COMPARISON OF HUMAN GROWTH HORMONE PRODUCTION PERFORMANCE OF TWO DIFFERENT METABOLICALLY ENGINEERED *Pichia pastoris*

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

BY

HALİME GÜL ZERZE

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

DECEMBER 2012

Approval of the thesis:

COMPARISON OF HUMAN GROWTH HORMONE PRODUCTION PERFORMANCE OF TWO DIFFERENT METABOLICALLY ENGINEERED *Pichia pastoris*

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ABSTRACT

COMPARISON OF HUMAN GROWTH HORMONE PRODUCTION PERFORMANCE OF TWO DIFFERENT METABOLICALLY ENGINEERED *Pichia pastoris*

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December 2012, 177 Pages

Recombinant human growth hormone (rhGH) production levels and bioprocess characteristics were investigated for two recombinant *Pichia pastoris* strains. In the first part of the study, feeding strategies for semi-batch operations were developed in pilot scale bioreactors to improve rhGH production under strong methanol inducible *alcohol oxidase* I (*AOX*1) promoter, by previously constructed *P. pastoris* M13 strain (pPICZαA*::hGH*-Mut+). Three different methanol feeding strategies together with mannitol co-feeding named as MM1, MM2 and MM3 were performed. In MM1, methanol was fed to the bioreactor with a pre-determined specific feeding rate of μ_{M0}=0.03 h^{-1}; whereas, three pulses of mannitol was introduced at t=0, 8, and 15 h and the mannitol concentration in the bioreactor increased to 50 g L^{-1}. In MM2, the semi-batch bioprocess was started with 50 g L^{-1} mannitol concentration at t=0 h and kept constant at this concentration until t=6 h; in the following period until t=19.5 h, methanol was fed with a pre-determined specific feeding rate for μ_{M0}=0.03 h^{-1}; and then in the third period, by t=20 h dynamic methanol feeding was employed

for constant μ=0.03 h^{-1}. In MM3, the same mannitol feeding strategy was performed as in MM2, together with the pre-determined methanol feeding for μ_{M0}=0.03 h^{-1}, until t=12 h; thereafter, for constant μ=0.03 h^{-1} dynamic methanol was feeding employed. Throughout the experiments, the cell, mannitol and methanol concentrations, rhGH, and organic acid concentrations; AOX and protease activities were experimentally determined. The highest rhGH concentration was obtained in the MM2 strategy as C_{rhGH}=1.2 g L^{-1}; whereas the highest cell concentration was reached in MM3 as C_X=157 g L^{-1}. Further, the highest overall product yields on substrate and cell, $Y_{P/St}$ and $Y_{P/X}$, were obtained in MM2 respectively as 4.02 mg g^{-1} and 10.67 mg g^{-1}.

In the second part of study, recombinant *P. pastoris* G7 strain, which provides constitutive rhGH expression under *glyceraldehyde-3-phosphate dehydrogenase* (GAP) promoter, was designed and constructed. After selection (how?) of the microorganism having the highest production potential (G7), two different glucose feeding strategy, namely G1 and G2, were employed to investigate and improve the rhGH production. In G1, glucose was fed with the pre-determined μ_{G0}=0.2 h^{-1} until t=6 h; where, with a step-down operation the pre-determined μ_{G0} decreased to μ_{G0}=0.03 h^{-1} throughout the bioprocess. Based on the findings of the G1 strategy, in G2, glucose was fed with the pre-determined specific growth rate of μ_{G0}=0.2 h^{-1} until t=3 h; and than proceeded with constant glucose feeding. The highest C_X and C_{rhGH} were obtained as 90 g L^{-1} and 0.2 g L^{-1} in G2; moreover, the overall yield coefficients $Y_{X/S}$, $Y_{P/S}$, and $Y_{P/X}$ were obtained, respectively, as 0.48 g g^{-1}, 1.21 mg g^{-1}, and 2.53 mg g^{-1}.

ÖZ

İKİ FARKLI METABOLİK MÜHENDİSLİK YAKLAŞIMIYLA GELİŞTİRİLEN Pichia pastoris HÜCRELERİNİN İNSAN BÜYÜME HORMONU ÜRETİM PERFORMANSLARININ KARŞILAŞTIRILMASI

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Aralık 2012, 177 sayfa

Bu çalışmada, iki farklı recombinant *Pichia pastoris* suşunun, rekombinant insan büyüme hormonu (rhGH) üretim performansları karşılaştırılmış ve biyoproses karakteristikleri araştırılmıştır. Bu kapsamda, birinci-araştırma programında, önceden geliştirilmiş, metanolle indüklenen *alkol oksidaz* I (*AOX*1) geni altında rhGH üretimi yapan *P. pastoris* M13 (pPICZαA*::hGH*-Mut+) suşu ile üç farklı mannitol ve metanol besleme stratejisi (MM1, MM2 ve MM3) uygulanmıştır. MM1 stratejisinde, yarı-kesikli metanol μ_{M0}=0.03 st^{-1} spesifik çoğalma hızında beslenirken, ikinci substrat mannitol t=0, 8, ve 15 st'lerde pulse-besleme ile (kesikli) eklenerek derişimi 50 g L^{-1} çıkarılmıştır. MM2 stratejisinde, t=0st'da 50 g L^{-1} başlangıç mannitol derişimi ile başlanmış ve t=6st'a kadar sürekli mannitol beslemesi yapılarak yarı-kesikli biyoreaktörde mannitol derişimi 50 g L^{-1}, de tutulmuş; sonraki periyotta t=19.5 st'e kadar önceden-belirlenmiş μ_{M0}=0.03 st^{-1} değeri için metanol beslemesi yapılmış; ve son periyotta t=19.5 st'ta kesikli metanol eklemesi yapıldıktan sonra

μ=0.03 st^{-1} değerinde sabit tutmak için, dinamik metanol beslemesi uygulanmıştır. MM3 stratejisinde, MM2'yle aynı mannitol beslemesi yapılmış; ancak, buna ek olarak metanol t=12 st'e kadar μ_{M0}=0.03 h^{-1} ile beslenmiş t>12 st'te μ=0.03 h^{-1} sabit olacak şekilde dinamik metanol beslemesi yapılmıştır. Bu deneylerin hücre, metanol, mannitol, organik asit ve rhGH derişimleri ile , AOX ve proteaz aktiviteleri deneysel olarak ölçülmüştür. En yüksek hücre derişimi MM3'te C_X=157 g L^{-1} olarak elde edilirken, en yüksek rhGH derişimi MM2 stratejisinde C_{rhGH}=1.2 g L^{-1} olarak elde edilmiştir. Veriler kullanılarak, substrat ve hücreye göre, en yüksek ürün verimleri MM2 stratejisinde, $Y_{P/St}$=4.02 mg g^{-1} ve $Y_{P/X}$=10.67 mg g^{-1} olarak bulunmuştur.

İkinci-araştırma programında, *gliseraldehit-3-fosfat dehidrojenaz* (*GAP*) geni altında yapısal rhGH ekspresyonu yapan rekombinant sistem tasarlanmış ve rekombinant *P. pastoris* G7 suşu oluşturulmuştur. En yüksek rhGH üretim potansiyeline sahip mikroorganizma seçildikten sonra, bu suşla rhGH üretimini araştırmak ve geliştirmek için G1 ve G2 olarak adlandırılan, glukoz ile iki farklı yarı-kesikli-biyoreaktör besleme stratejisi uygulanmıştır. G1 stratejisinde, t=6 st'e kadar μ_{G0}=0.2 st^{-1} değeri için kalma süresine karşı önceden-belirlenmiş glukoz çözeltisi değişken debisinde glukoz beslemesi yapılmış; sonraki periyotta t=6 st'ta basamak düşüş ile μ_{G0} değeri μ_{G0}=0.03 st^{-1} düşürülerek, t>6 st için önceden-hesaplanmış Q(t) debilerinde glukoz beslenmesine devam edilerek yarı-kesikli işletim sürdürülmüştür. G1 bulguları değerlendirilerek tasarlanan G2 stratejisinde, t=3st'e kadar μ_{G0}=0.2 st^{-1} değeri için önceden-hesaplanmış Q(t) debilerinde glukoz beslemesi sürekli yapılmış; ikinci periyotta, t>3 st için sabit akış hızında glukoz beslemesi yapılarak yarı-kesikli işletim uygulanmıştır. En yüksek C_X ve C_{rhGH}, 90 g L^{-1} ve 0.2 g L^{-1} olarak, ve en yüksek hücre ve ürün verimleri $Y_{X/S}$, $Y_{P/S}$, ve $Y_{P/X}$ değerleri, sırasıyla, 0.48 g g^{-1}, 1.21 mg g^{-1} ve 2.53 mg g^{-1} olarak G2 yarı-kesikli işletim stratejisinde elde edilmiştir.

To my spouse, Hasan

ACKNOWLEDGMENTS

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

My warmest thanks are to my family, Tülay-Mücahit, Mahmut Özer for loving, supporting and encouraging me all through my life. I would like to especially thank to Dr. Hasan Zerze for loving, encouraging and giving me extreme support in any way possible and also to Aslı-Efe Boran for their incredible friendship. Moreover, I am thankful to my invaluable friends Emre-Gizem Albayrak Durna for the days spent with them.

It is a great chance for me to have Bahar Bozkurt, Burcu Akdağ, Özge Ata Akyol, Burcu Gökbudak, Melda Eskitoros and Erdem Boy in my research group; without their great friendship and support even in sleepless nights, I would not have carried out the requirements of this thesis with success. Also I am thankful for their friendship out of laboratory.

I am grateful to friends among Chemical Engineering Department research assistants, Burcu Gökbudak, Necip Berker Üner, Okan Özkök, Gökhan Çelik, Atalay Çalışan, Mustafa Yasin Aslan, İbrahim Bayar and Güvenç Oğulgönen for their frienship. I would like to thank to all academic, administrative and technical staff of Department of Chemical Engineering, METU, for their help and support throughout my education.

Gül Zerze

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C	concentration in the medium	g dm^{-3} or mol m^{-3}
DO	dissolved oxygen	%
N	agitation rate	min^{-1}
Q	volumetric flow rate of the feed	dm^3h^{-1}
q	specific formation or consumption rate	g $g^{-1}h^{-1}$
r	formation rate or consumption rate	g dm^{-3} h^{-1}
t	cultivation (residence) time	h
T	bioreactor liquid medium temperature,	°C
U	one unit of an enzymatic activity	
V	bioreactor volume	dm^3
Y	yield (overall)	g g^{-1}

Greek Letters

ρ	density	g dm^{-3}
μ_0	pre-determined specific growth rate	h^{-1}
μ_t	total specific growth rate	h^{-1}

Subscripts

0	initial condition or pre-determined
AOX	alcohol oxidase
G	Glucose
M	Methanol
O	Oxygen
pro	Protease
S	Sorbitol or substrate
P	Product
X	Cell
fwd	Forward
rev	Reverse
T	Total

Abbreviations

*AOX*I	*Alcohol oxidase* I
CDW	Cell dry weight
GAP	*Glyceraldehyde-3-phosphate dehydrogenase*
GB	Glycerol batch phase
GFB	Glycerol fed-batch phase
hGH	Human growth hormone
HPLC	High performance liquid chromatography
MT	Methanol transition phase
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
rhGH	Recombinant human growth hormone

CHAPTER 1

INTRODUCTION

Being one of the fastest growing areas of science, biotechnology combines the use of the biological sciences, i.e., gene manipulation through metabolic engineering for recombinant biomolecule production, with the scientific fields, i.e., biochemical engineering, materials sciences, nanotechnological sciences, and computer software development, in order to discover, evaluate and develop products for bioindustry. As the leading constitutional research and application area of biotechnology, industrial biotechnology is the application of biological organisms and biological systems in the synthesis of bio-molecules for chemical and pharmaceutical industries, as well as for use in agriculture and veterinary, moreover in environmental operations. By the use of industrial microorganisms which are modified by mutations or metabolic engineering, industrial biotechnological processes uses cheap substrates and consume little energy for the biosynthesis of biomolecules. These superior features of biochemical processes, outweigh the traditional chemical processes.

Recombinant DNA technology which makes possible to manipulate DNA in order to identify, move and place genes into variety of microorganisms (Nielsen *et al*, 2003; Macauley-Patrick *et al*. 2005) brings recombinant production of therapeutically important proteins which has considerable commercial value. In pharmaceutical industry, human growth hormone (hGH) is one of the well known recombinant therapeutic proteins among human erythropoietin, insulin, interferon α, human granulocyte-macrophage colony-stimulating factor (hGM-CSF). Up to date more than 200 peptides and recombinant therapeutical proteins are approved by the Food and Drug

Administration (Demain and Vaishnav, 2009); therefore, biotechnological industry takes an important place in the market.

Among human therapeutics, human growth hormone has an important market share. Recombinant human growth hormone (rhGH) threapy is used to treat growth hormone deficiency both in adults and children (Dumas *et al*., 2006), pediatric growth disorders like idiopathic short stature (Hindmarch *et al*.,2006) also chronical renal insufficiency, low gestational weight and girls with Turner syndrome (Dumas *et al*., 2006, Haverkamp *et al*., 2008). It is estimated that treating one 30-kg children has the cost of approximately $15,000-$30,000 (2005 US $) with r-hGH. (Haverkamp *et al*., 2008).

Before the recombinant DNA technology, hGH used in treatments of above mentioned diseases was obtained from the pituitary glands of cadavers. In 1979, Goddel and co-workers reported the rhGH production by the organism *Escherichia coli* intracellularly. As a prokaryotic organism, recombinant production of human proteins was not efficient in *E. coli*. In following years, several alternative host microorganisms was used in rhGH production which are *Bacillus subtilis, Saccharomyces cerevisiae, Pseudomonas* and *Pichia pastoris* (Gray *et al*., 1985; Tokunaga *et al*., 1985; Treviño *et al*. 2000; Çalık *et al*., 2008; Özdamar *et al*., 2009).

Among the recombinant protein production hosts, *Pichia pastoris* has become more and more popular. Especially being a eukaryote, it is a potential and promising host microorganism for recombinant protein production, primarily for therapeutic protein production. It reaches considerable high cell densities which is higher than that of *S. cerevisiae*, but unlike *S. cerevisiae*, it makes correct glycosylation of human proteins. *S. cerevisiae* expresses hyperglycosylated recombinant proteins. The first rhGH production by *P. pastoris* was performed by Ecamilla-Trevino *et al*. (2000). By using a 2 dm^3 bioreactor, they obtained 49 mg dm^{-3} rhGH. Eurwilaichitr *et al.* (2002) investigated the optimal condition for high level expression of rhGH. They

obtained 190 mg dm^{-3} rhGH by induction with C_M=3% (v/v) throughout three days operation in a complex medium.

In our research group, Çalık *et al.* (2008) developed a novel expression system for extracellular rhGH under control of AOX promoter with p*PICZα*A vector. Then, in 2009, Orman *et al.* investigated the effects of carbon sources on rhGH production by two different *P. pastoris* strains; Mut^+ and Mut^S and they achieved 110 mg dm^{-3} rhGH by the Mut+ strain. Açık (2009) applied a feeding strategy for the rhGH production, abbreviated as MSS-0.03 by the same strain and they attained C_{rhGH}=301 mg dm^{-3} at t=30 h of production. Thereafter, Inankur (2010) developed further feeding strategy, namely as MSSS-0.03 and obtained 290 mg dm^{-3} rhGH concentration at t=39 h of the fermentation. In following feeding strategy development studies for rhGH production, Bozkurt (2012) obtained C_{rhGH}=640 mg dm^{-3}.

In this M.Sc. thesis, the aim is to compare r-protein production potentials of metabolically engineered two *P. pastoris* strains consisting of different expression promoters. For this purpose, in the first part of the study, feeding strategies were developed using previosly constructed *P. pastoris hGH*-Mut+ strain having methanol inducible *alcohol oxidase* (AOX) promoter (Çalık et al. 2008); where different methanol feeding strategies were employed with different co-feedings of mannitol. In the second part of the study, a new *hGH* gene expression system was developed in which *hGH* gene was expressed under the constitutive *glyceraldehyde-3-phosphate dehydrogenase* (GAP) promoter of *P. pastoris*. In order to compare the two metabolically engineered *P. pastoris* strains, the effects of glucose feeding strategies on rhGH production were investigated by the designed *P. pastoris* pGAPZαA*::hGH* having GAP promoter.

CHAPTER 2

LITERATURE SURVEY

In recombinant protein production, the biochemical properties and characteristics of the target protein and the gene encoding the protein, should be investigated in detail. Based on the selection of a suitable host microorganism, a smart expression system should be designed with an in-depth anaysis of the interactions of the designed recombinant system with the bioreactor operation conditions. After the construction of the recombinant system, bioreactor operation conditions, i.e., medium design, temperature, pH operation conditions, and oxygen transfer conditions, should be studied in order to determine the fermentation characteristics, i.e., the growth, induction mechanism, oxygen transfer characteristics, and yield coefficients, to conclude new strategies for the production.

In this context this chapter covers literature survey, on the target protein human growth hormone (hGH), the selected yeast *Pichia pastoris*, moreover promoters and induction mechanism of the yeast, the bioreactor operation conditions, and theoretical approach for the calculation of the fermentation characteristics.

2.1 Target Protein: Human Growth Hormone

Hormones are the group of proteins that constitutes signalling mechanisms between groups of cells in body. They are released from endocrine glands and affect many processes in body like growth, metabolism and reproduction functions. (Baulieu *et al*., 1990, Binkley, 1990) Hormone levels in blood are very sensitive and hormone disorders can be fatal to body. Being one of them growth hormone (GH) stimulates cell reproduction and generation and

growth in animal and human bodies and is secreted from somatotroph cells of anterior pituitary gland. Naturally secreted growth hormone in animals is called somatotropin (STH) whereas growth hormone produced by recombinant DNA technology is called somatropin.

2.1.1 Recombinant Human Growth Hormone

Growth hormone therapy also can be used in treatment of hypopituitary dwarfism, Turner‘s syndrome, human immune deficiency syndrome (HIV) growth hormone deficiency in adulthood as well as treatment of chronic renal insufficiency in children (Cazares-Delgadillo *et al*., 2010). Moreover, hGH is used in the treatment of burns, bleeding ulcers and bone fractures (Tritos and Mantzoros, 1998; Ecamilla-Trevino *et al*., 2000; Krysiak *et al*., 2007).

Growth hormone derived from pituitary glands was first described in 1921 (Evans and Long, 1921). Pituitary human growth hormone was isolated (Krysiak *et al*., 2007) from human pituitaries in 1956 but its structure was established in 1972 and extraction of GH from pituitaries of cadavers is the only way of obtaining GH for decades. Therefore, clinical application of the hormone was possible only for children suffering from growth retardation and short stature due to growth hormone deficiency (GHD). (Krysiak *et al*., 2007, Kopchick *et al*., 2003). By the time, extended hGH demand brings about the production of recombinant hGH. By genetic engineering techniques cDNA encoding hGH was used and cloned to a host microorganism for recombinant hGH production (Martial *et al*., 1979) and production of recombinant hGH was first achieved in a *E. coli* as host microorganism (Goeddel *et al*., 1979) In 1985 Gray *et al*. produced recombinant hGH in native form and in the same year clinical use of recombinant hGH was approved (Kopchick *et al*., 2003).

The first r-hGH production by *P. pastoris* was performed by Ecamilla-Trevino *et al*. (2000). By using a 2-L bioreactor, they obtained 49 mg L^{-1}. Eurwilaichitr *et al.* (2002) investigated the optimal condition for high level

expression of rhGH. They obtained 190 mg L^{-1} rhGH by induction with CMeOH=3% (v/v) throughout three days operation in a complex medium.

In our research group, Çalık *et al*. (2008) designed an expression system in *P. pastoris* (*P. pastoris hGH*-Mut+) for the production and purification of rhGH having methanol inducible AOX promoter. Orman *et al.* (2009) investigated the effects of carbon sources on rhGH production by two different *P. pastoris* strains; Mut^{+} and Mut^{S}. For this purpose, defined medium including 30 g L^{-1} glycerol with 1% (v/v) methanol was used. They achieved 110 mg L^{-1} rhGH by the Mut+ strain. Açık (2009) applied a feeding strategy for the rhGH production abbreviated as MSS-0.03 by the *P. pastoris* Mut^{+} strain. In this strategy fed-batch methanol feeding was performed with the specific growth rate of μ=0.03 h^{-1} with simultaneous sorbitol pulse feeding at t=0 h and at t=9 h of production to provide 50 g L^{-1} sorbitol in the bioreactor. The highest rhGH concentration was attained at t=30 h as C_{rhGH}=301 mg L^{-1} with this strategy. Thereafter, Inankur (2010) developed a feeding strategy with three-pulse sorbitol co-feeding at t=0 h, t=14 h and t=31 h with simultaneous methanol with μ=0.03 h^{-1}. By the each sorbitol pulse 50 g L^{-1} sorbitol concentration in the fermentation medium was provided. By this strategy, 290 mg L^{-1} rhGH concentration was reached at t=39 h of the fermentation. In studies of Çalık *et al*. (2010b) optimum biorector operation pH value value is reported as 5.0 for rhGH production by considering AOX enzyme activity and protease formation. In following feeding strategy development studies for rhGH production, Bozkurt (2012) obtained C_{rhGH}=640 mg L^{-1}. In that study, 50 g L^{-1} sorbitol concentration provided at the beginning of experiment and 50 g L^{-1} sorbitol concentration kept constant by fed-batch feeding of sorbitol until t=15 h of production keeping methanol feeding with μ=0.03 h^{-1} throughout the experiment.

2.1.2 Properties and Structure of Human Growth Hormone

hGH is a non-glycosylated 191 aminoacid single polypeptide chain produced by the anterior pituitary glands and has an apprimate mass of 22 kDa

