

ENHANCED RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION
BY SYNCHRONOUS POPULATIONS OF
TWO DIFFERENT STRAINS OF *Pichia pastoris* UNDER *GAP* PROMOTER

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

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SEPTEMBER 2016

Approval of the thesis:

**ENHANCED RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION
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ABSTRACT

ENHANCED RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY SYNCHRONOUS POPULATIONS OF TWO DIFFERENT STRAINS OF *Pichia pastoris* UNDER *GAP* PROMOTER

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September 2016, 166 Pages

Cell cycle synchronization used as a common procedure for understanding various genomic and metabolic phenomena in a cell is also an essential tool to optimize the production of biomolecules. In this work, the effect of cell cycle synchronization on the recombinant human growth hormone (rhGH) production by *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter was investigated. Starvation as a cost effective and readily applicable synchronization method was performed to investigate various cell cultures at age of $OD_{600} = 4, 8, \text{ and } 16$ to determine the most productive strategy. Thus, two different experimental setups were used, one of which was in air-filtered shake bioreactor cultures to consider the impact of semi-defined and complex pre-cultivation media on the efficiency of starvation concerned with the cell morphology and budding index. The second setup, a 3.0 L-bioreactor, was used to

compare the recombinant protein (r-protein) productions in both synchronous and asynchronous populations grown to $OD_{600} = 4, 8, \text{ and } 16$ by using exponential glucose feeding with pre-determined specific growth rate of $\mu_0 = 0.10 \text{ h}^{-1}$. The highest rhGH concentration was obtained from synchronous cell population at age of $OD_{600} = 16$ as $C_{rhGH} = 570 \text{ mg L}^{-1}$ at $t = 15 \text{ h}$ where the cell concentration was $C_X = 100 \text{ g L}^{-1}$. The overall product and cell yield on total substrate were obtained as $Y'_{p/S} = 2.06 \text{ mg g}^{-1}$ and $Y'_{X/S} = 0.13 \text{ g g}^{-1}$, respectively. We conclude that, this work demonstrates the impact of using aged synchronous populations of *P. pastoris* in r-protein production and paves the way for the design of the starvation based fed-batch operations to increase r-protein production by *P. pastoris*.

Keywords: Aged cell populations, cell cycle synchronization, *GAP* promoter recombinant human growth hormone, *PDC* knockout strain, *Pichia pastoris*, starvation

ÖZ

Pichia pastoris'in İKİ FARKLI SUŞUNUN SENKRONİZE POPÜLASYONLARI İLE *GAP* PROMOTÖRÜ ALTINDA İNSAN BÜYÜME HORMONU ÜRETİMİ

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Eylül 2016, 166 Sayfa

Bir hücredeki çeşitli genomik ve metabolik olayları anlamak için yaygın bir prosedür olarak kullanılan hücre döngüsü senkronizasyonu aynı zamanda çeşitli biyomoleküllerin üretimini optimize etmek için de gerekli bir araçtır. Bu çalışmada, *GAP* promotörü altında *Pichia pastoris* ile rekombinant insan büyüme hormonu (rhGH) üretiminde hücre döngüsü senkronizasyonunun etkisi araştırılmıştır. Uygun maliyetli ve kolay uygulanabilir senkronizasyon metodu olan açlık (starvasyon), en etkin üretimi bulabilmek için $OD_{600} = 4, 8, \text{ ve } 16$ yaşlarındaki popülasyonlara uygulandı. Bu nedenle iki farklı deney programı yönetildi. İlk deney programı, aşılama endeksi ve hücre morfolojisiyle ilgili starvasyon verimliliği üzerinde kompleks ve yarı-tanımlı proses ortamını değerlendirmek için hava filtreli çalkalamalı biyoreaktörlerde yürütüldü. İkinci deney programı, yarı kesikli işletimde önceden belirlenmiş çoğalma hızı $\mu_0 = 0.10 \text{ st}^{-1}$ göre üstel glukoz beslemesi ile $OD_{600} = 4, 8, \text{ ve } 16$ oluncaya kadar yaşlandırılan senkronize ve senkronize olmayan maya kültürlerinin rekombinant protein (r-protein) üretimlerini karşılaştırmak için

gerçekleştirildi. En yüksek rhGH derişimi $OD_{600} = 16$ oluncaya kadar yaşlandırılan senkronize maya popölasyonunda üretimin $t = 15$ st'te 570 mg L^{-1} olarak elde edildi ve hücre derişimi 100 mg L^{-1} 'ye ulaştı. Toplam substrat üzerindeki ürün ve hücre verimi $Y'_{P/S} = 2.06 \text{ mg g}^{-1}$ and $Y'_{X/S} = 0.13 \text{ g g}^{-1}$ olarak bulunmuştur. Bu çalışma, *P. pastoris*'in yaşlandırılmış senkronize popölasyonlarının r-protein üretiminde etkisini ortaya koymuştur ve *P. pastoris* tarafından r-protein üretimini artırmak için starvasyona dayalı yarı kesikli besleme stratejisinin tasarımına imkan sağlamıştır.

Anahtar Kelimeler: Yaşlandırılmış hücre popölasyonları, hücre döngüsü senkronizasyonu, *GAP* promotör, rekombinant insan büyüme hormonu, *PDC* geni silinmiş suş, *Pichia pastoris*, açlık (starvasyon)

To my beloved family

ACKNOWLEDGEMENTS

Foremost, I am deeply grateful to my supervisor Prof. Dr. Pınar Çalık, whose knowledge, guidance, generous advices and support me in all possible ways throughout my M.Sc. study and research program.

I would like to thank my co-supervisor, Prof. Dr. Mehmet İnan for his thoughtful advices and critics and Assoc. Prof. Dr. Çağdaş Devrim Son for his knowledge and guidance in confocal microscopy imaging.

The financial support provided by Technical Council of Turkey (TÜBİTAK- 114R091) and Middle East Technical University Research Fund Project are gratefully acknowledged.

I am grateful to my friends in Industrial Biotechnology and Metabolic Engineering Research Group: Bebeta Hoxha, Duygu Yalçınkaya, Yiğit Akgün, Erdem Boy, Abdullah Keskin, Onur Ersoy, Burcu Gündüz Ergün, Özge Kalender and Tuğçe Erdoğan for their contribution, friendship, help, support and advice. I would like to especially thank to Aslan Massahi, Özge Ata, Sibel Öztürk and Hande Güneş for their friendship and valuable contribution with their experience in my research studies.

I would also like to thank all academic, administrative and technical staff of Department of Biotechnology and Chemical Engineering, METU, for their support through my education.

My special thanks go to Gülfidan Aktaş, Melahat Aktaş, Çağdaş Aktaş and Selahattin Aktaş for their support and love throughout my life in Ankara. I am also grateful to Gülşah Dinçer and Erman Çandereli for their precious friendship, motivation and support.

Most of all, I would like to express my gratitude to my family Cemal Hücetoğulları, Nuran Hücetoğulları and Özge Hücetoğulları who always believed in me and encouraged me to follow myself with their love and support. I owe them so much.

Damla Hücetoğulları

20.09.2016

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NOMENCLATURE

C	Concentration	g L^{-1}
DO	Dissolved oxygen	%
N	Agitation rate	min^{-1}
Q	Volumetric flow rate	L h^{-1}
q	Specific formation or consumption rate	$\text{g g}^{-1} \text{h}^{-1}$ or $\text{mg g}^{-1} \text{h}^{-1}$
r	Formation or consumption rate	$\text{g L}^{-1} \text{h}^{-1}$
t	Cultivation (residence) time	h
T	Medium temperature	$^{\circ}\text{C}$
U	One unit of an enzymatic activity	
V	Volume of fermentation medium	L
Y	Yield	g g^{-1} or mg g^{-1}
Y'	Overall yield	g g^{-1} or mg g^{-1}
OUR	Oxygen uptake rate	$\text{mol m}^{-3} \text{sec}^{-1}$
OTR	Oxygen transfer rate	$\text{mol m}^{-3} \text{sec}^{-1}$

Greek Letters

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

Subscripts

0	Initial condition
O	Oxygen
p	Product
S	Substrate
X	Cell
G	Glucose
F	Fructose
pro	Protease

Abbreviations

<i>AOX1</i>	Alcohol oxidase 1
BMGY	Buffered glycerol-complex medium
BSM	Basal salt medium
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
EDTA	Ethylenedinitrilotetraacetic acid
<i>FLD1</i>	<i>Formaldehyde dehydrogenase 1</i>
<i>GAP</i>	<i>Glyceraldehyde-3-phosphate dehydrogenase</i>
GB	Glycerol batch phase
GFB	Glucose semi-batch phase
hGH	Human growth hormone
hGM	Human granulocyte-macrophage colony stimulating factor

HIV	Human immune deficiency virus
HPLC	High performance liquid chromatography
OD	Optical density
PCR	Polymerase chain reaction
<i>PGK1</i>	<i>Phosphoglycerate kinase</i>
rhGH	Recombinant human growth hormone
r-protein	Recombinant protein
PTM1	Pichia trace minerals
YPD	Yeast Extract Peptone Dextrose

CHAPTER 1

INTRODUCTION

In today's world, biotechnology is a buzz word in many areas, i.e., science, politics, industry, health care, daily life, social work, and also has gradually increased in importance since the Second World War (Verma et al., 2011). Although there are several definitions of biotechnology because of its common uses in these areas, Organization for Economic Cooperation and Development (OECD) defines biotechnology as “the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production or the modification of knowledge, goods and services” (<http://www.oecd.org>).

The applications of biotechnology based on ancient times have been proven by the vinegar, bread, alcoholic beverages, cheese, yogurt as the mostly known microbial products in Antiquity. The *classical biotechnology* era in which the awareness relevant biotechnology was firstly raised is characterized by “Laws of Inheritance” presented by Mendel, discovery of nucleus by Robert Brown, nuclein extracted from white blood cells by Fredrich Miescher, the description of bacterial colonies by Robert Koch, suggestion of chromosome as a term by Heinrich Wilhelm Gottfried Von Waldeyer-Hartz and development of first therapeutic proteins as vaccines against small pox and rabies by Edward Jenner and Louis Pasteur, respectively (Verma et al., 2011). Later, the microbial fermentation industry in early 1900s was made actual by the first large-scale anaerobic fermentations of chemicals such as acetone and butanol and then the aerobic production of citric acid was performed. The fermentation of penicillin was

not carried out until the early 1940 despite its finding in 1927 (Demain and Vaishnav, 2009).

The *modern biotechnology* era was initiated with discovery of the recombinant DNA (rDNA) technology by Berg, Cohen and Boyer in 1973 in California. Within the following two years, the biotechnology industry was founded by the establishment of Cetus Corporation followed by other corporations, i.e., Genentech, Amgen and Biogen (Demain and Vaishnav, 2009). In early 80's, the cooperation of rDNA technology and biotechnology industry put Humulin on the market, being the brand name of human insulin produced by recombinant *Escherichia coli* that is also first recombinant therapeutic protein approved by the U.S. Food and Drug Administration (FDA) in clinical use. Hence, the rDNA technology used in bacterium *E. coli* for the synthesis of both peptides and proteins of interest gave chance to handle probably adequate other organisms, i.e., bacteria and/or yeast (Ferrer-Miralles et al., 2009). In today's biotechnology under the guidance of science including genetic, protein and metabolic engineering, the use of various convenient organisms and biological compounds also releases all types of native and recombinant proteins to the market of agriculture, industry, textile industry, food industry, enzyme industry, pharmaceutical industry and biopharmaceutical industry etc. (Demain and Vaishnav, 2009).

The application of biotechnology in pharmacy is called as pharmaceutical biotechnology which also generates the biopharmaceuticals (Stryjewska et al., 2013) defined as a protein or nucleic acid based pharmaceutical substance used for therapeutic or in vivo diagnostic purposes, which is synthesized by rDNA technology excluding by the methods of chemical synthesis (Walsh, 2002). Hence, the biopharmaceutical industry is so significant owing to the ability to turn proteins into commercial products with high added value to benefit human health. The products of biopharmaceutical industry are categorized into monoclonal antibodies, vaccines, growth factors, cytokines, hormones, blood related products, and additional products including various peptide therapeutics, therapeutic enzymes, cell therapies, e.g., bone morphogenic proteins, and antisense drugs, e.g., RNA based product Macugen (Walsh, 2005). Since the approval of Humulin, the FDA and European Medicine Agency (EMA) have approved 211 protein-based recombinant pharmaceuticals called as

biologic medicines, i.e., human enzymes, hormones and monoclonal antibodies, consisting of 91 (43%) biopharmaceuticals produced by mammalian cells, 66 (31%) ones by *Escherichia coli*, 30 (15%) ones by *Saccharomyces cerevisiae* plus one by *Pichia pastoris* (Berlec and Štrukelj, 2013), 17 (11.2%) ones by hybridoma cells, one by insect cells and last one by transgenic goat (Ferrer-Miralles et al., 2009). Today, the candidate biopharmaceuticals are nearly 4000-5000 in research and development (R&D) in which there are over 10000 of both pharmaceutical and biopharmaceutical therapeutics. The world market being over \$200 billion of biopharmaceutical industry contributes to the worldwide market being approximately \$1 trillion of pharmaceutical industry (BioPlan Associates, 2015). Oddly enough, in biopharmaceutical industry, the over 90 percent of all sales in total market is resulted from about thirty protein-based recombinant medicines, plus the nearly 70 percent of this market share is created by the following seven r-proteins: erythropoietin, alpha interferon, hepatitis B vaccine, granulocyte stimulating factor, insulin, tissue plasminogen activator and human growth hormone (Palomares and Kuri-breña, 2007).

Hormones as chemical messengers synthesized by specific glands are secreted into circulatory system to induce target cells in long distance for the control and maintenance of metabolic activities (Campbell and Reece, 2001). In human body, 9 hormones specific major endocrine glands firstly produce and subsequently excrete 24 crucial hormones (Hiller-Sturmhöfel and Bartke, 1998) and the one among them is human growth hormone (hGH). The mature human growth hormone being non-glycosylated, anionic, peptide hormone consisting of 191 amino acid residues with molecular mass of 22 kDa and isoelectric point (pI) of 5.1 has stable tertiary structure with four helix bundle motif and two disulfide bonds between cysteines, C53-C165 and C182-C189. Through the life, the hGH known as “somatotropin” is in charge of the maintenance and control of body composition, human metabolism comprised of carbohydrate, lipid and protein metabolism, and intermediary metabolism, i.e., citric acid cycle, pentose phosphate pathway (Morowitz et al., 2000) as well as the body growth until the end of puberty.

Owing to the significant role of hGH on human metabolism and body growth, the hGH is used as therapeutic protein for the last 60 years to treat several diseases

such as GH insufficiency in both children and adults, Prader Willi Syndrome, Turner's syndrome, idiopathic short stature, and AIDS. In early 1940s, the first growth hormone (GH) therapy was applied to GH deficient children by GH extracted from the bovine or porcine. The hGH therapy for GH deficient children was initiated just after the first isolation of cadaveric hGH by Li and Rapkoff in 1956 and so was maintained with only cadaveric hGH from 1960 to 1985 in which the FDA received a report about the use of cadaveric hGH culminated in prion mediated Creutzfeldt–Jacob disease observed in four adult patients. Within the same year, the therapy with cadaveric hGH was abolished and then this therapy was preformed by the first recombinant human growth hormone (rhGH) expressed in the expression system *E. coli*. This product was released to market with its brand name, Protropin®, by Genentech in 1981 and also called as somatrem since N-terminal-methionylated form of hGH. The methionylated and non-methionylated form of rhGH are denominated as “somatrem” and “somatropin” together with brands name, respectively. Today, the 12 present brands delivered by 9 companies are only somatropin. Among them, the eight ones in *E. coli* and the one in *S. cerevisiae* are synthesized while the three ones are obtained from C127 mouse cells. For further rhGH production, *Bacillus subtilis*, *Pichia pastoris* and Chinese hamster ovary cells are other organisms used until today (Lindlom, 2006; Ayyar, 2011).

The *P. pastoris* introduced by Koichi Ogata in 1969 is one of methylotrophic yeasts being capable of utilization methanol as a sole carbon and energy source. To seize the opportunity relevant methylotrophic characteristic of *P. pastoris*, the Phillips Petroleum Company developed growth and fermentation medium with the suitable continuous operation to obtain high amount of single cell proteins (SCPs) in early 1970s. This system was more popular due to lower prices of methanol derived from natural gas methane until oil crisis in mid-1970s. In 1980s, the association between Phillips Petroleum Company and the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA, USA) brought a prestige via the use of *P. pastoris* derived expression systems for synthesis of recombinant proteins prior to 1993 in which current patent holder, Research Corporation Technologies (RCT) (Tucson, AZ) released the commercial kit of *P. pastoris* based expression systems for academy (Cregg, 2000; Cereghino, 2002)

There are over 1000 recombinant proteins produced by *P. pastoris* since its discovery to date. Additionally, it is convenient host organism for recombinant therapeutic proteins since the following advantages: (i) commercial kit of expression systems; (ii) the ease of genetic modification with ready protocols; (iii) the high stability of foreign gene inserted into genome; (iv) strong inducible and constitutive promoter; (iv) growth on inexpensive basal salt medium with high cell densities and high titer products (v) availability of recombinant proteins being the vast majority in fermentation medium because of less secreted homolog proteins; (vi) ability of post translational modifications such as disulfide bridge formation, folding and glycosylation; (vii) ease of downstream process related with secretion characteristic. Nevertheless, the inadequate glycosylation and the absence of chaperonins are drawbacks (Damasceno et al., 2012; Li et al., 2007; Fidan and Zhan, 2015; Jahic et al., 2006; James M Cregg et al., 2000)

Today, several recombinant protein-based therapeutics produced by *P. pastoris* are in either the market or R&D phase under the control of pharmaceutical companies. Human serum albumin, insulin, IFN- α and hepatitis B vaccine are released in India and/or Japan. The recombinant kallikrein inhibitor ecallantide (Kalbitor[®], Dyax, MA, USA) is the firstly approved recombinant protein in *P. pastoris* by FDA in 2009. ALD518, a humanized monoclonal antibody directed against human IL-6 (Alder Biopharmaceuticals, WA, USA) is in clinical pipeline. A fusion protein composed of an anticarcinoembryonic antigen single-chain fragment and the enzyme carboxypeptidase G2 is in clinical phase for drug therapy against breast cancer (MFECP1, Cancer Research Technology, UK) (Gasser et al., 2013)

The rhGH production in *P. pastoris* driven expression systems was firstly carried out by Ecamilla et al. (2000). The obtained rhGH concentration under the control of *AOX1* promoter was 49 mg L⁻¹ in 2 L bioreactor. Eurwilaichitr et al. (2002) studied the possibilities related to efficient secretion of rhGH in *P. pastoris* and obtained 190 mg L⁻¹ rhGH in 10 ml buffered minimal complex medium as an induction medium with induction every 24 h to maintain C_M=1% (v/v) during 144 h of cultivation. The rhGH production in *P. pastoris* was frequently carried out by Çalık and coworkers. Until 2012, the rhGH synthesis under the control of *AOX1* promoter

was tried to improve with different strategies of fed-batch operation conditions. The first study was the construction of a novel expression system including *AOX1* promoter leading hGH gene sequence and reached 350 mg L⁻¹ of rhGH (Çalık et al., 2008) and followed by batch operation yielding 110 mg L⁻¹ to evaluate the influence of carbon sources on the rhGH production (Orman et al., 2009). The researches concerned with the effect of pH (Çalık et al., 2010) and methanol feeding rate (Çalık et al., 2011) on rhGH synthesis were culminated in same concentrations of rhGH, 270 mg L⁻¹. In 2012, the first *GAP* promoter driven expression system allowing rhGH synthesis was developed and resulted in 220 mg L⁻¹ (Zerze, 2012). Up to today, the highest concentration of rhGH expressed by *P. pastoris* under the control of *GAP* promoter was acquired as 508 mg L⁻¹ (Keskin, 2014) whereas the highest one of rhGH expressed under the control of *AOX1* promoter was attained as 1300 mg L⁻¹ (Güneş et al., 2014).

Cell cycle synchronization as one of the molecular biology methods arrests the cells in a certain phase of cell cycle progression and also promotes the individual cells to behave just like one huge cell. This method facilitates to understand the phenomena concerned with intracellular actions, events in the nucleus, cell growth and metabolism. The methods relevant for cell cycle synchronization are principally classified into induction methods and selection methods. Induction methods block the cell cycle by any physical inducer, i.e., temperature, or chemical inducer, i.e., hydroxyurea, and then release cell cycle phase by removal inducer. Starvation among them is physical inducer that allows to minimize asymmetry resulted from the difference between bud size and the size of mother cell. On the other hand, selection methods separate the cell according to cell size indicating the phase involved by physical device. In *S.cerevisiae* being model organism for genetic studies, the recombinant protein production and secretion depend on cell cycle phases.

In this study, the construction of new gene knockout strain called as *P. pastoris* *pdca*Δ was investigated to enhance rhGH production. To characterize this new strain, different carbon sources, glucose, glycerol and mixing of glycerol and acetate, were used to evaluate the efficiency of r-protein synthesis under the control of *GAP* promoter. Later, the comparison in rhGH expression between two strains of *P. pastoris*, *PDC* deficient and non-deficient strain allowed to decide which strain should

be included in experiments relevant the synchronization. Starvation among several synchronization methods was used owing to its easy applicability to large volumes of yeast populations and to feeding strategies. The synchronization as a molecular tool was examined to yield high concentrations of recombinant protein produced when considering the effect of both aging and various pre-cultivation media on synchronization. To figure out the effect of synchronization on rhGH production, the yeast populations aging to $OD_{600} = 4, 8, \text{ and } 16$ were performed in fed-batch operation using exponential glucose feeding with a pre-determined specific growth rate of $\mu_0 = 0.10 \text{ h}^{-1}$. For this purpose, yield coefficients, substrate consumption rates and product formation rates resulted from the experiment data for each designed semi-batch bioreactor were calculated and compared with each other to determine the best strategy.

CHAPTER 2

LITERATURE SURVEY

2.1 Hormones

The cell responds other cells and changes in their environment to survive with the motion toward eligible places or away from dangerous situations. This cellular messaging between them and their environment is actualized by chemical signaling. In this type of signaling, the cellular conversion of chemical messengers is analyzed in three stages: reception, transduction and response. The extracellular information is detected by molecule specific receptor proteins and transduced by the changes in receptor protein and inside cells, and then a specific cellular response is triggered by this transduced signal. Additionally, the communication between cells is supported by several signaling ways such as paracrine, autocrine, synaptic or endocrine. The paracrine, autocrine and synaptic cell signaling are carried out by local regulators, i.e., neurotransmitters whereas the endocrine one induces the target cell by distal regulators, i.e., hormones (Campbell and Reece, 2001; Nelson and Cox, 2005). Hormones as chemical signal molecules in endocrine signaling, are produced by specific glands and then released into the circulatory system to reach the distal target cells. Hence, they serve as relatively long distance chemical messengers to evoke a receptor mediated response for the control and regulation of specific actions involved in target cell. In multicellular organisms, the integration and coordination of differentiated cell functions are promoted by hormones for their survival (Campbell and Reece, 2001; Bhavagan, 2002a). In general, these mentioned regulatory functions of hormones are maintenance of cell growth, development and metabolism; and the

reproduction (Wuttke, Jarry, and Seidlová-Wuttke, 2010; Hiller-Sturmhöfel and Bartke, 1998).

2.1.1 General Consideration of Hormones

The main principle of hormonal activity is based on the connection of two molecules, the hormone and its receptor, and on the types of them. Firstly, the hormone is released by the specialized cells in endocrine glands in response to stimuli resulted from the disturbances in homeostasis and then it reaches the target cell, tissue or organ. If the hormone is lipid soluble, it permeates through cell membrane and then it activates intracellular membrane receptor. Otherwise, if the hormone is water soluble, it interacts with cell surface receptor. Then, the way of signal transduction can be occurred according to this receptor molecule, large proteins. In case of activating intracellular membrane receptor, the dimer of receptor molecule and hormone adheres to the regulatory region of their target genes for stimulation of gene expression and cellular function. On the other hand, when the cell surface receptor binds its hormone, this interference initiates cascade events inside the cell to alter structure of mediatory messengers and thereby procures the desired enzyme activity or gene expression (Huhtaniemi and Martini, 2014). In addition, any receptor must have influential “capture” system for its hormone due to lack of relation same as lock and key model between substrate and enzyme and to low concentration of the hormone circulated in bloodstream (Norman and Litwack, 1997).

The hormonal activity in endocrine system is regulated with several stages under the control of central nervous system of brain. Firstly, the environmental signal is transduced by the neurosecretory cells of hypothalamus and then hormone specific releasing hormones (RH) are produced. Secondly, they are secreted by hypothalamus into the capillaries of median eminence and transported through circulatory system until the pituitary. Thirdly, when RH reaches pituitary, the production of pituitary hormones and transfer of them along endocrine glands are triggered. Lastly, the inherence between pituitary hormone and target cell in those glands culminates in the release of desired hormone called as target gland hormone that can move along the ultimate target of interest such as liver, heart etc. (Figure 2.1A). This regulation also is control by feedback mechanism. For instance, in negative feedback from target

gland to hypothalamus and pituitary, when the level of target gland hormone reaches the optimum predetermined value, hypothalamus and pituitary discontinue the its release (Figure 2.1B) (Kleine and Rossmanith, 2016; Hiller-Sturmhöfel and Bartke, 1998).

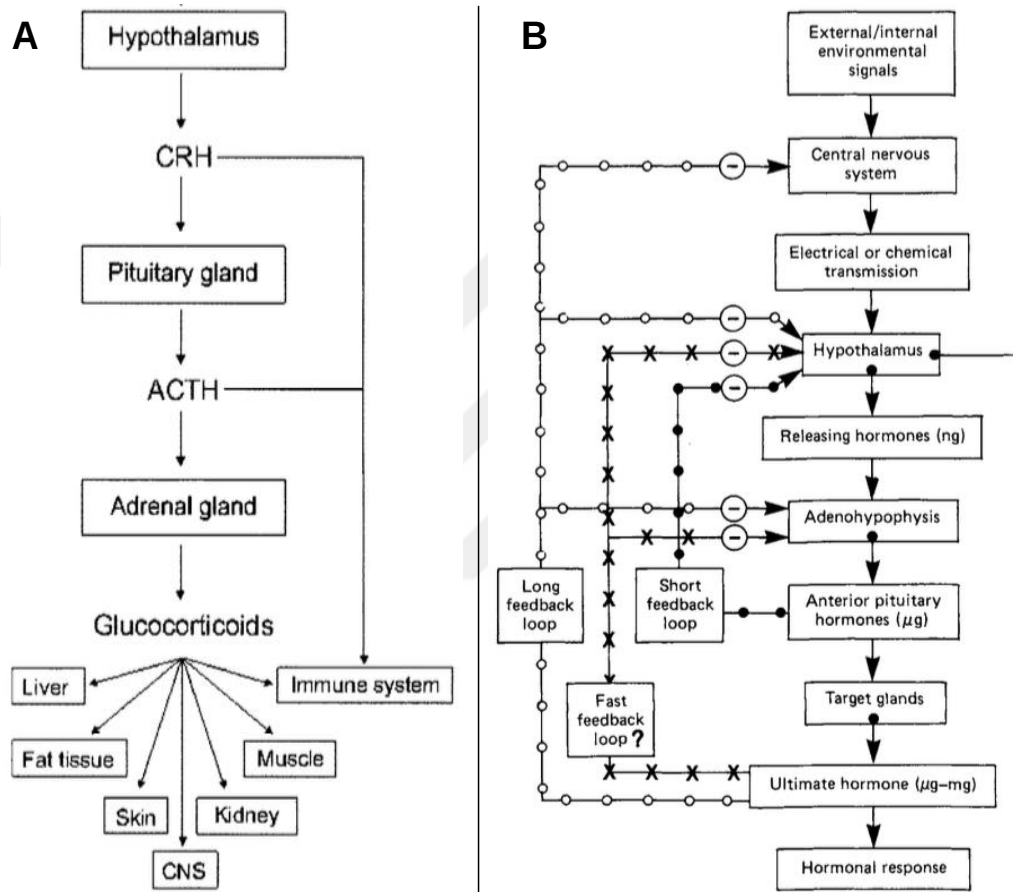


Figure 2. 1 Schematic diagram of hormone system (A) without feedback control (Paez-Pereda and Stalla, 2005) and (B) with feedback control (Norman and Litwack, 1997)

In general, the hormones are synthesized by three levels of endocrine glands. First level of endocrine glands is consisted of hypothalamus, adrenal, medulla, thyroid C-cell and gastrointestinal enterochromaffin cells which are tissues derived from nerve cells associated with nervous system. The second level of them includes anterior pituitary, pancreatic islet, and parathyroid that are relatively dependent on nervous

system. The last one is adrenal cortex, thyroid follicles and gonads which are stimulated by anterior pituitary. Additionally, the hormones secreted by endocrine glands are classified into four main groups based on chemical structure (Bhavagan, 2002a):

- **Hormone amines:** The hormones are simply modified forms of amino acids. These decarboxylated amines with the except of thyroid hormones are produced both in and out of nervous system. Also, these derivatives, i.e., dopamine, serve as neurotransmitters in nervous system (Bhavagan, 2002a). Tyrosine and tryptophan are primary amino acids for this category hormones. Triiodothyronine and thyroxine as thyroid hormones, and epinephrine are made from tyrosine as melatonin and serotonin are derived from tryptophan (Kleine and Rossmanith, 2016).

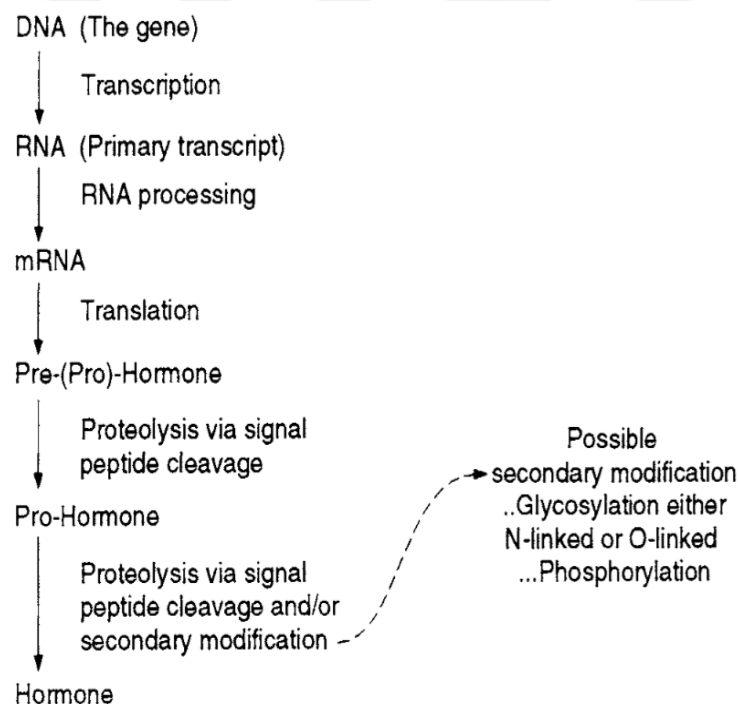


Figure 2. 2 Schematic diagram of steps involved in the synthesis of peptide hormone (Norman and Litwack, 1997).

