# EFFECTS OF pH ON HUMAN GROWTH HORMONE PRODUCTION BY *Pichia pastoris* CONSIDERING THE EXPRESSION LEVELS OF REGULATORY GENES

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**EFFECTS OF pH ON HUMAN GROWTH HORMONE PRODUCTION BY *Pichia pastoris* CONSIDERING THE EXPRESSION LEVELS OF REGULATORY GENES**

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

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# ABSTRACT


EFFECTS OF pH ON HUMAN GROWTH HORMONE PRODUCTION BY *Pichia pastoris* CONSIDERING THE EXPRESSION LEVELS OF REGULATORY GENES

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In this study, the aim was to investigate the effects of pH on therapeutically important protein, recombinant human growth hormone (rhGH), production by *Pichia pastoris* considering the expression levels of regulatory genes. In this frame, firstly the host microorganism was selected between two different methanol utilization phenotypes of *P. pastoris,* $Mut^+$ and $Mut^S$ on media containing glycerol/methanol or sorbitol/methanol. The highest rhGH production, 120 g $L^{-1}$, and *hGH* gene expression, $9.84x10^9$ copies $mg^{-1}$ CDW, were achieved in the medium containing 30 g $L^{-1}$ sorbitol and 1% (v/v) methanol by *P. pastoris hGH*-$Mut^+$ strain. Thereafter, effects of pH on rhGH production and stability were investigated in laboratory scale bioreactors. RhGH was more stable at pH 5.0. Throughout the production, it is seen that medium of pH decreased.

Thereafter, effects of pH on rhGH were investigated in pH controlled pilot-scale bioreactor. In addition to rhGH concentration, AOX intracellular enzyme activity, extracellular proteases concentrations; expression levels of *hGH*, *AOX*, *pep4, prb1* and *prc1* genes were determined. The highest cell concentration was obtained as 53 g $L^{-1}$ at pH 6.0 but hGH concentration was

found as 24 mg $L^{-1}$ at t=24 h. The highest rhGH concentration was obtained as 271 g $L^{-1}$ with 42 g $L^{-1}$ cell density at pH 5.0 in medium containing sorbitol at t=24 h. At this condition, the overall product and cell yield on total substrate were found as 2.08 mg $g^{-1}$ and 0.15 g $g^{-1}$. Furthermore, the highest expression levels of *hGH* and *AOX* were attained at pH 5.0. Moreover, by keeping pH at 5.0, expression levels of three types of vacuolar proteases were minimized.

**Keywords:** Recombinant human growth hormone, *Pichia pastoris*, optimization of pH, proteases

# öz

## *Pichia pastoris* İLE İNSAN BÜYÜME HORMONU ÜRETİMİNDE DÜZENLEYİCİ GENLERİN İFADE DÜZEYLERİNİ DİKKATE ALARAK pH ETKİSİNİN İNCELENMESİ

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Bu çalışmada, *Pichia pastoris* ile biyoreaktör işletim parametrelerinden pH'ın rekombinant insan büyüme hormonu (rhGH) üretimine etkisi düzenleyici genlerin ifade düzeyleri ile birlikte araştırılmıştır. İlk olarak, iki farklı metanol kullanım fenotipi, *P. pastoris* $Mut^+$ ve $Mut^S$ arasından konak mikroorganizma, gliserol/metanol veya sorbitol/metanol içeren ortamlar kullanılarak seçilmiştir. İncelenen koşullarda, en yüksek rhGH üretimi, 120 g $L^{-1}$, ve ifade düzeyi, $9.84x10^9$ kopya $mg^{-1}$ CDW, 30 g $L^{-1}$ sorbitol ve %1 (h/h) metanol içeren ortamda, *Pichia pastoris hGH*-$Mut^+$ suşu kullanılarak elde edilmiştir. Sonraki aşamada, pH'ın rhGH üretimine ve protein stabilitesine etkisi laboratuvar ölçekli biyoreaktörlerde incelenmiştir. Rekombinant insan büyüme hormonun pH 5.0' de daha stabil olduğu gözlenmiştir. Üretim süresince, ortamın pH değerinin düştüğü görülmüştür.

Üretim sırasında pH'ı kontrol altında tutabilmek için pH optimizasyon çalışmaları yarı kesikli biyoreaktörlerde gerçekleştirilmiştir. RhGH

konsantrasyonuna ek olarak, hücre içi AOX aktivitesi, hücre dışı proteaz konsantrasyonu; *hGH, AOX, pep4, prb1 ve prc1* genlerinin ifade düzeyleri incelenmiştir. En yüksek hücre konsantrasyonu pH 6.0 koşulunda t=24 st'te 53 g $L^{-1}$ olarak elde edilmiş fakat bu koşulda hGH derişimi 24 mg $L^{-1}$ seviyesinde kalmıştır. En yüksek hGH derişimi 271 g $L^{-1}$ olarak 42 g $L^{-1}$ hücre konsantrasyonunda sorbitol içeren ortamda pH 5.0 koşulunda t=24 st'te elde edilmiştir. İncelenen bu koşulda, toplam substrat üzerinden ürün ve hücre verimi 2.08 mg $g^{-1}$ and 0.15 g $g^{-1}$ olarak bulunmuştur. Buna ek olarak, incelenen koşullarda, en yüksek hGH ve alkol oksidaz ifade seviyesi pH 5.0 koşulunda elde edilmiştir. Ayrıca, pH 5.0 değerinde sabit tutularak, üç farklı proteazın ifade düzeylerinin azalması sağlanmıştır.

**Anahtar Kelimeler:** Rekombinant insan böyüme hormonu, *Pichia pastoris*, pH optimizasyonu, proteazlar

*To my mother*

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# TABLE OF CONTENTS

# LIST OF TABLES

TABLES

# LIST OF FIGURES

FIGURES

# 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) | |
| $\mu_0$ | Desired specific growth rate | $h^{-1}$ |
| $\mu_{s,max}$ | Maximum specific growth rate on sorbitol | $h^{-1}$ |

| | | |
|---|---|---|
| $\mu_t$ | Total specific growth rate | $h^{-1}$ |
| $\lambda$ | Wavelength | nm |

**Subscripts**

| | |
|---|---|
| 0 | Refers to initial condition |
| AOX | Refers to alcohol oxidase |
| G | Refers to glycerol |
| M | Refers to methanol |
| O | Refers to oxygen |
| p | Refers to protein |
| pro | Refers to protease |
| R | Refers to bioreaction medium |
| rp | Refers to recombinant protein |
| S | Refers to sorbitol or substrate |
| 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 |
| Pep4 | Proteinase A |
| Prb1 | Proteinase A |
| Prc1 | Carboxypeptidase Y |
| PCR | Polymerase chain reaction |
| rHuEPO | Recombinant human erythropoietin |
| rhGH | Recombinant Human growth hormone |
| SDS-PAGE | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |
| TCA | Tricarboxylic acid |

# CHAPTER 1

# INTRODUCTION

Biotechnology is one of the fastest growing areas of science based on the biological and engineering sciences. This interdisciplinary field is widely applied to industrial process and it has applications in four major industrial areas such as health care, environment, crop production and agriculture, non food uses of crops and other products (biodegradable plastics, vegetable oil, biofuels). This shifts towards industrial biotechnology results in development of new ways to optimize and increase the production of industrially important products. In this content, production of bio-molecules molecules in an organism other than its natural source was achieved by using recombinant DNA technology, which allows cloning of the target gene responsible for production of the desired product (Nielsen, 2003). A major use for many of these recombinant organisms is to produce recombinant therapeutic proteins in a functional and active form. Since many proteins are of immense commercial value.

The genetically engineered therapeutic proteins are generally hormones, which regulate the metabolic activities of a human body. Erythropoietin, insulin, interferon and human growth hormone (hGH) are the most widely produced hormones in industry. Human growth hormone being a non-glycosylated protein, is secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland (Norman, 1997). In the anterior pituitary gland, two different forms of human growth hormone are found. The native form of the hormone having a 22 kDa molecular weight and 191 amino acid residues play role in the development of skeletal and other tissues (Baulieu et al., 1990; Binkley, 1994). The recombinant form of human growth hormone supplies benefit in the treatment of hypopituitary dwarfism, children with growth hormone deficiency, girls with Turner's syndrome, children with chronic renal failure and adults with growth hormone deficiency or human

immune deficiency virus (HIV) syndrome. Furthermore, it is used in the treatment of injuries, bone fractures, bleeding ulcers, burns and maintaining health in the elderly (Tritos, 1998).

In 1956, human growth hormone was isolated from human pituitaries for the first time and biochemical structure of the protein was found in 1972. Until 1979, hormone was obtained by extracting from human pituitaries. Then, the protein had been tried to obtain recombinantly by using different microorganisms. Goeddel et al. (1979) was produced hGH as a recombinant product for the first time in 1979 and the native form of the protein was obtained by Gray et al. (1985) by using *Escherichia coli* as a host microorganism (Krysiak et al., 2007). However, being a prokaryotic cell, *E.coli* cannot undergo post-translational modifications such as glycosylation and disulfide bond formation. Moreover, by *E.coli*, rhGH has been produced intracellularly and secreted into culture medium using specialized secretion vectors. Therefore, other host microorganisms have been also used until now. *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Pseudomona*s are some microorganisms used to obtain rhGH (Gray et al., 1985; Tokunaga et al., 1985; Franchi et al., 1991; Özdamar et al., 2009).

Recently, *Pichia pastoris* has become a popular microorganism in the production of many recombinant proteins due to its advantages. There are several reasons that account for the popularity of the *P. pastoris* expression system: its ability to produce foreign proteins at high levels, either extracellularly or intracellularly; its facility in performing many post-translational modifications such as glycosylation, correct disulfide bond formation, and proteolytic processing; the availability of the alcohol oxidase I (AOX1) promoter (known to be one of the strongest and most tightly regulated eukaryotic promoters) for controlled gene expression; the ability to stably integrate expression plasmids at specific sites in the *P. pastoris* genome in either single or multiple copies; and its ability to grow to a very high cell density in bioreactors (Cereghino and Cregg 1999; 2000). In literature, the first study related with production of rhGH from *P.pastoris* was performed by Trevino et al. (2000). 11 mg $L^{-1}$ rhGH was obtained in shake tubes having 3 ml of defined culture medium and 49 mg $L^{-1}$ rhGH was produced in high cell density cultures by using a 2 L bioreactor. 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 by *P. pastoris*. It was found that, removal of glu-ala repeats from the hGH was not efficient and glu-ala repeats 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 $C_{MeOH}$=3% (v/v) after 3 day induction in complex medium, 190 mg $L^{-1}$ rhGH concentration was obtained. Orman et al. (2008) designed an expression system in *P. pastoris* for the production and purification of rhGH by cloning *hGH* cDNA sequence into *pPICZαA* vector under the control of AOX1 promoter. Moreover, Orman et al. (2009) 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 phenotypes of the microorganism. The highest amount of rhGH, 110 mg $L^{-1}$, was achieved in the medium containing $C_{Gly}$=30 g $L^{-1}$ and $C_{MeOH}$=1% (v/v) by using *Mut*$^S$ strain.

In this study, it was aimed to investigate effects of pH on therapeutically important protein, recombinant human growth hormone (rhGH), production by *Pichia pastoris* considering the expression levels of regulatory genes. In this frame, firstly the host microorganism was selected between two different methanol utilization phenotypes of *P. pastoris*, Mut$^+$ and Mut$^S$. For this purpose, as the carbon source glycerol, a repressing, and sorbitol, a non-repressing, were used besides methanol in the fermentation medium. The strain producing higher amount of rhGH was determined based on the rhGH concentration and expression level of *hGH*. Secondly, influence of pH on rhGH production and the stability of the protein were investigated by using chosen strain in laboratory scale air filtered shake bioreactors. In the third part of this study, to supply better control on pH and eliminate oxygen limitation, optimum pH was determined for rhGH production by using pilot scale bioreactor. Furthermore, cell growth, oxygen transfer and fermentation characteristics, effects of oxygen transfer on rhGH production and by-product formation were investigated and in addition to these to gain more insight on production and degradation of rhGH, expression levels of *hGH, AOX1, pep4, prb1* and *prc1* were determined by using real time-PCR analysis.

# CHAPTER 2

# LITERATURE SURVEY

In the bioprocess development of a specific biomolecule, based on the properties of the bioproduct selection of the host microorganism is one of the most important steps. Thereafter, the optimum medium composition and bioreactor operation conditions should be determined since they take crucial role in quality and quantity of the product. Finally, to be able to have a better insight in production of the biomolecule, the relationship between the expression level of the product and the enzymes should be investigated. In this context, this chapter reviews the literature on human growth hormone, host microorganism, medium design, bioreactor operation conditions and genetic engineering techniques.

## 2.1 Human Growth Hormone (hGH)

Human growth hormone (hGH), being a non-glycosylated protein, is secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland (Norman, 1997). In 1956, human growth hormone was isolated from human pituitaries for the first time and biochemical structure of the protein was found in 1972. Until 1979, hormone was obtained by extracting from human pituitaries. Then, the protein had been tried to be obtained recombinantly by using different microorganisms since it is one of pharmaceutically most important hormones. Goeddel et al. (1979) was produced hGH as a recombinant product for the first time in 1979 and the native form of the protein was obtained by Gray et al. (1985) by using *Escherichia coli* as a host microorganism (Krysiak et al., 2007).

The recombinant form of human growth hormone supplies benefit in the treatment of hypopituitary dwarfism, children with growth hormone

deficiency, girls with Turner's syndrome, children with chronic renal failure and adults with growth hormone deficiency or human immune deficiency virus (HIV) syndrome. Furthermore, it is used in the treatment of injuries, bone fractures, bleeding ulcers, burns and maintaining health in the elderly (Tritos, 1998).

### 2.1.1 Biological and Genetic Structure of hGH

In the anterior pituitary gland, two different forms of human growth hormone are found. The native form of the hormone having a 22 kDa molecular weight plays role in the development of skeletal and other tissues (Baulieu et al., 1990; Binkley, 1994). The molecular weight of other form is 20 kDa and it forms about 10% of growth hormone located in the pituitary gland (Norman, 1997).

The *hGH* genes are located in the q22-24 region of the chromosome 17 of human beings (http://www.ncbi.nlm.nih.gov). The native form of the hormone contains 191 amino acid residues, which fold into a four-helix bundle structure with two disulfide bonds formation. These bonds are formed by four cysteine biomolecules which are located on 35th, 165th, 182nd, and 189th positions of the hGH chain. The active form and tertiary structure of the protein are obtained as a result of disulfide bonds formation (Binkley, 1994). The nucleotide sequence, tertiary and covalent structure of hGH are given in the 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** Tertiary structure of hGH (http://www.ncbi.nlm.nih.gov).

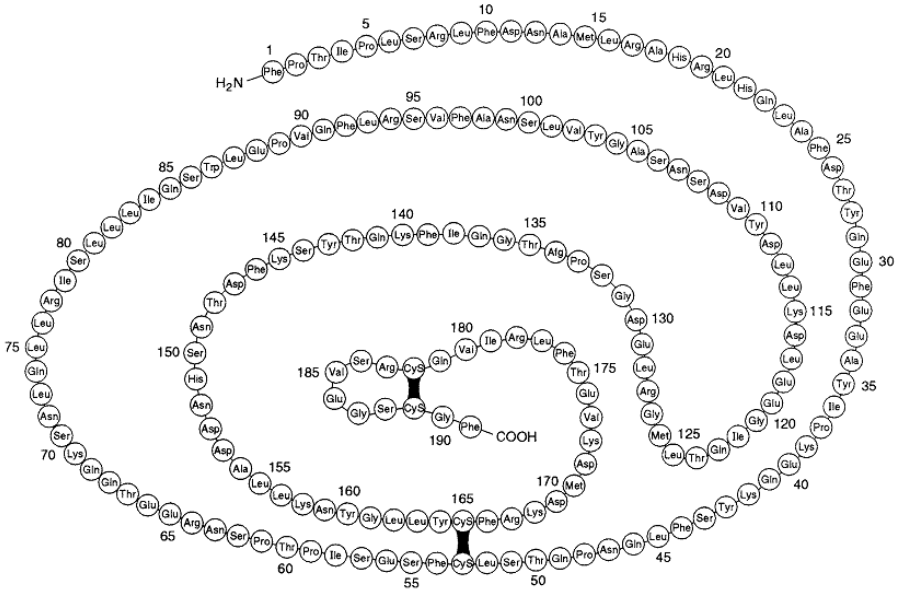



**Figure 2.3** Covalent structure of hGH (Norman, 1997).

## 2.2 Selection of Microorganism

Over the last few decades, geneticists have learned how to manipulate DNA to identify, move and place genes into a variety of organisms that are quite different from the source organism. A major usage of these recombinant organisms is to produce proteins. Since many proteins have immense commercial value, numerous studies have focused on finding ways to produce them efficiently and in a functional and active form. The production of a functional protein is directly related to the cellular machinery of the organism so selection of microorganism plays important role (Macauley-Patrick et al., 2005).

Until now, recombinant human growth hormone (rhGH) has been produced by using various host microorganisms. *Escherichia coli* has been extensively used one (Goeddel et al., 1979; Gray et al., 1985; Becker et al., 1986; Kato et al., 1987; Hsiung et al., 1986). However, *E.coli* cannot undergo post-translational modifications such as glycosylation and disulfide bond formation. Hence, protein can not be obtained in an active and functional form. Moreover, in *E.coli*, rhGH has been produced intracellularly and secreted into culture medium using specialized secretion vectors. *Bacillus subtilis, Saccharomyces cerevisiae* and *Pseudomonas* are the other recombinant strains used to obtain rhGH (Gray et al., 1985; Tokunaga et al., 1985; Franchi et al., 1991; Özdamar et al., 2009).

Recently, *Pichia pastoris* has become a popular microorganism in the production of many recombinant proteins due to its advantages for example, *P.pastoris* is a eukaryote so it can undergo post translational modifications and produce proteins in native form. In literature, the first study related with production of rhGH from *P.pastoris* was performed by Trevino et al. (2000). 11 mg $L^{-1}$ rhGH was obtained in shake tubes having 3 ml of defined culture medium and 49 mg $L^{-1}$ rhGH was produced in high cell density cultures by using a 2 L bioreactor. 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 by *P. pastoris*. It was found that, removal of glu-ala repeats from the hGH was not efficient and glu-ala repeats were not necessary for the removal of MFα-1signal sequence. In the same study, the optimal condition for high level production was investigated and by using $C_{MeOH}$=3% (v/v) after 3 day induction in complex

medium, 190 mg $L^{-1}$ rhGH concentration was obtained. Orman et al. (2008) designed an expression system in *P. pastoris* for the production and purification of rhGH by cloning *hGH* cDNA sequence into *pPICZαA* vector under the control of AOX1 promoter. Moreover, Orman et al. (2009) 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 phenotypes of the microorganism. The highest amount of rhGH, 110 mg $L^{-1}$, was achieved in the medium containing $C_{Gly}$=30 g $L^{-1}$ and $C_{MeOH}$=1% (v/v) by using *Mut*$^{s}$ strain.

### 2.2.1 *Pichia Pastoris*

*Pichia pastoris* is mesophilic yeast. Yeasts have typical eukaryotic cell structure and generally have a thick polysaccharide cell wall, and they are facultative anaerobes. Generally, the width and length of the yeasts are between 1-5 μm and 5-30 μm, respectively. It is taxonomically classified under the Kingdom Fungi, Division *Eumycota*, Subdivision *Ascomycotina*, Class *Hemoascomycetes*, Order *Endomycetales*, Family *Saccharomycetaceae* and Genus *Pichia* (www.ncbi.nlm.nih.gov).

*Pichia pastoris*, is a methylotrophic yeast which is able to utilize methanol as the sole carbon and energy source. The yeast was first evaluated for the production of single-cell protein for feed stock during the 1970's by the Phillips Petroleum Company (USA) due to ability of utilizing methanol as sole carbon source (Wegner et al., 1990; Cereghino and Cregg et al., 2000; Daly and Hearn, 2005).

Now, *P. pastoris* expression system is being used successfully for the production of various recombinant heterologous proteins. Over 400 proteins, from human endostatin to spider dragline silk protein, have been produced from *P. pastoris* (Cereghino et al., 2002). The increasing popularity of this expression system is attributed to several factors. These are the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly, the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing and the capability of growing to high cell density in a bioreactor (Cereghino et al., 2002; Jahic et al., 2006). The detailed list of advantages

and disadvantages of the microorganism are 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<br>• Strong promoter (AOX1)<br>• Chemically defined media-simple, inexpensive formulation<br>• Product processing like mammalian cells<br>• Stable production strains<br>• Low purification cost<br>• High levels of expression of intracellular and secreted proteins<br>• Eukaryotic post-translational modifications<br>• No endotoxin problem<br>• Non-pathogenic<br>• Broad pH range: 3- 7<br>• Ability of utilizing methanol<br>• Preference for respiratory growth rather than fermentative, a major advantage relative *S. cerevisiae.*<br>• Crabtree-negative<br>• Hyper-glycosylation is not as much as in *S. cerevisiae*. | • Potential of proteolysis, non-native glycosylation.<br>• Long time for cell cultivation compared to bacteria<br>• Monitoring methanol during a process is difficult in order to induce *AOX*1 promoter.<br>• 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*

In general, *P. pastoris* is grown first on glycerol used as carbon source according to standard *P. pastoris* fermentation protocols. This is due to biomass yield and maximum specific growth rate are higher for growth on glycerol than for growth on methanol and protein expression is repressed during growth on glycerol (Higgins and Cregg, 1998).

In glycerol utilization pathways (Figure 2.4), initially glycerol is phosphorylated to glycerol-3-phosphate by a glycerol kinase. Further oxidation of the product by a FAD-dependent glycerol-3-phosphate dehydrogenase results in dihydroxyacetone phosphate, which enters the glycolytic pathway (Nevoigt and Stahl, 1997). Then, pyruvate is formed as the outcome of glycolysis. Pyruvate is further oxidized to acetyl–CoA via pyruvate dehydrogenase. Subsequently, acetyl–CoA enters tricarboxylic acid (TCA) cycle, where many metabolites are produced and used for the synthesis of cellular constituents such as amino acids, nucleic acids as well as cell wall components (Ren et al., 2003).

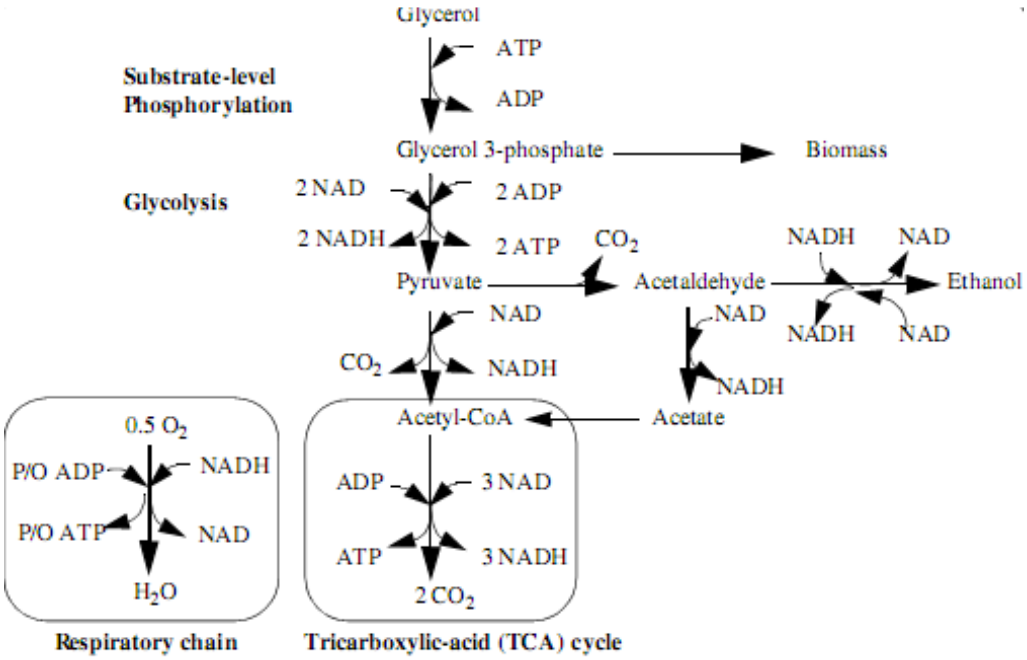



**Figure 2.4** Metabolic pathways of glycerol in *P. pastoris* (Ren et al., 2003).

Methylotrophic yeasts have a specific methanol utilization pathway (Figure 2.5) involving several unique enzymes, which are expressed at high levels when the cells are grown on methanol. The initial reactions take place in the peroxisomes, and are followed by subsequent metabolic steps in the

cytoplasm (Cereghino et al., 2000; Macauley-Patrick et al., 2005). The initial step in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide, is catalyzed by the enzyme alcohol oxidase (AOX). AOX is sequestered within the peroxisome along with catalase, which degrades hydrogen peroxide to oxygen and water. A portion of the formaldehyde generated by AOX leaves the peroxisome and is further oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases. The reactions are a source of energy for cells growing on methanol. The remaining portion of formaldehyde is assimilated to form cellular constituents by a cyclic pathway that starts with the condensation of formaldehyde with xylulose 5-monophosphate, a reaction catalyzed by a third peroxisomal enzyme dihydroxyacetone synthase (DHAS). The products of this reaction, glyceraldehyde 3-phosphate and dihydroxyacetone, leave the peroxisome and enter a cytoplasmic pathway that regenerates xylulose 5-monophosphate and other cell materials (Cereghino et al., 2000).

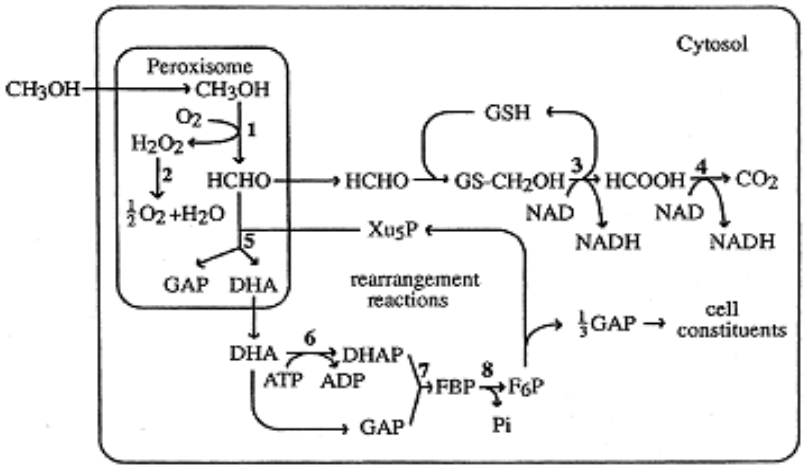



**Figure 2.5** The methanol pathway in *P. pastoris*. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase (Cereghino et al., 2000).

During the last decade, *P. pastoris* has become most popular expression system for production of many heterologous proteins. Therefore, besides methanol and glycerol, alternative repressing and non-repressing carbon sources have been investigated. Sorbitol is one of the most abundantly used alternative carbon sources. In sorbitol utilization pathway (Figure 2.6), firstly sorbitol enters the mannitol cycle where sorbitol is first oxidized to fructose by sorbitol dehydrogenase, and followed by phosphorylation via a sorbitol kinase. Then, sorbitol enters the glycolysis from fructose-6-phosphate branch point.

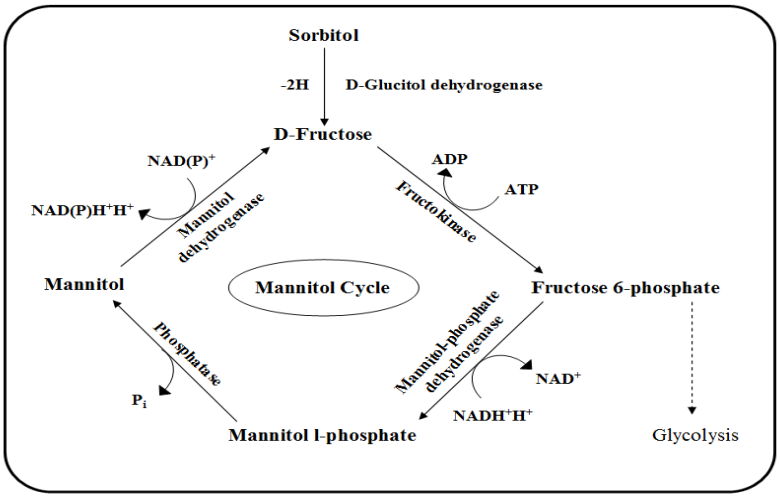



**Figure 2.6** Pathways of sorbitol and mannitol metabolism in yeasts (Walker, 1998).

### 2.2.1.2 Expression system of *Pichia pastoris*

The recombinant protein production in *P. pastoris* strongly depends on enzymes required for the metabolism of methanol and these enzymes are only present when cells are grown on methanol. This has been the most successful system under the control of the strong, tightly regulated, and methanol-induced alcohol oxidase promoter AOX1 (Cereghino et al. 2002; Jahic et al. 2006). AOX1 promoter is highly repressed in cells grown on glucose, glycerol,

and most other carbon sources, but it is strongly induced by methanol (Cereghino et al. 2002; Jahic et al. 2006). The advantages and disadvantages of using the AOX1 promoter are listed in Table 2.2.

In fact, there are two types of alcohol oxidase genes producing alcohol oxidase and ontology of these genes is given in Table 2.3. *AOX1* gene regulates 85% of the alcohol oxidase activity in the cell (Zhang et al., 2000). The second alcohol oxidase gene is *AOX2*, which yields 10–20 times less AOX activity than the *AOX1* gene (Macauley-Patrick et al., 2005). The total concentration of AOX promoter, in a methanol-limited fed-batch culture, reaches to more than 30% of total soluble protein (Cereghino and Cregg, 2000).

**Table 2.2** Advantages and disadvantages of using the AOX1 promoter system.

| **Advantages** | **Disadvantages** |
|---|---|
| • Transcription of the foreign protein is tightly regulated and controlled by a repression/derepression mechanism<br>• High levels of foreign proteins can be expressed<br>• The repression of transcription by the initial carbon source ensures that good cell growth is obtained before the gene product is overexpressed<br>• Induction of transcription is easily achieved by the addition of methanol | • Monitoring methanol during a process is often difficult, due to the unreliability of on-line probes and the complications of measuring off-line<br>• Methanol is a fire hazard; therefore, storing the large quantities required for these processes is undesirable<br>• Methanol is mainly derived from petrochemical sources, which may be unsuitable for use in the production of certain food products and additives<br>• Two carbon sources are required, with a switching over from one to the other at a precise time |

**Table 2.3** Ontology of alcohol oxidase genes, *AOX1* and *AOX2* (www.uniprot.org/uniprot/P04841).

| | |
|---|---|
| **Molecular Function** | Alcohol oxidase activity<br>FAD binding |
| **Biological process** | Methanol metabolic process<br>Oxidation reduction |
| **Cellular Component** | Peroxisome |

AOX1 is the most efficient promoter to express numerous foreign genes. However, in some conditions, such as food production, the usage of methanol inducing gene expression may not be appropriate since methane, a petroleum-related compound, is one source of methanol. Therefore, promoters that are not induced by methanol are attractive for expression of certain genes. Alternative promoters to the AOX1 promoter are the *P. pastoris* GAP, FLD1, PEX8, and YPT1 promoters (Cereghino and Cregg, 2000).

#### 2.2.1.3 Methanol Utilization Phenotypes of *Pichia pastoris*

Phenotypes of *P. pastoris* are divided into three groups with respect to methanol utilization. The first type is methanol utilization plus phenotype, $Mut^+$, which grow on methanol at the wild-type rate. The phenotype is under control of the both *AOX1* and *AOX2* genes. The maximum specific growth rate of $Mut^+$ on methanol is 0.14 $h^{-1}$ (Jungo et al., 2006). Methanol utilization slow phenotype, $Mut^s$, the second type, has a disruption in the *AOX1* gene. Methanol metabolism only depends on weaker AOX2 gene. The disruption in the *AOX1* gene occurs during transformation of the expression vector. 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. This disruption of the *AOX1* gene forces these strains to rely on the transcriptionally weaker *AOX2* gene for growth on methanol and, as a result, these strains have a $Mut^s$ phenotype (Cregg et al., 1987). By cultivating these strains on methanol, two strains are easily differentiated among transformed colonies since growing rates of two phenotypes on methanol differs from each other. The maximum specific growth rate of $Mut^s$ on methanol is 0.04 $h^{-1}$. The last phenotype is methanol

utilization minus phenotype, Mut$^-$, having disruption in both *AOX1* and *AOX2* genes so these strains are unable to grow on methanol. However, methanol is necessary for induction of recombinant protein expression (Stratton et al., 1998; Macauley-Patrick et al., 2005).

#### 2.2.1.4 Post-Translational Modifications and Secretion of Proteins

*P. pastoris*, unlike bacterial expression systems, has capability of performing the post-translational modifications usually performed in higher eukaryotes, such as, processing of signal sequence, correct folding, disulphide bond formation, addition of lipid required, O-and N-linked glycosylation (Macauley-Patrick et al., 2005; Cereghino and Cregg, 2000).

In production of heterologous proteins, it is favorable to produce protein extracellularly to be able to eliminate first steps of purification, such as cell lysis. In this aspect, *P. pastoris* is one of the most desirable expression systems since it has ability of producing foreign proteins both intracellularly and extracellulary (Macauley-Patrick et al., 2005). To be able to secret proteins into the culture medium, a specific signal sequence is needed. The signal sequence is usually a short amino-terminal pre-sequence that typically contains a charged amino-terminal region followed by numerous hydrophobic residues and a cleavage site recognized by signal peptidases (Paifer et al., 1994; Romanos et al., 1992). *S. cerevisiae* alpha-mating factor pre–proleader sequence (α-MF), the acid phosphatase signal sequence (PHO) and the invertase signal sequence (SUC2) are some of the native secretion signal (Li et al., 2001). The most widely and effectively used native signal is *S. cerevisiae* alpha-mating factor pre–proleader sequence, which is composed of a 19 amino acid signal peptide (pre-sequence), followed by a 60 amino acid pro-region. The signal sequence is removed by signal peptidase and pro-region cleavage site is recognized by the yeast kex2 protease, resulting in the release of the mature, fully processed protein (Raemaekers, et al. 1999).

The most crucial steps in the post translational modifications are folding of proteins, beginning with the formation of secondary structure, and disulphide bond formation in the endoplasmic reticulum. These steps play important role in obtaining mature and active form of proteins. The folding step determines the productivity of the *P. pastoris* expression system (Hlodan et al., 1994; Holst et al., 1996; Hohenblum et al., 2004). In prokaryotic

system, generally misfolded disulphide-bonds are formed due to the reducing environment of the cytoplasm. However, *P. pastoris* expression system has been successfully used to produce highly disulphide-bonded proteins (White et al., 1994).

One of the necessary post translational modifications to make proteins biologically active is glycosylation. There are two types of glycosylation that *P. pastoris* can perform, O- and N- linked glycosylation. It generates the glycosylated products generally having much shorter glycosyl chains than those expressed in *S. cerevisiae*, which makes *P. pastoris* a much more attractive host for the expression of recombinant proteins (Bretthauer et al., 1999).

Humanizing the glycosylation patterns of recombinant proteins, which is used as pharmaceuticals, is essential in order to get rid of the glycosylation or reduce the glycosyl chains. This may include changing the culture conditions, by either using mutant strains which are lack of some genes producing enzymes essential for the glycosylation, or using enzymes and adding some chemicals to remove the glycosyl chains (Daly and Hearn, 2005). Due to absence of the recognition, sequences for O- and N- linked glycosylation in hGH, there is no need any additional processes to get rid of the glycosylation or reduce the glycosyl chains. By using *P. pastoris* expression system, the mature form of the rhGH was produced without glycosyl chains by Trevino et al. (2000), Eurwilaichitr et al. (2002) and Orman et al. (2009).

#### 2.2.1.5 Proteolytic Degradation in *Pichia Pastoris*

*P.pastoris* is one of the most suitable expression systems for production of foreign proteins. However, in high cell density cultures, proteolytic degredation is an important problem (Van Den Hazel et al., 1996; Kobayashi et al., 2000; Gimenez et al., 2000; Sinha et al., 2004). Loss of biological activity of protein, reduction in product yield, and contamination of product by degradation of intermediates in downstream processing are emerged because of proteolysis (Kobayashi et al., 2000).

Yeast vacuoles contain various proteases whose levels vary according to the nutritional conditions (Hansen et al., 1977). In *P. pastoris,* the proteases are not well characterized. However, it is reported that extracellular proteases, cell-bound proteases and intracellular proteases from lysed cells

can be reasons of the proteolysis of secreted protein (Jahic et al., 2002 and 2003). In *Saccharomyces,* the main source of proteases is vacuolar proteases, followed by proteasome and the proteases of the secretory pathway (Yasuhara et al., 1994). Moreover, *P. pastoris* secretes only low levels of extracellular proteases (Jahic et al., 2006). Therefore, vacuolar proteases are probably the main reason of the proteolysis in the medium. The vacuolar proteases generally found in yeasts are endoproteinase A and B, or proteinase A and B, carboxypeptidase Y (*prc1*), and S, aminopeptidase I and yscCo (Jones et al., 1991 and 2002). Some of the vacuolar proteases, such as carboxypeptidase Y and proteinase B are activated by Proteinase A, being a vacuolar aspartyl protease. Proteinase B has about half the activity of the processed enzyme before being activated by proteinase A (Higgins and Cregg, 1998).

The vacuolar proteases have low substrate specificity and do not require ATP so they can act on any peptide bond in secreted protein structure, which causes degradation of the product. That makes cell viability an important parameter, since these proteases in the medium probably are released from dead cells that lyse (Jahic et al., 2006). Lysis of the yeast cell can be due to change of carbon sources, heat and pH changes, starvation or toxic chemicals since these factors creates stress on yeast cells. Proteins damaged by oxidative stress and heat-shock response also elicit a proteolytic response (Hilt and Wolf, 1992). Furthermore, overexpression of endogenous, vacuolar proteases results in a secretion of proteases to cytosoplasm and finally to the culture medium (Stevens et al., 1986).

Many strategies have been developed to deal with this problem. One of the most commonly used strategies is addition of amino acid reach component. A decrease in protein degradation is seen after addition of amino acid reach complement such as casamino acid and peptone and a defined nitrogen source such as ammonium sulfate into medium (Clare et al., 1991; Goodrick et al., 2001; Sinha et al., 2004). Addition of these components reduces degradation of proteins since they can eliminate nitrogen limitation. Also, they probably act as an alternative and competing substrate for proteases, which are less substrate specific enzyme (Brankamp et al., 1995).

Addition of inhibitors to the medium is also a way to reduce activity of proteases. In medium, generally, aspartic, cystine, and serine type proteases are found (Shi et al, 2003); in addition to these, metalloproteases can be also found in the medium (Sinha et al., 2004). Inhibition profiles by several kinds

of protease inhibitors such as antipain, chymostatin, diisopropyl fluorophosphate, elastatinal or phenyl methyl sufonyl fluoride (PMSF) and ethylenedinitrilotetraacetic acid (EDTA) were studied during production. Addition of inhibitors to the medium results in an increase in recombinant human serum albumin concentration (Kobayashi et al., 2000). Sinha et al. (2004) stated that addition of 1 mM EDTA or PMSF into medium reduced the total protease activity by 45% and 78%, respectively. Furthermore, Kobayashi et al. (2000) revealed that most of the proteases found in the medium are serine type proteases.

An alternative way to overcome to proteolysis is to use protease deficient strains having a disruption in the genes encoding proteases. These strains are SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 pep4*), and SMD1168 (*his4 pep4*), which have a disruption in the genes encoding proteinase A and proteinase B (Sreekrishna et al., 1997; Cereghino and Cregg, 2000). The *pep4* and *prb1* genes encode proteinase A and B, respectively (Jahic et al., 2006). A substantial decrease in activity of protease A and carboxypeptidase Y and a partial reduction of protease B activity are observed when *pep4* mutants are used since carboxypeptidase Y and proteinase B are activated by Proteinase A. Moreover, *prb1* mutant eliminated only protease B activity and the usage of *pep4 prb1* double mutant resulted in a substantial reduction or elimination of all three of these protease activities (Higgins and Cregg, 1998).