(Kasimova *et al*., 2002). In human, genes encoding hGH are located in q22-24 region of choromosome 17. Nucleotide sequence of hGH is given in Figure 2.1

```
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 hGH

Isoelectric point ranges in between 4.9 to 5.1 and net charge of hGH at pH 7.0 is -4.9 (Binkley, 1994). Native form of hGH was crystallized and its tertiary structure is given in Figure 2.2.

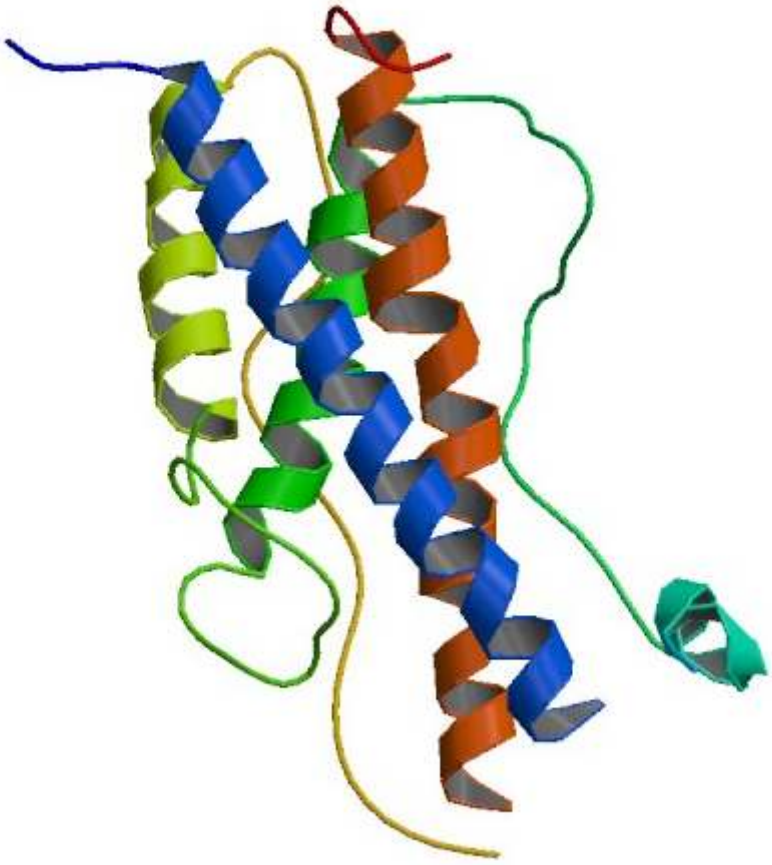

Figure 2.2 Tertiary structure of hGH (pdb file: 1hgu; www.rcsb.org)

For protein with accession number 1HGU, primary structure, amino acid sequence as well as secondary structure, disulfide bridges and alpha helices are indicated in Figure 2.3.

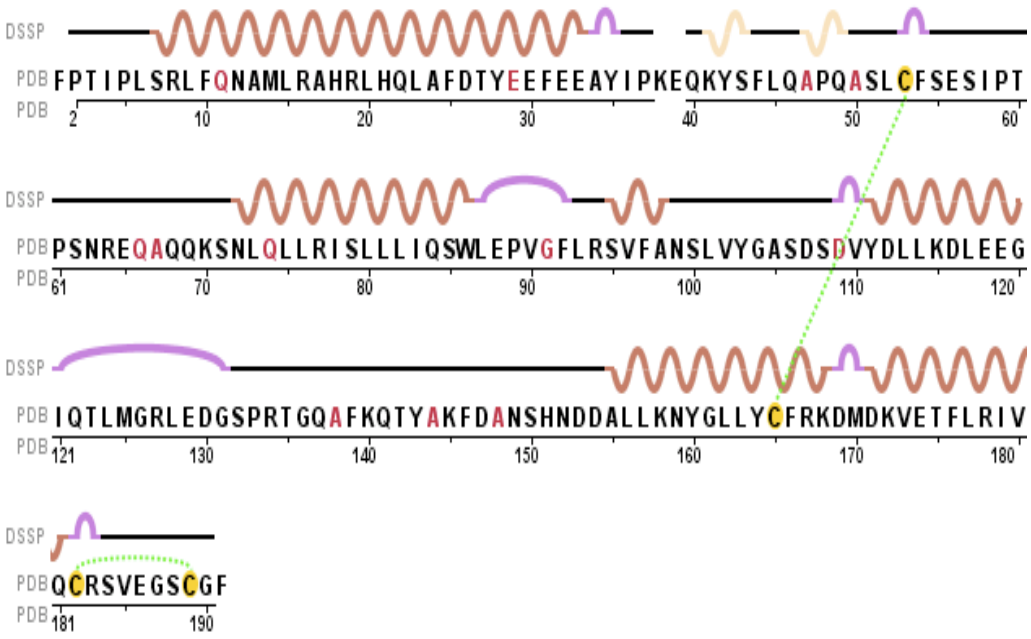

Figure 2.3 Primary and secondary structure of hGH; purple and brown curves indicate the major α-helices; pink curves are shorter connective α-helices; dashed green lines show the disulfide bridges between cystein residues. (http://www.pdb.org/pdb)

2.2 Microorganisms for rhGH Production

Microorganisms are microbioreactors of bioprocesses. Therefore, selection of microorganisms in recombinant protein production is crucial. Host microorganisms should achieve an appreciable cell density; give sufficient yields, not be toxic on products and be cheap on medium components. Being most identified organisms *Escherichia coli* in prokaryotic organisms and *Saccharomyces cerevisiae* in eukaryotic organisms are most preferred organisms in recombinant protein production. For production hGH most widely employed organism was *E.coli* (Goeddal *et al*., 1979; Gray *et al*, 1985; Becker and Hsiung., 1986; Kato *et al*., 1987; Shin *et al*, 1998; Patra *et al*., 2000; Tabandeh *et al*., 2004; Singh *et al*., 2009). Since *E. coli* is a prokaryotic organism in *E. coli* most of the productions were intracellular and/or in inclusion body form, therefore; separation and purification of proteins from fermentation broth is difficult.

Another expression host for rhGH is *Bacillus subtilis* (Nakayama *et al*., 1988; Franchi *et al*., 1991; Özdamar *et al*., 2009). One important disadvantage of this host is high protease formation rate and high proteolytic activity. This problem can be overcame by using protease inhibitor (Özdamar *et al*., 2009).

Being eukaryotic cells; yeasts are able to perform higher eukaryotic post-translational modifications and able to synthesize disulphide bonds. They show high density in cell concentration in fermentation broth and provide extracellular production of proteins therefore give higher fermentation efficiencies in protein production. The methylotropic yeast *Pichia pastoris (P. pastoris)* is an extensively used expression host for various heterologous protein production (Macauley-Patrick *et al*., 2005; Cos *et al*., 2006a). First rhGH production was achieved in 2L bioreactor by *P. pastoris* as 49 mg L^{-1} (Ecamilla-Trevino *et al*., 2000). In the latest study, *P. pastoris pPICZαA::hGH* strain, namely, *P. pastoris* M13 strain was constructed (Calik *et al*., 2008). Among two different strains Mut^+ and Mut^-; of P. pastoris, by using Mut^+ strain 110 mg L^{-1} rhGH production was achieved in batch cultivations (Orman *et al.*, 2009). Thereafter, by feeding strategy development 301 mg L^{-1} (Acik, 2009); 250 mg L^{-1} (Inankur, 2010) and 640 mg L^{-1} (Bozkurt, 2012) rhGH production was achieved with same *P. pastoris* strain.

2.2.1 Expression host: *Pichia pastoris*

Being a methylotropic yeast *P.pastoris* is facultative anaerobic, chemo-heterotroph and a mesophilic yeast which is able to live at mild temperatures between 25-35 $^{\circ}$C (Macauley-Patrick *et al*., 2005). By living at a broad pH range in between pH 3.0-7.0 *P.pastoris* provides an important advantage on protein production. (Cereghino and Cregg, 1999).

One of the notable advantages of *P.pastoris* over other expression hosts is that it reaches considerably high cell densities on minimal and simple chemically defined media with inexpensive formulation (Cos *et al*., 2006a). Being a non-pathogen expression host, it produces heterologous proteins

extracellularly at high levels which facilitates the subsequent purification of proteins. (Macauley-Patrick *et al*., 2005, Ragon *et al*., 2007). Other advantages of *P. pastoris*, it is easy to manipulate genetically and able to perform many post-translational modifications and provides scalable protein production (Cereghino *et al*., 2002, Khasa *et al*., 2010).

Most important feature of *P. pastoris* besides afore mentioned advantages, is the strongly regulated alcohol oxidase 1 (AOX1) promoter (Cereghino and Cregg, 2000). Inducer of this promoter is methanol which is also carbon source of the microorganism. First the Phillips Petroleum Company develop the media and procedure for growing *P. pastoris* during 1970s (Cereghino and Cregg, 2000).

Besides all these advantages of *P. pastoris* there are some disadvantages of it. Since *Pichia* fermentations shows slower cell growth rate compared to bacteria, cultivation time of this is longer. Moreover, like *Bacillus* species *Pichia pastor*is shows high protease activity and and hence it shows significant proteolytic activity (Kobayashi *et al*., 2000 and Sinha *et al*., 2004). Addition of protease inhibitor (Kobayashi *et al*., 2000 and Sinha *et al*., 2004) or using protease deficient strains (Sreekrishna *et al*., 1997) can be solutions of this problem. However, production efficiency should be checked when these solutions are used.

Another important disadvantage of it is necessity of using petrochemical hazardous agent methanol as inducer of AOX1 promoter. Difficulties in storage and transportation of methanol create a disadvantage for *Pichia* fermentations especially in large scale production (Zhang *et al*., 2009). However, AOX1 is not only promoter can be used in protein expression by *P. pastoris*. *P. pastoris* provides variety of expression vector systems like AOX1, glyceraldehyde-3-phosphate dehydrogenase (GAP) (Waterham *et al*., 1997), formaldehyde dehydrogenase 1 (FLD1) (Shen *et al*., 1998); isocitrate lyase (ICL1) (Menendez *et al*., 2003) and 3-phosphoglycerate kinase PGK1 (de Almeida *et al*., 2005). Since

there are other expression systems than AOX1, in this expression host, disadvantages of using methanol can be discarded. By availability of strongly inducible *AOX1* and constitutive *GAP* promoters-based expression vector systems of *P. pastoris* for host-vector design in recombinant protein expression provide great flexibility to this microorganism (Cos *et al*. 2006a).

2.2.2 Expression with different promoter based vectors of *P. pastoris*

There are two main expression systems AOX1 and GAP promoter based which are inducible and constitutive expression systems, respectively (Potvin *et al*. 2012). As an alternative to this expression vectors, there are several vectors designed which are FDL1, ICL1 and PGK1. Among them, FDL1 and ICL1 are alternative to inducible AOX1 promoter whereas PGK1 promoter is alternative to constitutive GAP promoter (Cos *et al*., 2006a). Among all these promoter systems, AOX1 is the most studied vector with a strongly methanol inducible promoter. As mentioned earlier one important disadvantage of *P. pastoris* is difficulties in storage and transportation of hazardous petrochemical substance methanol especially in large scale production for AOX promoter. However, by the discovery of GAP promoter in *P. pastoris* this disadvantage has been overcame (Waterham *et al*., 1997). Additionally GAP promoter derived expression system provides an effective system especially for continuous production of recombinant proteins since GAPDH enzyme is a constitutive enzyme (Zhang *et al*., 2009). Also it provides more cost-effective protein production for large scale by eliminating cost of delivery and storage of methanol. One another point is that as a standard protocol in AOX1 promoter derived expression system, due to low specific growth rate of cells on methanol before the production phase cells were grown on glycerol until cells reach a certain density, and then production starts by methanol induction and feeding. That is, AOX1 derived expression system requires two main phases. In contrast, in GAP promoter derived expression system biomass production and protein expression occur simultaneously in glycerol or glucose as single carbon source

(Zhang *et al*., 2009). GAP promoter is induced not only by the glucose; glycerol and sucrose can induce the pGAP (Fei *et al*., 2009). Additionally medium design plays an important role in recombinant protein expression in pGAP; in the study of Pal *et al*. (2006) defined, semi-defined and complex feed experiments were conducted and the highest product concentration was obtained in complex feed.

In this study for rhGH production a vector construct was developed by using GAP promoter for later comparison of production levels of rhGH by different promoter based expression systems. Depending on the protein produced; efficiency and production levels differ for GAP and AOX promoter systems. In some of the cases, GAP promoter expressed proteins gave higher production amount (Döring *et al*., 1998; Delroisse *et al*., 2005) whereas in some of the cases AOX promoter expressed proteins gave higher production amounts (Vassileva et at, 2001; Sears *et al*., 1998; Kim *et al*., 2009). A comparison table for production levels in AOX1 and GAP promoter produced protein by Table 2.1.

Table 2.1 GAP and AOX promoters productions (Cos *et al*. 2006a)

Protein	Promoter		Substrate	Total protein (mg/L)[1] (U/mL)[2]	Productivity (mg/L h)[1] (U/L h)[2]	Operational mode	Bioreactor
Insect esterase	*GAP*	Intracellular	Glucose	7[1]	--	Batch	Shake flasks 50 mL
		Extracellular		80[1]	--		
	AOX1	Extracellular	Methanol	40[1]	--		
Fructose exo-levanase	*GAP*	Extracellular	Glycerol	26.6[2]	682[2]	Fed-batch	Bioreactor 7.5 L
	AOX1	Extracellular	Methanol	21.1[2]	220[2]	Fed-batch	
Human trysinogen	*GAP*	Extracellular	Glycerol	--	--	Fed-batch	Bioreactor 2 L
	AOX1	Extracellular	Methanol	--	--	Fed-batch	
Vitellogenin	*GAP*	Intracellular	Glucose	12[2]	--	Fed-batch	Bioreactor 2 L
hGM-CSF	*GAP*	Extracellular	Glucose	90[1]	1.2[1]	Batch	Shake flasks 50 mL
	GAP+AOX1	Extracellular	Methanol	180[1]	2.4[1]	Batch	
Aqualysin I	*GAP*	Extracellular	Glucose	1000[1]		Batch	Shake flasks
h-Chitinase	*GAP*	Extracellular	Glucose	450[1]	2.8[1]	Fed-batch	Bioreactor 3 L
				--	15[1]	Continuous	
	AOX	Extracellular	Methanol	350[1]	2.9[1]	Fed-batch	
HBsAg	*GAP*	Extracellular	Glucose	--	--	Fed-batch	Shake flasks 500 mL
	GAP multicopy	Extracellular		--	--	Fed-batch	
				--	--	Cyclic batch	
	AOX1	Extracellular	Methanol	--	--	Fed-batch	
Cellobiohydrolase	*GAP*	Extracellular	Glycerol	--	--	Fed-batch	Bioreactor 7.5 L
	AOX1	Extracellular	Methanol	--	--	Fed-batch	

2.2.3 Carbon Source Utilization Pathways of *P.pastoris*

Before protein production, *P. pastoris* strains of AOX promoter requires a growth phase hence it requires two phases in fermentations, first one for growth and the other for production. In order to reach appreciable cell densities before protein production first phases was achieved by using glycerol as sole carbon source in AOX1 expression system. Glycerol represses the AOX1 expression system, but it is used in early growth phase because of the higher specific growth rate of *P. pastoris* on glycerol; 0.18 h L^{-1} (Cos *et al*., 2006a). After depletion of glycerol in fermentation medium, initiation of protein production is achieved by feeding methanol. Glycerol pathway of *P. pastoris* is illustrated in Figure 2.4.

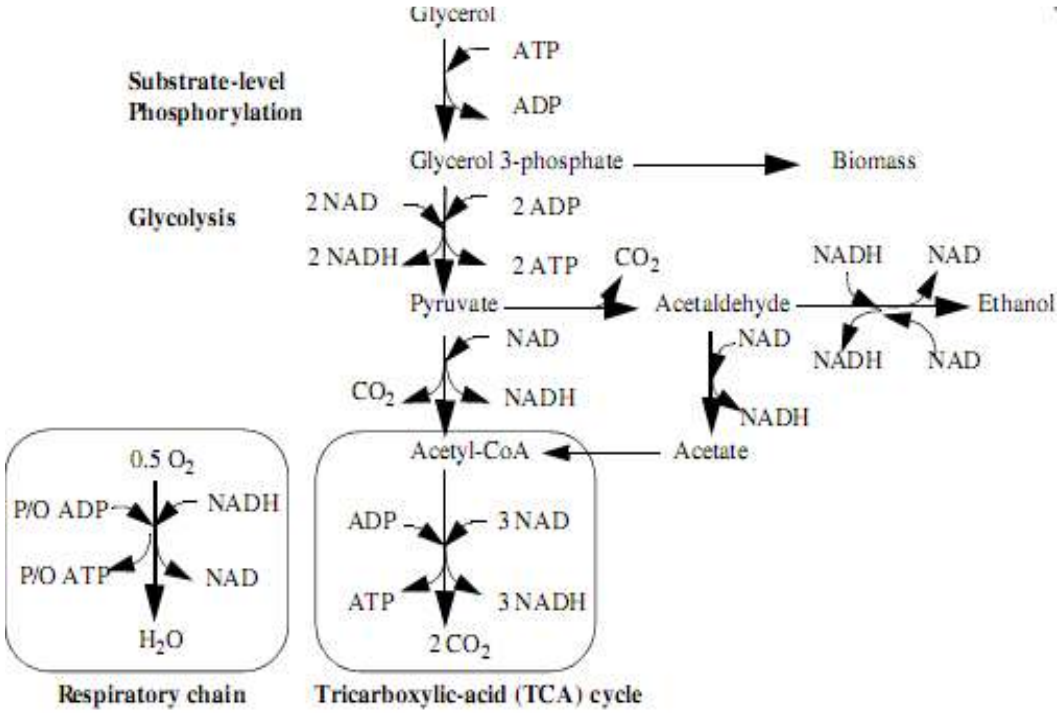

Figure 2.4 Glycerol metabolism in *P. pastoris* (Ren *et al*., 2003).

Conceptual basis for the expression system by *P. pastoris* depends on presence of substantial levels of some enzymes required for methanol metabolism only when cells are grown in methanol. (Veenhuis *et al*. 1983) Hence in Pichia expression system methanol metabolism emerges as an important pathway and shown in Figure 2.5.

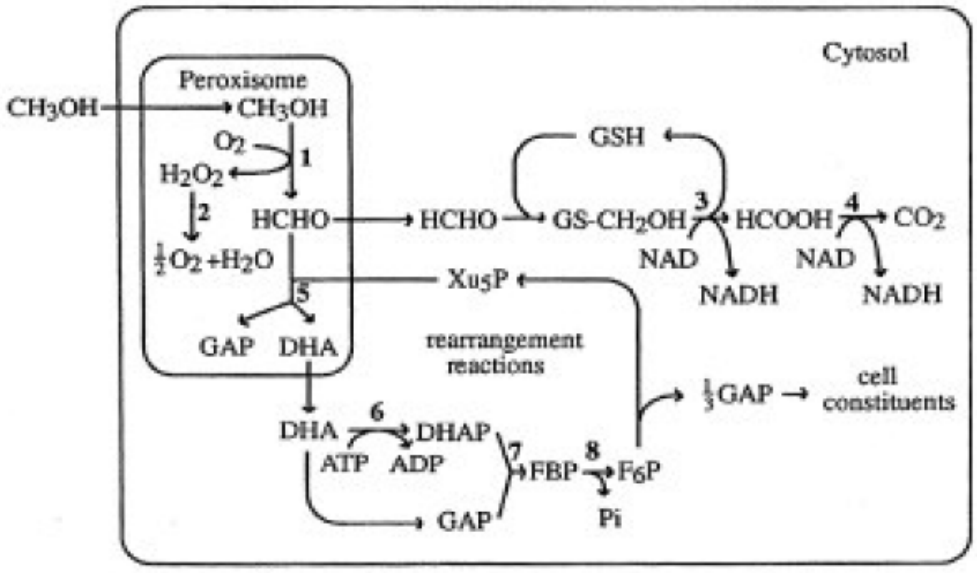

Figure 2.5 Methanol utilization pathways *in P.pastoris*. Enzymes indicated by numbers: 1- AOX; 2- Catalase; 3- formaldeyhde dehydrogenase; 4- formate dehydro-genase; 5-dihydroxyacetone synthase;6- dihydroxyacetone kinase; 7-fructose 1,6 biphosphate aldolase; 8-frustose 1,6-biphosphophatase (Cereghino and Cregg, 2000).

In recent studies for improvement of protein production other carbon sources in feeding strategy as co-substrate in addition to methanol have been investigated (Cos *et al*. 2006a). Glycerol like carbon sources repress the AOX1 promoter hence when choosing co-substrate for production phase it should be considered that it should not repress the AOX1 promoter. For this purpose and with this consideration several non-repressing co-carbon sources were investigated (Inan and Meagher, 2001). In our research group effect of sorbitol

on pilot scale production of rhGH was studied in detail. In this study a comparison will be given in co-carbon sources sorbitol and mannitol. Utilization pathway of sorbitol/mannitol is shown in Figure 2.6.

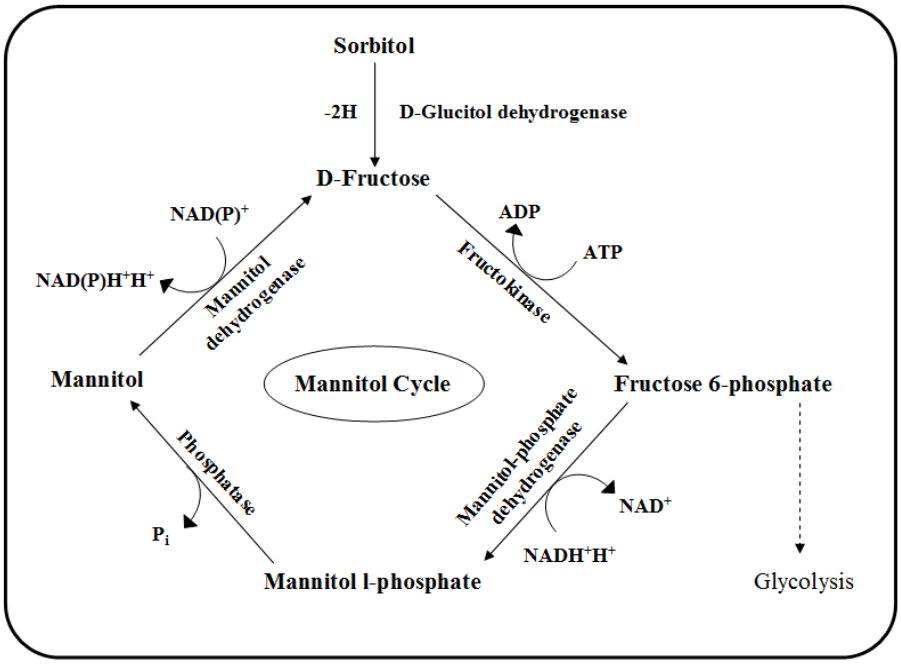

Figure 2.6 Sorbitol and mannitol utilization pathway of yeasts (Walker, 1998)

2.3 Bioreactor Operation Parameters and Medium Design

In fermentation processes, cell growth is an important factor as well as protein production. Both medium composition and bioreactor operation parameters were chosen by considering essential cell growth. Bioprocess operation parameters affect process yields very much. These parameters should be chosen by considering both optimum living conditions of expression host and optimum production conditions of recombinant protein to be produced.

2.3.1 Medium Design and Feeding Strategy

Medium composition should be determined by considering necessary enzymatic activity and required energy generation for cell maintenance. Concentrations of medium ingredients should be decided accordingly. In a well-designed fermentation medium vital components are carbon and nitrogen sources as well as other macronutrients and trace elements. The other consideration for medium design it should minimizes the downstream processing of protein production (Nielsen and Villadsen, 1994). Both macronutrients; carbon, nitrogen, oxygen, magnesium, sulphur, potassium etc., whose concentrations in the medium greater than 0.1 mM and micronutrients Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Na^{2+}, Cl^{-} which are less than 0.1 mM in fermentation medium. In medium design defined and complex media are possible. Complex media includes components which are naturally obtained without knowing the exact concentrations like yeast extract or molasses. In contrast to complex media, in defined media, concentrations of all components in the medium are exactly known. Generally, complex mediums are very rich in nutrients; hence high cell growth rates can be achieved. On the other hand in defined media it is possible to investigate effect of each component of on production efficiency and manipulate accordingly and analyze all produced and left components in fermentation medium. Therefore, by using defined medium in fermentation it is possible to analyze shifts in metabolic reaction networks of cells and manipulate accordingly. Additionally defined medium minimizes downstream processing of protein production. Hence, complex media can be used in precultivation steps due to higher cell growth rates whereas defined media can be preferred for production phases (Hohenblum *et al*., 2004; Calik *et al*., 2008; Dietzsch *et al*., 2011).

Basal salt medium (BSM) together with carbon source and *Pichia* trace salts (PTM) is the most commonly used medium for production by *P. pastoris* (Zhang *et al*., 2000a; Jungo *et al*., 2006; Dietzsch *et al*., 2011). Also modified BSM is used in literature (Stratton *et al*., 1998; Kobayashi *et al*., 2000; Sinha *et al*.,

2004). Being a macronutrient, nitrogen is an essential component of fermentation medium. The most commonly used one ammonium hydroxide; also used for pH adjustment of fermentation broth. Another essential medium component is the nitrogen. The most commonly used nitrogen source is ammonium hydroxide which is also used for pH adjustment (Wang *et al*., 2009; Soyaslan and Calik, 2011; Gao *et al*., 2012).

When using AOX1 promoter based expression vector in *P.pastoris* the specific carbon source, methanol is required as inducer of AOX1 promoter to produce r-protein. *P. pastoris* can be used methanol as sole carbon source (Macauley-Patrick *et al*., 2005); however, when methanol is used as sole carbon source it causes inhibition above 4 g L^{-1} methanol concentration (Zhang *et al*., 2000a) and 1-2%(v/v) can be toxic (Invitrogen, 2002). In methanol utilization pathway, methanol is first oxidized to formaldehyde and hydrogen peroxide hence excess methanol can cause accumulation of formaldehyde and hydrogen peroxide can poison the cells. To keep methanol concentration below the toxic limit and obtain higher efficiencies by increasing cell concentration using a carbon source other than methanol, stage-wise feeding employed. (Stratton *et al*., 1998; Zhang *et al*., 2000a; Sinha *et al*., 2004, Soyaslan and Çalık 2011). Generally four stage fermentation is employed in the first stage, by using glycerol in batch-wise manner cells were grown; in the second stage glycerol is fed in fed-batch manner according to a certain μ. This stage increase cell concentration further and prepare the cells for a new carbon source by derepressing the AOX1 promoter. In the next stage methanol is started to add in fermentation broth in batch-wise. The purpose of this stage is provide a transition phase in between glycerol and methanol, by this phase it is assured that before methanol fed batch phase there will be no AOX1 repressing carbon source. In last stage, production starts by adding methanol for induction of AOX1 promoter to fermentation medium in fed-batch.

Specific growth rate of the cells on methanol is very low with μ_{max} of 0.14 h^{-1} however; methanol is also inducing agent of AOX1 promoter, there is an

optimum of μ. Optimum methanol feeding μ for rhGH production was found as μ=0.03 h^{-1} (Çalık *et al*., 2011). Since this value is considerably low in order to increase product yields and decrease the process time, various co-carbon sources were tried with methanol in production phase such as, glycerol, ethanol, mannitol, sorbitol, acetate, glucose, trehalose and alanine (İnan and Meagher, 2001; Jungo *et al*. 2007; Paulova *et al*. 2012). Glycerol is one of the commonly used co-carbon source by *Pichia pastoris* cells as co-carbon source. However, it was reported that excess glycerol represses the AOX promoter (Xie *et al*., 2005). Instead of glycerol, sorbitol is used as non-repressive co-carbon source in various feeding strategies and satisfactory results have been achieved (Jungo *et al*. 2007; Çelik *et al*., 2009; Inankur, 2010; Bozkurt, 2012). Feeding strategies with co-feeding of sorbitol were studied by same strain used in this study and best rhGH production was reported by keeping sorbitol concentration at 50 g L^{-1} through first 15 hours of production phase and feeding of methanol with pre-determined specific growth rate of μ_{M0}=0.03 h^{-1} and keeping pH=5.0. (Bozkurt, 2012).

Unlike inducible AOX1 promoter expresion system, constitutive GAP promoter expression system does not require a specific carbon source, many alternative carbon sources like glycerol, glucose, oleic acid and methanol can be possible for r-protein production (Waterham et al., 1997). Additionally, accumulation of these carbon sources are not toxic as methanol, hence stage-wise fermentation does not required, neither. In GAP promoter expression system simultaneous cell growth and r-protein production can be possible.

2.3.2 Bioreactor Operation Parameters

2.3.2.1 Temperature

Since it affects intracellular enzymatic activities of cell, temperature is one the most critical parameters of bioreactor operation parameters. Levels of enzymatic activities determines cellular metabolism, cell growth and production pathways. That is bioreactor operation temperature should be decided by

considering cell metabolism and cell growth. Also temperature may cause misfolding of secreted recombinant proteins by affecting the secondary and tertiary structures of proteins (Georgiou and Valax, 1996). Therefore, when deciding temperature, properties of expressed protein should be considered.

In *Pichia* fermentations most of studies are carried out at 30^{o}C, due to cell growth profiles of microorganism (Thorpe *et al*., 1999; Calik *et al*. 2011; Gao *et al*., 2012). In literature it is noted over temperature over 32^{o}C can inhibit intracellular activities and lead to cell lysis resulting in cell death and cause higher extracellular protease activity. (Inan *et al*., 1999; Invitrogen 2002). For proteolytic activity, it is observed that when temperature decreases proteolytic activity decreases up to temperature 15°C (Li *et al*., 2001, Hong *et al*., 2002; Jahic *et al*. 2003; Macauley-Patrick *et al*. 2005). Hong and coworkers (2001) reported that they achieve higher antifreeze proteins and raised cell viability when dropping temperature from 30^{o}C to 20^{o}C. For rhGH production optimum temperature was reported as 30^{o}C (Inankur, 2010).

2.3.2.2 pH

Since hydrogen ion concentration influences cell membrane transport, intracellular and extracellular enzymatic reactions of the cell, pH is another key parameter in fermentation conditions. Microorganisms have capability to maintain their intracellular pH values; however, increased pH difference between intracellular and extracellular media increases maintenance energy requirement. Because cells attempt to keep the proton gradient across the membrane and it requires energy (Nielsen and Villadsen, 1994).

Tolerability of *Pichia pastoris* covers a broad range of pH between pH=3.0-7.0 (Macauley-Patrick *et al*., 2005). In this broad range pH value for bioreactor operation is determined mostly according to stability of the product protein and production yields. Final product concentration is affected mostly by protease activity in the medium and that is strogly related with medium pH. (Sreekrishna *et al*., 1997).

Having different isoelectric pI which is a pH value at which the net charge of the protein zero; different proteins is influenced form the pH differently. Hence different proteins have different optimum pH operation value depending on nature of the protein (Macauley-Patrick *et al*., 2005). Kobayashi and coworkers (2000) studied the effect of pH on human serum albumin production and investigated that at pH=5.6 less proteolytic activity was observed. At low pH values it was reported that at lower pH values higher protease activities observed and it may cause undetectable protein concentrations (Inan *et al*., 1999). For rhuEPO production pH 4.0, 4.5, 5.0, 5.5 and 6.0 was studied and investigated that pH 4.5 is the optimum for this protein by considering cell growth by considering AOX activity, oxygen demand and highest protein concentration achieved (Soyaslan and Çalık, 2011). For rhGH production optimum pH was reported as pH=5.0 (Bozkurt,2012).

2.3.2.3 Oxygen Transfer

Oxygen transfer parameters are other important bioreactor operation parameters because it is related with energy production in most of the microorganisms. Oxygen requirement depends on metabolism of microorganism hence it varies among different microorganisms. Being a facultative anaerobic microorganism *Pichia pastoris* prefers to produce energy by aerobic respiration in the presence of oxygen but also is able to produce switch its metabolism to produce energy without oxygen. Therefore, oxygen is an effective parameter influencing cellular metabolism. In presence of oxygen *P. pastoris* uses oxygen as terminal acceptor in oxidative phosphorylation for energy generation also uses as an essential growth factor in sterol or fatty biosynthesis (Walker, 1998). Being a facultative anaerobic microorganism it is a favourable expression host, in presence of oxygen it prefer respiratory pathways and does not switch to produce by-products ethanol and acetic acid (Cereghino *et al*., 2002). For *P. pastoris* studies especially with AOX1 promoter; oxygen transfer becomes more important because it uses oxygen in oxidation of methanol to formaldehyde in

methanol metabolism and it leads product formation. (Sibirny *et al*., 1990). In order to provide sufficient oxygen to organism, transfer resistances should be minimized by proper stirring and when needed oxygen enriched air should be supplied to the fermentation medium (Çelik *et al*., 2009).

In most of the fermentation studies with *P. pastoris* it is seen that dissolved oxygen (DO) concentration was kept around 10%-40%. (Thorpe *et al*., 1999; Çelik *et al*., 2009; Wang *et al*., 2009; Dietzsch *et al*., 2011; Soyaslan and Çalık, 2011).

Furthermore, monitoring DO provides information about carbon source limitation. When an oxygen spike is observed it means cells do not consume oxygen since carbon source was depleted (Invitrogen 2002). In early phases glycerol can be tracking by monitoring DO or in production phase methanol can be tracking by DO. DO monitoring as feedback control and methanol feeding accordingly could be a methanol feeding strategy in recombinant protein production (Lee at al., 2003).

2.4 Bioprocess Characteristics

2.4.1 Mass Balance Equations in Semi-Batch Bioreactor Systems

To evaluate the efficiency of a fermentation process it is important to calculate the yield coefficients and specific rates. The specific rate of production or consumption of a molecule at a certain cultivation time is defined, as the change in the amount of the molecule per unit time, either per unit volume of the cultivation medium or per unit cell dry-weight. The latter definition of the specific cell production or consumption rates based on the cell dry-weight is preferable, as it enables logical comparisons of the results.

Microbial growth can be defined as the increase in the cell population. The specific growth rate, μ, is an important parameter that characterizes the cell growth. The material balance for the cell in a completely mixed bioreactor is written considering the system is batch for the cell as follows, either in batch- or

semi-batch bioreactor operations, where there is no inlet of the cell with the feed in the semi-batch (fed-batch) bioreactor operations:

$$\frac{d(C_X V)}{dt} = r_X V \tag{2.1}$$

where r_x is the cell formation rate. The cell formation rate can be defined with a first order kinetic equation which is the function of C_X, where μ is the specific cell growth rate of the cells, defined as follows:

$$r_x = \mu C_x \tag{2.2}$$

The assumption related for the equations (2.1) and (2.2) is that the cell concentration $C_X(t)$ and bioreactor volume V(t) do not change by taking samples from the bioreactor, which indicates that the sampling should be carried out neatly by taking small volumes of samples as possible. By combining equations (2.1) and (2.2):

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

As the operation mode for the bioreactor in this work is semi-batch, based on continuous feeding of the selected substrate(s), volume of the liquid phase where the fermentation occurs, changes throughout the process. Assuming the density in the liquid phase is constant, the continuity equation for the semi-batch bioreactor having a feed inlet stream with the flow rate of Q_t is as follows:

$$Q_t = \frac{dV}{dt} \tag{2.4}$$

Equation (2.4) defines that the volume change in the bioreactor is due to feeding selected substrates with the Q_t inlet-stream. Thus, equation (2.3) is modified by inserting equation (2.4), as follows:

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

By rearranging the equation (2.5), the specific growth rate for semi-batch bioreactor, based on continuous feeding of selected substrates with the flow rate Q_t is obtained as follows:

$$\mu = \frac{d(C_X)}{dt} \frac{1}{C_X} + \frac{Q_t}{V} \quad (2.6)$$

The material balance for the continuously fed substrate (methanol) in the semi-batch process:

$$\frac{d(C_M V)}{dt} = Q_M C_{Mo} - 0 + r_M V \quad (2.7)$$

in which r_M which is the rate of consumption of methanol. The methanol consumption rate can be defined with a first order kinetic equation which is the function of C_X, where q_M is the specific methanol consumption rate of the cells, defined as follows:

$$-r_M = q_M C_X \quad (2.8)$$

where, q_M is the specific methanol consumption rate of the cells. By inserting equation(2.8) into equation (2.7), the ordinary differential equation (2.9) which includes two first order derivatives is obtained as follows:

$$V\frac{dC_M}{dt} + C_M\frac{dV}{dt} = Q_M C_{Mo} - q_M C_X V \tag{2.9}$$

In this M.Sc. thesis work, methanol was fed with a pre-calculated feeding rate of Q_M based on the predermined μ_{M0} value, according to the equation derived from the mass-balance equation for the component "*Pichia pastoris*"; consequently, the accumulation of methanol in the bioreactor was prevented; therefore:

$$\frac{dC_M}{dt} = 0 \tag{2.10}$$

Further, despite continuous feeding of methanol to the semi-batch bioreactor with a pre-calculated dynamic Q_M volumetric flow rate, the volume change in the bioreactor can be assumed negligible, due to use of concentrated methanol (100% w/v) which cumulative total volume is small:

$$\frac{dV}{dt} \sim 0 \tag{2.11}$$

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

$$0 = \frac{Q_M}{V}C_M - q_M C_X \tag{2.12}$$

Thus, the specific uptake rate of methanol can be defined as follows:

$$q_M = \left(\frac{Q_M}{V}\frac{C_{M0}}{C_X}\right) \tag{2.13}$$

Mannitol was used as the co-substrate in the experiments run by recombinant *P. pastoris* M13 strain (pPICZαA*::hGH*-Mut+). In the first pilot scale bioreactor experiment (MM1), mannitol was the batch-wise used substrate. Therefore, for the experiments designed based on batch mannitol use, the mass balance equation for mannitol is as follows:

$$r_{man}V = \frac{d(C_{man}V)}{dt} \tag{2.14}$$

where, r_{man} is the mannitol consumption rate. The mannitol consumption rate can be defined with a first order kinetic equation which is the function of C_X, where q_{man} is the specific mannitol consumption rate of the cells:

$$-r_{man} = q_{man}C_x \tag{2.15}$$

By inserting the equation (2.15) into (2.14), one can obtain:

$$-q_{man}VC_X = V\frac{d(C_{man})}{dt} + C_{man}\frac{d(V)}{dt} \tag{2.16}$$

Therefore, the specific uptake rate of mannitol, where mannitol is batch-wise used, is defined as follows:

$$q_{man} = -\left(\frac{dC_{man}}{dt} + \frac{Q_t}{V}C_{man}\right)\frac{1}{C_X} \tag{2.17}$$

The specific uptake rate of mannitol in batch- mannitol experiments is calculated from equation (2.17). In batch mannitol experiments, the volume change is merely due to exponential methanol feeding (Q_t), by removing negligible volume of sampling, is indeed noteworthy. Thus, Q_t is assumed equal to Q_M.

The recombinant protein production in the semi-batch operated bioreactor is batchwise; therefore, the material balance for rhGH is constructed as follows:

$$r_{rhGH}V = \frac{d(C_{rhGH}V)}{dt} \tag{2.18}$$

The recombinant protein formation rate (r_{rhGH}) can be defined with a first order kinetic equation which is the function of C_X, where q_{rhGH} is the specific rhGH formation rate, defined as follows:

$$r_{rhGH} = q_{rhGH}C_x \tag{2.19}$$

By inserting equation (2.19) into equation (2.18) the specific specific rhGH formation rate is obtained as follows:

$$q_{rhGH} = (\frac{dC_{rhGH}}{dt} + \frac{Q_t}{V}C_{rhGH})\frac{1}{C_X} \tag{2.20}$$

Thus, the specific rates μ, q_M, q_{man}, and q_{rhGH} in the semi-batch fermentations where mannitol used batch-wise are calculated, respectively, by using the equations 2.6, 2.13, 2.17, and 2.20.

In the semi-batch fermentation experiments where the co-substrate mannitol was also fed continuously besides methanol, the specific methanol consumption rate q_M is calculated also by the equation (2.12), as it was derived for continuous methanol feeding. Moreover, the parameters derived for the batch components of the semi-batch system, i.e., the cell and the product, the specific rates for the cell growth (μ) and the recombinant protein (q_{rhGH}) formation are calculated by, respectively, the equations (2.6) and (2.19). In the continuous methanol and mannitol fed semi-batch bioreactor experiments (MM2 and MM3), indeed,the total flow rate Q_t is the sum of the volumetric flow

rates of methanol and mannitol streams ($Q_t = Q_M + Q_{man}$). In this context, the mass balance equation for the component mannitol for the semi-batch bioreactor system is as follows:

$$Q_{man}C_{man0} + r_{man}V = \frac{d(C_{man}V)}{dt} = C_{man}\frac{dV}{dt} + V\frac{dC_{man}}{dt} \qquad (2.21)$$

Since mannitol concentration in the bioreactor was kept constant within the continuous mannitol feeding period, the time derivative of mannitol concentration is:

$$\frac{dC_{man}}{dt} = 0 \qquad (2.22)$$

As the mannitol consumption rate is defined by the first order kinetic equation 2.15, which is the function of C_X, where q_{man} is the specific mannitol consumption rate of the cells, as:

$$-r_{man} = q_{man}C_x \qquad (2.15)$$

by inserting the rate of mannitol consumption into q_S into the defining equation of mannitol (equation 2.21), the specific mannitol consumprtion rate (q_{man}) is derived for the mannitol fed semi-batch bioreactor, as follows:

$$q_{man} = -\frac{C_{man}}{VC_X}\frac{dV}{dt} + \frac{C_{man0}}{C_X}\frac{Q_{man}}{V} \qquad (2.23)$$

where,

$$\frac{dV}{dt} = Q_{man} + Q_M = Q_t \qquad (2.24)$$

as both mannitol and methanol are fed to the semi-batch bioreactor. Indeed, during the cultivation time interval where the co-substrate mannitol feeding is terminated (in this work: t>6 h), q_{man} is calculated based on the defining equation for batch mannitol use in the bioreactor, as derived by equation (2.17).

In the semi-batch bioreactor experiments by recombinant *P.pastoris* G7 cells producing rhGH under GAP promoter using glucose, as the cell formation and recombinant protein production occur batch-wise in the semi-batch system, their formations are also defined, respectively, i.e., by the mass balance equations (2.1) and 2.18. Thus, the specific cell growth rate (μ) and the specific recombinant protein formation rate (q_{rhGH}) are defined, respectively, by the equatios (2.6) and (2.20). The mass balance equation for the component glucose where glucose is fed continuously (C_{Go}) with the flow rate $Q_t = Q_G$ in a semi-batch bioreactor, is as follows similar to the equations (2.8) and (2.21):

