. well: t=39 h, 15. well: t=41 h, 16. well: t=44 h