- Peptide, protein or glycoproteins: These hormones are synthesized like any other proteins: transcription and translation. Preprohormone composes the backbone of all peptides and many proteins that act as hormone. This molecule is converted to prohormone by the cleavage of signal sequence included at its amino terminal end when it reaches endoplasmic reticulum (ER). The prohormone can be packaged into immature secretory granules by Golgi complex and then posttranslational modification of prohormone via proteolytic cleavage of prohormone can be resulted in mature hormone. Lastly, the mature hormone stored is released in mature vesicles (Figure 2.2). Furthermore, regulation of reproduction, energy balance, blood pressure and hormonal hierarchy is controlled by this class hormones such as gonadotropins, insulin, angiotensin and gonadotropin-releasing hormone, respectively (Bhavagan, 2002a; Kleine and Rossmannith, 2016)
- Steroids: There are many steroid hormones which they differ from each other according to structure and action despite the all are produced from cholesterol that is synthesized *de novo* from acetate (90%) or obtained from diet (10%) (Brandt, 2016). These hormones involved in this category are mainly classified into corticosteroids and sex steroids. Corticosteroids are typically produced in adrenal cortex as sex steroids are synthesized in gonad or placenta. In addition, corticosteroids are consisted of two sub-classes, glucocorticoids and mineralocorticoids while sex steroids include androgens, estrogens and progestogens. Each type of steroids has specific physiological effects. For instance, glucocorticoids are responsible for regulation of energy metabolism whereas mineralocorticoids are responsible to adjust electrolyte balance. Furthermore, sex steroids principally maintain the sexual development and reproductive functioning (Henley et al., 2005; Craigie et al., 2009)

- Eicosanoids (derivatives of fatty acids): The main eicosanoids are prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) that are mostly derived from essential fatty acid “arachidonic acid”. These hormones containing 18-22 carbon are bioactive, oxygenated long chain fatty acids. This group of hormones takes charge in regulation of ovulation, luteolysis, blood flow, aggregation, vasodilation etc. (Tolman, 2000; Krause and Dubois, 2000; Henry, 2014; Smith and Murphy, 2016)

Many hormones, their chemical structures and the target glands included in their production and secretion are shown in Table 2.1.

Table 2. 1 Classification of hormones according to their site of production and chemical structure (Huhtaniemi and Martini, 2014)

Site of Production	Name of Hormone	Chemical Structure
Hypothalamus	Growth Hormone Releasing Hormone (GHRH)	Peptide
	Dopamine	Tyrosine metabolite (Hormone amine)
Anterior Pituitary Gland	Thyroid Stimulating Hormone (TSH)	Glycoprotein
	Prolactin	Protein
	Growth Hormone (somatotropin)	Peptide
Posterior Pituitary Gland	Oxytocin	Peptide
Thyroid Gland	Thyroxine	Tyrosine derivative (hormone amine)
Ovary	Estradiol	Steroid
Eicosanoids	Prostaglandins	Fatty acid derivative

2.2 The Target Product: Human Growth Hormone

Growth hormone (GH) expressed in all vertebrates participates in the growth, reproduction and regeneration of somatic cells but its structure depends on species (Perez-Ibave et al., 2014). In human, the modulation of growth is directly linked to the human growth hormone (hGH) known as somatotropin until the completion of puberty whereas the body composition, somatic cell growth and intermediary metabolism are arranged by hGH throughout the life (Kato et al., 2002). The available amount of GH in circulatory system of human is mostly originated from the anterior pituitary gland whereas the remaining is supplied by extra-pituitary growth hormone produced within neural, immune, respiratory and reproductive tissues such as skin, ovary, uterus, liver etc. (Perez-Ibave et al., 2014). Nonetheless, the GH is synthesized by placenta in prenatal period due to repression of the gene expression in mother's pituitary gland. Therefore, multiple isoforms of human growth hormone are available throughout the life and so it is called heterogeneous protein (Baumann, 2009).

2.2.1 Gene Locus, Expression and Structure Human Growth Hormone

The gene locus of hGH resides on the long arm of human chromosome 17 at bands q22-24. It occupies 50 kilobases with highly conserved five genes: two of hGH (hGH-N and hGH-V), two of chorionic somatomotropin (hCSH; hCSH-A and hCSH-B) called as placental lactogen and one of chorionic somatomammotropin like hormone (CSH-L) as pseudogene. They are positioned from 5' to 3', hGH-N, hCS-L, hCS-1, hGH-V, hCS-2 (Figure 2.3). These genes are produced in different tissue in spite of high sequence similarity between them. The GH-N gene is transcribed by the somatotroph cells of anterior pituitary as the others are expressed by placental trophoblasts. The hGH-V gene is only expressed in prenatal period but somatotroph cells synthesize the hGH-N in entire life excepting prenatal one. The completely biological active products of hGH-V gene and hGH-N gene are peptide hormones with same molecular mass of 22 kDa consisting of 191 amino acids. However, the sequence of hGH-V differs from the other one at 13 amino acid positions and thus it is more basic rather the product of hGH-N (Baumann, 2009).

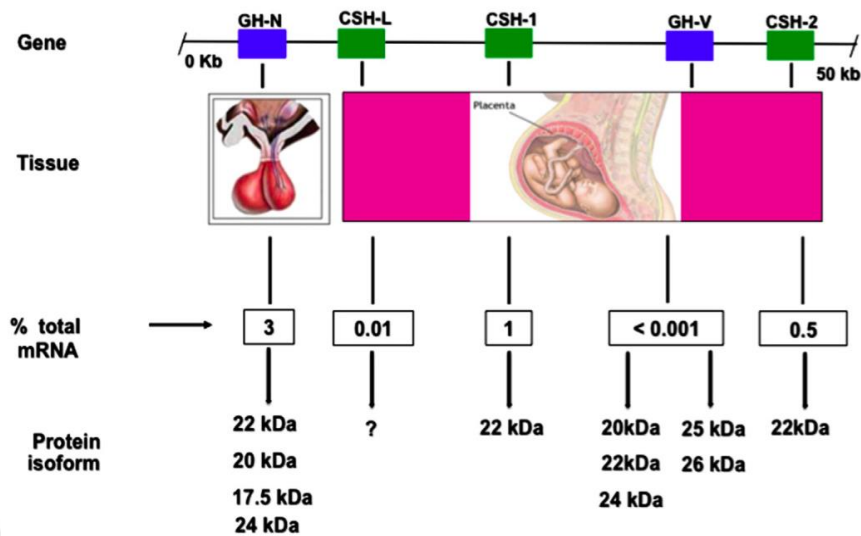


Figure 2. 3 The human GH locus relevant level of mRNA and protein isoforms (Perez-Ibave et al., 2014)

In human bloodstream, the most abundant form of hGH is the hGH-N gene transcript expressed in anterior lobe of pituitary gland by taking extra-pituitary growth hormone synthesis into consideration. Further to that, since the heterogeneity resulted from mRNA splicing and post-translational processing, the two distinct transcript of hGH-N gene in anterior pituitary are circulated. In bloodstream and anterior pituitary, 72% of the hGH is the primary abundant protein with molecular mass of 22 kDa as the product of full length sequence yielding 191 amino acids. The remainder of hGH is second abundant protein comprising 176 amino acids with molecular mass of 20 kDa due to alternative splicing of messenger RNA (mRNA) causing deletion of residues 32-46 and third abundant isoform having molecular mass of 17.5 kDa with lacking residues 32-71 by skipping exon 3. However, the second isoform promotes growth action similar to done by primary isoform but it lacks insulin like activity. The third isoform has not been synthesized in significant amounts under normal conditions.

Consequently, the hGH isoform responsible for therapeutic action throughout the life is mature hGH resulted from the proteolytic cleavage of 26 amino terminal signal peptide of preprohormone composed of 217 amino acids in anterior pituitary. This mature hGH comprising 191 amino acid residues (Figure 2.4) with molecular mass of

22 kDa is non-glycosylated, anionic, single polypeptide chain and it has an isoelectric point (pI) of 5.1 with the empiric formula of C₉₉₀H₁₅₂₉N₂₆₂O₃₀₀S₇.

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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

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Figure 2. 4 The hGH gene sequence

The tertiary structure of hGH is contributed by twist bundle of antiparallel four helixes located between residues 9–34, 72–92, 106–128 and 155–184, respectively (Figure 2.5A) and by two disulfide bridges due to the large loop of disulfide linkage between 53rd and 165th cysteine of the amino acid sequence and the small loop of that between 182nd and 189th cysteines of the amino acid sequence (Figure 2.6). Further, the helical structure of hGH makes protein very compact and stable owing to hydrophobic residues at inner core of four helix bundle. The localization of four helixes also manipulates receptor binding sites. It has two independent receptor binding sites located on opposite surfaces of GH (Figure 2.5B), site 1 including the central to the C-terminal region of helix 4 with loop 54-74 and the site 2 including helix 3 and helix 1. In case of low concentrations, hGH selectively binds its receptor through high affinity receptor binding site, site 1, and then the site 2, which has relatively low affinity compared to that of site 1, can sequentially interact with its receptor.

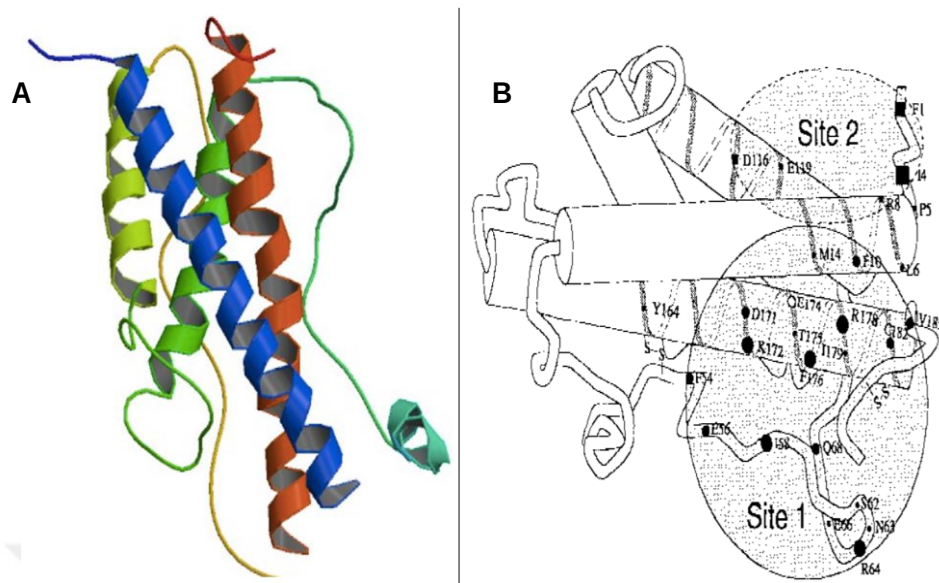


Figure 2. 5 The illustration (A) of tertiary structure of hGH, and (B) of receptor binding sites of hGH (Wells and de Vos, 1993)

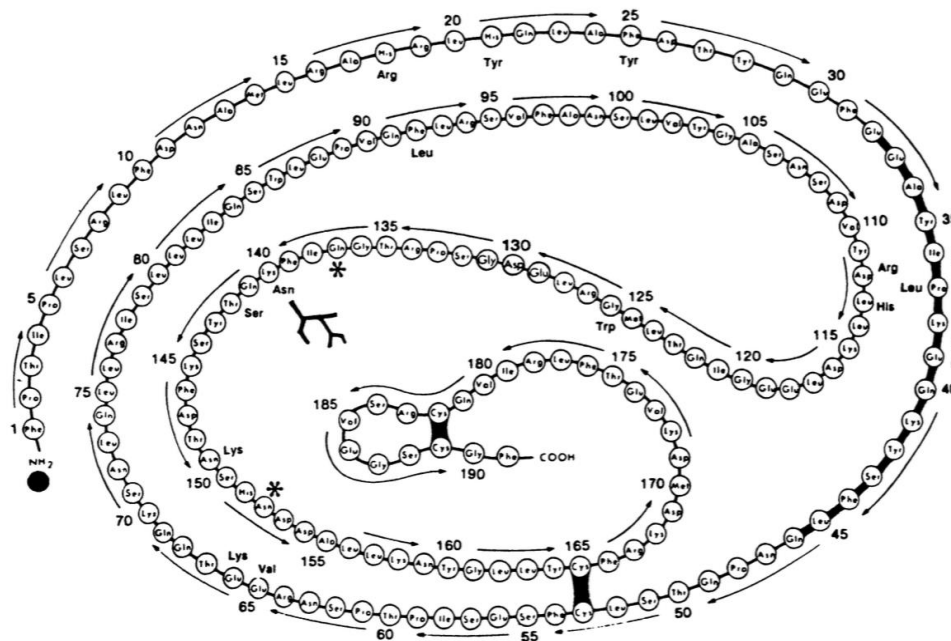


Figure 2. 6 The primary structure of hGH with two disulfide bridges and molecular weight of 22 kDa (Jorgensen, 1997)

2.2.2 Regulation of Human Growth Hormone Secretion

The hHG in blood is either in free state or in complex of GH and GH binding protein (GHP). The complex (GH/GHP) improves the half-life of GH in circulatory system by reducing its rate of degradation and also restricts the extravascular presence (Weigent 1996). The available amount of hGH is mainly regulated by reciprocal actions of two hypothalamic regulators, GH releasing hormone (GHRH) called as somatocinin promoting GH release and somatostatin (SS) named as somatotropin release inhibiting factor (SRIF) suppressing that action (Kochar, 2010), and by physiological stimulators and inhibitors.

When GHRH, peptide hormone including 44 amino acids, binds its receptor (GHRHR) on the anterior pituitary, which has amino terminal signal peptide and structure of G-protein coupled receptor indicating seven transmembrane helices, this interaction induces the expression of the GH gene through cyclic-adenosine 3', 5'-monophosphate (cAMP)-mediated action activated by GHRHR. Then, it excites GH secretion by calcium-mediated pathway activated by GHRHR. On the contrary, if SRIF, peptide hormone comprising 14 amino acids, interacts with its receptor on the anterior pituitary, these mechanisms are suppressed (Kato et al., 2002; Kopchick and Woodley, 1997).

Additionally, GH is also modulated by other hormones, i.e., insulin, insulin like growth factor (IGF-1), and even GH, that participate in negative feed back resulting in suppressing GH release, and by glucocorticoid hormones, thyroid hormone, and retinoic acid as a form of vitamin A, that upregulate the GH gene expression (Kopchick and Woodley, 1997). Under unstressed conditions, fine-tuned regulation of hGH via substrate feedback to hypothalamus demands cortisol and thyroid hormone to act hGH in amino acid uptake and protein synthesis (Bhavagan, 2002b). In addition, The IGF-1 plays an important role of hGH regulation. The synthesis of IGF-1, somatomedin 1, in liver is induced by GH that also controls the production of IGF-1 in other tissues (Clemmons, 2004). If the amount of IGF-1 is increased in blood, the negative feedback control displays and the GH secretion is ceased at the level of both the hypothalamus and the pituitary. Basal and GHRH-stimulated GH secretion at the level of pituitary and GH specific mRNA levels in

human hypothalamus are repressed by IGF-1 promoting release of somatostatin (Kopchick and Woodley, 1997). Figure 2.7 shows the regulation of GH by hormones and physiological factors.

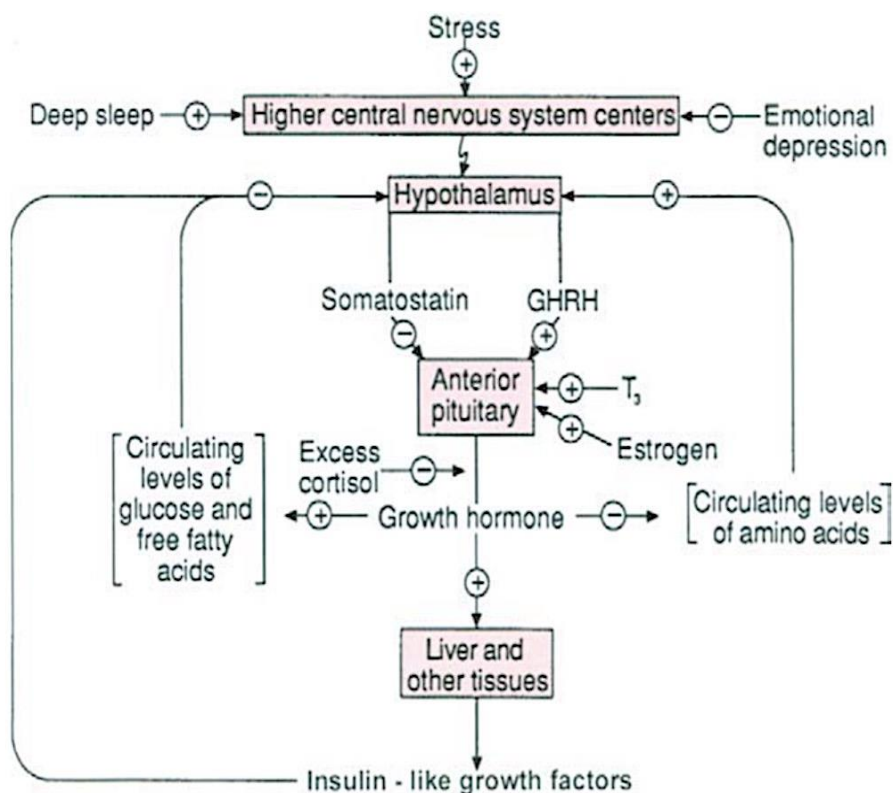


Figure 2. 7 The Regulation of GH by hormones and physiological factors (Bhavagan, 2002b).

2.2.3 Physiological effects of human growth hormone on metabolism

The physiological action of growth hormone in metabolism has been contended for about 80 years. Bernardo Houssay demonstrated first in 1930 that injection of purified GH improved the state of dogs had hyperglycemia. Up to today, the role of GH in postnatal growth and anabolism in body has been postulated and so the researches have shown that the metabolic action of GH is primary compared to the growth promoting action (Sperling, 2016; Møller et al., 2003). However, this order of importance in the role of GH changes according to age of human. For instance, the abnormalities in growth during childhood and metabolic disorders in adults are resulted from disorder of GH synthesis and secretion. Further, the relation of HG with

other hormones, neurotransmitter and growth factors alters the fate of body growth and function. During puberty, the GH with sex steroid secretes at high level to actuate gender specific body composition and then the level of GH synthesis and secretion reduces by aging.

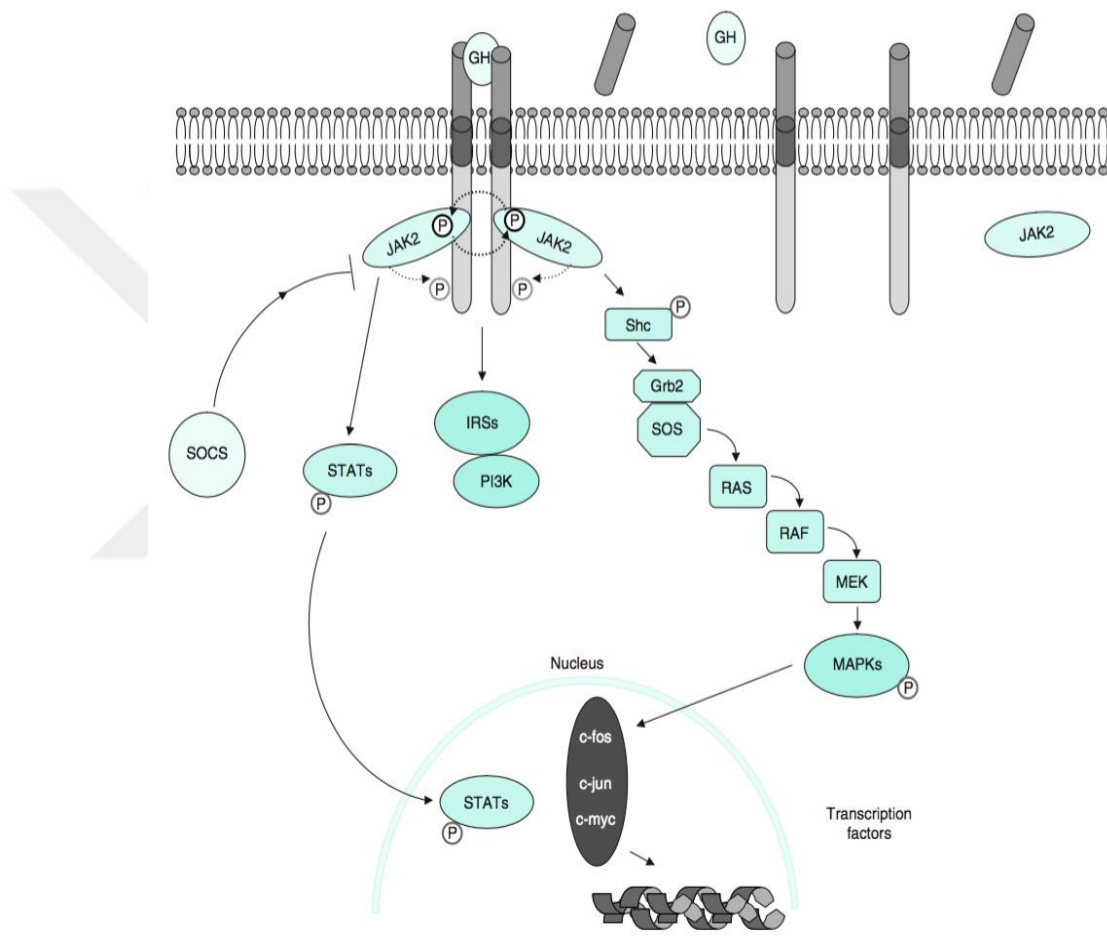


Figure 2. 8 The signaling pathway of GH regulation (Benedini et al., 2008)

To orientate mitogenic and metabolic actions concerned with GH, many signaling pathways should be stimulated and one of them is JAK2 (Janus kinase2)/STAT5 (the signal transducer and activator of transcription 5) signaling pathway. The signal transduction is initiated by activation of specific GH receptors (GHRs) via the binding of GH to them on the target cell surface. These specific binding on the plasma membrane conducts the activation of JAK2 and then it phosphorylates

STAT5. As a result of phosphorylated STAT5, the transcription of GH related genes are encouraged by the dimerization and translocation of phosphorylated STAT5 throughout the nucleus. This pathway regulates somatic growth and protein anabolism. The other major signaling pathways are shown in Figure 1.8. The insulin receptor substrates (IRSs) are phosphorylated by activation of receptors (GHRs) and then it binds and activates phosphatidylinositol 3-kinase (PI3K). Thus, it stimulates lipogenesis and anti-lipolysis. In the other signaling pathway similar to that in JAK2/STATs, Src homology domain containing protein (Shc) is phosphorylated and then it phosphorylates mitogen activated protein kinase (MAPK). Hence, it activates mitogenic action of GH. The last major signaling pathway is p125 focal adhesion kinase (FAK) induces the Jun amino terminal kinase (JNK) that manages cytoskeleton reorganization action of GH. However, The JAK2/STAT5 signaling by GH is negatively regulated by two group of inhibitors: the suppressor of cytokine signaling (SOCs) and protein tyrosine phosphates (PTPs) (Cázares-Delgadillo et al., 2011; Vijayakumar et al., 2009; Sonntag, Ramsey, and Carter, 2005).

Metabolic functions of GH are ordered as following (Vijayakumar et al., 2009; Benedini et al., 2008; Sperling, 2016) and shown in Figure 2.9:

- Metabolic effects on lipid metabolism
 - Stimulation of triglyceride (TG) lipolysis in adipose tissue by increased activity of hormone-sensitive lipase (HSL) and so resulting in increase of free fatty acids (FFA) circulated in bloodstream
 - Impact on adipogenesis
 - Repression of insulin action in adipose tissue by suppressing glucose uptake
 - Decrease in serum leptin levels due to its effects on adinopectin
- Metabolic effects on liver
 - Stimulation of TG uptake in liver by triggered transcription of hepatic lipase (HL) or lipoprotein lipase

- Reduction of liver glucose uptake
- Metabolic effects on protein metabolism
 - Stimulation of protein synthesis due to repression of proteolysis
 - Activation of mTOR signaling pathway required in protein synthesis
- Stimulation of myotube proliferation and extension resulting increase in muscle mass since synthesis and secretion of IGF-1
- Stimulation of division and multiplication of chondrocytes and osteoblasts responsible for epiphyses of long bone via synthesis and secretion of IGF-1
- Improvement of nitrogen and phosphate retention
- Promoting the immune system
- Regulating of body homeostasis

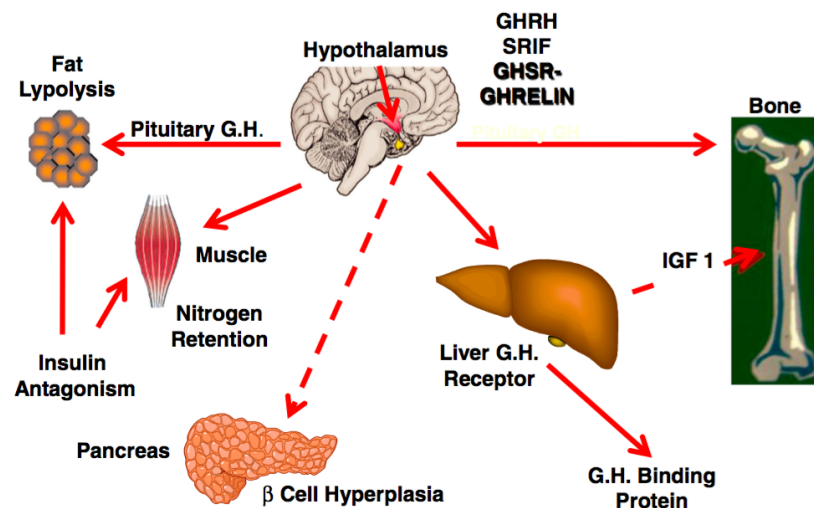


Figure 2. 9 The metabolic effects of GH (Sperling, 2016)

2.2.4 Therapeutic use

The clinical use of human growth hormone is chosen to treat insufficient growth since Prader Willi syndrome (PWS), Turner's syndrome (TS), chronic renal insufficiency (CRI), and syndromes of non-GH-deficiency such as idiopathic short stature (ISS), short children born small for gestational age (SGA), AIDS. The subcutaneous or intramuscular way as type of injection is used for FDA approved administration of liquid or powder formulized hGH to human. The recommended dosages for children and adults are 40 $\mu\text{g/kg/day}$ and 25 $\mu\text{g/kg/day}$, respectively (Pavlovic et al., 2008).

However, the dosages for GH therapy may be arranged at three level when considering the goal of the treatment. In replacement therapy for GH insufficiency, 100 $\mu\text{g/kg/day}$ and 25 $\mu\text{g/kg/day}$ are preferable for children and adults, respectively. To improve the poor growth in children since Turner's syndrome, chronic renal insufficiency, and idiopathic short stature, the dosage of hGH is nearly 50 $\mu\text{g/kg/day}$. A highest dosage as third level is around 100-200 $\mu\text{g/kg/day}$ for cure of short-bowel syndrome (SBS) or AIDS-associated syndrome (Cázares-Delgadillo et al., 2011).

Some diseases and impact of hGH therapy on them are clarified as following:

- Prader willi syndrome (PWS): It is genetic disorder since loss of several genes expression on chromosome 15q11 – q13. It is characterized by mental retardation, hypogonadism, hyperphagia, short stature, endocrine abnormalities, hypothalamic obesity, hypotonia, abnormal body composition with reduced lean body mass. The treatment with hGH yields linear increase in height and muscle mass (Watson and Rogol, 2013).
- Turner syndrome (TS): It is chromosomal defect seen in female sexual characteristic and not related GH regulation despite the levels of IGF-1 and GH are subnormal. Since it is associated with growth failure as short stature, the hGH therapy enhances patient situation (Watson and Rogol, 2013).

- Idiopathic short stature (ISS): ISS defines the states of height more than two standard deviations below the mean value according to age and sex. The hGH administration has positive effect on height growing despite it is a type of non-GH deficiency syndrome (Watson and Rogol, 2013).
- Adult growth hormone deficiency (AGHD): This deficiency implying inappropriate synthesis of GH can be arised from pituitary (i) adenomas with their treatment by surgery or radiotherapy, (ii) other tumors of the sella region, i.e., craniopharyngioma, meningioma, chordoma, (iii) congenital GH deficiency, i.e., idiopathic, genetic (iv) inflammatory, infectious or vascular pituitary disease, (v) cervical trauma, (vi) tumors. Symptoms are chronic fatigue, lack of energy and reduced resistance to effort, reduced sweating, intolerance to cold, psychosocial problems including anxiety, emotional lability, social isolation, sleep problems. The clinical signs are reduction in lean mass, muscle mass, bone mass, left ventricular mass, capacity for effort, HDL cholesterol, sensitivity to insulin and are increase in fat mass, total cholesterol, LDL cholesterol triglycerides, fibrinogen. The GH therapy increases lean and muscle mass, muscle strength, the body water content, bone mineral content, HDL cholesterol with decrease in fat mass, waist/hip ratio, total and LDL cholesterol (Alexpoulou et al., 2016).

Over the syndromes mentioned above, the syndromes based on skeletal dysplasia (SD), short bowel syndrome (SBS), Noonan syndrome (NS), short stature homeobox gene (SHOX) are treated by hGH (Watson and Rogol, 2013) while hGH therapy is favorable for the cure of bleeding ulcers, skin burns and wounds, cardiovascular disease, bone fractures owing to high healing efficacy (Liu et al., 2007). In addition, the use of GH in cystic fibrosis, inflammatory bowel disease, juvenile rheumatoid arthritis, osteoporosis, and in patients who require chronic glucocorticoid administration have been reported (Ayyar, 2011).