$$\frac{d(C_G V)}{dt} = Q_G C_{Go} - 0 + r_G V \tag{2.25}$$

where r_G is the glucose consumption rate defined as:

$$-r_G = q_G C_X \tag{2.26}$$

As glucose accumulation was prevented in the bioreactor ($(dC_G/dt)=0$), and the volume change is negligible ($(dV/dt)=0$), the semi-batch bioreactor system is assumed in quasi-steady state condition for the glucose, as follows:

$$\frac{Q_G}{V} C_G - q_G C_X = 0 \tag{2.27}$$

and the specific glucose consumption rate q_G is defined as follows:

$$q_G = \left(\frac{Q_G}{V}\frac{C_{G0}}{C_X}\right) \quad (2.28)$$

2.4.2 Selectivity Values: Overall and Instantaneous Yield Coefficients

The yield coefficients, which are the selectivity values (functions), are defined for the consumed and synthesised components of the bioprocess. The yield coefficients used in this study are presented in Table 2.2. The yield coefficient, e.g., $Y_{P/S}$, is defined, either, as the ratio of the mass (or, concentration) of the product formed per the amount of the selected substrate consumed,

i) within a finite cultivation time interval (Δt), that is "overall product yield on substrate ($Y_{P/S}$)", and formulated as follows:

$$Y_{P/S} = -(\Delta C_P/\Delta t)/\Delta C_S/\Delta t)$$

$$Y_{P/S} = -(\Delta C_P/\Delta C_S) \quad (2.29)$$

ii) or, at a cultivation time, that is "instantaneous product yield on substrate ($y_{P/S}$)", and formulated as follows:

$$y_{P/S} = -(dC_P/dt)/(dC_S/dt))$$

$$y_{P/S} = -(dC_P/(dC_S) \quad (2.30)$$

Table 2.2 Overall yield coefficients

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

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals were analytical grade and obtained from Sigma, Fluka and Merck.

3.2 Buffers and Stock Solutions

Formulations and preparations of all buffers and stock solutions are given in Appendix A. Sterilization of solutions was performed by autoclaving for 20 minutes at 121°C or filtering through 0.22 or 0.45 µm filters (Millipore Corporation Bedford, MA, USA) depending on the type of the solution.

3.3 Strains, Plasmids and Maintenance of Microorganisms

The *hGH* gene was amplified from *Escherichia coli* TOP10 strain carrying pPICZαA*::hGH* plasmid and antibiotic resistance gene to zeocin. Wild type *Pichia pastoris* X-33 strain and pGAPZαA shuttle vector containing zeocin resistance were purchased from Invitrogen. *E. coli* DH5α strain was used for both propagation of pGAPZαA vector and construction and amplification of pGAPZαA*::hGH* plasmid. *P. pastoris* M13 strain containing strongly inducible AOX1 promoter induced by methanol and *P. pastoris* G7 strain developed in this study containing constitutive GAP promoter induced by glucose were used in rhGH production. All strains and plasmids that were used in this study are represented in Table 3.1. The sequences and schematic representation of the plasmids are given in Appendix C.

Table 3.1 Strains and plasmids used in this study

Genus	Species	Strain	Genotype/plasmid	Source
Escherichia	*coli*	pPICZαA*::hGH*	pPICZαA*::hGH*	Calik et. al. (2008)
Escherichia	*coli*	DH5α	wild Type	Invitrogen (USA)
Escherichia	*coli*	DH5α-pGAPZαA	pGAPZαA	This study
Escherichia	*coli*	pGAPZαA*::hGH*	pGAPZαA*::hGH*	This study
Pichia	*pastoris*	X-33	wild type	Invitrogen (USA)
Pichia	*pastoris*	G7	pGAPZαA*::hGH*	This study
Pichia	*pastoris*	M13	pPICZαA*::hGH*	Calik et. al. (2008)

All microorganisms used in this study are stored in the microbanks (Pro-lab Diagnostics) or glycerol stocks containing 15% glycerol at -80°C.

3.4 Growth Media

Recombinant *E.coli* pPICZαA*::hGH* strains stored on microbanks at -80°C were cultivated on low-salt LB agar slants containing 25 μg ml^{-1} zeocin and incubated overnight at 37°C whereas wild type *E. coli* strains inoculated onto LB agar slants. Same antibiotic concentration was used when liquid media were used.

Wild type X-33 strain stored on microbanks at -80°C was inoculated onto YPD agar *P. pastoris* slants and incubated at 30°C for 48-60 hours. Recombinant

P. pastoris strain containing antibiotic resistance was cultivated on YPD agar slants containing 100 μg mL^{-1} zeocin.

All the media compositions, preparations and storage are given in detail in Appendix B.

3.5 Genetic Engineering Techniques

3.5.1 Enzymes, Kits, Molecular Size Markers

Pfu DNA polymerase, Taq DNA polymerase, dNTP mixture, DNase and and protease free RNase A, restriction enzymes (FastDigest® EcoRI, FastDigest®XbaI, PagI (BspHI)), T4 DNA ligase and their buffers were purchased from Fermentas Life Sciences. Lambda DNA/HindIII Markers and 6X loading dyes were also from Fermentas Life Sciences.

PCR Purification Kit: GeneJET™ PCR Purification Kit and Plasmid Purification Kit: GeneJET™ Plasmid Miniprep Kit were provided from Fermentas Inc. Gel Elution Kit is obtained from GeneMark Gene Molecular Biology Tools.

3.5.2 Plasmid Isolation From *E.coli*

From *E. coli* carrying pPICZαA*::hGH* plasmid which was used as template in PCR, plasmid isolation was carried out by plasmid purification kit. By the time shuttle vector pGAPZαA was isolated via alkaline lysis (midipreparation) method (Sambrook *et al*.,2001) from E. coli DH5α-pGAPZαA cells. Application of alkaline lysis method is given as follows and compositions of alkaline lysis solutions are given in Appendix A.

1. 10 mL of LB broth medium was inoculated with a single colony of bacteria. Incubate at 37°C with shaking of 200-225 rpm.
2. The culture was transferred to centrifuge tube and spin at 2000g for 10 minutes at 4°C to pellet the cells.
3. Supernatant was poured off and the tube was drained on a clean paper towel.

4. Cells were resuspended in 200 µL of ice-cold alkaline lysis I solution by vigorous vortexing until making sure that cells are dissolved completely.

5. 400 µL of freshly prepared alkaline lysis II solution was added. Tube was mixed gently by inversion five times and stored the tube on ice.

6. 300 µL of ice-cold alkaline lysis III solution was added, and the tube was inverted several times gently and incubated on ice for 3-5 minutes.

7. Tubes were centrifuged at 10000g for 5 minutes at 4°C. 600 µL of the supernatant was transferred to a fresh microcentrifuge tube. White precipitate (cell debris) was not disturbed while transferring.

8. Equal volume of phenol:chloroform (1:1) was added to tube and the tube was mixed by vortexing and centrifuged at maximum speed for 2 minutes at 4°C. The upper aqueous layer was transferred to a fresh microcentrifuge tube.

9. 600 µL of isopropanol was added, the contents were mixed and the tube were kept standed for 2 minutes at room temperature.

10. The tube was centrifuged at maximum speed for 5 minutes at room temperature.

11. The supernatant was poured off and the tube was drained on a paper towel.

12. The pellet was washed by with 1 mL of 70% EtOH, and the DNA was recovered by spinning the tube at maximum speed for 2 minutes at room temperature.

13. The supernatant was poured off and remaining ethanol in the tube was removed for 5-10 minutes.

14. Final pellet was dissolved in TE buffer; and RNAse was added at the final concentration of 20µg/mL. Isolated plasmids were stored at -20°C for further use.

3.5.3 Agarose Gel Electrophoresis

For visualization and quantification of DNA fragments or purification of specific DNA fragments gel electrophoresis were applied. In this study 0.8%

(w/v) agarose gel was preferred. For 0.8% (w/v) agarose; 0.4g agarose was dissolved in 50 mL 1X TBE buffer and heated until boiling. After cooling about 55°C, 3µL of ethidium bromide (Sigma-10mg/mL) was added for staining of DNA fragments under UV light. Then gel was poured off in an appropriate gel tray with and the appropriate gel comb for appropriate wells and gel was allowed to solidify. After solidification, combs are removed and gel was transferred to the electrophoresis tank filled with 1X TBE buffer. Formulation of 5X TBE stock solution is given in Appendix A. 5-10 µL of DNA samples were mixed with 1/5 volume of 6X loading dye then samples were loaded to wells. In order to distinguish the size of DNA fragments, DNA marker given in Appendix E was also loaded to an appropriate well of the gel. Electrophoresis was performed at 90 V for 60 minutes. At the end DNA bands were visualized by UV transilluminator. Also its photographs were taken by gel imaging system (Hamamatsu Digital CCD Camera) and stored by documentation system (UVP BioImaging System). Electrophoresis provides to separate DNA fragments according to their molecular weights. However in case of running of circular DNA it is expected that due to its compact structure it will run faster than its linear equivalents, that is, under UV light it seems ahead of its linear equivalents.

3.5.4 Determination of DNA Concentration

Concentration of DNA can be checked by the use of UV spectrophotometry when concentration of samples needed. DNA absorb UV light very efficiently at concentrations about 2.5 ng/µL. For quantification DNA samples readings from UV spectrophotometry (Thermo Spectronic, HEλIOS α) at 260 and 280 nm can be taken. Reading at 260 nm provides calculation of the concentration of nucleic acid in the sample. The absorbance at 260 nm in a 1-cm quartz cuvette of 50µg/mL solution of double stranded DNA is approximately equal to 1 (Sambrook *et al*. 2001). The ratio between readings at 260 and 280 nm (OD_{260}:OD_{280}) provides an estimate of purity of nucleic acids. Pure preparations DNA samples have OD_{260}:OD_{280} ratio of 1.8. If this ratio is much

different than this value, there might be significant contamination with protein or phenol. Therefore, an accurate measurement of DNA concentration could not be possible. Concentration of DNA in samples can be calculated by following equation:

$$DNA\ concentration\ (mg/ml) =$$

$$(OD_{260}) \times (dilution\ factor) \times (50\ \mu gDNA/mL)/(1\ OD_{260} unit) \qquad (3.1)$$

3.5.5 Primer Design

Primers were designed in order to amplify the cDNA of *hGH* from pPICZαA*::hGH* plasmid (Çalık *et al*., 2008) in accordance with the sequence of *hGH* and restriction enzymes recognition sites. Since same restriction enzyme sites have to found in pGAPZαA shuttle vector; restriction enzyme recognition sites were determined both considering non-cutters site of gene and cloning sites of vector pGAPZαA given in Appendix C. Restriction enzyme recognition sequences and non-cutter enzymes of gene were checked from Restriction Mapper web-page of USA Molecular Biology Resources (http://www.restriction mapper.org)

In this study primers were designed with EcoRI and XbaI restriction enzyme recognition sites, and stop codon (TCA) was also included in design of reverse primer also.

Forward Primer (FwdGhGH):

EcoR1 complementary to *hGH*

5’- GGAATTCTTCCCAACTATACCACTATCTCCGTC -3’

Reverse Primer (RevGhGH):

Xba1 complementary to *hGH*

5’- CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC -3’

Designed primers FwdGhGH which has 33 base pairs and RevGhGH which has 32 base pairs were synthesized in Thermo Fisher Scientific. Molecular weights, G-C contents, melting points and thermodynamic properties primers and also thermodynamic properties of formation of self annealing, loop formation like hair pin structure were checked via Oligo Explorer 1.2 software. Analysis is given in Appendix D.

3.5.6 Polymerase Chain Reaction (PCR)

Amplification of cDNA of *hGH* was carried out by PCR with the thermal cycling machine (Techgene, Flexigene). Isolated pPICZαA*::hGH* plasmid was used as template in PCR and final reaction mixture volume was set to 50 µL. Reaction mixture components and reaction operation parameters (Orman, 2008) are given in Table 3.2 and Table 3.3, respectively.

Table 3.2 Reaction mixture components of PCR

Component	*Amount*
10X PCR buffer (with Mg^{++})	5 µL
dNTPs (2mM)	5 µL
Forward Primer (10 µM)	1 µL
Reverse Primer (10 µM)	1 µL
Template DNA (0.01-1 µg)	1-5 µL
Nuclease-free dH_2O	Up to 49.5 µL
Pfu DNA Polymerase	0.5 µL (1.25u/50 µL)

Table 3.3 PCR Process Parameters

1 cycle	T1 = 94°C	4 min
30 cycle	T1 = 94°C	1 min
	T2 = 60°C	1 min
	T3 = 72°C	2 min
1 cycle	T3 = 72°C	10 min
	T4 = 4°C	10 min

3.5.7 Purification of PCR Products

In order to remove PCR buffer, salts, enzyme, nucleotides and primers; PCR product was purified by using PCR purification kit according to manufacturer's instructions. Final purified DNA was eluted with dH_2O from jet spin columns. Eluted DNA can be stored at -20°C for further use.

3.5.8 Restriction Enzyme Digestion Reaction

Both PCR products and shuttle vector are double digested with EcoRI and XbaI after isolation of vector and purification of the PCR products. Double digestion reaction mixture for plasmid DNA and PCR product are given in Table 3.4 and Table 3.5 respectively.

Table 3.4 Double digestion reaction mixture of plasmid DNA

Component	*Amount*
Nuclease-free water	14 µL
10X FastDigest® buffer	2 µL
DNA	2 µL (up to 1 µg)
FastDigest® EcoRI	1 µL
FastDigest® XbaI	1 µL
Total volume	20 µL

Table 3.5 Double digestion reaction mixture of PCR products

Component	*Amount*
Nuclease-free water	16 µL
10X FastDigest® buffer	2 µL
DNA	10 µL (~0.2 µg)
FastDigest® EcoRI	1 µL
FastDigest® XbaI	1 µL
Total volume	30 µL

All of the components were combined, mixed gently and spun down. Tubes were incubated at 37°C in water bath for 5 minutes for plasmid DNA; 20 minutes for PCR product. Then, enzymes were inactivated by heating for 5 minutes at 80°C.

For control purposes single digestion was carried out in many steps of cloning. Single digestions were conducted EcoRI or XbaI restriction enzymes and contents of the reaction mixture are given in Table 3.6.

Table 3.6 Single digestion reaction mixture

Component	*Amount*
Nuclease-free water	16 µL
10X FastDigest® buffer	2 µL
DNA	1 µL (~0.2 µg)
FastDigest® EcoRI	1 µL
Total volume	20 µL

3.5.9 DNA Purification after Digestion

Both digested PCR products and digested vectors were extracted from agarose gel in order to obtain pure DNA. For this purpose, DNA fragments were run in agarose gel by electrophoresis. And desired DNA bands were cut (up to 350 mg slices) and extracted from the gel by using gel elution kit according to the manufacturer's instructions.

3.5.10 Ligation Reaction

Ligation reaction was carried out in 20 µL total volume and contents are given in Table 3.7. Gene to vector molar ratio altered between 1 to 5 in order to attain ligation and *E. coli* transformation.

Table 3.7 Components of ligation reaction mixture

Component	*Amount*
10 X ligation buffer	2 µL
Insert DNA	20-90 ng
Vector DNA	100 ng
T4 DNA ligase	1 µL
Nuclease-free water	Up to 20 µL

In this study, at insert DNA:vector ratio 3:1, ligation and *E. coli* transformation was achieved. The amount of insert DNA used in ligation mixture is given by following equation:

$$100\ ng\ vector\ \times {Size\ of\ insert\ (bp)}/{Size\ of\ vector\ (bp)}\ \times {3}/{1} =$$

$$amount\ of\ insert\ (ng) \qquad (3.2)$$

The ligation reactions were carried out by incubation of mixture at 22°C for 1 hour. Ligation reaction was ended by incubation of mixture at 70°C for 10 minutes.

3.5.11 Transformation of Plasmid DNA to *E. coli*

For propagation of plasmids, wild type *E. coli* DH5α cells were made chemically competent by $CaCl_2$ method (Sambrook *et al*., 2001). Competent *E. coli* DH5α strain is prepared as follows;

1. *E. coli* DH5α cells were incubated overnight on LB-solid medium at 37°C.
2. A single colony was picked and transferred to 50 mL LB broth in 250 mL Erlenmeyer flasks and incubated at 37°C with vigorous shaking until OD_{600} reached 0.35-0.40.
3. The medium was transferred to a 50 mL polypropylene tube and the tube was kept on ice for 10 minutes.
4. After cooling the culture, the cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C.
5. The supernatant was poured off and the tube was drained on a paper towel for 1 minute.
6. The pellet was resuspended in 30 mL ice-cold $MgCl_2$-$CaCl_2$ solution by swirling. (80mM $MgCl_2$-20mM $CaCl_2$; filter sterilized)
7. Microorganisms were collected by centrifugation at 4000 rpm for 10 minutes at 4°C.
8. Supernatant was decanted and the tube was dried on a paper towel for 1 minutes.
9. The pellet was resuspended in 2 mL of ice cold 0.1M filter sterilized $CaCl_2$ solution was mixed by swirling.
10. At this point, chemically competent cells were used directly to the transformation or stored in aliquots containing 12% glycerol at -80°C.

For both fresh and frozen competent cells used in transformation, procedure is as follows,

11. 200 µL of competent cells were transferred into a sterile, ice-chilled propylene tubes using ice-chilled micropipette tips.

12. Ligation mixture was added as maximum 50 ng in 10 µL maximum volume to the competent cells. Contents were mixed by swirling gently. The tubes were stored on ice for 30 minutes.

13. The tubes were transferred to a rack place in preheated 42°C water bath. The tubes were stored in the rack for exactly 90 seconds without shaking.

14. The tubes transferred rapidly to an ice bath and incubated 1-2 minutes.

15. 800 µL of LB medium was added to each tube. The cultures were incubated at 37°C in water bath for 45 minutes to allow the bacteria to recover.

16. 25, 50, 100 and 200 µL of treated cells were transferred onto LSLB plate containing desired antibiotic at desired concentration. The cells were spread over the entire surface of plate and waited until all the liquid is absorbed.

17. The plates were inverted and incubated at 37° C overnight. Transformed colonies were visible in 12-24 hours.

3.5.12 DNA Sequencing

DNA sequences were checked after *E.coli* transformation was achieved. Sequencing was performed in METU Central Laboratory using ABI Prism 310 Genetic Analyzer. Sequence of pGAPZαA*::hGH* plasmid and sequence results from Genetic Analyzer is illustrated in Appendix C.

3.5.13 Transfection of *Pichia pastoris*

Pichia pastoris transfection was achieved by LiCl method as in manufacturer's instructions (Invitrogen, 2010) and all the solutions required for this method is given in Appendix A. Before the transfection, pGAPZα*::hGH* plasmid was digested with PagI (BspHI) enzyme to linearize the plasmid DNA. Digestion reaction components are given in Table 3.8. Solution was mixed and

spun down for a few seconds. Tubes were incubated at 37°C in water bath for 1-16 hours then enzyme was inactivated by incubation at 80°C for 20 minutes.

Table 3.8 Digestion reaction mixture components

Component	*Amount*
Nuclease-free water	16 µL
10X Buffer O	2 µL
DNA (0.5-2 µg/µL)	1 µL
PagI	0.5-2 µL

1. A 50 mL culture of *Pichia pastoris* X-33 strain was grown in YPD medium at 30°C and 225 rpm to OD_{600} of 0.8 to 1.
2. Cells were harvested and washed with 25 mL sterile water by centrifugation at 1500 g for 10 minutes at room temperature.
3. Water was poured off and cells were resuspended in 1 mL of 100 mM LiCl solution and cell suspension was transferred to a 1.5 mL microcentrifuge tube.
4. Cells were pelleted by centrifugation at maximum speed for 15 seconds and LiCl was removed with pipette.
5. Cells were resuspended in 400 µL of 100 mM LiCl and 50 µL of cells were dispensed into 1.5 mL microcentrifuge tubes for each transformation and used immediately, neither stored on ice nor frozen.
6. 1 mL of single stranded DNA was boiled for 5 minutes and chilled quickly in ice and kept on ice.

7. LiCl cells in step 5 was centrifuged and LiCl was removed with pipette.

8. For each transformation following components were added to the cells in order of given as,

- 240 µL of 50% PEG
- 36 µL of 1M LiCl
- 25 µL of 2 mg/mL single-stranded DNA
- 5-10 µg of plasmid DNA in 50 µL sterile water

9. Each tube was vortexed until the cell pellet was completely mixed.

10. Tubes were incubated at 30°C for 30 minutes without shaking.

11. Then tubes were incubated at 42°C in water bath for 20-25 minutes.

12. Tubes were centrifuged at 7000 rpm and transformation solution was removed with pipette.

13. Pellet was resuspended in 1 mL of YPD medium incubated at 30°C with shaking.

14. After 2 hours 25-100 µL of cell suspension were plated on YPD plates containing 100-200 µg/mL zeocin. Plates were incubated at 30°C for 2-3 days.

3.5.14 Isolation of Genomic DNA from Yeast

After transfection of *P. pastoris*, genomic DNA isolation was applied to the chosen transformants. For DNA isolation from *P. pastoris* cells following method (Burke *et al*., 2000) was applied:

1. 10 mL of yeast culture was grown to saturation in YPD in a 50 mL Falcon tube.
2. The cells were harvested by centrifugation at 5000 rpm for 6 minutes.

3. The cells were resuspended in a 0.5 mL of distilled water and transferred to the 1.5 mL microcentrifuge tube. The cells were harvested by centrifugation at maximum rpm for 2 minutes.

4. Supernatant was decanted and the pellet were vortexed in residual supernatant.

5. 200 µL of yeast lysis solution was added to the tubes and the tubes were mixed by inversion. Then 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g acid-washed glass beads were added.

6. The tubes were wrapped with parafilm and vortexed for 3-4 minutes.

7. 200 µL of TE (pH 8.0) was added and and the tubes were centrifuged at maximum speed for 5 minutes in a microfuge.

8. The upper aqueous layer was transferred to a fresh microcentrifuge tube.

9. 1 mL of 100% EtOH was and the contents were mixed by inversion.

10. Tubes were centrifuged at maximum rpm for 2 minutes and discard the supernatant.

11. The pellet was resuspended in 400 µL of TE buffer containing 10 mg/mL of RNase A.

12. The solution was incubated at 37°C for 10 minutes and 10 µL of 4M ammonium acetate and 1 mL of 100 % EtOH were added and the contents were mixed by inversion.

13. The DNA was pelletde by centrifugation at maximum rpm for 5 minutes in a microfuge and discard the supernatant. The pellet was air-dried and resuspended in 50 µL of sterile distilled water.

10 µL of isolated DNA corresponds approximately 2-4 µg of genomic DNA. All the solutions used in genomic DNA isolation from yeast is given in Appendix A.

3.6 Recombinant Human Growth Hormone Production

In this study recombinant human growth hormone was produced by using two different strains each carrying different promoters. For both strains both laboratory scale air filtered shake bioreactor experiments and pilot scale bioreactor experiments were carried out.

3.6.1 Precultivation

Before the production of recombinant human growth hormone it is necessary to grow recombinant cells until certain density to achieve protein production at high concentrations. In this study two different strains were used to produce recombinant protein. Precultivation differed for different strains and is given in following sections.

3.6.1.1 Precultivation of G7 strain

For the precultivation of G7 strain, *P. pastoris* G7 cells from microbanks were grown on YPD agar slants containing 0.1 g L^{-1} zeocin for 48 to 60 hours at 30°C. The precultivation method differs for the laboratory scale air filtered shake bioreactor experiments than that used for the pilot scale bioreactor experiments. For 50 mL laboratory scale experiments overnight precultivation was performed in 10 mL YPD medium by single colony inoculum in 50 mL sterile conical tubes. On the other hand, for the pilot scale bioreactor experiments precultivation was performed using BMGY medium. For the *P. pastoris* M13 strain pilot scale bioreactor experiments, the composition of the medium is given in the following section.

3.6.1.2 Precultivation of M13 strain

For the precultivation of M13 strain, *P. pastoris* M13 cells from microbanks were grown on YPD agar slants containing 0.1 g L^{-1} zeocin for 48 to 60 hours at 30°C. The precultivation method differs for the laboratory scale air filtered shake bioreactor experiments than that used for the pilot scale

bioreactor experiments. In the laboratory scale shake bioreactor experiments, a single colony chosen on the solid medium inoculated into BMGY (Buffered Glycerol Complex Medium) containing air-filtered 150 cc bioreactors with a working volume of V=20 cc for 20-24 hours at T= 30°C and N= 225 rpm until OD_{600} has reached 6-10. The cells were harvested by centrifugation at 4000g for 10 minutes. The composition of BMGY medium is given in Appendix B.

In the pilot scale experiments, a single colony was chosen on the solid medium and inoculated into BMGY medium in baffled and air-filtered 250 cc bioreactors with a working volume of V=50 cc for 20-24 hours at T=30°C , N= 225 rpm until OD_{600} has reached 2-6. The cells were harvested by centrifugation at 4000g for 10 minutes.

3.6.2 rhGH Production in Laboratory Scale Air Filtered Shake Bioreactors

Laboratory scale experiments were carried out in baffled and air-filtered 250 mL bioreactors with a working volume of V=50 cc.

3.6.2.1 rhGH Production in Laboratory Scale Air Filtered Shake Bioreactors with G7 strain

To select the best rhGH producing *Pichia pastoris* transformant carrying the GAP promoter, all the GAP promoter carrying *Pichia pastoris* colonies were tested separately in V=50 cc shake-bioreactor experiments, in YPD medium containing 2% (w/v) glucose. The fermentation experiments started by inoculating 100 µL inoculum from the overnight pre-cultivated medium of every transformant, into the shake-bioreactors and cultivated for t= 72 h.

3.6.2.2 rhGH Production in Laboratory Scale Air Filtered Shake Bioreactors with M17 strain

In the shake-bioreactor experiments by *Pichia pastoris* M17 strain, BSM (Basal Salt Medium) + PTM1 (Pichia Trace Salt) medium containing the alternative co-carbon sources, i.e., sorbitol and mannitol, were used. The cells

harvested from the precultivation medium were resuspended in the production medium in such a way that each bioreactor has an initial OD_{600} of 1. At t=0 h and in every 24 hours, 1% (v/v) methanol was added for induction. Batch fermentations were carried out for 60 hours at T= 30°C and N= 225 rpm. The composition of the nitrogen source was determined from the carbon/nitrogen and methanol/nitrogen ratios, which were 4.57 and 2.19 in the medium, respectively (Jungo *et al*. 2006). The compositions and methods for preparation of BSM (Jungo *et al*., 2006) and PTM1 (Sibirny *et al*., 1987) solutions are given in Appendix B. In order to compare the effects of the alternative co-substrates sorbitol and mannitol in shake-bioreactor experiments, mannitol and sorbitol concentrations, respectively, were set to 30 g L^{-1} and 50 g L^{-1} in the initial BSM medium.

3.6.3 rhGH Production in Pilot Scale Bioreactor

Pilot scale, recombinant human growth hormone production experiments using each recombinant *Pichia pastoris* strains, i.e., M13 and G7, were performed in a 3.0 L bioreactor (Braun CT2-2). The bioreactor consisted of a system of working volume V=1-2 dm^3, with temperature, pH, foam, dissolved oxygen, and stirring rate control; which was stirred with two four-blade Rushton turbines, and equipped with controlled positive-displacement pumps enabling continuous feeding of the substrate and co-substrates, separately, for semi-batch operation. Temperature control and sterilization was carried out by using the jacket around the bioreactor, using pressurized steam from a steam-generator and water at T= 30°C from an external cooler-circulator. pH was measured continuously by using a pH probe (Hamilton, Switzerland), and controlled by 25% NH_4OH (NH_3 in water) base and 2N phosphoric acid (H_3PO_3) acid solutions, each fed by peristaltic pumps on the bioreactor. Ammonium hydroxide is used also as the nitrogen source. Dissolved oxygen concentration was kept at a pre-set value, i.e., 20% of the saturation value, by pumping air using an air compressor (Larfon Top Silent 1.5, Italy), or pure oxygen from

pressurized oxygen-tubes, or by oxygen enriched air feed through a sparger; together by stirring at N= 900rpm. Oxygen inlet rate was adjusted by using mass flow controller. The schematic representation of cultivation steps and bioreactor system is illustrated on Figure 3.1.

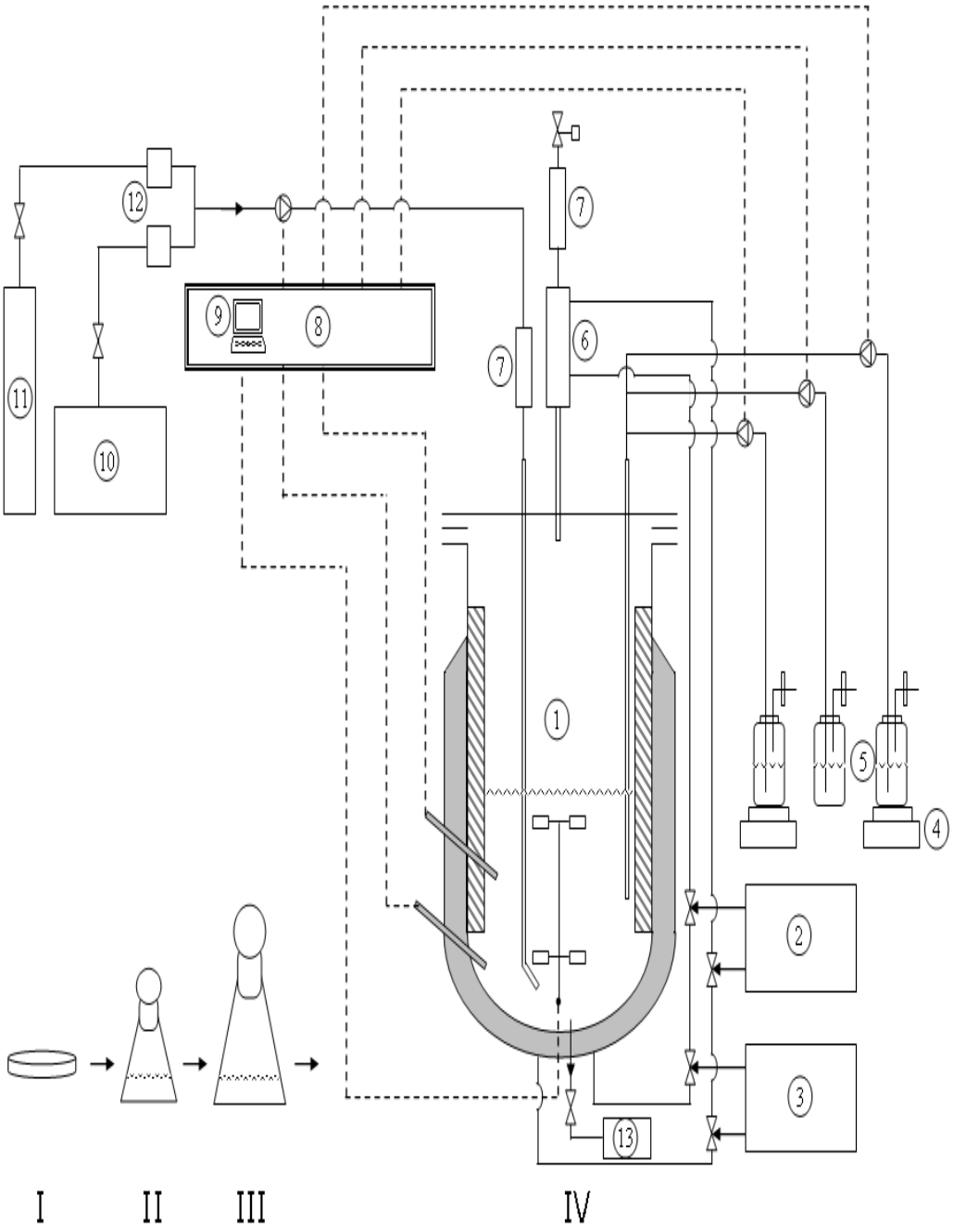

Figure 3.1 Cultivation scale up steps and pilot scale bioreactor setup. I: Solid medium inoculated from stock culture; II: First pre-cultivation medium; III: Second pre-cultivation medium IV: Pilot scale bioreactor setup composed of (1) Bioreaction vessel Biostat CT2-2 (2) Cooling circulator (3) Steam 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)

Production mediums, phases and bioreactor operation parameters fed batch given substrates were different according to genotypes and induction mechanisms of developed *P. pastoris* strains which are M13 and G7 strains.

3.6.3.1 rhGH Production in Pilot Scale Bioreactor by M13 strain

In rhGH production by *P. pastoris* carrying AOX1 promoter (M13 strain), after the pre-cultivation of the cells, the semi-batch fermentatation process proceeded through four phases, which are glycerol batch (GB), glycerol fed-batch (GFB), methanol transition (MT), and continuous methanol fed phases (MFB). For the expression of rhGH in *P. pastoris* under the control of AOX1 promoter, a standard protocol (Stratton *et al*., 1998) modified by Çelik *et al*. (2009) was used.

3.6.3.1.1 Bioreactor Operation Parameters for M13 strain

The bioreactor operation conditions for the experiments carried out by M13 strain, i.e., temperature, pH, dissolved oxygen concentration, were same as in the experiments carried out by Bozkurt (2012). Temperature was set and kept at T= 30°C isothermal condition, and pH was set and kept at 5.0 and contralles as mentioned in 3.6.3. The fermentations were followed by taking samples, taken at every 3 hours within the first 24 hours, and then at every 6 hours. The samples were centrifuged at +4°C and 4000g for 10 minutes. Supernatants were transferred to the fresh tubes and both pellets and supernatants were kept at -80°C.

3.6.3.1.2 Precultivation, Mannitol and Methanol Feeding in Fed-Batch Pilot Scale Bioreactor Operations

The cells harvested from the precultivation medium (section 3.6.1.2.) were inoculated and resuspended into the bioreactor where the initial OD_{600}=1, which corresponds to a cell concentration of 0.275 g dm^3, and the first phase of the fermentation, glycerol batch phase is started, as follows:

Glycerol batch phase (GB): This phase is initiated with the inoculation of the cells into the fermentation medium which contains 40 g dm^{-3} glycerol. The aim in this phase to increase *Pichia pastoris* M13 concentration, which act as the micro-bioreactor within the bioreactor. As can be understood from its name, the substrate is glycerol and used batch-wise.

Glycerol fed-batch phase (GFB): In this phase the bioreactor system is semi-batch for the substrate glycerol. Glycerol solution (50 %(v/v) glycerol in 12 cc dm^{-3} PTM1) is fed continuously to the semi-batch operated bioreactor, with a pre-calculated exponential feeding rate. The aim of GFB phase is to increase *P.pastoris* concentration further without glycerol accumulation in the medium, which prepare the cells to carbon source change, that is from glycerol to methanol. GBF phase proceeds for 6-12 hours until the cell concentration reached to a value within OD_{600}=80-90 which corresponds to C_X=20-23 g L^{-1}. Since glycerol is a repressive carbon source for AOX1 promoter, during the first two phases there is no recombinant protein synthesis in the cells.

Methanol Transition Phase (MT): In MT phase, methanol is used as the substrate, and the bioreactor system is semi-batch for methanol. The aim is to adapt the *P.pastoris* cells to methanol by activating its methanol metabolism. Methanol solution (100% (v/v) methanol in 12 cc dm^{-3} PTM1) is fed continuously to the semi-batch operated bioreactor, with a feeding profile provided by "Invitrogen Pichia Fermentation Process Guideline (Invitrogen 2002), i.e., 3.6 cc h^{-1} per dm^3 of initial fermentation volume within 4 hours.

Methanol fed-batch phase (MFB): MFB is the recombinant protein production phase of the bioprocess, where methanol is the first substrate which fed continuously (100% (v/v) methanol in 12 mL dm^{-3} PTM1) to the semi-batch bioreactor, with pre-calculated feeding rate based on the pre-determined μ_{M0}=0.03 h^{-1}. Moreover, in this work mannitol was used as the co-substrate based on the findings of the shake-bioreactor experiments. In the semi-batch operated bioreactor, mannitol was used batchwise in the experiment MM1, nevertheless, fed continuously in the experiments MM2 and MM3.

In MM1, mannitol was used batch-wise, using 250 g dm^{-3} sterile solution in BSM to attain C_{Man0}=50 g L^{-1} initial mannitol concentration in the bioreactor at t=0 h, where mannitol concentration in the medium was checked by HPLC in every three hours. In the second (MM2) and third (MM3) experiments, mannitol was fed continuously (250 g dm^{-3} sterile solution of mannitol in BSM) to keep its concentration at 50 g dm^{-3} in the bioreactor. Mannitol feeding in these experiments was performed by precalculation of mannitol feeding rate based on pre-determined specific mannitol uptake rate of the cells. Mannitol feeding was terminated at t=6 h for the second and third experiments by feeding a total of 100 g mannitol.

$$F(t) = \mu_0 V_0 C_{xo} / Y_{X/S} \exp(\mu_0 t) \quad (3.3)$$

or,

$$Q(t) = \mu_0 V_0 C_{xo} / C_{So} Y_{X/S} \exp(\mu_0 t) \quad (3.4)$$

In equation (3.3) μ_O is the pre-determined growth rate (h^{-1}), V_O is the initial fermentation volume (dm^3), C_{XO} (g dm^{-3}) is the initial cell concentration at t=0 h, $Y_{X/S}$ (g g^{-1}) is the cell yield on the given substrate. Parameters of equation 3.3 for glycerol, methanol are given in Table 3.9. Pre-determined exponential glycerol and methanol feeding profiles in of experiments were calculated in dm^3 h^{-1} by equation 3.3 and glycerol feeding profile is given in Figure 3.2.

Table 3.9 Parameters of Equation 3.3 for Glycerol, Methanol Fed-Batch Feeding

Parameter	Glycerol Fed-Batch Feeding	Methanol Fed-Batch Feeding
μ_0 (h^{-1})	0.18	0.03
$Y_{X/S}$ (g/g)	0.5*	0.42**
C_{S0}	630	790

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

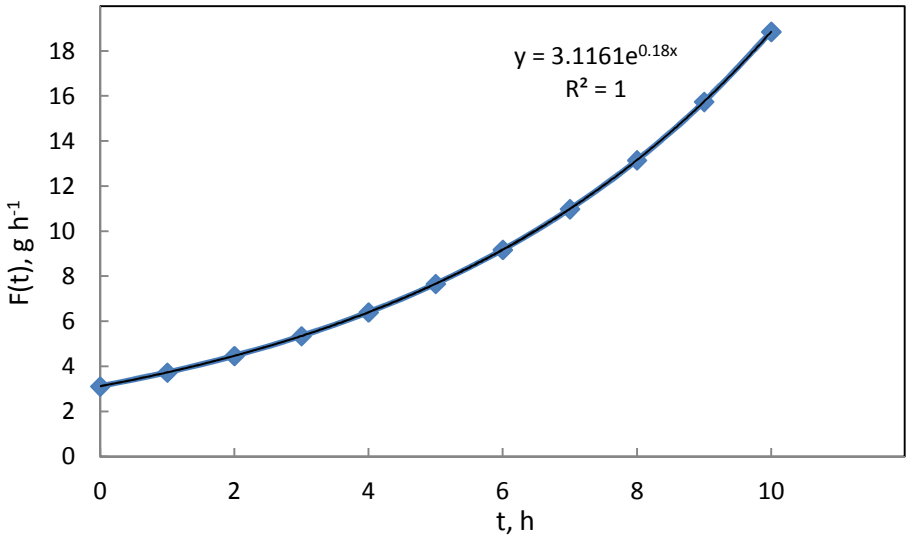

Figure 3.2 Pre-determined feeding profile for glycerol

3.6.3.2 rhGH Production in Pilot Scale Bioreactor with G7 strain

In rhGH production with *P. pastoris* G7 strain; constitutive GAP promoter induced rhGH production, there were two main phases of production after precultivation which are glycerol batch (GB), and glucose fed batch phases. As mentioned before, GAP is a strong constitutive promoter induced by many