Finding an optimum temperature for both production and growth is one of the developed strategies. The optimum temperature for growth of *P. pastoris* is 30°C (Wegner, 1983) and temperatures above 32°C can be detrimental to protein expression and may lead to cell death (Invitrogen, 2002). Thus, temperatures above 30°C were not appropriate for the production of recombinant microorganism because elevated temperatures result in cell death, which will lead to cell lysis and higher protease activity in fermentation media (Inan et al., 1999). It is observed that proteolysis is decreased when the cultivation temperature is lowered. This is due to the rate of proteolysis is lowered for kinetic reasons (Li et al., 2001; Hong et al., 2002; Macauley-Patrick et al., 2005).

One of the effective strategies developed for minimizing proteolytic degradation is to set the pH during the methanol induction phase at a value that is not optimal for protease activity (Jahic et al., 2002). *P. pastoris* is capable of growing over a relatively broad pH range from 3.0 to 7.0, more

preferably and usually about pH=3.5 to 5.5. This range has little effect on the growth rate (Wegner, 1983; Inan et al., 1999), which allows considerable freedom in adjusting the pH to one that is not optimal for a problem protease (Sreekrishna et al., 1997). Scorer et al. (1993) is found that dropping pH from 5.0 to 3.0 during the induction phase on methanol reduced the proteolytic degradation since alkaline and neutral proteases are generally responsible for damage to recombinant proteins in the culture broth. In literature, to be able to minimize degradation of the product, optimum pH values for different heterologous proteins were investigated (Inan et al., 1999; Kobayshi et al., 2000; Jahic et al., 2003). In this study, effect of pH on proteolytic degradation and protein concentration was investigated.

### 2.3 Medium Design and Bioreactor Operation Parameters

In production of a recombinant protein, the yields and protein production are affected from many parameters. Medium composition and bioreactor operation parameters such as pH, temperature and oxygen transfer are the most crucial ones since they show diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalık et al., 1999).

#### 2.3.1 Medium Design

In the improvement of the bioprocess, choice of fermentation medium is a key factor, since cell growth and formation of the product is the net result of the uptake and conversion of nutrients. Hence, a fermentation medium should fulfill following criteria (Nielsen and Villadsen, 1994):

- It should contain carbon, nitrogen and energy sources.
- It should contain all essential mineral required for growth.
- It should contain all necessary growth factors to ensure rapid growth and high yield of the desired product.
- It should have consistent quality and be readily available.
- It should cause minimum problem in the downstream processing.

Nutrients essential for the cell growth can be divided into two groups according to their required concentrations in the medium. Macronutrients are the first group and the required concentrations of them are generally larger than $10^{-4}$ M. Carbon, oxygen, nitrogen, hydrogen, sulfur, phosphorus, magnesium and potassium are main macronutrients. Micronutrients are the second group and the required concentrations of them are less than $10^{-4}$ M. Trace elements such as Ca, Cu, Fe, Na, Mn, Mo, Zn, and vitamins are some micronutrients and they are added to the culture medium as mineral salts (Fiecher et al., 1984).

There are three major types of growth media; defined medium, containing specific amounts of pure chemical compounds with known chemical compositions, semi-defined medium, and complex medium, containing natural compounds whose chemical composition is not exactly known (Shuler and Kargı, 2002). Complex media often contains an organic nitrogen source, essential minerals and different growth factors; however, variation in the composition can be seen (Nielsen and Villadsen, 1994). In industrial applications, it is desired to standardize the production process, validate the medium and process itself and, eliminate subsequent downstream processing (Nielsen and Villadsen, 1994; Macauley-Patrick et al., 2005). Therefore, defined medium is preferred in bioprocess.

The most common medium for high cell density fermentation of methylotrophic yeast *P. pastoris* is the basalt salt medium (BSM) along with its companion trace salts medium (PTM1). This is considered a standard medium, though it may not be the optimum. Hence, it may have some important problems, such as unbalanced composition, precipitates and high ionic strength (Cereghino et al., 2002; Cos et al., 2006). To be able to solve these problems, some modifications have been tried in several studies (Brady et al., 2001; Thorpe et al., 1999; Jungo et al., 2006) and the effect of each medium component have been investigated in detail by Plantz et al. (2007). Furthermore, alternative mediums have been formulated by Stratton et al. (1998) and d'Anjou et al. (2000).

Nitrogen is one of the essential micronutrients. In *P. pastoris* fermentation protocol, the nitrogen source is usually provided by the addition of ammonium hydroxide, which also has effect on controlling the pH to the desired level (Cos et al., 2006). Increased concentrations of ammonium in the medium can prolong the lag phase and thus inhibit cell growth and protein

production (Xie et al., 2003; Yang et al., 2004). The use of ammonium ions as a nitrogen source during recombinant protein production in *P. pastoris* is probably the most commonly used method for overcoming nitrogen-limitation problems such as proteolytic degradation. This is due to the fact that they probably act as an alternative and competing substrate for proteases, which are less substrate specific enzyme (Brankamp et al., 1995). A decrease in protein degradation is seen after addition of casamino acid and peptone and a defined nitrogen source such as ammonium sulfate into medium (Clare et al., 1991; Goodrick et al., 2001; Sinha et al., 2004).

In the recombinant protein production and cell growth, carbon sources play crucial roles. Most commonly used carbon sources are methanol, glycerol, sorbitol, glucose, mannitol, trehalose, etc. (Brierley et al., 1990; Sreekrishna et al., 1997; Thorpe et al., 1999; Inan and Meagher, 2001). Methanol is not used only as an inducer for the expression of recombinant protein, but also as a sole carbon source. Above certain concentrations, growth is substrate-inhibited by methanol (Zhang et al., 2000); therefore, a fed-batch protocol is generally used. The production of heterologous proteins in bioreactors by *P. pastoris* involves a three-stage high cell-density fermentation scheme. The first stage is the batch phase in which the culture is grown in a salt medium on a non-fermentable carbon source, such as glycerol. Upon glycerol depletion, the second phase (transition phase) is initiated by adding glycerol at a growth-limiting rate. The second phase is important since by-products such as, ethanol generated during batch phase is consumed and cells are primed for induction. The third phase is the induction phase, which is initiated by adding limited methanol (Higgins and Cregg, 1998).

By using the frame of the three-stage high cell-density fermentation scheme, many strategies have been developed to increase cell density and process productivity, as well as to reduce the induction time. A typical approach is the use of additional carbon sources beside methanol (Files et al., 2001). The first approach is mixed feeding of glycerol and methanol during the induction phase. This approach was firstly applied by Brierley et al. (1990) and Loewen et al. (1997). In the many studies, it is revealed that this strategy can increase the volumetric recombinant protein productivity as a result of higher cell densities and feeding rates (Cregg et al., 1993; Loewen et al., 1997; McGrew et al., 1997; Katakura et al., 1998; Zhang et al., 2003). However, glycerol excess represses the AOX1 promoter, which may result in lower

specific productivities of recombinant protein (Xie et al., 2005). The second approach is mixed feeding of sorbitol and methanol during the induction phase. According to the literature (Sreekrishna et al., 1997; Thorpe et al., 1999; Boze et al., 2001; Inan and Meagher, 2001; Xie et al., 2005), sorbitol is a non-repressing carbon source with respect to the AOX1 promoter, usually used for the expression of recombinant proteins in $Mut^s$ phenotype of *P. pastoris*. Thorpe et al. (1999) pointed out that by contrast with glycerol, one advantage with mixed feeds of sorbitol and methanol is that sorbitol accumulation during the induction phase does not affect the expression level of recombinant protein. Hence, control of residual sorbitol concentration during the induction phase is less critical than with mixed feeds of glycerol and methanol. Furthermore, use of mixed feeds of sorbitol and methanol supplies other advantages, for example, including the lower heat production rate and the lower oxygen consumption rate for growth on sorbitol than for growth on glycerol or methanol (Jungo et al., 2007). The first study related with both $Mut^+$ strains and sorbitol co feeding strategy was performed by Inan et al. (2001-b). In addition, optimization of sorbitol concentration in the feed was investigated at $\mu$=0.03 $h^{-1}$ and $\mu$=0.05 $h^{-1}$ with $Mut^+$ strain (Jungo et al., 2007). Çelik (2008), investigated non-inhibitory level of sorbitol for growth of $Mut^+$ phenotype of *P. pastoris* and found that 50 g $L^{-1}$ is optimum for both production and growth of the cell.

In the induction phase, it is important to keep methanol concentration below toxic limits, 4 g $L^{-1}$, since it is required to be supplied continuously to the growing culture to induce AOX1 promoter (Zhang et al., 2000b). Hence, investigating an optimum specific growth rate is one of the strategies developed. Cunha et al. (2004), Kobayashi et al. (2000), Ohya et al. (2005) and Zhang et al. (2005) revealed that the specific growth rate influence protein expression. Jungo et al. (2007) investigated the effect of specific growth rate ($\mu$), lower than 0.08 $h^{-1}$, on specific product productivity by using pre-determined exponential feeding profile with $Mut^+$ strain. It is observed that, for specific growth rates higher than about 0.02 $h^{-1}$, specific productivity increased slightly with $\mu$. However, a large decrease in specific productivities was observed at $\mu$ below 0.02 $h^{-1}$. Finally, Çelik et al., (2009) investigated the effect of specific growth rate on production in the presence of sorbitol with a predetermined exponential methanol feeding strategy by using $Mut^+$ strain. The highest protein concentration was achieved at $\mu$= 0.03 $h^{-1}$ and the highest

specific yield was achieved at = 0.02 $h^{-1}$. In this study, it is also found that methanol feeding rate had no effect on sorbitol consumption rate and, confirming the results of Jungo et al. (2007), sorbitol and methanol was found to be utilized simultaneously.

### 2.3.2 pH

Hydrogen ion concentration (pH) is stated as one of the most important bioreactor operation parameters, as it influences the microbial growth rate, the activity of enzymes, transport mechanisms and other extracellular and intracellular events. 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).

*P. pastoris* can tolerate a broad pH range between 3.0 and 7.0, more preferably and usually about 3.5 to 5.5. This range has little effect on the growth rate (Wegner, 1983; Inan et al., 1999), which allows considerable freedom in adjusting 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).

Optimum pH value strongly depends on the nature of the recombinant protein and its stability. Hence, different pH values were found to be optimal for different proteins (Macauley-Patrick et al., 2005). Clare et al. (1991) stated that pH 6.0 was optimal in production of recombinant mouse epidermal factor and pH 3.0 was optimal in production of insulin-like growth factor-I and cytokine growth-blocking peptide (50 mg $L^{-1}$) (Brierley et al., 1994; Koganesawa et al., 2002). Kobayashi et al. (2000) observed that, during recombinant human serine albumin production, protease activity rapidly increased below pH 5.0 and was minimum at pH 5.6 so degradation rate of HAS is minimized at pH 5.6. Proteolytic response increased at pH 5.0 (an average of 2836 U/mL protease or 1243.9 U of protease/mg protein in fermentation supernatant) and this caused a decrease in recombinant ovine interferon production. However, at pH 6.0 the proteolytic activity and protein degradation was substantially reduced (Sinha et al, 2004). The stability of a

fusion protein composed of a cellulose-binding module (CBM) from Neocallimastix patriciarum cellulase 6A and lipase B from Candida antarctica (CALB) was considerably increased at pH below 5. However, stability of the lipase activity decreased with pH below 4 and the results from degradation study demonstrated that pH 4 is optimal when both the cleavage reaction and the lipase activation are taken into consideration (Jahic et al., 2003). Production of rhGH was achieved at pH 6 (Trevino et al., 2000; Eurwilaichitr et al., 2002; Orman et al., 2009). In literature, there is no study related with optimization of pH for production of rhGH by *P. pastoris.* In this study, effects of pH on stability and production of rhGH, and proteolytic degradation were investigated.

### 2.3.3 Temperature

Temperature is an important environmental parameter for cell growth. Microorganisms do not have the ability to regulate their internal temperature. Hence, cell temperature is always equal to environmental temperature and all cellular biochemical reactions taking place depend directly on the external temperature. Moreover, not only the reaction rates, but also metabolic regulation, nutritional requirements, biomass composition and product formation will be affected by temperature. When optimum temperature is investigated, it must be taken into consideration that optimum temperature for growth and product formation can differ. In addition to that, the maintenance requirement of cells starts to increase when temperature is above the optimum temperature, like pH (Nielsen and Villadsen, 1994). Moreover, secondary and tertiary structures of the protein are influenced by temperature since lower temperature helps to reduce protein misfolding and to produce more properly folded proteins (Georgiou and Valax, 1996).

The effect of temperature on the maximum specific growth rate of a microorganism is similar to that observed for enzyme activity. An increase is observed when temperature increases 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, 2003).

The optimum temperature for growth of *P. pastoris* is 30°C (Wegner, 1983) and temperatures above 32°C can be detrimental to protein expression and

may lead to cell death (Invitrogen, 2002). Temperatures above 30°C were not appropriate for the production of recombinant peptides because elevated temperatures result in cell death, which will lead to cell lysis and higher protease activity in fermentation media (Inan et al., 1999). It is observed that proteolysis is decreased when the cultivation temperature is lowered. This is due to the rate of proteolysis is lowered for kinetic reasons (Li et al., 2001; Hong et al., 2002; Macauley-Patrick et al., 2005).

Li et al. (2001) showed that during the production of herring antifreeze proteins, decreasing the temperature from 30°C to 23°C increased the yield from 5.3 mg/l to 18.0 mg/l, and increased cell viability. Moreover, Hong et al. (2002) by decreasing temperature from 30°C to 20°C obtained more active laccase. Instead of methanol limited fed batch (MLFB) technique, temperature-limited fed-batch (TLFB) technique was tried to lower protease activity by Jahic and co-workers (2003) and it is revealed that protease activity and cell death rate decreased in the TLFB. This result can be related to increase in AOX activity due to adjustment of temperature. In addition to that, in TLFB technique, there is no oxygen limitation at high cell density, which prevents the cell lysis. As a result, secretion of proteases to the medium is prevented. It is observed that protein stability in intracellular production was improved when the temperature was at 25-27°C as compared to 30°C. Thus, optimization of production temperature may also minimize degradation (Zhang et al., 2000b). However, it has to be pointed out that there many other cases where 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). In this study, temperature was kept at 30°C during the process.

### 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., 1998, 1999 and 2000). The characteristics of metabolic pathway of microorganism, and carbon sources and other nutrition used play important role in the requirement of the oxygen by the cells in fermentation medium.

*P. pastoris* is an obligately aerobic organism, which needs high oxygen transfer rates when it grows on methanol since methanol metabolism utilizes oxygen at high rate. Furthermore, *P. pastoris,* unlike *Saccharomyces cerevisiae*, does not produce inhibiting products such as ethanol and acetic acid (Cereghino and Cregg, 2000). Molecular oxygen is not only used for the respiration but also for the initial oxidation of methanol to formaldehyde. This reaction, catalyzed by the AOX enzyme, generates hydrogen peroxide that an also be used by AOX for methanol oxidation. Thus, two potentially toxic metabolites are generated in the cell during the initial methanol oxidation. Formaldehyde could be expected to accumulate in the cells and create detrimental effect when the cells are exposed to oxygen limitation (Sibirny et al., 1990; Couderc et al., 1998). In the studies, generally, the dissolved oxygen level was kept above 20–30% during the whole induction phase on methanol (Jahic et al., 2006). Trentmann et al. (2004) compared two *P. pastoris* cultivation techniques, which are methanol-limited and methanol-saturated, with oxygen limitation cultures. It is observed that recombinant protein quality and productivity were higher in the methanol-saturated processes. Comparison of oxygen limited fed batch (OLFB) process with methanol limited fed batch (MLFB) process for the production of Thai Rosewood β-glucosidase was studied by Charoenrat et al, (2005). It is revealed that higher oxygen uptake rate, higher productivity and specific activity in OLFB were achieved when compared with MLFB. In this study, dissolved oxygen rate was kept above 20 %.

## 2.4 Computation of Bioprocess Characteristics

### 2.4.1 Yield Coefficients and Specific Rate

In fermentation process, computation of the yield coefficients and the specific rates plays important role in evaluation of bioprocess. Yield coefficient is stoichiometrically related parameters and defined as given in equation 2.1 where P and S are product and substrate, $Y_{P/S}$ is the overall yield coefficient. In this equation ΔP and ΔS represent the mass or moles of P produced and the mass or moles of S consumed, respectively.

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

Although, the definition gives an overall yield representing some sort of average value for the entire culture period, it is sometimes necessary to evaluate the instantaneous yield at a particular point in time. This is due to fact that the growth rate and metabolic functions of the microorganism cause variations in the yield coefficients throughout the process. The instantaneous yield can be calculated as follows:

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

Some of the yield coefficients, which demonstrate the efficiency of conversion of the substrate into biomass and product are listed in Table 2.4.

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). When cells are inoculated in batch cultivation, a characteristic sequence of events termed in the growth cycle takes place. In lag phase, physicochemical equilibration between the organism and the environment following inoculation occurs with very little growth. Growth starts to occur in acceleration phase and it achieves its maximum rate in growth phase. In stationary phase no net growth is observed since nutrients are depleted and cell deaths begin (Atkinson and Mavituna, 1991; Shuler and Kargı, 2002).

The rate of microbial growth is characterized by the specific growth rate, μ, and this term is derived from general mass balance for biomass for fed-batch processes. When equation is derived, main assumptions are that cells are batch-wise and are not lost through sampling and the system has constant density throughout the process.

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

**Table 2.4** 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 |

The biomass formation rate, $r_X$, is defined in terms of specific cell growth rate (μ), and cell concentration ($C_X$), which is given in equation 2.4.

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

By inserting equation 2.4 into 2.3, the below equation is obtained.

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

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

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

By inserting equation 2.6 into 2.5, the below equation is obtained.

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

When the equation 2.7 is rearranged, a term for specific growth rate, μ, in fed-batch processes is obtained.

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

Mass balance for methanol, the fed-batch substrate in this study, added with an exponential volumetric flow rate of Q, in fed-batch process with a reaction volume V, can be written as

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

The substrate consumption rate, $r_M$ can be defined in terms of specific substrate consumption rate, $q_M$ and cell concentration, $C_X$.

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

By inserting equation 2.10 into 2.9

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

The last term can be neglected since substrate can not be accumulated when nutrient consumption rate is nearly equal to nutrient feed rate (Shuler and Kargı, 2002). Thus,

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

By combining equations 2.9 and 2.12, and dividing by V, the general mass balance becomes,

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

By rearranging equation 2.13, specific methanol consumption rate, $q_M$ is obtained as follows:

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

In this study, sorbitol-methanol mixed feeding strategy is applied. Sorbitol is given to the system as batch so mass balance for sorbitol concentration becomes as follows:

$$r_s V = \frac{d(C_s V)}{dt} \tag{2.15}$$

The substrate consumption rate, $r_s$, can be defined in terms of specific substrate consumption rate, $q_s$ and cell concentration, $C_X$.

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

Inserting equation 2.16 into 2.15,

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

By rearranging equation 2.17, specific sorbitol consumption rate, $q_s$ is obtained as follows:

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

Using the same approach, the specific recombinant protein production rate, $q_{rp}$ can be determined.

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

In brief, the specific rates μ, $q_S$, $q_M$ and $q_{rp}$ can be calculated from experimental data by using the equations derived.

### 2.4.2 Oxygen Transfer Characteristics

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 in equation 2.20, where $K_La$ is the gas-liquid interfacial area, $C_O$ and $C_O^*$ are saturated dissolved oxygen concentration and actual dissolved oxygen concentration in the broth.

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

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). And the maximum possible mass transfer rate is defined as follows:

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

The oxygen uptake rate (OUR), $-r_O$, per unit volume of broth is defined in equation 2.22, where $q_O$ is the specific rate of oxygen consumption and $C_X$ is the cell concentration (Shuler and Kargı, 2002).

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

To be able to determine $K_La$ values experimentally, many methods have been developed. The most commonly used method 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:

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

To be able to apply this method, firstly the broth is de-oxygenated by stopping the air flow and lowering the agitation rate to a minimum level to prevent surface aeration. During this period, dissolved oxygen concentration

($C_O$) drops due consumption by the microorganism, and since there is no oxygen transfer, equation 2.23 becomes as follows:

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

Using equation 2.24 and drawing $C_0$ versus time graph, seen in Figure 2.7, oxygen uptake rate, $-r_O$, can be calculated from region-II of Figure 2.7.

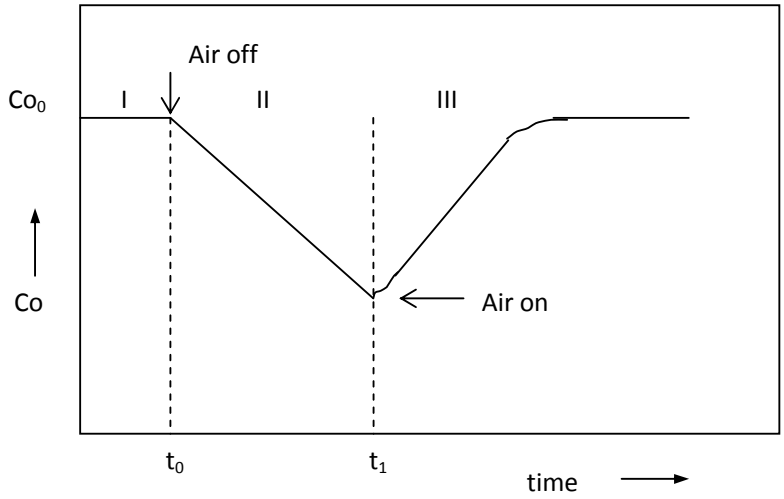



**Figure 2.7** Variation of dissolved oxygen concentration with time in dynamic measurement of $K_L a$.

After this step, by opening air inlet, oxygen concentration is increased and it is monitored as a function of time. By rearranging equation 2.23, equation 2.25 is obtained.

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

In this equation $r_O$ is known and by plotting $C_O$ versus ($dC_O/dt - r_O$) grap, $K_L a$ can be calculated from the slop of the plot.

The Dynamic Method can also be applied to conditions under which there is no reaction (no microorganism in the medium), i.e., $r_O=0$ (Nielsen,

2003). In this case, the broth is de-oxygenated by sparging nitrogen into the vessel. Air inlet is turned back on and again the increase in $C_O$ is monitored as a function of time. The equation 2.25 is changed as given,

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

By plotting $C_O$ versus $d(C_OV)/dt$, the physical mass transfer coefficient, $K_La_0$, can be calculated from the slope.

The maximum possible oxygen utilization rate (OD=oxygen demand) should be determined to be able to compare the relative rates of maximum oxygen transfer and biochemical reactions and find the rate limiting step of the bioprocess. Çalık et al. (2000) defines the maximum possible oxygen utilization rate as follows:

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

The oxygen uptake rate per maximum possible oxygen utilization rate, η (effectiveness factor) and maximum possible oxygen utilization rate per maximum mass transfer rate, Da (Damköhler number), should be calculated to express the oxygen limitation in an aerobic process. Çalık et al. (2000) defined these expressions as given in equation 2.28 and 2.29.

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

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

## 2.5 Real Time Polymerase Chain Reaction (Real time-PCR)

Reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of mRNA. Real-time PCR (or kinetic RT-PCR) is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range (Orlando et al., 1998; Pfaffl et al., 2001)

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitative transcription levels of specific genes has always been central to any research into gene function (Zamorano et al. 1996). The rapid development in PCR started with discovery of the thermophilic bacterium *Thermus aquaticus*. The thermally stable DNA polymerase extracted from this enzyme enabled replication of DNA template molecules during each PCR cycle (DeFrancesco et al., 2003). 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). In this study, to be able to better insight in production of hGH, real time PCR is performed to determine the expression levels of the specific genes such as, *hGH, AOX1, pep4, prb1* and *prc1*. These genes affect the structure and quantity of final product. There is no study directly related to expression levels of these genes. Ohi et al. (1995) used to PCR approach to discover the identity between the amino acid sequences of *P. pastoris prc1* and *S. cerevisiae prc1*. Hence et al. (2000) used reverse trancriptase PCR technique to detect *hGH* mRNA in total RNA prepared from glands in order to confirm that *hGH* was specifically expressed in transfected glands.

### 2.5.1 Basic Principles of Real Time-PCR

PCR, is a cyclical thermally driven in-vitro reaction that results in exponential replication of specific segments of DNA. The PCR can amplify DNA, when preceded by a reverse transcription (RT) incubation at 42–55$^{o}$C. The PCR process can be divided into three steps. First, double-stranded DNA (dsDNA) is separated at temperatures above 90$^{o}$C. Second, oligonucleotide primers generally anneal at 50–60$^{o}$C, and finally, optimal primer extension occurs at 70–78$^{o}$C (Figure 2.8). The temperature at which the primer anneals is usually referred to as the $T_M$. This is the temperature at which 50% of the oligonucleotide target duplexes have formed. A typical reaction consists of 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. Hence, the simultaneous monitoring of the reaction product by means of fluorescence detection is achieved as the PCR is in progress. This technique enables detection and

quantification of nucleic acids so it becomes an important tool (Schmittgen et al., 2000; Neumaier et al, 1995; Wittwer et al., 2001).

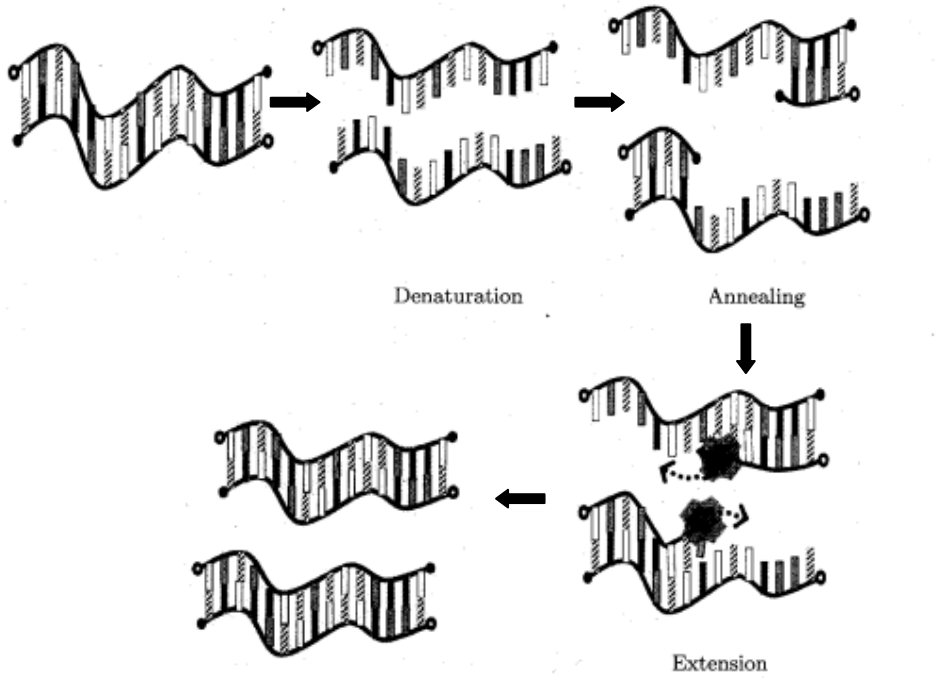



**Figure 2.8** The Polymerase Chain Reaction (Ngatchou, 2006).

In real time PCR analysis, concentration determination of the product is based on the concept of threshold cycle ($C_t$) or crossing point ($C_p$). During the reaction, the amount of fluorescence emitted is recorded at each cycle and this demonstrates the amount of product amplified. The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson et al. 1996). This point is defined as the $C_p$, and will always occur during the exponential phase of amplification. During the real time PCR assay, the target $C_p$ is compared directly with the standard $C_p$ and is recorded as containing either more or less mRNA.

To be able to determine concentration, quantification of mRNA transcription can be performed either relative or absolute. Absolute quantification allows the precise determination of copy number per cell, total RNA concentration, or unit mass of tissue by using absolute standard curve, constructed before. Relative quantification determines the changes in steady-state transcription of a gene. A relative standard is used to create a dilution series with arbitrary units to create a standard curve. The standard can be any nucleic acid, as long as its concentration and length of amplicon are known.

### 2.5.2 Reverse Transcription

Avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) are two commonly used reverse transcriptases.

In real time PCR application, one important problem occurs during the reverse transcription. This, RNA transcripts can exhibit significant secondary structure that affects the ability of the RNA dependent DNA polymerase, RT, to generate transcripts (Buell et al. 1978). Real time PCR quantification can be influenced from this problem and it should be minimized when comparing or quantifying diverse mRNA populations (Shimomaye & Salvato 1989). Therefore, to be able to eliminate problems related with RNA secondary structure, AMV-RT is preferred because significant polymerisation activity is achieved up to 55$^{o}$C (Freeman et al. 1996) and it is more durable than MMLV-RT (Brooks et al. 1995).

The reverse transcriptase step can be primed using specific primers, random hexamers and oligo-dT primers. The choice of primers requires careful consideration since yield and quality of cDNA are depend on 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 & Byrne 1999). In this study, oligo-dT primer is selected for cDNA synthesis.

### 2.5.3 Quantification Strategies in Real Time-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 it 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 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).

#### 2.5.3.2 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 (6.4 pg DNA), 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.

### 2.5.4 Principles of Fluorimetric Detection

Real time PCR application depends on detection of a fluorescent dye, and then correlation of this fluorescence signal with the amount of PCR product in a reaction. There are several methods for detecting and evaluating fluorimetric PCR reactions. The most commonly used fluorescence formats fall 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 achieved by means of melting curve analysis. The pure and homogeneous real time-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. Furthermore, to be able to eliminate primer-dimers, gene-specific fluorescent probes can be used. The best-know probe-based system is TaqMan. Thus, in this study, SYBR Green I assay for *hGH* and *AOX1* genes and hydrolysis probe assay for *pep4, prb1 and prc1* genes, are used to determine expression levels of the genes.

#### 2.5.4.1 SYBR Green I Assay

The method involves detection of the binding of a fluorescent dye (SYBR Green) to double-stranded DNA (dsDNA) (Morrison et al. 1998). The unbound dye exhibits little fluorescence in solution, but the fluorescence emission is greatly enhanced when it binds to DNA due to conformational changes in the dye. When SYBR Green I binds to dsDNA, its fluorescence emission increases over 100-fold and this increase in fluorescence is measured at 530 nm. 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. In the elongation phase, polymerization of PCR, PCR primers are extended and more SYBR Green I dye can bind. At the end of the phase, the entire DNA is double-stranded and a maximum amount of dye is bound (Buston 2000; www.roche-applied-science.com). All steps of this assay can be seen in Figure 2.9.

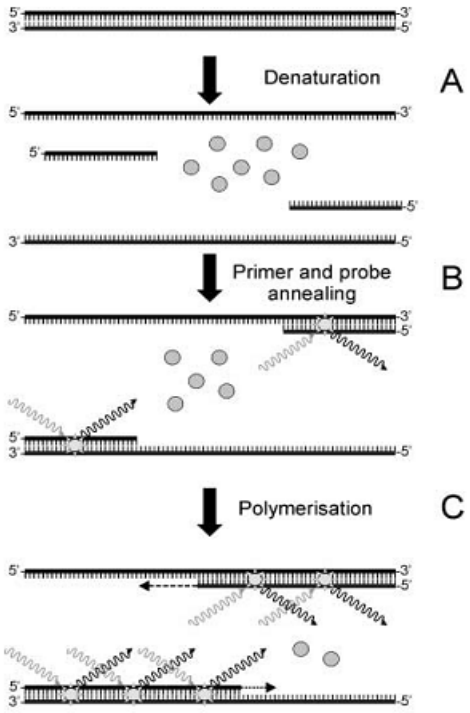



**Figure 2.9** Stages of SYBR Green I assay:(A) Denaturation. (B) Annealing. (C) Elongation (Buston 2000).

By using this method, both specific and non-specific, primer-dimer, PCR products are detected since SYBR Green I binds to any dsDNA and any double-stranded PCR artifact contributes to signal intensity, which may result in overestimation of the concentration of the target sequence. Therefore, the assay format must be carefully optimized. To able to discriminate product and primer-dimers, a melting curve analysis is applied. This is 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. A characteristic melting peak at the melting temperature ($T_m$) of the amplicon will distinguish it from primer-dimers that melt at lower temperatures in broader peaks (Buston 2000).

#### 2.5.4.2 Hydrolysis Probes Assay

The hydrolysis probe assay is also known as Taqman assay, utilizing the 5'-nuclease activity of the DNA polymerase to hydrolyse a hybridization probe bound to its target amplicon. Generally, Taq or Tth polymerase enzymes are used for amplification. The specificity of the assay comes from the usage of two template-specific primers and an oligonucleotide probe that hybridizes to the amplicon during the annealing and extension phase of the PCR (Buston 2000). The probe contains a fluorescent reporter dye at its 5' end and the emission spectrum of which is quenched by a second fluorescent dye at its 3' end. If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound. As the 5'-exonuclease activity of Taq and Tth polymerase is double-strand-specific (Heid et al. 1996), unbound probe remains intact and no reporter fluorescence is detected. Conversely, if the correct amplicon has been amplified, the probe can hybridize to that amplicon after the denaturation step. It remains hybridized while the polymerase extends the primers until it reaches the hybridized probe, when it displaces its 5' end to hold it in a forked structure. The enzyme continues to move from the now free end to the point, where cleavage takes place (Lyamichev et al. 1993). This separates the reporter and quencher dyes and releases quenching of reporter fluorescence emission. The largest fluorescence signal is obtained when the two labels are at the extreme 5' and 3' ends of the probe, probably because of more efficient cleavage by the polymerase (Livak et al. 1995). All steps of this assay can be seen in Figure 2.10.

The temperature conditions for annealing step should be adjusted to ensure probe binding. Most probes have a $T_m$ of around 70$^{o}$C. Hence, the Taqman system uses a combined annealing and polymerisation step at 60–62$^{o}$C. This ensures that the probe remains bound to its target during the primer extension step and maximum 5'–3' exonuclease activity of the Taq and Tth DNA polymerases is achieved (Tombline et al. 1996).

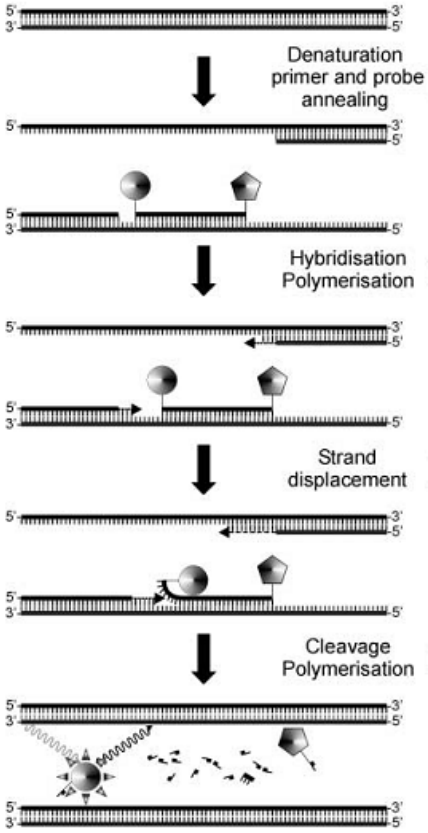



**Figure 2.10** Stages of hydrolysis probe assay (Buston 2000).

### 2.5.5 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.

#### 2.5.5.1 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).

#### 2.5.5.2 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).

#### 2.5.5.3 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 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 The Microorganism 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 the 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 cyrovial stored at -55$^{o}$C.

## 3.4 Growth Medium and Storage of Microorganism

### 3.4.1 Solid Medium

Recombinant *P. pastoris* strains were stored on microbanks at -55$^{o}$C or inoculated on YPD agar containing 0.1 g L$^{-1}$ Zeocin. The composition of solid medium is listed in Table 3.1. YPD agar containing *P. pastoris* was stored at 4$^{o}$C.

**Table 3.1** The composition of the YPD, solid medium.

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

### 3.4.2 Precultivation Medium and Glycerol Stock Solution

Recombinant *P. pastoris* strain grown on YPD agar was inoculated into first 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, second precultivation medium. The composition of BMGY is given in Table 3.4. The selective antibiotics, zeocin or chloramphenicol, were added to the precultivation mediums in amounts stated in Table 3.2 and Table 3.4 after sterilization.

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

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

**Table 3.4** The composition of BMGY, second precultivation medium**.**

| **Compound** | **Concentration, g $L^{-1}$** |
|---|---|
| Yeast extract | 10.0 |
| Peptone | 20.0 |
| Potassium phosphate buffer pH 6.0 | 0.1 M |
| YNB | 13.4 |
| Biotin | $4\times10^{-5}$ |
| 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. Production medium, containing glycerol or sorbitol together with methanol, basal salts solution and nitrogen sources, was used for air filtered shake bioreactors. It was a defined medium composition of which 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 used. 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 PTMJ and PTM1 (trace salt solutions), defined medium and BSM are listed Table 3.5, 3.6 and 3.7, respectively. All of the medium components except trace salts, sterilized with filter, were autoclaved at 121$^{o}$C for 20 min.

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

| **Compound** | **Concentration** | |
|---|---|---|
| | PTM1 g $L^{-1}$ | PTMJ mg L-1 |
| $CuSO_4.5H_2O$ | 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 $L^{-1}$** |
|---|---|
| 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 $L^{-1}$** |
|---|---|
| 85% $H_3PO_4$ | 26.7 ml |
| $CaSO_4.2H_2O$ | 1.17 |
| $MgSO_4.7H_2O$ | 14.9 |
| KOH | 4.13 |
| $K_2SO_4$ | 18.2 |
| Glycerol | 40.0 |

### 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, $Mut^+$ and $Mut^s$, carrying human growth hormone gene, was inoculated onto solid medium containing 0.1 g $L^{-1}$ 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 $L^{-1}$ 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 having a working volume capacities 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. This corresponds to a cell concentration between 0.55 and 1.65 g $L^{-1}$.

#### 3.5.2 RhGH Production in Laboratory Scale Air Filtered Shake Bioreactor

In laboratory scale air filtered shake bioreactor experiments, production was achieved in baffled and air filtered Erlenmeyer flasks V=250 mL in size having working volume capacities of $V_R$=50 ml. The cells taken from BMGY were harvested by centrifugation at 4000xg for 10 min and resuspended into production medium. Defined medium used 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 h, methanol was added to the medium to 1% or 3% (v/v). Production process was performed as batch 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 $dm^3$ bioreactor (Braun CT2-2), having a working volume of $V_R$=0.5-2.0 $dm^3$ 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-blade Rushton turbines. To achieve sterilization and temperature control a jacket around the bioreactor, an external cooler and steam generator were used. A compressor and a pure oxygen tube were utilized to supply air and oxygen. A mass flow controller was also used to adjust the oxygen concentration. Feed solutions placed on balances were transferred aseptically through inlet ports by using peristaltic pumps.

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 in controlled bioreactors. 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 $L^{-1}$. Firstly, the process was performed in batch mode until glycerol is totally consumed, which takes about 19 h, in order to achieve high cell densities rapidly. Secondly, glycerol fed-batch mode was applied by feeding 50% (v/v) glycerol containing 12 mL $L^{-1}$ PTM1 at limiting concentrations with a predetermined rate in order to increase biomass concentration further, 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 $L^{-1}$, (from 100% methanol containing 12 mL $L^{-1}$ PTM1). After 6 hours of transition phase, just before starting methanol fed-batch mode, sorbitol (500 g $L^{-1}$ sterile solution) was added batch-wise to the system, such that $C_{S0}$=50 g $L^{-1}$ was obtained. Methanol fed-batch process was achieved by feeding 100% methanol containing 12 mL $L^{-1}$ PTM1 to induce recombinant protein production. A predetermined exponential feeding profile was utilized for this purpose. The steps of scale up and the bioreactor system are shown in Figure 3.1.

