EFFECTS OF CARBON SOURCES AND FEEDING STRATEGIES ON HUMAN GROWTH HORMONE PRODUCTION BY METABOLICALLY ENGINEERED *Pichia pastoris*

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EFFECTS OF CARBON SOURCES AND FEEDING STRATEGIES ON HUMAN GROWTH HORMONE PRODUCTION BY METABOLICALLY ENGINEERED *Pichia pastoris*

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ABSTRACT

EFFECTS OF CARBON SOURCES AND FEEDING STRATEGIES ON HUMAN GROWTH HORMONE PRODUCTION BY METABOLICALLY ENGINEERED *Pichia pastoris*

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In this study, effects of different carbon sources and their feeding strategies on recombinant human growth hormone (rhGH) production by *Pichia pastoris* were investigated by means of cell growth, recombinant protein production and expression levels of *hGH* and alcohol oxidase (*AOX*) genes. In this content, firstly, the strain to be used for high level rhGH production was selected between the two phenotypes, i.e., *P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S}. In this selection both phenotypes were compared in two different media containing glycerol/methanol or sorbitol/methanol and *P. pastoris-hGH*-Mut^{+} strain grown on medium containing 30 g/L sorbitol with 1% (v/v) methanol was found to have the highest *hGH* expression level and rhGH production level, 9.84×10^{9} copies/mg CDW and 120 mg/L, respectively.

Thereafter, effects of sorbitol, mannitol, fructose, lactose, sucrose, citric acid, lactic acid and acetic acid were investigated by using *P. pastoris hGH*-Mut$^+$ strain in laboratory scale bioreactors. Among them sorbitol and sucrose were selected to be compared for production in pilot scale bioreactors by adding them batch-wise at the beginning of induction phase with fed batch methanol feeding scheme at μ=0.03h^{-1}. It was shown that sucrose does not support cell growth as sorbitol although it does not repress recombinant protein production. Then three different feeding strategies were applied to develop sorbitol/methanol mixed feeding i) single sorbitol addition at t=0, ii) besides at t=0, adding second batch-wise sorbitol at t=9 h, iii) giving pulse methanol at t=24 h to trigger AOX promoter. These three strategies were compared with a production without addition of co-substrate sorbitol. Substrate consumption, cell growth, recombinant protein production and expression levels of *hGH* and *AOX* were investigated for these different feeding strategies. The highest cell concentration was achieved in third strategy as 55 g/L where the highest extracellular rhGH production (301 mg/L) was achieved in the second strategy, with addition of two times of sorbitol. For this highest recombinant protein production case, overall cell and product yield on total substrate were found as 0.17 g/g and 1.71 mg/g, respectively. Moreover, the highest *hGH* and *AOX* expression levels were obtained in this strategy.

Keywords: Recombinant human growth hormone, *Pichia pastoris*, carbon source, feeding strategies, *AOX*

ÖZ

KARBON KAYNAKLARININ VE BESLEME STRATEJİLERİNİN REKOMBİNANT *Pichia pastoris* İLE İNSAN BÜYÜME HORMONU ÜRETİMİNE ETKİSİ

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Bu çalışmada, değişik karbon kaynaklarının ve beslenme stratejilerinin *Pichia pastoris* ile rekombinant insan büyüme hormonu (rhGH) üretimine etkileri hücre çoğalması, rekombinant protein üretimi ve *hGH* ve alkol oksidaz (*AOX*) genlerinin ifade düzeyleri belirlenerek araştırılmıştır. Bu kapsamda, öncelikle, yüksek seviyede insan büyüme hormonu üretimi için kullanılacak suş *Pichia pastoris-hGH'*nin iki farklı metanol kullanım fenotipi, *P. pastoris-hGH* Mut^+ ve *P. pastoris-hGH* Mut^S, arasından seçilmiştir. Bu seçim sırasında, iki suş da gliserol/metanol ya da sorbitol/metanol olmak üzere iki farklı karbon kaynağı içeren üretim ortamlarında karşılaştırılmış ve 30 g/L sorbitol ve %1 (h/h) metanol içeren ortamda *P. pastoris-hGH* Mut^+'nın daha yüksek *hGH* gen ifade düzeyine, $9.84x10^9$ copies/mg CDW, ve rhGH üretim seviyesine, 120 mg/L, sahip olduğu bulunmuştur.

Sonraki aşamada, sorbitol, mannitol, fruktoz, laktoz, sükroz, sitrik asit, laktik asit ve asetik asidin karbon kaynağı olarak etkisi *P. pastoris-hGH* Mut^+ kullanılarak laboratuvar ölçekli biyoreaktörlerde incelenmiştir. Bunlardan sorbitol ve sükroz seçilerek yarı-kesikli biyoreaktörde indükleme fazının başında, $\mu=0.03h^{-1}$'de yarı-kesikli metanol besleme programı ile birlikte kesikli olarak eklenmiştir. Sükrozun rhGH üretimini inhibe etmemesine karşın hücre çoğalmasını sorbitol kadar desteklemediği bulunmuştur. Daha sonra sorbitol/metanol karışık beslemesini geliştirmek için üç farklı besleme stratejisi uygulanmıştır. Bu stratejiler: i) t=0 st'te tek kesikli sorbitol eklemesi, ii) t=0 ve 9 st'te iki kesikli sorbitol eklemesi, iii) AOX'u tetiklemek amacıyla t=24 st'te ani metanol eklemesi olarak uygulanmıştır. Bu üç farklı besleme stratejisi sorbitolsüz ortamda yapılan üretim ile karşılaştırılmıştır. Bu farklı stratejiler için substrat tüketimi, hücre çoğalması, rekombinant protein üretimi ve *hGH* ve *AOX* genlerinin ifade düzeyleri incelenmiştir. En yüksek hücre derişimi olan 55 g/L'ye üçüncü strateji ile ulaşılırken en yüksek rekombinant protein derişimi (301 g/L) iki kez sorbitol eklenerek uygulanan ikinci strateji ile elde edilmiştir. Bu en yüksek üretim koşulunda toplam substrat üzerinden elde edilen en yüksek hücre ve ürün verimleri sırasıyla 0.17 g/g ve 1.71 mg/g olarak bulunmuştur. Ayrıca en yüksek *hGH* ve *AOX* ifade düzeylerine de bu strateji ile ulaşılmıştır.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, *Pichia pastoris*, karbon kaynağı, besleme stratejileri, *AOX*

To my family

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NOMENCLATURE

C	Concentration in the medium	g L^{-1} or mol m^{-3}
C_O^*	Saturated dissolved oxygen concentration	mol m^{-3}
Da	Damköhler number (=OD/OTR_{max} ; Maximum possible oxygen utilization rate per maximum mass transfer rate)	
DO	Dissolved oxygen	%
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient	
K_La	Overall liquid phase mass transfer coefficient	s^{-1}
K_La_0	Physical overall liquid phase mass transfer coef.	s^{-1}
N	Agitation rate	min^{-1}
OUR	Oxygen uptake rate	mol m^{-3} sec^{-1}
OTR	Oxygen transfer rate	mol m^{-3} sec^{-1}
OD	Oxygen demand	mol m^{-3} sec^{-1}
Q	Feed inlet rate	L h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
r	Formation or consumption rate	g L^{-1} h^{-1}
t	Cultivation time	h
T	Bioreaction medium temperature,	°C
U	One unit of an enzyme	
V	Volume of the bioreactor	L
Y	Yield (overall)	g g^{-1}

Greek Letters

ρ	Density	g L^{-1}
η	Effectiveness factor (=OUR/OD)	

μ_0	Desired specific growth rate	h^{-1}
$\mu_{s,max}$	Maximum specific growth rate on sorbitol	h^{-1}
μ_t	Total specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Refers to initial condition
AOX	Refers to alcohol oxidase
G	Glycerol
M	Refers to methanol
O	Refers to oxygen
rp	Refers to recombinant protein
S	Refers to sorbitol or substrate
X	Refers to cell

Abbreviations

AOX	Alcohol oxidase
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
EPO	Human erythropoietin hormone
hGH	Human growth hormone
HPCE	High pressure capillary electrophoresis
HPLC	High pressure liquid chromatography
PCR	Polymerase chain reaction
rhGH	Recombinant human growth hormone
rHuEPO	Recombinant human erythropoietin
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid

CHAPTER 1

INTRODUCTION

Industrial biotechnology is seen as one of the key technologies of the new age. It is mainly based on biological and engineering sciences and it develops new methods for the production of various important products for human health and environment. Biotechnology not only helps in producing important biomaterials but it also makes it possible to manufacture new products, which were once thought to be impossible. With the advances in recombinant DNA technology in the last few decades, it is now possible to produce bio-molecules in an organism other than its natural source (Nielsen, 2003) by cloning targeted gene, which is responsible for the production of the desired bio-molecule, into the genome of a different organism. The possibility of producing biologically active therapeutic proteins cause an increase in market values and in 2007, market of recombinant therapeutic protein reached a value about $42 billion where it was $23 in 2003 (www.researchandmarkets.com).

There are a number of recombinant therapeutic proteins which constitutes the lion's share in the market such as erythropoietin (EPO), human growth hormone (hGH), interferon, insulin, follicle stimulating hormone (FSH), thrombopoietin (TPO) agonists, thrombolytics and coagulation factors. Among them, hGH is a widely used hormone in treatments of various diseases like hypopituitary dwarfism, injuries, bone fractures, bleeding ulcers, and burns, furthermore it appears to be considerably beneficial to girls with Turner's syndrome, children with chronic renal failure and adults with growth hormone deficiency or human immunodeficiency virus (HIV) syndrome (Baulieu et al., 1990; Binkley, 1994; Trevino et al., 2000).

Human growth hormone was first isolated from human pituitaries in 1956. For decades, the only way of obtaining GH was by its extraction from human pituitaries (Krysiak et al. 2007). Later, production of hGH from *Escherichia coli* was first reported in 1979 (Goddel et al., 1979). Then, after 1985, growth hormone whose amino acid sequence and conformation is identical to the native molecule was produced again from *E.coli* (Gray et al, 1985, Becker et al, 1986, Kato et al, 1987, Hsiung et al., 1986). Since *E. coli* is a well-known and easily handled microorganism, most of the studies have been reported from *E. coli* by applying different promoters, signal peptides for extracellular protein production and bioprocess operation parameters (Ikehara et al., 1984; Gray et al., 1985; Kato et al., 1987; Shin et al., 1998b; Tabandeh et al., 2004). In addition to *E.coli*, *Bacillus subtilis*, *Pseudomonas* and a eukaryote host - the baker's yeast, *Saccharomyces cerevisiae* strains were used to produce rhGH (Gray et al., 1985; Tokunaga et al., 1985; Franchi et al., 1991; Özdamar et al., 2009).

Alternatively, *Pichia pastoris*, a methylotrophic yeast has shown to be a promising host for high-level heterologous gene expression because of its several advantages (Cregg et al, 1993; Romanos 1995; Sreekrishna et al., 1997). Its molecular genetic manipulations are simple and protocols are available. Fermentation protocols yielding high cell densities on simple, inexpensive media have been developed (Thorpe et al., 1999). It has a good secretion capacity for recombinant proteins due to the availability of strong alcohol oxidize 1 (AOX1) promoter which initializes the synthesis of the first enzyme in methanol utilization pathway (Jahic et al., 2006). Furthermore *P. pastoris* has the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing. Although, *P. pastoris* is one of the strong candidates for the production of hGH, in literature there are only three studies on rhGH production by this microorganism. Travino et al. (2000) was the first group who produced rhGH by *P. pastoris* through manipulating and inserting the cDNA of hGH into the genome of this methylotrophic yeast is. In this study, 11 mg/L rhGH was obtained in shake tubes having 3 ml of defined culture medium and 49 mg/L rhGH was produced in high cell density cultures by using a 2-L bioreactor. Then, Eurwilaichitr et al.

(2002) constructed three different vectors to investigate whether glutamic acid and alanine spacer was necessary for the removal of MFα-1 signal sequence fused to the hGH produced from *P. pastoris*. It was found that, removal of glu-ala repeats from the hGH were not efficient and they were not necessary for the removal of MFα-1 signal sequence. In the same study, the optimal condition for high-level production was investigated and by using 3% (v/v) methanol concentration after 3 days of induction in complex medium, 190 mg/L rhGH concentration was obtained. Then, Orman et al. (2008) designed an expression system in *P. pastoris* for production and purification of rhGH by cloning cDNA sequence of *hGH* into *pPICZαA* vector under the control of AOX1 promoter. In the same study, they investigated the effects of carbon sources on rhGH production by using *P.pastoris hGH*-Mut^+ and *P.pastoris hGH*-Mut^S strains, which are two methanol utilization phenotypes of the microorganism. The highest amount of rhGH, 110 mg/L, was achieved in the medium containing C_{Gly}=30 g/L and C_{MeOH}=1% (v/v) by using Mut^S strain.

In this study, it was aimed to investigate the effects of different carbon sources and feeding strategies on recombinant human growth hormone production by *Pichia pastoris*. In the first part, the strain to be used for hGH production was selected between the two strains having different methanol utilization phenotypes (*P. pastoris hGH*-Mut^+ and *P. pastoris hGH*-Mut^S). After that, using the selected strain, effects of several different carbon sources on recombinant protein production were investigated. Among them, the ones which support cell growth and do not repress the recombinant protein production were determined. In the second part of the study, first the selected carbon sources was used in pilot scale bioreactors then effect of feeding strategies of carbon sources on cell growth, oxygen transfer and fermentation characteristics, rhGH production and by-product formation together with and expression levels of *rhGH* and *AOX1* were investigated again by using pilot scale bioreactor.

CHAPTER 2

LITERATURE SURVEY

For efficient production of a recombinant protein, properties of the protein itself, characteristics of the selected host microorganism and the optimum bioprocess operation parameters should be investigated in detail. Furthermore, in order to understand the response of the microorganism to the external stimulus some basic computational methods for yield coefficients, oxygen transfer characteristics, specific production and consumption rates, and genetic engineering techniques to realize the relationship between the transcriptional levels of desired product and enzymes affecting the production should be covered. In this sense, this chapter reviews the literature on these core topics.

2.1 Product: Human Growth Hormone (hGH)

Hormones are chemical messengers from a cell or a group of cells and serve as a signal to the target cells. Hormones have wide effects on human body such as stimulation or inhibition of growth, induction or suppression of apoptosis (programmed cell death), activation or inhibition of the immune system, regulation of the metabolism and preparation for a new activity or phase of life (Baulieu et al., 1990; Binkley, 1994). One of the crucial hormones produced by human body is growth hormone. It is secreted by the somatotroph cells of the anterior pituitary gland.

Growth hormone (GH) was isolated for the first time from human pituitaries in 1956. For decades, the only way of obtaining GH was by its extraction from human pituitaries (Krysiak et al. 2007). Later, production of GH from *E.coli* was first reported in 1979 (Goddel et al., 1979). Then, after 1985, growth hormone whose amino acid sequence and conformation is identical to the native molecule was produced again from *E.coli* (Gray et al., 1985, Becker et al.,

1986, Kato et al., 1987, Hsiung et al., 1989). Therefore, its application was widened from growth hormone deficiency to various diseases such as Turner or Noonan Syndromes, chronic renal failure, children born small for gestation age (SGA) and Prader-Willi syndrome (PWS). Nowadays, not only children but also adults benefit from GH therapy (Krysiak et al., 2007).

2.1.1 Structure of hGH

The *hGH* genes are located in the q22-24 region of chromosome 17 of human beings (http://www.ncbi.nlm.nih.gov). It is synthesized, stored, and secreted by the somatotroph cells of the anterior pituitary gland. There are two isoforms of the hormone in the human body. The largest one has a molecular weight of 22 kDa. Although a smaller variant of 20 kDa, accounts for about 10% of GH in the pituitary gland, its function is not clear yet (Norman, 1997). The hGH that is required in order to perform the body functions is the one that has a molecular weight of 22 kDa. This non-glycosylated protein consisting of 191 amino acid residues folds into a four-helix bundle structure with two disulfide bonds (De Vos et al., 1992). The four cysteine biomolecules which are found on 35^{th}, 165^{th}, 182^{nd}, and 189^{th} positions of the hGH chain result in two disulfide bonds which enable it to form tertiary structure or active form of protein and the helices are necessary for interaction with the hGH receptors (Binkley, 1994). Nucleotide sequence, covalent structure and tertiary structure of hGH are shown in Figures 2.1, 2.2 and 2.3, respectively.

```
ttc cca act ata cca cta tct cgt cta ttc gat aac gct atg ctt cgt gct
cat cgt ctt cat cag ctg gcc ttt gac acc tac cag gag ttt gaa gaa gcc
tat atc cca aag gaa cag aag tat tca ttc ctg cag aac ccc cag acc tcc
ctc tgt ttc tca gag tct att ccg aca ccc tcc aac agg gag gaa aca caa
cag aaa tcc aac cta gag ctg ctc cgc atc tcc ctg ctg ctc atc cag tcg
tgg ctg gag ccc gtg cag ttc ctc agg agt gtc ttc gcc aac agc cta gtg
tac ggc gcc tct gac agc aac gtc tat gac ctc cta aag gac cta gag gaa
ggc atc caa acg ctg atg ggg agg ctg gaa gat ggc agc ccc cgg act ggg
cag atc ttc aag cag acc tac agc aag ttc gac aca aac tca cac aac gat
gac gca cta ctc aag aac tac ggg ctg ctc tac tgc ttc agg aag gac atg
gac aag gtc gag aca ttc ctg cgc atc gtg cag tgc cgc tct gtg gag ggc
agc tgt ggc ttc tag ctg ccc ggg tgg cat ccc tgt gac ccc tcc cca gtg
cct ctc ctg gcc
```

Figure 2.1 Nucleotide sequence of human growth hormone (Baulieu et al., 1990; Binkley, 1994).

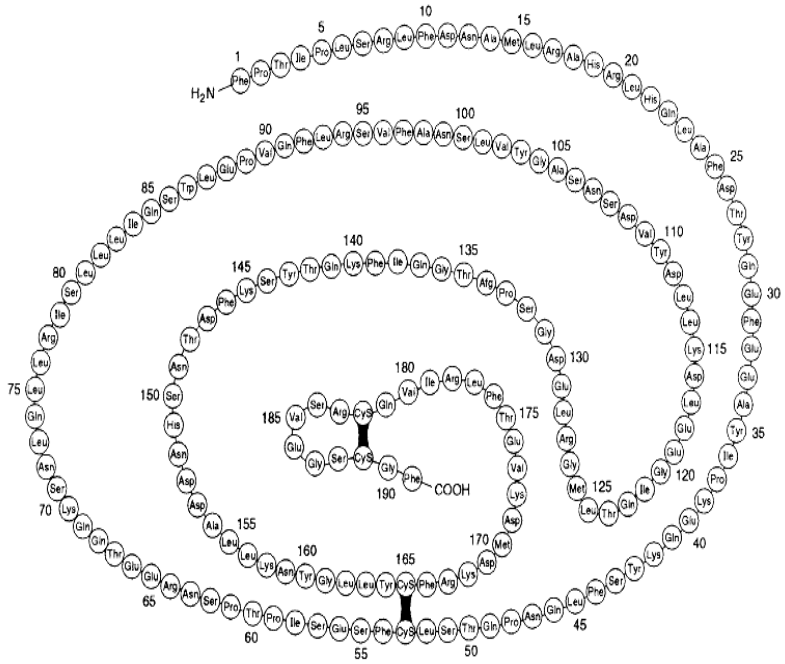

Figure 2.2 Covalent structure of hGH (Norman, 1997)

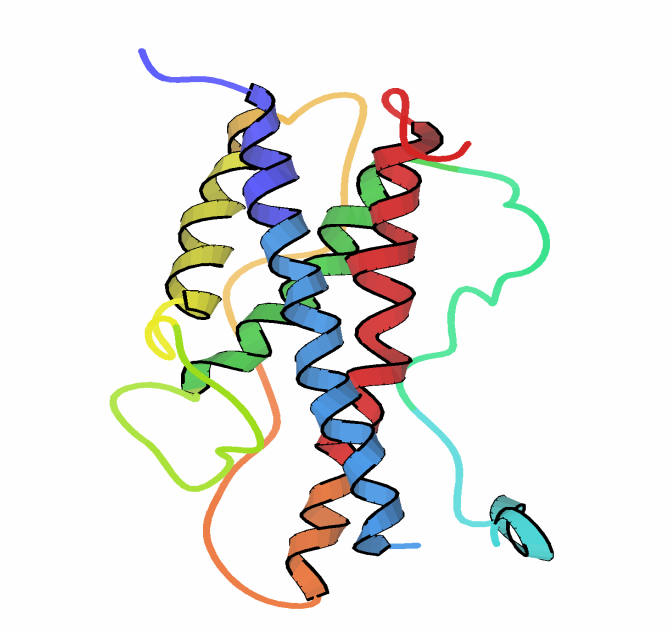

Figure 2. 3 Tertiary structure of hGH (http://www.ncbi.nlm.nih.gov).

2.2 Microorganism Selection

The industrial production of biomolecules, i.e., as amino acids, organic acids, nucleic acid related compounds, antibiotics, carbohydrates, lipids, and proteins as well as alcoholic beverages and foods, is carried out using microorganisms as microbioreactors. For the production of recombinant biomolecules, selection of the appropriate host microorganism is the first and one of the most important steps in bioprocess development. Since recombinant protein production technology began to be developed, *E. coli* and *S. cerevisiae* were the mostly preferred organisms although they have some drawbacks. *E. coli* does not possess the ability to perform many post-translational modifications such as glycosylation, while *S. cerevisiae* hyper-glycosylates proteins (Walker, 1998) which reduces secretion rates and potentially alters protein functionality.

In particular, in literature, *E. coli* cells are most commonly used for hGH production as they require inexpensive medium and can be easily handled (Goddel et al., 1979; Gray et al., 1985; Becker et al., 1986; Kato et al., 1987;

Hsiung et al., 1989; Shin et al., 1998b; Tabandeh et al., 2004); on the other hand, formation of inclusion bodies is one of the advantages of rhGH production by *E. coli.* Furthermore, rhGH has been produced intracellularly and specialized secretion vectors are needed for the protein to be secreted to periplasm or culture medium. In addition to *E.coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Pseudomonas* strains were used to produce rhGH (Gray et al., 1985; Tokunaga et al., 1985; Franchi et al., 1991; Özdamar et al., 2009).

Alternatively, *Pichia pastoris*, a methylotrophic yeast has shown to be a promising host for high-level heterologous gene expression (Cregg et al., 1993; Romanos, 1995; Sreekrishna et al., 1997). Although, *P. pastoris* is one of the strong candidates which can be used for the production of hGH, in literature there are only three studies on rhGH production by this microorganism. The first group who produced rhGH by *P. pastoris* through manipulating and inserting the cDNA of hGH into the genome of this methylotrophic yeast is Travino et al. (2000). In this study, 11 mg/L rhGH was obtained in shake tubes having 3 ml of defined culture medium and 49 mg/L rhGH was produced in high cell density cultures by using a 2-L bioreactor. Then, Eurwilaichitr et al. (2002) constructed three different vectors including no glu-ala, one glu-ala and two glu-ala repeats, respectively to investigate whether glutamic acid and alanine spacer was necessary for the removal of MFα-1 signal sequence fused to the hGH produced from *P. pastoris*. It was found that, removal of glu-ala repeats from the hGH were not efficient and they were not necessary for the removal of MFα-1 signal sequence. In the same study, the optimal condition for high-level production was investigated and by using 3% (v/v) methanol concentration after 3 days of induction in complex medium, 190 mg/L rhGH concentration was obtained. Orman et al. (2008) designed an expression system in *P. pastoris* for production and purification of rhGH by cloning cDNA sequence of *hGH* into *pPICZαA* vector under the control of AOX1 promoter. Following that, they recently investigated the effects of carbon sources on rhGH production by using *P.pastoris-hGH-Mut*$^{+}$ and *P.pastoris-hGH-Mut*^{S} strains, which are two methanol utilization phenotypes of the microorganism. The highest amount of rhGH, 110

mg/L, was achieved in the medium containing C_{Gly}=30 g/L and C_{MeOH}=1% (v/v) by using Mut^S strain (Orman et al., 2009)

2.2.1 *Pichia pastoris*

About forty years ago, ability of certain yeast species to utilize methanol as sole source of carbon and energy was first described (Ogata et al., 1969); and they are named as methylotrophic yeasts. Amongst, only four genera were identified up to now; namely, *Hansenula*, *Pichia*, *Candida* and *Torulopsis* (Faber et al., 1995). During 1970s, media and protocols for one of the most popular methylotrophic yeast from *Pichia* genera, *P. pastoris*, was first developed by Phillips Petroleum Company and in the last three decades, interest on *P. pastoris* has increased dramatically. Over 400 proteins, from human endostatin to spider dragline silk protein, have been produced by *P. pastoris* (Cereghino et al., 2001).

P. pastoris is a mesophilic microorganism from the yeast species. Yeasts are unicellular fungi which are usually oval cells of 1-5 µm wide by 5-30 µm long. They have typical eukaryotic cell structure and generally have a thick polysaccharide cell wall, and they are facultative anaerobes. *P. pastoris* is taxonomically classified under the Kingdom *Fungi*, Division *Eumycota*, Subdivision *Ascomycotina*, Class *Hemoascomycetes*, Order *Endomycetales*, Family *Saccharomycetaceae* and Genus *Pichia* (http://www.ncbi.nlm.nih.gov).

P. pastoris, a promising expression host, has many advantages. Its molecular genetic manipulations are simple and protocols are available. Fermentation protocols yielding high cell densities on simple, inexpensive media have been developed (Thorpe et al, 1999). Furthermore, it has a good secretion capacity for proteins (Jahic et al., 2006). A detailed list showing advantages and disadvantages of *P. pastoris* is given in Table 2.1 (Cregg, 1999; Daly and Hearn, 2005; Macauley-Patrick et al., 2005).

Table 2.1 Advantages and disadvantages of *P. pastoris*

Advantages	Disadvantages
• High yield and productivity • Strong promoter (AOX1) • Chemically defined media-simple, inexpensive formulation • Product processing like mammalian cells • Stable production strains • Low purification cost • High levels of expression of intracellular and secreted proteins • Eukaryotic post-translational modifications • No endotoxin problem • Non-pathogenic • Broad pH range: 3- 7 • Ability of utilizing methanol • Preference for respiratory growth rather than fermentative, a major advantage relative *S. cerevisiae.* • Crabtree-negative • Hyper-glycosylation is not as much as in *S. cerevisiae*.	• Potential of proteolysis, non-native glycosylation. • Long time for cell cultivation compared to bacteria • Monitoring methanol during a process is difficult in order to induce AOX1 promoter. • Since methanol is a petrochemical substance, it may be unsuitable for use in the food industry and also storing of this in industrial scale is undesirable because it is a fire hazard.

2.2.1.1 Metabolism of *Pichia pastoris*

The most common strategy in high productivity *P. pastoris* fermentation uses a two-stage feeding protocol. Cell mass is quickly generated by batch and/or fed-batch growth on glycerol. Once glycerol is depleted, a production phase is initiated by methanol feeding.

The glycerol catabolic pathway (Figure 2.4) includes phosphorylation by a glycerol kinase, resulting in the formation of glycerol 3-phosphate which is then followed by oxidation to dihydroxyacetone phosphate (DHAP) by a FAD-dependent glycerol-3-phosphate dehydrogenase. The dihydroxyacetone phosphate formed enters the glycolytic pathway. A few yeast species have an alternative pathway for dissimilating glycerol which involves a NAD-dependent glycerol dehydrogenase and a dihydroxyacetone kinase (Nevoigt and Stahl, 1997). Pyruvate is formed as an outcome of glycolysis and is further oxidized to acetyl-CoA, via pyruvate dehydrogenase. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, where many metabolites are produced and used for the synthesis of cellular constituents such as amino acids, nucleic acids and cell wall components (Ren et al, 2003).

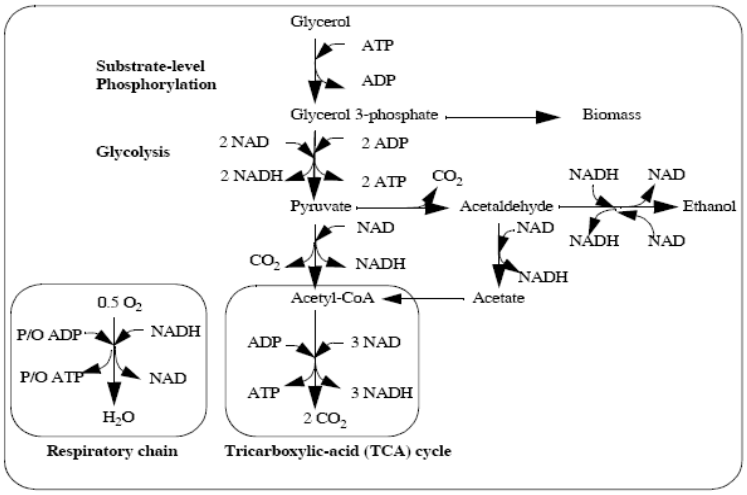

Figure 2.4 The glycerol catabolic pathway in *P. pastoris* (Ren et al, 2003)

The conceptual basis for the *P. pastoris* expression system relies on the observation that some of the enzymes required for methanol metabolism are present at substantial levels only when cells are grown on methanol (Veenhuis et

al., 1983; Egli et al., 1980). Therefore methanol metabolism emerges as an important pathway to be covered in the metabolism of *P. pastoris*.

The initial reactions occur in the peroxisomes and followed by a number of reactions in the cytoplasm (Macauley-Patrick et al., 2005). The enzyme alcohol oxidase (AOX) catalyzes the first step of in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide (Figure 2.5). The hydrogen peroxide formed in the first reaction is degraded to oxygen and water by catalase. A portion of the formaldehyde leaves peroxisome and further oxidized to formate and carbon dioxide. These reactions are the source of energy for cells growing on methanol. The remaining portion of the formaldehyde is assimilated to form cell constituents by cyclic pathway that starts with the reaction of formaldehyde and xylulose 5-monophosphate. The products of this reaction leave the peroxisome and enter a cytoplasmic pathway for the reproduction of xylulose 5-monophosphate and other cell materials (Cereghino et al., 2000; Charoenrat et al., 2005).

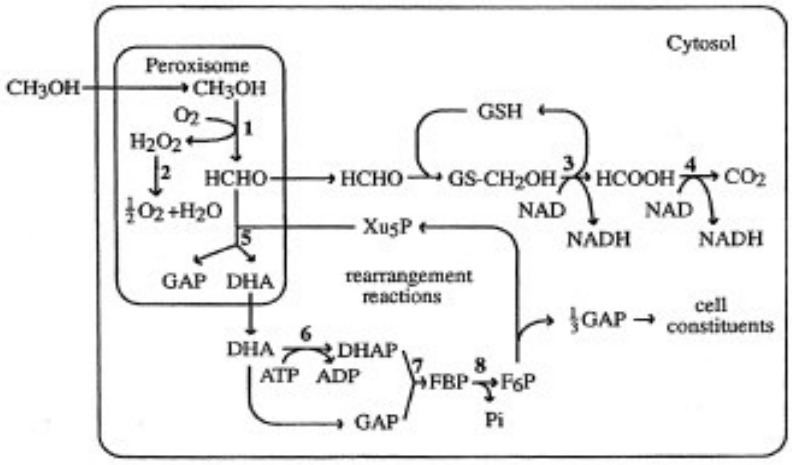

Figure 2.5 The methanol pathway in *P. pastoris*. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase (Cereghino et al., 2000).

Another promising carbon source used in the fermentations of *P. pastoris* is sorbitol which is considered as less repressive to methanol utilization pathway (Sreekrishna et al., 1997). Sorbitol enters the glycolysis pathway from fructose-6-phosphate branch point. Before that, it first enters the mannitol cycle where sorbitol is first oxidized to fructose by sorbitol dehydrogenase, and followed by phosphorylation via fructokinase (Figure 2.6).

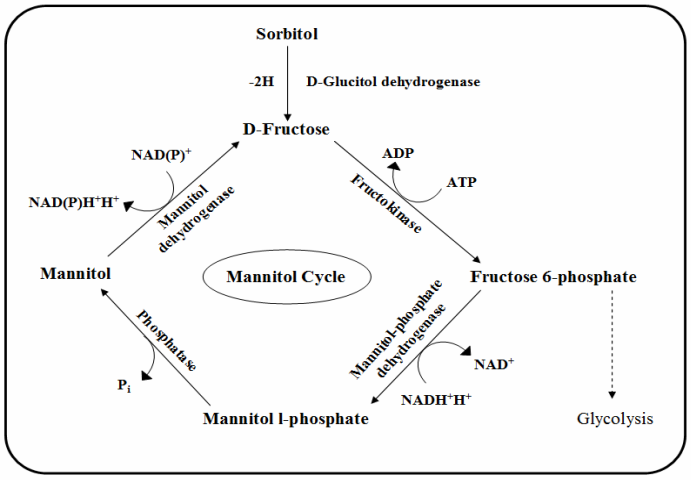

Figure 2.6 Pathways of sorbitol and mannitol metabolism in yeasts (Walker, 1998)

2.2.1.2 *P. pastoris* Expression System

A growing number of researchers are employing *P. pastoris* to produce foreign proteins. Among the advantages of using *P. pastoris* as host, the most remarkable one is its tightly regulated promoter alcohol oxidase I (AOX1). Expression of it, in fact, is tightly regulated at the level of transcription (Ellis et al., 1985) and is controlled by both substrate repression/derepression and induction mechanisms (Tschopp et al., 1987; Ozimek et al., 2005) The AOX1 promoter is strongly repressed in cells grown on glycerol or glucose and induced over 1000-fold when cells are shifted to a medium containing methanol as a sole carbon source (Cereghino et al., 2002). To illustrate, the AOX enzyme can

account for up to 30% of the total cell protein in cells fed with methanol at growth-limiting rates but it is virtually absent in cells grown on glucose, glycerol or ethanol (Cereghino et al., 2000).

The first group who isolated the *AOX1* gene is Ellis et al. (1985). Ontology of this alcohol oxidase gene is in Table 2.2.

Table 2.2 The *AOX* gene ontology (www.uniprot.org)

Molecular Function	Alcohol oxidase activity FAD binding
Biological process	Methanol metabolic process Oxidation reduction
Cellular Component	Peroxisome

Actually, *P. pastoris* contains two alcohol oxidase enzyme genes, *AOX1* and *AOX2* (Koutz et al., 1989). *AOX1* is responsible for the greater part of alcohol oxidase activity in the methanol metabolism (Cregg et al., 1989). Thus, AOX1 as a strong promoter can be used to drive the expression of recombinant proteins to high levels, up to 12 g/L (Cregg et al. 1993). The *AOX2* gene also produces alcohol oxidase; however, it yields 10-20 times less AOX activity than that of *AOX1* gene (Cregg et al., 1988) As a result, expression levels of foreign proteins under the control of the AOX1 promoter have been much higher than those reported for the AOX2 promoter (Cereghino et al., 2001a).

Even though the AOX1 promoter has been successfully used to express numerous foreign proteins; there are cases in which this promoter may not be suitable. For example, for the production of some food products, the use of methanol to induce gene expression may not be appropriate since methane, a petroleum-related compound, is one source of methanol. Additionally, methanol is a potential fire hazard, especially in quantities needed for large-scale fermentations. Therefore, for expression of certain genes, promoters that are not induced by methanol are attractive. Those alternative promoters used with the *P.*

pastoris expression systems are GAP, FLD1, PEX8, and YPT1 (Cereghino et al., 2000)

2.2.1.3 Methanol Utilization Phenotypes of *P. pastoris*

There are three phenotypes of *P. pastoris* with regard to methanol utilization. The methanol utilization plus phenotype, Mut^+, grow on methanol at the wild-type rate and require high feeding rates of methanol in large-scale fermentations (Cereghino et al., 2001a). This phenotype has a maximum specific growth rate of 0.14 h^{-1} (Macauley-Patrick et al., 2005) on methanol. The methanol utilization slow phenotype, Mut^S, has a disruption in the *AOX1* gene. Therefore the cells must rely on the weaker *AOX2* for methanol metabolism resulting slower growing and slower methanol utilization. This phenotype formation may occur during transformation events. Approximately 10-20% of transformation events are the result of a gene replacement event in which the *AOX1* gene is deleted and replaced by the expression cassette and marker gene. Due to this disruption of the *AOX1* gene, these strains should rely on the transcriptionally weaker *AOX2* gene for growth on methanol (Cregg et al., 1987). As a result, these strains have a Mut^S phenotype. Different methanol utilization phenotypes are easily identified among transformed colonies by selecting those growing on methanol slowly since specific growth rates of Mut^+ and Mut^S phenotypes on methanol differ significantly i.e., the specific growth rate of Mut^S is 0.04 h^{-1} (Jungo et at., 2006) In addition to these two phenotypes, the methanol utilization minus phenotype, Mut^-, is unable to grow on methanol as both *AOX* genes are deleted. These strains cannot metabolize methanol. Currently, the majority of researchers use the Mut^+ phenotype (Hohenblum et al., 2004; Slibinskas et al.,2004; Yu et al., 2002) but some researchers are also using the Mut^S phenotype (Aoki et al., 2003; McKinney et al., 2004; Paramsivam et al., 2002; Peng et al., 2004; Yang et al., 2004a).

2.2.1.4 Post-translational Modifications of Secreted Proteins

One major advantage of *P. pastoris* expression system over the bacterial ones is its potential to perform many of the post-translational modifications typically associated with higher eukaryotes such as processing of signal sequences, folding, disulfide bond formation, certain types of lipid addition and *O*- and *N*- linked glycosylation (Cereghino et al., 2000).

Since *P. pastoris* secretes low amounts of native proteins to the medium it is usually preferable to make the desired protein secreted to the medium. This also constitutes the first step of downstream processing (Cereghino et al., 2000; Macauley-Patrick et al., 2005). In order to direct proteins into the secretory pathway, a specific signal sequence is required. There are various secretion signals used in literature. One alternative is to use the native secretion signal of the foreign protein but α-factor prepro peptide of *S. cerevisiae* can be considered as the most widely and successfully used one and in some cases it is better than the native one (Macauley-Patrick et al., 2005). This signal sequence contains a 19-amino acid signal (pre) sequence followed by a 66-residue (pro) sequence (Kurjan et al., 1982). The acid phosphatase signal sequence (PHO) or the invertase signal sequence (SUC2) are also commonly used with *P. pastoris*. It is possible to use different secretion signals with different recombinant proteins but the most suitable one should be determined experimentally because of many possible interactions that could result between the signal peptide and the protein itself.

Other crucial steps, determining the productivity of the expression system used, are folding and disulphide bond formation. These have been identified in some case as the 'rate-limiting' step in the production of foreign protein from the microorganism (Hohenblum et al., 2004). The presence of disulphide bonds may have an effect on the binding activity of certain proteins as in the case of hGH. Prokaryotic systems have been generally unsuccessful in achieving this. The *P. pastoris* expression system has been successfully used to produce proteins that are highly disulphide-bonded (White et al., 1994).

Glycosylation is the other most common post-translational modification performed by *P. pastoris* (Eckart et al., 1996). There are two types of glycosylations both of which can be performed by *P. pastoris*; O- and N- linked glycosylations. Since almost 0.5–1.0% of the translated proteins in eukaryotic genomes are glycoproteins (Daly et al., 2005), it is necessary to have the correct glycosylation patterns on recombinant proteins to ensure their biological activity. The glycosylated products of *P. pastoris* generally have much shorter glycosyl chains than those expressed in *S. cerevisiae*, making this microorganism a much more attractive host for the expression of recombinant proteins (Bretthauer et al., 1999).

Eukaryotic cells add O-oligosaccharides onto the hydroxyl groups of serine and threonine of the secreted protein. In mammals, O-linked oligosaccharides are composed of a variety of sugars, including N-acetylgalactosamine, galactose (Gal), and sialic acid (NeuAc). In contrast, lower eukaryotes such as *P. pastoris* add O-oligosaccharides composed solely of mannose (Man) residues (Cereghino et al., 2000).