2.2.5 Background and Production of Recombinant Human Growth Hormone (rhGH)

The existence of dwarfs and giants has been attractive since antique ages but the relation between these disorders and human pituitary gland was revealed a century ago. Until the first hGH therapy in 1960s, there were many researches about acromegaly, dwarfism and gigantism. Table 2.2 shows these researches to clarify period pre-1960s and pro-1960s (Lindlom, 2006). For growth hormone deficient children, the first therapy through extracted pituitary GH from other sources were acquired in pre-1940s. Then, the extracted hGH from cadaveric human pituitary gland in 1958 lead to treat children having hGH deficiency between 1960-1985 under supervision of the National Pituitary Agency (NPA) and the Food and Drug Administration (FDA). In 1985, the situation of four adult patient having Creutzfeldt–Jacob disease was reported to the FDA. Since Creutzfeldt–Jacob disease resulted prion mediated from cadaveric hGH, FDA suspended cadaveric hGH therapy as a treatment in 1985. Later, the administration of recombinant human growth hormone was carried out as a cure owing to first rhGH with its trade name “Protropin” developed by Genentech in 1981 (Blizzard, 2012).

In today’s world, two forms of recombinant hGH are supplied as synthetic drug, somatropin and somatrem. Both forms display similar biological activity and potency in spite of somatrem having an extra amino acid on the N-terminus compared to somatropin being identical to the inherent hGH synthesized by somatotroph cells in human anterior pituitary gland. Up to today, eleven pharmaceutical company have introduced eleven different brands into the pharmaceutical market in accordance with the aim of syndrome specific treatment (Table 2.3). Over the past 30 years with brands, the rhGH therapy for the children growth hormone deficiency, chronic renal insufficiency, adult growth hormone deficiency, Turner syndrome, Prader Willi syndrome, small for gestational age, idiopathic short stature, short stature homeobox-containing gene deficiency, Noonan syndrome were approved by FDA in 1985, 1993, 1996, 1997, 2000, 2001, 2003, 2006 and 2007, respectively (Ayyar, 2011).

The eight of commercial recombinant rhGH are produced by *E. coli* while three of them and the last one are synthesized by C127 mouse cells and *S. cerevisiae*,

respectively. rhGH is also produced by *Bacillus subtilis* and *P. pastoris* under the scope of researches. There are three type of organism, bacteria, yeast and mammalian cell as it is seen from the instances mentioned previously.

Table 2. 2 The chronology of studies about human growth hormone (Lindlom, 2006; Ayyar, 2011)

Year	Scientist (s)	Research Study
1772	Sancerotte	First note about the availability of enlarged sella in patient who has acromegaly
1884	Pier Marrie	First detailed report including designation of acromegaly in several patients
1887	Minkowski	Demonstration of relation between acromegaly and enlarged sella in almost all published cases
1893	Caton and Paul	Operation of acromegalic patient
1905	Lewis	Suggestion of six different theories on the reason of acromegaly
1908	Hochenegg	Successful operation of acromegalic woman patient
1908	Cushing	Operation of acromegalic patient who survived next 21 years
1908	Houssay and Biasotti	Removal of pituitary leading amelioration of hyperglycemia in diabetic dog
1909	Krause	First transcranial operation on acromegalic woman who was alive next 17 years
		Injection of pituitary extract improving diabetes mellitus
1922	Evans and Long	Injection of beef pituitary extract to rats and then excess growth observed
-	Smith	Cessation of growth by hypophysectomy in rats and re-growth by subsequent implantation of pituitary tissue
1932	-	First results of treating children with extracted GH from other sources
1944	Li and Evans	First isolation of bovine growth hormone

Table 2. 2 The chronology of studies about human growth hormone (Lindlom, 2006; Ayyar, 2011) (Continued)

Year	Scientist (s)	Research Study
1944	Raben and Westermeyer	First isolation of porcine growth hormone
1947	Houssay	Nobel Prize for study called as “Houssay Phenomenon” in 1908
1956	Li and Rapkoff	First isolation of GH from the human pituitary gland
1956	Li and coworkers	Demonstration of differences between structure of monkey and human GH (hGH) and structure of bovine GH
1958	Raben	Exhibition of complete structure of hGH containing 191 amino acids
1960		Success of first complete hGH synthesis
1960		Prooving the effect of hGH on growth
1960		Cure of hGH deficiency by extracted pituitary growth hormone from cadavers for GH deficient children
1960	National Pituitary Agency (NPA)	Formation of goals for coordinating pituitary collection and extraction
1960-1985	NPA	Supervision of NPA for almost all therapy via extracted hGH
1972		Identification of biochemical structure of human GH
1979		First cloning hGH gene by recombinant DNA technology
1981	Genentech	Protropin®, First recombinant human GH (rhGH) production by Escherichia coli via biosynthetic process
1985	Food and Drug Administration (FDA)	Receiving report about four adult patient having prion mediated Creutzfeldt Jacob disease that is fatal and slow viral
1944	Li and Evans	First isolation of bovine growth hormone

Table 2. 3 The commercial rhGH products

Trade name	Manufacturer	Aim of treatment	Administration	Host organism	Approval Year
Accretropin	Cangene Corporation	AGDH, CGDH, TS,	s.c	<i>E. coli</i>	2008
Genotropin	Pharmacia and Upjohn, Pfizer	AGDH, CGDH, TS, PWS, ISS, SGA	s.c	<i>E.coli</i>	1995
Humatrope	Eli Lilly and Co	AGDH, CGDH, TS, ISS, SHOX, SGA	s.c, i.m. s.c.	<i>E. coli</i>	1987
Norditropin, Nordiflex	Novo Nordisk Inc.	AGDH, CGDH, TS, SGA, NS	s.c	<i>E.coli</i>	2000
Nutropin	Genentech	AGDH, CGDH, TS, CRI, ISS	s.c	<i>E. coli</i>	1993
Nutropin AQ	Schwartz Pharma, Genentech	AGDH, CGDH, TS, CRI, ISS	s.c.	<i>E.coli</i>	1995
Omnitrope	Sandoz	AGDH, CGDH	s.c.	<i>E.coli</i>	2006
Serostim	Merck Serono Inc.	AIDS-associated weight loss or cachexia	s.c., i.m.	<u>C127 mouse cell</u>	1996
Saizen	Merck Serono Inc.	AGDH, CGDH	s.c., i.m.	<u>C127 mouse cell</u>	1996
Tev-tropin	TEVA-Pharmaceuticals	CGDH (pediatric only)	s.c.	<i>E.coli</i>	2002
Valtropin	Biopartners	AGDH, CGDH, TS,	s.c.	<u>S. cerevisiae</u>	2007
Zorbtive	Serono/Novartis	SBS	s.c.	<u>C127 mouse cell</u>	2003

2.3 The Selection of Host Microorganism: *Pichia pastoris*

In today's biopharmaceutical industry, many host organisms are available to produce recombinant protein-based therapeutics since the discovery of Humulin, the first product of rDNA technology. Application of the current techniques involved in this technology is significant for the yield and productivity of bioprocess because these techniques such as foreign gene insertion disturb cell functions of the host organisms at many levels (i) DNA replication, (ii) regulation of transcription, (iii) ribosome functions, (iv) RNA turnover, (v) activities of regulatory proteins, (vi) chaperone and protease levels, (vii) membrane energetics, (viii) posttranslational processing, (ix) energy and intermediary metabolism. Therefore, these hosts and its adequate expression vectors are chosen by taking the following points into consideration:

- the source of foreign gene related glycosylation process
- the toxicity of foreign gene on the host organism
- intrinsic r-product characteristics (size, codon usage, gene copy number, efficient transcription by suitable promoters, translation signals, translocation determined by the secretion signal, post-translational modifications including folding, disulfide bridge formation, glycosylation) (Cos et al., 2006).
- product performance (protein turnovers by proteolysis, stability, activity, authenticity)
- financial considerations (final use, quantity required, cost/added value, time for development, market)
- many production parameters (cultivation mode, medium composition, environmental conditions and others) (Palomares and Kuri-breña, 2007).

According to the above criteria, the most commonly used hosts such as bacteria, yeast and mammalian cells are convenient whereas the others are human cells, insect cells, plant cells, and transgenic plants and animals. The key difference between these organisms are principally resulted from post translational modifications and secretion characteristics. In general, non-glycosylated therapeutic proteins are produced in *E.coli* as glycoproteins and antibodies are expressed by mammalian cells. Despite the mammalian cells being able to adequate glycosylation for human proteins, mammalian cells are sensitive to environmental changes; source of viral

contaminations (Fidan and Zhan, 2015); requires complex and expensive media; and have greater doubling times, 12-28 h, resulted in both slowly growing cultures and low product concentration with long cultivation times (Kim et al., 2015). *E. coli* has the lack of posttranslational modifications due to their insufficient organelles compared with eukaryotic cells (Chiba, 2007). However, the bacterial and mammalian cells are more applicable in the biopharmaceutical industry owing to the well-defined systems.

Of these expression systems, yeast since used for thousands of years in fermentation process is favorable host due to its several advantages over all remainders for the production of heterologous proteins (Porro et al., 2005) and *S. cerevisiae* as a conventional yeast attain a place in the biopharmaceutical market and supplies half of the present insulin demand since 1987 in which Novo-Nordisk distributed Novolin®, being the first recombinant form of insulin from *S. cerevisiae* (Meehl and Stadheim, 2014). Yeast as an unicellular eukaryote being generally recognized as safe (GRAS) organisms has ability for glycosylation of proteins and has secretory pathway similar to mammalian cells (Chiba, 2007). However, this class of organisms has several bottlenecks including inability to perform prolyl hydroxylation, amidation, some types of phosphorylation and glycosylation among eukaryote specific complex post-translational modifications (Cereghino and Cregg, 1999; Hohenblum et al., 2004). During last 20 years, there are various non-conventional yeast species developed as alternative hosts such as *Hansenula polymorpha*, *Pichia pastoris*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, and *Kluyveromyces lactis* (Fidan and Zhan, 2015) in addition to the most abundant yeast *S. cerevisiae* as host organism for 30 therapeutic proteins followed by secondly important yeast *P. pastoris* in biopharmaceutical industry (Kim et al., 2015).

2.3.1 Background and General Properties

The rising methylotrophs in the world were firstly defined by Koichi Ogata in 1969 and the term “methylotrophs” implies yeast species having the ability to metabolize methanol as a sole source of carbon and energy. There are very few methylotrophic yeast species, i.e., *Pichia pastoris* being first determined one followed by *Hansenula polymorpha* (*Pichia angusta*), *Candida boidinii*, and *Pichia methanolica* during next decade (Gellissen, 2000).

The methylotrophic yeast *P. pastoris* was attractive for the production of single cell proteins (SCPs) for feedstock because of methanol yielding high growth as well as of methanol being inexpensive carbon source synthesized by natural methane until mid-1970s. In 1970, the Phillips Petroleum Company developed cultivation and fermentation medium with suitable continuous operation to attain high cell density culture (>130 g/L dry cell weight) of *P. pastoris* to fulfil SCPs (Higgins and Cregg, 1998). In mid-1970s, the oil crisis caused the obsolescence of *P. pastoris* while soybean rose as the major feedstock source. Thus, in the 1980s, Phillips Petroleum Company associated with the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA, USA) to provide an advantage in favor of *P. pastoris* based expression systems with regard to recombinant protein production. In 1993, Research Corporation Technologies (RCT) (Tucson, AZ) buying out the patent of *P. pastoris* expression systems from Phillips Petroleum Company put the ready to use kit of *P. pastoris* expression system on the market for academic research laboratories. Today, Invitrogen Corporation™ being a part of Thermo Fischer Scientific (Carlsbad, CA) is licensed to sell components of the system under the supervision of Research Corporation Technologies as the current patent holder (Cereghino and Cregg, 2000). Most of patents from Phillips Petroleum Company can already expired today.

Since 1993 to date, the knowledge about *P. pastoris* have been conveyed and disseminated through both academia and industry even though the genome sequence, coding genes, promoters, the intracellular actions and its metabolism have been partially clarified. During last 20 years, the genome of *P. pastoris* consisting of four chromosomes and its being in the range of 9.43-9.7 Mbp determined by pulse-field gel electrophoresis have been revealed (De Schutter et al., 2009). Additionally, *P. pastoris*, the yeast species of interest is taxonomically classified under the Kingdom Fungi, Division *Eumycota*, Subdivision *Ascomycotina*, Class *Hemoascomycetes*, Order *Endomycetales*, Family *Saccharomycetaceae*, and Sub-family *Saccharomycetoideae* involving genera of *Komagataella* and *Hansenula* (Damasceno et al., 2012). The *P. pastoris* known as *Komagataella pastoris* is included in the genera of *Komagataella* comprising following species *Komagataella phaffii*, *Komagataella populi*, *Komagataella pseudopastoris*, *Komagataella ulmi*, *Ogataea glucozyma* and

Pichia membranifaciens (Kurtzman, 2012). However, other *Pichia* strains are reassigned to different genera, e.g., *Pichia stipitis* in genera of *Scheffersomyces*.

P. pastoris being one of the most favorable and robust host organism to produce r-protein production has following several advantages over other eukaryotic and prokaryotic expression systems from different point of view (Damasceno et al., 2012; Li et al., 2007; Fidan and Zhan, 2015; Jahic et al., 2006; Cregg et al., 2000):

- its products being generally recognized as safe (GRAS) status,
- useful model systems for the import and assembly of peroxisomes, the selective degradation of peroxisomes via autophagy, and the organization and function of the secretory pathway in eukaryotes,
- commercial availability of *P. pastoris* derived expression system with well known protocols,
- ease of genetic manipulation of commercially available well-characterized yeast expression vectors,
- being tolerant to low pH,
- the absence of fermentative metabolism under high glucose concentration resulting in higher by-product formation, i.e., ethanol
- rapid growth rate on inexpensive mineral salt medium, coupled with ease of high cell-density fermentation and high titer product formation,
- presence of both strong constitutive promoters regulated by carbon sources and strong inducible promoters triggered by inducers,
- high levels of productivity in an almost protein-free medium owing to low level secretion of endogenous protein,
- elimination of contamination derived from endotoxins, oncogenic and viral DNA,
- the high stability of foreign gene inserted into yeast genome by homolog recombination
- absence of known human pathogenicity in the spectrum of lytic viruses that prey on *P. pastoris*,

- diverse posttranslational modifications that include polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments,
- the ease of downstream processing without harvesting the yeast cells because of recombinant proteins secreted into fermentation medium as well as of vast majority of fermentation medium being r-protein.

2.3.2 Expression systems

The expression system should be considered with regard to the type of host strain and expression vector composed of marker gene, secretion signal sequence and promoter that all have significant impact on the expression and secretion of foreign gene of interest.

The all *P. pastoris* expression strains are derived from parental organism called as NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL) (Cereghino and Cregg, 2000; Li et al., 2007). *P. pastoris* GS115, DSMZ 70382 known as CBS704, X33 and CBS7435 known as NRRL-Y 11430 (Ahmad et al., 2014) are four mainly used host strains concerned the either genome scale model constructed (Dikicioglu et al. 2014). According to these genome scale models, 5313 and 5450 gene coding sequences are included in *P. pastoris* strain GS115 and DSMZ 70382, respectively as both *P. pastoris* strain CBS 7435 and X33 have 5007 open reading frames (ORFs). Besides, the first genome-wide metabolic model of *P. pastoris* demonstrates that eight compartments, 668 metabolic genes, 1361 reactions and 1171 metabolites (Damasceno et al., 2012).

The host strains are also divided into wild types, methanol utilization phenotypes, auxotrophic strains and protease-deficient strains. The genotype of wild strain does not contain any mutant allele at current locus without causing any change in its phenotype. DSMZ 70382 (CBS704), X33 and NRRL-Y 11430 (CBS7435) are wild type strains of *P. pastoris*. The methanol utilization phenotypes are classified into methanol utilization plus one (Mut⁺) including both of *AOX1* and *AOX2* genes, methanol utilization slow one (Mut^S) resulting from deletion of *AOX1* gene or methanol utilization minus one (Mut⁻) resulting from both *AOX* genes (Cos et al.,

2006). KM71 strain and MC100-3 are protease deficient strains with Mut^S and Mut⁻ phenotype, respectively while all wild types and *HIS4* deficient auxotrophic strain GS115 show Mut⁺ phenotype (Gonçalves et al., 2013). Auxotrophic strains that have inability to synthesize the amino acids and organic compounds required for its growth are various. The mostly known auxotrophic one is strain *GS115* due to the absence of *HIS4* gene encoding hydroxyhistidinase comprising of three independent domains resulting in three functional enzymes (Bigelis, Keesey and Fink, 1981) catalyzing the second (phosphoribosyl-ATP pyrophosphohydrolase), third (phosphoribosyl-AMP cyclohydrolase), and tenth (histidinol dehydrogenase) steps in histidine biosynthesis (Crane and Gould, 1994). In addition, auxotrophic characteristics are used for the selection of transformants via expression vectors including synthetic forms of these genes. Protease deficient strains are preferable to minimize the proteolysis of heterologous proteins secreted into fermentation medium. For this purpose, some gene concerned the activity of protease are deleted. Nonetheless, the lower chance in viability, slow growth and the difficulty in biotransformations are several drawbacks of protease deficient strains. Of them, SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prc1*) and SMD1168 (*his4 pep4*) are commercially available (Cereghino and Cregg, 2000; Cereghino, 2002).

The principle in the choice of selectable marker depends the resistance to any antibiotic or the transfer of substantial gene deficient through organism by expression vector. In the first one, the transformants including resistance gene can grow on solid media containing the antibiotic while the transformant including synthetic gene can survive on essential amino acid or organic compound deficient solid media related relevant auxotrophic strain. In selection via resistance to any drug, the bacterial kanamycin gene resistant to geneticin (G418), the *Sh Ble* gene from *Streptoalloteichus hindustanus* resistant to bleomycin related drug zeocin, Blastocidin S deaminase gene from *Aspergillus terreus* resistant to blastocidin and acetyl-CoA carboxylase from *Sorangium cellulosum* resistant to macrocyclic soraphen A are favorable for *P. pastoris* based expression systems. On the other hand, in the case of biosynthetic markers, the *HIS4* (hydroxyhistidinase), *ADE1* (PR-amidoimidazole succinocarboamide synthase), *ARG4* (argininosuccinate lyase) and *URA3* (orotidine

5'-phosphate decarboxylase) encourage cells to grow on gene specific product free medium (Macauley-Patrick et al., 2005; Cereghino and Cregg, 2000)

The choice of secretion signal sequence is most significant to secrete the heterologous protein into the extracellular space. There are many preferable signal sequences such as α -MF from α -mating factor of *S. cerevisiae*, PHO1 from acid phosphatase of *P. pastoris*, SUC2 from invertase of *S. cerevisiae*, PHA-E from phytohemagglutinin of *Phaseolus vulgaris*, KILM1 from K1 toxin, the 128-kDa pGKL from pGKL killer toxin and the leader sequence of the *Pichia acaciae* killer toxin etc (Ahmad et al., 2014; Li et al., 2007)

The last part of expression system is choice of a suitable promoter regulating the expression and secretion of foreign gene of interest in *P. pastoris* that also has several strong inducible and constitutive promoters as well as both moderate and weak ones of them. The fate of heterologous protein production is directed by expression of enzyme cascades within metabolic pathways controlled by promoters. Therefore, the decision about the promoter option based on the characteristics of both bioprocess and the r-protein desired is either constitutive or inducible with the strength of them (Weinhandl, 2014). The table 2.4 indicates both constitutive and inducible promoters involved in *P. pastoris* by taking into consideration their strong, moderate and weak characteristic (Vogl et al., 2013).

The constitutive promoter based expression systems produce and then secrete heterologous proteins until the end of exponential growth. In addition, the strength of r-protein expression under constitutive promoters strictly depends the type of carbon sources. However, inducible promoter based systems are induced/repressed by carbon and/or nitrogen sources, and by other environmental conditions, i.e., stress, lack or excess of amino acids, intracellular ion concentration. Thus, the synthesis of r-protein is initiated by these inducers when predetermined cell concentration is reached. When considering the principles of operation under both promoters, the inducible promoters are more preferred than constitutive ones since toxic effect of r-protein decreased by the r-protein accumulation initiated during the production phase in the event of its high production and secretion rate. In both cases of bioprocess under carbon source

dependent constitutive and inducible promoter, there is two major phases, growth phase maximizing cell growth and protein production phase triggered by promoter specific carbon source.

Table 2. 4 The constitutive and inducible promoters in *Pichia pastoris*

Constitutive Promoters			Inducible Promoters			
Gene name	Gene product	Expression level	Gene Name	Gene product	Expression level	Inducer
GAP	Glyceraldehyde 3-phosphate dehydrogenase	Strong	AOX1	Alcohol oxidase 1	Strong (naturally 5% of mRNA and 30% of total protein)	methanol
ENO1	Enolase	20-70% of P_{GAP}	AOX2	Alcohol oxidase 2	5–10% of P_{AOX1}	methanol
GPM1	Phosphoglycerate mutase	15–40% of P_{GAP}	DAS	Dihydroxyacetone synthase	Strong (similar to P_{AOX1})	methanol
HSP82	Cytoplasmic chaperone (Hsp90 family)	10–40% of P_{GAP}	FLD1	Formaldehyde dehydrogenase	Strong (similar to P_{AOX1})	Methanol and methylamine
ILV5	Acetohydroxy acid isomeroreductase	15% of P_{GAP}	ICL1	Isocitrate lyase	Not compared to P_{AOX1} or P_{GAP}	Derepression and ethanol induction
KAR2	ER resident chaperone (also termed Bip)	10–70% of P_{GAP}	PEX8	Peroxisomal matrix protein	Weak	Methanol or oleate
KEX2	Endopeptidase involved in the processing of secreted proteins	10% of P_{GAP}	PHO89 or NSP	Sodium-coupled phosphate symporter	Strong (similar to P_{GAP})	Phosphate limitation
PET9	ADP/ATP carrier of the inner mitochondrial membrane	10–1700% of P_{GAP}	THI11	Protein involved in thiamine biosynthesis	70% of P_{GAP} on medium lacking thiamin	Completely repressed by thiamin
PGK1	Phosphoglycerate kinase	10% of P_{GAP}				
SSA4	Heat shock protein	10–25% of P_{GAP}				
TEF1	Translation elongation factor 1 alpha	Strong (similar to P_{GAP})				
TPI1	Triose phosphate isomerase	10–80% of P_{GAP}				
YPT1	GTPase involved in secretion	weak				

Consequently, to attain recombinant protein production in desired concentration, the adequate host strain requires commercially available expression vector system composed of the following elements : (i) 5'- the desired promoter upstream; (ii) a secretion signal sequence; (iii) a multiple cloning site with many, preferably unique, endonuclease sites; (iv) a transcription termination site; (v) a marker for selection; (vi) for select ion with antibiotic for *E. coli*; and (vii) a replication element for plasmid (Li et al., 2007).

2.3.3 *GAP* promoter (P_{GAP})

The strength of *GAP* promoter consisted of 477 bp was first displayed by Waterham and coworkers in 1997. It regulates the expression of glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) and its homolog protein synthesized as a gene product, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is one of the substantial enzymes in the glycolytic pathway. Hence, it also modulates glycolysis pathway and constitutively expresses its homolog protein during the growth. Nevertheless, its strength varies from one carbon source to another one although it is as strong as *AOX* promoter (Waterham et al., 1997). Therefore, this strong constitutive promoter is strictly regulated by the carbon sources such as glucose, glycerol, methanol and oleic acid among many carbon sources. The researches show that glucose and glycerol enhances higher r-protein concentration compared to what methanol obtain the least product

The P_{GAP} driven expression system has more advantages over P_{AOX} based one despite P_{GAP} driven one being inadequate expression system for toxic r-protein production. The most important drawback of P_{AOX} based systems is requirement of methanol as inducer, being hazardous and expensive chemical, and problematical with the storage in large volumes. The P_{GAP} driven system overcomes this problem with the use of eco-friendly carbon sources such as glucose or commercial glycerol solution recycled from waste of many industries. In addition, as P_{GAP} driven expression systems does not require strict process control, since the straightforward growth of the microorganism. Lastly, another problem of methanol utilization is the limitation of oxygen imposed in culture conditions, which limits cell proliferation. The maintenance

of the dissolved oxygen concentration (C_{DO}) at a certain level is difficult with high cell densities; and with the increase in the cell concentration (C_X) in which the specific oxygen utilization rate remains constant, the volumetric oxygen uptake rate eventually increases. Moreover, the use of P_{GAP} —driven systems solves the problem of the accumulation of by-products of methanol metabolism, such as formaldehyde and hydrogen peroxide (Çalık et al., 2015).

2.3.4 Protein secretion and post translational modifications

The one reason why *P. pastoris* is one of the most preferable organisms is vast majority of heterologous proteins in the fermentation medium owing to only low level of endogenous proteins secreted into medium. Nonetheless, *P. pastoris* has ability to secrete proteins either intracellularly or extracellularly. In addition, *P. pastoris* is capable of post translational modification including protein folding, proteolytic processing, disulfide bond formation and glycosylation. The signal sequence processing in ER is proceeded prior to post translational modifications (Cereghino and Cregg, 2000; Cereghino et al., 2001; Cereghino et al., 2002)

The success of glycosylation determines the characteristics of protein containing the reaction kinetics such as enzyme activity, solubility, serum half-life, thermal stability, *in vivo* activity known as biological activity, immunogenicity and receptor binding (Demain and Vaishnav, 2009). *P. pastoris* is competent to assemble both *O*- and *N*-linked carbohydrate moieties to secreted proteins. In *O*-glycosylation, the *O*-linked carbohydrates are added on the hydroxyl groups of serine and threonine. The lower eukaryote *P. pastoris* links *O*-oligosaccharides consisting merely of mannose (Man) residues even though higher eukaryote mammals add *O*-oligosaccharides composed of N-acetylgalactosamine, galactose (Gal), and sialic acid (NeuAc). Nevertheless, the availability of these residues are significant because the half life and efficacy of therapeutic proteins depends on the existence of terminal sialic acid (Hamilton et al., 2006).

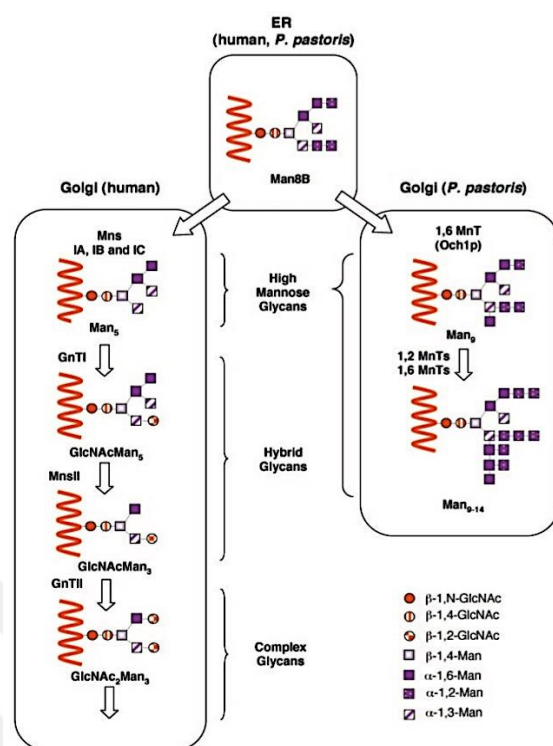


Figure 2. 10 N-linked glycosylation in humans and in *P. pastoris* (Hamilton et al., 2003)

N-glycosylation is especially substantial for proper folding, pharmacokinetic stability, tissue targeting and efficacy for a large number of proteins (Choi et al., 2003; Sethuraman and Stadheim, 2006). In *N*-glycosylation, the assemble of oligosaccharides is occurred subsequently in endoplasmic reticulum (ER) and golgi apparatus. The difference in *N*-glycosylation between *P. pastoris* and mammals is derived from the process occurred in golgi apparatus even if the assembling in ER of *P. pastoris* is similar to that in ER of mammals. In both ER, a lipid-linked oligosaccharide unit, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Glc = glucose; Man = mannose; GlcNAc = N-acetylglucosamine) is transported to asparagine at the recognition sequence Asn-X-Ser/Thr and so the core, $\text{Man}_8(\text{GlcNAc})_2$, is provided for *N*-glycosylation. Later, this core is trimmed in golgi apparatus that differentiates oligosaccharides depending on the type of organism (Figure 2.10). As a result of *N*-glycosylation in golgi, this core is trimmed down mainly to $\text{Man}_{5-6}\text{GlcNAc}_2$, $\text{Man}_{8-14}\text{GlcNAc}_2$ and $\text{Man}_{50-150}\text{GlcNAc}_2$ in humans, *P. pastoris* (Grinna and Tschopp, 1989) and *S. cerevisiae*, respectively. In the lower eukaryotes *P. pastoris* and *S. cerevisiae*, this core is hyperglycosylated by Golgi

mannosyltransferases but *P. pastoris* is more favored rather than *S. cerevisiae* from the point of *N*-glycosylation. To overcome this hyperglycosylation, the humanized *N*-glycosylation is tried to obtain via both protein engineering and genetic engineering (Figure 2.11).

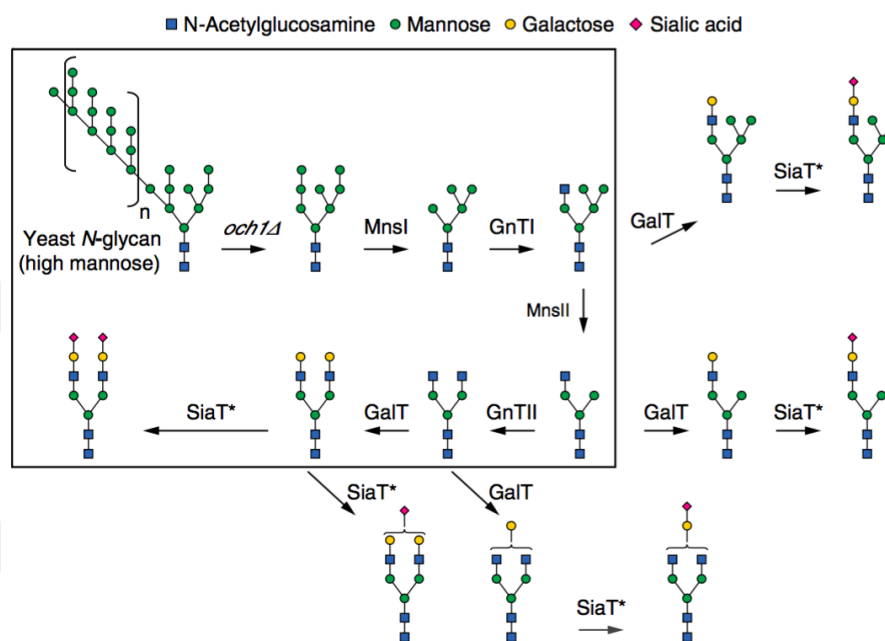


Figure 2. 11 The illustration of yeast-N-glycan engineering (Meehl and Stadheim, 2014)

Finally, terminal α -1,3-linked mannosylated glycans responsible for highly antigenic characteristic of *S. cerevisiae* glycoproteins is not appeared in *P. pastoris* oligosaccharides (Cregg et al., 1993). The antigenicity is not tolerable for recombinant therapeutic proteins.