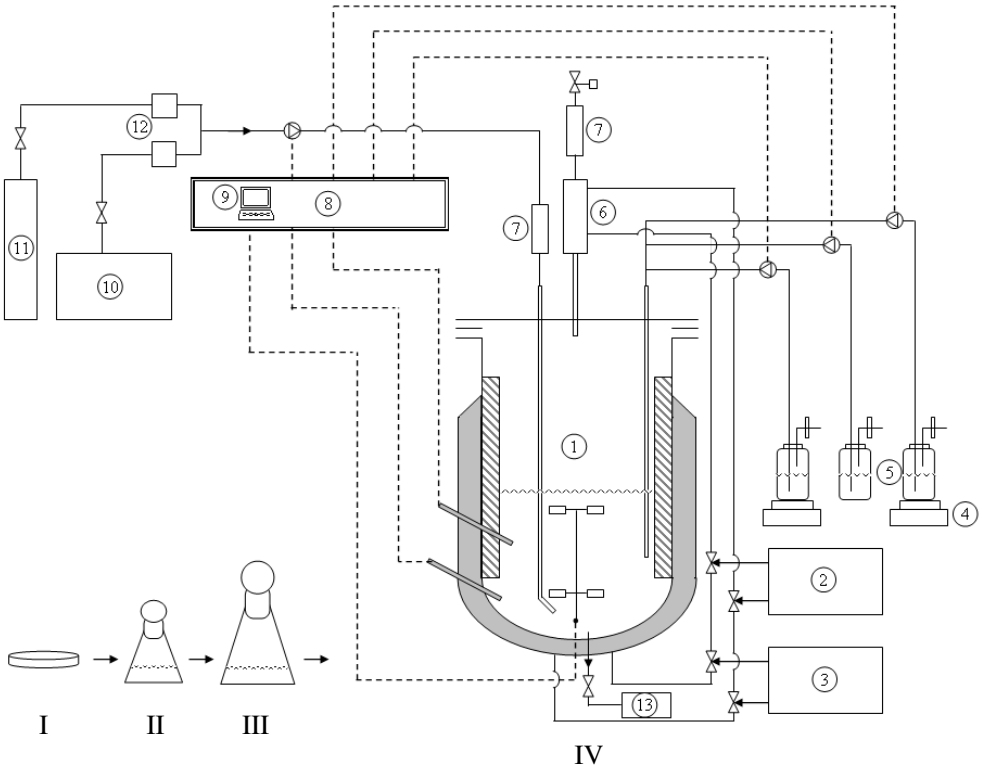



**Figure 3.1** Scale up steps and the pilot scale bioreactor system. I: Solid medium inoculated from stock culture; II: 1st Precultivation medium, V = 10 mL; III: 2nd Precultivation medium, V = 50 mL; IV: Pilot scale bioreactor system, $V_o$=1 L, which is composed of (1) Bioreaction vessel, Biostat CT2-2 (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).

### 3.6 Analysis

Throughout the production, samples were collected with a certain time interval to determine cell concentration and to perform all analysis. The medium collected was centrifuged for 10 min at 13200xg and 4°C and, stored at -55°C for further analysis. The supernatant was used to determine methanol, sorbitol, amino acid, organic acid, proteases and human growth hormone concentrations. The harvested cells were used to determine AOX activity and 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 $OD_{600}$. Therefore, sample taken from medium was diluted with $dH_2O$. To convert absorbance to cell concentration, $C_X$ (g $L^{-1}$), 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 $mL^{-1}$ (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 60 cm x 75 µm silica capillary and 50 mM borate buffer (pH=10) containing zwitter ion (Z1-Methyl reagent, Waters) as the separation buffer. The 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 bar), 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-polyacrlamide Gels:

1. Clean the glasses with ethanol. And assemble the glass plates according to the manufacturer's 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 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 $min^{-1}$, 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 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 $min^{-1}$ |
| 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 $0.62\times10^{-3}$% (v/v) $H_3PO_4$ (İleri and Çalık, 2006) as the mobile phase at a flow rate of 0.8 mL $min^{-1}$, and Dual absorbance detector (Waters 2487) were used to determine organic acid concentrations. The analysis were 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 $min^{-1}$ |
| 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 $min^{-1}$ |
| Column temperature | :38 °C |
| 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 should be extracted by using yeast lysis buffer (Appendix A) since AOX1 is an intracellular enzyme. For this, 500 µL yeast lysis buffer and a spoon of glass beads are added to cells obtained from centrifugation of 1 mL production medium. These cells are mixed up three times for 20 sec by vortex. After each cycle, the cells are kept on ice for 30 sec. Then, they are at 3000xg and +4ºC for 2 min. The supernatant

obtained is centrifuged at 12000xg for 5 min at +4°C. After second centrifugation, the supernatant obtained is used for AOX 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. 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 2 U $mL^{-1}$ HRP in 0.1M phosphate buffer, 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, 30 µL HRP, 375 µL methanol and 75 µl sample are put in cuvette and mixed. The increase in absorbance at 500 nm is monitored for 3 min and the absorbance is collected with 30 sec time intervals. To convert absorbance to AOX concentration (mg $mL^{-1}$), equation 3.2 was used and calibration curve is given in Appendix B.

$$C_{AOX}(U/g\,CDW) = 18.8\left(\frac{U/mL}{absorbance}\right) * OD_{500} * \frac{1}{C_X} \qquad (3.2)$$

### 3.6.6 Protease Activity Assay

Proteolytic activity was measured by hydrolysis of casein. The supernatant of the culture collected during the experiments was centrifuged at 13500xg for 10 min. Hammerstein casein solution (2 mL of 0.5% w/v) was prepared in either 0.05 M borate buffer (pH 10), 0.05 M sodium acetate buffer (pH 5) or 0.05 M sodium phosphate buffer (pH 7). Then the casein solution was mixed with 1 mL of diluted sample and hydrolyzed at T=37°C, pH=10 for 20 min. By adding 10% (w/v) trichloroacetic acid (TCA), the reaction was ceased and the reaction mixture was centrifuged at 10500xg for 10 min at +4°C. After waiting the mixture at room temperature for 5 min, the absorbance of the supernatant was measured at 275 nm in UV-Vis spectrophotometer. One unit protease activity was defined as the activity that liberates 4 nmole tyrosine $min^{-1}$. (Moon and Parulekar, 1991). The calibration equation used for converting absorbance to protease activity (U $cm^{-3}$) (Çalık, 1998) is given in equation (3.3).

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

### 3.6.7 Oxygen Uptake Rate and Liquid Phase Mass Transfer Coefficient

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

The physical mass transfer coefficient ($KLa_0$) 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 level oxygen on the microorganisms.

### 3.7 Genetic Engineering Techniques

In this study, it is aimed to determine the expression levels of the specific genes such as, *hGH, AOX1, pep4, prb1* and *prc1*. To achieve this purpose absolute quantification was applied by using real time PCR technique. SYBR Green I assay for *hGH* and *AOX1* genes and hydrolysis probe assay, TaqMan, for pep4, prb1 and prc1 genes, were preferred to supply more reliable and effective quantification. Expression levels of the desired genes were calculated by using the standard curves formed. To prepare standard curve, *pPICZaA::hGH* plasmid was used as a template for *hGH* and *AOX1* and, cDNA synthesized from total RNA of *P. pastoris* was used as a standard for pep4, prb1 and prc1. Orman et al. (2008) was constructed *pPICZaA::hGH* plasmid (Figure 3.2) by cloning *hGH* cDNA sequence into *pPICZaA* vector under the control of AOX1 promoter, which included a polyhistidine-tag on the amino terminal end.

#### 3.7.1 Enzymes, Kits and Primers

High Pure RNA Isolation Kit, Transcriptor High Fidelity cDNA Synthesis Kit, LightCycler TaqMan Master, 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.

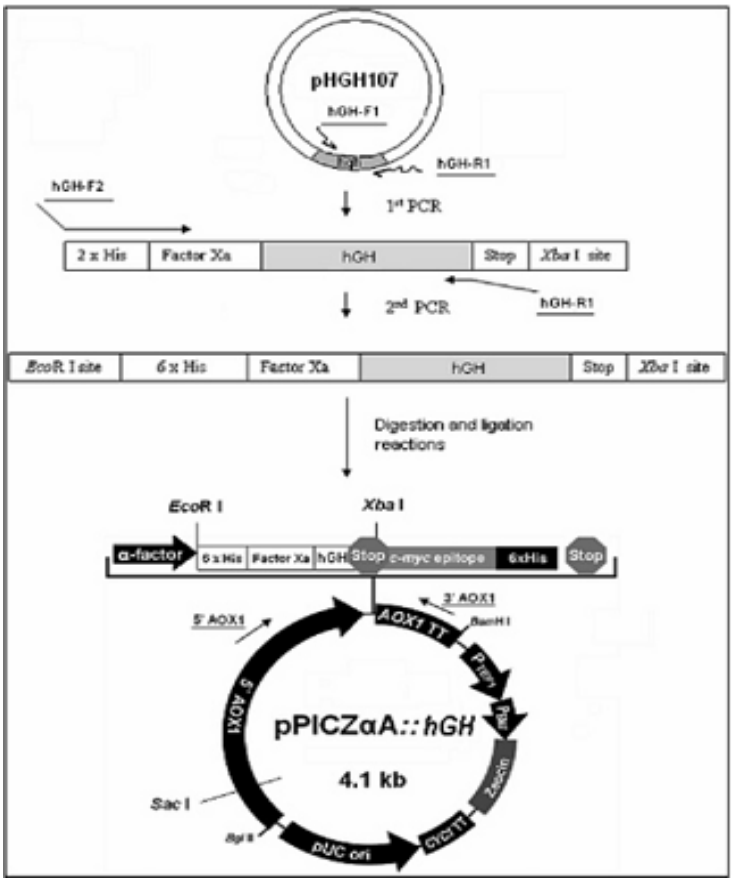



**Figure 3.2** Schematic representation of *hGH* amplification, integration of specific recognition sites by two-step PCR, and construction of the *pPICZaA::hGH* plasmid. Shown are the EcoRI and XbaI sites used in ligation of the insert to the vector, the SacI site used to linearize the plasmid before 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).

### 3.7.2 Total RNA Isolation from *Pichia pastoris*

High Pure RNA Isolation Kit (Roche GmbH, Mannheim, Germany) was used to perform 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 $OD_{600}$=0.1–0.15/mL which corresponds to $2x10^6$ cell).
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 $mL^{-1}$) 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 RNA samples obtained from RNA 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 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 the ratio of A260 to A280 is between 1.9 and 2.1, the samples were diluted at different ratios to reach the same amount of RNA, which is 50 ng for *AOX* and *hGH* and, 500 ng for *pep4, prb1* and *prc1* 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.

**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 | |

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.

**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 | |

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.

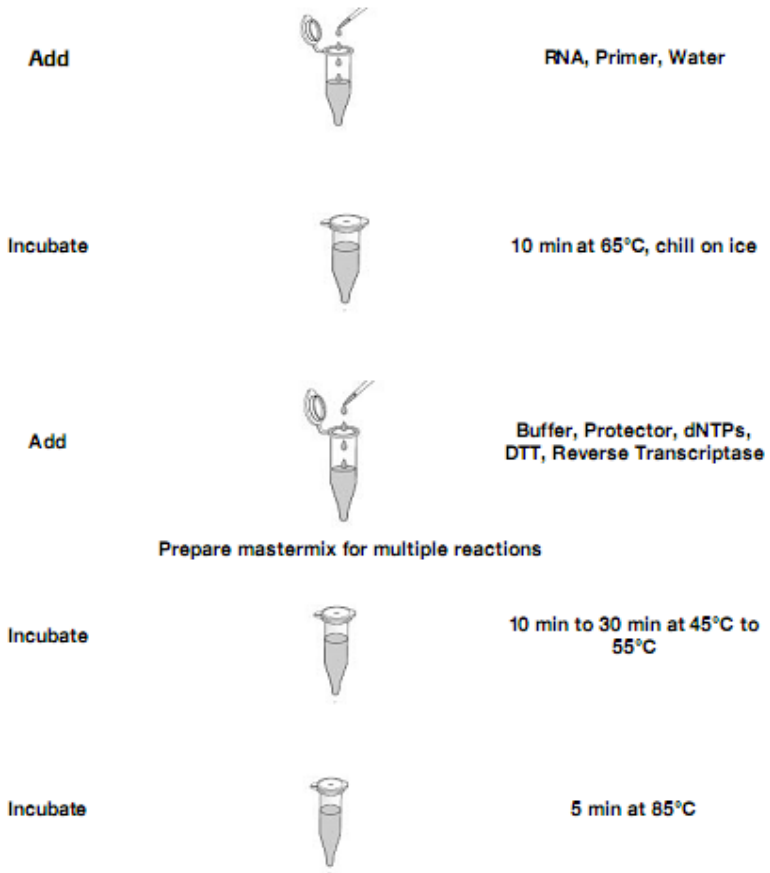



**Figure 3.3** Overview of cDNA synthesis procedure (www.roche-applied-science.com).

### 3.7.4 Determination of Gene Expression Level with Real Time-PCR

In this study, absolute quantification for expression levels of five different genes was performed by real-time PCR (Roche Lightcycler 1.5,

Mannheim, Germany). *AOX1, hGH, pep4, prb1* and *prc1* genes were selected for quantifications.

### 3.7.4.1 Primer Design

Primers were designed to amplify the cDNA of *hGH, AOX1, pep4, prb1* and *prc1* genes and the sequences of the genes are listed in Appendix F. For efficient amplification in real-time-PCR, it is important to design primer pairs so that the size of the amplicon should be smaller than 150 base pairs. By considering this, ProbeFinder 2.43 from Roche Universal Probe Library (https://www.roche-applied-science.com) and Primer 3 programmes (Rozen and Skaletsky, 2000) were used. The detailed results of the programmes are listed in Appendix G and the sequences of designed primer pairs for each gene are given in Table 3.14.

**Table 3.14** Primers used for amplifications of *hGH, AOX1, pep4, prb1* and *prc1* genes.

| Gene | | Sequence | Melting Temperature (°C) | % 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 |
| *pep4* | Left Primer | tcaacaggtcggagcctcta | 62.00 | 55.00 |
| | Right Primer | aacctgtactatcagcttgttcca | 68.00 | 41.67 |
| *prb1* | Left Primer | tccaagggacaccctaagc | 60.00 | 57.89 |
| | Right Primer | agggtggccttctttcatct | 60.00 | 50.00 |
| *prc1* | Left Primer | tgggaagaatgtgtatgatattcg | 66.00 | 37.50 |
| | Right Primer | tgaattcaaggtccttatagcaca | 66.00 | 37.50 |

### 3.7.4.2 Plasmid Isolation

Plasmid DNA, *pPICZaA::hGH*, containing *AOX1* and *hGH* genes is isolated from *Escherichia coli.* The procedure for isolation is given 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°C, with vigorous shaking (~200 rpm)
2. Pour 1mL of culture into microcentrifuge tube and centrifuge at 13200 $min^{-1}$, 4°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

To prepare standard curve, *pPICZaA::hGH* plasmid was used as a template for *hGH* and *AOX1* and, cDNA synthesized from total RNA of *P. pastoris* was used as a standard for *pep4, prb1* and *prc1*. Nucleic acid concentrations of standards were determined by AlphaSpect µL spectrophotometer (AlphaInnotech Inc, USA) at 260 nm. Purity of the nucleic acids were also determined by using ratio of $A_{260}/A_{280}$ which must be around 1.9 for DNA. After that, copy number of the each standard was calculated by using equations 3.4 and 3.5.

$$Copy\,number/\mu L = \frac{initial\,concentration\,of\,isolated\,plasmid\,(or\,genome)}{M_{plasmid}/\#copies\,of\,desired\,gene\,in\,plasmid\,(or\,genome)} \quad (3.4)$$

Where $M_{plasmid} = mass\,of\ plasmid = n\left(1.096 * 10^{-21}\ {}^{g}/_{base\ pair}\right)$ (3.5)

Where n= genome size (base pair)

For each PCR amplification standards for *hGH* and *AOX1* were made daily from the plasmid working stock ($8.5x10^{10}$ copies/µL). Dilutions of the standards from $10^8$ copies/ µL down to $10^4$ copies/ µL were then used to generate the standard curves. Standards for the *pep4, prb1* and *prc1* were made from genomic DNA (gDNA) synthesized from total RNA of $Mut^+$ phenotype of *P. pastoris.* Dilutions of the standards from $3x10^7$ to $3x10^5$ copies/µL were then used to generate the standard curve. Standard curves created for quantification of various genes are given in Results and Discussion part in Section 4.5.1.3 and 4.5.1.4.

### 3.7.4.4 Quantification of Expression Level of the Desired Genes

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

SYBR Green I assay for *hGH* and *AOX1* genes and hydrolysis probe assay, TaqMan, for *pep4, prb1* and *prc1* genes, are used to absolute quantifications of the genes. In order to analyze and quantitate the data obtained from these assays, LightCycler® Software 4.0 was used. This programme provides easy setting for required cycling parameters and enhanced data management. The protocol given by Roche to prepare reaction mixture for both assays 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 precipitateis 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. Pipet 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 10×concentration solution of PCR primers. Concentration of each primer should be 0.3 - 1 µM for LightCycler FastStart DNA Master SYBR Green I kit and 0.1 - 1 µM for LightCycler TaqMan Master kit in the reaction mixture.
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 and Table 3.16.
4. Mix carefully by pipetting up and down (do not vortex) and pipet 18 µL for the first kit and pipet 15 µL for the second kit PCR mixture into each precooled capillary. Then add 2 µL of the DNA template for SYBR Green method and 5 µL of the DNA template for TaqMan method. Then seal each capillary with a stopper.
5. Centrifuge capillaries at 700×g for 5 s.
6. Place the capillaries in the real time-PCR and program insert the PCR parameters to the program. A standard real time-PCR program is given in Table 3.17 and Table 3.18.

Amplification temperature of every primer pairs is different since number of base pairs, and A and C contents of the primers are various. Therefore, amplification of each gene was achieved at various temperatures. $MgCl_2$ concentration was also optimized for AOX and hGH primer pairs to achieve the specific and efficient amplification. Amplification temperature and $MgCl_2$ concentration used for quantification of each gene during real time-PCR reaction are given in Table 3.19

**Table 3.15** Components of the reaction mixture for real time-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** Components of the reaction mixture for real time-PCR for 20 µL reaction mixture for LightCycler® TaqMan Master kit.

| Component | Volume µL | Final Concentration |
|---|---|---|
| $H_2O$ PCR grade | 8.8 | |
| Probe (10 µM) | 0.2 | 0.05-0.1 µM |
| PCR primer, 10xconc. (4-10 µM) | 2 | 0.2–0.5 µM |
| Master Mix, 5×conc. | 4 | |
| DNA template | 5 | |
| **Total Volume** | 20 | |

**Table 3.17** Real time-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 |

**Table 3.18** Real time-PCR program for TaqMan method for LightCycler® 1.5 System.

| Analysis Mode | Cycles | Segment | Target Temperature | Hold Time | Acquisition Mode |
|---|---|---|---|---|---|
| **Pre-Incubation** | | | | | |
| None | 1 | | 95°C | 15 min | none |
| **Amplification** | | | | | |
| Quantification | 45 | Denaturation | 95°C | 10 s | none |
| | | Annealing | primer dependent | 20-40 s | none |
| | | Extension | 72°C | 1 s | single |
| **Cooling** | | | | | |
| None | 1 | | 40°C | 30 s | none |

**Table 3.19** Amplification temperature and $MgCl_2$ concentration for each gene during real time-PCR reaction.

| Gene | Amplification Temperature °C | $MgCl_2$ Concentration mM |
|---|---|---|
| AOX1 | 60 | 2 |
| hGH | 55 | 4 |
| Proteinase A | 62 | - |
| Proteinase B | 60 | - |
| Carboxypeptidase Y | 62 | - |

# CHAPTER 4

# RESULTS AND DISCUSSION

In this study, it was aimed to investigate effects of pH on therapeutically important protein, recombinant human growth hormone (rhGH), production by *Pichia pastoris* considering the expression levels of regulatory genes. In the first part of this study, effects of the use of glycerol and sorbitol as a second carbon source besides methanol, was investigated by *P. pastoris hGH-Mut*$^{+}$ and *P. pastoris hGH-Mut*$^{s}$, and the strain producing higher amount of rhGH was determined based on the rhGH concentration and expression level of *hGH*. Secondly, influence of pH on rhGH production and the stability of the protein were investigated by using chosen strain in laboratory scale air filtered shake bioreactors. In the third part of this study, to supply better control on pH and eliminate oxygen limitation, optimum pH was determined for rhGH production by using pilot scale bioreactor. Furthermore, cell growth, oxygen transfer and fermentation characteristics, effects of oxygen transfer on rhGH production and by-product formation were investigated and in addition to these to gain more insight on production and degradation of rhGH, expression levels of *rhGH, AOX1, pep4, prb1* and *prc1* were determined.

## 4.1 Selection of Microorganism

Before investigating the effects of pH on production and stability of rhGH, rhGH production and *hGH* expression levels of *Pichia pastoris*, Mut$^{+}$ and Mut$^{S}$, were determined. For this purpose, a repressing, glycerol, or a non repressing, sorbitol, carbon source was used besides methanol.

The strains grown on the solid medium were inoculated into precultivation medium, BMGY containing glycerol, and incubated at T=30ºC and N=225 min$^{-1}$ for 20 h in laboratory scale air filtered shake bioreactors.

Then, the harvested cells were resuspended in production medium including glycerol or sorbitol and incubated at T=30ºC and N=225 $min^{-1}$ for 47 h.

### 4.1.1 Microorganisms Grown on Glycerol

In recent times, although glycerol repress AOX1 promoter, glycerol and methanol co feeding strategy has been applied. In order to determine and compare expression levels of *hGH* on glycerol/methanol and sorbitol/methanol media, first of all fermentation using the optimum conditions, $C_{gly}$=30 g $L^{-1}$ and $C_{MeOH}$=3% (v/v) for *P.pastoris hGH-Mut*$^{+}$ strain and $C_{gly}$=30g/l and $C_{MeOH}$=1% (v/v) for *P.pastoris hGH-Mut*$^{S}$ strain, reported by Orman et al. (2009) was conducted. Two fold higher rhGH production was achieved with *P.pastoris hGH-Mut*$^{s}$, $C_{rp}$=110 mg $L^{-1}$ (Figure 4.1), similar to those reported by Orman et al. (2009).

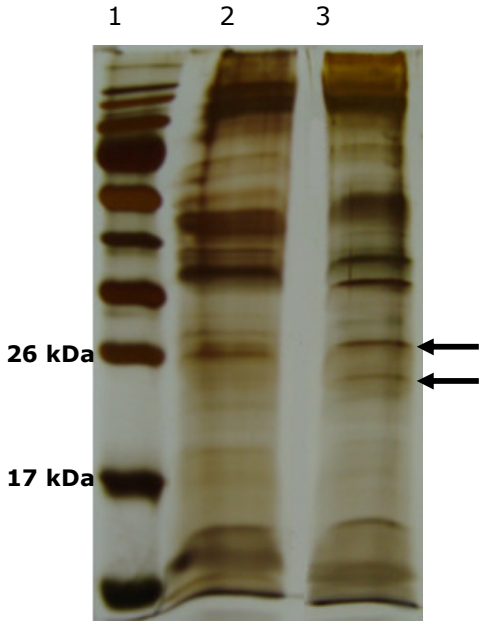



**Figure 4.1** Silver stained SDS-PAGE gel view of extracellular proteins produced in laboratory scale air filtered shake bioreactors to observe the difference in rhGH production between *P.pastoris hGH-Mut*$^{+}$ and *P.pastoris hGH-Mut*$^{S}$ phenotypes grown on optimized conditions in medium containing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

Amount of *hGH* found in extracellular medium is directly related with expression level of *hGH*. Determination of expression level can be achieved only by analyzing expression at transcriptional level. Investigation of the expression level of desired gene was performed by real time-PCR analysis using absolute quantification method. To be able to perform absolute quantification, calibration curves were formed before the analysis and formation of the curves is given in Appendix I. Before analysis, cDNA synthesis was performed by using total RNA isolated from the cells, which were collected at certain times during the production. While selecting the time interval for real time-PCR analysis, methanol addition schemes were taken into consideration. Throughout shake flask experiments, methanol was added to medium at t=0 h and t=24 h, where entire methanol added at the beginning of production phase was assumed to be consumed. To be able to observe effect of methanol induction, cells were collected both before and after addition of methanol and at the end of the process for real time-PCR analysis (t=23 h, t=26 and t=47 h). The results obtained from real time-PCR for the expression level of *hGH* gene (Figure 4.2) are consistent with SDS-PAGE results. Expression level of *hGH* in *P.pastoris hGH-Mut*$^S$ phenotype is higher than *P.pastoris hGH-Mut*$^+$ at every selected period of the induction phase. The expression level in Mut$^S$ strain, $6.76 \times 10^9$ copies $mg^{-1}$ CDW ($5.34 \times 10^5$ copies $ng^{-1}$ total RNA), is 3.25 fold higher than in *P.pastoris hGH-Mut*$^+$ strain, $2.08 \times 10^9$ copies $mg^{-1}$ CDW , at the end of the production phase in medium with glycerol/methanol (Figure 4.2).

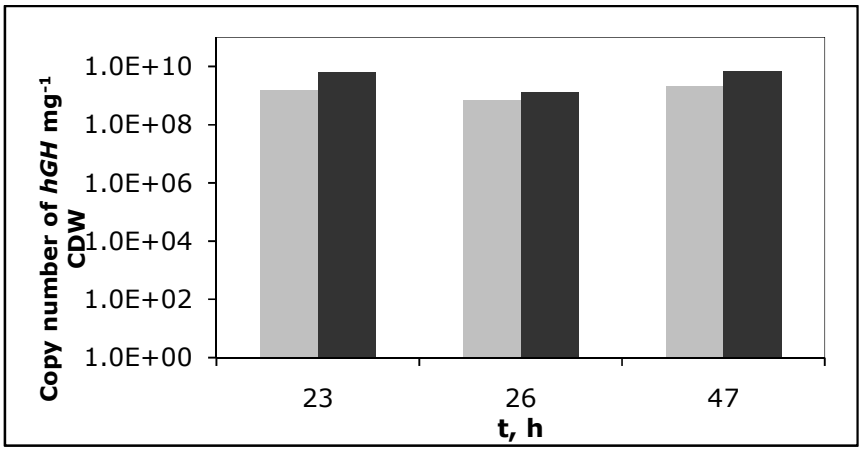



**Figure 4.2** Variation in expression level of *hGH* with cultivation time in *P.pastoris hGH-Mut*$^{+}$ and *P.pastoris hGH-Mut*$^{S}$ phenotypes grown on optimized conditions in medium containing glycerol and methanol in a defined medium. (■) *P.pastoris hGH-Mut*$^{+}$ phenotype, (■) *P.pastoris hGH-Mut*$^{S}$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

### 4.1.2 Microorganisms Grown on Sorbitol

Sorbitol a non-repressing carbon source with respect to the AOX1 promoter is usually used for the expression of recombinant proteins in Mut$^{S}$ phenotype of *P. pastoris*. 30 g $L^{-1}$ sorbitol concentration in the production medium was used since Orman et al. (2009) found that optimum glycerol concentration is 30 g $L^{-1}$ and medium containing equivalent amount of carbon molecules with 30 g $L^{-1}$ glycerol is 30 g $L^{-1}$ sorbitol. On the other hand, related with the methanol concentration, Çelik (2008) used 1% (v/v) methanol for Mut$^{+}$ concentration in medium containing sorbitol; whereas, Orman et al. (2009) showed that rhGH production is the highest in 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 sorbitol was investigated and it was found that above 1% (v/v), methanol exhibits inhibitory effect on cell growth of Mut$^{+}$ (Figure 4.3).

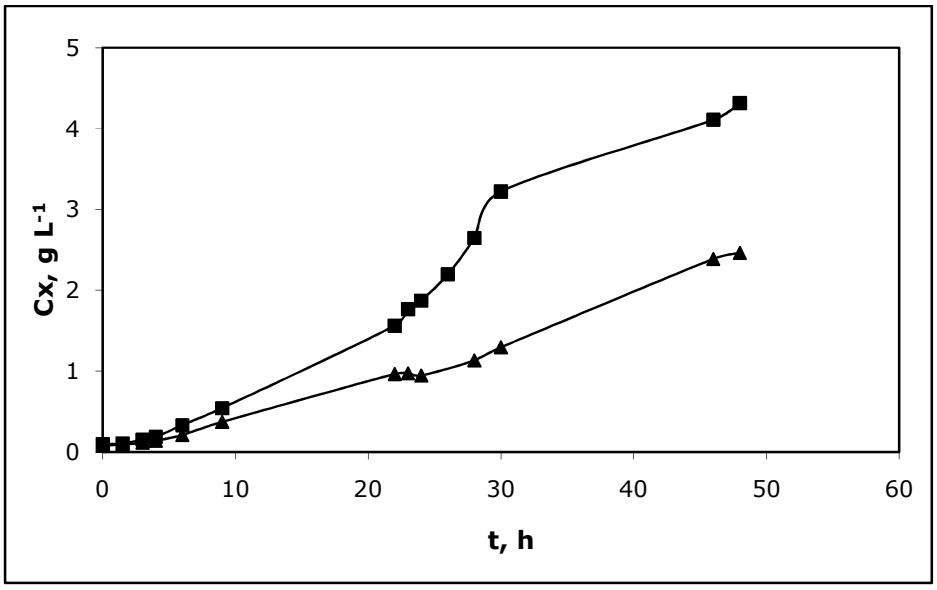



**Figure 4.3** Variation in *P.pastoris hGH-Mut*$^{+}$ cell concentration with time in defined medium containing 30 g $L^{-1}$ sorbitol. Initial methanol concentrations: (■) 1%, (▲) 3% (v/v).

In medium having 1% (v/v) methanol concentration and 30 g $L^{-1}$ sorbitol, rhGH concentration in *P.pastoris hGH-Mut*$^{+}$ phenotype was found as 120 mg $L^{-1}$ which is 1.9-fold higher than *P.pastoris hGH-Mut*$^{S}$ phenotype (62 mg $L^{-1}$) (Figure 4.4).

In medium containing sorbitol, it is observed that expression levels of both phenotypes are close to each other at t=23 and 26 h; whereas at t=47 h, hGH expression level of Mut$^{+}$ phenotype ($9.84x10^{9}$ copies $mg^{-1}$ CDW or $1.22x10^{6}$ copies $ng^{-1}$ total RNA) was 5.82-fold higher than that of Mut$^{S}$ phenotype (Figure 4.5), resulted in higher rhGH production.

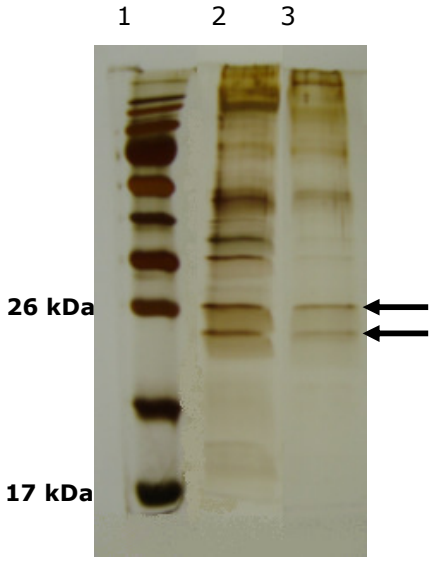



**Figure 4.4** Silver stained SDS-PAGE gel view of extracellular proteins produced in laboratory scale air filtered shake bioreactor to observe the difference in rhGH production between *P.pastoris hGH-Mut*$^{+}$ and *P.pastoris hGH-Mut*$^{S}$ phenotypes grown on sorbitol at t=47 h. 1. well: protein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

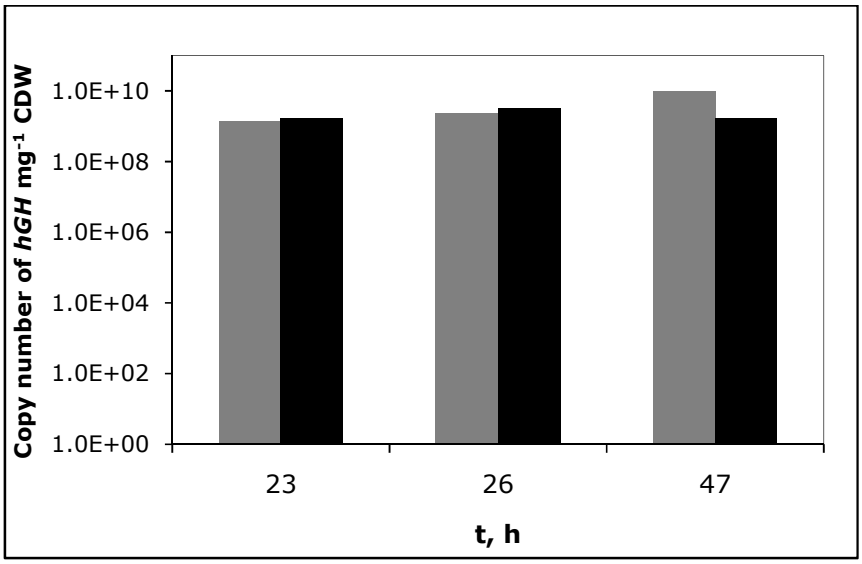



**Figure 4.5** Variation in expression level of *hGH* with cultivation time in *P.pastoris hGH-Mut*$^{+}$ and *P.pastoris hGH-Mut*$^{S}$ phenotypes grown on medium containing sorbitol and methanol in a defined medium. (■) *P.pastoris hGH-Mut*$^{+}$ *phenotype* (■) *P.pastoris hGH-Mut*$^{S}$ phenotype.

### 4.1.3 Comparison of Mut+ and MutS Phenotypes of *Pichia pastoris*

$Mut^S$ strain produces rhGH 2-fold higher than $Mut^+$ strain when glycerol and methanol co feeding strategy is applied and the result is consistent with the Orman et al., (2009) ones. However, $Mut^+$ strain produced rhGH 1.9-fold higher than $Mut^S$ strain as sorbitol and methanol co feeding strategy is used. This is an expected result when repression mechanism of AOX1 promoter is taken into consideration this promoter is tightly regulated by the presence of repressing carbon sources. In same SDS-PAGE gel (Figure 4.6), both strains and medium were compared 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. Furthermore, expression levels of *hGH* at t=23, 26 and 47 h were compared (Figures 4.7). These points were selected to observe differentiation in expression levels of the desired gene after the second induction applied at t=24 h. The comparison was not performed before second induction since until t=9 h, the cell growth is slow and up to that point significant increase in expression levels are not expected. According to Figure 4.7, generally an increase was seen in expression levels of *hGH* at t= 47 h for all conditions when it is compared to the ones obtained at t=23 and 26 h. Similarly SDS-page results (Figure 4.6), *hGH* in $Mut^S$ strain was expressed 3.25-fold higher and produced 2 fold higher than the ones in $Mut^+$ strain in medium with glycerol at t=47 h. However, $Mut^+$ strain had 5.82-fold higher copies and 1.9-fold higher product concentration than $Mut^S$ strain at t=47 h as sorbitol and methanol co feeding strategy was applied. Furthermore, the highest *hGH* expression level, $9.9x10^9$ copies $mg^{-1}$ CDW, obtained in medium containing sorbitol and 1% (v/v) methanol by using $Mut^+$ strain. This result is consistent with SDS-PAGE results.

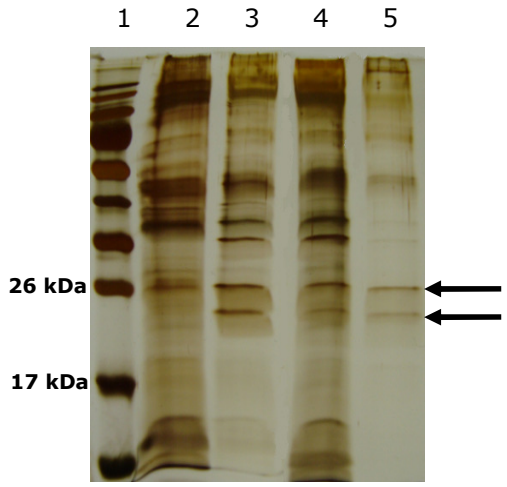



**Figure 4.6** Silver stained SDS-PAGE gel view of extracellular proteins produced in laboratory scale air filtered shake bioreactor to observe the difference in rhGH production between *P.pastoris hGH-Mut*$^{+}$ and *P.pastoris hGH-Mut*$^{S}$ phenotypes at t= 47 h. 1. well: protein marker, 2. well: 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.

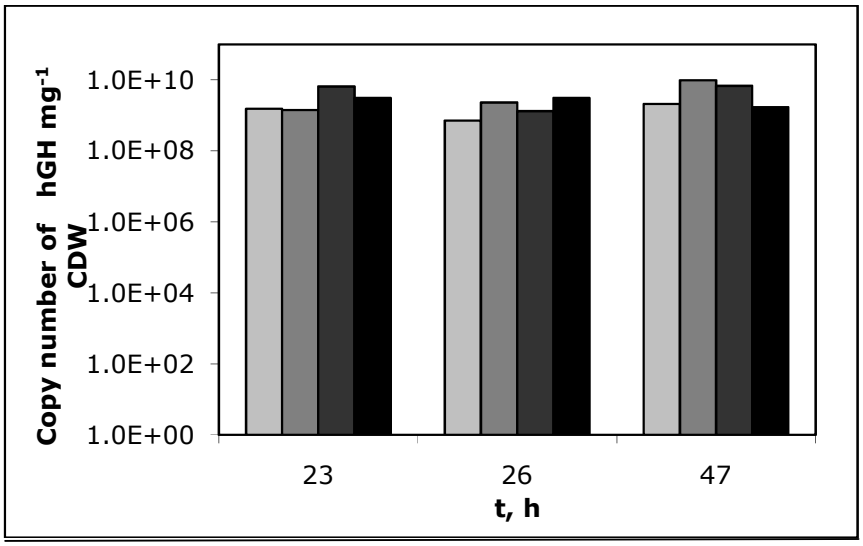



**Figure 4.7** Variation in expression levels of *hGH* at t=23, 26 and 47 h to observe the difference in *hGH* expression of *P.pastoris hGH-Mut*$^{+}$ and *P.pastoris hGH-Mut*$^{S}$ phenotypes in different medium. (■) Mut$^{+}$ phenotype grown on glycerol and 3% methanol, (■) Mut$^{+}$ phenotype grown on sorbitol and 1% methanol (■) Mut$^{S}$ phenotype grown on glycerol and 1% methanol, (■) Mut$^{S}$ phenotype grown on sorbitol and 1% methanol.

As hGH production and expression level are taken into consideration, the best result obtained by using $Mut^+$ strain (Figure 4.8). Therefore, expression level of *AOX* was only determined for $Mut^+$ strain. In medium containing 30 g $L^{-1}$ glycerol and 3% (v/v) methanol, higher AOX ($1.24x10^{10}$ copies $mg^{-1}$ CDW or $4.34x10^5$ copies $ng^{-1}$ total RNA) was expressed than in medium with 30 g $L^{-1}$ sorbitol and 1% (v/v) methanol throughout the process although, higher hGH production expression was achieved in sorbitol/methanol medium. This is an expected result since higher methanol content results in an increase in the induction of alcohol oxidase.

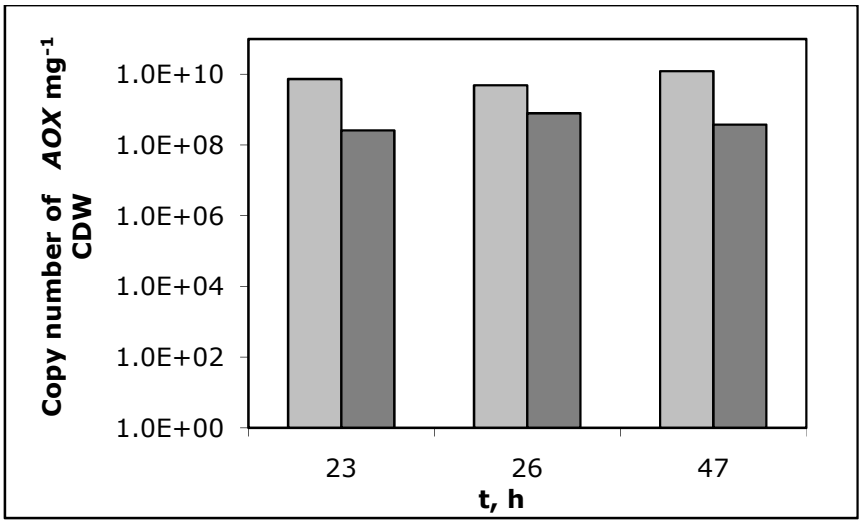



**Figure 4.8** Variation in expression levels of *AOX* at t=23, 26 and 47 h to observe the difference in *AOX* expression of *P.pastoris hGH-Mut*$^+$ and *P.pastoris hGH-Mut*$^S$ phenotypes in different medium. (▫) *P.pastoris hGH-Mut*$^+$ phenotype grown on glycerol and 3% methanol, (▪) *P.pastoris hGH-Mut*$^+$ phenotype grown on sorbitol and 1% methanol.