N-linked glycosylation, in eukaryotes, begins on the cytoplasmic side of the endoplasmic reticulum (ER) with the generation of a branched heptasaccharide intermediate. As in the case of O-linked glycosylation, N-linked glycosylation patterns changes from higher eukaryotes to lower ones. The mammalian Golgi apparatus performs a series of trimming and addition reactions that generate oligosaccharides composed of high-mannose type (Man5-6GlcNAc2), a mixture of several different sugars (complex type), or a combination of both (hybrid type) . In lower eukaryotes, N-linked core units are elongated in the Golgi through the addition of mannose outer chains (Cereghino et al., 2000).

Humanizing the glycosylation patterns of foreign proteins secreted by *P. pastoris* is desirable, since the extent and positioning of the glycosides may affect the activity of the protein (Macauley-Patrick et al., 2005). This may include changing some of the process parameters such as using mutant strains, using enzymes removing the glycosyl chains or adding some chemicals such as

tunicamycin which is a competitive inhibitor of UDP–GlcNAc dolichol P-GlcNAc transferase, an enzyme in glycosylation machinery (Daly et al., 2005).

Because of the absence of the recognition sequences for O- and N- linked glycosylation in hGH, there is no need for any additional processes to get rid of the glycosylation or reduce the glycosyl chains. Trevino et al. (2000), Eurwilaichitr et al. (2002), Orman et al. (2009) were able to produce mature rhGH without glycosyl chains by applying recombinant *P. pastoris*.

2.3 Medium Design and Bioreactor Operation Parameters

Efficient production of a recombinant protein relies on both the genetic engineering techniques used and the optimal bioprocess parameters such as composition of the fermentation medium, medium temperature and pH and lastly the oxygen transfer characteristics of the medium.

2.3.1 Medium Design and Feeding

Growth and production of metabolites by an organism in a bioprocess are results of the interactions between intracellular and extracellular effectors (Fiecher, 1984). The first step of the extracellular medium development is to decide on the necessary components then their concentrations in the medium. These necessary nutrients contain the chemical elements which constitute the cellular materials and structures, as well as elements which are required for membrane transport, enzyme activity, and generation of the energy required for biosynthetic processes (Scragg, 1988). Nutrients required for growth can be classified in two categories. The first one is macronutrients, generally needed in concentrations larger than 10^{-4} M, for example carbon, oxygen, nitrogen, hydrogen, sulfur, phosphorus, magnesium and potassium. The second one is micronutrients, compounds needed essentially for growth in the concentrations of less than 10^{-4} M such as trace elements such as Ca, Cu, Fe, Na, Mn, Mo, Zn, and vitamins, etc. These micronutrients are added to the culture medium as mineral salts (Fiecher, 1984).

There are three major types of growth media, defined medium containing specific amounts of pure chemical compounds with known chemical compositions, complex media containing natural compounds whose chemical compositions are not exactly known and semi-defined medium. Complex medium often results in higher cell and protein yields due to its rich ingredients; on the other hand, defined medium allows better control over the fermentation and leads to easier and cheaper recovery and purification of a product (Shuler and Kargı, 2002). Indeed, the use of yeast extracts and peptones, whose chemical composition is not exactly known, causes variations from batch to batch. Therefore it is desirable to eliminate any complex components from the medium so that making it easier to standardize the production process and to validate the medium and process itself. Production of large quantities of the heterologous protein, either for characterization or structural studies, or for manufacture, requires the use of a defined medium (Macauley-Patrick et al., 2005).

Optimized growth medium concentrations play important role on the production of recombinant proteins at high levels; therefore, trace salt solution, the amount of nitrogen sources, carbon sources affecting the amount of biomass as well as the recombinant protein produced within the cells are some important nutritional parameters which should be taken into consideration. Generally, basal salt medium (BSM) along with its companion trace salts medium (PTM1) are used for high cell density fermentation of *P. pastoris*. This is considered as a standard medium, though it may not be the optimum and may have some important problems, such as unbalanced composition, precipitates and high ionic strength (Cereghino et al., 2002; Cos et al., 2006). There are some studies to modify this standard medium (Brady et al., 2001; Thorpe et al., 1999; Jungo et al., 2006) and the effects of each medium component have been investigated in detail by Plantz et al. (2007) but it is still the mostly employed fermentation medium for *P. pastoris*.

One of the important medium components is the nitrogen source. In the standard medium ammonium hydroxide solution is mainly used which also has the effect of controlling the pH at the desired level (Cos et al., 2006). This method has the advantage of avoiding nitrogen accumulation, which can provoke

the inhibition of growth and enlarge the lag phase (Yang et al., 2004). The disadvantage arises in the case of inadequate feeding of nitrogen source, causing nitrogen starvation and consequently increased protease secretion. Other nitrogen sources such as yeast extract, casamino acids, L-arginine and EDTA are also used to increase the production level (Macauley-Patrick et al., 2005) since they inhibit or repress the protease production and secretion. In particular, ammonium ions seem to have the greatest effect on the extent of proteolysis (Daly et al., 2005).

Another parameter in medium design for fermentation of *P. pastoris* is the carbon source which plays an important role on recombinant protein production and cell growth. Most commonly used carbon sources for *P. pastoris* are methanol, glycerol, sorbitol, glucose, mannitol, and trehalose (Brierley et al., 1990; Sreekrishna et al., 1997; Thorpe et al., 1999; Inan and Meagher, 2001-b).

A three-stage process is typically utilized for the production of foreign proteins in fermenter cultures of *P. pastoris* (Higgins and Cregg, 1998). In the first stage, the microorganism is batch-cultured in a simple defined medium with a non-fermentable but repressing carbon source such as glycerol to accumulate biomass. In the second stage, a fed-batch transition phase is applied in which glycerol is fed to the culture at a growth-limiting rate to further increase the biomass concentration and to prepare (derepress) the cells for induction. The third stage is induction phase which is started by adding methanol to the culture at a slow rate, which facilitates the adaptation of cell culture to methanol and initiates the synthesis of the recombinant protein (Cregg et al., 2002).

Methanol is used as an inducer for the expression of recombinant proteins as well as a carbon and energy source. However, above certain concentrations, growth is substrate-inhibited by methanol (Zhang et al., 2000b); therefore a fed-batch protocol, in which its concentration is kept below toxic limits, 4 g/L, is generally used. A number of fed-batch strategies for methanol addition have been established (Zhang et al., 2000b). These strategies can be based on metabolism related parameters or predetermined methanol feeding programs. Metabolism related strategies can be based on parameters such as methanol consumption (Curvers et al., 2001a; Guarna et al., 1997; Hellwig et al., 2001;

Katakura et al., 1998; Kobayashi et al., 2000-a), oxygen consumption (Byrne et al., 2000; Chung, 2000; Minning et al., 2001), CO_2 concentration or pH control where predetermined methanol feeding strategies can be at constant, linear, or exponential rates (Chauhan et al., 1999; Freyre et al., 2000; Inan et al., 1999; Murasugi et al., 2000, Zhang et al., 2000a). In predetermined feeding strategies the feeding rate determines the growth rate. Cunha et al. (2004), Kobayashi et al. (2000), Ohya et al. (2002) and Zhang et al. (2005) revealed that the specific growth rate influence protein expression. In a comparative study performed by Trinh et al. (2003) different methanol feeding strategies were investigated for increased cell and recombinant protein productivities. A predetermined exponential methanol feeding profile controlling the growth rate at 0.02 h^{-1} was found to be more efficient than constant and linear feedings. Jungo et al. (2007) investigated the effect of specific growth rate (μ), lower than 0.08 h^{-1}, on specific product productivity by using pre-determined exponential feeding profile. In that study, it was observed that, for specific growth rates higher than 0.02 h^{-1}, specific productivity increased slightly with μ. However, a large decrease in specific productivities was observed at μ below 0.02 h^{-1}. Recently, Çelik et al. (2009) reported that with a predetermined exponential methanol feeding strategy as much or higher amounts of recombinant protein can be produced in shorter induction periods in the presence of sorbitol than in the usage of only methanol. The highest protein concentration was achieved at μ= 0.03 h^{-1} and the highest specific yield was achieved at = 0.02 h^{-1} in this study.

The use of multi-carbon substrates in addition to methanol in fermentations of methylotrophic yeasts is another strategy to increase cell density and process productivity, as well as to reduce the induction time. The use of mixed feeds of methanol and glucose was first investigated in 1980's by Egli et al. with the methylotrophic yeasts *Hansenula polymorpha* and *Candida boidinii* (Egli et al., 1980; Egli et al., 1982b; Egli et al., 1982a; Egli et al., 1983; Egli et al., 1986). One of the remarkable results is that methanol was utilized at dilution rates significantly higher than the maximum specific growth rate achieved on methanol as sole carbon source providing higher productivities than methanol as sole carbon source (Egli et al., 1982a). However, derepression of methanol

dissimilating enzymes was observed during glucose-limited continuous cultures (Egli et al., 1980).

For *P. pastoris*, usage of multi-carbon substrates together with methanol has been mostly employed for fermentations using Mut^S strains because of their genetically reduced capacity to assimilate methanol (Ramon et al., 2007). Even so, because of its various advantages increased attention on mixed substrate growth has been seen on Mut^+ strains. A fed-batch strategy using mixed substrates of glycerol and methanol was first investigated by Brierley et al. (1990) using a *P. pastoris* Mut^- strain. Working with Mut^S phenotypes, Files and coworkers (2001) showed that the addition of glycerol during the induction phase increased the concentration and productivity of cystatin in the early stages of induction in a fed-batch production with a constant methanol feeding rate and different glycerol feeding rates. However, they found out that the concentration of heterologous protein reached a maximum, leveled off and, when high glycerol feed rates were used, it decreased (Files et al., 2001). There are also some studies on mixed feeds of glycerol and methanol in Mut^+ strain. Katakura and coworkers (1998) showed that the specific growth rate in the presence of glycerol feeding was about 20% higher than that in absence of glycerol feeding in the production of human β2-glycoprotein I domain V in fed-batch process at a constant methanol concentration of 5.5 g/L. The specific production rate was also 2.3 times higher. Hellwig et al. (2001) tried different constant glycerol feeding rates while maintaining constant the methanol concentration at 5 g/L. The highest level of recombinant protein obtained at the lowest glycerol feeding rate tested an it was two fold higher than fermentation without glycerol addition. Then, Zhang et al. (2003) analyzed the influence of mixed-feeds of glycerol and methanol with a *P. pastoris* Mut^+ phenotype. They designed a fed-batch strategy at an optimal growth rate for maximal production (μ = 0.015 1/h) on methanol feed alone and a preprogrammed feeding strategy with glycerol growth rate ratios ranging from 1 to 4. They concluded that during growth on a mixed feed with $\mu_{Gly} \leq 0.06\ h^{-1}$ for this strain, the supplementary feeding of glycerol enhanced the overall growth rather than functioned as a repressor. This observation indicates that it is feasible to use a mixed feed in Mut^+ fermentations

without growth inhibition by glycerol when the feeding strategy is properly designed. Glycerol/methanol mixed feeding strategy was followed by several other groups, to increase the volumetric protein productivity as a result of higher cell densities and feeding rates possible with growth on glycerol (Cregg et al., 1993; Loewen et al., 1997; McGrew et al., 1997). However, still in some studies, it is claimed that the optimal level of protein expression is not achievable with mixtures of glycerol and methanol, due to a partial repression of the AOX1 promoter by glycerol, which may result in lower specific productivities of recombinant protein (Sreekrishna et al., 1997; Hellwig et al., 2001; Xie et al., 2005).

In mixed feeds of methanol/glycerol the partial repression of the AOX1 promoter by glycerol leads scientists to use carbon sources that support growth but do not repress the AOX1 promoter which is also essential to fermentation process control and scale-up. Sreekrishna et al. (1997) suggested that sorbitol might be less repressive to AOX1 promoter than glycerol. Then, a comparative study was performed by Thorpe et al. (1999) between mixed-feed strategies of methanol/glycerol and methanol/sorbitol with a Mut^S phenotype of *P. pastoris*. They reported that the presence of residual sorbitol in the growth medium appears to be less repressive to the alcohol oxidase promoter and although cell yields are lower on sorbitol higher specific product formation rates are obtained. Inan et al. (2001-b), then, was the first showing the advantage of sorbitol as a co-substrate in the induction phase of Mut^+ strains. In a study on expression and purification of ancrod, an anticoagulant drug, methanol/sorbitol co-feeding was applied to *P. pastoris*. As a result of this study, it was suggested that the ancrod produced by *P. pastoris* with a sorbitol as a co-substrate in the fermentation medium has the potential to be used clinically. Studies on sorbitol were resulted two conflicting comments on consumption mechanism of sorbitol and methanol. Ramon et al. (2007) claimed that these substrates were consumed sequentially where Jungo et al. (2007) concluded that they exhibit a simultaneous consumption manner. Additionally, Jungo et al. (2007) optimized the sorbitol content in the feed in a single continuous bioreactor experiment, and at this feeding ratio, by performing two fed-batch bioreactor experiments, at μ=0.03 h^{-1}

and μ=0.05 h^{-1} resulted that at μ=0.05 h^{-1}, accumulation of sorbitol did not affect the specific productivity. In a recent study, Çelik et al. (2009) investigated methanol induction strategies in the medium containing sorbitol by adding this substrate batch wise to the induction phase of *P. pastoris* Mut^+ strain producing EPO. In this study, methanol feeding rate found to have no effect on sorbitol consumption rate and, confirming the results of Jungo et al. (2007), sorbitol and methanol was found to be utilized simultaneously.

There are some additional advantages of mixed substrate growth on sorbitol and methanol such as significant reductions in heat production and oxygen consumption rates. From the heat production point of view, since the enthalpy of combustion of sorbitol is about 8% lower than that of glycerol and about 30% lower than that of methanol, for a given growth rate, less heat will be released in cultures with mixed carbon sources of sorbitol and methanol than with mixed carbon sources of glycerol and methanol or with methanol alone. Considering the oxygen need, since the degree of reduction of sorbitol is lower than those of glycerol and methanol, less oxygen will be consumed during mixed substrate growth on sorbitol and methanol (Jungo et al., 2007).

There are some other studies on non-repressing carbon sources that can be used as a co-substrate with methanol. Inan and Meagher (2001) compared different carbon sources; alanine, sorbitol, mannitol, trehalose, in terms of their ability to support growth and expression of β-Gal for *P. pastoris* Mut^- strain in shake flasks. They attained the result that Mut^- strain growing in media containing these carbon sources with methanol (0.5%) expressed as much or higher amount of β-Gal as compared to the Mut^+ grown on methanol containing media. Thereafter, Xie et al. (2005) studied the effects of glycerol, sorbitol, acetic acid and lactic acid as mixed feeds of methanol using a Mut^S strain and obtained the lowest angiostatin production in the fermentation fed with acetate and methanol. The highest recombinant protein production level of 191 mg/L was achieved with lactic acid even though accumulated lactic acid reached 6.3 g/L. Although alanine, mannitol, trehalose and lactic acid was reported as non-repressing carbon sources, no one was investigated as much detailed as sorbitol probably because they are much more costly.

2.3.2 Temperature

Temperature is one of the most important physical parameters which influence yeast growth. Microbial cells impose temperature conditions, since they have an optimum temperature for their metabolic activity and they do not have the ability to regulate their internal temperature. Temperatures over the optimum will accelerate cell death and at the opposite direction will slow down metabolic reaction kinetics (Donati, 2007). Therefore it is preferred to keep the medium temperature at the optimum value throughout the bioprocess. Otherwise, not only the reaction rates, but also metabolic regulations, nutritional requirements, biomass composition, product formation and yield coefficients will be affected by temperature; however, the optimum temperature for growth and product formation may be different. The influence of temperature on the maximum specific growth rate of a microorganism is similar to that observed for enzyme activity such that an increase is observed with increasing temperature up to a certain point where protein denaturation starts, and a rapid decrease beyond this point. For temperatures below the onset of protein denaturation the maximum specific growth rate increases much the same way for a normal chemical rate constant, explained by Arrhenius equation (Nielsen et al., 2003). On the other hand, when temperature is increased above the optimum temperature, the maintenance requirements of cells will also increase.

Fermentation processes with *P. pastoris* are usually run at an optimum temperature for growth of 30°C (Wegner, 1983). It has been stated that temperatures above 32°C can be detrimental to protein expression and may lead to cell death (Invitrogen, 2002), and temperatures above 30°C were not appropriate for the production of a recombinant peptide (Inan et al., 1999), since elevated temperatures result in cell death, which will lead to cell lysis and higher protease activity in fermentation media. On the other hand, lower cultivation temperature generally influences the yield of recombinant protein produced by *P. pastoris* because protease release to the medium is reduced due to lower cell death, decreasing the degradation of proteins (Macauley-Patrick et al., 2005). Additionally, expression of foreign proteins at lower temperatures helps reducing

protein misfolding and producing more properly folded proteins (Georgiou and Valax, 1996). There many studies showing that lowering the temperature below 30°C does not significantly influence the production of recombinant proteins expressed by *P. pastoris* strains (Inan et al., 1999; Curvers et al., 2001-b; Hong et al., 2002; Kupesulik and Sevella, 2005) although there are some in which increased yields of recombinant proteins at lower temperatures have been reported (Chen et al., 2000; Whittaker and Whittaker, 2000; Li et al., 2001; Hong et al., 2002; Sarramegna et al., 2002; Jahic et al., 2003a; Jahic et al., 2003b; Li et al., 2003; Shi et al., 2003).

2.3.3 pH

Other physical growth requirement for yeast cells is pH, the hydrogen ion concentration. It plays important role in the activity of enzymes, transport mechanisms and other extracellular and intracellular events as in the case of temperature therefore it influences the growth rate. Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level, even with large variations in the pH of the extracellular medium, but only at the expense of an increase in the maintenance energy demands, since Gibbs free energy has to be used for maintaining the proton gradient across the cell membrane. (Nielsen and Villadsen, 1994).

Most yeast species grow very well between pH values of 4.5 and 6.5 (Walker, 1998). *P. pastoris*, particularly, is capable of growing across a relatively broad pH range from 3.0 to 7.0 which actually does not affect the growth significantly (Macauley-Patrick et al., 2005). This makes it easier to choose the pH for recombinant protein production and stability and, minimizing activity of proteases in the medium (Sreekrishna et al., 1997; Macauley-Patrick et al., 2005). Studies show that different pH values were found to be optimal since recombinant proteins can be stable at different pH values and proteolysis of recombinant protein can be decreased by adjusting the pH values. Clare et al. (1991) found that for production of recombinant mouse epidermal factor optimal pH was 6.0 where it is 3.0 for production of insulin-like growth factor-I and

cytokine growth-blocking peptide (Brierley et al., 1994; Koganesawa et al., 2002). Most commonly the pH value has been fixed around 5.5 to reduce protease effects in the medium, and to improve the stability of the foreign protein (Cos et al., 2006). For the production of rhGH, pH 6.0 was used (Trevino et al., 2000; Eurwilaichitr et al., 2002; Orman et al., 2009).

2.3.4 Oxygen Transfer Rate

Oxygen shows diverse effect on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999). Yeasts need oxygen not just as the terminal electron acceptor in respiratory growth, but also as an essential growth factor for membrane fatty acids and sterol biosynthesis (Walker, 1998). Oxygen need of the microorganism is actually affected by the nature and cultivation conditions such as characteristics of metabolic pathway of the microorganism, and carbon sources and other nutrition used.

Strongly fermentative organisms produce high amounts of ethanol and acetic acid which quickly reach toxic levels in the cultivation medium. One of the principle factors why *P. pastoris* is such a popular expression system is that this microorganism prefers a respiratory growth rather than fermentative (Cereghino et al., 2002). Molecular oxygen is not only used for the respiration but also for the initial oxidation of methanol to formaldehyde. Hence, high oxygen transfer rates are needed when it grows on methanol since methanol metabolism utilizes oxygen at high rate. This can be achieved by aeration of pure oxygen or enrichment of air with pure oxygen which is one of the drawbacks of this expression system due to safety and economic aspects (Jungo, 2007)

In most of the studies carried out with *P. pastoris* use supplementation with pure oxygen to keep the dissolved oxygen level above 20 – 30% of saturation during the whole induction phase on methanol (Jahic et al., 2006). Exceptionally, there are some studies in which oxygen limited fed batch processes were tried (Trentmann et al., 2004; Trinh et al., 2003). A comparative study between oxygen limited fed batch (OLFB) process and methanol limited fed batch

(MLFB) process was performed by Charoenrat et al. (2005). They found 35% higher oxygen uptake rate, higher productivity and specific activity in OLFB when compared to MLFB.

2.4 Computation of Bioprocess Characteristics

2.4.1 Yield Coefficients and Specific Rates

Computation of the yield coefficients and the specific rates is an important step in evaluating the bioprocess correctly. Yield coefficients are stoichiometrically related parameters generally defined as;

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

where, $Y_{P/S}$ is the overall yield coefficient with P and S representing the product and the substrate, respectively. On the right hand side of the equation, ΔP is the mass or moles of P produced, and ΔS is the mass or moles of S consumed. This definition expresses an overall yield giving an average value for the entire culture period. However, in batch and fed-batch operations, since the yield coefficients may differ during the process for a given microorganism in a given medium, due to the growth rate and metabolic functions of the microorganism, it is sometimes necessary to evaluate the instantaneous yield at a particular point in time which can be computed as given in equation (2.2). When yields for fermentation are reported, the time or time period to which they refer should be stated (Doran, 1995).

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

Some of the frequently used yield coefficients are listed in Table 2.3.

Table 2.3 Definition of yield coefficients

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

Microbial growth can be considered as an increase in the number of individuals in the population as a result of both replication and change in cell size due to the chemical reactions occur inside the cell (Nielsen and Villadsen, 1994; Scragg, 1988). The rate of microbial growth is characterized by the specific growth rate, μ. Assuming that the cells are batch-wise and are not lost through sampling, general mass balance for biomass can be written as:

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

where r_x, the biomass formation rate, is defined as the product of specific cell growth rate (μ), and cell concentration (C_x), i.e.,

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

Equation for μ, the specific growth rate, is then obtained by combining equations (2.3) and (2.4) as,

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

In fed-batch systems, volume changes throughout to process due to methanol feed with a predetermined rate and variation in the volume is derived from general mass balance.

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

Thus, knowing the cell concentration (C_X) and volume (V) data throughout the bioprocess, μ can be calculated easily by inserting equation (2.5) into (2.6),

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

Rearranging equation (2.7) gives

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

In this study the substrate given batch-wise to the medium is sorbitol. Mass balance of sorbitol for this fed-batch system becomes,

$$r_s V = \frac{d(C_s V)}{dt} \tag{2.9}$$

Similar to equation (2.4); the substrate consumption rate for sorbitol, r_S, can be defined in terms of specific substrate consumption rate, qs and cell concentration, C_X ;

$$r_S = q_S C_X \tag{2.10}$$

Inserting equation (2.10) into (2.9),

$$\frac{dC_S}{dt} = -\frac{Q}{V}C_S + q_S C_X \tag{2.11}$$

and rearranging gives,

$$q_S = \left(\frac{dC_S}{dt} + \frac{Q}{V}C_S\right)\frac{1}{C_X} \tag{2.12}$$

Mass balance for the fed-batch substrate, methanol, which is added with an exponential volumetric flow rate of Q, can be written as

$$QC_{Mo} - 0 + r_M V = \frac{d(C_M V)}{dt} \tag{2.13}$$

The substrate consumption rate for methanol, r_M, can be defined as the product of specific substrate consumption rate (q_M) and cell concentration (C_X),

$$r_M = q_M\, C_X \tag{2.14}$$

Inserting equation (2.13) into (2.14)

$$QC_{M0} + q_M C_X V = V\frac{dC_M}{dt} + C_M\frac{dV}{dt} \tag{2.15}$$

When the nutrient consumption rate is nearly equal to nutrient feed rate, a fed-batch system can be assumed to operate at quasi-steady state. At quasi-steady state, no significant level of the substrate can accumulate (Shuler and Kargı, 2002). Therefore, the terms on the right hand side are physically negligible, but mathematically the term on the right hand side should be considered carefully.

$$C_M\frac{dV}{dt} \sim 0 \tag{2.16}$$

Combining equations (2.13) and (2.16), dividing by V and rearranging, the general mass balance simplifies to,

$$\frac{dC_M}{dt} = \frac{Q}{V}(C_{M0}) + q_M C_X \tag{2.17}$$

The specific methanol consumption rate, q_M can be calculated from equation (2.17), where the last term on right hand side is also negligible in quasi-steady states as,

$$q_M = \frac{Q}{V}\frac{C_{M0}}{C_X} - \frac{1}{C_X}\frac{dC_M}{dt} \tag{2.18}$$

Using similar mass balance equations, the recombinant protein balance becomes,

$$\frac{dC_{rp}}{dt} = q_{rp} C_X - \frac{Q}{V} C_{rp} \tag{2.19}$$

The specific recombinant protein production rate, q_{rp}, can be calculated after rearranging the equation above;

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

As a result of these mass balances and simplifications, the specific rates μ, q_S, q_M and q_{rp} can be calculated from experimental data using equations (2.8), (2.12), (2.18), (2.20), respectively.

2.4.2 Oxygen Transfer Characteristics

The transfer of oxygen into the microbial cell in aerobic fermentation processes strongly affects product formation by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999). For *P. pastoris* oxygen transfer characteristics are highly important since it needs significantly high amounts of oxygen due to its methanol metabolism. The transfer of oxygen from the fermentation medium to microorganism takes place in several steps (Bailey

and Ollis, 1986). When cells are dispersed in the liquid, and the bulk fermentation broth is well mixed, the major resistance to oxygen transfer is the liquid film surrounding the gas bubbles. Therefore the rate of oxygen transfer from gas to liquid is of prime importance. An expression for oxygen transfer rate (OTR) from gas to liquid is given by,

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

where, C_O^* is saturated dissolved oxygen concentration and C_O is the actual dissolved oxygen concentration in the broth and a is the gas-liquid interfacial area. Since solubility of oxygen in aqueous solutions is very low, the liquid phase mass transfer resistance dominates, and the overall liquid phase mass transfer coefficient, K_La, is approximately equal to liquid phase mass transfer coefficient, k_La (Shuler and Kargı, 2002). Using equation (2.21), the maximum possible mass transfer rate can be defined as,

$$OTR_{\max} = K_L a C_O^* \tag{2.22}$$

Oxygen uptake rate (OUR), $-r_O$, is another important parameter of oxygen transfer characteristics. It is defined as per unit volume of broth and expressed as,

$$OUR = -r_O = q_O C_X \tag{2.23}$$

where, q_O is the specific rate of oxygen consumption and C_X is the cell concentration (Shuler and Kargı, 2002).

K_La values in the OTR expressions should be determined experimentally and there are a number of methods. A widely used one is the dynamic method (Bandyopadhyay and Humprey, 1967) which can be applied during the fermentation process. The method is based on a material balance for oxygen,

$$\frac{dC_O}{dt} = OTR - OUR = K_L a(C_O^* - C_O) + r_O \tag{2.24}$$

In this method, at some time t_0, the broth is first de-oxygenated by stopping the air flow and lowering the agitation rate to a minimum level to prevent surface aeration. During this time period, dissolved oxygen concentration (C_O) drops due consumption by the microorganism, and since there is no oxygen transfer. As a result, the equation (2.24) becomes:

$$\frac{dC_O}{dt} = r_O \tag{2.25}$$

This process represents the region-II of Figure 2.7 and oxygen uptake rate, $-r_O$, in can be determined using Equation 2.25 with the experimental data.

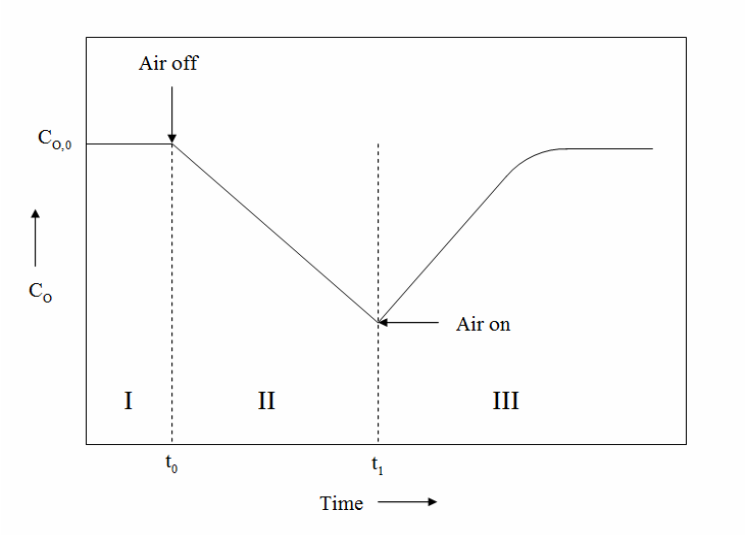

Figure 2.7 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La

Air inlet is then turned on at time t_1, and the increase in C_O is recorded as a function of time. This period is represented as region-III in Figure 2.7 and equation (2.24) is valid here. r_O in region-III is assumed to have the same value as calculated from region-II for the same analysis set. After rearranging equation (2.24), equation (2.26) is obtained.

$$C_O = -\frac{1}{K_L a}\left(\frac{dC_O}{dt} - r_O\right) + C_O^* \qquad (2.26)$$

Thus, from a plot of C_O versus ($dC_O/dt - r_O$), K_La can be determined from the slope as shown in Figure 2.8.

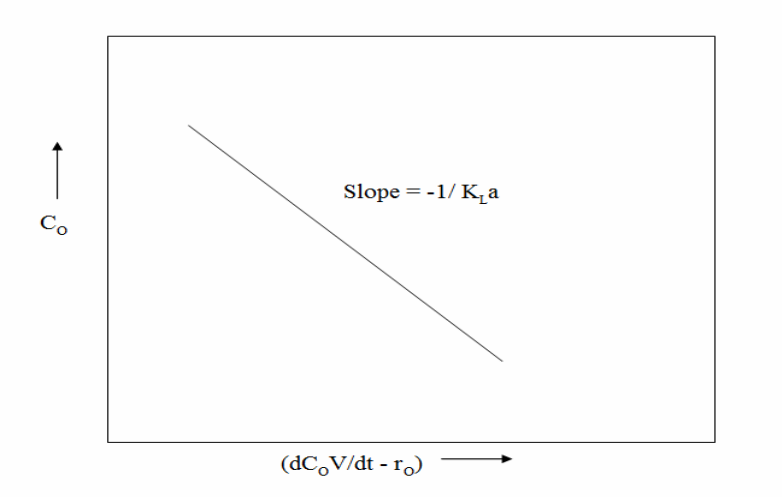

Figure 2. 8 Evaluating K_La using the Dynamic Method.

The Dynamic Method can also be applied to conditions under which there is no reaction, that is no microorganism in the medium, i.e., r_O=0 (Nielsen et al., 2003). In order to do this, the broth is de-oxygenated by giving nitrogen into it. After that, Air inlet is turned on and again the increase in C_O is monitored as a function of time. Modifying equation (2.26) such that there is no r_O term,

$$C_O = -\frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \qquad (2.27)$$

This time, from the slope of a plot of C_O versus $d(C_OV)/dt$, the physical mass transfer coefficient, K_La_0, can be determined.

In order to compare the relative rates of maximum oxygen transfer and biochemical reactions and find the rate limiting step of the bioprocess the maximum possible oxygen utilization rate (OD=oxygen demand) should be determined. It is defined as follows (Çalık et al., 2000),

$$OD = \frac{\mu_{\max} C_X}{Y_{X/O}} \tag{2.28}$$

In order to express the oxygen limitation in an aerobic process, the effectiveness factor, η and Damköhler number, Da are defined. The effectiveness factor, η, is the oxygen uptake rate per maximum possible oxygen utilization rate and Damköhler number, Da, is the maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2000). These terms have the following formulations:

$$\eta = \frac{OUR}{OD} \tag{2.29}$$

$$Da = \frac{OD}{OTR_{\max}} \tag{2.30}$$

2.5 Genetic Engineering Techniques: Real Time Polymerase Chain Reaction

Quantitative nucleic acid sequence analysis has an important role in many fields of biological research (Heid et al., 1996). Measurement of gene expression (RNA) has been used extensively in monitoring biological responses to various stimuli (Tan et al. 1994; Huang et al., 1995a, b; Prud'homme et al., 1995). Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has always been important in making these decisions (Zamorano et al., 1996). Reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of mRNA. Real-time RT-PCR (or kinetic RT-PCR) is widely and

increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range (Orlanso et al., 1998; Pfaffl et al., 2001).

The polymerase chain reaction (PCR) has been in use for almost two decades (Mullis et al., 1986) but its rapid development started with discovery of the thermophilic bacterium *Thermus aquaticus* in 1969 (Brock et al., 1969). DNA polymerase was first isolated from this microorganism in 1976 (Chien et al., 1976) Then, it is discovered that this enzyme could be used in the polymerase chain reaction (PCR) process for amplifying short segments of DNA (Saiki et al., 1988). The first practical kinetic PCR technology, the 5'-nuclease assay, was established 1993 and combines the exponential PCR amplification of a specific transcript with the monitoring of newly synthesized DNA in each performed PCR cycle (Higuchi et al., 1993; Gibson et al., 1996). Today, real-time PCR is used to detect nucleic acids from food, vectors used in gene therapy protocols, genetically modified organisms, and areas of human and veterinary microbiology and oncology (Klein, 2002; Mhlanga et al., 2001).

In this study, real time PCR is used to determine the expression levels of the *hGH* and *AOX1* genes. These genes were selected for investigation since they are directly related to the production of rHGH and their response to the external stimulus like medium composition in the transcriptional level is important. Hideyuki et al. (1995) used PCR approach to discover the identity between the amino acid sequences of a protease PRC1 in *P. pastoris* and *S. cerevisiae*. Hence et al. (2000) used reverse transcriptase PCR technique to detect hGH mRNA in total RNA prepared from glands in order to confirm that hGH was specifically expressed in transfected glands. Resina et al. (2007) investigated the transcriptional levels of some genes related to the unfolded protein response (UPR) and central metabolism of *Pichia pastoris* during batch and fed-batch cultivations using strain expressing a *Rhizopus oryzae* lipase under control of the formaldehyde dehydrogenase promoter (*FLD1*), namely the alcohol oxidase gene *AOX1*, the formaldehyde dehydrogenase *FLD1*, the protein disulfide isomerase *PDI*, the *KAR2* gene coding for the BiP chaperone, the 26S rRNA and the *R. oryzae* lipase gene *ROL* by means of relative quantification.

2.5.1 Basic Principles of Real Time PCR

PCR is the most commonly used nucleic acid amplification technique which relies on thermal cycling, consisting of cycles of repeated heating and cooling of an *in-vitro* reaction for DNA melting and enzymatic replication of the DNA. High temperature above 90^{o}C is applied to separate (melt) the strands of the double helical DNA, then temperature is lowered to let primers anneal to the template and finally the temperature is set around 72^{o}C, which is optimum for the polymerase that extends the primers by incorporating the dNTPs (Figure 2.9). Polymerase chain reaction is performed on a DNA template, which can be single or double stranded. Also needed are two oligonucleotide primers that flank the DNA sequence to be amplified, dNTPs, which are the four nucleotide triphosphates, a heat-stable polymerase, and magnesium ions in the buffer (Kubista et al., 2006).

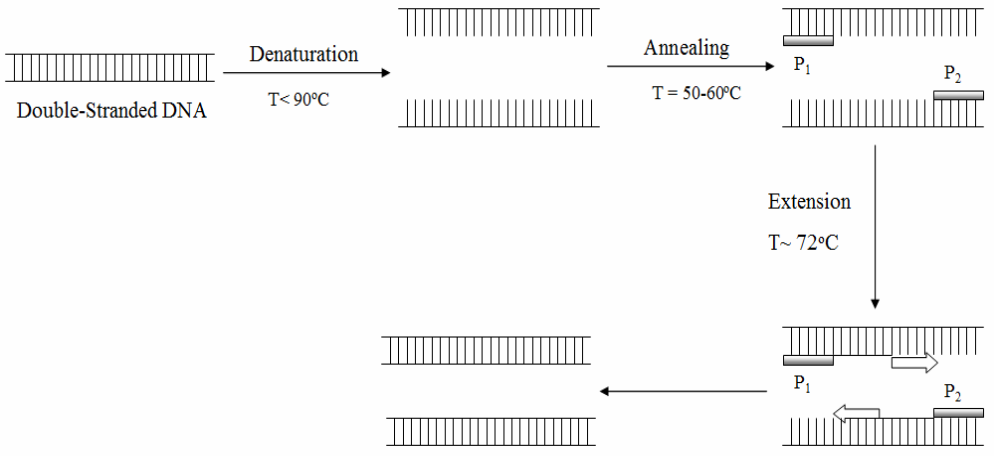

Figure 2.9 The Polymerase Chain Reaction

A typical PCR analysis involves 30 to 50 cycles, and the product concentration doubles after each cycle. In real time PCR, the reaction mixture contains fluorescent markers, which are designed to interact with reaction product, thus, the simultaneous monitoring of the reaction product by means of fluorescence detection is achieved as the PCR is in progress (Schmittgen et al., 2000; Neumaier et al, 1995; Wittwer et al., 2001). Therefore, real time PCR is the most sensitive method for the detection and quantification of mRNA (Pfaffl et al., 2002; Gause et al., 1994)

2.5.2 Reverse Transcription

Before a gene expression measurement can be performed by real-time PCR, the mRNA in the sample must be copied to cDNA by reverse transcription (RT). The RT step is critical for sensitive and accurate quantification, since the amounts of cDNAs produced must correctly reflect the input amounts of the mRNAs (Kubista et al., 2006).

The two commonly used reverse transcriptases are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Bustin, 2000). Generally, AMV-RT is employed rather than MMLV-RT since it retains significant polymerization activity up to 55^{o}C (Freeman et al., 1996) and can help in eliminating problems associated with RNA secondary structure.

Specific primers, random hexamers and oligo-dT primers can be used for the reverse transcriptase step. The choice of primers requires careful consideration since yield and quality of cDNA are depending on these primers. The use of mRNA-specific primers decreases background priming. On the other hand, the use of random and oligo-dT primers maximizes the number of mRNA molecules. (Zhang and Byrne, 1999). oligo-dT primers were used for cDNA synthesis in this study.

2.5.3 Real-Time Monitoring of RT-PCR

Real-time PCR, in addition to DNA sequence to be amplified, dNTPs, and a heat-stable polymerase, also needs a fluorescent reporter that binds to the product formed and reports its presence by fluorescence. The reporter generates a fluorescence signal that reflects the amount of product formed. During the initial cycles the signal is weak and cannot be distinguished, however, as the amount of product accumulates a signal develops that initially increases exponentially. Thereafter the signal levels off and saturates. The signal saturation is due to the reaction running out of some critical component. This can be the primers, the reporter, or the dNTPs (Kubista et al., 2001). In a typical real-time PCR experiment all response curves saturate at the same level. Thus, end-points of PCR measurements give no information about the initial amounts of target molecules. On the other hand the response curves are separated in the growth phase of the reaction. This reflects the difference in their initial amounts of template molecules. The number of amplification cycles required for the response curves to reach a particular threshold fluorescence signal level is compared between different samples to quantify the initial template concentrations. The number of cycles required to reach threshold value is called the threshold cycle, C_T or namely crossing point, C_p.

2.5.3.1 Quantification Strategies in Real Time RT-PCR

The amount of template in a sample can be described either relatively or absolutely. Relative quantitation is the simpler approach and it is based on the expression levels of a target gene versus a housekeeping gene (reference or control gene) and in theory is adequate for most purposes to investigate physiological changes in gene expression levels. Absolute quantitation is more demanding but states the exact number of nucleic acid targets present in the sample (Mackay, 2004). It requires a standard curve constructed before the quantification of the sample.