Consequently, the therapeutic proteins obtained from *P. pastoris* are more reliable due to less hyperglycosylation and absence of terminal α -1,3-linked mannosylated glycans.

2.3.5 Proteolytic degradation

In general, the high cell density cultures of *P. pastoris* meet with difficulties since the proteolytic degradation of secreted recombinant proteins (Clare et al., 1991) poses a problem relevant to (i) decrease in product yield because of the availability of degraded product; (ii) loss of biological activity due to denatured conformational structure; and (iii) contamination of the product via outspread intermediates resulted from proteolysis owing to their physicochemical and/or affinity characteristics similar to that of product (Jahic et al., 2003). The predicted sources of proteolysis may (i) the extracellular or cell wall associated proteases; (ii) intracellular proteases released into fermentation medium as a result of cell lysis due to high cell density fermentation and (iii) vacuolar proteases involved in the secretory pathway during vesicular transport. However, the vacuolar proteases are indicated as the major source of proteases since low level secretion of endogenous proteins (Ahmad et al., 2014). These vacuolar proteases composed of endoproteinase A and B, carboxypeptidase Y and S, aminopeptidase I and yscCo have low substrate affinity and do not require ATP (Jones, 1991).

To minimize proteolysis in *P. pastoris*, many strategies based on following distinct principles can be employed (Macauley-Patrick et al., 2005):

- modifying cultivation parameters such as pH, temperature, specific growth rate and media composition,
- application of genetic engineering to host organism to obtain protease deficient strain (Gleeson et al., 1998),
- application of both genetic and protein engineering to recombinant protein (Gustavsson et al., 2001).

In the scope of modifying cultivation parameters, each parameter can be optimized to reduce proteolytic activity. Both the adjustment of pH and addition of suitable nitrogen source are mostly applicable procedures to control proteolysis. Since *P. pastoris* grows in wide range of pH 3.0 to 7.0, the cell growth is not affected by this range leading to optimize pH that should provide the best stability of r-protein during cultivation period (Jahic et al., 2006). The value of optimized pH can be r-protein

specific: pH 6 being optimal in production of recombinant mouse epidermal factor and human serum albumin as pH 3.0 being optimal in production of insulin-like growth factor-I and cytokine growth-blocking peptide (50 mg/l) (Macauley-Patrick et al., 2005).

The second way is addition of several substances responsible for various effects on proteolysis. Of them, the addition of amino acid or amino acid rich supplements such as casamino acids, peptone (Clare et al., 1991) and yeast extract decrease proteolysis because they are competitive substrates for proteases and prevent nitrogen limitation causing proteolysis activity (Daly and Hearn, 2005; Jahic et al., 2006). The reduction in proteolysis activity by addition of ammonium ions is shown and these ions are involved in the ammonium hydroxide used as preferable nitrogen source during fed-batch operation (Daly and Hearn, 2005). The another way is addition of protease specific inhibitors such as serine protease inhibitor or aspartic protease inhibitor which reduces 53% and 30% of protease activity, respectively, in the culture medium of *P. pastoris* expressing a single-chain antibody (scFv) with aspartic and serine-type proteases (Shi et al., 2003).

The third way of modifying cultivation parameters is the temperature-limited fed-batch technique (TLFB). The lower cultivation temperature yields higher production rate and cell viability with decreased proteolysis activity since the lower cell death rate. For instance, the higher cell viability was attained with the amount of herring antifreeze proteins rose from 5.3 mg/l to 18.0 mg/l when the temperature was decreased from 30°C to 23°C (Macauley-Patrick et al., 2005; Daly and Hearn, 2005).

The another distinct principle is use of protease deficient strains in the cultivation operations (Jahic et al. 2006). The responsible genes for expression of proteinase A and proteinase B are *PEP4* and *PRB1*, respectively. The *PBR1* is also partially dependent *PEP4* to completely activate. In protease deficient strains, these genes are deleted and so mutant strains (*pep4/prb1*) are obtained. The disadvantages of these mutant strains are mentioned above. In the production of insulin-like growth factor-I, ghilanten and laccase, these protease-deficient strains are not efficient in spite of being combined with other strategies (Macauley-Patrick et al., 2005).

To reduce proteolytic activity, the last principle is that the linker site sequence recognized by native proteases is replaced with new synthetic stable linker site in fusion proteins. The fusion protein comprised of a cellulose binding domain from *Neocallimastix patriciarum* cellulase A and *Candida antarctica* lipase B is produced by *P. pastoris* under the control of *AOXI* promoter. The designed one insensitive to native proteases concludes the reduced binding capacity to substrate even as its higher stability (Gustavsson et al., 2001).

2.3.6 PDC knockout strain of *P. pastoris*

Pyruvate decarboxylase (EC 4.1.1.1) is a prominent enzyme located at the branch point between fermentative and respiratory pathways of central metabolism in many organisms (van Maris et al., 2004) and catalyzes non-oxidative decarboxylation of pyruvate to acetaldehyde in the presence of its cofactors, Mg^{2+} and thiamine pyrophosphate (TPP) (Flikweert et al., 1999). The fate of pyruvate at the branch point is determined either by the affinity of pyruvate decarboxylase (PDC) leading fermentative pathway or by affinity of pyruvate dehydrogenase (PDH) leading respiratory pathway. In addition, the affinity of both enzymes to pyruvate varies from Crabtree positive yeasts to Crabtree negative ones. They are also classified by the difference in the changing affinities of these enzymes under two conditions, oxygen availability and the concentration of glucose. In Crabtree positive yeasts, even if oxygen is available, the fermentative pathway is carried under the presence of high glucose concentration since higher substrate affinity of PDC in comparison with that of PDH. On the contrary, Crabtree negative yeasts as respiratory ones, have lower substrate affinity of PDC than that of PDH (Urak et al., 1990). Thus, the PDC is so significant enzyme for the central carbon metabolism of yeast.

In model eukaryote *S. cerevisiae* as Crabtree-positive yeast, three of *PDC* genes among six ones express PDC1, PDC5 and PDC6 isozymes responsible for the catalytic activity in fermentative pathways. The deletion of these three genes creates *PDC* knockout (*Pdc⁻*) strains that does not grow on glucose containing mineral media even though slowly grows glucose containing complex media due to blocking of cytosolic-CoA cycle. The cytosolic-CoA being essential for lysine and lipid synthesis is provided by the conversion of pyruvate into acetyl-CoA subsequently occurred by

pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ADH) and acetyl-CoA synthetase (ACS). Therefore, the added small amount of ethanol and acetate stimulates growth in glucose-limited chemostat cultures owing to conversion of them into cytosolic-CoA cycle by ADH and ACS (Flikweert et al., 1999; Abbott et al., 2009).

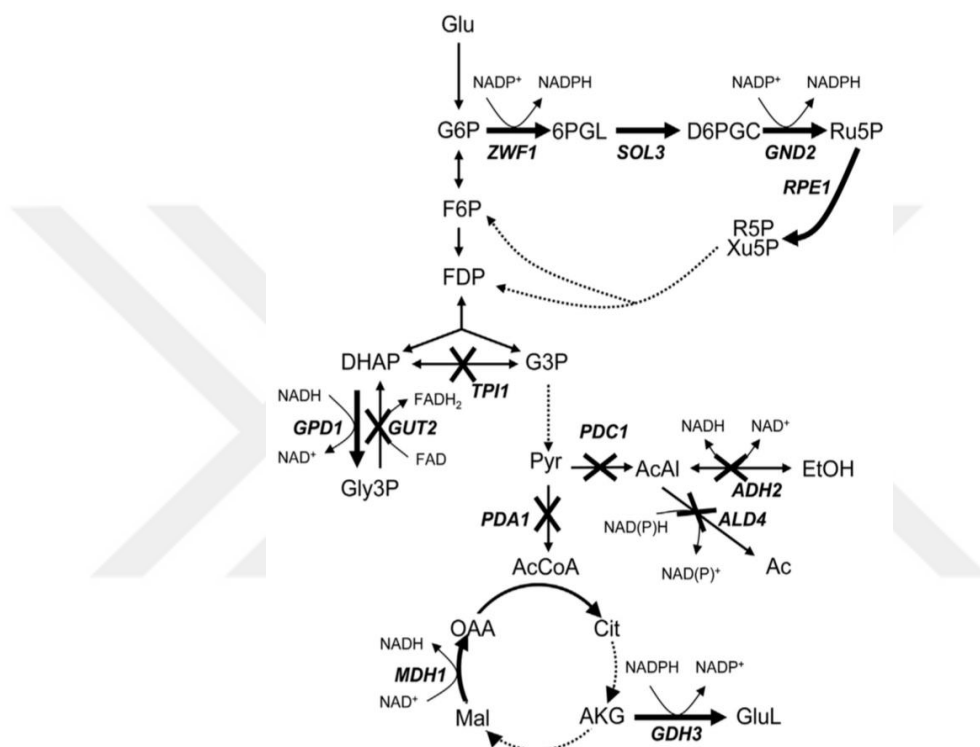


Figure 2. 12 The illustration of *PDC* knockout target in metabolic pathway (Nocon et al., 2014)

However, PDCs have not only been found in fermentative yeasts but also in respiratory yeasts being also Crabtree negative ones like *Kluyveromyces lactis*, *Ogataea polymorpha*, *Scheffersomyces stipites*, and *Wickerhamomyces anomalus* (Agarwal et al., 2013). The crucial role of PDC is not observed for *Pdc*⁻ knockout strain of *K. lactis* (Breunig et al., 2000).

P. pastoris as Crabtree-negative yeast species does not produce ethanol under aerobic conditions and has one confirmed putative *PDC* gene (De Schutter et al., 2009; Mattanovich et al., 2009). In study about kinetic characteristics of PDC produced in

P. pastoris (SpPDC), the maximum PDC activity is reached in directed metabolism by glucose and glycerol whereas it is not detectable in directed metabolism by methanol and ethanol. Hence, the PDC activity depends on the type of carbon sources. On the other hand, the affinity of SpPDC for pyruvate is less than all three PDCs in *S. cerevisiae* (ScPDCs) and thus the dissimilation of pyruvate through fermentative pathway is catalyzed by SpPDC at higher pyruvate concentration under anaerobic conditions (Agarwal et al., 2013). There are not many data about Pdc⁻ strains of *P. pastoris*.

2.4 Key Parameters in Bioreactor Operation

The aim of the bioprocess performed in a bioreactor is to attain high productivity with the desired quality, activity, and amount of the recombinant products of interest. For this purpose, the culture medium composition, pH, temperature, dissolved oxygen demand and agitation rate as key parameters in bioreactor operation should be optimized first according to the kind of strain used and heterologous protein produced (Cos et al., 2006). These non-fixed and optimal conditions are guidelines that ensure the anticipated alterations in fluxes of metabolic pathways and so lead to improve the favorable productivity and yields for a current bioprocess (Çalık et al., 1999; Cos et al. 2006).

2.4.1 The Composition and Design of Culture Medium

The considerations related with the design of culture medium composition and the choice of culture medium type vary depending on industrial and/or laboratory scale bioprocess and pre-cultivation and/or production media despite all types of microbiological media must involve water and sources of carbon, nitrogen, minerals, and growth factors to supply the requirements of an organism (Okafor, 2016).

The culture media are mainly classified into three main group as defined, semi-defined and complex medium in terms of composition. Defined or synthetic medium as a well designed recipe contains exact amounts of pure and off-the-shelf organic and inorganic chemicals added to water and also is categorized into defined minimal medium and defined mineral medium according to the needs of an organism (Heijnen,

2009; Ertola et al., 1994). For most of organisms synthesizing all cell components, the defined minimal medium composed of one organic compound (glucose) as a carbon source and one relevant inorganic compound (ammonia) as a nitrogen source with salts (MgSO_4) and buffers (KIP) as mineral sources is used (Heijnen, 2009; Ertola et al., 1994; Nielsen et al., 1994) while the defined mineral medium consisting solely of inorganic compounds is preferred for the organisms consuming CO_2 as a carbon source. The composition of semi-defined or semi-synthetic medium is almost known since single complex constituent as carbon and/or nitrogen source at a level of 1% (w/v) or less involved (Connors, 2002). However, complex medium is rich in all essential nutrients and also completely different one in which exact chemical constitution is not known because of inexpensive and undefined mixtures of organic and inorganic components included (Heijnen, 2009).

The medium used whether for laboratory purposes or for industrial purposes makes first choice about the composition of culture medium. Thus, the significant points including (i) the yield and concentration of product or biomass per gram of substrate in medium, (ii) the rate of product formation, (iii) the yield of undesired byproduct formation (iv) consistent quality and availability of medium throughout the year, (v) transportation, filtration and sterilization of medium, (vi) equipment design, (vii) flow rates (i.e., retention time), (viii) oxygen demand, (ix) ease and potential problems of downstream processing and (x) total cost of bioprocess related to previously mentioned factors should be taken into consideration (Nielsen et al., 1994). In industrial scale bioprocess, relatively cheap complex media, i.e., molasses, corn steep liquor, whey, originated from food and agricultural wastes and byproducts are traditionally used for the production of single cell protein (SCP), ethanol, organic acids, amino acids and some microbial gums as a type of high volume/low value products (Table 2.5) (Stanbury et al., 2016; Nielsen et al., 1994). Nevertheless, the complex media are inappropriate for use in membrane type bioreactors since the characteristics of medium, e.g., viscosity, and the interactions between medium and membrane (Ertola et al., 1994). Additionally, the complex media are avoided in recombinant protein production as a type of low volume/high value products because the major loss of product in downstream processing, variability in elemental composition and pH of complex media cause the difference in quality of product and

unpredictable biomass and/or product yields from one batch to another batch (Ertola et al., 1994; Heijnen, 2009; Stanbury et al., 2016) although they have organic nitrogen source and various growth factors (Nielsen et al., 1994). Thus, the regulatory authorities, as the Food and Drug Administration (FDA), demand defined media to assure excellent product quality (Heijnen, 2009). In laboratory scale bioprocess, these three culture media are also used. Defined and semi-defined media containing number of minerals, e.g., Cu, Zn, Fe, Co, Mn, Mo, in trace amounts (Ertola et al., 1994) are more preferred for cell growth, reproducibility of bioprocess and consistency of medium for next bioprocess for recombinant protein production. Furthermore, the defined- and semi-defined mediums enable the easier recovery, separation, purification steps and effluent treatment as well as facilitating detection of improvement in bioprocess (Stanbury et al., 2016).

To improve the capacity of bioprocess, the other preference of microbial medium constitution changes with purpose of pre-cultivation medium for culture growth or production medium for product formation because the composition directly affects the microbial growth rate and production rate of product. The pre-cultivation stage induces rapid propagation of cells in pure culture from stocks stored while the production stage triggers the synthesis of desired product (Rai, 2016). Therefore, the richer medium in nutrient level is widely used in pre-cultivation stage since higher growth rate is obtained in the complex medium encouraging better cell proliferation compared to that in defined medium (Ertola et al., 1994). Otherwise, the minimal defined medium is appropriate to stimulate product formation with high biomass level in such a way starved by carbon or nitrogen sources or induced by any compound (Rai, 2016). Maximized amount of product by the highest volumetric productivity with the highest specific productivity of the smallest amount of biomass is satisfactory with regard to proper production medium (Connors, 2002). For these reasons, the favorable option and design of medium composition should be supreme when considering all factors mentioned above for the individual bioprocess.

Table 2. 5 Complex fermentation media often applied in the fermentation industry

Medium	Contents	Origin	Typical application
Corn steep liquor	Lactate, amino acids, minerals, vitamins	Starch processing from corn	Antibiotics
Corn starch	Starch, glucose	Corn	Ethanol, industrial enzymes
Barley malt	Starch, sucrose	Barley	Beer, whiskey
Molasses	Sucrose, raffinose, minarels, fructose, glucose, betain	Sugar cane or sugar beet	Bakers yeast, ethanol
Pharmamedia	Carbonhydrates, minerals, aminoacids, vitamins, fats	Cotton seed	Antibiotics
Serum	Amino acids, growth factors	Serum	Recombinant proteins
Whey	Lactose, proteins	Milk	Lactic acid
Yeast extract	Peptides, amino acids, vitamins	Yeast	Enzymes

2.4.1.1 Nutritional requirements of *Pichia pastoris* and medium optimization

The chemical composition of an organism is also composed of carbon and nitrogen molecules as well as hydrogen and oxygen molecules involved in water constituting approximately seventy percent of a cell. The way to satisfy the carbon requirement of an organism is determined by choosing carbon source from its organic and inorganic forms. The widely used microorganisms in the bioprocess are heterotrophic ones which mainly needs organic carbon source and additional source of nitrogen, macro minerals (phosphate, sulfate and magnesium), and trace minerals (zinc, iron, copper, manganese). The other needs such as growth factors (vitamins, amino acids and hormones) and the precursors of desired product are resulted from the inability in metabolism of strain of interest (Connors, 2002).

The suitable carbon source serves as building block(s) for targeted product and biomass as well major biomolecules included in biomass and product formation (Connors, 2002). Hence, it should be carefully decided according to the promoter controlling the expression of foreign gene of interest and the presence of hydrolytic enzymes synthesized by the kind of strain used. To date, the various carbon sources originated from pure chemical and complex media have been examined with regards to their effects on the yields of biomass and product formation. Table 2.6 shows these carbon sources and classification of them into carbohydrates, lipids, proteins, organic acids and hydrocarbons and derivative of hydrocarbons. Similar to the all yeasts species, *Pichia pastoris* can also metabolize the most of carbon sources listed to produce energy via assimilation of simple sugars (glucose and fructose), disaccharides (maltose and trehalose), sugar alcohols/polyols (glycerol, mannitol and sorbitol), organic acids (citric acid, lactic acid, acetic acid), hydrocarbons and derivatives (methanol and ethanol). Contrary to that in *Saccharomyces cerevisiae*, *P. pastoris* can not metabolize sucrose and galactose since the absence of invertase enzyme and specific transporter, respectively. Similar to that in *S. cerevisiae*, lactose can not be utilized because of lacking the α -galactosidase or β -galactosidase enzymes (Çalık et al., 2015). The nitrogen source participates as a nitrogen molecule in the structure of all nitrogen containing compounds such as amino acids in proteins, and purines and pyrimidines in nucleic acids (Okafor, 2016; Connors, 2002). Hence, it is the second

key compound to supply in culture media. The amount required of nitrogen in fermentation medium should be calculated according to the average composition of microorganism used and the predictable biomass formation. On the basis of dry weight, the average nitrogen content of bacteria, yeast and mold is 12.5, 7.5 and 6%, respectively, whereas the carbon content of all is same and equal to 48% (Okafor, 2016). The C/N ratio of *P. pastoris* in biomass C-molecular composition varies in the range between 5.9-7.1 dependent on type of carbon source (Jordà et al., 2012). In the design of fermentation medium, the high C/N ratio, which means a large excess of carbon source in the presence of a limiting amount of nitrogen, must be adjusted to enhance the production of microbial products targeted (Connors, 2002). In addition, that balance between carbon and nitrogen sources controls the changes in pH through buffering capacity provided by the proteins, peptides and amino acids (Stanbury et al., 2016). Therefore, the quantity and type of N-source are so significant and also supplied as inorganic form of nitrogen included in ammonia gas, ammonia/ammonium hydroxide, ammonium salts, nitrates and nitrite, and organic form of nitrogen included in amino acids, urea, yeast extract (YE), peptone (P) and casamino acids (Table 2.6).

Peptone, yeast extract and yeast nitrogen base without amino acids (YNB) are mostly used as nitrogen sources in both of BMGY and YPD as usual pre-cultivation media and BMMY as usual induction medium for *P. pastoris* (Li et al., 2007). For the formulation of medium in laboratory-scale fermenter (3L-10L), ammonium hydroxide provides the nitrogen need of *P. pastoris* as well as higher biomass formation while it is also added to control the pH of medium known as BSM and FM22. Conversely, the all amounts required of nitrogen is added at the initial formulation of the d'Anjou medium (Cos et al., 2006). Besides, the nitrogen depletion causes the increased proteolytic activity resulted in the degradation of extracellular protein (Kobayashi et al., 2000) as the excess amount of ammonium ion prolongs the lag phase and thus suppresses the microbial growth (Macauley-Patrick et al., 2005).

Table 2. 6 Nutritional requirements in both complex and defined media

		Derivatives in Defined Medium	Derivatives in complex medium
Carbon source	Carbohydrates	Simple sugars, e.g., fructose, galactose, glucose, Disaccharides, e.g., lactose, maltose, sucrose Sugar polymers, e.g., dextrans, starch Sugar alcohols/polyols as glycerol, mannitol, sorbitol	Molasses for glucose and fructose Whey for lactose, molasses for sucrose Barley malt for starch
	Proteins		
	Lipids	Fatty acids, linoleic, linoleic and oleic acids included in oils of olive, maize, cotton seed, soya bean	
	Hydrocarbons and derivatives	n-alkanes, e.g., methane Ethanol Methanol	Molasses, corn steep liquor for proteins Wine must for decanoic and octanoic acid
Nitrogen source	Organic acids or their salts	Acetic acid/acetate, citric acid/citrate, lactic acid/lactate, malic acid/malate	Corn steep liquor for lactic acid, whey for citric acid
	Organic nitrogen	Available forms in complex media	Soybean meal, yeast extract, casein hydrolysate, pharma media
	Inorganic nitrogen	Ammonia gas, ammonium nitrates or ammonium salts, e.g., ammonium sulphates, succinate	Beer wort for amino acids, ammonium ions, amino nitrogen compounds
	Macro elements	Calcium, magnesium, phosphorus, potassium, sulphur	Wine must for phosphorus, potassium, magnesium, sulfur (often sulfide)
Minerals	Trace elements	Calcium, copper, iron, manganese, molybdenum, nickel, zinc,	Cheese whey for iron, zinc, manganese, copper
		Amino acids, e.g., asparagine, glutamic acid, methionine Purines, e.g., guanine and pyrimidines, e.g., uracil Vitamins, e.g., Biotin (B7), choline, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxine (B6), riboflavin (B2), thiamin (B1)	
Signaling molecules			
		Pure chemical	Beer wort, wine must for amino acids
	Growth factors	Pure chemical	Corn steep liquor for biotin, pyridoxine, thiamin

The diversity in micronutrients consisting of trace minerals and growth factors is dependent on the genus and species of an organism. For instance, inositol, pantothenate, biotin, thiamine, pyridoxine, thiamine and nicotinic acid were determined as the required vitamins by nearly sixty yeasts whereas only biotin and thiamine necessitates for molds (Koser, 1968). Iron, manganese, copper of trace minerals and biotin of growth factor are supplemented as micronutrient needs of *P. pastoris* by PTM1 trace salts introduced by Invitrogen. The effect of each component in PTM1 on the metabolism of *P. pastoris* for the recombinant protein production and biomass formation has not been completely clarified (Cos et al., 2006). Nevertheless, the greater/more positive impact of seven vitamins and two-trace minerals added in BSM medium on the recombinant porcine-follicle stimulating hormone (rFSH) by *P. pastoris* has been demonstrated by comparison with that in combination of BSM+PTM1 trace salts (Hélène et al., 2001). In *Pichia* medium, the availability of minimum 200 μM copper providing optimal laccase activity (O'callaghan et al., 2002), and of 100 μM ferric ion increasing the expression of recombinant porcine lactoferrin rPLF (Wang et al., 2002) have indicated the significance of salt trace elements.

2.4.2 pH

The pH as a symbol of hydrogen ion concentration implies the relative acidity and alkalinity. This relativity is so critical for the activities of enzymes involved in chemical and biochemical reactions. Each biochemical reaction of an organism requires many enzymes to catalyze several reactions simultaneously. Since the diversity in biochemical reactions directed by environmental conditions, some enzymes activate as the others inactivate and so the microorganism adapts the changes in environment. Therefore, each organism has crucial maximum, optimum and minimum pH values to survive. The optimum pH value ensures maximum cell growth while the values above maximum and/or below minimum pH inhibit cell proliferation. On the other hand, these crucial values of pH and its range between from minimum to maximum vary from one species to another one, for instance, the pH = 3-8 for bacteria, pH = 3-6 for yeast and pH = 3-7 for molds. Besides, the pH values in the range 6.5 to 7 stimulates optimal growth for bacteria while optimum range of pH is between 4 to 6

for fungi. In general, the acceptable deviation from the optimum pH is 1 to 2 units of pH. In the case of larger deviation than that, the required energy for cell maintenance increases (Liu, 2013).

In any bioprocess, the optimum pH is so significant not only for cell growth but also formation, stability, secretion and maintenance of recombinant proteins since proteolysis activity (Brady et al., 2001). To minimize protease activity in fermentation broth for each mode of bioreactor operation, the pH value must be optimized operation parameter which also must ensure correct tertiary structure with the highest biochemical and biological activity of recombinant enzymes and therapeutic proteins, respectively. In any fermentation process performed by *P. pastoris*, which grows over pH range from 3 to 7, the control of optimum pH is primarily requisite to avoid the proteolytic degradation of secreted heterologous proteins because of minimal effect of pH on its growth rate. To control and determine optimum pH in bioreactor cultures of *P. pastoris*, there are many principles conducted by type of carbon and nitrogen sources, individual r-protein and promoter regulating its synthesise, and mode of bioreactor operation.

The studies about the effect of pH on cell growth and carbon utilization in batch operations with uncontrolled pH demonstrate that rapid decrease in pH from 5 to 2.2 limits cell growth and cause accumulation of glycerol. The explanation indicates the more energy spent to compensate the great difference between the pH of intracellular and extracellular medium rather than being in cell proliferation. Conversely, high cell density culture of *P. pastoris* is achieved under controlled pH = 5.0 (Chiruvolu et al., 1998).

When considering the highest productivity of target r-protein, the optimum pH should satisfy the highest formation and stability of protein with the minimum biomass formation. The effect of pH on total secretion of recombinant human granulocyte colony-stimulating factor (rhG-CSF) produced by *S. cerevisiae* is investigated at three different pH = 5.0, 5.5 and 6.0. The the highest monomer percentage of extracellular rhG-CSF obtained as 76% at pH 6.5 shows that increase in pH improves secretion of rHG-CSF (Bae et al., 1999). Similar to what research is done in *S. cerevisiae*, the effect of pH on recombinant human growth hormone (rhGH) produced by *P. pastoris* under

the control of *AOX* promoter is studied at four pH = 4.2, 5.0, 5.5, 6.0 for initial batch phase and fed-batch phase. According to data resulted from study, the all cell growths are same in batch phase at four different values of pH while the highest rhGH concentration obtained at pH = 5.0 with lower cell growth because of higher amino acid demand. Further, the higher stability of r-protein secreted at pH = 5.0 rather than that at pH = 5.5 is observed due to minimum protease activity although the highest product concentrations obtained are so similar at these points of pH (Çalık et al., 2010). Further, the favorable pH for expression of influenza virus A (hemagglutinin) is determined as pH = 8.0 (Garcia-Ortega et al., 2013). The high levels expression, stability and activity of α -amylase are achieved at pH 6.0 (Choi and Park, 2006). In fed batch operation for r-protein production by *P. pastoris* under the control of promoter of interest, the production phase for induced r-protein production is adjusted to desired value of pH dependent on target r-protein while the initial batch phase for cell growth is traditionally maintained at pH = 5. Otherwise, the fed-batch phase is performed at pH = 3.0 under *AOX* promoter while the optimum pH value of the initial batch phase is same as that above. The fall in pH of induction phase is one of the methods to repress both of protease activity and bacterial contamination (Chiruvolu et al., 1998).

The key point of optimized pH is its strict control by enhanced buffer capacity of the medium. The way of control is determination of appropriate nitrogen source between various nitrogen sources such as yeast extract, peptone, ammonium, nitrate etc. In bioreactors, complex media is not favored although yeast extract and peptone based media drops protease activity (Daly and Hearn, 2005). In the case of preference between ammonium and nitrate as a nitrogen source, the use of ammonium is resulted in lower pH by releasing hydrogen ions since microbial consumption of ammonia. On the contrary, the higher pH is obtained due to reduction of nitrate to ammonia causing removal of hydrogen ions from the fermentation broth (Liu, 2013). Also, ammonium ions included in ammonium phosphates facilitate to decrease proteolytic activity. The traditional bioprocess with *P. pastoris*, ammonium hydroxide is used as nitrogen source with buffering capacity of decreasing pH resulted from metabolic activities.

2.4.3 Temperature

Temperature plays an important role for the growth and performance of any microorganism because the integrity of cell membrane and the activity of proteins involved in cellular reactions are affected by temperature. The behavior of lipids in cell membrane and of the hydrogen bonds providing unique three dimensional structure of individual proteins is influenced by current temperature. The rigidity and fragility of membrane increase due to low temperatures whereas it loses its compounds and cellular organelles since lipids becomes fluid at high temperatures. Also, the hydrogen bonds between molecules of proteins tend to be break at higher temperatures. Therefore, temperature is also crucial parameter just as pH (Bauman, 2007).

The temperatures at which the enzymes of any organism are functionally active determines the temperature range over which it grows (Black and Black, 2015). In a similar characteristic to pH, there are minimum, optimum and maximum points of temperature, called as cardinal temperatures, at which the microorganism distinctly conduct metabolism (Madigan and Martinko, 2010). Within this temperature range, the optimum growth temperature stimulates the maximum growth rate with the shortest doubling time. On the contrary, both of minimum and maximum ones indicate the limiting temperatures at which microorganism can be able to survive. In particular, the points over the maximum temperature is resulted in thermal death because of permanent denaturation of all essential proteins (Madigan and Martinko, 2010). However, regardless of type of organism, growth gradually increases from the minimum to optimum temperature and decreases very sharply from the optimum to maximum temperature. These growth properties are due to changes in enzyme activity. Enzyme activity generally doubles for every 10°C rise in temperature until the high temperature begins to denature all proteins, including enzymes (Black and Black, 2015).

The one of ways to enhance r-protein production is bioprocess performed in temperature below optimal temperature promoting cell growth. This approach is widely used for the culture of bacteria, yeast and mammalian cells. In bacterial production, the lower growth temperature contributes into the facilitated protein folding and consequently concludes higher yields of correctly folded soluble product.

