METABOLIC ENGINEERING WITH A NOVEL PROMOTER IN *Pichia pastoris* FOR RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION: EFFECTS OF OXYGEN TRANSFER CONDITIONS

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ABSTRACT

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The objectives of this thesis are investigation of, ***i)*** the effects of oxygen transfer conditions on recombinant protein production in *Pichia pastoris* strains designed with novel naturally occuring pyruvate kinase (PYK) promoter (P_{PYK}) which is a potential promoter for recombinant protein production under low to moderate oxygen transfer conditions, and ***ii)*** influences of engineering with single- and multi- copy genes, in fed-batch fermentation processes. Production of recombinant human growth hormone (rhGH) by *P. pastoris* strains under P_{PYK} carrying single- and multi- copies of *hGH* gene were carried out to determine the effect of gene copy number. For the characterization of the performances of the engineered *P. pastoris* strains, effects of the oxygen transfer conditions were studied at three oxygen transfer conditions at constant dissolved oxygen concentrations (C_{DO}) of C_{DO}= 1%, 5%, and 15% of air saturation. With the single copy strain, the highest

cell concentrations were obtained as ca. C_x = 110 g L^{-1} at C_{DO} = 5% and C_{DO} = 15%; however, the highest rhGH concentration was produced as C_{rhGH} = 28.54 mg

L^{-1} at C_{DO} = 5%. Therefore, the highest productivity was achieved at the oxygen transfer condition of C_{DO} = 5%. Based on the results with single-copy strain, with the two-copy strain, a pilot-scale experiment was performed at C_{DO} = 5% condition. The highest cell and rhGH concentrations were achieved as C_x = 143 g L^{-1} and C_{rhGH} = 50 mg L^{-1} with the strain carrying two copies of *hGH* gene. The results conclusively demonstrate that the oxygen transfer condition at constant C_{DO} = 5% is the superior bioreactor operation condition; and, with the multi-copy strain when the gene copy number was doubled, 1.8- fold increase in the rhGH production was obtained.

Keywords: Recombinant human growth hormone, *Pichia pastoris*, fed-batch operation, oxygen transfer conditions, pyruvate kinase (PYK) promoter, metabolic engineering.

ÖZ

REKOMBİNANT İNSAN BÜYÜME HORMONU ÜRETİMİ İÇİN YENİ BİR PROMOTOR İLE *Pichia pastoris*'TE METABOLİK MÜHENDİSLİK TASARIMLARI: OKSİJEN AKTARIM KOŞULLARININ ETKİSİ

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Ocak 2018, 171 sayfa

Bu tezin amaçları, ***i)*** *Pichia pastoris*'te doğal olarak bulunan pyruvate kinase promotoru (P_{PYK}) başlatımında tasarlanan rekombinant sistemlerle rekombinant protein üretimine düşük- ile orta- oksijen aktarım koşulları arasında oksijen aktarımının, ile ***ii)*** tek- ve çoklu- gen sistemleri oluşturarak yapılan metabolik mühendislik tasarımlarının, etkilerinin yarı-kesikli biyoreaktör işletimiyle araştırılmasıdır. *P. pastoris*'te P_{PYK} altında tek- ve çok- *hGH* geni taşıyan sistem tasarımları yapılarak, rekombinant insan büyüme hormonu (rhGH) üretimi araştırılmıştır. Metabolik tasarımları yapılan ve oluşturulan *P. pastoris* suşlarının karakterizasyonu için, oksijen aktarım koşullarının etkisi sabit çözünen-oksijen derişimlerinde (C_{DO}): C_{DO}= %1, %5, ve %15'te, üç oksijen aktarım koşulunda araştırılmıştır. Tek- kopya suşla, en yüksek hücre derişimi, yaklaşık, C_x = 110 g L^{-1} olarak C_{DO} = %5 ve C_{DO} = %15 oksijen aktarım koşullarında; ancak, maksimum

rhGH, C_{rhGH} = 28.54 mg L^{-1} olarak, C_{DO} = %5 koşulunda üretilmiştir. Böylece, rhGH verimliliği de en yüksek C_{DO} = % 5'te elde edilmiştir. Tek- kopya gen sonuçları kullanılarak, iki- kopya gen taşıyan suşla, C_{DO} = % 5 koşulunda pilot-ölçek biyoreaktör deneyi yapılmıştır. İki- kopya *hGH* geni taşıyan suşla, en yüksek hücre ve rhGH derişimleri, sırasıyla, C_x = 143 g L^{-1} ve C_{rhGH} = 50 mg L^{-1} elde edilmiştir. Sonuçlar, C_{DO} = % 5 sabit oksijen aktarım koşulunun üstün oksijen aktarım koşulu olduğu ve, çok- gen sistemi kullanarak gen kopya sayısı 2- katına çıkarıldığında, rhGH üretiminin 1.8-kat arttığı, bulunmuştur.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, *Pichia pastoris*, yarı-kesikli işletim, oksijen aktarım koşulları, piruvat dekarboksilaz (PYK) promotoru

To my beloved family

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NOMENCLATURE

C	Concentration	g L^{-1} or mol m^{-3}
CO*	Saturated dissolved oxygen concentration	mol m^{-3}
DO	Dissolved oxygen	%
K_La	Overall liquid phase mass transfer coefficient	s^{-1}
K_La_0	Physical overall liquid phase mass transfer coefficient	s^{-1}
N	Agitation rate	min^{-1}
OD	Oxygen demand	mol m^{-3} sec^{-1}
OTR	Oxygen transfer rate	mol m^{-3} sec^{-1}
OUR	Oxygen uptake rate	mol m^{-3} sec^{-1}
Q	Volumetric flow rate	L h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
t	Process time	h
T	Medium temperature	^oC
V	Volume	L
Y	Yield	g g^{-1}

Greek Letters

μ	Specific growth rate	h^{-1}
μ_0	Pre-determined specific rate	h^{-1}
λ	Wavelenght	nm
ρ	Density	g L^{-1}

Subscripts

0	Initial condition
P	Product
S	Substrate
X	Cell

Abbreviations

Ala	Alanine
AOX1	Alcohol oxidase 1
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
BMGY	Buffered glycerol complex medium
BSM	Basal Salt Medium
GAP	Glyceraldehyde-3 phosphate dehydrogenase
Gln	Glutamine
Glu	Glutamate
Gly	Glycine

hGH	Human growth hormone
His	Histidine
HPLC	High-performance liquid chromatography
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
PYK	Pyruvate kinase
Ser	Serine
TCA	Tricarboxylic acid
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
YPD	Yeast extract-peptone-dextrose

CHAPTER 1

INTRODUCTION

Biotechnology is not a sector, but a broad generic technology. It had a slow, emprical development during centuries until the middle of the 19th century. Modern research and development, with the discovery of the sructure of DNA with a breakthrough in 1953, consecuently increasing knowledge on the genome particularly metabolic engineering and genetic engineering constructions in the last three decades, has turned it into an efficient and rapidly growing set of tools and applications a modern biotechnology with vast implications have been influencing almost all the sectors by opening new avenues to the future. Production of biochemicals by microbial cells for the sectors of industrial biotechnology is the leading research and application area in biotechnology. In 1858, Louis Pasteur proved that living organisms take part in these processes by demonstrating that fermentation is caused by the growth of microorganisms (Barnett, 2000). In the following years, a great number of important biochemicals were produced by the help of the activities of various microorganisms. Biotechnology can be defined simply as the usage of a living organism, or some component of a living system, to produce beneficial products. According to Organization for Economic Co-operation and Development (OECD), the definition of biotechnology stated as "the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services" (Bud, 1993). After the DNA molecule was discovered in 1953, a branch of genetic engineering has been developed and began to be used in the applications of industrial biotechnology. Biochemical compounds are produced in living organisms by

applying different techniques which cause alterations in the genetic structure of an microorganism. In line with these developments, recombinant DNA technology has been progressed and started to be used widely for the production of many pharmaceutical compounds by inserting the desired gene to the genome of the microorganism.

Therapeutic proteins form a large part of the drugs which are used against various diseases such as hemophilia, anemia, hepatitis B/C and cancer. With the applications of recombinant DNA technology, production of these proteins can be achieved in specific host cells under defined conditions. Up to date, Food and Drug Administration (FDA) and European Medicines Agency (EMA) have approved about 400 recombinant proteins for pharmaceutical use and other 1300 recombinant proteins are under development (Sanchez-Garcia *et al*., 2016).

Human growth hormone (hGH) is a peptide hormone which stimulates growth and cell regeneration in humans and other animals. It is used against diseases such as dwarfism, Turner syndrome and chronic renal failure. Although recombinant hGH (rhGH) has been expressed by using prokaryotic organisms, it is produced more efficiently in *P. pastoris* due to its eukaryotic post-translational processing system. It was first produced intracellularly by *Escherichia coli* cells in 1979 (Goeddel *et al*., 1979). In the following years, it was also produced by various microorganism such as *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Pichia pastoris (*Nakayama *et al*., 1988; Tokunaga *et al*., 1985; Treviño *et al*., 2000; Çalık *et al.,* 2008; Özdamar *et al*., 2009; Çalık *et al*., 2016).

Selection of the suitable expression system is an important step in recombinant protein (r-protein) production therefore the characteristics of the desired protein molecule should be taken into consideration when the host cell is chosen. *P. pastoris* is a distinguished expression host for r-protein production because of its advantageous characteristics. High cell concentrations are obtained in a simple and

inexpensive medium, it can produce proteins intracellularly and extracellularly at high amounts and it allows various genetic manipulations with well-known protocols. In addition to these features, its strongly regulated expression system plays also a major role in r-protein production. P_{AOX1} was the first isolated promoter of *P. pastoris* which is used extensively for the production of various recombinant proteins owing to its regulatable expression characteristics and ability to express proteins at significant amounts (Cregg *et al*., 1993). After that, another strong promoter P_{GAP} was discovered and started to be used without methanol induction unlike P_{AOX1}, which facilitates the production at high-scale (Waterham *et al*, 1997). With the discoveries of promising promoters and optimizations in fermentation systems *P. pastoris* expression system has developed in a short time (Shen *et al*., 1998; Menendez *et al*., 2003; Ahn *et al*., 2007; Massahi *et al*., 2017; Liu *et al*., 1995; Sears *et al*., 1998; Cregg *et al*., 2012; Almeida *et al*., 2005).

Production of rhGH was succeeded by our research group by using expression system of *P. pastoris* under the control of P_{AOX1} first in 2008 (Çalık *et al.,* 2008). Açık (2009) obtained 301 mg/L rhGH at first, Bozkurt (2012) increased this amount to 640 mg/L and Zerze (2012) achieved 1200 mg/L rhGH by using different fermentation feeding strategies. Production under the constitutive GAP promoter was also studied firstly by Zerze (2012) by feeding glucose with a pre-determined specific growth rate of μ=0.2 h^{-1} until t= 3h of the fermentation operation and then by applying constant glucose feeding. At the end of the process 200 mg/L rhGH was reached. Güneş (2015) studied the expression of glucose isomerase under P_{GAP} by investigating oxygen transfer conditions and found that dissolved oxygen concentration at 15% saturation gives maximum recombinant protein activity. In order to improve the fermentation strategy, defined and complex medium based feeding strategies were also tried for the expression sytem under the control of P_{GAP} (Hoxha, 2016). Discoveries of novel naturally occuring promoters, pyruvate kinase (PYK) and pyruvate decarboxylase (PDC), which are

induced at low to moderate oxygen transfer conditons have led the studies in this field recently (Massahi, 2017). Yalçınkaya (2017) constructed a double-promoter expression system and observing the simultaneous effects of GAP and PDC promoters on recombinant human growth hormone production.

Discovery of novel strong promoters is crucial to achieve successful metabolic system designs in *P. pastoris* that can operate under low- to moderate- oxygen transfer conditions. In this context, the objectives of this thesis are investigation of, ***i)*** effects of oxygen transfer conditions on recombinant protein production in *Pichia pastoris* strains designed with novel naturally occurring pyruvate kinase (PYK) promoter (P_{PYK}) which is a potential promoter for recombinant protein production, and ***ii)*** influences of engineering with single- and multi- copy genes, in fed-batch fermentation processes. Production of recombinant human growth hormone (rhGH) by *P. pastoris* strains under P_{PYK} carrying single- and multi-copies of *hGH* gene were carried out to determine the effect of gene copy number. For the characterization of the performances of the engineered *P. pastoris* strains, effects of the oxygen transfer conditions were studied under low to moderate oxygen transfer conditions.

CHAPTER 2

LITERATURE SURVEY

2.1. Product: Human Growth Hormone (hGH)

Hormones are defined as chemical messengers which are produced and used by endocrine system to achieve specialized targets in human body. They are secreted by glands and organs for vital requirements of the body such as blood pressure control, digestion, glucose and calcium level control in the blood, menstrual cycle regulation. Hormones can be grouped into three types: steroids, amines and peptides. Most steroids are lipids which are synthesized from cholesterol and amines are secreted from thyroid. Peptides are secreted as proteins consisting of amino acid chains and they form a large part of hormones.

hGH is a peptide hormone which is secreted by somatotroph cells in pituitary gland. The *hGH* gene is located at the growth hormone locus of chromosome 17, in the q22-24 region, of human beings (http://ncbi.nlm.nih.gov). It was first isolated from human pituitary gland in 1956 (Li and Papkoff, 1956) and its biochemical structure was assigned in 1972. Designation of the biochemical structure has led to insight for recombinant production of this hormone (Goeddel *et al*., 1979). In 1985, Food and Drug Administration (FDA) approved rhGH as a drug and studies for producing this hormone by recombinant DNA technology has stepped up (Cronin,1997). After its approval, the hormone was used as a drug for children suffering from growth hormone deficiency (GHD) in United States (Ayyar, 2011). Today, it has also been used for treatment of various diseases such as "Prader-Willi syndrome, chronic renal insufficiency, Turner syndrome,

idiopathic short stature both in children and adults" (Richmond *et al*.,2010, Nissel *et al*., 2008, Baxter *et al*., 2007, Carrel *et al*., 2002, Cohen *et al*., 2008).

Somatotropin is the trade name of human growth hormone and it is one of the mostly marketed pharmaceutical products in worldwide. The annual sales of hGH has reached to $1.8 billion by the year of 2004 and expected to rise under the leadership companies of Novo Nordisk and Genentech (Demain, 2007).

2.1.1. Structure of hGH

191 amino acids come together and form a single polypeptide chain which gives the shape of human growth hormone. It has two disulfide bonds for stabilizing its structure; one between Cys-53 and Cys-65 and the other one between Cys-182 and Cys-189 (Junnila and Kopchick, 2013). Its molecular weight is 22,125 Da and empirical formula is $C_{990}H_{1529}N_{262}O_{300}S_7$ (Becker *et al*., 1995). Primary structure of hGH is presented in Figure 2.1.

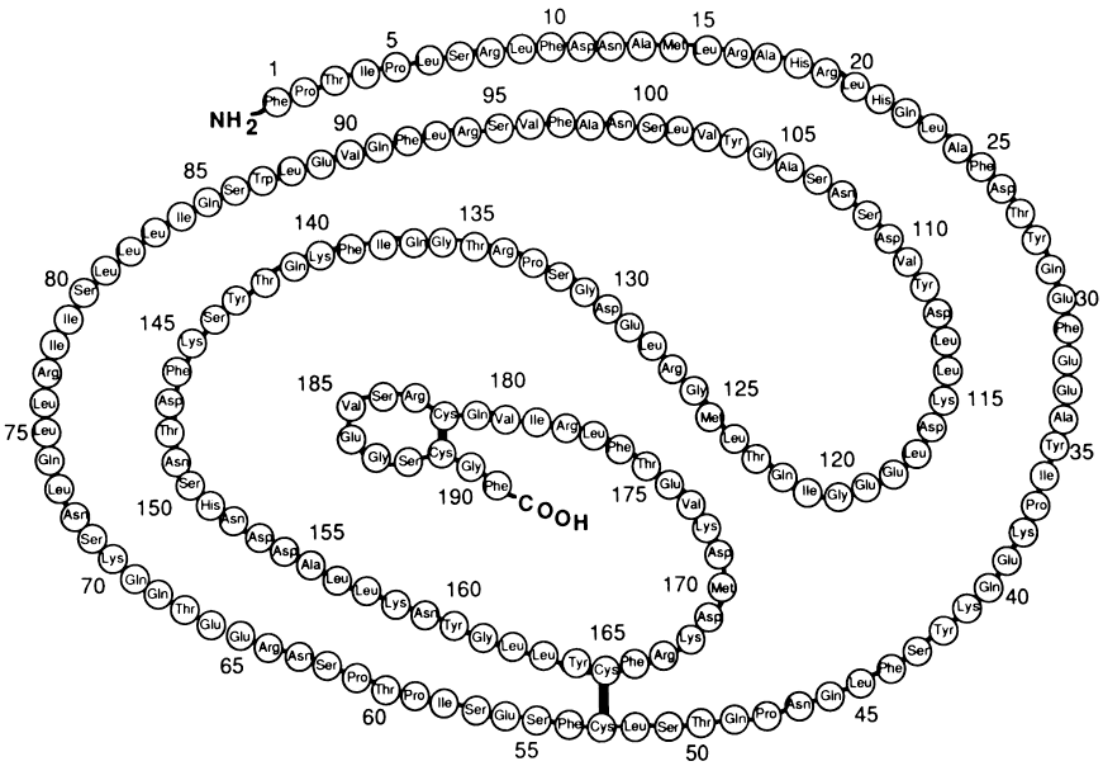


Figure 2. 1 Primary structure of hGH including disulfide bonds (Cogan and Phillips, 1995)

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. 2 Nucleotide sequence of hGH (Baulieu et al., 1990; Binkley 1994)

In Figure 2.2, nucleotide sequence of hGH is presented. For effective functioning of hGH, the hormone should bind to its receptor and tertiary structure of the protein plays an essential role for achieving this complex. Tertiary structure of the hormone consists of four-helix bundle and when the structure of the hormone-receptor complex is investigated it is seen that the complex comprises one molecule of human growth hormone per two molecules of receptor (Abraham *et al.*, 1991). Tertiary structure of hGH is presented in Figure 2.3.

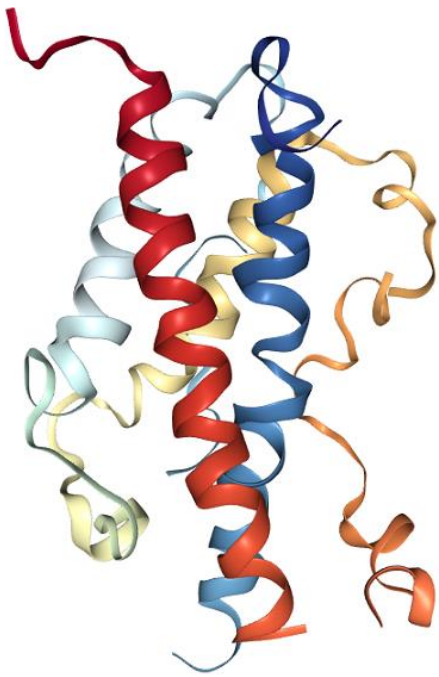


Figure 2. 3 Tertiary structure of hGH (http://rcsb.org)

2.2. Microorganism Selection

Selection of host microorganism among available expression systems is a crucial step for the production of r-proteins. Although, bacteria and yeast cells are widely used as host organisms, mammalian and insect cells can also be used for various protein expression. Host cell selection should be made by taking into consideration of the characteristics of the product molecule. The cost of growing and growth patterns of the cell, the level of expression that the cell type can achieve, the ease of purification provided by the cell, ability of proper folding and modification of the product protein for full activity and availability of suitable vectors for transformation have also great impact on host cell selection.

hGH was firstly expressed by bacterial systems since the cells can reach to high cell concentrations in an inexpensive medium. *E. coli* cells were mostly preferred for the expression of this protein (Goeddel *et al*., 1979, Martial *et al*., 1979).

Although production at high amounts was accomplished in fed-batch fermentations, the product molecule was expressed in the form of inclusion bodies within the bacterial cytoplasm and it was necessary to purify it with various methods such as acid precipitation or urea solubilization (Khodabandeh *et al*., 2003, Patra *et al*., 2000).

B. subtilis is another microorganism that is used for the expression of hGH due to its favorable characteristics such as the ability of secretion of the molecules into the medium, lack of pathogeneity and having well documented genetic manipulation protocols (Nakayama *et al*., 1988; Çalık *et al*., 2016). However, proteolytic degradation was observed after production and adding protease inhibitors into the medium became necessary (Özdamar *et al*., 2009).

Yeasts are mostly utilized microorganisms among other eukaryotic expression systems especially for the production of eukaryotic proteins. Efficient expression of human growth hormone was achieved by using *S. cerevisiae* as a host organism but codon optimization need to be done for more effective production (Tokunaga *et al*., 1985).

P. pastoris has became an ideal host for many heterologous protein production including human growth hormone. It enables both intracellular and extracellular production at high levels. Also secretion of endogenous proteins at low levels makes the purification of the desired product easier. The steps, thus the high cost of downstream processes can be eliminated by taking the advantage of efficient secretion levels. Since hGH is a pharmaceutical product, it is important to have it with at least 99.9% purity and free of endotoxins. *P.pastoris* is a commonly preferred organism for recombinant protein production mostly for its strong regulated expression system resulting in high levels of recombinant proteins. At first, Treviño *et al*. (2000) expressed rhGH extracellularly by using *P. pastoris* expression system. The expression was achieved under alcohol oxidase 1 (AOX)

promoter and *S. cerevisiae* α-factor signal sequence was used for the secretion of the protein. In this study, 11 mg/L rhGH was obtained with 3 mL culture medium in shake flask under oxygen limited induction conditions while production of 49 mg/L rhGH was achieved with 2 L culture medium in the bioreactor. Eurwilaichitr *et al*. (2002) managed to obtain higher amount of rhGH as 190 mg/L by using the codons preferred by *P. pastoris* for the encoding gene of human growth hormone. The length of induction and methanol concentration were also changed during the experiment. In another study (Çalık *et al*., 2008), hGH was expressed again under P_{AOX1} and this time polyhistidine-tag was added to the amino terminal end of the hormone in order to carry out an easy separation. 115 mg/L rhGH was produced after 24th hour of induction and without medium optimization. Expression of 500 mg/L rhGH was succeeded by using multi-copy, non-codon optimized *hGH* gene in a fermentation system with the addition of surfactants to basal salt medium (BSM) in the study of Deshpande *et al*.(2009). High copy-number and high secretion efficiency might be responsible from the higher yields for this experiment. Also it was stated that Tween 20, which was used as a surfactant, improves the stability of the product molecule. Orman *et al*. (2009) investigated the effect of carbon sources on rhGH production by using two different phenotypes for *P. pastoris*. The results were in the range of 32 to 60 mg/L for Mut^+ strain and 110 to 160 mg/L for Mut^S strain since they depend on the consumed carbon source.

2.2.1. Host Organism: *Pichia pastoris*

Methylotrophic yeasts can use methanol as the single carbon and energy source. Although the presence of methylotrophic bacteria have been known for many years, methylotrophic yeast species was discovered in 1969 (Ogata *et al*., 1969). *Candida boidinii, Hansenula polymorpha, Pichia methanolica* and *Pichia pastoris* were accepted as methylotrophic yeasts and studies for utilizing them as host

organisms in recombinant protein production processes have been proceeding (Gellissen, 2000).

Pichia pastoris is a unicellular fungus and taxonomically ranked under the" Kingdom *Fungi*, Division *Eumycota,* Subdivision *Ascomycotina*, Class *Hemoascomycetes,* Order *Endomycetales*, Family *Saccharomycetaceae* and Genus *Pichia"* (http://ncbi.nlm.nih.gov). Besides its methylotrophic character, *Pichia pastoris* is a mesophilic yeast which lives at temperatures between 25 °C and 35 °C. Also, it can grow in media having a wide pH range from 3 to 7 (Cregg *et al*., 2000). It is a facultative anaerobe and displays the typical characteristics of other yeast species having 1-5 μm width and 5-30 μm length. It belongs to the family of eukaryotic cells and has a cell wall made from polysaccharide.

Since the yeasts belonging to the genera of *Pichia*, along with several yeast species, can grow on methanol; the idea of using them as a source of single-cell protein (SCP) has appeared. The 1970s was the decade for the discovery and development of *P. pastoris* as a potential expression host for many heterologous production. Firstly, Phillips Petroleum Company started to use various yeast strains for producing high-protein animal feed as a SCP by scanning different strains growing on methanol. *P. pastoris* has outshined the most with its superior characteristics such as having higher protein content and stable fermentation traits. Moreover, the highest cell mass yield from methanol was obtained for the case of *P.pastoris* and was improved by applying high oxygen and heat transfer conditions resulting in highest cell density at around 125 g dry weight/L of culture broth (Seetharam *et al*., 1991). However, a decrease in the cost of soybeans was observed depending on the increased methane price as a result of the oil crisis in 1970s. Since soybeans are mainly used as animal feed, producing high-protein animal feed by using *P.pastoris* strains became an inefficient procedure and this led to new opportunities for the usage of *P. pastoris* for recombinant protein expression (Cereghino *et al*., 2000). During the research, it was detected that

induction of certain pathway enzymes is necessary for proper functioning of methanol metabolism (Veenhuis *et al*., 1983). Alcohol oxidase (AOX) was discovered as the first enzyme in the methanol-consumption pathway and it makes up to 30% of total soluble proteins in the cells growing on methanol while it could not be determined for the cells growing on carbon sources other than methanol. Thus, regulation of the synthesis of AOX enzyme was found to occur at the transcription level and promoter from this gene was isolated. With generation of various vectors and strains, high level of foreign protein expression was achieved by using strong regulated AOX promoter (Cregg *et al*., 1993).

P. pastoris expression system has evolved in a short time with the discoveries of new promoters and optimizations in fermentation systems. It is still widely used for production of heterologous proteins at large scale; various heterologous proteins expressed in *P. pastoris* are listed in Table 2.1.

Table 2. 1 Heterologous proteins expressed in *P.pastoris*

Protein	Mode	Amount	Signal sequence	Reference
Bacillus licheniformisα-amylase	Extracellular	2.5 g/L	SUC2	Paifer *et al*.. 1994
Bordetella pertussis pertussis pertactin (P69)	Intracellular	3 g/L	-	Romanos *et al*., 1991
Clostridium tetani tetanus toxin fragment C	Intracellular	12 g/L	-	Clare *et al*., 1991
Staphylococcus aureus staphylokinase	Extracellular	50 mg/L	α-MF	Miele *et al*., 1999
Streptomyces viridosporus T7A peroxidase, endoglucanase	Extracellular	2.47 g/L	α-MF	Thomas *et al*.. 1998
Aspergillus fumigatus catalase L	Extracellular	2.3 g/L	PHO1	Calera *et al*.. 1997
Rhizopus oryzae lipase	Extracellular	60 mg/L	α-MF	Minning *et al*., 1998
Saccharomyces cerevisiae invertase	Extracellular	2.5 g/L	native	Tschopp *et al*., 1987
Allium sativum (garlic) alliin lyase	Intracellular	2.167 U/g	-	Cregg *et al*.. 1989

Table 2.1 Heterologous proteins expressed in *P.pastoris* (cont'd)

Protein	Mode	Amount	Signal sequence	Reference
Cynodon dactilon (Bermuda grass) Cyn d1	Extracellular	1.5 g/L	PHO1	Smith *et al*., 1996
Hevea brasiliensis hydroxynitrile lyase	Intracellular	22 g/L	-	Hasslacher *et al*., 1997
Boophilus microplus (cattle tick) Bm86	Intracellular and Extracellular	1.5 g/L	SUC2	Gallet *et al*., 1998
Hirudo medicinalis (leech) hirudin	Extracellular	1.5 g/L	α-MF	Rosenfeld *et al*., 1996
Bovine β-casein	Intracellular	1 g/L	-	Choi *et al*., 1996
Bovine β-lactoglobulin	Extracellular	> 1 g/L	α-MF	Denton *et al*., 1998
Bovine tissue-type plasminogen activator (tPA)	Extracellular	1.1 mg/L	α-MF	Johnsen *et al*., 1998
Mouse epidermal growth factor	Extracellular	450 mg/L	α-MF	Clare *et al*., 1991
Rabbit monoclonal single-chain Fv specific for recombinant human leukemia inhibitory factor	Extracellular	100 mg/L	α-MF	Ridder *et al*., 1995

Table 2.1 Heterologous proteins expressed in *P.pastoris* (cont'd)

Protein	Mode	Amount	Signal sequence	Reference
Chimeric B7-2 antibody fusion protein	Extracellular	15 mg/L	α-MF	Gerstmayer *et al.*, 1997
Endostatin	Extracellular	20 mg/L	α-MF	Boehm *et al.*, 1999
Fibrinogen, 143-411, 143-427	Extracellular	100 mg/L 75 mg/L	α-MF	Cote *et al.*, 1997
Insulin-like growth factor-1 (IGF-1)	Extracellular	600 mg/L	α-MF	Brierley, 1998
Interleukin-17 (hIL-17)	Extracellular	0.35 mg/L	α-MF	Murphy *et al.*, 1998
Leukemia inhibitory factor (LIF)	Extracellular	17 mg/L	α-MF	Zhang *et al.*, 1997
Monoclonal single-chain Fv	Extracellular	50 mg/L	α-MF	Luo *et al.*, 1995
Serum albumin	Extracellular	3 g/L	native	Ohtani *et al.*, 1998
Tumor necrosis factor α (TNF)	Intracellular	10 g/L	-	Sreekrishna *et al.*, 1988

Table 2.1 Heterologous proteins expressed in *P.pastoris* (cont'd)

Protein	Mode	Amount	Signal sequence	Reference
Hepatitis B virus surface antigen	Intracellular	400 mg/L	-	Cregg *et al*., 1987
Superoxide dismutase	Intracellular	750 mg/L	-	Holtz *et al*., 1993
Human interleukin-2	Intracellular	4 g/L		Davis *et al*., 1993
Bovine lysozyme	Extracellular	300 mg/L	native	Digan *et al*., 1989
Human lysozyme	Extracellular	700 mg/L	ND	Davis *et al*., 1993
Aprotinin analog	Extracellular	800 mg/L	α-MF	Vedvick *et al*., 1991
Kunitz protease inhibitor	Extracellular	1 g/L	α-MF	Wagner *et al*., 1992
Subtisilin inhibitor (*Streptomyces*)	Extracellular	500 mg/L	native	Markaryan *et al*., 1996
Glycolate oxidase (spinach)	Intracellular	> 250 U/g	-	Payne *et al*., 1995

Table 2.1 Heterologous proteins expressed in *P.pastoris* (cont'd)

Protein	Mode	Amount	Signal sequence	Reference
Bm86 antigen (tick)	Intracellular	1.5 g/L	-	Rodriguez *et al.*, 1994
Human Lewis fucosyltransferase (Fuc-TIII)	Extracellular	30 mg/L	α-MF	Gallet *et al.*, 1998
Human sex steroid-binding protein (SBP)	Extracellular	4 mg/L	α-MF	Sui *et al.*, 1999

Signal sequences: α-MF (*S. cerevisiae* α-mating factor); SUC2 (*S. cerevisiae* invertase); PHO1 (*Pichia pastoris* acid phosphatase)

To achieve a heterologous gene expression in *P. pastoris*, the gene encoded the desired protein must be inserted into a suitable expression vector at first. Then this vector is integrated into the genome of *P. pastoris* with a variety of methods. After these steps, a screening for the potential productive strains is made. In Figure 2.4, the components of the *P. pastoris* expression vector can be seen.