2.5.3.1.1 Relative Quantification

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This reference gene is often a housekeeping gene and can be co-amplified in the same tube in a multiplex assay or can be amplified in a separate tube (Wittwer et al., 2001; Pfaffl, 2002). Therefore, relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known (Bustin, 2002) Relative quantification is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels. To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established. Calculations are based on the comparison of the distinct cycle determined by various methods, e.g., crossing points (C_P) and threshold values (C_t) at a constant level of fluorescence; or C_P acquisition according to established mathematic algorithm (Wittwer et al.,2001; Tichopad et al., 2003 and 2004).

2.5.3.1.2 Absolute Quantification

Absolute quantification requires a calibration curve constructed before the quantification of the sample. Calibration curves should be reproducible and allow the generation of highly specific, sensitive and reproducible data (Reischl et al., 1995; Bustin, 2000; Pfaffl, 2001; Pfaffl, 2002). The dynamic range of the performed calibration curve can be up to nine orders of magnitude from 10^1 to 10^{10} start molecules, depending on the applied standard material (Pfaffl, 2001; Pfaffl, 2002).The calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules, e.g., recombinant plasmid DNA (recDNA), genomic DNA, RT-PCR product, and commercially synthesized big oligonucleotide (Reischl at al., 1995; Morrison et al., 1998; Bustin 2000; Pfaffl, 2001; Rasmussen et al., 2001).

In absolute quantification, the final result is always reported relatively compared to a defined unit of interest, e.g., copies per defined ng of total RNA, copies per genome, copies per cell, copies per gram of tissue, copies per ml blood, etc (Pfaffl, 2004). The quality of your gene quantification data cannot be better than the quality of the denominator. Any variation in denominator will obscure real changes, produce artificial changes, and wrong quantification results.

2.5.3.2 Principles of Fluorimetric Detection

Real-time PCR relies on fluorimetric detection in which a fluorescent reporter that binds to the product formed and reports its presence by fluorescence. There are various methods for detecting and evaluating fluorimetric PCR results. The most commonly used fluorescence formats are divided into two classes (www.roche-applied-science.com).

1. Sequence-Independent Detection Assays: This method relies on fluorophores that bind to all double-stranded DNA molecules regardless of sequence. There are two types of sequence-independent detection assays;
   - SYBR Green I
   - Ethidium bromide
2. Sequence-Specific Probe Binding Assays: In these assays, fluorophores are coupled to sequence-specific oligonucleotide hybridization probes that only detect certain PCR product. The assays are;
   - Single labeled probes
   - Hybridization probes
   - Hydrolysis probes

The major problem in real time PCR applications is the formation of non-specific products like primer-dimers. Real-time assays using SYBR Green I can easily reveal the presence of primer-dimers, which are the product of nonspecific

annealing and primer elongation events (Morrison et al., 1998). The discrimination between the specific product and non-specific ones is performed by means of melting curve analysis. The pure and homogeneous RT-PCR products produce a single, sharply defined melting curve with a narrow peak. In contrast, the primer dimers melt at relatively low temperatures and have broader peaks. Therefore, in this study, SYBR Green I assay was used to determine expression levels of the *hGH* and *AOX1* genes.

2.5.3.2.1 SYBR Green I Assay

This method involves detection of the binding of a fluorescent dye, SYBR Green, to DNA (Morrison et al., 1998). Fluorescent dyes have virtually no fluorescence when they are free in solution. On the other hand the dyes become brightly fluorescent when they bind to DNA (Nygren et al., 1998). In PCR the fluorescence of these dyes increases with the amount of double stranded product formed. The increase in SYBR Green I signal, which is measured at 530 nm, correlates with the amount of product amplified during PCR.

During the various stages of PCR, the intensity of the fluorescence signal will vary, depending on the amount of dsDNA that is present. After denaturation, all DNA becomes single-stranded. At this stage of the reaction, SYBR Green I dye will not bind and the fluorescence intensity is low. During annealing, the PCR primers hybridize to the target sequence, creating small regions of dsDNA that SYBR Green I dye can bind, thereby leading to increased fluorescence (www.roche-applied-science.com).

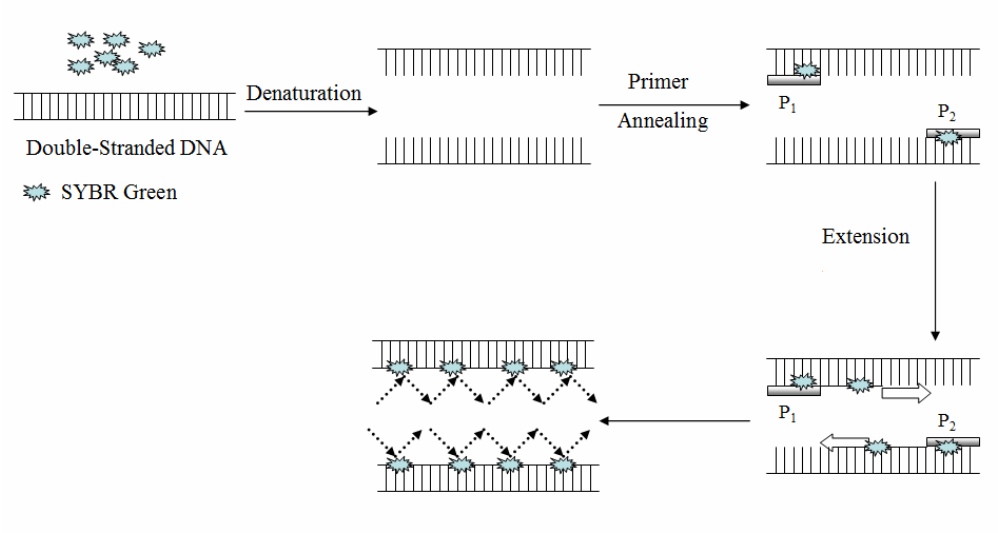

Figure 2.10 Stages of SYBR Green I assay

The SYBR Green I format cannot discriminate between different dsDNA species since SYBR Green I binds to any dsDNA. Hence, the specific product, nonspecific products and primer-dimers are detected equally well. Additional specificity and product verification can be achieved by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (Ririe et al., 1997). This is done by slowly increasing the temperature above the T_m of the amplicon and measuring the fluorescence. As the T_m of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product (Bustin, 2002).

2.5.4 Data Analysis

In real-time PCR data analysis, there are numerous methods. Cycle threshold method (C_t method), second derivative maximum method (SDM) and non-linear regression analysis (NLR) are the most commonly used ones.

2.5.4.1 Cycle Threshold Method

In this method, threshold fluorescence is calculated from the initial cycles, and in each reaction C_t value is defined by the fractional cycle at which the fluorescence intensity equals the prior set threshold fluorescence. This method is based on an assumption of equal PCR efficiency in all reactions, and accuracy may be compromised if this condition is not met (Pfaffl, 2009). The strength of this method is that, it is extremely robust. The weakness is that it is not easily automated and so requires a lot of user interaction, which can be arbitrary (Rasmussen, 2001; LightCycler Software, 2001).

2.5.4.2 Second Derivative Maximum Method

Applying the SDM, the quantification point is automatically identified and measured at the maximum acceleration of fluorescence (Rasmussen, 2001; Tichopad et al., 2003). LightCycler software (Roche Diagnostics) is using this method although its exact mathematical algorithm is still unpublished. It is possible to fit sigmoidal and polynomial curve models (Liu et al., 2002; Tichopad et al., 2003 and 2004), which can be differentiated, and the second-derivate maximum can be estimated (Tichopad et al., 2003 and 2004).

2.5.4.3 Non-Linear Regression Analysis

NLR has been suggested as an alternative to the C_t method for absolute quantitation (Goll et al., 2006). The advantages of NRL are that, the individual sample efficiency is stimulated by the model and that absolute quantitation is possible without a standard curve, releasing reaction wells for unknown samples. NLR is advantageous since it can be fully automated and it saves both time and resources. However, it is reported that further work is needed to improve the precision of the fluorescence copy number conversion factor (Pfaffl, 2009).

In this study, Roche LightCycler Software 4.0 was used for data analysis, in which second derivative maximum method is applied.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

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

3.2 Buffers and Stock Solutions

All buffers and stock solutions were prepared with distilled water and sterilized by autoclaving at 121^{o}C for 20 minutes or by filtering with 0.20 μm filters (Sartorius AG, Gottingen, Germany) and stored at +4^{o}C or room temperature. All buffers and stock solutions used are listed in Appendix A.

3.3 Microorganisms and Plasmids

Pichia pastoris hGH-Mut^{+} and *Pichia pastoris hGH*-Mut^{S} (Çalık et al., 2008; Orman et al., 2009) were used for rhGH production. The recombinant microorganisms are stored in microbanks (PRO-LAB), by inoculating young colonial growth into cyropreservative fluid present in the vial. After providing the adsorption of microorganisms into the porous beads, excess cryopreservative was aspirated and inoculated into cyrovial stored at -55^{o}C.

3.4 Growth Medium and Storage of Microorganisms

3.4.1 Solid Medium

Recombinant *P. pastoris* strains were stored in microbanks at -55^{o}C or inoculated on YPD agar containing 0.1 g/L Zeocin at 4^{o}C. The composition of solid medium is listed in Table 3.1.

Table 3.1 The composition of the YPD, solid medium.

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

3.4.2 Precultivation Medium and Glycerol Stock Solution

Recombinant *P. pastoris* strain grown on YPD agar was first inoculated into percultivation medium, YPD (Table 3.2). Harvested cells from YPD were resuspended in glycerol stock solution (Table 3.3) and stored at -55^{o}C or directly inoculated into BMGY, the second precultivation medium. The composition of BMGY is given in Table 3.4. The selective antibiotics, zeocin or chloramphenicol, were added to the precultivation media with concentrations given in Table 3.2 and Table 3.4 after sterilization.

Table 3.2 The composition of YPD, the first precultivation medium.

Compound	Concentration, g/L
Yeast extract	10
Peptone	20
Glucose	20
Zeocin	0.1

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

Compound	Concentration, g/L
NaCl	9
Glycerol	20

Table 3.4 The composition of BMGY, second precultivation medium.

Compound	Concentration, g/L
Yeast extract	10.0
Peptone	20.0
Potassium phosphate buffer pH 6.0	0.1 M
YNB	13.4
Biotin	$4x10^{-5}$
Glycerol	10.0

3.4.3 Production Medium

The recombinant *P. pastoris* strain grown on second precultivation medium was inoculated into production medium after cells were harvested by centrifugation. A defined medium production medium, which contains glycerol or sorbitol together with methanol, basal salts solution and nitrogen sources, was

used in air filtered shake bioreactor experiments whose composition was reported by Jungo et al. (2006). However, the following modifications were performed in the medium. Ammonium sulfate was used instead of ammonium chloride and two different trace salt solutions were tried. Methanol and sorbitol at different concentrations were added to production medium in shake flask experiments. Basal salt medium (BSM) was used as a production medium for pilot scale fed-batch bioreactor experiments. The compositions of the PTM1 and PTMJ (trace salt solutions), defined medium and BSM are listed Table 3.5, 3.6 and 3.7, respectively. All of the medium components were autoclaved at 121^{o}C for 20 min, except trace salts which was sterile filtered.

Table 3.5 The composition of the trace salt solutions PTM1 and PTMJ (Sibirny et al., 1987; Jungo et al., 2006).

Compound	**Concentration**	
	PTM1 g/L	PTMJ mg/L
$CuSO_4.5H_2O$	6	8
KI	-	1.2
NaI	0.08	-
$MnSO_4.H_2O$	3	28
$Na_2MoO_4.2H_2O$	0.2	5.2
H_3BO_3	0.02	8
$ZnSO_4.7H_2O$	-	44
$ZnCl_2$	20	-
$FeCl_3.6H_2O$	-	75
$FeSO_4.7H_2O$	65	-
$CoCl_2$	0.5	-
$CoCl_2.6H_2O$	-	8
H_2SO_4 (mL)	5	-
Biotin	0.2	1.74
Add to medium	4.35 mL/L	2.00 mL/L

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

Compound	Concentration g/L
Glycerol / methanol	40 / 20
$(NH_4)_2SO_4$	18.84
KH_2PO_4	5.62
$MgSO_4.7H_2O$	1.18
$CaCl_2.2H_2O$	0.11

Table 3.7 The composition of Basal Salt Medium (BSM) (Sibirny et al., 1987).

Compound	Concentration g/L
85% H_3PO_4	26.7 mL
$CaSO_4.2H_2O$	1.17
$MgSO_4.7H_2O$	14.9
KOH	4.13
K_2SO_4	18.2
Glycerol	40.0

3.5 Recombinant Human Growth Hormone Production

Protein production was performed either in batch cultures using laboratory scale air filtered shake bioreactors or pilot scale fed-batch bioreactors.

3.5.1 Precultivation

P. pastoris strains carrying human growth hormone gene, was inoculated onto solid medium containing 0.1 g/L Zeocin, YPD agar, and incubated for t=48-60 h at 30°C. After that two or three colonies were inoculated into 100 mL YPD medium containing 0.1 g/L Zeocin and the culture was incubated at 30ºC and

N=225 min^{-1} for 19 h in agitation and heating rate controlled orbital shakers (B.Braun, Certomat BS-1) using air-filtered Erlenmeyer flasks of 150 mL in size with a working volume of 10 mL. After 19 h either the culture was centrifuged 10 min at 4000xg and resuspended in glycerol stock solution and stored or directly inoculated into second precultivation medium, BMGY, and were grown at 30ºC and N=225 min^{-1} for t= 20-24 h until cell concentration reaches to OD_{600} = 2-6 which corresponds to cell concentrations of C_X=0.55-1.65 g/L.

3.5.2 RhGH Production in Laboratory Scale Air Filtered Shake Bioreactors

In laboratory scale air filtered shake bioreactor experiments, production was achieved in baffled and air filtered Erlenmeyer flasks of 250 mL in volume (V) having working volume capacities (V_R) of 50 mL. The cells grown on BMGY were harvested by centrifugation at 4000xg for 10 minutes and resuspended into production medium. Defined medium given by Jungo et al. (2006) was used as a production medium. Glycerol or sorbitol at different concentration was also added to production medium and every 24 hours, methanol was added to the medium to 1% or 3% (v/v). Production process was performed batch-wise and continued for 50 h.

3.5.3 RhGH Production in the Pilot Scale Bioreactor

In the pilot scale bioreactor experiments, production was achieved in V=3.0 L bioreactor (Braun CT2-2), having a working volume of V_R=0.5-2.0 L and consisting of temperature, pH, foam, stirring rate, feed inlet rate and dissolved oxygen control systems. The bioreactor consisting of four baffles and a sparger was stirred with two four-bladed Rushton turbines. Sterilization and temperature control was achieved using a jacket around the bioreactor, an external cooler and steam generator. A compressor and a pure oxygen tube were employed to supply air and oxygen. A mass flow controller was also used to adjust the inlet oxygen flow rate. Feed solutions placed on balances were transferred through inlet ports by using peristaltic pumps.

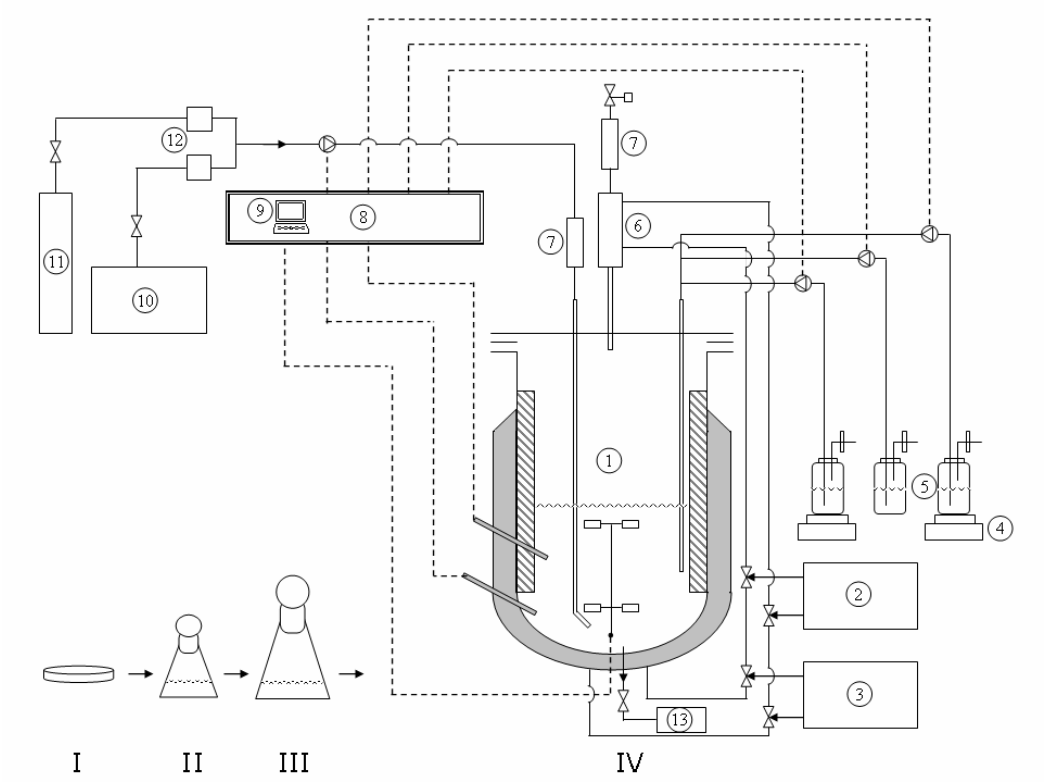

Figure 3.1 Scale up steps and the pilot scale bioreactor system. I: Solid medium inoculated from stock culture; II: 1st Precultivation medium, V = 10 mL; III: 2nd Precultivation medium, V = 50 mL; IV: Pilot scale bioreactor system, Vo=1 L, which is composed of (1) Bioreaction vessel, Biostat CT2-2 (2)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

A standard protocol for expression of recombinant proteins in *P. pastoris* under the control of the AOX1 promoter was followed (Stratton et al., 1998). Some modifications were done for the production at high cell densities. The cells, harvested from the precultivation medium, were resuspended in BSM inside the bioreactor such that the initial OD_{600} =1. This corresponds to a cell concentration of 0.275 g/L. In order to achieve high cell densities, the first part of the process was performed batch-wise until glycerol was totally consumed, which takes about 19 h. Secondly, glycerol fed-batch strategy was applied by

feeding 50% (v/v) glycerol containing 12 mL/L PTM1 at limiting concentrations with a predetermined rate in order to increase biomass concentration while derepressing the AOX enzyme (necessary for the dissimilation of methanol) gradually. After that, a transition phase was performed by giving a short pulse of methanol feed, C_{M0}=1.5 g/L, (from 100% methanol containing 12 mL/L PTM1). After 6 hours of transition phase, just before starting methanol fed-batch mode, co-substrate (500 g L^{-1} sterile solution) was added batch-wise to the system, such that C_{S0}=50 g/L was obtained. Methanol fed-batch process was achieved by feeding 100% methanol containing 12 mL/L PTM1 to induce recombinant protein production. A predetermined exponential feeding profile was utilized for this purpose. The steps of scaling up and the bioreactor system are shown in Figure 3.1.

3.6 Analysis

Throughout the production process, samples were collected with a certain time interval to determine cell concentration. The medium collected was centrifuged 10 min at 13200xg and +4°C. Cells and the supernatant were stored separately at -55°C for further analysis. The supernatant was used to determine methanol, sorbitol, amino acid, organic acid and hGH concentrations. The harvested cells were used to determine AOX activity and expression levels in real time-PCR analysis.

3.6.1 Cell Concentration

Cell concentration was measured using a UV-Vis Spectrophotometer (Thermo Spectronic, Heλiosα) at 600 nm. The range is between 0.1 and 0.9 to read OD_{600}. Hence, in most of the cases samples taken from medium were diluted with dH_2O. To convert absorbance to cell concentration, C_X (g/L), equation 3.1 was used.

$$C_X = 0.275 * OD_{600} * \text{Dilution Ratio} \quad (3.1)$$

3.6.2 Protein Analysis

3.6.2.1 Total Protein Concentration

Total protein concentration was measured spectrophotometrically using Bradford assay (Bradford, 1976). 50 µL of sample was mixed with 1.5 mL of Bradford reagent (BioRad) and incubated at room temperature for 5-15 min. The absorbance was read at 595 nm by UV-spectrophotometer. The calibration curve was obtained using BSA in the concentration range of 0-2 mg/mL (Appendix B).

3.6.2.2 hGH Concentration

RhGH concentrations were measured using a high-performance capillary electrophoresis (Capillary Electrophoresis System, Agilent). Samples filtered with 0.45 µm cellulose acetate filters were analyzed at 12 kV and 15°C with a positive power supply using 60cm x 75 µm silica capillary and 50 mM borate buffer (pH=10.0) containing zwitter ion (Z1-Methyl reagent, Waters) as the separation buffer. The zwitter ion is used to prevent protein adsorption to the capillary column. Proteins were detected by UV absorbance at 214 nm, as mentioned elsewhere (Çalık et al., 1998). A sample electropherogram which belongs to a hGH standard can be seen in Appendix C.

3.6.2.3 Ultrafiltration

The production medium was concentrated and desalted by ultrafiltration using 400 mL stirred cells (Amicon) and 10 kDa cut-off regenerated cellulose ultrafiltration membranes (Millipore). The process was carried in cold room (2-8°C) using N_2 gas pressure of maximum 55 psi (3.8 bars), until at least 10-fold concentration of the medium was obtained

3.6.2.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970). The sample buffer and protein samples were mixed and heated in boiling water for 5 min. 3

μL of a dual color prestained protein MW marker (Appendix D) and 15 μL of the samples were loaded to gel and were run simultaneously at 40 mA of constant current. The buffers used are given in Appendix A.

Pouring SDS-polyacrylamide Gels:

1. Clean the glasses with ethanol and assemble the glass plates according to the manufacturers' instructions. To check whether the glasses are properly sealed, pour distilled water between glasses. If water level does not decrease, glasses are properly sealed. Then, pour out the water and let the glasses to dry.
2. In an Erlenmeyer flask, prepare appropriate volume of solutions containing the desired concentration of monomer solution for 12% separating gel, using the values given in Appendix A. Mix the solutions in the order shown. Polymerization will begin as soon as the NNN'N'-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) have been added.
3. Swirl the mixture rapidly and immediately pour the solution into the gap between the glass plates. Leave sufficient space for the stacking gel. Add some water to overlay the monomer solution and leave the gel in a vertical position until polymerization is completed.
4. After 30 min, pour off the water and dry the area above the separating gel with filter paper before pouring the stacking gel Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerize 30-45 minutes.

Preparation of Samples and Running the Gel:

1. While stacking gel is polymerizing, prepare samples by diluting at least 1:1 with sample buffer and heated at 95°C for 5 minutes.
2. After polymerization is complete (30 min), mount the gel in electrophoresis apparatus and fill the reservoir with running buffer.

3. Load up 20 µL of each sample into the wells and start running with 30 mA. After the dye front has moved into the separating gel increase the applied current. The usual run time is approximately 45 minutes. This electrophoresis cell is for rapid separation and is not recommended for runs over 60 minutes long.

3.6.2.5 Staining the SDS-PAGE Gels

Staining procedure for the SDS-Polyacrylamide Gel Electrophoresis is given below:

Staining SDS-Polyacrylamide Gels with Coomassie Brilliant Blue:

1. After running is completed, immerse the gel in 5 volumes of staining solution and place on a slowly rotating platform for 4h at room temperature.
2. Remove the stain and save it for future use. Destain the gel by soaking it in the methanol-acetic acid solution without the dye on a slowly rotating platform for 4-8 h, changing the destaining solution 3-4 times. After destining store the gel in dH_2O.

Staining SDS-Polyacrylamide Gels with Silver Salts:

The gels were silver stained using the procedure of Blum et al. (1987) which is given in Table 3.8.

Table 3.8 Procedure for silver staining

	STEP	SOLUTION	TIME OF TREATMENT	COMMENTS
1	Fixing	Fixer	≥ 1 hr	Overnight incubation is all right
2	Washing	50% Ethanol	3 x 20 min	Should be fresh
3	Pre-treatment	Pretreatment Solution	1 min	Should be fresh
4	Rinse	Distilled water	3 x 20 sec	Time should be exact
5	Impregnate	Silver Nitrate Solution	20 min	
6	Rinse	Distilled water	2 x 20 sec	Time should be exact
7	Developing	Developing Solution	~ 5 min	After a few minutes add some distilled water to proceed the reaction slowly. Time should be determined by observation of color development
8	Wash	Distilled water	2 x 2 min	
9	Stop	Stop Solution	≥ 10 min	The gels can be kept in this solution overnight

3.6.3 Methanol, Sorbitol and Organic Acid Concentrations

Methanol, sorbitol and organic acid concentrations were measured with reversed phase HPLC (Waters HPLC, Alliance 2695, Milford, MA) on Capital Optimal ODS-5µm column (Capital HPLC, West Lothian, UK) (Çelik et al., 2009). The method is based on reversed phase HPLC, in which their concentrations were calculated from the chromatogram, based on the chromatogram of the standard solutions. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to the analysis system. 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min, and refractive index detector (Waters-2414) at 30°C were used to determine methanol and sorbitol concentrations. The analysis was performed under the specified conditions given in Table 3.9.

Table 3.9 Conditions for HPLC system for methanol and sorbitol analysis

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
Column temperature	: 30°C
Detector and temperature	: Waters 2414 Refractive Index detector, 30°C
Injection volume	: 5 µL
Analysis period	: 10 min

3.12% (w/v) NaH_2PO_4 and 0.62×10^{-3}% (v/v) H_3PO_4 (İleri and Çalık, 2006) as the mobile phase at a flow rate of 0.8 mL/min, and dual absorbance detector (Waters 2487) were used to determine organic acid concentrations. The analysis was performed under the specified conditions in Table 3.10.

Table 3.10 Conditions for HPLC system for organic acids analysis

Column	: Capital Optimal ODS, 5µm
Column dimensions	: 4.6×250 mm
System	: Reversed phase chromatography
Mobile phase flow rate	: 0.8 mL/min
Column temperature	: 30°C
Detector and wavelength	: Waters 2487 Dual absorbance detector, 254 nm
Injection volume	: 5 µL
Analysis period	: 15 min

The calibration curves used to determine concentrations are give in Appendix B.

3.6.4 Amino Acids Concentrations

Amino acid concentrations were measured with an amino acid analysis system (Waters, HPLC), using the Pico Tag method (Cohen, 1983). The method is based on reversed phase HPLC, using a pre-column derivation technique with a gradient program developed for amino acids. The amino acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard amino acids solution. 6.0% (v/v) acetonitrile as a mobile phase was used. The analysis was performed under the conditions specified in Table 3.11.

Table 3.11 Conditions for HPLC system for amino acids analysis

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

3.6.5 Determination of AOX Activity

3.6.5.1 Yeast Lysis to Get Inracellular Medium

Intracellular medium of the cells harvested from the fermentation medium should be extracted by using yeast lysis buffer (Appendix A) since AOX is an intracellular enzyme. In order to get the intracellular medium of the cells, 500 μL yeast lysis buffer and a spoon of glass beads are added to cells obtained from centrifugation of 1 mL of production medium. These cell are

mixed up three times for 20 sec and after each cycle they are kept on ice for 30 sec. Then, the cells are centrifuged at 3000xg for 2 min at +4^{o}C the supernatant obtained is centrifuged again at 12000xg for 5 min at +4^{o}C. The supernatant obtained from this second centrifugation is used for AOX activity assay.

3.6.5.2 AOX Activity Assay

A bi-enzymatic assay comprising alcohol oxidase (AOX) and horseradish peroxidase (HRP) was used to monitor the oxidation of methanol to formaldehyde by AOX enzyme. A colorimetric system based on the combination of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) was chosen to measure the concentration of H_2O_2 produced by AOX. In this particular system, two moles of H_2O_2 react with one mole of PSA and one mole of 4-AAP, yielding three moles of water, one mole of sodium hydrogenosulfate and one mole of a quinoneimine dye.

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

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

This dye has a characteristic magenta color with maximum absorption around 500 nm. The activity of AOX was determined by monitoring the associated increase in absorbance at 500 nm with UV-Vis spectrophotometer. This increase is proportional to the rate of H_2O_2 production and, consequently, to the rate of methanol consumption. All kinetic studies were performed at 25°C using a standard assay reaction mixture, containing 0.4mM 4-AAP, 25mM PSA, and 2U/mL HRP in 0.1M phosphate buffer with pH 7.5. One unit of activity (U) was defined as the number of μmol of H_2O_2 produced per minute at 25°C (Azevedo et al., 2004). The reaction mixture is prepared as follows; 3 ml standard assay reaction mixture is put into a cuvette and 30 μL HPR, 375 μL methanol and 75 μL sample are added and mixed with that standard assay reaction mixture . Then increase in absorbance at 500 nm is monitored for 3 min and recorded with 30 sec of time intervals. To convert absorbance to AOX

concentration (mg/mL), equation 3.2 was used and the calibration curve is given in Appendix B.

$$C_{AOX}\left[\frac{U}{gCDW}\right] = 18.8\left[\frac{U/mL}{absorbance}\right] * OD_{500} * \frac{1}{C_X} \quad (3.2)$$

3.6.6 Oxygen Uptake Rate and Liquid Phase Mass Transfer Coefficient

In order to determine the liquid phase mass transfer coefficient and oxygen uptake rate in the rhGH production process, a dynamic method was used (Bandyopadhyay and Humprey, 1967) which was explained in Section 2.4.2.

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

3.7 Genetic Engineering Techniques

The expression levels of the *hGH* and *AOX1*genes were investigated in this study. In this context, absolute quantification with real time PCR technique was applied using SYBR Green I assay. Expression levels of the desired genes were calculated by using the standard curves formed. To prepare standard curve, *pPICZαA::hGH* plasmid was used as a template. Orman et al. (2008) was constructed *pPICZαA::hGH* plasmid (Figure 3.2) by cloning hGH cDNA sequence into *pPICZαA* vector under the control of AOX1 promoter, which included a polyhistidine-tag on the amino terminal end.

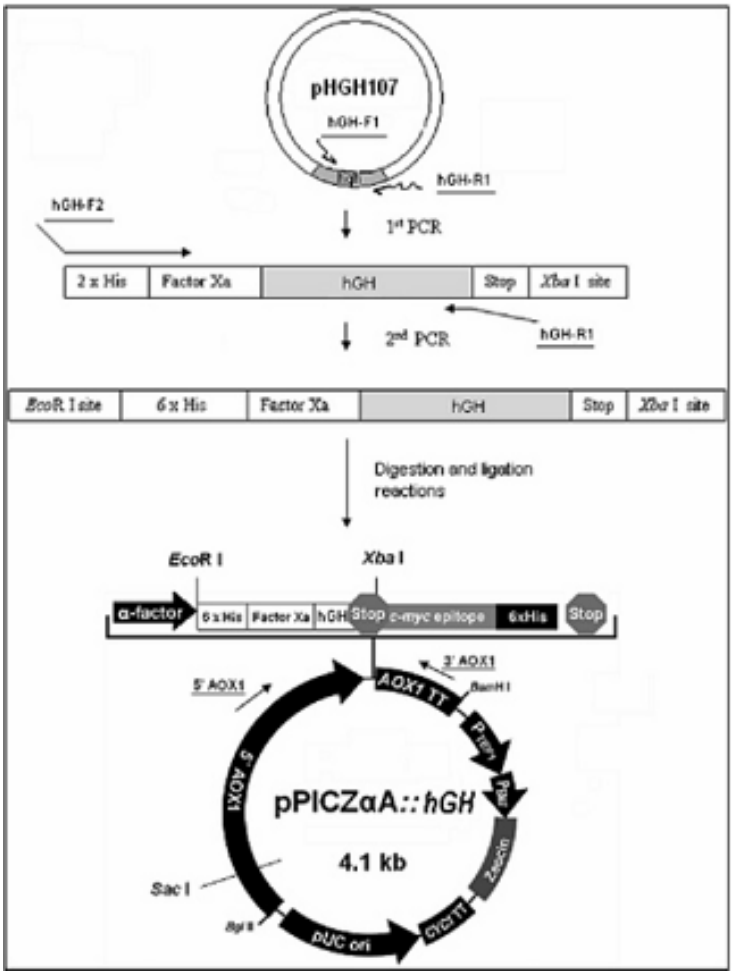

Figure 3.2 Schematic representation of *hGH* amplification, integration of specific recognition sites by two-step PCR, and construction of the *pPICZαA::hGH* plasmid. Shown are the EcoRI and XbaI sites used in ligation of the insert to the vector, the SacI site used to linearize the plasmid before transformation, and the primers used for sequencing. There are 976 nucleotides between the 5′AOX1 primer and hGH-R1 primer, and 793 nucleotides between the hGH-F2 primer and the 3′AOX1 primer (Orman et al., 2008).

3.7.1 Enzymes, Kits and Primers

High Pure RNA Isolation Kit, Transcriptor High Fidelity cDNA Synthesis Kit, LightCycler® Fast Start DNA Master SYBR Green I and lyticase enzyme required for lysis were purchased from Roche. Contents of the all kits are listed in Appendix E.

Primers and probes designed for various genes were purchased from Roche, and Iontek.

3.7.2 Total RNA Isolation from *Pichia pastoris*

High Pure RNA Isolation Kit (Roche GmbH, Mannheim, Germany) was used for total RNA isolation. RNA isolation procedure given by Roche for yeast is as follows:

1. Dilute the harvested cells during mid log or late log phase of growth until reaches the proper cell concentration (Use a dilution which gives OD600=0.1–0.15/mL which corresponds to $2x10^6$ cells).
2. Collect the yeast by centrifugation at 2000xg for 5 min in a standard tabletop centrifuge.
3. Resuspend the pellet in 200 µL of PBS and transfer the suspension to a sterile 1.5 mL microcentrifuge tube.
4. Add 10 µL lyticase solution (0.5 mg/mL) to each microcentrifuge tube. Incubate the tube for 15 min at 30°C.
5. Add 400 µL Lysis/Binding Buffer to the resuspended cells. Mix the contents of the tube well.
6. To transfer the sample to a High Pure Tube, insert one High Pure Filter Tube into one Collection Tube and pipette entire sample into upper buffer reservoir of the Filter Tube (max. 700 µL).
7. Insert the entire High Pure Tube assembly into a standard tabletop microcentrifuge and centrifuge the tube assembly for 15 s at approximately 8000xg.
8. After centrifugation, remove the Filter Tube from the Collection Tube, discard the flowthrough liquid and reinsert the Filter Tube in the same Collection Tube.
9. In a separate, sterile tube, mix 100 µL DNase solution (90 µL DNase Incubation Buffer + 10 µL reconstituted DNase I) for each sample and then add 100 µL DNase solution to the upper reservoir of the Filter Tube.
10. Incubate the Filter Tube for 15 min at 15 to 25°C.

11. After the DNase incubation, add 500 µL Wash Buffer I to the upper reservoir of the Filter Tube and centrifuge 15 s at 8000xg.
12. After centrifugation, remove the Filter Tube from the Collection Tube, discard the flowthrough liquid and reinsert the Filter Tube in the same Collection Tube.
13. Add 500 µL Wash Buffer II to the upper reservoir of the Filter Tube, centrifuge 15 s at 8000xg and then discard flowthrough and combine Filter Tube with the used Collection tube.
14. Add 200 µL Wash Buffer II to the upper reservoir of the Filter Tube and centrifuge the tube assembly for 2 min at maximum speed (approximately 13,000xg) to remove any residual Wash Buffer.
15. Discard the Collection Tube and insert the Filter Tube in a clean, sterile 1.5 mL microcentrifuge tube.
16. To elute the RNA, add 50 – 100 µL Elution Buffer to the Filter Tube and centrifuge the tube assembly for 1 min at 8000xg.
17. The microcentrifuge tube now contains the eluted total RNA, which may be used directly in a variety of procedures or stored at -80^{o}C for later analysis.

3.7.3 cDNA Synthesis from Total RNA

The total RNAs obtained from isolation were used as templates for cDNA synthesis. After RNA isolation, RNA concentration and the ratio of the RNA to protein concentration were measured by AlphaSpect µL Spectrophotometer (AlphaInnotech Inc., USA). The ratio of the RNA to protein concentration is given by A260/A280. A260 and A280 are the optical spectrometer measurement of absorbance at the wavelengths of 260 nm and 280 nm respectively. A260 is frequently used to measure RNA concentration and A280 is used to measure protein concentration. To minimize protein contamination, ratio of A260/A280 should be between 1.9 and 2.1. After ensuring that A260/A280 is in the desired range for all samples, the samples were diluted at different ratios to have the same amount of RNA in each sample, which is 50 ng for both of the selected genes.

Transcriptor High Fidelity cDNA Synthesis Kit (Roche GmbH, Mannheim, Germany) was used for cDNA synthesis. The overview of cDNA synthesis is given Figure 3.3 and the protocol given by Roche is as follows:

1. Thaw all frozen reagents, briefly centrifuge them before starting the procedure and keep all reagents on ice while setting up the reactions.
2. In a sterile, nuclease-free, thin-walled PCR tube on ice, prepare the template-primer mixture for one 20 µL reaction by adding the components in the order listed in Table 3.12.
3. Denature the template-primer mixture by heating the tube for 10 min at 65°C in a thermal block cycler with a heated lid (to minimize evaporation). This step ensures denaturation of RNA secondary structures. Then immediately cool the tube on ice.
4. To the tube containing the template-primer mix, add the remaining components of the RT mix in the order listed in Table 3.13.
5. Mix the reagents in the tube carefully and place the tube in a thermal block cycler with a heated lid (to minimize evaporation).
6. Incubate the reaction for 10 to 30 min at 45°C to 55°C. Depending on the RNA target chosen, optimal reaction temperature and time may vary. Transcriptor High Fidelity Reverse Transcriptase can be used for temperatures between 45°C and 55°C, also for GC-rich targets. The recommended incubation time is 30 min. For many reactions however 10 min are sufficient.
7. Inactivate Transcriptor High Fidelity Reverse Transcriptase by heating to 85°C for 5 min and stop the reaction by placing the tube on ice.
8. At this point the reaction tube may be stored at +2 to +8°C for 1-2 h or at -15 to -25°C for longer periods for further analysis.