carbon sources including glucose. GB phases were applied to increase cell density before the production.

3.6.3.2.1 Bioreactor Operation Parameters for G7 strain

The bioreactor operation conditions for the rhGH production by *P. pastoris* M13 strain were the same for the experiments by *P. pastoris* G7 strain.

3.6.3.2.2 Precultivation and Glucose Feeding in Fed-Batch Pilot Scale Bioreactor Operations

When the cell concentration reaches OD_{600}=2-6 (section 3.6.1.1.), the cells harvested from the precultivation medium were inoculated and resuspended into the bioreactor where the initial OD_{600}=1, which corresponds to a cell concentration of 0.275 g dm^{-3}, and the first phase of the fermentation, glycerol batch phase is started, as follows:

Glycerol batch phase (GB): This phase is initiated with the inoculation of the cells into the fermentation medium which contains 40 g dm^{-3} glycerol. The aim in this phase to increase *Pichia pastoris* G7 population, which act as the micro-bioreactor within the bioreactor. As can be understood from its name, the substrate is glycerol and used batch-wise. GB phase lasts when the optical density reaches to a value within OD_{600}=30-35; which corresponds to to ca. 10 g dm^{-3}cell concentration that take 15-18 hours.

Glucose fed-batch phase (GFB): In GFB phase the bioreactor system is semi-batch for the substrate glucose. Glucose solution (50 %(w/v) glucose in 12 mL dm^{-3} PTM1) is fed continuously to the semi-batch operated bioreactor, with a pre-calculated exponential feeding rate. The aim of GFB phase is to increase *P.pastoris* G7 concentration further without glucose accumulation in the medium, together with the production of the recombinant protein rhGH without glucose inhibition.

3.7 Analysis

Through the fermentation samples were taken for every 3 hours for first 24 hours after 24 hours samples were taken for every 6 hours and centrifuged at +4°C and 1500 g for 10 minutes. Supernatants were transferred to the fresh tubes and both pellets and supernatants were kept at -55°C. From supernatants, protein concentration, carbon source concentration, by-products concentration and extracellular protease activity were measured. Also from the harvested cells AOX enzyme activity was measured.

3.7.1 Cell Concentration

Cell concentration was measured using a UV-Vis Spectrophotometer (Thermo Spectronic, Hελιosα) at 600 nm as soon as sample was taken. The range is between 0.1 and 0.9 to read OD_{600}. Hence, in most of the cases samples taken from medium were diluted with dH_2O. To convert absorbance to cell concentration, C_X (g L^{-1}), equation (3.2) was used (Orman, 2008).

$$C_X = 0.275 \times OD_{600} \times \text{Dilution Ratio} \quad (3.2)$$

3.7.2 rhGH Concentration

rhGH concentrations were measured by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli (1970). The sample loading buffer and protein samples were mixed in 1:3 ratio and boiled for 4 minutes. 3 µL of a prestained protein molecular weight marker (Appendix E) and 15 µL of the samples were loaded to each well of the SDS gel. SDS gels were run at constant current of 40 mA for 45min and protein bands were visualized by silver staining.

Analysis has three main parts. All the buffers and solutions used in SDS-PAGE analysis are given in Appendix A.

Preparation of SDS polyacrylamide gel:

1) The glasses were cleaned and gel cast was set up according to manufacturer's instructions.

2) 12% resolving gel and 5% stacking gel were prepared without adding 10% (w/v) ammonium persulphate (APS) and NNN'N'-Tetramethylethylenediamine (TEMED) solutions in Erlenmeyer flasks. Resolving gel percent is decided according to molecular weight range. 12% resolving gel is appropriate for 10 kDa-200kDa range. Contents of gels are given in Appendix A.

3) Required amounts of APS and TEMED solutions were added in resolving gel and the contents were mixed vigorously. Then the solution was poured into gel cast by using a micropipette as quickly as possible to prevent bubble formation and polymerization. The gel cast was filled with resolving gel so that sufficient place left for stacking gel was left.

4) Thin layer of isopropanol was poured onto resolving gel to smooth the surface of gel.

5) The gel was left for polymerization at least 45 minutes. Then isopropanol on the gel was poured off and the space between glasses was washed with distilled water and dried with filter paper.

6) Required amounts of APS and TEMED solutions were added in stacking gel and the contents were mixed vigorously. Then the gel solution was poured into left space on the resolving gel as quickly as possible and the comb was put immediately with an angle so that no bubble is formed. Then, the stacking gel was left for polymerization at least 20 minutes.

7) After completion of polymerization, gels are used immediately or stored up to one week with wetted paper towels at 4°C.

Gel Electrophoresis:

1) The loading buffer and protein samples were mixed in 1:3 ratio by volume and boiled for 4 minutes and kept on ice for 5 minutes. Thereafter, the samples were vortexed and spun down.

2) Meanwhile the gels were placed in electrophoresis apparatus and the reservoir was filled with 1X SDS running buffer.

3) 15 µL of samples and 3 µL of prestained protein marker were loaded into wells of gels and the gels were run at 40 mA at constant current for 45 minutes.

Staining the SDS Gels (Blum et al, 1987):

Gels were silver stained as given as Table 3.9. Compositions and preparations of all required solutions were given in Appendix A.

Table 3.10 Silver staining for SDS-PAGE (Blum *et al*., 1987)

	Treatment	*Solution*	*Duration*	*Application*
1	Fixing	Fixer	≥1 hour	Overnight incubation acceptable
2	Washing	50% Ethanol	3X20 min	Should be fresh
3	Pretreatment	Pretreatment Solution	1 min	Should be fresh
4	Rinsing	Distilled water	3X20 sec	
5	Impregnation	Silver Nitrate Solution	20 min	
6	Rinsing	Distilled water	2X20 sec	
7	Developing	Developing Solution	~5min	After image starts to appear water can be added to slow down developing
8	Stop	Stop Solution	>10 min	Gels can be stored in this solution

3.7.4 Dot blot Analysis

The analysis was carried out with supernatant samples of shaker experiment to choose best rhGH producing colony of transfected *P. pastoris* cells. Pretreated Immobilon^{TM} PVDF membrane (Millipore, Bedford, MA) (0.45µm) was used for this purpose. All the required solutions are given in Appendix A.

1. The membrane was cut-off to size and one corner was to mark the sequence of samples.
2. The membrane was pre-wetted with methanol for 15 seconds then immersed in distilled water for 2 minutes and placed on a smooth clean surface.
3. 5 µL of samples was dropped with a micropipette in pre-determined order. Dropping of samples was repeated for three rounds until total amount of each sample on the membrane reached 15 µL.
4. The membrane was washed 2-3 times with TBS-T and immersed in TBS-T milk solution for 1 hour at room temperature with shaking to block unbound surface of membrane.
5. The membrane was washed with TBS-T three times with shaking at room temperature (15 minutes, 5 minutes, 5 minutes) with fresh changes of large volumes of TBS-T. Tween in TBS-T prevents unspecific binding of antibodies.
6. The primary antibody (monoclonal Anti-human growth hormone antibody, Fitzgerald, MA, USA) was diluted to the concentration of 1 µg/mL in TBS-T.
7. The membrane was transferred into a lid of 96-well microtiter plate or a similar low volume container and incubated in the diluted primary antibody for one hour with shaking.
8. The membrane was transferred to gel box and washed as in Step 5.

9. The secondary antibody was diluted as 1:5000 (antimouse IgG horseradish peroxidase-linked whole antibody, Amersham Biosciences, Uppsala, Sweden) in TBS-T.
10. The membrane was transferred to gel box and washed three times as 10 minutes per wash with shaking and fresh changes of large volumes of TBS-T at room temperature.
11. Dots were visualized by using Substrate-Chromagen Kit (S10 HRP, Biomeda, ABD).

3.7.5 Total Protein Concentration

Total protein concentration was measured by Bradford assay (Bradford, 1976). 100 µL of sample was incubated in 3 mL Bradford Reagent (BioRad) at room temperature in a dark place for 10 minutes. Color change was read at 595 nm by spectrophotometer. The calibration curve given in Appendix H was obtained by using bovine serum albumin (BSA) in the range of 0-0.75 mg mL^{-1}.

3.7.6 Methanol and Mannitol Concentrations

Concentrations of methanol and mannitol were measured by using reversed phase high pressure liquid chromatography (HPLC) (Waters HPLC, Alliance 2695, Milford, MA) on Capital Optimal ODS-5µm column (Capital HPLC, West Lothian, UK) (Çelik *et al*. 2009). The concentrations were calculated from HPLC chromatograms of standart solutions. Calibration curves both for methanol and mannitol were given in Appendix H. Samples and mobile phases were filtered by the 45 µm filter (ACRODISC CR PTFE) and dilution of samples were performed by the mobile phase of the system. 100-200 µL of samples were loaded to vials for the injections. All buffers and the ultra pure water was degassed by ultrasonic water bath for 15 minutes. Conditions and the system are given in Table 3.11.

Table 3.11 Conditions for HPLC system for methanol, glycerol and mannitol analyses

Column	: Capital Optimal ODS, 5 µm
Column dimensions	: 4.6 x 250 mm
System	: Reversed phase chromatography
Mobile phase	: 5 mM H_2SO_4
Mobile phase flow rate	: 0.5 mL/min
Column temperature	: 30°C
Detector type and wavelength	: Waters 2414 Refractive Index detector, 214 nm
Detector temperature	: 30°C
Injection volume	: 5 µL
Analysis period	: 10 min
Space time	: 5 min

3.7.7 Organic Acid Concentrations

Concentrations of methanol and mannitol were measured by using reversed phase high pressure liquid chromatography (HPLC) (Waters HPLC, Alliance 2695, Milford, MA) on Capital Optimal ODS-5µm column (Capital HPLC, West Lothian, UK). The concentrations were calculated from HPLC chromatograms of standart solutions. Calibration curves for all organic acids were given in Appendix I.. Samples and mobile phases were filtered by the 45 µm filter (ACRODISC CR PTFE) and dilution of samples were performed by the mobile phase of the system. 100-200 µL of samples were loaded to vials for the injections. All buffers and the ultra pure water was degassed by ultrasonic water bath for 15 minutes. Conditions and the system are given in Table 3.12.

Table 3. 12 Condition for HPLC system for organic acid analyses (Ileri and Calik, 2006)

Column	: Capital Optimal ODS, 5 µm
Column dimensions	: 4.6 x 250 mm
System	: Reversed phase chromatography
Mobile phase	: 0.312% (w/v) NaH_2PO_4 and 0.062% (v/v) H_3PO_4
Mobile phase flow rate	: 0.8 mL/min
Column temperature	: 30°C
Detector type and wavelength	: Waters 2487 Dual absorbance detector, 210 nm
Detector temperature	: 30°C
Injection volume	: 5 µL
Analysis period	: 15 min
Space time	: 5 min

3.7.7 Protease Activity Assay

Extracellular protease activity was evaluated by the hydrolysis of the casein. For three different protease activites, which are alkali, neutral and acidic, Hammerstein casein solution (0.5% w/v) was prepared in either 0.05 M borate buffer (pH=10.0), 0.05 M sodium phosphate buffer (pH=7.0) or 0.05 M sodium acetate buffer (pH=5.0), respectively. The compositions of all three buffers are given in Appendix A. The samples were diluted in related casein solution at required dilution. 2 mL of related casein solution was mixed with 1 mL of diluted sample. After that, hydrolysis was performed at T=30°C, 200 rpm for 20 minutes. To terminate the reaction 2 mL of 10% (w/v) trichloroacetic acid (TCA) solution was added to the mixture of reaction. The final solution was kept on ice for 20 minutes and after 20 minutes, centrifuged at 10500rpm for 10 minutes at +4°C.

Thereafter, the mixture with separated layers was kept at room temperature for 5 minutes. After five minutes, the absorbance of supernatant part was read at 275 nm in UV-Vis spectrophotometer. The absorbance should be between 0.2-0.6 and the dilution at the beginning of the analysis was accordingly.

In order to convert the measured absorbance value to protease activity, the correlation based on tyrosine liberated was used (Moon and Parulekar, 1991). One unit protease activity was defined as the activity that generates 4nmole tyrosine per minute. (U cm^{-3}) and the related equation was given in (3.4) (Çelik *et al*., 2008) .

$$A = \left(\frac{Absorbance}{0.8x1/\mu mol.cm^{-3}}\right)\left(\frac{1U}{4nmol/\min}\right)\left(\frac{1}{20\min}\right)\left(\frac{1000nmol}{1\mu mol}\right)\left(\frac{Dilution}{Ratio}\right) \quad (3.4)$$

3.7.8 AOX Activity

AOX activity was measured from cell pellet of 1 mL of the bioreactor medium. Since AOX is an intracellular enzyme of *P. pastoris*, cells of 1 mL medium was lysed to get contents of intracellular medium. The AOX activity assay was performed with intracellular medium. Steps of assay are given as follows:

Yeast Lysis:

Lysis of cells was achieved by adding yeast lysis buffer (Appendix A) on pellet at a final volume of 0.6 to 0.7 mL. Then cells were vortexed for 20 seconds and kept on ice for 30 seconds and these were repeated for three times. Thereafter, half spoon of glass beads were added on cells again cells were vortexed for 20 seconds and kept on ice for 30 seconds and these were repeated for three times. After last cycle, cell lysate were centrifuged at 3000 rpm and +4°C for 2 minutes. Supernatant was transferred to a fresh tube and centrifuged at 12500 rpm and +4°C for 5 minutes. After second centrifugation supernatant obtained was used in activity assay.

Activity Determination:

In this assay oxidation of methanol to formaldehyde by the AOX enzyme was monitored by the bi-enzymatic reaction of alcohol oxidase (AOX) and horseradish peroxidase (HRP). H_2O_2 concentration produced by the AOX enzyme was measured by the colorimetric system based on the combination of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP). The assay was performed at 25°C using the standard assay reaction mixture. The standard assay mixture contains 0.4 mM 4-AAP, 25 mM PSA, and 2 U/mL HRP in 0.1 M potassium phosphate buffer with pH 7.5. The reaction system in this assay are given as follows:

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

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

The quinoneimine dye which is liberated at the end of this two step series reaction. It gives a characteristic magenta color and it has maximum absorption around 500 nm. Color changes during the course of reaction was monitored by UV-Vis spectrophotometer at 500 nm. The increase in absorbance is proportional to the rate of liberation of H_2O_2 or to the rate of consumption of methanol. One unit of the AOX enzyme activity (U) was defined as the number of µmol of H_2O_2 produced per minute at 25°C (Azevedo *et al*., 2004).

The procedure of the analysis is as follows; 3 ml standard assay reaction mixture was put into a cuvette and 30 µL HPR, 375 µL methanol and 75 µL sample are added and mixed with that standard assay reaction mixture. As soon as sample was added the absorbance values at 500 nm is recorded in every 30 seconds of time for 4 minutes.

For conversion of absorbance value to specific AOX activity, equation (3.5) was applied. Calibration curve to obtain equation (3.5) is given in Appendix J.

$$C_{AOX}\left(\frac{U}{gCDW}\right) = 15.67\left(\frac{UmL^{-1}}{Absorbance}\right) \times OD_{500} \times \frac{1}{C_X} \qquad (3.5)$$

3.7.9 Glucose Concentrations

Glucose concentrations (C_G) were measured spectrophotometrically by using glucose analysis kit (Biasis, Ankara) by the glucose oxidation method (Boyacı, 2005). In this method D-glucose is oxidized to gluconate in the presence of the enzyme glucose oxidase and liberates H_2O_2. Liberated H_2O_2 is measured by the reaction with 4-aminoantiprine and phenol. This reaction gives iminoquinone dye rpoprotional to glucose concentration and observed color change UV/Vis Spetrophotometer at 505 nm. The reaction system including srie reactions is given as follows:

$$D - glucose + O_2 + H_2O \xrightarrow{Glucose\ oxidase} Gluconate + H_2O_2$$

$$H_2O_2 + 4 - Aminoantiprine + Phenol \xrightarrow{Peroxidase} Iminoquinine + H_2O$$

Analysis kit includes a glucose analysis reactive including the enzymes glucose oxidase and peroxidase; and 4-aminoantiprine; and a glucose analysis buffer including potassium dihydrogen phosphate and phenol. Samples containing more than 2 g L^{-1} glucose were diluted with water. 0.05 mL sample was mixed with 0.05 mL analysis reactive and 0.4 mL analysis buffer and 2.05 distilled water. The reaction mixture then incubated at 37°C for 20 minutes. Thereafter, absorbance values were measured by UV/Vis Spectrophotometer at 505 nm. In order to determine concentrations of samples, calibration curve obtained by glucose standarts is given in Appendix K.

CHAPTER 4

RESULTS AND DISCUSSION

The scope of this M.Sc. thesis is, to design and construct a new expression system for rhGH production by *P. pastoris* carrying hGH gene in the GAP promoter locus (G7); and then to compare the newly designed recombinant *P. pastoris* G7 with the previously designed (Orman *et al*, 2009) recombinant *P. pastoris Mut*$^{+}$ carrying hGH gene in the AOX1 promoter region (M13), with the originally designed semi-batch bioreactor strategies using appropriate substrates and co-substrates. In this context, in the first part of the study three feeding strategies were designed using methanol and mannitol as dual substrates in the pilot scale bioreactor system using previously constructed *P. pastoris* M13 strain. In the second part of the thesis, *P. pastoris* carrying hGH gene in the GAP promoter locus (G7) was constructed. Lastly, in the third part of the work, two semi-batch feeding strategy designed for the *P. pastoris* G7 strain using glucose as the substrate. The fermentation characteristics for the both recombinant systems were determined. In order to to make proper comparisons and to investigate the effects of the strategies on the cell growth, substrate consumption and rhGH production rates, alcohol oxidase (AOX) and protease activities were followed by taking samples.

4.1 rhGH Production by *P. pastoris* M13 strain

To enhance rhGH production, as an alternative co-substrate to sorbitol, influences of mannitol besides continuous feeding of the substrate methanol in a semi-batch bioreactor system were investigated; consequently, new semi-batch strategies were designed and compared for recombinant *P. pastoris Mut*$^{+}$ carrying hGH gene in the AOX1 promoter region (M13). In this context first, the substrates, i.e., mannitol and methanol, were used together in air filtered shake bioreactor experiments and compared with the results of the sorbitol and

methanol. The results revealed that with the co-substrate mannitol higher cell rhGH concentrations were obtained than that of sorbitol. On the bases of shake bioreactor experiments, three feeding strategy for the substrates methanol and mannitol were designed as semi-batch operations of the bioreactor.

4.1.1 Production of Recombinant Human Growth Hormone by *Pichia pastoris* M13 strain in batch bioreactors

To compare the alternative co-substrates and to determine the optimum initial concentration; using the initial 30 g L^{-1} and 50 g L^{-1} sorbitol and mannitol concentrations, the effects of the alternative co-substrates were investigated in batch bioreactors, separately. In all the batch shake-bioreactor experiments, methanol induction was conducted for every 24 hours as 1% (v/v). After the precultivation of the cells in BMGY media, the cells were harvested and inoculated into the production media (BSM), where the initial cell concentration was 0.55 g/L or initial optical density OD_{600} = 2. The batch fermentations proceeded for 48 hours and the effects of different co-substrates at different concentrations were determined.

The cell concentration profiles show that the highest cell concentration is obtained at 50 g L^{-1} initial mannitol concentration (Figure 4.1). The results reveal that the cell concentration is not directly proportional with the r-protein production that indicates the influence of the choice of the substrate on the intracellular reaction network, consequently, on the recombinant protein syntheses. However, higher cell concentrations give higher amounts of recombinant protein, as the cells are the micro-bioreactors within the bioreactor system. Recombinant hGH production amounts were determined by SDS-PAGE analysis and results are illustrated on Figure 4.2.

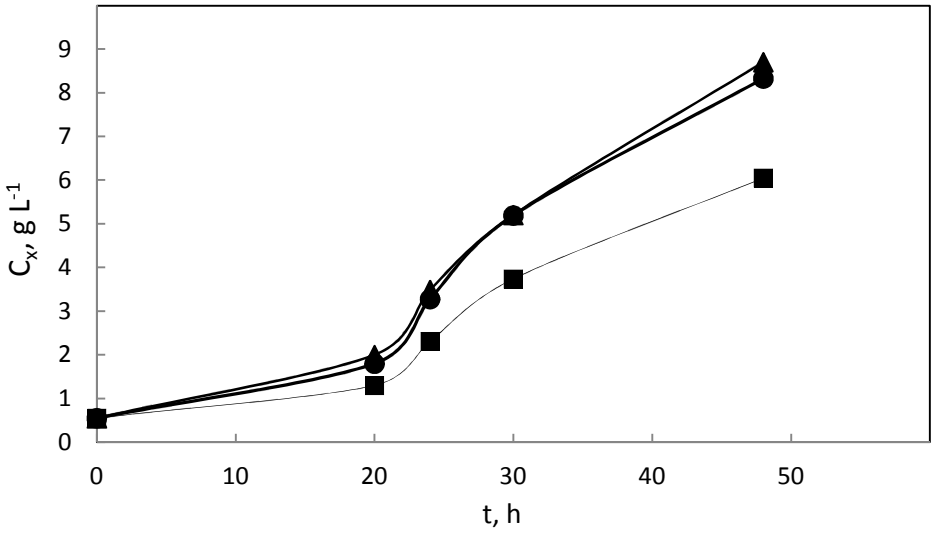

Figure 4.1 The variations in the cell concentration by the cultivation time at different mannitol concentrations, together with 1% (v/v) methanol. 30 gL^{-1} mannitol (●), 50 gL^{-1} sorbitol (■) , 50 gL^{-1} mannitol (▲)

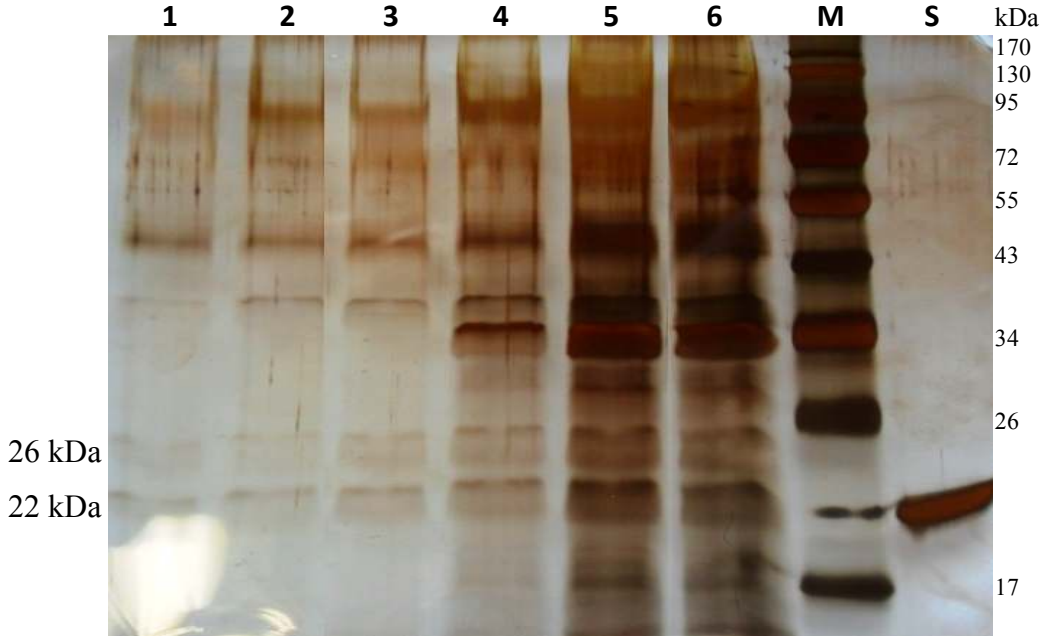

Figure 4.2 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in batch shake bioreactors to observe the effects of sorbitol/mannitol on the rhGH production; double band region indicated by arrow shows rhGH regions in protein bands Lane M: protein marker; Lane S: 0.2 g L^{-1} hGH standard, Lane 1: 50 g L^{-1} sorbitol-24 h,; Lane 2: 50 g L^{-1} mannitol-24 h; Lane 3: 30 g L^{-1} mannitol-24 h Lane 4: 50 g L^{-1} sorbitol-48 h, Lane 5: 50 g L^{-1} mannitol-48 h, Lane 6: 30 g L^{-1} mannitol-48 h

The maximum rhGH production was achieved at t=48 h in the medium containing C_{Man0}= 50 g L^{-1} (Figure 4.2). The highest cell concentration was also achieved in this medium. Therefore, the results of batch shake-bioreactor experiments revealed that mannitol appears as a good alternative co-substrate to sorbitol. Therefore; for the alternative pilot scale semi-batch strategy design, 50 g L^{-1} mannitol concentration value was used.

4.1.2 Semi-Batch Strategy Alternatives by *Pichia pastoris* M13

Three semi-batch strategy alternatives for the fourth-phase of the bioprocess which proceeds through four phases as mentioned in section 3.6.3.1.1, were designed which are namely, MM1, MM2 and MM3 (Table 4.1) by using the substrates methanol and mannitol. Glycerol batch (GB) phase was performed with the initial 40 g L^{-1} glycerol concentration; glycerol fed-batch (GFB) phase was performed by continuous feeding of glycerol solution with a precalculated feeding rate based on the predetermined specific growth rate of μ_0=0.18 h^{-1}; the third phase, which is the last phase perior to the production phase, is methanol transition (MT) phase, where 100% methanol in 12 mL L^{-1} PTM1 solution was fed continuously with rate of 3.6 mL h^{-1} or 2.8 g h^{-1} per liter of reaction volume. All designed alternative semi-batch operations performed in this thesis and the previously performed semi-batch operations experiments designed in our laboratory are summarized with their abbreviations in Table 4.1 and 4.2, respectively, for comparison of the results.

4.1.2.1 Semi-Batch Strategy Design with Mannitol and Methanol

In MM1, mannitol was the batch component started with C_{Man0}= 50 g L^{-1} concentration at t=0 h; further, at the two consequtive cultivation times of t=8 h and t=15 h by pulse-mannitol feeding the mannitol concentration in the bioreactor was increased to C_{Man}= 50 g L^{-1}. In MM1 methanol was fed

continuously with a pre-calculated feeding rate based on the pre-determined specific growth rate of μ_{M0}=0.03 h^{-1}. Methanol feeding profile is presented in Figure 4.3. In MM1 it was observed that after mannitol consumed in the fermentation medium, as the cell concentration was increased to higher amounts than expected, methanol feeding became insufficient probably not only for rhGH production but also for the obligatory cellular metabolism for the maintanance. This observation was based on the oxygen spikes and low oxygen demand during the period when mannitol was consumed. The cell concentration profile was fluctuating showing a tendency to decrease especially after t=21 h (Figure 4.7); besides, after t=18 h rhGH concentration was drasticly decreased (Figure 4.10), indicating that the semi-batch MM1 process should be ended at t=21h. The experimentally determined total specific cell growth rate, μ_t, was higher than the pre-determined μ_{M0} (0.03 h^{-1}), which is indeed the influence mannitol (Table 4.3). On the other hand, with the co-substrate sorbitol methanol insufficiency does not occur because of lower specific uptake rate of sorbitol as reported by Bozkurt (2012) (within q_S=0.025-0.165 g g^{-1} h^{-1}), compared to the specific mannitol uptake rates which were calculated within q_{Man}= 0.148-0.299 g g^{-1} h^{-1}. The results reveal that mannitol consumption rate ca. 2-3 -fold faster than sorbitol, and provided higher cell growth.

The cells show high demand to oxygen; and pH has a tendency to decrease when cells grow especially on methanol and synthesize both the cellular and recombinant proteins. Therefore, it is concluded that low oxygen demand and pH increase in the bioreactor should be the signal of insufficiency of carbon source even for the survival of cells. Therefore, the next semi-batch strategy was designed on the basis of the MM1 results, and precautions were taken accordingly due to the course of the dissolved oxygen concentration.

In MM2, the semi-batch process started by batch-wise mannitol with the concentration C_{Man0}= 50 g L^{-1} at t=0; besides, mannitol concentration in the bioreactor was kept at C_{Man}= 50 g L^{-1} by continuous mannitol feeding with a pre-

calculated feeding rate, based on the specific mannitol consumption rate of MM1, which is q_{Man} =0.27 g g^{-1} h^{-1} (Table 4.3). Mannitol was kept at 50 g L^{-1} until 50 g L^{-1} cell concentration was reached, as was in the experiment SSM1 (Bozkurt, 2012). Therefore, mannitol feeding was terminated at t=6 h when 50 g L^{-1} cell concentration obtained; where, cumulatively 100 g mannitol was fed. Mannitol feeding profile is illustrated in Figure 4.4. As was mentioned methanol feeding was continued according to pre-calculated feeding profile beginning from t=0 h until t=19.5 h. As, oxygen spikes and a pH increase were observed at t=19.5 h, a pulse methanol feed was introduced within half an hour. Since a sharp increase in cell concentration was seen when mannitol was consuming by cells, it can be said that a so-called second methanol transition was applied for half an hour between t=19.5 and 20 h of production especially to keep bioprocess running. In MT phase before the production methanol was fed as 3.6 mL h^{-1} (2.8 g h^{-1}) per initial liter of fermentation medium when Cx was approximately 25 g L^{-1}. At t=18 h of production cell concentration was approximately 100 g L^{-1} that is four-fold of MT phase. Hence, 14.4 mL h^{-1} or 11.4 g h^{-1} methanol; that is 5.7 g methanol was fed to medium batch-wise per liter of fermentation medium for half an hour. After second methanol transition was completed at t=20 h of production, dynamic feeding strategy was employed for the methanol. New methanol profile which was higher than that of calculated at t=0 h, was calculated with again according to predetermined specific growth rate of μ_{M0}=0.03 h^{-1} but with new Cx_0 at that time and this time was taken as t=0 h for calculation new feeding profile. Methanol feeding profile is illustrated in Figure 4.3.

In the third experiment; MM3, same as MM2; again 50 g L^{-1} initial mannitol pulse was fed and then, mannitol was kept at 50 g L^{-1} until cell concentration reached 50 g L^{-1}. Different than MM2, in MM3 dynamic methanol feeding profile began earlier without applying a second methanol transition, meaning that, recalculation of methanol profile began earlier, since strong positive effects of changing and increasing methanol was observed in late hours

of MM2. Dynamic methanol feeding was employed after t=12 h, as soon as mannitol was totally consumed, by recalculating methanol feeding profile in every three hours by changing Cx_0 and setting feeding time t=0 h in every three hours again according to pre-determined specific growth rate of 0.03 h^{-1}. In usual fed-batch methanol feeding, feeding was performed according to parameters at t=0 h and pre-determined μ_{M0}=0.03 h^{-1}. However, the specific cell growth rate was much higher than pre-determined μ_{M0} due to mannitol co-feeding (Table 4.3). Therefore, methanol feeding with initial parameters became inadequate and needed to increase. Since new profiles of methanol feeding were recalculated as soon as mannitol was consumed; decrease in oxygen demand and pH increase tendency were never observed. Methanol feeding profile was shown in Figure 4.3. All the feeding strategies applied with co-feeding of mannitol are summarized in Table 4.1.

As mentioned before in MM2 in order to prevent early ending of bioprocess a so-called methanol transition is applied between t=19.5 and 20 h of production by 5.7 g batch-wise feeding of methanol to the medium. After that new methanol profiles were recalculated for every three hours by considering response of cell growth to co-carbon source mannitol and methanol feeding. However, it should be pointed out that the reason for this increased methanol feeding at late hours of production was mostly to prevent early ending of bioprocess due to much more increased cell mass than expected by pre-determined methanol profile by μ_{M0}=0.03 h^{-1}. The reason for this much cell increase can be very fast consumption of co-carbon source mannitol. Methanol limitation was not observed in experiments with sorbitol co-carbon source whereas there were clearly carbon source limitations in mannitol experiments because sorbitol consumption is not higher as mannitol. However, protein concentration analysis showed that batch-wise methanol feeding and increased methanol feeding profile in late hours of MM2 gave 4.8-fold increased protein production when compared to maximum amount before the second methanol transition phase in MM2, further discussion will be given in section 4.1.2.4.

Since 1.9 fold increased rhGH production was obtained in MM2 when compared to maximum rhGH production up to date which was 640 mg/L (Bozkurt, 2012), in order to understand effect of changing methanol deeply, a further experiment was carried out which was the MM3. The main purpose of this experiment to understand whether positive effect of increased methanol on rhGH production was due to batch-wise feeding of methanol in production hours or just increased feeding of methanol with respect to cell growth response.

Table 4.1 Definition of feeding strategies developed in this study

Strategy Name	***Strategy Definition***
MM1	Pulse mannitol feeding at t=0, t=8 and t=15 h to increase C_{man}= 50 g L^{-1} and methanol feeding with μ_{M0}=0.03 h^{-1}.
MM2	Pulse mannitol feeding at t=0 h to increase C_{man}= 50 g L^{-1} mannitol concentration at 50 g L^{-1} between t=0-6 h and methanol feeding with μ_{M0}=0.03h^{-1} until t=19.5h, at t=19.5h second methanol transition phase for 0.5 hour after t=20 h dynamic methanol feeding; to keep μ=0.03 h^{-1}.
MM3	50 g L^{-1} pulse feeding of mannitol at t=0 h and keeping mannitol concentration at 50 g L^{-1} between t=0-6 h and methanol feeding with μ_{M0}=0.03 until t=12h (when all mannitol was consumed), after t=12 h dynamic methanol feeding; to keep μ=0.03 h^{-1}.

The first strategy was compared to the strategy MSSS-0.03 (Inankur, 2010) in all aspects whereas the strategy 2nd was compared to SSM1 (Bozkurt, 2012). Until t=18 h of production hours of two experiments only parameter differs in two experiments was only co-carbon source, however, in rest of the production hours of two experiments methanol feeding differed also. The descriptive tabulation of these two experiments is given in Table 4.2.

Table 4.2 Definiton of feeding strategies used for comparison

Strategy name	***Strategy definition***
MSSS-0.03	50 g L^{-1} pulse feeding of sorbitol at t=0 h, t=14 and t=31 h of production and methanol feeding with μ_{M0}=0.03 h^{-1}
SSM1	50 g L^{-1} pulse feeding of sorbitol at t=0 h and keeping sorbitol concentration at 50 g L^{-1} between t=0-15 h; methanol feeding with μ_{M0}=0.03 h^{-1}

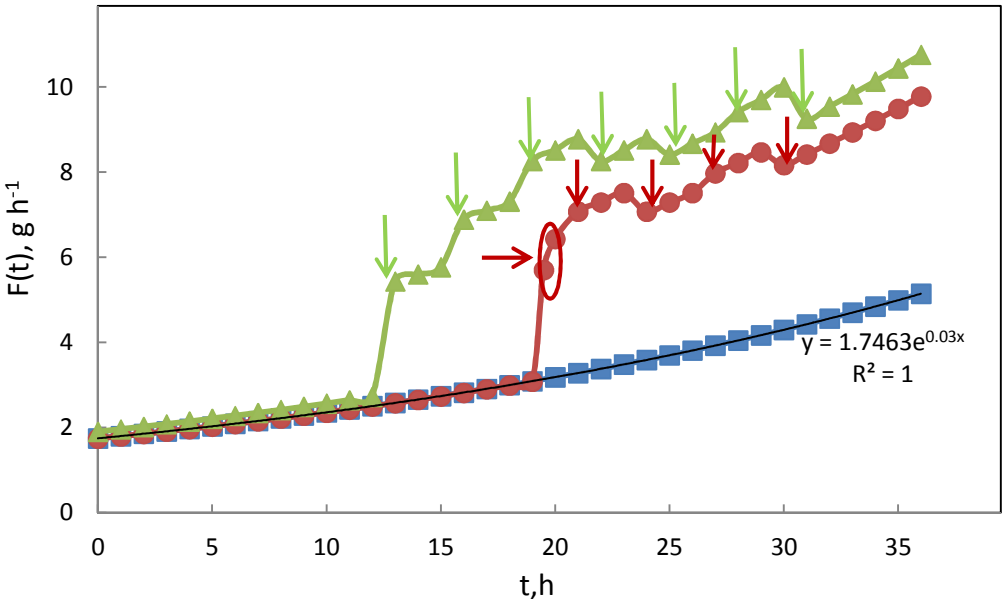

Figure 4.3 Methanol feeding profiles of MM1 (■), MM2 (●) and MM3 (▲). Blue dots show pre-determined methanol feeding profile in MM1 for μ_{M0} of 0.03 h^{-1}. Horizontal red arrow shows second methanol transition phase of MM2 and red vertical arrows indicate the times at which methanol feeding was changed in MM2. Vertical green arrows indicate the times at which methanol feeding was changed in MM3.

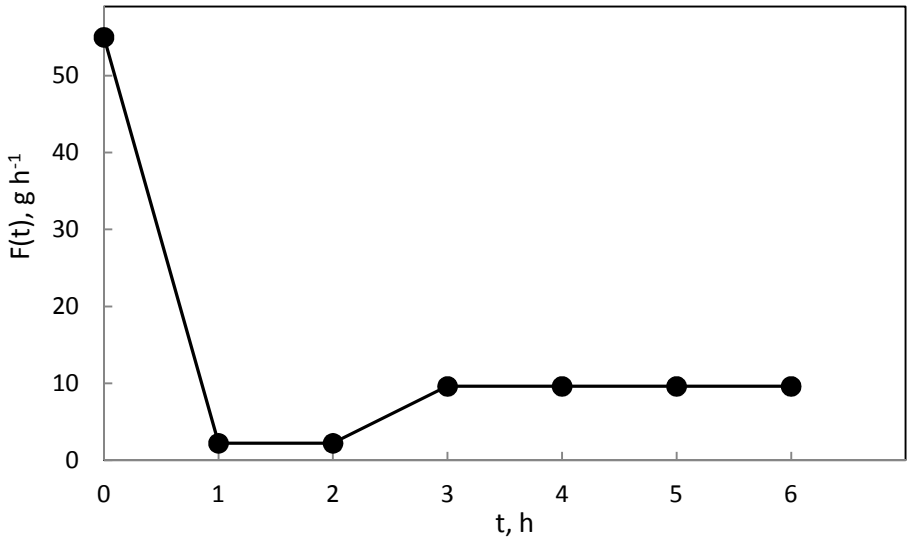

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Figure 4.4 Mannitol feeding profile in MM2 and MM3 to keep mannitol concentration at 50 g L^{-1} constant until t=6 h; 50 g L^{-1} cell concentration attained.

The last experiment; MM3 was applied to see effect of increased methanol feeding on rhGH production without applying methanol transition and results of the strategy third were compared to results of the strategy MM2. Hence the strategy MM3 is compared to MM2 in bioprocess characteristics.

4.1.2.2 Mannitol Consumption

As mentioned before, in MM1 mannitol feeding was performed batch-wise as 50 g L^{-1} pulses whereas in MM2 and MM3 mannitol concentration was set 50 g L^{-1} at the beginning of experiments and kept constant at this level, until t=6 h of the production phase.

In MM1, mannitol was fed from 250 g L^{-1} stock solution first at t=0 h of production to set initial mannitol concentration to 50 g L^{-1} in the bioreactor and when mannitol was totally consumed it was fed to the biorector (t=8 h; t=15 h) to increase concentration to 50 g L^{-1} in the medium (Figure 4.5). Mannitol was totally consumed at t=21 h. The specific mannitol consumption rate (q_{Man}) values were between 0.065-0.267 g g^{-1} h^{-1} for MM1 (Table 4.3). Related with q_{Man} values, it can be said that as mannitol concentration decreased in the medium, the values increased, too. Additionally, as cell concentration increased q_{Man} decreased, depending on mass transfer limitations due to high cell density in the medium. However, the results showed that q_{man} values depend mostly on concentration of mannitol rather than cell concenration.