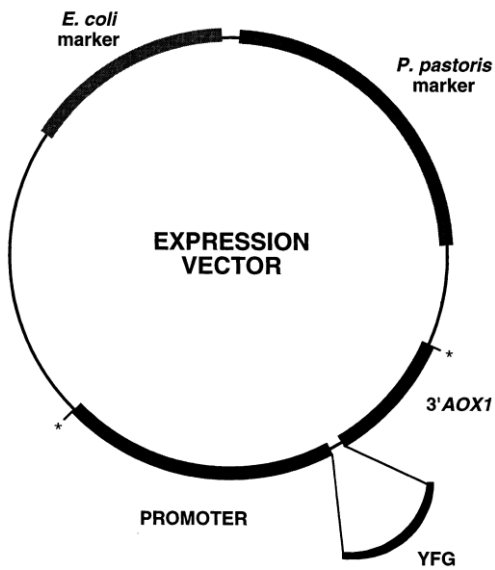


Figure 2. 4 Components of the *P. pastoris* expression vector (Cereghino *et al*., 2000) *[YFG: your favorite gene]*

All expression vectors have been designed as shuttle vectors which can function both in *E. coli* and *P. pastoris* cells thus they contain markers for the selection in both organisms. Genes of ADE1, ARG4, G418, HIS4, URA3, Kanamycin^r and Zeo^r are used as selectable markers in *P.pastoris*. Moreover, expression vectors carry a short fragment from the 3′ end of P_{AOX1} as a transcription termination site. In Table 2.2 and 2.3, commonly used expression vectors and host strains are presented for *P. pastoris*.

Table 2. 2 Commonly used expression vectors in *P. pastoris*

Plasmid	Expression cassette	Selection marker	Reference
pGAPZ	GAP-promoter; AOX1-terminator	ZEOCIN^R	Waterham *et al*., 1997
pPIC9K	AOX1-promoter; AOX1-terminator	HIS4; Kanamycin^R	Scorer *et al*., 1993
pPICZ	AOX1-promoter; AOX1-terminator	ZEOCIN^R	Otterbein *et al*., 2000

Table 2. 3 Host strains used for gene expression in *P. pastoris*

Host strain	Genotype	Phenotype	Reference
GS115	*his4*	Mut$^+$ His$^-$	Cregg *et al*., 1985
KM71	*aox1Δ::SA RG4his4his4arg4*	Mut^S His$^-$	Cregg and Madden, 1987
MC100-3	*aox1Δ::SA RG4aox2Δ::Phis4his4arg4*	Mut$^-$ His$^-$	Cregg *et al*., 1989
SMD1168	*pep4Δhis4*	Mut$^+$ His$^+$; protease$^-$	White *et al*., 1995

Despite all of its advantages, some problems may arise in fermentation systems where *P. pastoris* is used as a host organism. When compared with prokaryotes, it has slower growth rate so generally longer cultivation time is needed. It shows high proteolytic activity which causes degradation of the product molecules and methanol can be a problem at large scale productions when P_{AOX1} is used for

expression. In order to eliminate these handicaps, research is still made such as the usage of protease free strains or discovery of new promoters that do not require methanol as inducer.

2.3. Genetic Parameters of P. pastoris

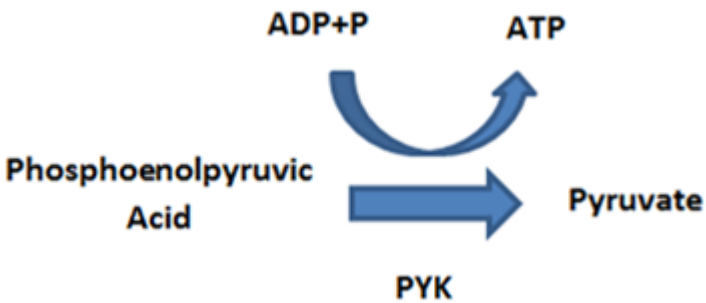
2.3.1. Promoters

RNA polymerase binds to some special nucleotide sequences of DNA and starts the transcription. These nucleotide sequences are designated as promoter regions and they are the major elements of a protein synthesis process. Their response can initiate and even adjust the expression of the genes. The activity of some promoters are induced by the existence or absence of various factors; these type of promoters are defined as inducible promoters. The gene under control is not expressed unless the inducer compound is present. On the other hand, continuous expression is achieved by constitutive promoters since their activity is independent of environmental and developmental factors.

In many fermentation systems, selection of the suitable promoter for protein expression is the crucial step since it affects the characteristics of the whole process. An effective promoter should minimize the necessary carbon source concentration and it should not need many carbon source shifts in the operation. Also, a promoter that can perform at low and even limited oxygen transfer conditions is desirable for fermentation systems. Continuous operations are much more cost-effective at large-scale productions so preferring constitutive promoters for these type of processes can be a reasonable choice. Furthermore, elimination of undesired by-products can also be achieved by using the viable promoters for specific expression systems.

2.3.1.1. PYK Promoter

The enzyme of pyruvate kinase is located in the glycolysis pathway in *P. pastoris*. It catalyzes the following reaction in the presence of K^+ and Mg^{2+} or Mn^{2+} ions:



Activity of the enzyme is enhanced by phosphoenolpyruvic acid (PEP) and fructose-1,6-bisphosphate while the presence of ATP, long-chain fatty acids and acetyl-CoA at high concentrations inhibit all isozymes of pyruvate kinase. If alanine accumulates in the pathway, it also decelerates the production of pyruvate by inhibiting enzyme activity (Xu *et al*., 2012; Veelen *et al*., 1979; Grüning *et al*., 2011). Its promoter sequence was characterized recently and the studies showed that the promoter is induced significantly at oxygen-limitation conditions (Massahi *et al*., 2017).

The determination of the promoter nucleotide sequence was based on the *P. pastoris* GS115 strain whose genome scale can be found at (www.ncbi.nlm.nih.gov). At first, a BLAST analysis was performed between the pyruvate kinase gene in *P. pastoris* DSMZ strain and entire genome of the GS115 strain. Then a nucleotide sequence which has 95% analogy was identified and annotated as pyruvate kinase in GS115 strain. 92% identity was also obtained for the preceding gene regions between DSMZ and GS115 strain thus the "*putative promoter region*" was assumed to be the segment between these two genes. The region between these two genes was obtained by using the nucleotide sequences of

the genes in sequenced strand of chromosome 2; the complementary sequence was evaluated and rearranged in 5' to 3' direction to obtain the following sequence:

5'…TTCAGTGTGCGGGATACTGTATTCCGCTCGGGGTTCTAAAGAA ATTGTTTAAACTAAACCAAATCGGATCAGAGGTTCCGTACGTTTTT CACATTCAAGGATGAGGGTTTTCCACGAGTGAACTATTACTCCGGT CTCCCACCATCATTTGCGGAATGAAACCTTTTGTGCTGAGATTGTA TAGGGCGTGGGGACGGACGCTTCTTAACCGTTCCCCTAGAATGTC GTCCCCTGATCAAAATTTAATGGCATCCAACTTTGCTGTAATAGGT ATATATAACCTAGCAGGCGACCGTTCATGTACAGTAAATTGTTTTA GACTTTTTTTTAACTGAAATCAATCCA…3'

Figure 2. 5 Nucleotide sequence of the putative promoter region peculiar to pyruvate kinase gene with 345 bp length in *P. pastoris* GS115 strain (Massahi *et al*.,2017)

2.3.1.2. AOX1 Promoter

In *P. pastoris*, the genes *AOX1* and *AOX2* encode alcohol oxidase and *AOX1* gene is mostly responsible from the alcohol oxidase activity in the cells (Ellis *et al*., 1985). The genes on the methanol pathway in *P. pastoris* are repressed by glucose, ethanol and glycerol while they are induced by methanol (Hartner *et al*., 2006).

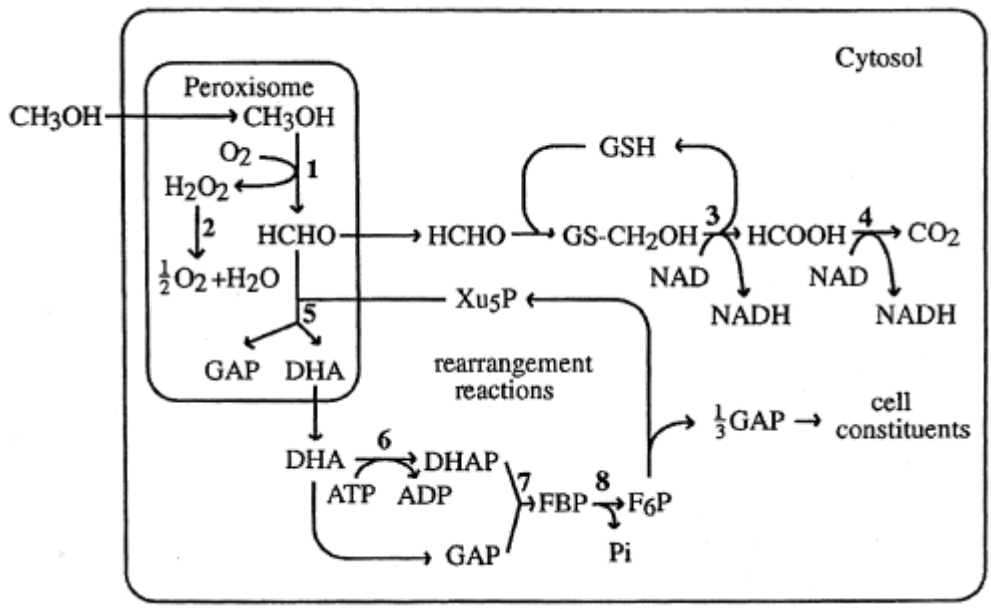


Figure 2. 6 *P. pastoris* methanol pathway 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-phosphate aldolase; 8, fructose 1,6-bisphosphatase (Cereghino *et al*., 2000).

In Figure 2.6, methanol pathway of *P. pastoris* is presented. Alcohol oxidase was found as the first enzyme in the methanol pathway and it takes part in the oxidation of methanol to hydrogen peroxide and formaldehyde. This reaction takes place in peroxisome and hydrogen peroxide is degraded to oxygen and water by the enzyme catalase. Some amount of formaldehyde passes to cytosol and is oxidized again to formate and carbon dioxide. These reactions occuring at cytosol are the sources of energy for the cells consuming methanol as a carbon source. The rest of the formaldehyde is used up through a cell cycle in order to generate cell constituents (Cereghino *et al.,* 2000).

Since it allows a strict methanol control, the gene was isolated and P_{AOX1} was started to be used for the expression of foreign genes by designing commercial vectors (Cregg *et al*., 1985). In fermentation systems, 14.8 g/L product

concentration can be obtained for extracellular production while at intracellular production product concentrations can be as high as 22 g/L (Hasslacher *et al*., 1997; Wertel *et al*., 1999). For the expression of recombinant proteins under the control of P_{AOX1}, a glycerol batch phase is generally applied followed by a glycerol or glucose fed-batch phase with exponential feeding for a short period of time to derepress the P_{AOX1}. Then methanol is fed to the bioreactor for induction with a gradual increment resulting in an increased oxygen uptake rate. When the dissolved oxygen tension (DOT) has reached to a certain level, the rate of methanol feed is kept constant in order to prevent oxygen-limitation (Jahic *et al*., 2002). For optimizing the procedure, oxygen-limited conditions with higher methanol concentrations was tried (Charoenrat *et al*., 2005). 16% higher product concentration and 5% higher cell density were achieved for oxygen-limited conditions compared with the traditional methanol-limited processes. Higher methanol consumption rate resulting from the high oxygen uptake rate could improve the productivity of the process. Moreover, mixed feed strategies were tried to observe the growth patterns of the cells and comparison of different carbon sources was made in order to obtain higher productivities (Egli *et al.*, 1980; Sakai *et al*., 1987; Sibirny *et al*., 1988; İnan *et al.,* 2001). However, addition of different carbon sources to the methanol medium made a delay in the induction of the alcohol oxidase gene and none of the fermentation systems using various carbon sources could reach to expression levels of the system which is induced by methanol.

2.3.1.3. GAP Promoter

Despite the high expression levels of P_{AOX1}, storage problems and potential hazards of methanol can not be ignored especially at large scale productions. Methanol also can not be used for the production of foodstuffs because it is synthesized from methane which is a petroleum product.

These negative properties have led the scientist, to find new promoters that do not require methanol for induction. In 1997, glyceraldehyde 3-phosphate dehydrogenase (GAP) gene was isolated and its promoter reached expression levels almost as high as the expression levels of P_{AOX1} (Waterham *et al*, 1997).

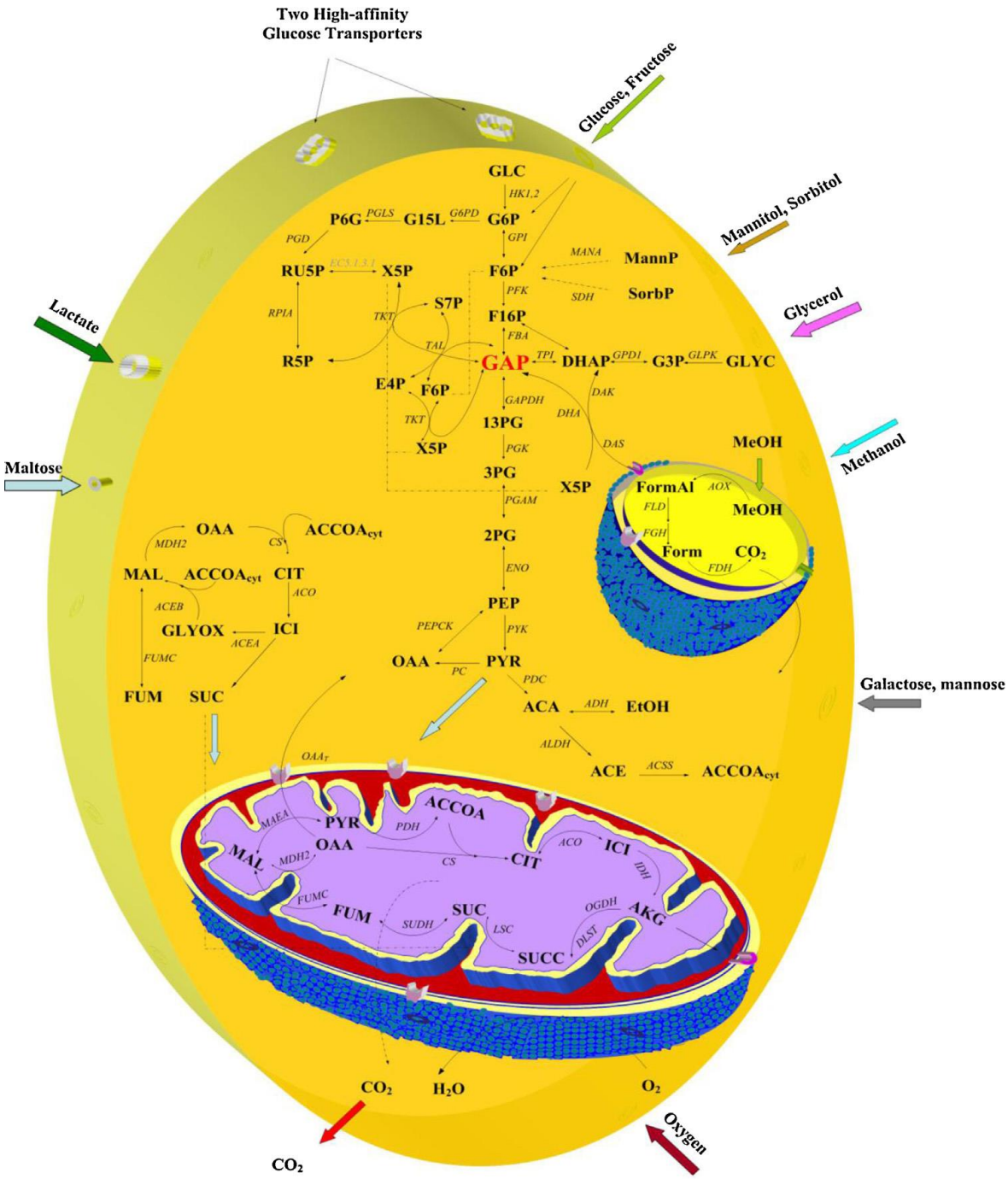


Figure 2. 7 Carbon metabolism of *P. pastoris* (Çalık *et al*., 2015)

Central carbon metabolism of *P. pastoris* is presented in Figure 2.7. Glyceraldehyde 3-phosphate dehydrogenase enzyme is placed in the glycolysis and gluconeogenesis pathways in the carbon metabolism of *P. pastoris*. It is expressed constitutively under the control of its promoter which means it does not require any substrate for induction. Recombinant protein production is achieved in parallel with the growth of the cells. Glucose or glycerol can be used as a carbon source for the expression with P_{GAP} (Cos *et al*., 2006).

The activity levels of GAP promoter was found higher for the cells growing on glucose thus it can be said that carbon source regulates the promoter's activity in a strong manner (Cereghino *et al*., 2000; Döring *et al*., 1998). Compared with the P_{AOX1}, P_{GAP} expression levels are still controversial. Heterologous protein expression levels of P_{GAP} were found higher than the expression levels of the P_{AOX1} in some publications (Delroisse *et al*., 2005; Menendez *et al*., 2003; Waterham *et al*., 1997; Döring *et al*., 1998). Menendez *et al*., used glycerol as a carbon source and found that recombinant protein expression was 3-fold more effective than the one with methanol-inducible AOX promoter expression system. Delroisse *et al*., has also reported that P_{GAP} could be substituted for P_{AOX1} since 2-fold expression levels were reported for shaking flask batch fermentation. Goodrick *et al*., (2001) obtained similar expression levels for AOX1 and GAP promoter but a continuous fermentation system was developed for enhanced protein production in this study. On the other hand, higher expression levels for P_{AOX1} were stated by some authors. For the heterologous expression of *T. reesei* cellobiohydrolase Cel7A, yield of the production was found higher for P_{AOX1} although efficient folding and N-glycosylation was observed when it was expressed with P_{GAP} (Boer *et al*., 2000). Also Hepatitis B surface antigen was highly expressed when single copy of the gene was expressed under the control of P_{AOX1} (Vassileva *et al*., 2001). Although the results are conflicting, it can not be denied that P_{GAP} is a strong alternative for P_{AOX1} since it does not need an induction and carbon source shifts during the

process. Furthermore it allows a continuos operation which improves product yield. Nevertheless it has a limitation in the production of compounds which are toxic to the cell since it is expressed directly proportional with the cell growth.

2.3.1.4. FLD1 Promoter

Glutathione-dependent formaldehyde dehydrogenase (FLD) enzyme is located in the methanol pathway of *P. pastoris* and it requires either methanol or methylamine as a sole carbon or nitrogen source for its activity. The gene was isolated and its promoter was investigated in terms of expression levels for foreign protein production. Equal or higher expression levels were reported for the production of β-lactamase when compared with the expression levels under the control of P_{AOX1} (Shen *et al*., 1998). In the following studies, it was seen that sorbitol, when using as a carbon source, does not repress the activity of the FLD1 promoter rather causes the induction of the promoter when used with methylamine in a fed-batch process (Resina *et al*., 2005). This shows that production of recombinant proteins in fed-batch fermentations can be achieved by using methylamine as an inducer along with a carbon source such as sorbitol by eliminating the need for methanol. Since it allows a broad range of operating conditions, optimization in this fed-batch process was done by testing different specific growth rates and promising results were obtained for efficient production under the control of FLD1 promoter (Resina *et al*., 2005).

2.3.1.5. ICL1 Promoter

Isocitrate lyase gene of the *P.pastoris* was isolated and characterizations were done in order to use its promoter for heterologous protein expression studies. Expression of dextranase gene was achieved under the control of ICL1 promoter by applying glucose or ethanol as carbon sources. According to study, it was found that activity of ICL1 promoter is repressed in the presence of glucose while it is induced when ethanol is used as a carbon source (Menendez *et al*., 2003). Thus

this promoter can also be a promising alternative for P_{AOX1} if further studies and optimizations are conducted for its fermentation system.

2.3.1.6. TEF1 Promoter

Translation elongation factor 1-α (TEF) gene was cloned and the promoter was derived by using the DNA microarray data based on the experiments. The promoter does not require additional substrates for induction in a similar way of GAP promoter. When the expression levels of TEF1 promoter system was compared with the expression levels of GAP promoter system; nearly equal expression levels were obtained for batch fermentation. Additionally, two-fold higher enzyme activity was observed for the production under TEF1 promoter in carbon-limited fed-batch fermentations (Ahn *et al*., 2007). Glucose or glycerol can be used as a carbon source since TEF1 promoter shows an activity in a constitutive manner. However derepression of the promoter was observed for high glucose concentraions (Nakari *et al*., 1993).

2.3.1.7. PDC Promoter

In anaerobic conditions, some yeast species produce ethanol by fermentation and pyruvate decarboxylase enzyme is placed in this fermentative pathway. It removes CO_2 from pyruvate to generate acetaldehyde with the help of cofactors. Thiamine pyrophosphate (TPP) and Mg^{2+} are the cofactors needed for pyruvate decarboxylase activity (Juni *et al*., 1968). It was reported that the activity of PDC in *P.pastoris* is induced by the presence of glucose or glycerol, higher activity was observed on glucose (Agarwal *et al*., 2013). The PDC promoter sequence was identified recently and it was seen that the promoter works effectively under oxygen-limited conditions (Massahi *et al*., 2017).

2.3.1.8. PEX8, YPT1, ENO1 and PGK1 Promoters

Until this section, the promoters which give high expression levels of foreign proteins have been summarized. However, in some certain situations expression at high levels can be unfavorable. For the case of some proteins, overexpression causes a saturation in the secretion pathway of the cells and as a result, misfolded or unfolded proteins might be produced (Thill *et al*., 1990; Brierley *et al*., 1998). To circumvent these issues, promoters having moderate expression levels can be preferred.

P.pastoris PEX8 gene is responsible for the synthesis of a peroxisomal matrix protein that takes part in the peroxisome biogenesis. It is expressed when the cells are grown on glucose and induced when methanol is introduced to the system at moderate levels (Liu *et al*., 1995).

GTPase enzyme has a function in secretion system and is encoded by the *YPT1* gene in *P. pastoris*. Glucose, methanol or mannitol can be used as a carbon source for its constitutive expression (Sears *et al*., 1998).

ENO1 gene encodes enolase enzyme which is present in the glycolytic pathway in methylotrophic yeasts (Uemura *et al*., 1985). High expression levels were reported for glucose, glycerol or ethanol-grown cells while its expression was repressed by methanol (Cregg *et al*., 2012).

Molecular characterization of the 3-phosphoglycerate kinase (PGK1) gene of *P. pastoris* was performed and its promoter was derived based on this data (Almeida *et al*., 2005). When the cells are allowed to grow on glucose, they give higher expression levels compared to cells grown on glycerol or methanol (Arruda *et al*., 2015).

In Table 2.4, various promoters used in *P. pastoris* expression systems are listed with some information about their fermentation characteristics.

Table 2. 4 Promoters used in *P. pastoris* expression systems for recombinant protein production

Protein	Strain	Promoter	Operation Mode	Substrate	Volume (L)	μ (1/h)	Production titer (mg/L)	Highest Cx (g/L)	Reference
β-*glucosidase*	Y-11430	AOX1	Fed-batch	Glycerol, then methanol	3	–	6 U/mL	130	Charoenrat *et al*., 2005
β-galactosidase	MC100-3	AOX1	Shake -flask	Sorbitol, then methanol	50 mL	–	1116 Miller units	–	İnan *et al*., 2001
Candida antarctica lipase B	SMD 1168	AOX1	Fed-batch	Glycerol, then methanol	3	0.18	1.4 g/L	160	Jahic *et al.*, 2002
HBsAg	GS115	AOX1	Batch	Glycerol, then methanol	240	–	0.4 g/L	59	Cregg *et al*., 1987
Invertase	KM71	AOX1	–	Glycerol, then methanol	–	–	2.5 g/L	40	Tschopp *et al*., 1987
Candida antarctica lipase B	SMD 1168	AOX1	Shake -flask	Glycerol, then methanol	500 mL	–	25	–	Rotticci-Mulder *et al*., 2001
Candida rugosa LIP2	X-33	AOX1	Fed-batch	Glycerol, then methanol	1	–	750	20	Ferrer *et al*., 2009
Laccase	SMD 1168	AOX1	Fed-batch	Glycerol, then methanol	1	0.012	11 U/mL	70	Hong *et al*., 2002
Mouse endostatin	GS115	AOX1	Fed-batch	Glycerol, then methanol	3	0.02	400	434	Trinh *et al*., 2003
E. coli phytase	Km71-61	AOX1	Fed-batch	Glycerol, then methanol	2	–	4946 U/mL	–	Chen *et al*., 2004
Human chitinase	SMD 1168	GAP	Continuous	Glycerol	1.5	–	450	330	Goodrick *et al.*, 2001
H-chitinase	X-33	GAP	Continuous	Glucose	–	0.042	250	110	Schilling *et al*., 2001
Fructose-releasing exo-levanase (LsdB)	X-33	GAP	Fed-batch	Glycerol	3.5	–	26.6 U/mL	59.7	Menendez *et al*., 2004
2F5Fab	SMD1168H	GAP	Fed-batch	Glucose	1.75	0.055	41	160	Gasser *et al*., 2006
hGM-CSF	GS115	GAP	Fed-batch	Glucose	1	0.2	250	–	Pal *et al*., 2006
D-amino acid oxidase	GS115	GAP	Fed-batch	Glucose	3	–	7.29 U/mL	110	Zheng *et al*., 2006
Bacillus stearothermophilus lipase	GS115	GAP	Fed-batch	Glycerol	1.8	–	201 U/mL	–	Ahn *et al*., 2007
hGM-CSF	–	GAP	Continuous	Glucose	1	0.2	82	33.5	Khasa *et al*., 2007
Human angiostatin	GS115	GAP	Continuous	Glycerol	10	–	176	–	Zhang *et al*., 2007
3H6 Fab	X-33	GAP	Fed-batch	Glucose	17	0.0317	46.85	112.07	Baumann *et al*., 2007
Rhizopus oryzae lipase	X-33	FLD1	Fed-batch	Glycerol, then sorbitol and methylamine	3.5	0.02	385 AU/mL	–	Resina *et al.*, 2005
Rhizopus oryzae lipase	X-33	FLD1	Fed-batch	Glycerol, then sorbitol and methylamine	3.5	0.015	170 UA/mL	230	Resina *et al*., 2005
Rhizopus oryzae lipase	X-33	FLD1	Fed-batch	Glycerol, then sorbitol and methylamine	3.5	0.005	40 AU/mL	31	Resina *et al*., 2009
Translation elongation factor 1-α	GS115	TEF1	Fed-batch	Glycerol	1.8	–	410 U/mL	–	Ahn *et al*., 2007

2.3.1.9. Double-Promoter Expression Systems

To achieve an enhanced degree of protein expression, studies are still proceeding and combining the synergistic function of two promoters is another choice for this aim (Öztürk *et al*., 2017). In most of the double-promoter expression systems, a constitutive promoter was used along with an inducible promoter and recombinant proteins are produced simultaneously or sequentially under the control of these promoters (Wu *et al*., 2003; Wu *et al.,* 2003; Callewaert *et al*., 2001). In the study of Wu *et al*. (2003) two-fold higher expression was achieved for combined use of P_{GAP} and P_{AOX1} than the system under the control of P_{GAP} alone. Duan *et al.* (2009) has also proved that simultaneous expression of the two different genes is possible under two inducible promoters.

2.3.2. Gene Copy Number

Copy number of the expression cassette is another important parameter that affects the recombinant protein expression. In Table 2.5, some studies that investigated the effect of gene copy number on r-protein production is presented, the increase show the fold values with respect to single copy strains. For many cases, it has been reported that recombinant protein expression is greatly enhanced by increasing gene copy number (Vassileva *et al*., 2001).

Table 2. 5 Effect of copy number on the r-protein expression

Gene	Copy Number	Increase	Reference
Tumor necrosis factor	>20	200-fold	Sreekrishna *et al*., 1987
Tetanus toxin fragment C	14	6-fold	Clare *et al*., 1991
Murine epidermal growth factor	19	13-fold	Clare *et al*., 1991
Aprotinin	5	7-fold	Thill *et al*., 1990
Insulin-like growth factor-1	6	5-fold	Brierley et al., 1994

However Thill *et al*. (1990) has showed that increasing gene copy number may affect the recombinant protein synthesis negatively for some cases and the effect is mainly unpredictable. In the study of Cregg *et al*. (1985) no significant increase in the protein synthesis was observed when the copy number is increased. If the produced recombinant protein is toxic to the host cell, increasing gene copy number might have destructive effects for the cells (Athmaram *et al*., 2012). Except those, overexpression of the proteins by increasing gene copy number can lead to saturation in the secretion pathway of the eukaryotic cells and this step becomes rate-limiting. Since post-translational modifications of the proteins are mainly carried out in the endoplasmic reticulum and golgi apparatus, these organelles can fail to satisfy proper post-translational modifications of the produced proteins resulting in an ineffective process (Macauley-Patrick *et al*., 2005; Hohenblum *et al*., 2003; İnan *et al.*, 2005). It was also seen that the effect of the copy number on recombinant protein production depends on the type of the

promoter used for expression (Hohenblum *et al*., 2003). By considering these inferences, experimental determination of the optimum copy number for the production of specific proteins becomes inevitable.