Similar to the culture of bacterial cells, the mammalian cell culture is improved by extended period of viability with higher product yield in case of the cultivation temperature reduced from 37°C to less than 30°C. Further, the higher cell viability and less accumulation of unfolded protein leading more secreted products are obtained the cultivation of *S.cerevisiae* meanwhile the optimal cultivation temperature, 30°C is decreased to suboptimal temperature, 20°C. The slow growth as a result of reduction in temperature, leads the decline in the rates of metabolic reactions with protein folding (Dragosits et al., 2011).

The temperature limited operations are also used to enhance r-protein production in *P. pastoris* that optimally grows at 30°C and gradually decreases the protein expression above 32°C. From the inspiration derived from the results mentioned above, when the cultivation temperature decreased from 30°C to 23°C yields three-fold protein expression even the cell growth was decreased (Li et al., 2009). In the bioprocess carried out by *P. pastoris* under the control of *AOXI* promoter, the lower temperature in the range of 15°C to 25°C is more favorable to induce efficient protein expression. Moreover, the cells grown under these suboptimal temperatures need less oxygen. Nevertheless, this is not easily applicable for industrial scale bioprocess due to higher cost of cooling (Gao et al., 2012). Additionally, the decreased cultivation temperature solved the problem about the proteolysis of r-proteins in *P. pastoris*. For this purpose, Shi et al. (2003) showed the effect of temperature on proteolytic activity and examined cultivation temperatures of 30, 27, 25, 23 and 15°C. The cells efficiently produced scFv with minimum protease activity at 15°C while this performance was lost slowly by increased temperatures from 15°C to 30°C

2.4.4 Oxygen transfer

The viability of aerobic heterotrophic (chemoheterotrophic) organisms used in a bioprocess depends on the presence of oxygen since cellular energy obtained from the catabolism of carbon sources via oxidative phosphorylation. Therefore, the oxygen is one of the essential compounds such as carbon, nitrogen and micronutrients for metabolic pathways responsible for cell growth and maintenance, and formation of targeted product and byproduct.

In all bioreactors as a model of multiphase systems, the mass transfer of any component through cell is the rate limiting step of biochemical reactions occurred in a cell because of the lower maximum transport rates as compared with the maximum reaction rates (Nielsen et al., 1994). In aerobic systems, the cells must supply oxygen from the gas bubble in liquid while the overall mass transfer of oxygen from gas bubble to cell involves following eight steps as a series of resistance which oxygen must overcome (Figure 2.13)(Doran, 2012):

1. Diffusion of oxygen from the bulk gas in the interior of the bubble, gas film, to the gas-liquid interface
2. Transport of oxygen along the gas-liquid interface
3. Diffusion of oxygen through the relatively stagnant liquid film around the gas bubble
4. Transfer along the bulk fluid
5. Diffusion of oxygen throughout the relatively stagnant liquid film surrounding the cells
6. Motion along the liquid-cell interface and the following step similar to step 8
7. In other respects, diffusion from the liquid-cell aggregate interface to the individual cells since the cells involved in a type of cell aggregate such as floc, clump, or solid particle.
8. Movement along cytoplasm and then towards the site of oxidative phosphorylation in the cytoplasm, e.g., mitochondria

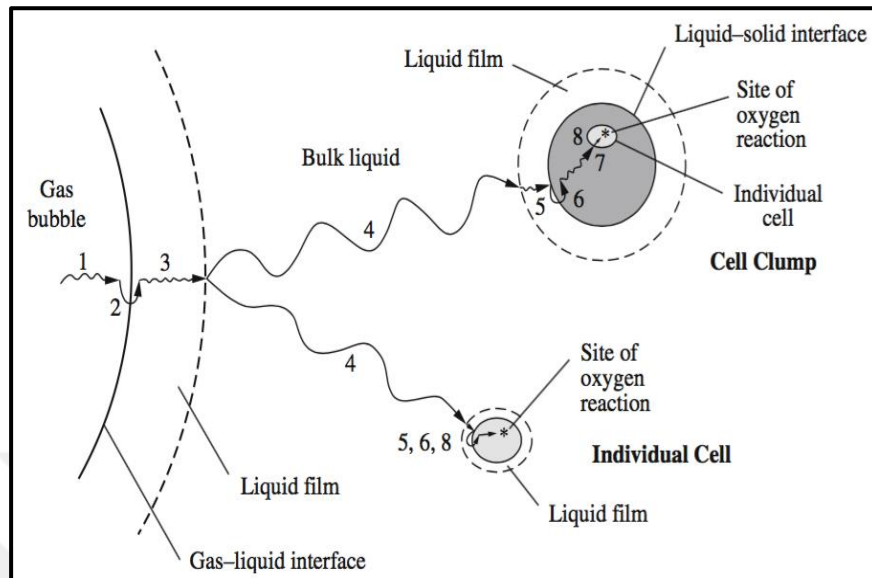


Figure 2. 13 Steps for oxygen transfer from gas bubble to microorganism (Doran, 2012)

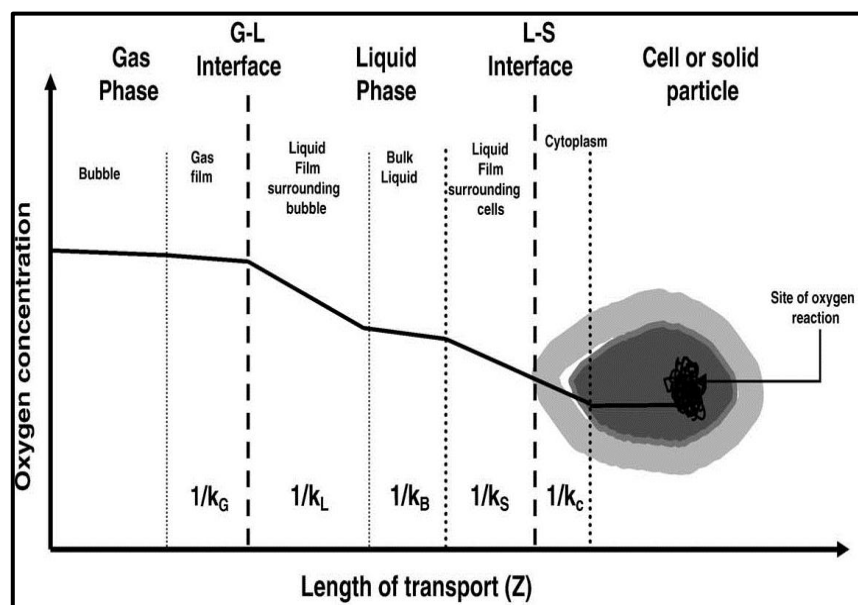


Figure 2. 14 Steps with resistances and oxygen concentration profile through the oxygen transfer from gas bubble to microorganism (Garcia-Ochoa and Gomez, 2009)

When considering the above steps with resistances (Figure 2.14) in terms of high diffusivity of oxygen in gas phase, and of low solubility in liquid phase since high Henry's constant, the resistance in gas film of bubble, step 1, is quite small compared to that in stagnant liquid film surrounding a gas bubble, step 3. Additionally, the resistance in stagnant liquid film of a gas bubble, step 3, is much greater in proportion to that in stagnant liquid film around a cell, step 5 if the dimensions of gas bubble and microorganism are taken into account. However, the resistance during the motion of oxygen along the bulk liquid is negligible since well mixing in systems. Therefore, step 3 is the rate controlling step of oxygen mass transfer. Consequently, the rate of oxygen transferred by molecular diffusion through the stagnant liquid film around bubble is defined as oxygen transfer rate (OTR) of these multiphase systems (Clarke, 2013).

In bioreactors, a phenomenon of oxygen transfer and determination of OTR for aerobic processes has been derived from “two film theory” governed by Fick's Law. According to Fick's Law, the molecular diffusion of oxygen through the stagnant liquid film is quantitatively defined by the term of molar flux per unit area (J) as a function of oxygen concentration gradient (ΔC), the thickness of film (δ) and the diffusivity (D) in Equation 2.1.

$$J = -\frac{D}{\delta} (C_{sat} - C) \quad (2.1)$$

where the oxygen concentration gradient (ΔC) as driving force equals the difference between the C as bulk oxygen concentration in liquid, C_{sat} as the oxygen concentration in liquid equilibrated with that in gas phase and the term of D/δ represents k_L as “the local oxygen transfer coefficient”. In addition, diffusivity is assumed constant under steady state condition in case of no reaction in stagnant film.

The two film theory developed by Whitman (1923) is mainly used if the gas-liquid mass transfer mainly occurs from both sides of the interface. The gas-liquid mass transfer rate is modeled according to this theory, describing the flux through each film as the product of the driving force by the mass transfer coefficient, according to:

$$J^o = k_G(p_G - p_i) = k_L(C_i - C_L) \quad (2.2)$$

where J^0 is the molar flux per unit area ($\text{mol.m}^{-2}\text{s}^{-1}$), k_G and k_L are the local mass transfer coefficients, p_G is the oxygen partial pressure in the gas bubble and C_L is the dissolved oxygen concentration in the bulk liquid, index i refers to values at the gas-liquid interface

Since the the interfacial concentrations are not directly measurable and considering the mass transfer coefficient, the Equation 2.2 can be rewritten as:

$$J^0 = K_G(p_G - p^*) = K_L(C^* - C_L) \quad (2.3)$$

where the K_L called as “overall oxygen transfer coefficient”, p^* is the oxygen pressure in equilibrium with the liquid phase, C^* is the oxygen saturation concentration in the bulk liquid in equilibrium with bulk gas phase according to Henry’s Law ($p^* = HC^*$)

Combining Equation 2.2 and 2.3, the following expression can be obtained

$$\frac{1}{K_L} = \frac{1}{HK_G} + \frac{1}{k_L} \quad (2.4)$$

where H is Henry’s constant.

Taking into account that oxygen is only slightly soluble in water (H is very large), it is commonly accepted that the greatest resistance for mass transfer is on the liquid side of the interface and the gas phase resistance can usually be neglected and thus the overall mass transport coefficient is equal to the local coefficient: $K_L = k_L$.

In general, the molar flux per unit volume (N) has been measured since the difficulty about measurement of molar flux per unit area of a gas bubble. Thus, the further Equation demonstrating molar flux per unit volume can be obtained from Equation 2.3 by the incorporation of the interfacial area per unit volume as follows:

$$N = J^0 \cdot a \quad (2.5)$$

If the equation 2.3 can be inserted in Equation 2.5, it can be expressed as:

$$N = K_G a(p_G - p^*) = K_L a(C^* - C_L) \quad (2.6)$$

where the lumped K_{La} parameter called as “overall volumetric oxygen transfer coefficient” is universally used (Clarke, 2013).

$$N = K_L a (C^* - C_L) \quad (2.7)$$

Equation 2.6 describes the oxygen transfer rate in the volume of bioreactor

The OTR is affected by many factors following:

- physicochemical properties of liquid, i.e, viscosity, density, pH, chemical oxygen demand (COD) and biological oxygen demand (BOD) related its composition
- tank design including reactor geometry and aeration design, i.e, diameter and length of bioreactor and blades; diameter of sparger.
- operational conditions, i.e, airflow rate, agitation rate and power input
- the oxygen uptake rate (OUR) of biomass

The first three point related influences the residence time and diameter of bubble, which influences interfacial area and mass transfer coefficient whereas OUR directly affects the concentration gradient of dissolved oxygen in liquid (Garcia-Ochoa and Gomez, 2009; Pittoors et al., 2014; Löffelholz et al., 2013)

The oxygen consumption rate called as oxygen uptake rate, OUR is specified by oxygen demand of the cell culture. Further, the oxygen demand of cell culture mainly varies with the kind of strain and the nature of carbon sources as well as operation mode of bioreactor, biomass concentration and the proliferation phase of culture growth (Löffelholz et al., 2013; Doran, 2012). For instance, in early phase of cell culture, “the specific oxygen uptake rate, q_{O_2} ” known as the rate of oxygen consumption per cell is maximum and thus OUR is relatively high. The OUR is other significant characteristic as well k_{La} for oxygen transfer in aerobic process and can be formulated as (Doran, 2012):

$$OUR = -r_0 = q_{O_2} \cdot C_X \quad (2.8)$$

where $-r_0$ is the oxygen consumption rate ($\text{mol m}^{-3} \text{s}^{-1}$), q_{O_2} is the specific oxygen consumption rate per cell ($\text{mol kg}^{-1} \text{s}^{-1}$) and C_x is the cell concentration (kg m^{-3})

To maintain metabolic activities of the cell for desired cell growth and product formation, OUR must be balanced with OTR. In well mixed liquid phase provided by bioreactors, the simplified mass balance consisting of OUR and OTR for the dissolved oxygen can be written as (Garcia-Ochoa et al., 2010; Doran, 2012):

$$\frac{dC_{O_2}}{dt} = OTR - OUR \quad (2.9)$$

If the function of OTR in Equation 2.7 and of OUR in Equation 2.8 are substituted in Equation 2.9, it can be denoted as:

$$\frac{dC_{O_2}}{dt} = K_L a (C_{O_2}^* - C_{O_2}) - q_{O_2} \cdot C_x \quad (2.10)$$

where dC_0/dt is the oxygen accumulation in the liquid phase.

In both of air-filtered shake bioreactors and laboratory scale bioreactors, it must be evaluated carefully whether gas-liquid mass transfer for oxygen is sufficient or not in the aqueous fermentation media. The solubility of oxygen in salt-based media and culture media varies in the range of $1\text{-}10 \text{ mgL}^{-1}$ and also is inadequate. This low solubility of oxygen in broths, its lower transfer rate and high oxygen demand of aerobic cells during fast growth are challenges of these systems (Pittoors et al., 2014; Mandenius, 2016). However, the way of oxygen transfer from gas to liquid phase is different in two systems. In air-filtered ones, the oxygen in air is transferred by diffusion through total liquid surface area. The capability of system for oxygen transfer may be enhanced by increase in aeration rate, shaking frequency and diameter as well as larger surface area gained by decrease in broth volume or specialized flask geometry including baffles. Furthermore, the oxygen transfer capacity of this system relies on the physical properties of bulk liquid, i.e., viscosity and diffusivity (Mandenius, 2016).

However, the oxygen supply in laboratory scale bioreactors is provided by spargers, membranes or open channels (Löffelholz et al., 2013). The tank design, antifoam agents, temperature, fermentation broth, stirrer speed, gas flow rate, gas pressure and oxygen partial pressure are main factors influencing the value of k_L and a , and driving force as variables of OTR. In bioreactor operation systems, the commonly accepted value of k_L is available and also depends on the size and rigidity of bubbles. As a general rule of thumb, for bubbles bigger than 2 to 3 mm diameter, the value of k_L is nearly $4 \times 10^{-4} \text{ m s}^{-1}$ as it can be $1 \times 10^{-4} \text{ m s}^{-1}$ for smaller ones (Doran, 2012).

2.5 Bioprocess Kinetics in Semi-Batch Operation

Performance and maintenance of the bioprocess are defined by specific growth rate of the cell, consumption rate of the substrate, and specific production rate. To compare different semi-batch operation strategies with each other, the absolute values of specific rates and yields are calculated. The mathematical models applied for these calculations are derived from the material balances of cell, substrate and product.

2.5.1 Mass Balance Equation for the Cell

The cell growth is defined as the increase in number of microorganisms during bioprocess. At the beginning of bioprocess, the cells are inoculated into the fermentation medium and then there is no input and output streams related cell. Therefore, if no cell loss and the generation of microorganisms are assumed, mass balance equation for the cell can be written as:

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

where C_x is the cell concentration, V is the working volume of bioreactor, t is the cultivation time and r_x is the cell growth rate that can be introduced as:

$$r_x = \mu \times C_x \quad (2.12)$$

where μ is the specific growth rate of microorganism. Thus, the Equation 2.12 is inserted in Equation 2.11, the further equation is represented as:

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

The derivative on the left side of Equation 2.13 should be separated into terms to evaluate the changes of each term with respect to time and it can be described as:

$$\mu C_x V = C_x \frac{dV}{dt} + V \frac{dC_x}{dt} \quad (2.14)$$

In semi-batch operation, the feed of substrate stream consistently increases the working volume of bioreactor with time. If no change in density of fermentation medium is assumed, the change in the working volume can be expressed as:

$$Q_{in} - Q_{out} = \frac{dV}{dt} \quad (2.15)$$

where Q_{in} is the volumetric flow rate of input stream and Q_{out} is the volumetric flow rate of output stream. In semi batch operation, there is no output and so Equation 2.15 is written again as:

$$Q_{in} = \frac{dV}{dt} \quad (2.16)$$

If the derivative term related working volume in Equation 2.16 is substituted in Equation 2.6 to demonstrate the effect of alteration in working volume on the bioprocess dynamic, the derived equation can be described as:

$$\mu C_x V = C_x Q + V \frac{dC_x}{dt} \quad (2.17)$$

When the derivative term is left alone on the left side of equation, the equation can be simplified as

$$\frac{dC_x}{dt} = \left(\mu - \frac{Q}{V} \right) C_x \quad (2.18)$$

Consequently, in semi-batch operation, the specific growth rate of microorganism can be described by rearrangement of Equation 2.18 as follows:

$$\mu = \frac{dC_x}{dt} \frac{1}{C_x} + \frac{Q}{V} \quad (2.19)$$

2.5.2 Mass Balance Equation for the Substrate

In semi-batch operation, when considering the feed of substrate stream without an output stream and the substrate consumption rate of microorganism, the mass balance equation can be written as:

$$Q_s C_{s_0} + r_s V = \frac{d(C_s V)}{dt} \quad (2.20)$$

where Q_s is the volumetric feed rate of the substrate, C_{s_0} is the concentration of substrate in the feed or stock medium and C_s is the concentration of the substrate at a particular time in the bioreactor and r_s is the substrate utilization rate. The first order kinetic equation can be applicable to describe substrate utilization rate being a function of both cell concentration and specific substrate utilization rate as follows:

$$-r_s = q_s C_x \quad (2.21)$$

where q_s is the specific substrate utilization rate. If Equation 2.21 is inserted in equation 2.20, the further Equation 2.22 yielding the relation between the substrate concentration and cell concentration at same particular time can be denoted as:

$$Q_s C_{s_0} - q_s C_x V = C_s \frac{dV}{dt} + V \frac{dC_s}{dt} \quad (2.22)$$

If q_s is left alone on the left side of Equation 2.22, the next one can be expressed as:

$$q_s = \frac{1}{C_x} \left(\frac{Q_s}{V} C_{s_0} - \frac{C_s}{V} \frac{dV}{dt} - \frac{dC_s}{dt} \right) \quad (2.23)$$

Under quasi-steady state condition in semi-batch operation, The operation of semi-batch concerning the concentration of substrate in the cultivation medium of the bioreactor, provides a which means that: $dC_s/dt = 0$. In such condition, consumption of substrate is only associated with cell formation. To create a correlation

between cell formation rate and substrate utilization rate, cell yield on the substrate parameter is introduced:

$$-r_s = (r_x/Y_{x/s}) \quad (2.24)$$

Furthermore, if Equation 2.24 is inserted into Equation 2.20 the derived equation is written as:

$$Q_s C_{s_0} - \frac{r_x V}{Y_{x/s}} = C_s \frac{dV}{dt} \quad (2.25)$$

Additionally, to include μ term in Equation 2.25, r_x is replaced with μC_x to give:

$$Q_s C_{s_0} - \frac{\mu C_x V}{Y_{x/s}} = C_s Q_s \quad (2.26)$$

The differential Equation 2.11 can be written as a linear equation by using Equation 2.23 and the obtained expression is:

$$C_x V = C_{x_0} V_0 e^{\mu t} \quad (2.27)$$

Equation 2.28 is further inserted to Equation 2.27 which is arranged to present the volumetric feed rate of the substrate as function of time:

$$Q_s = \frac{\mu C_{x_0} V_0}{Y_{x/s} C_0} \exp(\mu t) \quad (2.28)$$

2.5.3 Material Balance Equation for the Product (rhGH)

In semi-batch operation, mass balance of product is only represented by the production rate and the accumulation rate in the medium since there is no input or output streams involving product.

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

Where C_p is the product concentration in the cultivation medium, and r_p is the product formation rate which is written as a function of cell concentration by means of a first order kinetic equation

$$r_p = q_p C_x \quad (2.29)$$

Where q_p is the specific formation rate of the product ($\text{g}_{\text{product}} \text{g}_{\text{cell}}^{-1} \text{h}^{-1}$). Specific formation rate of the product is obtained by rearranging the above equations:

$$q_p = \frac{1}{C_x} \left(\frac{C_p}{V} Q + \frac{dC_p}{dt} \right) \quad (2.30)$$

2.5.4 Yield Coefficients

Yield coefficients represent the mass of cell or product formed per unit mass of substrate:

$$Y_{X/S} = \frac{r_x}{-r_s} = \frac{dC_x/dt}{-dC_s/dt} \quad (2.31)$$

$$Y_{P/S} = \frac{r_p}{-r_s} = \frac{dC_p/dt}{-dC_s/dt} \quad (2.32)$$

To obtain the overall yield coefficients for the entire bioprocess, Equation 2.31 and 2.32 are presented as linear equations for finite period of time as follows:

$$\bar{Y}_{X/S} = \frac{\Delta C_x / \Delta t}{-\Delta C_s / \Delta t} \quad (2.33)$$

$$\bar{Y}_{P/S} = \frac{\Delta C_p / \Delta t}{-\Delta C_s / \Delta t} \quad (2.34)$$

In aerobic processes, microorganisms consume oxygen intensively to carry out their oxidative reactions and to synthesise the recombinant protein. For the consumed oxygen, the cell and product yields on oxygen are also calculated:

$$Y_{X/O} = \frac{r_x}{-r_o} \quad (2.35)$$

$$Y_{P/O} = \frac{r_P}{-r_O} \quad (2.36)$$

The following table summarizes the most used yield coefficients with their symbols and the particular definitions:

Table 2. 7 Definition of calculated yield coefficients

Symbol	Definition
$Y_{X/S}$	Mass of cell produced per unit mass of substrate consumed
$Y_{X/O}$	Mass of cell produced per unit mass of oxygen consumed
$Y_{S/O}$	Mass of substrate consumed per unit mass of oxygen consumed
$Y_{P/X}$	Mass of product produced per unit mass of cell produced
$Y_{P/S}$	Mass of product produced per unit mass of substrate consumed
$Y_{P/O}$	Mass of product produced per unit mass of oxygen consumed

2.6 Synchronization

Synchronization concerned with the subsequently used terms, *i.e.* rhythm/oscillation, cycle/periodicity, clock-oscillators, is encountered with the different applications in various fields of the life including science, engineering, and social behavior (Pikovsky et al., 2003). The relation between these terms is significant to find out the scope and principles of this phenomenon. Oscillation seems as oscillatory or rhythmic behavior which can be commonly found in biological systems, mechanical and electrical oscillators, chemical reactions, and behavioral processes (Gustavsson et al. 2014; Richard 2003; Guemkam Ghoms et al., 2014) Cycle is innate to nature and related to oscillation with circadian cycle (period time close to 24 h) that ensures the adaptation of organism to environmental shifts, annual cycle, reproductive cycle and other common biological cycles, *e.g.*, heartbeat, that are certainly intrinsic to a specific function to which its frequency and intensity are associated (Gustavsson et al., 2014; Richard, 2003; Romagnoli et al., 2011). Thus, synchronization can be

defined as the rhythm harmony of oscillating objects to arrest in a common frequency in a certain cycle (Pikovsky et al., 2003; Guemkam Ghomsi et al., 2014) as the concept is evaluated as a whole with the mentioned terms above. However, the individual cells included in a population that are in different phases of their cycles do not produce relevant coordinated dynamics at the population level (Romagnoli et al., 2011) whereas synchronization is ubiquitous characteristic of the nature (Guemkam Ghomsi et al., 2014). Therefore, synchronization provides the synchronous populations at the same reproduction and physiological states to behave a like a single cell instead of the many individual cells when this phenomenon is especially considered in terms of biological systems.

Budding yeast, *e.g.*, *Saccharomyces cerevisiae*, among the various biological oscillators, is an ideal model system to study “the dynamics of black box-cell-” by synchronization that allows the investigation of cell cycle control (Rupeš, 2002), *i.e.*, cyclin-dependent kinases (CDKs)/cyclin complexes (Aggarwal et al., 2011), and cell cycle dependent regulatory and metabolic activities, *i.e.*, DNA replication and the synthesis of cell wall, membrane and protein, (Fox, 2004; Neymann, Wegerhoff, and Engell, 2011; Cooper, 2004). Additionally, the optimization of yeast metabolites and cell-cycle modulated recombinant protein (r-protein) production (Frykman and Srienc, 2001; Uchiyama et al., 1997) are researched via synchronization within the scope of biotechnology (Walker, 1999). The explanation of cell synchrony observed in cell populations of yeast is tried to understand by cellular metabolism coupled with its environment . Cellular metabolism consists of continuous events known as cellular growth (increase in cell mass of individual cell by synthesis of ribosomes and proteins) and discontinuous events (DNA replication, mitosis and cell division) (Humphrey and Pearce, 2005). These eukaryotic growth and division cycles considered as a type of biochemical oscillations (Alberghina et al., 2012) are historically modeled as non-linear process “cell cycle progression” composed of four sequential phases (Lee et al. 2011): G₁ (gap required for generation of new cells), S (DNA synthesis), G₂ (gap required for initiation of mitosis), M (mitosis) (Neymann, Wegerhoff, and Engell, 2011; Choi et al., 2009; Lee et al., 2011). Both the internal determinants, *e.g.*, reach of critical size, and the environmental determinants, *e.g.*, temperature and availability of

nutrients and pheromones, influence the rate of cell cycle progression while they do not affect equivalently each phase in it (Alberghina et al., 2012).

2.6.1 Synchronization Methods for Yeast Populations

Many synchronization procedures are performed for yeast cell populations and these procedures can be classified into selection methods and induction methods by taking internal and environmental determinants into consideration (Table 2.8) (Walker, 1999). In addition, these methods are subclassified into batch synchrony and continuous synchrony according to differences in their applications. The principle of induction synchrony known as *block and release* method involves that the particular phase of cell cycle being arrested by the addition of chemicals or depletion of growth factors, which causes inhibiting of crucial gene product included in transition of cell cycle division, and then being released by removal of these blocking situations (Amon, 1990; Choi et al., 2009; Shaw et al., 2012). Conversely, the selection method, e.g., centrifugal elutriation, is a separation process collecting cells in G₁ phase from asynchronous cell culture, based on cell size, shape, and mass to attain uniform yeast cell culture (Amon, 1990; Day et al., 2004; Breeden, 1997; Choi et al., 2009). However, each synchronization method has advantages and drawbacks (Breeden, 1997). For instance, their limitations, batch synchrony called as whole-culture synchronization leading synchronization of almost all cell population (Choi et al., 2009), is used to study cell cycle processes while continuous synchrony in chemostats ensures to explore the oscillation of metabolism based on the relation between cell cycle and environmental shifts in partially synchronized culture of yeast cells (Walker, 1999).

2.6.2 Starvation as a Synchronization Method and Its Relation with Budding Yeast

When considering the cell division of budding yeast with regard to critical cell size that correlates with cell stages (Amon, 1990; Navarro et al., 2012), budding is asymmetrical and produces larger mother cells and smaller daughter cells (Rupeš, 2002; Beuse et al., 1998). To fulfill the inherent suppression of this asymmetry (Rupeš,

2002), the bud spends a longer time in G₁ phase and grow more than the larger parent to reach a critical size for initiation of the subsequent bud formation in size

dependent cell cycle transition phase between G₁ and S (Rupeš, 2002; Turner et al., 2012). As a result of this, the cell cycle related oscillations and heterogeneity in cell culture occur spontaneously (Beuse et al., 1998). Also, the nutritional availability stimulates the cell cycle progression and asymmetry while it determines the fate of descendants of the cell by the entry into new cycle or stationary phase, sporulation etc. In budding yeast, poor media yields large mother cells and very small buds since the cells in G₁ phase are more sensitive to perturbation in nutrients. From the synchronization point of view, these determinants should be evaluated to arrest the yeast cells in one of these phases mentioned as well as they determine the classification principle of synchronization methods.

Starvation is also the one of these available methods and arrests cells in early G₁ phase (Dechant and Peter, 2008) by inhibition of regulatory proteins known as the highly conserved kinases target-of-rapamycin (TOR) and protein kinase A (PKA) activated by availability of carbon and nitrogen sources (Turner et al., 2012; Dechant and Peter, 2008). Prior to start (in early G₁), the cells are responsive to the changes in environmental conditions (Humphrey and Pearce, 2005) and so the nutritional availability coupled to the cell cycle progression and asymmetry (Alberghina et al., 2012) determines the fate of descendants of the cell by the entry into new cycle, stationary phase or sporulation *etc.* (Humphrey and Pearce, 2005). In budding yeast, poor media yields large mother cells and very small buds since the cells in G₁ phase are more sensitive to perturbation in nutrients. As a consequence of survival program termed as “starvation response”, cells exit the cell cycle and arrest in early G₁ phase (Dechant and Peter, 2008).

In the literature, there are several studies about cell cycle progression and the synchronization of in batch and/or chemostat cultivations of *S.cerevisiae* and its modeling whereas a few research is present about cell cycle model of *P. pastoris* (Jia and Yuan, 2007; Love et al., 2010) and synchronization of its cultivation. Jia and Yuan (2007) found that a multi-bud phenomenon related with cell morphology was observed when *P. pastoris* was grown on methanol contrary to the cell morphology observed in

the glycerol growth phase. Furthermore, Uchiyama et al. (1997) showed that higher intracellular production of rice amylase by *S.cerevisiae* in M phase rather than other phases while Love et al. (2010) reported the stochastic secretion of human Fc fragment by *P. pastoris*

Table 2. 8 The synchronization methods for yeast cell populations

The cell cycle phase arrested	Induction Methods					
	Batch					Continuous
	<i>Nutritional starvation</i>	<i>α-Mating factor</i>	<i>Hydroxyurea</i>	<i>Nocodazole</i>	<i>Restrictive temperature /cdc mutants</i>	Feeding-starving of key nutrition
G₀	Inhibition of regulatory proteins					Inhibition of regulatory proteins
G₁	Blocking of haploid <i>S.cerevisiae</i> MATa cells					<i>cdc28</i>
S	Blocking of ribonucleotide reductase					<i>cdc27</i>
G2/M or M	Degradation of microtubules					<i>cdc14, cdc15, cdc20</i>
The cell cycle phase arrested	Selection Methods					
G₀, G₁, S G2/M or M	Centrifugal elutriation	Velocity separation	Density separation	Continuous-flow separation	Zonal centrifugation	



CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals and commercially available solutions used in this research were analytical grade products and supplied from many companies such as Fluka Co., Difco Laboratories, Sigma Aldrich Co., Merck and Co. Inc listed in Appendix A.