### 4.2 Effects of Sorbitol Concentration

Effects of sorbitol concentration on rhGH production were investigated at $C_S$= 20, 30, 40, 50, 60, 70 g $L^{-1}$ at $C_M$=1% (v/v) initial methanol concentration. The methanol and sorbitol consumption profiles are given in Figures 4.9 and 4.10, respectively. The cell concentration profiles were very close to each other below $C_S$= 50 g $L^{-1}$ (Figure 4.11). This can be due to fact

that the amount of sorbitol consumed does not change with the initial amount of sorbitol put into the medium (Figure 4.10). A decrease in the cell growth rate was found (Figure 4.11) when initial sorbitol concentration above 50 g $L^{-1}$ so the non-inhibitory limit for rhGH production from $Mut^+$ phenotype of *P. pastoris* is 50 g $L^{-1}$. Çelik (2008), investigated the optimum sorbitol concentration for laboratory scale shakes bioreactors and non-inhibitory sorbitol concentration for production of human erythropoietin (EPO), and found that optimum initial sorbitol concentration for laboratory scale shake bioreactors was 7.5 g $L^{-1}$. 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. For EPO production, non-inhibitory sorbitol concentration was found as 50 g $L^{-1}$ (Çelik et al., 2009). Hence, the results obtain in this study are consistent with the ones obtained by Çelik et al. (2009).

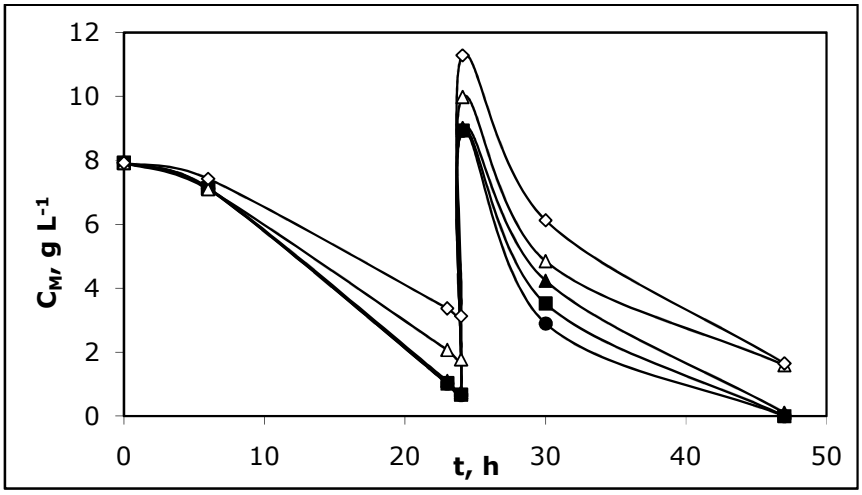



**Figure 4.9** Methanol utilization diagrams of *P. pastoris-hGH-Mut*$^+$ strain in defined medium with initial sorbitol concentrations of 20 g $L^{-1}$ (●), 30 g $L^{-1}$ (■), 40 g $L^{-1}$ (▲), 50 g $L^{-1}$ (Δ), 60 g $L^{-1}$ (◇).

As it seen Figure 4.9, methanol consumption rate decreases as sorbitol concentration found in the medium increases when initial sorbitol concentration above 50 g $L^{-1}$. However, methanol utilization rate remains almost constant when initial sorbitol concentration below 50 g $L^{-1}$. Although,

presence of sorbitol does not repress AOX, utilization of additional sorbitol may put an additional stress on cells, affecting the methanol utilization pathway. This may be due the fact than cells may prefer utilizing sorbitol with glycolysis rather than utilizing toxic methanol with peroxisomal reactions.

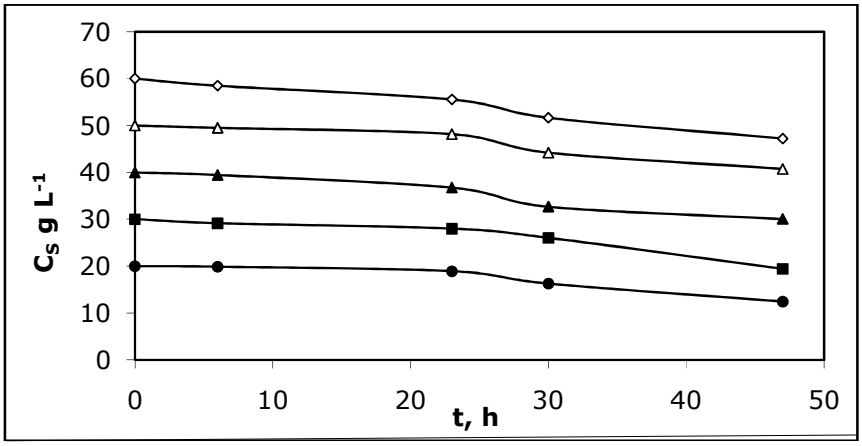



**Figure 4.10** Sorbitol utilization diagrams of *P. pastoris-hGH-Mut*$^+$ strain in defined medium with initial sorbitol concentrations of 20 g $L^{-1}$ (●), 30 g $L^{-1}$ (■), 40 g $L^{-1}$ (▲), 50 g $L^{-1}$ (Δ), 60 g $L^{-1}$ (◇).

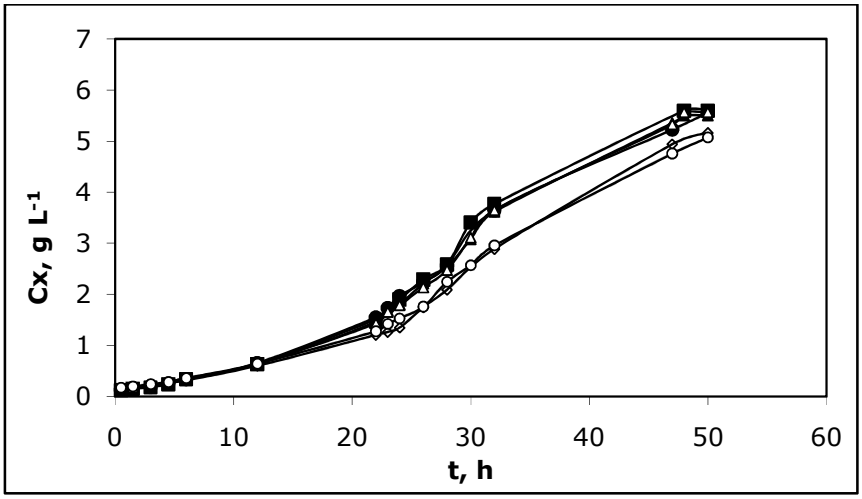



**Figure 4.11** Variation in cell concentration with time in defined medium containing 20 (●), 30 (■), 40 (▲), 50 (Δ), 60 (◇), 70 (o) g $L^{-1}$ sorbitol with 1% (v/v) methanol.

RhGH concentration increased with the increase in initial sorbitol concentration below 50 g $L^{-1}$ and the concentration almost remains constant at 130 mg $mL^{-1}$ above 50 g $L^{-1}$ (Figure 4.12). As a result, 50 g $L^{-1}$ sorbitol concentration was found optimum for both cell growth rate and rhGH production.

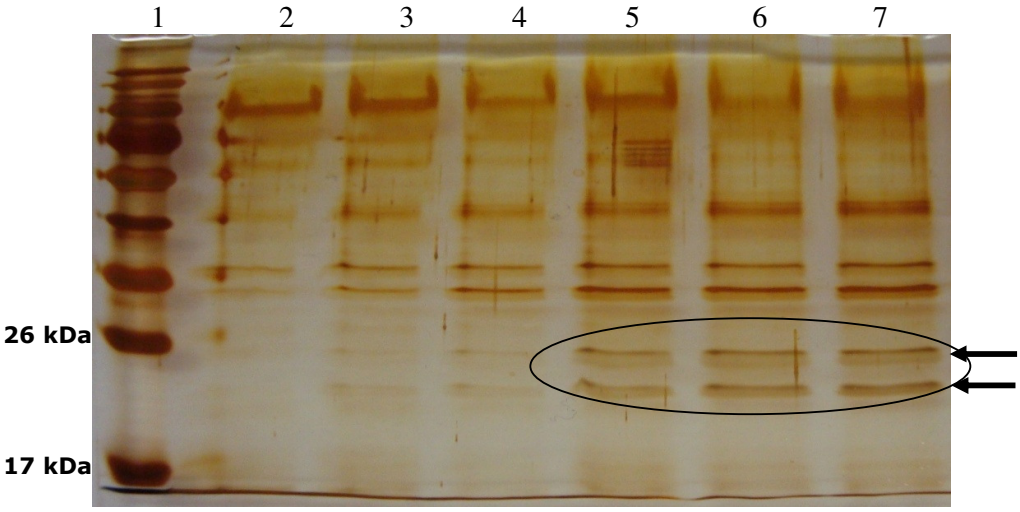



**Figure 4.12** Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor to observe the difference in rhGH production between different initial sorbitol concentrations at t=47 h. 1. well: protein marker, 2. well: 20 g $L^{-1}$,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

### 4.3 Effect of pH on Production and Stability

Effects of pH on cell growth, rhGH production and stability were investigated in medium containing 50 g $L^{-1}$ sorbitol and 1% (v/v) methanol by using laboratory scale air filtered shake bioreactors. 47 h production was performed. In order to search, pH range between 3.0 and 7.0 was used and, medium of pH was adjusted by using 0.5 M $Na_2HPO_4.7H_20$ and 0.25 M citric acid.$H_2O$. For investigation of stability, after production, supernatant of the culture having pH 6.0 was separated and divided into 7 samples. Their pH values were adjusted between 3.0 and 7.0 and the samples were kept at room temperature for 20 and 48 h.

### 4.3.1 Effect of pH on Cell Growth

Firstly, effect of initial medium pH on cell growth was investigated. Generally, cell concentration increased with the increase in pH within the range 3.0 to 7.0; and, there was no considerable difference between the final cell concentrations, especially in the pH range between 5.0 and 7.0 (Figure 4.13). This is an expected result since *P. pastoris* can tolerate a broad pH range between 3.0 and 7.0, more preferably and usually about 3.5 to 5.5. This range has little effect on the growth rate (Wegner, 1983; Inan et al., 1999).

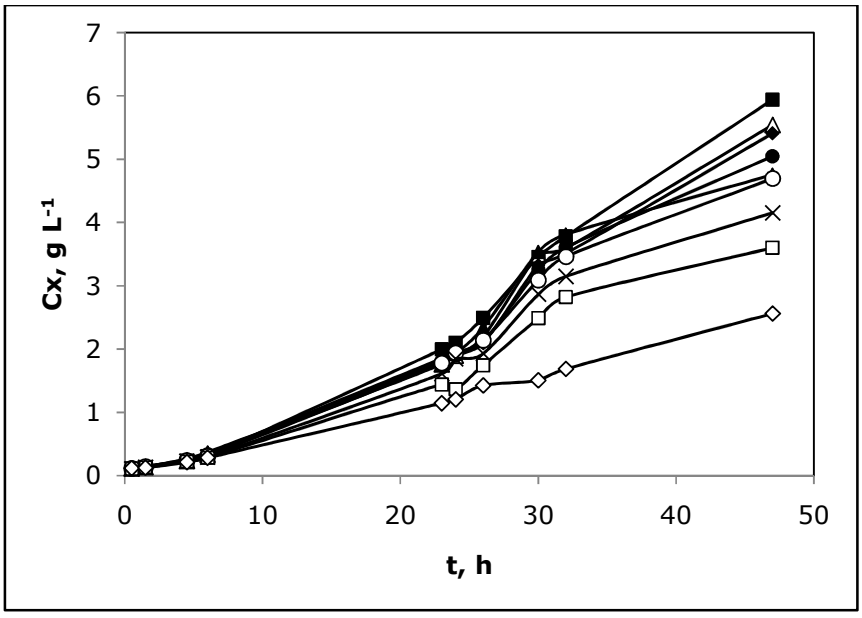



**Figure 4.13** Variation in cell concentration with respect to time and medium pH. pH values: (◇) pH 3.0, (□) pH 3.5, (x) pH 4.0, (o) pH 4.5, (●) pH 5.0, (◆) pH5.5, (▲) pH 6.0, (Δ) pH 6.5, (■) pH 7.0.

### 4.3.2 Effect of pH on rhGH stability

In order to search, samples having different pH value were kept at room temperature for 20 and 48 h and their rhGH concentrations were compared with the one stored at - 20ºC. When the results obtained at t=20 h were compared with one stored at - 20ºC, protein degradation was observed in the samples kept at room temperature. Furthermore, there is no drastic difference in the concentrations of rhGH between medium having different pH values (Figure 4.14). A considerable amount of degradation was observed, again, in the samples kept for t=48h. However, in this case, more rhGH remained stable at pH 5.0. As a result, rhGH is found to be more stable at pH 5.0 (Figure 4.15).

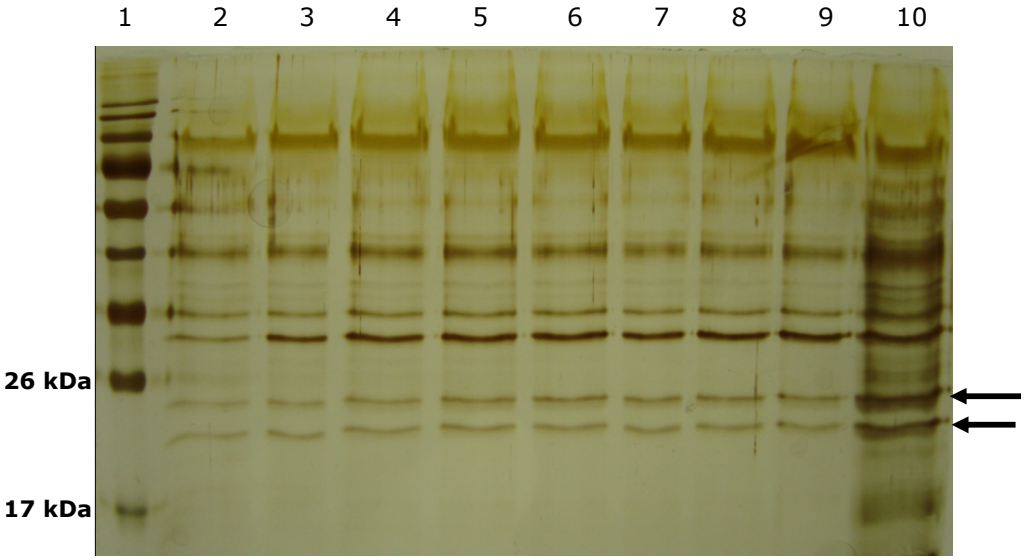



**Figure 4.14** Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor to observe effects of pH on rhGH stability. The samples kept at room temperature for 20 h. 1. well: protein marker, 2. well: pH 3.5, 3. well: pH 4.0, 4. well: pH 4.5, 5. well: pH 5.0, 6. well: pH 5.5, 7. well: pH 6.0; 8. well: pH 6.5, 9. well: pH 7.0, 10. well: samples kept at – 20ºC.

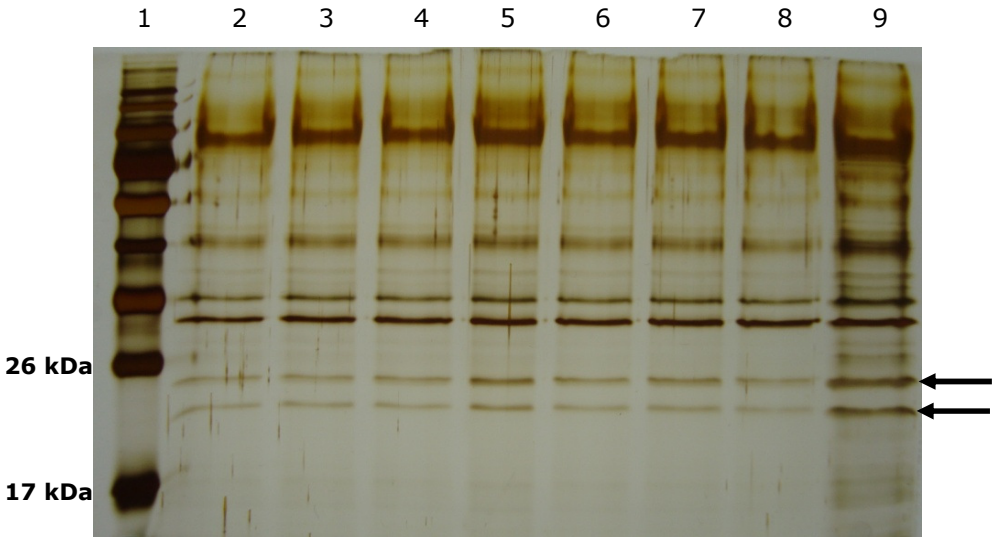



**Figure 4.15** Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor to observe effects of pH on rhGH stability. The samples kept at room temperature for 48 h. 1. well: protein marker, 2. well: pH 3.5, 3. well: pH 4.0, 4. well: pH 4.5, 5. well: pH 5.0, 6. well: pH 5.5, 7. well: pH 6.0; 8. well: pH 6.5, 9. well: samples kept at – 20ºC.

### 4.3.3 Effect of pH on rhGH production

Effect of pH on rhGH production was investigated and shown in Figure 4.16; there was no significant variation in the production levels at different pH values, especially between 5.0 and 7.0. This can be due to fact that, pH can not be controlled throughout the process since it is batch and, the final pH measured in all media was around pH=2.4. This shows that, further investigation is needed to determine optimum pH. Therefore, optimization of pH should be determined by using pilot scale bioreactor, which supplies a better pH and oxygen control.

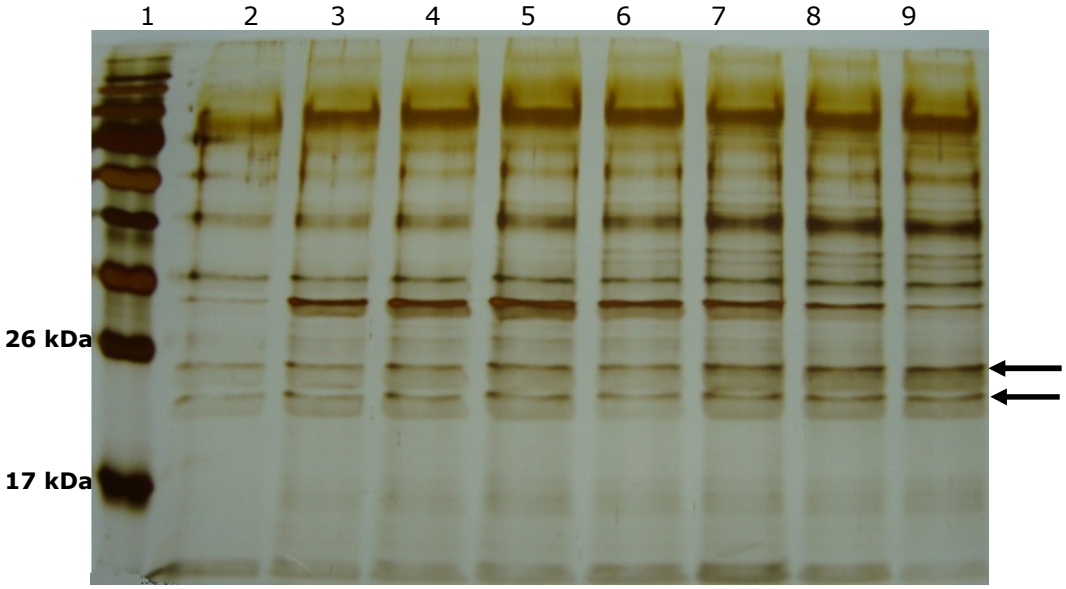



**Figure 4.16** Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in laboratory scale air filtered shake bioreactor to observe effect of pH on rhGH production at t=47 h. 1. well: protein marker, 2. well: pH 3.5, 3. well: pH 4.0, 4. well: pH 4.5, 5. well: pH 5.0, 6. well: pH 5.5, 7. well: pH 6.0; 8. well: pH 6.5, 9. well: pH 7.0.

### 4.4 Expression of Recombinant Human Growth Hormone by *P. pastoris* in Pilot Scale Bioreactors

Having selected the strain to be used throughout the process, determined the second carbon source and having investigated the effects of pH on cell growth rate, stability and production of rhGH in laboratory scale air filtered shake bioreactors, the next step is to determine optimum pH considering proteolytic degradation by using pilot scale bioreactor. The reactor is equipped with temperature, pH, foam, stirring rate, feed inlet rate, dissolved oxygen control and a mass flow controllers to adjust the dissolved oxygen concentration in the medium.

It is known that, addition of sorbitol batch wise to the medium, was beneficial in terms of eliminating the long lag-phase for the cells and therefore cell yields were greatly improved (Çelik et al., 2009). Therefore, optimization of pH was performed in medium containing sorbitol. To be able to determine optimum pH, three sets of fed-batch bioreactor experiments were performed. The sorbitol was added to medium as a batch at the beginning of the methanol fed-batch phase. After investigation of optimum pH, one more set of fed-batch

bioreactor experiment was performed where only methanol was used as a carbon source to be able to observe the effectiveness of the sorbitol/methanol mixed feeding strategy.

### 4.4.1 Control of Bioreactor Operation Parameters in Pilot-Scale Bioreactor

In this study, it is important to keep pH constant throughout the bioprocess. For this, 25% ammonia solution, $NH_3OH$, was used. Furthermore, by setting the PI control parameters as $X_p$=30% and TI= 30s, and keeping the base-pump-valve open at 10%, the pH of the medium was controlled at desired pH ± 0.1. The other most important parameter is dissolved oxygen concentration and oxygen transfer rate since *P. pastoris* is an aerobic microorganism and, when methanol is used as the carbon source it has extremely high oxygen demand. Therefore, dissolved oxygen (DO) has been maintained above 20% saturation to prevent oxygen limitation. At the beginning of glycerol batch phase, only air was used to control DO. However, after $12^{th}$ of glycerol batch phase, supplying only air was not sufficient to keep DO levels above 20% saturation. Therefore, air was enriched with oxygen using mass flow controllers.

The temperature was kept at T=30°C by the PI controller of the system. The agitation rate was maintained constant at N=900 rpm. Agitation rate higher than N=900 rpm can cause shear damaging the cells, increase in temperature and further foam formation; and as lower agitation rate can not be enough since *P. pastoris* yeast consumes oxygen at a very high rate. Foaming was prevented by adding antifoam solution to the initial medium (0.01% v/v) and maximum 1 or 2 ml antifoam was used during all process.

### 4.4.2 Glycerol, Sorbitol and Methanol Feeding Rate in Fed-Batch Pilot Scale Bioreactor Operations

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

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

where, $\mu_0$ is the specific growth rate ($h^{-1}$), $V_0$ is the initial culture volume (L), $C_{X0}$ is the initial cell concentration (g $L^{-1}$), and $Y_{X/S}$ (g $g^{-1}$) is the cell yield on substrate.

Fed-batch pilot scale bioreactor experiments include four phases, which are glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT) and methanol fed-batch phase (MFB). In glycerol and methanol fed- batch phases, predetermined feeding profiles were calculated by using equation 4.1. For glycerol fed-batch phase specific growth rate was kept constant at 0.18 g $L^{-1}$, which is the maximum specific growth rate of *P.pastoris hGH-Mut*$^+$ strain on glycerol. The cell yield on glycerol ($Y_{X/G}$) is 0.5 g $g^{-1}$ (Cos et al., 2005). The predetermined feeding profile for this phase can be seen in Figure 4.17.

At the beginning of the methanol fed-batch phase, induction phase, $C_s$=50 g $L^{-1}$ was added to medium as a batch-wise. The specific growth rate on methanol was selected as 0.03 $h^{-1}$, as the specific recombinant protein productivity does not depend on the growth rate above specific growth rates of $\mu$=0.025 $h^{-1}$ (Cunha et al., 2004). Furthermore, the maximum specific growth rate of *P. pastoris* on methanol is $\mu_{max}$=0.14 $h^{-1}$, while that on sorbitol is $\mu_{max}$=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. In addition, Jungo et al. (2007) investigated the effect of specific growth rate ($\mu$), lower than 0.08 $h^{-1}$, on specific product productivity by using pre-determined exponential feeding profile. It was observed that, for specific growth rates higher than about 0.02 $h^{-1}$, specific productivity increased slightly with $\mu$. However, a large decrease in specific productivities was observed at $\mu$ below 0.02 $h^{-1}$. Finally, Çelik et al., (2009) investigated the effect of specific growth rate on production in the presence of sorbitol and the highest protein concentration was achieved at $\mu$= 0.03 $h^{-1}$. Therefore, $\mu$= 0.03 $h^{-1}$ was used for all bioreactor experiment. The cell yield on methanol ($Y_{X/M}$) is 0.42 g $g^{-1}$ (Jungo et al., 2006) and the predetermined feed profiles for this phase can be seen in Figure 4.18.

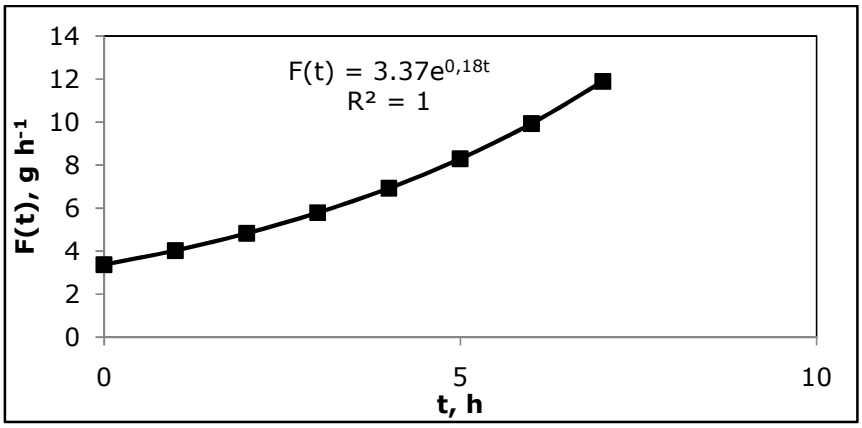



**Figure 4.17** The predetermined feeding profile for glycerol, calculated for specific growth rate ($\mu_o$) 0.18 $h^{-1}$.

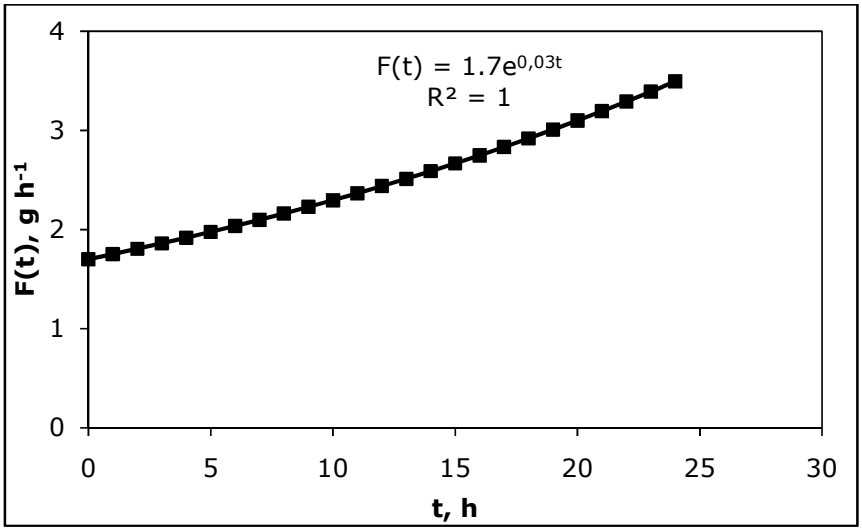



**Figure 4.18** The predetermined feeding profile for methanol, calculated for specific growth rate ($\mu_o$) 0.03 $h^{-1}$.

### 4.4.3 Effects of pH on Sorbitol and Methanol Consumption Rates

According to sorbitol consumption profiles (Figure 4.19), sorbitol was consumed more rapidly in media with pH 4.2 and 5.0. However, at pH 6.0, sorbitol was totally consumed at t=21 h. This case can be related to the higher biochemical reaction rates towards cell synthesis instead of r-protein synthesis at pH 6.0. In the medium, methanol has never been detected. This shows

simultaneous utilization of sorbitol with methanol, similar those reported by Jungo et al. (2007) and Çelik et al. (2009). The limited methanol feeding and high methanol consumption rate of *P. pastoris* can be reasons of that situation.

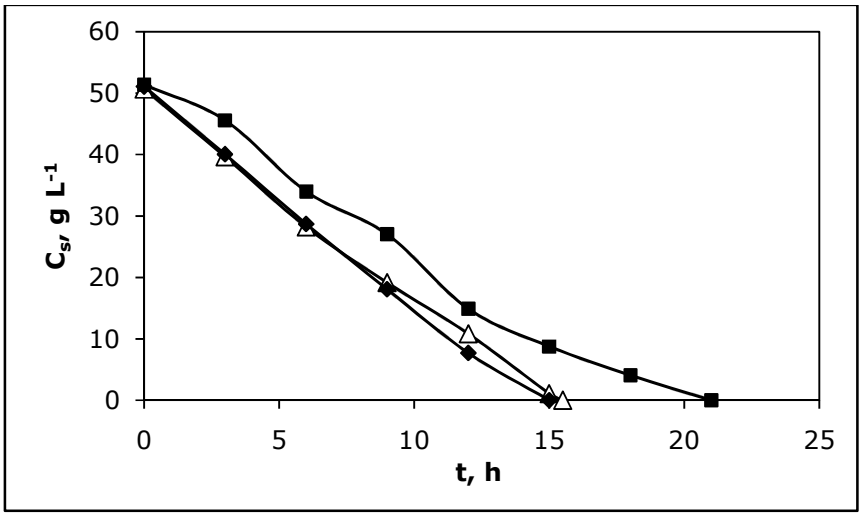



**Figure 4.19** Variation in sorbitol concentration with cultivation time at different pH. pH values: (△) pH 4.2, (◆) pH 5.0, (■) pH 6.0.

### 4.4.4 Cell Growth

#### 4.4.4.1 Effect of pH Cell Growth

According to *P. pastoris* fermentation protocol, to be able to reach desired cell concentration, GB, GFB and MT phases were performed before induction phase. The medium pH was adjusted at the beginning of the GB phase and maintained at the same value throughout the process. It is observed that, cell growth profiles obtained at pH 4.2, 5.0 and 6.0 are almost same for the first three phases (Figure 4.20). This is an expected result since *P. pastoris* can tolerate a broad pH range between 3.0 and 7.0, more preferably and usually about 3.5 to 5.5. This range has little effect on the growth rate (Wegner, 1983; Inan et al., 1999). Furthermore, there is no considerable protein, amino acid formation and etc. in these phases and

hydrogen ions ($H^+$) are consumed only for cell growth. Therefore, initial $H^+$ concentration does not affect the growth of the cell in these phases.

The methanol fed batch phase, induction phase, starts around t=31.5 h of the precultivation period, for simplicity in calculations, commence of the production phase was taken as t=0 h (Figure 4.21). In production phase, by starting with the same cell concentration, the highest and lowest final cell concentrations were obtained at pH 6.0 (52.53 g $L^{-1}$) and 5.0 (42.3 g $L^{-1}$), respectively (Figure 4.21). At pH 5.0, probably energy and substrate were mostly used for rhGH production rather than cell growth.

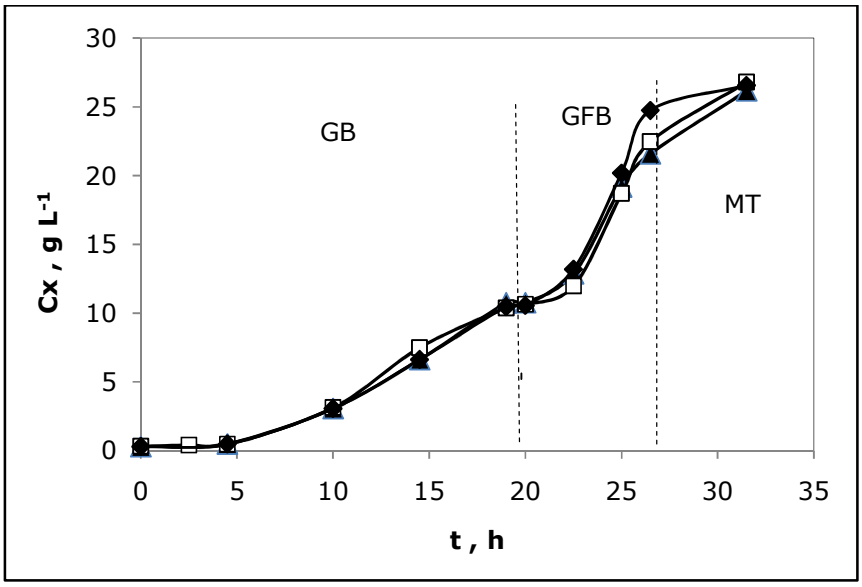



**Figure 4.20** Variation in cell concentration with cultivation time in glycerol batch phase (GB), glycerol fed-batch phase (GFB) and methanol transition phase (MT) at different pH. pH values: (▲) pH 4.2, (◆) pH 5.0, (□) pH 6.0.

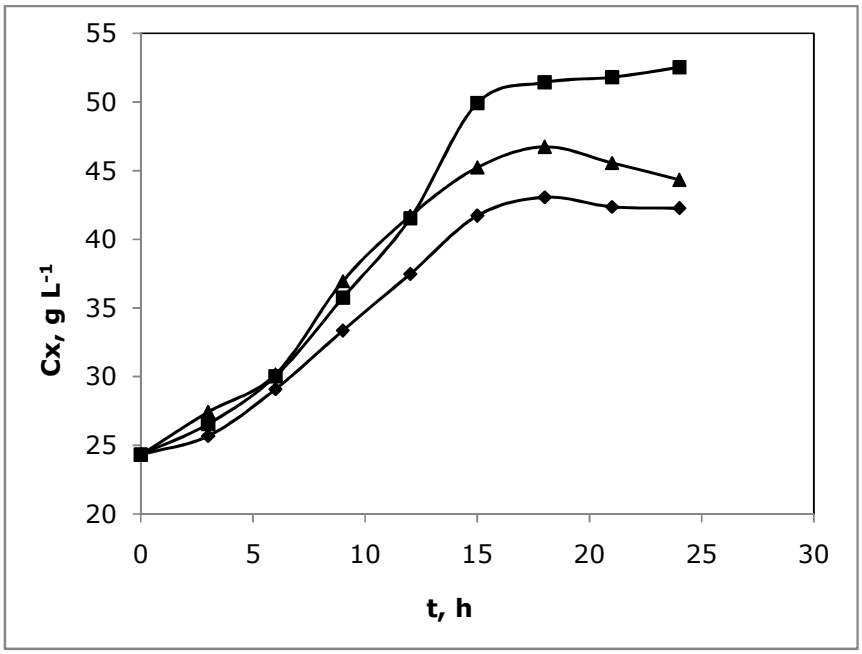



**Figure 4.21** Variation in cell concentration with cultivation time in production phase at different pH. pH values: (▲) pH 4.2, (◆) pH 5.0, (■) pH 6.0.

#### 4.4.4.2 Effect of Sorbitol/Methanol Mixed Feeding on Cell Growth

In order to determine the effectiveness of the methanol/sorbitol mixed feeding strategy, firstly cell growth rates are compared. Variation in cell concentrations in sorbitol/methanol medium and methanol medium are given in Figure 4.22. It is observed that addition of sorbitol as batch wise to the medium eliminates the long lag-phase for the cells (Figure 4.22), similar to the results reported by Çelik et al. (2009). Moreover, at t=24 h, the final cell concentration in medium containing sorbitol (at pH 5.0) is almost 1.4 fold higher than final cell concentration (at pH 5.0) obtained by using methanol as a sole carbon source (Figure 4.22) although the same methanol feeding profile ($\mu$=0.03 $h^{-1}$) was used. This can be due to elimination of negative effects of methanol on cell metabolism in the presence of sorbitol. Çelik et al., (2009) revealed that 1.7-fold higher cell concentration was obtained in medium with sorbitol compared to that without sorbitol by using same methanol feeding profile. These results are verified by this study. In addition to that Çelik et al., (2009) obtained 66.9 g $L^{-1}$ cell concentration at t=24 h in medium containing sorbitol and at $\mu$=0.03 $h^{-1}$ whereas by using same condition, 42.27 g $L^{-1}$ cell

concentration was acquired in this study. This can be due to variation in production level of the desired protein since in this study, 271 g $L^{-1}$ rhGH was produced, which is higher than 130 g $L^{-1}$ rHuEPO.

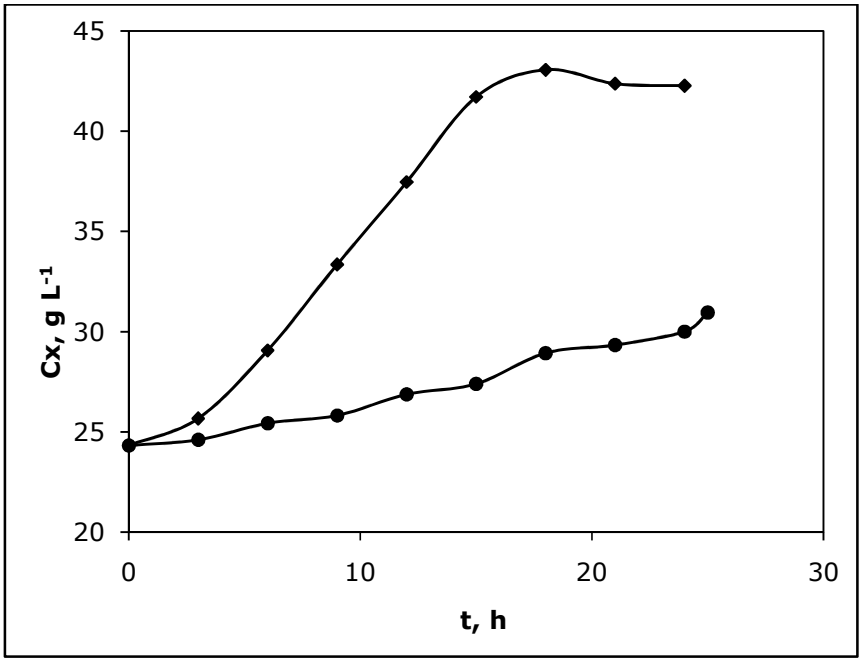



**Figure 4.22** Variation in cell concentration with cultivation time in production phase at pH 5.0 with different production medium for an exponential feeding at μ=0.03 $h^{-1}$. (◆) Methanol/sorbitol mixed feeding, (●) Methanol feeding.

### 4.4.5 Recombinant Human Growth Hormone Production

#### 4.4.5.1 Effect of pH on Recombinant Human Growth Hormone Production

Recombinant human growth hormone production level is the most important factor while determining an optimum pH for production. Firstly, rhGH production levels at t=24 h, where highest rhGH obtained at all conditions, were compared by SDS-PAGE analysis (Figure 4.23) and then the concentrations were measured by HPCE (Figure 4.24). In addition to these, expression level of the *hGH* gene was determined by real time-PCR (Figure 4.25). The highest rhGH concentration, $C_{rp}$= 271 mg $L^{-1}$, was acquired at pH 5.0. Thus, 1.22-fold higher protein was obtained at t=24 h at pH 5.0

compared to the one at pH 4.2. The rhGH concentration in medium with pH 6.0 is 24 mg $L^{-1}$ at t=24 h, which is the lowest one although the highest cell concentration (52.53 g $L^{-1}$) was obtained. This can be related to the expression level of *AOX1* since AOX activity obtained at pH 6.0 probably was so low.

In literature, using $C_{MeOH}$=3% (v/v) after 3 day induction in complex medium, 190 mg $L^{-1}$ rhGH concentration was obtained by Eurwilaichitr et al. (2002). RhGH produced in this work, $C_{rp}$= 271 mg $L^{-1}$, is 1.43-fold higher than that reported by Eurwilaichitr et al. (2002); and the highest rhGH concentration obtained until now.

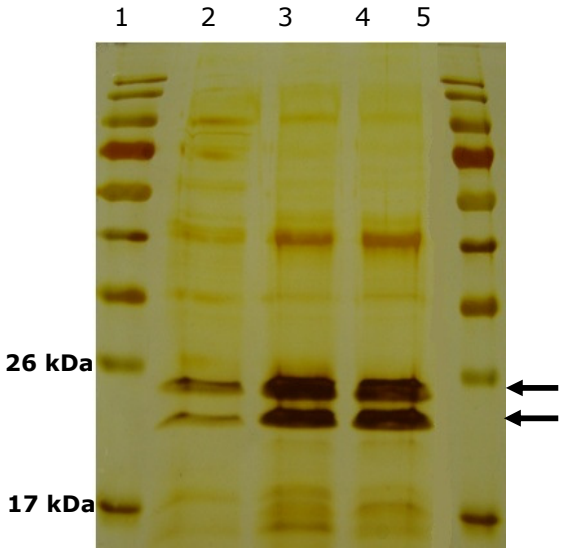



**Figure 4.23** Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* in pilot scale bioreactor to observe effects of pH on rhGH production at t= 24 h. 1. well: protein marker, 2. well: pH 6.0, 3. well: pH 5.0, 4. well: pH 4.2, 5. well: protein marker.