2.3.3. Protein Secretion

One of the advantages of using eukaryotic yeast cells for recombinant protein production is that they can secrete heterologous proteins in their native form. Signal sequences are used for secretion of the proteins and mostly used signal sequences in *P.pastoris* are listed in Table 2.1 for extracellular production of some heterologous proteins. Extracellular production is often desirable in recombinant protein production for eliminating the purification steps of the downstream processes (Porro *et al*., 2005). A lot of studies have been carried out to improve the secretion machinery of the yeast expression systems including vector system modifications, engineering of the fermentation systems and host strains. Studies have showed that 100 to 1000-fold lower values were recorded in secretion titers than theoretical ranges (Schröder, 2007). The reasons for that can be the improper choices of host strain, cultivation conditions, vector systems, leader sequences, translation signals for the desired protein. Selection of the proper host strain and promoter/signal peptide optimization have found to be potential remedies for this issue primarily. However many proteins still could not reach to theoretical expression levels after these modifications and it was understood that several factors affect the secretion of the proteins such as translocation of the proteins to the endoplasmic reticulum, protein folding, glycosylation and intracellular protein trafficking (Idiris *et al.,* 2010).

2.3.4. Proteolytic Degradation

Proteases are the enzymes that degrade the proteins and result in a decrement in the amount of produced recombinant proteins in fermentation medium. Vacuoles are the main sources for protease enzymes in cell structure of the yeasts and the

levels of these enzymes mainly depend on the nutritional conditions (Hansen *et al*., 1977). In order to prevent proteolytic activity in fermentation broths, different feeding strategies can be tried. Kobayashi *et al*. (2000) succeeded in decreasing protease activity by increasing the concentrations of phosphoric acid and ammonia in the fermentation medium for the production of recombinant human serum albumin. They also adjusted the pH of the medium to eliminate degradation. Jahic *et al*. (2003) also reported an optimum pH and temperature value for improving the yield of the operation by reducing protease activitiy. Addition of casamino acids to the fermentation medium or using protease inhibitors were also suggested as a solution to this issue (Goodrick *et al*., 2001; Kurokawa *et al*., 2002). The choice of using protease-deficient strains in which the gene encodes protease is disrupted can also be preferred for high level expression without proteolytic activity (Brankamp *et al*., 1995).

2.4. Bioprocess Operation Parameters

2.4.1. Temperature

Since *P. pastoris* lives in a narrow temperature range, a wide range of temperature set points can not be tried in bioreactor experiments. Most fermentation experiments were carried out at a temperature value of 30 °C (Chen *et al.,* 2004; Files *et al*., 2001; İnan *et al*., 2001; Chiruvolu *et al.,* 1997; Wang *et al*., 2010; Cunha *et al*., 2004; Guo *et al.,* 2014; Yang *et al*., 2004) while studies that conducted at T=20 °C (Ndayambaje *et al*., 2014) and T=25 °C (Damasceno *et al*., 2004) are also present. Decreasing the temperature may increase oxygen solubility in fermentation broth and thus oxygen transfer rate is increased in the bioreactor. However, it may cause a decrease in the intracellular reaction rates and cell growth kinetics (Dragosits *et al*., 2010). Optimum temperature for the production of a recombinant protein is also closely related with the expressed protein itself since the activity and stability of the proteins highly depend on temperature. Hong *et al*. (2002), examined the effect of temperature on the expression of laccase enzyme by

conducting two experiments at T=20 °C and T=30 °C. Higher enzyme activity was achieved when temperature was set to 20 °C and enhanced stability, release of protease enzymes at lower levels and efficient folding at lower temperatures were listed as the reasons for this high specific activity. A similar result was also reported for the production of galactose oxidase; nearly four-times higher yield was obtained when the cultivation temperature was reduced from 30 °C to 25 °C (Whittaker *et al*., 2000). Eissazadeh et al. (2017) has recently analyzed the combinatorial effect of pH and temperature on the production of human epidermal growth factor and found the optimum temperature as 29 °C for this process. Furthermore, a significant decrease on the expressed protein levels was observed for the temperatures above 31 °C. In another study, 28 °C was found as an optimum cultivation temperature for the expression of human granulocyte-colony stimulating factor by Bahrami et al. (2007). As can be understood from the examples above, temperature is one of the important parameters in fermentation systems and optimum temperatures for cell growth and recombinant protein expression can differ from each other making the decision more challenging.

2.4.2. pH

Providing an optimal pH during fermentation processes has a significant importance in terms of cell growth, protein expression and protein stability. In most cases, pH is adjusted below 7.0, mostly in the range of 4.0-6.5. There are some studies which kept the pH value at 5.0 for recombinant protein production (Zhang *et al*., 2003; Brierley *et al*., 1990; Guo *et al*., 2014; Wang *et al*., 2014). Besides, pH of 6.0 (Anasontzis *et al*.,2014; Terrazas *et al*., 2014) and pH of 5.5 (Phuoc *et al*., 2009; Zhao *et al*., 2008) was also applied for various recombinant protein expression. Zhang *et al.* (2009) carried out a fermentation process at pH=4.0 and Ndayambaje *et al*. (2014) conducted an experiment when pH was kept at 4.8. It was reported that the kinetics of proteolytic reactions and viability of the cells are significantly affected by the pH (Li *et al*., 2007). Damasceno *et al.* (2004)

and Werten *et al*. (1999) have stated that higher product yield was obtained when pH is decreased from 6.0 to 3.0 since proteolytic degradation was measured at minimum levels for pH=3.0. On the other hand, İnan et al. (1998) has presumed that maximum protein expression can be achieved at pH values between 6.8 and 7.0. This might be due to the different stability levels of particular proteins since some proteins are unstable under acidic conditions. At pH=8.0, particular proteins displays higher stability but cell growth is inhibited at this condition. In order to overcome this problem, Hu *et al*. (2014) developed a feeding strategy to improve the cell growth under alkaline conditions. For the heterologous expression of rhGH, the effect of pH was investigated in details at different pH values (Çalık *et al*., 2010). According to this study, cell concentration was higher at pH=6.0 and the highest recombinant protein concentration was obtained at pH=5.0 as 0.27 g/L. It was seen that oxygen uptake rate and total protease secretion increase with increasing pH. When the expression level of recombinant human growth hormone is considered, an optimum value for the pH was reported as 5.0. Also, the studies have showed that pH is a critical parameter for bioreactor experiments and maintaining the optimum pH throughout the whole process is also vital for both cell viability and r-protein production (Chiruvolu *et al*., 1998).

2.4.3. Oxygen Transfer

Maximal oxygen transfer rate is defined as one of the most limiting parameters (Çalık *et al*., 2000) for large scale high cell density cultivations (Porro *et al*., 2005).

In aerobic bioprocesses; gas, liquid and solid phase exist together in the bioreactor and oxygen is transferred from air (gas phase) to the microorganism (solid phase). This phenomenon is explained as follows and named as two-film theory:

1. Transfer from the inner part of the gas bubble to the gas-liquid interface
2. Movement through the gas-liquid interface

3. Diffusion through the stagnant liquid film which surrounds the gas bubble
4. Movement in the bulk liquid
5. Diffusion through the stagnant liquid film which surrounds the cell
6. Motion across the liquid-solid interface (If the any clumps or aggregate is present, diffusion through the solid particle to the individual cell.)
7. Movement into the cytoplasmic part where metabolic reactions take place
8. Biochemical reactions in which oxygen is depleted, CO_2 and other gases are produced
9. Transfer of the generated gases in the opposite direction

In Figure 2.8, transfer of oxygen from gas bubble to solid particle is presented.

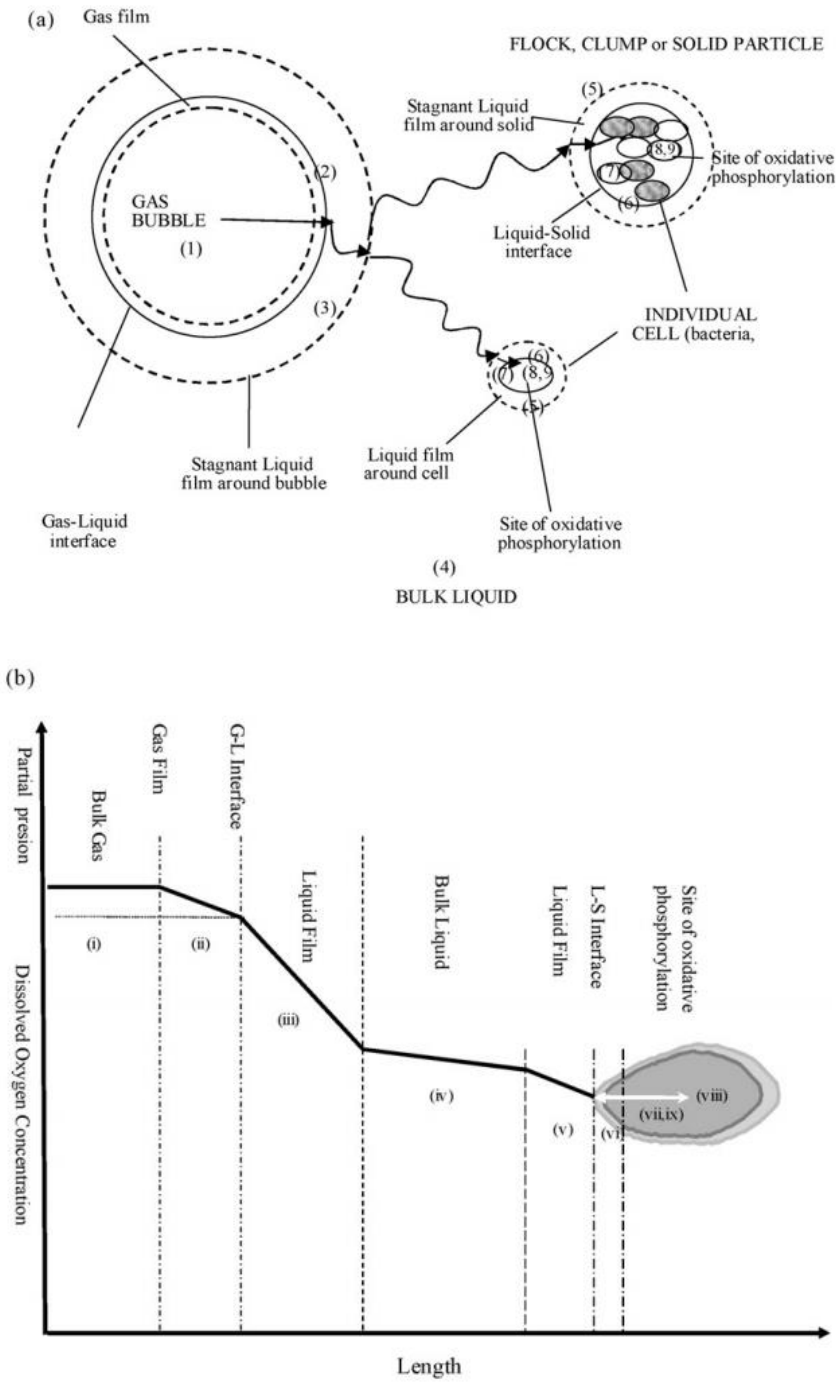


Figure 2. 8 a) Oxygen transfer steps from gas bubble to solid particle

b) Profile of oxygen concentration from gas bubble to solid particle (Garcia-Ochoa *et al*., 2010)

Çalık *et al*. (1999) has specified that air flow (Q_0/V) and agitation rates (N) mostly affect oxygen transfer rate and dissolved oxygen concentration in the bioreactor. If the cells are found in a homogeneously dispersed form in the fermentation medium, movement of the oxygen molecule in the bulk liquid is very fast. Also the transfer of the oxygen particle across the cell membrane by passive diffusion is easy if the cell clusters are not formed. Since the size of the cell is small, the resistance against the transport in the cytoplasm can also be neglected (Nielsen and Villadsen, 1994). Thus, "oxygen transfer from the gas bubble to the stagnant region of the bulk liquid which surrounds the gas bubble" and biochemical reactions in the cell are generally accepted as the limiting steps in aerobic bioprocesses (Garcia-Ochoa *et al*., 2010). These steps control the overall rate of the mass transfer and if the biochemical reactions take place faster than the diffusion, oxygen transfer rate (OTR) from gas to liquid is considered as the main limitation and is explained by two-film theory:

$$N_o = k_G (P_G - P_i) = k_L (C_{O,i} - Co) \quad (2.1)$$

In this equation, N_o is defined as the molar mass transfer flux of oxygen, k_G and k_L are defined as the gas side and liquid side mass transfer coefficients respectively, P_G is the partial pressure of oxygen in the gas bubbles and C_O is the dissolved oxygen concentration in the bulk liquid, subscript *i* represents the interface.

For the overall mass transfer, the following equation can be written:

$$N_O = K_G (P_G - P^*) = K_L (C_O^* - C_O) \quad (2.2)$$

Where P^* is the oxygen pressure in equilibrium with the liquid phase and C_O^* is the oxygen saturation concentration in the bulk liquid in equilibrium with the bulk gas phase, K_G and K_L are overall mass transfer coefficients. According to Henry's Law:

$P^* = H\, C_O^*$ (2.3)

Combining equations (2.1) and (2.2);

$$\frac{1}{K_L} = \frac{1}{H\, k_G} + \frac{1}{k_L} \quad (2.4)$$

Mass transfer resistance in liquid phase is much greater than the mass transfer resistance in gas phase because of the low solubility of oxygen in aqueous solutions, the first term on the right hand side becomes negligible. Thus K_La is almost equal to the k_La, liquid phase mass transfer coefficient. Oxygen transfer rate can be obtained by the following equation:

$OTR = N_oa = K_La\,(C_0^* - C_0)$ (2.5)

In this equation, a is the gas-liquid interfacial area per unit volume ($m^2\ m^{-3}$), K_La is the overall mass transfer coefficient (s^{-1}), C_0^* is the saturated dissolved oxygen concentration (mol m^{-3}) and C_0 is the actual dissolved oxygen concentration (mol m^{-3})

The maximum oxygen transfer rate (OTR_{max}) is defined as:

$OTR_{max} = (N_o)_{max}a = K_La\, C_0^*$ (2.6)

Oxygen transfer rate is influenced by many different parameters such as the rheology of the medium, temperature, agitation and aeration rates, oxygen uptake rate (OUR) and the type of the microorganism. Oxygen uptake rate can be written as:

$OUR = -r_o = -r_o'''C_x = q_o\, C_x$ (2.7)

In this equation, $-r_o$ is the rate of oxygen consumption (mol $m^{-3}\ s^{-1}$), $-r_o'''$ is the rate of oxygen consumption per unit cell dry weight (mol $kg^{-1}\ s^{-1}$), C_x is the cell concentration (kg m^{-3}) and q_o is defined as the specific oxygen consumption rate

(mol kg^{-1} s^{-1}). The growth phase of the cell and source of carbon in the medium influence the oxygen uptake rate.

Previously, enhanced productivity of heterologous proteins could be obtained by controlling the growth with carbon limitation but now some optimizations for fed-batch bioreactor systems can be done to increase the productivity and production levels. For example, a continuous feed stream design method was developed with the optimization of two parameters, transition time and specific growth rate, for *B. subtilis* fermentation process. The performance of the process was increased by 36% for the extracellular rhGH production and the cultivation time was shortened with these optimizations (Çalık *et al*., 2016). However, oxygen-limitation was suggested as another choice for optimizing heterologous protein production in recent studies. For *P. pastoris* expression systems, the effects of oxygen limitation on recombinant protein production and disulfide bridge formation have not been thoroughly investigated yet. Studies investigating different oxygen transfer conditions on r-protein production is listed in Table 2.6.

Güneş and Çalık (2016) have investigated the effects of oxygen transfer on r-protein production under glyceraldehyde-3-phosphate dehydrogenase promoter and compared two strategies. Constant oxygen transfer rate was chosen as the basis for one strategy while in the other strategy, dissolved oxygen concentrations were kept constant. The highest cell concentration was obtained at C_{DO}=20% although the highest volumetric and and specific enzyme activities were reported at C_{DO}=15%.

Ponte *et al.* (2016), conducted fed-batch bioreactor experiments at four different dissolved oxygen (DO) set points which were 7%, 10%, 25% and 45% in respect to air saturation. DO around 25% was found as optimal for the *Rhizopus oryzae* lipase production while overall product yield was higher when DO was set to 10%. In this study, oxygen transfer rate (OTR) was not limited and it changed along the

process in order to satisfy desired DO set points. According to results, oxygen tension has a positive effect on specific growth rate and specific productivity was higher at aerobic conditions in contrast to P_{GAP}-driven strain (Baumann *et al*., 2008). From this work, it can be concluded that productivity and the yield of the heterologous proteins can be optimized by controlling the oxygen tension in the fermentor.

Since conflicting arguments are present related with the optimum DO concentration for recombinant protein production, a further investigation was suggested considering with the oxygen transfer effects together with oxygen uptake rate (Çalık *et al*., 2015).

Table 2. 6 List of studies investigating different oxygen transfer conditions on r-protein production for *P. pastoris* fermentation system

Strain	Plasmid	Inducible Promoter	Recombinant protein (rP)	Operation strategy in production phase (PP)	C_{rP}	C_{DO}	Aeration Rate (vvm)	N (rpm)	T (°C)	$pH_{o,PP}$	$V_{o,PP}$ (L)	$C_{Xo,pp}$ (OD_{600})	Reference
X-33	pPICZa	AOX1 (Mut$^+$)	*Rhizopus oryzae lipase*	Methanol Non-limited Fed Batch (C_M= 3 g/L)	127 mg/L	7%	-	800 – 1000	30	5.5	2	2.5	Ponte *et al.*, 2016
					166 mg/L	10%							
					174 mg/L	25%							
					158 mg/L	45%							
Strain	**Plasmid**	**Constitutive Promoter**	**Recombinant protein (rP)**	**Operation strategy in production phase (PP)**	**C_{rP}**	**C_{DO}**	**Aeration Rate (vvm)**	**N (rpm)**	**T (°C)**	**$pH_{o,PP}$**	**$V_{o,PP}$ (L)**	**$C_{Xo,pp}$ (OD_{600})**	**Reference**
X-33	pGAPZαA	GAP	Human antibody fragment 2F5 Fab	Chemostat Dilution rate of D=0.1 h^{-1}	8 µg/L	>20%	0.8	700	25	5	1	-	Garcia-Ortega *et al.*, 2017
					12 µg/L	0%							
X-33	pGAPαA	*GAP*	Antibody fragment, Fab	Chemostat µ=0.1 h^{-1}	~50 extracellular 10> intracellular (µg Fab/g DW h)	21%	1.5	700	25	5	2	1 (25 g/L)	Carnicer *et al.*, 2009
					~40-50 extracellular ~10 intracellular (µg Fab/g DW h)	11%							
					~80 extracellular ~10 intracellular (µg Fab/g DW h)	8%							
GS115	-	*GAP*	Methionine adenosyltransferase (MAT)	Glycerol Fed-batch	3.45 g/L	50%	0.2-1.4	800	30	6	7.5	(20 g/L)	Hu *et al.*, 2008
					4.14 g/L	25%							
					5.22 g/L	0%							
GS115	pPIC9	*PGK*	L-lactic acid	Batch C_{GLY}=40g/L	10 g/L	3%	0.05	350 500 900	30	5	0.5	2	de Lima *et al.*, 2016
					8 g/L	5%							
				Fed-Batch C_{GLY}=20g/L	22 g/L	30%	0.05	500	30	5	0.5	2 (20 g/L)	
CBS7435 MutS strain (Δoch1)	-	-	Horseradish peroxidase	Exponential glycerol fed-batch µ = 0.08 h^{-1}	56.5 U/mg	10%	1	900	20	5	2.2 (max.)	-	Gmeiner *et al.*, 2015
					0.54 U/mg				30	5			
					47.2 U/mg				20	7			
					0.29 U/mg				30	7			
					7.41U/mg	20%			25	6			
					15.9 U/mg				25	6			
					61.7 U/mg	30%			20	5			
					0.21 U/mg				30	5			
					38.7 U/mg				20	7			
					0.20 U/mg				30	7			

2.5. Bioprocess Kinetics in Fed-batch Bioreactor Operation

To fully understand any kind of a bioprocess, mathematical representation of the system behavior including the specific growth rate, biomass generation rate, substrate consumption rate and production rate should be defined in detail. Mass conservation equations are written for every species in the bioreactor according to the verbal equation, as follows:

Input-Output + Generation = Accumulation (2.8)

By keeping sample volumes small, perturbation effects of sampling on the cell generation can be minimized; thus, in fed-batch fermentations the positive value of the cultivation time derivative of fermentation volume (V) is caused by the volume increase due to continuous feeding. Therefore, the volume change because of acid-, base, antifoam- additions, and sampling can be negligible.

$$\frac{dV}{dt} \approx Q(t) \tag{2.9}$$

For the batch, first phase of the bioreactor operation the volume change is, as follows:

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

2.5.1. Mass Conservation Equation for the Cell Growth

In general, following inoculation at t = 0 h in batch and fed-batch bioreactor operations, fermentation proceeds without cell feed throughout the process. Thus, in fed-batch and batch- operations, mass conservation equations are identical for the cell (Çalık et al., 2016):

$$r_x V = \frac{d(C_x V)}{dt} \tag{2.11}$$

where, is the residence time (t) (so called cultivation time in fermentation processes) representing the performance of batch- and fed-batch bioreactors, is the bioreactor design parameter. By keeping sample volumes small, perturbation effects of sampling on the cell generation can be minimized; thus, in fed-batch fermentations the positive value of the cultivation time derivative of fermentation volume (V) is caused by the volume increase due to continuous feding.

In equation 2.11; r_x is the cell formation rate, V is the volume of the fermentation medium, C_x is the cell concentration in the bioreactor and t is the cultivation or residence time. The cell formation rate can be expressed with a first order equation in the production domain:

$$r_x = \mu C_x \tag{2.12}$$

Thus equation (2.11) becomes;

$$\frac{d(C_x V)}{dt} = \mu C_x V \tag{2.13}$$

By separation of the variables in equation (2.13), and integration from the initial condition-1 (t=0, $C_x = C_{x0}$) to initial condition-2 (t= t, $C_x = C_x$):

$$\int_0^t \mu dt = \int_{C_{x0}}^{C_x} \frac{dC_x}{C_x} \tag{2.14}$$

Under the assumption of constant fermentation broth density, and using the continuity equation for fed-batch operation having a continuous feed inlet stream with the flow rate Q(t), the following equation is derived for the specific growth rate:

$$\mu = \frac{dC_x}{dt}\frac{1}{C_x} + \frac{Q(t)}{V} \tag{2.15}$$

For batch phase as Q(t)= 0, specific growth rate of the cells is represented in the following form:

$$\mu = \frac{dC_x}{dt}\frac{1}{C_x} \quad (2.16)$$

2.5.2. Mass Conservation Equation for the Substrate

The second group of equations required in fed-batch bioreactor operation are mass conservation equations for each continuously fed substrate with a time varying feed stream flow rate of Q(t) without withdrawal of a corresponding effluent stream, is constructed as (Çalık et al, 2016):

$$Q(t)C_s{}^o + r_s V = \frac{d(C_s V)}{dt} \quad (2.17)$$

where, $C_S{}^o$ is the constant substrate feed concentration; and the substrate consumption rate r_S, can be defined with a first order kinetic equation in the production domain:

$$-r_s = q_s C_x \quad (2.18)$$

where, q_s is the specific substrate consumption rate. By substituting Eq-2.18 into Eq-2.17, the following is derived for q_S:

$$q_s = -\frac{1}{C_x}\left(\frac{dC_s}{dt} + \frac{C_s}{V}Q(t) - \frac{Q(t)}{V}C_S{}^o\right) \quad (2.19)$$

2.5.3. Mass Conservation Equation for the Product

The third group of equations required in fed-batch bioreactor operations are mass conservation equations for the product and each by-product synthesised in the cell which can be excreted or secreted into the fermentation broth, considering their batchwise formations (Çalık *et al*., 2016):

$$r_p V = \frac{d(C_p V)}{dt} \quad (2.20)$$

The product formation rate r_p, can be defined with a first order kinetic equation:

$$r_p = q_p C_x \tag{2.21}$$

where, q_p is the specific product formation rate. By substituting Eq-2.21 into Eq-2.20, and by rearranging q_p is formulated as:

$$q_p = \frac{1}{C_x}\left(\frac{dC_p}{dt} + \frac{Q(t)}{V} C_p\right) \tag{2.22}$$

The mathematical model of a fed-batch bioreactor with a continuous feed flow rate, Q(t), the followings can be derived (Çalık *et al*., 2016). For the cell, by combining Eq-2.11 and Eq-2.12:

$$\frac{d(C_x V)}{dt} - \mu C_x V = 0 \tag{2.23}$$

For each substrate carried into fed-batch bioreactor by continuous feed stream, rearranging Eq-2.19 gives:

$$\frac{dC_s}{dt} + \frac{Q(t)}{V} C_s + q_s C_x = \frac{Q(t)}{V} C_s{}^o \tag{2.24}$$

For the product or each by-product, by rearranging Eq-2.22:

$$\frac{dC_p}{dt} + \frac{Q(t)}{V} C_p - q_s C_x = 0 \tag{2.25}$$

If cultivation time derivative of C_s is assumed to be zero, Eq-2.17 becomes:

$$Q(t) C_s{}^o + r_s V = C_s \frac{dV}{dt} \tag{2.26}$$

Using the definitions of the yield coefficient $Y_{X/S}$ and by substituting Eq-2.18 into Eq-2.19:

$$Q(t) C_s{}^o - \frac{r_x}{Y_{x/s}} V = C_s \frac{dV}{dt} \tag{2.27}$$

By substituting Eq-2.12 into Eq-2.11, after separation of the variables, integration from the initial condition-1(t = 0 $C_x = C_{x0}$) to initial condition-2 (t = t $C_x = C_x$) yields:

$$C_x V = C_{x0} V_0 e^{\mu t} \tag{2.28}$$

Therefore, by substituting Eq-2.12 and Eq-2.28 into Eq-2.27, Q(t) is formulated as:

$$Q(t) = \frac{\mu V_o C_{xo}}{Y_{x/s}(C_s{}^o - C_s)} \exp(\mu t) \tag{2.29}$$

Further, assuming $C_s{}^o >> C_s$, continuous feed stream flow rate is formulated as:

$$Q(t) = \frac{\mu V_o C_{xo}}{C_s{}^o Y_{x/s}} \exp(\mu t) \tag{2.30}$$

where, μ is the pre-determined specific growth rate, V_o is the initial fermentation volume, $C_{X,o}$ is the initial cell concentration, $C_S{}^o$ is the feed substrate concentration, and $Y_{X/S}$ is the cell yield on the substrate glucose.

2.5.4. Yield Coefficients

The cell mass and product formation, and substrate consumption can be quantitatively defined by yield coefficients in order to have a clear understanding of a bioprocess (Hong, 1988). Yield coefficients can either be calculated as overall parameters or as instantenous parameters which may vary during the fermentation.

$Y_{x/s}$ is defined as the cell mass generated per mass of the utilized substrate, it is the cell yield on substrate. $Y_{p/s}$ is described as the mass of the product formed per mass of the substrate consumed and $Y_{p/x}$ represents the mass of the product formed per cell mass generated at the same time interval. The instantenous parameters are expressed as follows:

$$Y_{P/S} = \frac{r_p}{(-r_s)} = \frac{dC_p/dt}{-dC_s/dt} \tag{2.31}$$

$$Y_{P/x} = \frac{r_p}{r_x} = \frac{dC_p/dt}{dC_x/dt} \tag{2.32}$$

$$Y_{x/s} = \frac{r_x}{(-r_s)} = \frac{dC_x/dt}{-dC_s/dt} \tag{2.33}$$

Overall yield coefficients can be described as follows between defined time intervals:

$$\bar{Y}_{x/s} = \frac{\Delta X}{-\Delta S} \tag{2.34}$$

$$\bar{Y}_{P/s} = \frac{\Delta P}{-\Delta S} \tag{2.35}$$

$$\bar{Y}_{P/x} = \frac{\Delta P}{\Delta X} \tag{2.36}$$

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals

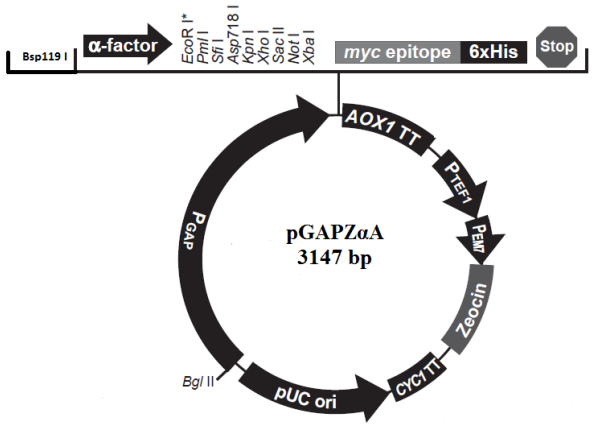
All chemicals used in this study were analytical grade and purchased from Sigma-Aldrich Co. and Merck Millipore.

3.2. Buffers and Stock Solutions

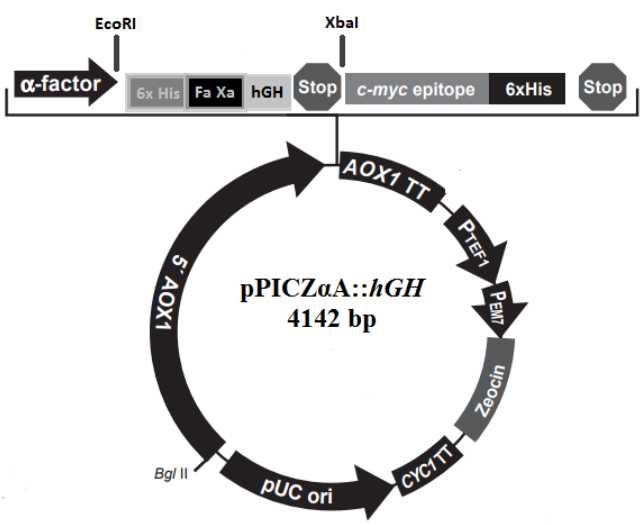
Contents and preparations of all buffer and stock solutions can be found in Appendix A. The sterilization of the solutions was achieved by autoclaving for 20 minutes at 121 °C or filtering the solution through 0.45 μm filters (Millipore Corporation Bedford, MA, USA).

3.3. Strains, Plasmids and Maintenance of Microorganisms

Escherichia coli DH5α strain was used for cloning and amplification of the constructed plasmids. *Pichia pastoris* X-33 strain was chosen as the expressing strain (Invitrogen, Carlsbad, Ca, USA). For the construction of the desired plasmid, pGAPZαA (Invitrogen) and pPICZαA::*hGH* (Çalık *et al*., 2008) plasmids were employed as parent plasmids (Massahi, 2017). Schematic representation of these plasmids can be seen in Figure 3.1.