3.2 Buffers, Stock solutions and Kits

All buffers, stock solutions and kits that are given with their recipes in Appendix B were prepared with ultra purified water and sterilized either by autoclaving at 121°C for 20 minutes or by filter sterilization through filters with 0.20 or 0.45 µm pore size (Sartorius AG, Gottingen, Germany) according to the type of substance and the intended purpose of solutions, and stored at +4°C or room temperature.

3.3 The Microorganism, Plasmids and Maintenance of Microorganisms

Three strains of *P. pastoris* were used in this study. To visualize the morphology of the synchronous and asynchronous cells by confocal microscopy, the strain expressing intracellular eGFP under the control of *GAP* promoter was used. The other strain was a previously developed recombinant strain of *P. pastoris* which expresses *hGH* gene under the control of *GAP* promoter (Zerze, 2012). The recombinant *PDC* knockout strain expressing rhGH was acquired via transformation of pGAPZαA::hGH constructed by Massahi and Çalık (2015). The all strains were

originated from a single colony stored in microbanks (Pro-Lab Diagnostics) at -80°C to ensure its viability. It was firstly grown on YPD agar containing 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 20 g L⁻¹ D-glucose, 20 g L⁻¹ agar and 100 µg/ml ZeocinTM for 48 h at 30°C.

Table 3. 1 The composition of YPD agar for growth of recombinant *P. pastoris* strains

Component	Concentration (g L ⁻¹)
Yeast Extract	10
Peptone	20
Agar	20
Glucose	20
Zeocin TM	1 ml
dH ₂ O	to 1 L

3.4 Pre-cultivation Media and Growth Conditions

The pre-cultivations in air-filtered shake flask experiments were carried simultaneously on two media, YPD broth and BMGY (buffered glycerol complex medium), until the cells grew to three OD₆₀₀ values of approximately 4, 8, and 16, to investigate the effect of cell age on the synchronization of yeast cell population. The designed operation conditions for air-filtered shake flask bioreactor experiments were given in Fig. 1. The cell ages were evaluated according to the middle-late exponential growth phase, *i.e.*, OD₆₀₀ = 4-8, and stationary growth phase, *i.e.*, OD₆₀₀ = 12-16. The culture grown on solid medium was inoculated into YPD broth containing 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 20 g L⁻¹ D-glucose, and 100 µg/ml ZeocinTM, and BMGY including 20 g L⁻¹ peptone, 10 g L⁻¹ yeast extract, 1.34 g L⁻¹ yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 10 g L⁻¹ ammonium sulfate, 10 g L⁻¹ glycerol, 4x10⁴ g L⁻¹ biotin, 100 mM potassium phosphate buffer (pH = 6.0) and 100 µg/ml ZeocinTM. Pre-cultivation followed by starvation process was performed in 250 ml baffled, air-filtered shake bioreactors with working volume of 50 ml for 15-20 hours in an orbital shaker (Sartorius, Germany) with agitation rate of 200 rpm at 30°C. Otherwise, BMGY was only used as a pre-cultivation medium in the laboratory scale bioreactor experiments.

Table 3. 2 The composition of BMGY medium

Component	Concentration (g L ⁻¹)
Yeast Extract	10
Peptone	20
Potassium phosphate buffer (pH = 6.0)	0.1 M
Yeast nitrogen base (YNB)	13.4
Ammonium sulfate (NH ₄) ₂ SO ₄	10
Glycerol	10
Chloramphenicol	1ml L ⁻¹
Biotin	4 x 10 ⁻⁵
dH ₂ O	to 1 L

Table 3. 3 The composition of YPD medium

Component	Concentration (g L ⁻¹)
Yeast Extract	10
Peptone	20
Glucose	20
Chloramphenicol	1ml L ⁻¹
Biotin	4 x 10 ⁻⁵
dH ₂ O	to 1 L

3.5 Starvation Media and Growth Conditions

The previously developed starvation procedure (Manukyan et al., 2011) was executed with following modified steps:

- 1- Growth of yeast cultures to desired OD₆₀₀ values (OD_{age} = 4, 8, and 16 h) in pre-cultivation conditions.
- 2- Centrifugation of whole cell culture at 2000xg for 2-3 min at 4 °C.
- 3- Discarding the supernatant and repeating of centrifugation to remove small cells with tendency to bud.
- 4- To starve by deprivation of carbon source, resuspension of the pellet in 50 ml of YEP containing only 10 g L⁻¹ yeast extract and 20 g L⁻¹ peptone, and

incubation of whole cell culture for 6 hours in the orbital shaker with agitation rate of 200-220 rpm at 30°C.

- 5- This step is same as in the second one but needs the amount of initial cell concentration OD = 1.0 (5×10^7 cells/ ml) or OD= 2.0 (1×10^8 cells/ ml) according to working volume varies with 250 ml baffled, air-filtered shake bioreactor and 3.0 L bioreactor.
- 6- Inoculation of cells resuspended in pre-warmed production media to initiate a synchronously dividing culture.

3.6 Production Media and Growth Conditions

Air-filtered shake bioreactor experiments and laboratory scale bioreactor experiments were done to investigate the effect of synchronization on the production efficiency of rhGH as a model protein. Additionally, the laboratory scale bioreactor experiments and their parameters were performed according to the results obtained from shaker experiments.

In air-filtered shake bioreactor experiments, two production media of 50 ml were used by taking same carbon moles included into consideration, containing 20 g L⁻¹ of glucose and 20.26 g L⁻¹ of glycerol, respectively. Additionally, the operation conditions were as same in the both of pre-cultivation and starvation phases.

In 3.0-L laboratory scale bioreactor (B.Braun Biostat CT2.2, Germany) with a working volume capacity of 0.8-2.2 L, the production phases with or without synchronization were conducted in two subsequent steps: glycerol batch phase and glucose fed-batch phase. The medium of glycerol batch phase known as basal salts medium (BSM) contained 45 g L⁻¹ glycerol (100%), 26.7 ml L⁻¹ H₃PO₄ (85%), 14.9 g L⁻¹ MgSO₄·7H₂O, 0.93 g g L⁻¹ CaSO₄, 18.2 g L⁻¹ K₂SO₄, 4.13 g L⁻¹ KOH and 4.35 ml L⁻¹ of filter sterilized PTM1 solution as 50 % (w/v) glucose and or glycerol solution as fed-batch stock solution involved only 12 ml L⁻¹ PTM1 and 2.35 ml L⁻¹ PTM1.

Table 3. 4 The composition of BSM

Components	Concentration (g L⁻¹)
CuSO ₄ .5H ₂ O	6
H ₃ BO ₃	0.02
NaI	0.08
Na ₂ MoO ₄ .2H ₂ O	0.2
MnSO ₄ .H ₂ O	3
ZnCl ₂	20
FeSO ₄ .7H ₂ O	65
CoCl ₂ .6H ₂ O	0.916
H ₂ SO ₄	5 mL
Biotin	0.2 mL
dH ₂ O	to 1 L

Table 3. 5 The composition of PTM1 solutions

Components	Concentration (g L⁻¹)
CuSO ₄ .5H ₂ O	6
H ₃ BO ₃	0.02
NaI	0.08
Na ₂ MoO ₄ .2H ₂ O	0.2
MnSO ₄ .H ₂ O	3
ZnCl ₂	20
FeSO ₄ .7H ₂ O	65
CoCl ₂ .6H ₂ O	0.916
H ₂ SO ₄	5 mL
Biotin	0.2 mL
dH ₂ O	to 1 L

In the case of with synchronization, the cells were obtained according to starvation procedure mentioned above whereas the asynchronous cells were centrifuged at 1500xg for 10 minutes at 4°C and then inoculated into BSM. The glycerol batch phase was carried on until the cells reached approximately 24 g L⁻¹

concentration at which time the glycerol was totally consumed and the process was shifted from glycerol batch to glycerol fed-batch phase. Since the overall process time in bioreactor is about 40 h, the glycerol batch phase takes the first 18 h when C_{X_0} also reaches approximately 24 g L⁻¹. Then, the glycerol fed batch phase starts at ≈ 18 h regarded as $t = 0$ for start time of glycerol fed-batch phase. Exponential feeding of the glucose solution was accomplished by dynamic flow rate [Q_s] calculated according to following Equation (1) based on a desired pre-determined specific growth rate:

$$Q_s = \frac{\mu_o \cdot C_{X_0} \cdot V_o}{Y_{X/S} \cdot (C_S^o - C_S)} \exp(\mu_o \cdot t) \quad (1)$$

where, Q_s (L h⁻¹) is volumetric flow rate of the feed stream, μ_o (h⁻¹) is the pre-determined specific growth rate, C_{X_0} (g L⁻¹) is the initial cell concentration at the beginning of glucose fed-batch operation, V_o (L) is the initial volume of the fermentation medium at the beginning of glucose-fed batch operation, $Y_{X/S}$ (g g⁻¹) is cell yield on substrate, C_S^o (g L⁻¹) is feed substrate concentration and C_S is substrate concentration in the bioreactor medium at any time. Since glucose accumulation prevented, C_S is equal to zero during all times in glucose fed-batch phase. Eventually, Equation (1) becomes:

$$Q_s = \frac{\mu_o \cdot C_{X_0} \cdot V_o}{Y_{X/S} \cdot C_S^o} \exp(\mu_o \cdot t) \quad (2)$$

In fed-batch operation, the pre-determined parameters of equation (2) were $V_o = 0.9$ L, $C_{X_0} = 24$ g L⁻¹, $\mu_o = 0.1$ h⁻¹; $Y_{X/S} = 0.48$ g g⁻¹ and $C_S^o = 500$ g L⁻¹ for glucose and $Y_{X/S} = 0.55$ g g⁻¹ and $C_S^o = 500$ g L⁻¹ for glycerol.

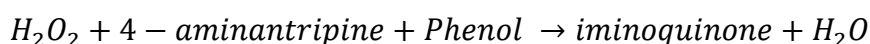
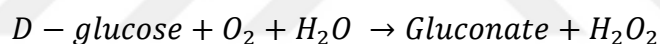
In laboratory scale bioreactor experiments, both synchronous and asynchronous cultivations of OD₆₀₀ = 4, 8, and 16, were performed under strictly controlled conditions. All bioreactor operations were conducted at C_{DO}=20% controlled by Hamilton VisiFerm DO 120 probe (Switzerland), N=900 rpm agitation rate, T = 30°C, pH = 5.0. pH of the fermentation broth was maintained by NH₄OH which also served as the nitrogen source.

3.7 Analyses

Imaging of cells was carried out with confocal microscopy. Cell concentrations were determined at 600 nm with a UV-Vis spectrophotometer (Spectroquant, Merck, Germany). The equation (3) resulted from calibration curve was used to convert absorbance values to dry cell concentration (g L^{-1}).

$$C_x = 0.244 \times OD_{600} \times DF \quad (3)$$

Glucose Analysis Kit (Biasis, Turkey) based on principles of glucose oxidation method was used to decide glucose concentration in fermentation medium. In the presence of glucose oxidase catalyst, two subsequent reactions occur in series for conversion of glucose to gluconate and hydrogen peroxide. The second reaction's product, iminoquinone has the same concentration with glucose converted and its production is accompanied with a pink colour.



To draw the calibration curve given in APPENDIX C, OD_{505} of standard glucose solutions with known concentrations was measured.

To determine the concentration of rhGH, SDS-PAGE described by Laemmli was performed with some modifications such as use of TGX-Stain Free FastCast Acrylamide Kit, 12% (BioRad) and silver nitrate staining. The preparation of gels and silver staining solutions were presented in APPENDIX B. The SDS gels were visualized by imaging system (UVP).

Organic acid concentrations were measured by using Reverse phase high pressure liquid chromatography (HPLC) (Waters HPLC, Alliance 2695, Milford, MA). Organic acids were separated according to the stationary phase consisted of the column which is filled with an adsorbent material and the mobile phase was composed

of 3.12% (w/v) NaH_2PO_4 and $0.62 \times 10^{-3}\%$ (v/v) H_3PO_4 . Mobile phase flowed through column and carried the samples made of filtered supernatants.

Before loading the samples in HPLC, first, solution standards for each organic acid were read in the system and the respective retention times were determined to identify the organic acids. Area of the peaks displayed presented the concentration of the components. Calibration curves given in APPENDIX C, provide the relation of peak areas with concentration of the organic acids.

Table 3. 6 HPLC operation characteristic and condition for identifying and quantifying the organic acids

Column	Capital Optimal ODS
Column dimensions	4.6 mm x 250 mm x 5 m
System	Reversed phase chromatography
Mobile phase	3.12% (w/v) NaH_2PO_4 , $0.62 \cdot 10^{-3}$ (v/v) H_3PO_4
Flow rate of mobile phase	0.8 ml min ⁻¹
Column temperature	30°C
Detector type and wavelength	Waters 2487 Dual Absorbance Detector, 210 nm
Detector temperature	30°C
Injection volume	5 µl
Analysis period	15 min
Delay time	5 min

CHAPTER 4

RESULTS AND DISCUSSION

The objective of this study was to investigate the effect of both aging and starvation as a cell cycle synchronization method to enhance recombinant human growth hormone production (rhGH) production by *P. pastoris* under control of *GAP* promoter. For this purpose, four sub-research studies were performed. The first stage in the research was the evaluation of new recombinant strain of *P. pastoris*, being *PDC* (pyruvate decarboxylase) knockout *P. pastoris* strain *GAP::hGH*. In the literature, the ethanol production in the presence of excess glucose was showed in the bioreactor operations despite *P. pastoris* is Crabtree negative yeast (Heyland et al., 2011). Therefore, the recombinant protein production was improved when the gene sequence of enzyme, pyruvate decarboxylase, responsible for fermentative pathway and so the increase in r-protein production was acquired in this gene deficient strain (Nocon et al., 2014). In the view of such information, this new gene deficient strain was evaluated to improve rhGH production and thus the growth characteristics were defined for three different carbon sources, glucose, glycerol and glycerol plus 40% acetate in batch bioreactor operations. However, the low rhGH production in this *PDC* gene deficient strain, the experiments were maintained with the following strains, *P. pastoris* *GAP::eGFP* and *P. pastoris* *GAP::hGH*.

In the second stage of experiments, among the several synchronization procedures, starvation was chosen as a suitable method owing to easy application in pre-cultivation phase, cost- and labor-effective method, not disturbing cell cycle as chemicals, and probable application in large-scale bioreactor experiments in semi-

batch and continuous operations by feeding and starving in a current time interval during bioprocess. For this purpose, the optimization of starvation was carried out when considering the influence of aging to $OD_{600} = 4, 8, \text{ and } 16$ on on starvation process relevant type of pre-cultivation medium, buffered glycerol complex medium (BMGY) and YPD broth including yeast extract, peptone and dextrose. The efficiency of starvation procedure was interpreted according to results obtained from confocal microscopy imaging indicating the physiological states of *P. pastoris* strains expressing recombinant enhanced green fluorescent protein (eGFP) under *GAP* promoter.

Later, these optimized synchronization procedures were regarded from the point of expressing and secretion of rhGH to find out how synchronization influences the productivity of rhGH produced by *P. pastoris* strain under *GAP* promoter. In the last stage of research, either optimized conditions and their negative controls with only aging to $OD_{600} = 4, 8 \text{ and } 16$ were performed in the seven semi-batch operations by feeding of carbon source with predetermined specific growth rate $\mu = 0.1 \text{ h}^{-1}$. The efficiency of each strategy was performed by specific rates of growth, substrate utilization and r-protein production, by the yield coefficients of biomass and product on substrate and of product on biomass and their overall yields according to time at which the highest product concentration reached , and by the cell metabolism described by organic acids.

4.1 Strategy Design

The abbreviations and explanations of all strategies designed in shaker and bioreactor experiments within the scope of three sub-research studies. All strategies designed were summarized:

- Investigation of *PDC* (pyruvate decarboxylase) knockout *P. pastoris* strain *GAP::hGH* through adequate carbon sources and the productivity of rhGH
- Optimization of starvation procedure in a current pre-cultivation medium with the optimum of both age and pre-cultivation time

- Evaluation of rhGH production under the influence of synchronization and its combination with aging

Table 4. 1 Definitions of strategies designed for batch and semi-batch rhGH production by *PDC* knockout *P. pastoris* *GAP::hGH* in laboratory scale bioreactor

Strategy	Operation mode	The Design of Strategy
B1	Batch	Determination of growth characteristic during 18 h of cultivation in defined medium containing glucose of 20 g L ⁻¹ at t = 0 h
B2	Batch	Determination of growth characteristic during 21 h of cultivation in defined medium containing glycerol of 20 g L ⁻¹ at t = 0 h
B3	Batch	Determination of growth characteristic during 25 h of cultivation Defined medium containing glycerol of 20 g L ⁻¹ and acetate of 7.8 g L ⁻¹
B4	Semi-Batch	Defined medium containing glucose of 500 g/L and PTM1 of 12 ml exponentially fed with $\mu = 0.1 \text{ h}^{-1}$ into the fermentation medium during 21 h of cultivation
B5	Semi-Batch	Defined medium containing glycerol %50 and PTM1 of 2.5 ml exponentially fed with $\mu = 0.1 \text{ h}^{-1}$ into the fermentation medium during 21 h of cultivation

For investigation of *PDC* knockout *P. pastoris* strain *GAP::hGH*, five strategies were performed with three ones in batch bioreactor operation and two ones in semi-batch operation and also showed in Table 4.1

To optimize starvation as a synchronization method in air-filtered shake bioreactors, six strategies were developed in terms of factors such as aging and type of pre-cultivation media, affecting maintenance and efficiency of method concerned with the pre-cultivation time. Additionally, the morphological states and intracellular

expression of enhanced green fluorescent protein (eGFP) were viewed by confocal microscopy. The designed strategies were given in Table 4.2.

Table 4. 2 Definitions of strategies designed for batch operation by *P. pastoris* *GAP::eGFP* in air-filtered shake bioreactors

Strategy	Pre-cultivation medium	Pre-cultivation time	Aging	Starvation	Imaging
S1	YPD broth	24-30 h	OD ₆₀₀ = 4	Cultivation in YEP medium for 6 hours	Evaluation of both the physiological states of cell and the intracellular distribution of expressed eGFP viewed by confocal microscopy
S2	YPD broth	24-30 h	OD ₆₀₀ = 8		
S3	YPD broth	24-30 h	OD ₆₀₀ = 16		
S4	BMGY	24-30 h	OD ₆₀₀ = 4		
S5	BMGY	24-30 h	OD ₆₀₀ = 8		
S6	BMGY	24-30 h	OD ₆₀₀ = 16		
S7	YPD broth	15-18 h	OD ₆₀₀ = 4		
S8	YPD broth	15-18 h	OD ₆₀₀ = 16		
S9	BMGY	15-18 h	OD ₆₀₀ = 4		
S10	BMGY	15-18 h	OD ₆₀₀ = 16		

According to the results obtained from air-filtered shaker experiments, the optimized conditions were fulfilled to interpret the impact of synchronization on rhGH produced by *P. pastoris* *GAP::hGH* strain. Hence, the optimized conditions within the scope of aging with optimum pre-cultivation time and of type of production medium in air-filtered shake bioreactors were summarized in Table 4.3

Table 4. 3 Definitions of strategies designed for batch operation by *P. pastoris* *GAP::hGH* in air-filtered shake bioreactors

Strategy	Pre-cultivation phase	Pre-cultivation time	Aging	Starvation	Production phase for 30 h
BMGY-Glucose	BMGY	24 h	OD ₆₀₀ = 4	Cultivation in YEP medium for 6 hours	Glucose
BMGY-Glucose	BMGY	18 h	OD ₆₀₀ = 8		Glucose
BMGY-Glucose	BMGY	18 h	OD ₆₀₀ = 16		Glucose
BMGY-Glycerol	BMGY	24 h	OD ₆₀₀ = 4		Glycerol
BMGY-Glycerol	BMGY	18 h	OD ₆₀₀ = 8		Glycerol
BMGY-Glycerol	BMGY	18 h	OD ₆₀₀ = 16		Glycerol
YPD-Glucose	YPD	18 h	OD ₆₀₀ = 4		Glucose
YPD-Glucose	YPD	18 h	OD ₆₀₀ = 8		Glucose
YPD-Glucose	YPD	18 h	OD ₆₀₀ = 16		Glucose
YPD-Glycerol	YPD	18 h	OD ₆₀₀ = 4		Glycerol
YPD-Glycerol	YPD	18 h	OD ₆₀₀ = 8		Glycerol
YPD-Glycerol	YPD	18 h	OD ₆₀₀ = 16		Glycerol

In air-filtered shake bioreactor experiments, both aging and synchronization had adverse impact on the amount of rhGH produced by recombinant *P. pastoris* strain in production phase containing glucose as the sole carbon source in mineral medium. Thereby, the laboratory scale bioreactor experiments were carried out to decide whether or not any effect of both type of production medium and strictly controlled environmental conditions on synchronization relevant rhGH formation. For this reason, seven strategies of fed-batch operation were conducted and also figured out in Table 4.4. When taking design strategies into consideration, the BR2 was negative control owing to its recommended inoculation age of $OD_{600} = 2-6$ by Invitrogen procedure. There was both differently aged synchronized and desynchronized populations at age of $OD_{600} = 4, 8$, and 16. Thus, asynchronous populations were also negative controls of synchronized populations at same age as well as the sole indicator of aging. All differently aged synchronous populations were considered from the point of view synchronization. Nevertheless, the synchronous population of negative control was demonstrator of only synchronization. In the other cases, the influence of both aging and synchronization should be considered. In further parts, the efficiency of designed strategies regarding synchronization and aging was explained within this concept. In Figure 4.1, the designed seven strategies were demonstrated.

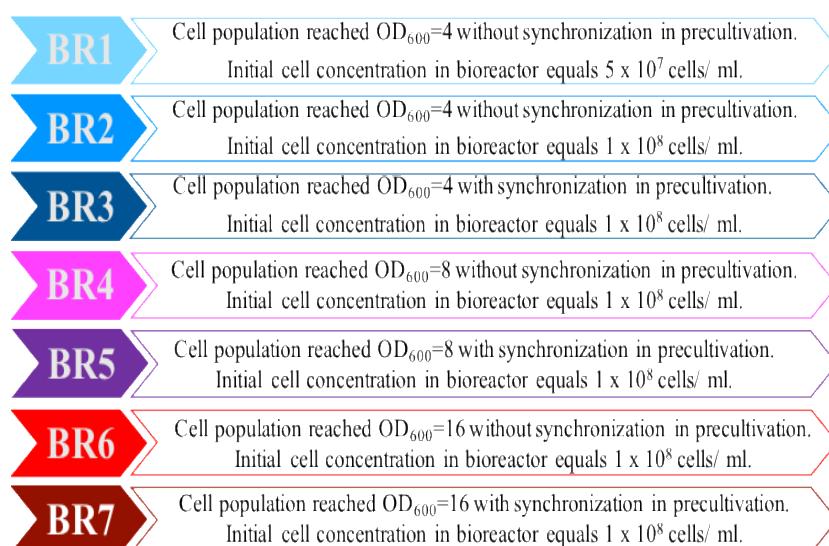


Figure 4. 1 Definitions of strategies designed for rhGH production by *P. pastoris* *GAP::hGH* in semi-batch operations by feeding of glucose with predetermined specific growth rate $\mu = 0.1 \text{ h}^{-1}$ in laboratory scale bioreactors

4.2 Determination of Growth Characteristics of *PDC* Knockout *P. pastoris* strain *GAP::hGH* in Batch Operation

In previous studies related with *PDC* knockout *P. pastoris* strain, its metabolic flux analyses and the concentration of recombinant human superoxide dismutase (hSOD) obtained indicates the increase in product concentration (Nocon et al., 2014). To enhance rhGH production in this research, *PDC* knockout *P. pastoris* strain was investigated. For this purpose, the specific growth rate and specific product formation rate for glycerol, glucose and glycerol plus 40% acetate as a sole carbon source were determined in batch operation under controlled growth conditions. In batch operation, the amounts of glucose and glycerol were adjusted to satisfy 0.66 mole C L⁻¹, being equal to 20 g L⁻¹ in B1 and 20.26 g L⁻¹ in B2. In addition, the amounts of glycerol and acetate in the substrate mixture were determined as C_{Gly}= 20.26 g L⁻¹ and C_{Ace}= 7.8 g L⁻¹ in B3 according to shaker results in which 0.66 mole C L⁻¹ from glycerol and 0.264 mole C L⁻¹ from acetate yielded the higher rhGH production. Nevertheless, the co-utilization of glycerol and acetate was reported despite the combination of glucose and acetate was not preferable because of glucose catabolite repression.

The variations in cell concentration with cultivation time were given in Figure 4.2. For *P. pastoris* strains, the biomass yield coefficient on glucose and glycerol are 0.31-0.50 and 0.5-0.6 g g⁻¹, respectively (Garcia Ortega et al, 2013; Ghosalkar et al., 2008). When viewed from this aspect, the concentration of yeast population reached to 9.4 g DCW L⁻¹ in glucose containing media at t = 20 h⁻¹ of cultivation and 12 g DCW L⁻¹ in glycerol containing media. In addition, the highest cell concentration was attained as C_x = 15.5 g L⁻¹ in glycerol and acetate containing medium owing to contribution of either biomass yield coefficient. In literature there is no available data relevant biomass yield coefficient on acetate for *P. pastoris*. As a result of this experiment, Y_{X/S,Ace} was nearly 1.5 g g⁻¹

These cell growth profiles facilitated to determine specific growth rates on these carbon sources for *PDC* knockout *P. pastoris* strain.

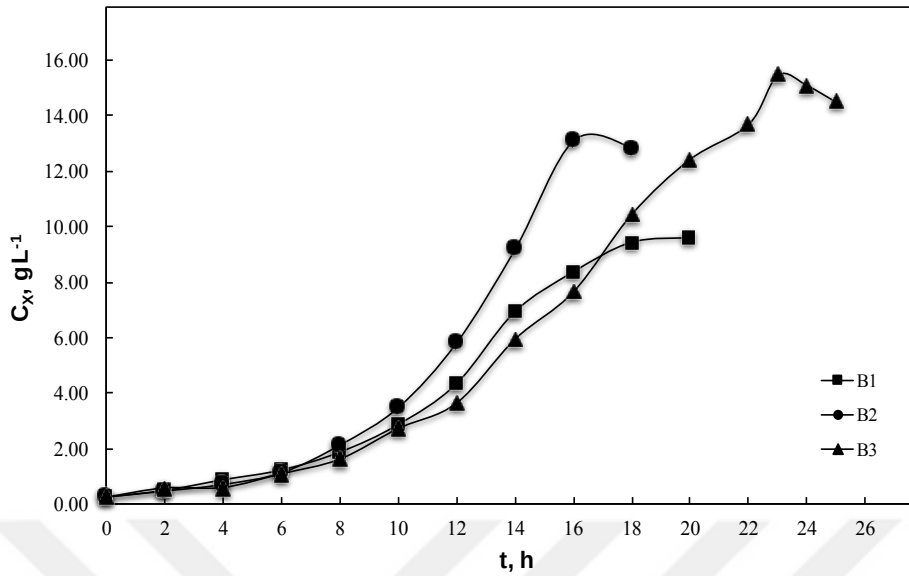


Figure 4. 2 The variations in cell concentration profiles with the cultivation time for different carbon sources

The theoretical maximum specific growth rates ($\mu_{\max}$) on glucose and glycerol at fed-batch operation for *P. pastoris* cultures are 0.19 h^{-1} and $0.20\text{--}0.26 \text{ h}^{-1}$, respectively. The variations in specific growth rate were shown in Figure 4.3. In strategy B1, the μ on glucose was 0.17 h^{-1} prior to $t = 14 \text{ h}$ of bioprocess at which the highest theoretical value of $\mu_{\max}$ was obtained as $\mu_{\max, \text{Glu}} = 0.19 \text{ h}^{-1}$ and then rapidly decreased. On the contrary, the $\mu_{\max}$ on glycerol was acquired as $\mu_{\max, \text{Gly}} = 0.24 \text{ h}^{-1}$ at the beginning of cultivation $t = 8 \text{ h}$, and then gently reduced in B2. The alteration in the specific growth rate on the mixture of glycerol and acetate was so complicated. In previously performed shaker experiments, the specific growth rate of *P. pastoris* on the acetate concentration being equal to 7.8 g L^{-1} was determined as $\mu_{\max, \text{Ace}} = 0.12\text{--}0.13 \text{ h}^{-1}$. In the early exponential growth phase, the value of μ increased from 16 h^{-1} to 20 h^{-1} and then decreased to 13 h^{-1} , again rising to 19 h^{-1} . Hence, the behavior of specific growth rate was like as sinusoidal wave which showed that the use of carbon sources was in order. Moreover, the use of acetate extended the cultivation time more than four hours.