Table 3.12 Template-primer mixture for one 20 µL reaction

Component	**Volume , µL**	**Final Concentration**
Total RNA	variable	50 ng
Anchored-oligo(dT) Primer	1	2.5 µM
Water, PCR grade	variable	to take total volume=11.4
Total Volume	11.4	

Table 3.13 Remaining components of the reaction mixture for 20 µl reaction

Component	**Volume µL**	**Final Concentration**
Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 5× conc	4	1x(8mM $MgCl_2$)
Protector RNase Inhibitor, 40 U/µL	0.5	20 U
Deoxynucleotide Mix, 10 mM	2	1 mM
DTT (100 mM)	1	5 mM
Transcriptor High Fidelity Reverse Transcriptase	1.1	10 U
Total Volume	20	

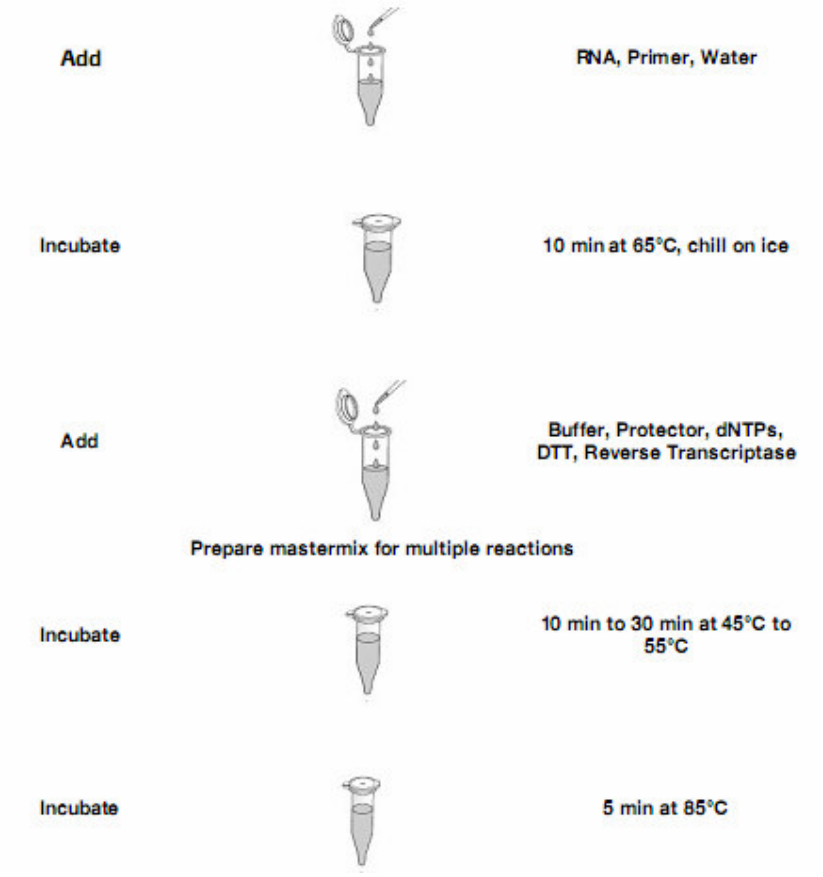

Figure 3.3 Overview of cDNA synthesis procedure (www.roche-applied-science.com)

3.7.4 Determination of Gene Expression Level with Real Time-PCR

In this study, absolute quantification of expression levels of two different genes was performed by real-time PCR (Roche Lightcycler® 1.5, Mannheim, Germany). *AOX1* and *hGH* were selected genes for quantification.

3.7.4.1 Primer Design

Primers were designed to amplify the cDNA of *AOX1* and *hGH* of *pPICZαA::hGH* and *Pichia pastoris*. The sequences of these genes are given in Appendix F. For efficient amplification in real-time RT-PCR, it is important to

design primer pairs such that the size of the amplicon is smaller than 150 base pairs. Primer 3 programme (Rozen and Skaletsky, 2000) was used by considering this fact. Detailed results of the programme are listed in Appendix G and the sequences of designed primer pairs for both genes are given in Table 3.14.

Table 3.14 Primer pairs used in this study and their sequences

Gene		Sequence	Melting Temperature (^oC)	% GC Content
hGH	Left Primer	gcctttgacacctaccagga	60.11	55.00
	Right Primer	acactaggctgttggcgaag	60.45	55.00
AOX1	Left Primer	gtcggcataccgtttgtctt	60.00	50.00
	Right Primer	ttcccaccagaatcttggaa	60.43	45.00

3.7.4.2 Plasmid Isolation

Plasmid DNA containing *AOX1* and *hGH* genes is isolated from *Escherichia coli.* The procedure for isolation is as follows:

1. Pick a single colony from a selective plate and inoculate a starter culture of 30 mL LB medium. Grow for 12h (overnight) at 37^{o}C, with vigorous shaking (~200 rpm)
2. Pour 1mL of culture into microfuge tube and centrifuge at 13200 min^{-1}, 4^{o}C, for 30 s,
3. Remove the supernatant and add again 1mL of culture and repeat the centrifugation step,
4. Remove the supernatant and take off all fluid by micropipette; place the tube on ice,

5. Resuspend the bacterial pellet in 100 µL of ice-cold alkaline lysis solution I one by vigorous vortexing. Make sure that the bacterial pellet is completely dispersed in alkaline lysis solution I,
6. Add 200 µL of freshly prepared alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the content by inverting the tube gently 5 times and store at room temperature for 5 minutes,
7. Add 150 µL of ice-cold alkaline lysis solution III. Close the tube and disperse alkaline lysis solution III through the viscous bacterial lysate by inverting the tube 5 times. Store the tube on ice for 10 minutes,
8. Centrifuge the bacterial lysate at 13200 min^{-1}, 4°C, for 10 minutes. Transfer the supernatant to a fresh tube.
9. Add 1/10 volumes of NaAc and 2 volumes of EtOH. Mix the solution by inverting and then allow the mixture to stand for at least 10 minutes at -20°C,
10. Collect the precipitated plasmid DNA by centrifugation at 13200 min^{-1}, 4°C, for 10 minutes.
11. Remove the supernatant gently and stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.
12. Dissolve the plasmid DNA in suitable amount of dH_2O and store the solution at -20°C (Sambrook, 2001).

3.7.4.3 Standard Curve Preparation

In order to prepare standard curves, *pPICZαA::hGH* plasmid was used as a standard for *hGH* and *AOX1*. Nucleic acid concentration of standards was determined by AlphaSpect µL Spectrophotometer (AlphaInnotech Inc., USA) at 260 nm. Purity of the nucleic acid was determined by using ratio of A_{260}/A_{280} which must be around 1.9 for DNA. After that, copy number of each standard was calculated by using equations 3.3 and 3.4.

$$Copy\ number/\mu L = \frac{initial\ concentration\ of\ isolated\ plasmid}{M_{plasmid}/\#\ of\ copies\ of\ desired\ gene\ in\ plasmid} \qquad (3.3)$$

where $M_{plasmid}$ is the mass of plasmid and it was calculated using the following equation.

$$M_{plasmid} = n\left[1.096 * 10^{-21} \frac{g}{base\ pair}\right] \quad (3.4)$$

where n is the number of base pairs in the plasmid.

For each PCR amplification standards for *hGH* and *AOX1* were made daily from the plasmid working stock ($8.5x10^{10}$ copies/µL). Dilutions of these standards from $1x10^{8}$ copies/µL down to $1x10^{4}$ copies/µL were then used to generate the standard curve. Standard curves created for quantification of the selected genes are given in Results and Discussion Section 4.5.

3.7.4.4 Quantification of Expression Level of the Desired Genes

In order to perform absolute quantification for determination of expression levels of the genes, cDNA of each sample obtained from total RNA isolation was used as a templates for PCR reaction. Roche LightCycler® 1.5 real time-PCR was used to achieve amplification and quantification.

SYBR Green I assay was used for both genes, *hGH* and *AOX1*, for absolute quantification. In order to analyze and quantitate the data obtained from this assay, LightCyler® Software 4.0 was used. This programme provides easy set up of required cycling parameters and enhanced data management.

The protocol given by Roche for preparing reaction mixture for this particular assay is as follows:

<u>Preparation of the Master Mixture:</u>

1. Thaw one vial of "Reaction Mix" (vial 1b). A reversible precipitate may form in vial 1b during storage. If a precipitate visible, place the reaction mix at 37°C and mix gently from time to time until the precipitate is completely dissolved. Recentrifuge to collect the reagent at the bottom of the tube, then put the vial back on ice. This treatment does not influence the performance in PCR.

2. Briefly centrifuge one vial “Enzyme” (vial 1a) and the thawed vial of “Reaction Mix” (from Step 1).
3. Pipette 10 µL from vial 1a into vial 1b and mix gently by pipetting up and down (do not vortex). Then re-label vial 1b with the new labels (vial 1) that are provided with the kit.

Preparation of the PCR Mixture:

1. Depending on the total number of reactions, place the required number of capillaries in precooled centrifuge adapters or in a sample carousel in a precooled carousel centrifuge bucket.
2. Prepare a 10x concentration solution of PCR primers. Concentration of each primer should be 0.3 - 1 µM.
3. In a 1.5 mL reaction tube on ice, prepare the PCR mixture for one 20 µL reaction by adding the following components in the order mentioned in Table 3.15.
4. Mix carefully by pipetting up and down (do not vortex) and pipette 18 µL PCR mixture into each precooled capillary. Then add 2 µL of the DNA template and then seal each capillary with a stopper.
5. Centrifuge capillaries at 700xg for 5 s.
6. Place the capillaries in the real time RT-PCR and program insert the PCR parameters to the program. A standard real time RT -PCR program is given in Table 16.

Table 3.15 Components of the reaction mixture for real time RT-PCR for 20 µL reaction mixture for LightCycler® FastStart DNA Master SYBR Green I kit.

Component	Volume µL	Final Concentration
H_2O PCR grade	x	
$MgCl_2$ stock solution (25 mM)	y	Used optimized conc. (1–5 mM)
PCR primer, 10xconc. (4-10 µM)	2	0.2–0.5 µM
LightCycler® FastStart DNA Master SYBR Green I, 10×conc.	2	
DNA Template	2	
Total Volume	20	

Table 3.16 Real Time RT-PCR program for SYBR Green method for LightCycler® 1.5 System.

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95°C	10 min	none
Amplification					
Quantification	45	Denaturation	95°C	10 s	none
		Annealing	primer dependent	0-10 s	none
		Extension	72°C	bp/25 s	single
Melting Curve					
Melting Curves	1	Denaturation	95°C	0 s	none
		Annealing	65°C	15 s	none
		Melting	95°C slope=0.1°C/s	0 s	continuous
Cooling					
None	1		40°C	30 s	none

Amplification temperature of every primer pairs is different since number of base pairs, and A and C contents of the primers are different. Hence, amplification of each gene was achieved at different temperatures. $MgCl_2$ concentration was also optimized for the primer pairs to achieve specific and efficient amplification. Amplification temperature and $MgCl_2$ concentration used for quantification of each gene during real time RT-PCR reaction are given in Table 3.17.

Table 3.17 Amplification temperature and $MgCl_2$ concentration for each gene during real time RT-PCR reaction

Gene	Amplification Temperature, ^{o}C	$MgCl_2$ Concentration, mM
AOX1	60	2
hGH	55	4

CHAPTER 4

RESULTS AND DISCUSSION

In this study, different carbon sources and feeding strategies were investigated for recombinant human growth hormone production by *Pichia pastoris*. In the first part, the strain to be used for high level human growth hormone production was selected between the two strains having different methanol utilization phenotypes (*P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S}). Thereafter, using the chosen strain, effects of a number of different carbon sources on recombinant protein production were investigated. Among these carbon sources, the ones which support cell growth and do not repress the recombinant protein production were determined. In the second part of the study, using the selected carbon sources and optimized defined medium concentrations, effects of feeding strategies on cell growth, oxygen transfer and fermentation characteristics, rhGH production and by-product formation together with expression levels of *hGH* and *AOX1* were investigated by using pilot scale bioreactor.

4.1 Comparison of Mut^{+} and Mut^{S} Phenotypes of *Pichia pastoris*

During the investigation of alternative carbon sources which do not repress the AOX promoter while supporting the cell growth, a comparison and choice should be made between the two available methanol utilization phenotypes, namely *P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S}. These phenotypes are the result of integration of *pPICZαA::hGH* vector into *P. pastoris* and they were differentiated from each other by Orman et al. (2009) with dot-blot analysis among a number of transformants. In this study, comparison was done by using two different carbon sources. Glycerol is the one which has

repressing effect on *AOX*, and sorbitol is the second one which shows non-repressing effect on *AOX*.

4.1.1 Microorganisms Grown on Glycerol

Glycerol is the most commonly used carbon source for both strains although it is a repressor of AOX1 promoter. The optimized conditions for Mut^+ strain are 30 g/L glycerol and 3% (v/v) methanol and 30g/L glycerol and 1% (v/v) methanol for *P. pastoris hGH*-Mut^S strain (Orman et al., 2007). It was shown that in the case of glycerol/methanol mixed feeds in a defined medium with optimized conditions for both methanol utilization phenotypes; production of rhGH (C_{rp}=110 mg/L) is almost two-fold higher in *P. pastoris hGH*-Mut^S. SDS-PAGE image of these results can be seen in Figure 4.1.

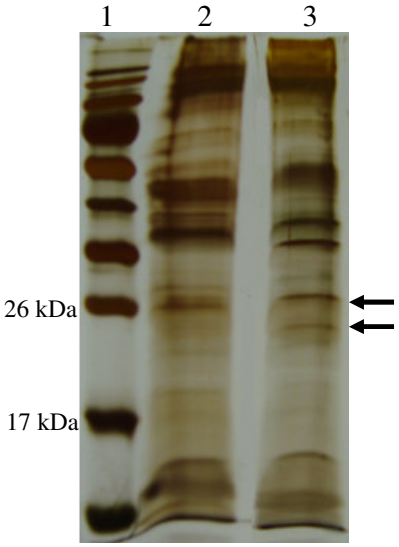

Figure 4.1 Silver stained SDS-PAGE gel view of extracellular proteins produced in laboratory scale air filtered shake bioreactors to observe the difference in rhGH production between *P. pastoris hGH*-Mut^+ and *P. pastoris hGH*-Mut^S phenotypes grown on optimized conditions in medium containing glycerol and methanol in a defined medium at t = 47 h. 1. well: protein marker, 2. well: Mut^+ phenotype, 3. well: Mut^S phenotype.

Amount of rhGH found in extracellular medium is directly related to the expression level of *hGH* gene which can be investigated in the transcriptional level. For this purpose, real time RT-PCR was used with absolute quantification method. Absolute quantification with real time PCR requires calibration curve before the analysis of samples. Construction of calibration curves is given in Appendix I.

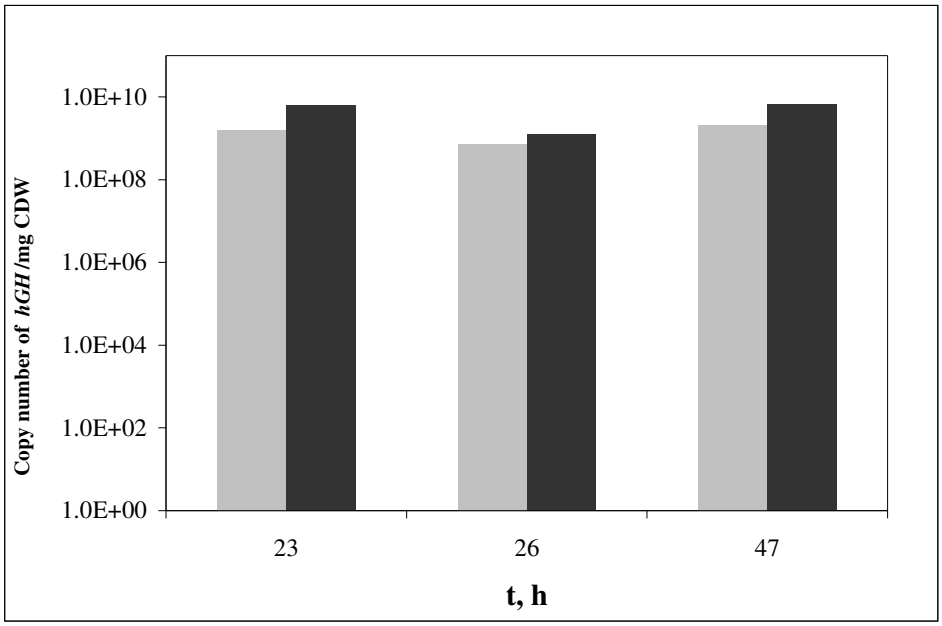

Figure 4.2 Variation in expression levels of *hGH* gene with cultivation time in *P. pastoris hGH*-Mut$^+$ and *P. pastoris hGH*-Mut^S phenotypes grown on optimized conditions in medium containing glycerol and methanol in a defined medium; Mut$^+$ (■), Mut^S (■).

Expression level of *hGH* gene was determined using cDNA synthesized from total RNA isolated from the cells taken at certain hours of the induction period. The time of sampling for real time PCR analysis was chosen according to the methanol addition schemes. In shaking bioreactor experiments microorganisms were induced two times; first at the beginning of the production phase (t=0 h) then at t=24 h where the methanol added at the beginning of the

production phase was assumed to be totally consumed. Thus, to determine the effect of inductions on *hGH* and *AOX* gene expression levels clearly the points before and after the second induction, and the last hour of the process was selected (t = 23, 26, and 47 h) for real time PCR analysis. Real time PCR results for the expression level of *hGH* gene (Figure 4.2) were in good agreement with the SDS-PAGE results. Expression level of *hGH* gene in Mut^S phenotype is higher than Mut^+ at every selected period of the induction phase. At the end of production, expression level in Mut^S phenotype ($6.76x10^9$ copies/mg CDW or $5.34x10^5$ copies/ng total RNA) reaches to a value 3.25-fold higher then Mut^+ ($2.08x10^9$ copies/mg CDW) grown on glycerol/methanol.

4.1.2 Microorganisms Grown on Sorbitol

As an alternative to glycerol, sorbitol is used as a carbon source for the comparison of two phenotypes of *Pichia pastoris* since it is reported that sorbitol is a non-AOX-repressing carbon source (Sreekrishna et al., 1997). Since non-repressing carbon sources are being investigating, it is better to make the comparison and selection between the strains using a non-AOX-repressing carbon source. Optimum carbon source concentration for the co-substrate glycerol was determined as 30 g/L (Orman et al., 2009). Therefore sorbitol is used such that production medium contained equivalent amount of carbon molecules to the optimized glycerol concentration, which makes 30 g/L. Related with the methanol concentration, Çelik et al., (2009) used 1% (v/v) methanol in medium containing sorbitol for Mut^+; whereas Orman et al. (2009) showed that rhGH production was the highest in the medium containing glycerol and 3% (v/v) methanol for Mut^+ and 1% (v/v) methanol for Mut^S. Therefore, effect of methanol concentration on cell growth of Mut^+ in medium containing co-substrate sorbitol was investigated and it was found that above 1% (v/v), methanol exhibits inhibitory effect on cell growth of Mut^+ (Figure 4.3). Thus, 1% (v/v) methanol concentration was used for both strains for selection on medium containing sorbitol.

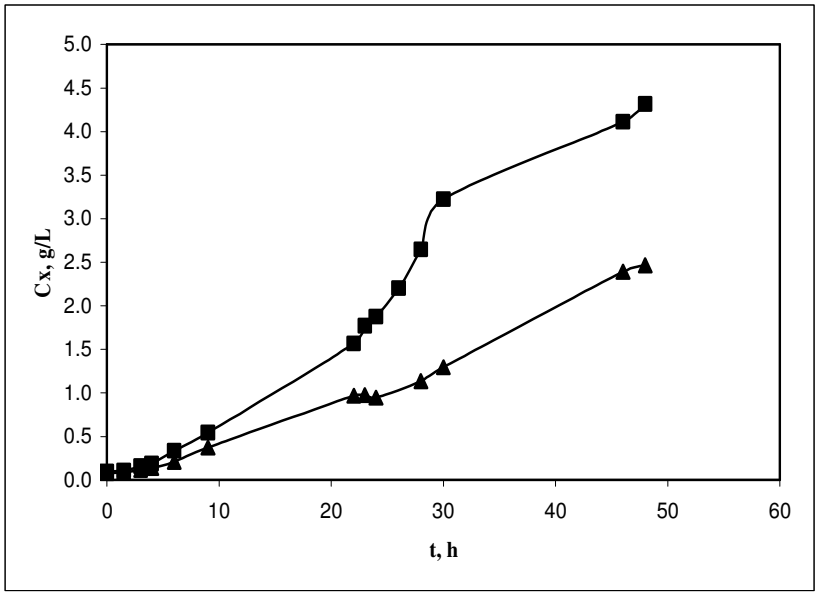

Figure 4.3 Variation in *P. pastoris hGH*-Mut$^+$ cell concentration with time in defined medium containing 30 g/L sorbitol and initial methanol concentrations of 1% (■), 3% (▲) (v/v).

In the production medium containing initially 30 g/L sorbitol together with 1% (v/v) methanol, rhGH production in *P. pastoris hGH*-Mut$^+$ phenotype is 120 mg/L, which is 1.9-fold higher than that of Mut^S phenotype in the same medium (Figure 4.4). The results obtained from real time PCR for these conditions are consistent with SDS-PAGE results (Figure 4.4 and 4.5). In medium containing sorbitol, it was seen that expression levels in both strains were close to each other until t = 47 h. After the second induction with methanol at t = 24 h, a significant increase was observed in *hGH* expression in Mut$^+$ ($9.84x10^9$ copies/mg CDW or $1.22x10^6$ copies/ng total RNA), 5.82 fold higher than Mut^S, which resulted in an increase in amount of final product.

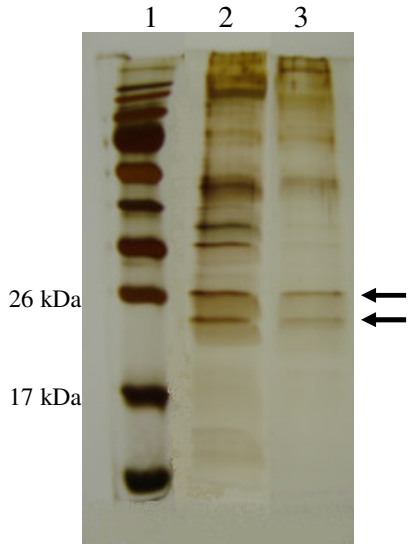

Figure 4.4 Silver stained SDS-PAGE gel view of extracellular proteins produced in laboratory scale air filtered shake bioreactors to observe the difference in rhGH production between *P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S} phenotypes grown on sorbitol at t=47 h. 1. well: protein marker, 2. well: Mut^{+} phenotype, 3. well: Mut^{S} phenotype.

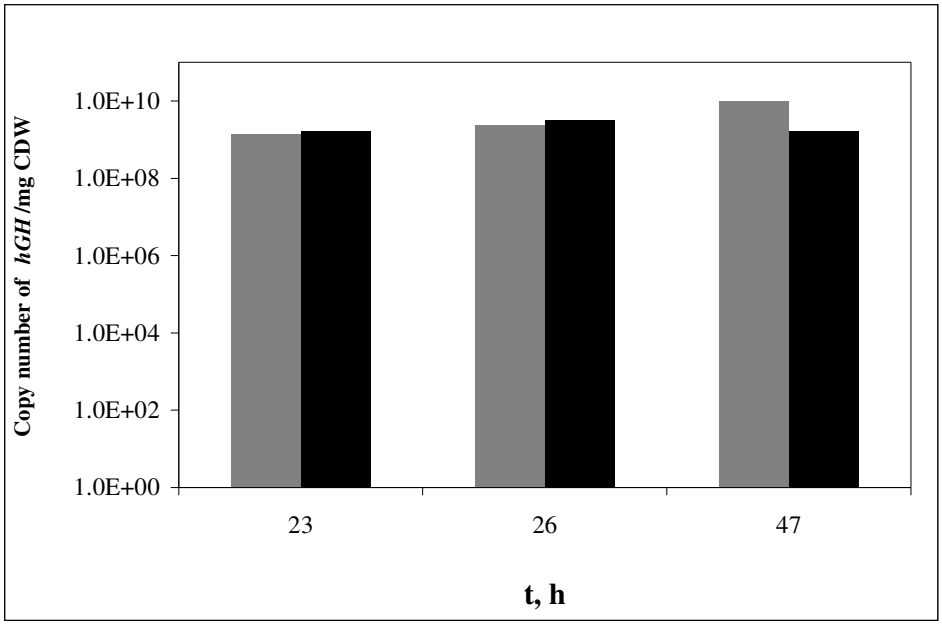

Figure 4.5 Variation in expression levels of *hGH* gene with cultivation time in *P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S} phenotypes grown on medium containing sorbitol and methanol in a defined medium; Mut^{+} (■), Mut^{S} (■).

4.1.3 Selection of the Microorganism

It is known that Mut^S produces almost two-fold higher rhGH than Mut^+ phenotype on glycerol from both results obtained in this study and the previous study (Orman et al., 2009). However, when sorbitol was employed as the co-carbon source, 1.9-fold higher recombinant protein is obtained by *P. pastoris hGH*-Mut^+ phenotype than that of Mut^S. Since Mut^+ phenotype relies mostly on AOX1 promoter and since this promoter is tightly regulated in the presence of repressing carbon sources this was an expected result. However, in order to see this difference more clearly, both strains and medium were compared in same SDS-PAGE gel and the highest amount of rhGH was obtained by using Mut^+ phenotype of *P. pastoris* in medium containing 30 g L^{-1} sorbitol and 1% (v/v) methanol (Figure 4.6). Besides the amount of the final product, these strains were compared considering the expression levels of *hGH* gene at t= 23, 26 and 47 h (Figure 4.7 and 4.8). These instants of fermentation were selected to determine the variation of expression levels of related genes after the second induction applied at t = 24h. This comparison was not made before the second induction since until t = 9 h cell growth is slow and up to this point no considerable *hGH* or *AOX* expression were expected.

Comparing the expression levels, it was seen that generally for both phenotypes and carbon sources expression levels of *hGH* show increasing behavior from t =23 h to 47 h. Then, the production levels at t = 47 h is compared with the SDS-PAGE results. As expected, Mut^S grown on glycerol/methanol achieved higher expression level and protein production than that of Mut^+ grown on glycerol/methanol; in parallel to this, on glycerol/methanol, expression level of *hGH* in Mut^S is 3.25-fold higher than Mut^+. This difference in expression levels was reflected to production such that 2-fold higher r-protein was obtained in the extracellular medium of Mut^S. However, when sorbitol is used in the medium as a co-substrate, although the amount of methanol was lower than the amount used with glycerol for Mut^+, this strain reached higher expression levels ($9.84x10^9$ copies/mg CDW) and protein production (C_{rp}=120 mg/L). On sorbitol, Mut^+ achieves 5.82-fold higher hGH

expression and 1.9-fold higher extracellular recombinant protein concentration than Mut^S in same conditions.

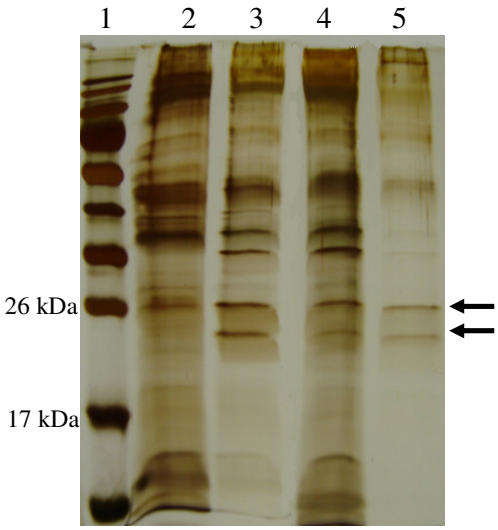

Figure 4.6 Silver stained SDS-PAGE gel view of extracellular proteins produced in laboratory scale shake bioreactors to observe the difference in rhGH production between *P. pastoris hGH*-Mut^+ and *P. pastoris hGH*-Mut^S phenotypes at t = 47 h. 1. well: protein marker, 2. well: Mut^+ phenotype grown on glycerol and 3% methanol, 3. well: Mut^+ phenotype grown on sorbitol and 1% methanol, 4. well: Mut^S phenotype grown on glycerol and 1% methanol, 5. well: Mut^S phenotype grown on sorbitol and 1% methanol.

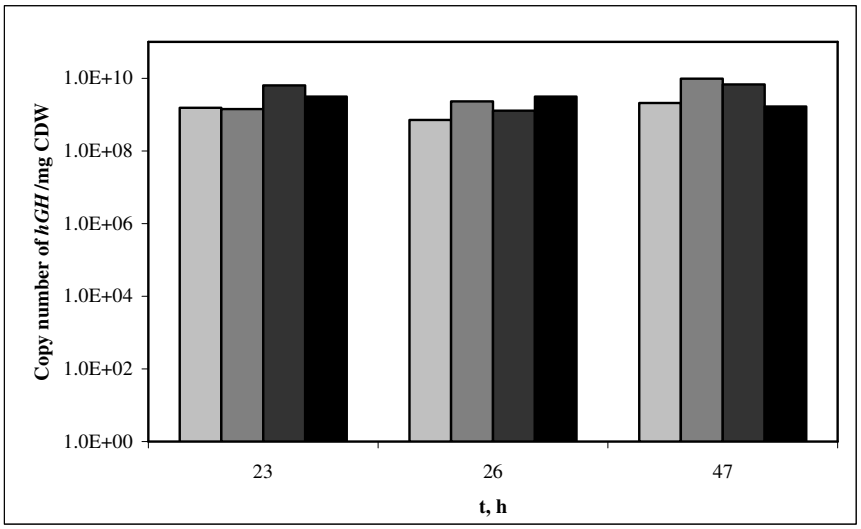

Figure 4.7 Variation in expression levels of *hGH* gene in *P. pastoris hGH*-Mut$^+$ and *P. pastoris hGH*-Mut^S phenotypes grown on medium containing glycerol or sorbitol in a defined medium at t =23, 26 and 47 h.; Mut$^+$ grown on glycerol (■), Mut$^+$ grown on sorbitol (■), Mut^S grown on glycerol (■), Mut^S grown on sorbitol (■).

Considering both *hGH* expression levels and extracellular rhGH productions of the two strains, it was decided that Mut$^+$ strain grown on sorbitol resulted in higher expression and production levels. Therefore *AOX* expression levels were investigated only for Mut$^+$ strain (Figure 4.8). Naturally in medium containing 30 g/L glycerol and 3% (v/v) methanol, higher *AOX* expression level was attained ($1.24x10^{10}$ copies/mg CDW or $4.34x10^5$ copies/ng total RNA) than Mut$^+$ grown on sorbitol ($9.84x10^9$ copies/mg CDW). This is probably due to the increasing demand of alcohol oxidase enzyme in a medium due to high methanol content. However, this increase was not reflected to hGH production and both *hGH* expression level and recombinant protein production remain at lower values than Mut$^+$ strain grown on sorbitol.

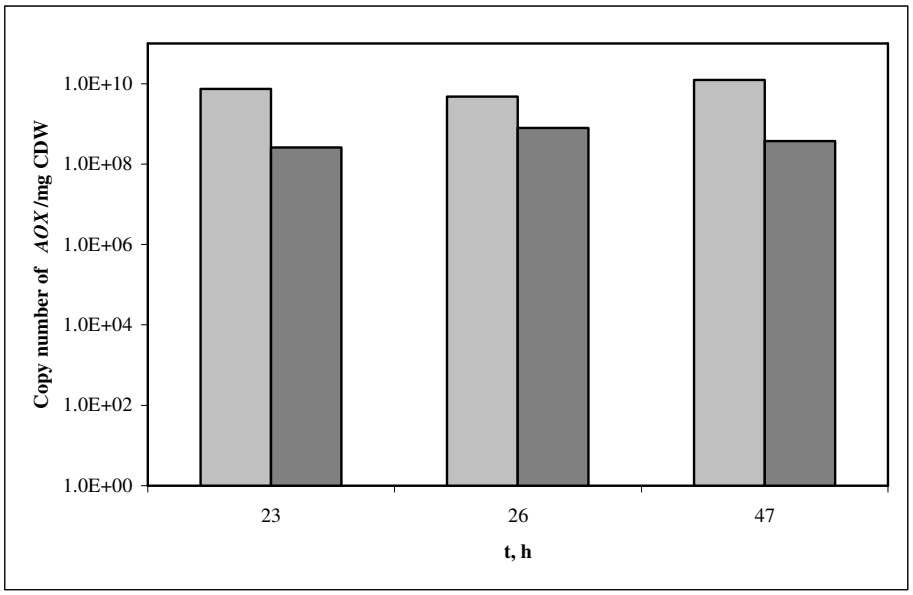

Figure 4.8 Variation in expression levels of *AOX* gene in *P. pastoris hGH*-Mut$^+$ grown on medium containing glycerol or sorbitol in a defined medium at t =23, 26 and 47 h; Mut$^+$ grown on glycerol (■), Mut$^+$ grown on sorbitol (■).

Considering rhGH concentrations together with real time PCR analysis, it is suitable to perform rhGH production with Mut$^+$ phenotype in the presence of sorbitol in the medium. As a result, further investigations were performed by *P. pastoris hGH*-Mut$^+$ and sorbitol was taken as basis in evaluating the alternative carbon sources.

4.2 Effects of Sorbitol Concentration

Selection of the phenotype was made according to the predetermined concentrations of the carbon sources however it is required to inquire if the optimum concentration of sorbitol is different from that of glycerol. Since the utilization pathways and effects on the AOX promoter of these two carbon sources are different; their optimum concentrations might also be different. Sorbitol does not repress the AOX promoter so that the residual sorbitol concentration does not affect the production level of recombinant protein production (Çelik, 2008). With the addition of a second utilizable carbon source,

the amount of cells generated is expected to increase until an inhibitory level of this new substrate is reached. In order to search this non-inhibitory concentration for *P. pastoris hGH* strain carrying *pPICZαA::hGH* plasmid; initial sorbitol concentrations of C_S = 20, 30, 40, 50, 60, 70 g/L were investigated with an initial methanol concentration of 1% (v/v). Ammonium sulfate was used as the nitrogen source in these media with concentrations of 17.0, 22.6, 26.5, 31.3, 36.0 and 40.8 g/L, respectively. Figure 4.9 and 4.10 show the methanol and sorbitol consumption profiles and Figure 4.11 shows the cell growth profiles obtained at different initial sorbitol concentrations, respectively.

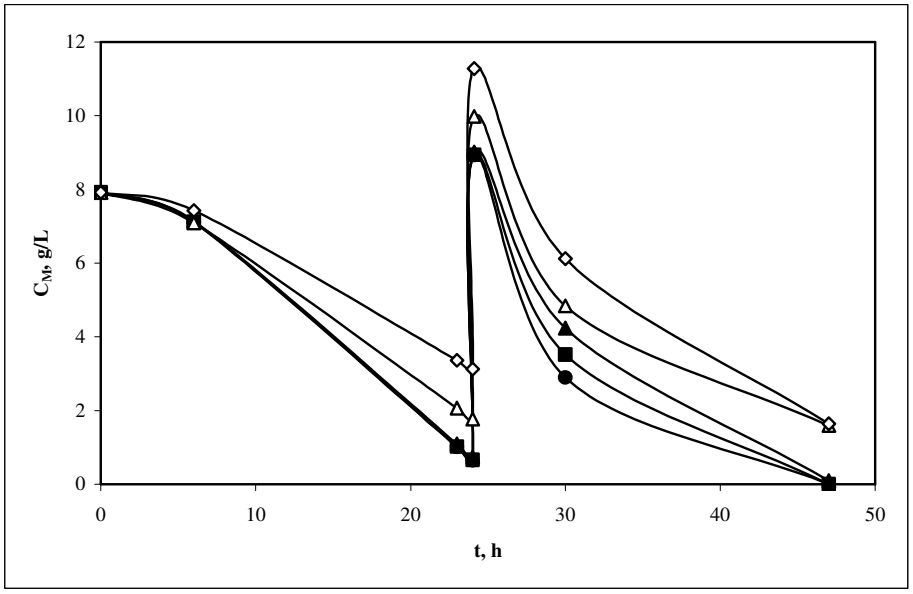

Figure 4.9 Methanol utilization diagrams of *P. pastoris hGH*-Mut$^+$ in defined medium containing 20 (●), 30 (■), 40 (▲), 50 (Δ), 60 (◊) g/L sorbitol with 1% (v/v) initial sorbitol concentrations.

Methanol utilization rate of *P. pastoris hGH*-Mut$^+$ changed with the initial amount of sorbitol in the medium. Below 50 g/L initial sorbitol concentration, methanol utilization rates do not change significantly, however at 50 g/L sorbitol and above a decrease in methanol utilization was observed.

Although presence of sorbitol does not repress AOX, utilization of additional sorbitol may put and additional stress on cells, affecting the methanol utilization pathway. Hence, yeast cells prefer utilizing sorbitol with glycolysis rather than utilizing the toxic methanol with peroxisomal reactions.

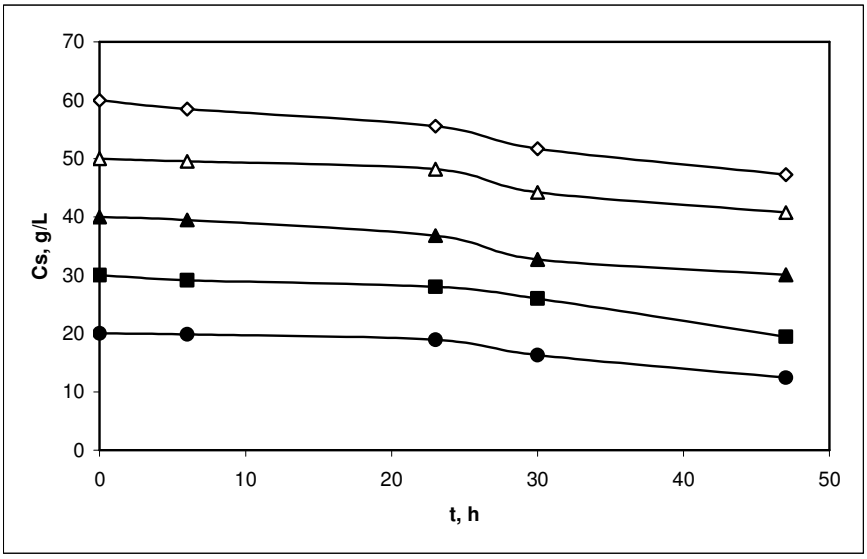

Figure 4.10 Sorbitol utilization diagrams of *P. pastoris-hGH-Mut*$^+$ strain in defined medium with initial sorbitol concentrations of 20 g/L (●), 30 g/L (■), 40 g/L (▲), 50 g/L (Δ), 60 g/L (◊)

The initial sorbitol concentration was optimized for laboratory scale shake bioreactors as C_{S0}= 7.5 g/L by Çelik et al. (2009) for *P. pastoris* Mut$^+$ strain producing human erythropoietin (EPO). This amount is essentially close to amount consumed in shake bioreactors regardless of the initial concentrations. However, this concentration would be insufficient to be used in high-cell density fed-batch pilot scale bioreactor operations since sorbitol will be added to the fermentation medium batch-wise during the production phase. The non-inhibitory sorbitol concentration was 50 g/L for *P. pastoris* strain producing human erythropoietin (EPO) (Çelik et al., 2009)

Cell concentration profiles show that cell growth is inhibited above 50 g/L initial sorbitol concentration. Below 50 g/L there is no significant change in cell concentration profiles probably because of the amount of sorbitol consumed does not change with the initial amount of sorbitol in laboratory scale shake bioreactors (Figure 4.11).

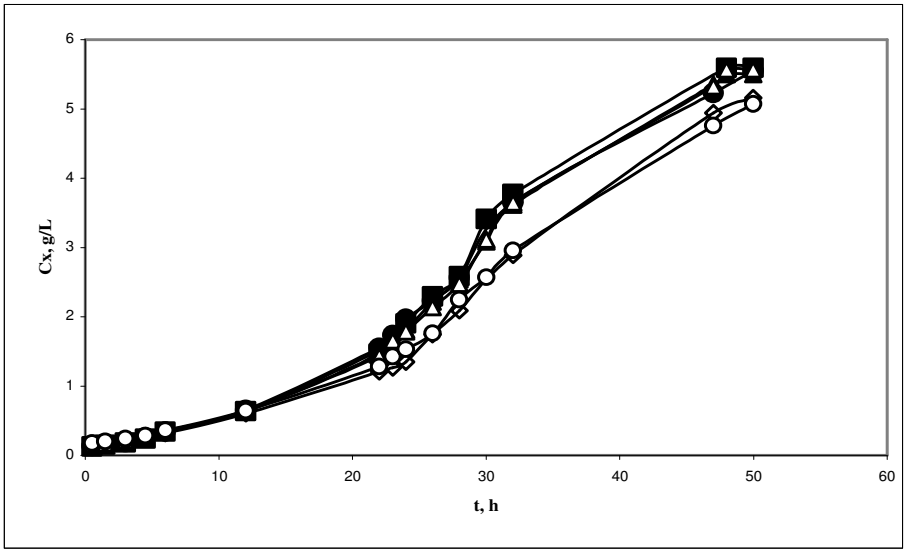

Figure 4.11 Variation in cell concentration with time in defined medium containing 20 (●), 30 (■), 40 (▲), 50 (Δ), 60 (◊), 70 (o) g/L sorbitol with 1% (v/v) methanol.