(a)



(b)

Figure 3. 1 (a) Schematic representation of pGAPZαA **(b)** Schematic representation of pPICZαA::*hGH*

All microbial cultures were stored at -80 °C in a microbank preservation system. With this system, the strains can be stored without the risk of contamination, loss or alteration for a long time. After cultivation of the strains on agar plates, the plates can be stored at +4°C for 15 or 30 days depending on the presence of antibiotic in the plate. The loss of rDNA and contamination of the cultures may

arise resulted from the sub-culturing from one plate to another. All the plasmids were stored at -20°C.

3.4. Media

Media used for the growth of the microorganisms and the media used for the shake-flask and bioreactor experiments differ from each other in terms of their ingredients. Therefore they are explained as two separate parts in this study.

3.4.1. Growth Media

E. coli strains were grown either in LB agar or LB broth as solid and liquid medium in which LB describes Lysogeny broth. Recombinant *E. coli* pPYKZαA*::hGH* strains were grown on LB agar plates which contain 25 μg mL^{-1} zeocin and they were incubated overnight at 37°C. Same antibiotic concentration was used for the liquid medium.

P. pastoris strains were grown in yeast extract peptone dextrose (YPD) medium which can be prepared either in solid or liquid form. Recombinant *P. pastoris* pPYKZαA*::hGH* strains were cultivated on YPD agar plates including 25 μg mL^{-1} zeocin and incubated for 48-60 hours at 30°C.

All media preparations, composition details and storage conditions are listed in Appendix B.

3.4.2. rhGH Production Media

Production of rhGH was achieved by conducting experiments both in laboratory scale shake flask air-filtered bioreactors and pilot scale bioreactor. The cells were precultivated before production to obtain high amount of cell.

Production mediums differ from each other although precultivation medium is same for both production processes.

3.4.2.1. Pre-cultivation Medium

Buffered minimal glycerol-complex medium (BMGY) was used for precultivation of the cells for both shake-flask and pilot-scale bioreactor experiments. Preparation and composition details of the BMGY medium can be found in Appendix B.

3.4.2.2. Production Media

3.4.2.2.1. Production Medium Used in Shake flask Air-filtered Bioreactors

The composition and preparation of the production medium for shake flask air-filtered bioreactor experiments can be found in Appendix B. Shake-flask bioreactor fermentation medium was designed by replacing NH_4Cl with $(NH_4)_2SO_4$ by determination of its concentration based on the C/N = 4.57 ratio, reported by Jungo *et al*.(2006). Pichia Trace Salt Solution (PTM1) was also added to production medium in order to fulfill the mineral requirements of the yeast. The preparation, storage conditions and composition of PTM1 is presented in Appendix B.

3.4.2.2.2. Production Medium Used in Pilot-scale Bioreactor

In batch phase of the pilot-scale bioreactor experiments, basal salt medium (BSM) was used as the production medium. The composition and preparation of the medium is given in Appendix B. In the fed-batch phase, glucose solution with a concentration of 500 g L^{-1} containing 12 mL L^{-1} PTM1 was fed as the continuous feed stream into bioreactor.

3.5. Genetic Engineering Techniques

3.5.1. Enzymes, Kits, Molecular Size Markers

Taq DNA polymerase, protease-free RNase A, gene ruler express DNA ladder and lambda DNA/HindIII marker were purchased from ThermoFisher Scientific. Restriction enzyme *Nsi*I, dNTP Mix and 6X loading dye solution were purchased from Fermentas Life Sciences.

GeneJet PCR Purification Kit was purchased from ThermoFisher Scientific and Wizard® Genomic DNA Purification Kit was provided from Promega.

3.5.2. Plasmid Isolation From *E. coli*

Plasmid isolation from *E.coli* DH5α cells which were carrying pPYKZαA::*hGH* plasmid was performed by alkaline lysis (midipreparation) method (Sambrook *et al*., 2001). The contents of alkaline lysis solutions were given in Appendix A and the procedure was as follows:

Preparation of the cells:

1) A single colony of the transformed *E. coli* cells was inocculated of 10 mL of the LB Broth medium containing Zeocin.
2) The cells were incubated at 37°C with vigorous shaking at 200 rpm overnight, approximately 16 hours,.
3) The cells were centrifuged at 3000 g for 10 minutes at 4°C.
4) Supernatant was removed by gentle aspiration and the remainig medium in the tube was positioned properly on a clean towel paper in order to keep the cells as dry as possible.

Cell lysis:

5) The pellet in the tube was re-suspended with 200 µl ice-cold alkaline lysis I solution by efficient mixing until the cells are

dispersed completely. The medium was transferred to 2 mL microfuge tube.

6) Alkaline lysis II solution of 400 µl was added to the mixture and the tube was inverted for five times for efficient mixing. Then the tube was stored on ice.
7) 300 µl ice-cold alkaline lysis III solution was added and gently mixed by inversion then the tube was again kept on ice for 3-5 minutes.
8) Centrifugation was carried out at 14000 g at 4°C for 5 minutes and 600 µl supernatant was carefully transferred to a fresh tube in order not to disturb the cell debris during transfer.
9) Equal volumes of phenol/chloroform were added to supernatant and mixed by vortexing. Then the sample was centrifuged at 14000 g for 2 minutes at 4°C. Supernant was again transferred to a fresh tube.

Recovery of the plasmid DNA:

10) 600 µl iso-propanol was added and the solution was vortexed, the tube was kept for 2 minutes at room temperature in order to precipitate nucleic acids from the supernatant.
11) Centrifugation was carried out at 14000 g for 5 minutes at room temperature.
12) Supernatant was poured off by gentle aspiration and the tube was placed in a manner that the remaining medium drains on a clean towel paper. Any fluid droplets present in the tube wall was also removed.
13) 70% ethanol of 1 mL was added for washing the pellet and the DNA was precipitated by centrifugation for 2 minutes at 14000 g at room temperature.

14) Supernatant was poured off by gentle aspiration.
15) Remaining ethanol droplets were removed from the tube walls and the tube was held at room temperature for 10-15 minutes to let the ethanol to be evaporated without dehydrated the DNA.
16) Final pellet was dissolved in 30-100 µl TE Buffer containing RNAse at the final concentration of 20 µg/mLand the solution was gently vortexed for a few seconds and isolated plasmids were stored at -20°C.

3.5.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) was performed for control, visualization and quantification of the isolated plasmids and genomic DNA, PCR products, digested or purified DNA fragments. DNA fragments were separated of varying sizes from 100 bp to 25 kb with this method. The system which was used for the analysis was Mini-sub® Cell GT Cell system (Bio-Rad, CA, USA). The common procedure can be described as follows:

1) 5X TAE buffer was prepared and diluted to 1X before the usage.
2) Required amount of agarose was dissolved in 1X TAE buffer in order to obtain the best resolution. In this study, 0.4 g agarose was dissolved in 50 mL buffer and heated until boiling.
3) After the solution has cooled down, EtBr (Sigma-10 mg/mL) was added to reach a final concentration of 0.4 µg/mL.
4) The solution was poured off to the plastic trays which have been assembled before and the combs were placed into the gel to form the wells.
5) The gel was left for complete drying for 20 minutes and the comb was removed carefully. Then the gel was transferred to electrophoresis tank filled with 1X TAE buffer.

6) 5-10 µl of DNA sample was mixed with 0.2 volume of 6X loading dye solution and the samples were loaded to the wells on the gel. The samples can be diluted with filter-sterilized ultrapure water when necessary.
7) Appropriate DNA ladder or marker was also loaded to the well in order to make a comparison of the size of the DNA fragments.
8) The lid of the electrophoresis tank was closed and electrical leads were attached. Electrophoresis was conducted at 90V for 30-100 minutes depending on the desired DNA length and gel concentration.
9) DNA bands were screened under UV light and images can also be taken and stored by the system (Hamamatsu Digital CCD Camera).

3.5.4. Digestion of the Plasmid DNA

In order to perform transfection of the desired plasmid to *Pichia pastoris* cells, the plasmid must be linearized by restriction endonuclease enzymes. These enzymes cut out the DNA fragments in specific nucleotide sequences. In this study, single digestion was carried out by *Nsi*I restriction ezyme according to following reaction mixture:

Table 3. 1 Reaction mixture for single-digestion with *Nsi*I restriction enzyme

Component	Amount
Nuclease-free water	30 µl
*Nsi*I unique buffer 10X	5 µl
DNA	1 µg
*Nsi*I	1 µl
Total volume	**50 µl**

All components in the Table 3.1 were combined and gently mixed. Reaction tubes were incubated at 37°C for 18 hours then the enzymes were inactivated by holding the reaction mixture at 65°C for 20 minutes. The control was made by agarose gel electrophoresis method by visualizing the linearized DNA under UV light. When DNA was circular, it runs faster than the linearized form in the gel due to its compact structure thus linearized and circular forms of the same plasmid can be distinguished by looking at their scale under the UV light.

3.5.5. Purification of the Digestion Products

The DNA fragments which were subjected to digestion or polymerase chain reaction (PCR) should be purified in order to get rid of any interfering agents which may inhibit following experiments. The procedure was described as follows:

1) 1:1 volume of binding buffer was added to digestion mixture and mixed efficiently.
2) The color of the solution must be checked, a yellow color illustrates optimal pH for DNA binding.

3) Up to 800 µl of the mixture was transferred to the GeneJet purification column supplied by kit and centrifuged at 14000 g at room temperature for 30-60 seconds. The supernatant was removed.
4) 700 µl of wash buffer was added to the mixture and centrifuged again for 30-60 seconds.
5) The supernatant was discarded and empty purification column was centrifuged at 14000 g at room temperature for 1 minute to completely remove all of the wash buffer.
6) GeneJet purification column was transferred to a clean 1.5 mL microcentrifuge tube and 50 µl elution buffer/autoclaved and filter-sterilized water was added to the center of the GeneJet purification column membrane.
7) The tubes were centrifuged for 1 minute and the column was discarded.
8) The eluted DNA was stored at -20°C.

3.5.6. Transfection of the *Pichia pastoris* X-33 Cells

After all these steps, the plasmids were ready for transfection if 5-10 µg of plasmid per each transfection is acquired successfully. Transfection was accomplished by lithium chloride method (LiCl) as explained in EasySelect™ *Pichia* Expression Kit. All solutions used in this method are listed in Appendix A including their preparation and compositions. The procedure can be written as:

1) *Pichia pastoris* cell culture was cultivated in 50 mL YPD medium at 30°C and 200-225 rpm until OD_{600} has reached to 0.8-1.0 ($\sim 10^8$ cells/mL).
2) The cells were harvested by centrifugation at 4000 g for 5 minutes at +4°C.

3) The pellets were washed with 25 mL sterile distilled water by inversion then the tubes were centrifuged at room temperature at 1500 g for 10 minutes.
4) Water was discarded and the cells were re-suspended in 1 mL 100 mM LiCl solution at room temperature.
5) The suspended cells were centrifuged again at 16000 g for 15 seconds and LiCl was removed with a pipette.
6) The cells were re-suspended again in 400 µl of 100 mM LiCl solution by a pipette.
7) 50 µl of the cell suspension was transferred into a 1.5 mL microcentrifuge tube (for each transfection) and it must be immediately used without storing on ice or freezing at -20°C.
8) 1 mL of single-stranded DNA was boiled for 5 minutes and quickly placed on ice.
9) The solution was centrifuged at maximum speed for 15 seconds and LiCl was removed by using a pipette.
10) The reagents were added very slowly to solution in exact sequence given:
    - 240 µl 50% (w/v) PEG
    - 36 µl 1 M LiCl
    - 25 µl Salmon sperm (2 mg/mL)
    - Linearized plasmid DNA (5-10 µg) in 50 µl sterile water
11) The tubes were vortexed until thorough mixing (~ 1 minute)
12) The tubes were tightly sealed and incubated at 30°C for 30 minutes.
13) They were incubated at 42°C for 20-25 minutes.
14) The cells were centrifuged at 4000 g for 15 seconds and the solution was discarded.

15) The collected cells were re-suspended in 1 mL YPD medium and incubated at 30°C at 150-200 rpm for 4 hours.
16) The cells were placed on YPD plates containing 100-200 µg/mL zeocin and incubated at 30°C for 48 hours.

3.5.7. Isolation of Genomic DNA from Yeast

In order to select the positive transformants genomic DNA should be isolated according to the following method (Wizard® Genomic DNA Purification Kit):

1) The cell culture was grown for 20 hours in YPD broth and 1 mL of this culture was placed into a clean 1.5 mL microcentrifuge tube.
2) Pellets were obtained after centrifugation at 16000 g for 2 minutes and the supernatant was poured off.
3) The cells were re-suspended thoroughly in 293 µl of 50 mM EDTA.
4) 7.5 µl of 5 units/µl lyticase was added and gently pipetted 4 times to mix.
5) The sample was incubated at 37°C for 30-60 minutes to digest the cell wall and cooled down to room temperature.
6) Centrifugation was carried out at 13000-16000 g for 2 minutes, then supernatant was poured off.
7) 300 µl of nuclei lysis solution was added to the cell pellet and gently pipetted to mix.
8) 100 µl of protein precipitation solution was added and the mixture was vortexed vigorously at high speed for 20 seconds.
9) The sample was left on ice for 5 minutes.
10) Centrifuge was carried out at 13000-16000 g for 3 minutes.

11) Supernatant containing the DNA was transferred to a clean 1.5 mL micro centrifuge tube containing 300 µl of iso-propanol at room temperature.
12) The mixture was gently mixed by inversion until the thread-like strands of DNA became visible.
13) The solution was centrifuged at 13000-16000 g for 2 minutes.
14) The supernatant was poured off and the tube was placed on a clean absorbent paper. 70% ethanol was added to the pellet at room temperature and the tube was gently inverted for several times to wash the DNA pellet.
15) Centrifuge was performed again at 13000-16000 g for 2 minutes. Then all ethanol was carefully aspirated.
16) The tube was placed on a clean absorbent paper and the pellet was left for drying for 10-15 minutes.
17) 50 µl of DNA rehydration solution was added.
18) 1.5 µl of RNase solution was added to the purified DNA and the sample was vortexed for 1 second.Then it was centrifuged for 5 seconds and incubated at 37°C for 15 minutes.
19) The DNA was rehydrated by incubating it in water bath at 65°C for 1 hour. The sample should be periodically mixed by gently tapping the tube during this step. Alternatively, it can be incubated overnight at room temperature or at +4°C.
20) The DNA should be stored at 2-8°C.

3.5.8. Verification of the Genomic Integration in Isolated Genomes

PCR (polymerase chain reaction) was performed in order to verify the insertion of the desired construct in the genome of *Pichia pastoris*. The verification was conducted with a promoter-specific forward primer and a gene-specific reverse

primer. In Table 3.2, primers used in PCR can be shown with their nucleotide sequences.

Table 3. 2 The primers used in PCR for verification of the desired construct

Primer Name	Primer Nucleotide Sequence
PYRK-F	5'…TAAATGCATGAGATCTTCAGTGTGCGG…3'
***hGH*-R**	5'…AATGTCTCGACCTTGTCCATGTCCTTCCTGAA…3'

Isolated genomic DNA's of the samples were used as templates for PCR and the reaction was performed in total volume of 25 µl. A typical PCR reaction mixture components and operation parameters are given in Table 3.3 and Table 3.4.

Table 3. 3 Typical amounts of PCR components

Component	Amount
10X amplification buffer	5 µl
2 mM solution of dNTPs	5 µl
5 µM forward primer	1-2 µl (can change)
5 µM reverse primer	1-2 µl (can change)
25 mM $MgCl_2$	2-4 µl (can be optimized)
Taq DNA polymerase	1-2 U
Template DNA	Variable
dH_2O	Variable
Total volume	**50 µl**

Table 3. 4 PCR process parameters

Number of cycles	Temperature	Time
1 cycle primary denaturation	94°C	3 min.
30 cycles	94°C Variable 72°C	1 min. 1 min. variable
1 cycle final extension	72°C	10 min.

Variable constituents and parameters depend on the melting temperatures of the primers, length of the amplified DNA fragment and the type of the polymerase enzyme. The parameters can be modified according to specific requirements of the reactions. PCR experiments were performed in a thermal block cycler (Techne®, Flexigene and TC-3000X).

3.5.9. qPCR for Copy Number Determination

In this study, quantitative PCR (qPCR) was chosen as a copy number determination method. With this method quantification of the target gene is made by measuring it in parallel with a reference gene. For this purpose, a single-copy gene, *ARG4* (argininosuccinate lyase) was used as reference to determine the unknown *hGH* gene in genomic DNA sample. Thus *hGH* copy number can be calculated as follows:

$$\text{Copy number}_{hGH} = \frac{\text{Assayed copy quantity}_{hGH}}{\text{Assayed copy quantity}_{ARG4}}$$

For the analysis, two standard curves must be prepared for *ARG4* and *hGH* genes so these genes were amplified by PCR at first. The primers for preparation of standard DNA for both of the genes are listed in Table 3.5.

Table 3. 5 Primers for preparation of the standard DNA for *ARG4* and *hGH* genes

Primer Name	Primer Nucleotide Sequence
ARG4-Std-F	5'…CTTGAACATTGATGCCGAACGA…3'
ARG4-Std-R	5'…GACTCTAGCTTTTCATTCAGTGC…3'
GAP forward	5'…GTCCCTATTTCAATCAATTGAA…3'
AOX reverse	5'…GCAAATGGCATTCTGACATCC…3'

Standards for the qPCR experiment were prepared by conducting PCR experiments with the primers in Table 3.5 and PCR products were purified. Another set of primers was used for the amplification of the desired amplicon in qPCR experiment. These primers were designed to bind to a specific segment inside the prepared standard DNA and amplify it. In Table 3.6, primers used in qPCR experiment can be found.

Table 3. 6 Primers used in qPCR experiment

Primer Name	Nucleotide Sequence
*Eda-q*PCR-F	5'…GCCTTTGACACCTACCAGGA…3'
*Eda-q*PCR-R	5'…ACACCAGGCTGTTGGCGAAG…3'
ARG-F	5'…TCCATTGACTCCCGTTTTGAG…3'
ARG-R	5'…TCCTCCGGTGGCAGTTCTT…3'

For the qPCR experiment, all genomic DNA samples and prepared standards were placed in reaction tubes with specified primers. Negative controls of the samples were also prepared to control the contamination during the experiment. The mixtures were prepared by using the amounts in Table 3.7.

Table 3. 7 Reaction mixture for qPCR experiment

Component	Amount (µl)
dH_2O	6.4
SYBR Green Master 2X	10
Forward primer (5 µM)	0.8
Reverse primer (5µM)	0.8

3.6 Recombinant Human Growth Hormone Production

Recombinant hGH (rhGH) was produced both in laboratory scale shake flask bioreactor experiments and pilot scale bioreactor experiments.

3.6.1 Precultivation

For the precultivation of X-33 strain, *Pichia pastoris* X-33 cells were grown on YPD agar plates containing 25 µg mL^{-1} Zeocin for 48-60 hours after inoculation from microbanks. Then the cells were transferred to another YPD agar plate and grown there for 24 hours. Precultivation step was the same for the laboratory scale shake flask bioreactor experiments and pilot scale bioreactor experiments. Both of the experiments were carried out by using BMGY medium. The cells were inoculated into 250 mL shake-flask bioreactors (baffled erlenmayer flasks) into a working volume of 50 mL to an initial OD_{600} of 2 and cultivated for 15-18 hours at 30°C at 200 rpm. OD_{600}=16 is the required value for starting pilot scale bioreactor experiments. After reaching the desired cell concentration, the cells were harvested by centrifugation at 2000 g for 2 minutes.

3.6.2 rhGH Production in Laboratory Scale Shake Flask Bioreactors

Production step of the laboratory scale experiments were carried out in 250 mL shake bioreactors with 50 mL working volume. The cells were cultivated in production medium mentioned in section 3.4.2.2.1 for 24-48 hours at 30°C and

200 rpm. At desired hour, the samples were taken by centrifugation of culture medium at 1500 g for 10 minutes.

3.6.3 rhGH Production in Pilot Scale Bioreactor

rhGH production experiments using recombinant *P. pastoris* strains carrying single- and two copies of *hGH* gene were performed in a laboratory scale 7.0 L bioreactor (Biostat CPlus). The bioreactor is consisted of a system of working volume up to 5 L with temperature, pH, antifoam, dissolved oxygen (DO), and agitation rate (N) controls. It is equipped with a 6-blade disk impeller, integrated with external substrate pumps for fed-batch operations. Temperature control and sterilization was performed by using pressurized steam from a steam generator before loading 1.9 dm^3 production medium described in section 3.4.2.2.2. Variations in proton concentrations (pH) was measured continuously during the process by a pH probe (Hamilton, Switzerland) and was kept constant at pH_C = 5.5 ± 0.1 by addition of 25% NH_4OH solution. DO concentration (C_{DO}) was adjusted to 20% of air saturation in the batch phase; while, it was set to the constant C_{DO} value of each investigated oxygen transfer condition in fed-batch phase of experiments. DO concentration value in bioreactor was regulated by pumping air with an air compressor (Larfon Top Silent 1.5, Italy), if required oxygen enriched air was supplied when air is not sufficient to keep the DO concentration at preset C_{DO} levels. The schematic represantation of the bioreactor system is shown in Figure 3.2.

Production medium, operation parameters and phases are the same for all pilot scale bioreactor experiments except the DO concentration in the fed batch phase. In batch-fermentation phase, bioreactor operation conditions were set as T = 30°C, pH_C = 5.5, C_{DO} = 20 % , N = 600 rpm in all experiments. In fed-batch fermentation phase, bioreactor operation conditions were set as T = 30°C, pH_C = 5.5, N = 700 rpm. In the first part of the thesis, rhGH production with the engineered *P. pastoris*

strains designed and constructed with P_{PYK} carrying single-copy *hGH* gene at three oxygen transfer conditions at constant DO concentrations of C_{DO} = 1%, C_{DO} = 5%, C_{DO} = 15% were investigated to determine superior bioreactor operation condition.

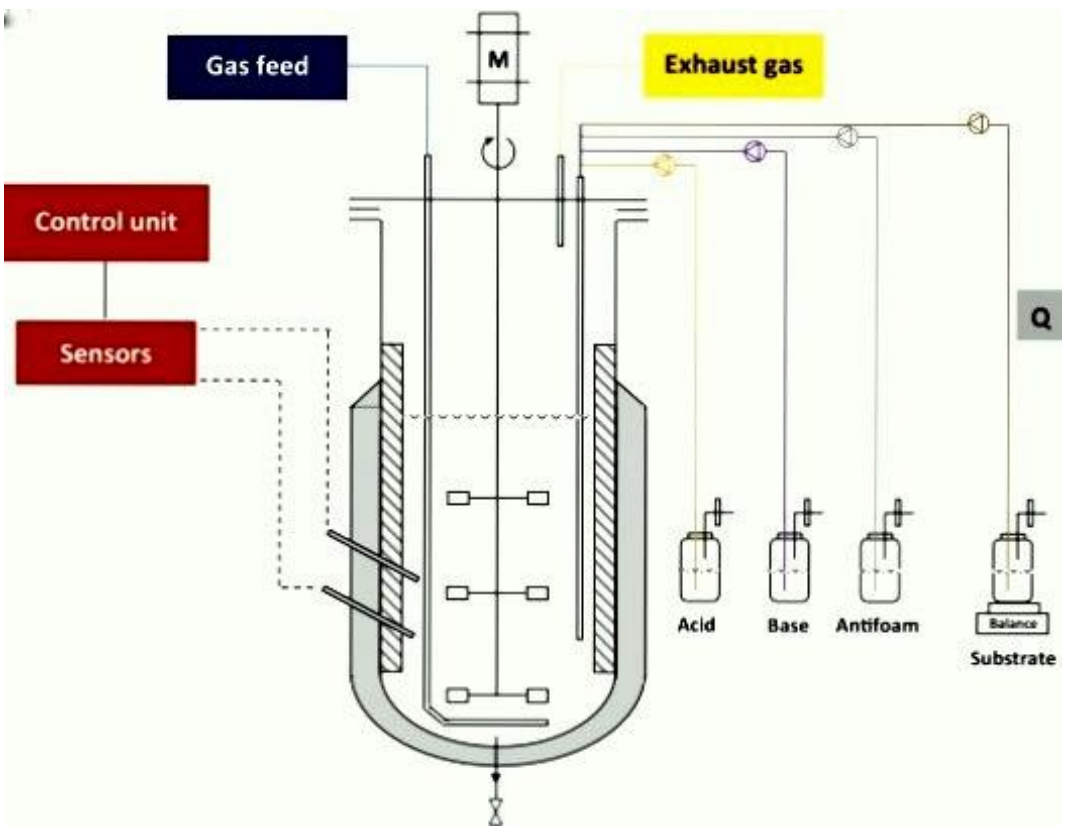


Figure 3. 2 Scheme of the pilot scale bioreactor system (Öztürk *et al*., 2016)

In fed-batch fermentation phase, glucose solution was fed to the bioreactor exponentially according to a pre-determined specific growth rate of μ = 0.1 h^{-1}. The volumetric feed flow rate of the glucose solution was calculated by using equation (2.23), derived in section 2.5 in Chapter 2.

$$Q(t) = \frac{\mu C_{x0}.V_0.e^{\mu t}}{C_s{}^{o}Y_{x/s}} \qquad (2.23)$$

Where Q(t) is the substrate feed rate (L h^{-1}), μ is the pre-determined specific growth rate (h^{-1}), C_{x0} is the initial cell concentration (g L^{-1}), C_S^o is the initial substrate concentration (g L^{-1}), $Y_{x/s}$ is the cell yield on substrate (g g^{-1}) and t is defined as the cultivation time (h). The values of the parameters: $Y_{x/s}$ =0.47 g g^{-1}, C_S^o = 500 g L^{-1}, V_0 = 1.9 L and C_{x0} = 24 g L^{-1}. The samples were removed from bioreactor at every 3 hours during the fed-batch phase, and stored at -80°C in the forms of pellets, medium, supernatants and filtrates. To prepare pellets, supernatants and filtrates; samples were centrifuged at 4°C and 1500 g for 10 minutes. Filtrates were collected by filtrating supernatant with a 0.45 μm porous filter (Sartorius, AG).

3.7 Analyses

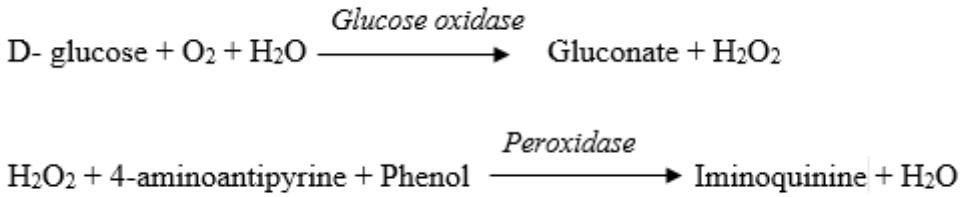
3.7.1 Cell Concentration

Cell concentrations were measured by using a UV-Vis Spectrophotometer (Thermo Spectronic, Hελios α) at 600 nm. The acceptable range for the measurement of cell concentration was selected as 100-800 nm. The samples were diluted with distilled water in some cases to achieve an absorbance value within the range. The concentration of the cells (g L^{-1}) were calculated by usign the following equation:

$$C_x = 0.275 \times OD_{600} \times \textit{Dilution Ratio} \qquad (3.2)$$

3.7.2 Glucose Concentration

Glucose analysis kit (Biasis, Ankara) was used to measure glucose concentrations. The analysis was based on the glucose oxidation method which follows the reactions below:



First, D-glucose is oxidized to gluconate and liberates H_2O_2 with catalysis of the enzyme *glucose oxidase*. Then this H_2O_2 molecule reacts with phenol and 4-amionantipyrine to give an iminoquinine dye which is proportional to glucose concentration. The color change is observed spectrophotometrically at 505 nm and glucose concentrations were calculated from these absorbance values.

3.7.3 rhGH Concentration

To measure the extracellular rhGH concentration in the supernatants SDS-PAGE analysis was performed. Silver staining method was applied for the samples taken from laboratory scale shake flask experiments while Comassie blue dye was used for the samples taken from pilot scale bioreactor experiments. The reason for this difference in the analysis methods is that silver staining method has higher sensitivity and generally is used for the determination of protein concentrations at low amounts while Comassie blue method is applied for the detection of higher concentrations. For both methods, the procedure is the same until the staining of the gels.

SDS-PAGE Gel Preparation:

1) The gel drying apparatus, the glasses and the comb were cleaned and the glasses were assembled in accordance with the gel preparation kit.
2) In 50 mL falcon tubes, the Resolver and the Stacker solutions were prepared by following the instructions in the gel preparation kit. 10 (w/v) Ammonium persulfate (APS) and NNN'N'-

Tetramethylethylenediamine (TEMED) were added to these solutions at given amounts in the gel preparation kit.

3) The Resolver solution was poured off between the glasses by using a pipette up to the green line in the apparatus and the Stacker solution was loaded onto it up to the glass border.
4) The comb was placed between the glasses carefully and the gels were left for drying for about 45 minutes under the fume cupboard.
5) After 45 minutes, the comb was withdrawn and the wells were formed.

SDS-PAGE Gel Electrophoresis:

1) The gels were assembled to the gel electrophoresis device and 1X Running Buffer was loaded up to the surface.
2) 13 µl of the samples were mixed with 5 µl 4X Loading Dye and 2 µl of 0.1 M Ditiotreitol (DTT) solution. If necessary, the samples were diluted with distilled water before the mixing.
3) The prepared samples were incubated at 95°C for 5 minutes.
4) 15 µl of the samples and 15 µl of the rhGH standard were loaded into the wells in order.
5) 3 µl Protein Ladder can be loaded into one of the wells. (Optional)
6) Running Buffer was added up to the reference line in the gel electrophoresis tank.
7) The electrodes were placed and the gels were run at 200V for approximately 50 minutes.

SDS-PAGE Staining:

Silver Staining Method:

After the electrophoresis, the gels were discarded from the apparatus and transferred from one solution to another in the following order and for given periods:

- Fixer Solution – (1-16 hours)
- 50% Ethanol Solution- (3x20 minutes)
- dH_2O- (3x20 seconds)
- Pre-treatment Solution- (1 minute)
- dH_2O- (3x20 seconds)
- Silver nitrate Solution- (20 minutes)
- dH_2O- (3x20 seconds)
- Developing Solution-(approximately 5 minutes)
- Stop Solution (for storage)

Comassie Blue Method:

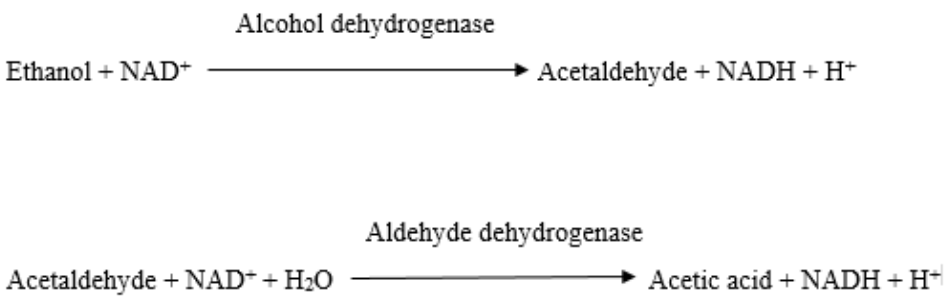
After the electrophoresis, the gels were discarded from the apparatus and transferred from one solution to another in the following order and for given periods:

- Fixing Solution- (1 hour)
- Comassie Blue Dye- (1 hour)
- Destain Solution I- (It should be changed periodically until the gel background is clear.)
- Destain Solution II- (for storage)

All staining procedures were applied at room temperature in covered plastic trays with slowly shaking. The compositions of the solutions can be found in Appendix A.