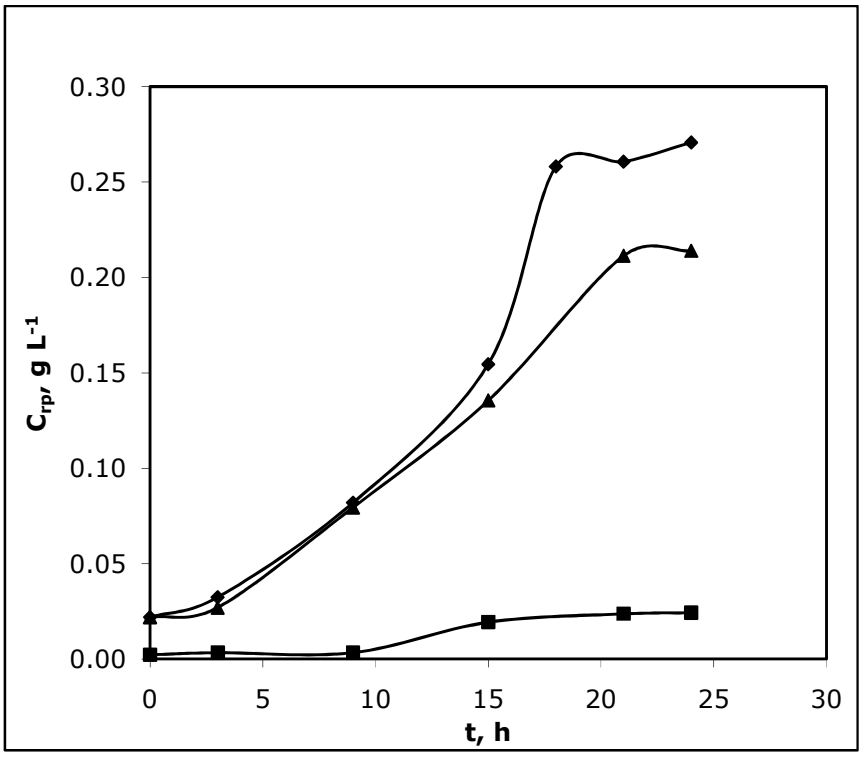



**Figure 4.24** Variation in rhGH concentration with cultivation time in production phase at different pH. pH values: (▲) pH 4.2, (◆) pH 5.0, (■) pH 6.0.

The expression level profiles of *hGH* are not parallel to rhGH concentration profiles since rhGH concentration increased exponentially and then reached a stable point whereas fluctuations were observed at *hGH* expression levels (Figure 4.24 and 4.25). Furthermore, in expression level profiles, hGH expression drops suddenly after reaching a maximum point, which is t=15 h for pH 5.0 and 6.0 and, t=18 h for pH 4.2, corresponding to the end of the cell growth phase. Time required to attain maximum point is different for different pH values. This can be directly related to the expression level and consequently AOX activity profiles since the production of the recombinant protein depends on the induction of AOX promoter.

It is observed that highest *hGH* expression, $4.01 \times 10^{10}$ copies $mg^{-1}$ CDW ($8.78 \times 10^{5}$ copies $ng^{-1}$ total RNA), was obtained at pH 5.0 at t= 15 h, which verifies the results obtained by SDS-PAGE and HPCE. According to HPCE result, at the end of the production phase, rhGH concentration is so low, 24 mg $L^{-1}$, at pH 6.0 whereas maximum expression level reached at pH 6.0,

$1.30x10^{10}$ copy number $mg^{-1}$ CDW, is very close to $1.10x10^{10}$ copy number $mg^{-1}$ CDW obtained at pH 4.2 (Figure 4.25). This can be due to low efficiency of translation at pH 6.0 In a well-designed system, all transcripted genes should undergo translation but in most cases this can not be achieved due to some limitations occurred in amino acid synthesis and translation and, expression level analysis was performed at transcriptional level. Therefore, results obtained at real time-PCR analysis and HPCE may differ from each other.

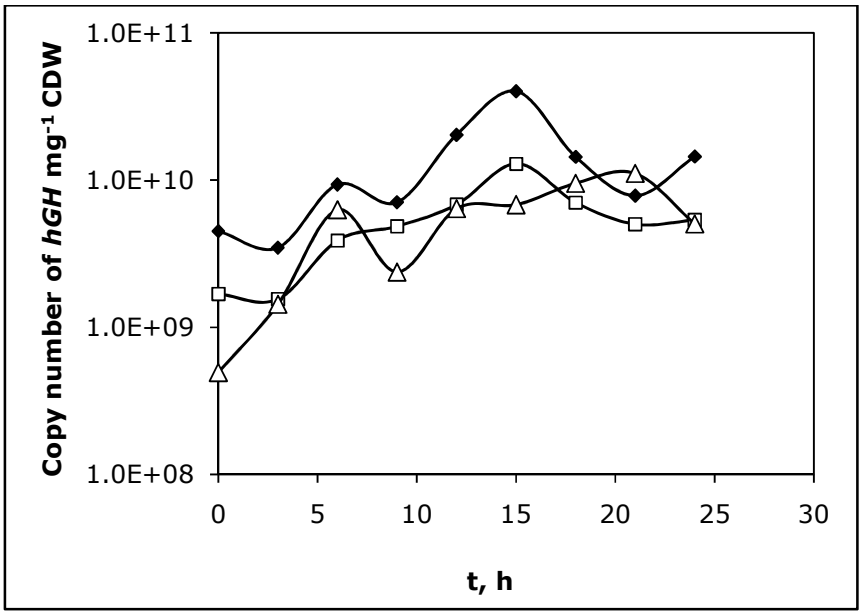



**Figure 4.25** Variation in *hGH* expression level with cultivation time in production phase at different pH. pH values: (△) pH 4.2, (◆) pH 5.0, (□) pH 6.0.

#### 4.4.5.2 Effect of Sorbitol/Methanol Mixed Feeding on Recombinant Human Growth Hormone Production

To investigate effect of addition of sorbitol on production, rhGH concentration profile acquired at pH 5.0 was compared to the one containing only methanol in medium at pH 5.0 (Figure 4.26). It is seen that at t= 24 h rhGH concentration in medium with sorbitol is 270 mg $L^{-1,}$ which is 1.5 fold higher than 180 mg $L^{-1}$ obtained in medium containing only methanol. This is

an expected result since almost 1.4 fold higher cell concentration was achieved by using sorbitol. Furthermore, addition of sorbitol as batch wise to the medium eliminates the long lag-phase and this minimizes toxic effects of methanol. Hence, repression of AOX promoter may become faster and this results in higher rhGH production.

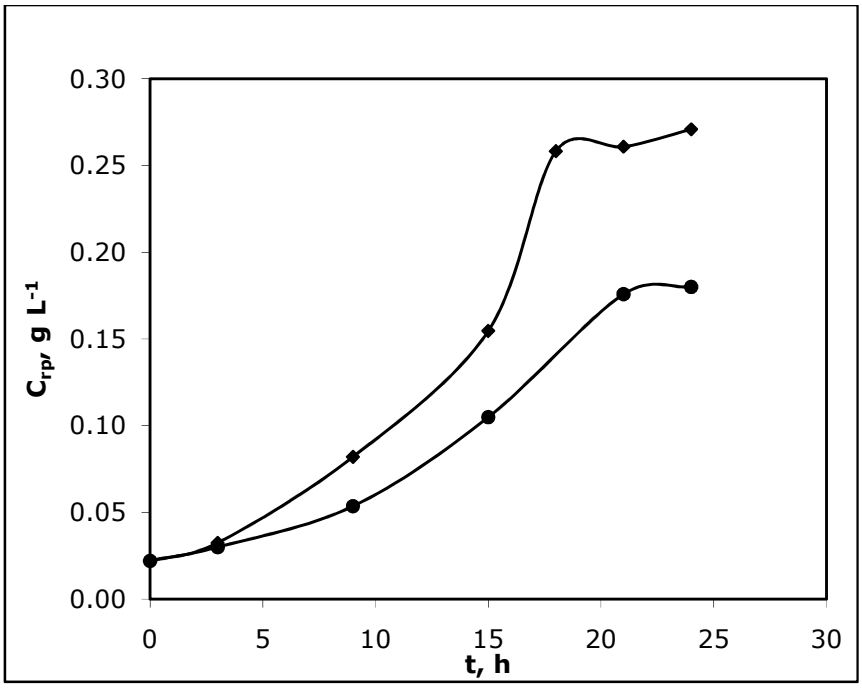



**Figure 4.26** Variation in rhGH concentration with cultivation time in production phase at pH 5.0 with different production medium for an exponential feeding at μ=0.03 $h^{-1}$. (◆) methanol/sorbitol mixed feeding, (●) Methanol feeding.

In addition to rhGH concentration profiles, *hGH* expression level profiles were compared (Figure 4.27). It is revealed that addition of sorbitol into the medium influenced expression positively and 8.6 fold higher expression was achieved at t=15 h.

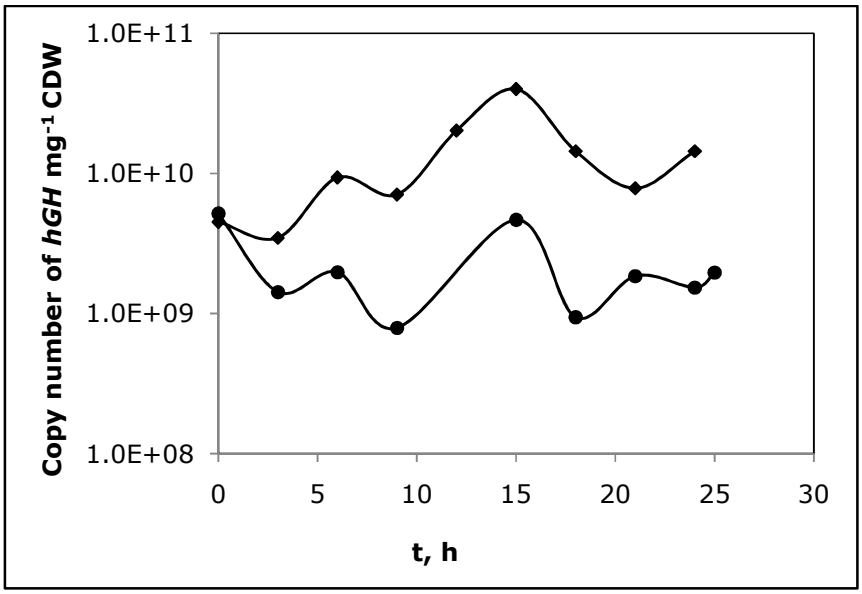



**Figure 4.27** Variation in *hGH* expression level with cultivation time in production phase at pH 5.0 with different production medium for an exponential feeding at μ=0.03 h-1. (◆) methanol/sorbitol mixed feeding, (●) Methanol feeding.

### 4.4.6 Alcohol Oxidase Enzyme Activity

#### 4.4.6.1 Effect of pH on Alcohol Oxidase Production

The specific AOX activity profiles obtained at pH 5.0 and 6.0 demonstrated that there is direct relationship between substrate consumption and AOX activity. In medium, sorbitol was totally consumed at t=15 h for pH 5.0 (Figure 4.19) and, AOX activity increased until t= 15 h (Figure 4.28). After that point, in the absence of sorbitol, AOX activity reduced suddenly, since the cells enter the lag phase again. For pH 6.0, similar behavior was observed. However, in that case, AOX activity went on increasing until t=18 h since there was 4 g $L^{-1}$ sorbitol in medium and it was not detected at t=21 h. On the other hand, specific AOX activity profile for pH 4.2 showed a different behavior and significant fluctuations were observed (Figure 4.28). During the experiment, it was observed that pH altered the yeast physiology and the cells were not found as homogenous in the culture. Therefore, samples required for determination of AOX activity assay could not be collected at the desired amount. This can affect the result considerably.

The highest specific activity was obtained as 40.7 U $g^{-1}$ CDW at pH 5.0 and t=15 h and, the lowest one was obtained as 13.8 U $g^{-1}$ CDW at pH 6.0 at t=18 h. This is an expected result since the highest and lowest protein concentration, 271 mg $L^{-1}$ and 24 g $L^{-1}$, was achieved at pH 5.0 and 6.0, respectively. RhGH production is relevant to the induction of AOX promoter. Hence, the level of intracellular *AOX* or relevantly its activity, should demonstrate *hGH* translation levels as well.

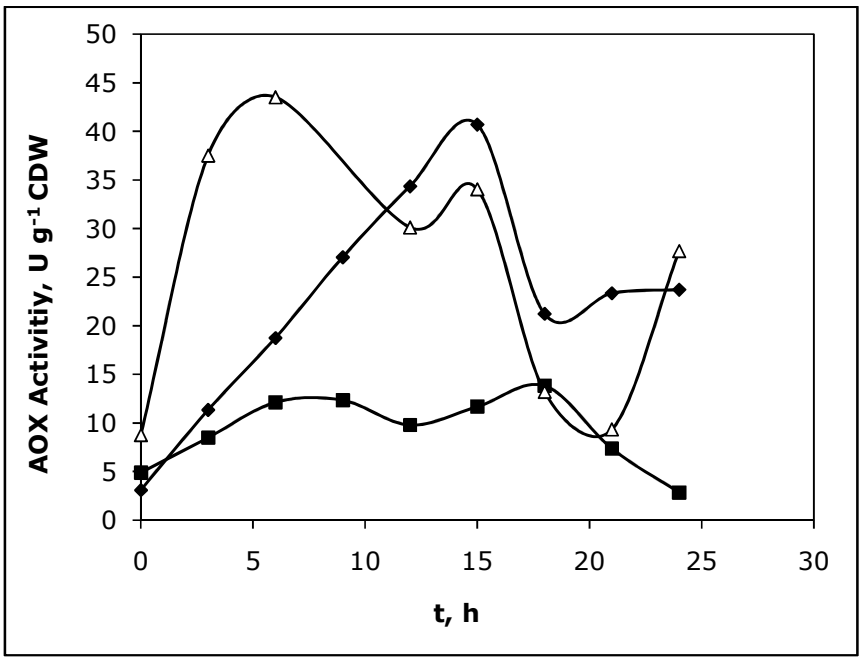



**Figure 4.28** Variation in specific AOX activity with cultivation time in production phase at different pH. pH values; (■) pH 6.0, (◆) pH 5.0, (△) pH 4.2.

To be able to have a better insight in production, the relationship between the expression levels of the product and *AOX* was investigated. For expression level profile obtained at pH 5.0, a similar manner to AOX activity was observed. AOX expression increased until t=15 h and then it started to decrease (Figure 4.29). For pH 4.2, again similar behavior to AOX activity was obtained. However, in this case, increase in *AOX* expression level at t=6 h is not as sharp as the one in AOX activity. For pH 6.0, a different profile was

observed and the highest expression level was obtained at t=9 h (Figure 4.29), whereas the highest AOX activity was seen at t=21 h (Figure 4.28). Furthermore, during the production, the expression of *AOX* at pH 6.0 was higher than obtained at pH 4.2. However, this case is opposite the one for AOX activity. This can be due to fact all transcripted *AOX* could not undergo translation at pH 6.0, which likes expression profile obtained for *hGH.* The highest *AOX* expression level, $9.38 \times 10^{10}$ copy number $mg^{-1}$ CDW or $2.05 \times 10^{6}$ copies $ng^{-1}$ total RNA, was obtained at pH 5.0 and at t=15 h, similar to AOX activity.

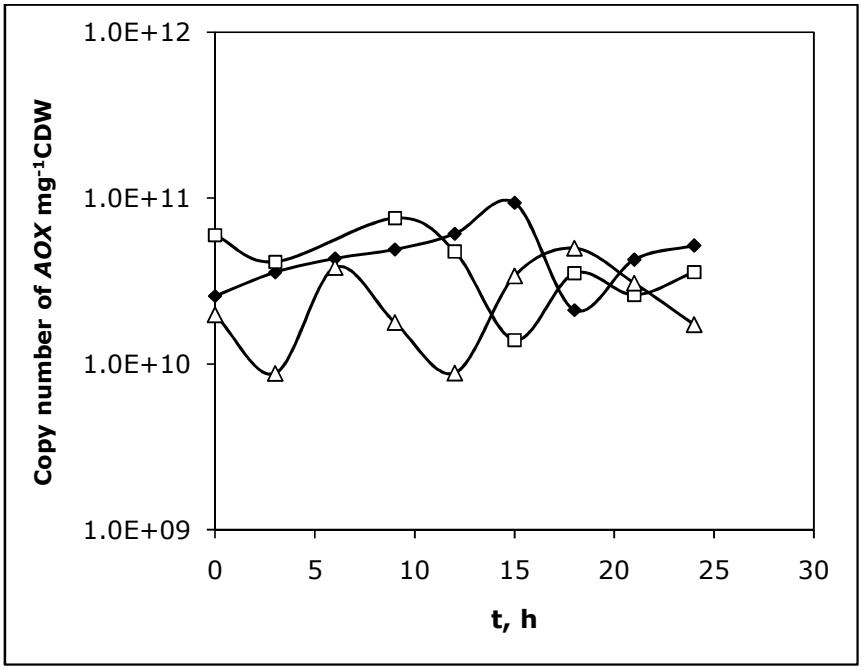



**Figure 4.29** Variation in *AOX* expression level with cultivation time in production phase at different pH. pH values; (□) pH 6.0, (◆) pH 5.0, (△) pH 4.2.

#### 4.4.6.2 Effect of Sorbitol/Methanol Mixed Feeding on Alcohol Oxidase Production

According to Figure 4.30, it is seen that specific AOX activity for medium containing only methanol, fluctuated at the beginning of the production and then a sudden increase was observed after t=15 h. This is an

expected result since the cells entered the growth phase at t=18 h. However, addition of sorbitol into the medium shorten the lag phase so specific AOX activity increased until t= 15 h and then decreased. The specific AOX activity for cells grown on only methanol at t=24 h is 39.6 U $g^{-1}$ CDW, which is very close to 40.7 U $g^{-1}$ CDW obtained in presence of sorbitol at t=15 h (Figure 4.30).

When AOX activity and expression level profiles (Figures 4.30 and 4.31) were compared, and it is seen that they were parallel to each other. For cells grown on methanol, *AOX* expression level was started to increase after t=15 h but, more slowly in this case. Furthermore, the highest expression of *AOX*, $9.38x10^{10}$ copy number $mg^{-1}$ CDW, was achieved for cell grown on sorbitol at t=15 h. Thus, *AOX* expression profiles are in good agreement with the ones obtained by using specific AOX activity.

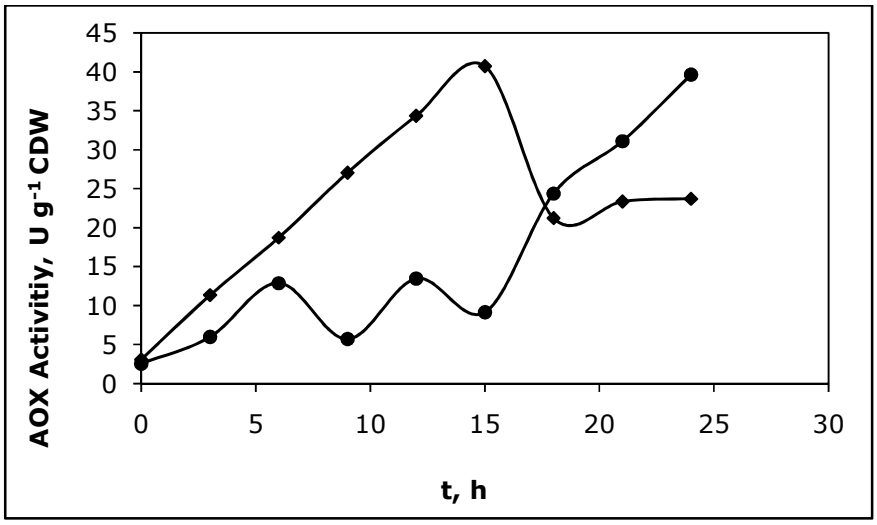



**Figure 4.30** Variation in specific AOX activity with cultivation time in production phase at pH 5.0 with different production medium for an exponential feeding at μ=0.03 $h^{-1}$. (◆) methanol/sorbitol mixed feeding, (●) methanol feeding.

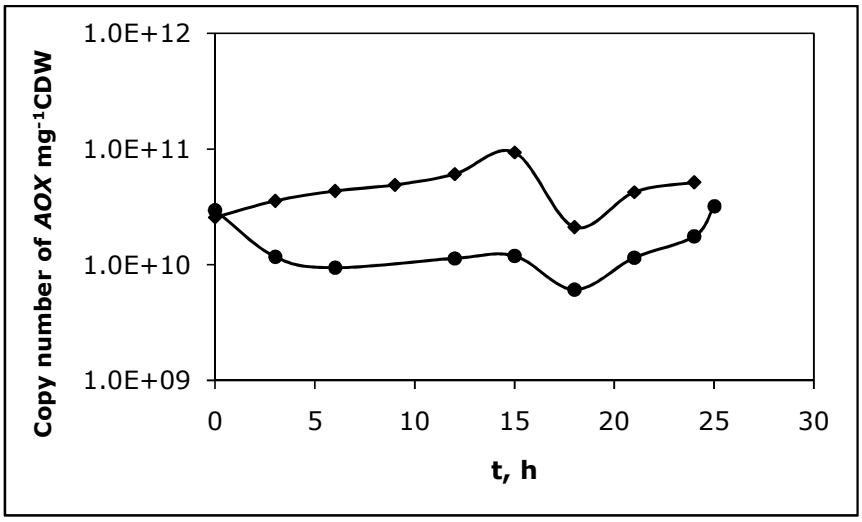



**Figure 4.31** Variation in *AOX* expression level with cultivation time in production phase at pH 5.0 with different production medium for an exponential feeding at μ=0.03 $h^{-1}$. (◆) methanol/sorbitol mixed feeding, (●) methanol feeding.

### 4.4.7 Total protease Concentration in Extracellular Medium

#### 4.4.7.1 Effect of pH on Total Protease Concentration

To determine the optimum pH, investigation of effect of pH on protease concentration is important because proteolytic degradation of the product can be minimized by finding a proper pH value. For this, activities of acidic, neutral and alkali proteases were measured separately and their activity was converted to protease concentration. It is observed that, at all pH values, acidic, neutral and alkali proteases concentrations are almost within the same range and they showed similar behavior in protease concentration profiles. To illustrate, in medium with pH 5.0, final acidic, neutral and alkali proteases concentrations are 0.0293, 0.0292 and 0.0282 g $L^{-1}$, respectively (Figure 4.32).

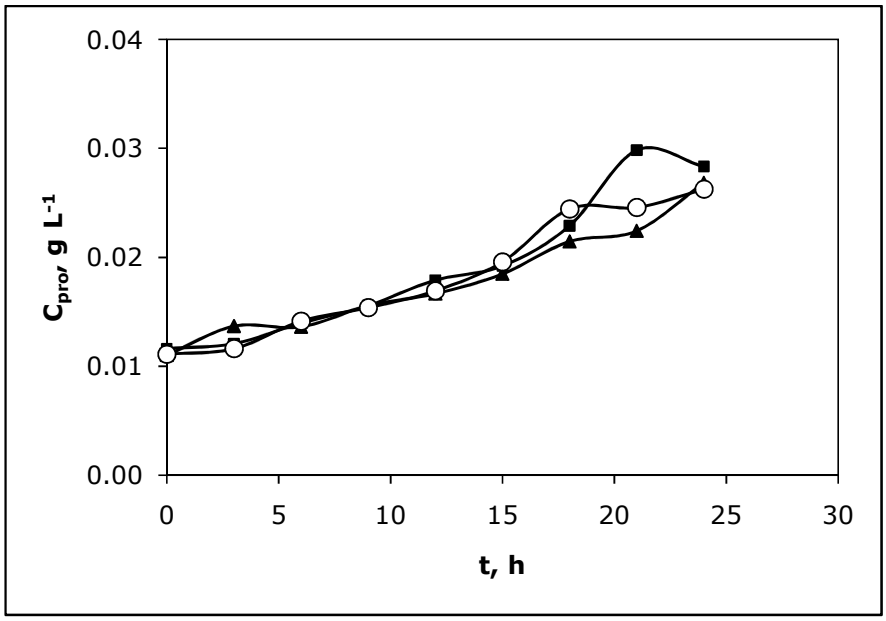



**Figure 4.32** Variation in alkali, acidic and neutral proteases concentrations with cultivation time in production phase at pH 5.0. (▲) alkali proteases, (■) acidic proteases, (○) neutral proteases.

The total protease concentration was also calculated and the profiles obtained were compared. It is seen that, total protease concentration profiles obtained at different pH values have similar behavior such that an exponential increase was observed (Figure 4.33). Moreover, total protease concentrations at t= 24 h for pH 6.0 is 0.1 g $L^{-1}$, which is the highest one. This result can be due to highest cell concentration obtained at this pH value. Therefore, it will be better to compare results based on specific protease concentrations. For pH 4.2, 5.0 and 6.0 at t=24 h specific protease concentrations are 1.92, 1.94 and 1.90 mg $g^{-1}$ cell dry weight, respectively. They are really close to each other so change in pH does not affect total protease concentration considerably in extracellular medium.

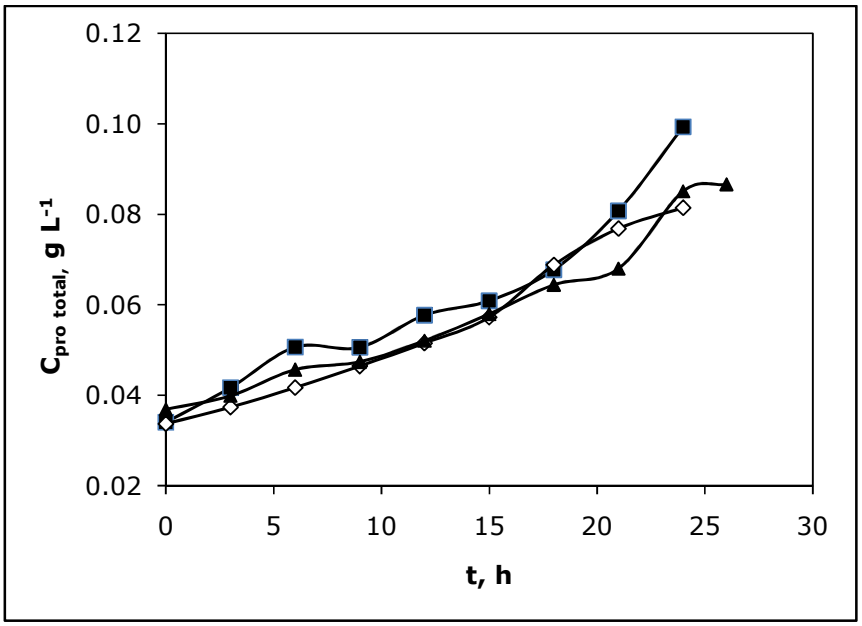



**Figure 4.33** Variation in total proteases concentrations with cultivation time in production phase at different pH. pH values; (■) pH 6.0, (◇) pH 5.0, (▲) pH 4.2.

#### 4.4.7.2 Effect of Sorbitol/Methanol Mixed Feeding on Total Protease Concentration

According to total protease concentration profile (Figure 4.34), at t=24 h total protease concentration for medium containing only methanol at pH 5.0 is 0.053 g $L^{-1}$, which is much less than 0.082 g $L^{-1}$ obtained for medium containing sorbitol with pH 5.0. However, it will be better to compare the results based on specific protease concentrations since the final cell concentration for medium with sorbitol is almost 1.4 fold higher. At t=24 h, 1.94 mg protease $g^{-1}$ cell dry weight for medium with sorbitol is really close to 1.76 mg protease $g^{-1}$ cell dry weight for medium with only methanol. As a result, addition of the sorbitol into the medium does not create significant difference on the protease production level.

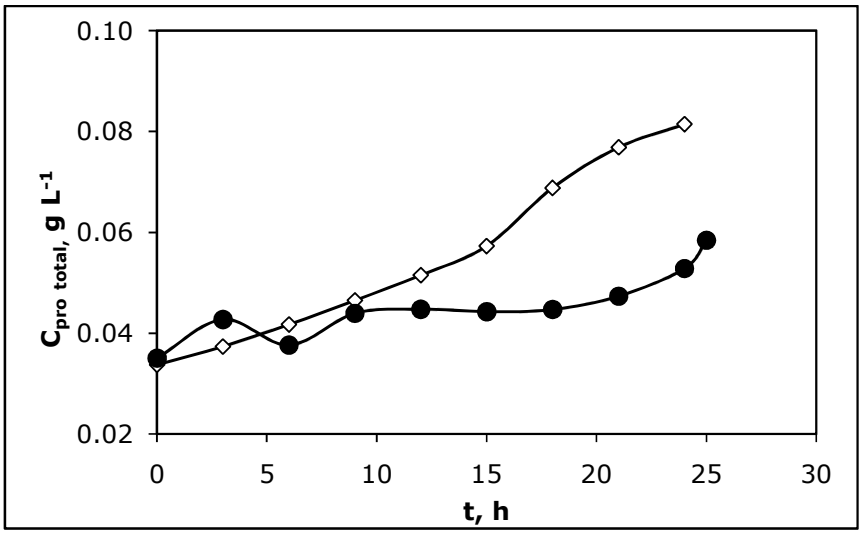


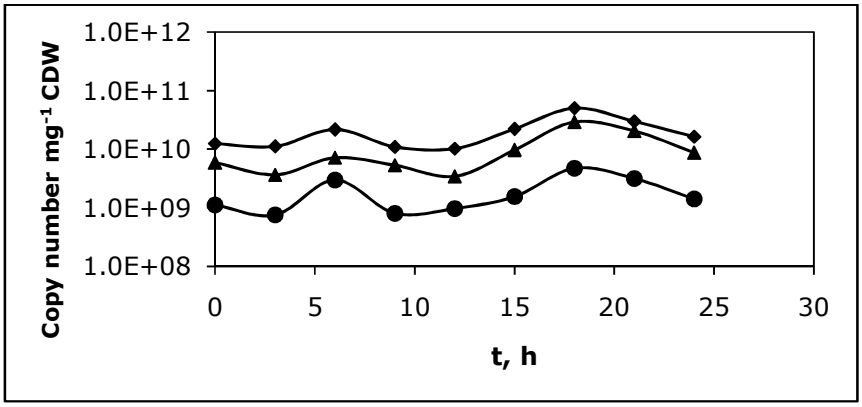

**Figure 4.34** Variation in total proteases concentrations with cultivation time in production phase at pH 5.0 with different production medium for an exponential feeding at μ=0.03 $h^{-1}$. (◇) methanol/sorbitol mixed feeding, (●) methanol feeding.

#### 4.4.7.3 Effect of pH on Expression level of *pep4*, *prb1* and *prc1* Genes

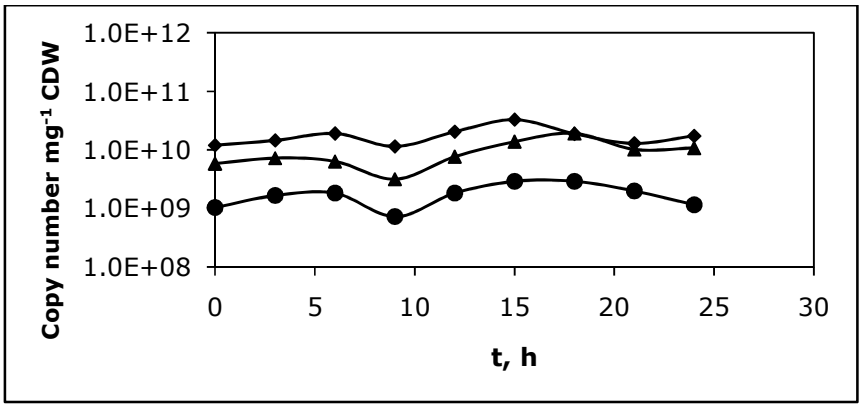
In *P. pastoris* expression system, vacuolar proteases are probably the main reason of the proteolysis in extracellular medium. The vacuolar proteases generally found in yeasts are proteinase A (*pep4*) and B (*prb1*) and carboxypeptidase Y (*prc1*), (Jones et al., 1991 and 2002). Hence, to gain more insight in relationship between expression level of product and the enzymes having influence on product concentration in the medium, expression levels of the genes (*pep4, prb1* and *prc1*) responsible from production of proteinase A and B and, carboxypeptidase Y were determined by real time-PCR.

In each production condition, expression level profiles of the genes were compared with each other. The manners of the expression levels of *pep4, prb1* and *prc1* with respect to time are parallel to each other in each different pH value (Figure 4.35). This is an expected result since it is known that carboxypeptidase Y and proteinase B are activated by Proteinase A. Proteinase B has about half the activity of the processed enzyme before being activated by proteinase A (Higgins and Cregg, 1998). Furthermore, it was revealed that expression level of *prc1* is much higher than the others in each condition.

a)




b)




c)

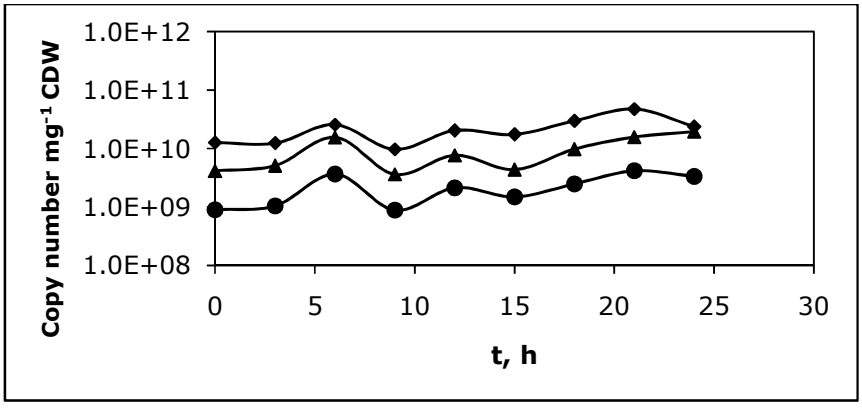



**Figure 4.35** Variation in *pep4, prb1 and prc1* expression levels with cultivation time in production phase. a) pH 4.2, b) pH 5.0, c) pH 6.0. (●) pep4, (▲) prb1, (◆) prc1.

The expression levels of the *pep4* obtained for each pH value were compared. Some fluctuations was observed in the profiles but the maximum *pep4* expression was generally achieved at t=18 h (Figure 4.36). $4.73x10^9$ copies $mg^{-1}$ CDW ($5.9x10^4$ copies $ng^{-1}$ total RNA), highest *pep4* expression was obtained at pH 4.2 and it is 1.6 fold higher than the one at pH 5.0. Furthermore, it is seen that throughout the process, the lowest expression was attained at pH 5.0. For *prc1*, similar manner was observed. During the production, pH 5.0 had the lowest expression level and at pH 4.2 and 6.0 reached the highest expression level, $5.01x10^{10}$ and $4.75x10^{10}$ copies $mg^{-1}$ CDW, at t=18 h and t=21 h, respectively (Figure 4.37). Thus, the expression level at pH 4.2 and 6.0 is almost 1.6 fold higher than one obtained at pH 5.0 at t=15 h. Moreover, at t=18 h *prb1* concentration obtained at pH 4.2 is 2.0-fold higher than the one at pH 5.0 (Figure 4.38), again. This result is same with the ones obtained for *pep4* and *prc1.* This is natural since *pep4* supplies the activation of *prb1* and *prc1.* As a result, pH 5.0 seems to be the best choice when expression levels of the different proteases are taken into consideration.

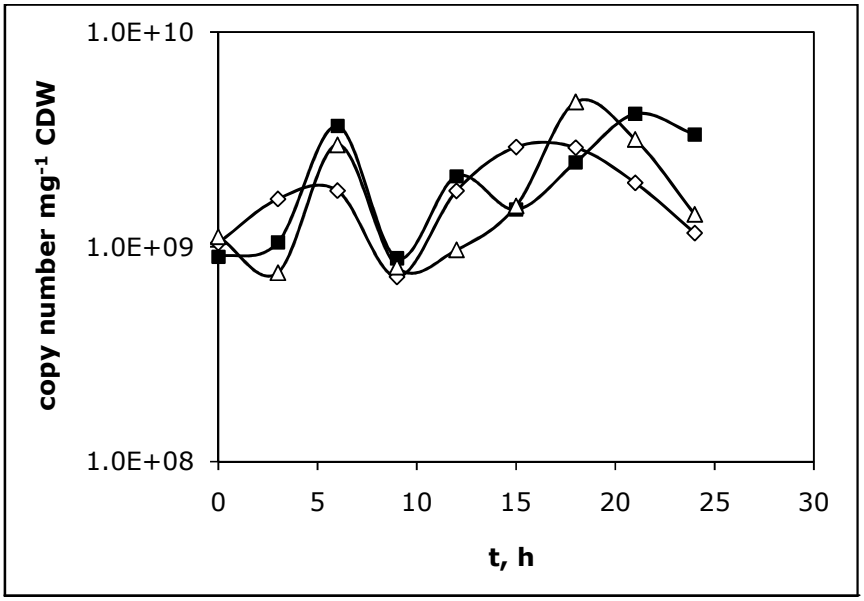



**Figure 4.36** Variation in *pep4* expression level with cultivation time in production phase at different pH. pH values; (△) pH 4.2, (◇) pH 5.0, (■) pH 6.0.

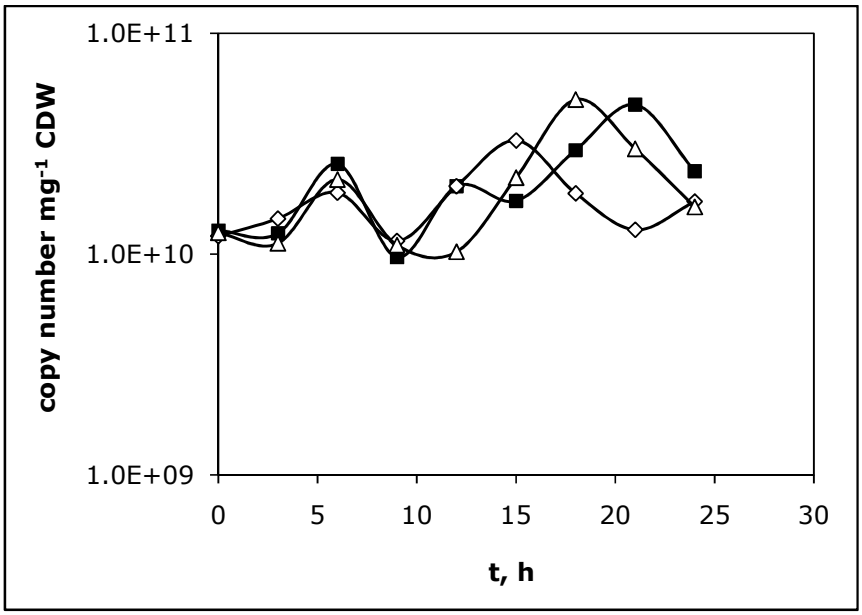



**Figure 4.37** Variation in *prc1* expression level with cultivation time in production phase at different pH. pH values; ( △) pH 4.2, ( ◇) pH 5.0, (■) pH 6.0.