From the aspect of recombinant protein produced, 50 g/L sorbitol concentration is also suitable. SDS-PAGE results shown in Figure 4.12 represent that amount of rhGH increases significantly as the initial sorbitol concentration increases until 50 g/L. Above this non-inhibitory limit, recombinant protein production is almost constant at 130 mg/L.

Thus, the non-inhibtory sorbitol concentration was found as 50 g/L which is the same found for by Çelik et al., (2009) for the Mut^+ phenotype of *P. pastoris* producing human erythropoietin (EPO)

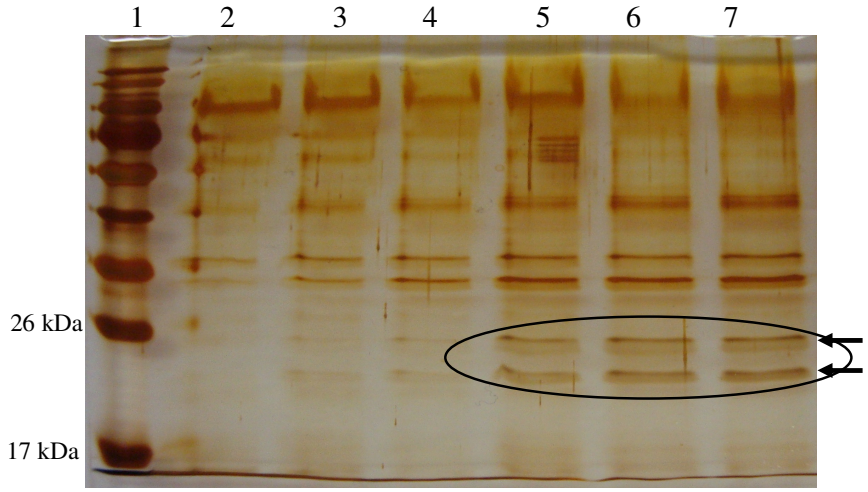

Figure 4.12 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor to observe the difference in rhGH production between different initial sorbitol concentrations at t=47 h. 1. well: protein marker, 2. well: 20 g/L, 3. well: 30 g/L, 4. well: 40 g/L, 5. well: 50 g/L, 6. well: 60 g/L, 7. well: 70 g/L.

4.3 Effects of Different Carbon Sources

In this study, one of the main objectives is to investigate non-repressing carbon sources that can be used with methanol in the production phase of the process. Glycerol is one of the most popular carbon sources used as mixed substrates with methanol. However, in some studies, it is claimed that the optimal level of protein expression is not achievable with mixtures of glycerol and methanol, due to a partial repression of the AOX1 promoter by glycerol. In order to avoid repression of the AOX1 promoter usage of non-AOX-repressing carbon sources is essential. Sorbitol is the mostly employed carbon source of that kind and it is the strongest candidate to be used in recombinant protein production. Then, mannitol, alanine, trehalose and lactic acid were suggested (Inan et al., 2001; Xie et al., 2005). For the production of rhGH by *P. pastoris* some of these reported non-repressing carbon sources were tried as well as some newly suggested ones. Sorbitol, mannitol, and lactic acid were tried among the known alternatives. Fructose, lactose, sucrose and citric acid were the new

alternatives which have no results reported in literature. Glucose was also used in the production medium once in order to be able to make a comparison with fructose, although it is known to be an AOX repressing carbon source. Acetic acid was also tried once although it was also considered to be repressing (Xie et al., 2005).

Experiments with different carbon sources in the production medium were performed in laboratory scale air filtered shake bioreactors. Concentrations of the selected carbon sources in the production medium were determined according to the previous optimizations performed for both glycerol and sorbitol. Concentrations were adjusted such that equivalent carbon atoms to the optimized concentrations were present in growth medium for each carbon source tried.

First, glucose and fructose were used in the production medium. Concentrations were adjusted by taking the optimized glycerol case (Orman et al., 2008) as basis since glucose is also an AOX repressing carbon source. This concentration corresponds to 30 g/L for both glucose and fructose since they have the same molecular formula. In order to make a more reliable comparison, medium containing sorbitol with equivalent carbon atoms (which also corresponds to 30 g/L) was also considered. Variation in cell concentration in each of these media can be seen in Figure 4.13.

Similar growth curves were obtained for media containing glucose and fructose. On the other hand, although cells grown on sorbitol, reached the same cell concentration at the end, they have grown slower. It was known that glucose supports cell growth very well even if it represses AOX promoter. The growth curves show that fructose also supported the cell growth very well. However, we are mainly interested in the recombinant protein production in the presence of co-substrates. SDS-PAGE analysis (Figure 4.14) showed that no rhGH production occurred in the presence of glucose and fructose where significant amounts of hGH is produced in medium containing sorbitol. As a result, it can be concluded that fructose is not a suitable carbon source to be used in the production of recombinant proteins under control of AOX promoter of *P. pastoris*.

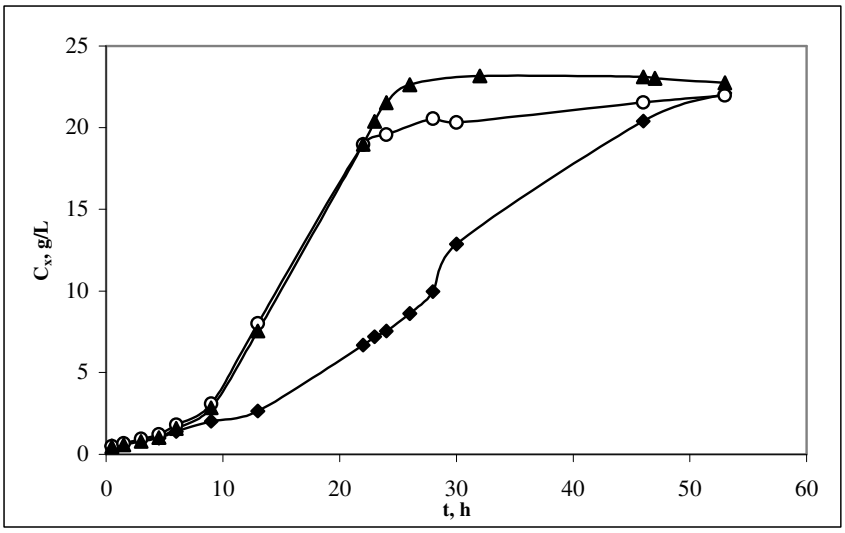

Figure 4.13 Variation in cell concentration with time in defined medium containing 30 g/L sorbitol (♦), glucose (o), fructose (▲) with 1% (v/v) methanol.

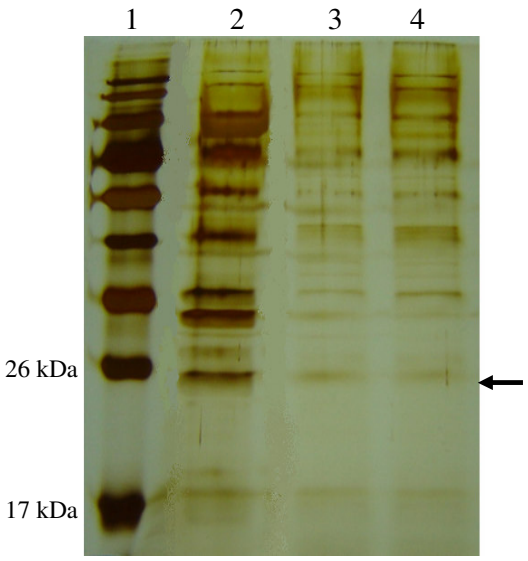

Figure 4.14 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor. 1. well: protein marker, 2. well: 30 g/L sorbitol, 3. well: 30 g/L glucose, 4.well: 30 g/L fructose.

Thus, fructose is not a suitable carbon source to be used in the production of recombinant proteins under control of AOX promoter of *P. pastoris*. Therefore, there is no need to try the non-inhibitory concentration found for sorbitol since even less amounts of fructose represses the production.

The other carbon sources selected to be used in the production medium were decided to be added in the equivalent concentration to the non-inhibitory sorbitol case, which was 50 g/L. This due to the fact that co-substrate that will be used in the production will be given batch-wise to the medium and it is important that the yeast cells can tolerate as much or higher amounts of sorbitol to prevent need of addition of the co-substrate during the fermentation. Hence, production media containing 50 g/L sorbitol, 50 g/L mannitol, 47 g/L lactose, 47 g/L sucrose, 52.7 g/L citric acid, 50 g/L acetic acid, 50 g/L lactic acid were used to cultivate *P. pastoris* cells in laboratory scale air filtered shake bioreactors. Growth curves obtained from each medium can be seen in Figure 4.15.

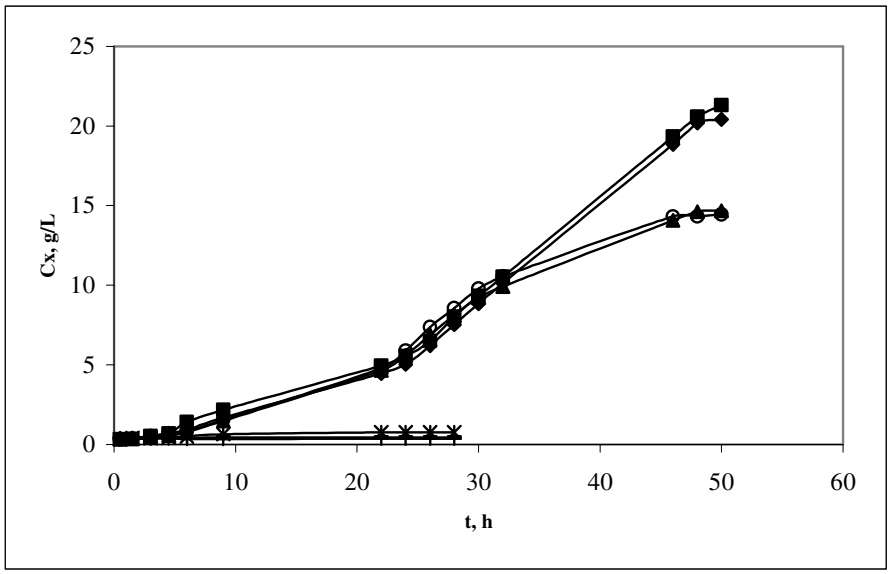

Figure 4.15 Variation in cell concentration with time in defined medium containing 50 g/L sorbitol (♦), 50 g/L mannitol (■), 47 g/L lactose(▲), 47 g/L sucrose (o), 50 g/L lactic acid (*), 53 g/L citric acid (-), 50 g/L Acetic acid (+) with 1% (v/v) methanol.

From cell growth point of view, there were two couples which show the same behavior; sorbitol/mannitol and lactose/sucrose. It was seen that sorbitol and mannitol, which are isomers, supported the cell growth in the same extent. However, if a choice should be made sorbitol would be more economical since it is commercially cheaper than mannitol. When they are compared considering recombinant protein production with SDS-PAGE, although there is no significant difference, in medium containing sorbitol there is more rhGH in the extracellular medium (Figure 4.16).

Sucrose and lactose supported the growth in the same extent which is less that those of sorbitol and mannitol. When the rhGH production levels were compared for sucrose and lactose, it can be seen from the SDS-PAGE image that there is more rhGH production in the presence of sucrose (Figure 4.17). Therefore it can be concluded that lactose may have a partial repressive effect on AOX promoter.

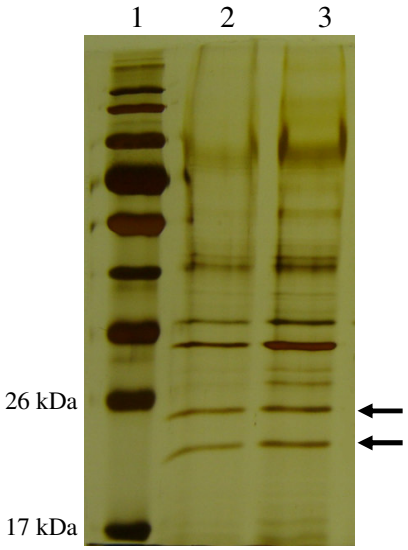

Figure 4.16 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor. 1. well: protein marker, 2. well: 50 g/L mannitol, 3. well: 50 g/L sorbitol

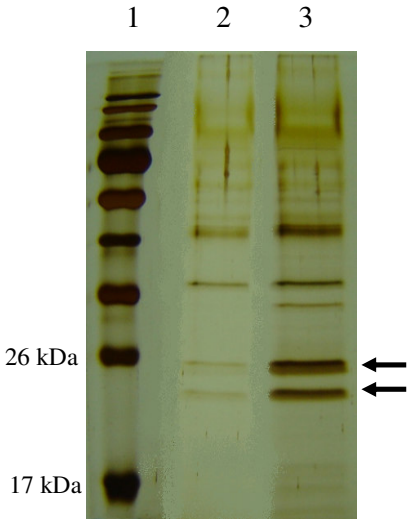

Figure 4.17 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor. 1. well: protein marker, 2. well: 47 g/L lactose, 3. well: 47 g/L sucrose

In media containing lactic acid, citric acid and acetic acid cell growth was totally inhibited therefore no rHGH was found in the extracellular medium. Inhibition of cell growth was most probably due to the high concentrations of these carbon sources which have high acidic content.

Among the different carbon sources used, sorbitol, mannitol, and sucrose seemed to be suitable for using in production medium. Sorbitol is much more economical than mannitol, therefore mannitol can be eliminated. Hence, pilot scale bioreactor experiments can be performed using the two remaining carbon sources; sorbitol and sucrose. Furthermore, since sucrose works well in the concentration equivalent to non-inhibitory sorbitol case, it was determined to use this concentration as a starting point in pilot scale bioreactor experiments.

4.4 Production of Human Growth Hormone by *Pichia pastoris* in Pilot Scale Bioreactor

After selecting the strain to be used, determining the possible co-substrates of methanol and deciding on their concentrations in laboratory scale air filtered shake bioreactors, in the next step, effects of these selected carbon

sources on rhGH production were investigated in pilot scale bioreactor, as oxygen limitation in laboratory scale bioreactors has diverse effects on metabolism of yeast and product formation. The bioreactor is equipped with temperature, pH, foam, stirring rate, feed inlet rate, dissolved oxygen and mass flow controllers to provide efficient control of the production process.

To determine the best co-substrate, first two fed batch bioreactor experiments were performed with selected carbon sources in the shake flask experiments. Additionally, a pilot scale bioreactor experiment in which there was no co-substrate was performed. Comparing the results of these bioreactor experiments, the best way to produce rhGH was chosen and focused in order to improve it with different feeding strategies.

4.4.1 Bioreactor Operation Parameters

To construct an efficient bioprocess in order to obtain high productivity and yields, determining the ideal bioprocess operation parameters such as pH, temperature and dissolved oxygen level is important. In this study, where the main aim is to investigate the effects of carbon sources and feeding strategies for the production of hGH by *P. pastoris* cells, the temperature has decided to be kept at 30°C throughout the process. This is the most frequently applied temperature condition for *P. pastoris* processes, regardless of the product.

P. pastoris is capable of growing across a relatively broad pH range from 3.0 to 7.0 which actually does not affect the growth significantly (Macauley-Patrick et al., 2005). This makes it easier to choose the pH for recombinant protein production and stability and, minimizing activity of proteases in the medium (Sreekrishna et al., 1997; Macauley-Patrick et al., 2005). Çelik et al., (2009) reported that for *P. pastoris* Mut^+ strain producing rhEPO, in the production media containing the pH=4.0-5.0 buffers, the higher product concentrations are achieved, while after pH=5.0 the productivity decreases. Particularly, for the production of rhGH, pH 6.0 was used (Trevino et al., 2000; Eurwilaichitr et al., 2002; Orman et al., 2009). However, as Bayraktar (2009)

found pH 5.0 optimum for rhGH production by *P. pastoris*, in each pilot scale bioreactor experiments pH was kept at pH 5.0.

Another crucial parameter in high density *P. pastoris* fermentations is dissolved oxygen in the broth since *P. pastoris* is an aerobic microorganism and, has extremely high oxygen consumption rates. Therefore, dissolved oxygen (DO) has been maintained above 20% saturation to prevent oxygen limitation. At the beginning, only air was used to control DO. However, after 12 hours of fermentation, only air was not enough to keep DO levels at the desired value. Therefore, air was enriched with increasing amounts of oxygen by using mass flow controllers.

The agitation rate was maintained constant at N=900 rpm for pilot scale bioreactor experiments where it was N=250 rpm for laboratory scale shake bioreactors. Agitation used in shake flask experiments would not be enough since *P. pastoris* yeast consumes oxygen at a very high rate in high density fermentations. Furthermore, a higher agitation rate can cause shear damaging the cells, increase in temperature and foam formation.

4.4.2 Control of Bioreactor Operation Parameters in Pilot-Scale Bioreactor

Previously determined bioreactor operation conditions such as temperature, pH, dissolved oxygen level, and agitation rate should be controlled as accurately as possible to obtain reliable results and provide reproducibility.

The temperature was accurately controlled at T=30±0.1°C using the PI controller of the bioreactor system. For pH adjustment only the base (25% NH_3OH) was supplied to the fermentation medium (Çelik, 2008). pH was also controlled using the PI controller of the bioreactor system with parameters as Xp=30% and TI= 30s, and keeping the base-pump-valve open at 10%, the pH of the medium was automatically controlled at pH=5.0±0.2. Foaming was prevented by adding antifoam solution to the initial medium (0.01% v/v), and 1 or 2 mL of 10% antifoam solution was required during the process.

DO has been maintained above 20% saturation using only air for the first 12 hours of the process. Then, air was enriched with increasing amounts of

oxygen by using mass flow controllers since air is not enough to keep DO levels at the desired value.

4.4.3 Precultivation, Co-Substrate Addition and Methanol Feeding Rate in Fed-Batch Pilot Scale Bioreactor Operations

In fed-batch fermentations specific growth rate determines the exponential feed inlet rate of the limiting substrate, in this study methanol. This exponential feeding profile is determined according to equation 4.1 (Weigand et al., 1979)

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

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

In this study, a bioprocess including four phases was applied. These phases are glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and methanol fed-batch phase (MFB). During the GFB and MFB phases, predetermined exponential feeding profiles were applied by using equation 4.1 and the parameters used in this equation are given in Table 4.1.

Table 4.1 Parameters used in equation 4.1

Parameter	Glycerol Fed-Batch Phase	Methanol Fed-Batch Phase
μ_0 (h^{-1})	0.18	0.03
$Y_{X/M}$ (g/g)	0.5 *	0.42**

* Ref: Cos et al., 2005

** Ref: Jungo et al., 2006

Using the parameters given in Table 4.1 for GFB phase, a sample glycerol feeding scheme is shown in Figure 4.18.

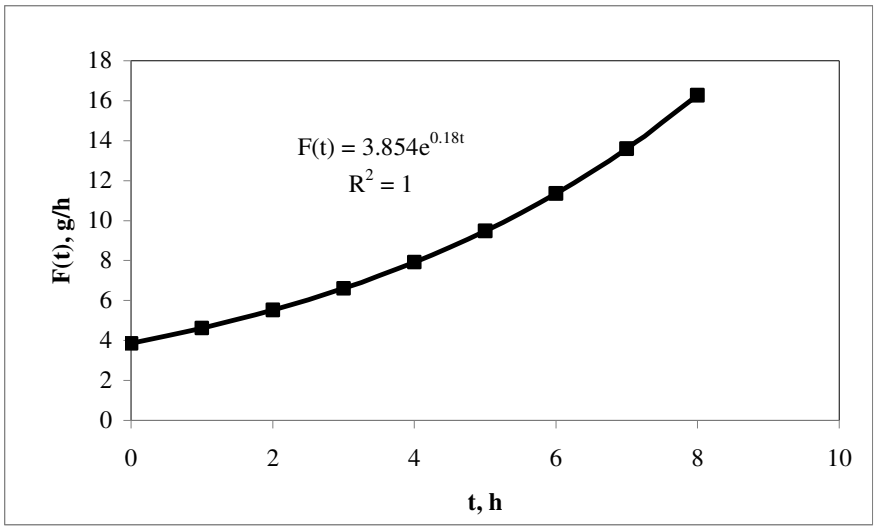

Figure 4.18 The predetermined feed profiles for glycerol, calculated for specific growth rate (μ_o) of 0.18 h^{-1}.

For each bioreactor experiment performed in this study, the first three phases; GB, GFB and MT, the same cell growth was achieved (Figure 4.19).

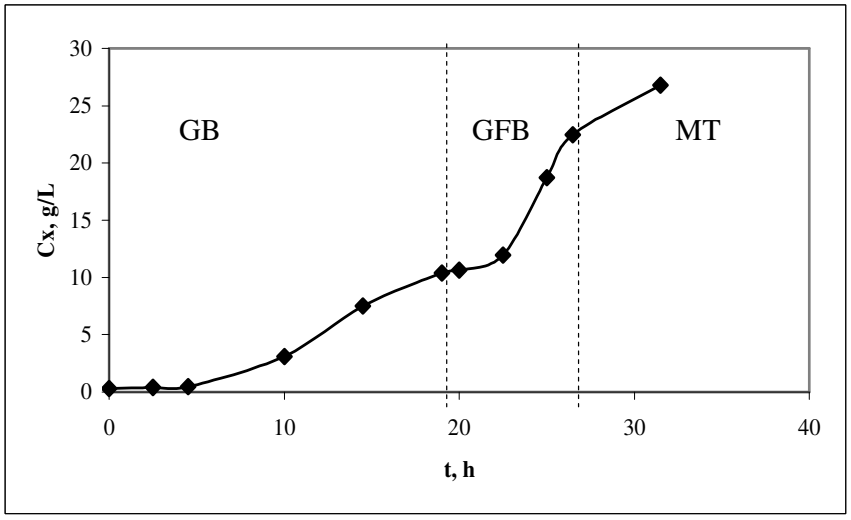

Figure 4.19 Variation in cell concentration with cultivation time in the precultivation phases: glycerol batch phase (GB), glycerol fed-batch phase (GFB) and methanol transition phase (MT).

At the beginning of MFB phase, the co-substrate to be used was added to medium batch-wise in the previously determined concentration. Although the process is continuous and the production (induction) phase starts at around t=32h of the precultivation period, for simplicity in calculations, beginning of the production phase was taken as t=0h.

In the first bioreactor experiment, the co-substrate was sorbitol with an initial concentration of 50 g/L. In the second fed-batch experiment, the carbon source used instead of sorbitol was sucrose at the same concentration with sorbitol. Then, in the third bioreactor experiment, no co-substrate was used; therefore methanol was added to the production medium as sole carbon and energy source. In each of these experiments, 0.03 h^{-1} was selected as specific growth rate on methanol for a number of reasons. Firstly, Cunha et al. (2004) found that above specific growth rates of μ=0.025 h^{-1}, specific recombinant protein productivity does not depend on the growth rate. Moreover, the maximum specific growth rate of *Pichia pastoris* on methanol is μ_{max}=0.14 h^{-1}, while that on sorbitol is μ_{max}=0.032 h^{-1} (Jungo et al., 2007), thus the specific growth rate was intended to be kept close to the maximum specific growth rate on sorbitol. Since the maximum specific growth rate on sucrose was not known, the value selected for sorbitol was a good starting point. With these parameters selected for predetermined exponential feeding strategy, the methanol feeding scheme applied in the production phase can be seen in Figure 4.20.

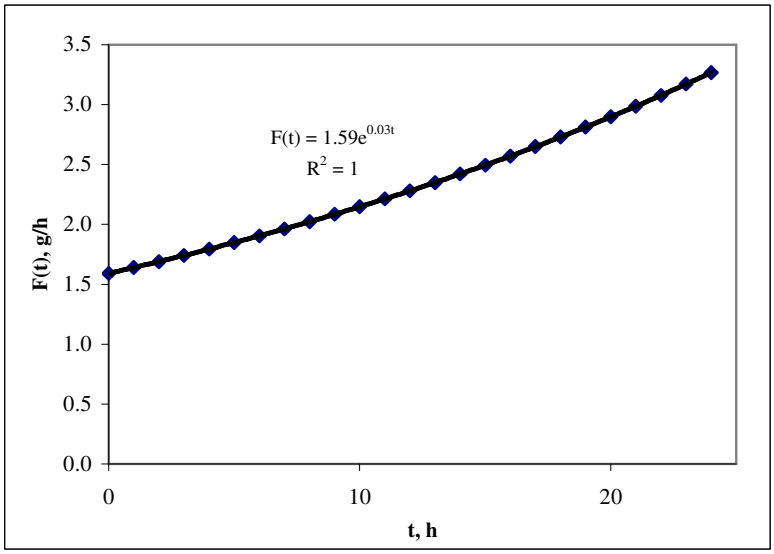

Figure 4.20 The predetermined feeding profile for methanol, calculated for specific growth rate (μ_o) of 0.03 h^{-1}.

4.4.4 Effect of Co-Substrate Addition and Methanol Feeding Rate on Cell Growth

The effect of carbon sources should be examined firstly on cell growth. When sorbitol was added as a co-substrate at the beginning of the induction phase, the long lag-phase (t=0-9 h) seen in two other cases was eliminated and cells started to proliferate immediately. After 24 hours of induction, cell concentration reached to 42.3 g/L in medium with sorbitol, which was 1.4-fold higher than that of no co-substrate addition. This was an expected result when compared to the ones obtained by Çelik et al., (2009) where 1.7-fold higher cell concentration was obtained in medium with sorbitol compared to that without sorbitol with *P. pastoris* producing EPO at t=24 h of production. When the medium containing sucrose is considered, it was seen that sucrose does not support the cell growth as expected and the cell concentration profile is parallel to the one containing only methanol as sole carbon source. In shaking flask experiments, it was seen that although there are significant amounts rhGH in the extracellular medium according to the SDS-PAGE results, cell growth curve lies

under that of medium with sorbitol. This reveals that even though presence of sucrose in the medium does not represses AOX promoter, it does not support the cell growth as well as sorbitol and the cell growth seen in shaking flask experiments might be due to the presence of methanol in the medium. Moreover, elimination of lag phase in the production of recombinant proteins is important on the basis of economical aspects. However, sucrose has no effect on eliminating the lag-phase seen in feedings of methanol without a second carbon source.

Comparing the results of three different pilot scale bioreactor experiments with different initial conditions in the induction phase, it was decided to focus on sorbitol containing production medium in order to develop a better feeding strategy for the production of rhGH.

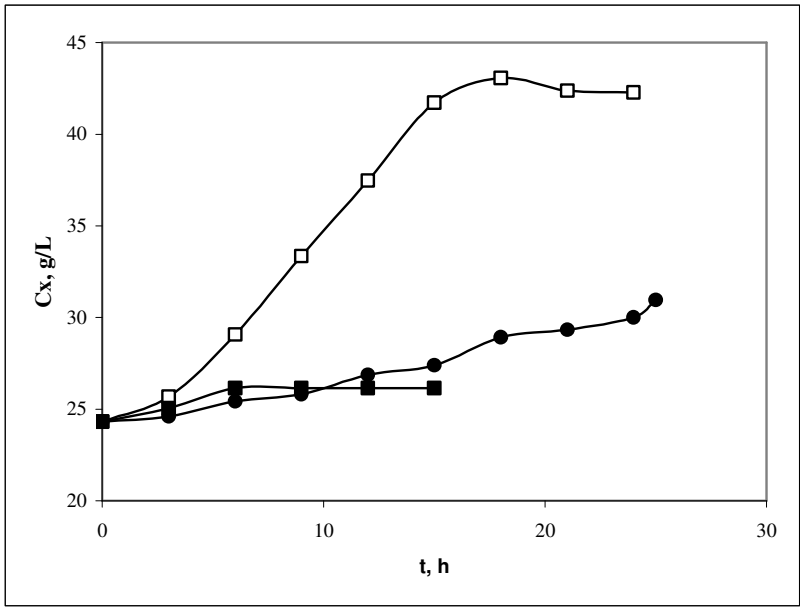

Figure 4. 21 Variation in cell concentration with time in the production phase of bioprocesses with different initial conditions: 50 g/L sorbitol (□); 50 g/L sucrose (■); only methanol (●).

4.4.5 Development of Carbon Source Feeding Strategies

According to the results of laboratory scale shake bioreactor experiments, sorbitol and sucrose was chosen to be used in the pilot scale bioreactor experiments as a co-substrate of methanol in the induction phase. In addition to those, a bioreactor experiment having neither of these co-substrates was performed. Comparing the results of these three pilot scale bioreactor experiments with different initial conditions in the MFB phase, it was decided that production medium containing sorbitol would be better for fed-batch production processes. Thereafter, results obtained from the first bioreactor with sorbitol as a mixed substrate with methanol was analyzed in terms of cell growth, sorbitol and methanol consumptions and recombinant protein production. According to these results, two different strategies were developed to improve the first one.

In the first production of rhGH in bioreactor, sorbitol was added to the medium batch-wise just before the induction with methanol. Its concentration was adjusted such that the initial concentration was equal to the non-inhibitory limit. From cell concentration point of view, it was seen that yeast cells started to proliferate almost without showing a lag phase. However the cell concentration stopped increasing just after 15 hours of induction and although the methanol addition was continued it did not increase for the last 9 hours of the process (Figure 4.20). At the end of 24 hours of induction 271 mg/L rhGH was obtained. Sorbitol utilization started immediately when added to the medium, and lasted for 15 hours (Figure 4.22). Methanol, on the other hand, was never detected in the medium. Therefore, it was assumed that cells consume all methanol fed as far as it was added to the medium. This reveals the simultaneous utilization of sorbitol with methanol, confirming the results of Jungo et al. (2007) and Çelik et al. (2009).

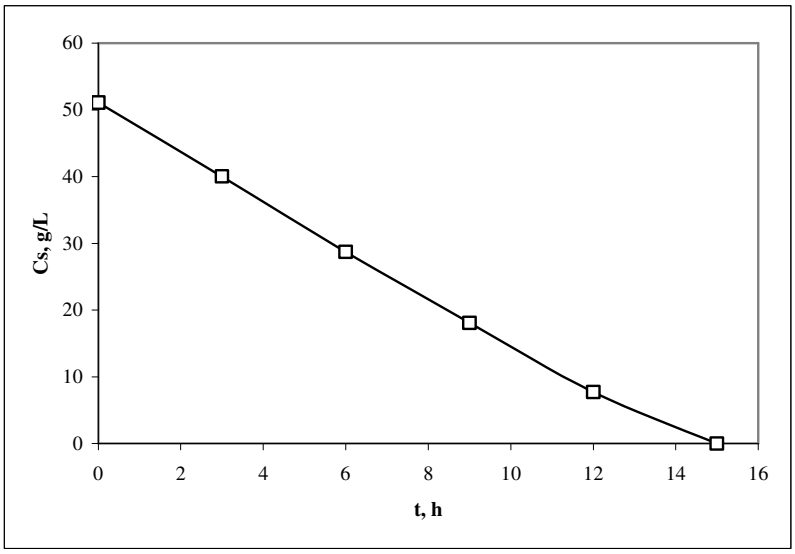

Figure 4.22 Variation in sorbitol concentration with cultivation time for exponential methanol feeding with μ=0.03 h^{-1}.

Considering the sorbitol consumption profile together with the cell growth profile, it was seen that the cell growth stopped just at the same time where sorbitol in the medium was totally depleted. Hence, it was decided to increase the sorbitol concentration in the medium to its initial value before its depletion. Thus in a second bioreactor experiment, sorbitol concentration, C_S, was increased approximately to its initial concentration at the ninth hour by taking the sorbitol profile of the first experiment as a basis. This prolongs the exponential cell growth phase and increased the rhGH concentration in the extracellular medium to 301 mg/L (Detailed results given in Sections 4.4.7 and 4.4.8). In a third bioreactor experiment, with the second sorbitol addition, methanol feeding scheme was also altered such that just after the cessation of cell growth (t=24 h) a pulse of methanol was given to the medium and then the feeding rate was kept constant at a value above the predetermined exponential scheme and below the inhibitory limits. This was made in order to trigger the AOX promoter at the last hours of fermentation. Then, the results of these strategies were compared in order to choose the best one. For simplicity in comparison the experiments performed were named as follows (Table 4.2).

Table 4.2 The abbreviations used for experiments

Experiment Name	Experiment Definition
M-0.03	No addition of co-substrate sorbitol, only methanol feeding with μ=0.03 h^{-1}
MS-0.03	Batch-wise sorbitol addition at t=0 h and methanol feeding with μ=0.03 h^{-1}
MSS-0.03	Batch-wise sorbitol addition at t=0 h and t=9 h and methanol feeding with μ=0.03 h^{-1}
MSSM-0.03	Batch-wise sorbitol addition at t=0 h and t=9 h and methanol feeding with μ=0.03 h^{-1} up to t=24 h, then constant methanol feeding at 6.35 g/h (3 g/h above the last exponential value)

4.4.6 Effect of Different Feeding Strategies on Sorbitol and Methanol Consumptions

Sorbitol consumption profile was investigated in the first bioreactor experiment (MS-0.03) in which this co-substrate was added to the medium batch-wise at a concentration of 50 g/L at the beginning of induction phase. It lasts 15 hours for the whole sorbitol to be depleted (Figure 4.22). The other two strategies were actually built on this sorbitol consumption profile. With the addition of second batch-wise sorbitol to the medium by increasing its concentration to the non-inhibitory level (MSS-0.03), it was seen that the sorbitol consumption rate was almost not changed and the sorbitol in the medium was totally used up at t=25 h. In a third strategy, with the second sorbitol addition, methanol feeding scheme was also altered such that just after the cessation of cell growth (t=24 h); a pulse of methanol was given to the medium such that its concentration reaches to 3 g/L and then the feeding rate was kept constant at a value of above the 3 g/h above the last predetermined exponential scheme flow rate (MSSM-0.03). The sorbitol consumption profiles of these pilot scale bioreactor experiments can be seen in Figure 4.23.

From the sorbitol consumption profiles, it was concluded that the sorbitol utilization starts immediately at the beginning of the induction phase. Before addition of the second sorbitol to the medium, profile is almost the same for all three experiments. Moreover, sorbitol consumption trends of MSS-0.03 and MSSM-0.03 were expected to be the same since these experiments differ only after t=24 h of the production phase and the results obtained from these experiments were as expected.

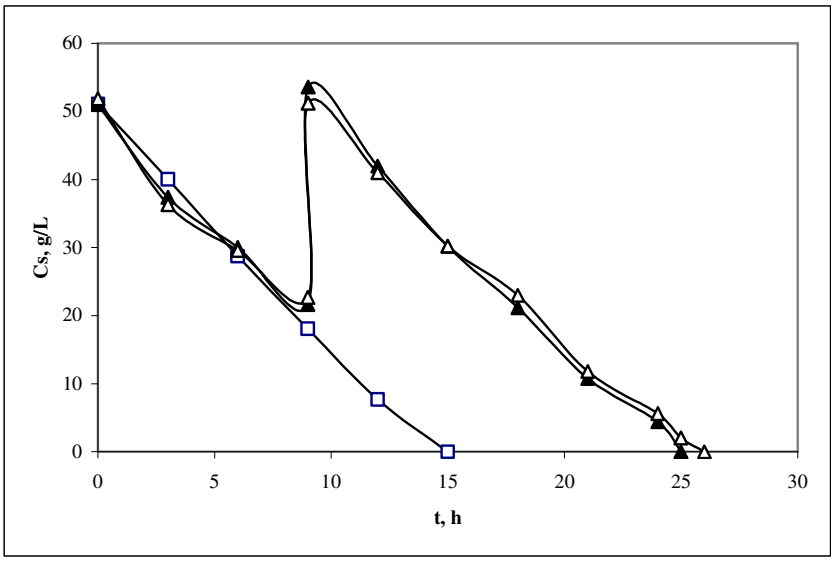

Figure 4.23 Variation in sorbitol concentration with cultivation time for different feeding profiles: MS-0.03 (□); MSS-0.03 (▲); MSSM-0.03 (Δ).

With the analysis of samples taken from the fermentation medium, it was seen that there were no residual methanol in the medium. It might be expected to see some methanol in the last six hours of MSSM-0.03 since the feeding rate was above the exponential one. However, it seems that cells consumed all the methanol as far as it was added to the medium although it was higher than the amount added in exponential feeding scheme of selected specific growth rate.

4.4.7 Effect of Different Feeding Strategies on Cell Growth

Cell growth is the first parameter to be considered during the evaluation of different carbon sources and feeding strategies. The cell growth profiles achieved in the pre-cultivation phase of all bioreactor experiments were the same since there are no differences in the pre-cultivation phases (Figure 4.19). The only difference is in the induction phases.

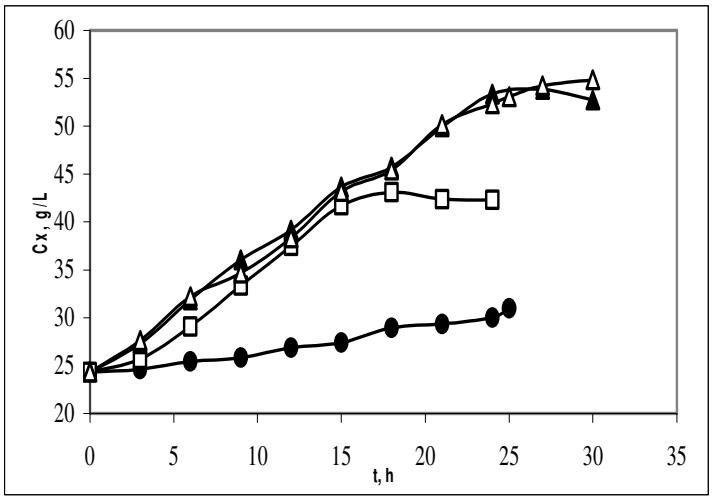

Figure 4.24 Variation in cell concentration with cultivation time in the production phase of bioprocesses with different feeding profiles: M-0.03 (●); MS-0.03 (□); MSS-0.03 (▲); MSSM-0.03 (Δ).

As indicated in Section 4.4.4; when sorbitol was added as a co-substrate at the beginning of the production phase, the lag-phase seen in cells grown only on methanol was eliminated and cells started to proliferate immediately. After 24 hours of induction, cell concentration reached 42.3 g/L in medium with sorbitol (MS-0.03), which is 1.4-fold higher than that of no co-substrate addition. Furthermore, it was seen that in the medium containing sorbitol, cell growth ceases with the total consumption of sorbitol. When the sorbitol concentration in the medium was reincreased to its initial value at the ninth hour of induction

(MSS-0.03); it was seen that the cell growth continues after the depletion of all sorbitol in the medium, t=25 h. The highest cell concentration was achieved at t=27 h as 53.8 g/L. However, in MSSM-0.03, the addition of extra methanol after t=24 h of the fermentation did not cause any significant change in the cell growth after that hour (Figure 4.24) and the highest cell concentration was attained at t=30 h as 55 g/L.

4.4.8 Effect of Different Feeding Strategies on Recombinant Protein Production

In this study, since the main aim is to produce rhGH, production level of different feeding strategies is an important parameter to evaluate them. In order to compare rhGH production levels, firstly SDS-PAGE analysis was performed using extracellular parts of the samples taken at the last hours of induction (Figure 4.25a). Then, using HPCE recombinant protein concentrations were determined and protin production profiles were given in Figure 4.25b. In addition to these, expression level of the *hGH* gene was determined by real time PCR. The highest rhGH concentration was obtained in MSS-0.03 with C_{rp}=301 mg/L at t=30 h of the induction phase. Therefore, rhGH production was increased by 1.11-fold by adding sorbitol two times to the medium. The lowest rhGH concentration, C_{rp}=180 mg/L, was obtained in the strategy with no co-substrate addition where the only carbon and energy source was methanol. In MSSM-0.03, the total rhGH concentration was lower than MSS-0.03 although the aim was suddenly increasing the AOX activity with the addition of excess methanol to the medium. Cells showed diverse response to the excess methanol added to the medium and therefore the highest recombinant protein production was C_{rp}=232 mg/L in MSSM-0.03.