3.7.4 Ethanol Concentration

Extracellular ethanol concentration in the fermentation broth was measured by using an ethanol analysis kit (Megazyme, Ireland). The ethanol measurement was done spectrophotometrically and based on the following two reactions:



The NADH amount produced at the end of these reactions is directly propotional to twice the amount of ethanol because of the stoichiometry. NADH was measured by the absorbance values at 340 nm and the concentration of ethanol was calculated by applying the formula of:

$$C = \frac{\text{V x MW}}{\varepsilon \text{ x d x v x 2}} \text{ x } \Delta A_{ethanol} \quad (3.1)$$

$$\Delta A_{ethanol} = (A_2 - A_1)_{Sample} - (A_2 - A_1)_{Blank} \quad (3.2)$$

where; V, final volume [mL]; MW, molecular weight of ethanol [g/mol]; Ɛ, extinction coefficient of NADH at 340 nm [L mol^{-1} cm^{-1}]; d, light path [cm]; v, sample volume ; [mL]; ($A_2 - A_1$), absorbance difference.

3.7.5 Organic Acid Concentrations

Organic acid concentrations were measured by using reverse phase high pressure liquid chromatography (Waters HPLC, Alliance 2695, Milford, MA) with Capital Optimal ODS- 5 µm column (Capital HPLC, West Lothian, UK). 3.12% (w/v) NaH_2 PO_4 and $0.62x10^{-3}$ % (v/v) H_3PO_4 composed the mobile phase for the analysis. The mobile phase was filtered by using 45 µm filters (ACRODISC CR PTFE) just before the experiment. The filtrates taken from the pilot scale bioreactor experiments were used as samples for this analysis and they were diluted with distilled water when necessary. The samples were loaded into the system by filling the vials with 200 µl of the samples. Before the experiment, all buffers and solutions should be degassed in ultrasonic water bath for 15 minutes. The operating conditions of the system are shown in Table 3.8.

Table 3. 8 HPLC operation conditions

Column	Capital Optimal ODS, 5 µm
Column Dimensions	4.6 x 250 mm
Mobile Phase	3.12% (w/v) NaH_2 PO_4 and $0.62x10^{-3}$ % (v/v) H_3PO_4
Mobile Phase Flow Rate	0.8 mL min^{-1}
Column Temperature	30°C
Detector and Wavelenght	Waters 2487 Dual absorbance detector, 210 nm
Injection Volume	5 µl
Analysis Period	15 min
Space Time	5 min

The concentrations of organic acids were calculated from the areas under the corresponding peaks in the chromatogram. The area values were then converted to concentrations by using standard curves. All standard calibration curves for the measured organic acids are presented in Appendix C.

CHAPTER 4

RESULTS AND DISCUSSION

The objectives of the thesis are investigation of, ***i)*** effects of oxygen transfer conditions on recombinant protein production in *Pichia pastoris* strains designed with novel naturally occuring pyruvate kinase (PYK) promoter (P_{PYK}) which is a potential promoter for recombinant protein production (Massahi and Çalık, 2018), and ***ii)*** influences of engineering with single- and multi- copy genes, in fed-batch fermentation processes. Production of recombinant human growth hormone (rhGH) by *P. pastoris* strains under P_{PYK} carrying single- and multi- copies of *hGH* gene were carried out to determine the effect of gene copy number. For the characterization of the performances of the engineered *P. pastoris* strains, effects of the oxygen transfer conditions were studied at three oxygen transfer conditions within a range from low to moderate oxygen transfer conditions at constant dissolved oxygen concentrations (C_{DO}) of C_{DO}= 1%, 5%, and 15%. Based on the results with single-copy *P. pastoris* strain, a pilot-scale experiment was performed with the two-copy *P. pastoris* strain, at the superior oxygen transfer condition in fed-batch bioreactor operation. The cell, glucose, rhGH, ethanol, organic acid concentrations were determined together with the fermentation characteristics and yield coefficients to determine the efficiency of each oxygen transfer condition.

For in-depth analysis of the oxygen transfer characteristics in the pilot scale fed-batch bioreactor experiment program, the physical mass transfer coefficients, K_La_0, was calculated during the course of the processes by using the dynamic method (Bandyopadhyay et al., 1967), to be used as a part of an extensive oxygen transfer

analysis in *P. pastoris* fermentations in order to make a reassesment of the findings reported in the literature. Moreover, in the last part of the thesis, two laboratory scale shake flask batch bioreactor experiments were carried out to determine the effects of peptone as nitrogen source, and phosphate as additional phosphorus source on the rhGH production. Furthermore, another set of laboratory scale shake-flask bioreactor experiments were performed by the addition of amino acids into media in different concentrations to test the impact on the r-protein production.

4.1 Oxygen Transfer Effects in Fed-Batch Phase of rhGH Fermentations

The pilot-scale fed-batch bioreactor experiments were conducted at constant DO concentrations by recombinant *P. pastoris* strain carrying single copy *hGH* gene under P_{PYK} . The second-phase of fermentations under the fed-batch bioreactor operation conditions were carried out at constant DO concentrations of 1%, 5%, and the third one at 15% air saturation in order to compare with the data under P_{GAP} (Güneş and Çalık, 2015).

As the r-protein is synthesised and secreted from *P. pastoris* cells which act as the micro-bioreactors, the cell concentration need to be increased which is achieved by a two-phase fermentation, where gycerol batch-phase is the initial phase of the fermentation process, carried out to increase *P. pastoris* cell concentration and then shifted to the fed-batch phase. Even though *P. pastoris* is able to grow on many different carbon and energy sources, the choice of the carbon source depends on the promoter (Looser *et al*., 2015). Therefore, as P_{PYK} is a promoter around the branch-point pyruvate in the glycolysis pathway, glucose was selected as the carbon source to synthesise rhGH. Hence, glycerol batch phase was the first phase of the fermentation process with initial glycerol concentration of 40 g L^{-1}; and then shifted to fed-batch phase when glycerol was depleted in the bioreactor broth, by feeding 500 g L^{-1} glucose containing 12 mL L^{-1} PTM1 (Massahi *et al*., 2018).

The pilot-scale bioreactor experiments performed in 5 L pilot-scale bioreactor carried out at T=30°C, pH_C=5.5, N=700 min^{-1} while the working volume varied within the range of 1.9-3.7 L, due to substrate solution feeding in the fed-batch phase of the fermentations. Q_0/V_R was dynamically changed to keep the DO concentration constant at the preset value. Oxygen enriched air was supplied to control the C_{DO} at the preset level and, to preserve the hydrodynamic regime of the gas (air)- liquid-intracellular/enzyme catalytic three-phase mixed reaction system if aeration became insufficient because of the requirement to high oxygen transfer rates. 25% NH_4OH solution was used to maintain pH constant at pH_C= 5.5. The pre-determined specific growth rate μ = 0.1 h^{-1} was used for the calculation of the continuous feed flow rate Q(t) by using equation (2.23).

4.1.1 Effects of Oxygen Transfer Conditions on the Cell Growth

The variations in the cell concentration with the fed-batch cultivation time are presented in Figure 4.1. When the highest cell concentrations are considered, nearly the same values were obtained at C_{DO} = 5% and C_{DO} = 15% and almost followed the same loci until t=15 h. After t=15 h, a sharp increase in the cell growth of C_{DO} = 15% is observed, and gave the maximum at t=18 h as 115 g L^{-1}; thereafter, decreased after t = 18 h either because of the cell lysis or flocculation of the cells. At C_{DO} = 5%, a smooth exponential increase in the cell growth was experienced, and reached to C_X = 111 g L^{-1} at t = 21 h. The lowest cell concentration was obtained at the oxygen transfer condition set to C_{DO} = 1% as 53 g L^{-1} at t=12 h.

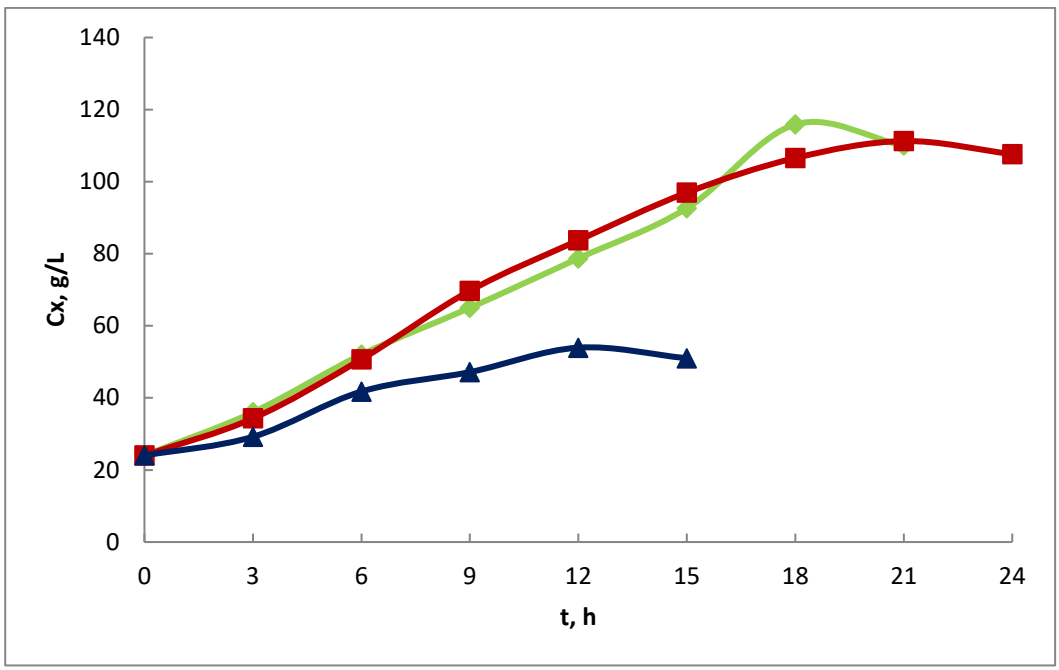


Figure 4. 1.Variations in the cell concentrations with the cultivation time in fed-batch bioreactor experiments; C_{X0}= 24 g L^{-1}, T = 30°C, pH_C = 5.5. C_{DO} = 1% (▲), C_{DO} = 5% (■), C_{DO} = 15% (♦)

Therefore, the oxygen transfer condition at C_{DO} = 1% is an insufficient condition for *P. pastoris* cell growth. Since oxygen availability is a crucial factor in eukaryotic cells, low oxygen transfer conditions can cause important metabolic, functional and structural changes in the cell that affect the cell survival and cause energy deprivation and affect the core metabolism (Adelantado *et al*., 2017).

4.1.2 Effects of Oxygen Transfer Conditions on rhGH Production

The variations in the rhGH concentrations with the cultivation time in fed-batch bioreactor experiments are presented in Figure 4.2. Since P_{PYK} is a constitutive promoter, rhGH concentration is expected to increase in parallel with the cell growth. As can be seen in Figure 4.2, the highest rhGH concentration was obtained as 28.54 mg L^{-1} at t = 21 h at C_{DO} = 5%.

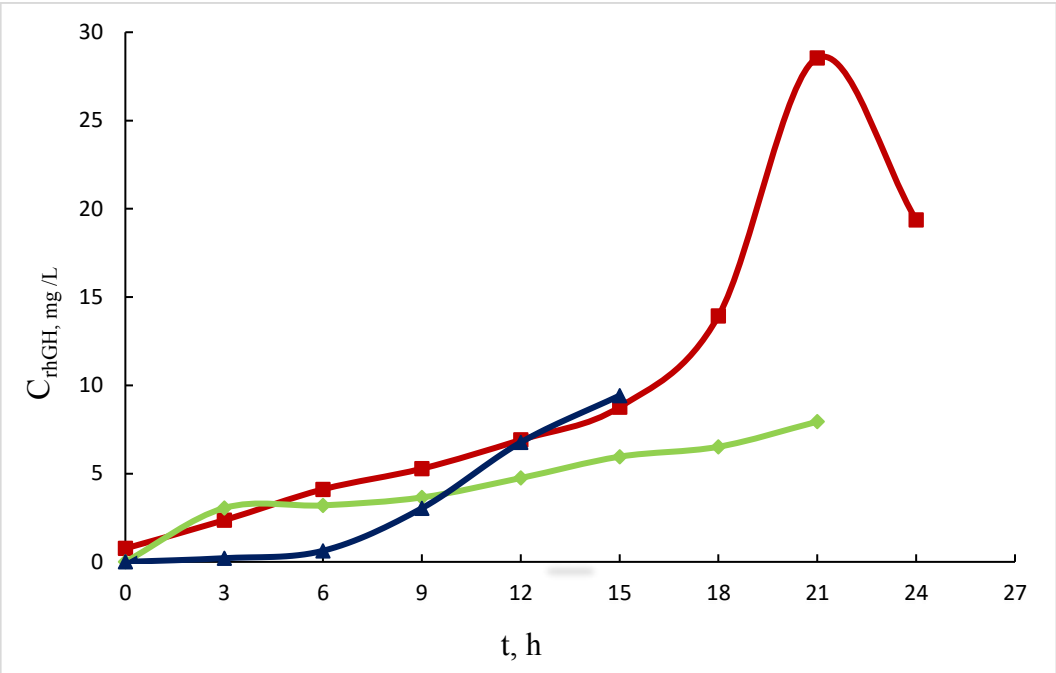


Figure 4. 2 Variations in rhGH concentrations with the cultivation time in fed-batch bioreactor experiments; C_{X0}= 24 g L^{-1}, T = 30°C, pH_C = 5.5. C_{DO} = 1% (▲), C_{DO} = 5% (■), C_{DO} = 15% (♦)

The maximum rhGH production achieved at C_{DO} = 5% oxygen transfer condition is 3- fold and 3.6- fold, respectively, higher than produced at C_{DO} = 1% and C_{DO} = 15%. The decrease in the rhGH concentration after the maxima is because of the protease synthesis in the cell. Since ca. 3- fold lower rhGH productions were determined as 9.42 mg L-1 and 7.94 mg L^{-1}, respectively, at C_{DO} = 1% and C_{DO} = 15% oxygen transfer conditions, the former condition which represents low oxygen transfer condition and the latter which represent high oxygen transfer condition, are not appropriate *P. pastoris* fermentation conditions, where the glycerol batch- phase was shifted to glucose fed-batch phase at C_{X0}= 24 g L^{-1} high cell density *P. pastoris* concentration. At C_{DO} = 15% the OTR was high enough for the cells to grow efficiently however r-protein production was at lower levels compared to C_{DO} =1% and C_{DO} = 5% conditions. Although C_{DO} = 5% condition

gave the highest r-protein concentration in this study, a two-phase strategy could be applied after investigating the oxygen transfer conditions within the range of C_{DO} = 1% to C_{DO} = 6% in order to increase the product yields further.

In our research group recently, the maximum rhGH production under the control of P_{PYK} was reported as 101 mg L^{-1} (Massahi., 2017); however, DO concentration in the fermentation broth could not be kept constant at the preset levels within 1% < C_{DO} < 4%; and the extracellular rhGH production was analyzed by SDS-PAGE analysis with silver staining. Meanwhile, due to the low reproducibility of silver staining method, classical Coomassie dye method has been evaluated as more reliable and replaced in the SDS-PAGE analysis. Since the light sensitivity of silver staining is higher than the classical Coomassie staining, a drastic difference between the rhGH concentrations could be observed.

4.1.3 Effects of Oxygen Transfer Conditions on Substrate Consumption

The formation rate of the cells mainly depends on substrate concentration. Substrate accumulation in the broth should be avoided since it can cause inhibition on the cell. In this context, the design of the continuous feed stream for glucose feeding is important in design of fed-batch bioreactor operations for enhancing the r-protein production by increasing the production capacity of the cells. Thus, the variations in glucose concentration with respect to cultivation time in fed-batch bioreactor experiments are presented in Figure 4.3.

Glucose accumulation in the bioreactor was found at negligible levels at all the oxygen transfer conditions with the highest value of 0.066 g L^{-1} at C_{DO} = 15%. Indeed interesting, the loci of the glucose consumption profiles are similar for the oxygen transfer conditions at C_{DO} = 5% and C_{DO} = 15%. There was no glucose accumulation: ***i)*** at C_{DO} = 1%, until t= 3 h; whereas, ***ii)*** at C_{DO} = 5%, until, t= 9 h; and, ***iii)*** at C_{DO} = 15%, also until t= 9 h. The glucose accumulation and consumption rates alternating after t= 9 h, was lower at C_{DO} = 5% condition than

that of the C_{DO} = 15%; nevertheless, related with the 1% condition, the cells utilized the substrate neither for the cell formation nor the rhGH synthesis and secretion.

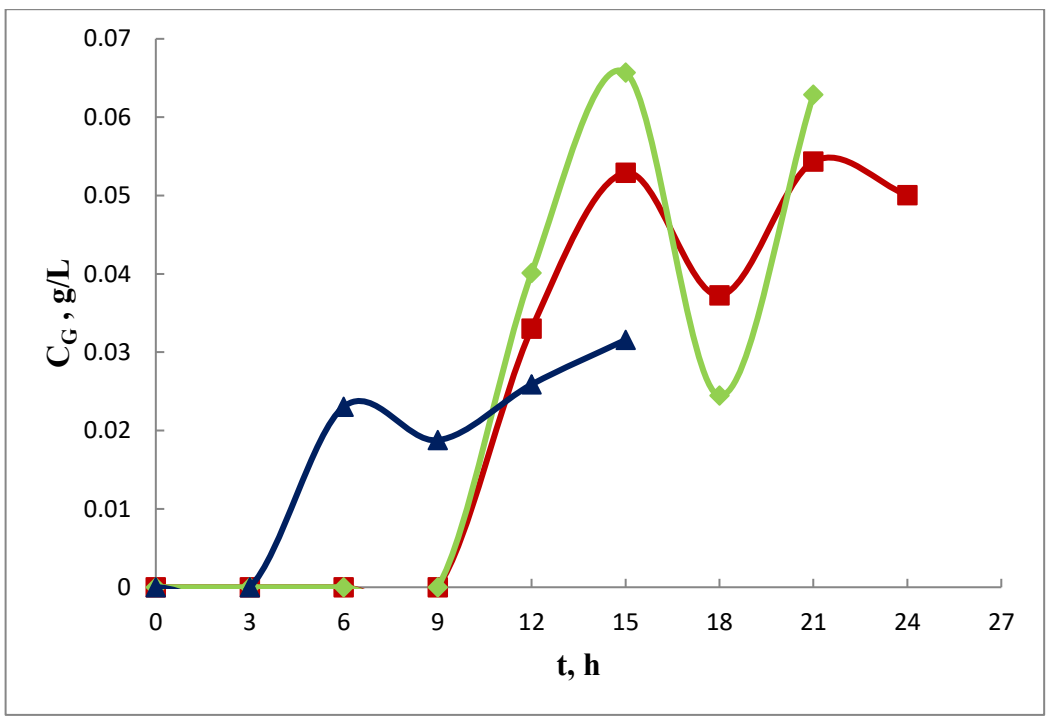


Figure 4. 3 Variations in glucose concentrations with the cultivation time in fed-batch bioreactor experiments; C_{X0}= 24 g L^{-1}, T = 30°C, pH_C = 5.5. C_{DO} = 1% (▲), C_{DO} = 5% (■), C_{DO} = 15% (♦)

Despite the low amounts of glucose accumulated in the fermentation broth, specific growth rate of the cells could not be kept at preset values and decreased after certain amount of time. The reason of this might be the accumulated ethanol in the fermentation broth which is an end-product of the fermentative pathway and inhibits the cell growth above certain limits.

4.1.4 Effects of Oxygen Transfer Conditions on Specific Rates and Yield Coefficients

In order to determine fermentation process characteristics, the specific growth rate (μ), specific substrate utilization rate (q_s), specific product formation rate (q_p) and yield coefficients including cell yield on substrate ($Y_{X/S}$), product yield on substrate ($Y_{P/S}$) and product yield on cell ($Y_{P/X}$) are needed. Thus, all these components were calculated for three fed-batch bioreactor experiments and presented in Table 4.1.

Table 4. 1 Variations in the fermentation characteristics with the cultivation time and oxygen transfer conditions in fed-batch bioreactor experiments

Oxygen Transfer Condition, at	t h	μ h^{-1}	q_s $g\ g^{-1}\ h^{-1}$	q_p $mg\ g^{-1}\ h^{-1}$	$Y_{x/s}$ $g\ g^{-1}$	$Y_{p/s}$ $mg\ g^{-1}$	$Y_{p/x}$ $mg\ g^{-1}$
C_{DO} = 1%	3	0.128	0.183	0.004	-	-	0.025
	6	0.088	0.193	0.012	0.383	0.012	0.031
	9	0.064	0.219	0.023	0.288	0.093	0.321
	12	0.038	0.241	0.023	0.298	0.118	0.397
	t_{max}=15	0.008	0.269	0.022	0.036	0.078	2.138
C_{DO} = 5%	3	0.140	0.156	0.017	-	-	0.150
	6	0.132	0.159	0.011	0.496	0.051	0.104
	9	0.100	0.148	0.008	0.822	0.053	0.064
	12	0.080	0.155	0.009	0.561	0.060	0.106
	15	0.072	0.166	0.015	0.490	0.059	0.121
	18	0.062	0.184	0.036	0.386	0.121	0.313
	t_{max}=21	0.048	0.209	0.020	0.292	0.274	0.936
	24	0.035	0.214	-	0.170	-	-
C_{DO} = 15%	3	0.140	0.149	0.016	-	-	0.244
	6	0.109	0.155	0.003	0.487	0.008	0.016
	9	0.089	0.159	0.005	0.597	0.023	0.039
	12	0.085	0.165	0.006	0.544	0.040	0.074
	15	0.099	0.174	0.005	0.496	0.039	0.078
	18	0.064	0.169	0.005	0.649	0.023	0.036
	t_{max}=21	0.022	0.180	0.007	0.133	0.037	0.278

In fed-batch phase of bioreactor experiments, the calculated specific growth rate values (μ) were calculated in order to evaluate the performance of the designed fed-batch bioreactor operation with with the pre-determined specific growth rate μ = 0.1 h^{-1}. The highest specific growth rates were calculated as 0.128 h^{-1} at C_{DO} =1%, and 0.140 h^{-1} at C_{DO}=5% and C_{DO} = 15% after shifting the bioprocess to fed-batch initially. Thereafter, the observed μ values decreased with the cultivation time, except the value of 0.099 at t=15-18 at C_{DO} = 15%.

In general, q_s values were increased throughout the bioprocess since accumulation of glucose was negligible. The highest values at C_{DO} = 1%, C_{DO} = 5%, C_{DO} = 15% were calculated as 0.269, 0.214 and 0.180 g g^{-1} h^{-1}, after the t_{max} where the maximum r-protein synthesis and secretion were performed; meanwhile the highest values for q_p were calculated as 0.023, 0.036 and 0.016 mg g^{-1} h^{-1} respectively.

The maximum$Y_{x/s}$ values were calculated as 0.383, 0.822 and 0.649 g g^{-1} at C_{DO} = 1%, C_{DO} = 5% and C_{DO} = 15 % respectively. The values are close to each other for C_{DO} = 5% and C_{DO} = 15% condition since cell growth follow similar loci during the bioprocesses.

The overall cell yields on total substrate ($\bar{Y}_{x/s}$) were calculated as 0.41, 0.48, and 0.62; and the overall product yields on total substrate ($\bar{Y}_{p/s}$) were found as 0.08, 0.14, and 0.04 at C_{DO} = 1%, C_{DO} = 5% and C_{DO} = 15%, respectively.

4.2 Development of Multi-Copy *Pichia pastoris* X-33 Strain

The multi copy strain was developed mainly in two steps. In the first step, the wild type *Pichia pastoris* was transfected with the plasmid pPYKZαA*::hGH* and transformants were selected. Thereafter, positive colony selection was conducted by PCR experiments and verified in laboratory scale air filtered shake flask

bioreactor experiments. The detailed summary of the construction of the multi copy strains are presented in Figure 4.4.

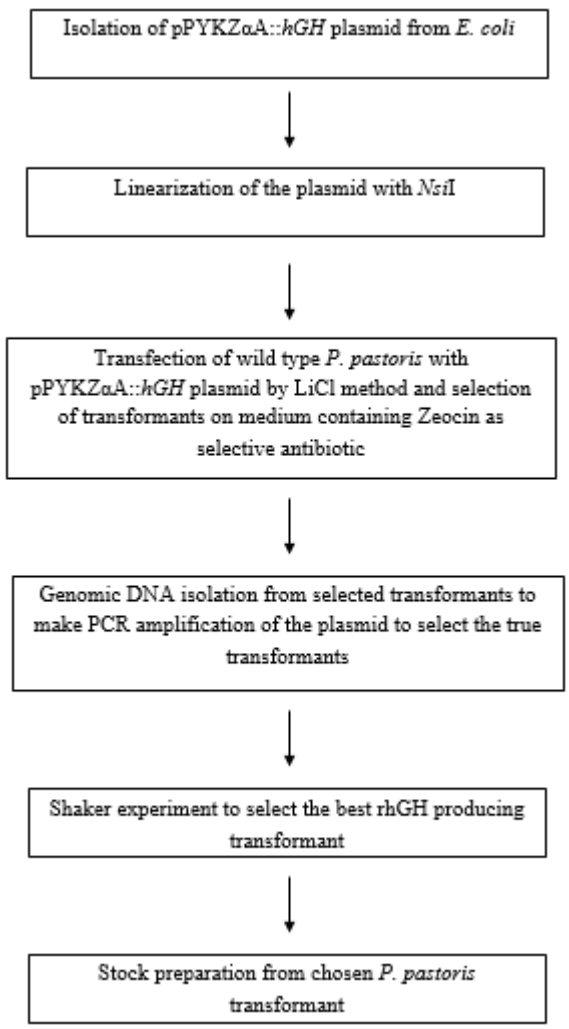


Figure 4. 4 Construction steps of the multi copy *Pichia pastoris* strain

4.2.1 Plasmid Isolation from *E. coli*

Isolation of the pPYKZαA::*hGH* plasmid was carried out by alkaline lysis method for ten samples and agarose gel electrophoresis results of the process is presented in Figure 4.5. Eight plasmids were isolated successfully. The plasmids were observed within 3000-2000 base pair (bp) range although the size of the constructed plasmid was expected as 3600 bp. Since the plasmids are circular, they run faster than the linearized forms because of their compact structure.

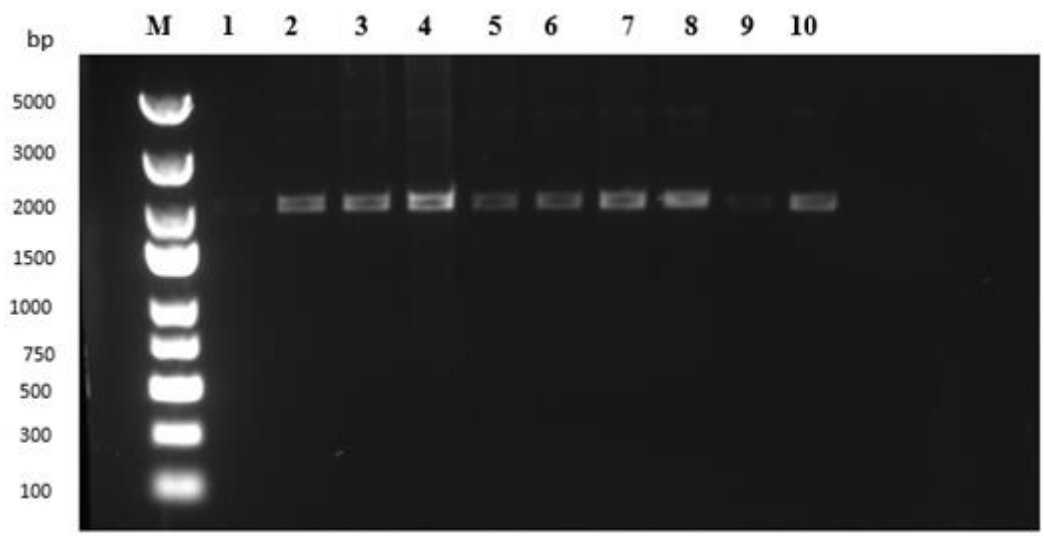


Figure 4. 5 Agarose gel electrophoresis of isolated pPYKZαA::*hGH* plasmid. M:GeneRuler Express DNA Ladder; Lane 1-10: circular pPYKZαA::*hGH*

4.2.2 Linearization of the Plasmid DNA

For genomic integration of pPYKZαA::*hGH* plasmid, the constructed plasmid was linearized by single digestion from its origin. The digestion was confirmed by agarose gel electrophoresis of the products. Since plasmids were linear, they were detected at their exact bp which is 3600 as shown in the Figure 4.6.

After single digestion, the products were combined and purified in order to remove the residual components from digestion rection. PCR purification kit was used and the purified products were visualized under UV-light after agarose gel

electrophoresis, Figure 4.7. Concentrations of the products were determined for transfection.