In MM2 and MM3 mannitol was fed to bioreactor batch-wise at t=0 h to set initial mannitol concentration to 50 g L^{-1} and t=0-6 h of the production phase its concentration was kept constant at 50 g L^{-1} using the specific mannitol consumption rate values obtained in MM1 strategy. In MM2 and MM3 strategies, mannitol was totally consumed t=15 h. q_{Man} values were between 0.148-0.299 g g^{-1} h^{-1}. Here it is clear that concentration of mannitol affects q_{man} values, since, in these two experiments mannitol concentration did not change in t=0-6 h. Also, again as cell concentration increased, q_{man} values decreased.

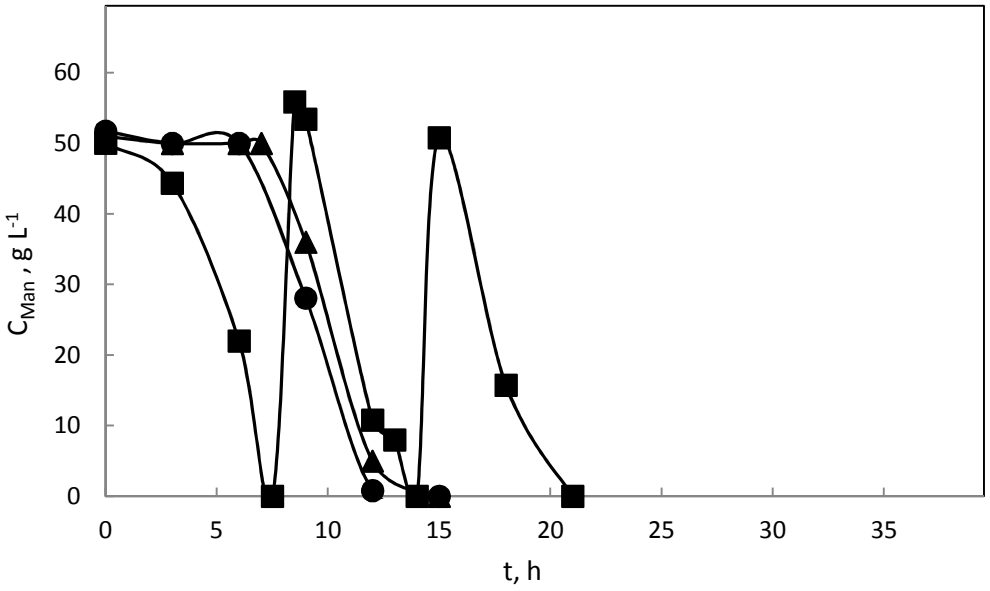

Figure 4.5 Variation in mannitol concentration within cultivation time of production phase: MM1 (■), MM2 (●) , MM3 (▲)

The experiment MM1 was compared to the MSSS-0.03 (Inankur, 2010) in terms of co-carbon consumption profiles; and comparison indicates that mannitol was metabolized significantly faster than sorbitol (Figure 4.6). One of the possible reasons for this much difference in consumption rates can be related with consumption pathway differences of these carbon sources. Both sorbitol and mannitol consumed in glycolysis pathway which is one of the basic carbon pathway of cells. Sorbitol enters the glycolysis pathway first by being oxidized to D-fructose by the enzyme D-Glucitol dehydrogenase and then phosphorylated to fructose-6-phosphate by the enzyme fructokinase (Figure 2.6). On the other hand mannitol can enter the glycolysis pathway in two different ways: first like sorbitol, by being oxidized to D-fructose and then phosphorylated to fructose-6-phosphate or in another way by being phosphorylated to mannitol-1-phosphate and then oxidized to fructose-6-phosphate to enter glycolysis pathway. Another possible reason can be difference in expression levels of enzymes used in mannitol and sorbitol

conversion. The enzymes converting mannitol can be expressed higher than the enzymes converting sorbitol in *Pichia pastoris* cells.

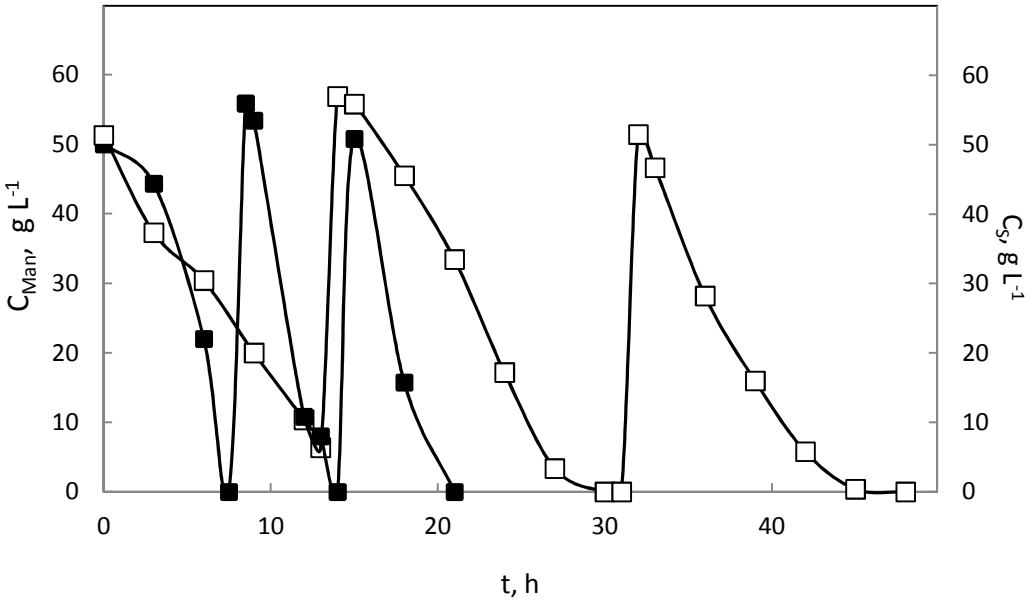

Figure 4.6 Co-carbon consumption profiles of *P. pastoris* M13 cells. MM1 (■) belongs to mannitol (primary axis) and MSSS-0.03 (□) belongs to sorbitol (secondary axis)

When the specific rates are compared both for sorbitol and mannitol it is concluded that mannitol is metabolized by cells much faster than sorbitol. The highest sorbitol uptake rate (q_s) found was 0.205 g g^{-1} h^{-1} at t=0 h in MSSS-0.03 (İnankur, 2010) and 0.144 g g^{-1} h^{-1} in SSM1 at t=0 h (Bozkurt, 2012) whereas the highest mannitol uptake rate (q_{Man}) found was 0.30 g g^{-1} h^{-1} at t=6 h (Table 4.3).

4.1.2.3 The Cell Growth

In all three feeding strategies GB, GFB and MT phases were performed in the same way. The cell growth profiles were given in Figure 4.7 both showing production phase and identical earlier phases for MM1, MM2 and MM3.

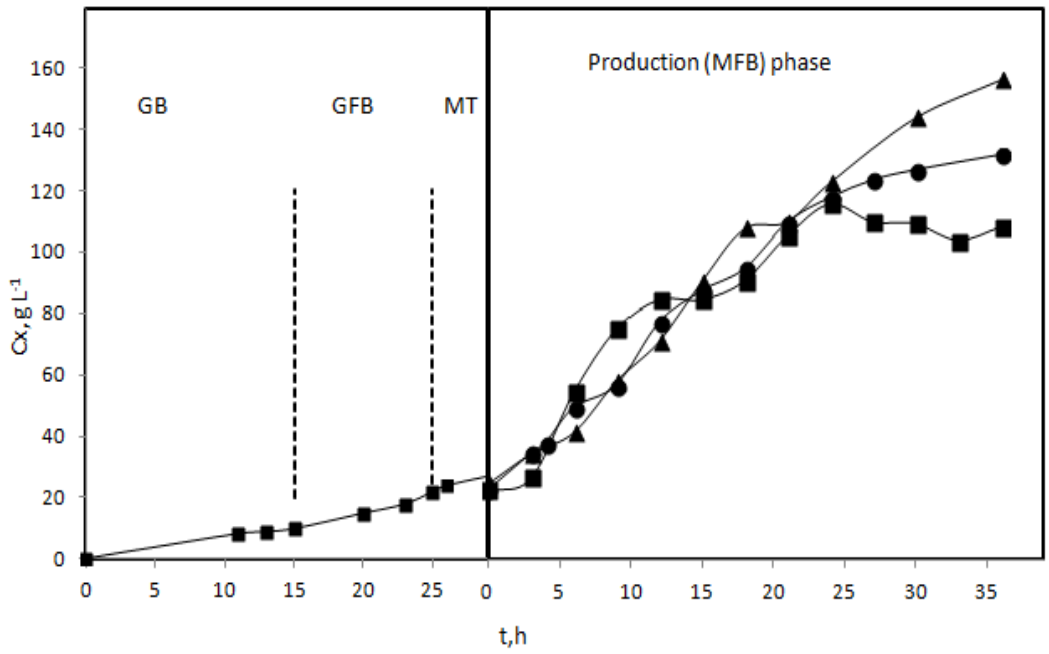

Figure 4.7 Variation in cell concentration with the cultivation time for feeding strategies MM1 (■), MM2 (●) , MM3 (▲)

The highest cell concentrations reached in MM1, MM2, and MM3 were 115.8 g L^{-1}, 127.1 g L^{-1} and 156.5 g L^{-1} which are 2, 2.2 and 2.7 fold higher than that of MSSS-0.03 (Inankur, 2010) and 1.1, 1.2 and 1.5 fold higher than that of SSM1 (Bozkurt, 2012), respectively. Related with cell concentration, growth profiles of MM1, MM2 and MM3 show similar profiles until t=21 h of processes. Until t=21 h, a total of 150 g mannitol was consumed in MM1, whereas this amount was 100 g in MM2 and MM3. However, additional 50 g mannitol did not affect the cell growth in MM1 (Figure 4.7). After t=21 h methanol feeding was changed and increased in MM2 and increase in cell growth was maintained as an effect of changing methanol profile. In MM3, the highest cell concentration was attained as an effect of increase methanol feeding.

Cell growth profiles of MM1 and MM2 are compared respectively with the cell profiles of MSSS-0.03 (Inankur, 2010) and SSM1 (Bozkurt, 2012) and shown in Figure 4.8 and Figure 4.9. When cell growth profiles are compared for

three pulses of sorbitol and mannitol feeding (Figure 4.8), it can be stated that mannitol is metabolized efficiently and it gives two-fold increased cell concentration even in early hours of production.

As mentioned before, in SSM1 (Bozkurt, 2012) sorbitol was fed until t=30 h of production where C_X= 55 g L^{-1} was generated. In MM2 mannitol was fed until approximately C_X= 50 g L^{-1} reached which corresponds t=6 h of production. So, MM2 reached almost same cell concentration of SSM1 24 hours earlier than SSM1. Mannitol shortened the bioprocess up to 24 hours when SSM1 and MM2 are compared in point of cell growth.

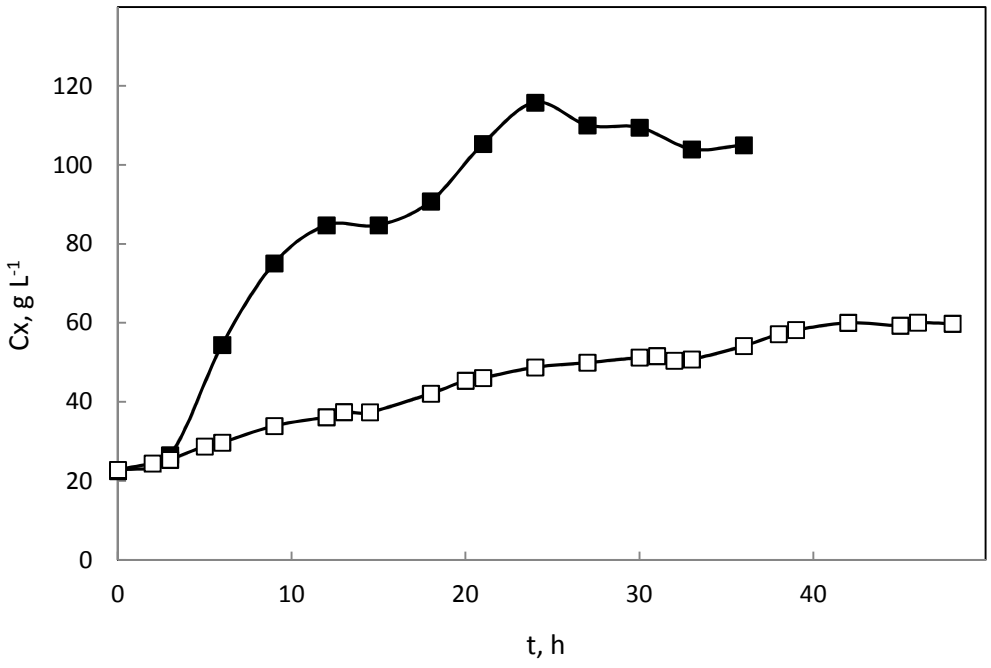

Figure 4.8 Cell growth profiles of *P. pastoris* M13 cells; MM1 (■) and MSSS-0.03 (□)

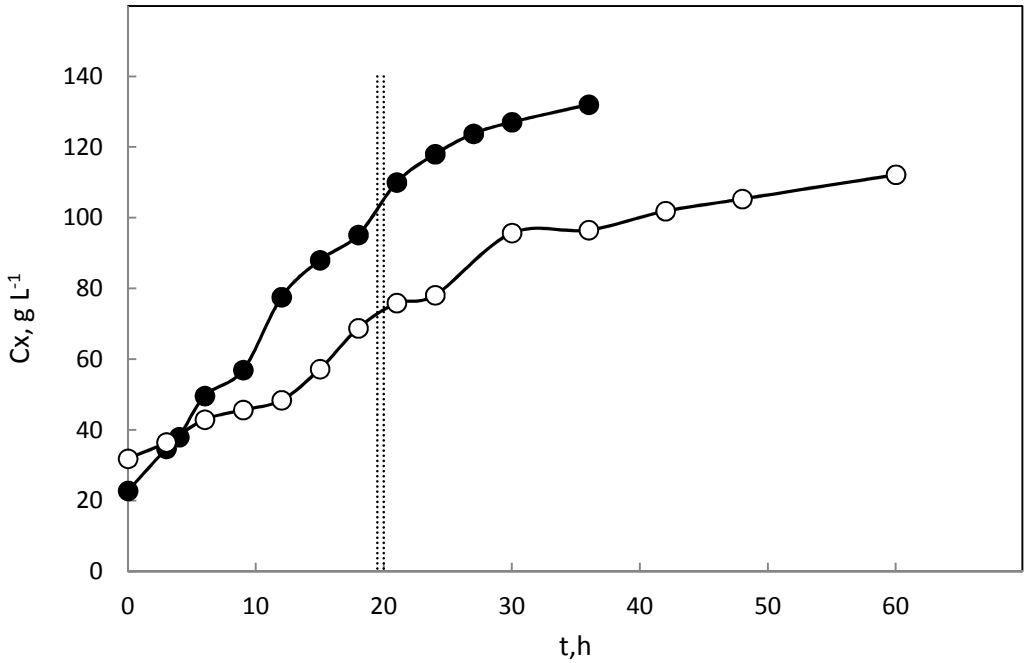

Figure 4.9 Cell growth profiles of *P. pastoris* M13 cells; MM2 (●) and SSM1 (o). Dashed lines indicate the hours second methanol transition phase of MM2.

Until t=12 h of production for MM2 and MM3 are the same in all process parameters including mannitol and methanol feeding. Until t=12 h of production MM2 and MM3 shows very similar cell growth profiles (Figure 4.7); however, after t=12 h, MM3 shows higher trend than MM2. The reason why MM3 showed higher trend in cell growth is the difference in methanol feeding after t=12 h of these bioreactors. Amount of methanol fed was higher for MM3 than for MM2 throughout the whole course of production after t=12 h (Figure 4.3).

The highest specific growth rates, μ_t, achieved in MM1, MM2 and MM3 were 0.343, 0.180 and 0.138 h^{-1}, respectively. Depending on the higher uptake rate of mannitol, maximum specific growth rates in MM1 was almost 6-fold of MSSS-0.03 (İnankur, 2010) and that of MM2 was approximately 1.5-fold of SSM1 (Bozkurt, 2012). However a drastic decrease even negative value in the specific growth rate was observed in late hours of MM1 which indicates the ending of bioprocess after total consumption of mannitol.

Table 4.3 Variation in specific rates within cultivation time

Experiment Name	t h	μ_t h^{-1}	q_{man} $g\ g^{-1}\ h^{-1}$	q_M $g\ g^{-1}\ h^{-1}$	q_{rp} $mg\ g^{-1}\ h^{-1}$
	3	0.343	0.276	0.029	0.471
	6	0.148	0.269	0.039	0.131
	9	0.060	0.191	0.027	0.395
	12	0.014	0.069	0.025	-
MM1	15	0.031	0.138	0.026	-
	18	0.042	0.065	0.029	-
	21	0.030		0.027	0.026
	24	-		0.027	-
	30	-		0.036	-
	36	-		0.047	-
	3	0.162	0.269	0.048	0.148
	6	0.042	0.299	0.034	0.088
	9	0.113	0.148	0.034	0.174
	12	-	0.164	0.028	0.251
	15	0.071		0.032	0.208
MM2	18	0.042		0.029	1.102
	21	-		0.046	0.798
	24	0.037		0.068	0.339
	27	0.005		0.061	0.347
	30	0.007		0.068	0.130
	36	0.056		0.070	0.261
	3	0.080	0.267	0.053	0.286
	6	0.095	0.299	0.046	0.135
	9	0.085	0.190	0.037	0.096
	12	0.076	0.150	0.034	0.252
	15	0.068		0.058	0.333
MM3	18	0.030		0.064	0.052
	21	0.022		0.077	-
	24	0.031		0.071	-
	30	0.023		0.067	-
	36	0.039		0.067	-

4.1.2.4 rhGH Production

Effects of feeding strategies designed on rhGH production amounts were analyzed by SDS-PAGE analyses of the samples. Variation in rhGH amounts by the cultivation time is plotted in Figure 4.10 by several parallel SDS-PAGE analyses. Screening of proteins in SDS-PAGE analyses were illustrated in Appendix F. The highest concentrations obtained are 250 mg L^{-1}, 1200 mg L^{-1} and 307 mg L^{-1} in MM1, MM2 and MM3, at t=15 h, t=36 h and t=18 h, respectively. They are 0.86, 4.1 and 1.1 fold of the highest rhGH concentration in MSSS-0.03 (Inankur, 2010) and 0.4, 1.9 and 0.5 fold of that of SSM1 (Bozkurt, 2012), respectively. The highest value achieved in MM2 as 1200 mg L^{-1} was 85% of the total protein at that hour whereas the highest value (250 mg L^{-1}) in MM1 is 75% and the highest value in MM3 (307 mg L^{-1}) is 43% of total proteins.

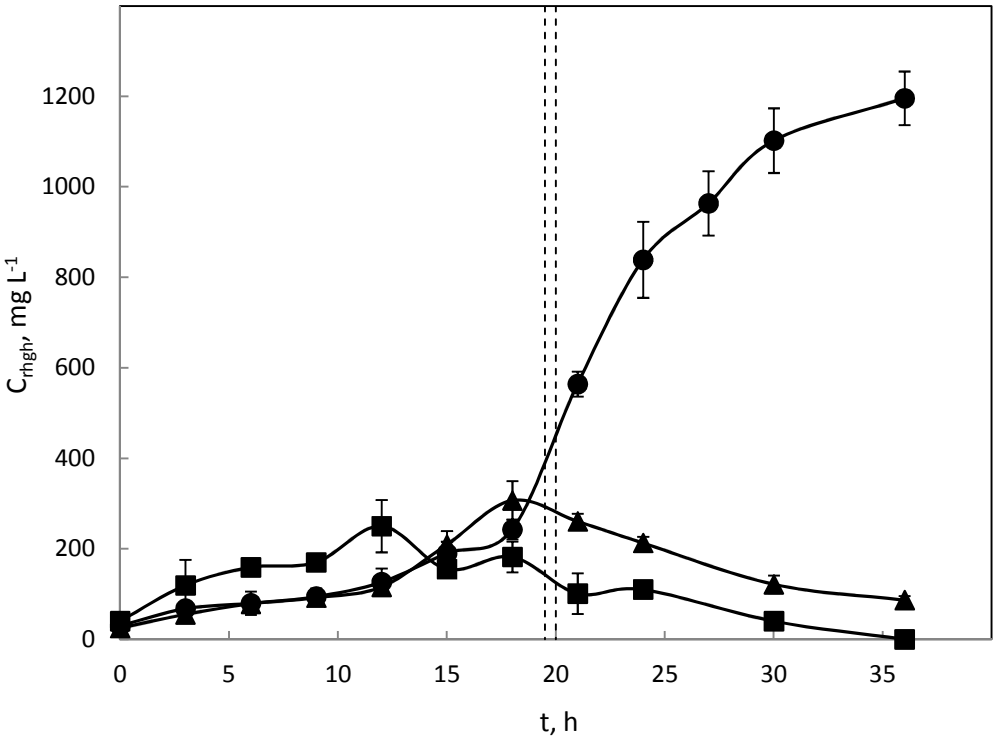

Figure 4.10 Variation in rhGH concentration with cultivation time: MM1 (■), MM2 (•) and MM3 (▲) (standard errors are calculated seperately for each data point). Dashed lines indicate the hours of second methanol transition in MM2.

The highest specific recombinant protein production rate (q_{rhGH}) is obtained as 1.1 g g^{-1} L^{-1} in MM2 which is 2.2-fold of that of SSM1 (Bozkurt, 2012). The maximum q_{rhGH} was obtained at t=21 h of MM2 as an effect of second methanol transition between t=19.5 and 20 h. Additionally, the maximum q_{rhGH} obtained in MM1 is 1.057 g g^{-1} L^{-1} at t=0 h and 0.409 g g^{-1} L^{-1} in MM3 again at t=0 h.

The highest concentration attained in MM1 C_{rhGH} =250 mg L^{-1} at t=12 h of production whereas maximum C_{rhGH}=290 mg L^{-1} attained at t=39 h in MSSS-0.03 (Inankur, 2010) (Figure 4.11). Although the highest concentration attained in MM1 is not high as in MSSS-0.03 (Inankur, 2010); C_{rhGH} =250 mg L^{-1} achieved at t=12 h of production, so mannitol co-feeding shortened the bioprocess up to 27 h.

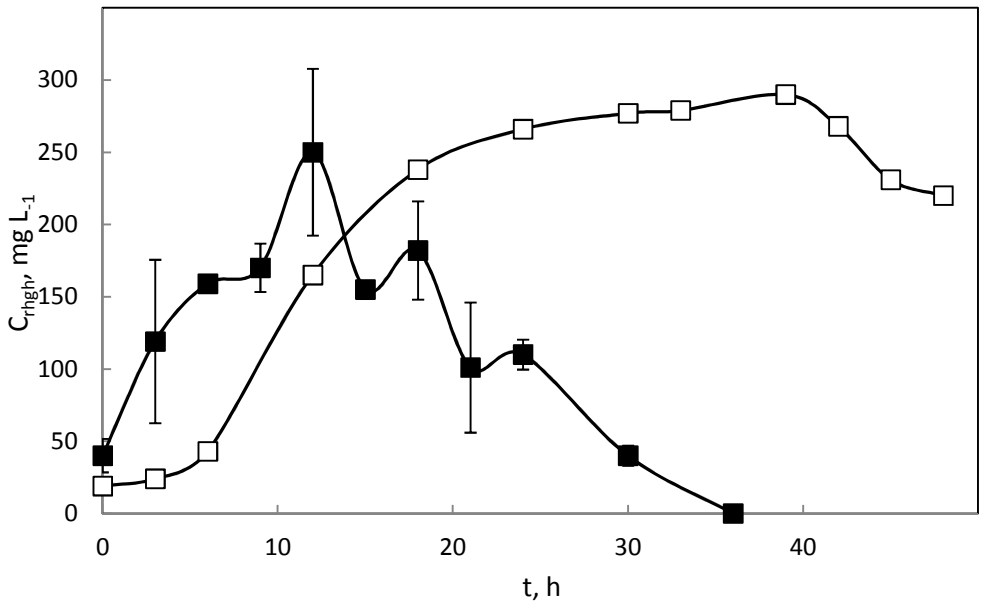

Figure 4.11 rhGH concentration profiles of *P. pastoris* M13 cells; MM1 (■) and MSSS-0.03 (□)

Until the second methanol transition phase of MM2; rhGH concentration profiles were similar to SSM1 (Bozkurt, 2012) however, recombinant protein concentrations in MM2 are almost 1.2-fold higher than that of SSM1 until second methanol transition phase as an effect of mannitol (Figure 4.12). Mannitol shortened the fermentation time when compared to the sorbitol. After second methanol transition recombinant protein concentration is increased approximately two-fold of SSM1.

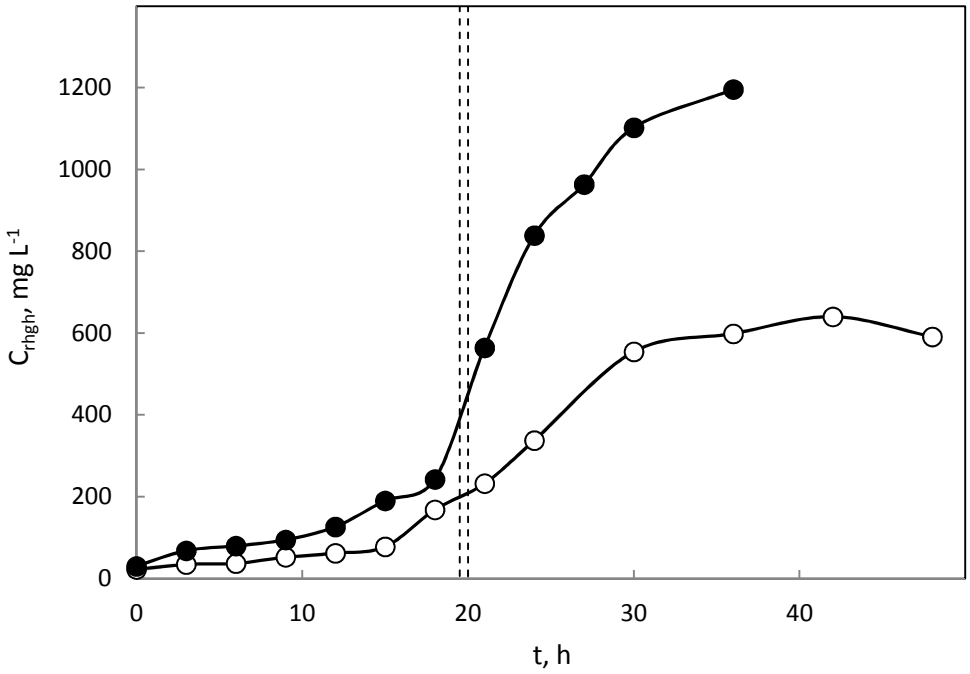

Figure 4.12 rhGH concentration profiles of *P. pastoris* M13 cells; MM2 (●) and SSM1 (o). Dashed lines indicate the hours second methanol transition phase of MM2.

In MM3 rhGH concentration was changed a little after change in methanol feeding (t=12 h). However, in later hours of the strategy MM3, decrease in rhGH concentration was seen (Figure 4.10). Although cell

concentration profiles show that the highest profile in MM3, recombinant protein production in MM3 was much lower than in MM2. Therefore, when recombinant protein productions of MM2 and MM3 compared, it can be said that how to feed increased methanol is much more important than to increase methanol feeding with co-feeding of mannitol.

4.1.2.5 Alcohol Oxidase Activity

Alcohol oxidase (AOX) initializes the methanol metabolism and is the key enzyme in methanol utilization of *Pichia pastoris*. Recombinant protein production begins with the induction of AOX1 promoter by the methanol. As an important parameter of recombinant protein production AOX activities were measured and presented in Figure 4.13 for MM1, MM2 and MM3.

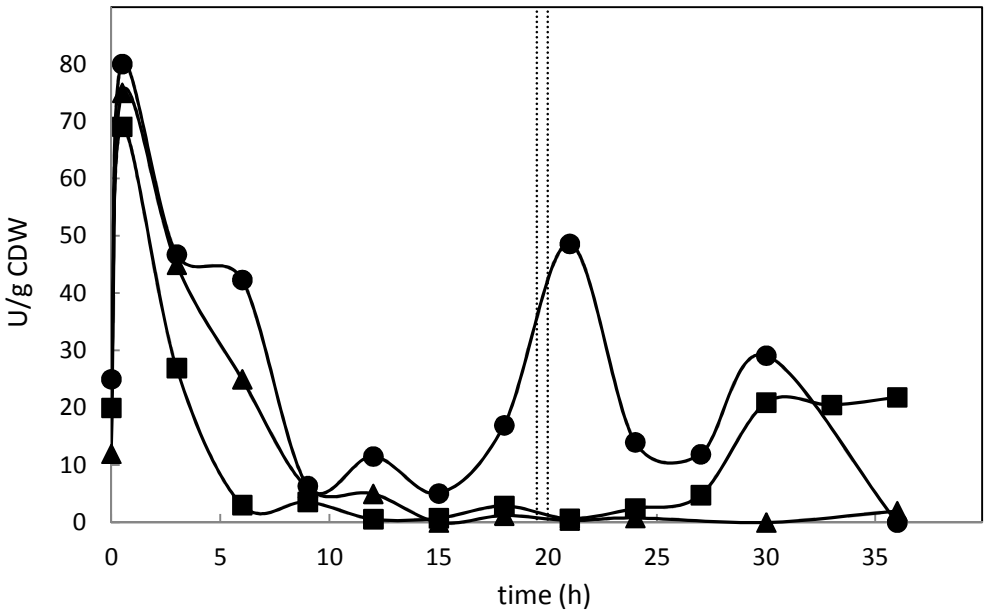

Figure 4.13 Variation in AOX1 activity with cultivation the time; MM1 (■), MM2 (•) and MM3 (▲). Dashed lines indicate the hours of the second methanol transition phase of MM2.

In all three feeding strategy experiments, AOX activities show their maxima at the beginning of experiment being 69, 80 and 75 U/g CDW for MM1, MM2 and MM3 respectively. In all the strategies after AOX activities gave their maxima at the beginning, they decayed rapidly to very low values. The highest specific rhGH production rates were also achieved at the beginning of the MM1 and MM3 also as an effect the AOX activities. Therefore, AOX activity profiles support the highest recombinant protein production was achieved earlier than MSSS-0.03 as an effect of mannitol. The highest activity of the MM1 was 1.8-fold of the MSSS-0.03 (İnankur, 2010) (Figure 4.14).

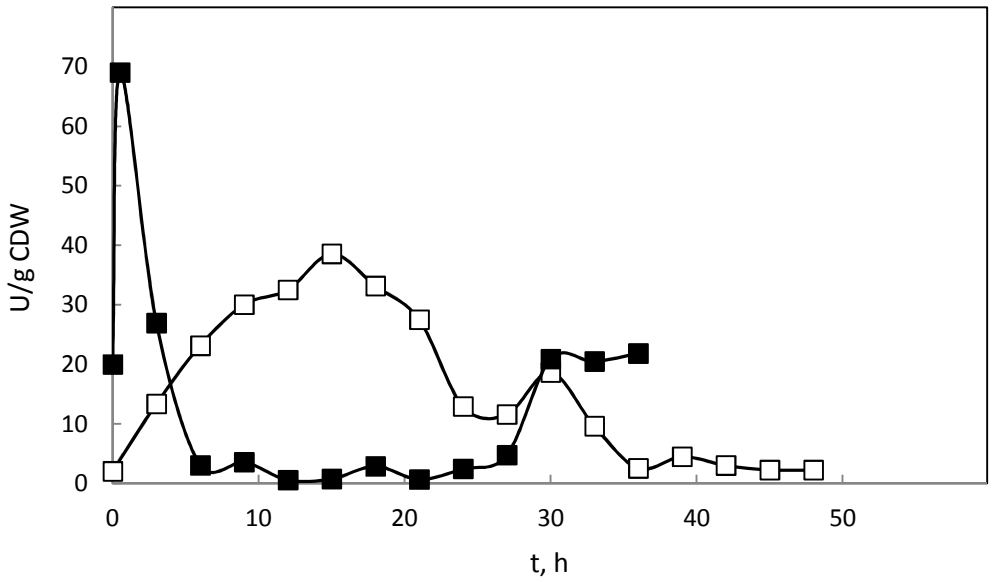

Figure 4.14 AOX activity profiles of *P. pastoris* M13 cells. MM1 (■) and MSSS-0.03 (□).

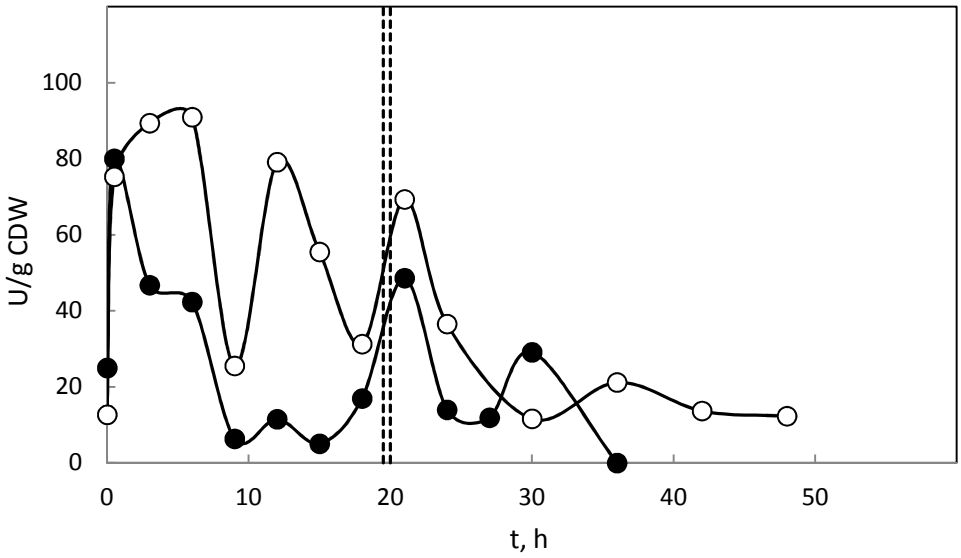

Figure 4.15 AOX activity profiles of *P. pastoris* M13 cells. MM1(●) and SSM1 (○). Dashed lines indicate the hours of the second methanol transition phase of MM2.

Related with MM2, again the highest AOX activity was measured at the beginning of experiment, however as an effect of the second methanol transition phase, again it increases at t=21 h and rhGH production increases after t=21 h of MM2. When compared to the SSM1 (Bozkurt, 2012) AOX activity shows a lower profile throughout the whole fermentation except t=30 h (Figure 4.15).

In MM3 again the highest AOX activity was measured at the beginning of the production phase; however, in the following hours of production it shows a sharp decay and stayed at the low values throughout the fermentation. After t=12 h of production, fed-batch methanol feeding was changed in MM3, but it did not increase the AOX activity of the cells. Accordingly, the highest rhGH production was obtained at the early hours of fermentation in MM3. However, in MM2 batch-wise methanol feeding increased the AOX activity and rhGH production. Batch-wise methanol feeding (MM2) increases the AOX activity

which affecting the recombinant protein production but increasing fed-batch methanol feeding (MM3) did not increase AOX activity. Therefore, to increase recombinant protein production, how to fed methanol with co-feeding of mannitol gains importance when AOX activity profiles are considered.

4.1.2.6 Protease Concentration

Total protease concentration in the extracellular medium was measured from activity of acidic, basic and neutral proteases in the medium and illustrated on Figure 4.16 for all of the strategies MM1, MM2 and MM3; as the extracellular proteases degrade the extracellular proteins in the medium including the rhGH. The highest protease concentrations measured were 0.115 g L^{-1}, 0.148 g L^{-1}, and 0.128 g L^{-1} for MM1, MM2 and MM3, respectively. In all three strategies, similar protease concentration profiles were observed; beginning at lower values and increasing with respect to time. Among the three experiments the highest protease concentration was observed at the end of MM2.

Protease concentration in MM1 begins at t=0 h at a higher level than MSSS-0.03 (İnankur, 2010), however, as cultivation continues proteases comes approximately same levels in both strategies (Figure 4.17). When compared with sorbitol, mannitol does not have significant effect on protease concentration.

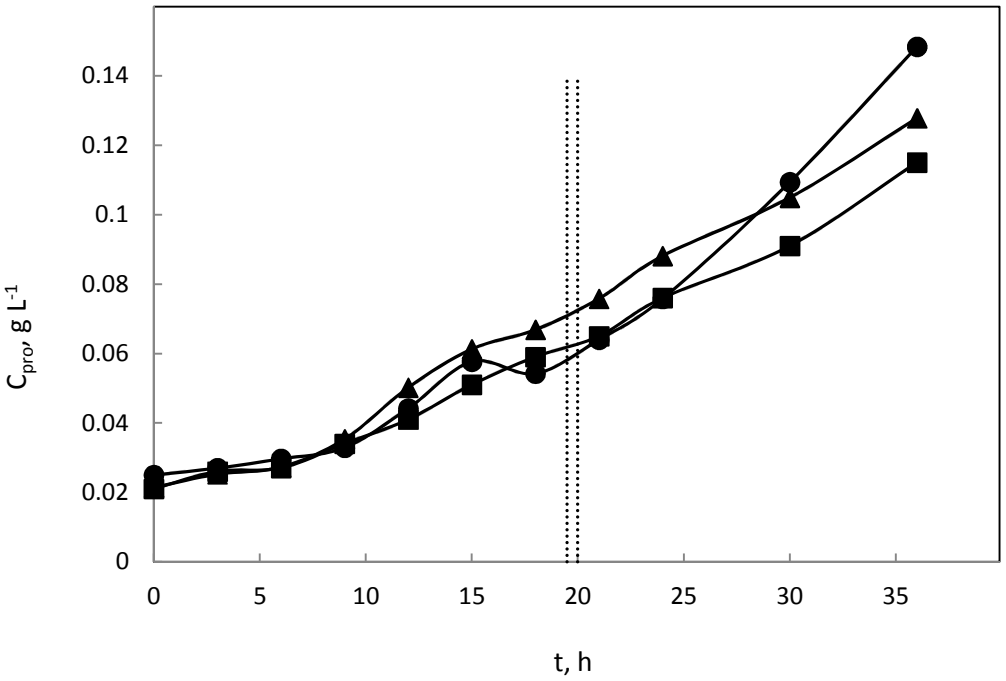

Figure 4.16 Variation in protease concentration within cultivation time; MM1 (■), MM2(●) and MM3 (▲). Dashed lines indicate the second methanol transition hours of MM2.

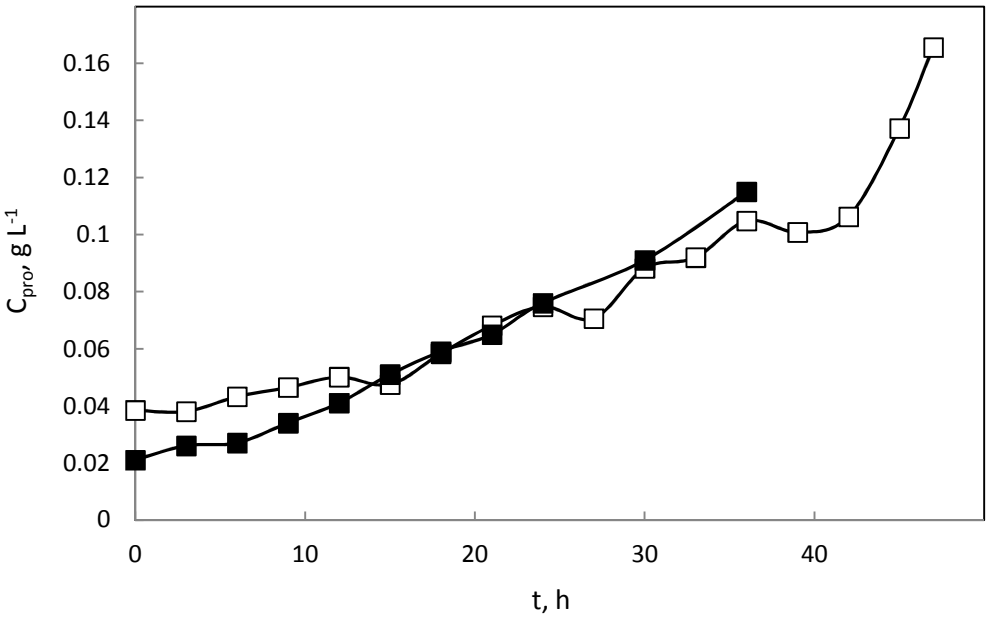

Figure 4.17 Protease concentration profiles of MM1 (■) and MSSS-0.03 (□)

In MM2 and SSM1 (Bozkurt, 2012) protease concentrations begins at t=0 h at the same levels and continues with similar profiles; however, in MM2 the profile shows higher trend than in SSM1 (Figure 4.18). On the other hand, at the end of the processes which are t=36 h for MM2 and t=48 h for SSM1, final protease concentrations are close to the each other.

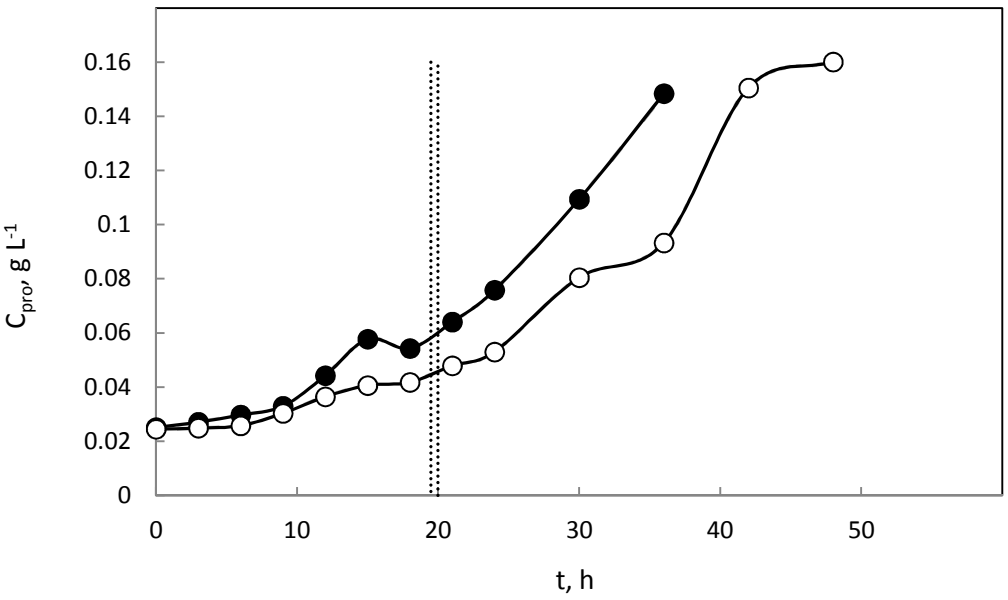

Figure 4.18 Protease concentration profiles of MM1(●) and SSM1 (○)

4.1.2.7 Yield Coefficients

In order to evaluate efficiency of bioprocesses it is essential to determine overall yields of bioprocesses. The major overall yield coefficients include, the overall yield of cell generated per mass of total substrate consumed ($Y_{X/St}$), the overall yield of product per mass of total substrate ($Y_{P/St}$), and the overall yield of product formed per mass of cells generated ($Y_{P/X}$). All of them were calculated for all three different feeding strategies (Table 4.5).

In all of the experiments, yield coefficients were calculated based on the the cultivation times at which the the highest rhGH concentrations were reached because it was aimed to increase the highest C_{rhGH}. Depending on that, all yields calculated at t=12 h, t=36 h and t=15 h of the respectively MM1, MM2 and MM3. Total substrates (mannitol and methanol) fed were 117 and 290 and 146 g in MM1 (t=12h), MM2 (t=36h) and MM3 (t=15h), respectively. Total cell yield on substrates was calculated as 0.53 g g^{-1}, 0.38 g g^{-1} and 0.45 g g^{-1} in MM1, MM2 and MM3, respectively (Table 4.4). The lowest cell yield was obtained and the highest substrate was used in MM2 depending on the long cultivation time, hence process does not seem an effiecient one in point of cell yield. However highest C_{rhGH} obtained in MM2 which was the aim, so, cell yields are not much important. Total product yields on substrates were 1.79 mg g^{-1}, 4.02 mg g^{-1} and 1.92 mg g^{-1} (Table 4.4). Both the highest $Y_{P/St}$ and the highest C_{rhGH} were obtained in MM2. Additionally, the highest $Y_{P/X}$ was obtained in MM2 as 10.7 mg g^{-1}. When compared to SSM1 (Bozkurt, 2012), MM2 gave same $Y_{X/St}$, whereas 2.3- and 1.5-fold higher $Y_{P/St}$ and $Y_{P/X}$ than that of SSM1. Moreover, by comparing to MSSS-0.03 (Inankur, 2010) $Y_{X/St}$, and $Y_{P/St}$ values are 3- and 10-fold higher in MM1, respectively, and $Y_{P/X}$ value is approximately same. Hence by comparing sorbitol and mannitol as co-carbon sources, strategy with mannitol co-feeding, MM1 was more efficient than MSSS-0.03.

Table 4.4 Overall yield coefficients

Strategy	***$Y_{X/St}$ (g g^{-1})***	***$Y_{P/St}$ (mg g^{-1})***	***$Y_{P/X}$ (mg g^{-1})***
MM1	0.53	1.79	3.38
MM2	0.38	4.02	10.67
MM3	0.45	1.92	4.28

4.1.2.8 Organic Acid Concentrations

Organic acids in the extracellular medium can provide better understanding of intacellular metabolic network. They can give an insight about demand and supply of metabolites. Organic acid concentrations were measured at t=0 h, t=6 h, t=12 h, t=18 h, t=24 h, t=30 h and t=36 h. Detected organic acids in extracellular medium which are gluconic, formic, malic, lactic, acetic, maleic, citric, fumaric, succinic and pyruvic acids are given in Table 4.5.

Lactic acid among them, increases as the cultivation time increases and detected even in early hours of production in all experiments MM!, MM2 and MM3. Depending on the lactic acid concentrations it can be said that oxygen limitations occured during the production. This can happen due to effect of metabolism of mannitol by the cells. Mannitol was upttaken and utilized very fast by the cells, depending on that cells grew fast on mannitol. Therefore, cellular metabolism may require much more oxygen. Oxygen feeding may became inadequate and cells may require anaerobic aspiration. Hence, it may cause lactic acid formation.

Fumaric acid, acetic acid, citric acid, pyruvic acid and succinic acids are metabolites of tricarboxylic acid (TCA) cycle in all of the experiments. Concentrations of all of them increases as cultivation time increases. Among them, succinic acid and acetic acid were not detectable and concentrations of the others were significantly low in early hours of production, however increases with time. Therefore, it can be said that the TCA cycle organic acids were not consumed efficiently as cultivation time increases. Since TCA cycle belong to the respiratory pathway, the reason why TCA cycle organic acids accumulated may be the oxygen insufficiency.

In methanol utilization pathway of *P. pastoris*, first methanol is oxidized to formaldehyde and gives hydrogen peroxide. Thereafter, some portion of the formaldehyde enters the central carbon pathways whereas some of them is oxidized to formic acid. In all of the experiments MM1, MM2 and MM3

significant concentration of formic acid was detected. In MM3 formic acid concentration was increased as cultivation time increases, probable reason can be dynamic methanol feeding. In MM1 formic and MM2 acid concentration fluctuates.

Among other organic acids, concentration of gluconic acid fluctuates but generally it was in high levels in any of the cultivation time of all of the experiments. Moreover, malic and maleic acids were detected as in late hours of production in all of the experiments and their concentrations increased as cultivation time increased.

Table 4.5 Variations in organic acid concentrations with respect to cultivation time for different feeding strategies in g L^{-1}.

	Experiment							
	t(h)	**0**	**6**	**12**	**18**	**24**	**30**	**36**
	Gluconic acid	0.4640	0.5408	0.9014	1.1358	0.8293	0.9555	0.9212
	Formic acid	1.0462	1.1409	0.8615	1.3712	1.6682	0.7019	1.1890
	Malic acid	-	-	-	-	0.1829	0.1817	0.2406
	Lactic acid	0.1551	0.3210	0.3256	0.3087	0.5115	0.4833	0.5865
MM1	Acetic acid	-	-	-	-	0.0166	0.0157	0.0310
	Maleic acid	-	-	-	-	0.0002	0.0001	0.0004
	Citric acid	-	0.0514	0.0521	0.0955	0.1932	0.1645	0.2720
	Fumaric acid	-	-	0.0005	0.0013	0.0029	0.0036	0.0057
	Succinic acid	-	-	-	-	-	0.0587	0.1045
	Pyruvic acid	0.0028	-	-	0.0038	0.0375	0.0289	0.0356
	Experiment							
	t(h)	**0**	**6**	**12**	**18**	**24**	**30**	**36**
	Gluconic acid	1.0462	1.1409	0.8615	1.3712	1.6682	0.7019	1.1890
	Formic acid	0.0050	0.0744	0.1105	0.0083	0.0326	0.0604	0.0661
	Malic acid	-	0.0010	0.0279	0.0356	0.0500	0.0827	0.1635
	Lactic acid	0.2731	0.2932	0.3200	0.4351	0.6879	0.8792	1.7638
MM2	Acetic acid	-	-	-	-	0.0056	0.0214	0.0818
	Maleic acid	-	-	-	-	0.0001	0.0004	0.0010
	Citric acid	-	-	0.0245	0.0661	0.0963	0.1778	0.2546
	Fumaric acid	-	0.0004	0.0005	0.0012	0.0028	0.0050	0.0113
	Succinic acid	-	-	0.0016	0.0365	0.0355	0.0349	0.0488
	Pyruvic acid	0.0017	0.0045	0.0067	0.0137	0.0148	0.0173	0.0485
	Experiment							
	t(h)	**0**	**6**	**12**	**18**	**24**	**30**	**36**
	Gluconic acid	1.0021	0.6280	0.7246	1.0276	2.0432	0.9014	1.0817
	Formic acid	-	0.0347	0.0198	0.0827	0.1630	0.1909	0.4134
	Malic acid	-	-	-	0.0465	0.0508	0.0827	0.1444
	Lactic acid	0.2152	0.2156	0.4472	0.6209	0.6894	0.8792	1.5738
MM3	Acetic acid	-	-	-	-	0.0268	0.0254	0.0557
	Maleic acid	-	-	-	0.0001	0.0001	0.0004	0.0010
	Citric acid	-	-	0.0294	0.0446	0.0963	0.1777	0.3676
	Fumaric acid	-	0.0002	0.0007	0.0018	0.0026	0.0050	0.0113
	Succinic acid	-	-	-	0.0193	0.0317	0.0238	0.0476
	Pyruvic acid	0.0019	0.0031	0.0075	0.0132	0.0148	0.0173	0.0480

4.2 Development of *Pichia pastoris* G7 Strain

This part of study is mainly conducted in three steps. In the first step *hGH* gene as target gene was amplified and vector DNA was propagated. Then, they were ligated to construct recombinant plasmid pGAPZαA*::hGH* and transformed into *E. coli*. In the last step, after selection of true transformant among *E. coli* transformants; the wild type *P. pastoris* was transfected with recombinant plasmid and selected under Zeocin resistance. Detailed summary of construction of rhGH producing *P. pastoris* G7 strain is illustrated in Figure 4.19.

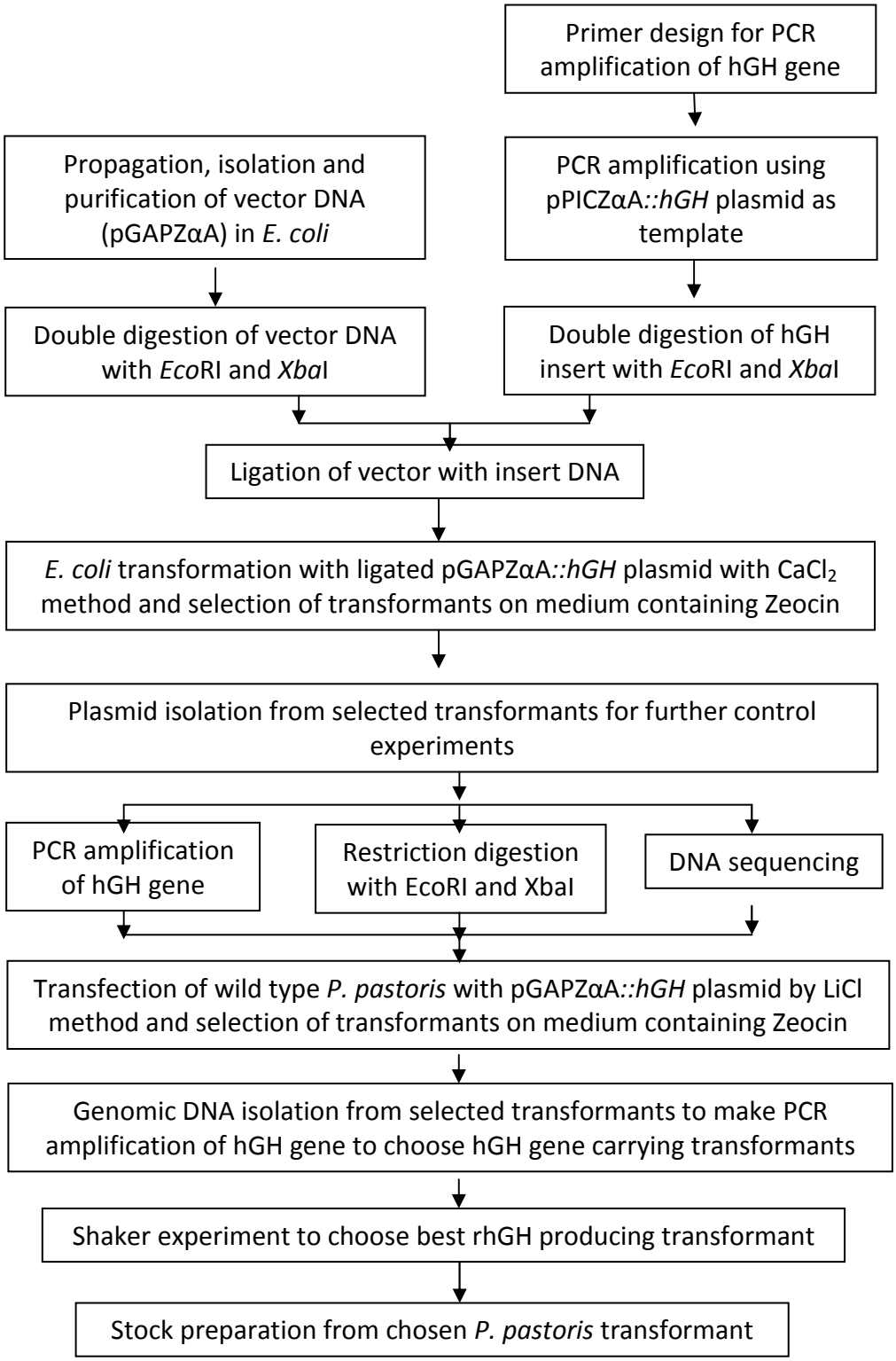

Figure 4.19 Flowchart of construction of rhGH producing *P. pastoris* G7 strain

4.2.1 Primer Design for Amplification of *hGH* Gene

As mentioned in section 3.5.5, two primers were designed in order to amplify the *hGH* gene by considering the restriction sites. Two designed primers were abbreviated as FwdGhGH and RevGhGH, respectively. Forward primer was designed by considering the *EcoR* I recognition site (6 bp) and *hGH* gene sequence at 5' end. Reverse primer was designed in regard to *Xba* I recognition site and *hGH* sequence at 3' end. Primers used in amplification of *hGH* gene and control of cloning steps are given in Table 4.6.

Table 4.6 Primers used for amplification of desired gene fragments

Abbreviation	Name	Sequence (5'-3')
FwdGhGH	Forward primer	GGAATTCTTCCCAACTATACCACTATCTCCGTC
RevGhGH	Reverse primer	CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC
5'GAP	GAP forward primer	GTCCCTATTTCAATCAATTGAA
3'GAP	AOX1 reverse primer	GCAAATGGCATTCTGACATCC

4.2.2 Amplification of *hGH* Gene by Polymerase Chain Reaction (PCR)

E.coli cells that carrying *hGH* gene and Zeocin resistance from microbanks was plated and grown onto LSLB+zeocin plates and then inoculated into LSLB liquid medium containing 0.25 μg ml^{-1} zeocin and cultivated overnight with vigorous shaking. Afterwards plasmids were isolated via alkaline lysis method and then were run on agarose gel with empty pPICZαA plasmid and results were given in Figure 4.20. Empty pPICZαA vector was used as control in agarose gel electrophoresis.

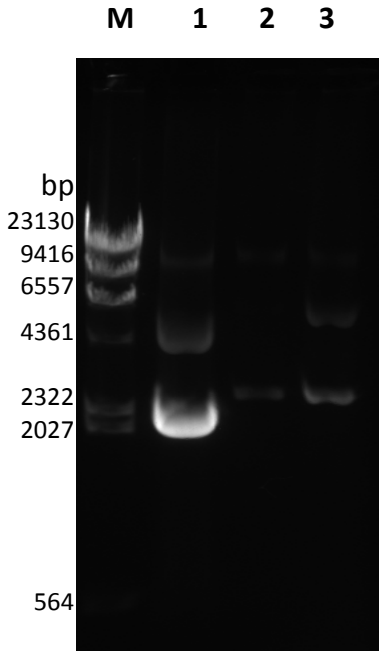

Figure 4.20 Agarose gel electrophoresis of isolated pPICZαA and pPICZαA*::hGH* plasmid extracted from *E.coli* M: λDNA/HindIII; Lane 1: circular pPICZαA; Lane 2 and 3: circular pPICZαA*::hGH*

PCR was performed by using 1 µL of isolated pPICZαA*::hGH* plasmid and the reaction mixture described in Table 3.5 and process parameters given in Table 3.6. PCR products were purified by using PCR purification kit. Results are shown in Figure 4.21.

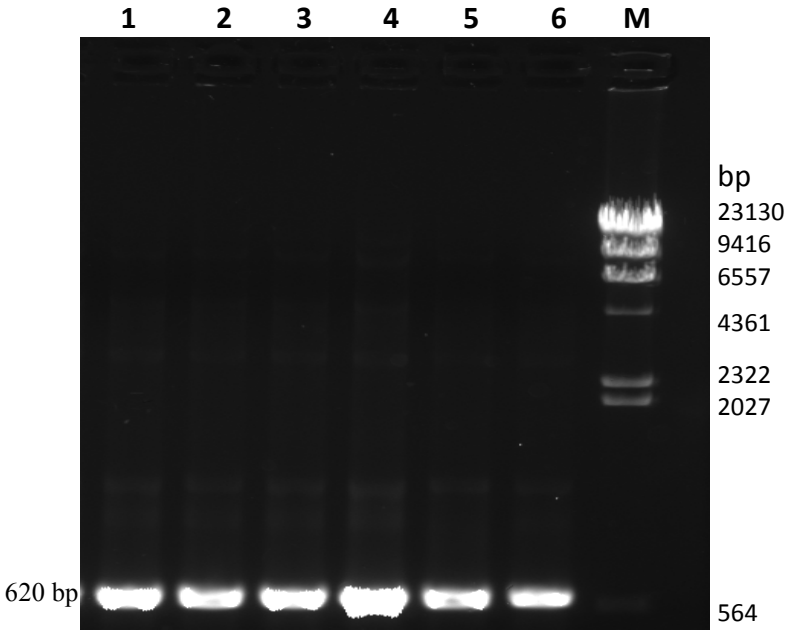

Figure 4.21 Agarose gel electrophoresis results of *hGH* gene with size of 620 bp amplified by PCR using designed primers and pPICZαA*::hGH* plasmid as template. Lane 1-6: PCR amplified *hGH* gene; Lane M: λDNA/HindIII Marker

4.2.3 Propagation of pGAPZαA vector

pGAPZαA vector was propagated in *E.coli* DH5α cells. Single colony was chosen from an overnight incubated LSLB plate containing 25 µg mL^{-1} and cells were grown overnight in liquid LSLB + zeocin medium. Then plasmids of cells were isolated via alkaline lysis method described in section 3.6.2. Since circular vector runs faster on agarose gel it was not possible to evaluate the size of the plasmid from the circular one. Therefore in order to understand the size of isolated vector, it was single digested with *EcoR*I restriction enzyme and both digested and undigested plasmids were run on agarose gel. Results are illustrated on Figure 4.22. Digestion reaction is carried out as explained in section 3.6.8.

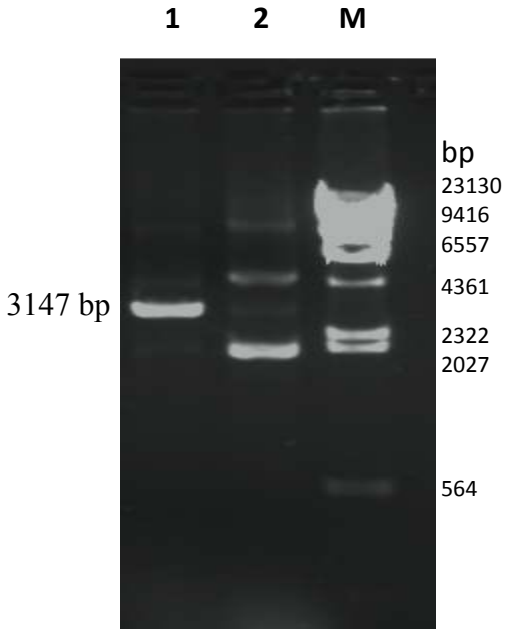

Figure 4.22 Agarose gel electrophoresis of vector pGAPZαA. Lane 1: Single digested linear vector pGAPZαA Lane 2: Undigested circular pGAPZαA M: λDNA/HindIII Marker

4.2.4 Restriction enzyme digestion reactions

After checking whether EcoRI and XbaI enzymes are non-cutters of *hGH* gene from NEB cutter V2.0 software (http://tools.neb.com/NEBcutter2/index.php); both PCR amplified and purified *hGH* gene and shuttle vector pGAPZαA were double digested with *Eco*RI and *Xba*I restriction enzymes by using a common buffer of enzymes and reaction mixtures defined in Table 3.8 and 3.7, respectively and results are given in Figure 4.23 A. Also for control of the enzymes, single digestions were carried out by both enzymes and results were given in Figure 4.23 B. Size of pGAPZαA vector is 3127 bp and digested part of vector is 60 bp by double digestion hence, difference between single digested and double digested vector seems very small (Figure 4.23 B). Furthermore, since digested part of *hGH* gene is only a couple of nucleotides, the difference could not be visualized from screening of agarose gel.

After double digestion of PCR products and vector, in order to prepare them to ligation, both were run on agarose gel and extracted from the gel as described in section 3.6.9. In order to check their purity and concentration they extracted DNA fragments were again run on agarose gel and results are shown in Figure 4.24.

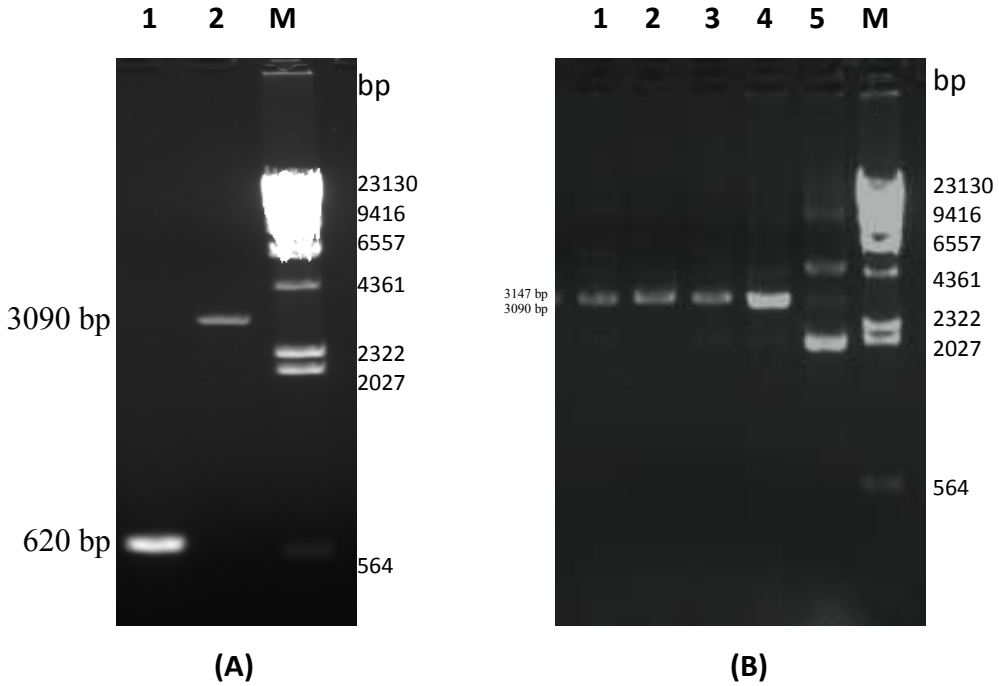

Figure 4.23 AGE screening of products of digestion reactions. (A) Double digestion of *hGH* gene and plasmid pGAPZαA; M: λDNA/HindIII Marker Lane 1: *hGH* gene Lane 2: pGAPZαA. (B) Control digestion reaction products of pGAPZαA; M: λDNA/HindIII Marker, Lane 1 and 4: Double digested pGAPZαA, Lane 2: *EcoR*I digested pGAPZαA, Lane 4: *Xba*I digested pGAPZαA, Lane 5: Undigested pGAPZαA.

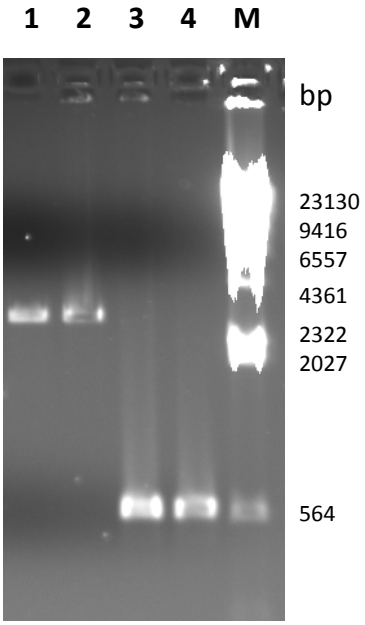

Figure 4.24 AGE screening of double digested gene insert and vector DNA after gel elution Lane 1-2: Vector DNA, Lane 3-4: Gene insert M: λDNA/HindIII Marker

4.2.5 Ligation Reaction

Ligation reaction was conducted by using double digested and eluted products and concentrations were determined by the method described in section 3.6.4 and the reaction carried out as described in section 3.6.10. Ligation reaction was achieved at gene to vector ratio of 3:1 and final concentrations of contents are given in Table 4.7. Constructed plasmid is schematically represented in Figure 4.25 by showing cloning steps starting from template.

Table 4. 7 pGAPZαA*::hGH* ligation reaction contents

Component	*Amount*
10 X ligation buffer	2 µL
Insert DNA	90 ng
Vector DNA	100 ng
T4 DNA ligase	1 µL
Nuclease-free water	Up to 20 µL

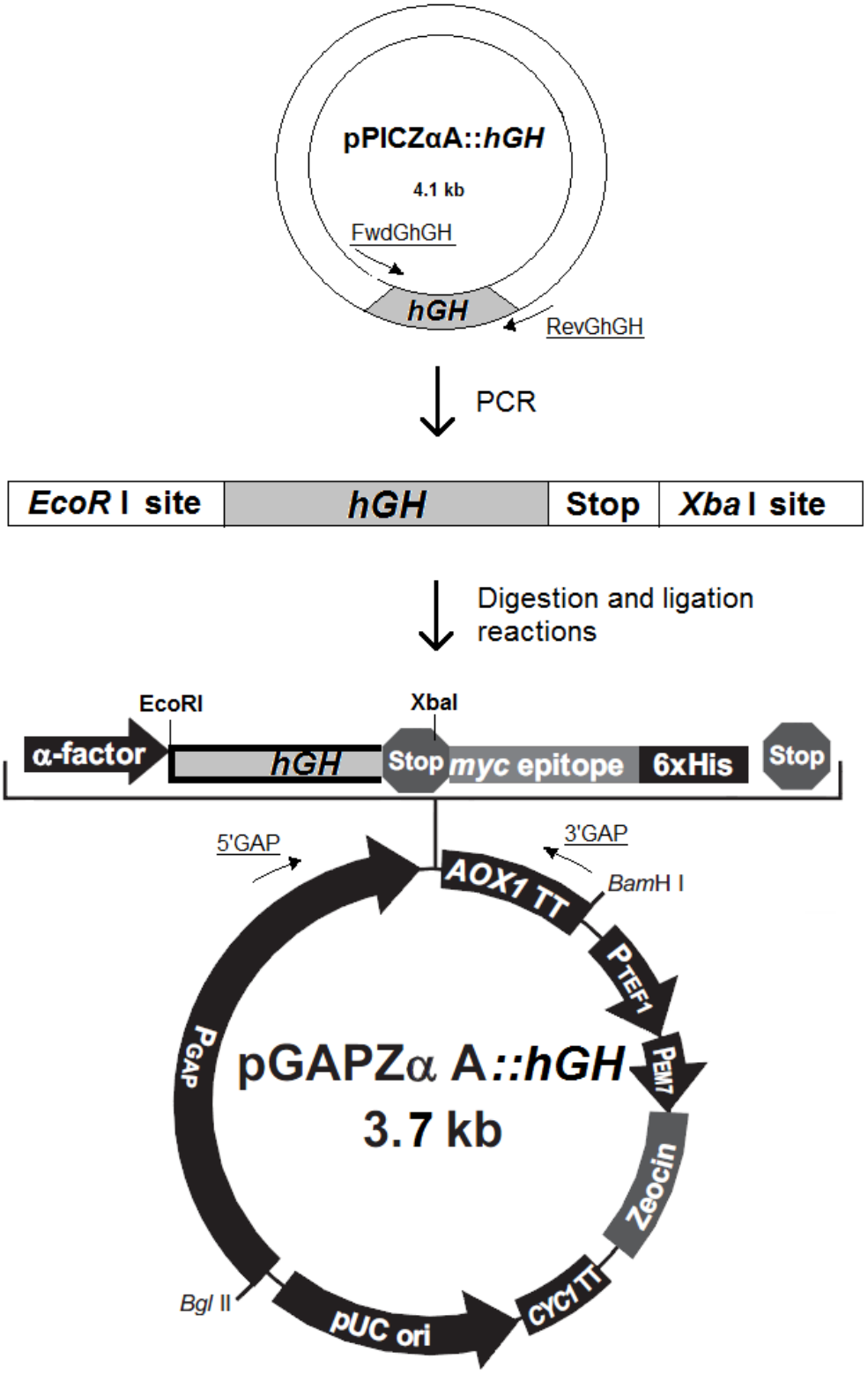

Figure 4.25 Schematic representation of construction of pGAPZαA::*hGH* plasmid

4.2.6 Transformation of pGAPZαA::*hGH* into *E.coli* cells and Selection of True Transformants

Ligation products were transformed into *E.coli* DH5α strain by the $CaCl_2$ method given in section 3.6.1. After 16 hours, transformants appeared on LSLB plates containing 25 μg mL^{-1} zeocin were plated once on fresh LSLB + zeocin plate for further tests and short term storage of colonies. Isolated colonies also grown in liquid LSLB + zeocin medium and plasmids were isolated via alkaline lysis method and results are illustrated in Figure 4.26.

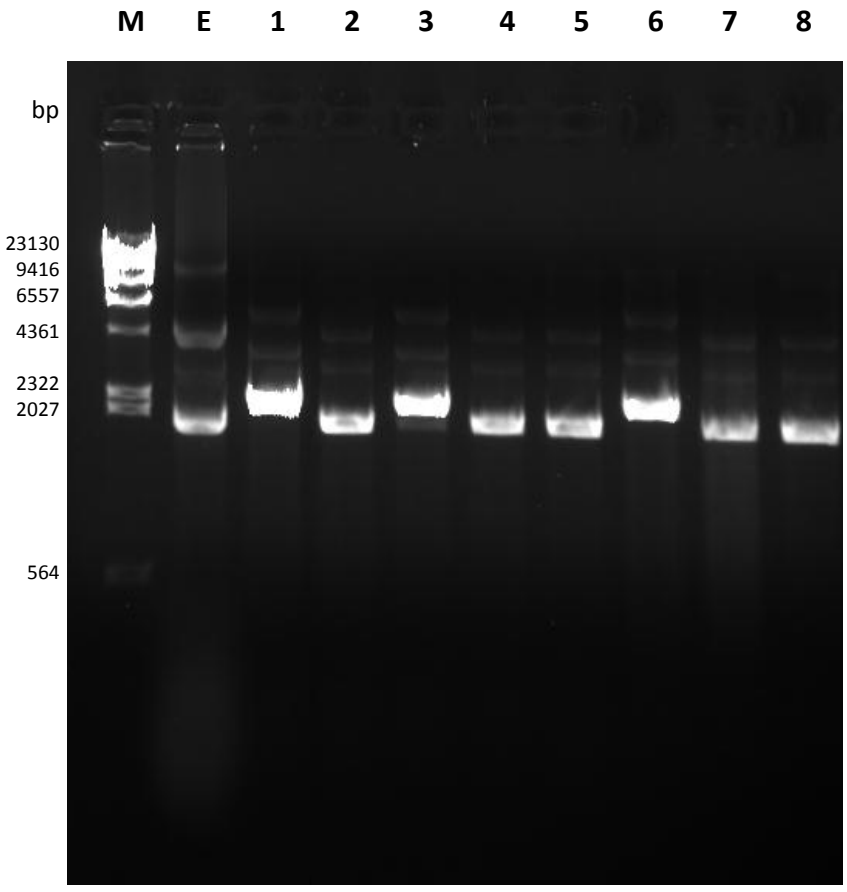

Figure 4.26 AGE screening of circular plasmids isolated from *E.coli* transformants. M: λDNA/HindIII Marker, E: Empty pGAPZαA vector, Lane 1-8: *E. coli* transformants.

Plasmids of colonies with numbers of 1, 3 and 6 in Figure 4.6 considered that they carry *hGH* gene since they run slower than empty plasmid on agarose gel.

Plasmids of selected colonies were digested with restriction enzymes for further control analysis and results were illustrated on Figure 4.27. Additionally, PCR were conducted with plasmids of selected colonies in order to check whether these plasmids carry the *hGH* gene with same conditions stated in section 4.2.2.

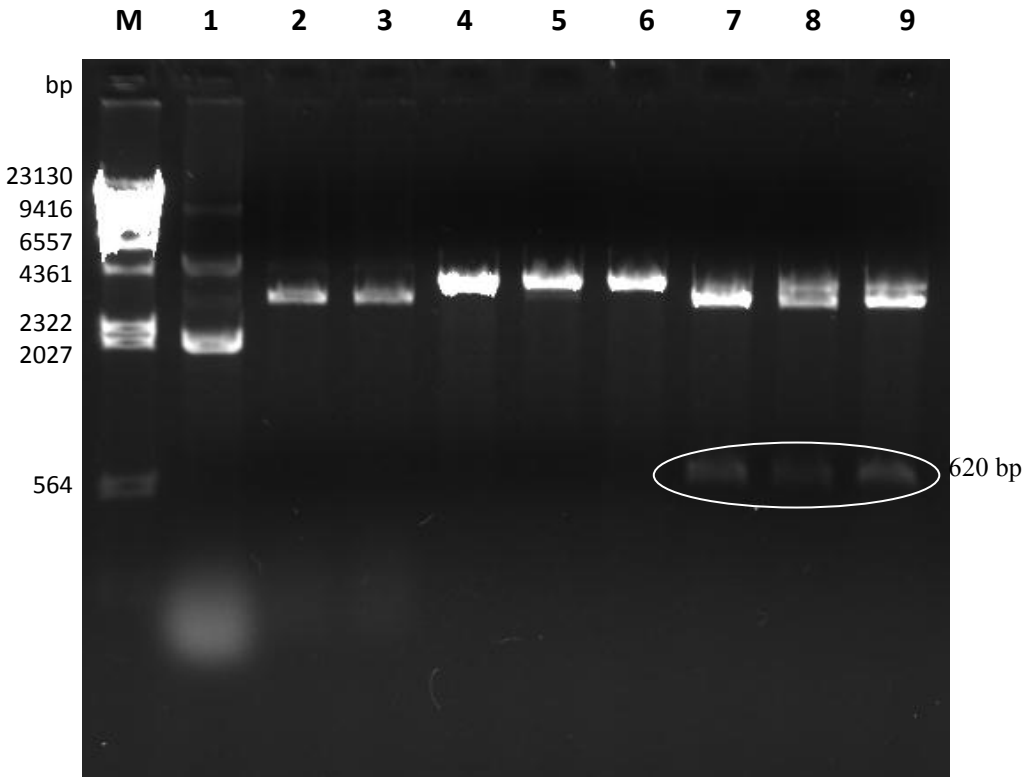

Figure 4.27 AGE screening of digestion reactions of plasmids of selected colonies. M: λDNA/HindIII, Lane 1: Circular empty vector, Lane 2: Single digested empty vector, Lane 3: Double digested empty vector, Lane 4-6: Single digested plasmids of selected colonies (1, 3 and 6, respectively) Lane 7-9: Double digested plasmids of selected colonies (1, 3 and 6, respectively)

After double digestion of selected plasmids 620 bp *hGH* gene was seen on agarose gel (Figure 4.27). Hence plasmids of selected colonies are verified that they carry *hGH* gene. Single and double digested empty vector were used as negative controls.

620 bp DNA fragments are also seen in AGE of PCR products (Figure 4.28). Plasmids of selected colonies were used as template in PCR. In negative control, empty vector is used as template in PCR, in positive control pPICZαA*::hGH* plasmid was used as template. Gene sequence of constructed plasmid is given in Figure (C.6).

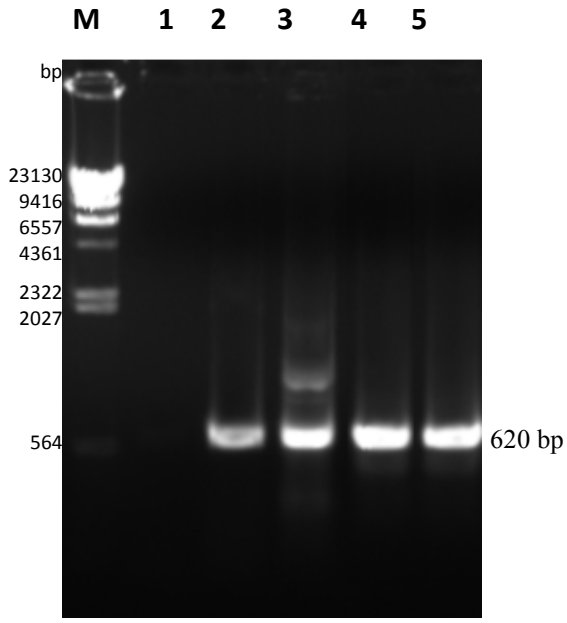

Figure 4.28 AGE screening of PCR products. M: λDNA/HindIII, Lane1: Negative control, Lane 2-4: PCR products of selected colonies (1,3 and 6 respectively), Lane 5:positive control

After controls were carried out DNA sequence analysis of plasmids of selected colonies was conducted by METU Central Laboratory (ABI Prism 310 Genetic Analyzer) using designed primers and chromatogram data and obtained

sequence is given in Appendix C. Sequence from DNA sequencing were analyzed by Basic Local Alignment Tool (BLAST) by comparing them exact sequence of *hGH* (Figure 2.1). All of them gave exact sequence and then microbanks were prepared and selected colony named as *E. coli* pGAPZαA*::hGH* and stored at -80°C for long term storage of pGAPZαA::*hGH* plasmid.

4.1.7 Transfection of Pichia pastoris cells with pGAPZαA*::hGH* plasmid

For genomic integration of pGAPZαA*::hGH* plasmid into the genome at GAP locus the constructed plasmid was linearized in its GAP locus by digestion as described in section 3.5.13 (Table 3.8). Full digestion was verified by AGE of digestion products. Digested product was purified by the ethanol precipitation. Its concentration was determined by the spectrophotometer and stored at -20°C until it is required. Transfection was carried out as explained in section 3.5.13.

After 48-60 h of incubation, eleven single colonies were selected from YPD+Zeocin (200 µg mL^{-1}) plates and restreaked onto YPD+Zeocin plates for further control and and short term strorage. Thereafter, they were inoculated into liquid medium YPD+Zeocin. Cell were harvested then, and their genomic DNA was isolated as descibed in section 3.5.14 to make PCR controls. Isolated genomic DNA of selected colonies was used as template in PCR. Positive control was performed by using constructed pGAPZαA::*hGH* plasmid whereas the negative kontrol was performed by using water as template. The *hGH* gene was amplified in all PCR mixtures, hence all of the colonies were carries the *hGH* gene (Figure 4.29). Moreover, the PCR was performed by using GAP forward and GAP reverse (AOX1 reverse) primers and positive control was performed by using constructed pGAPZαA::*hGH* plasmid whereas the negative kontrol was performed by using water as template. DNA fragments with approximately 1100 bp were amplified in PCR which verifies the true transformants (Figure 4.30).

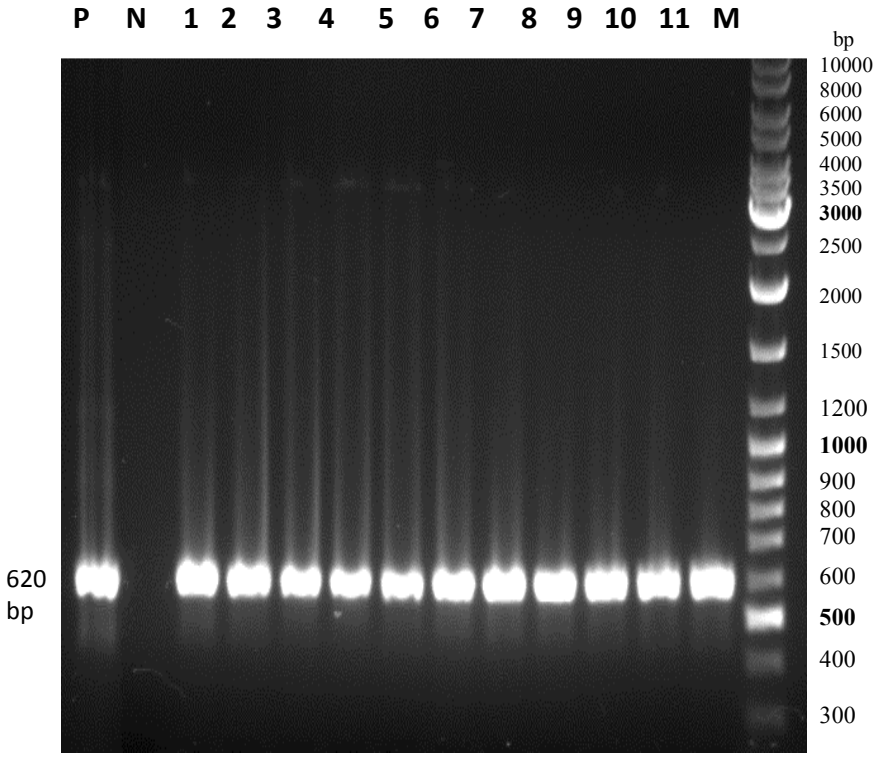

Figure 4.29 AGE screening of PCR products. M: λDNA/HindIII, N: Negative control, P: Positive control, Lane 1-11: PCR products of selected colonies

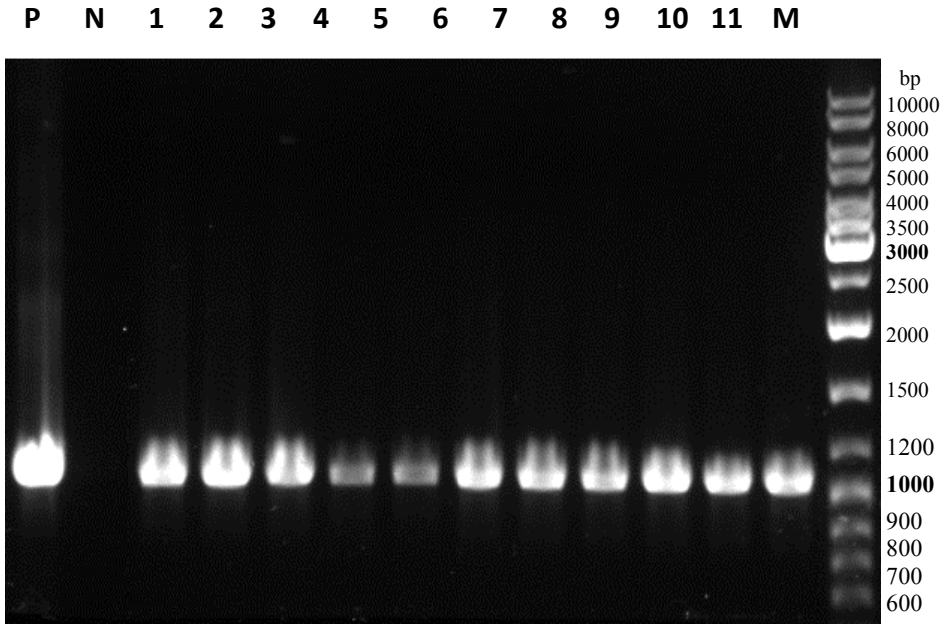

Figure 4.30 AGE screening of PCR products. M: λDNA/HindIII, N: Negative control, P: Positive control, Lane 1-11: PCR products of selected colonies

4.1.8 Expression of Recombinant Human Growth Hormone by G7 strain

After *P. pastoris* transfection, all of the further tested colonies are positive for *hGH* gene. Among them to choose best potential to produce rhGH 50 mL shake flask experiments were conducted. All of eleven colonies were inoculated into 10 mL YPD+Zeocin (100 mg L^{-1}) medium and incubated overnight in agitation and temperature controlled orbital shakers at 30°C and 200 rpm. Thereafter, 100µL inoculation was done into fresh 50 mL YPD medium in air filtered 250 mL Erlenmeyer flasks at 30°C and 200 rpm and samples were taken in every 24 hours until t=72 h. Taken samples were harvested by the centrifugation at maximum rpm for 2 minutes at room temperature and 0.5 mL supernatants were stored at -55°C for SDS-PAGE and Dot-blot analysis. Same cultivation procedures were applied for wild type *P. pastoris* X-33 and samples of wild type strain were used as negative control in SDS-PAGE and Dot-blot analysis.

Firstly, SDS-PAGE was performed by the supernatants of t=48 h to verify production of rhGH by its molecular weight. rhGH concentrations (22 kDa) of colonies which have numbers of 2, 3, 7, 8 and 11 have significantly higher than the others (Figure 4.31). Additionally, Figure 4.31 illustrates that sample taken from the wild type did not show any protein at about 22 kDa. Hence, existence of rhGH in these colonies was verified.

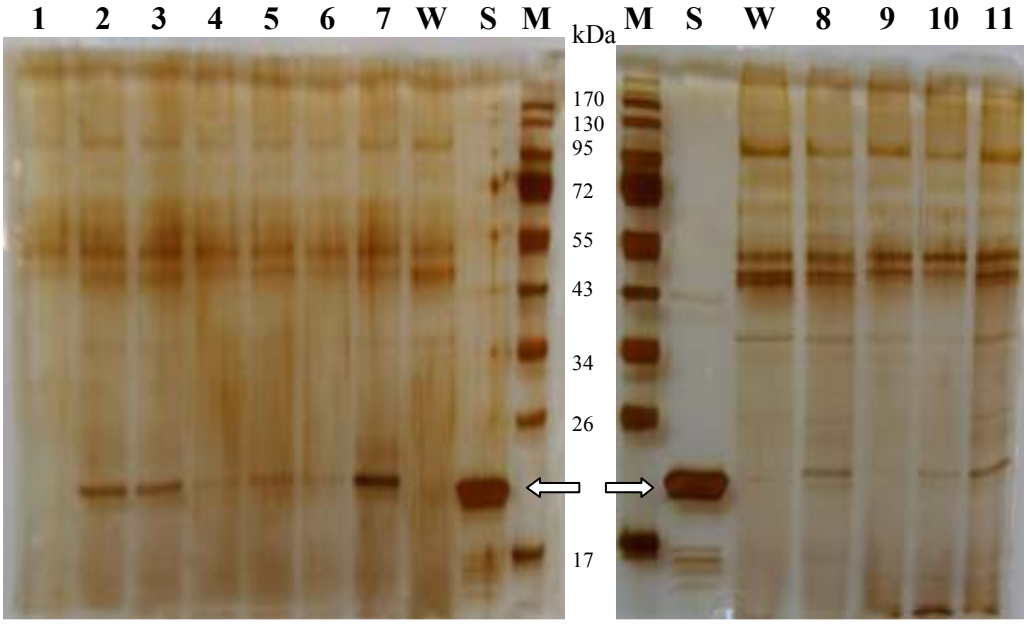