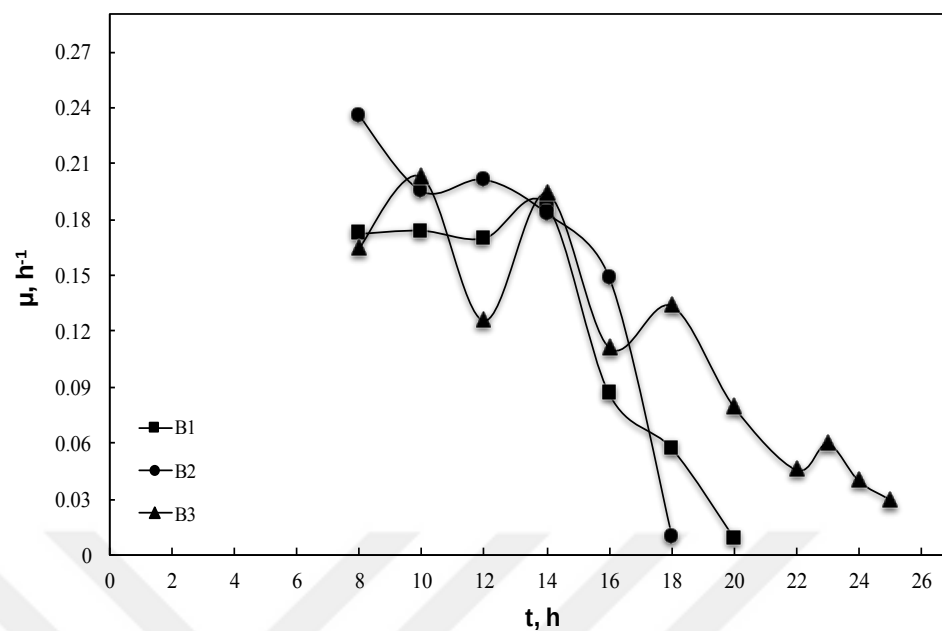


Figure 4. 3 The variations in the specific growth rates with the cultivation time for different carbon sources

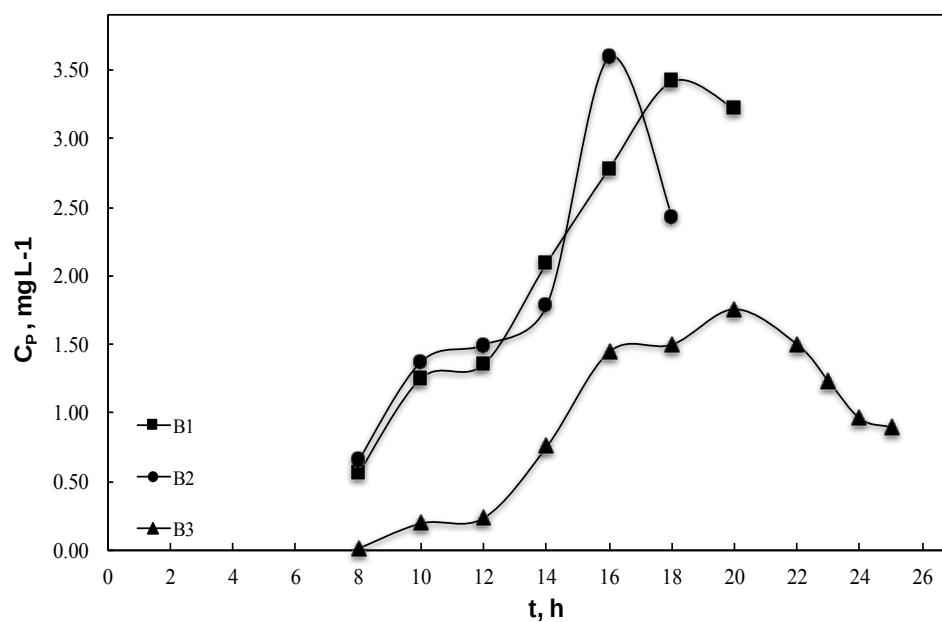


Figure 4. 4 The variations in the rhGH concentration profiles with cultivation time for different carbon sources

Since the r-protein expression under the constitutive *GAP* promoter related with cell growth is triggered by type of carbon sources, the choice of one carbon source among them depends on the concentration of rhGH produced during bioprocess. Among batch operations, the highest C_{rhGH} was acquired as $C_{rhGH,max} = 3.6 \text{ mg L}^{-1}$ in B2 at $t_{max} = 16 \text{ h}$ which is end of late-exponential growth phase and was followed by $C_{rhGH,max} = 3.4 \text{ mg L}^{-1}$ at $t_{max} = 18 \text{ h}$ in B1 containing glucose as a sole carbon source. Thereby, the characteristic of *GAP* promoter commanded the r-protein production being on the rise until the stationary phase under the strictly controlled reactor parameters (Figure 4.4). However, the lowest $C_{rhGH,max} = 1.76 \text{ mg L}^{-1}$ at $t_{max} = 20 \text{ h}$ in the mid-exponential growth phase because the acetate was not enter glycolytic pathway which involves *GAP* promoter and catabolism of both glucose and glycerol and so it changes the metabolic fluxes through TCA cycle, glycolytic, fermentative and pentose phosphate pathway.

The specific production rate ($q_{p,max}$) is the indicator for conversion of substrate to product in a cell metabolism. The highest value of $q_{p,max}$ was strikingly acquired in B3. Nevertheless, the profile of specific production rate with time was not in order in contrast to that in B1 and B2. The variations in specific production rates was presented in Table.

Table 4. 4 The variations in specific rhGH production rates with the cultivation time for different carbon sources

t, h	$q_{p,B1}$	$q_{p,B2}$	$q_{p,B3}$
10	0.24	0.21	0.33
12	0.10	0.02	0.09
14	0.06	0.03	0.45
16	0.08	0.04	0.81
18	0.07		0.04
20			0.26
22			

4.3 The Determination of Growth Characteristics of *PDC* Knockout *P. pastoris* strain *GAP::hGH* in Fed-Batch Operation

Since the lower product concentrations in batch operation, the semi-batch operation was fulfilled by feeding of glucose with predetermined specific growth rate $\mu = 0.1 \text{ h}^{-1}$ to make accurate comparison with the results obtained as $C_{\text{rhGH}} = 508 \text{ mg L}^{-1}$ in semi-batch operation $\mu = 0.15 \text{ h}^{-1}$ and $C_{\text{x0}} = 10 \text{ g L}^{-1}$ from the experiments in previous studies performed by Keskin (2014) in our research group. For this purpose, the glucose and glycerol as a sole carbon source fed to bioreactor with $\mu = 0.1 \text{ h}^{-1}$ in each strategy that designated best suitable carbon source in terms of rhGH production.

4.3.1 The variations in cell concentration profiles, specific growth rate profiles, specific substrate consumption rates and rhGH concentration profiles

The variations in profile of both cell growth and rhGH concentration production were presented in Figure 4.5. The maximum cell concentration reached to $C_X = 95.9$ and 111.4 mg L^{-1} at $t = 21 \text{ h}$ in B4 and B5, respectively. Nevertheless, The highest product concentration was obtained as $C_{\text{rhGH, max}} = 21.62 \text{ mg L}^{-1}$ at $t_{\text{max}} = 21 \text{ h}$ of cultivation in B4 as a result of glucose feed with $\mu = 0.1 \text{ h}^{-1}$ in semi-batch operation meanwhile the highest $C_{\text{rhGH, max}} = 13.40 \text{ mg L}^{-1}$ at $t_{\text{max}} = 21 \text{ h}$ of cultivation in B5 that was semi-batch strategy with glycerol fed by satisfying $\mu = 0.1 \text{ h}^{-1}$.

The variations in specific rates of growth (μ), substrate utilization (q_s) and product formation (q_{rhGH}) were shown in Figure 4.6. The specific growth rates were in the range of 0.10-0.90 until the late exponential growth phase in both strategies. The similar specific carbon consumption rates were observed. Additionally, the specific production rate in B4 was higher compared to that in B5 in the further hours of bioprocess. Hence, the higher productivity was obtained in B4.

Because the lower concentrations of rhGH, the study relevant synchronization and its effect on rhGH production was carried on with well-studied recombinant strain of *P. pastoris* not being gene deficient in our research group.

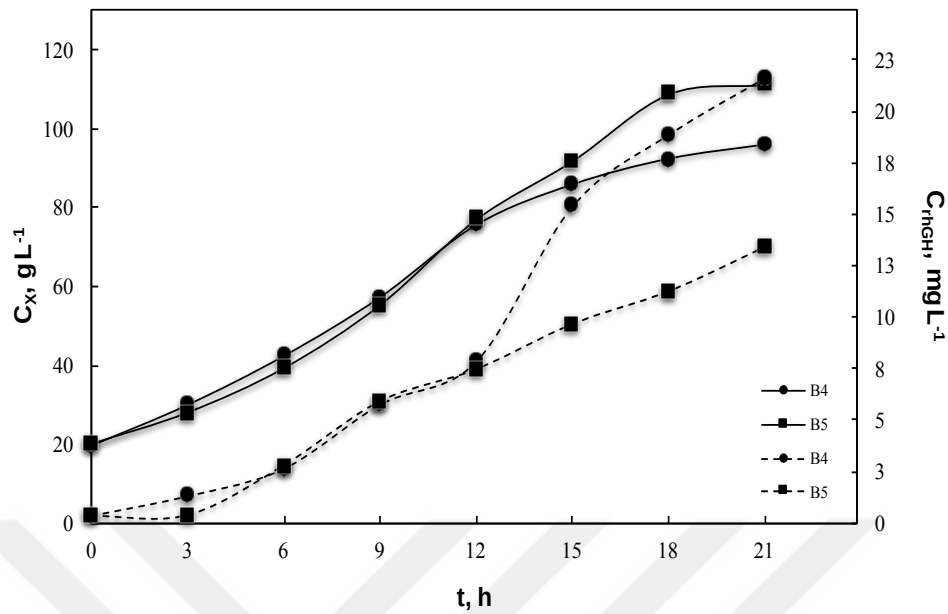


Figure 4. 5 The variations in cell concentration (solid line) and the concentration of rhGH produced (dashed line) with the cultivation time

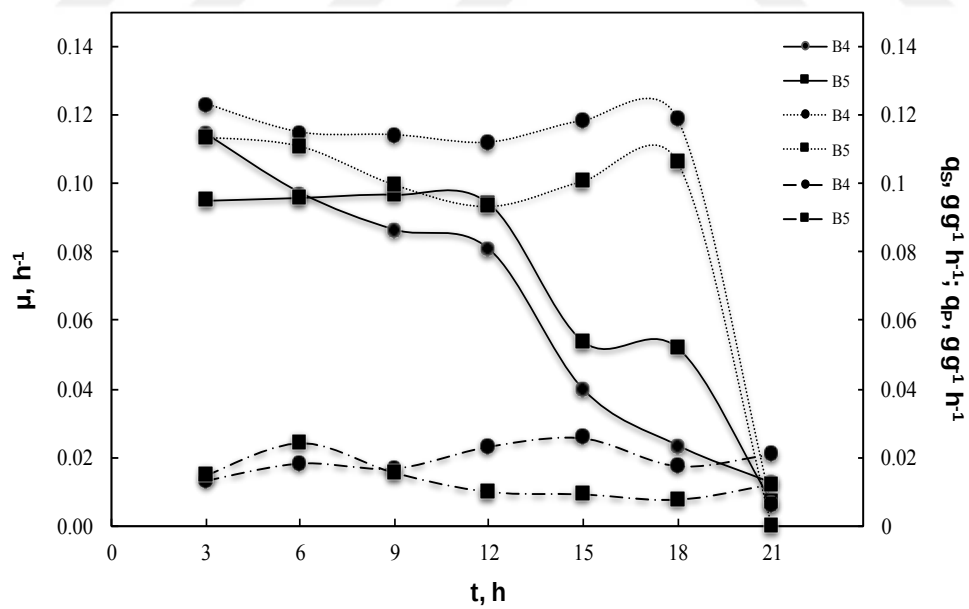


Figure 4. 6 The variations in specific growth rate (solid line), specific substrate utilization rate (dashed line) and specific protein production rate with the cultivation time

4.4 Optimization of Starvation as a Synchronization Method in Air-Filtered Shake Bioreactors for The Batch Cultures of *P. pastoris* strain *GAP::eGFP*

The influence of cell age and pre-cultivation medium on synchronization method, starvation, based on carbon source deficiency was firstly investigated and then the optimized condition was determined according to synchronization degree that also defines the physiological state designated by the shape of cells, and intracellular production and localization of eGFP as the model protein.

In this study, the best physiological state of synchronous yeast cultures can be specified by completely round cell without bud and vacuole formation, and by uniformly dispersed r-proteins throughout the cytosol. To obtain well synchronized yeast populations in air-filtered shake bioreactors, two widely used pre-cultivation media for *P. pastoris*, YPD broth and BMGY, were simultaneously experimented with cell ages at the mid-exponential growth phase (OD_{600} of 4), at the late-exponential growth phase (OD_{600} of 8) and at the stationary phase (OD_{600} of 16) (Basanta et al., 2010). In the perspective of this experiment design based on optimization of synchronization, the efficiency of starvation procedure was evaluated by the intracellular green fluorescent distribution pattern revealing inside the cell, the shape of cells, and the existence of budding. Hence, the expression level of r-protein was ignored.

The cells were grown in two media until the cell density reached to $OD_{600} = 4$, 8, and 16 for 24 h that is also recommended time for starvation in synchronization procedure. Then, the cultures were inoculated to YEP and cultivated during 6 h to synchronize with starvation. At the end of starvation, the confocal microscopy images of the cells were taken to check whether they can be synchronized or not (Figure 4.7). In response to starvation, the yeast cultures grown in YPD broth at three different ages always satisfied the size, bud, vacuole and uniformity related criteria mentioned above (Figure 4.7A). On the contrary, the cells grown in BMGY at $OD_{600} = 16$ were not synchronized even though the cells grown in BMGY at $OD_{600} = 4$ and 8 were synchronous. Further, the vacuole formation in cells at $OD_{600} = 16$ demonstrated that they were more aged as a function of time (Figure 4.7B).

As a result of the difference between the physical situations of cells at $OD_{600} = 16$ and of cells at $OD_{600} = 4$; 8 in BMGY, the cultivation time was decreased to 15-18 h that is also preferable pre-cultivation time for *P. pastoris*. The cultivation time was decreased not only for cells grown in BMGY until $OD_{600} = 4$ and 16 but also for cells grown in YPD broth until $OD_{600} = 4$ and 16. The cells grown in both BMGY and YPD broth, at age $OD_{600} = 16$, had vacuoles but were in same size even if the others were desynchronized since budding (Figure 4.7C).

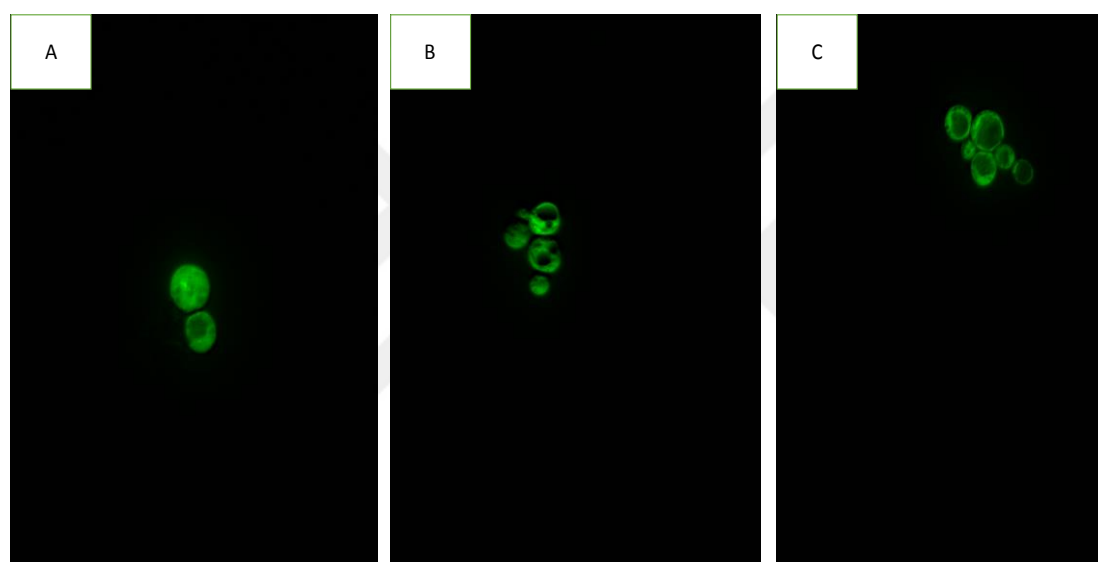


Figure 4. 7 The confocal microscopy images of synchronous cells at the end of starvation process to control cell morphology in (A) The image of cell population grown to $OD_{600} = 16$ in YPD medium for 24 h; (B) The image of cell population grown to $OD_{600} = 16$ in BMGY medium for 24 h; (C) The image of cell population grown to $OD_{600} = 16$ in BMGY medium for 15-18 h.

Consequently, the achievement of synchronization with starvation was considerably influenced by the choice of pre-cultivation medium associated with cell age and pre-cultivation time. The synchronization in YPD was always accomplished under all situations whereas the synchronization in BMGY required different optimization relevant cell age and time. In other words, for the probability of

successful cell cycle synchronization in BMGY, the older cells were cultivated for the time below 24 h to avoid vacuole formation whereas the younger ones required longer time in pre-cultivation phase to reduce budding index that disturbs cell cycle synchronization. Therefore, to obtain and arrest the healthy cells in early G₁ phase via starvation, the younger ones required to be grown to OD₆₀₀ = 4 for 24-30 h while the older ones should be grown to OD₆₀₀ = 16 for 15-18 h in BMGY.

4.5 The Effect of Starvation as a Synchronization Method on rhGH production in air-filtered shake bioreactors for batch operation of *P. pastoris*

GAP::hGH

Extracellular secretion of any r-protein influences the intrinsic dynamics of a cell. For this reason, the effect of cell age related synchronization on rhGH production and secretion into fermentation medium was determined by taking previously optimized conditions into consideration for starvation in two pre-cultivation media mentioned above. In addition, two chemically defined media containing either glycerol or glucose as carbon source were used as production media to understand how they affect the efficiency of synchronization

Two pre-cultivation media, three cell age of OD₆₀₀ = 4; 8; 16 for synchronization plus one cell age of OD₆₀₀ = 4, as a negative control, and two production media were combined with each other. As result of this combination, the sixteen different strategies were performed in triplicate within the scope of this phase in the experimental design. The samples were taken at t = 30 h of cultivation time in all strategies and their replications.

To make a point of growth kinetics at the end of starvation process in all conditions, the exponential growth was observed and cell concentrations were 2- or 3-fold higher than that prior to starvation and thus the lack of carbon source in the medium did not suppress microbial growth. Further, regardless of both pre-cultivation and production media, the cell concentrations were similar as C_x = 10-11 g/L for the cell density of synchronous populations that reached to OD₆₀₀ = 4 and 8, and of asynchronous ones to OD₆₀₀ = 4. However, the divergent results were obtained in mostly aged synchronous cell populations grown to OD₆₀₀ = 16. In the synchronous

populations inoculated from YPD and BMGY to glucose containing media, their cell concentrations reduced. On the contrary, the higher C_x was reached as 15 g/L and 13 g/L in synchronous cultures transferred from YPD broth and BMGY, respectively, to the production medium containing glycerol. This interference was expected because of the maximum specific growth rate, μ , on glycerol being higher than that on glucose for *P. pastoris*. Nevertheless, the difference between cell concentrations originated from the maximum specific growth rate on glucose and glycerol was not distinguished. Therefore, the values of r-protein concentration and overall product yield on total cell are more meaningful to consider the contribution of synchronization in r-protein production.

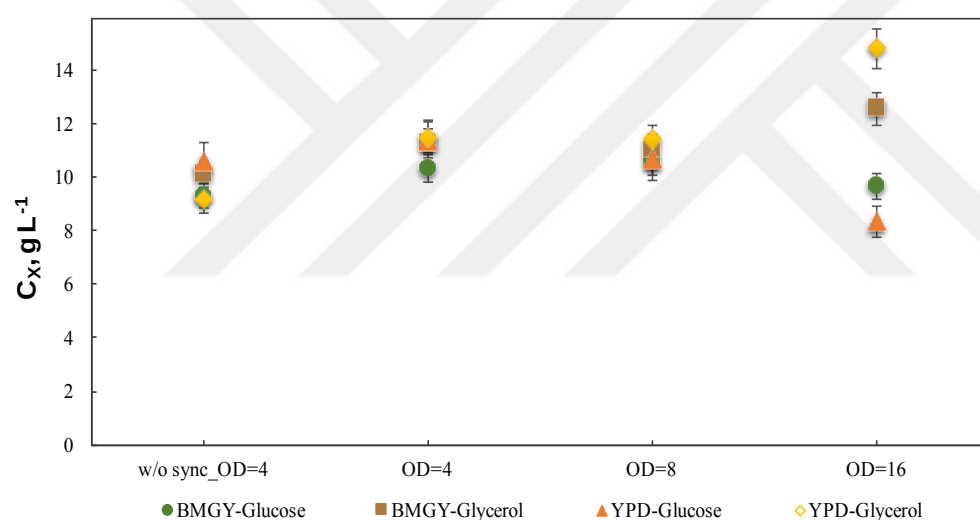


Figure 4. 8 The profile of asynchronous cell population at age $OD_{600} = 4$, and of synchronous population at age $OD_{600} = 4, 8$, and 16 at $t = 24$ h at which the highest cell concentration was obtained

In favor of the synchronized yeast populations, the maximum increase in rhGH production between negative control and synchronous one was acquired as 3.61-fold in populations transferred from YPD to glycerol containing media at $OD_{600} = 16$, and 2.13-fold followed that in the cultures transferred from BMGY to glycerol including

medium at $OD_{600} = 16$. Nevertheless, in synchronous cultures inoculated from BMGY to glucose involving medium at $OD_{600} = 4$, the maximum C_{rhGH} was reached to 55 mg/L, and 1.8-fold higher than its negative control. The product concentrations of synchronous populations softly reduced or increased compared to their negative controls in all cases excepting that at $OD_{600} = 16$.

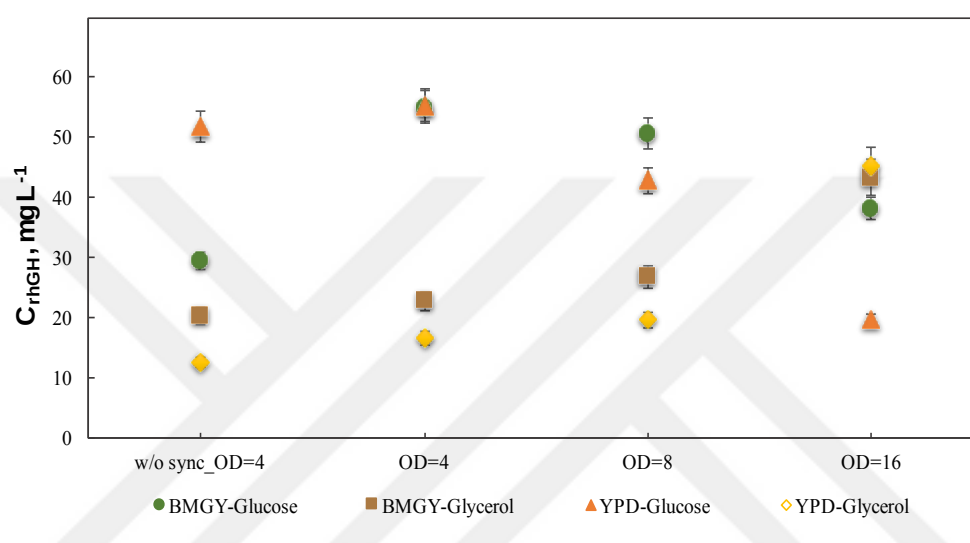


Figure 4. 9 The rhGH profile of asynchronous cell population at age of $OD_{600} = 4$, and of synchronous population at age of $OD_{600} = 4, 8$, and 16 at $t = 24$ h at which the highest rhGH concentration obtained

Similar to the values of rhGH concentration, in synchronous cultures inoculated from BMGY to glucose involving medium at $OD_{600} = 4$, the highest overall product yield on total cell concentration was $5.3 \text{ g g}^{-1}\text{h}^{-1}$ and 1.7-fold higher than negative one and the maximum increment in that overall yield between synchronous one and asynchronous one was 2.2-fold higher on the side of synchronous populations transferred from YPD to glycerol containing media at $OD_{600} = 16$.

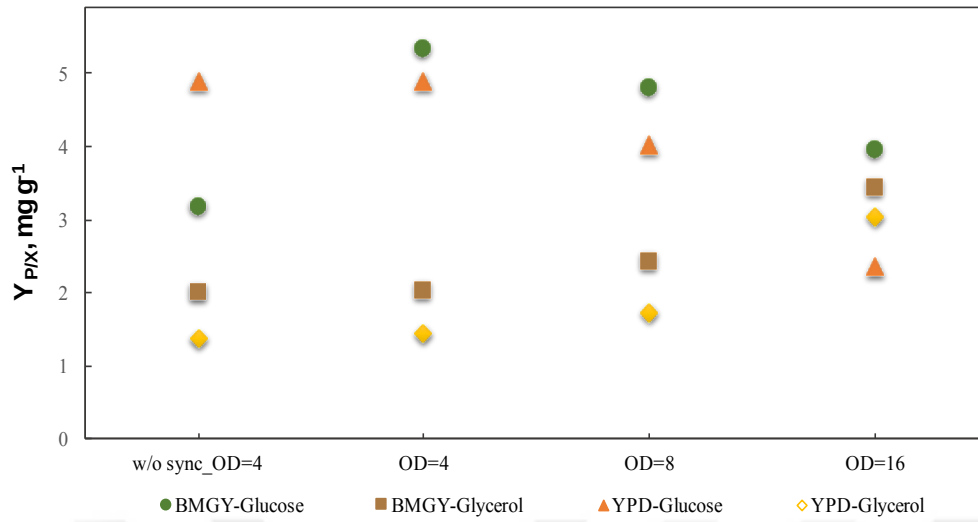


Figure 4. 10 The profile in overall product yield on total cell of asynchronous cell population at age of $OD_{600} = 4$, and of synchronous population at age of $OD_{600} = 4$, 8, and 16 at $t = 24$ h at which the highest rhGH concentration obtained

Consequently, all highest values of C_{rhGH} , and increment fold were obtained in same strategy because cell concentrations did not differ significantly. Further, the loci of the curves of overall product yield ($\bar{Y}_{P/X}$) on total cell for all designed and performed operation conditions were identical to that of curve for rhGH concentration by virtue of no difference between their cell concentrations in cases of with and without starvation excepting $OD_{600} = 16$. Nevertheless, the rhGH production was modulated by the type of the carbon source used in synchronous cultures of yeast cells similar to that in conventional bioprocess in *P. pastoris*, and also enhanced by glycerol under the impact of aging and synchronization.

4.6 The Effect of Starvation as a Synchronization Method on rhGH Production in Fed-Batch Operation of *P. pastoris* strain *GAP::hGH*

To enhance cell growth and r-protein production in laboratory scale bioreactor operation, the desired metabolic activity in isogenic population of microbial cells is manipulated by favorable supply of nutrient and oxygen under strictly controlled key parameters in bioreactor operation, including temperature, pH, mixing and aeration. Unlike laboratory scale bioreactor operation, the air-filtered shake bioreactor operation provokes oscillations in oxygen, pH and substrate levels due to absence of control system, and so triggers the synchronized populations to be desynchronized. Therefore, the metabolic activity of synchronous cell populations should be particularly examined under the conditions in laboratory scale bioreactor operation rather than those in the air-filtered shake bioreactor operations. On this basis, laboratory scale bioreactor operations were performed with the widely used pre-cultivation medium BMGY, BSM in batch operation, and glucose as a sole carbon source plus PTM1 in fed-batch operation whether the yeast culture was synchronized or not. However, the experiments performed in the air filtered shake bioreactor demonstrated the adverse effects of both cell aging and synchronization on rhGH production in production medium containing glucose that also provokes r-protein synthesis under *GAP* promoter.

To comprehend how cell aging and synchronization affect the level of r-protein expression without the impact of the poor conditions in the air filtered shake bioreactor, laboratory scale bioreactor experiments were carried out by exponential glucose feeding with pre-determined specific growth rate of $\mu_0 = 0.10 \text{ h}^{-1}$ for heterologous protein expression under *GAP* promoter in *P. pastoris*.

4.6.1 Effect of initial cell concentration on cell growth and rhGH production in batch operation

Prior to investigation of the influence of synchronization on rhGH production, the effect of inoculation cell densities (C_{X_0}) in batch operation was investigated at 0.24 g DCW L⁻¹ ($OD_{600} = 1$) in BR1 and 0.50 g DCW L⁻¹ ($OD_{600} = 2$) in BR2 (Fig. 2). Since the limitation of carbon amount being same in batch phases of all strategies, the final

cell concentrations reached to $C_X = \sim 24 \text{ g DCW L}^{-1}$ ($OD_{600} = 100$) at the end of batch phases in all strategies. Nevertheless, two different inoculation cell densities changed the elapsed time in batch operation as a result of cultivation time required being 20 h in BR1 and 17 h in BR2 respectively. Furthermore, the time difference between two batch phases was nearly equal the doubling time, 2.5–3.0 h, of this recombinant strain, and so the generation of cell population in BR2 was younger than that in BR1. In this case, the intracellular dynamic of cell can show an alteration. Thus, the highest concentrations of cell and r-protein produced in fed-batch phases of both BR1 and BR2, were evaluated whether there is the effect of various inoculation cell densities on rhGH production.

In comparison with fed-batch phase of BR1 and BR2, the maximum cell concentration was obtained as $C_X = 142 \text{ g DCW L}^{-1}$ in BR2, and also was 1.27-fold higher than that obtained in BR1. Additionally, the highest rhGH concentration was acquired as $C_X = 357 \text{ mg L}^{-1}$ in BR2 and 2.2-fold higher compared to that in BR1 (Fig. 5B). Thus, the inoculation density of batch phase in laboratory scale bioreactor experiments was favored as $C_{X0} = 0.5 \text{ g DCW L}^{-1}$ ($OD_{600} = 2$) to investigate the effect of synchronization on rhGH production.

4.6.2 The variations in cell concentration profiles of differently aged synchronous and asynchronous cell populations

The effect of both cell age and synchronization on the variations of cell growth profiles with cultivation time was presented in Figure 4.11. In all designed strategies of semi-batch operation, the highest cell concentration was ever reached at $t = 15 \text{ h}$ of bioprocess plus the strategy of BR2 was aimed as negative control in which OD_{600} of 2–6 as the recommended log-phase growth that cells need to reach (Invitrogen) was used. When considering negative control, the effect of aging was only evaluated according to results obtained from strategies, BR4 and BR6, in which no synchronization was while the strategy of BR3 which had synchronous cells at same age, determined how synchronization influenced the dynamics of cell. Additionally, the impact of their combination was judged via strategies, BR6 and BR7, having both of synchronization and aging.

In the strategies excepting BR2 and BR7, the cell growth profiles were similar and maximum cell concentrations varied in the range of 115-125 g DCW L⁻¹. However, the cell concentrations after t = 9 h in BR2 were the highest values whereas the cell concentrations after t = 6 h in BR7 were the lowest ones compared to those in other strategies.