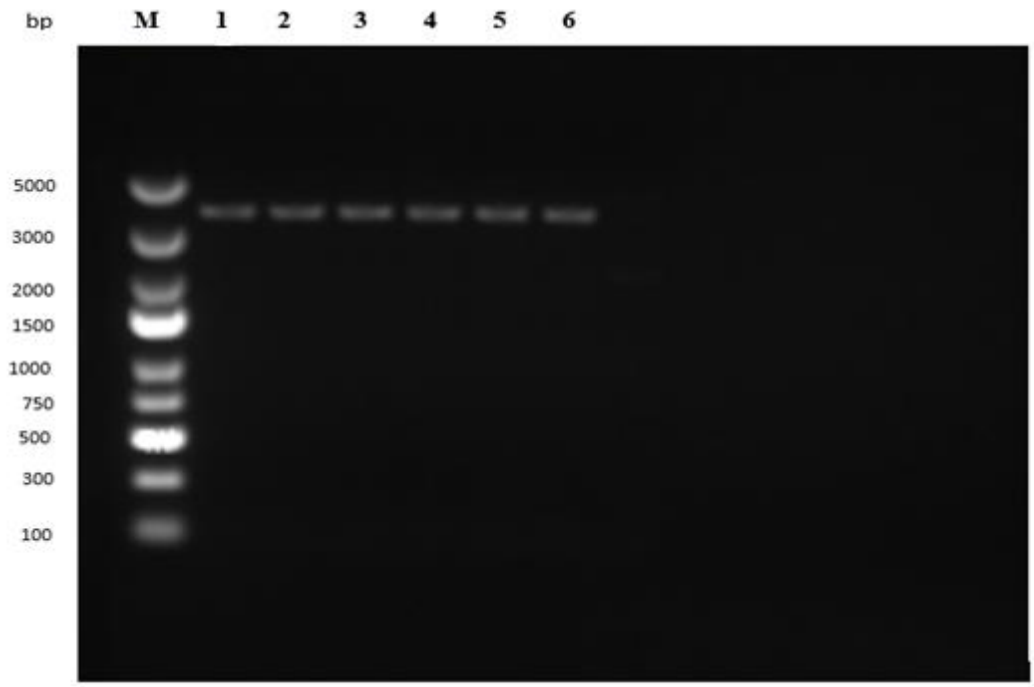


Figure 4. 6 Agarose gel electrophoresis of linearized pPYKZαA::*hGH* plasmid. M:GeneRuler Express DNA Ladder; Lane 1-6: linear pPYKZαA::*hGH*

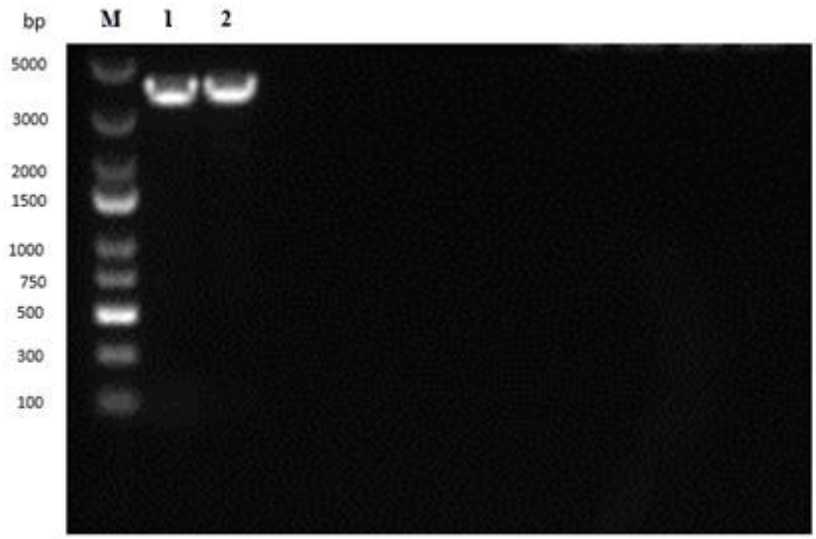


Figure 4. 7 Agarose gel electrophoresis screening of purified products.
M:GeneRuler Express DNA Ladder; Lane 1-2: purified products

4.2.3 Transfection of the *Pichia pastoris* X-33 Cells

Transfection was achieved by the method explained in section 3.5.6. After transfection, some of the cells were grown in YPD agar plates containing 200 µg mL^{-1} Zeocin while some of them were grown in plates containing 100 µg mL^{-1} Zeocin. Since the level of Zeocin resistance roughly correlates with vector copy number (Scorer *et al*., 1994), the colonies grown in higher Zeocin concentration were believed to have high copy numbers. 48 hours later from incubation, six single colonies were selected from YPD plates containing 200 µg mL^{-1} Zeocin and another six single colonies were chosen from YPD plates containing 100 µg mL^{-1} Zeocin. These cells were again inoculated into agar plates containing Zeocin for further control and short-term storage.

4.2.4 Genomic DNA Isolation and PCR Verification

Genomic DNA isolation of all selected colonies was carried out by the method in section 3.5.7. The agarose gel electrophoresis results of six of them are shown in

Figure 4.8. Since molecular weight of the genomic DNA is very high, the bands are above the detectable range.

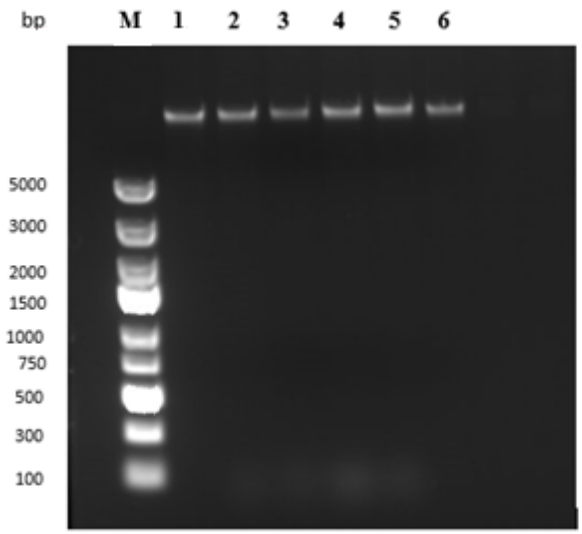


Figure 4. 8 Agarose gel electrophoresis results of genomic DNA isolation of the selected colonies. M:GeneRuler Express DNA Ladder; Lane 1-6: Genomes of the selected colonies

Isolated genomic DNAs of the samples were used as templates for PCR. PCR was accomplished by using PYK forward (PYK-F) and *hGH* reverse (*hGH*-R) primers. The constructed plasmid, pPYKZαA::*hGH*, was used as positive control and water was used as template for negative control in the PCR experiment. At the end of the PCR experiments, the *hGH* gene and PYK promoter were amplified in twelve colonies at 1241 bp as can be seen in Figure 4.9 and Figure 4.10.

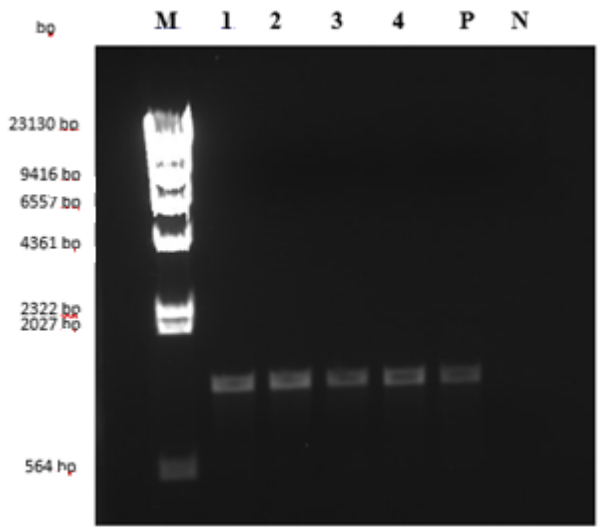


Figure 4. 9 Agarose gel electrophoresis results of PCR products. M: λDNA/HindIII; P: Positive control; N: Negative control; Lane 1-4: PCR products

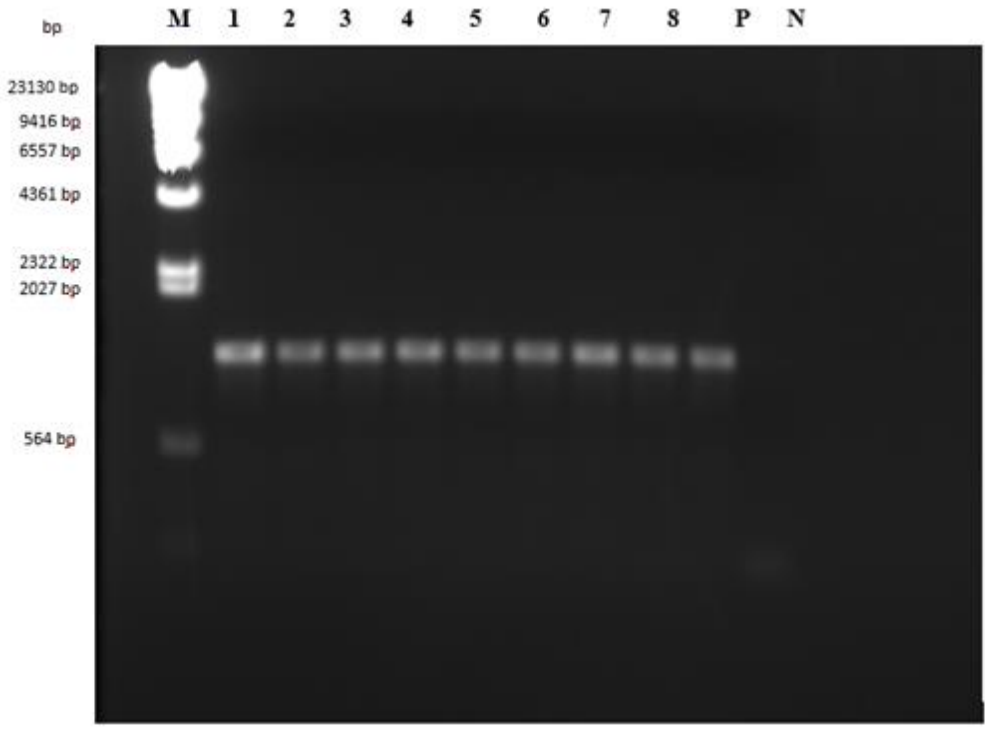


Figure 4. 10 Agarose gel electrophoresis results of PCR products.M: λDNA/HindIII; P: Positive control; N: Negative control; Lane 1-8: PCR products

4.2.5 Expression of Recombinant Human Growth Hormone by Multi-copy Strain

Although P_{PYK} and *hGH* gene were amplified successfully for twelve transformants, the colonies grown in higher Zeocin concentration were chosen for laboratory scale shake flask bioreactor experiments. It was expected that high copy numbers give high r-protein expression levels thus these colonies were primarily selected and their expression levels were compared.

The cells were cultivated in 50 mL precultivation medium in air filtered shake flask bioreactors at 30°C and 200 rpm. When initial OD_{600} of 2 was reached, the cells were transferred into 50 mL production medium and allowed to grow at 30°C and 200 rpm. The samples were removed at 21st, 24th and 27th hours during the

production phase and were harvested by the centrifugation at 1500 g for 10 minutes at 4°C; and, ca. 1 mL of supernatants were stored at -80°C for SDS-PAGE analysis.

SDS-PAGE analysis was carried out with the supernatants of t = 24 h to verify the expression of rhGH by its molecular weight and the results are presented in Figure 4.11. rhGH concentrations of the colonies 2,8,11,13,12 and 15 were compared. Colony 8 produced higher amounts of rhGH compared with the other colonies; thus, was chosen as the best transformant and stock preparation was made.

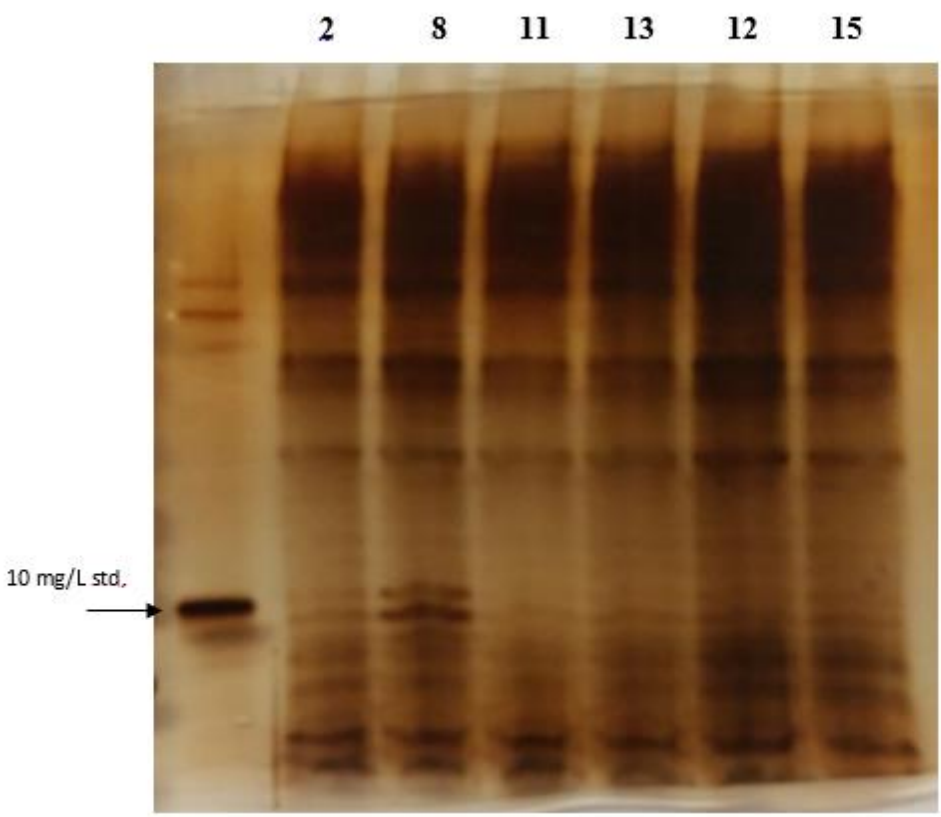


Figure 4. 11 SDS-PAGE results of the air filtered shake flask bioreactor experiment. **Column 2-15**: Proteins of t = 24 h of selected colonies

4.2.6 qPCR for Copy Number Determination

The copy number of the strain was determined by Roche LightCycler® 480 Real-Time PCR System. For the precision of the experiment, standards and samples were prepared in duplicate. Two non-template control tubes were also prepared with water for negative control. Thereafter, four serial dilutions of each standard each in duplicate was performed. The copy numbers of the prepared serial dilutions of the standard samples are presented in Table 4.2.

For the comparison, the qPCR experiment was performed with the target strain and two other strains whose copy numbers were determined and reported (Massahi, 2017). The target strain was named as colony 8 and other strains were named as PDC and PYK respectively. The final results of the experiment are shown in Table 4.3.

Table 4. 2 Copy numbers of ARG4 and hGH genes in serial dilutions of the corresponding standard DNA samples.

***hGH* standard dilution**	**copy/µl**	***ARG4* standard dilution**	**copy/µl**
$1/10^2$	$3.92x10^6$	$1/10^2$	$1.67x10^6$
$1/10^3$	$3.92x10^5$	$1/10^3$	$1.67x10^5$
$1/10^4$	$3.92x10^4$	$1/10^4$	$1.67x10^4$
$1/10^5$	$3.92x10^3$	$1/10^5$	$1.67x10^3$

Table 4. 3 Summarized results of the qPCR experiment

Basic Relative Quantification			
Set 1		**Set 2**	
Strain Number	**Copy**	**Strain**	**Copy Number**
Colony 8	2.37	**Colony 8**	1.75
PDC	0.57	**PDC**	0.69
PYK	1.07	**PYK**	0.89
Advanced Relative Quantification			
Set 1		**Set 2**	
Strain Number	**Copy**	**Strain**	**Copy Number**
Colony 8	1.23	**Colony 8**	1.11
PDC	0.53	**PDC**	0.44
PYK	0.53	**PYK**	0.54

LightCycler® 480 Real-Time PCR system analyzes the gene copy number quantities by two methods which are basic relative quantification and advanced relative quantification. The data obtained from basic relative quantification are based on an assumption that the efficiency equals to 2. On the other hand, the data acquired from advanced relative quantification are based on the actual efficiency of each reaction, which was derived from serial dilutions of the target and reference genes. The efficiency (E) of the PCR can be defined as the overall quality of the experiment which is expressed as a single number. The highest quality PCRs run at the efficiency of two (E = 2), which means that the number of target molecules doubles with every PCR cycle. However, many factors affect PCR process such as sample preparation and nucleic acid purification; thus, most PCRs run at an efficiency less than 2.

By taking into consideration all these information, advanced relative quantification method was found more reliable and the results were interpreted according to this method. For both sets, the ratio between the target gene copy number and reference strains was found as 2. Since copy numbers of the PYK and PDC strains were reported as 1 before, the *hGH* copy number of the selected transformant was specified as 2 in this experiment.

4.3 Comparison of Production Levels of Strains

For the comparison of production levels of rhGH by *P. pastoris* strains carrying single- and two- copies of *hGH* gene; with the multi-copy strain, another pilot scale bioreactor experiment was conducted at C_{DO} = 5% and the results were compared with that of the single-gene copy results at C_{DO}= 5%. With the single-copy *hGH* gene results at C_{DO}= 5%, the maximum rhGH concentration was determined as 28.54 mg L^{-1}. For comparison, the bioreactor experiment was performed under the same conditions. Definition of the name of the strains and bioreactor operation conditions are presented in Table 4.4.

Table 4. 4 Definition of the name of the strains and bioreactor operation conditions

Strain Name	Fed-batch Strategy Definition
SC (Single copy strain)	Exponential feeding of glucose with $\mu = 0.1\ h^{-1}$, T = 30°C, pH=5.5, C_{DO} = 5%, N=700 min^{-1}
MC (Multi copy strain)	Exponential feeding of glucose with $\mu = 0.1\ h^{-1}$, T = 30°C, pH=5.5, C_{DO} = 5%, N=700 min^{-1}

4.3.1 Substrate Consumption and Cell Growth

In both experiments, glucose was used as sole carbon source in fed-batch operation. The variations in glucose concentrations with the cultivation time in fed-batch bioreactor experiments are presented in Figure 4.12.

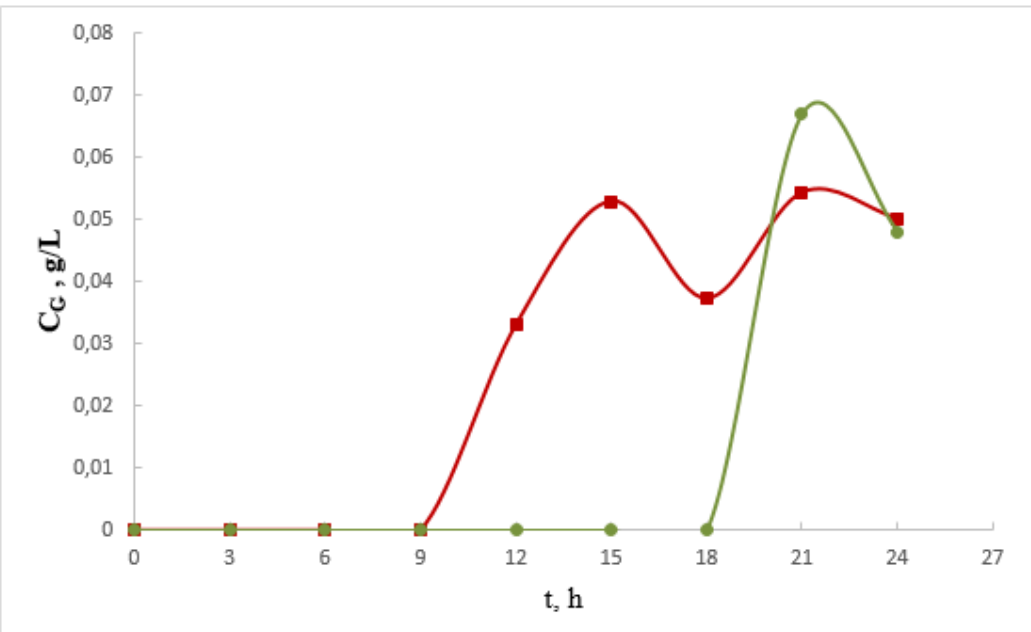


Figure 4. 12 Variations in glucose concentrations with the cultivation time in fed-batch bioreactors; C_{X0}= 24 g L^{-1}, T = 30°C, pH_C = 5.5, C_{DO} = 5%. SC (■), MC (●)

The results show that accumulation of glucose in bioreactor was negligible due to precise design of the continuous feed stream in both fed-batch bioreactor runs. The variations in the cell concentrations with the cultivation time in fed-batch bioreactor experiments are presented in Figure 4.13. The highest cell concentration was reported as C_X = 143 g L^{-1} for MC strain while it was C_X = 111 g L^{-1} for SC. The loci of the cell growth was similar until t = 15 h for both experiments; however, after t= 15 h higher cell concentrations were obtained for MC strain.

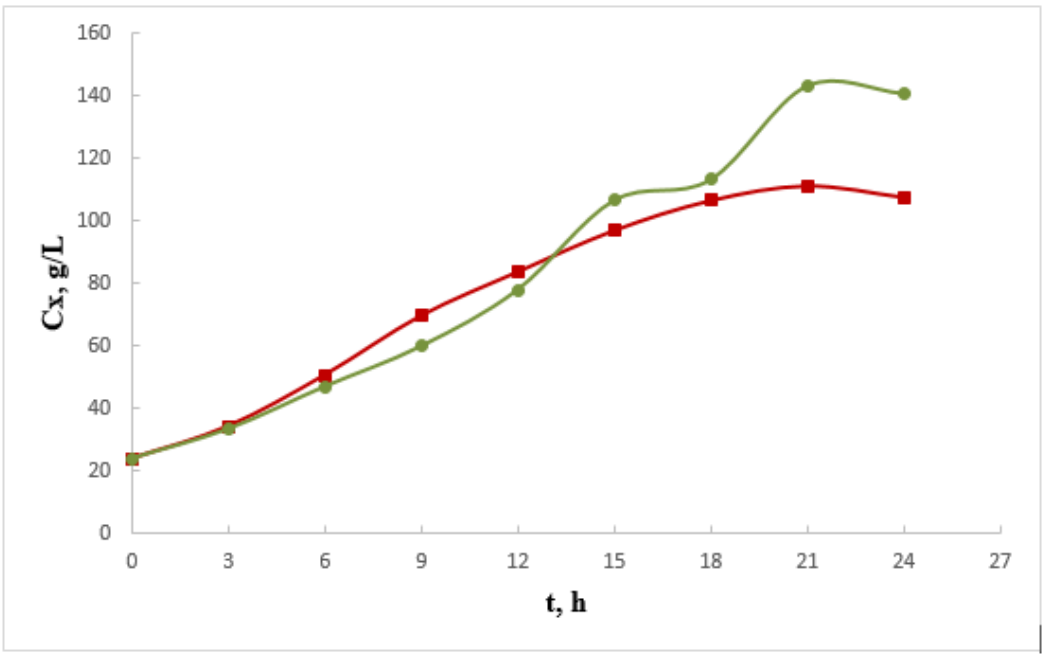


Figure 4. 13 Variations in the cell concentration with the cultivation time in fed-batch bioreactors; C_{X0}= 24 g L^{-1}, T = 30°C, pH = 5.5, C_{DO} = 5%. SC (■), MC (●)

Since all the operation parameters were same, the maximum cell concentrations could be expected close for the both strains. Contrarily, increasing the gene copy number increased the cell growth which is the first time finding and has not been reported in the literature yet.

4.3.2 rhGH Production

The highest rhGH concentrations were reported as 50 mg L^{-1} at t=24 h and 28.54 mg L^{-1} at t= 21 h for MC and SC respectively. The variations in rhGH concentrations with the cultivation time in fed-batch bioreactor experiments are presented in Figure 4.14. As expected, rhGH production was increased with the increased gene copy number. When the gene copy number was doubled, 1.8-fold increase in the rhGH production was obtained. Until t = 15 h, rhGH concentration profiles were nearly the same for both experiments; after this time, rhGH production was increased sharply with MC strain and gave its maximum at t= 24 h

while with SC strain, the maximum value was obained at t= 21 h and then decreased. Thus, the results reveal that, t = 15 h is the cultivation time when significant amounts of rhGH started to be produced and the effect of the gene copy number on rhGH production can be observed.

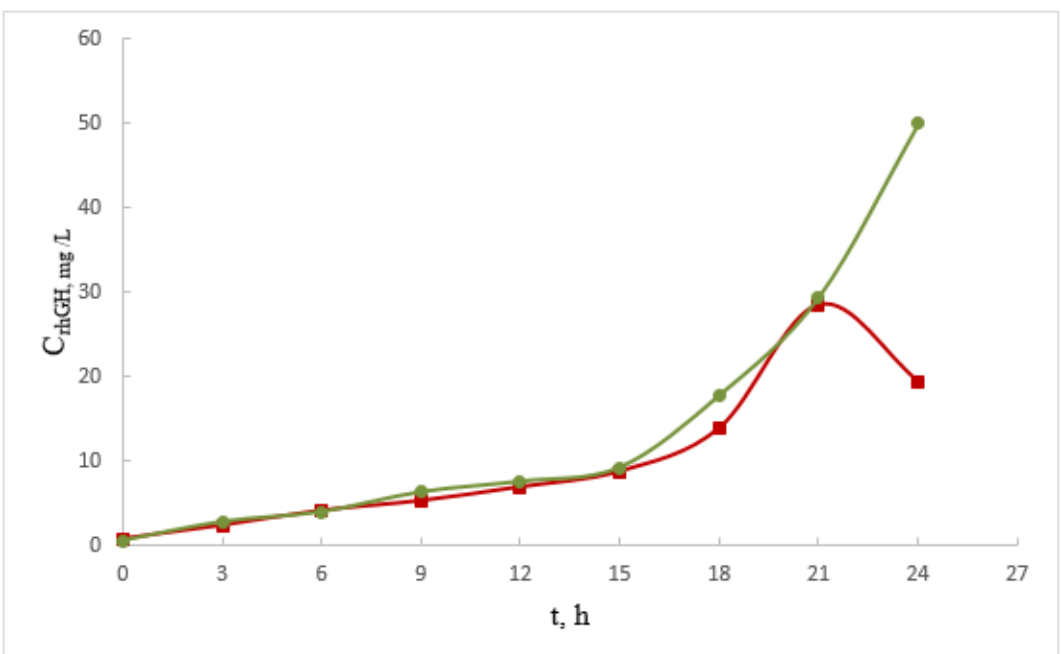


Figure 4. 14 Variations in rhGH concentrations with the cultivation time in fed-batch bioreactors; C_{X0}= 24 g L^{-1}, T = 30°C, pH = 5.5, C_{DO} = 5%. SC (■), MC (●)

4.3.3 Specific Rates and Yield Coefficients

All parameters related with the bioprocess experiments were calculated for the two fed-batch bioreactor experiments and presented in Table 4.5.

Table 4. 5 Variations in the fermentation characteristics of engineered *P. pastoris* strains carrying single- and multi- copy *hGH* genes with the cultivation time in fed-batch bioreactor experiments (t_{max} is the time where maximum rhGH production was performed)

Strategy	t h	μ h^{-1}	q_s $g\ g^{-1}\ h^{-1}$	q_p $mg\ g^{-1}\ h^{-1}$	$Y_{x/s}$ $g\ g^{-1}$	$Y_{p/s}$ $mg\ g^{-1}$	$Y_{p/x}$ $mg\ g^{-1}$
SC	3	0.140	0.156	0.017	-	-	0.150
	6	0.132	0.159	0.011	0.496	0.051	0.104
	9	0.100	0.148	0.008	0.822	0.053	0.064
	12	0.080	0.155	0.009	0.561	0.060	0.106
	15	0.072	0.166	0.015	0.490	0.059	0.121
	18	0.062	0.184	0.036	0.386	0.121	0.313
	t_{max}=21	0.048	0.209	0.020	0.292	0.274	0.936
	24	0.035	0.214	-	0.170	-	-
MC	3	0.125	0.160	0.018	-	-	0.226
	6	0.110	0.172	0.014	0.416	0.036	0.087
	9	0.107	0.172	0.012	0.583	0.097	0.167
	12	0.126	0.167	0.009	0.662	0.050	0.076
	15	0.087	0.151	0.019	0.851	0.056	0.066
	18	0.093	0.173	0.036	0.346	0.189	0.548
	21	0.079	0.163	0.047	0.731	0.232	0.317
	t_{max}=24	0.040	0.164	0.065	0.252	0.356	1.413

The observed specific growth rates (μ) at MC condition were close to pre-determined value $\mu_0 = 0.1\ h^{-1}$ for the first twelve hours. At t=12-15 h, a decrease in observed μ value is probably due to the increase in r-protein production which increased further within t = 15-18 h. The highest specific growth rates were reported as 0.140 h^{-1} and 0.126 h^{-1} for SC and MC strains respectively. For SC, the cell concentration increased slightly faster and the observed μ values decreased earlier.

With both strains, the specific substrate utilization rates (q_s) were not changed much throughout the bioprocess since the accumulation of glucose is in low levels in the media. The highest q_s values for SC and MC were calculated as 0.214 $g\ g^{-1}$

h^{-1} and 0.173 g g^{-1} h^{-1} at t = 24 h and t = 18 h respectively. The specific product formation rates (q_p) were increased as the cultivation time and were determined as 0.036 and 0.065 mg g^{-1} h^{-1} for SC and MC strains respectively.

The maximum$Y_{x/s}$ values were calculated as 0.822 and 0.851 g g^{-1} for SC and MC respectively. The values are close to each other since the cell growth profiles were mostly similar during the course of the bioprocess. $Y_{p/s}$ and $Y_{p/x}$ values were determined close to each other for SC and MC with the maximum values at t_{max} = 21 h and t_{max} = 24 h.

The overall cell yields on total substrate ($\bar{Y}_{x/s}$) were determined as 0.48 and 0.53 g g^{-1} and the overall product yields on total substrate ($\bar{Y}_{p/s}$) were calculated as 0.14 and 0.21 g kg^{-1} for SC and MC respectively. The results reveal that glucose was more efficiently used for the r-protein formation with MC.

4.3.4 Organic Acid Concentrations

Four-, five- and six-carbon organic acids are important metabolites which affect the activity of metabolic pathways in the cell, as they take part in some biochemical pathways in the TCA cycle. When the product is a recombinant protein, the concentrations of these by-products should be controlled during the bioprocess. In this regard, the variations in the concentrations of organic acids with the cultivation time were analyzed for fed-batch bioreactor experiments and are presented in Table 4.6. Pyruvic acid concentration was the highest while acetic acid, fumaric acid and succinic acid concentrations were the lowest with SC. With MC, formic acid has the highest concentration and the lowest concentrations were obtained for fumaric and succinic acids.