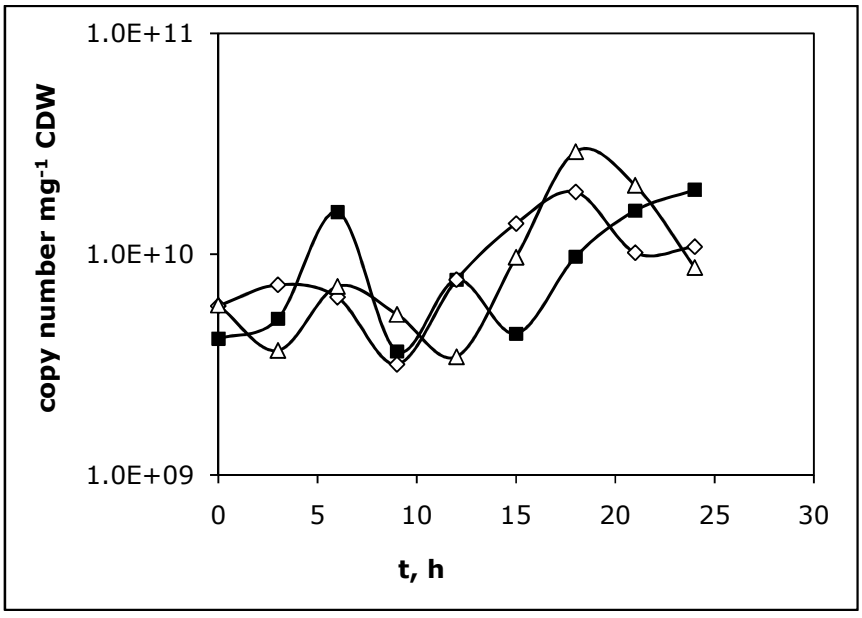


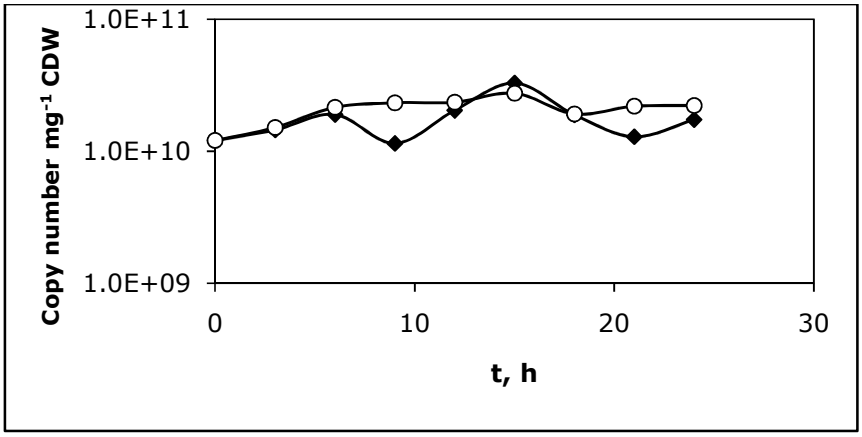

**Figure 4.38** Variation in *prb1* expression level with cultivation time in production phase at different pH. pH values; ( △) pH 4.2, ( ◇) pH 5.0, (■) pH 6.0.

#### 4.4.7.4 Effect of Sorbitol/Methanol Mixed Feeding on level of *pep4, prb1* and *prc1* Genes

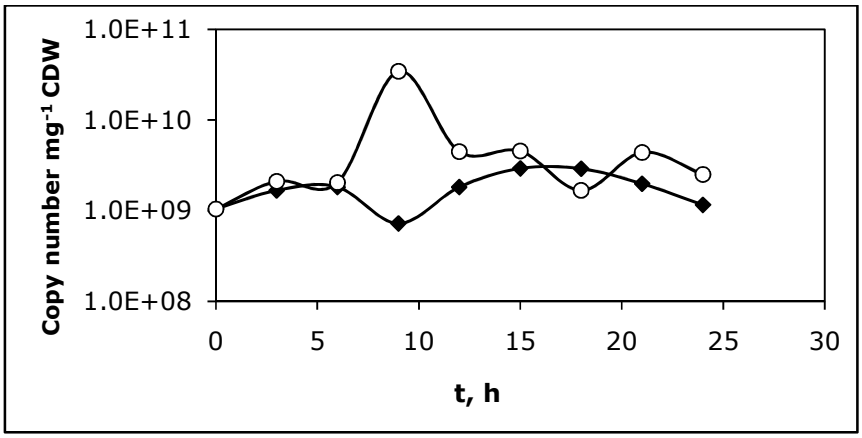
The expression level profiles of the genes were compared and almost same behavior was observed in each condition. Generally, for medium containing sorbitol, expression levels of the genes slightly increased until t=15 h except t=9 h, where a small decrease was observed. After t=15 h, a sudden drop in expression observed (Figure 4.39). This drop can be related with the absence of the sorbitol in the medium. For the cells grown on only methanol, the maximum concentration was reached at t=9 h and after this point a decrease was observed. Then, the expression level of all genes remained stable (Figure 4.39).

According to Figure 4.39, generally expression levels of the proteases in medium with methanol were almost same the ones with sorbitol although 1.4-fold lower final cell concentration was obtained when cells were grown on only methanol. As a result, addition of sorbitol did not affect expression of proteases considerably. This results is similar to one obtained for total protease concentration.

a)



b)




c)

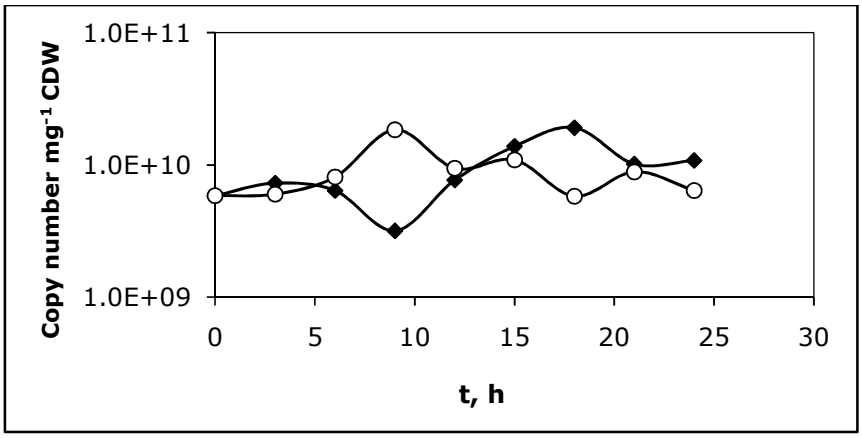



**Figure 4.39** Variation in *pep4, prb1 and prc1* expression levels with cultivation time in production phase with different production medium for an exponential feeding at μ=0.03 $h^{-1}$. a) *prc1*, b) *pep4*, c) *prb1*. (◆) methanol/sorbitol mixed feeding, (○) methanol feeding.

### 4.4.8 Amino and Organic Acid Concentration Profiles

Throughout the process, amino and organic acid concentrations profiles give an idea about the supply and demand for these metabolites, which are regulated by the metabolic reaction network. The variations in amino acid and organic acid concentrations detected in production phase with cultivation time are given in Table 4.1 and Table 4.2, respectively. Asparagine, aspartic acid, cysteine, glycine, histidine, lysine, proline, tryptophan, valine, methionine, glutamic acid and leucine are amino acids detected in the fermentation broth.

**Table 4.1** Variation in amino acid concentration (g $L^{-1}$) with cultivation time in production phase at different pH with different production medium.

| pH 4.2 (sorbitol/methanol mixed feed) | | | | |
|---|---|---|---|---|
| **Time (h)** | **3** | **9** | **15** | **21** |
| **Asp** | 0.0090 | 0.0150 | 0.0141 | 0.0157 |
| **Glu** | | | | 0.0010 |
| **Asn** | 0.0047 | 0.0172 | 0.0033 | 0.0239 |
| **Gly** | 0.0083 | 0.0222 | | 0.0208 |
| **Pro** | | 0.0004 | | 0.0004 |
| **Val** | 0.0162 | | | |
| **Met** | 0.0041 | 0.0040 | 0.0048 | 0.0051 |
| **Cys** | | | | 0.0054 |
| **Phe** | | 0.0067 | 0.0075 | 0.0060 |
| **Trp** | | 0.0013 | | |
| **Lys** | 0.0026 | 0.0166 | 0.0135 | 0.0131 |
| **Total** | 0.0449 | 0.0835 | 0.0431 | 0.0913 |
| **pH 5.0 (sorbitol/methanol mixed feed)** | | | | |
| **Time (h)** | **3** | **9** | **15** | **21** |
| **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 |
| **pH 6.0 (sorbitol/methanol mixed feed)** | | | | |
| **Time (h)** | **3** | **9** | **15** | **21** |
| **Asp** | 0.0091 | 0.0138 | 0.0142 | 0.0121 |
| **Asn** | 0.0090 | 0.0163 | 0.0173 | 0.0131 |
| **Gly** | | 0.0218 | 0.0178 | 0.0153 |
| **Val** | 0.0317 | 0.0251 | 0.0135 | |
| **Met** | 0.0110 | 0.0082 | 0.0068 | 0.0077 |
| **Cys** | | 0.0010 | 0.0031 | 0.0065 |
| **Phe** | 0.0038 | 0.0034 | 0.0045 | 0.0041 |
| **Lys** | 0.0082 | 0.0130 | 0.0145 | 0.0119 |
| **Total** | 0.0729 | 0.1027 | 0.0917 | 0.0707 |
| **pH 5.0 only methanol** | | | | |
| **Time (h)** | **3** | **9** | **15** | **21** |
| **Asp** | 0.0075 | 0.0108 | 0.0098 | 0.0163 |
| **Glu** | 0.0000 | | | |
| **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 |

**Table 4.2** Variation in organic acids concentration (g $L^{-1}$) with cultivation time in production phase at different pH with different production medium.

| pH 4.2 (sorbitol/methanol mixed feed) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Time (h)** | **0** | **3** | **6** | **9** | **12** | **15** | **18** | **21** | **24** |
| **Formic Acid** | 0.0639 | 0.0643 | 0.0782 | 0.0819 | 0.0969 | 0.1131 | 0.1364 | 0.1585 | 0.1843 |
| **α-keto Glutaric Acid** | 0.0219 | 0.0242 | 0.0258 | 0.0275 | 0.0267 | 0.0256 | 0.0266 | 0.0297 | 0.0342 |
| **Citric Acid** | 0.0067 | 0.0067 | 0.0083 | 0.0092 | 0.0109 | 0.0152 | 0.0178 | 0.0224 | 0.0282 |
| **Fumaric Acid** | 0.0005 | 0.0006 | 0.0009 | 0.0012 | 0.0014 | 0.0016 | 0.0018 | 0.0023 | 0.0027 |
| **Succinic Acid** | 0.0651 | 0.0691 | 0.0683 | 0.0670 | 0.0773 | 0.0871 | 0.0968 | 0.1120 | 0.1302 |
| **pH 5.0 (sorbitol/methanol mixed feed)** | | | | | | | | | |
| **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 |
| **pH 6.0 (sorbitol/methanol mixed feed)** | | | | | | | | | |
| **Time (h)** | **0** | **3** | **6** | **9** | **12** | **15** | **18** | **21** | **24** |
| **Formic Acid** | 0.1893 | 0.1639 | 0.1860 | 0.1884 | 0.1957 | 0.1771 | 0.2296 | 0.2372 | 0.3986 |
| **α-keto Glutaric Acid** | 0.0118 | 0.0124 | 0.0148 | 0.0158 | 0.0161 | 0.0132 | 0.0138 | 0.0136 | 0.0152 |
| **Citric Acid** | 0.0067 | 0.0067 | 0.0067 | 0.0067 | 0.0067 | 0.0067 | 0.0071 | 0.0072 | 0.0097 |
| **Fumaric Acid** | 0.0000 | 0.0000 | 0.0002 | 0.0002 | 0.0006 | 0.0005 | 0.0008 | 0.0010 | 0.0015 |
| **Succinic Acid** | 0.0215 | 0.0243 | 0.0249 | 0.0250 | 0.0228 | 0.0190 | 0.0184 | 0.0141 | 0.0132 |
| **pH 5.0 (only methanol)** | | | | | | | | | |
| **Time (h)** | **0** | **3** | **6** | **9** | **12** | **15** | **18** | **21** | **24** |
| **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 |

In the cells, all essential amino acids are synthesized for production of proteins and enzymes. In extracellular medium, sometimes amino acids are detected and fluctuation in their concentrations can be observed. There are two main reasons for that. Firstly, degradation of proteins in extracellular medium by proteases results in a decrease in the concentration of amino acids. Secondly, if amino acid production exceeds the demand, they will be excreted to extracellular medium. During the production, amino acids can be required again and in this case, the requirement can be supplied from the medium. In all conditions, leucine (Leu), glutamine (Gln), serine (Ser) and glutamic (Glu) acids were not detected in the medium (Table 4.1). This is probably due to high Leu, Ser, Glu and Gln content of hGH, which contains 13.6% Leu, 9.4% Ser, 7.3% Glu and 6.8% Gln. Furthermore, histidine (His) was only detected in medium containing only methanol and its concentration reached 0.0017 g $L^{-1}$ at t=21 h. Valine (Val) was also detected sorbitol containing media only and its concentration decreased with respect to time in the medium. It is seen that valine concentration generally increased with increasing pH and 0.0319 g $L^{-1}$ was the highest valine concentration obtained at pH 5.0 and t=0 h. In addition to these, it is observed that phenylalanine (Phe) concentration decreased with increasing pH. 0.041 g $L^{-1}$ Phe was the lowest concentration obtained at t=21 h and pH 6.0.

Formic, fumaric, glutaric, citric, succinic and lactic acids are the organic acids detected in the medium. *P. pastoris* strains cannot tolerate high methanol concentration due to the accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which are the oxidized products of methanol by AOX and are toxic to the cell (Zhang et al., 2000), therefore analyzing formaldehyde production throughout the fermentation process is important. For all condition, formaldehyde was not detected in the medium throughout the process. This is due to the fact that formaldehyde is readily oxidized to formic acid or goes into the assimilatory pathway and enters glycolysis. Therefore, formic acid was detected at all conditions. It is observed that amount of formic acid in extracellular medium increased with respect to time. Furthermore, formic acid concentration increased as pH increases formic acid concentration, 0.3986 g $L^{-1}$, was the highest at pH 6.0.

Lactic acid is also another important organic acid, which should be analyzed in the fermentation broth since it is the main metabolic product formed when oxygen is insufficient in the medium. Lactic acid was only

detected in medium containing only methanol as a carbon source and the concentration at t=24 h is 0.1623 g $L^{-1}$.

The other organic acids detected in the medium are fumaric, α-keto glutaric, citric and succinic acids and their concentrations increased in extracellular medium with respect to time. It is revealed that fumaric and succinic acid concentration in the medium increased with decreasing pH and, 0.027 fumaric acid and 0.1302 g $L^{-1}$ succinic acid were obtained at t=24 h and pH 4.2.

### 4.4.9 Yield Coefficients and Specific Rates of the Bioprocess

The overall yield coefficients should be determined in order to have a better insight in the efficiency and profitability of a bioprocess. 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 performed at different pH values with different production medium. For medium containing both sorbitol and methanol, while calculating the overall yield coefficients related to substrate, both carbon sources were taken into consideration. All overall yield coefficients are summarized in Table 4.3.

**Table 4.3** Overall yield coefficients.

| Experiment name | $Y_{x/s}$ g $g^{-1}$ | $Y_{P/X}$ mg $g^{-1}$ | $Y_{P/S}$ mg $g^{-1}$ |
|---|---|---|---|
| pH 4.2 | 0.17 | 9.80 | 1.63 |
| pH 5 | 0.15 | 13.86 | 2.08 |
| pH 6 | 0.25 | 0.78 | 0.20 |
| pH 5 only methanol | 0.10 | 23.81 | 2.50 |

The cell yield on substrate at pH 6.0 is $Y_{X/S}$=0.25 g $g^{-1}$, which is higher than the ones obtained at pH 4.2 and 5.0. This is an expected result since the highest final cell concentration (52.5 g $L^{-1}$) was obtained at pH 6.0 although initial cell concentrations at all conditions were the same. The overall product yield on cell at pH 5.0 with medium containing sorbitol is $Y_{P/X}$=13.86 mg $g^{-1}$,

which is 1.4 and 17.8 fold higher than the ones obtained at pH 4.2 and 6.0, respectively. Similarly, by adjusting pH to 5.0, the overall product yield on the total carbon source ($Y_{P/S}$=2.08 mg $g^{-1}$) increased 1.3 and 10.4 fold with respect to pH 4.2 and 6.0, respectively.

The addition of sorbitol into the medium as batch wise increased the cell yield on substrate 1.7 fold and $Y_{X/S}$=0.10 g $g^{-1}$, the lowest cell yield on substrate, was obtained for medium containing methanol as a sole carbon source. The addition of sorbitol decreased the both the overall product yield on cell and total carbon sources about 1.7 and 1.2-fold, respectively. 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. Human growth hormone is a protein having high values for low volumes so increasing the amount of final product is the main aim. Therefore, addition of sorbitol is more profitable since it increased final amount of product (270 mg $L^{-1}$) 1.5-fold.

In Table 4.4, the variation in total specific growth rate ($\mu_t$), 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 ($Y_{X/O}$) obtained during the production processes performed at different pH values are summarized.

It is observed higher specific growth rate obtained at pH 6.0 during the process although a specific growth rate of $\mu_0$=0.03 $h^{-1}$ was selected for all experiments. This result can be related to energy required for maintenance of the cells and production of r-protein. At pH 4.2 and 5.0, energy mostly used for r-protein production. This results in lower specific growth rate throughout the process.

For the cells grown on methanol, the average of specific growth rate was lower than 0.03 $h^{-1}$. This is because cells undergo a long lag-phase and the induction was stopped just at the beginning of exponential growth. Furthermore, it is revealed that addition of sorbitol into the medium increased the specific growth rates obtained during the process. This result is consistent with the one obtained by Çelik (2008). Çelik (2008) found that in the presence of sorbitol higher total specific growth rate values were obtained and it was resulted that the deviation from the desired value was explained with the growth on sorbitol such that $\mu_t = \mu_0 + \mu_S$.

The specific sorbitol consumption rates were the highest at the beginning of the process and then they were decreased for all conditions. The sorbitol was consumed more rapidly at pH 4.2 and 5.0 with respect to pH 6.0. This can be related to their specific protein formation rates, which results in higher sorbitol consumption rates. It is observed that, there was no significant difference between the methanol consumption rates at different pH values. However, slightly higher values obtained at pH 5.0. This is an expected result since the lower cell concentration obtained at the end of the process a specific growth rate of $\mu_0$=0.03 $h^{-1}$ was selected for all experiments. In addition to that, generally, for all pH values, there was no notable change in $q_M$ during the process. At the end of the process, $q_M$ slightly increased. It was logical since although cell growth nearly ceased, increasing amount of methanol was added to medium. The highest specific rhGH formation rate, 0.463 mg $g^{-1}$ $h^{-1}$, was obtained at pH 5.0 at t=15 h. This was a natural result since highest AOX activity obtained at these conditions and protein production is coupled to AOX enzyme in the methanol utilization pathway. The lowest $q_{rp}$ was observed at pH 6.0. This can be due to lowest AOX activity seen at this pH value.

Unlike cells grown on sorbitol, for cells grown on only methanol, $q_M$ increased continuously. This is an expected result since cell growth is very slow although increasing amounts of methanol was added to the medium. Moreover, addition of sorbitol at pH 5 increased specific rhGH formation rate when it is compared to medium containing only methanol.

The highest specific oxygen uptake rate, 0.153 g $g^{-1}$ $h^{-1}$, was obtained in the medium with sorbitol at pH 5.0 when it is compared the one obtained at pH 4.2 and 6.0. This can be related to high protein production since it accelerates the amino acid production. The increase in amino acid synthesized from oxidative pathway may result in higher oxygen demand.

It is observed that addition of sorbitol into the medium increased the specific oxygen uptake rate. There can be two reasons for that. First, the addition of sorbitol shortens the lag phase and accelerates the cell growth, which results in higher oxygen uptake rate. Secondly, higher production accelerates the amino acid production and increase in amino acid synthesized from oxidative pathway may cause higher oxygen uptake.

**Table 4.4** Variation in specific rates throughout the bioprocesses in production phase at different pH with different production medium.

| Time | Cx | $\mu_t$ | $q_S$ | $q_M$ | $q_{rp}$ | $q_0$ | $Y_{x/o}$ |
|---|---|---|---|---|---|---|---|
| h | g $L^{-1}$ | $h^{-1}$ | g $g^{-1}$ $h^{-1}$ | g $g^{-1}$ $h^{-1}$ | mg $g^{-1}$ $h^{-1}$ | g $g^{-1}$ $h^{-1}$ | |
| **pH 4.2 (sorbitol/methanol mixed feed)** | | | | | | | |
| 3 | 27.43 | 0.049 | 0.128 | 0.067 | 0.152 | 0.039 | 1.19 |
| 6 | 30.16 | 0.054 | 0.110 | 0.066 | | 0.065 | 0.79 |
| 9 | 36.96 | 0.046 | 0.084 | 0.059 | 0.297 | 0.060 | 0.73 |
| 12 | 41.70 | 0.037 | 0.070 | 0.057 | | 0.037 | 0.92 |
| 15 | 45.23 | 0.025 | 0.060 | 0.058 | 0.266 | 0.054 | 0.41 |
| 18 | 46.74 | 0.011 | | 0.061 | | 0.049 | 0.15 |
| 21 | 45.57 | | | 0.069 | 0.163 | 0.048 | |
| 24 | 44.33 | | | 0.077 | 0.063 | 0.047 | |
| **pH 5.0 (sorbitol/methanol mixed feed)** | | | | | | | |
| 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 | | | 0.086 | 0.220 | 0.045 | |
| 24 | 42.3 | | | 0.091 | 0.025 | 0.059 | |
| **pH 6.0 (sorbitol/methanol mixed feed)** | | | | | | | |
| 3 | 26.5 | 0.043 | 0.120 | 0.070 | 0.014 | 0.096 | 0.42 |
| 6 | 30.0 | 0.059 | 0.098 | 0.068 | | 0.074 | 0.76 |
| 9 | 35.8 | 0.058 | 0.074 | 0.062 | 0.046 | 0.044 | 1.26 |
| 12 | 41.5 | 0.050 | 0.058 | 0.059 | | 0.056 | 0.84 |
| 15 | 49.9 | 0.036 | 0.043 | 0.053 | 0.038 | 0.040 | 0.81 |
| 18 | 51.4 | 0.022 | 0.037 | 0.057 | | 0.053 | 0.35 |
| 21 | 51.8 | 0.006 | 0.031 | 0.062 | 0.009 | 0.042 | 0.06 |
| 24 | 52.5 | 0.009 | | 0.066 | 0.006 | 0.045 | 0.10 |
| **pH 5.0 only methanol** | | | | | | | |
| 3 | 24.61 | 0.002 | | 0.072 | 0.071 | 0.116 | 0.06 |
| 6 | 25.44 | 0.005 | | 0.076 | | 0.097 | 0.09 |
| 9 | 25.83 | 0.009 | | 0.082 | 0.316 | 0.086 | 0.11 |
| 12 | 26.88 | 0.012 | | 0.086 | | 0.058 | 0.17 |
| 15 | 27.40 | 0.015 | | 0.093 | 0.379 | 0.113 | 0.09 |
| 18 | 28.94 | 0.018 | | 0.096 | | 0.078 | 0.12 |
| 21 | 29.33 | 0.020 | | 0.104 | 0.286 | 0.062 | 0.15 |
| 24 | 30.02 | 0.023 | | 0.111 | 0.190 | 0.083 | 0.10 |

In the presence of sorbitol, higher cell yields on oxygen were obtained throughout the process. This can be due to fact that sorbitol + methanol supported cell growth better than methanol therefore higher cell concentrations were attained in the presence of sorbitol then in the presence of only methanol. Furthermore, the cell yields on oxygen decrease suddenly since cells enter again a lag phase and oxygen demand increases.

Some total specific growth rate values and cell yields on oxygen cannot be calculated (at t=21 and 24 h for pH 5.0 and pH 4.2) since cell growth terminates in the last hours of process and variation in cell concentration is negative.

### 4.4.10 Oxygen Transfer Characteristics of Bioprocess

Throughout the rhGH production by *P. pastoris* in fed-batch media at different pH and in different production medium, dynamic method was applied to find the oxygen transfer parameters. Oxygen uptake rate (OUR), oxygen transfer rate (OTR), and oxygen transfer coefficient, $K_La$ are the oxygen transfer parameters. 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 were calculated. They are listed in Table 4.5.

Throughout the fermentation process, oscillation was observed in $K_La$ and generally higher oxygen transfer coefficients were obtained at pH 5.0 (ranges 0.067-0.136 $s^{-1}$). This can be related to the change in rheological properties of the fermentation medium and presence of fine particles in the mass transfer zone. Temperature and agitation rate are the other factors affecting the $K_La$. However, these parameters were kept constant during the process so they cannot be the reason in the variation of $K_La$. At pH 4.2, it was observed that pH affects the physiology of the cell, which alters rheological properties of the fermentation medium. This could be result in an increase in mass transfer resistant. Furthermore, OUR at pH 4.2 (ranges 0.0094-0.0211 mol $m^{-3}.s^{-1}$) throughout the process was lower than the one at pH 5 (ranges 0.0166-0.0341 mol $m^{-3}.s^{-1}$). Mutual effect of mass transfer resistance and OUR determines the inclination of $K_La$ throughout the fermentation process.

Therefore, lower $K_La$ values can be obtained at pH 4.2. At pH 6.0 (ranges 0.042-0.093 $s^{-1}$), again lower mass transfer coefficients were acquired than at pH 5 (ranges 0.067-0.136 $s^{-1}$), although their oxygen uptake rates were close to each other during the process. This can be due to higher cell concentration achieved at pH 6 since this cause cell coalescence, which should increase mass transfer resistant in the fermentation medium.

The oxygen uptake rate, OUR, and oxygen transfer rate, OTR, demonstrated similar manner during the process for all conditions. Moreover, they were really close to each other. This means that cells consumed nearly all oxygen transferred to the medium. Therefore, keeping dissolved oxygen level above 20% seems to be best choice. Relatively higher OUR, ranges between 0.0166-0.0341 mol $m^{-3}.s^{-1}$, was observed throughout the fermentation for medium with sorbitol at pH 5.0. This is related with higher oxygen demand because of higher protein production.

Throughout the process, the maximum possible oxygen utilization rate, $OD=\mu max C_X/Y_X/O$, generally increases with respect to time for all conditions. Damköhler number, Da, defined as maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık et al., 2000). When Damköhler number is much greater than 1, the mass transfer resistances are effective at all times whereas as it is smaller than 1 oxygen transfer is biochemical reaction limited. For all conditions, Da is greater than 1 (Table 4.5) and it is not so much greater than 1 until t=15 h so oxygen transfer is both mass transfer and biochemical reaction limited. After t=15 h and at the beginning of the process for pH 6 and medium without sorbitol, generally, Da is much greater than 1. Therefore, oxygen transfer is mass transfer limited. The effectiveness factor, η, defined as the oxygen uptake rate per maximum possible oxygen utilization rate values (Çalık et al., 2000) for the ideal condition it should be equal to 1. According to Table 4.5, the highest η=0.4 achieved at pH 6.0 and at t=6, 9 h and effectiveness factor (η) values for all conditions smaller than 1. This means that the cells were consuming lower oxygen than the oxygen demand (OD).

**Table 4.5** The variations in oxygen transfer parameters with cultivation time in production phase at different pH with different production medium.

| t | $K_La$ | E | $OTRx10^3$ | $OTR_{max}x10^3$ | $OURx10^3$ | OD $x10^3$ | Da | η |
|---|---|---|---|---|---|---|---|---|
| (h) | ($s^{-1}$) | $K_La/K_La_0$ | (mol $m^{-3}s^{-1}$) | (mol $m^{-3}s^{-1}$) | (mol $m^{-3}s^{-1}$) | (mol $m^{-3}s^{-1}$) | | |
| **pH 4.2 (sorbitol/methanol mixed feed)** | | | | | | | | |
| 0 | 0.065 | 5.86 | 16.5 | 22.4 | 15.0 | 65.6 | 2.9 | 0.23 |
| 3 | 0.047 | 4.25 | 11.2 | 16.2 | 9.4 | 28.0 | 1.7 | 0.34 |
| 6 | 0.078 | 6.98 | 18.8 | 26.7 | 17.1 | 46.2 | 1.7 | 0.37 |
| 9 | 0.080 | 7.22 | 20.6 | 27.7 | 19.1 | 61.8 | 2.2 | 0.31 |
| 12 | 0.066 | 5.96 | 15.6 | 22.8 | 13.4 | 55.1 | 2.4 | 0.24 |
| 15 | 0.093 | 8.37 | 23.3 | 32.0 | 21.1 | 134.5 | 4.2 | 0.16 |
| 18 | 0.088 | 7.95 | 21.7 | 30.4 | 20.1 | 385.2 | 12.7 | 0.05 |
| 21 | 0.083 | 7.47 | 20.3 | 28.6 | 18.9 | | | |
| 24 | 0.078 | 6.98 | 19.7 | 26.7 | 18.2 | | | |
| **pH 5.0 (sorbitol/methanol mixed feed)** | | | | | | | | |
| 0 | 0.092 | 8.28 | 29.0 | 36.6 | 27.8 | 244.4 | 6.7 | 0.11 |
| 3 | 0.119 | 10.71 | 36.4 | 47.5 | 34.1 | 132.6 | 2.8 | 0.26 |
| 6 | 0.100 | 8.97 | 30.7 | 39.7 | 28.0 | 90.8 | 2.3 | 0.31 |
| 9 | 0.119 | 10.71 | 33.5 | 47.5 | 28.8 | 97.7 | 2.1 | 0.30 |
| 12 | 0.136 | 12.24 | 38.2 | 54.1 | 33.4 | 135.0 | 2.5 | 0.25 |
| 15 | 0.121 | 10.89 | 34.7 | 48.2 | 33.6 | 193.3 | 4.0 | 0.17 |
| 18 | 0.093 | 8.37 | 26.6 | 37.0 | 25.0 | 287.9 | 7.8 | 0.09 |
| 21 | 0.067 | 6.03 | 18.6 | 26.7 | 16.6 | | | |
| 24 | 0.091 | 8.15 | 26.0 | 36.0 | 21.5 | | | |
| **pH 6.0 (sorbitol/methanol mixed feed)** | | | | | | | | |
| 0 | 0.078 | 6.98 | 24.9 | 30.7 | 23.9 | 557.6 | 18.2 | 0.04 |
| 3 | 0.078 | 6.98 | 23.5 | 30.7 | 22.2 | 76.5 | 2.5 | 0.29 |
| 6 | 0.073 | 6.55 | 21.6 | 28.7 | 19.4 | 48.2 | 1.7 | 0.40 |
| 9 | 0.053 | 4.75 | 15.5 | 20.8 | 13.7 | 34.4 | 1.7 | 0.40 |
| 12 | 0.042 | 3.80 | 12.0 | 16.7 | 20.3 | 60.1 | 3.6 | 0.34 |
| 15 | 0.066 | 5.96 | 19.5 | 26.1 | 17.4 | 75.0 | 2.9 | 0.23 |
| 18 | 0.089 | 7.99 | 25.6 | 35.1 | 23.6 | 176.1 | 5.0 | 0.13 |
| 21 | 0.081 | 7.26 | 20.9 | 31.9 | 19.0 | 1084.7 | 34.0 | 0.02 |
| 24 | 0.090 | 8.07 | 22.3 | 35.4 | 20.5 | 628.1 | 17.7 | 0.03 |
| **pH 5.0 only methanol** | | | | | | | | |
| 0 | 0.070 | 6.34 | 23.4 | 31.6 | 21.0 | 643.0 | 20.3 | 0.03 |
| 3 | 0.080 | 7.22 | 26.6 | 36.0 | 24.8 | 514.1 | 14.3 | 0.05 |
| 6 | 0.070 | 6.34 | 24.2 | 31.6 | 21.5 | 363.6 | 11.5 | 0.06 |
| 9 | 0.067 | 6.01 | 23.0 | 30.0 | 19.2 | 283.9 | 9.5 | 0.07 |
| 12 | 0.052 | 4.66 | 17.4 | 23.3 | 13.5 | 190.5 | 8.2 | 0.07 |
| 15 | 0.089 | 7.99 | 28.1 | 39.8 | 26.8 | 367.9 | 9.2 | 0.07 |
| 18 | 0.069 | 6.21 | 21.8 | 31.0 | 19.7 | 284.8 | 9.2 | 0.07 |
| 21 | 0.053 | 4.75 | 18.0 | 23.7 | 15.8 | 239.7 | 10.1 | 0.07 |
| 24 | 0.072 | 6.47 | 23.0 | 32.3 | 21.5 | 360.8 | 11.2 | 0.06 |

# CHAPTER 5

# CONCLUSION

In this study, it was aimed to investigate effects of pH on therapeutically important protein, recombinant human growth hormone (rhGH), production by *Pichia pastoris* considering the expression levels of regulatory genes. In the first part of this study, effect of the use of glycerol and sorbitol as a second carbon source besides methanol, was investigated by using *P. pastoris hGH-Mut*$^{+}$ and *P. pastoris hGH-Mut*$^{S}$, and the strain producing higher amount of rhGH was determined based on the cell, rhGH concentration and expression level of *hGH*. Secondly, influence of pH on rhGH production and the stability of the protein were investigated by using chosen strain in laboratory scale air filtered shake bioreactors. In the third part of this study, to supply better control on pH and eliminate oxygen limitation, optimum pH was determined for rhGH production by using pilot scale bioreactor. Furthermore, cell growth, protease formation, oxygen transfer and fermentation characteristics, effects of oxygen transfer on rhGH production and by-product formation were investigated and in addition to these to gain more insight in production and degradation of rhGH, expression levels of *hGH, AOX1, pep4, prb1* and *prc1* were determined.

To be able to determine the most suitable host strain *P. pastoris hGH-Mut*$^{+}$ and *P. pastoris hGH-Mut*$^{S}$ were compared. For this purpose, as the carbon source glycerol, a repressing, and a non-repressing carbon source sorbitol were used besides methanol in the fermentation medium. In defined medium (5.62, $KH_2PO_4$; 1.18, $MgSO_4 \cdot 7H_2O$; 0.83, $CaSO_4 \cdot 2H_2O$; 0.0080 $CuSO_4 \cdot 5H_2O$, 0.0012, KI, 0.0750, $FeCl_3 \cdot 6H_2O$; 0.0280, $MnSO_4 \cdot H_2O$; 0.0044, $ZnSO_4 \cdot 7H_2O$; 0.0080, $CoCl_2 \cdot 6H_2O$; 0.0052, $Na_2MoO_4 \cdot 2H_2O$, 0.0080, $H_3BO_3$, and 0.00174 biotin) at pH 6, 110 mg $L^{-1}$ rhGH was achieved using $C_{gly}$=30 g $L^{-1}$ and $C_{MeOH}$=1% (v/v) for *P.pastoris hGH-Mut*$^{S}$ strain. It was 2-fold higher than the one obtained with $C_{gly}$=30g $L^{-1}$ and $C_{MeOH}$=3% (v/v) for *P.pastoris hGH-*

*Mut*$^{+}$ strain. Parallel to production level, expression level of *hGH* in Mut$^{S}$ phenotype was higher than Mut$^{+}$ at every selected period of the induction phase. Furthermore, the expression level in Mut$^{S}$ phenotype, 6.76x10$^{9}$ copies mg$^{-1}$ CDW, was 3.25-fold higher than in Mut$^{+}$ phenotype at the end of the production phase.

In medium containing 30 g L$^{-1}$ sorbitol, effect of methanol was investigated and it was found that above $C_{MeOH}$ = 1% (v/v), methanol exhibits inhibitory effect on cell growth of Mut$^{+}$. In $C_{MeOH}$ = 1% (v/v) and $C_{Sorb}$ = 30 g L$^{-1}$ medium rhGH concentration in Mut$^{+}$ phenotype was found as $C_{rhGH}$ =120 g L$^{-1}$, which is 1.9-fold higher than Mut$^{S}$ phenotype. In medium containing sorbitol, it is observed that expression levels of both phenotypes are close to each other at t=23 and 26 h; whereas at t=47 h, *hGH* expression level of Mut$^{+}$ phenotype (9.84x10$^{9}$ copies mg$^{-1}$ CDW) was 5.82-fold higher than that of Mut$^{S}$ phenotype (Figure 4.5), resulted in higher rhGH production.

When rhGH concentration is considered, the best result, 120 mg L$^{-1}$, was obtained by using Mut$^{+}$ phenotype of *P. pastoris* in medium containing 30 g L$^{-1}$ sorbitol and 1% (v/v) methanol. Thereafter, expression level of *AOX* was only determined for Mut$^{+}$ strain. In medium containing 30 g L$^{-1}$ glycerol and 3% (v/v) methanol, higher *AOX* (1.24x10$^{10}$ copies mg$^{-1}$ CDW) was expressed than in medium with 30 g L$^{-1}$ sorbitol and 1% (v/v) methanol throughout the process although, higher hGH production and expression achieved in sorbitol/methanol medium.

After selection of the host strain, effect of sorbitol concentration on rhGH production was investigated at $C_S$= 20, 30, 40, 50, 60, 70 g L$^{-1}$ at $C_M$=1% (v/v) initial methanol concentration. The cell concentration profiles were very close to each other below $C_S$= 50 g L$^{-1}$. A decrease in the cell growth rate was found when initial sorbitol concentration above 50 g L$^{-1}$ so the non-inhibitory limit for rhGH production from Mut$^{+}$ phenotype of *P. pastoris* is 50 g L$^{-1}$. RhGH concentration increased with the increase in initial sorbitol concentration below 50 g L$^{-1}$ and the concentration almost remains constant at 130 mg mL$^{-1}$ above 50 g L$^{-1}$. As a result, 50 g L$^{-1}$ sorbitol concentration was found optimum for both cell growth rate and rhGH production.

Effects of pH on cell growth, rhGH production and stability were investigated in medium containing 50 g L$^{-1}$ sorbitol and 1% (v/v) methanol by using laboratory scale air filtered shake bioreactors. Generally, cell concentration increased with the increase in pH within the range 3.0 to 7.0;

and, there was no considerable difference between the final cell concentrations, especially in the pH range between 5.0 and 7.0. In order to search, samples having different pH value were kept at room temperature for 20 and 48 h and their rhGH concentrations were compared with the one stored at -20ºC. When the results obtained at t=20 h were compared with one stored at -20ºC, protein degradation was observed in the samples kept at room temperature. Furthermore, there was no drastic difference in the concentrations of rhGH between medium having different pH values. A considerable amount of degradation was observed, again, in the samples kept for t= 48 h. However, in that case, more rhGH remained stable at pH 5.0. As a result, rhGH was found to be more stable at pH 5.0. Furthermore, there was no significant variation in the production levels at different pH values, especially between 5.0 and 7.0. The final pH measured in all the media was around pH=2.4. This showed that, further investigation is needed to determine optimum pH.

The main objective of this study is to find a proper pH by means of protein production, cell and proteases concentration. For this purpose optimization of pH studies were performed in pilot scale bioreactor which supplies a better oxygen and pH control. Furthermore, to supply better insight in production, relationship between *hGH* and *AOX*, a tightly regulated promoter and *pep4, prb1 and prc1*, responsible from proteolytic degradation, were investigated at transcriptional level. The temperature and agitation rate were kept at T=30°C and N=900 rpm, respectively, throughout the process. The dissolved oxygen (DO) has been maintained above 20% saturation to prevent oxygen limitation during the fermentation. To be able to determine optimum pH, three sets of fed-batch bioreactor experiments were performed at pH 4.2, 5.0 and 6.0, respectively. The results are summarized below.

- Sorbitol was consumed more rapidly in media with pH 4.2 or 5.0 However, at pH 6.0, sorbitol was totally consumed at t=21 h because of a shift in metabolism of cells at pH 6.0. In the medium, methanol has never been detected. This shows simultaneous utilization of sorbitol with methanol.
- It is observed that, cell growth profiles obtained at pH 4.2, 5.0 and 6.0 were almost same for glycerol batch, glycerol fed batch and methanol transition phases. However, in production phase by starting with the

same cell concentration, the highest and lowest final cell concentrations were obtained at pH 6.0 (52.53 g $L^{-1}$) and 5.0 (42.3 g $L^{-1}$), respectively.