Figure 4.31 SDS-PAGE screening of selected colonies. M: PageRuler Protein ladder, W: Negative control (Wild type), S: 200 g L^{-1} hGH standart, Lane 1-11: Extracellular proteins of t=48 h of selected colonies.

Additionally, dot-blot analysis was performed. Dot-blot can be more reliable than SDS-PAGE, since it is performed by using protein specific antibodies. The analysis was carried out as described in the section 3.7.4 by using 15 µL samples of t=24 h, t=48 h and t=72 h. Also, 15 µL samples of wild type colony was used as negative control in the analysis. After samples were transferred to the PVDF membrane; membrane was treated first by monoclonal human growth hormone antibody (Fitzgerald, MA, USA) and then antimouse IgG horseradish peroxidase-linked whole antibody (Amersham Biosciences, Uppsala, Sweden). Finally, visualization of the protein dots on the membrane was achieved by Substrate-Chromogen Kit (S10 HRP, Biomeda, USA). Results are exhibited in Figure 4.32. At t=48 h, the 2nd, 3rd and 7th colonies have significantly higher rhGH concentrations as in the SDS-PAGE analysis . At t=72 h of production, all of the colonies have high rhGH concentrations except 1st, 8th, 10th and 11th colonies. However, any one of the colonies shows significant dot intensity at t=24 h except the 7th colony. Additionally, it should be noted that

samples of wild type as negative control do not show dot intensity at any of the hours.

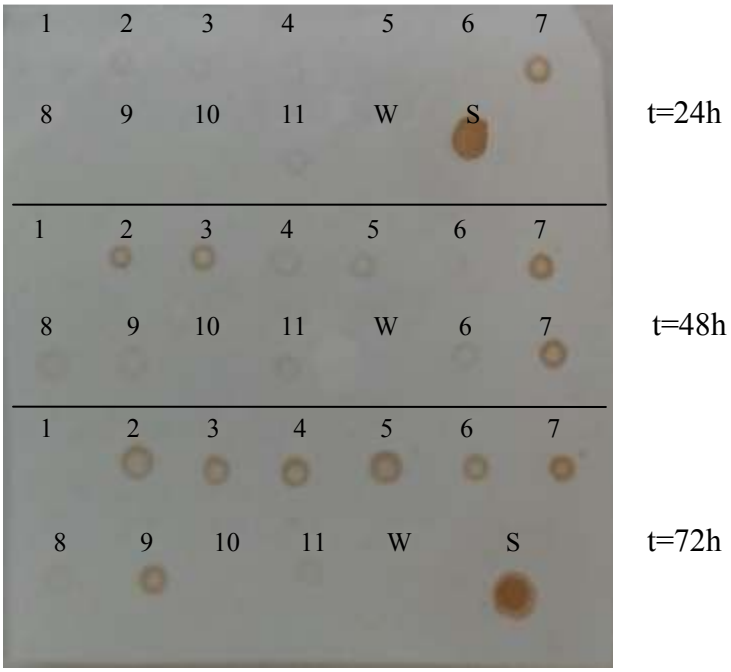

Figure 4.32 Dot blot analysis of 15 µL samples of extracellular mediums of 11 selected colonies of t=24 h, t=48 h and t=72 h. S and W indicate hGH standart and extracellular mediums of wild type *P. pastoris* X-33 of related hours, respectively.

4.3 Comparison of Production Levels of Strains

For comparison of production levels of recombinant hGH, two different strains of same microorganism were used. G7 strain carrying constitutive GAP promoter and M13 strain carrying strongly regulated AOX promoter were used. In the first part of study, by the feeding strategy development 1200 mg L^{-1} rhGH concentration was reached by the M13 strain. In this part of study G7 strain was developed and used to produce rhGH by two different feeding strategies. In these experiments GFB phase was removed. Because one of the most important

advantage of GAP promoter is simultaneous cell growth and recombinant protein production.

In both experiments glucose feeding was performed with 50% (w/v) glucose solution containing 12 mL L^{-1} PTM1 solution. In the first strategy which is abbreviated as G1, glucose was fed with predetermined μ=0.2 h^{-1} until t=6 h to achieve cell growth profile like in the GFB phase. After 29.1 g L^{-1} cell concentration was reached in the medium (t=6 h); in the following hours of production glucose was fed with predetermined μ=0.03 h^{-1} as methanol feeding in M13 strain. In this strategy limiting glucose feeding was performed. Glucose feeding profile in the strategy G1 was shown in Figure 4.33.

In the second strategy, which is abbreviated as G2, glucose feeding was performed with predetermined μ=0.2 h^{-1} until t=3 h of production. Since in G1 maximum rhGH concentration was achieved at t=3 h of production, at that hour exponential feeding was switched to constant feeding in which flow rate was maintained constant at the ultimate value of the exponential feeding. Glucose feeding profile was illustrated in Figure 4.33. Feeding strategies applied in this part of the study was summarized in Table 4.8.

Table 4.8 Definiton of feeding strategies with G7 strain

Strategy name	***Strategy definition***
G1	Glucose feeding with μ=0.2 h^{-1} until t= 6 h and glucose feeding with μ=0.03 h^{-1} in following hours of production.
G2	Glucose feeding with μ=0.2 h^{-1} until t=3 h then in following hours constant feeding in which flow rate was maintained constant at the ultimate value of the exponential feeding.

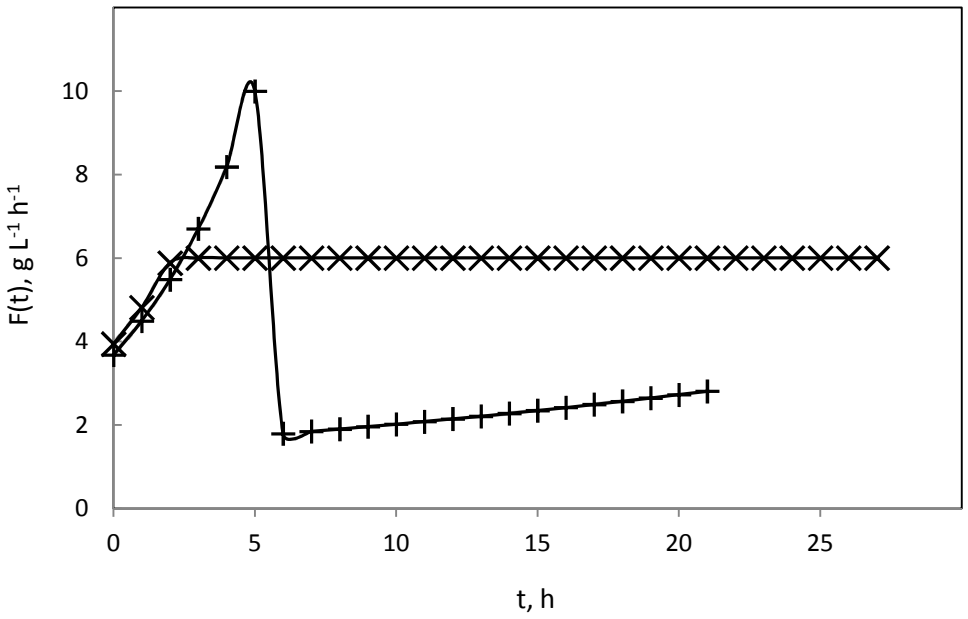

Figure 4.33 Glucose feeding profiles of G1 (+) and G2 (X)

4.3.1 The Glucose Consumption and Cell Growth

Glucose concentration in the medium was measured by the method given in section 3.7.9 and calibration curve is illustrated in Appendix K. In neither experiment glucose was detected in the medium, in any hour of the experiments. That means glucose was efficiently uptaken by the cells. The highest q_G values were attained as 0.428 g g^{-1} h^{-1} in G1 at t=3 h and 0.378 g g^{-1} h^{-1} again at t=3 h in G2 (Table 4.9). In following hours of G1, q_G values decreased approximately 0.055 g g^{-1} h^{-1} due to limiting feeding after t=6 h. Related with G2, q_G values decreased, too, but, the decrease was not so drastic as in G1. The values decreased steadily in the G2 and reached 0.046 g g^{-1} h^{-1} at t=27 h as in the G1. That may mean if experiment was continued with same constant feeding, glucose feeding may be limiting for following hours.

The highest cell concentration attained in G2 was 90 g L^{-1} at t=27 h of production whereas in G1 it was 36.9 g L^{-1} at t=21 h of production. In both

strategies cell concentration was increasing as cultivation time increases. Variation in cell concentration with respect to time is given in Figure 4.34. Since glucose feeding was limiting in G1, the maximum cell growth achieved is much lower than those of G2. Additionally, the maximum μ values obtained were 0.225 h^{-1} and 0.196 h^{-1} at t=3 h in both experiments (Table 4.9). μ was set 0.2 h^{-1} between t=0-6 h of G1 and 0.03 h^{-1} for rest of the experiment. It was observed that the pre-determined μ values held for early hours, however, it decreased under pre-determined value for following hours; meaning that μ-controlled feeding can be better as in methanol feeding in M13 strain productions. In G2, μ values started with its highest value and decreased steadily until t=21 h as expected due to constant feeding. Nevertheless, for following hours it increased again; the reason could be intracellular glucose accumulation at the beginning of experiment, then, in the following hours glucose was being converted into cell as can be observed from cell growth trend in the late hours (Figure 4.34) and μ (Table 4.9).

In M13 strain production cell growth was much lower when single carbon source, methanol was fed to the bioreactor. This lower cell growth is due to limiting methanol feed. Since higher methanol feeding will be toxic to the cells, methanol feeding cannot exceed a limiting value. Increase in cell concentration in M13 strain fermentations can be achieved by the co-carbon feeding like glycerol, sorbitol or mannitol. On the other hand, being one of the advantages of GAP promoter, single carbon source, glucose, provides significant cell growth in shorter cultivation times.

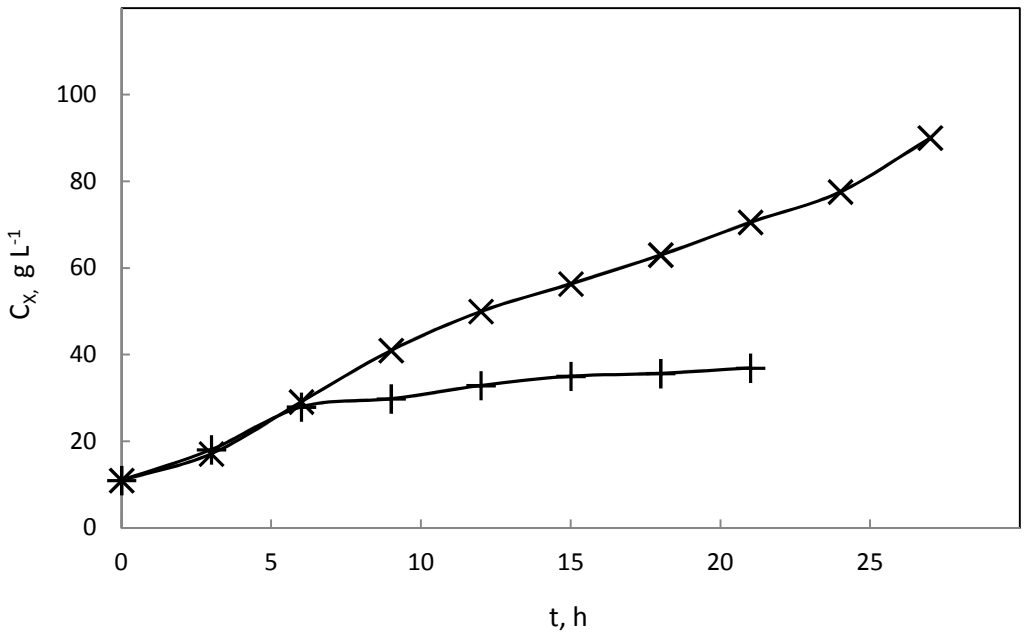

Figure 4.34 Variation in cell concentration with respect to cultivation time. G1 (+); G2 (X)

Table 4.9 Variations in specific rates within cultivation time.

Experiment Name	T H	μ h^{-1}	q_G g g^{-1} h^{-1}	q_{rp} mg g^{-1} h^{-1}
G1	0	0.225	0.400	1.213
	3	0.172	0.428	0.316
	6	0.073	0.052	-
	9	0.031	0.053	-
	12	0.030	0.052	-
	15	0.017	0.053	-
	18	0.013	0.056	-
	21	0.016	0.059	-
G2	0	0.196	0.400	0.494
	3	0.190	0.378	0.210
	6	0.149	0.214	0.102
	9	0.097	0.146	0.072
	12	0.063	0.116	0.093
	15	0.050	0.099	0.170
	18	0.048	0.086	0.189
	21	0.045	0.074	0.164
	24	0.052	0.065	0.175
	27	0.056	0.052	0.181

4.3.2 rhGH Production

The maximum rhGH concentration attained in the strategy G1 was 40 mg L^{-1} at t=3 h of production whereas those of G2 was 200 mg L^{-1} at t=27 h of production. Variation in recombinant protein concentration with respect to cultivation time was illustrated in Figure 4.35. Due to the much lower cell concentrations in G1, much lower (8-fold lower) recombinant protein was obtained. Moreover, unlike the cell concentration, rhGH concentration was not incresing in G1 by the time, probably as an effect of limiting glucose feeding. In G1 exponential glucose feeding was performed until t=6 h, however the highest rhGH concentration were achieved at t=3 h of production. Depending on this finding in G1, in G2 exponential feeding was ceased at t=3 h and constant feeding was performed in following hours of production.

When comparing to M13 strain, recombinant protein production was lower in G7 strain. In this work, by the MM2 strategy 1200 mg L^{-1} rhGH concentration was attained whereas 200 mg L^{-1} maximum rhGH concentration was reached by G2 which is approximately six-fold lower. However, it should be pointed out that feeding strategies were developed over the years by M13 strain (Orman et al., 2009; Inankur, 2010 and Bozkurt, 2012) even in this work whereas these are the first bioreactor operations by G7 strain.

Since recombinant protein production is growth associated, in M13 strain feeding strategies co-carbons were fed to increase recominant protein production. However, single carbon source glucose provides an increasing recombinant protein production as cell concentration increases again as an advantage of GAP promoter. Fermentations based on single carbon feedings eases the processes.

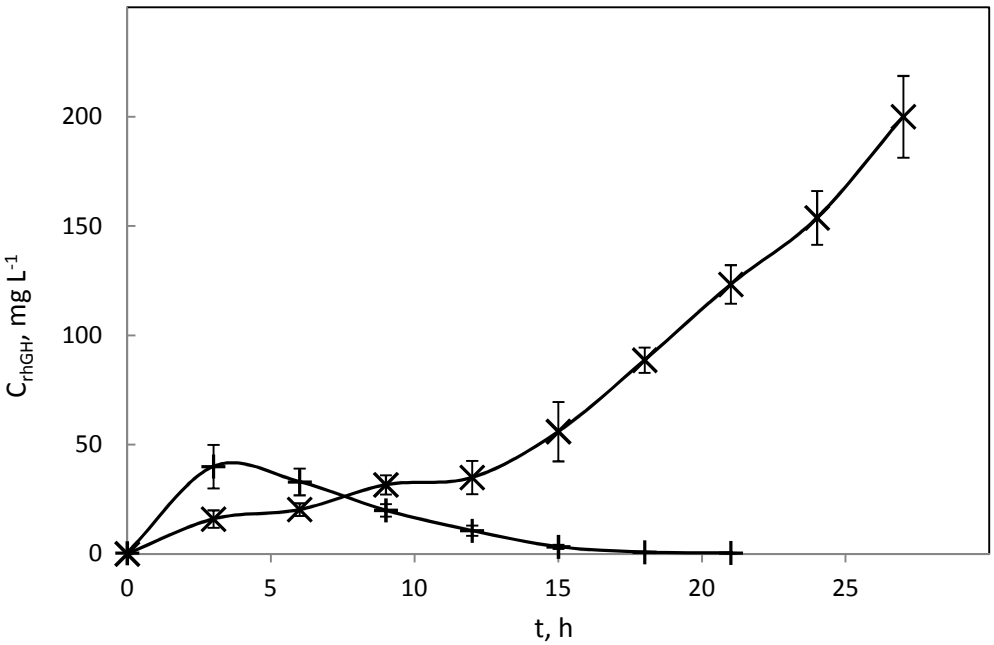

Figure 4.35 Variation in rhGH concentration with respect to cultivation time. G1 (+); G2 (X). (standard errors are calculated seperately for each data point).

4.3.3 Protease Concentration

Total protease concentration in the extracellular medium was again measured from activity of acidic, basic and neutral proteases in the medium and illustrated on Figure 4.36 for both of the strategies G1 and G2. The highest protease concentrations measured were 0.058 g L^{-1} and 0.102 g L^{-1} for G1 and G2, respectively. In both strategies the highest protease activities were obtained at the last hours of the processes, probably depending on the cell growth. The highest activity in G1 was almost half of that of G2, as G2 reached higher cell densities at the end of the process. By comparing the M13 strain cultivations, the protease activity levels were similar in both strain's cultivations. Changing the strain, carbon source and feeding did not affect the protease activity level significantly.

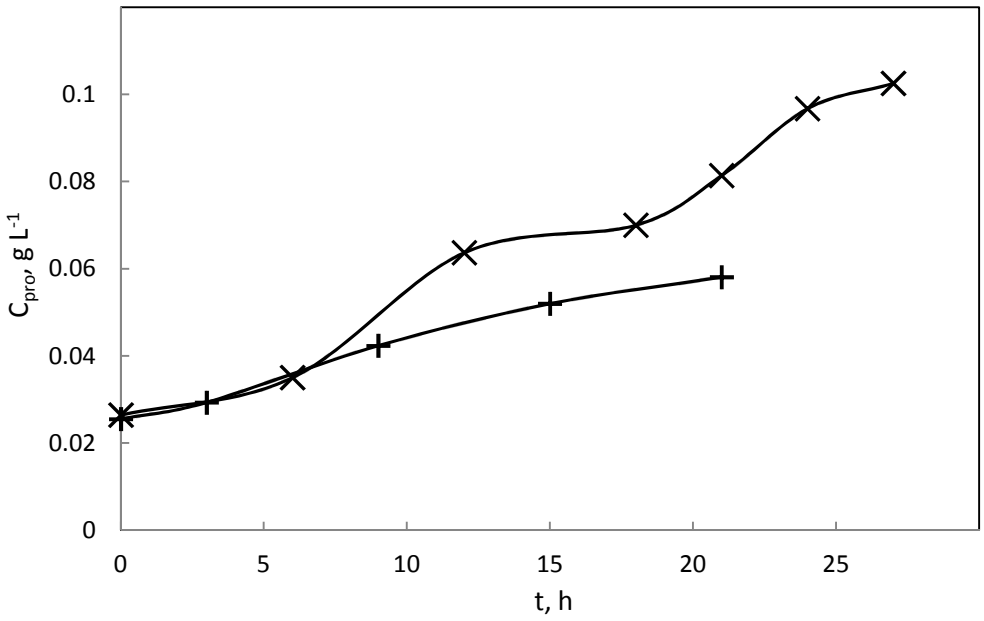

Figure 4.36 Variation in extracellular protease concentration with respect to cultivation time. G1 (+); G2 (X).

4.3.4 Yield Coefficients

The overall yield coefficients are calculated for cultivation times at which the highest C_{rhGH} were reached. Hence, $Y_{X/S}$, $Y_{P/St}$ and $Y_{P/X}$ values were calculated at t=3 h and t=27 h of G1 and G2, respectively. The overall cell yield values on glucose which are 0.47 and 0.48 g g^{-1}, respectively for G1 and G2 are very close to 0.5 g g^{-1} as set in pre-determined feeding profile. $Y_{P/S}$ and $Y_{P/X}$ values are higher in G1, probably depending on lower cultivation time of G1 (Table 4.10). When comparing to M13 strain productions, yield values are closer to MM1 and MM3, whereas 2-3- fold lower than MM2. Therefore, it can be said that G7 strain cultivation was more efficient than MM1 and MM3, whereas MM2 is still the most efficient one. However, it should be taken into account that G7 strain fermentations were carried out single substrate whereas the other included mannitol co-feeding.

Table 4.10 Yield coefficients

Strategy	$Y_{X/S}$ (g g^{-1})	$Y_{P/S}$ (mg g^{-1})	$Y_{P/X}$ (mg g^{-1})
G1	0.47	2.83	5.97
G2	0.48	1.21	2.53

4.3.5 Organic Acid Concentrations

When compared to the fermentations of M13 strains organic acid concentrations are much lower (Table 4.11). Unlike the M13 strain fermentations, maleic acid and fumaric acid were not detected in G7 strain fermentations. Additionally, formic acid was either not detected in the medium or significantly low in G7 fermentations. This is an expected result due to differences in carbon sources of both strains. Since methanol metabolism causes the formic acid formation as explained in section 4.1.2.7, formic acid accumulation were not observed in G7 strain productions. Similarly, gluconic acid concentrations of G7 strain productions were significanly lower than that of M13 strain productions. Gluconic acid was formed by the oxidation of gluconate and gluconate was formed by the oxidation of the some portion of the glucose. Depending on the low gluconic acid concentrations it can be said that intracellular glucose is more efficiently consumed by glycolytic cycle, so lower portion of the glucose converted to gluconic acid.

Table 4.11 Variation in organic acid concentrations within cultivation time

	Experiment t(h)	**3**	**9**	**15**	**21**	
	Gluconic acid	-	-	-	-	
	Malic acid	-	-	-	-	
	Lactic acid	-	0.0617	0.1940	0.3051	
G1	Acetic acid	-	0.0000	0.0578	0.0705	
	Formic acid	-	-	-	-	
	Citric acid	-	0.0053	0.0091	0.0113	
	Succinic acid	-	-	-	-	
	Pyruvic acid	-	0.0458	0.0037	0.0795	
	Experiment t(h)	**3**	**9**	**15**	**21**	**27**
	Gluconic acid	0.1278	0.1800	0.3167	0.3239	0.3599
	Malic acid	-	0.3439	0.3397	0.5747	0.9519
	Lactic acid	-	-	-	0.3669	0.5327
G2	Acetic acid	0.4208	0.6613	0.7022	1.0181	1.1675
	Formic acid	-	-	-	0.0579	0.0827
	Citric acid	-	-	0.0071	0.0114	0.0160
	Succinic acid	-	0.3696	0.2649	0.1792	0.3249
	Pyruvic acid	-	-	0.0198	0.0350	0.0375

CHAPTER 5

CONCLUSION

In this M.Sc. thesis, rhGH production levels and bioprocess characteristics were investigated for two different *P. pastoris* strains. For this purpose, in the first part of the study, to enhance rhGH production, feeding strategy development was performed with previously constructed *P. pastoris* M13 (*hGH*-Mut+) strain with methanol inducible AOX promoter. Effects of mannitol co-feeding on cell growth and rhGH production were investigated and compared with sorbitol in laboratory scale experiments. Thereafter, three different methanol feeding strategies, namely, MM1, MM2 and MM3 were employed in pilot-scale by fed-batch operations together with co-feeding of mannitol and effects on bioprocess characteristics were investigated. In the second part of study *P. pastoris* G7 strain carrying *hGH* gene under constitutive *GAP* promoter was constructed to overcome undesirable methanol requirement of AOX promoter and to enhance rhGH production and to provide continuous production of hGH in further studies.

For the first part, in all experiments temperature was kept at T=30°C, pH was kept at pH=5.0 and stirring was kept at N=900 rpm also four-phase fermentation which are GB, GFB, MT and production phases was employed. In any of them GB, GFB and MT phases did not differ. Production phases were performed as follows; in MM1, pre-determined methanol (μ=0.03 h^{-1}) was fed together with three pulses of mannitol (t=0, 8 and 15 h) to increase its concentration to 50 g L^{-1}. In MM2, 50 g L^{-1} mannitol concentration was set at 50 g L^{-1} by pulse feeding of mannitol at t=0 h and kept until t=6 h and pre-determined (μ=0.03 h^{-1}) methanol was fed until t=19.5 h, batch methanol was added at that hour, after t=20 h, dynamic methanol feeding to keep μ=0.03 h^{-1}

was employed. Required mannitol feeding to keep mannitol concentration at constant level was calculated from q_{man} values of MM1, calculated mannitol feeding was performed in fed-batch manner. Additionally batch methanol addition was performed as 5.7 g L^{-1} for half an hour, similar to MT phase before the production. In MM3 same mannitol feeding was performed as in MM2 together with pre-determined (μ=0.03 h^{-1}) methanol feeding until t=12 h, after that hour dynamic methanol feeding to keep μ=0.03 $h^{-1}$was employed. The major findings obtained by these strategies can be listed as follows:

- The highest C_X was reached in MM3 as 156.6 g L^{-1} which is 1.35- and 1.23-fold higher than that of MM1 (115.8 g L^{-1}) and MM2, (127.1 g L^{-1}), respectively. In all strategies, μ values decreased drastically after mannitol was totally consumed in the medium. However by the dynamic methanol feeding μ can be kept around μ=0.03 h^{-1} in MM3 after t=12 h.
- Mannitol consumption rate was found as 2-3 fold higher than sorbitol from q_{man} values. q_{man} values found as in between 0.299-0.150 g g^{-1} h^{-1} and it was observed that they were affected from mannitol concentration primarily, but from cell concentration also.
- In any of experiments methanol was not detected in the medium and q_M values were found in between 0.025-0.072 g g^{-1} h^{-1}.
- Although the highest cell concentration was reached in MM3; the highest C_{rhGH} and q_{rhGH} values were obtained in the strategy MM2 as an effect of batch methanol addition to the medium at t=19.5 h. The C_{rhGH} value attained in MM2 was 1200 mg L^{-1} which is 4.8- and 3.9-fold higher than that of MM1 (250 mg L^{-1}) and MM3 (307 mg L^{-1}), respectively. In MM2 the q_{rhGH} values increased after batch methanol addition. Hence, it can be concluded that how to feed methanol affects r-protein production more than to increase methanol feeding to keep μ=0.03 h^{-1}.
- The highest $Y_{P/S}$ and $Y_{P/X}$ were obtained in MM2 respectively as 4.02 mg g^{-1} and 10.67 mg g^{-1} whereas the lowest $Y_{X/St}$ obtained as 0.38 g g^{-1}, based on total substrate. Since to enhance cell growth is not the major

aim, MM2 was the most efficient strategy in point of product yields among three of them.

- In all MM group experiments the highest AOX activities was obtained at the beginning of the experiments as 69, 80 and 75 U/g CDW for MM1, MM2 and MM3, respectively and decreased rapidly in following hours and stay at approximately zero level expect MM2. In MM2, again as an effect batch methanol addition to the broth AOX activity profile gave a peak at t=21 h then it oscilated.
- The total protease concentrations in all three strategies began at lower levels and increased continuously to reach their highest values at t=36 h of experiments which are 0.115 g L^{-1}, 0.148 g L^{-1}, and 0.128 g L^{-1} for MM1, MM2 and MM3, respectively.

In the second part of the study *P. pastoris* G7 strain carrying *hGH* cDNA under the control of GAP promoter was constructed for further enhance rhGH production by constitutive expression without methanol. For this purpose forward and reverse primers were designed for amplification of *hGH* cDNA, by considering the *EcoR* I and *Xba* I restriction enzyme recognition sites. After *hGH* gene was amplified by PCR by using pPICZαA*::hGH* plasmid and pGAPZαA vector, including α-factor for extracellular production, was propagated in *E. coli* DH5α cells, both are double digested with related restriction enzymes. By the ligation reaction, the plasmid pGAPZαA*::hGH* was constructed. After verification of plasmid, wild type *P. pastoris* X-33 cells were transfected with linearized plasmid. Then, the best potential for rhGH production was chosen from the laboratory scale experiments by SDS-PAGE and dot-blot analysis. After selection of colony two different feeding strategies namely, G1 and G2 were performed by fed-batch single carbon source, glucose, feeding. Again, in all experiments temperature was kept at T=30°C, pH was kept at pH=5.0 and stirring was kept at N=900 rpm. As pre-cultivation, unlike M13 strain cultivations, only GB phase was performed, then production phase started with glucose feeding. In G1, as first

cultivation with this strain, glucose was fed with pre-determined $\mu=0.2h^{-1}$ until t=6 h to increase cell concentration to 23-28 g L^{-1} as in M13 strain cultivations just before the production phase. For following hours of production, pre-determined μ decreased to $\mu=0.03\ h^{-1}$ as methanol in M13 strain cultivation. In G2 strategy, depending on findings of G1, glucose was fed with pre-determined $\mu=0.2\ h^{-1}$ until t=3 h, for following hours constant feeding of glucose was employed. Major findings from G1 and G2 can be summarized as follows,

- Pre-determined $\mu=0.03\ h^{-1}$ was too low for glucose feeding as can be observed from much lower cell concentrations and decrease in C_{rhGH} for $\mu=0.03\ h^{-1}$ glucose feeding hours.
- The highest C_X is obtained in 90 g L^{-1} at t=27 h of production in G2. Due to limiting glucose feeding in G1 the highest C_X= 36.9 g L^{-1} at t=21 h which is 2.4-fold lower than that of G2. μ values were lower than pre-determined values in pre-determined glucose fed hours of cultivations, probably depending on maintanance factor.
- Glucose was not detected in neither experiments, related with q_G values it was higher in G2 strategy. In G2, it decreased until to reach same low value as in G1 due to constant glucose feeding.
- The highest C_{rhGH} was attained in t=27 h of G2 as 200 mg L^{-1}, and product formation was observed as growth associated. If cultivation were continued further, probably rhGH production will increase further, at least until cell concentration will reach stationary phase. Related with q_{rhGH} values they achieve their maxima at the beginning of experiment depending on carbon source shift from glycerol to glucose. At t=0 h of both productions, there were not any significany extracellular protein in the medium although r-protein production is known as possible with glycerol feeding under GAP promoter.
- The overall yield coefficient $Y_{X/S}$ values of G1 and G2 was found as 0.47 and 0.48 g g^{-1} as consistent with the value used in calculation of pre-determined feeding. Furthermore, in G1 $Y_{P/S}$ and $Y_{P/X}$ values are found as

approximately 2-fold of that in G2, respectively, depending on shorter cultivation time (t=3 h) in G1.

- Protease concentration profile was lower in G1 depending on lower cell concentrations. When comparing to M13 strain cultivations, it was observed that protease activity level did not differ much among them.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Agarose Gel Electrophoresis Solutions

5X TBE — Dissolve 54 g of Tris base, 27.5 of boric acid in 900 mL dH_2O. Add 20 mL 0.5 M EDTA (pH 8.0) Complete the volume up to 1 L. Store at room temperature.

1X TBE — Dilute the 5X stock solution to 1X with dH_2O.

Alkaline Lysis Solutions

Alkaline Lysis I — 50mM glucose, 25mM Tris-Cl (pH 8.0) and 10mM EDTA (pH 8.0) Prepare 100 mL with dH_2O. Autoclave for 15 minutes at 15 psi on liquid cycle and store at 4°C.

Alkaline Lysis II — Dissolve 1 % (w/v) SDS in 0.2 N NaOH (freshly diluted from 10 N stock) Prepare fresh and use at room temperature.

Alkaline Lysis III — Mix 60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid and 28.5 mL dH_2O. Store the solution at 4°C and transfer to an ice bucket just prior to use.

TE — Mix 1 mL 1M Tris-HCl (pH 8.0) and 0.5 mL of 0.5M EDTA, and complete to 100mL with dH_2O.

Genomic DNA Isolation Solutions

Yeast Lysis Solution	Dissolve 2 g of Triton X-100, 1 of SDS, 0.1 mole of NaCl, 0.01 mole of Tris-Cl and 0.001 mole of Na_2EDTA in 1 liter of dH_2O.
4M Sodium Acetate	Dissolve 30.84 g of ammonium acetate in 100 mL of distilled water.
TE	Mix 1 mL 1M Tris-HCl (pH 8.0) and 0.5 mL of 0.5M EDTA, and complete to 100mL with dH_2O.

Lithium Chloride Transformation Solution

1M LiCl	Dissolve 4.24 g of LiCl in distilled water and filter sterilize. Dilute with sterile water when needed.
PEG	Dissolve 50 % polyethylene glycol (PEG-3350) in distilled water and filter sterilize. Store in tightly capped bottle.
Single-stranded DNA	2 mg/mL denaturated, fragmented salmon sperm DNA in TE (pH 8.0) buffer, store at -20°C.

AOX Assay Solutions

Yeast Lysis Buffer	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 M potassium phosphate, pH=7.5	1M KH_2PO_4, 1M K_2HPO_4 was dissolved in dH_20 and titer KH_2PO_4 with K_2HPO_4 while controlling pH. The buffer was autoclaved and stored at room temperature.

SDS-PAGE Solutions

10%(w/v) APS (Ammonium PerSulfate)	Add 0.1g APS to 1 mL dH_2O , freshly prepared.
1.5 M 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.
Resolving Buffer (12%) (for 2 gels)	3.4mL dH_20, 4mL 30% Acrylamide-bis, 2.5 mL 1.5M Tris–HCl pH=8.8, 100µL 10%SDS, prior to gel preparation add 50µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine .
Stacking Buffer (5%) (for 2 gels)	2.8mL dH_20, 0.85mL 30% Acrylamide-bis, 1.25 mL 0.5M Tris –HCl pH=6.8, 50µL 10%SDS, prior to gel preparation add 25µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine
4 x Sample Loading Buffer for 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 can be stored at 2-8°C.
1x SDS-PAGE Running	Diluted from 5X buffer solution prior to use and can

Buffer	be used three times.
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 stored up to one month.
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.

Dot-Blot Solutions

10X TBS, pH=7.6	Dissolve 12.11 g Tris-base and 87.66 g NaCl in 900 mL distilled water and pH adjusted to 7.6. Then volume adjusted to 1 L. Store at room T.
TBS-T solution	Dilute 10X TBS to 1X . Add 0.1%(v/v) Tween-20. Prepare on the day of use.
TBS-T Milk	Dissolve 5%(w/v) non-fat milk powder in TBS-T. Prepare on the day of use and store at 2-8°C.

Protease Assay solutions

Borate buffer (for Alkali proteases)	2.381 g Boraks ($Na_2B_4O_7.10\ H_2O$) dissolved in 250 ml dH_2O. pH is 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

GROWTH MEDIA

Table B.1 Composition of LB (Luria-Bertani)

Compound	*Concentration (kg m^{-3})*
Tryptone	10.0
Yeast extract	5.0
NaCl	10.0

pH is adjusted to 7.5 with NaOH, then final volume is adjusted with distilled water. 15 g L^{-1} agar is used if solid medium is required. The medium is autoclaved and stored at room temperature.

Table B.2 Composition of LSLB (Low Salt Luria-Bertani)

Compound	*Concentration (kg m^{-3})*
Tryptone	10.0
Yeast extract	5.0
NaCl	5.0

pH is adjusted with NaOH, then final volume is adjusted with distilled water. 15 g L^{-1} agar is used if solid medium is required. The medium is autoclaved and stored at room temperature. For the media referred as LSLB+Zeocin, Zeocin was added after sterilization when the medium has cooled to below 55°C, at a final concentration of 25 µg mL^{-1} Zeocin.

Table B.3 Composition of YPD (Yeast Extract Peptone Dextrose)

Compound	*Concentration (kg m^{-3})*
Peptone	20.0
Yeast extract	10.0
Glucose	20.0

For 1 liter medium, dissolve 20 g of peptone and 10 g of yeast extract in 900 mL water, include 20 g of agar if solid medium is required. Autoclave for 20 minutes at 121°C on liquid cycle, store at room temperature. Separately autoclave 100 mL of the 20 % (w/v) glucose. Add the glucose to the yeast extract peptone medium when required. In the media referred as YPD +zeocin, zeocin was added after sterilization of medium when cooled about 55°C with a final zeocin concentration of 100 to 200 μg mL^{-1}.

Table B.4 BMGY (Buffered Glycerol Complex Medium)

Compound	*Concentration (kg m^{-3})*
Yeast extract	10.0
Peptone	20.0
Potassium phosphate buffer pH=6.0	0.1 M
YNB (Yeast Nitrogen Base)	3.4
$(NH_4)SO_4$	10.0
Biotin	4×10^{-4}
Glycerol	10.0

For 500 mL BMGY medium 5 g of Yeast extract and 10 g of peptone were dissolved in 325 mL distilled water and autoclaved. After cooled to the room temperature 50 mL of 10X YNB stock (autoclaved separately), 50 mL of 1M Potassium phosphate buffer pH=6.0 (autoclaved separately), and 50 mL of 10% glycerol solution (autoclaved separately) was added. Thereafter 1 mL from 500X filter sterilized biotin solution and 0.5 mL from chloramphenicol (antibiotic)

stock solution was added for precultivation medium. Chloramphenicol is prepared as 34mg/ml stock in pure ethanol, kept in sterile dark bottle at -20^{o}C.

Table B.5 BSM (Basal Salt Medium) for Laboratory Scale Shake Flask Experiments

Compound	*Concentration (kg m^{-3})*
Methanol (mL)	10
Potassium phosphate buffer pH=6.0	0.1 M
$(NH_4)_2SO_4$	C/N=4.57; M/N=2.19
PTM1 (mL)	4.35
$MgSO_4.7H_2O$	7.30
$CaSO_4.2H_2O$	0.57
Chloramphenicol (mL from stock)	1

For 1 liter basal salt medium 7.30 g of $MgSO_4.7H_2O$ and 0.57 g of $CaSO_4.2H_2O$ was dissolved

Table B.6 BSM (Basal Salt Medium) for Pilot Scale Bioreactor Experiments

Compound	*Concentration (kg m^{-3})*
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
Chloramphenicol (mL from stock)	1
30% antifoam (mL)	1
PTM1 (mL)	4.35

All the compounds except chloramphenicol, antifoam and PTM1 solution were dissolved in distilled water and volume is adjusted to 1 liter.

Table B.7 PTM1 (Pichia Trace Salts)

Compound	*Concentration (g/100mL)*
$CuSO_4.5H_2O$	0.6
NaI	0.008
$MnSO_4.H_2O$	0.3
$Na_2MoO_4.2H_2O$	0.02
H_3BO_3	0.002
$ZnCl_2$	2
$FeSO_4.7H_2O$	6.5
$CoCl_2.6H_2O$	0.09
H_2SO_4 (mL)	0.5
Biotin*	0.02

Dissolve all the compounds in distilled water, adjust volume to 100 mL and sterilize by filtering and store at +4°C. Solution results in turquoise color clear solution. Discard when the color of solution turns into yellowish-green.

Glycerol Feed

100 mL of pure glycerol was diluted to 200 mL with distilled water and sterilized by autoclaving. Add 12 mL PTM1 solution per liter of solution when feeding.

Methanol Feed

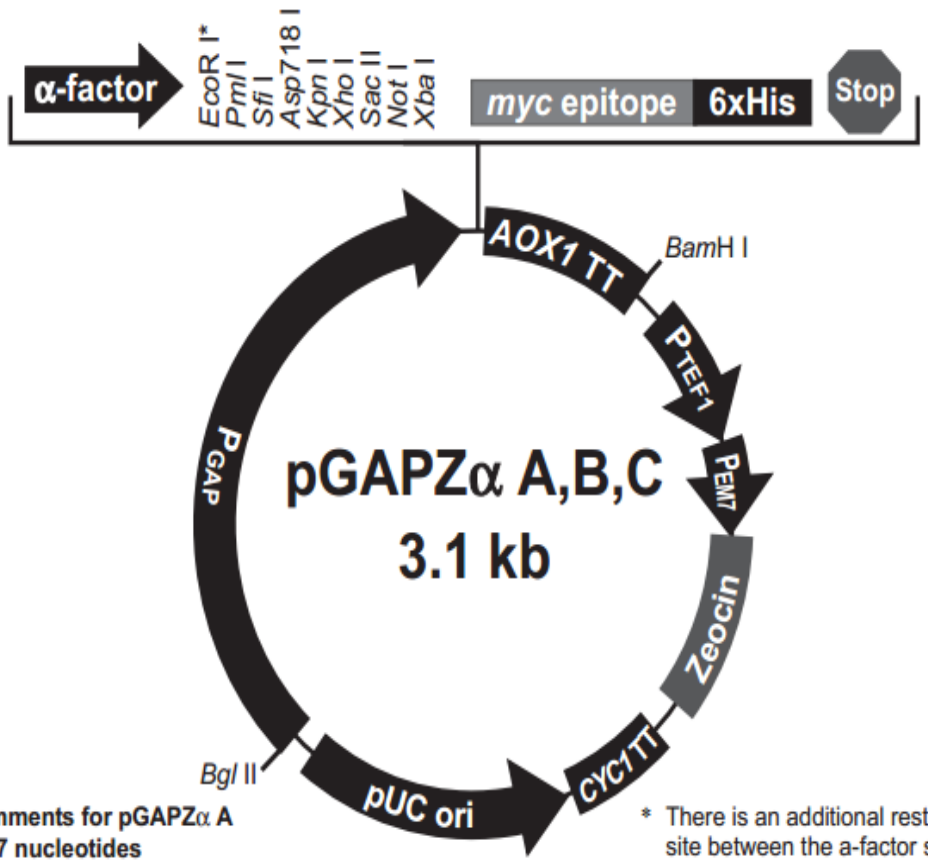
Add 12 mL PTM1 per liter of pure methanol just before feeding. No need to sterilize.

Mannitol Feed

Dissolve 50 g of mannitol in BSM medium adjust volume to 200 mL and sterilize by autoclaving.

APPENDIX C

NUCLEOTIDE SEQUENCES AND PLASMIDS



Comments for pGAPZα A
3147 nucleotides

GAP promoter region: bases 1-483
α-factor signal sequence: bases 493-759
Multiple cloning site: bases 760-828
myc epitope tag: bases 827-856
Polyhistidine tag: bases 872-889
AOX1 transcription termination region: bases 893-1233
TEF1 promoter region: bases 1234-1644
EM7 promoter: bases 1645-1712
Sh ble ORF: bases 1713-2087
CYC1 transcription termination region: bases 2088-2405
pUC origin: bases 2416-3089

* There is an additional restriction site between the a-factor signal sequence and the *Eco*R I site in versions B and C of pGAPZa:

Pst I in pGAPZα B
Cla I in pGAPZα C

Figure C.1 Schematic representation of map of pGAPZαA vector supplied from Invitrogen

Sequence of pGAPZαA (3147 bp)

AGATCTTTTTTGTAGAAATGTCTTGGTGTCCTCGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGT
TGCAACTCCGAACGACCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAACCA
GAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAAATTTTACTCTGCTGGAGAG
CTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCAGCATTACGTTGCGGGTAAAACGGAGGTCGTGTACC
CGACCTAGCAGCCCAGGGATGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGA
GATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTGGTTTCTCCTGACCCAA
AGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAATTGAACAACTATTTCGAAACGATGAGATTTCCT
TCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGAT
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TTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAA
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ggtacctcgagccgcggcggccgccagcttTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGC
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TACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATT
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TGCGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACT
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TGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAA
AAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTG
ACCTCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTAT
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GTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCT
CCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCC
AGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGT
GGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGT
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ACGTCCGACGGCGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAA
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CGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGC
TCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTG
CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTT
TCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA
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ACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTT
TGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGAC
GCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCATGAGATC

Figure C.2 Sequence of pGAPZαA vector.

Multiple Cloning Site of pGAPZαA:

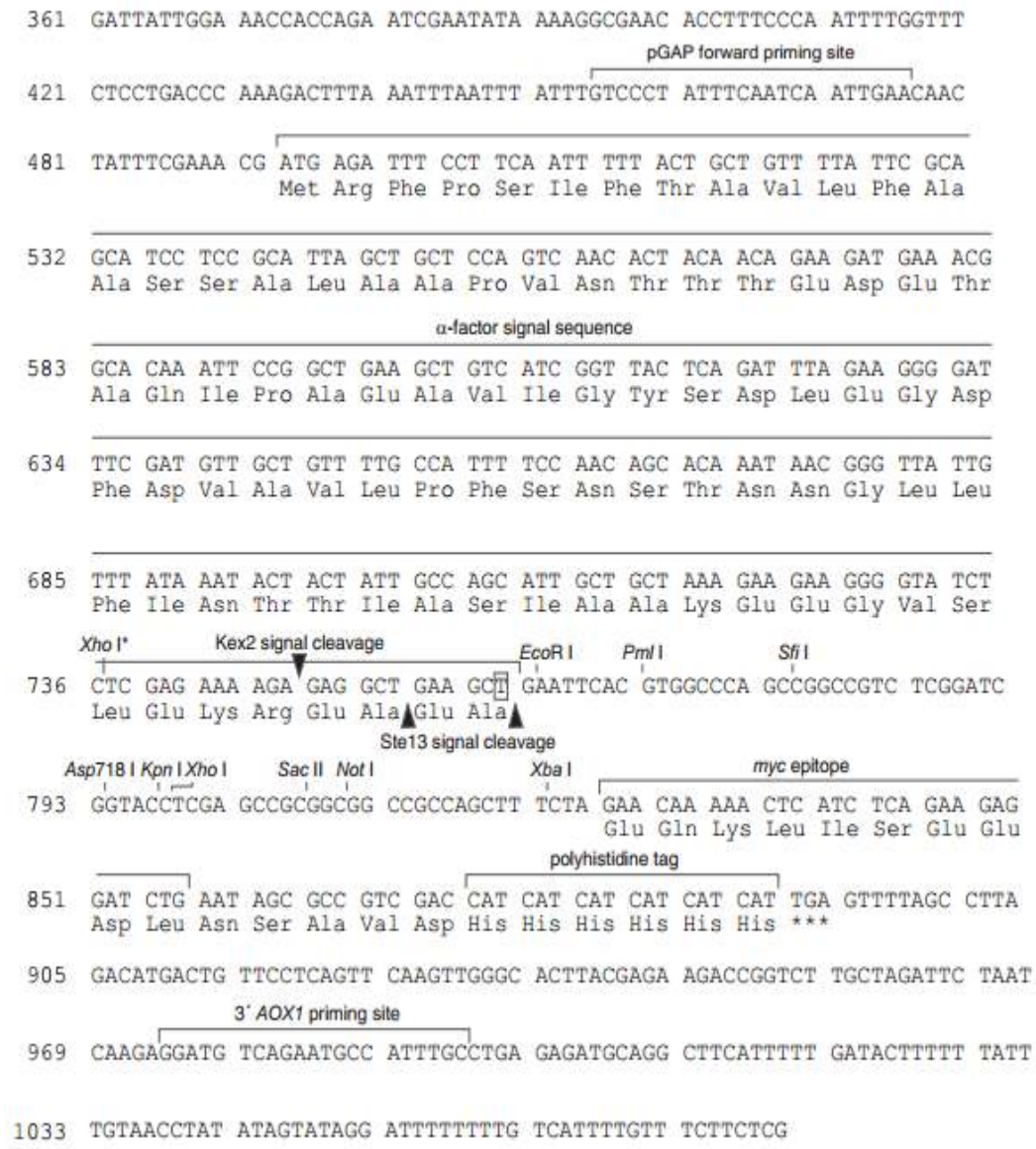

Figure C.3 Nucleotide sequence of multiple cloning site of pGAPZαA.

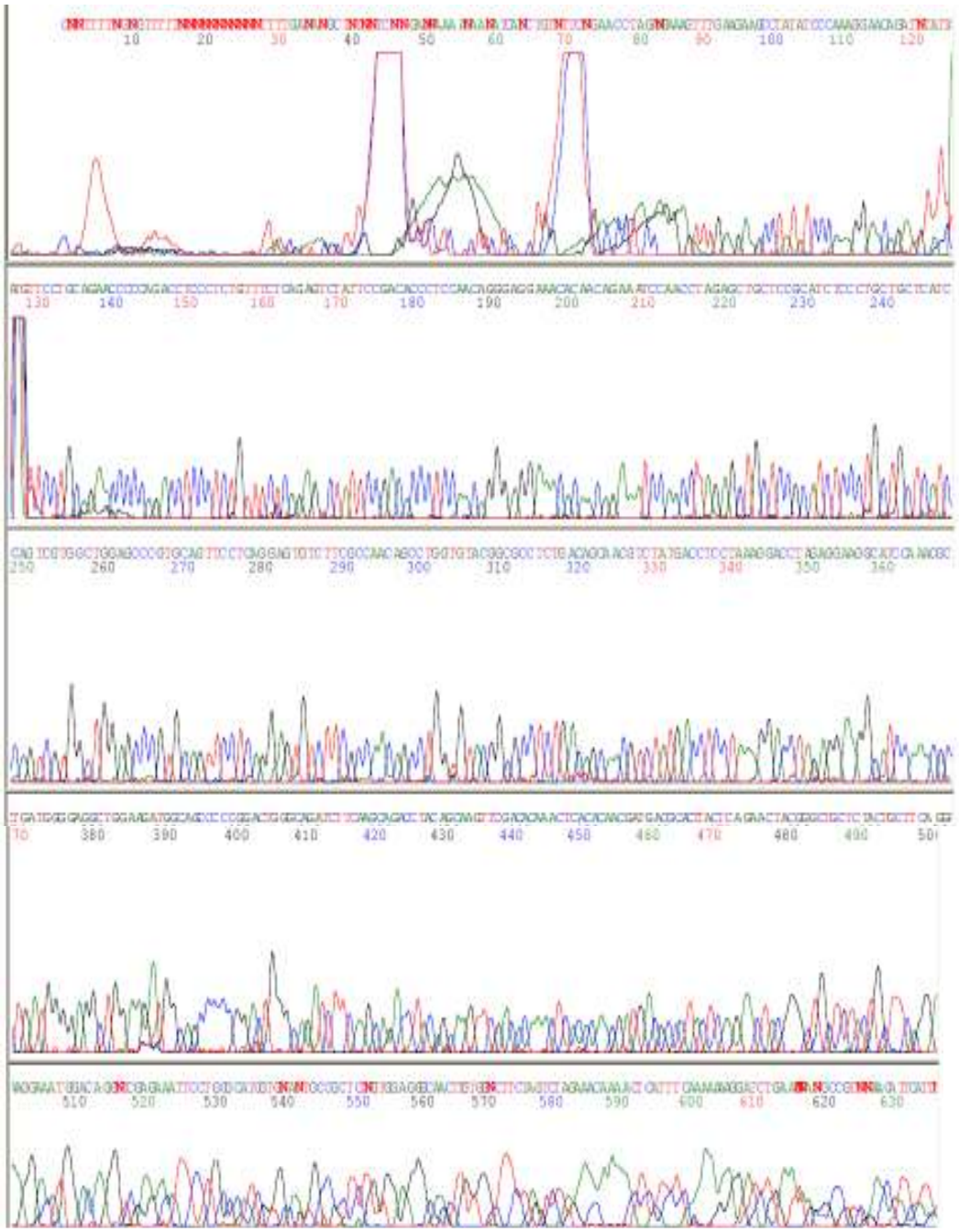

Figure C.4 Nucleotide sequence from forward reading

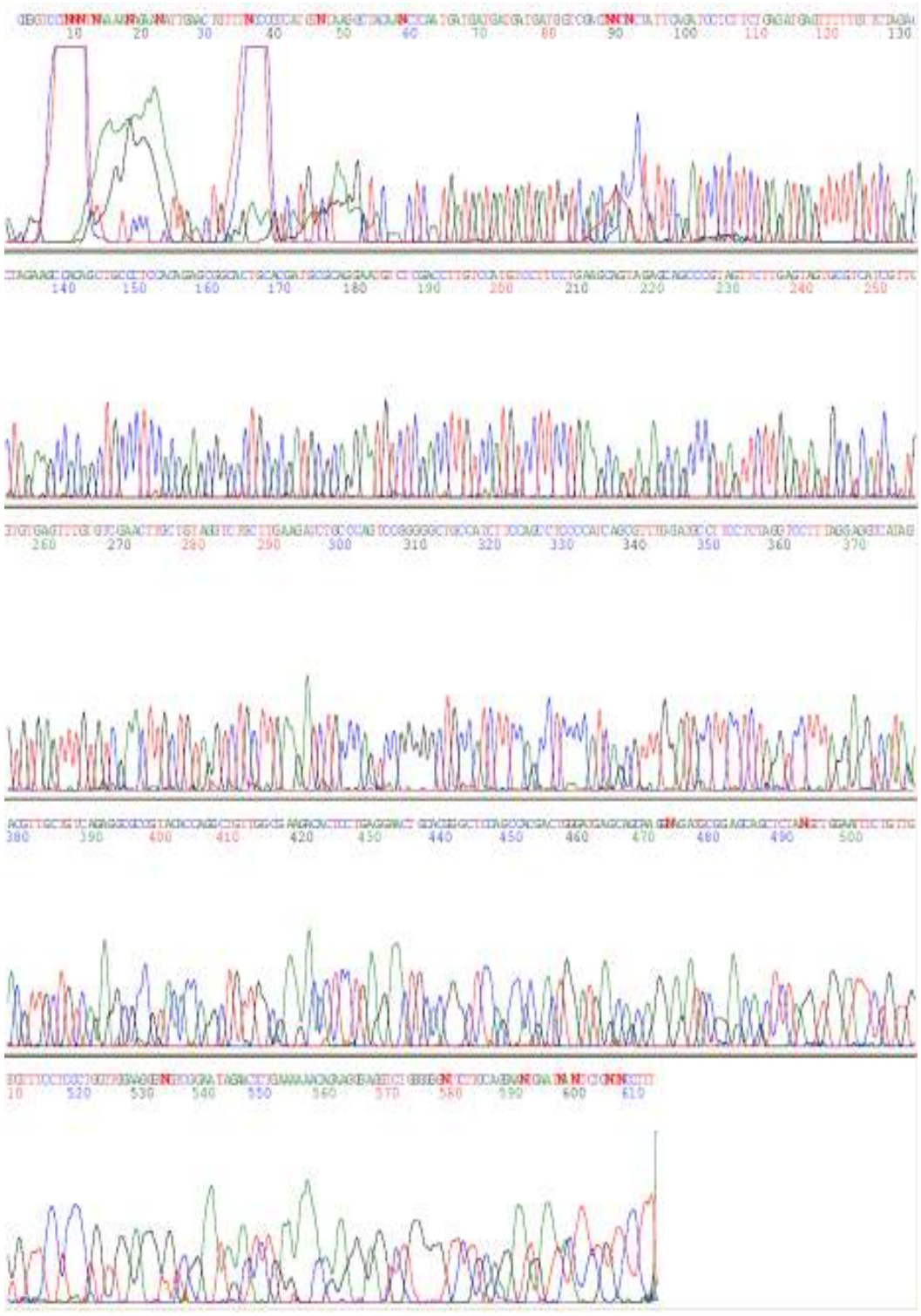

Figure C.5 Nucleotide sequence from reverse reading

AGATCTTTTTTGTAGAAATGTCTTGGTGTCCTCGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGT
TGCAACTCCGAACGACCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAACCA
GAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAAATTTTACTCTGCTGGAGAG
CTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCAGCATTACGTTGCGGGTAAAACGGAGGTCGTGTACC
CGACCTAGCAGCCCAGGGATGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGA
GATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTGGTTTCTCCTGACCCAA
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GAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTT
TTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAA
GAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCttcccaactataccactatctcgtcta
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attccgacaccctccaacagggaggaaacacaacagaaatccaacctagagctgctccgcatctccctgctg
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TACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATT
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TGCGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACT
CCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTTCCCTCTTTCTTCCTCTAGGG
TGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAA
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TCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTG
CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTT
TCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA
CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGAC
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TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTT
ACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTT
TGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGAC
GCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCATGAGATC

Figure C.6 Sequence of constructed pGAPZαA*::hGH* plasmid (3715bp); nucleotides written in bold italics show GAP forward and AOX reverse primers recognition sites, nucleotides written underlined show restriction enzyme recognition sites, nucleotides written in lower case letters show hGH gene added to pGAPZαA plasmid.

APPENDIX D

THERMODYNAMIC PROPERTIES OF DESIGNED PRIMERS

Oligo Analyzer - 1.1.2 05/10/2011

FwdGHgh