Table 4. 6 The variations in organic acid concentrations with the cultivation time detected during the fed-batch bioreactor operations with engineered *P. pastoris* strains carrying single- (SC) and multi- copy (MC) *hGH* genes

Concentration g L^{-1}						
t, h	**6**	**12**	**15**	**18**	**21**	**24**
SC						
Oxalic Acid	0.000	0.000	0.009	0.000	0.000	0.015
Formic Acid	0.000	0.0033	0.000	0.076	0.000	0.000
Pyruvic Acid	0.294	0.000	0.013	0.000	0.263	0.190
Malic Acid	0.000	0.000	0.027	0.000	0.055	0.000
Acetic Acid	0.000	0.000	0.000	0.000	0.000	0.000
Fumaric Acid	0.000	0.000	0.000	0.000	0.000	0.000
Succinic Acid	0.000	0.000	0.000	0.000	0.000	0.000
MC						
Oxalic Acid	0.000	0.001	0.015	0.015	0.010	0.022
Formic Acid	0.000	0.012	0.104	0.245	0.361	0.195
Pyruvic Acid	0.342	0.061	0.013	0.014	0.008	0.180
Malic Acid	0.000	0.000	0.017	0.060	0.008	0.117
Acetic Acid	0.049	0.061	0.029	0.041	0.017	0.086
Fumaric Acid	0.000	0.000	0.000	0.000	0.000	0.000
Succinic Acid	0.000	0.000	0.000	0.000	0.000	0.000

Oxalic acid is a TCA cycle metabolite and it was not within detectable limits for SC strain until the last hour of the fermentation. The concentrations were also low for the MC strain and increased slightly throughout the process. Therefore, accumulation of oxalic acid was not observed both cases.

Formic acid is the conjugate pair of the formate and usually found in this form in nature. In both experiments, formic acid concentration increased from the

beginning of the fed-batch operation and gave the maximum at t = 18 h for SC, and at t= 21 as 0.361 g L^{-1} for MC, before the t_{max}.

Pyruvic acid is also a metabolite of the TCA cycle and the concentrations were high at the beginning in both fermentations. When pyruvate conversion either towards the TCA cycle or ethanol is increased, its concentration in the broth decreases.

Malic acid is formed in the TCA cycle and by anaplerotic reactions from pyruvate; and was negligible except during the last hours of the bioprocesses.

Acetic acid, fumaric acid and succinic acid were also not detected in the fermentation medium for the experiment with single-copy strain. With MC, low concentrations of acetic acid were detected. In general, TCA cycle metabolites were not accumulated in fermentation medium at significant amounts. This can be the sign of efficiency in aerobic respiration.

In low oxygen availability, the cells shift their metabolism from respiratory pathway to the fermentative pathway in order to regenerate the NAD^+ required for effective and proper functioning of the glycolysis pathway. Ethanol is one of the end-products of the fermentative pathway. Because of the low amounts of metabolites detected in the TCA cycle, the verification of this shift was performed by monitoring the accumulated ethanol in the fermentation broth during glucose fed-batch phase for both experiments. The variations in ethanol concentration with the cultivation time in fed-batch bioreactor experiments are presented in Figure 4.15. An increase in the produced ethanol concentration was detected at t =12 and found as 27 g L^{-1} with SC while the value of 23.8 g L^{-1} was attained with MC.

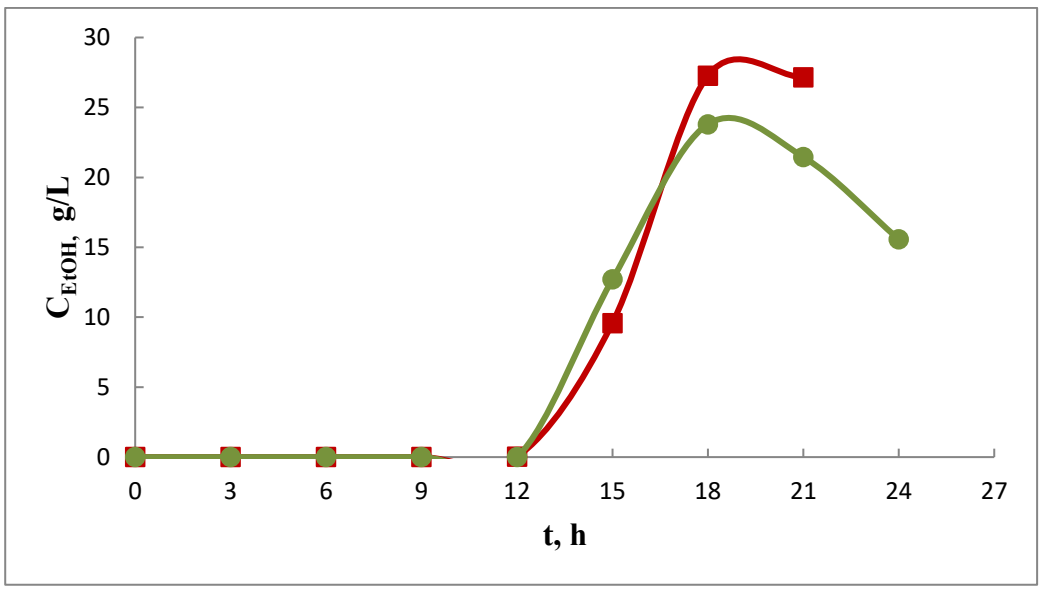


Figure 4. 15 The variations in ethanol concentration with the cultivation time in fed-batch bioreactors; C_{X0}= 24 g L^{-1}, T = 30°C, pH = 5.5, C_{DO} = 5%. SC (■), MC (●)

4.4 Determination of Physical Mass Transfer Coefficients in 5-L Lab-Scale Bioreactor

As discussed earlier, mass transfer coefficient is important parameter that must be evaluated for the specification of oxygen transfer characteristics. Mass transfer coefficient (K_La) and oxygen tranfer rate (OTR) is mainly depend on the relevant physical properties of the fluid, the geometry used along with relevant dimensions, on the microorganism and intracellular reaction rates, aeration rate, and agitation rate.

For K_La measurements, different methods can be applied such as the static gassing-out method, dynamic gassing-out method and the oxygen balance method (Rainer, 1990). The dynamic method (Bandyopadhyay et al., 1967) is most widely used and the measurements are based on the respiration of the microorganisms in the reactor. To apply the method, inlet gas flow to the bioreactor is stopped for a while and agitation rate is lowered as much as possible to block the surface

aeration. Then the decrease in dissolved oxygen concentration was detected and the rate of decline gives the oxygen uptake rate (OUR); where K_La equals to zero. Thereafter, the gas is allowed to flow into the bioreactor again under the operation conditions for obtaining the same OTR in the system.

The mass balance equation for the dissolved oxygen in the well-mixed bioreactor is constructed by the mass conservation equation for disolved oxygen (Bandyopadhyay *et al*., 1967):

$$K_La(C_0^* - C_0) + r_0 = \frac{dC_0}{dt} \quad (4.1)$$

$$K_La(C_0^* - C_0) + q_0\, C_x = \frac{dC_0}{dt} \quad (4.2)$$

The first term is the OTR and second term is OUR while the term (dC_0/dt) represents the oxygen accumulation in the liquid phase, q_o is the specific oxygen uptake rate. K_La values can be determined from the slope of the graph when (dC_0/dt-ro) versus C_0 was plotted. In order to investigate the case without oxygen consumption, K_La_0 values were determined by dynamic method.

For determination of the physical K_La_0 values in the absence of the fermentation, working volume of the reactor was chosen as 2 L and the bioreactor was filled with BSM medium without the microorganism. Firstly, nitrogen flow was sent to the system and dissolved oxygen concentration decreased to zero. Then air flow was supplied to the system and C_{DO} was recorded at every five seconds until it reached the saturation point. The general mass balance equation reduces to following form since OUR is equal to zero:

$$K_La_0(C_0^* - C_0) = \frac{dC_0}{dt} \quad (4.3)$$

When the graph of (dC_0/dt) versus C_0 was plotted, physical K_La_0 values were calculated from the slope of this graph. The analysis was carried out at different aeration rates as Q/V_R= 0.5, 1, 2, 3, 4, 5 vvm and at various agitation rates which were N =150, 300, 450, 600 and 750 rpm. The variations in K_La_0 values with respect to agitation and aeration rates are presented in Figure 4.16 and 4.17 respectively.

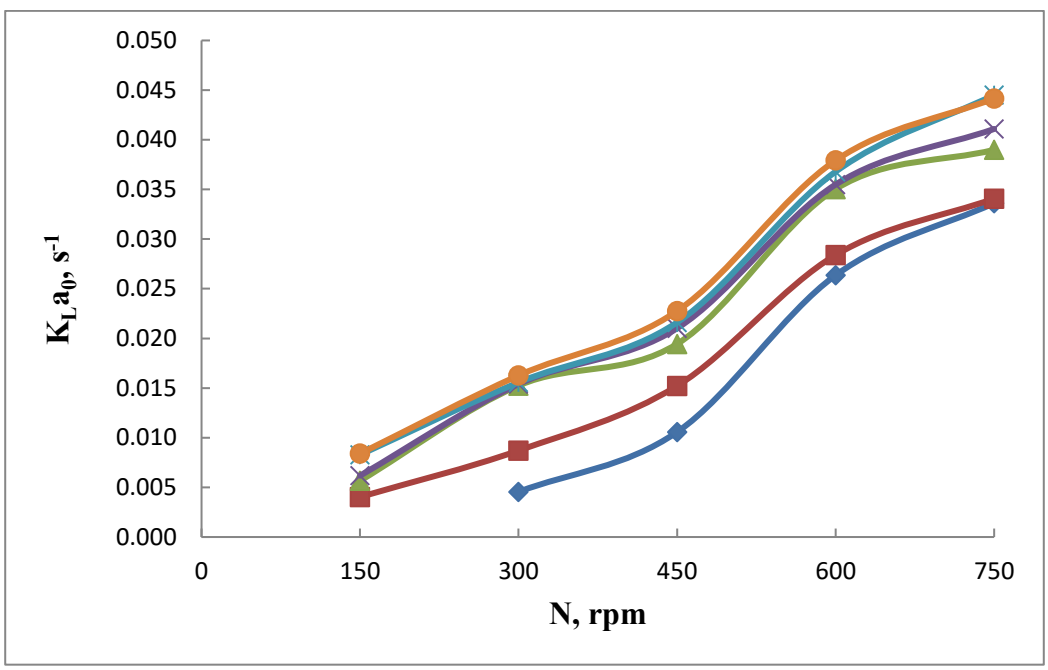


Figure 4. 16 The variations in K_La_0 values with agitation rates. V= 2 L, T = 30°C, pH = 5.5. 0.5 vvm (♦), 1 vvm (■), 2 vvm (▲), 3 vvm (×), 4 vvm (*), 5 vvm (●)

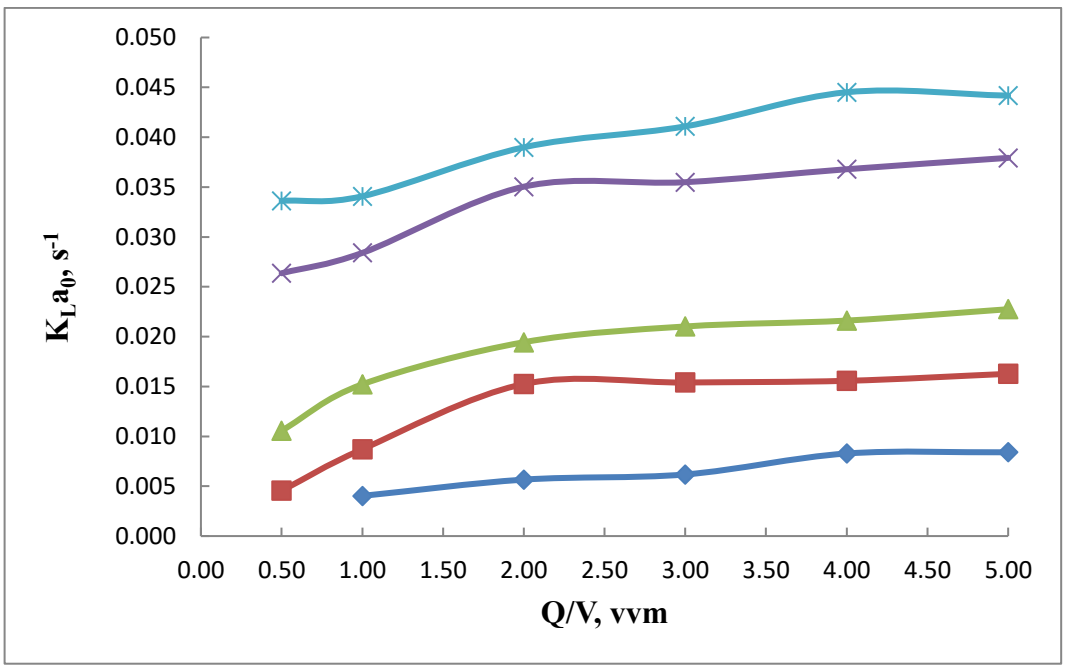


Figure 4. 17 The variations in K_La_0 values with aeration rates. V= 2 L, T = 30°C, pH = 5.5. 150 rpm (♦), 300 rpm (■), 450 rpm (▲), 600 rpm (×), 750 rpm (*)

As expected, K_La_0 values as well as OTR increased with increased agitation rate since agitation strongly affects the bubble size, bubble retention time and liquid mixing (Roberts *et al*., 1992). However the increase in K_La_0 values became smaller after 2 vvm which means that aeration rates higher than 2 vvm have no effect on oxygen transfer rate in the bioreactor. When the aeration rate was increased, the gas bubbles in the fermentation medium became smaller thus the surface area per volume decreased. This resulted a decrease in K_La_0 values and oxygen transfer rate. This result was also verified by the graph drawn in Figure 4.17.

4.5 Effect of Supplements on rhGH Production

The medium used for the growth of microorganisms is generally categorized into two groups: complex and defined medium. For the preparation of defined medium, precise amounts of higly purified inorganic and organic substances are used while impure compounds with unknown contents are generally used for the preparation

of complex medium. Digest of some microbial products is commonly preferred for complex medium (Nielsen *et al*., 2003). By using a complex medium, high cell densities and product yields can be obtained in fermentations however separation and purification of the desired product is easier in defined medium. In general, complex medium is used for precultivation steps while defined medium is preferred for production steps. There is also an intermediate form of these mediums named as semi-defined medium and it contains only one or two complex components in the composition.

In eukaryotic cells, ca. 7% of the cell weight is composed of nitrogen thus this element has the second highest percentage after carbon and reported as one of the most abundant components in eukaryotic cells (Carnicer *et al*., 2009). Microorganisms require nitrogen for the synthesis of vital compounds such as nucleic acids and proteins thus supplying the culture medium with sufficient amount of this element is essential for fermentation processes.

In order to increase the product yields, nitrogen can be supplied into the medium and generally peptone is used as the complex nitrogen and carbon source for this purpose (Loewen *et al*., 1997, Gou *et al*., 2012, Terrazas *et al*., 2014, Öztürk, 2014). 1.7-fold increase in total protein production was observed for the study of Terrazas and 2.3-fold increase in rhGH production was reported in Öztürk's thesis. Therefore, a laboratory scale shake flask bioreactor experiment was conducted for investigating the influence of peptone on rhGH production. The influence of peptone was studied at three different concentrations; production mediums including 2 g L^{-1} peptone, 5 g L^{-1} peptone and 10 g L^{-1} peptone with a medium that did not contain peptone as reference. The samples were removed at t = 24 h and t = 48 h of the production, and the results are presented in Figure 4.18. The results reveal that the increase in peptone concentration improved the rhGH production and the highest rhGH concentration was determined as 5 mg L^{-1} at t = 48 h in the medium containing 10 g L^{-1} peptone. rhGH concentrations analyzed at different

hours of production and at different peptone concentrations are presented in Table 4.7.

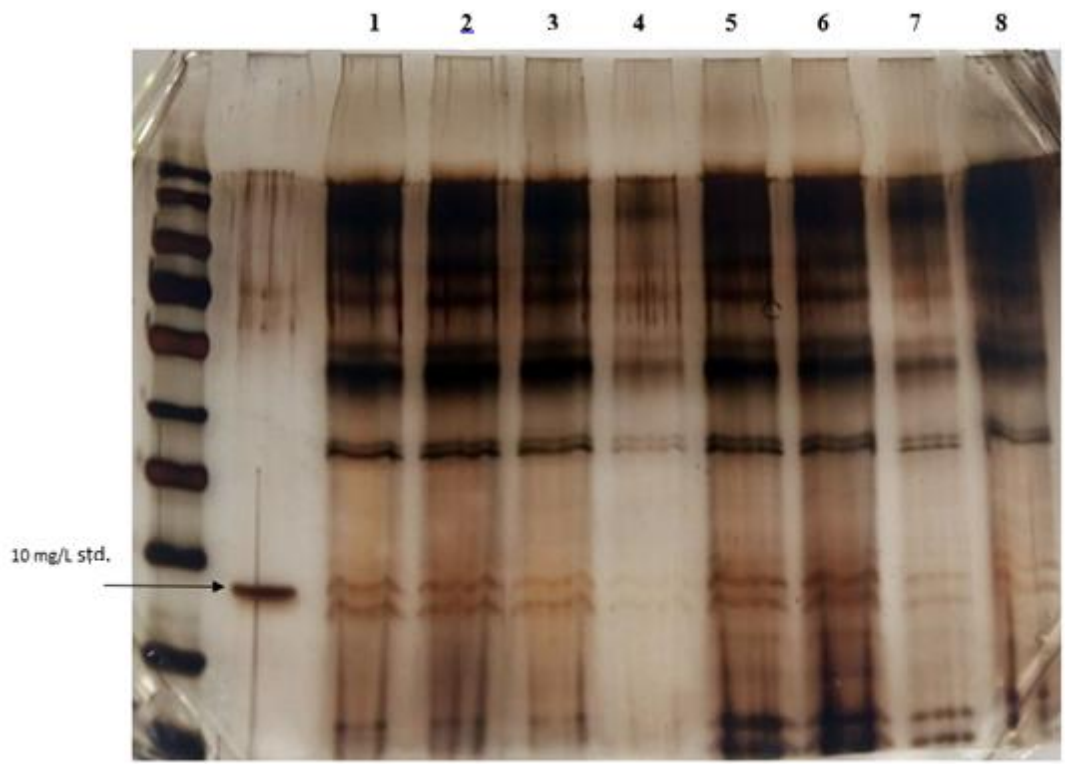


Figure 4. 18 SDS-PAGE screening of the samples containing various amounts of peptone. **Lane 1**: 5 g L^{-1} peptone (t = 24h), **Lane 2**: 10 g L^{-1} peptone (t = 24h), **Lane 3**: 2 g L^{-1} peptone (t = 24h), **Lane 4**: without peptone (t =24h), **Lane 5**: 5 g L^{-1} peptone (t = 48h), **Lane 6**: 10 g L^{-1} peptone (t = 48h), **Lane 7**: 2 g L^{-1} peptone (t= 48h), **Lane 8**: without peptone (t = 48h)

Table 4. 7 rhGH concentrations in laboratory scale shake flask bioreactor experiment performed with various peptone concentrations

	rhGH Concentration, mg L^{-1}	
Medium	**24 h**	**48 h**
2 g L^{-1} peptone	1.2	2.33
5 g L^{-1} peptone	2.5	4.23
10 g L^{-1} peptone	3.2	5
without peptone	0.16	0.96

At high cell density fermentations, nutrient limitations can be challenging since the cells may suffer from environmental stress or they have to adapt different metabolic states. In a recent research, strong transcriptional regulations were discovered related with sulfur, phosphorus and nitrogen metabolism of *P. pastoris.* In this context, several biomarker genes were selected and their transcriptional pattern was investigated. A significant increase in the transcript levels of phosphorus limitation marker genes was observed for non-supplemented culture. Additionally, it was proved that phosphorus supplementation has a positive impact on biomass generation and r-protein production; 9% higher biomass and 60% more protein activity were reported for the cultures containing additional phosphorus source (Burgard *et al*., 2017). To investigate the effect of phosphorus addition to the production medium, a laboratory scale shake flask bioreactor experiment was conducted. In the production phase of laboratory scale shake flask bioreactor experiments, 0.1 M potassium phosphate buffer (KIP) is used as the main phosphorus source. To analyze the effect of phosphorus amount in the medium, the molarity of the KIP solution was changed to 0.05 M and 0.4 M and the results are presented in Figure 4.19. A dramatic improvement on the rhGH production was observed when the molarity of the phosphate buffer was increased up to 4–fold. 11.6 mg L^{-1} rhGH concentration was obtained in SDS-PAGE analysis for the

medium containing 0.4 M phosphate buffer while only 2.3 mg L^{-1} rhGH concentration was attained for the medium containing 0.1 M phosphate buffer.

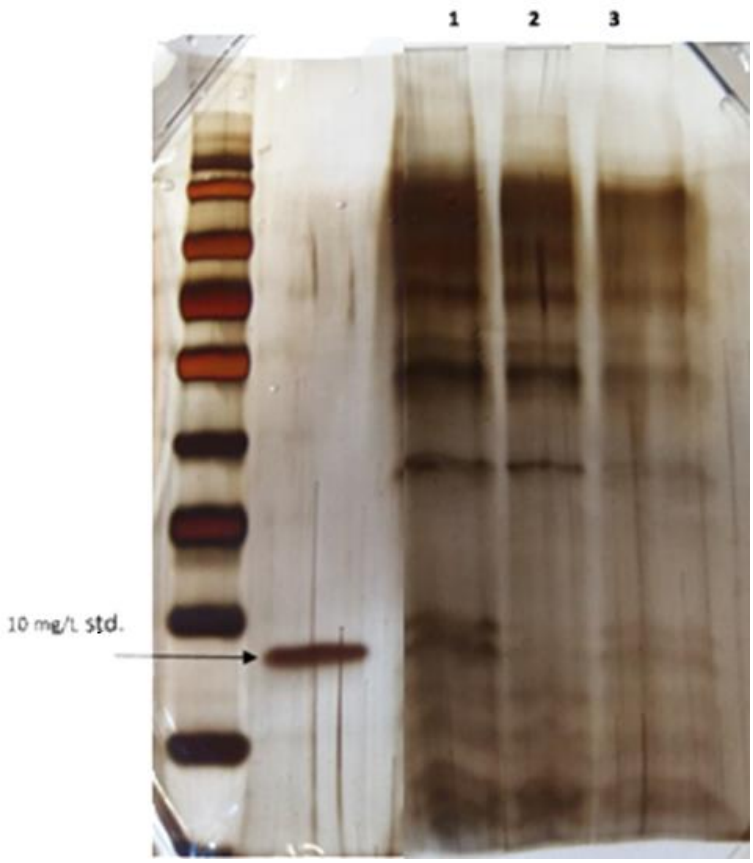


Figure 4. 19 SDS-PAGE screening of the samples containing various amounts of phosphate. **Lane 1**: 0.4 M KIP, **Lane 2**: 0.05 M KIP, **Lane 3**: 0.1 M KIP

Supplementation of a chemically defined medium with amino acids is another choice for the optimization of nutritional medium to stimulate heterologous r-protein production. In many studies, positive effects of these additives on r-protein activity and production were reported (Shang et al., 2017, Zhang et al., 2017, Görgens et al., 2005). In Table 4.8, intracellular amino acid concentrations are presented for *P. pastoris*.

Table 4. 8 Intracellular amino acid concentrations gathered from literature

	Russmayer *et al.* 2015	**Carnicer *et al.* 2012**
Amino Acid	**Intracellular Metabolite Concentration [µmol/g DCW]**	**Intracellular Metabolite Concentration [µmol/g DCW]**
Alanine	16.10	23.5
Valine	1.44	1.97
Leucine	0.84	1.48
Isoleucine	0.34	0.53
Methionine	1.12	0.97
Phenylalanine	0.19	0.51
Proline	33.7	10.8
Serine	6.89	-
Threonine	2.36	3.94
Asparagine	4.46	7.1
Glutamine	143	177
Aspartate	33.2	26.4
Glutamate	172	200
Lysine	7.76	12.8
Arginine	94.9	-
Histidine	6.57	7.4
Tyrosine	-	0.8
Tryptophan	-	0.24
Glycine	-	1.83

Proteins contain structural amino acids at various percentages thus amino acid composition of the hGH molecule was also obtained for determining the major amino acids that form the whole composition. The amino acid composition of hGH is presented in Table 4.9.

Table 4. 9 Amino acid composition of hGH

Amino acid	Weight percent (%)	Amino acid	Weight percent (%)
Ala	3.5	Leu	12.9
Arg	5.9	Lys	4.5
Asn	4.5	Met	2.0
Asp	5.4	Phe	6.4
Cys	2.0	Pro	4.0
Gln	6.4	Ser	8.9
Glu	7.4	Thr	5.0
Gly	4.5	Trp	0.5
His	4.5	Tyr	4.0
Ile	4.5	Val	3.5

When the intracellular amino acid concentrations in *P. pastoris* and structural amino acid percentages for hGH are taken into consideration; Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), Tryptophan (Trp) and Tyrosine (Tyr) were chosen as the supplements for the laboratory scale shake flask bioreactor experiment. The concentrations of these amino acids in the production medium were determined by using the data reported by Sherman (2002) and are presented in Table 4.10.

Table 4. 10 Concentration of amino acids in the production medium

Amino acid	Concentration (mM)
Ile	0.25
Leu	0.6
Phe	0.4
Trp	0.2
Tyr	0.3

To achieve the highest rhGH production, the experiment was performed with multi- copy strain carrying pPYKZαA::*hGH* plasmid, and the phosphate buffer concentration in the production medium was adjusted to 0.4 M. The amino acids were supplied into the production medium at t = 16 h and the supernatants were collected at t = 22 h. The SDS-PAGE results of the samples are presented in Figure 4.20.

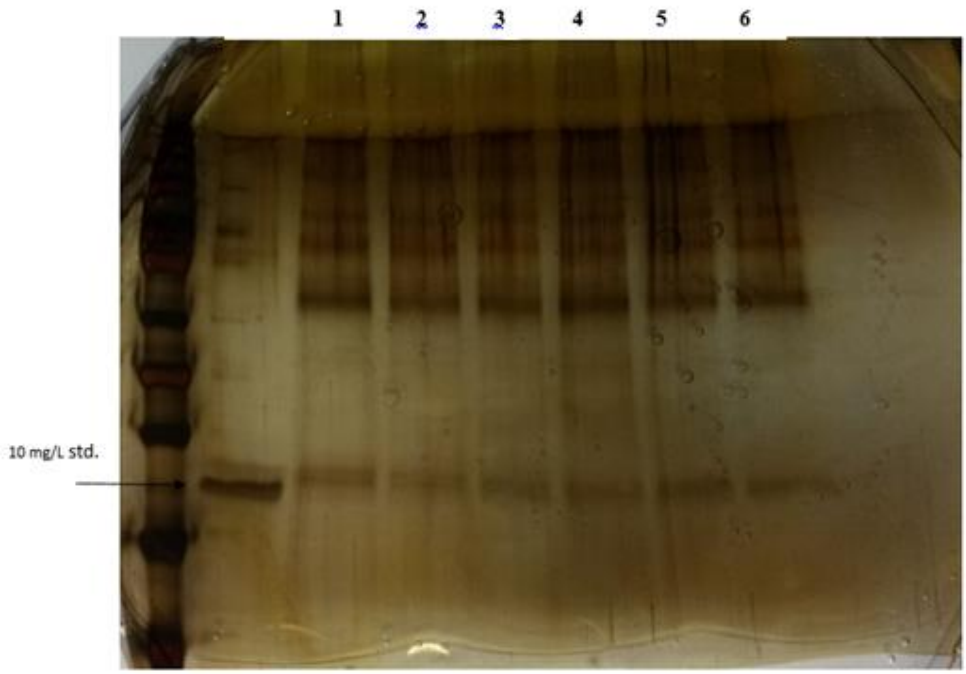


Figure 4. 20 SDS-PAGE screening of the samples with amino acid supplements. **Lane 1**: 0.25 mM Ile, **Lane 2**: 0.6 mM Leu, **Lane 3**: 0.2 mM Trp, **Lane 4**: 0.3 mM Tyr, **Lane 5**: 0.4 mM Phe, **Lane 6**: without amino acid

Table 4. 11 rhGH concentrations in laboratory scale shake flask bioreactor experiment performed with various amino acid supplements

Amino Acid	**rhGH Concentration, mg L^{-1}**
0.25 mM Ile	8.92
0.6 mM Leu	4
0.2 mM Trp	6.55
0.3 mM Tyr	3.3
0.4 mM Phe	3.91
without amino acid	2.15

rhGH concentrations in laboratory scale shake flask bioreactor experiment performed with various amino acid supplements are presented in Table 4.11. In this experiment, rhGH concentration was increased 4-fold with Ile supplementation and 3-fold with the addition of Leu compared to reference medium.

These strategies can be applied to enhance the r-protein production at high cell density *P. pastoris* fermentations however further analysis and research are needed to eliminate some inconsistencies.

CHAPTER 5

CONCLUSIONS

In the first part of this thesis, an investigation was made to observe the effects of oxygen transfer conditions on r-protein production in *Pichia pastoris* strains designed with novel naturally occuring pyruvate kinase (PYK) promoter (P_{PYK}) which is a potential promoter for recombinant protein production under low to moderate oxygen transfer conditions. After deciding the superior bioreactor operation condition in fed-batch operation, the influences of engineering with single- and multi- copy genes on the productivity was also studied by performing a pilot scale bioreactor experiment with *P. pastoris* strain under P_{PYK} carrying two copies of *hGH* gene. To achieve these goals, three pilot scale bioreactor experiments were firstly conducted at three oxygen transfer conditions at constant dissolved oxygen concentrations. Dissolved oxygen concentrations in the fermentation broth were set to 1%, 5% and 15% for these experiments and production levels of rhGH were compared as well as the calculation of other process parameters such as yield coefficients, specific rates and substrate accumulation. Thereafter, another pilot scale bioreactor experiment was carried out under P_{PYK} with the *P. pastoris* strain carrying multi-copies of *hGH* gene under the same operation conditions with the strain carrying single-copy of *hGH* gene at consant dissolved oxygen concentration which gave the highest production in the first part. At the end of the experiments, rhGH production, cell growth and substrate consumption characteristics were investigated. In addition to this, specific rates, yield coefficients and organic acid concentrations were also evaluated and compared for both of the strains. For in-depth analysis of oxygen

transfer characteristics in the 5 L pilot scale bioreactor, physical mass transfer coefficients, $K_La_{0,}$ were calculated in batch operation by using the dynamic method, to be used as a part of an extensive oxygen transfer analysis in *P. pastoris* fermentations in order to maka a reassesment of the findings reported in the literature. As future work, laboratory scale air filtered shake bioreactor experiments were carried out to optimize the growth medium by investigating the effects of peptone, phosphorus and amino acid supplements on rhGH production.