- The highest rhGH concentration, $C_{rp}$= 271 mg $L^{-1}$, was acquired at pH 5.0. Thus, 1.22-fold and 11.3-fold higher protein was obtained at t=24 h at pH 5.0 compared to the one at pH 4.2 and 6.0, respectively. The rhGH concentration in medium with pH 6.0 was 24 mg $L^{-1}$ at t=24 h, which is the lowest one although the highest cell concentration (52.53 g $L^{-1}$) was obtained. This is related to the expression level of *AOX1* since AOX activity obtained at pH 6.0 was so low as compared to obtained at pH 4.2 and 5. In terms of expression levels, it is observed that the best result, $4.01x10^{10}$ copy number $mg^{-1}$ CDW, was obtained at pH 5.0 at t= 15 h, which verifies the results obtained by SDS-PAGE and HPCE. According to HPCE result, at the end of the production phase, rhGH concentration was so low, 24 mg $L^{-1}$, at pH 6.0 whereas maximum expression level reached at pH 6.0, $1.30x10^{10}$ copy number $mg^{-1}$ CDW, was very close to $1.10x10^{10}$ copy number $mg^{-1}$ CDW obtained at pH 4.2. This demonstrates a limitation occurred at translation, amino acid synthesis and secretion steps.
- The highest specific AOX activity was obtained as 40.7 U $g^{-1}$ CDW at pH 5.0 and t=15 h and, the lowest one was obtained as 13.8 U $g^{-1}$ CDW at pH 6.0 at t=18 h. For pH 4.2 and 5.0, a similar behavior to AOX activity observed in expression level of *AOX.* For pH 6.0, a different profile was observed and the highest expression level was obtained at t=9 h whereas the highest AOX activity was seen at t=21 h. Furthermore, during the production, the expression of *AOX* at pH 6.0 was higher than obtained at pH 4.2. This shows, again, a limitation occurred at translation, amino acid synthesis and secretion steps. The highest *AOX* expression was attained at pH 5.0 and t= 15 h, similar to AOX activity.
- It is seen that, total protease concentration profiles obtained at different pH values have similar behavior such that an exponential increase was observed. Moreover, for pH 4.2, 5 .0 and 6.0 at t=24 h specific protease concentrations were 1.92, 1.94 and 1.90 mg $g^{-1}$ CDW, respectively. They are really close to each other so change in pH does not affect total protease concentration considerably in extracellular medium. In each production condition, expression level profiles of the genes were compared with each other. The manners of the expression

levels of *pep4, prb1* and *prc1* are parallel to each other with respect to time in each different pH value (Figure 4.35) and expression level of *prc1* was much higher than the others in each condition. $4.73x10^9$ copies $mg^{-1}$ CDW, highest *pep4* concentration was obtained at pH 4.2 and it is 1.6 fold higher than the one at pH 5.0. Similarly, prc1 expression levels at pH 4.2 and 6.0 were almost 1.6 fold higher than one obtained at pH 5.0. Moreover, *prb1* concentrations obtained at pH 4.2 and 6.0 are much higher than at pH 5.0, again. As a result, pH 5.0 seems to be the best choice when expression levels of different proteases are taken into consideration to eliminate the risk of proteolytic degradation.

- In all conditions, leucine (Leu), glutamine (Gln), serine (Ser) and glutamic (Glu) acids were not detected in the medium. This is probably due to high Leu, Ser, Glu and Gln content of hGH, which contains 13.6% Leu, 9.4% Ser, 7.3% Glu and 6.8% Gln. Moreover, it is observed that phenylalanine (Phe) concentration decreased with increasing pH. 0.041 g $L^{-1}$ Phe was the lowest concentration obtained at t=21 h and pH 6.0.
- For all condition, formaldehyde was not detected in the medium throughout the process. However, formic acid was detected at all conditions. It is observed that amount of formic acid in extracellular medium increased with respect to time and formic acid concentration was higher at pH 6.0. Furthermore, formic acid concentration increased as pH increases and 0.3986 g $L^{-1}$ was the highest concentration obtained at pH 6.0. The other organic acids detected in the medium were fumaric, α-keto glutaric, citric and succinic acids. It is revealed that fumaric and succinic acid concentration in the medium increased with decreasing pH.
- The cell yield on substrate at pH 6.0 is $Y_{X/S}$=0.25 g $g^{-1}$, which is higher than the ones obtained at pH 4.2 and 5.0. The overall product yield on cell at pH 5.0 with medium containing sorbitol is $Y_{P/X}$=13.86 mg $g^{-1}$, which is 1.4 and 17.8 fold higher than the ones obtained at pH 4.2 and 6.0, respectively. Similarly, by adjusting pH to 5.0, the overall product yield on the total carbon source ($Y_{P/S}$=2.08 mg $g^{-1}$) was increased 1.3 and 10.4 fold with respect to pH 4.2 and 6.0, respectively.

- The highest specific rhGH formation rate, 0.463 mg $g^{-1}$ $h^{-1}$, was obtained at pH 5.0 at t=15 h. This was a natural result since highest AOX activity obtained at these conditions and protein production is coupled with AOX enzyme in the methanol utilization pathway. The lowest $q_{rp}$ was observed at pH 6.0. This is due to lowest AOX activity seen at this pH value.
- Throughout the process, higher oxygen transfer coefficients were obtained at pH 5.0. At pH 4.2, it was observed that pH affects the physiology of the cell, which alters rheological properties of the fermentation medium, which results in an increase in mass transfer resistant. Furthermore, OUR at pH 4.2 throughout the process was lower than the one at pH 5.0. Mutual effect of mass transfer resistance and OUR determines the inclination of $K_La$ throughout the fermentation process. At pH 6.0, again lower mass transfer coefficients were acquired than at pH 5. This can be due to higher cell concentration achieved at pH 6.0 since this cause cell coalescence which should increase mass transfer resistant in the fermentation medium.

As gene expression levels of *hGH, AOX, pep4, prb1* and *prc1* and rhGH production are considered, the most suitable extracellular pH is found as 5. In addition to that adjustion of pH to 5 affects expression levels of *hGH* and *AOX* positively, whereas it negatively affects expression levels of protease related genes. This positive and negative effects of pH on gene expressions can be global, in other words, pH can influence expression levels of these genes indirectly. This means that, change in pH affects expression via metabolism of cell and proton regulated genes. To be able to ensure that, further investigation based on all genome of the microorganism should be performed.

After determining pH 5.0 as a optimum pH for rhGH production, one more set of fed-batch bioreactor experiment was performed where only methanol was used as a carbon source to be able to observe the effectiveness of the methanol/sorbitol mixed feeding strategy. The results were compared with the one obtained at pH 5.0 with medium containing sorbitol and they are summarized below.

- It is observed that addition of sorbitol as batch wise to the medium eliminates the long lag-phase for the cells. Moreover, at t=24 h, the final cell concentration, 42.3 g $L^{-1}$, in medium containing sorbitol was almost 1.4 fold higher than final cell concentration obtained by using methanol as a sole carbon source although the same methanol feeding profile ($\mu$=0.03 $h^{-1}$) was used.
- It is seen that at t= 24 h rhGH concentration in medium with sorbitol is 270 mg $L^{-1,}$ which is 1.5 fold higher than 180 mg $L^{-1}$ obtained in medium containing only methanol. In addition to rhGH concentration profiles, *hGH* expression level profiles were compared. It is revealed that addition of sorbitol into the medium influence expression positively and 8.6 fold higher expression was achieved at t=15 h.
- The specific AOX activity for cells grown on only methanol at t=24 h is 39.6 U $g^{-1}$ CDW, which is very close to 40.7 U $g^{-1}$ CDW obtained in presence of sorbitol at t=15 h. Furthermore, the highest expression of *AOX*, 9.38x$10^{10}$ copy number $mg^{-1}$ CDW, was achieved for cell grown on sorbitol at t=15 h. Thus, *AOX* expression profiles are in good agreement with the ones obtained by using specific AOX activity.
- At t=24 h total protease concentration for medium containing only methanol at pH 5.0 is 0.053 g $L^{-1}$, which is much less than 0.082 g $L^{-1}$ obtained for medium containing sorbitol with pH 5.0. In addition to that, specific protease concentrations were compared since the final cell concentration for medium with sorbitol is almost 1.4 fold higher. At t=24 h, 1.94 mg protease $g^{-1}$ cell dry weight for medium with sorbitol is really close to 1.76 mg protease $g^{-1}$ cell dry weight for medium with only methanol. As a result, addition of the sorbitol into the medium does not create significant difference on the protease production level. By means of expression levels of *pep4, prc1* and *prb1*, generally expression levels of the proteases in medium with methanol were almost same the ones with sorbitol although 1.4-fold lower final cell concentration was obtained when cells were grown on only methanol. As a result, addition of sorbitol did not affect expression of proteases considerably. This results is similar to one obtained for total protease concentration.

- Histidine (His) was only detected in medium containing only methanol and its concentration reached 0.0017 g $L^{-1}$ at t=21 h. Furthermore, valine (Val) was also detected sorbitol containing media only.
- Lactic acid was only detected in medium containing only methanol and the concentration at t=24 h is 0.1623 g $L^{-1}$.
- The addition of sorbitol into the medium as batch wise increased the cell yield on substrate 1.7 fold and $Y_{X/S}$=0.10 g $g^{-1}$ was cell yield on substrate for medium containing methanol as a sole carbon source. The addition of sorbitol decreased the both the overall product yield on cell and on total carbon sources about 1.7 and 1.2-fold, respectively. However, human growth hormone is a protein having high values for low volumes so increasing the amount of final product is the main aim. Therefore, addition of sorbitol is more profitable since it increased final amount of product (270 mg $L^{-1}$) 1.5-fold.
- It is revealed that addition of sorbitol into the medium increased the specific growth rates obtained during the process.

Besides bioreactor experiments, to be able to perform absolute quantification to determine expression levels of regulatory genes, standard curves were constructed. For *hGH* and *AOX,* SYBR Green I assay was used in real time PCR analysis. Plasmid DNA, *pPICZaA::hGH*, was used for quantification of genomic DNA or complementary DNA extracted from samples taken during the production of rhGH from *P. pastoris*. By using optimized conditions, standard curves with efficiencies of 1.723 ± 0.0261 and 1.523 ± 0.0623 were constructed for *hGH* and *AOX*, respectively. The hydrolysis probe assay, TaqMan, was used for *pep4, prb1* and *prc1* genes and genomic DNA was used for quantification of samples. Standard curves with efficiencies of 2.271 ± 0.0164, 1.609 ± 0.0294 and 1.666 ± 0.0251 were constructed for *pep4, prb1 and prc1,* respectively.

Thus, this work contributes not only to the optimization of pH for rhGH production by recombinant *P. pastoris,* but also to further understanding the relationship between expression levels of *hGH* and *AOX*, a tightly regulated promoter and *pep4*, *prb1* and *prc1*, responsible from proteolytic degradation. Furthermore, in this study, the effectiveness of sorbitol/methanol cofeeding strategy was verified.

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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**

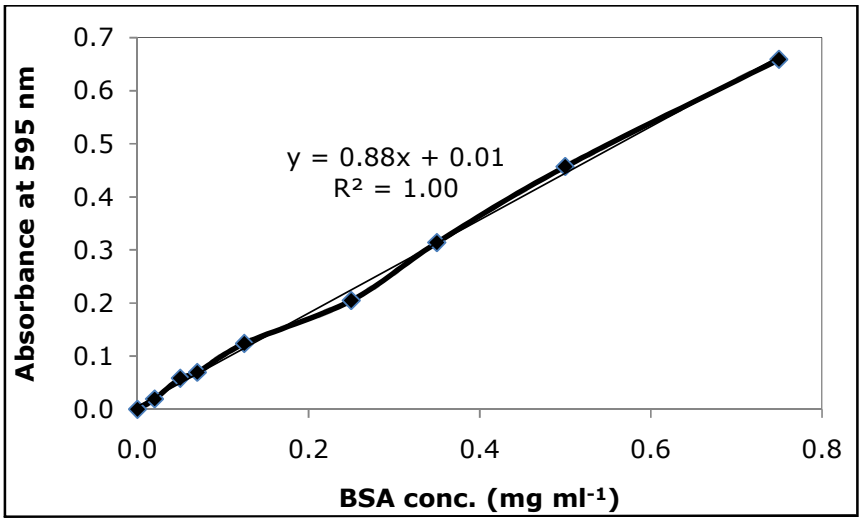


**Figure B.1** Calibration curve for Bradford Assay

**Calibration Curve for Sorbitol Concentration**

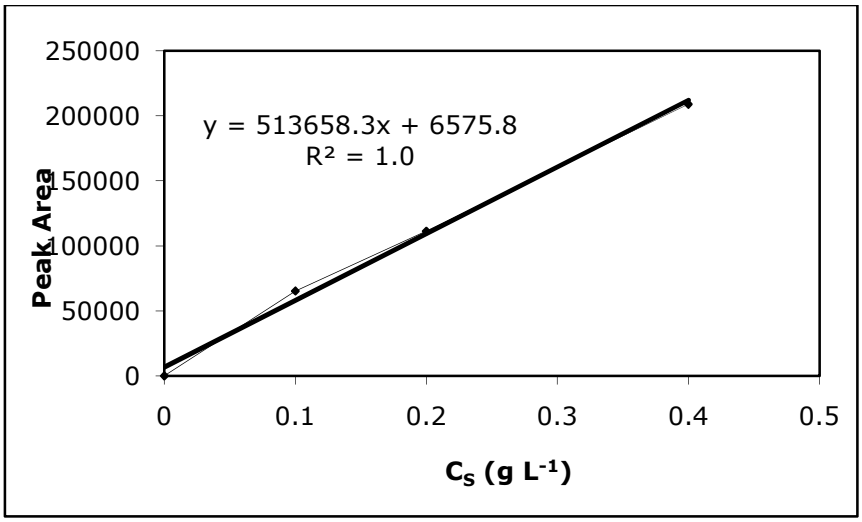


**Figure B.2** Calibration curve obtained for sorbitol concentration; analysis was performed by HPLC.

**Calibration Curve for Methanol Concentration**

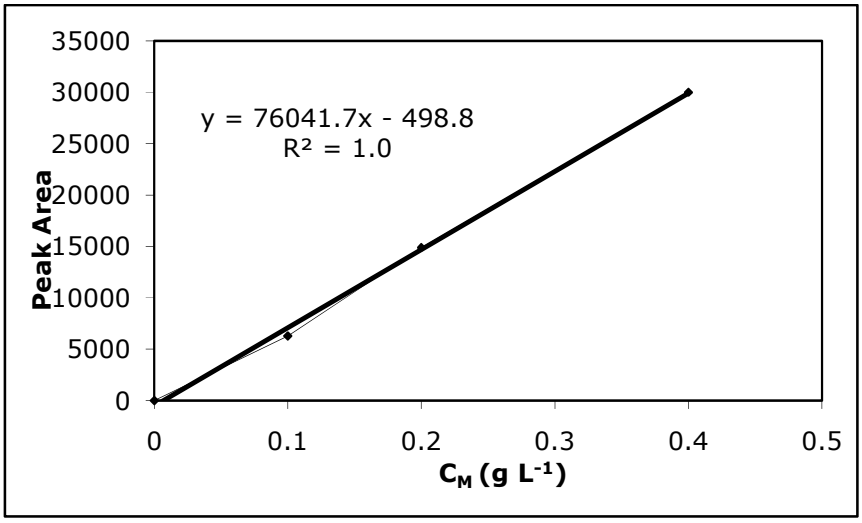


**Figure B.3** Calibration curve obtained for methanol concentration; analysis was performed by HPLC.

**Calibration Curve for Succinic Acid Concentration**

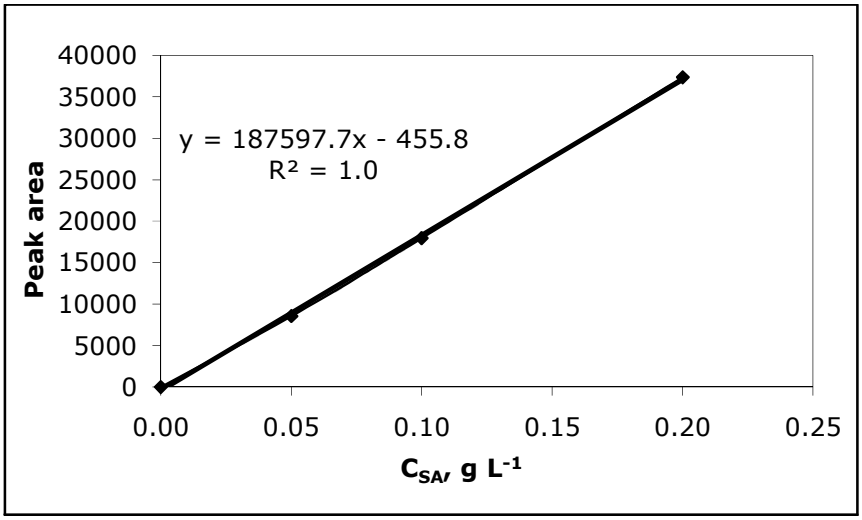


**Figure B.4** Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

**Calibration Curve for Meleic Acid Concentration**

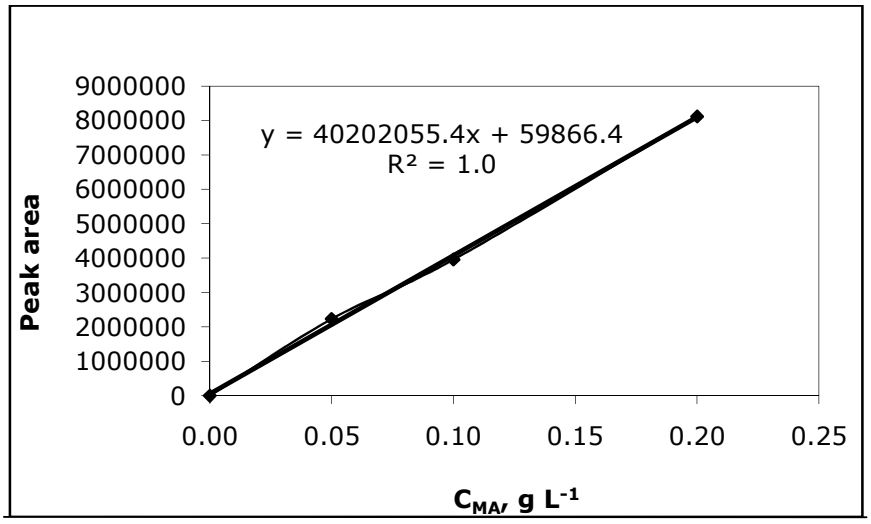


**Figure B.5** Calibration curve obtained for maleic acid concentration; analysis was performed by HPLC.

**Calibration Curve for Glutaric Acid Concentration**

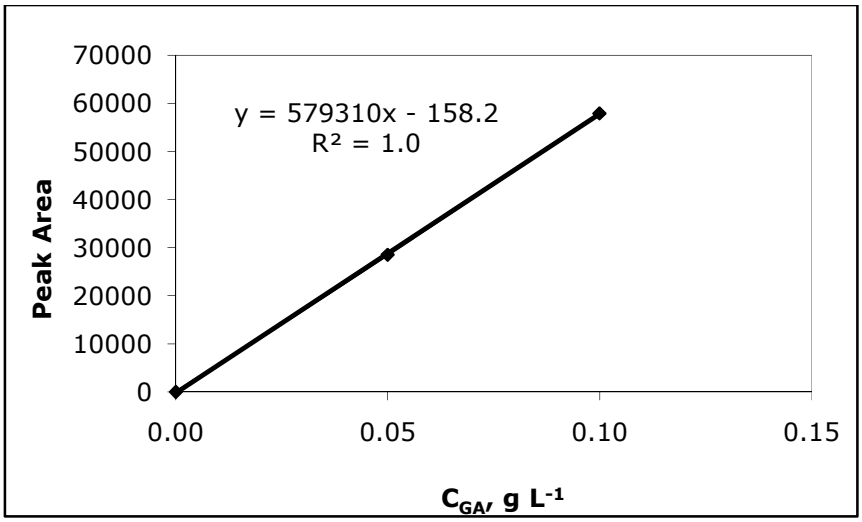


**Figure B.6** Calibration curve obtained for glutaric acid concentration; analysis was performed by HPLC.

**Calibration Curve for Lactic Acid Concentration**

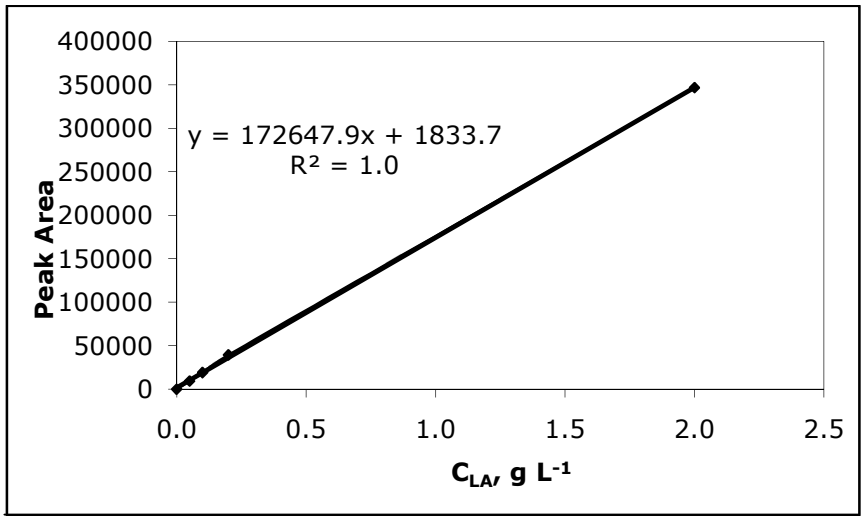


**Figure B.7** Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

**Calibration Curve for Formic Acid Concentration**

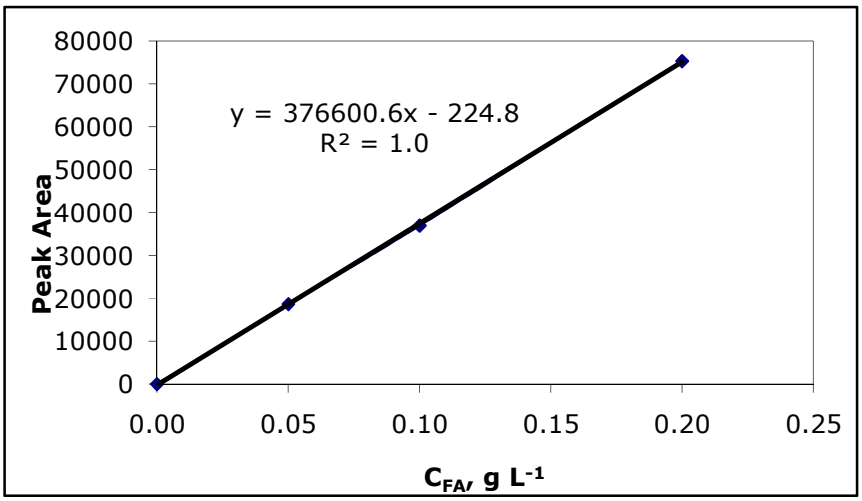


**Figure B.8** Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

**Calibration Curve for Citric Acid Concentration**

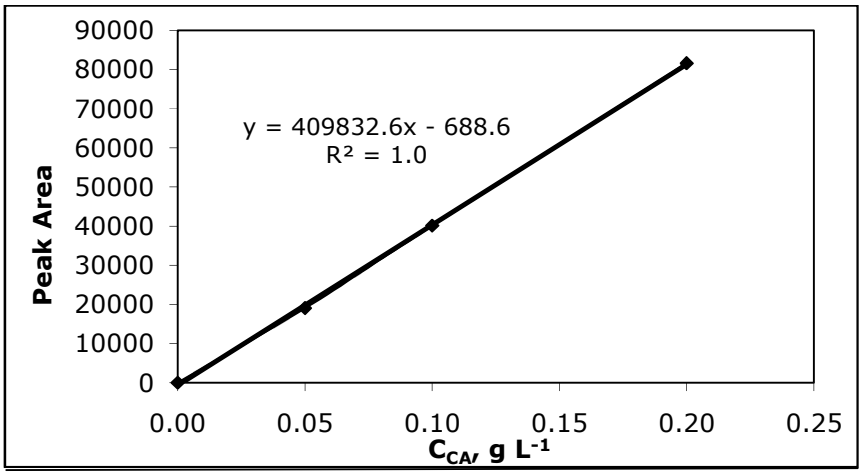


**Figure B.9** Calibration curve obtained for citric acid concentration; analysis was performed by HPLC.

**Calibration Curve for Fumaric Acid Concentration**

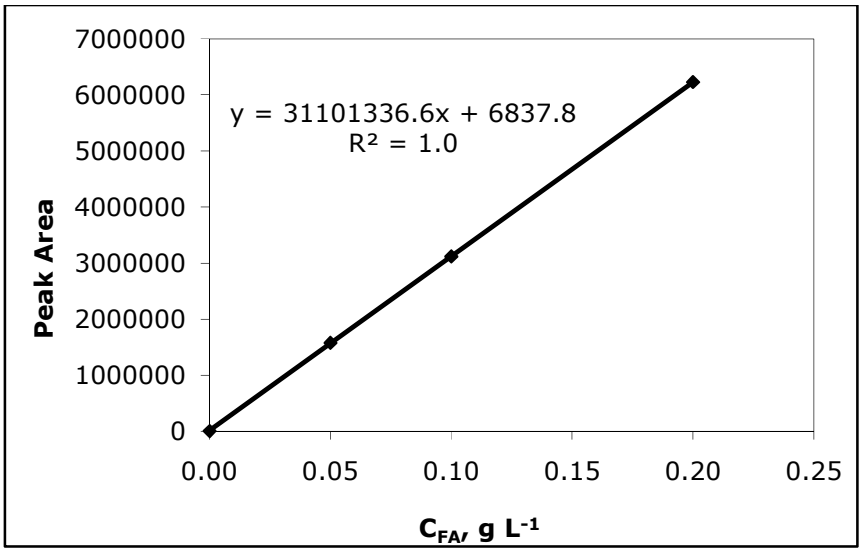


**Figure B.10** Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

**Calibration Curve for AOX Activity**

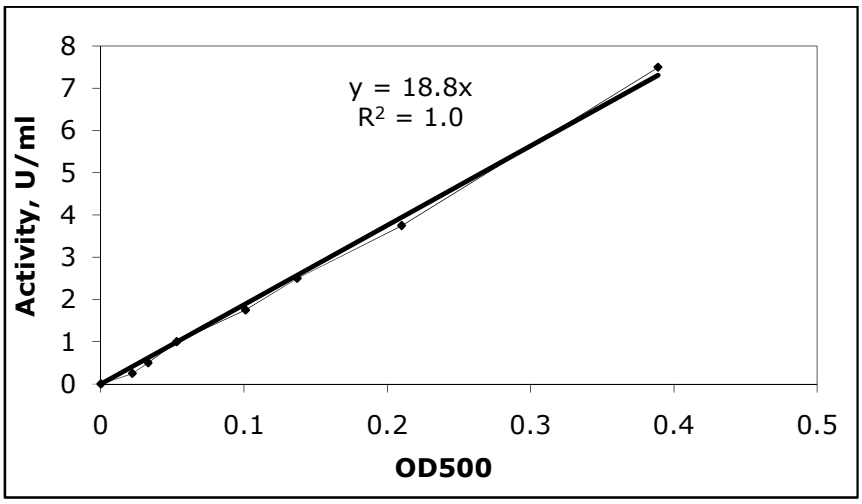


**Figure B.11** Calibration curve obtained for AOX activity.

# APPENDIX C

## ELECTROPHEROGRAM OF hGH STANDARD

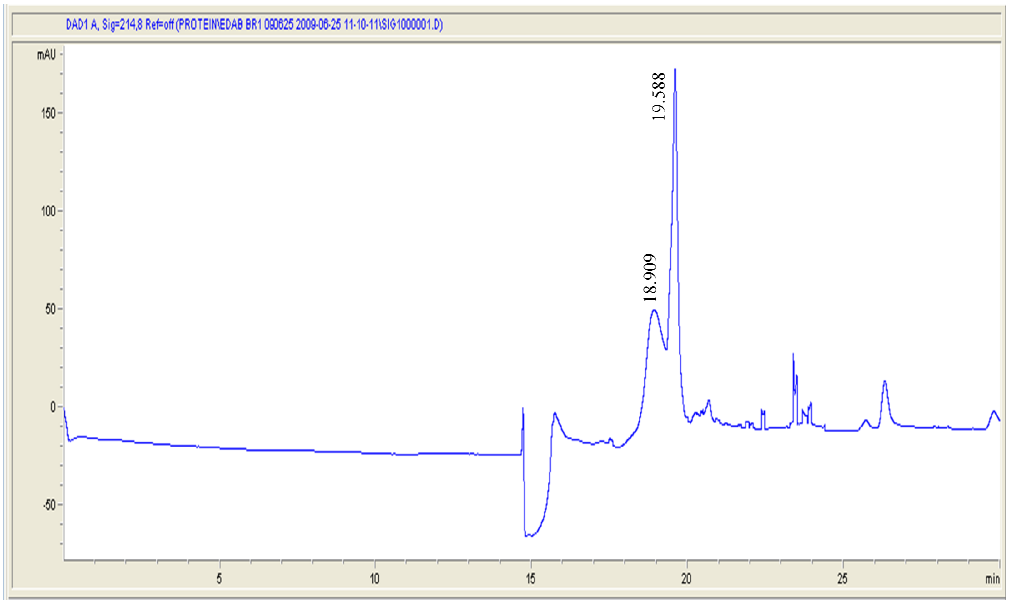


**Figure C.1** Electropherogram of 0.05 g $L^{-1}$ standard hGH

# APPENDIX D

## MOLECULAR WEIGHT MARKER

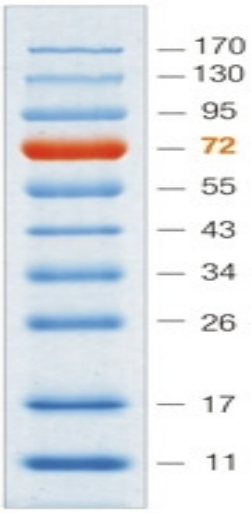


**Figure D.1** PageRuler™ Prestained Protein Ladder (Fermentas)

# 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 LightCycler TaqMan Master Kit**

| Vial/Cap | Label | Contents/Function<br>a) Cat. No. 04 535 286 001 (96 reactions)<br>b) Cat. No. 04 735 536 001 (480 reactions) |
|---|---|---|
| 1a<br>white cap | Enzyme | a) 1× vial 1a, 3× vial 1b, for 3× 128 µl Master Mix (5x conc.)<br>b) 5× vial 1a, 15× vial 1b, for 15× 128 µl Master Mix (5× conc.)<br>• Ready-to-use hot start PCR reaction mix (after pipetting 10 µl from vial 1a into one vial 1b)<br>• Contains FastStart Taq DNA Polymerase, reaction buffer, $MgCl_2$ and dNTP mix (with dUTP instead of dTTP) |
| 1b<br>red cap | Reaction Mix | |
| 2<br>colorless cap | Water, PCR grade | a) 2× 1 ml<br>b) 7× 1 ml<br>• to adjust the final reaction volume |

**Figure E.1** Contents of Roche LightCycler TaqMan Master Kit.

**Roche Trancriptor High Fidelity cDNA Synthesis Kit**

| Vial/ Cap | Label | Content<br>a) Cat. No. 05 081 955 001 (50 reactions)<br>b) Cat. No. 05 091 284 001 (100 reactions)<br>c) Cat. No. 05 081 963 001 (200 reactions) |
|---|---|---|
| **1 red** | Transcriptor High Fidelity Reverse Transcriptase | a) 1 vial, 55 µl<br>b) 1 vial, 110 µl<br>c) 1 vial, 220 µl<br>• 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<br>b) 1 vial, 1 ml<br>c) 2 vials, each 1 ml<br>• 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)<br>b) 1 vial, 50 µl (40 U/µl)<br>c) 2 vials, each 50 µl (40 U/µl)<br>• 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<br>b) 1 vial, 200 µl<br>c) 2 vials, 200 µl<br>• 10 mM each dATP, dCTP, dGTP, dTTP |
| **5 blue** | Anchored-oligo$(dT)_{18}$ Primer | a) 1 vial, 100 µl (50 µM)<br>b) 1 vial, 200 µl (50 µM)<br>c) 2 vials, each 200 µl (50 µM) |
| **6 blue** | Random Hexamer Primer | a) 1 vial, 100 µl (600 µM)<br>b) 1 vial, 200 µl (600 µM)<br>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<br>b) 2 vials, 1 ml<br>c) 3 vials, 1 ml |
| **9 green** | Control RNA | a) only Cat. No. 05 081 955 001<br>1 vial, 20 µl (50 ng/µl)<br>• 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<br>1 vial, 40 µl<br>• 5 µM forward and reverse primer specific for human porphobilinogen deaminase (PBGD) |

**Figure E.2** Contents of Roche Trancriptor High Fidelity cDNA Synthesis Kit.

**Roche LightCycler FastStart DNA Master SYBR Green I kit**

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

**Figure E.3** Contents of Roche LightCycler FastStart DNA Master SYBR Green I kit

# APPENDIX F

## DNA SEQUENCES AND PLASMIDS

**Sequence of pPICZαA::*hGH* plasmid**

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**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**

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**Note:**

Highlighted sequences are primers designed for *AOX1* gene

**Proteinase A (*pep4*) BLAST HIT (includes STOP)**

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tggtctgcttttccttttgagcaatctcatttgagaacttatcgctggggagaggatggactagct
ggagtctcagacaatcatcaactaatttgtttctcaatggcactgtggaatgagaatgatgatatt
ttgaaggagcgattatttggggtcactggagaggctgcaaatcatggagaggatgttaaggagctt
tattattatcttgataatacaccttctcactcttatatgaaataccttttacaaatatccacaatcg
aaatttccttacgaagaattgatttcagagaaccgtaaacgttccagattagaaagagagtacgag
attactgactctgaagtactgaaggataacagatattttgatgtgatctttgaaatggcaaaggac
gatgaagatgagaatgaactttactttagaattaccgcttacaaccgaggtcccacccctgcccct
ttacatgtcgctccacaggtaacctttagaaatacctggtcctggggtatagatgaggaaaaggat
cacgacaaacctatagcttgcaaggaataccaagacaacaactattctattcggttagatagttgg
aagtatggctcaaatagattggtatttgctccttcgcccgccttcagtgaagaatcatcagacatt
gaacctaagcttcttttcactaataatgaaagtaataaacaaaaactatggaatcaaaaaaatgcg
tcaccctacaccaaggatgctttccatgagtacattgtcaatgaagatagctctgcaattaaccca
caacaaaagggtacgaaggcctgtgcctggttttcttttgatgaaaatggtggtgttccccctggt
gattatgtaactatccgttacagattcacaagaacagatagtgaccagcctgtaattgatgaagag
gctttcgataaagtatttgctcgtagacaactagaggctgacgagttttattggaggatttctccc
ttgccaatcagtgatgagctcagaagtgtccagcggcaagcttttgccggtctcctttggaacaaa
cagttctatcagtttatccatgattattggtacagaggagaccctgactcaaaaataaagcctact
attaacagagctaatggacgaaacaaagattggaagcatctttatataaatgatgtactatctctc
cctgacaaatgggagtatccttttttctgcacgtgggacactgccttccactgcataccaatagcca
tgatagatccagattatgcgaagaatcaattagacattttgactcgggaatggtatatgcatccca
atggacaaatgcccgcgtacgaatggaatttcagtgacgtcaaccctccagtccatgcatgggcca
ccttcagaacttataaaattgaaagaaaaatgtacgggcgtcaggatattccatttttggaacgtg
tttttcagaagcttttactaaacttcacttggtgggtcaatagaaaggatactgagggcaaggggc
tttttgagggaggtttcttgggacttgataacattggcatattcaacaggtcggagcctctaccca
ctggtggaacactggaacaagctgatagtacaggttggatggctttttttttctttgcaaatgttga
atattgcattggaactagcacaatacaatcctgtctatgaagatattgcttcgaagttttttgaac
atttcattctaatttctgatgcgatgacagttaacggaggcactggggagaaatcgttatggaatg
acgacgacggcttttattatgatgccataagttggggagagtccacatcaatgcagttgcctattc
gttcattagtgggcctgatcccattgtatgctagtctgactttggagccagagttaattaagaagt

ttccctcattcacgaaacgattagattggtttatcaaaatgaaaggtaatgttgcagagcgaaata
ttgaatccatgagtgcccgaggggttggcgaacggttacttctctcacttgtcagtaaagaacgtt
tagtttcaattttggaaaaaatgctagacgaggatgagtttttgtcccaatacggtattagatctt
tatcaaagtaccacgaagagcatccctttgagatggaagttcatggccagaattatgaagtaaaat
atcttccaggagagtcggatagtggactgtttggtggaaattccaattggagaggtccaatttggc
ttcctgttaatttccttatcgttgaatcattacaaaagttctatctatactacggctcgtccttga
aggtagaatgcccgaaagggtccggaaaatacttgaatttggctcaagttgcagaggagattcaac
atcgcttgattcatttgttcattagggatgtcaatggaaagagagcttcaaacggagactgtgatc
tgcttaatgaggacaagcatttcaaaaactatgtattgttctatgagtattttgaaggagacaccg
gaagaggtttaggagcgggacatcaaacaggttggacggcacttgtagctaagtggattcatgaca
ctggaatatcatgccgggtacccaaaactcccaaaacagatgacagaagaccgtcttactttctac
aaaaacacggtacaaacgatgaagacatttatcctgacgaccaagacgatttatcccagagtttct
caccctacatgtcaactcccttccctggaaacctcactagaagaaggtcgggtaaatcactgctga
atctgacgactgacgtcctagatatgacagatgaagagcttaaagctcatcgaagaaatcttcctg
gattgagtcgtactgccagcttagctagctccaaatctgggtatgtatctgctggtggcgctagtg
gaagtgaagtgctctcacgcaagagtagttgcagtgaaagtgacgaaattgatttttttaaccaag
ttcgagacgccatcagtaattatgagctcaaagatgatgataatcttagtggggatgaattagaga
gtcacattaattagttt

**Note:**

Highlighted sequences are primers designed for *pep4* gene

**Proteinase B (*prb1*) BLAST HIT(includes STOP)**

atgcaattgcgtcattccgttggattggctatcttatctgccatagcagtccaaggattgctaatt
cctaacattgagtcattacccagccagtttggtgctaatggtgacagtgaacaaggtgtattagcc
caccatggtaaacatcctaaagttgatatggctcaccatggaaagcatcctaaaatcgctaaggat
tccaagggacaccctaagcttgccctgaagctttgaagaagatgaaagaaggccaccctcggct
ccagtcattactacccattccgcttctaaaaacttaatcccttactcttatattatagtcttcaag
aagggtgtcacttcagaggatatcgacttccaccgtgaccttatctccactcttcatgaagagtct
gtgagcaaattaagagagtcagatccaaatcactcatttttcgtttctaatgagaatggcgaaaca
ggttacaccggtgacttctccgttggtgacttgctcaagggttacaccggatacttcacggatgac
actttagagcttatcagtaagcatccagcagttgctttcattgaaagggattcgagagtatttgcc
accgattttgaaactcaaaacggtgctccttggggtttggccagagtctctcacagaaagcctctt
tccctaggcagcttcaacaagtacttatatgatggagctggtggtgaaggtgttacttcctatgtt
atcgatacaggtatccacgtcactcacaaagaattccagggtagagcatcttggggtaagaccatt
ccagctggagacgttgatgacgatggaaacggtcacggaactcactgtgctggtaccattgcttct
gaaagctacggtgttgccaagaaggctaatgttgttgccatcaaggtcttgagatctaatggttct
ggttcgatgtcagatgttctgaagggtgttgagtatgccacccaatcccacttggatgctgttaaa
aagggcaacaagaaatttaagggctctaccgctaacatgtcactgggtggtggtaaatctcctgct
ttggaccttgcagtcaatgctgctgttaagaatggtattcactttgccgttgcagcaggtaacgaa
aaccaagatgcttgtaacacctcgccagcagctgctgagaatgccatcaccgtcggtgcatcaacc
ttatcagacgctagagcttacttttctaactacggtaaatgtgttgacattttcgctccaggttta
aacattctttctacctacactggttcggatgacgcaactgctaccttgtctggtacttcaatggcc
tctcctcacattgctggtctgttgacttacttcctatcattgcagcctgctgctggatctctgtac
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gtcggagtattagaggatgttccagaagacactccaaacctcttggtttacaatggtggtggacaa
aacctttcttctttctggggaaaggagacagaagacaatgttgcttcctccgacgatactggtgag
tttcactcttttgtgaacaagcttgaatcagctgttgaaaacttggcccaagagtttgcacattca
gtgaaggagctggcttctgaacttatttagatt