```
Name           : FwdGHgh
Primer         : 5´-GGAATTCTTCCCAACTATACCACTATCTCGTC-3´
Reverse        : 3´-CTGCTCTATCACCATATCAACCCTTCTTAAGG-5´
Length         : 32 nt

Tm (basic)     : 92.0 °C
Tm (salt)      : 71.2 °C
Tm (NN)        : 67.0 °C

GC %           : 43.8 %
dG             : -62.1 kCal/mol

3'-tail GC %   : 57.1 %
3'-tail dG     : -11.5 kCal/mol

Molecular weight : 9716.3 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 3.13 µM
and contains 30.4 µg ssDNA
```

FwdGHgh self annealing:

```
                          5'-GGAATTCTTCCCAACTATACCACTATCTCGTC-3'
                             ||||||
3'-CTGCTCTATCACCATATCAACCCTTCTTAAGG-5'
 dG: -5.54 kcal/mol

                       5'-GGAATTCTTCCCAACTATACCACTATCTCGTC-3'
                          ::::   ||||
3'-CTGCTCTATCACCATATCAACCCTTCTTAAGG-5'
 dG: -3.97 kcal/mol

5'-GGAATTCTTCCCAACTATACCACTATCTCGTC-3'
      :           ||||           :
  3'-CTGCTCTATCACCATATCAACCCTTCTTAAGG-5'
 dG: -0.59 kcal/mol

5'-GGAATTCTTCCCAACTATACCACTATCTCGTC-3'
                   |||    :::
```

```
          3'-CTGCTCTATCACCATATCAACCCTTCTTAAGG-5'
 dG: 0.77 kcal/mol
```

FwdGHgh loops:

```
                      5'-GGAAT
                         |||| T
3'-CTGCTCTATCACCATATCAACCCTTC
 dG: -3.55 kcal/mol

5'-GGAATTCTTCCCAACTATACC
                    |||  )
          3'-CTGCTCTATCA
 dG: 1.19 kcal/mol
```

Oligo Analyzer - 1.1.2 05/10/2011

RevGHgh

```
Name          : RevGHgh
Primer        : 5´-CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC-3´
Reverse       : 3´-CACCTCCCGTCGACACCGAAGATCAGATCTCC-5´
Length        : 32 nt

Tm (basic)    : 102.0 °C
Tm (salt)     : 77.6 °C
Tm (NN)       : 75.8 °C

GC %          : 59.4 %
dG            : -72.6 kCal/mol

3'-tail GC %  : 71.4 %
3'-tail dG    : -12.7 kCal/mol

Molecular weight : 9721.3 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 3.10 µM
and contains 30.2 µg ssDNA
```

RevGHgh self annealing:

```
5'-CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC-3'
                    ||||||
          3'-CACCTCCCGTCGACACCGAAGATCAGATCTCC-5'
 dG: -8.77 kcal/mol

                       5'-CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC-3'
                            ||||||
3'-CACCTCCCGTCGACACCGAAGATCAGATCTCC-5'
 dG: -7.35 kcal/mol

                 5'-CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC-3'
                    : ||||| ::::: :
```

```
3'-CACCTCCCGTCGACACCGAAGATCAGATCTCC-5'
 dG: -4.62 kcal/mol

5'-CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC-3'
         :    : |||    ::: :    :
    3'-CACCTCCCGTCGACACCGAAGATCAGATCTCC-5'
 dG: -2.46 kcal/mol

            5'-CCTCTAGACTAGAAGCCACAGCTGCCCTCCAC-3'
                  :     ||||     :
3'-CACCTCCCGTCGACACCGAAGATCAGATCTCC-5'
 dG: -1.90 kcal/mol
```

RevGHgh loops:

```
                  5'-CCTCTAG
                     : |||| A
3'-CACCTCCCGTCGACACCGAAGATC
 dG: -2.33 kcal/mol

5'-CCTCTAGACTAGAAGCCA
         :    : |||  )
    3'-CACCTCCCGTCGAC
 dG: -2.04 kcal/mol
```

APPENDIX E

MOLECULAR WEIGHT MARKERS

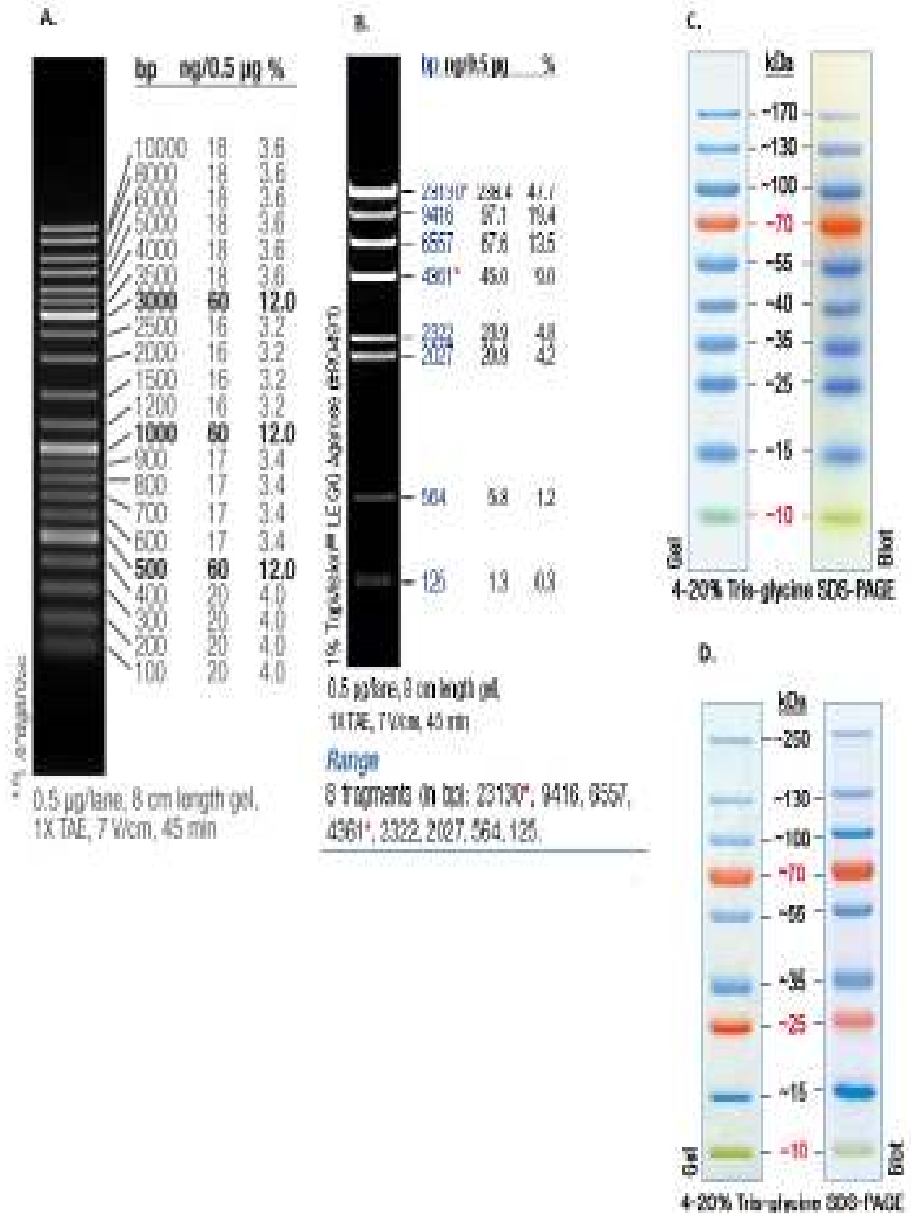

Figure E.1 A. GeneRuler™ DNA Ladder Mix (Fermentas) B. Lambda DNA/HindIII Marker (Fermentas) C. PageRuler™ Prestained Protein Ladder (Fermentas) D. PageRuler™ Plus Prestained Protein Ladder (Fermentas)

APPENDIX F

SDS-PAGE PROTEIN ANALYSES

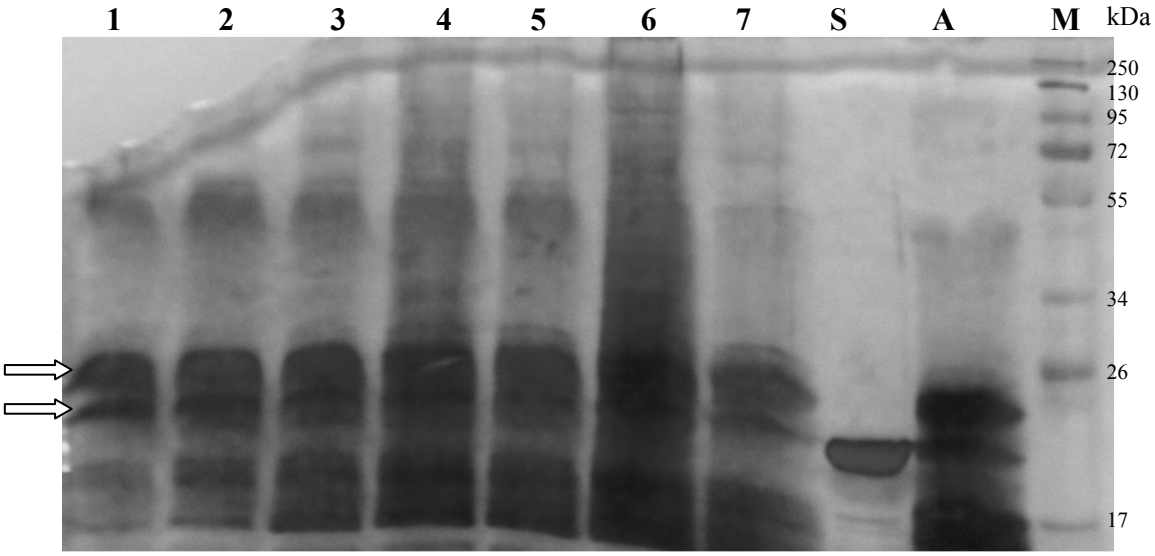

Figure F.1 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM1 double band region indicated by arrows shows rhGH regions in protein bands. Lane M: PageRuler Plus protein ladder; Lane S: 0.2 g L^{-1} hGH standard, Lane A: t=48 h of SSM1, Lane 1: t=3 h, Lane 2: t=6 h, Lane 3: t=9 h, Lane 4: t=12 h, Lane 5: t=15 h, Lane 6: t=18 h, Lane 7: t=0 h.

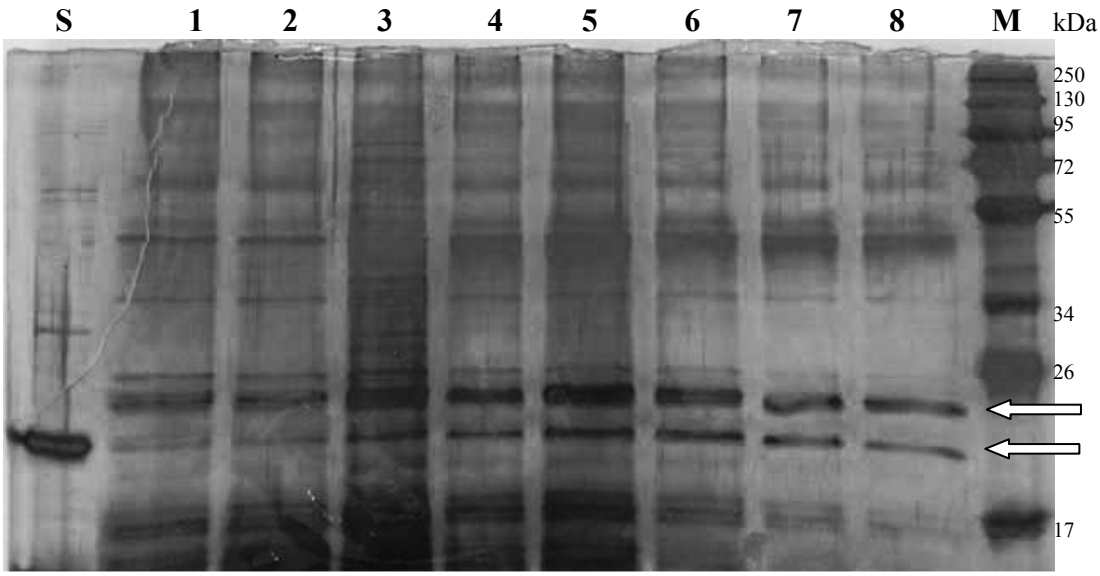

Figure F.2 Silver stained SDS-PAGE gel view of extracellular proteins produced by *Pichia pastoris* M13 strain in MM. Double band region indicated by arrows shows the rhGH regions in the gel. LaneM: PageRuler Plus protein ladder, Lane S: 0.2 g L^{-1} hGH standart, Lane 1: t= 6 h, Lane 2: t=9 h, Lane-3: t=12 h, Lane 4: t=15 h, Lane 5: t=21 h, Lane 6: t=24 h, Lane 7: t=30h.

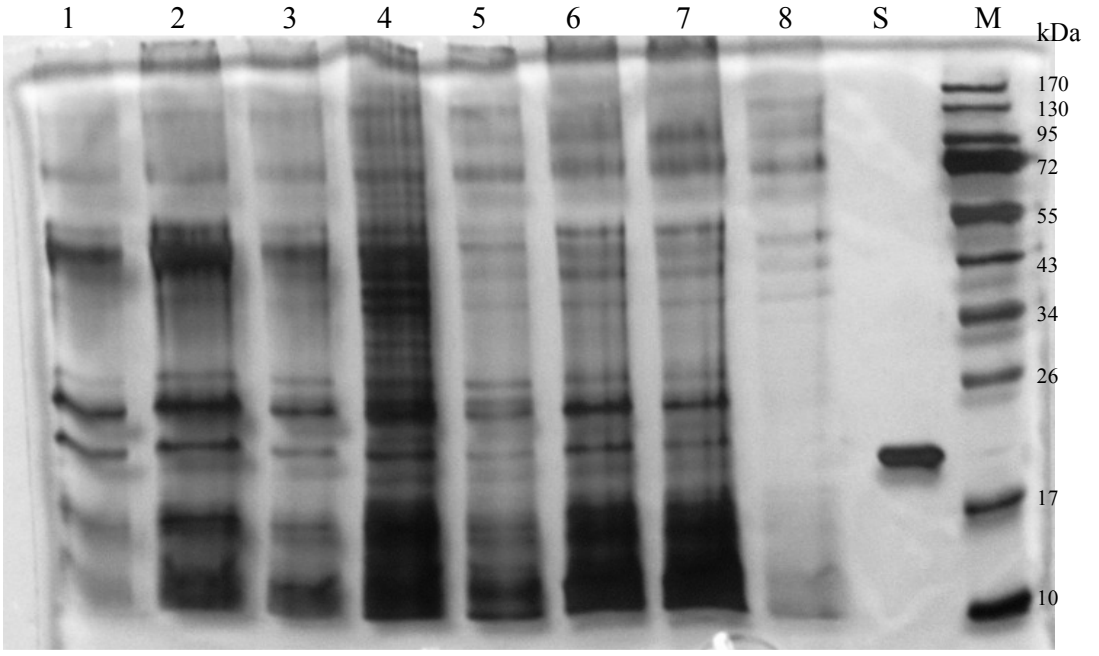

Figure F. 3 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM1. Lane M: PageRuler protein ladder; Lane S: 0.2 g L^{-1} hGH standard, Lane 1: t=9 h, Lane 2: t=12 h, Lane 3: t=15 h, Lane 4: t=18 h, Lane 5: t=21 h, Lane 6: t=24 h, Lane 7: t=30 h, Lane 8: t=36 h.

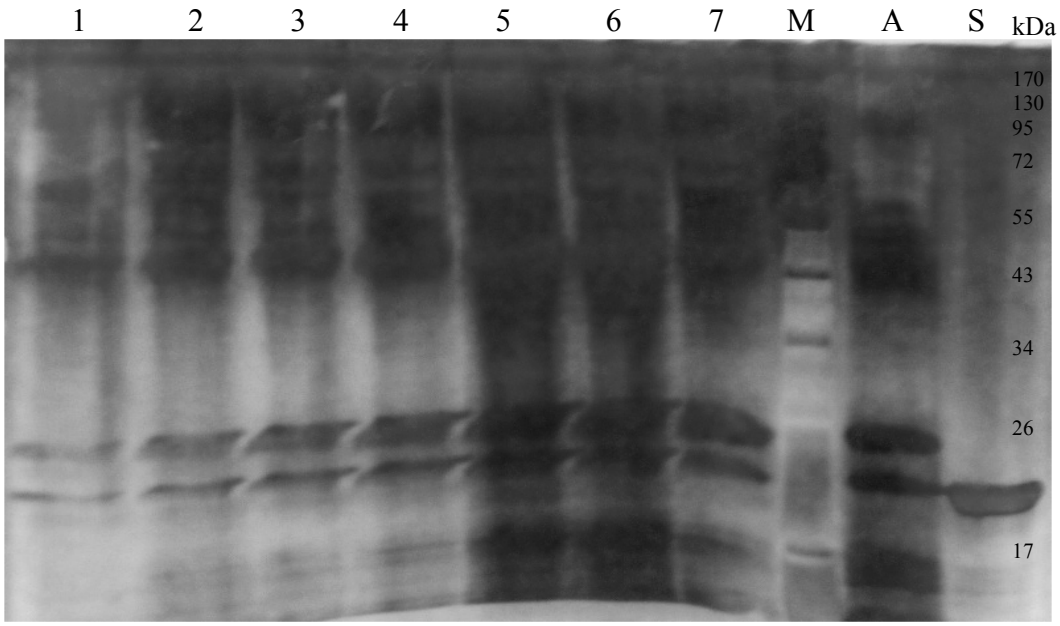

Figure F.4 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM2. Lane M: PageRuler protein ladder; Lane S: 0.2 g L^{-1} hGH standard, Lane A: t=48 h of SSM1, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18 h

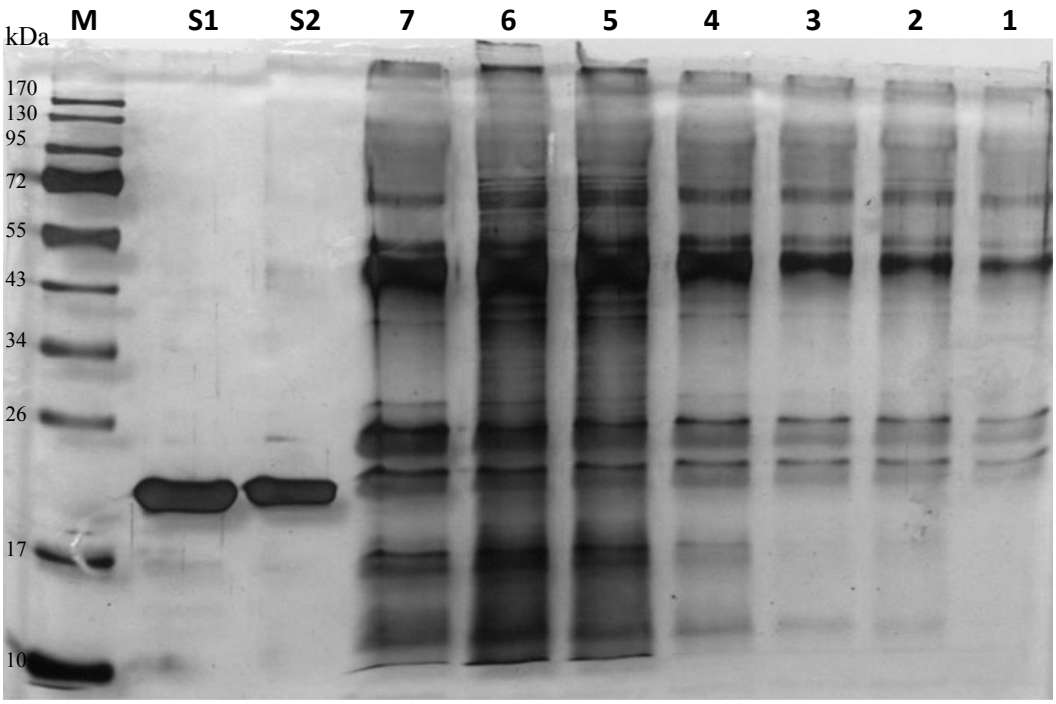

Figure F.5 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM2, 1:2 diluted samples. M: PageRuler protein ladder; S1: 0.2 g L^{-1} hGH standard, S2: 0.2 g L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18 h.

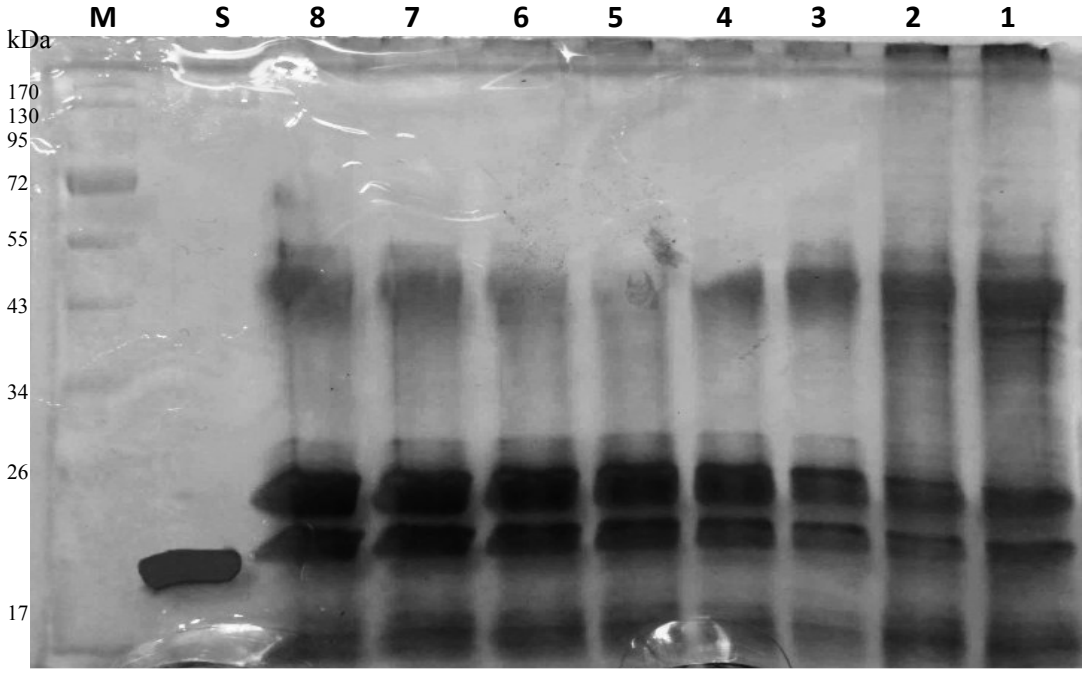

Figure F.6 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM2, 1:2 diluted samples. M: PageRuler protein ladder; Lane S: 0.2 g L^{-1} hGH standard, Lane 1: t=12 h, Lane 2: t=15 h, Lane 3: t=18 h, Lane 4: t=21 h, Lane 5: t=24 h, Lane 6: t=27 h, Lane 7: t=30 h, Lane 8: t=36 h.

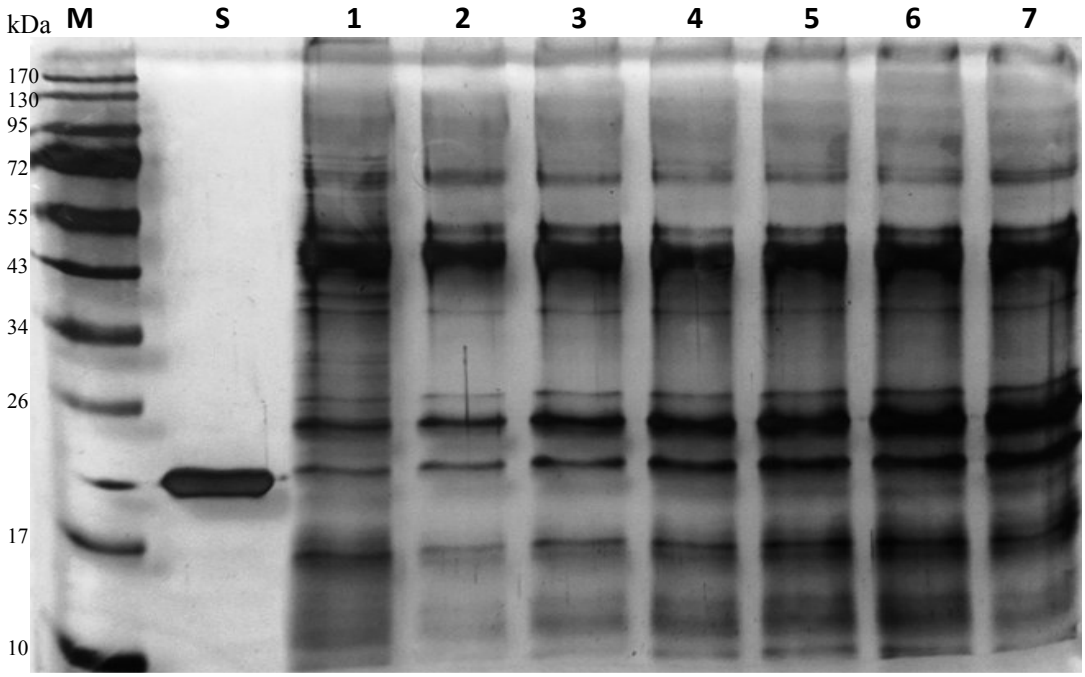

Figure F.7 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM2, 1:2 diluted samples. M: PageRuler protein ladder; S: 0.3 g L^{-1} hGH standard, Lane 1: t=15 h, Lane 2: t=18 h, Lane 3: t=21 h, Lane 4: t=24 h, Lane 5: t=27 h, Lane 6: t=30 h, Lane 7: t=36 h.

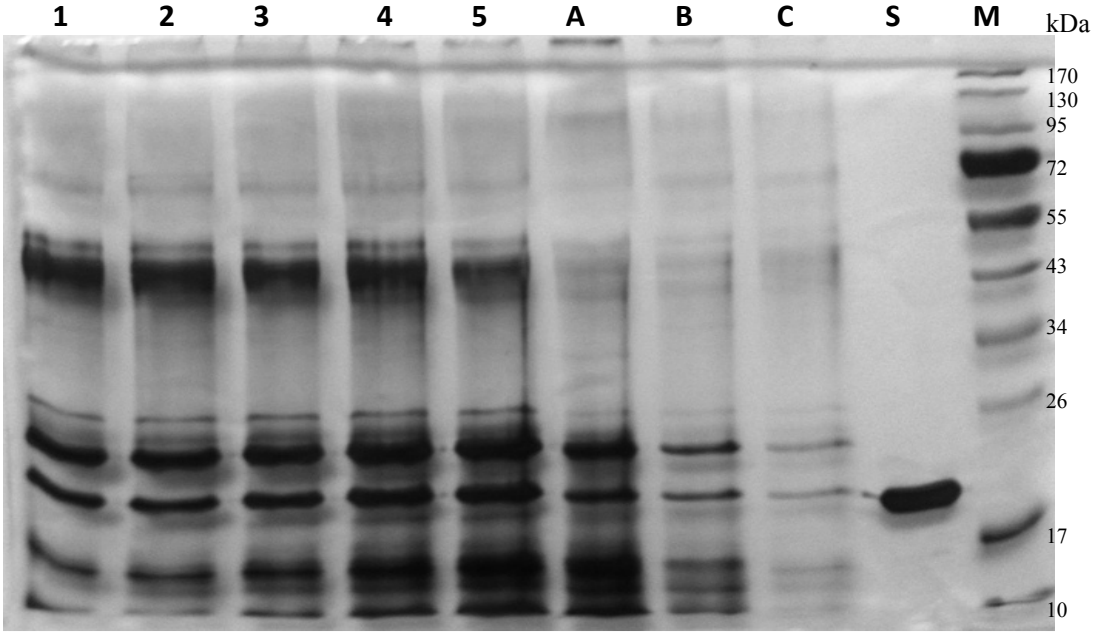

Figure F.8 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM2, 1:2 diluted samples. M: PageRuler protein ladder; S: 0.4 g L^{-1} hGH standard, Lane A: t=48 h of SSM1, Lane B: t=18 h of MM3, Lane C: t=12 h of MM1, Lane 1: MM2 t=21 h, Lane 2:MM2 t=24 h, Lane 3: MM2 t=27 h, Lane 4: MM2 t=30 h, Lane 5: MM2 t=36 h.

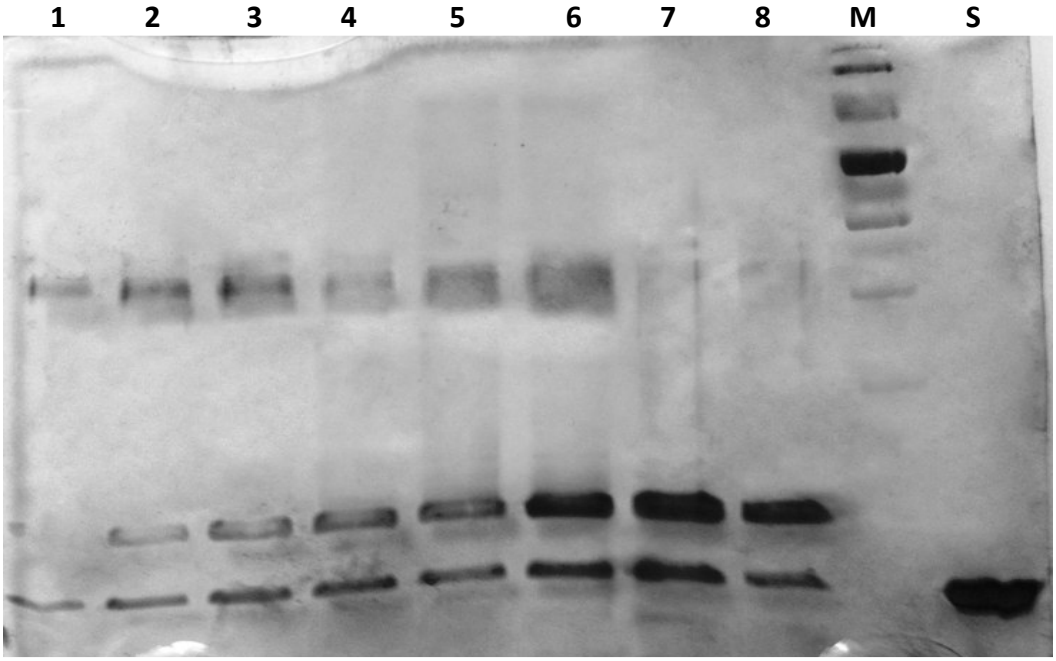

Figure F.9 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM3, 1:2 diluted samples. M: PageRuler protein ladder; S: 0.2 g L^{-1} hGH standard Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18 h, Lane 8: t=21 h.

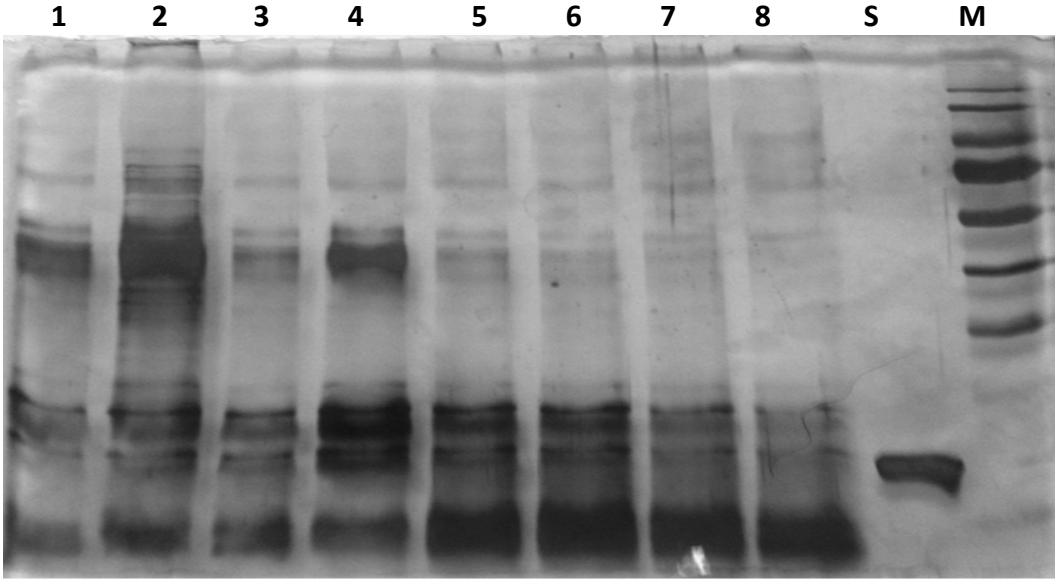

Figure F.10 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM3, 1:2 diluted samples. M: PageRuler protein ladder; S: 0.2 g L^{-1} hGH standard, Lane 1: MM3 t=12 h, Lane 2: MM2 t=18 h, Lane 3: MM3 t=15 h, Lane 4: MM2 t=21 h, Lane 5: MM3 t=18 h, Lane 6: MM3 t=21, Lane 7: MM3 t=24, Lane 8: MM3 t=30h.

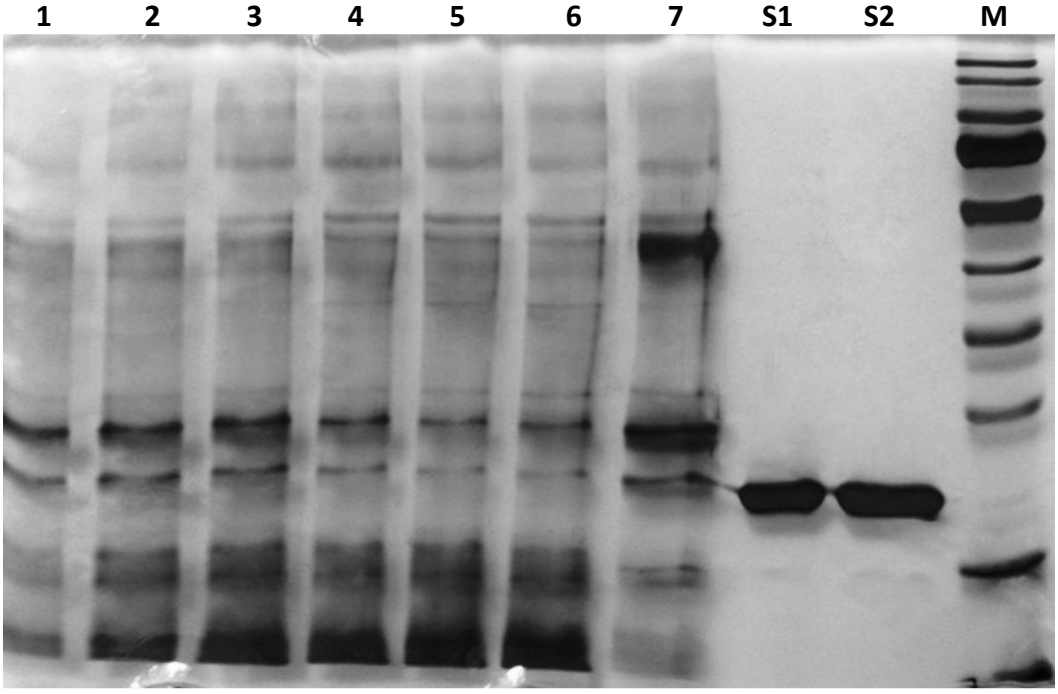

Figure F.11 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM3, 1:2 diluted samples. M: PageRuler protein ladder; S1: 0.1 g L^{-1} hGH standard, S2: 0.2 g L^{-1} hGH standard, Lane 1: t=15 h, Lane 2: t=18 h, Lane 3: t=21 h, Lane 4: t=24 h, Lane 5: t=30 h, Lane 6: t=36, Lane 7: t=21 h of MM2.

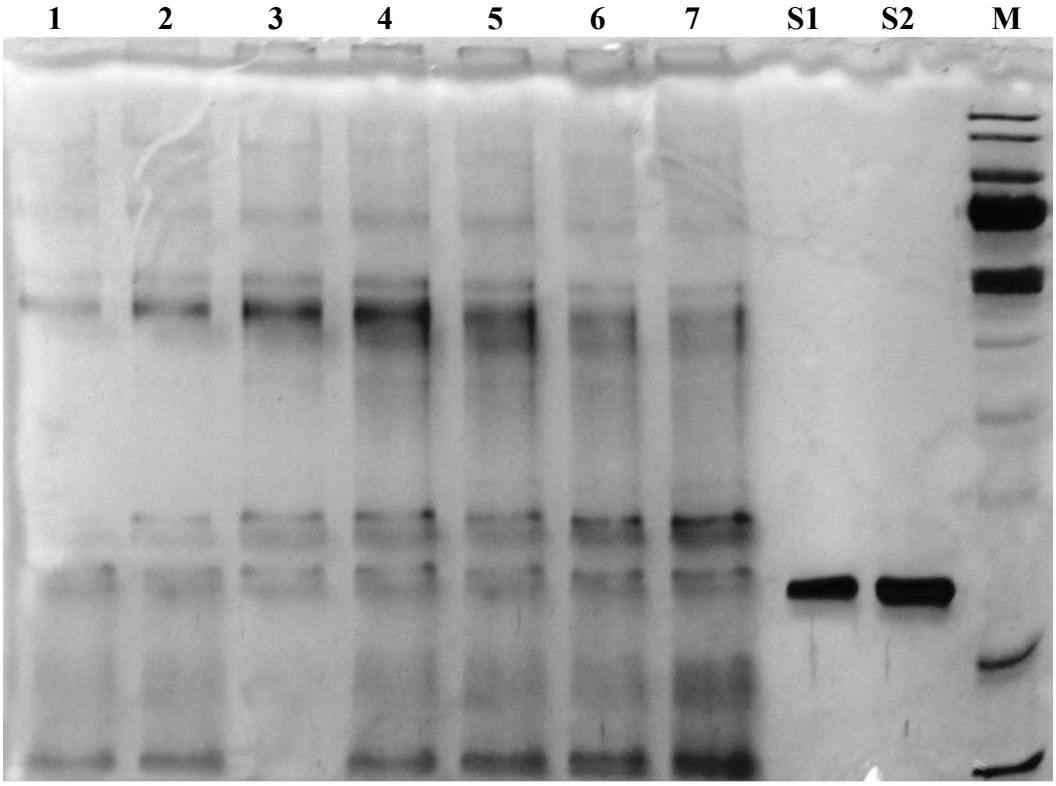

Figure F.12 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* M13 strain in the experiment MM3, 1:2 diluted samples. M: PageRuler protein ladder; S1: 0.1 g L^{-1} hGH standard, S2: 0.2 g L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15, Lane 7: t=18 h.

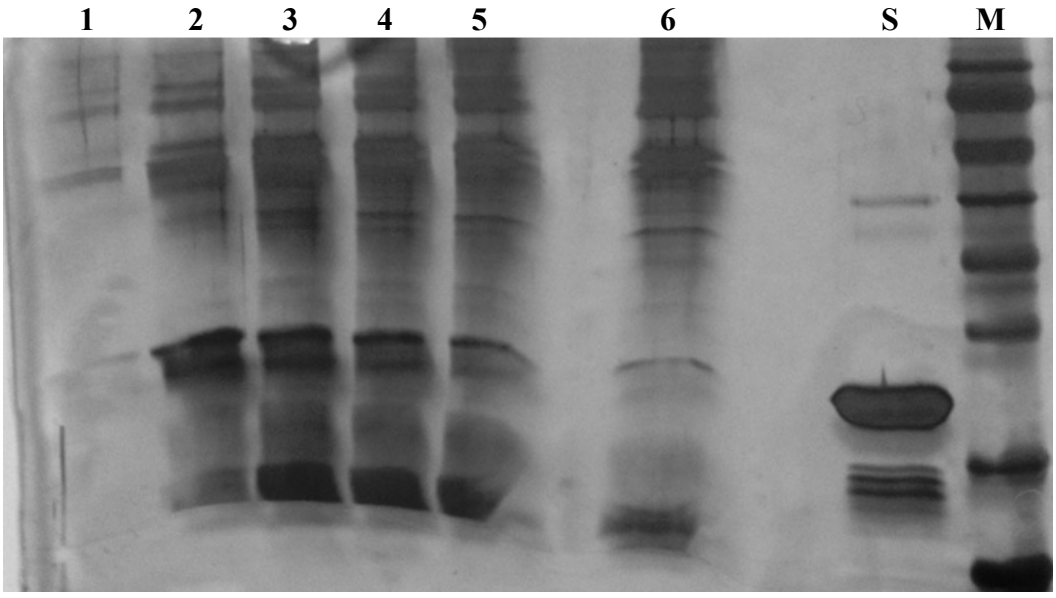

Figure F.13 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G1. M: PageRuler protein ladder; S: 0.2 g L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h.

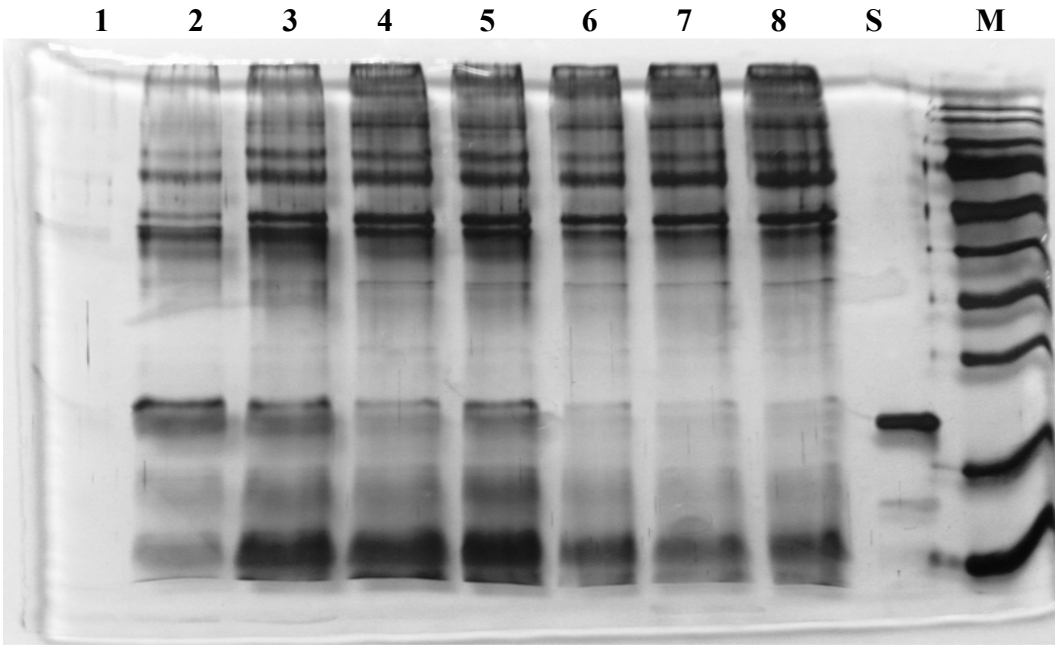

Figure F.14 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G1. M: PageRuler protein ladder; S: 0.1 g L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18 h and Lane 8:t=21 h.

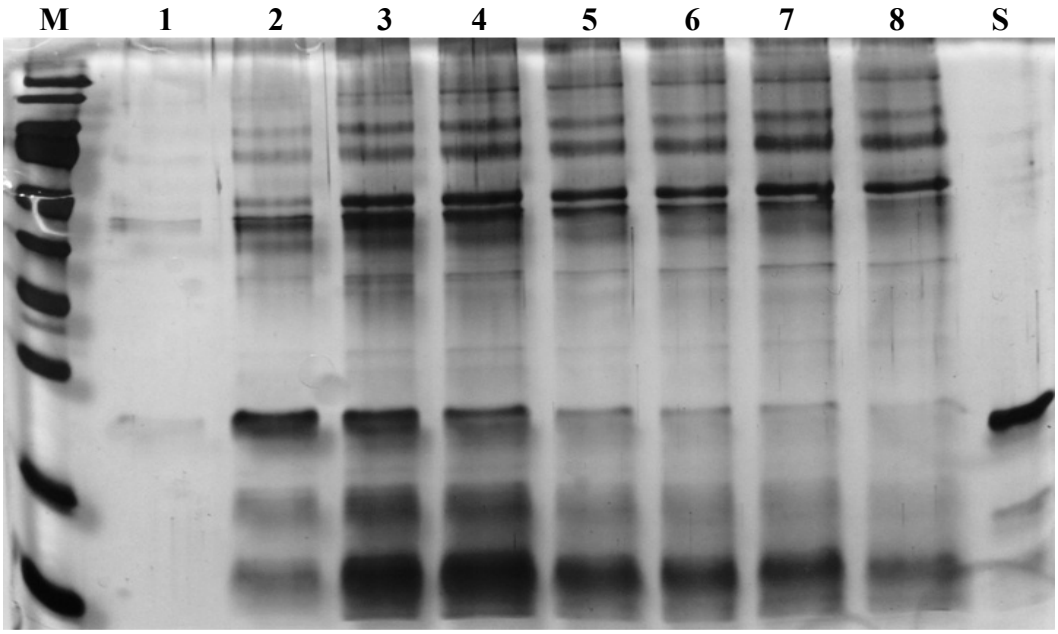

Figure F.15 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G1. M: PageRuler protein ladder; S: 0.1 g L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18 h and Lane 8:t=21 h.

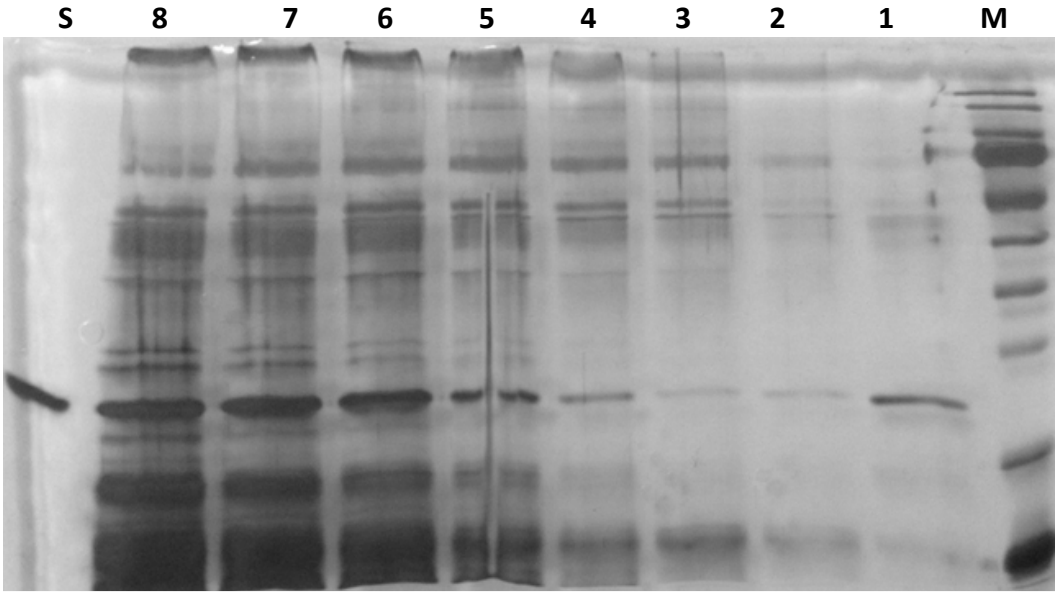

Figure F.16 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G2. M: PageRuler protein ladder; S: 0.1 g L^{-1} hGH standard, Lane 1: t=3 h of experiment G1, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=21 h and Lane 8:t=24 h.

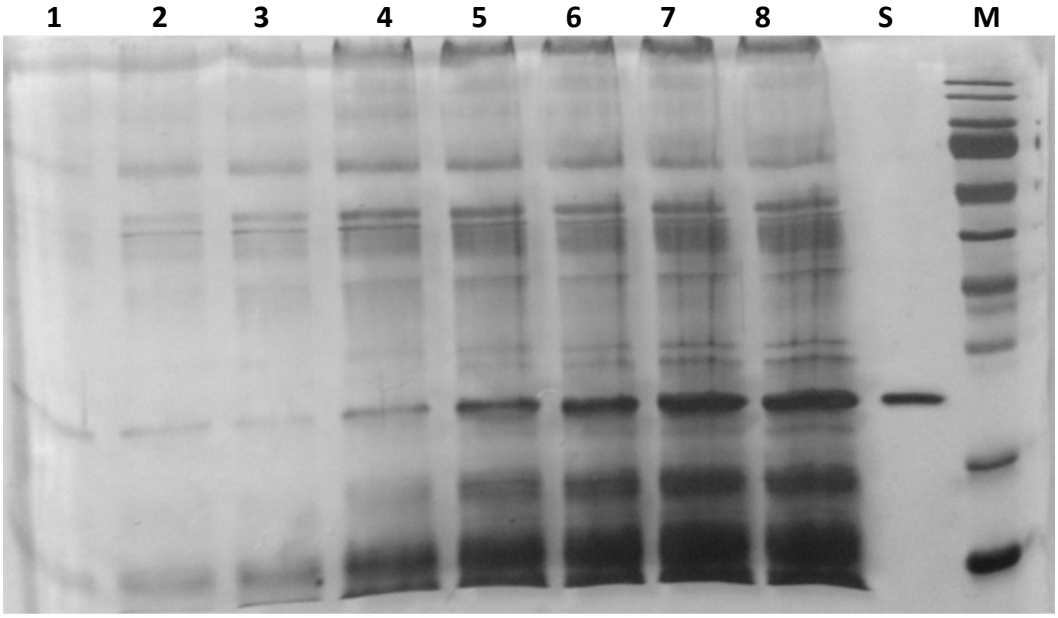

Figure F.17 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G2. M: PageRuler protein ladder; S: 0.1 g L^{-1} hGH standard, Lane 1: t=0 h, Lane 2: t=3 h, Lane 3: t=6 h, Lane 4: t=9 h, Lane 5: t=12 h, Lane 6: t=15 h, Lane 7: t=18 h and Lane 8:t=21 h.

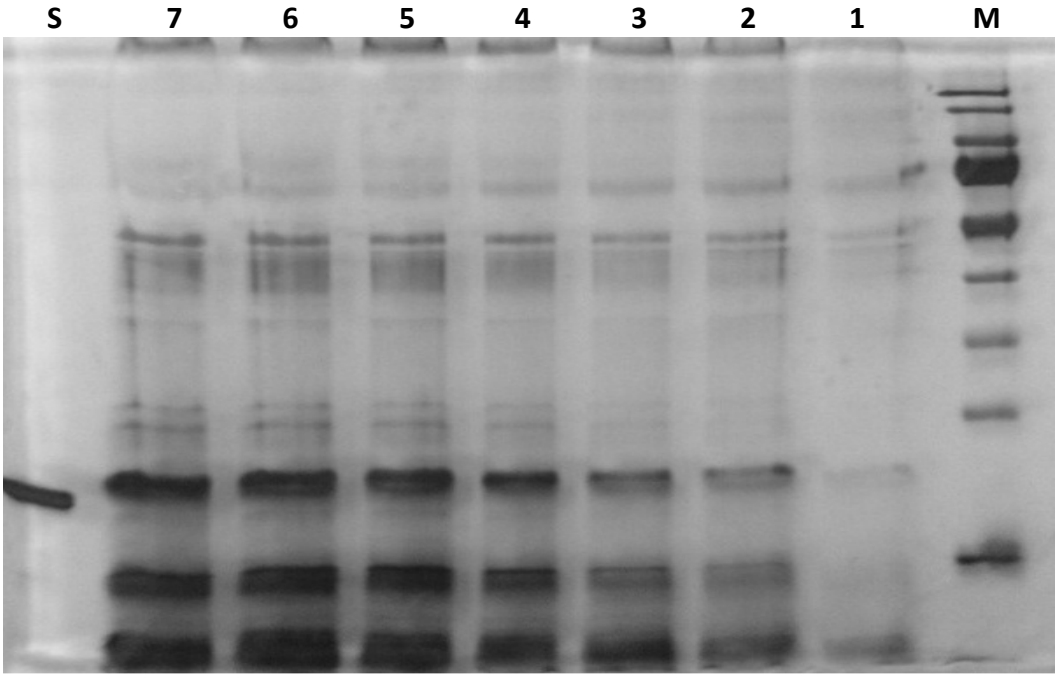

Figure F.18 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G2. M: PageRuler protein ladder; S: 0.1 g L^{-1} hGH standard, Lane 1: t=9 h, Lane 2: t=12 h, Lane 3: t=15 h, Lane 4: t=18 h, Lane 5: t=21 h, Lane 6: t=24 h, Lane 7: t=27 h.

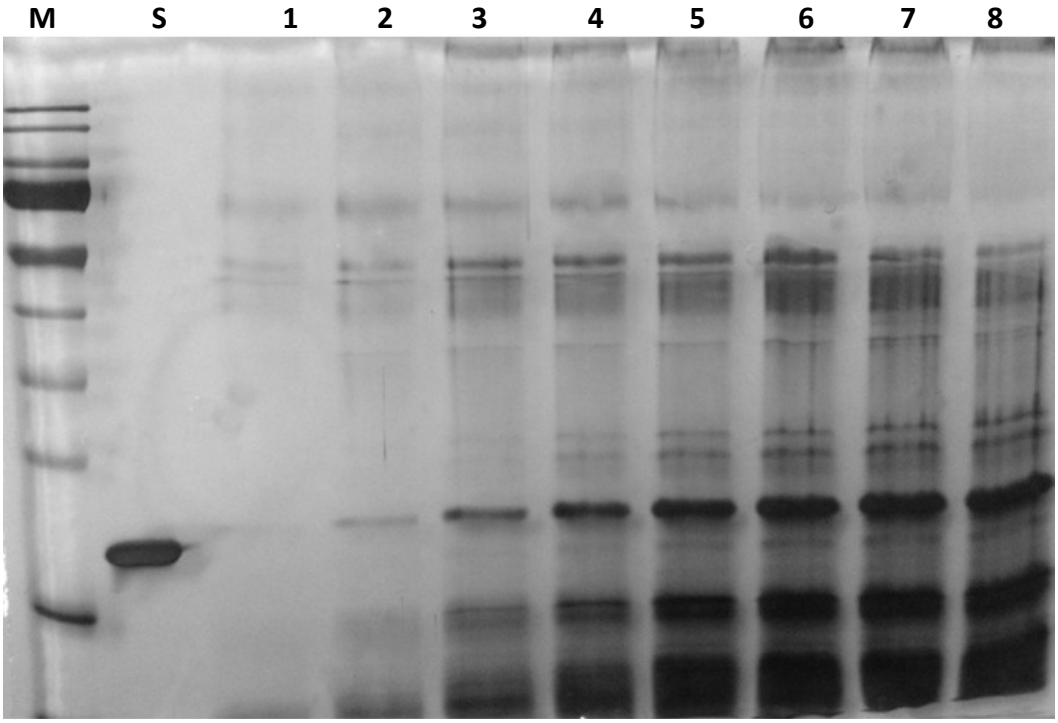

Figure F.19 Silver stained SDS-PAGE view of proteins produced by *Pichia pastoris* G7 strain in the experiment G2. M: PageRuler protein ladder; S: 0.1 g L^{-1} hGH standard, Lane 1: t=6 h, Lane 2: t=9 h, Lane 3: t=12 h, Lane 4: t=15 h, Lane 5: t=18 h, Lane 6: t=21 h, Lane 7: t=24 h and Lane 8:t=27 h.

APPENDIX G

CALIBRATION CURVE FOR BRADFORD ASSAY

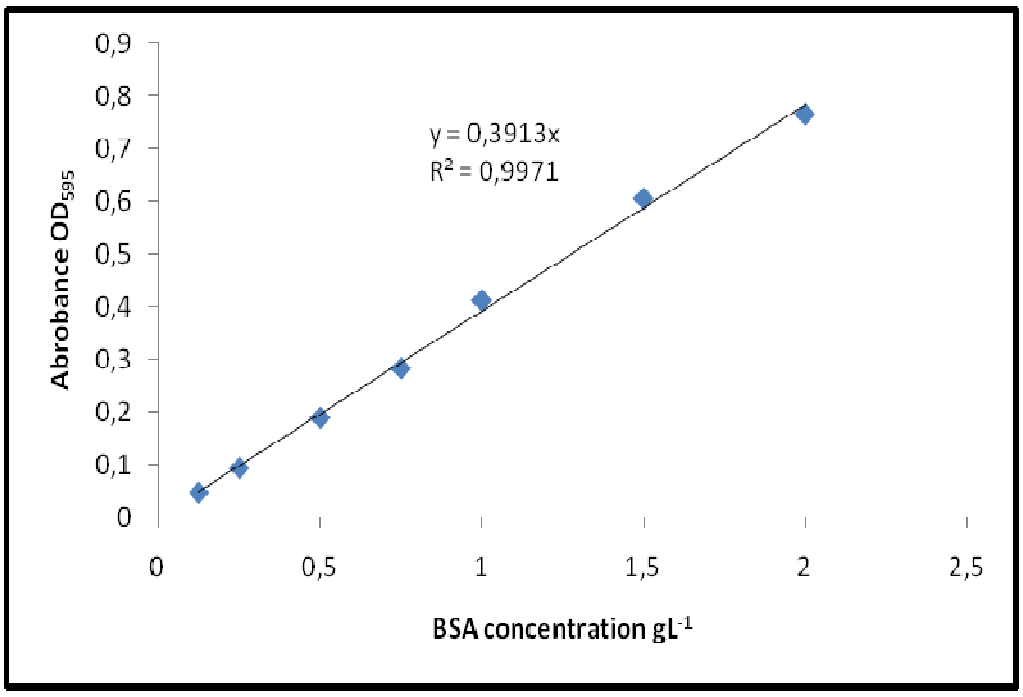

Figure G.1 Calibration curve for Bradford Assay

APPENDIX H

CALIBRATION CURVES FOR METHANOL AND MANNITOL

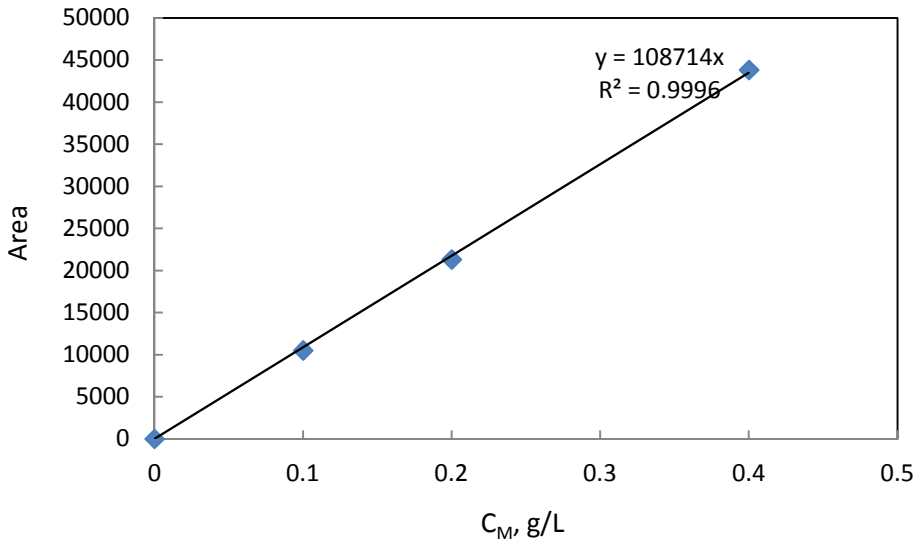

Figure H. 1 Calibration curve for methanol concentration

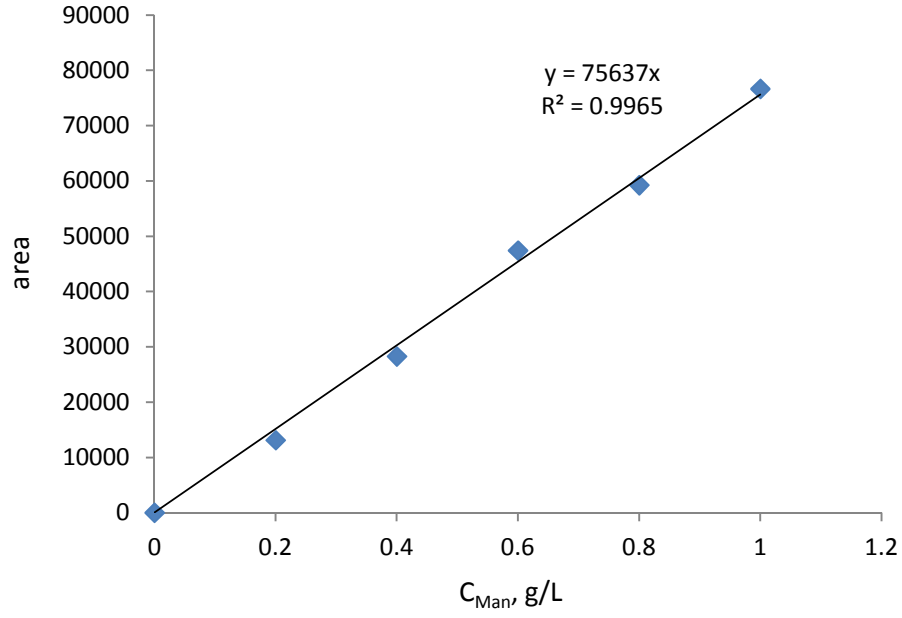

Figure H.2 Calibraion curve for mannitol concentration

APPENDIX I

CALIBRATION CURVES FOR ORGANIC ACIDS

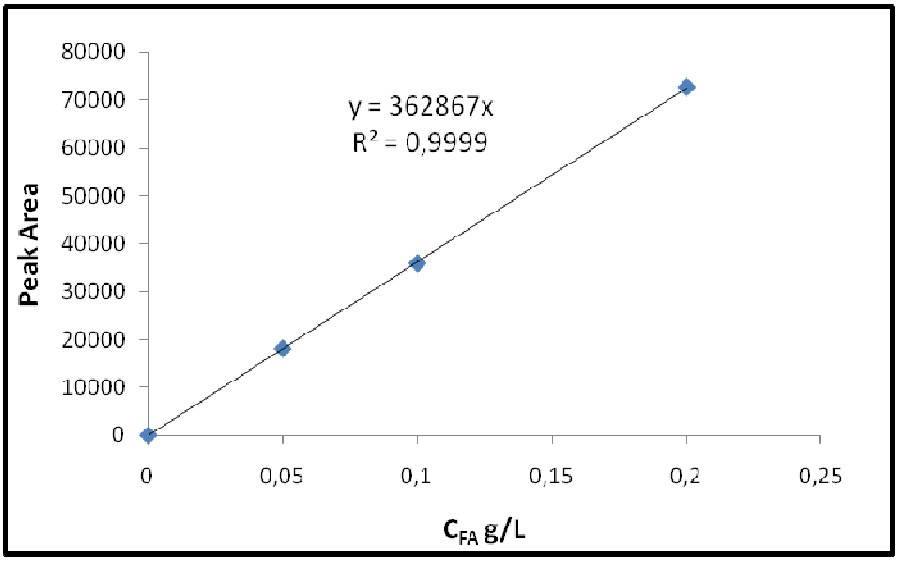

Figure I.1 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

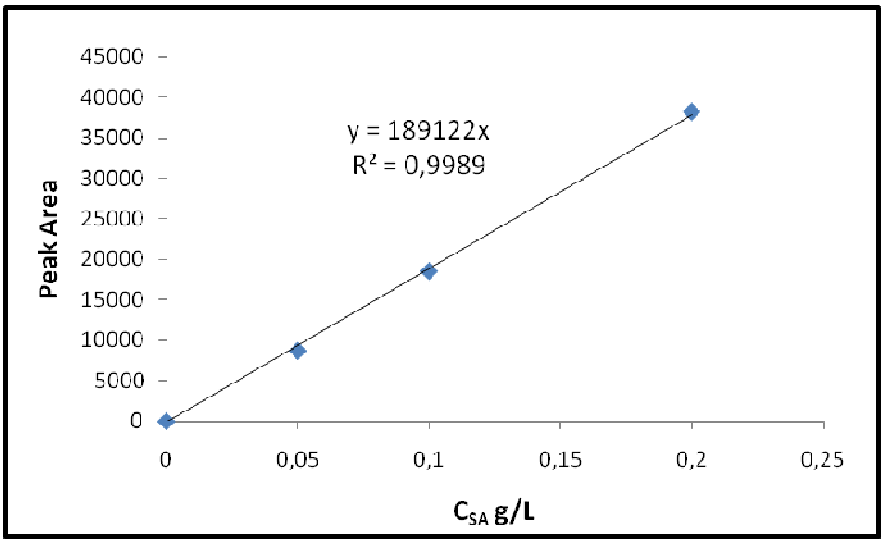

Figure I.2 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

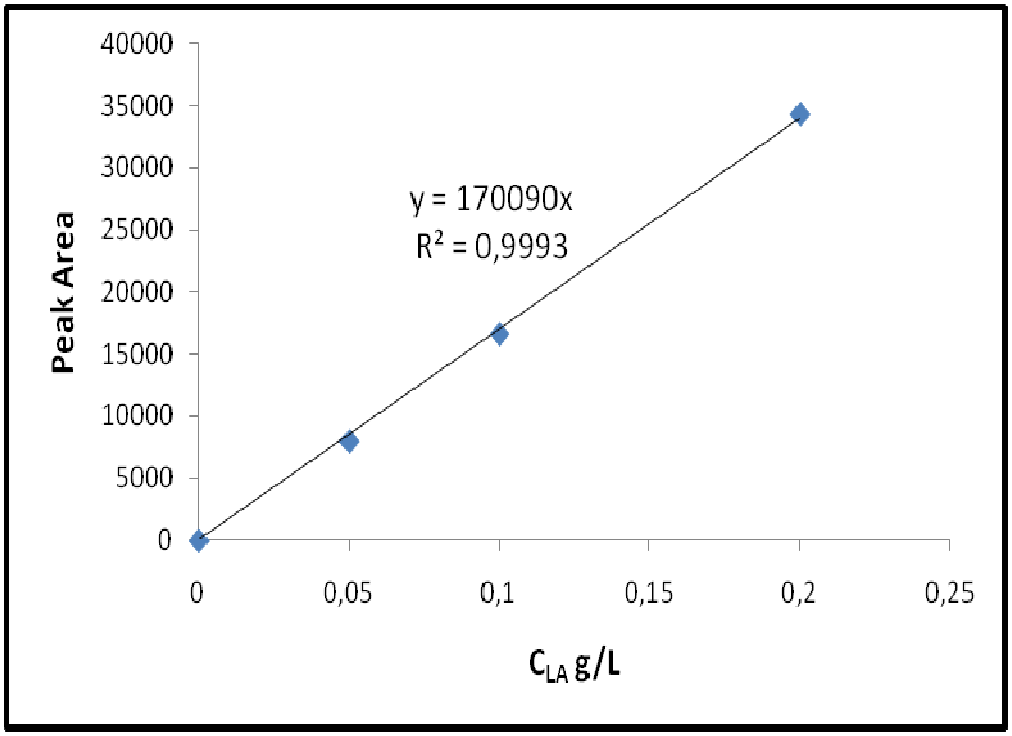

Figure I.3 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

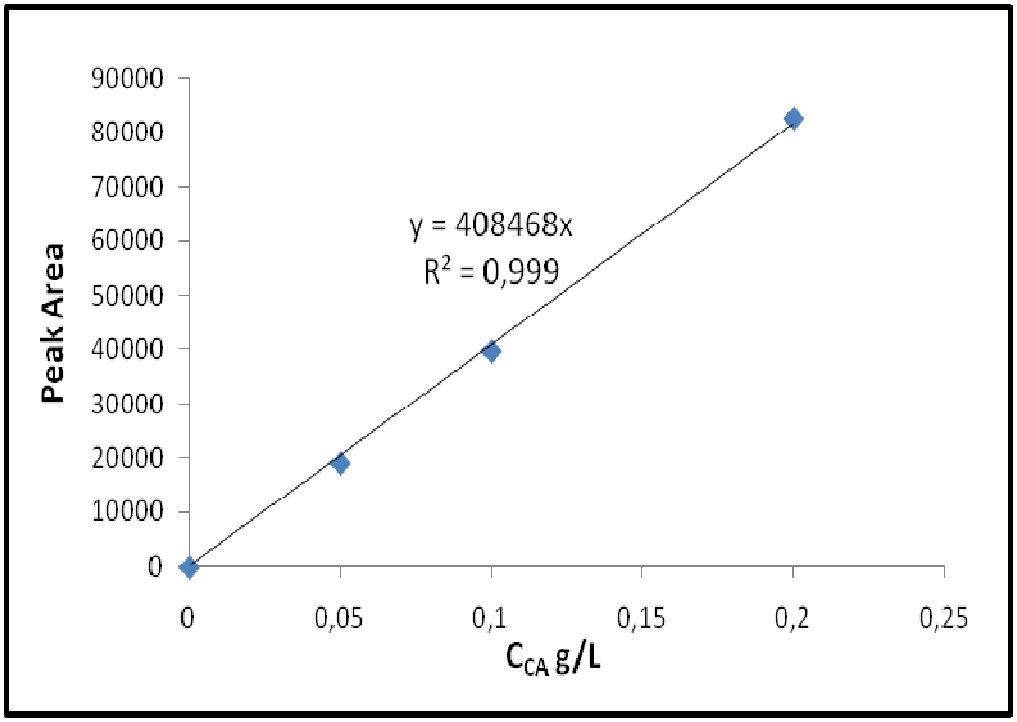

Figure I.4 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC

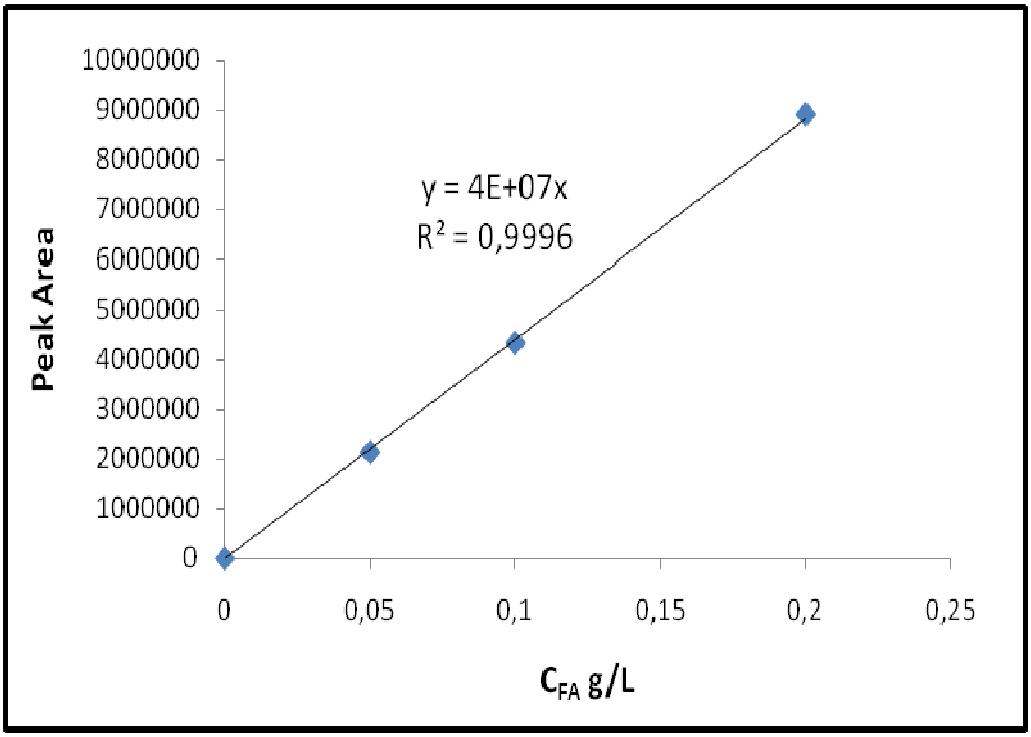

Figure I.5 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

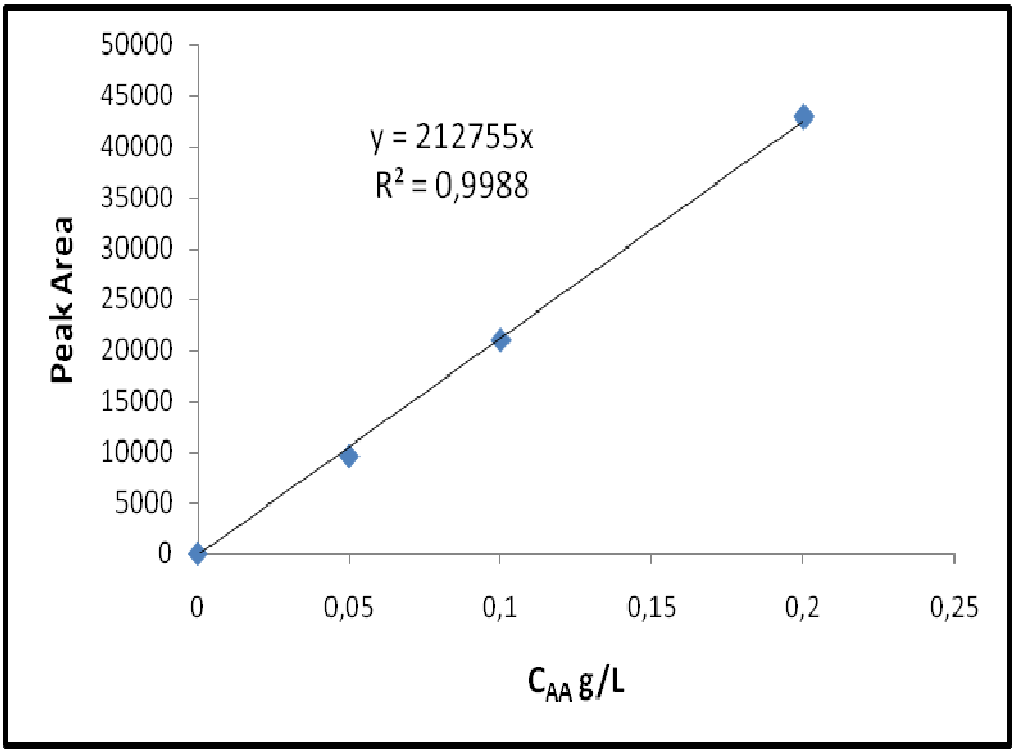

Figure I 6 Calibration curve obtained for acetic acid concentration; analysis was performed by HPLC**.**

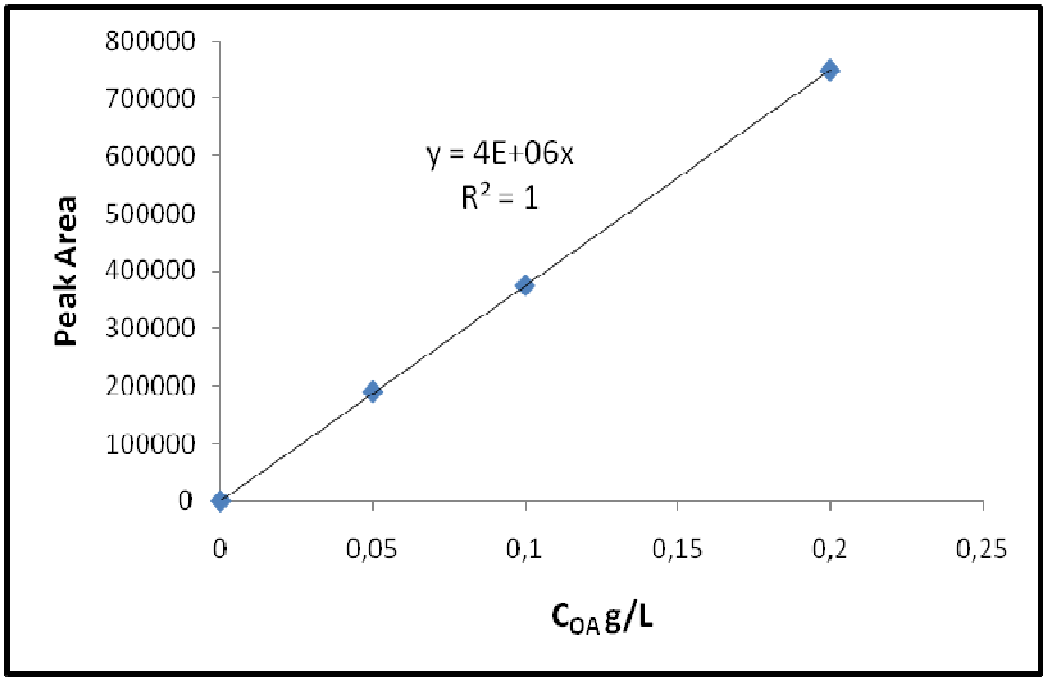

Figure I.7 Calibration curve obtained for oxalic acid concentration; analysis was performed by HPLC.

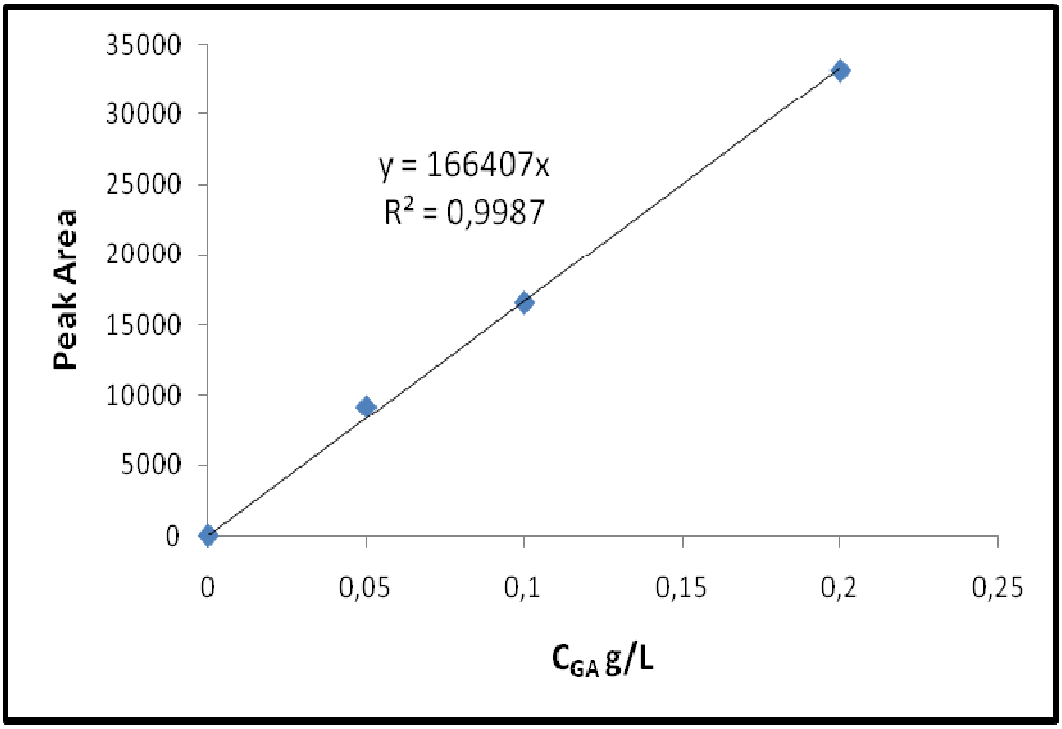

Figure I.8 Calibration curve obtained for gluconic acid concentration; analysis was performed by HPLC

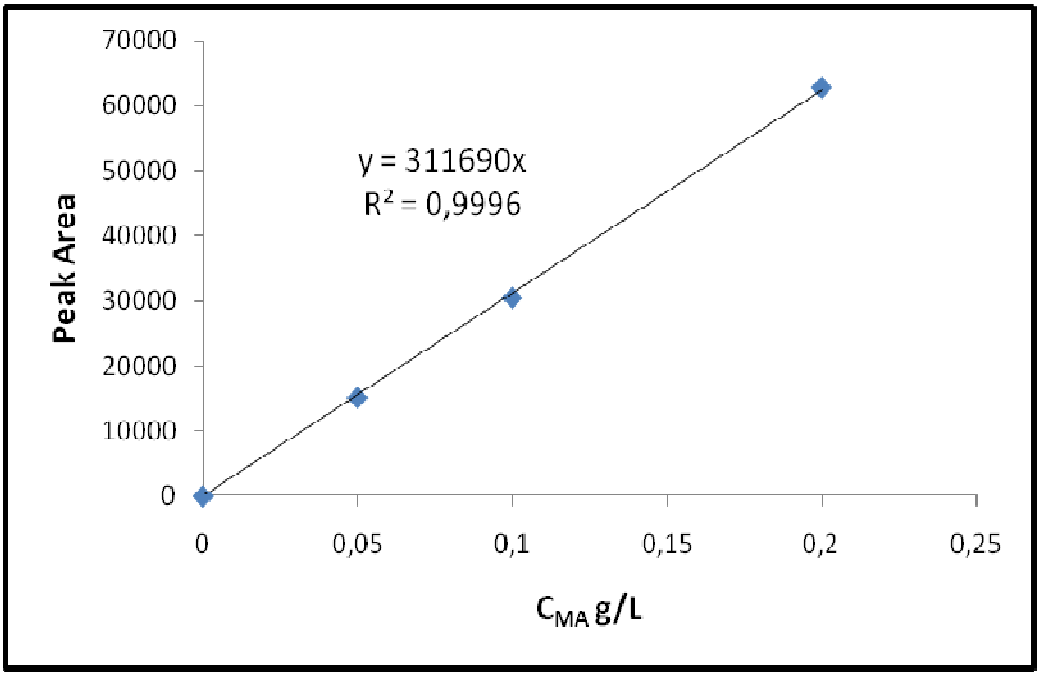

Figure I.9 Calibration curve obtained for malic acid concentration; analysis was performed by HPLC

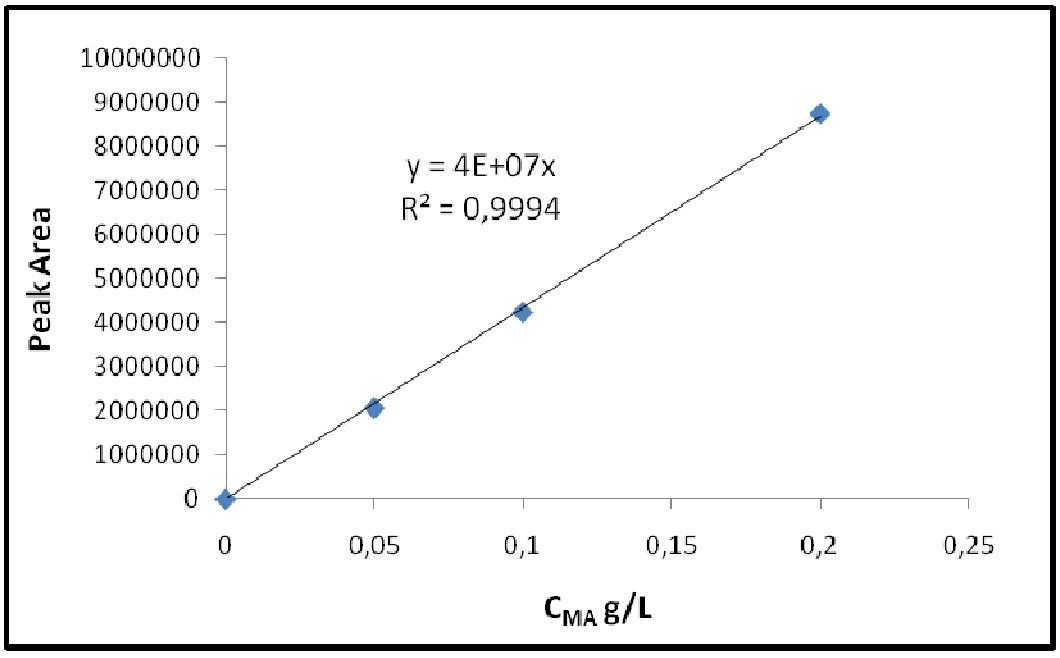

Figure I.10 Calibration curve obtained for maleic acid concentration; analysis was performed by HPLC**.**

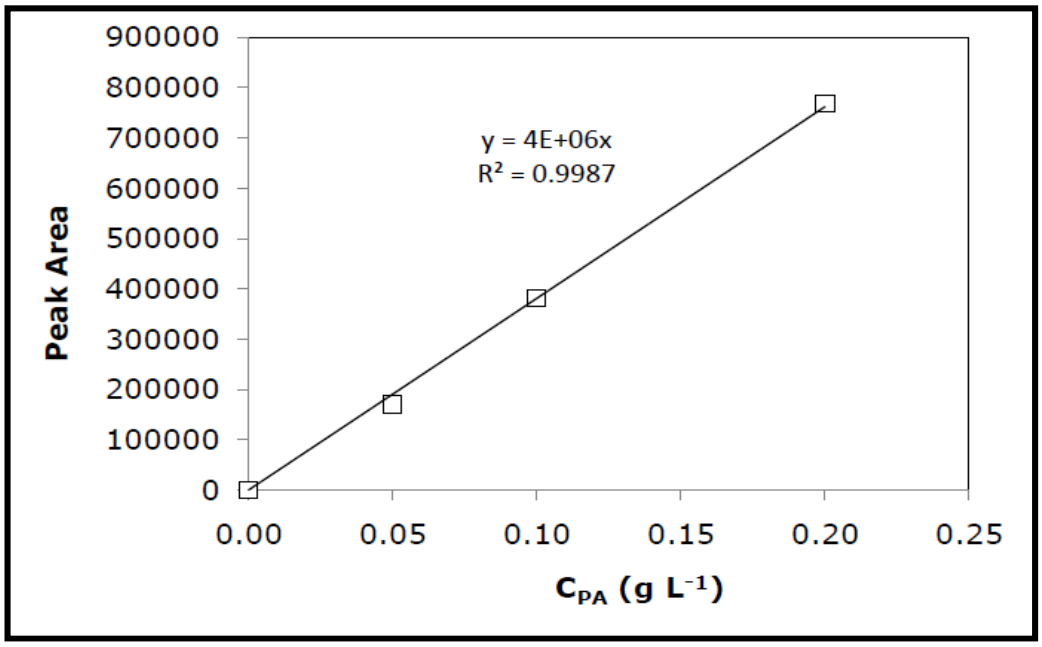

Figure I.11 Calibration curve obtained for pyruvic acid concentration; analysis was performed by HPLC

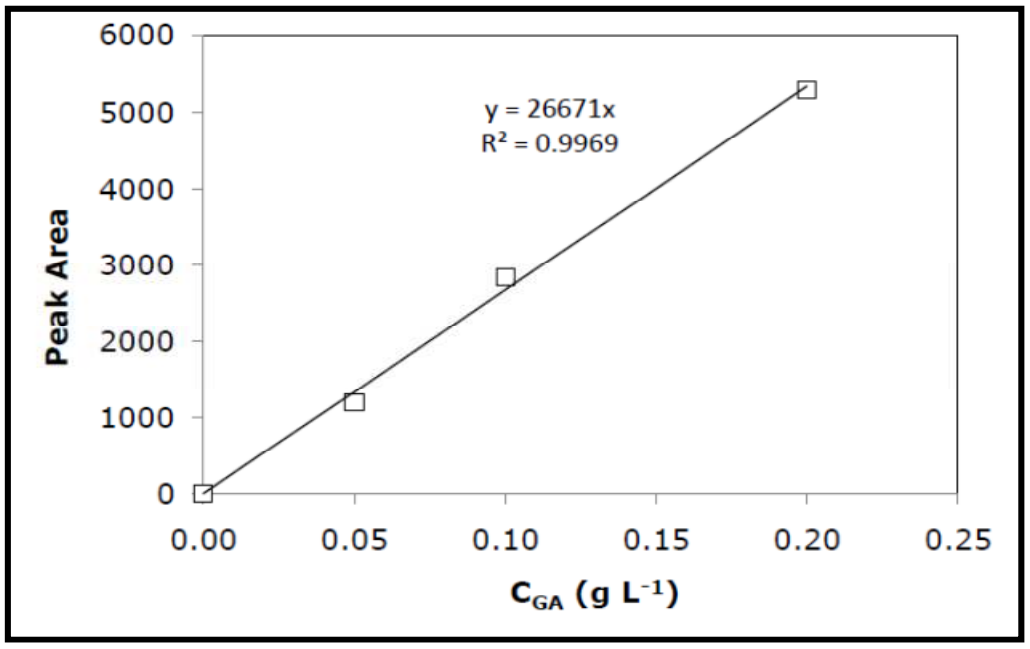

Figure I.12 Calibration curve obtained for glutaric acid concentration; analysis was performed by HPLC.

APPENDIX J

CALIBRATION CURVE FOR AOX ACTIVITY

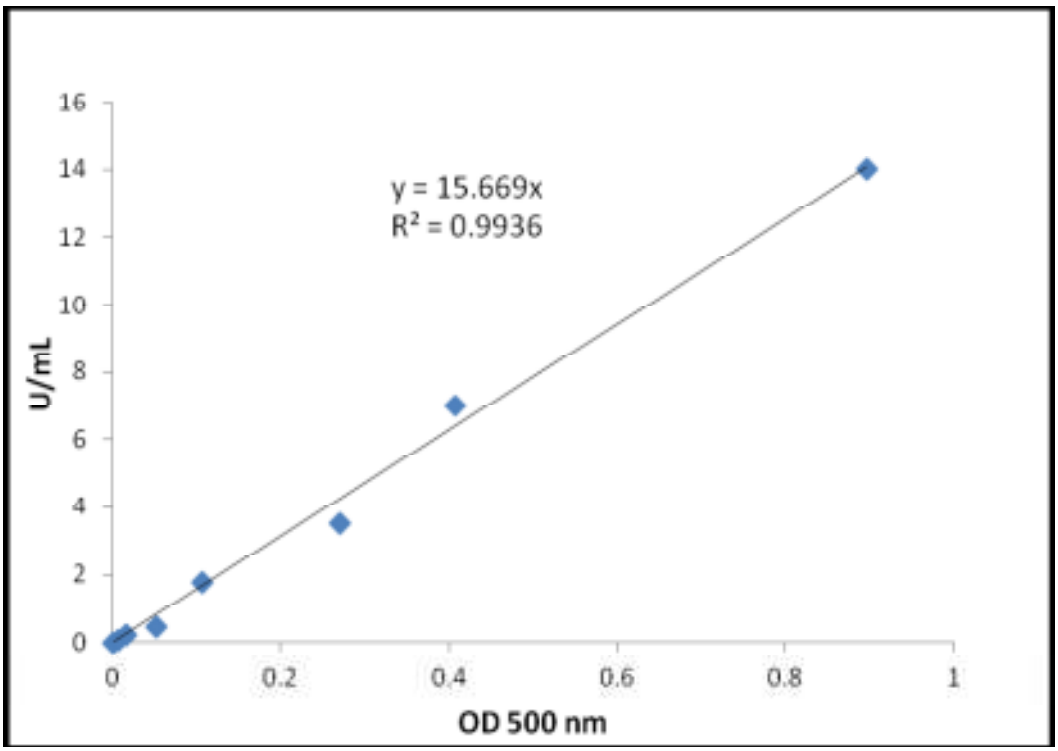

Figure J.1 Calibration curve for AOX activity

APPENDIX K

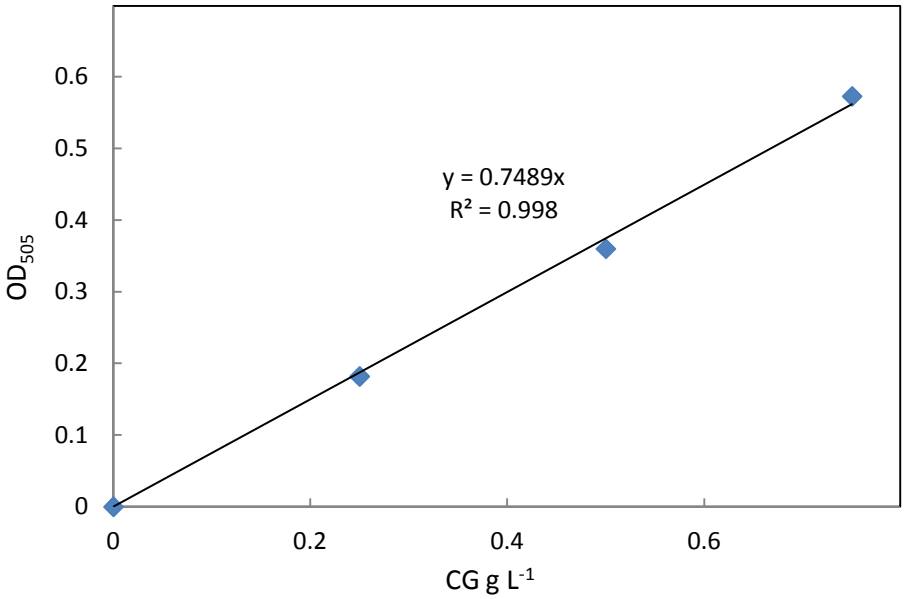

Figure K.1 Calibration curve for glucose concentration