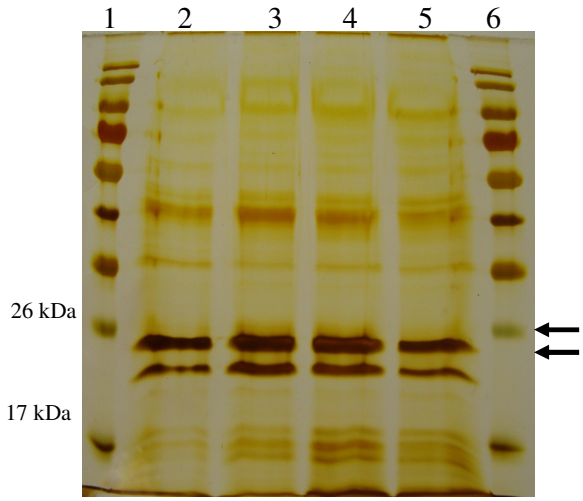

Figure 4.25a Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in pilot scale bioreactors 1. well: protein marker, 2. well: M-0.03-t=24 h; 3. well: MS-0.03- t= 24 h; 4. well: MSS-0.03- t=30 h; 5. well: MSSM-0.03- t= 30 h.

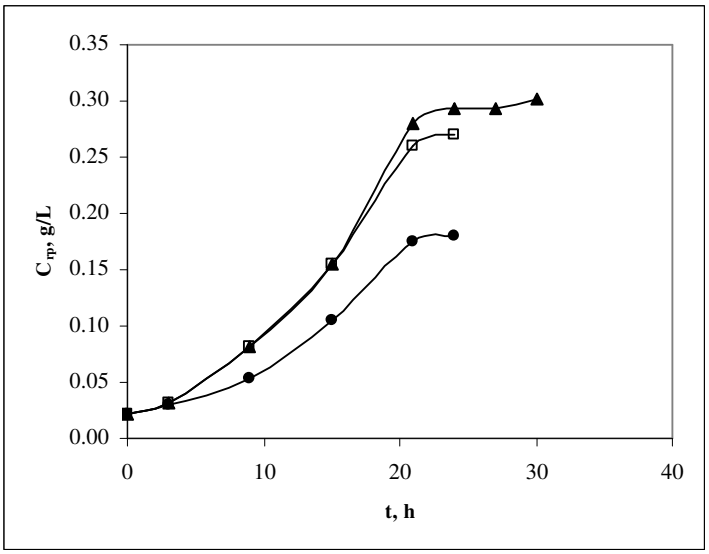

Figure 4.26b Variation in recombinant protein production with cultivation time in differerent feeding strategies. M-0.03 (●); MS-0.03 (□); MSS-0.03 (▲)

Besides directly measuring the amount of hGH in the extracellular medium, expression levels of gene coding hGH was investigated using real time PCR (Figure 4.26). Results showed that expression level of *hGH* gene did not change during experiment M-0.03, where there was only methanol in the medium as carbon and energy source. Expression levels of the gene of interest is higher in all conditions containing sorbitol in the medium than the one with only. To illustrate, the highest *hGH* expression levels achieved in MS-0.03, MSS-0.03 and MSSM-0.03 are $4.01x10^{10}$, $4.17x10^{10}$ and $4.07x10^{10}$ copies/ mg CDW where that of M-0.03 is $3.11x10^{9}$ copies/ mg CDW. Addition of sorbitol to the medium definitely affects the expression levels positively. Amount of rhGH found in extracellular medium also reveals this positive effect. In an ideal expression system, it is expected that all transcripted genes are also translated. Even if this could not be achieved in many expression systems, changes in level of transcription gives an idea about the final product. Thus, the final product is the lowest in experiment M-0.03 (C_{rp} = 180 mg/L) where the expression levels are lowest. For MS-0.03, it is seen that the maximum level of transcription was achieved at t=15 h. After that, expression level of *hGH* decreases and this decrease starts at the same time with depletion of sorbitol and termination of cell growth. As all the sorbitol in the medium was used up, the yeast cells undergo a lag phase since the positive effect of co-substrate sorbitol on cell growth disappears and cells should depend only on methanol. The same profile was observed in MSS-0.03. Although there are some fluctuations in the *hGH* expression level profile of MSS-0.03, the maximum level of *hGH* expression is achieved at t=24 h. Just as MS-0.03, the decrease in *hGH* expression starts at the same time with depletion of sorbitol and termination of cell growth. The maximum *hGH* expression levels in MS-0.03 and MSS-0.03 are very close to each other, $4.01x10^{10}$ copies/mg CDW ($8.78x10^{5}$ copies/ng total RNA) in MS-0.03 and $4.17x10^{10}$ copies/mg CDW ($5.38x10^{5}$ copies/ng total RNA) in MSS-0.03. However this maximum value was attained at a later instant of the fermentation in MSS-0.03. The reason of this delay is most probably the addition of second sorbitol to the medium. Since cells continue to proliferate until entire sorbitol in the medium was depleted, the point where maximum expression level

was also delayed. Although same expression levels per cell were attained in MS-0.03 and MSS-0.03, the higher cell concentration achieved in MSS-0.03 resulted in higher rhGH production (271 mg/L in MS-0.03 and 301 mg/L in MSS-0.03). MSSM-0.03 shows a similar expression level profile as expected. In fact MSS-0.03 and MSSM-0.03 reaches almost the same *hGH* expression levels in an supervening manner. However, in MSSM-0.03, after addition of pulse methanol to the medium, its negative effects on expression level can clearly be seen and instead of the second highest peak observed in MSS-0.03, a continuous decrease was obtained.

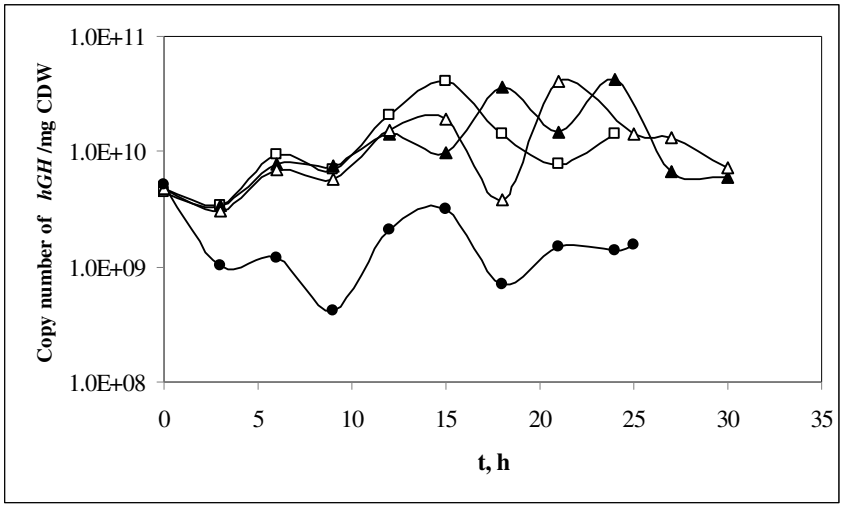

Figure 4.27 Variation in expression level of *hGH* with cultivation time in the production phase of bioprocesses with different feeding profiles. M-0.03 (●); MS-0.03 (□); MSS-0.03 (▲); MSSM-0.03 (Δ).

In literature, the highest extracellular rhGH concentration obtained up to today was 190 mg/L using C_{MeOH}=3% (v/v) after 3 day induction of *P. pastoris* in complex medium (Eurwilaichitr et al., 2002). In this study, C_{rp}= 301 mg/L, was achieved using double batch-wise sorbitol addition together with an exponential methanol mixed feeding strategy. This 1.58-fold higher production was reached after 30 hours of induction. Even with single sorbitol addition, 1.43-fold higher rhGH was obtained in this study. Hence, with addition of sorbitol to

the medium, higher rhGH concentrations can be obtained with shorter induction periods. In fact, in almost one third of process time required for fermentation without a co-substrate, 1.58-fold higher r-protein can be produced in the presence of sorbitol. Therefore using sorbitol in the medium results in significant reduction in total cost of fermentation since addition of sorbitol shortens the fermentation time required to produce desired amount of recombinant protein.

4.4.9 Effect of Different Feeding Strategies on Alcohol Oxidase Production

The specific AOX activity profiles for media containing sorbitol (MS-0.03, MSS-0.03, MSSM-0.03) show similar trends (Figure 4.27). In the presence of sorbitol, AOX activity shows an increasing behavior until the total depletion of sorbitol. Thereafter, it starts to decrease. This decrease might be a result lag phase that cells undergo due to the shift from medium containing both sorbitol and methanol to a medium with only methanol. In MS-0.03, AOX activity reaches its maximum value at t = 15 h, where sorbitol in the medium was just exhausted. In MSS-0.03 and MSSM-0.03, the maximum activity was reached at t=21 h, just before the depletion of sorbitol in the medium. Extra methanol added in MSSM-0.03 at t = 24 h did not trigger AOX as expected.

The highest specific activity was obtained as 40.7 U/g CDW t=15 h of MS. Both in MSS-0.03 and MSSM-0.03, the maximum specific activity was less than this value; 34.8 and 32.9 U/g CDW, respectively. This might be a result of second sorbitol addition to the medium. Although presence of it does not repress AOX, utilization of additional sorbitol may put an additional stress on cells, affecting the methanol utilization pathway. Although it was found that *P. pastoris* yeast cells utilize sorbitol and methanol simultaneously, in the presence of sorbitol they might prefer relying on glycolysis to form cell constituents rather than methanol utilization pathway taking place in peroxisomes. Thus, the microorganism itself minimizes the need of methanol in the presence of a co-substrate (sorbitol in this case). Additionally the specific AOX activity when there is only methanol in the medium started increasing after 15 hours of induction, where the lag-phase ends. This was an expected result since in the

absence of a non-repressing carbon source, the lag-phase needed for the cells to acclimate methanol prolongs. Therefore, addition of sorbitol in the production phase helps shortening the lag-phase while providing to reach high AOX activities in shorter times.

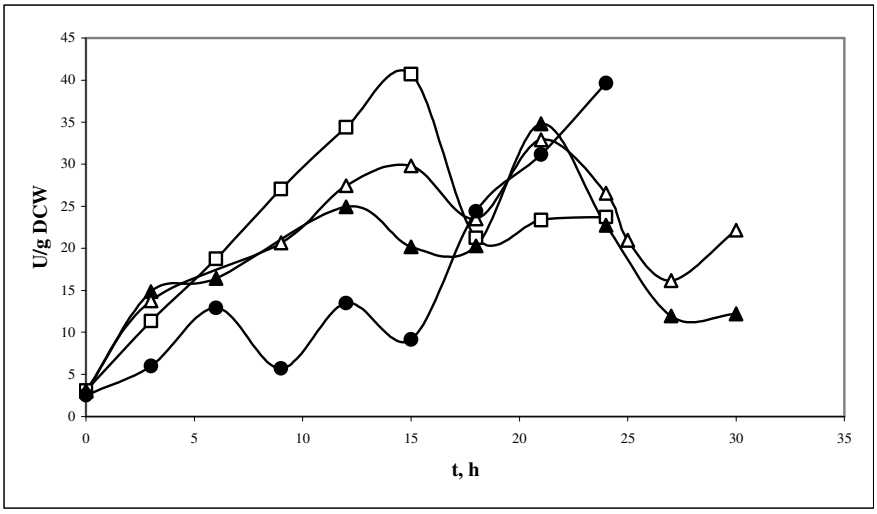

Figure 4.28 Variation in alcohol oxidase activity with cultivation time for different feeding profiles: M-0.03 (●); MS-0.03 (□); MSS-0.03 (▲); MSSM-0.03 (Δ).

As in the case of *hGH* gene, besides measuring the activity of AOX enzyme in the yeast cells, expression levels of *AOX1* gene coding AOX1 enzyme was investigated using real time PCR (Figure 4.28). Behavior of expression level profiles in each experiment show similarities with AOX activity profiles. For experiment M-0.03, expression level of *AOX1* gene starts to increase after t = 18 h. AOX activity in this experiment also shows and increasing manner however it starts after 15 hours of induction. In MS-0.03, the highest level of expression ($9.38x10^{10}$ copies/mg CDW or $2.05x10^{6}$ copies/ng total RNA) was achieved at t = 15 h where AOX activity also reaches its maximum value. In this experiment, the highest expression level for *hGH* gene was also seen at t=15 h. In MSS-0.03 and MSSM-0.03, almost the same copy numbers with maximum value of MS-

0.03 were attained 3 hours later, at t =18 h ($8x10^{10}$ copies/mg CDW). This shift might be a result of addition of extra sorbitol to the medium. These two bioreactors differ in methanol feeding schemes applied at t=24h. Extra methanol added to the medium obviously has diverse effect on *AOX1* expression. Just after addition of extra methanol to medium *AOX1* expression level decreases where without this methanol addition in MSS-0.03, the highest transcriptional level was reached. This extra methanol was added to trigger *AOX* expression however it negatively affects the cells and expression decreases although methanol concentration in the medium did not reach toxic limits stated in literature, 4g/L (Zhang et al., 2000b).

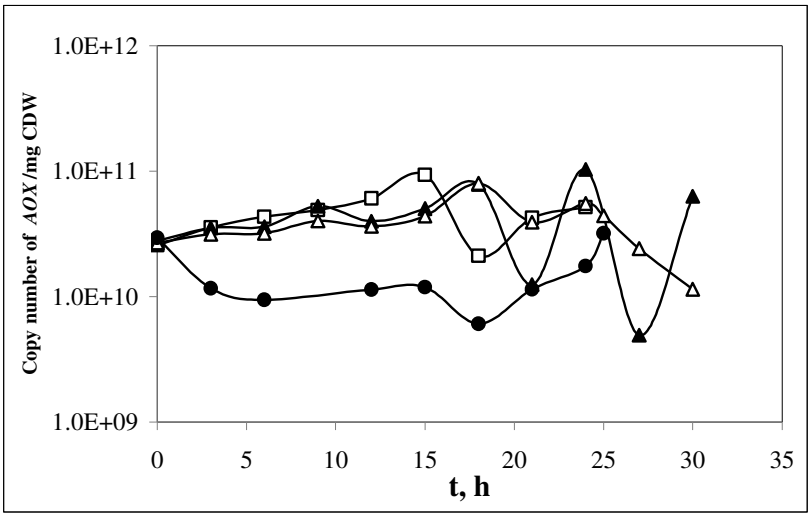

Figure 4. 29 Variation in expression level of *AOX* with cultivation time in the production phase of bioprocesses with different feeding profiles. M-0.03 (●); MS-0.03 (□); MSS-0.03 (▲); MSSM-0.03 (Δ).

4.4.10 Effect of Different Feeding Strategies on Amino and Organic Acid Concentration Profiles

The amino and organic acid profiles give an idea about the supply and demand for these metabolites, which is regulated by the metabolic reaction

network. The amino acid and organic acid concentrations are given in Tables 4.3 and 4.4, respectively.

Both oxygen and methanol concentrations in *Pichia* processes affecting the cell growth and product formation may theoretically result in elevated formaldehyde concentrations in the cell if they generate an imbalance between the initial oxidation of methanol and the formaldehyde consuming reactions in anabolism and catabolism (Charoenrat et al., 2006). Formaldehyde was detected in neither of the bioreactor media. This might have mainly two reasons; it can be immediately oxidized to formic acid or it can go into the assimilatory pathway and enters glycolysis. Formic acid was detected in the medium in all conditions and its concentration increases suddenly in the last hours of fermentation where cell growth stops. Since slow growth was observed in this phase, most of the formaldehyde may be converted to formate instead of cell constituents. Therefore, in the log phase of induction, formic acid concentration is lower than the plateau phase at the end of the fermentation.

Most microorganisms have the metabolic machinery to synthesize all essential amino acids from carbon and nitrogen sources for the production of proteins. Regulation of the metabolic reaction network should cause good coupling of supply and demand for the amino acids. If the supply exceeds the demand, the amino acids would be excreted to the extracellular medium. If they are required again, they could be supplied from the medium. Moreover, the secreted proteins in the extracellular medium are degraded by the extracellular proteases, which also cause an increase in the amino acid concentration in the medium. For all feeding strategies, leucine (Leu), serine (Ser), glutamic acid (Glu) and glutamine (Gln) were not detected in the extracellular medium. This may be a result of amino acid content of hGH which contains 13.6 % Leu, 9.4% Ser, 7.3 % Glu and 6.8% Gln in its structure. Furthermore, valine (Val) is present only in cases where there is sorbitol in the medium and its concentration decreases with time. This shows that demand of the yeast cells to this metabolite increases during the of production phase.

Table 4.3 Variation in amino acid concentration* profiles with cultivation time for runs with different feeding strategies

Experiment	Time (h)	3	9	15	21	
M-0.03	Asp	0.0075	0.0108	0.0098	0.0163	
	Asn	0.0143	0.0074	0.0031	0.0085	
	Gly	0.0303	0.0244	0.0147	0.0253	
	His	0.0049	0.0017		0.0017	
	Met	0.0044	0.0022	0.0020	0.0051	
	Leu	0.0030				
	Phe	0.0225	0.0074	0.0071	0.0078	
	Lys	0.0184	0.0125	0.0134	0.0175	
	Total	0.1053	0.0664	0.0502	0.0823	
	Time (h)	**3**	**9**	**15**	**21**	
MS-0.03	Asp	0.0126	0.0181	0.0274	0.0168	
	Asn	0.0152	0.0167	0.0160	0.0082	
	Gly	0.0229	0.0182	0.0160		
	Pro		0.0006	0.0005		
	Val	0.0319	0.0245		0.0049	
	Met	0.0049	0.0064	0.0077	0.0081	
	Phe	0.0028	0.0045	0.0043	0.0051	
	Trp		0.0009			
	Lys	0.0022	0.0183	0.0150	0.0140	
	Total	0.0925	0.1081	0.0868	0.0572	
	Time (h)	**3**	**9**	**15**	**21**	**27**
MSS-0.03	Asp	0.0126	0.0181	0.0118	0.0317	0.0206
	Asn	0.0152	0.0167	0.0190	0.0167	0.0150
	Gly	0.0229	0.0182	0.0202	0.0151	0.0157
	Pro		0.0006	0.0010	0.0004	0.0006
	Val	0.0319	0.0245	0.0340	0.0267	0.0026
	Met	0.0049	0.0064	0.0034	0.0046	0.0029
	Phe	0.0028	0.0045	0.0398	0.0123	0.0094
	Trp		0.0009			
	Lys	0.0022	0.0183	0.0133	0.0123	0.0092
	Total	0.0925	0.1081	0.1425	0.1198	0.0760
	Time (h)	**3**	**9**	**15**	**21**	**27**
MSSM-0.03	Asp	0.0126	0.0181	0.0092	0.0159	0.0212
	Asn	0.0152	0.0167	0.0102	0.0108	0.0160
	Gly	0.0229	0.0182	0.0245	0.0231	0.0146
	His			0.0016	0.0017	0.0021
	Pro		0.0006			
	Val	0.0319	0.0245	0.0269	0.0146	
	Met	0.0049	0.0064	0.0041	0.0049	0.0030
	Cys			0.0026	0.0041	0.0119
	Phe	0.0028	0.0045	0.0047	0.0090	0.0096
	Trp		0.0009			
	Lys	0.0022	0.0183	0.0016	0.0107	0.0104
	Total	0.0925	0.1081	0.0853	0.0947	0.0889

*Concentrations given in g/L.

Table 4.4 Variation in organic acid concentration* profiles with time for runs with different feeding strategies

Experiment	Time (h)	0	3	6	9	12	15	18	21	24			
M-0.03	Formic Acid	0.0889	0.1195	0.1298	0.1486	0.1564	0.1553	0.1546	0.2437	0.2166			
	α-keto-glutaric Acid	0.0231	0.0254	0.0262	0.0278	0.0302	0.0317	0.0289	0.0356	0.0373			
	Citric Acid	0.0075	0.0082	0.0092	0.0089	0.0106	0.0129	0.0159	0.0201	0.0224			
	Fumaric Acid	0.0009	0.0011	0.0009	0.0008	0.0012	0.0013	0.0011	0.0015	0.0015			
	Succinic Acid	0.0544	0.0595	0.0524	0.0529	0.0563	0.0549	0.0547	0.0702	0.0756			
	Lactic acid	0.0469	0.0652	0.0654	0.0683	0.0883	0.0868	0.0867	0.1352	0.1623			
MS-0.03	Time (h)	0	3	6	9	12	15	18	21	24			
	Formic Acid	0.0851	0.1163	0.1155	0.1299	0.1616	0.1743	0.2627	0.3015	0.3021			
	α-keto-glutaric Acid	0.0252	0.0269	0.0288	0.0319	0.0342	0.0364	0.0368	0.0402	0.0440			
	Citric Acid	0.0067	0.0067	0.0074	0.0089	0.0112	0.0145	0.0199	0.0261	0.0323			
	Fumaric Acid	0.0002	0.0010	0.0010	0.0012	0.0014	0.0017	0.0017	0.0017	0.0020			
	Succinic Acid	0.0635	0.0721	0.0788	0.0751	0.0812	0.0882	0.0798	0.0815	0.0941			
MSS-0.03	Time (h)	0	3	6	9	12	15	18	21	24	27	30	
	Formic Acid	0.0939	0.0671	0.0756	0.0824	0.0837	0.1323	0.1289	0.1701	0.1428	0.2231	0.3094	
	α-keto-glutaric Acid	0.0258	0.0285	0.0256	0.0244	0.0210	0.0214	0.0220	0.0209	0.0204	0.0206	0.0231	
	Citric Acid	0.0067	0.0067	0.0078	0.0089	0.0106	0.0125	0.0164	0.0198	0.0235	0.0273	0.0348	
	Fumaric Acid	0.0007	0.0016	0.0015	0.0016	0.0016	0.0017	0.0023	0.0025	0.0030	0.0033	0.0040	
	Succinic Acid	0.0699	0.0703	0.0679	0.0663	0.0633	0.0674	0.0721	0.0736	0.0790	0.0867	0.1099	
MSSM-0.03	Time (h)	0	3	6	9	12	15	18	21	24	25	27	30
	Formic Acid	0.0878	0.0716	0.0728	0.0820	0.0929	0.1093	0.1599	0.1497	0.1600	0.2523	0.2539	0.3154
	α-keto-glutaric Acid	0.0227	0.0247	0.0240	0.0282	0.0247	0.0247	0.0227	0.0238	0.0210	0.0241	0.0241	0.0280
	Citric Acid	0.0067	0.0067	0.0067	0.0071	0.0078	0.0096	0.0117	0.0156	0.0175	0.0224	0.0233	0.0302
	Fumaric Acid	0.0008	0.0014	0.0012	0.0017	0.0017	0.0018	0.0020	0.0025	0.0024	0.0034	0.0035	0.0034
	Succinic Acid	0.0538	0.0639	0.0593	0.0694	0.0618	0.0626	0.0656	0.0707	0.0681	0.1197	0.1233	0.1089

* Concentrations given in g/L

Lactic acid is the main metabolic product formed in case of insufficient oxygen, when the TCA cycle cannot take place efficiently. This condition was seen only in medium without sorbitol and lactic acid (Lac) was detected as a metabolic byproduct. It achieves its maximum concentration at t=24 h as 0.16 g/L. Lactic acid formation in medium without sorbitol was also reported by Çelik et al. (2009). In that study, lactic acid formation was also seen in medium containing sorbitol although it attained relatively low concentrations.

α-keto-glutaric, citric, fumaric and succinic acids are the other organic acids detected in the medium, which are mostly the TCA cycle metabolites. This is an expected result since metabolism of yeast, thus TCA cycle worked better in the presence of sorbitol. As a general trend, concentrations of these metabolites did not show significant variations between different production strategies and also during the fermentation in each experiment although there were small increases through the end of the processes. The most obvious increase was seen in citric acid concentration and the highest citric acid concentrations was observed in the last hours of MS-0.03 and MSS-0.03 as 0.0323 and 0.0348 g/L, respectively.

4.4.11 Yield Coefficients and Specific Rates of the Bioprocess

To evaluate the bioprocess from efficiency and profitability points of view, the overall yield coefficients should be determined. The overall yield of cell generated per mass of substrate consumed ($Y_{X/S}$), the overall yield of product formed per mass of cells generated ($Y_{P/X}$) and the overall yield of product formed per mass of substrate consumed ($Y_{P/S}$) were calculated for the rhGH production processes with different feeding profiles (Table 4.5). For yield coefficients related to substrate, both sorbitol and methanol were considered together since both are used as carbon sources by the microorganism. Sorbitol was added to the medium batch-wise at the beginning of the induction phase where methanol was fed with a predetermined exponential feeding scheme with a specific growth rate of μ_o=0.03 h^{-1}. Both carbon sources were utilized by the microorganism simultaneously.

Table 4.5 Overall yield coefficients

Experiment Name	$Y_{X/S}$ g/g	$Y_{P/X}$ mg/g	$Y_{P/S}$ mg/g
M-0.03	0.10	23.81	2.49
MS-0.03	0.15	13.86	2.08
MSS-0.03	0.17	9.83	1.71
MSSM-0.03	0.17	6.76	1.17

The cell yields on substrate in conditions containing sorbitol are close to each other. Doubling the sorbitol addition to the medium did not have a significant effect on cells' carbon source utilization metabolism. The highest cell yields on substrate was obtained at MSS-0.03 and MSSM-0.03 conditions, as $Y_{X/S}$=0.17 g/g. The lowest $Y_{X/S}$ value, on the other hand, is obtained in medium containing methanol as sole carbon and energy source. The highest overall product yield on cell ($Y_{P/X}$=23.81 mg/g) was obtained in the medium without sorbitol. Furthermore, the highest overall product yield on the total carbon source ($Y_{P/S}$=2.49 mg/g) was obtained again in M-0.03 condition. High yields with no co-substrate addition were expected since with relatively low cell concentration (30.95 g/L) 180 mg/L rhHG was obtained. In MSS-0.03, the highest rhGH concentration, 301 mg/L, was obtained with a cell concentration of 52.7 g/L. Although the overall yield coefficients are important parameters to evaluate the efficiency and profitability of the bioprocess, it should be kept in mind that for products having high commercial values, the final amount of product is more crucial. hGH is a protein having high values for low volumes therefore increasing the amount of final product is the major aim of this study. Hence, the aim of increasing the amount of final product was achieved. 1.58-fold higher rhGH was produced than the highest amount reported in literature (190 mg/L) (Eurwilaichitr et al., 2002). Furthermore, by adding second sorbitol to the medium 1.1-fold higher rhGH was obtained than with addition of single sorbitol. Furthermore, in literature the highest rhGH production was achieved in 3 days of induction in complex medium (Eurwilaichitr et al., 2002). By adding batch-wise sorbitol to a defined medium, even once, 1.43-fold higher rhGH was obtained in

24 hours, which is one third of the case in literature. Therefore in shorter fermentations with *P. pastoris*, higher amounts of r-protein can be produced with sorbitol as co-carbon source, which can decrease the cost of the bioprocess significantly by means of both substrate and energy demand.

The variation in total specific growth rate (μ_t), specific sorbitol consumption rate (q_S), specific methanol consumption rate (q_M), specific recombinant product formation rate (q_{rp}), specific oxygen uptake rate (q_O) and cell yields on oxygen throughout the production processes with different feeding profiles are given in Table 4.6. Although a specific growth rate of μ_0=0.03 h^{-1} was selected for all experiments, it can only be achieved in average, but not strictly constant at the desired value. Especially, for the case when only methanol was used; the average of specific growth rate was lower than 0.03. This is because cells grown only on methanol undergoes a long lag-phase and the induction was stopped just at the beginning of exponential growth. Comparing the specific growth rates of all experiments, results evidently reveal that adding sorbitol batch-wise to the bioreactor medium resulted in higher specific growth rates. Similar results were obtained by Çelik (2008). In that study, in the presence of sorbitol higher total specific growth rate values were obtained and the deviation from the desired value was explained with the growth on sorbitol such that $\mu_t = \mu_0 + \mu_S$.

Naturally, the specific sorbitol consumption rates were the highest at the beginning of the process (0.145 g g^{-1} h^{-1} for MS-0.03, 0.129 g g^{-1} h^{-1} for MSS-0.03 and 0.137 g g^{-1} h^{-1} for MSSM-0.03) and then they decreased since cell concentration increases continuously while sorbitol concentration decreases. When the sorbitol concentration was increased after t=9 h in MSS-0.03 and MSSM-0.03, increases in specific sorbitol consumption rates was obviously seen at t=12 h. Just before total depletion of sorbitol q_S decreased to 0.068, 0.042 and 0.047 g g^{-1} h^{-1} for MS-0.03, MSS-0.03 and MSSM-0.03, respectively. Specific methanol consumption rates did not change notably during the processes up to the cessation of cell growth. After that point small increases were observed since increasing amounts of methanol were added to the medium although there was no increase in cell growth. The stability of specific methanol consumption rates

has two apparent exceptions. First one is the process where there is no sorbitol in the medium. q_M increases continuously in the absence of sorbitol from 0.072 to 0.111 g g^{-1} h^{-1}. This was an expected result since cell growth is very slow although increasing amounts of methanol was added to the medium. The second case is in MSSM-0.03, after addition of extra methanol at t = 24h. The specific methanol consumption rate increases from 0.064 g g^{-1} h^{-1} to 0.120 g g^{-1} h^{-1}. This two-fold increase in q_M is also logical since the methanol feed rate was almost doubled after termination of cell growth. The highest specific recombinant protein production rate was obtained in MS-0.03 strategy as q_{rp}=0.463 mg g^{-1} h^{-1}. The specific oxygen uptake rate values are generally higher in medium containing sorbitol, especially at the beginning of the production phase. This was logical since same amount of methanol was fed to M-0.03 and MS-0.03. Therefore same amount of methanol was consumed by the cells together with sorbitol in MS-0.03. When there were two substrates, metabolism works better and without undergoing a lag phase, they immediately started to proliferate and produce proteins. Many of the amino acids required for the protein synthesis are produced from oxidative pathway metabolites. Therefore addition of sorbitol caused an increase in r-protein production resulting an increase in oxygen demand due to the increase in amino acid demand. Furthermore, the cell yields on oxygen throughout the production processes for medium containing sorbitol is higher than the medium only with methanol. This may be due to the fact that sorbitol supports cell growth better than methanol therefore higher cell concentrations were attained in the presence of sorbitol then in the presence of only methanol, without undergoing into a lag-phase.

Some total specific growth rate values and cell yields on oxygen cannot be computed (MS-0.03: t=21 h and 24 h; MSS-0.03: t=30 h) since cell growth terminated and even cell death started in these last hours of fermentation and negative values of variation in cell concentration with respect time were obtained. To illustrate, in MS-0.03 condition, cell concentration was C_X=43.1 g/L at t=18 h where it decreases to C_X=42.4 and 42.3 g/L at t=21 and 24 h, respectively. Similarly, in MSS-0.03 condition, at t=27 h cell concentration was measured as C_X=53.8 g/L and than at t=30 h it was found as C_X=52.7 g/L.

Table 4.6 Variation in specific rates throughout the bioprocesses

Exp. Name	t h	C_X g L^{-1}	μ_t h^{-1}	q_s g g^{-1} h^{-1}	q_M g g^{-1} h^{-1}	q_{rp} mg g^{-1} h^{-1}	q_o g g^{-1} h^{-1}	$Y_{x/o}$
M-0.03	3	24.6	0.002		0.072	0.071	0.116	0.06
	6	25.4	0.005		0.076		0.098	0.09
	9	25.8	0.009		0.082	0.316	0.086	0.11
	12	26.9	0.012		0.086		0.058	0.17
	15	27.4	0.015		0.093	0.379	0.113	0.09
	18	28.9	0.018		0.096		0.078	0.12
	21	29.3	0.020		0.104	0.286	0.062	0.15
	24	30.0	0.023		0.111	0.190	0.083	0.10
MS-0.03	3	25.7	0.039	0.145	0.080	0.112	0.153	0.24
	6	29.1	0.046	0.120	0.077		0.111	0.39
	9	33.4	0.045	0.098	0.076	0.463	0.100	0.41
	12	37.5	0.038	0.082	0.072		0.103	0.34
	15	41.7	0.028	0.068	0.073	0.420	0.093	0.26
	18	43.1	0.016		0.075	0.346	0.067	0.18
	21	42.4	0.000		0.086	0.220	0.045	
	24	42.3			0.091	0.025	0.059	
MSS-0.03	3	27.3	0.045	0.129	0.064	0.121	0.260	0.17
	6	31.8	0.043	0.084	0.060		0.187	0.22
	9	36.1	0.040	0.051	0.058	0.380	0.089	0.42
	12	39.1	0.037	0.101	0.058		0.096	0.36
	15	43.6	0.032	0.080	0.057	0.392	0.077	0.37
	18	45.7	0.027	0.067	0.060		0.057	0.42
	21	49.9	0.020	0.053	0.060	0.274	0.074	0.23
	24	53.3	0.014	0.042	0.061	0.171	0.045	0.21
	27	53.8	0.007		0.066	0.052	0.091	0.03
	30	52.7			0.074	0.070	0.068	
MSSM-0.03	3	27.6	0.043	0.137	0.065	0.173	0.127	0.32
	6	32.2	0.041	0.073	0.061		0.216	0.18
	9	34.7	0.040	0.027	0.062	0.296	0.145	0.26
	12	38.3	0.036	0.085	0.061		0.091	0.37
	15	43.2	0.032	0.072	0.059	0.265	0.037	0.78
	18	45.4	0.028	0.064	0.062		0.052	0.47
	21	50.2	0.022	0.055	0.061	0.170	0.053	0.35
	24	52.4	0.017	0.049	0.064	0.102	0.079	0.16
	25	53.1	0.019	0.047	0.120		0.051	0.22
	27	54.2	0.015		0.117	0.033	0.049	0.15
	30	54.8	0.008		0.116	0.049	0.097	0.04

4.4.12 Oxygen Transfer Characteristics of the Bioprocess

Dynamic Method was applied to find the oxygen transfer parameters throughout the rhGH production by *P. pastoris* in fed-batch media with different feeding rates. These oxygen transfer parameters are oxygen uptake rate (OUR), oxygen transfer rate (OTR), and oxygen transfer coefficient, K_La. The physical oxygen transfer coefficient, K_La_0, was measured in the medium without microorganism. The variations in volumetric mass transfer coefficient (K_La), the enhancement factor E (K_La/K_La_0), oxygen transfer rate (OTR), oxygen uptake rate (OUR), maximum possible oxygen utilization rate or oxygen demand (OD), Damköhler number (Da) and effectiveness factor (η) throughout the bioprocesses are given in Table 4.7.

Overall liquid phase mass transfer coefficient, K_La, depends on agitation rate, temperature, rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. Expectedly, K_La, first increased at the beginning of the fermentation process (t=0-12 h), and then it decreased with the cultivation time when there was sorbitol in the medium. For example, in MS-0.03, K_La is 0.092 h^{-1}, then it continuously increase and achieve its highest value at t=12 h as 0.136 h^{-1}. After that, although there are some small oscillations it decreases continuously to 0.091 h^{-1} at t=24 h. Temperature and agitation rate were kept constant throughout the production process, therefore the reason for the changes in K_La is due to the change in properties of the fermentation medium. As the cells grow and secrete metabolites and proteins to the extracellular medium resistance zone for mass transfer increases. Additionally, high cell density processes with *Pichia pastoris* causes cell coalescence. However, K_La profile in M-0.03 is not similar to the ones containing sorbitol in the medium. In fact, K_La values for M-0.03 do not change significantly during the production process most probably because low cell concentration in M-0.03 did not cause significant resistance for oxygen transfer. So, K_La ranges between 0.052 to 0.089 h^{-1} in M-0.03. The change in rheology of medium also results in an increase in resistance for oxygen transfer. Obviously, oxygen transfer rates show similar variations with K_La with cultivation time. The

oxygen uptake rate and oxygen transfer rate show similar manner during the process for all conditions and their values are close to each other, and as a general trend OTR is a little larger than OUR. This indicates that cells consumed nearly all oxygen transferred to the medium. Therefore keeping dissolved oxygen level above %20 was appropriate choice. Generally, OUR values in medium containing sorbitol as co-substrate are higher than M-0.03. In MS-0.03, MSS-0.03 and MSSM-0.03, the highest OUR values are $33.6x10^{-3}$, $61.5x10^{-3}$ and $60.3x10^{-3}$ mol m^{-3} s^{-1} where in M-0.03 it is $26.8x10^{-3}$ mol m^{-3} s^{-1}. This may be due to the increasing oxygen demand of cells in the presence of sorbitol due to higher cell concentrations and higher amounts of protein production.

The maximum possible oxygen utilization rate or oxygen demand (OD), a kind of Damköhler number, Da, and effectiveness factor, η, were also found as defined by Çalık et al. (2000). As a general trend, OD increases as cell concentration increases expectedly. Damköhler number greater than 1 (Da>>1) refers to a process where mass-transfer resistances are effective. Conversely, Damköhler number smaller than 1 (Da<1) means that the mass transfer is biochemical reaction limited. For all strategies used for rhGH production with *P. pastoris*, Da is close to or greater than 1. This means that both resistances are effective in oxygen transfer. In high cell density fermentations it is expected to observe mass transfer resistances due to rheology of the fermentation medium. However it was also expected to observe biochemical reaction limitation since methanol is fed to the medium at growth limiting rates. At t=27 and 30 h of MSS-0.03 and MSSM-0.03 conditions, an increase in Da was observed. At these hours, mass transfer resistance in oxygen transfer is dominant. For the effectiveness factor, η =1 is the ideal condition. However, from Table 4.7, it can be seen that η is lower than 1 for all cases indicating that the cells are consuming lower oxygen than the oxygen demand. The highest effectiveness factor value was attained in both MS-0.03 and MSS-0.03 as η=0.31.