In this context, three pilot scale fed-batch bioreactor experiments were firstly conducted at three constant dissolved oxygen concentrations; since P_{PYK} is effective at low to moderate oxygen transfer conditions, C_{DO} = 1%, C_{DO} = 5% and C_{DO} =15% were selected as the operation parameters. The pilot scale operations were conducted at T = 30°C, N = 700 min^{-1}, pH = 5.5 with the volume of 1.9 L for the fed-batch phase. With the single-copy strain, the highest cell and rhGH concentrations were obtained as C_x = 115 g L^{-1} and C_{rhGH} = 28.54 mg L^{-1} at C_{DO} = 15% and C_{DO} = 5%, respectively. Although the cell concentration was higher at C_{DO} = 15%, the highest productivity was achieved at C_{DO} = 5%. Thus, C_{DO} = 5% was found as the superior bioreactor operation condition for glucose fed-batch phase for the production of rhGH under P_{PYK}.

To investigate the influences of engineering with single- and multi- copy genes, a pilot scale experiment was performed again at C_{DO} = 5% with the *P. pastoris* strain carrying two-copies of *hGH* gene. The highest cell and rhGH concentrations were achieved as C_x = 143 g L^{-1} and C_{rhGH} = 50 mg L^{-1}. As expected, rhGH production was enhanced with the increased gene copy number. When the gene copy number was doubled, 1.8-fold increase in rhGH production was obtained.

For in-depth analysis of oxygen transfer characteristics in the 5 L pilot scale bioreactor, physical mass transfer coefficients (K_La_0) were also evaluated. For determination of the physical K_La_0 values in the absence of the fermentation, Q/V

values were set to 0.5, 1, 2, 3, 4, 5 vvm while agitation rates were adjusted to 150, 300, 450, 600 and 750 rpm. K_La_0 values, thus OTR, was found to increase with the increased agitation rate. However the variation in K_La_0 values became smaller after 2 vvm; this value was chosen as the maximum aeration rate for the future experiments since operation at the Q/V values higher than 2 vvm have no effect on oxygen transfer rate in the bioreactor.

Lastly, laboratory scale shake flask bioreactor experiments were performed to investigate the influence of peptone, phosphorus and amino acid supplementation on rhGH production. 5.2-fold increase in rhGH production was observed for the medium containing peptone as nitrogen source and 4-fold increase was reported for the mediums where phosphorus and various amino acids were used as supplements.

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A. BUFFERS AND STOCK SOLUTIONS

SDS-PAGE Solutions

10% (w/v) APS (Ammonium PerSulfate)	0.1 g APS is dissolved in 1 mL distilled water.
0.1 M Ditiotreitol (DTT)	0.0154 g DTT is dissolved in 1 mL distilled water
5X SDS-PAGE Running Buffer	7.5 g Tris base, 36 g glycine and 2.5 g SDS are dissolved in 500 mL distilled water. After preparation, it can be stored at 2-8°C.
1X SDS-PAGE Running Buffer	It is diluted from 5X buffer prior to use and can be used three times.
4X Loading Buffer	It contains 200 mM Tris-HCl (pH=6.8), 40% glycerol, 6% SDS, 0.013% Bromophenol blue. It can be stored at room temperature in a foiled tube.
Fixer Solution	100 µl 37% formaldehyde, 100 mL methanol and 24 mL acetic acid are mixed and distilled water is added to this mixture until the total volume has reached to 200 mL. It can be stored up to one month.

Pretreatment Solution 0.05 g $Na_2S_2O_3.5H_2O$ is dissolved in 200 mL distilled water. This solution should be freshly prepared prior to use.

Silver Nitrate Solution 0.2 g silver nitrate is dissolved in 100 mL distilled water and 75 µl 37% formaldehyde is added to this solution. It should be freshly prepared prior to use.

Developing Solution 2.25 g potassium carbonate is dissolved in 100 mL distilled water. 2 mL pretreatment solution and 75 µl 37% formaldehyde are added to the mixture. It should be freshly prepared prior to use.

Stop Solution 50 mL methanol and 12 mL acetic acid are mixed and the volume is completed to 100 mL by adding distilled water.

Fixing Solution 4 mL 25% glutaraldehyde and 150 mL ethanol are mixed. The volume is completed to 500 mL with distilled water. 13.61 g sodium acetate trihydrate is dissolved in this mixture.

Coomassie R250 Staining Solution 1 g of Coomassie R250 is dissolved in 300 mL methanol. Then 650 mL distilled water and 50 mL acetic acid are added. The solution is stirred on a magnetic stirrer for 2 hours. Then it is filtered by a Whatman No. 1 paper.

	The solution can be stored at 20°C for several months.
Destain Solution I	300 mL methanol, 50 mL acetic acid and 650 mL distilled water are mixed. It can be stored at 20°C for several months.
Destain Solution II	880 mL distilled water, 70 mL acetic acid and 50 mL methanol are mixed. The solution can be stored at 20°C for several months.

Alkaline Lysis Solutions

Alkaline Lysis I	It contains 50 mM glucose, 25 mM Tris-Cl (pH=8.0) and 10 mM EDTA (pH=8.0). It is prepared as 100 mL by adding distilled water and sterilized by autoclaving. It can be stored at +4°C.
Alkaline Lysis II	1% (w/v) sodium dodecyl sulfate (SDS) is dissolved in 0.2 N NaOH. It should be freshly- prepared and used at room temperature.
Alkaline Lysis III	60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid and 28.5 mL distilled water are mixed. It can be stored at +4°C and should be transferred to an ice-bucket before use.

TE Buffer	1 mL 1 M Tris-Cl (pH=8.0) and 0.2 mL 0.5 M EDTA are mixed and pH was adjusted to 8.0 by the addition of NaOH or HCl. Final volume was completed to 100 mL by the addition of distilled water. It is sterilized by autoclaving and can be stored at room temperature.

Lithium Chloride Transfection Solutions

1 M LiCl	0.424 g LiCl was dissolved in 10 mL distilled water and filter-sterilized. It can be diluted to 0.1 M with sterile water and stored at +4 °C.
50% (w/v) PEG	25 g polyethylene glycol (PEG-3350) was dissolved in 50 mL distilled water by gentle heating and filter-sterilized. It must be stored at tightly capped bottle. It can be stored at -20°C.
Single-stranded DNA	2 mg/mL denaturated, fragmented salmon sperm DNA was prepared in TE buffer (pH=8.0). It can be stored at -20°C.

B. GROWTH MEDIA

For the preparation of LB agar plates, 35 g LB agar which is provided from Sigma is dissolved in 1 L ultrapure water. For sterilization, it is autoclaved at 121°C for 20 minutes. After it allowed to cool, 25 μg mL^{-1} zeocin is added as an antibiotic and the solution is poured into Petri dishes. These plates can be stored at +4°C or the microorganism can be cultivated into them directly.

20 g LB broth is dissolved in 1 L ultrapure water for the liquid medium. It is also autoclaved for sterilization and 25 μg mL^{-1} zeocin is added after it is cooled below 55°C.

Table B. 1 Composition of YPD (Yeast Extract Peptone Dextrose)

Compound	**Concentration (g L^{-1})**
Yeast extract	10
Peptone	20
Agar	20
Dextrose	20

For the preparation of 1 liter medium, 10 g yeast extract, 20 g peptone and 20 g dextrose are dissolved in ultrapure water. If solid medium is required 20 g agar is also added to this mixture. Then the mixture is autoclaved at 121 °C on liquid cycle for 20 minutes. 25 μg mL^{-1} zeocin is added as an antibiotic after sterilization when it is cooled below 55°C.

Table B. 2 Composition of BMGY, precultivation medium

Compound	Concentration (g L^{-1})
Yeast extract	10
Peptone	20
Potassium phosphate buffer (pH=6.0)	0.1 M
Yeast nitrogen base (YNB) (w/o amino acids)	3.4
Ammonium sulfate	10
Glycerol	10
Biotin	$4x10^{-5}$
Chloramphenicol (34 mg mL^{-1})	1 mL L^{-1}
dH_2O	to 1 L

For 250 mL BMGY medium, 2.5 g yeast extract and 5 g peptone is dissolved in 125 mL ultrapure water. 1 M potassium phosphate buffer (KIP) is prepared by dissolving 1.16 g K_2HPO_4 and 4.52 g KH_2PO_4 in 40 mL ultrapure water. 0.85 g yeast nitrogen base (w/o amino acids) and 2.5 g ammonium sulfate is dissolved in 50 mL ultrapure water. Glycerol solution is prepared by adding 2.87 g glycerol to 50 mL ultrapure water. Yeast extract peptone solution, potassium phosphate buffer, yeast nitrogen base ammonium sulfate mixture and glycerol solution are autoclaved separately at 121 °C on liquid cycle for 20 minutes. After the solutions are allowed to cool for a certain time they are combined and 1 M potassium phosphate buffer is diluted to 0.1 M in this step. 500 µl 500X Biotin and 250 µl chloramphenicol are added to the final mixture. Chloramphenicol is used as an antibiotic for precultivation medium and prepared as 34 mg/mL stock solution in absolute ethanol. It is stored at -20 °C in a sterile dark bottle.

Table B. 3 Composition of production medium

Compound	Composition (g L^{-1})
Glucose	20 g
Ammonium sulfate	9.54 g
Potassium phosphate buffer (pH=6.0)	0.1 M
Magnesium sulfate heptahydrate	14.9 g
Calcium sulfate dihydrate	1.17 g
Chloramphenicol	1 mL
PTM1	4.35 mL

Table B. 4 Composition of BSM (Basal Salt Medium)

Compound	Amount per liter
Phosphoric acid (85%)	26.7 mL
Calcium sulfate dihydrate	1.17 g
Potassium sulfate	18.2 g
Magnesium sulfate heptahydrate	14.9 g
Potassium hydroxide	4.13 g
Glycerol (100%)	40 g
Antifoam 10%	1 mL
PTM1	4.35 mL
Chloramphenicol	1 mL

All chemicals except antifoam, PTM1 and chloramphenicol are dissolved in ultrapure water and autoclaved at 121 °C on liquid cycle for 20 minutes. After the mixture is cooled below 55 °C, antifoam, PTM1 and chloramphenicol are added.

Table B. 5 Composition of PTM1 solution

Compound	Amount per liter
Cupric sulfate-pentahydrate ($CuSO_4.5H_2O$)	6.0 g
Sodium iodide (NaI)	0.08 g
Manganase sulfate monohydrate ($MnSO_4.H_2O$)	3.0 g
Sodium molybdate dehydrate ($Na_2MoO_4.2H_2O$)	0.2 g
Boric acid (H_3BO_3)	0.02 g
Cobalt chloride ($CoCl_2$)	0.5 g
Zinc chloride ($ZnCl_2$)	20.0 g
Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	65.0 g
Biotin	0.2 g
Sulfuric acid (H_2SO_4)	5.0 mL
dH_2O	to 1 L

All compounds are dissolved in ultrapure water and final volume is adjusted to 1 liter. It is filtered for sterilization and store at +4 °C. The solution should have clear turquoise colour and it must be discarded when the colour is changed into yellowish-green.

Glucose Feed

500 g glucose anhydrous or 550 g glucose monohydrate is dissolved completely in 1 liter ultrapure water. It is autoclaved at 121 °C on liquid cycle for 20 minutes. 12 mL PTM1 solution is added per liter of glucose solution after it is cooled below 55 °C.

C. CALIBRATION CURVES

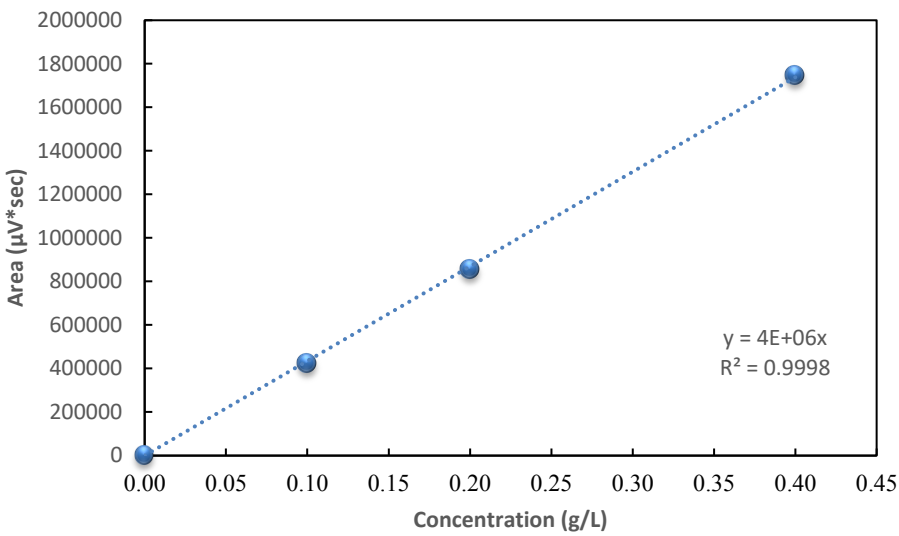

Figure C. 1 Calibration curve of oxalic acid

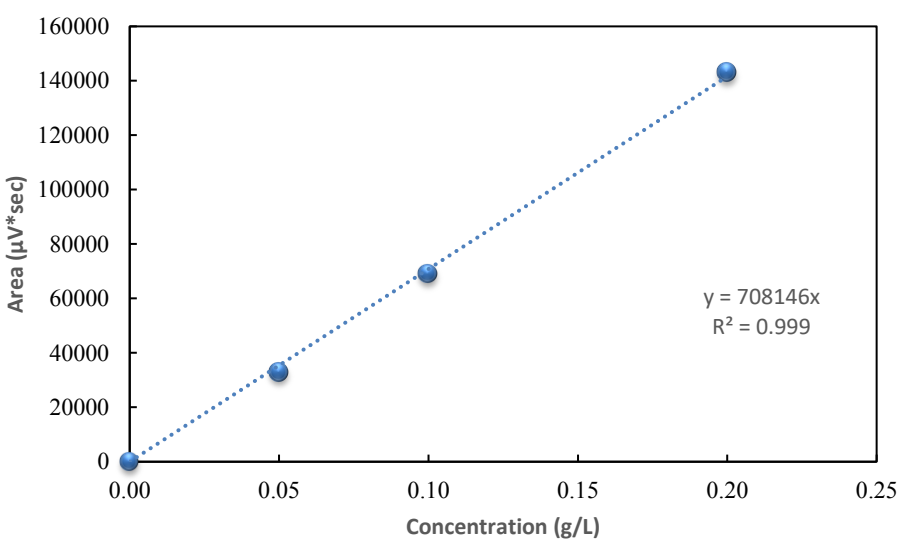

Figure C. 2 Calibration curve of formic acid

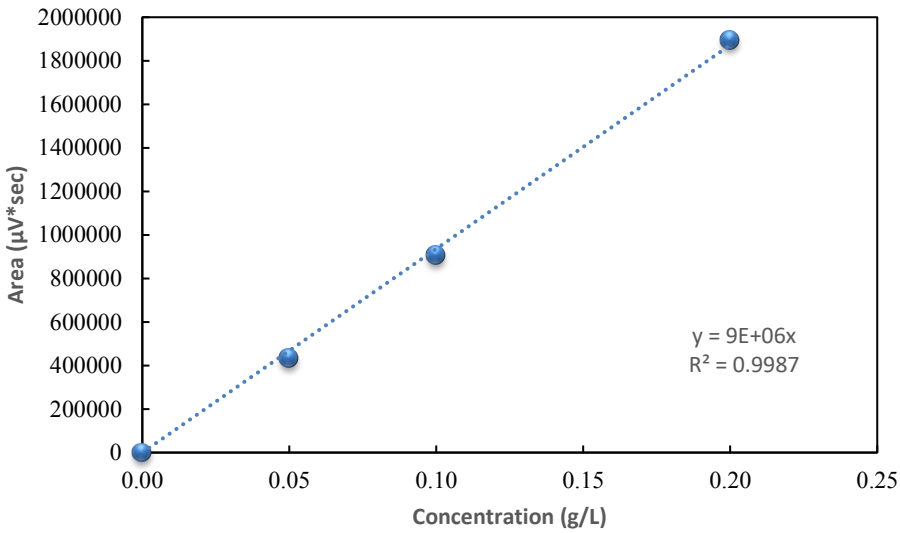

Figure C. 3 Calibration curve of pyruvic acid

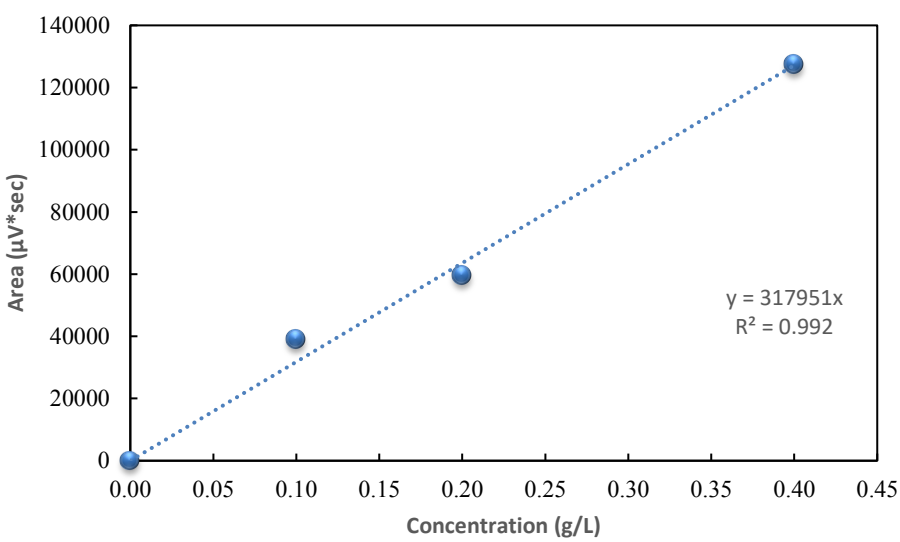

Figure C. 4 Calibration curve of malic acid

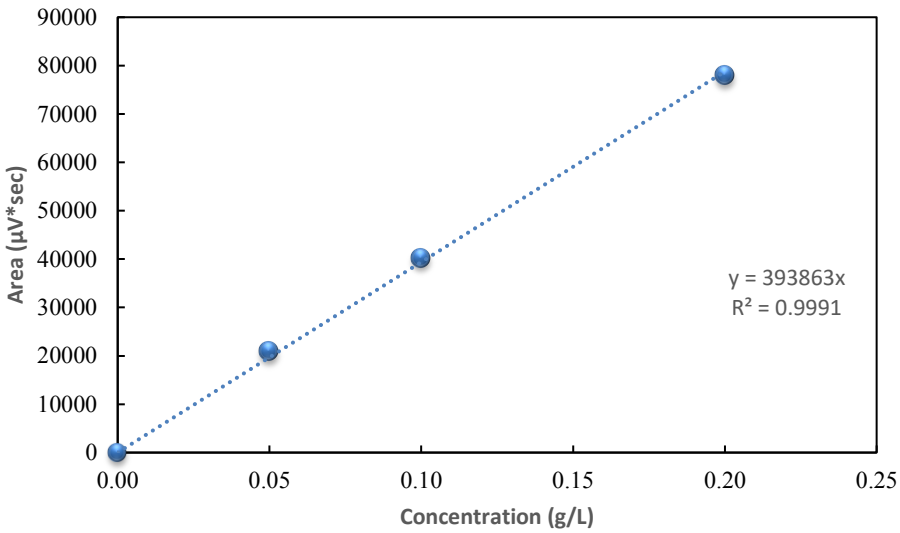

Figure C. 5 Calibration curve of acetic acid

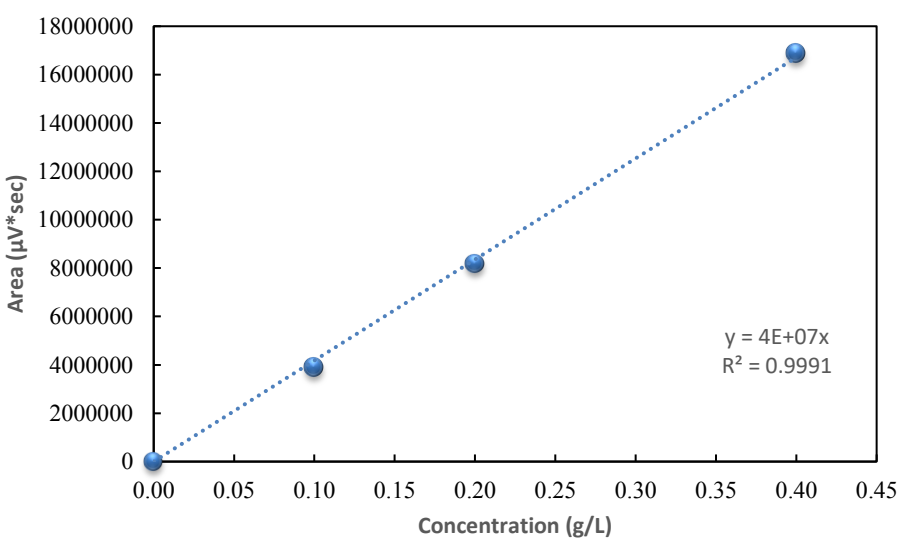

Figure C. 6 Calibration curve of fumaric acid

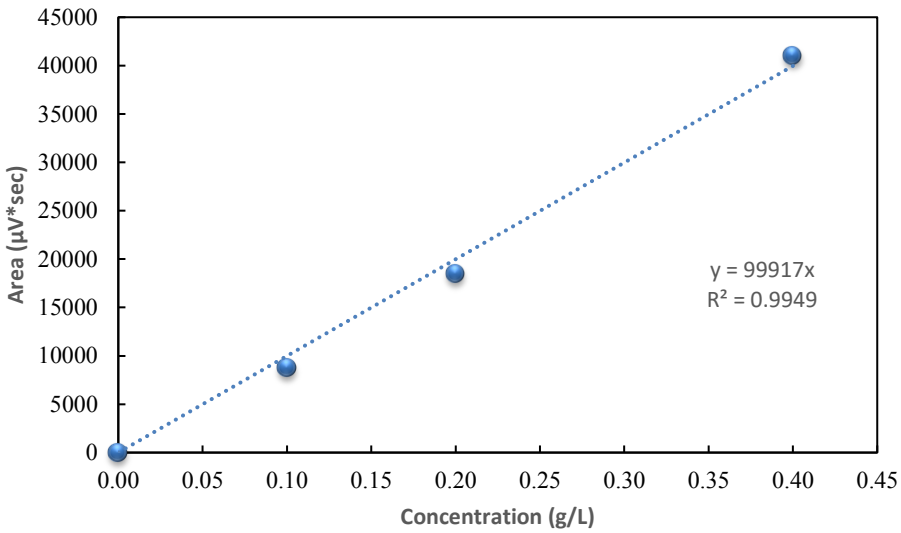

Figure C. 7 Calibration curve of succinic acid

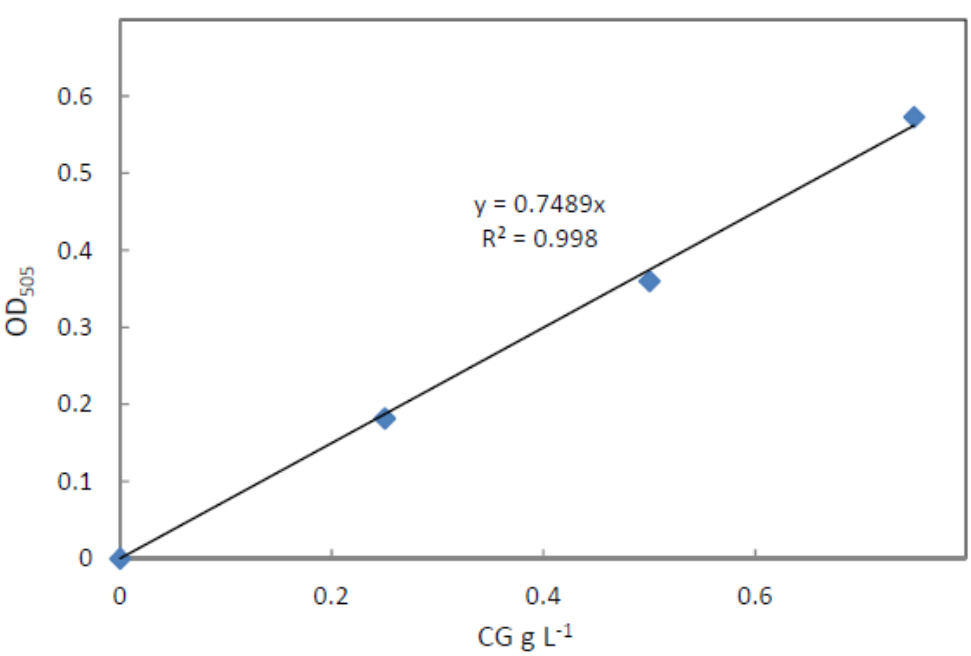

Figure C. 8 Calibration curve for glucose concentration

D. SDS-PAGE PROTEIN ANALYSIS

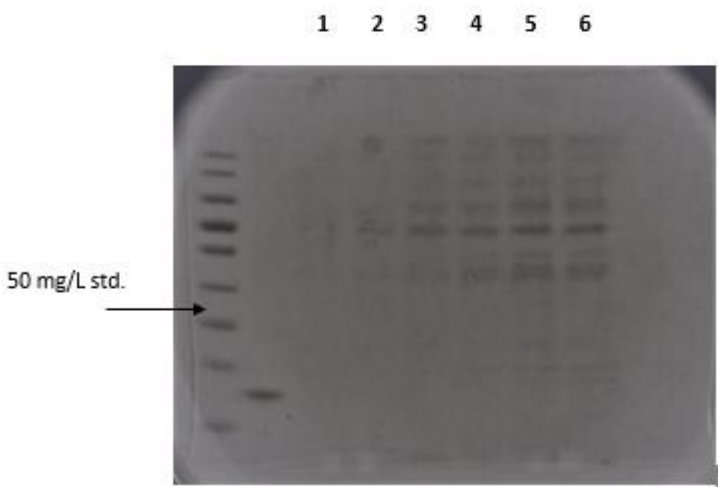

Figure D. 1 Coomassie dye stained SDS-PAGE gel image of proteins produced at CDO = 1%. Lane 1: t=0h, Lane 2: t=3h, Lane 3: t=6h, Lane 4: t=9h, Lane 5: t=12h, Lane 6: t=15h.

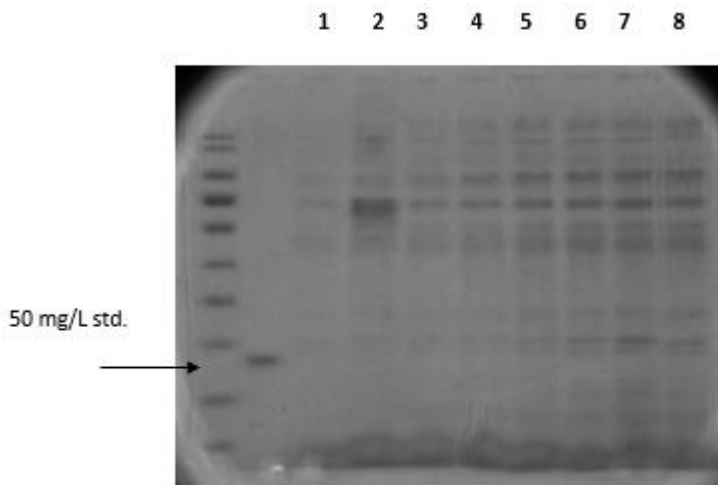

Figure D. 2 Coomassie dye stained SDS-PAGE gel image of proteins produced at CDO = 5%. Lane 1: t=3h, Lane 2: t=6h, Lane 3: t=9h, Lane 4: t=12h, Lane 5: t=15h, Lane 6: t=18h, Lane 7: t=21h, Lane 8: t=24h.

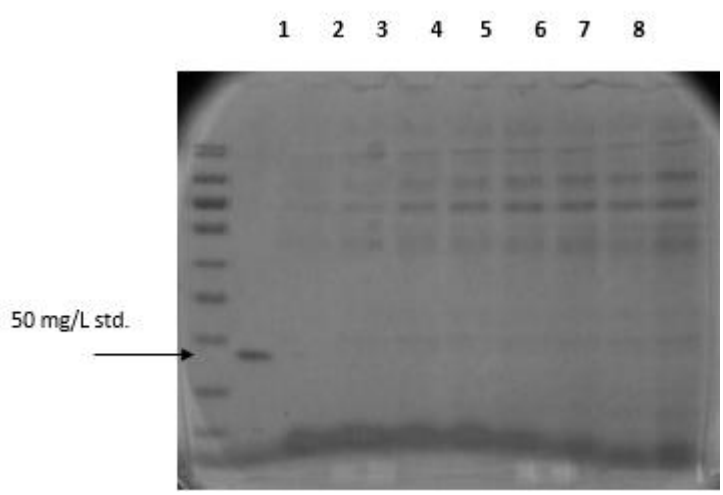

Figure D. 3 Coomassie dye stained SDS-PAGE gel image of proteins produced at CDO = 15%. Lane 1: t=0h, Lane 2: t=3h, Lane 3: t=6h, Lane 4: t=9h, Lane 5: t=12h, Lane 6: t=15h, Lane 7: t=18h, Lane 8: t=21h.

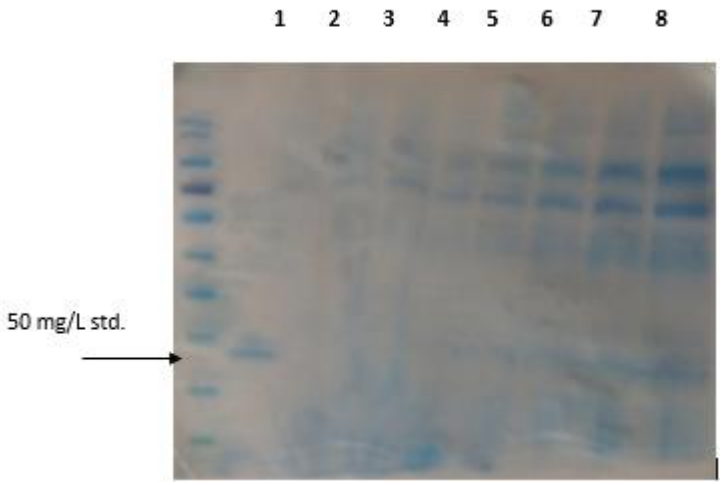

Figure D. 4 Coomassie dye stained SDS-PAGE gel image of proteins produced at CDO = 5% with multi-copy *P. pastoris* strain. Lane 1: t=3h, Lane 2: t=6h, Lane 3: t=9h, Lane 4: t=12h, Lane 5: t=15h, Lane 6: t=18h, Lane 7: t=21h, Lane 8: t=24h.

E. pPYKZαA::*hGH* PUTATIVE PLASMID NUCLEOTIDE SEQUENCE

Green highlight is the P_{PYK}. Blue highlight is α-factor. Grey highlight is *hGH*.

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