**Note:**

Highlighted sequences are primers designed for *prb1* gene

***P.pastoris PRC1* gene**

gaattcacctgctcaatgactttacttaagtgagtgtaagcctcttgtggagacaatttcagttcg
gtgattaaacgatcaactttgttcaatacaagaatagctttcaacttgtctgaccaaacttgtcgt
agcacactaattgtctgagaacaaactccttccacgacgtcgactaatacaactgccccatcacat
aatctcgaagctgtactaacttctgagctaaagtcaacatgacctggagaatcaattaaattaata
agatgttcattgactgtgatatcactatcagaatcaccctcatttctgtgtatcaccctaaaatac
agcgaaacagcgctactctccattgttatgccccgtaactgctcatcctcccttgagtccaagtat
ctaatcttgcccgccatttttgtgtgagattatgccatttgtcgctaagagactatcgcttaaagaa
gtcttaccatggtccacatgagcaactatacatatatttcgaacacaatcggggtcattttgaaga
gtgttcagttgttctttagttaaccgcatagttaccaaaactcggtaacgaacaaatggtatcacg
ctcctccaataactagtaaaaaaaaaaactaatccgcgcacaagcttaaatcgattatataatgat
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aaattccaagtcttcagttgtaatcatgtcccaatacttgtacagcatcatcactgcgaatagatt
gcaaagtgtaccaaaaaacagtccatcaaggtaatgattaacaccctcgcaaatctgatttgaaaa
acgatacatgagaacctgtgctgcagtgaaaaagaaacatcctaacatgattgcacccaaagccca
ccagttttcaaaatagtcagtataagaatgatttgagagagtacgtaaatgccaatagtgaccgc
attgaggaagtacatggtcacgaaaagagcgagtgtatttgtgttatcgacaattgagttactgga
ccaattgtgaaaagtgaaaagcgaaaggataaagttaagggaaaaggcacataaagcaatcaatct
agtaaagttaatagattttcttgtgccatcttcccaaaactgaaaccccagaaaaccattcagcat
cagagaccagcagcaggctgatgccaggccaatttgaactgccacgaagtatccgtatgttgcact
gctgggtggagaaacaccagcatcaacgaccaatgaggcgatggtgttggcaatgtacaggtagaa
aaaaaaaagcatttcgatccttccaattgctgtgtatttgcttctaacatggaacacaataatcat
caagacgacaatagcgctgatgtgtataaaacagttccctacctggaaaatcattgtgttggttac
cgatatacttctagcgtaacaagtagaaataattcccttgaagtttgtaccgtggttgggcagtaa
aacattacacagaggtaatggagtttttgacaaatctcataaaagttcccaaatgacataacttg
tttcagttaatgagctacttctatgccttcgttataactttatatatggttgtcaaattcctcgcc
tgaatctgaaaccaagatatattatatacttatgtaagcatccttacctctgtcctgcttcacttt
ttatgtacctcagccaataaaaaggcgaagactggatcaactcctctcttgaaggttgatctcaaa
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agttcaaccccgtaatccagccttatcggatgctatagatagtttcatactatttacaagtaaaat
tcgtttcacattgaatcaatgatattacacacctatattattctctcgttattgactatatttcct
aaagctattggtctgtccttgcagatgccaatggccttggaagctagttatgcctcattagtggag
aaagcaaccctcgctgttggacaagaaattgatgccatacaaaagggtattcagcaaggttggttg
gaagtagagacaagatttccaactatagtgtcacagttatcctatagtactggcccaaaatttgcg
atcaagaagaaagatgcaacttttttgggatttctatgttgaaagtcaagagttgccaaactaccgg
ttacgcgtgaaaaggaacaatcccgaagttttgaaagttgattttactaaacagtatagcgggtat
ttggacgtagaggctgatgacaagcatttttttttactggttttttgaatcgaggaatgacccacag
aatgacccaattatttttatggttgaatggtggccctggctgctcttccttgactggtctctttttc
gaattaggatcgtctagaattaatgaaaatctgaaaccaatttttcaaccccctattcgtggaatggt
aatgcttcaatcatctacttagatcaaccggtcaatgttgggttttcttattcttcatcatcggtg
agtaacactgttgttgcgggagaagatgtgtatgcatttcttcagctttttttttcaacacttcccg
gaatatcaaactaatgactttcatattgccggtgaatcttatgcaggacattacattccggtgttt
gcagacgaaattttgagtcaaaagaacagaaatttcaatcttacttcagtcttgatcggaaatgga
ttaactgacccttttgactcaataccgatattacgagccaatggcttgtggtgaaggtggtgccccg
tcagtactgcctgccgatgagtgcgaaaatatgctagttacccaagataaatgtttgtctttaatt
caagcatgctacgactcacagtcggcattcacatgcgcaccggctgccatttattgtaataacgct
cagatgggaccctatcagagaactgggaagaatgtgtatgatattcgtaaggaatgtgatggtgga
tccttgtgctataaggaccttgaattcatcgatacctacttaaatcaaaagtttgttcaagatgct
ttgggcgccgaggtcgatacctatgaatcttgcaattttgaaatcaacagaaacttttttatttgct
ggagattggatgaaaccttatcatgaacatgtcagcagtctcttgaacaaaggtttgcccgttttg
atttacgcaggggacaaagatttcatttgcaactggttgggtaatcgagcatggactgatgtcttg
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ccatatgatcagccagagaattctcatgaaatggtcaatagatggatatccggagactttagcttt
cactagggtcgctgaattgcaaatatagtcagtaaattaagccttggggtgctctttattgcacga

aaaactaatactttcaaagttgaccatgagactgattattgaatttatattgctgaatctgaatta
ttgaaattacactattgttacttgtgtaaccatgctggtctggactgggcttgctgagtcagtact
gtatatcgtacacgggaagatc

**Note:**

Highlighted sequences are primers designed for *prc1* 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 attcctgcagaaccccagacctccctctgtttctcagagtctattccgacaccctccaa
  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


KEYS (in order of precedence):
>>>>>> left primer
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)
```

## Results from Roche ProbeFinder Vesion 2.43 for *pep4* gene:

**ProbeFinder has designed an optimal real-time PCR assay for:**
**gene_1**

Assay rank 1
Use probe **#31** (cat. no. 04687647001)

| Primer | Length | Position | Tm | %GC | Sequence |
|---|---|---|---|---|---|
| Left | 20 | 3277 - 3296 | 60 | 55 | tcaacaggtcggagcctcta |
| Right | 24 | 3313 - 3336 | 59 | 42 | aacctgtactatcagcttgttcca |

Amplicon (60 nt)

tcaacaggtcggagcctctacccactggtggaacactggaacaagctgatagtacaggtt

This assay has: All criteria met.



## Results from Roche ProbeFinder Vesion 2.43 for *prb1* gene:

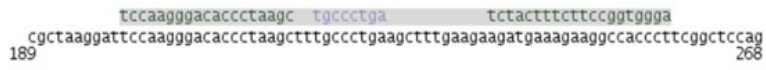
Assay rank 1
Use probe **#122** (cat. no. 04693566001)

| Primer | Length | Position | Tm | %GC | Sequence |
|---|---|---|---|---|---|
| Left | 19 | 199 - 217 | 60 | 58 | tccaagggacaccctaagc |
| Right | 20 | 239 - 258 | 60 | 50 | agggtggccttctttcatct |

Amplicon (60 nt)

tccaagggacaccctaagctttgccctgaagctttgaagaagatgaaagaaggccaccct

This assay has: All criteria met.

**Results from Roche ProbeFinder Vesion 2.43 for *prc1* gene:**

Assay rank 1

Use probe **#138** (cat. no. 04694228001)

| Primer | Length | Position | Tm | %GC | Sequence |
|---|---|---|---|---|---|
| Left | 24 | 1062 - 1085 | 60 | 38 | tgggaagaatgtgtatgatattcg |
| Right | 24 | 1109 - 1132 | 60 | 38 | tgaattcaaggtccttatagcaca |

Amplicon (71 nt)

tgggaagaatgtgtatgatattcgtaaggaatgtgatggtggatccttgtgctataaggaccttgaattca

This assay has: All criteria met.

# 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 |
| Glutamic Acid | 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 for *hGH* and *AOX***

Mass of plasmid was calculated using Equation 3.5. The plasmid used in this study involves 4146 base pairs. Then mass of plasmid becomes;

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

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

The initial concentration of isolated plasmid, which will be used as standard, was measured as 388 ng $\mu L^{-1}$ with AlphaSpect μL Spectrophotometer (AlphaInnotech Inc., USA). Using Equation 3.4 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 $8.5 \times 10^{10}$ copies $\mu L^{-1}$ were used. These samples contained $10^8$ to $10^4$ copies $\mu L^{-1}$.

**Construction of Standard Curves for *pep4, prb1* and *prc1***

Mass of genome was calculated using Equation 3.5. The genome size of *P. pastoris* is 9.5-9.8x$10^6$ base pairs. Then mass of genome becomes;

$$M_{plasmid} = (9.5*10^6\ bp)*\left[1.096*10^{-21}\frac{g}{base\ pair}\right] = 1.042*10^{-5}\ ng$$

Then it was assumed that one genome involves only one copy of desired gene.

The initial concentration of isolated plasmid, which will be used as standard, was measured as 310 ng $\mu L^{-1}$ with AlphaSpect μL Spectrophotometer (AlphaInnotech Inc., USA). Using Equation 3.4 number of copies of the gene of interest was calculated.

$$Copy\ number/\mu L = \frac{310\,ng/\mu L}{1.042*10^{-5}\ ng/1 copy\ of\ desired\ gene} = 3*10^7\ copies/\mu L$$

In order to construct the standard curves, serially diluted samples from the initial genome stock containing 3x$10^7$ copies $\mu L^{-1}$ were used. These samples contained 3x$10^7$ to 3x$10^5$ copies $\mu L^{-1}$.

**Real Time-PCR**

To be able to have a better insight in production of the biomolecule, the relationship between the expression level of the product and the enzymes should be investigated. Recombinant human growth hormone (rhGH) production is related to alcohol oxidase (AOX) production since alcohol oxidase plays important role in methanol utilization pathway and is tightly regulated promoter enhancing production. Hence, the level of intracellular AOX or relevantly its activity should demonstrate expression level of *hGH* as well. Furthermore, it is important to investigate expression levels of certain types of proteases since they take role in degradation of hGH in intracellular and extracellular media which decreases the amount of product. In this content, expression levels of *hGH*, *AOX*, proteinase A (*pep4*), proteinase B (*prb1*) and carboxypeptidase (*prc1*) genes were determined by real time-PCR. In this study, to determine the expression levels of desired genes, absolute

quantification method was performed by using SYBR Green I and hydrolysis probe, TaqMan assays. For SYBR Green I method, specificity of the amplified PCR product was assessed by performing a melting curve analysis. The resulting melting curves allow discrimination between primer-dimers and specific product since specific product melts at a higher temperature than the primer-dimers. However, melting curve analysis is not required for TaqMan method, since formation of primer-dimers can be eliminated by using of two template-specific primers and an oligonucleotide probe.

**Optimization for Real Time-PCR**

Real time-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 fidelity of the reaction. To achieve this, after designing primers pairs for each gene, $MgCl_2$ (for SYBR Green I assay) and primer concentrations, annealing temperatures, annealing times, extension times and the other cycling conditions were carefully optimized. Then, the calibration curves were formed in order to quantitate desired genes in the samples. SYBR Green I assay was used for both formation of calibration curves and analysis of the samples for *hGH* and *AOX* genes. Plasmid DNA was used to form calibration curves for these two genes. For both formation of calibration curves and analysis of the samples for investigation of *pep4*, *prb1* and *prc1* genes, TaqMan assay was used. The calibration curves were obtained by using genomic DNA (gDNA).

**Real Time-PCR conditions for *AOX* and *hGH***

One important step of optimization is to determine the concentrations of reaction mixture components such as primer and $MgCl_2$. The concentration range between 1 mM and 5 mM was investigated for $MgCl_2$ concentration whereas the range between 0.2 µM and 0.5 µM was investigated for left and right primers concentrations. It is found that 0.2 µM primer concentration for both left and right primers is optimum for amplification of both genes and 2 mM and 4 mM $MgCl_2$ concentrations are found as optimum for *AOX* and *hGH*, respectively. The optimum conditions found for the reaction mixture are listed in Table I.1.

**Table I.1** Optimum concentrations for components of the reaction mixture for real time-PCR for 20 µL reaction mixture for LightCycler® FastStart DNA Master SYBR Green I kit.

| Component | hGH | | AOX1 | |
|---|---|---|---|---|
| | **Volume µL** | **Final Conc.** | **Volume µL** | **Final Conc.** |
| $H_2O$ PCR grade | 11.6 | | 13.2 | |
| $MgCl_2$ stock solution (25 mM) | 2.4 | 4 mM | 0.8 | 2 mM |
| PCR Primer (4 µM) | 2 | 0.2 µM | 2 | 0.2 µM |
| DNA Master SYBR Green I | 2 | 1x | 2 | 1x |
| DNA template | 2 | $10^4$-$10^8$ copies $\mu L^{-1}$ | 2 | $10^4$-$10^8$ copies $\mu L^{-1}$ |
| **Total volume** | **20** | | **20** | |

The annealing temperature chosen for amplification should be 3-5ºC lower than the primer melting point temperature (Table 3.14). The proper annealing temperatures for hGH and AOX primer pairs were found as 55 and 60ºC, respectively. Furthermore, the annealing time was determined as 4s and 3s for hGH and AOX primer pairs, respectively. Besides these, the other cycling parameters were optimized and results are given in Table I.2 for both *hGH* and *AOX*.

**Table I.2** Real time-PCR conditions used for both *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: 0.10ºC/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: 0.10ºC/sec), 0s, continuous | |
| | | Primer dimers inhibition: 83ºC, 1s single | | |

**Real Time-PCR conditions for *pep4, prb1 and prc1***

To quantification of *pep4*, *prb1* and *prc1* genes, hydrolysis probe assay was preferred. In the reaction mixture supplied from the kit, $MgCl_2$ concentration is almost optimized for primer combinations so additional $MgCl_2$ was not required for this assay. In this case, primers and probes concentrations were optimized between the range 0.2-0.5 μM for left and right primers and 0.05-0.1 μM for probes. 0.2 μM primer concentration both left and right primers and 0.1 μM probe concentration were found as optimum for three genes. The optimum conditions found for the reaction mixture are listed in Table I.3.

**Table I.3** Optimum concentrations for components of the reaction mixture for real time-PCR for 20 μL reaction mixture for LightCycler® TaqMan Master kit.

| **Component** | **Volume μL** | **Final Concentration** |
|---|---|---|
| $H_2O$ PCR grade | 8.8 | |
| Probe (10 μM) | 0.2 | 0.1 μM |
| PCR primer, 10xconc. (4 μM) | 2 | 0.2 μM |
| Master Mix, 5×conc. | 4 | |
| DNA template | 5 | $10^4$-$10^8$ copies $\mu L^{-1}$ |
| **Total Volume** | 20 | |

The proper annealing temperatures for *pep4, prb1* and *prc1* primer pairs were found as 60, 62 and 60ºC, respectively. Furthermore, for typical primers, an incubation time of 30 s was chosen for the annealing step. Besides these, the other cycling parameters were optimized and results are given in Table I.4 for *pep4, prb1* and *prc1* genes.

**Table I.4** Real time-PCR conditions used for *pep4, prb1* and *prc1* genes.

| Gene name | Denaturation | Amplification | Cooling |
|---|---|---|---|
| *Pep4* | Cycle :1 | Cycles :45 | Cycle :1 |
| | 95ºC, 10 min, none | Denaturation:95ºC,10s, none | 40ºC,30s, none |
| | | Annealing : 62ºC, 30s, none | |
| | | Extension : 72ºC, 1s, single | |
| *Prb1* | Cycle :1 | Cycles :45 | Cycle :1 |
| | 95ºC, 10 min, none | Denaturation:95ºC,10s, none | 40ºC,30s, none |
| | | Annealing : 60ºC, 30s, none | |
| | | Extension : 72ºC, 1s, single | |
| *Prc1* | Cycle :1 | Cycles :45 | Cycle :1 |
| | 95ºC, 10 min, none | Denaturation:95ºC,10s, none | 40ºC,30s, none |
| | | Annealing : 62ºC, 30s, none | |
| | | Extension : 72ºC, 1s, single | |

**Standardization for *hGH* and *AOX***

In this study, plasmid DNA was used for quantification of genomic DNA or complementary DNA, synthesized from total RNA extracted from samples taken during the production of rhGH from *P. pastoris*.

To construct standard curves for *hGH* and *AOX* genes, *pPICZaA::hGH* plasmid (Çalık et al., 2008) containing both *hGH* and *AOX* genes, was used. Reactions were performed on plasmid DNA series containing $10^4$-$10^8$ copies $\mu L^{-1}$ 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 a dilution series of *pPICZaA::hGH* vector DNA. The crossing point values for each concentration were found as follows; (Copies $\mu L^{-1}$: Crossing point); $10^8$ : 15.09, $10^7$ : 19.51, $10^6$ : 24.04, $10^5$ : 28.27, $10^4$ : 31.64 and $10^8$:16.08, $10^7$:20.98, $10^6$:26.40, $10^5$:31.86, $10^4$:40.09 for *hGH* and *AOX* genes, respectively. The calibration curves obtained by using these crossing point data are given in Figures I.3 and I.4 for *hGH* and *AOX*, respectively.

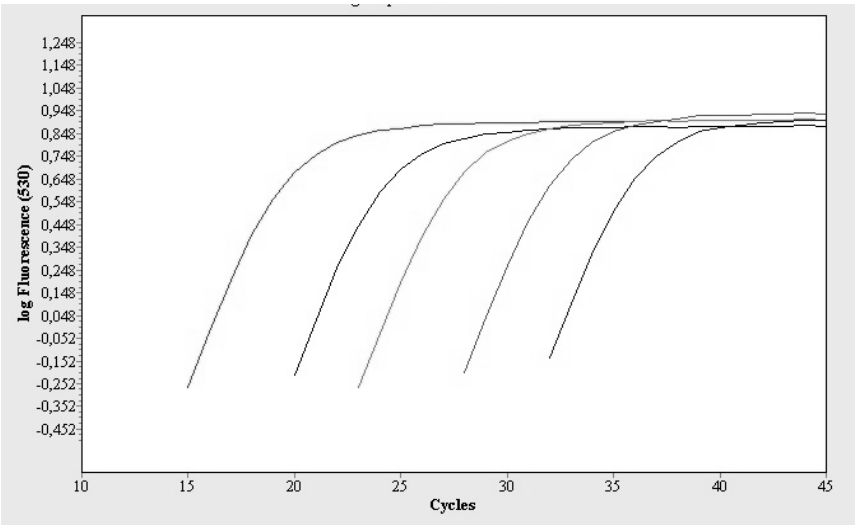



**Figure I.1** Serially diluted samples containing $10^8$ copies/ µL, $10^7$ copies/ µL, $10^6$ copies/ µL, $10^5$ copies/ µL and $10^4$ copies/ µL plasmid DNA as starting template were amplified using the LightCycler DNA Master SYBR Green I for *hGH*. As a negative control, template DNA was replaced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

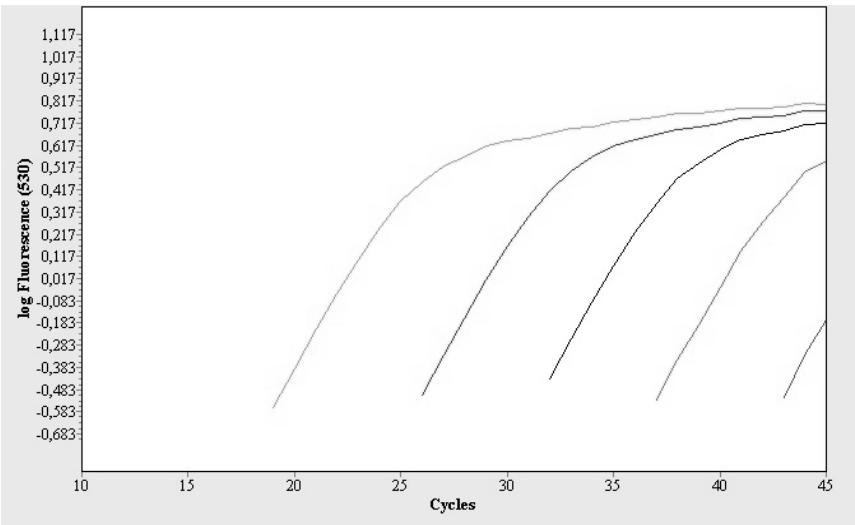



**Figure I.2** Serially diluted samples containing $10^8$ copies/ µL, $10^7$ copies/ µL, $10^6$ copies/ µL, $10^5$ copies/ µL and $10^4$ copies/ µL plasmid DNA as starting template were amplified using the LightCycler DNA Master SYBR Green I for *AOX*. As a negative control, template DNA was replaced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

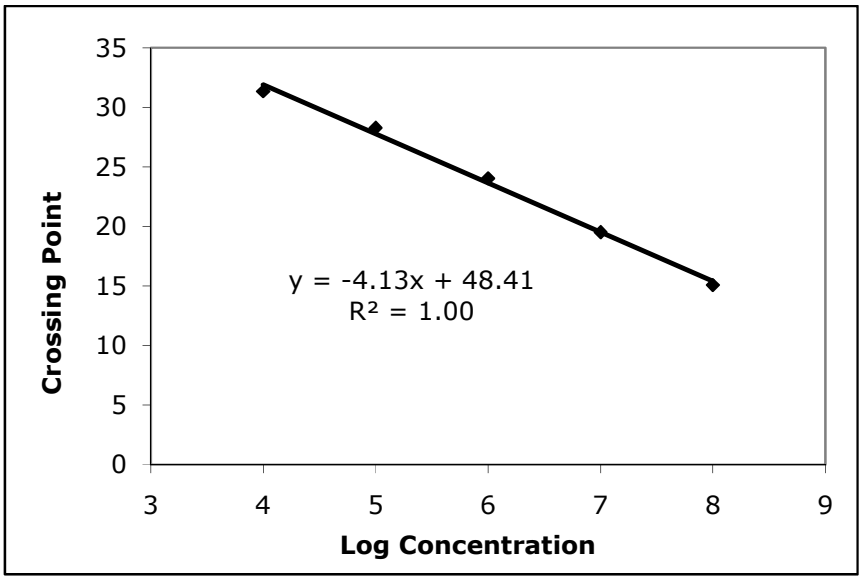



**Figure I.3** Standard curve of a dilutional series of plasmid DNA standards for *hGH* with Efficiency : 1.723 ± 0.0261.

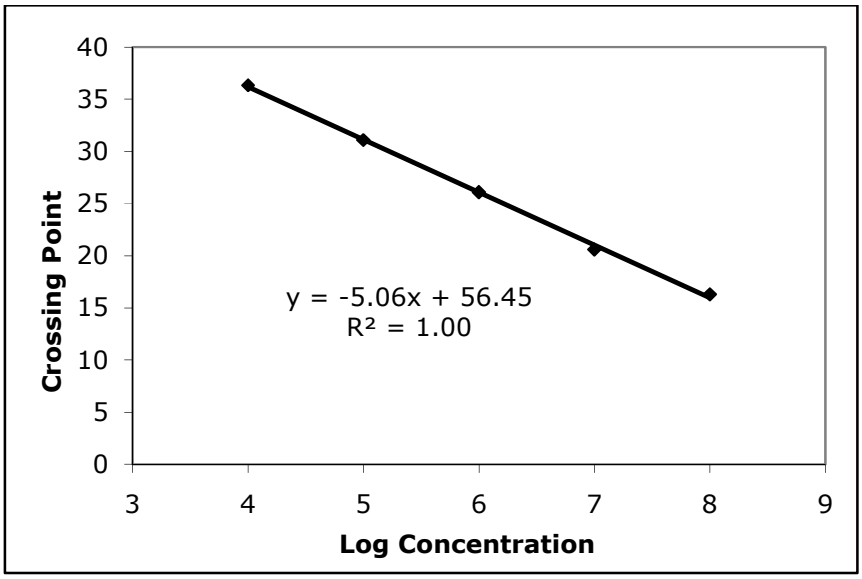



**Figure I.4** Standard curve of a dilutional series of plasmid DNA standards for *AOX* with Efficiency : 1.523 ± 0.0623.

In order to determine reproducibility of the assays, all experiments were repeated with same conditions and efficiency values were obtained as follows: 1.759 ± 0.0479 for *hGH* and 1.607 ± 0.0678 for *AOX*. Thus, the results are consistent with each other. Moreover, real time-PCR efficiency was calculated from the calibration curve slope by the Lightcycler Softare 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). This condition is satisfied for both genes.

The specificity of the amplified PCR product should be determined by using melting curve analysis. The resulting melting curves allow differentiation between primer-dimers and specific product. The specific hGH and AOX products melt at about 88 and 85ºC, respectively. The primer-dimers formation was observed in Figure I.5. However, the specific hGH product melts at a higher temperature than the primer-dimers so the formation primer-dimers will not affect quantification. There are no primer-dimers products in the melting peaks of *AOX* (Figure I.6).

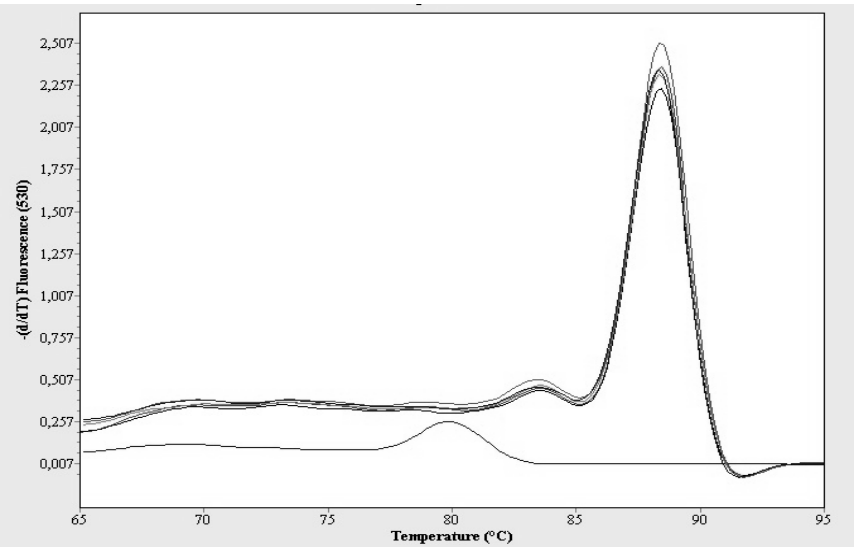



**Figure I.5** Melting curves of amplified samples containing dilutional series of plasmid DNA standards as starting template for *hGH*. As a negative control, template DNA was replaced by PCR grade water.

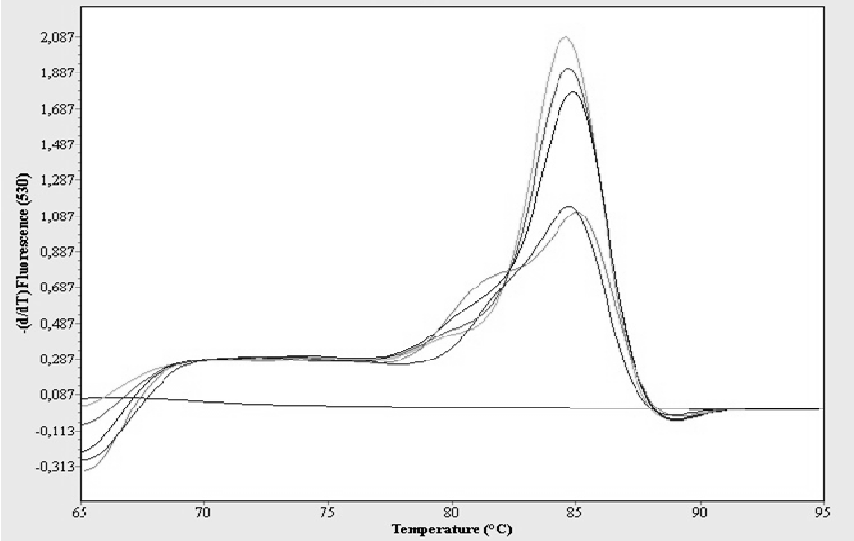



**Figure I.6** Melting curves of amplified samples containing dilutional series of plasmid DNA standards as starting template for *AOX*. As a negative control, template DNA was replaced by PCR grade water.

**Standardization for *pep4, prb1* and *prc1***

In the study, to be able to investigate presence of *pep4, prb1* and *prc1* genes in complementary DNA extracted from samples taken during the production, genomic DNA of $Mut^+$ phenotype of *P. pastoris* was used to create standard curves. To form standard curves for the three genes, i.e., *pep4, prb1* and *prc1*, genomic DNA was obtained by using 4000 ng total RNA in cDNA synthesis and gDNA series contain $3x10^5$-$3x10^7$ copies $\mu L^{-1}$ genomic DNA. The following amplification curves (Figures I.7, I.8 and I.9) were obtained using a dilution series of genomic DNA of $Mut^+$ strain. The crossing point values for each concentration were found as follows; (Copies $\mu L^{-1}$: Crossing point); $3x10^7$ : 23.07, $6x10^6$: 25.24, $1.5x10^6$: 26.70, $6x10^5$: 27.82 and, $3x10^7$: 22.55, $6x10^6$: 25.93, $3x10^6$: 27.24, $6x10^5$: 28.54, $3x10^5$: 29.49 and $3x10^7$: 23.13, $6x10^6$: 26.29, $3x10^7$: 27.99, $1.5x10^6$: 28.48, $6x10^5$: 29.99 for *pep4, prb1* and *prc1* genes, respectively. The calibration curves obtained by using these crossing point data are given in Figures I.10, I.11 and I.12 for *pep4, prb1 and prc1*, respectively.

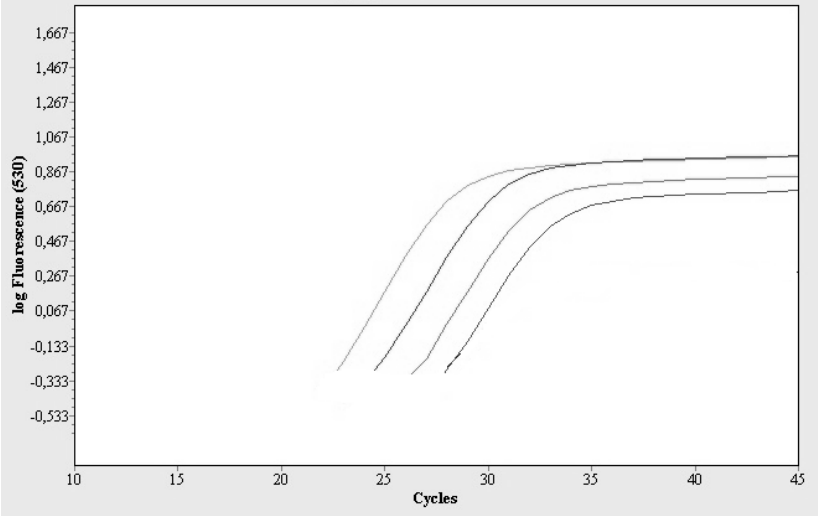



**Figure I.7** Serially diluted samples containing $3x10^7$ copies $\mu L^{-1}$, $6x10^6$ copies $\mu L^{-1}$, $1.5x10^6$ copies $\mu L^{-1}$, and $6x10^5$ copies $\mu L^{-1}$ genomic DNA as starting template were amplified using the LightCycler TaqMan Master for *pep4*. As a negative control, template DNA was replaced by PCR grade water.

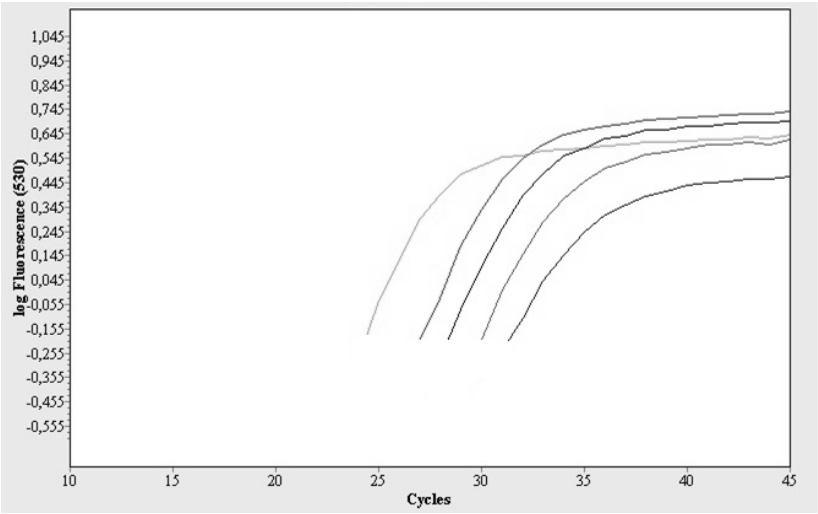



**Figure I.8** Serially diluted samples containing $3x10^7$ copies $\mu L^{-1}$, $6x10^6$ copies $\mu L^{-1}$, $3x10^6$ copies $\mu L^{-1}$, $6x10^5$ copies $\mu L^{-1}$, and $3x10^5$ copies $\mu L^{-1}$ genomic DNA as starting template were amplified using the LightCycler TaqMan Master for *prb1*. As a negative control, template DNA was replaced by PCR grade water.

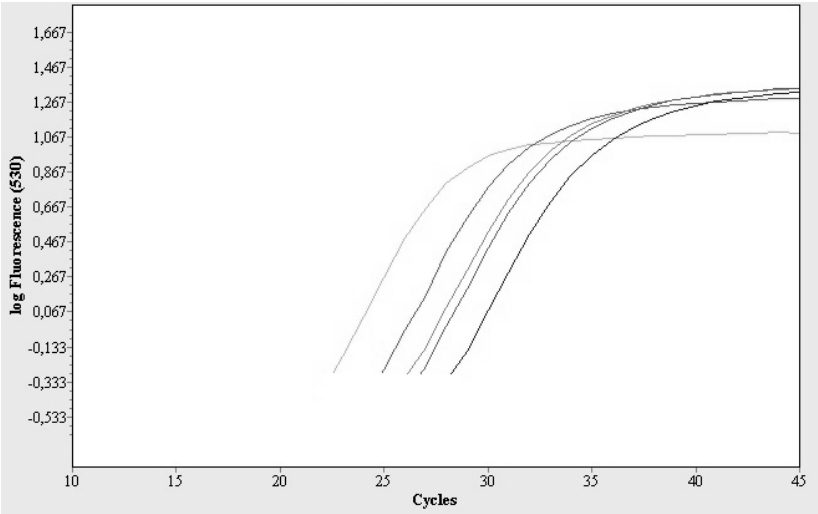



**Figure I.9** Serially diluted samples containing $3x10^7$ copies $\mu L^{-1}$, $6x10^6$ copies $\mu L^{-1}$, $3x10^6$ copies $\mu L^{-1}$, $1.5x10^6$ copies $\mu L^{-1}$, and $6x10^5$ copies $\mu L^{-1}$ genomic DNA as starting template were amplified using the LightCycler TaqMan Master for *prc1*. As a negative control, template DNA was replaced by PCR grade water.

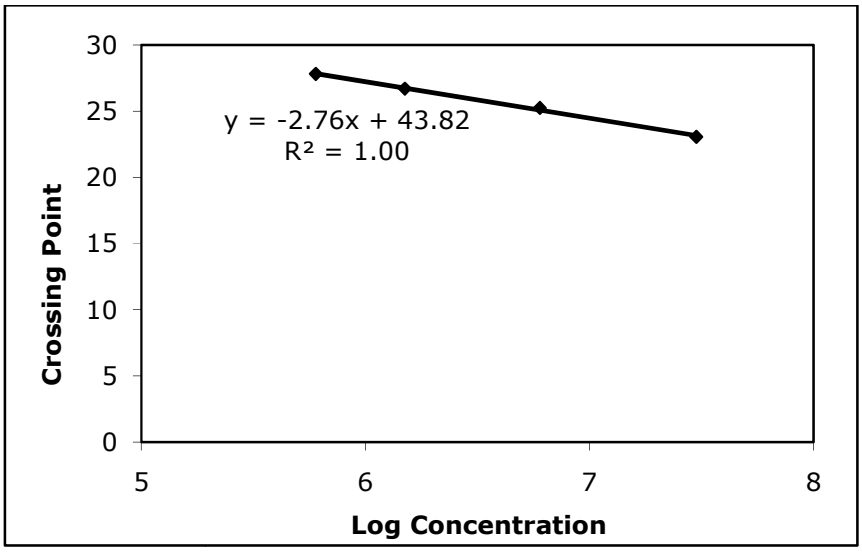



**Figure I.10** Standard curve of a dilutional series of genomic DNA standards for *pep4* with Efficiency: 2.271 ± 0.0164.

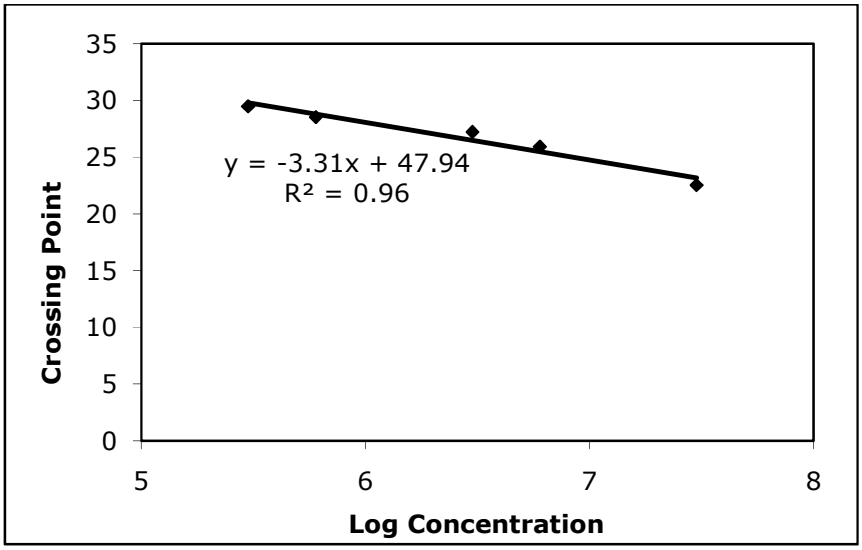



**Figure I.11** Standard curve of a dilutional series of genomic DNA standards for *prb1* with Efficiency: 1.609 ± 0.0294.

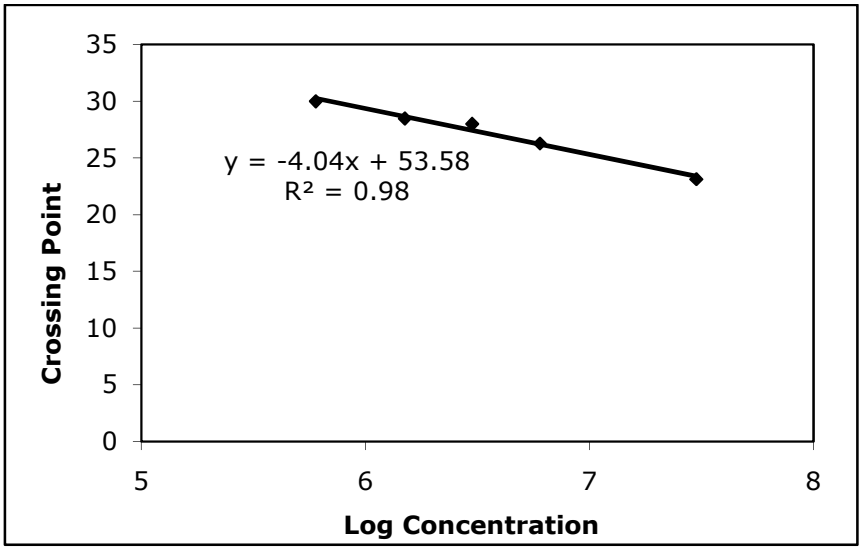



**Figure I.12** Standard curve of a dilutional series of genomic DNA standards for prc1 with Efficiency: 1.666 ± 0.025.

There are no primer-dimers formations in the amplification curves of pep4, *prb1* and *prc1* (Figures 4.43, 4.44 and 4.45).

**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 500 ng of those total RNA for *pep4, prb1* and *prc1* genes and the final RNA concentration in cDNA reaction mixture is 25 ng $\mu L^{-1}$. 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 $\mu L^{-1}$. Then these were converted to copies/mg CDW or copies $ng^{-1}$ total RNA. A sample calculation can be seen below.

For medium with sorbitol at pH 5 and t=18 h concentration of *pep4* given by LightCycler Software 4.0 was $2.66x10^6$ copies $\mu L^{-1}$. 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=3.76 Furthermore, the amount of cell used for RNA isolation was $C_X$= 0.00344 mg $\mu L^{-1}$.

*pep4* expression
in copies $mg^{-1}$ CDW =(2.66x106 copies $\mu L^{-1}$)/3.76/(0.00344 mg $\mu L^{-1}$)
$=2.9x10^9$ copies $mg^{-1}$ CDW

*pep4* expression
in copies $ng^{-1}$ total RNA = ($2.66x10^6$ copies $\mu L^{-1}$) / 25 ng total RNA $\mu L^{-1}$
= $1.06x10^5$ copies $ng^{-1}$ total RNA