Table 4.7 The variations in oxygen transfer parameters with different feeding strategies

Exp. Name	t (h)	K_La (s^{-1})	E K_La/K_La_0	OTRx10^3 (mol $m^{-3}s^{-1}$)	$OTR_{max}x10^3$ (mol $m^{-3}s^{-1}$)	OURx10^3 (mol $m^{-3}s^{-1}$)	OD x10^3 (mol $m^{-3}s^{-1}$)	Da	η
M-0.03	0	0.070	6.30	23.4	31.4	21.0	643.0	20.3	0.03
	3	0.080	7.20	26.6	36.0	24.8	514.3	14.3	0.05
	6	0.070	6.30	24.2	31.4	21.5	363.8	11.5	0.06
	9	0.067	6.03	23.0	30.0	19.2	284.0	9.5	0.07
	12	0.052	4.66	17.4	23.3	13.5	190.6	8.2	0.07
	15	0.089	7.99	28.1	39.8	26.8	368.1	9.2	0.07
	18	0.069	6.21	21.8	31.0	19.7	284.9	9.2	0.07
	21	0.053	4.75	18.1	23.7	15.8	239.8	10.1	0.07
	24	0.072	6.48	23.0	32.3	21.5	360.9	11.2	0.06
MS-0.03	0	0.092	8.28	29.0	36.5	27.8	244.4	6.7	0.11
	3	0.119	10.71	36.4	47.2	34.1	132.6	2.8	0.26
	6	0.100	8.97	30.7	39.6	28.0	90.8	2.3	0.31
	9	0.119	10.71	33.5	47.2	28.8	97.7	2.1	0.30
	12	0.136	12.24	38.2	54.0	33.4	135.0	2.5	0.25
	15	0.121	10.89	34.7	48.0	33.6	193.3	4.0	0.17
	18	0.093	8.37	26.6	36.9	25.0	287.9	7.8	0.09
	21	0.067	6.03	18.6	26.6	16.6			
	24	0.091	8.19	26.0	36.0	21.5			
MSS-0.03	0	0.072	6.48	24.0	29.7	24.2	85.7	2.9	0.28
	3	0.162	14.58	56.8	67.2	61.5	200.3	3.0	0.31
	6	0.162	14.58	42.2	67.2	51.7	176.7	2.6	0.29
	9	0.116	10.45	28.6	48.2	27.9	103.2	2.1	0.27
	12	0.118	10.65	34.1	49.1	32.7	133.3	2.7	0.25
	15	0.122	10.98	30.1	50.6	29.1	141.9	2.8	0.21
	18	0.099	8.92	24.5	41.1	22.5	133.0	3.2	0.17
	21	0.120	10.77	33.2	49.6	32.0	266.2	5.4	0.12
	24	0.091	8.19	22.6	38.0	21.1	302.2	8.0	0.07
	27	0.154	13.88	38.1	64.0	42.5	2562.2	40.0	0.02
	30	0.135	12.12	33.2	55.9	31.0			
MSSM-0.03	0	0.105	9.47	35.1	45.9	33.2	121.1	2.6	0.27
	3	0.095	8.55	31.6	41.4	30.5	106.0	2.6	0.29
	6	0.149	13.44	46.9	65.1	60.3	221.2	3.4	0.27
	9	0.154	13.83	47.9	67.0	43.5	164.2	2.5	0.27
	12	0.103	9.23	31.4	44.7	30.4	126.8	2.8	0.24
	15	0.059	5.34	18.2	25.9	13.7	67.7	2.6	0.20
	18	0.080	7.20	25.8	34.9	20.5	118.2	3.4	0.17
	21	0.076	6.81	24.4	33.0	23.1	175.9	5.3	0.13
	24	0.117	10.54	37.9	51.0	36.1	385.8	7.5	0.09
	25	0.094	8.43	26.0	40.8	23.4	293.6	7.2	0.08
	27	0.084	7.56	24.1	36.6	22.9	448.3	12.2	0.05
	30	0.117	10.54	49.5	51.0	46.4	1807.5	35.4	0.03

CHAPTER 5

CONCLUSION

In this study, different carbon sources and feeding strategies were investigated for recombinant human growth hormone production by *Pichia pastoris*. The strain to be used for rhGH production was selected between the two phenotypes having different methanol utilizations (*P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S}). Thereafter, using the chosen phenotype, effects of a number of different carbon sources on r-protein production were investigated. Among these carbon sources, the ones which support cell growth and do not repress the recombinant protein production were determined. Then, using the selected carbon sources and optimized defined medium concentrations, effect of feeding strategies on cell growth, oxygen transfer and fermentation characteristics, rhGH production and by-product formation together with expression levels of *hGH* and *AOX1* were investigated by using pilot scale bioreactor.

Two phenotypes, *P. pastoris hGH*-Mut^{+} and *P. pastoris hGH*-Mut^{S}, which were results of integration of *pPICZαA::hGH* vector into *P. pastoris* (Orman et al. (2009)), were used during the investigation of alternative carbon sources. A comparison between those phenotypes was done by using two different carbon sources; AOX repressing glycerol, and non-repressing sorbitol as co-carbon sources with methanol in shake flask bioreactors. For glycerol, the optimized conditions by Orman et al. (2008) were used (30 g/L glycerol and 3% (v/v) methanol for *P. pastoris hGH*-Mut^{+} strain and 30 g/L glycerol and 1% (v/v) methanol for *P. pastoris hGH*-Mut^{S}). In the case of glycerol/methanol mixed feeds in a defined medium; production of rhGH (C_{rp}=110 mg/L) is almost two-fold higher in *P. pastoris hGH*-Mut^{S}. Furthermore, expression levels of *hGH* gene in Mut^{S} phenotype is higher than Mut^{+} at every selected period of the

induction phase. At the end of production, expression level in Mut^S phenotype ($6.76x10^9$ copies/mg CDW) reaches to a value 3.25-fold higher then Mut^+ ($2.08x10^9$ copies/mg CDW) grown on glycerol/methanol.

The alternative production medium contained sorbitol such that there were equivalent amount of carbon molecules to the optimized glycerol concentration, which makes 30 g/L. It was found that in the presence of sorbitol, above 1% (v/v) methanol exhibits inhibitory effect on cell growth of Mut^+. In the medium containing initially 30 g/L sorbitol and 1% (v/v) methanol, rhGH production in *P. pastoris hGH*-Mut^+ phenotype is 120 mg/L, which is 1.9-fold higher than that of Mut^S phenotype in the same medium. Expression level of *hGH* in both strains were close to each other at t=23 and 26 h where at t=47 h a significant increase was observed in *hGH* expression in Mut^+ ($9.84x10^9$ copies/mg CDW), 5.82 fold higher than Mut^S, which resulted in a 1.9-fold increase in amount of final product.

In shake flask bioreactors, the highest amount of rhGH (120 mg/L) was obtained by using Mut^+ phenotype of *P. pastoris* in medium containing 30 g L^{-1} sorbitol and 1% (v/v) methanol. On sorbitol, Mut^+ achieves 5.82-fold higher *hGH* expression and 1.9-fold higher extracellular recombinant protein concentration. Therefore, *AOX* expression levels were investigated only for Mut^+. In medium containing 30 g/L glycerol and 3% (v/v) methanol, a higher *AOX* expression level was attained throughout the process, however; this increase was not reflected to hGH production and on sorbitol more extracellular hGH was obtained. As a result of these investigations, it was decided to perform further investigations for rhGH production with *P. pastoris hGH*- Mut^+ and to take sorbitol as reference carbon source in evaluating alternative carbon sources.

Then, optimum and non-inhibitory concentration of sorbitol was investigated. Initial sorbitol concentrations of C_S = 20, 30, 40, 50, 60, 70 g/L were used with an initial methanol concentration of 1% (v/v) in laboratory scale bioreactors. Cell growth was inhibited above 50 g/L initial sorbitol concentration and amount of rhGH increases significantly as the initial sorbitol concentration increases until 50 g/L. Therefore 50 g/L sorbitol concentration was found to be the non-inhibitory sorbitol concentration.

To see the effects of alternative carbon sources mannitol, glucose, fructose, lactose, sucrose, citric acid, lactic acid and acetic acid were used in production medium in shake flask bioreactors and concentrations of these co-substrates were adjusted such that they involve equivalent carbon atoms to the optimized concentrations of glycerol or sorbitol. Although cells grown on sorbitol, reached the same cell concentration with glucose and fructose at the end, they have grown slower. When rhGH production was considered, it was seen that no rhGH production occurred in the presence of glucose and fructose. Therefore, it was concluded that fructose is not a suitable carbon source to be used in the production of recombinant proteins under control of AOX1 promoter of *P. pastoris*. Among the other alternative carbon sources, two couples show the same cell growth behavior; sorbitol/mannitol and lactose/sucrose. Sorbitol and mannitol supported the cell growth in the same extent and there were no significant difference in rhGH productions. Sucrose and lactose supported the growth in the same extent which is less that those of sorbitol and mannitol. However, in the presence of lactose rhGH production was less than sucrose. In media containing lactic acid, citric acid and acetic acid cell growth was totally inhibited therefore no rHGH was found in the extracellular medium. Among the different carbon sources used, sorbitol, mannitol, and sucrose seemed to be suitable for using in production medium. Sorbitol is much more economical than mannitol, therefore mannitol was eliminated. Hence, first pilot scale bioreactor experiments was decided to be performed using the two remaining carbon sources; sorbitol and sucrose.

Production of rhGH was then performed in pilot scale bioreactors having a working volume of V_R=1 L. In all bioreactor experiments, pH and temperature were kept at 5.0 and 30^{o}C, respectively, dissolved oxygen has been maintained above 20% of saturation to prevent oxygen limitation, and the agitation rate was maintained constant at N=900 rpm. A bioprocess including four phases was applied; glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and methanol fed-batch (MFB) phases. In the first three phases of each bioreactor experiment the same cell growth was achieved. At the beginning of MFB phase, the co-substrate to be used was added to medium batch-wise. In the

first three bioreactor experiments, cases in which sorbitol or sucrose was used as co-substrates of methanol were compared with the case in which there was no co-substrate addition. In medium containing sorbitol the long lag-phase (t=0-9 h) seen in two other cases was eliminated and cells started to proliferate immediately. At the end of the process, t=24 h, cell concentration reached to 42.3 g/L in medium with sorbitol, which is 1.4-fold higher than that of no co-substrate addition. Sucrose, on the other hand, did not support the cell growth and the cell concentration profile is parallel to the one containing only methanol. Although presence of sucrose in the medium does not repress AOX promoter, it does not support the cell growth as well as sorbitol. Thus, it was decided to focus on sorbitol containing production medium in order to develop a better feeding strategy for the production of rhGH. Results obtained from bioreactor medium containing sorbitol were analyzed in terms of cell growth, sorbitol and methanol consumptions and recombinant protein production. According to these results, two different strategies were developed to improve the first one.

In the bioreactor medium containing sorbitol cell growth stopped just at the same time where sorbitol in the medium was totally depleted (t=15 h). In the second strategy, it was decided to increase the sorbitol concentration in the medium to its initial value before its depletion. In a third bioreactor experiment, with the second sorbitol addition, methanol feeding scheme was also altered such that just after the cessation of cell growth (t=24 h) a pulse of methanol was given to the medium and then the feeding rate was kept constant at a value above the predetermined exponential scheme and below the inhibitory limit (4 g/L).

Effects of these different strategies on sorbitol and methanol consumption profiles were investigated. It takes 15 hours for the whole sorbitol to be depleted when there was single sorbitol addition. With the addition of second batch-wise sorbitol (MSS-0.03), sorbitol consumption rate was almost not changed and the sorbitol in the medium was totally used up at t=25 h. For all conditions, it was seen that there were no residual methanol in the medium since cells consumed all the methanol as far as it was added to the medium. This reveals the simultaneous utilization of sorbitol with methanol.

When sorbitol was added as a co-substrate at the beginning of the production phase, the lag-phase seen in cells grown only on methanol was eliminated and cells started to proliferate immediately. In MS-0.03 at t=24 h, cell concentration reached to 42.3 g/L, which is 1.4-fold higher than that of no co-substrate addition. In the medium containing sorbitol, cell growth ceases with the total consumption of sorbitol. When the sorbitol concentration in the medium was reincreased to its initial value at t=9 h (MSS-0.03); cell growth continues until the depletion of all sorbitol molecules in the medium, t=25 h. The highest cell concentration was achieved at t=27 h as 53.8 g/L. In MSSM-0.03, the addition of extra methanol at t=24 h did not cause any significant change in the cell growth after that hour and the highest cell concentration was attained at t = 30 h as 55 g/L.

The highest rhGH concentrations achieved in MS-0.03 and MSS-0.03 were C_{rp} =271 mg/L at t= 24 h and C_{rp} = 301 mg/L at t= 30 h, respectively. Therefore, rhGH production was increased for 1.11-fold by adding sorbitol two times to the medium. In MSSM-0.03, cells showed diverse response to the excess methanol added to the medium and therefore the highest recombinant protein production was C_{rp}=232 mg/L, which is lower than both MS-0.03 and MSS-0.03. The lowest rhGH concentration, C_{rp} = 180 mg/L, was obtained in the strategy with no co-substrate use where the only carbon and energy source was methanol.

Real time PCR results showed that expression level of *hGH* gene does not change during experiment M-0.03 and it attained a maximum value of $3.11x10^9$ copies/mg CDW at t=15 h. Addition of sorbitol to the medium definitely affects the expression levels positively. For MS-0.03, it was seen that the maximum level of transcription ($4.01x10^{10}$ copies/mg CDW) was achieved after 15 hours of induction. A close expression level ($4.17x10^{10}$ copies/mg CDW) was attained in MSS-0.03 however a later instant of the fermentation (t=24 h) since cells continue to proliferate until entire sorbitol in the medium was depleted, the point where maximum expression level was also delayed. Although almost the same expression levels per cell were attained in MS-0.03 and MSS-0.03, the higher cell concentration achieved in MSS-0.03 resulted in higher

rhGH production (271 mg/L in MS-0.03 and 301 mg/L in MSS-0.03). MSS-0.03 and MSSM-0.03 reach almost the same *hGH* expression levels in an supervening manner however the negative effects of extra methanol added to the medium was also observed in expression levels and after the pulse methanol addition expression of *hGH* decreased continuously.

AOX expression levels were investigated together with AOX activity assay results. The highest specific activity was obtained as 40.7 U/g CDW t=15 h of MS. Both in MSS-0.03 and MSSM-0.03, the maximum specific activity was less than this value; 34.8 and 32.9 U/g CDW, respectively. The specific AOX activity for M-0.03 started increasing after t=15 h, where the lag-phase ends. Therefore, addition of sorbitol in the production phase helps shortening the lag-phase while it provides reaching high AOX activities in shorter times. Behaviors of expression level profiles in each experiment were parallel to AOX activity profiles. The highest level of expression ($9.38x10^{10}$copies/mg CDW) was achieved in MS-0.03, at t = 15 h where AOX activity also reaches its maximum value. In MSS-0.03 and MSSM-0.03, almost the same copy numbers ($8x10^{10}$ copies/mg CDW) with maximum value of MS-0.03 were attained 3 hours later, at t =18 h.

Amino and organic acid profiles were also investigated for all conditions. For all feeding strategies, leucine (Leu), serine (Ser), glutamic acid (Glu) and glutamine (Gln) were not detected in the extracellular medium. This may be a result of amino acid content of hGH which contains 13.6 % Leu, 9.4% Ser, 7.3 % Glu and 6.8% Gln in its structure. Formaldehyde was detected in neither of the bioreactor media since it can be immediately oxidized to formic acid or it can go into the assimilatory pathway and enters glycolysis. Formic acid was detected in the medium in all conditions and its concentration increases suddenly in the last hours of fermentation where cell growth stops. Lactic acid is the main metabolic product formed in case of insufficient oxygen, when the TCA cycle cannot take place efficiently. This condition was seen only in medium without sorbitol. α-keto-glutaric, citric, fumaric and succinic acids were the other organic acids detected in the medium.

The highest cell yields on substrate was obtained at MSS-0.03 and MSSM-0.03 conditions, as $Y_{X/S}$=0.17 g/g. The lowest $Y_{X/S}$ value, on the other hand, was obtained in medium containing methanol as sole carbon and energy source ($Y_{X/S}$=0.10 g/g). The highest overall product yield on cell ($Y_{P/X}$=23.81 mg/g) was obtained in the medium without sorbitol. Furthermore, the highest overall product yield on the total carbon source ($Y_{P/S}$=2.49 mg/g) was obtained again in M-0.03 condition. Although the highest overall yields were mostly obtained in the absence of a co-substrate the aim of increasing the amount of final product was achieved. In MSS-0.03 (C_{rp}=301 mg/L) 1.67-fold higher rhGH than M-0.03 (C_{rp}=180 mg/L) was produced.

Among the oxygen transfer characteristics, overall liquid phase mass transfer coefficient, K_La, was first increased in the beginning of all fermentation processes (t=0-12 h), and then it decreased with the cultivation time. The oxygen uptake rate and oxygen transfer rate show similar manner during the process for all conditions and their values were close to each other, and as a general trend OTR is a little larger than OUR. This indicates that cells consumed nearly all oxygen transferred to the medium. Therefore keeping dissolved oxygen level above %20 was appropriate choice. For all strategies used for rhGH production with *P. pastoris*, Da was close to or greater than 1. This means that both mass transfer resistances and reaction rate limitation were effective in oxygen transfer.

In order to perform absolute quantification for expression level analysis, in a simultaneous work performed with bioreactor experiments, standard curves for both *hGH* and *AOX* genes were constructed using SYBR Green I assay in real-time PCR. Plasmid DNA, *pPICZαA::hGH*, was used for quantification of genomic DNA or complementary DNA extracted from samples taken during the production of rhGH from *P. pastoris*. Optimization of real time PCR process parameters for both genes were successfully performed and standard curves with efficiencies of 1.723 ± 0.0261 and 1.523 ± 0.0623 were constructed for *hGH* and *AOX*, respectively which should be in the range from E = 1.0 (minimum value) to E = 2.0 (theoretical maximum and efficiency optimum). Specificity of the amplified PCR products, whose amplification results were used to construct standard curves, were also verified with melting curve analysis

As a result of carbon sources and feeding strategies used in rhGH production with *Pichia pastoris hGH*-Mut$^+$, the highest rhGH production was achieved in pilot scale bioreactor in which fed-batch methanol feeding was applied at a growth limiting rate with μ=0.03 h^{-1} and co-substrate sorbitol was added twice as batch-wise (MSS-0.03). At the end of 30 hours of production 52.7 g/L cell concentration and 301 mg/L extracellular rhGH concentration was obtained. At this condition the highest *hGH* and *AOX* expression levels were $4.14x10^{10}$ and $8x10^{10}$ copies/mg CDW and the highest AOX activity was 34.8 U/g CDW. Overall yield coefficients in MSS-0.03 were $Y_{X/S}$=0.17 g/g, $Y_{P/X}$=9.83 mg/g and $Y_{P/S}$=1.71 mg/g. With the addition of second sorbitol to the production medium, rhHG production was 1.1-fold increased when compared to single sorbitol addition. In order to further increase the rhGH production three or more batch-wise sorbitol addition might be considered. However, in that case the fermentation would be longer and cell viability should be checked. Furthermore, *hGH* and *AOX* expressions were affected positively in the presence of sorbitol, so that higher gene expression levels were achieved. This positive effect of the used carbon source might be global such that not only recombinant gene and *AOX* but also the whole carbon source metabolism and related genes of the yeast cells might be affected. However, in order to clarify whether the carbon source effect is global or direct, wider investigations should be performed in genome scale or including the changes in metabolic fluxes and expression levels of many other important genes.

Thus, this work contributes not only to the investigation of alternative carbon sources and their feeding strategies that can be used in recombinant protein productions by *P. pastoris*, but also to further understanding of the relationship between production and expression levels of *hGH* and *AOX* genes. Furthermore, advantages of sorbitol addition to the production medium were verified by means of producing recombinant proteins in high cell density fermentations in shorter fermentation durations.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

0.125 M (or 0.5 M) EDTA, pH 8.0	4.65 g (or 18.61 g) Ethylenediaminetetra acetic acid disodium salt dihydrate was was dissolved in 80 mL dH_2O. NaOH was added until EDTA was dissolved. The final pH was further adjusted to pH 8.0 and the final volume was adjusted to 100 mL. The buffer was autoclaved and stored at room temperature.
1 M Tris-Cl, pH 8.0	12.1 g Tris base was dissolved in 80 mL dH_2O and the pH was adjusted to 8.0 by adding concentrated HCl. The volume was made up to 100 mL. The buffer was autoclaved and stored at room temperature.
Yeast Lysis Solution	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl-pH8.0, 1mM Na_2EDTA. The solution was autoclaved and stored at room temperature.
1.5 M Tris-HCl, pH 8.8	36.3 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 8.8 with 6N HCl. The buffer was made up to 200 mL with dH_2O. The buffer was autoclaved and stored at 2-8°C.
0.5 M Tris-HCl, pH 6.8	12.1 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 6.8 with 6N HCl. The buffer was made up to 200 mL with dH_2O. The buffer was autoclaved and stored at 2-8°C.
4 x Sample Loading Buffer for SDS-PAGE	200 mM Tris-HCl,pH 6.8; 40% glycerol; 6% SDS;

	0.013% Bromophenol blue; 10% 2-mercaptoethanol. Distributed into microcentrifuge tubes and stored at -20°C.
5x SDS-PAGE Running Buffer	15 g Tris Base, 72 g glycine, 5 g SDS, dH2O to 1 liter. The buffer was stored at 2-8°C and diluted 1:5 with dH_2O prior to use.
1 M potassium phosphate, pH 6.0	56.48 g KH_2PO_4, 14.8 g K_2HPO_4 was dissolved in dH_20 and the volume made upto 500 mL. The pH was controlled. The buffer was autoclaved and stored at room temperature.
20x YNB Stock solution	17 g Yeast Nitrogen Base without amino acids, 50 g $(NH_4)_2SO_4$ was dissolved in dH_20 and the volume made upto 500 mL. The solution was autoclaved, aliquoted into 50 mL Falcon® tubes and stored at room temperature in dark.
Fixer Solution	Mix 150 mL methanol + 36 mL acetic acid + 150 µL 37% formaldehyde and complete to 300 mL with distilled water. This solution can be used several times.
Pretreatment Solution	Dissolve 0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 400 mL distilled water by mixing with a glass rod. Take 8 mL and set aside for further use in developing solution preparation.
Silver Nitrate Solution	Dissolve 0.8 g silver nitrate in 400 mL distilled water and add 300 µL 37% formaldehyde
Developing Solution	Dissolve 9 g potassium carbonate in 400 mL distilled

	water. Add 8 mL from pretreatment solution and 300 µL 37% formaldehyde.
Stop Solution	Mix 200 mL methanol + 48 mL acetic acid and complete to 400 mL with distilled water
Antifoam	10 % (v/v) antifoam solution, prepared with dH_2O. Can be autoclaved once.
Base for Bioreactor	25 % NH_3OH (Sigma). No need to sterilize.
Phosphate Buffered Saline (PBS)	Dissolve 8 g of NaCl , 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.27 g of KH_2PO_4 in 800 ml of distilled H_2O. Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter, autoclave and store at room temperature.
Borate buffer (for Alkali proteases)	2.381 g Boraks ($Na_2B_4O_7$.10 H_2O) dissolved in 250 ml dH_2O. pH adjusted to 10 by 1 M NaOH (6-7 ml) and add dH2O till 500 ml. Filter and store at +4^oC.
0.05 M Sodium Acetate buffer (For acidic proteases)	Dissolve 0.713 ml acetic acid in 25 ml total dH_2O. Dissolve 2.052 g sodium acetate in 50 ml dH_2O. Titrate sodium acetate solution with acetic acid solution to pH 5.0, and final V= 50 ml. Then dilute to 500 ml. Autoclave and store at +4^oC.
0.05 M Sodium Phosphate Buffer (for neutral proteases)	Dissolve 6.70 g $Na_2HPO_4.7H_2O$ 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 CURVES

Calibration Curve for Bradford Assay

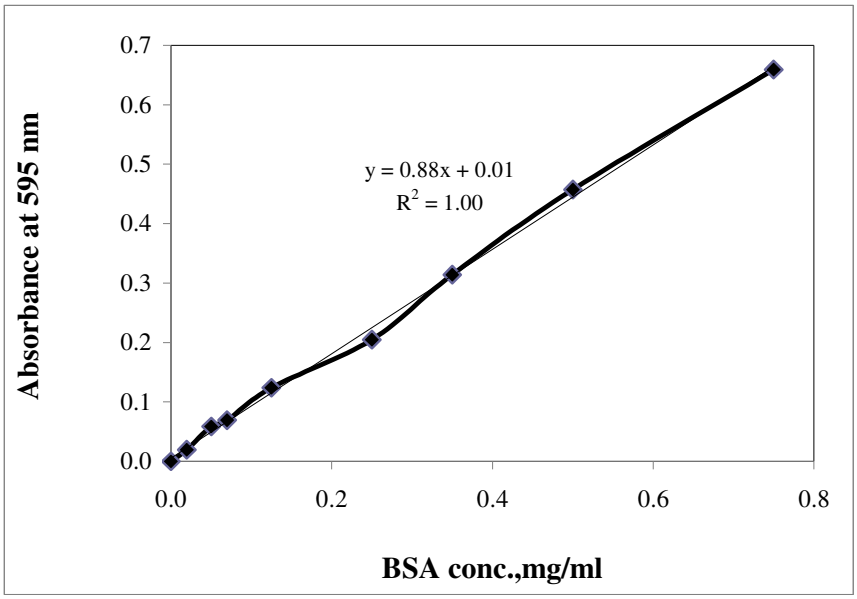

Figure B.1 Standard curve for Bradford Assay

Calibration Curve for Sorbitol Concentration

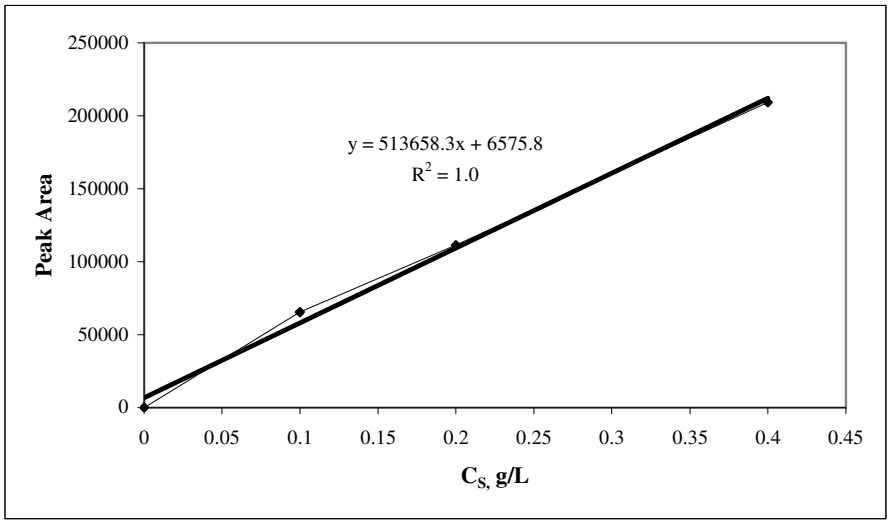

Figure B.2 Calibration curve obtained for sorbitol concentration; analysis was performed by HPLC

Calibration Curve for Methanol Concentration

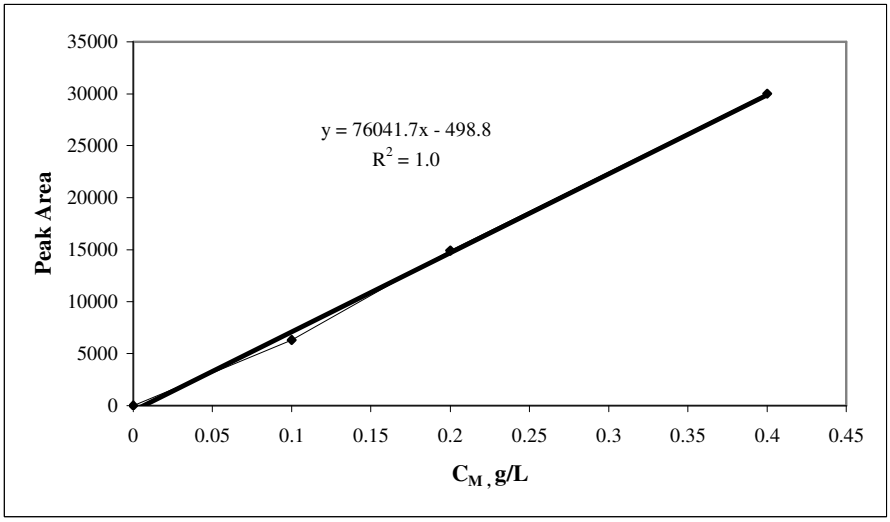

Figure B.3 Calibration curve obtained for methanol concentration; analysis was performed by HPLC

Calibration Curve for Succinic Acid Concentration

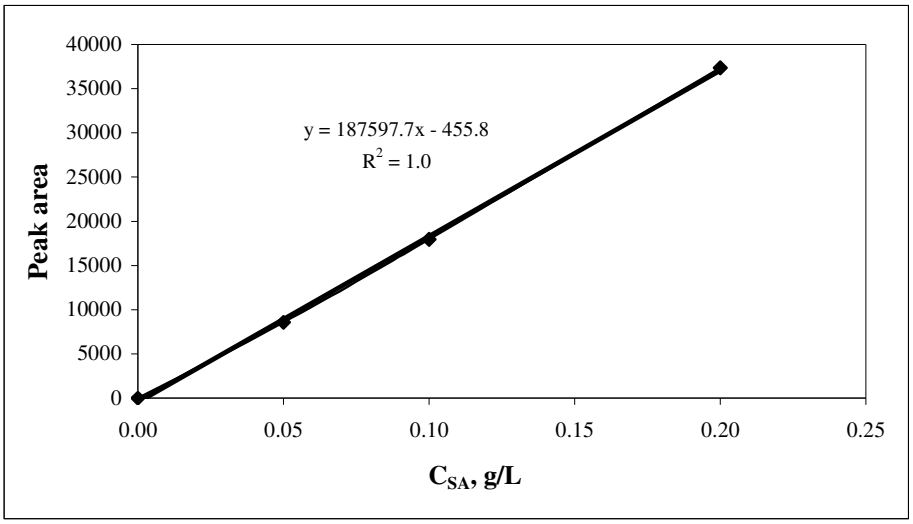

Figure B.4 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC

Calibration Curve for Meleic Acid Concentration

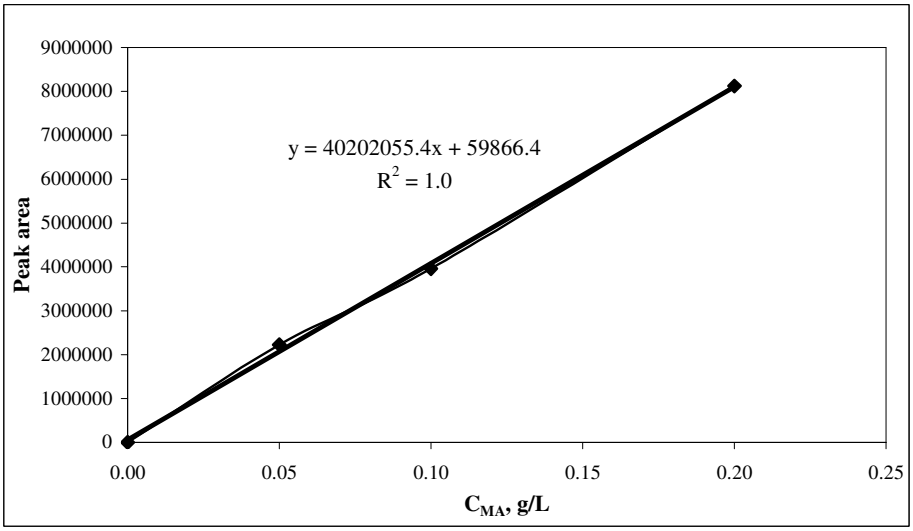

Figure B.5 Calibration curve obtained for maleic acid concentration; analysis was performed by HPLC

Calibration Curve for Glutaric Acid Concentration

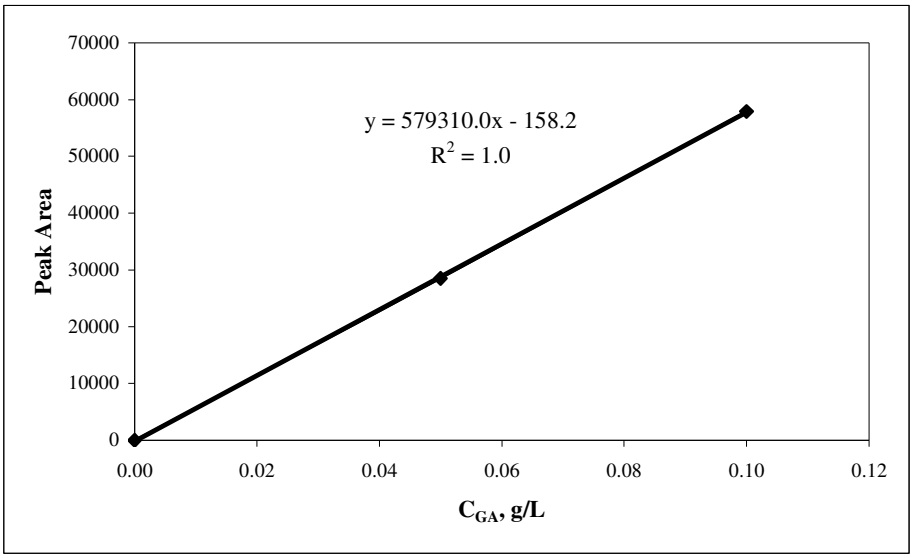

Figure B.6 Calibration curve obtained for glutaric acid concentration; analysis was performed by HPLC

Calibration Curve for Lactic Acid Concentration

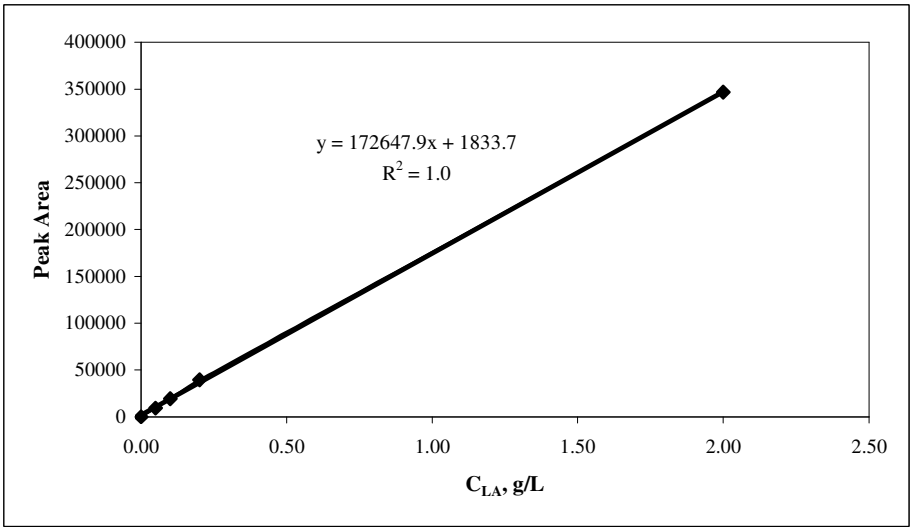

Figure B.7 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC

Calibration Curve for Formic Acid Concentration

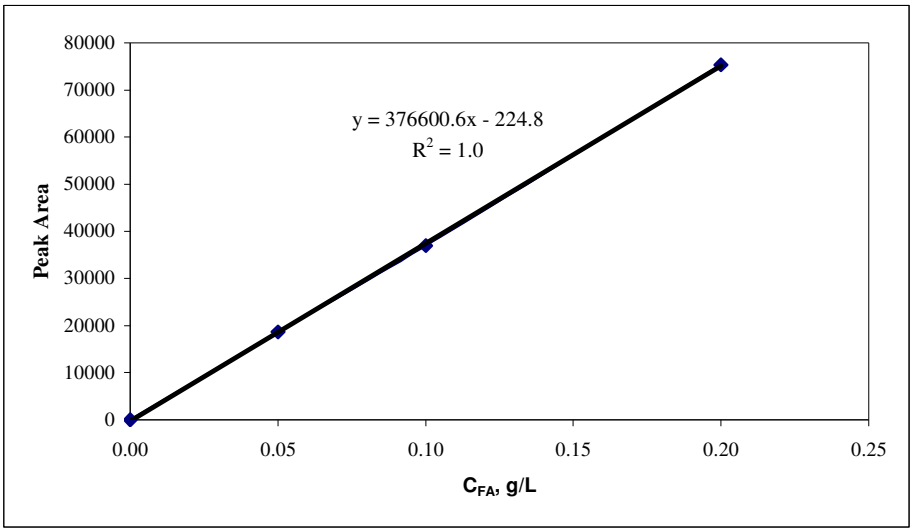

Figure B.8 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC

Calibration Curve for Citric Acid Concentration

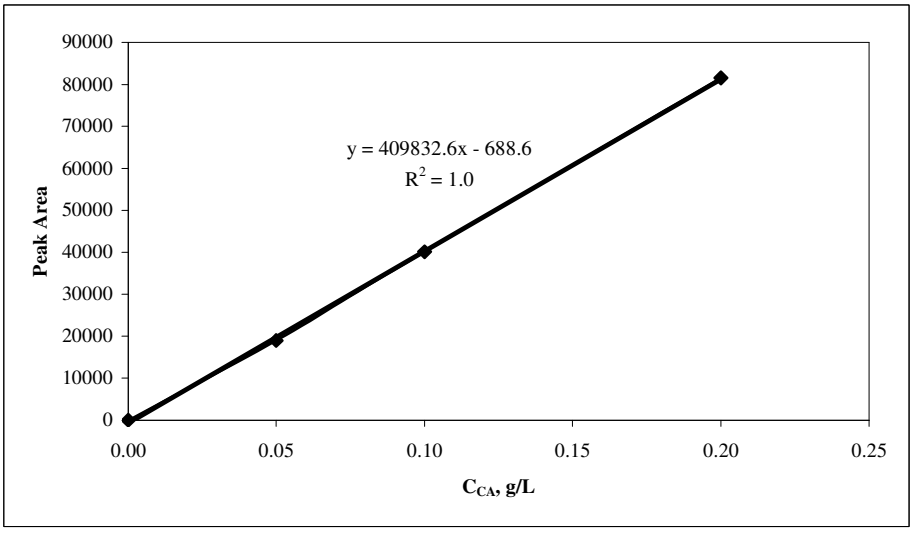

Figure B.9 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC

Calibration Curve for Fumaric Acid Concentration

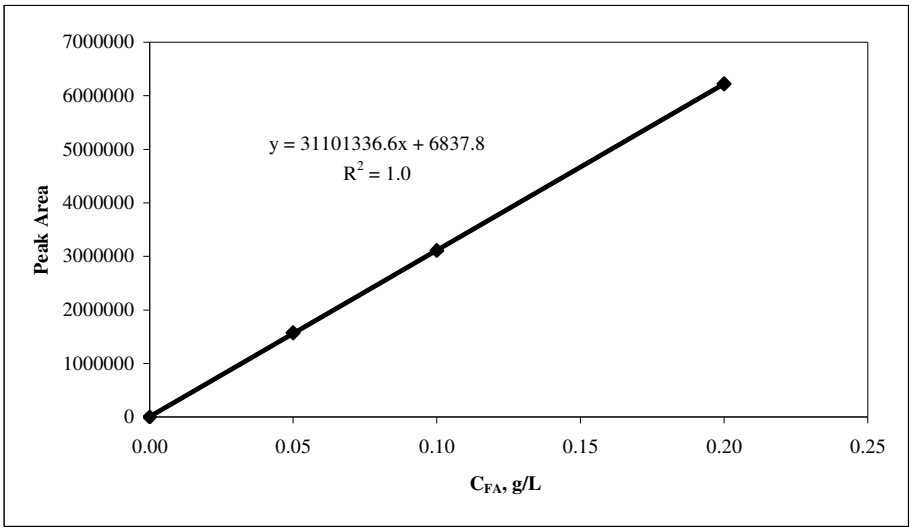

Figure B.10 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC

Calibration Curve for AOX Activity Assay

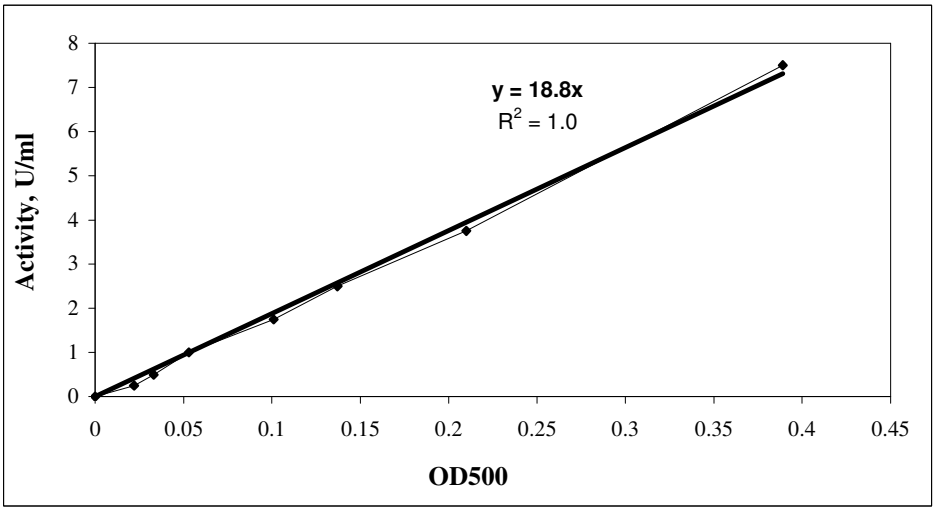

Figure B. 11 Calibration curve for AOX activity assay

APPENDIX C

ELECTROPHEROGRAM OF hGH STANDARD

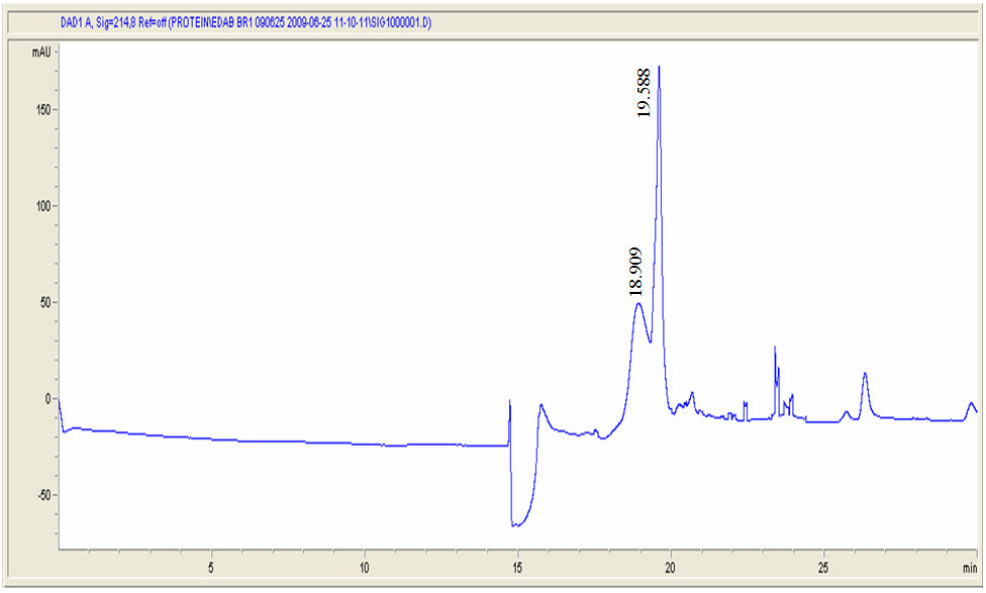

Figure C.1 Electropherogram of 0.05 g/L hGH standard.

APPENDIX D

MOLECULAR WEIGHT MARKER

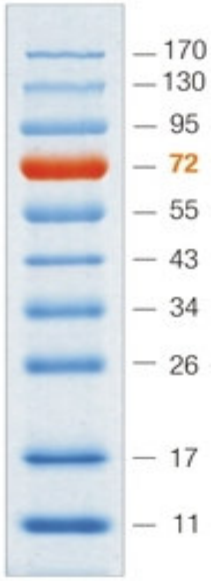

Figure D.1 PageRuler™ Prestained Protein Ladder (Fermentas)

APPENDIX E

CONTENTS OF THE KITS

Roche High Pure RNA Isolation Kit

- Lysis/Binding Buffer containing guanidine HCl and Triton X-100 (25 ml).
- DNase I, lyophilized (10 kU) (Dissolve DNase I in 0.55 ml Elution Buffer and store in aliquots at –15 to –25°C. A 0.11 ml aliquot is enough to process 10 samples).
- DNase Incubation Buffer (10 ml).
- Wash Buffer I containing guanidine HCl (33 ml) (Add 20 ml absolute ethanol to Wash Buffer I before use).
- Wash Buffer II (10 ml) (Add 40 ml absolute ethanol to Wash Buffer II before use).
- Elution Buffer (30 ml)
- High Pure Filter Tubes (50 tubes)
- Collection Tubes, 2 ml (50 tubes)

Roche Trancriptor High Fidelity cDNA Synthesis Kit

Vial/ Cap	Label	Content a) Cat. No. 05 081 955 001 (50 reactions) b) Cat. No. 05 091 284 001 (100 reactions) c) Cat. No. 05 081 963 001 (200 reactions)
1 red	Transcriptor High Fidelity Reverse Transcriptase	a) 1 vial, 55 µl b) 1 vial, 110 µl c) 1 vial, 220 µl • Storage buffer: 200 mM potassium phosphate, 2 mM dithiothreitol, 0.2% Triton X-100 (v/v), 50% glycerol (v/v), pH approx. 7.2
2 color-less	Transcriptor High Fidelity Reaction Buffer (5×)	a) 1 vial, 1 ml b) 1 vial, 1 ml c) 2 vials, each 1 ml • 5× conc.: 250 mM Tris/HCl, 150 mM KCl, 40 mM $MgCl_2$, pH approx. 8.5 (25°C)
3 color-less	Protector RNase Inhibitor	a) 1 vial, 50 µl (40 U/µl) b) 1 vial, 50 µl (40 U/µl) c) 2 vials, each 50 µl (40 U/µl) • Storage buffer: 20 mM Hepes-KOH, 50 mM KCl, 8 mM dithiothreitol, 50% glycerol (v/v), pH approx. 7.6 (at 4°C)
4 purple	Deoxynucleotide Mix	a) 1 vial, 200 µl b) 1 vial, 200 µl c) 2 vials, 200 µl • 10 mM each dATP, dCTP, dGTP, dTTP
5 blue	Anchored-oligo$(dT)_{18}$ Primer	a) 1 vial, 100 µl (50 µM) b) 1 vial, 200 µl (50 µM) c) 2 vials, each 200 µl (50 µM)
6 blue	Random Hexamer Primer	a) 1 vial, 100 µl (600 µM) b) 1 vial, 200 µl (600 µM) c) 2 vials, each 200 µl (600 µM)
7 color-less	DTT	1 vial, 1 ml, 0.1 M
8 color-less	Water, PCR grade	a) 1 vial, 1 ml b) 2 vials, 1 ml c) 3 vials, 1 ml
9 green	Control RNA	a) only Cat. No. 05 081 955 001 1 vial, 20 µl (50 ng/µl) • contains a stabilized solution of a total RNA fraction purified from an immortalized cell line (K562)
10 green	Control Primer Mix PBGD	a) only Cat. No. 05 081 955 001 1 vial, 40 µl • 5 µM forward and reverse primer specific for human porphobilinogen deaminase (PBGD)

Roche LightCycler FastStart DNA Master SYBR Green I kit

Vial/Cap	Label	Contents/Function a) Cat. No. 03 003 230 001 (96 reactions) b) Cat. No. 12 239 264 001 (480 reactions)
1a colorless cap	LightCycler® FastStart Enzyme	a) 1 vial 1a, 3 vials 1b for 3 vials, 64 µl each LightCycler® FastStart DNA Master SYBR Green I (10× conc.) b) 5 vials 1a, 15 vials 1b for 15 vials, 64 µl each LightCycler® FastStart DNA Master SYBR Green I (10× conc.) • Ready-to-use hot start PCR reaction mix (after pipetting 10 µl from vial 1a into one vial 1b). • Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye and 10 mM $MgCl_2$
1b green cap	LightCycler® FastStart Reaction Mix SYBR Green I, 10× conc.	
2 blue cap	$MgCl_2$ stock solution, 25 mM	a) 1 vial, 1 ml b) 2 vials, 1 ml each • To adjust $MgCl_2$ concentration
3 colorless cap	H_2O, PCR-grade	a) 2 vials, 1 ml each b) 7 vials, 1 ml each • To adjust the final reaction volume

APPENDIX F

DNA SEQUENCES AND PLASMIDS

Sequence of pPICZαA*::hGH* plasmid

agatctaacatccaaagacgaaaggttgaatgaaacctttttgccatccgacatccacaggtcca
ttctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgca
ggacctccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattggg
cttgattggagctcgctcattccaattccttctattaggctactaacaccatgactttattagcc
tgtctatcctggcccccctggcgaggttcatgtttgtttatttccgaatgcaacaagctccgcat
tacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtttcatgttcccc
aaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctca
tccaagatgaactaagtttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttcca
aaagtcggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataatctcattaa
tgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaatgggga
aacacccgctttttggatgattatgcattgtctccacattgtatgcttccaagattctggtggga
atactgctgatagcctaacgttcatgatcaaaatttaactgttctaacccctacttgacagcaat
atataaacagaaggaagctgccctgtcttaaacctttttttttatcatcattattagcttacttt
cataattgcgactggttccaattgacaagcttttgattttaacgacttttaacgacaacttgaga
agatcaaaaaacaactaattattcgaaacgatgagatttccttcaatttttactgctgttttatt
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cggctgaagctgtcatcggttactcagatttagaaggggatttcgatgttgctgttttgccattt
tccaacagcacaaataacgggttattgtttataaatactactattgccagcattgctgctaaaga
agaaggggtatctctcgagaaaagagaggctgaagctgaattc**caccatcaccatcaccatattg**
aagggagattcccaactataccactatctcgtctattcgataacgctatgcttcgtgctcatcgt
cttcatcagctggcctttgacacctaccaggagtttgaagaagcctatatcccaaaggaacagaa
gtattcattcctgcagaacccccagacctccctctgtttctcagagtctattccgacaccctcca
acagggaggaaacacaacagaaatccaacctagagctgctccgcatctccctgctgctcatccag
tcgtggctggagcccgtgcagttcctcaggagtgtcttcgccaacagcctagtgtacggcgcctc
tgacagcaacgtctatgacctcctaaaggacctagaggaaggcatccaaacgctgatggggaggc
tggaagatggcagcccccggactgggcagatcttcaagcagacctacagcaagttcgacacaaac
tcacacaacgatgacgcactactcaagaactacgggctgctctactgcttcaggaaggacatgga
caaggtcgagacattcctgcgcatcgtgcagtgccgctctgtggagggcagctgtggcttctagt
ctagaacaaaaactcatctcagaagaggatctgaatagcgccgtcgaccatcatcatcatcatca
ttgagtttgtagccttagacatgactgttcctcagttcaagttgggcacttacgagaagaccggt
cttgctagattctaatcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttt
tgatacttttttatttgtaacctatatagtataggatttttttgtcattttgtttcttctcgta
cgagcttgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggggtttgggaaaa

tcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtga
gaccttcgtttgtgcggatcccccacacaccatagcttcaaaatgtttctactccttttttactc
ttccagattttctcggactccgcgcatcgccgtaccacttcaaaacacccaagcacagcatacta
aattttccctctttcttcctctagggtgtcgttaattacccgtactaaaggtttggaaaagaaaa
aagagaccgcctcgtttctttttcttcgtcgaaaaaggcaataaaaatttttatcacgtttcttt
ttcttgaaatttttttttttagttttttctctttcagtgacctccattgatatttaagttaata
aacggtcttcaatttctcaagtttcagtttcatttttcttgttctattacaacttttttacttc
ttgttcattagaaagaaagcatagcaatctaatctaaggggcggtgttgacaattaatcatcggc
atagtatatcggcatagtataatacgacaaggtgaggaactaaaccatggccaagttgaccagtg
ccgttccggtgctcaccgcgcgcgacgtcgccggagcggtcgagttctggaccgaccggctcggg
ttctcccgggacttcgtggaggacgacttcgccggtgtggtccgggacgacgtgaccctgttcat
cagcgcggtccaggaccaggtggtgccggacaacaccctggcctgggtgtgggtgcgcggcctgg
acgagctgtacgccgagtggtcggaggtcgtgtccacgaacttccgggacgcctccgggccggcc
atgaccgagatcggcgagcagccgtgggggcgggagttcgccctgcgcgacccggccggcaactg
cgtgcacttcgtggccgaggagcaggactgacacgtccgacggcggcccacgggtcccaggcctc
ggagatccgtccccctttccttgtcgatatcatgtaattagttatgtcacgcttacattcacg
ccctcccccacatccgctctaaccgaaaaggaaggagttagacaacctgaagtctaggtcccta
tttatttttttatagttatgttagtattaagaacgttatttatatttcaaatttttctttttttt
ctgtacagacgcgtgtacgcatgtaacattatactgaaaaccttgcttgagaaggttttgggacg
ctcgaaggctttaatttgcaagctggagaccaacatgtgagcaaaaggccagcaaaaggccagga
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aatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccc
tggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttc
tcccttcgggaagcgtggcgctttctcaatgctcacgctgtaggtatctcagttcggtgtaggtc
gttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccgg
taactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggta
acaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactac
ggctacactagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaag
agttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttttttgtttgcaagc
agcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgac
gctcagtggaacgaaaactcacgttaagggattttggtcatgagatc

Note:

Red colored sequence is sequence belonging *hGH* gene.

Highlighted sequences are primers designed for *hGH* gene.

P. pastoris **Strain NRRL Y-11430 alcohol Oxidase (*AOX1*) Gene, Complete cds**

tttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagtcggcataccgt
ttgtcttgtttggtattgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtctc
tctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaatggggaaacacccgctttttgg
atgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgctgatagcct
aacgttcatgatcaaaatttaactgttctaacccctacttgacagcaatatataaacagaaggaa
gctgccctgtcttaaacctttttttttatcatcattattagcttactttcataattgcgactggt
tccaattgacaagcttttgattttaacgacttttaacgacaacttgagaagatcaaaaaacaact
aattattcgaaacgatggctatccccgaagagtttgatatcctagttctaggtggtggatccagt
ggatcctgtattgccggaagattggcaaacttggaccactccttgaaagttggtcttatcgaagc
aggtgagaacaacctcaacaacccatgggtctaccttccaggtatttacccaagaaacatgaagt
tggactccaagactgcttccttctacacttctaacccatctcctcacttgaatggtagaagagcc
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caaagatccggttttggtgacccaatcaagttgagagccgctggtgttaagcctttggtcaactt
gccaggtgtcggaagaaacttccaagaccactactgtttcttcagtccttacagaatcaagcctc
agtacgagtctttcgatgacttcgtccgtggtgatgctgagattcaaaagagagtctttgaccaa
tggtacgccaatggtactggtcctcttgccactaacggtatcgaagctggtgtcaagatcagacc
aacaccagaagaactctctcaaatggacgaatccttccaggagggttacagagaatacttcgaag
acaagccagacaagccagttatgcactactccatcattgctggtttcttcggtgaccacaccaag
attcctcctggaaagtacatgactatgttccacttcttggaatacccattctccagaggttccat
tcacattacctccccagacccatacgcagctccagacttcgacccaggtttcatgaacgatgaaa
gagacatggctcctatggtttgggcttacaagaagtctagagaaaccgctagaagaatggaccac
tttgccggtgaggtcacttctcaccaccctctgttcccatactcatccgaggccagagccttgga
aatggatttggagacctctaatgcctacggtggacctttgaacttgtctgctggtcttgctcacg
gttcttggactcaacctttgaagaagccaactgcaaagaacgaaggccacgttacttcgaaccag
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tatagtataggatttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctat
ctcgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttctt
ggtatttcccactcctcttcagagtacagaagattaagtgagacgttcgtttgtgcaagctt

Note:

Highlighted sequences are primers designed for *AOX1* gene

APPENDIX G

DETAILED RESULTS OF THE PRIMER DESIGN PROGRAMMES

Results of Primer 3 for *hGH* gene:

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER          2   20   60.11   55.00  3.00  1.00 gcctttgacacctaccagga
RIGHT PRIMER       239   20   60.45   55.00  4.00  2.00 acactaggctgttggcgaag
SEQUENCE SIZE: 255
INCLUDED REGION SIZE: 255

PRODUCT SIZE: 238, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

    1 ggcctttgacacctaccaggagtttgaagaagcctatatcccaaaggaacagaagtattc
       >>>>>>>>>>>>>>>>>>>>
   61 attcctgcagaacccccagacctccctctgtttctcagagtctattccgacaccctccaa
  121 cagggaggaaacacaacagaaatccaacctagagctgctccgcatctccctgctgctcat
  181 ccagtcgtggctggagcccgtgcagttcctcaggagtgtcttcgccaacagcctagtgta
                                          <<<<<<<<<<<<<<<<<<<<
  241 cggcgcctctgacag

KEYS (in order of precedence):
>>>>>> left primer
ADDITIONAL OLIGOS
                 start  len      tm     gc%   any   3' seq

 1 LEFT PRIMER       2   20   60.11   55.00  3.00  1.00 gcctttgacacctaccagga
   RIGHT PRIMER    237   20   59.50   55.00  4.00  2.00 actaggctgttggcgaagac
   PRODUCT SIZE: 236, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

 2 LEFT PRIMER       2   20   60.11   55.00  3.00  1.00 gcctttgacacctaccagga
   RIGHT PRIMER    244   20   60.71   60.00  4.00  1.00 gccgtacactaggctgttgg
   PRODUCT SIZE: 243, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

 3 LEFT PRIMER       2   20   60.11   55.00  3.00  1.00 gcctttgacacctaccagga
   RIGHT PRIMER    220   21   59.90   57.14  7.00  2.00 gacactcctgaggaactgcac
   PRODUCT SIZE: 219, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

 4 LEFT PRIMER       2   20   60.11   55.00  3.00  1.00 gcctttgacacctaccagga
   RIGHT PRIMER    165   19   60.13   57.89  7.00  2.00 atgcggagcagctctaggt
   PRODUCT SIZE: 164, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

Statistics
         con   too    in    in          no    tm    tm  high  high        high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab    ok
Left    1321     0     0     0     0     0   432   487     0     0     0    33   369
Right   1326     0     0     0     0     0   208   804     0     5     0    30   279
Pair Stats:
considered 483, unacceptable product size 469, high end compl 1, ok 13
primer3 release 1.1.4


(primer3_results.cgi release 0.4.0)
```

Results of Primer 3 for *AOX1* gene:

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER          5   20   60.00   50.00  6.00  1.00 gtcggcataccgtttgtctt
RIGHT PRIMER       197   20   60.43   45.00  5.00  2.00 ttcccaccagaatcttggaa
SEQUENCE SIZE: 306
INCLUDED REGION SIZE: 306

PRODUCT SIZE: 193, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00

    1 aaaagtcggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataatct
        >>>>>>>>>>>>>>>>>>>
   61 cattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaac
  121 gcaaatggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttc
                                                              <<<
  181 caagattctggtgggaatactgctgatagcctaacgttcatgatcaaaatttaactgttc
      <<<<<<<<<<<<<<<<<
  241 taacccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctttt
  301 ttttat

ADDITIONAL OLIGOS
                 start  len      tm     gc%   any   3' seq

 1 LEFT PRIMER       5   20   60.00   50.00  6.00  1.00 gtcggcataccgtttgtctt
   RIGHT PRIMER    198   20   59.34   45.00  5.00  3.00 attcccaccagaatcttgga
   PRODUCT SIZE: 194, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

 2 LEFT PRIMER       5   20   60.00   50.00  6.00  1.00 gtcggcataccgtttgtctt
   RIGHT PRIMER    206   20   59.24   50.00  2.00  0.00 tcagcagtattcccaccaga
   PRODUCT SIZE: 202, PAIR ANY COMPL: 6.00, PAIR 3' COMPL: 2.00

 3 LEFT PRIMER       4   20   59.62   50.00  6.00  3.00 agtcggcataccgtttgtct
   RIGHT PRIMER    197   20   60.43   45.00  5.00  2.00 ttcccaccagaatcttggaa
   PRODUCT SIZE: 194, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

 4 LEFT PRIMER     135   20   59.41   40.00  3.00  2.00 acccgctttttggatgatta
   RIGHT PRIMER    285   20   60.38   50.00  6.00  0.00 agggcagcttccttctgttt
   PRODUCT SIZE: 151, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

Statistics
         con   too    in    in          no    tm    tm  high  high        high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab    ok
Left    1911     0     0     0    16     0   562   753     0     5     0    21   554
Right   1889     0     0     0     9     0   661   707     0     3     9    22   478
Pair Stats:
considered 40, unacceptable product size 26, high end compl 1, ok 13
primer3 release 1.1.4


(primer3_results.cgi release 0.4.0)
```

APPENDIX H

AMINO ACID CODONS AND ABBREVIATIONS

Table H.1 Amino acid codons and abbreviations

Amino acid	One-letter code	Three-letter-code	Codons
Alanine	A	Ala	GCU, GCC, GCA, GCG
Cysteine	C	Cys	UGU, UGC
Aspartic Acid	D	Asp	GAU, GAC
GlutamicAcid	E	Glu	GAA, GAG
Phenylalanine	F	Phe	UUU, UUC
Glycine	G	Gly	GGU,GGC,GGA,GGG
Histidine	H	His	CAU, CAC
Isoleucine	I	Ile	AUU, AUC, AUA
Lysine	K	Lys	AAA, AAG
Leucine	L	Leu	CUU, CUC, CUA, CUG
Methionine	M	Met	AUG (start codon)
Asparagine	N	Asn	AAU, AAC
Proline	P	Pro	CCU, CCC, CCG,CCA,
Glutamine	Q	Gln	CAA, CAG
Arginine	R	Arg	CGU, CGC,CGA, CGG
Serine	S	Ser	AGU, AGC
Threonine	T	Thr	ACU, ACC, ACA, ACG
Valine	V	Val	GUU, GUC, GUA, GUG
Tryptophan	W	Trp	UGG
Tyrosine	Y	Tyr	UAU, UAC
Stop Codons			UGA, UAA, UAG

APPENDIX I

SAMPLE CALCULATIONS AND STANDARD CURVES FOR REAL TIME PCR ANALYSIS

Construction of Standard Curves

Mass of plasmid was calculated using Equation 3.4 given in Section 3.7.4.3. The plasmid used in this study involves 4146 base pairs (Orman, 2008). Then mass of plasmid becomes;

$$M_{plasmid} = (4166\ bp) * \left[1.096 * 10^{-21} \frac{g}{base\ pair} \right] = 4.54 * 10^{-9}\ ng$$

It was assumed that one plasmid involves only one copy of *AOX* and/or *hGH* genes.

The initial concentration of isolated plasmid, which was used as standard, was measured as 388 ng/μL with AlphaSpect μL Spectrophotometer (AlphaInnotech Inc., USA). Using Equation 3.3 number of copies of the gene of interest was calculated.

$$Copy\ number\ /\ \mu L = \frac{388\ ng\ /\ \mu L}{4.54 * 10^{-9}\ ng\ /1\ copy\ of\ desired\ \ gene} = 8.5 * 10^{10}\ copies\ /\ \mu L$$

In order to construct the standard curves, serially diluted samples from the initial plasmid stock containing 8.5×10^{10}copies/μL were used. These samples contained 10^8 to 10^4 copies/μL.

Real-Time RT-PCR

In order to have a better insight in production of the desired biomolecule; the relationship between the expression levels of the product and the important enzymes should be investigated. In this study, the product is rhGH and the enzyme is AOX, which have a crucial role in the methanol utilization pathway and hence in the production due to its tightly regulated promoter. To understand their relationship, their expression levels in the cell were investigated and in this context real time RT-PCR was employed. The expression levels of the genes of interest were determined with absolute quantification method using SYBR Green I assay then the specificity of the amplified PCR product was assessed by performing a melting curve analysis since melting curves allow discrimination between primer-dimers and specific product which melts at a higher temperature than the primer-dimers.

Optimization of Real Time-PCR for *hGH* and *AOX*

The RT-PCR is a complex assay and all physical and chemical components of the reaction are interdependent. They must be considered carefully when optimizing the specificity, sensitivity, reproducibility or reliability of the reaction. During this optimization process, after designing suitable primers for both genes, primer and $MgCl_2$ concentrations in the reaction mixture, annealing temperatures and times, extension times and the other cycling parameters were considered. Thereafter, in order to quantitate the expression levels of desired genes, calibration curves were formed. Both for *hGH* and *AOX*, plasmid DNA carrying these genes were used.

Real Time-PCR Conditions for hGH and AOX

One important step of optimization is to determine the concentrations of reaction mixture components such as primers and $MgCl_2$. Concentrations between 1mM-5mM was tried for $MgCl_2$ where 0.2 μM-0.5 μM was tried for each primers. Optimum concentrations for both left and primers of both genes

were found as 0.2 µM while optimum concentrations for $MgCl_2$ for *hGH* and *AOX* genes were found as 4 mM and 2mM, respectively. Summary of these optimized concentrations are given in Table I.1.

Table I.1 Optimized concentrations of components of the LightCycler® FastStart DNA Master SYBR Green I kit reaction mixture for real time RT-PCR analysis of *hGH* and *AOX* genes' expression levels

Component	*hGH*		*AOX1*	
	Volume, µL	**Final Conc.**	**Volume**	**Final Conc.**
H_2O, PCR grade	11.6		13.2	
$MgCl_2$ stock solution (25mM)	2.4	4 mM	0.8	2 mM
PCR Primer (4µM)	2	0.2 µM	2	0.2 µM
LightCycler® DNA Master SYBR Green I	2	1x	2	1x
DNA Template	2	10^4-10^8 copies/µL	2	10^4-10^8 copies/µL
Total volume	20		20	

The annealing temperature chosen for amplification should be 3-5^oC lower than the primer melting point temperature (Table 3.14). For *hGH* the annealing temperature is 55^oC where it is 60^oC for *AOX*. Furthermore, for typical primers, an incubation time of 0-10 s is usually chosen for the annealing step. For *hGH* the optimum annealing duration is found as 4s where it is 3s for *AOX*. These incubation times for annealing are in suggested range. In addition to these, all other cycling parameters were also optimized. These optimized values are summarized in Table I.2 for both genes.

Table I.2 Real time PCR conditions used for *hGH* and *AOX* genes

Gene name	Denaturation	Amplification	Melting Curve	Cooling
hGH	Cycle :1	Cycles :45	Cycle :1	Cycle :1
	95ºC, 10 min, none	Denaturation:95ºC,10s, none	Denaturation:95ºC, 0s, none	40ºC,30s, none
		Annealing : 55ºC, 4s, none	Annealing : 65ºC, 15s, none	
		Extension : 72ºC, 2s, none	Melting:95ºC (slope: 0.10C^{o}/sec), 10 s, continuous	
		Primer dimers inhibition: 83ºC, 1s single		
AOX1	Cycle :1	Cycles :40	Cycle :1	Cycle :1
	95ºC, 10 min, none	Denaturation:95ºC,10s, none	Denaturation:95ºC, 0s, none	40ºC,30s, none
		Annealing : 60ºC, 3s, none	Annealing : 65ºC, 10s, none	
		Extension : 72ºC, 1s, none	Melting:95ºC (slope: 0.10C^{o}/sec), 0s, continuous	
		Primer dimers inhibition: 83ºC, 1s single		

Standardization for *hGH* and *AOX* Genes

In this study, plasmid DNA was used for quantification of genomic DNA or complementary DNA extracted from samples taken during the production of rhGH from *P. pastoris*. Hence, standard curves were constructed using a plasmid containing both *hGH* and *AOX* genes, *pPICZαA::hGH*, which was isolated by Çalık et al. (2008). Reactions were performed on plasmid DNA series containing 10^4-10^8 copies/µL for both genes to set up standard curves. These standard curves were used for quantification by means of absolute quantification. The following amplification curves (Figures I.1 and I.2) were obtained using dilutional series of plasmid *pPICZαA::hGH*. The crossing point values for each concentration are as follows for *hGH,* Copies/µL : Cp (Crossing point); 10^8 : 15.09, 10^7 : 19.51, 10^6 : 24.04, 10^5 : 28.27, 10^4 : 31.64 and for *AOX1*; 10^8 : 16.08, 10^7 : 20.98, 10^6 : 26.40, 10^5 : 31.86, 10^4 : 40.09. The calibration curves constructed using these crossing point data are given in Figures I.3 and I.4 for *hGH* and *AOX*, respectively.

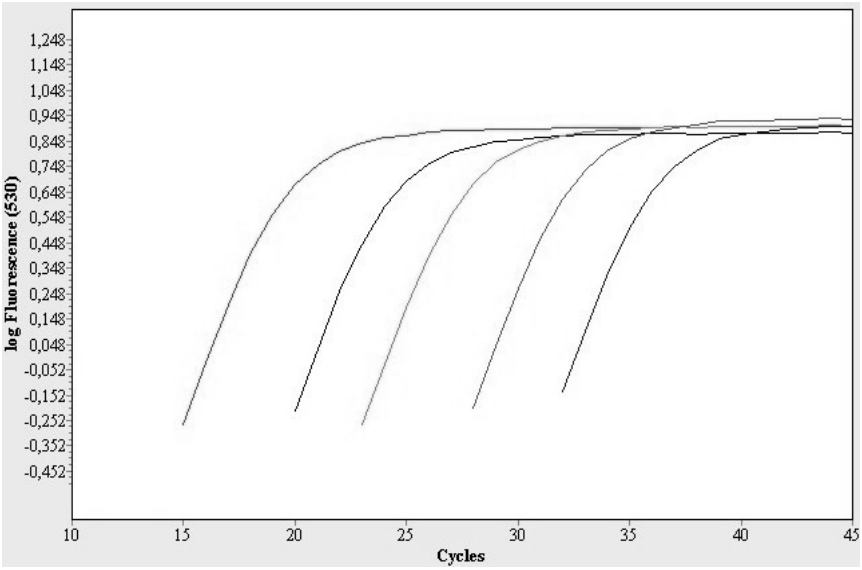

Figure I.1 Serially diluted samples containing 10^8 copies/µL, 10^7 copies/µL, 10^6 copies/µL, 10^5 copies/µL and 10^4 copies/µL plasmid DNA as starting template; amplified using the LightCycler DNA Master SYBR Green I for *hGH*. As a negative control, template DNA was replaced by PCR grade water.

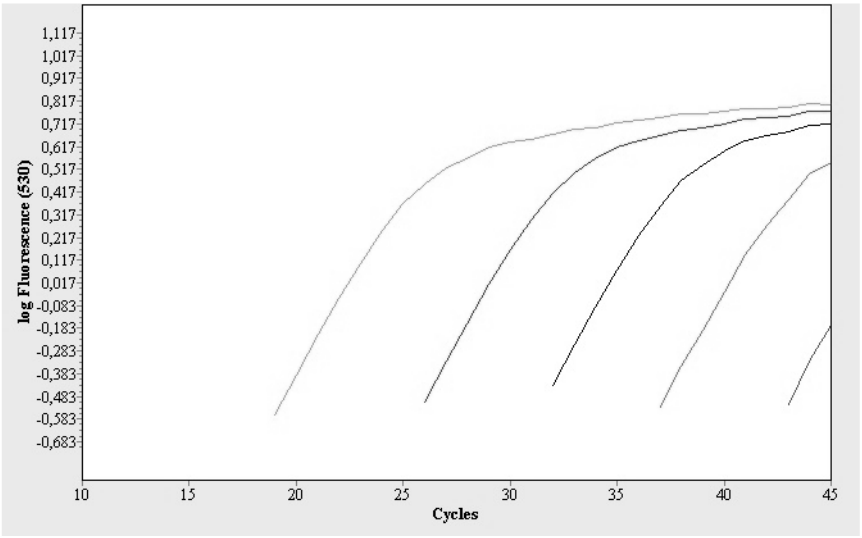

Figure I.2 Serially diluted samples containing 10^8 copies/µL, 10^7 copies/µL, 10^6 copies/µL, 10^5 copies/µL and 10^4 copies/µL plasmid DNA as starting template; amplified using the LightCycler DNA Master SYBR Green I for *AOX*. As a negative control, template DNA was replaced by PCR grade water.

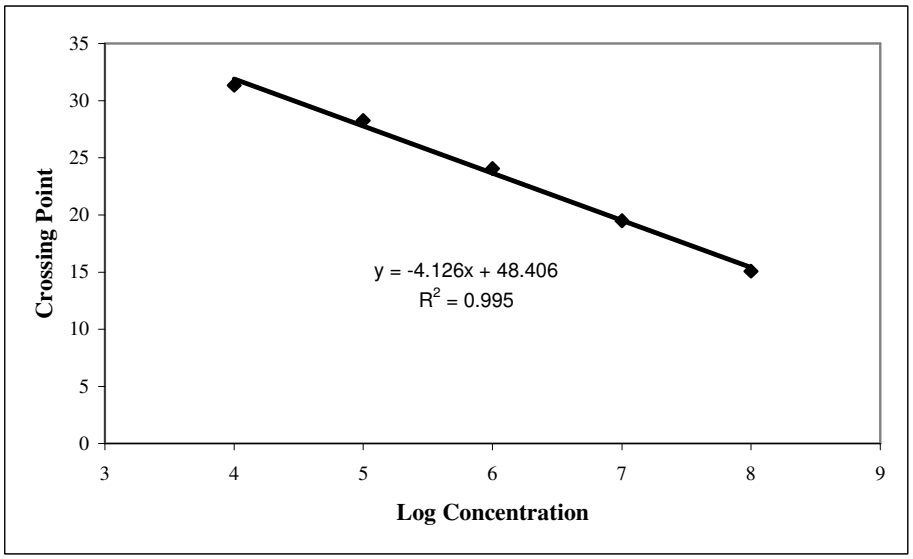

Figure I.3 Standard curve of a dilutional series of plasmid DNA standards for *hGH* with efficiency: 1.723 ± 0.0261

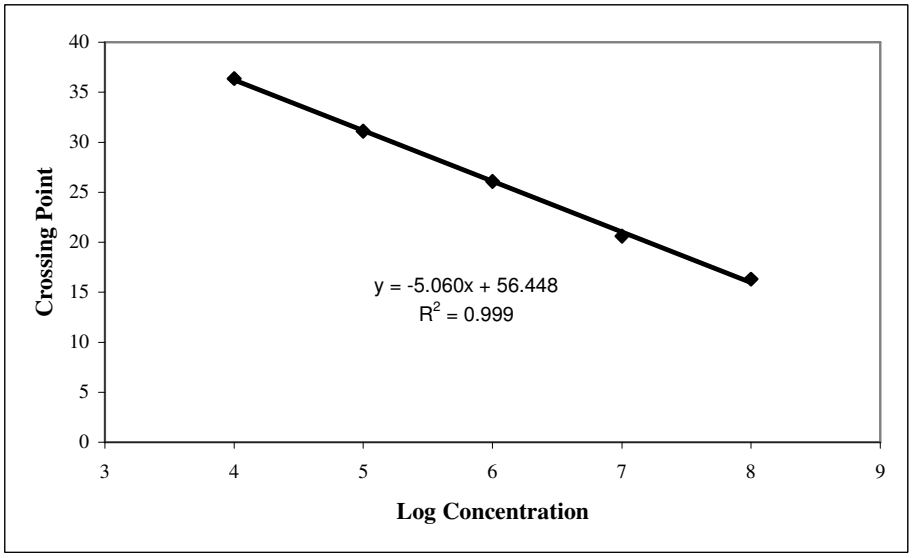

Figure I.4 Standard curve of a dilutional series of plasmid DNA standards for *AOX* with efficiency: 1.523 ± 0.0623

In order to determine reproducibility of the assays all experiments were rerun with same conditions and efficiency values were obtained as 1.759 ± 0.0479 for *hGH* and as 1.607± 0.0678 for *AOX*. Hence, the results of reruns have consistent efficiency values with the previous runs. Furthermore, real-time PCR efficiency is calculated from the calibration curve slope by the LightCycler Software 4.0, according to the established equation $E = 10^{[-1/slope]}$. This efficiency should be in the range from E = 1.0 (minimum value) to E = 2.0 (theoretical maximum and efficiency optimum) (Pfaffl, 2004) which is satisfied for both genes.

Specificity of the amplified PCR product, whose amplification results were used to construct standard curves, should be determined and for this purpose melting curve analysis was performed. The melting curves allow differentiation of primer-dimers and the specific product since the product melts at a higher temperature than the primer-dimers. The specific *hGH* and *AOX* products melt at about 88 and 85°C, respectively (Figure I.5 and I.6). Primer-dimer formation was observed in *hGH* melting curves; however, there were no primer-dimer products in the melting peaks of AOX assay.

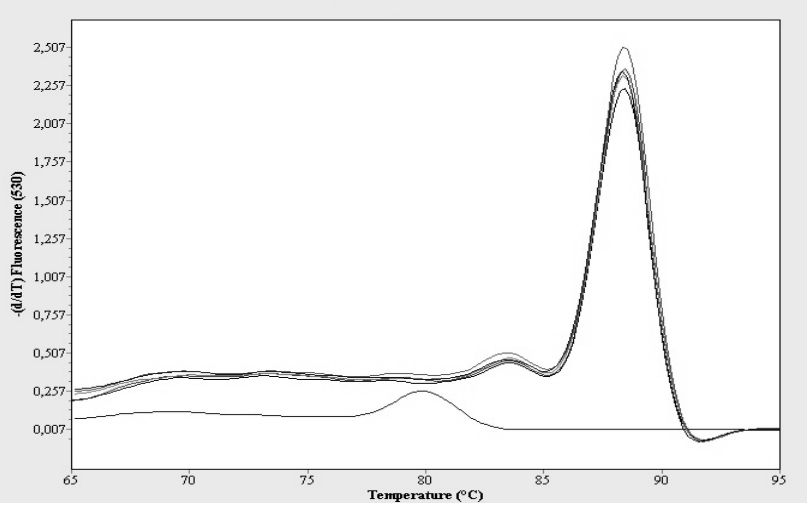

Figure I. 5 Melting curves of amplified *hGH* samples containing dilutional series of plasmid DNA standards as starting template and PCR grade water as a negative control

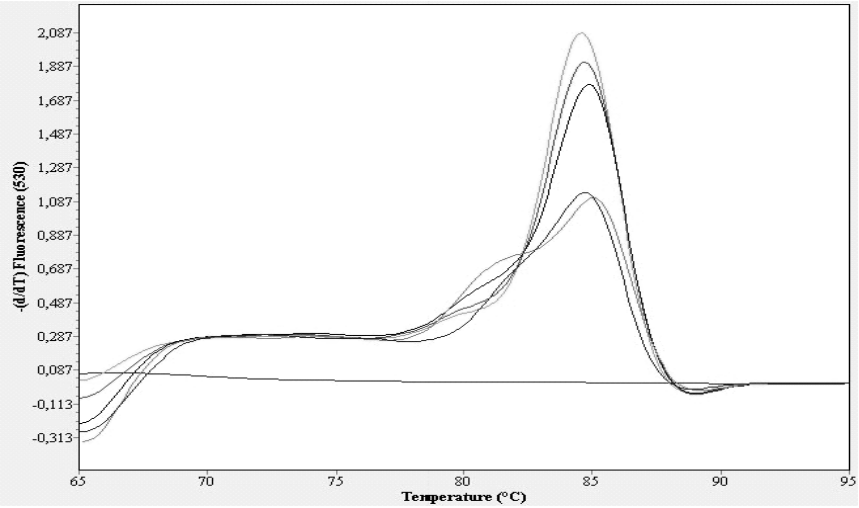

Figure I. 6 Melting curves of amplified *AOX* samples containing dilutional series of plasmid DNA standards as starting template and PCR grade water as a negative control

Preparation of Samples

For the samples to be analyzed in real-time PCR total RNA was isolated from a known amount of cell. Then cDNA was synthesized from 50 ng of those total RNA and the final RNA concentration in cDNA reaction mixture is 2.5 ng/ μL. The synthesized cDNA samples were used for analysis in real-time PCR. Initial concentrations of desired genes were calculated by LightCycler Software 4.0 using the standard curves constructed previously and these concentrations were in units of copies/μL. Then these were converted to copies/mg CDW or copies/ng total RNA. A sample calculation can be seen below.

For MS-0.03 at t=15 h concentration of *hGH* given by LightCycler Software 4.0 was 2.195×10^6 copies/μL. From total RNA isolation to real-time PCR analysis this sample was diluted for several times. For each sample this dilution ratio changes and for this sample it was DR=54.1 Furthermore, the amount of cell used for RNA isolation was C_X= 0.00296 mg/μL.

hGH expression
in copies/mg CDW = 54x(2.195x10^6 copies/µL) / (0.00296 mg/µL)

= 4.01x10^{10} copies/mg CDW

hGH expression
in copies/ng total RNA = (2.195x10^6 copies/µL) / (2.5ng total RNA/µL)

= 8.78x10^5 copies/ng total RNA