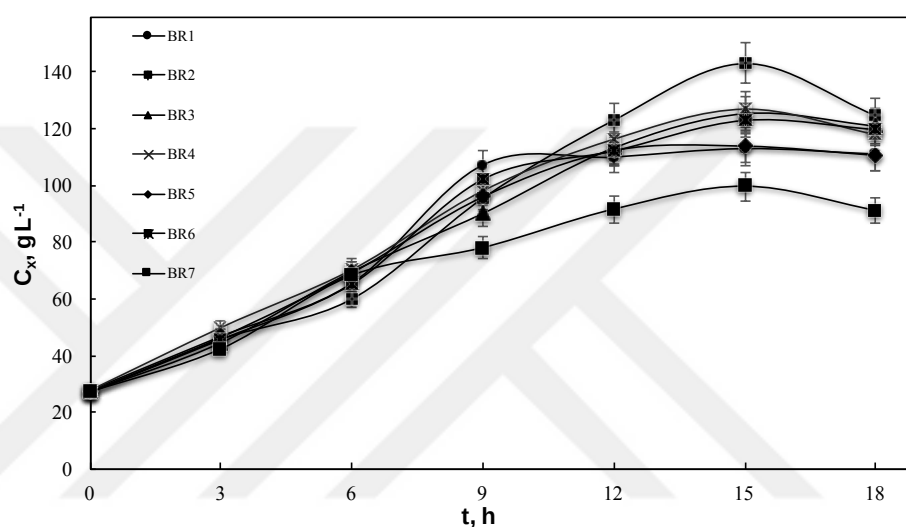


Figure 4. 11 The variations in cell concentration in the fermentation medium with the cultivation time for both of asynchronous and synchronous cell populations at age of OD₆₀₀ = 4, 8 and 16

In comparison with synchronous and asynchronous cultures, the growth profiles differed from each other starting from t = 6 h in BR3, BR5 and BR7 of strategies including synchronous cell populations, and from t = 9 h in BR2, BR4 and BR6 of strategies containing asynchronous ones. Additionally, the highest cell concentration of synchronous population at current age was always lower than those of asynchronous ones at same age in all aging-associated conditions. The population aging to OD₆₀₀ = 4 in BR2 among asynchronous populations reached the highest cell concentration as $C_X = 142 \text{ g DCW L}^{-1}$ that was 1.14- and 1.27-fold higher than the cell concentration of asynchronous population aging to OD₆₀₀ = 8 in BR4, and to OD₆₀₀ = 16 in BR6, respectively. Since the similar trend was obtained in synchronous

populations, the lowest cell concentration was attained as $C_X = 100 \text{ g DCW L}^{-1}$ in the population at age of $OD_{600} = 16$, being followed by 114 g DCW L^{-1} and 125 g DCW L^{-1} at age of $OD_{600} = 8$ and 4 , respectively.

According to results originated from negative control BR2, the cell concentrations decreased with aging whether synchronization or not and there was the equal contribution of synchronization and aging into cell growth. Nonetheless, the application of both synchronization and aging was 1.26-fold more effective than that alone of either synchronization or aging. Therefore, the maximum difference between cell concentration of synchronous and asynchronous populations was observed at age $OD_{600} = 16$. Consequently, the synchronization influenced the cell growth via aging, and the cell growth profile of BR7 differed from other conditions considerably.

4.6.3 The variations in glucose concentration profiles and specific glucose utilization rates of differently aged synchronous and asynchronous cell populations

At all operations, the glucose as a sole carbon source fed to the bioreactor was totally consumed until $t = 15 \text{ h}$; and then it accumulated. The minimum amount of accumulated glucose (C_S) in fermentation medium was 2.2 g L^{-1} in BR2, being 4-, 8- and 2-fold lower than that in BR4, BR6 and BR3, whereas the highest glucose accumulation was detected as $C_S = 17.1 \text{ g L}^{-1}$ in BR7, which was almost same in BR6 and also 1.3- and 3.2-fold higher than that in B5 and B3, respectively.

These values show that the amount of accumulated glucose increased from younger cell population to older ones, irrespective of synchronization. Further, the increase coefficient from younger ones to older ones for asynchronous cultures was 4 and 2 between cultures at $OD_{600} = 4$ and $OD_{600} = 8$, and between cultures at $OD_{600} = 8$ and $OD_{600} = 16$, respectively. However, this coefficient for synchronous cultures was lower than that for asynchronous ones. From the point of view of synchronization effect, the difference in accumulated glucose between synchronous and asynchronous cultures at same age were same in aging cultures to $OD_{600} = 4$ and 8 , and this difference was not observed between both cultures aging to $OD_{600} = 16$. As a result of comparison with negative control, aging process was more perturbational rather than only

synchronization while differently aged asynchronous population more effectively metabolized carbon source compared to synchronized populations at same age with regards to glucose accumulation (Figure 4.12). In addition, the variations in the amount of accumulated glucose between both aged and synchronized populations were lower than that between only aged asynchronous ones.

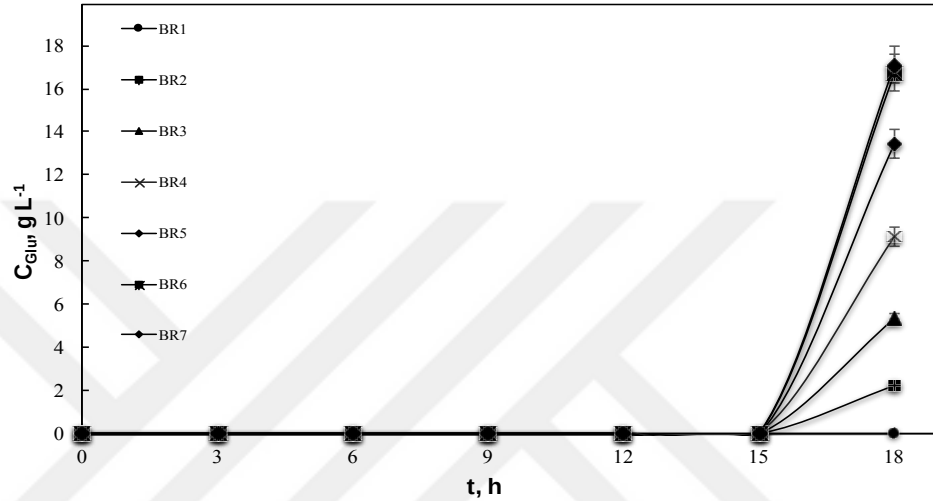


Figure 4. 12 The variations in glucose concentration in the fermentation medium with the cultivation time for both of asynchronous and synchronous cell populations at age of $OD_{600} = 4, 8$ and 16

The specific substrate consumption rates (q_s) relevant cell concentration were considerably differed from each other although the volumetric substrate utilization rates (r_s) were the same in each strategy at $t = 0-15$ h of the fed-batch operation owing to completely consumed glucose. Also, the maximum specific substrate utilization rate ($q_{s,max}$) was attained at $t = 15$ h of bioprocess in all strategies (Figure 4.13). On the other hand, in all fed-batch operations, the specific substrate consumption rates were similar at $t = 0-3$ h and then they were diverged. During all process, the q_s of BR2 and BR3 was so linear whereas the others were in same oscillated behavior with the inflection point at $t = 9$ h (Fig. 7). The highest specific glucose consumption rate among all them with and/or without synchronization was acquired in BR7 as $q_s = 0.32$ $g\ g^{-1}\ h^{-1}$.

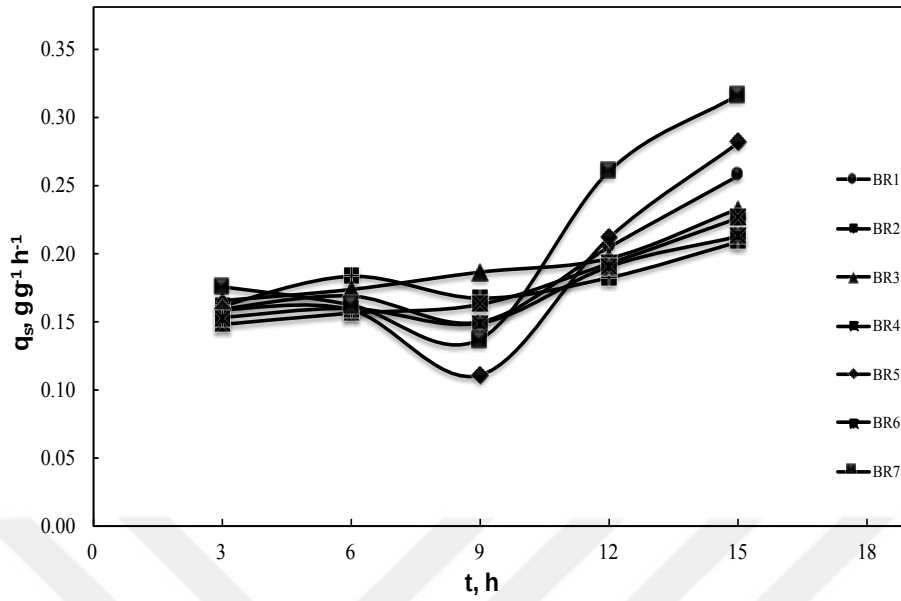


Figure 4. 13 The variations in specific substrate utilization rates in the fermentation medium with the cultivation time for both of asynchronous and synchronous cell populations at age of $OD_{600} = 4, 8$ and 16

When considering aging, the highest one among maximum specific glucose consumption rates in differently aged asynchronous populations was obtained as $q_{S,max} = 0.23 \text{ g g}^{-1} \text{ h}^{-1}$ in population aging to $OD_{600} = 8$ in B4, being approximately close to other ones. However, the highest $q_{S,max}$ in differently aged synchronous populations reached to $0.32 \text{ g g}^{-1} \text{ h}^{-1}$ in population aging to $OD_{600} = 16$ in BR7 that was 1.12-, 1.36- and 1.51-fold higher than that in in B5, B3 and B2, respectively. Thus, the maximum specific glucose consumption rates increased with respect to aging in the case which synchronization was applied meanwhile the $q_{S,max}$ in asynchronous population increased in middle-aged ones and then decreased in older ones with small differences.

From the point of view of synchronization, the specific consumption rates in synchronous cell populations at age $OD_{600} = 4, 8$, and 16 , were respectively 1.11-, 1.24- and 1.48-fold higher than that of asynchronous ones owing to lower cell concentration compared to asynchronous ones. In comparison with negative control BR2, the fed-batch operation should be enhanced by adequate substrate limitation for both aged and synchronized population in BR5 and BR7 because of the maximum specific substrate consumption rate related to specific growth rate.

4.6.4 The variations in rhGH concentration profiles and specific rhGH production rates of differently aged synchronous and asynchronous cell populations

The variation in rhGH concentration whether there is cell age and synchronization or not were presented in Figure 4.14. In all strategies of fed-batch operation, each maximum r-protein concentration ($C_{rhGH,max}$) was attained at different times (t_{max}) of bioprocess and there was no similar behavior between the rhGH concentration profiles unlike the cell growth profiles. The highest rhGH concentration among maximum r-protein concentrations in all strategies was obtained as $C_{rhGH,max} = 571 \text{ mg L}^{-1}$ at $t_{max} = 12 \text{ h}$ in BR7 in which synchronous yeast population aging to $OD_{600} = 16$ was involved, and also was followed by $C_{rhGH,max} = 357 \text{ mg L}^{-1}$ at $t_{max} = 12 \text{ h}$ in BR2 as negative control. Contrariwise, the lowest one among them was equal to 189 mg L^{-1} at $t_{max} = 6 \text{ h}$ in BR3 in which synchronous yeast population aging to $OD_{600} = 4$ was included.

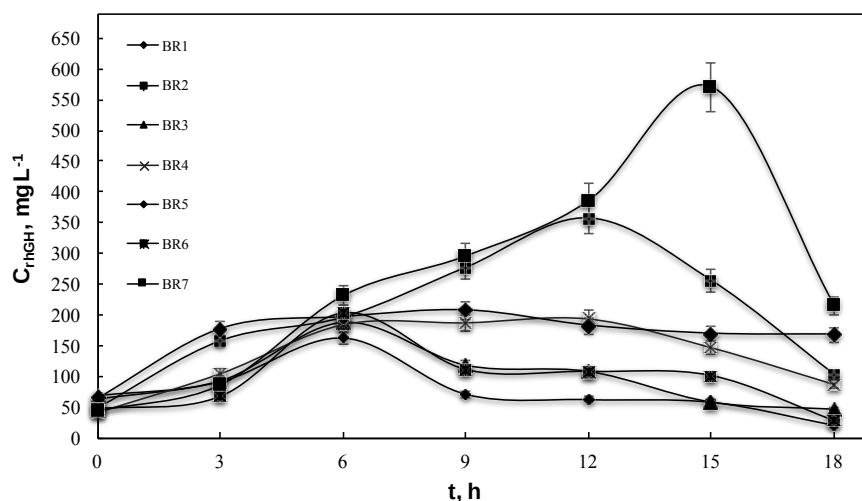


Figure 4. 14 The variations in the concentration of rhGH produced with the cultivation time for both of asynchronous and synchronous cell populations at age of $OD_{600} = 4$, 8 and 16.

In differently aged synchronous populations, the highest value of rhGH concentration increased from older ones to younger ones, being 571 mg L⁻¹ at t = 15 h in BR7, 208 mg L⁻¹ at t = 9 h in BR5 and 185 mg L⁻¹ at t = 12 h in BR3, respectively. Conversely, the maximum rhGH concentrations reduced from younger asynchronous yeast populations to older ones, being 357 mg L⁻¹ at t = 12 h in BR2, 194 mg L⁻¹ at t = 12 h in BR4 and 173 mg L⁻¹ at t = 6 h in BR6, respectively. On the other hand, acquired amount of rhGH synthesized in mostly aged synchronized population was 2.81- and 1.1-fold higher than asynchronous one that at age OD₆₀₀ = 16 and OD₆₀₀ = 8, respectively. When considering maximum time (t_{max}) at which the highest product concentration was obtained, the aging within the scope of synchronization led to the attainment of product in further hours. Conversely, t_{max} in asynchronous ones was shorten only by aging to OD₆₀₀ = 16.

In the case of synchronization, the rhGH concentration in BR3 decreased in comparison with the its negative control, BR2 at same age. In both populations aging to OD₆₀₀ = 8, the synchronization was not altered rhGH production. However, synchronization had the adverse effect on rhGH production in younger cell population at age of OD₆₀₀ = 4, but positive impact on that in mostly aged cell culture to OD₆₀₀ = 16. As a result of this, the rhGH production was enhanced with aging in the synchronous populations unlike that in asynchronous populations. On the other hand, there was no detectable co-inference concerned with association of aging and synchronization and so they were separately evaluated each other. The highest rhGH concentration attained in synchronous populations at age of OD₆₀₀ = 16 was 1.6-fold higher than that asynchronous population at age of OD₆₀₀ = 4 as negative control while any enhancement was not detected in other strategies.

The rhGH concentration profiles of all strategies were presented in Figure 4.14. This figure showed that both of the synchronized and desynchronized populations at age of OD₆₀₀ = 4 and OD₆₀₀ = 16 had a breakthrough for rhGH production though there was stable production in both populations at age of OD₆₀₀ = 8 during all process. Furthermore, the younger cell populations without synchronization produced higher amount of rhGH whereas a complicated profile of r-protein concentration was observed in populations synchronized. Additionally, there was a divergent point at t =

6 h when the curve of cell growth in synchronous one was examined. When considering both variations in cell and rhGH concentration profiles of synchronous populations, the differentiation in cell concentration influenced the rhGH production.

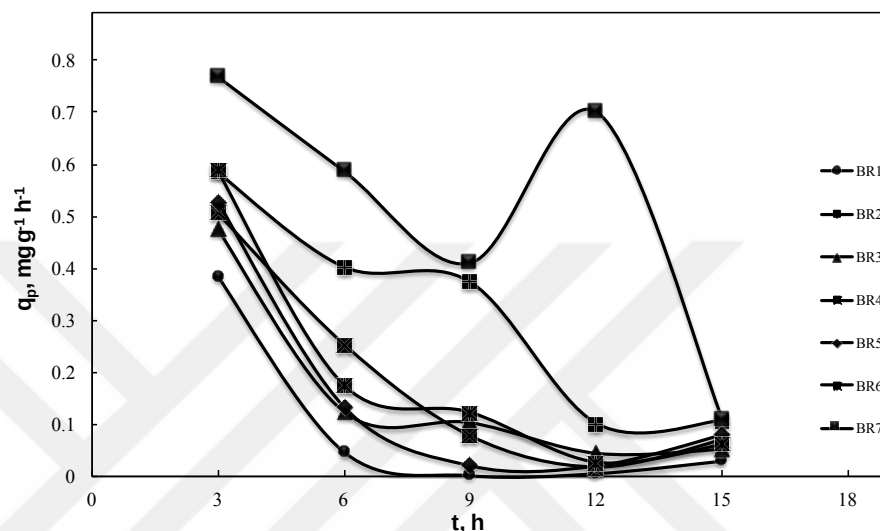


Figure 4. 15 The variations in the specific rhGH production rates (q_p) with the cultivation time for both of asynchronous and synchronous cell populations at age of $OD_{600} = 4, 8$ and 16

In all strategies excepting BR7, the specific rhGH production rates (q_p) decreased with time and the highest value was obtained in BR7 $q_{rhGH} = 0.77 \text{ mg g}^{-1} \text{ h}^{-1}$ at $t = 0 \text{ h}$ of cultivation when the all specific rhGH production rates were compared (Figure 4.15). The specific rhGH production rates increased with respect to aging in synchronous populations, $0.47 \text{ mg g}^{-1} \text{ h}^{-1}$ in BR3, $0.53 \text{ mg g}^{-1} \text{ h}^{-1}$ in BR5 and $0.77 \text{ mg g}^{-1} \text{ h}^{-1}$ in BR7, while the q_p of asynchronous populations decreased from $0.59 \text{ mg g}^{-1} \text{ h}^{-1}$ as q_p of younger population in BR2 to $0.51 \text{ mg g}^{-1} \text{ h}^{-1}$ as q_p of middle-aged one in BR4, and then increased again to $0.59 \text{ mg g}^{-1} \text{ h}^{-1}$ as q_p of the mostly aged one in BR6. The variation in q_p of asynchronous populations was not dependent from aging to the contrary of the increase in q_p of synchronous populations with aging.

In the synchronous population at age of $OD_{600} = 16$, the q_p value was 1.31-fold higher than both asynchronous ones at same age and $OD_{600} = 4$, being as negative control whereas no big difference in q_p of synchronized and desynchronized populations at $OD_{600} = 8$. However, the q_p of synchronized population at $OD_{600} = 4$ was 1.23-fold lower compared to asynchronous one as negative control. Accordingly, the association of aging and synchronization positively influenced the rhGH production in the cellular level.

Figure 4.15 displayed that desynchronized populations at age of $OD_{600} = 4$ and the synchronized one at age of $OD_{600} = 16$ had different trends of q_p curve and the milestone at $t = 9$ h and 12 h at which the highest rhGH concentrations of them were obtained, respectively.

Consequently, the main outcome of this research was resulted in that rhGH production by *P. pastoris* was affected not only from the cell age but also synchronization. Contrarily, Love et al., (2010) showed that the secretion of recombinant crystallizable region (Fc) fragment by *P. pastoris* was constitutive and independent from cell cycle while Frykman et al., (2001) indicated that the secretion behavior of recombinant human cytokine in synchronous cultures was different from that in asynchronous cultures. Otherwise, the intracellular production of recombinant rice amylase by *S. cerevisiae* was higher in M phase compared to other phases (Uchiyama et al., 1997). There was a difference between the synchronization effect on the intracellular production and extracellular secretion of heterologous protein in *S. cerevisiae* due to the trafficking in endoplasmic reticulum, golgi and vacuole. Thus, the effect of synchronization on r-protein production concerned with the secretion, cell cycle dependency and the choice of method might be protein specific in *P. pastoris* similar to *S. cerevisiae*. Prior to make a generalization, the further research that determines the effect of synchronization on intracellular recombinant protein production is required whereas we have demonstrated the favorable effect of synchronization via aging on the productivity of extracellular produced r-protein. Additionally, starvation as a synchronization method should be applied in semi-batch operation because it was applied in chemostat cultivations of *S. cerevisiae* with periodic feeding and/or starving by carbon and nitrogen source and also it was defined

as spontaneous synchronization (Creanor and Toyne, 1993; Boiateaux, 1995). Additionally, Ortega et al., (2016) performed starvation in fed-batch operations in *P. pastoris* and improved r-protein production

4.6.5 The variations in yield coefficients with cultivation time and in respective overall yield coefficients

The variations in yield coefficients through the bioprocess and strategy specific overall yield coefficient was given in Table 4.5. In batch operation for Fab production, the biomass yield on glucose was determined as 0.31 g g^{-1} while the value of $Y_{x/s}$ increased to 0.45 g g^{-1} with the predetermined specific growth rate in semi-batch operations (Garcia-Ortega, 2013). In this study, the mean values of cell yield on substrate varied from one strategy to another one, being 0.47, 0.46, 0.58, 0.48, 0.42, 0.51 and 0.31 g g^{-1} in BR1, BR2, BR3, BR4, BR5, BR6 and BR7, respectively. However, the biomass yield was oscillated in a certain range in all strategies and decreased through the end of bioprocess. This coefficient could not change greatly in the event of the adjusted volumetric flow satisfying pre-determined specific growth rate and thus was evidence that sufficient amount of substrate was supplied for cell growth.

The product yield on cell was higher at the beginning of exponential growth rather than that through its end. The highest mean value of product yield on cell was attained as 9.53 g g^{-1} in BR7, being followed by 3.89 g g^{-1} in BR2. This indicated that these two strategies improved the productivity of cell populations. Similar to the product yield on cell, the highest product yield on substrate was 2.80 g g^{-1} and 1.80 g g^{-1} in BR7 and BR2, respectively while the values in other strategies changed in the range of $0.34\text{-}0.77 \text{ g g}^{-1}$. Thus, these strategies promoted cell population to efficiently utilize glucose for r-protein production.

The overall yield coefficient determines the system characteristic related with cultivation time at which the highest r-protein concentration reached. According to these relative values, among the all systems, the best system of cell populations as producer was obtained in BR7, that has highest overall product yield with the lowest overall biomass yield on cell, being as 15.32 g g^{-1} and 0.13 g g^{-1} , respectively.

Table 4. 5 The variations in yield coefficients with the cultivation time, and in respective overall yield coefficients with the cultivation time for both of asynchronous and synchronous cell populations at age of $OD_{600} = 4, 8$ and 16

Yield Coefficients	t, h	Strategies						
$Y_{X/S}, g\ g^{-1}$		BR1	BR2	BR3	BR4	BR5	BR6	BR7
	6	0.57	0.45	0.52	0.54	0.52	0.54	0.38
	9	0.62	0.54	0.70	0.57	0.43	0.58	0.22
	12	0.38	0.47	0.64	0.46	0.39	0.51	0.28
	15	0.32	0.38	0.46	0.36	0.36	0.44	0.33
$Y_{P/S}, mg\ g^{-1}$		BR1	BR2	BR3	BR4	BR5	BR6	BR7
	6	0.03	2.70	0.86	1.97	0.98	1.30	4.67
	9	1.55	2.71	0.85	0.50	0.23	0.74	2.57
	12	0.00	0.51	0.36	0.00	0.03	0.12	3.46
	15	0.19	1.31	0.33	0.49	0.26	0.44	0.52
$Y_{P/X}, mg\ g^{-1}$		BR1	BR2	BR3	BR4	BR5	BR6	BR7
	6	0.05	6.02	1.65	3.67	1.88	2.39	12.32
	9	1.33	5.05	1.22	0.87	0.53	1.28	11.93
	12	0.01	1.08	0.56	0.00	0.06	0.23	12.31
	15	0.59	3.43	0.73	1.36	0.73	1.01	1.58
Overall Yield Coefficients		Strategies						
		BR1	BR2	BR3	BR4	BR5	BR6	BR7
$\bar{Y}_{X/S}, g\ g^{-1}$		0.36	0.19	0.43	0.38	0.42	0.54	0.13
$\bar{Y}_{P/S}, mg\ g^{-1}$		2.79	1.19	2.77	1.11	1.71	2.44	2.06
$\bar{Y}_{P/X}, mg\ g^{-1}$		7.82	6.29	6.43	2.89	4.05	4.51	15.32

4.6.6 The variations in organic acids concentration profiles of differently aged synchronous and asynchronous cell populations

In the case of recombinant protein production causing metabolic burden in a cell, the energy, NADPH/NADP⁺ pool and *de novo* synthesis of amino acids are supplied by tricarboxylic acid cycle (TCA) and so the organic acids called as carboxylic acids secreted in fermentation medium are so significant to criticize the fate of metabolic pathways, cell growth and product formation and their relations.

The comparison between wild type strain and recombinant strain, the adverse influence of heterologous protein production on the cell growth is known since the energy drain from biomass formation towards product formation. In yeast *P. pastoris*, overexpression of r-protein is performed by increased in TCA cycle flux with reduced by-product formation such as ethanol, acetate or following organic acids detected in rhGH production medium: oxalic acid, gluconic acid, formic acid, pyruvic acid, malic

acid, lactic acid, acetic acid, maleic acid, citric acid, fumaric acid and succinic acid. Among all these organic acids, the most abundant in the fermentation medium was gluconic acid followed by succinic acid, acetic acid and pyruvic acid (Table 4.7). Contrariwise, the lowest ones of them was maleic acid and fumaric acid in all semi-batch operations. The total concentration of all carboxylic acids increased with respect to time as well as aging.

Gluconic acid is a product resulted from glucose dehydrogenated by glucose oxidase. Hence, the amount was increased in the presence of both glucose and oxygen. Towards the late exponential growth phase, the cell expressing r-protein under *GAP* promoter did not effectively consume both oxygen and glucose accumulated after $t = 15$ h of bioprocess. The amount of gluconic acid in synchronous populations was higher compared to asynchronous ones at same age.

The glucose is converted pyruvate at the end of glycolytic pathway called as pyruvate node. According to oxygen availability and the carbon source concentration, the pruvate branches oxidative pathway, fermentative pathway and biomass. The accumulation in pyruvic acid with acetic acid demonstrated that flux distribution through the oxidative TCA cycle was more than estimated amounts. The emergence of succinic acid at the end of bioprocess, being the excess amount of succinic for redox potential required by cell, corroborated the outcome of metabolic burden during cultivation.

Although, *P. pastoris* is Crabtree-negative yeast, the smaller amounts of ethanol was detected in cultivaion medium of *P. pastoris* since excess glucose. Additionally, the activity of fermentative pathway in synchronous cell population was lower than that in asynchronous ones because synchronous cell populations rapidly metabolize carbon source with higher specific subtrate consumption rate and thus, surplus amounts of glucose was not noticed inside cells.

Table 4.6 showed that reason about the difference in BR1 and BR2 in terms of rhGH concentration obtained. The yeast population in BR2 effectively metabolized the carbon source due to lower concentrations of gluconic acid, acetic acid and succinic acid.

Table 4. 6 The variations in organic acid concentrations with the cultivation time in strategies of semi-batch bioreactor operation, BR1 and BR2

t, h	Concentration, g L ⁻¹										
	Oxalic acid	Gluconic acid	Formic acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Fumaric acid	Succinic acid
BR1											
0	0.280	0.368	-	0.393	-	-	0.867	-	-	-	0.637
3	0.333	0.393	0.070	0.544	-	-	0.795	-	-	-	0.592
6	0.407	0.377	0.094	0.073	0.002	0.180	1.012	0.001	0.020	0.021	0.794
9	0.361	0.628	0.092	0.080	0.004	-	1.186	0.001	0.044	0.023	0.603
12	0.830	1.699	0.150	1.789	0.059	-	-	0.001	0.207	-	1.411
15	3.156	0.525	0.556	-	0.179	-	-	0.001	0.464	0.019	0.274
18	0.987	1.742	0.716	-	0.466	-	-	0.003	0.822	0.043	-
Total											
											2.550
											2.727
											2.941
											3.022
											6.146
											5.147
											4.779
t, h	Concentration, g L ⁻¹										
	Oxalic acid	Gluconic acid	Formic acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Fumaric acid	Succinic acid
BR2											
0	0.350	0.424	0.091	0.446	-	-	-	-	-	0.001	0.286
3	0.248	0.389	0.144	0.112	-	0.713	-	-	0.053	0.001	0.039
6	0.337	0.339	0.075	0.022	0.039	0.164	-	0.001	0.080	0.001	0.022
9	0.548	0.396	0.293	0.137	0.049	0.399	1.021	0.001	0.172	0.003	0.073
12	0.400	0.469	0.244	0.498	0.004	0.211	1.111	0.001	0.172	0.001	0.114
15	0.123	0.078	0.489	1.724	-	-	-	-	0.109	0.001	0.458
18	0.957	0.293	0.732	-	-	-	-	-	0.082	0.003	0.324
Total											
											1.598
											1.726
											1.08
											3.092
											3.224
											2.982
											3.792

Table 4. 7 The variations in organic acid concentrations with the cultivation time in semi-batch bioreactor operation strategies

t, h	Concentration, g L ⁻¹										
	Oxalic acid	Gluconic acid	Formic acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Fumaric acid	Succinic acid
BR2	0	0.350	0.424	0.091	0.446	-	-	-	-	0.001	0.286
	3	0.248	0.389	0.144	0.112	-	0.713	-	0.053	0.001	0.039
	6	0.337	0.339	0.075	0.022	0.039	0.164	-	0.001	0.001	0.022
	9	0.548	0.396	0.293	0.137	0.049	0.399	1.021	0.001	0.003	0.073
	12	0.400	0.469	0.244	0.498	0.004	0.211	1.111	0.001	0.001	0.114
	15	0.123	0.078	0.489	1.724	-	-	-	0.109	0.001	0.458
	18	0.957	0.293	0.732	-	-	-	-	0.082	0.003	0.324
Total											
1.598											
1.726											
1.08											
3.092											
3.224											
2.982											
3.792											
t, h											
Concentration, g L ⁻¹											
BR3	0	0.113	0.270	0.016	0.192	-	-	0.422	-	-	-
	3	0.133	0.207	0.019	0.016	0.067	0.075	0.482	-	-	-
	6	0.019	0.426	0.745	0.429	0.742	0.164	0.938	-	0.011	-
	9	0.175	0.296	0.026	0.004	0.202	0.200	0.848	-	0.292	-
	12	0.647	0.429	0.336	1.595	-	-	-	-	0.005	-
	15	0.714	0.824	0.537	-	1.286	-	-	-	0.001	0.117
	18	0.651	1.306	0.385	-	1.571	-	-	0.159	0.007	0.972
									0.382	0.002	1.778
Total											
1.013											
1.01											
3.76											
1.884											
4.145											
5.523											
5.328											
t, h											
Concentration, g L ⁻¹											
BR4	0	0.196	0.566	-	0.300	-	-	0.577	-	0.001	-
	3	0.152	0.399	-	0.029	0.063	0.074	0.364	-	0.001	-
	6	0.158	0.517	0.019	0.016	0.179	0.098	0.687	-	0.001	-
	9	0.104	0.223	0.013	0.145	-	0.139	1.858	-	-	-
	12	0.701	1.241	0.242	1.136	-	0.922	-	-	0.026	-
	15	0.071	1.551	0.899	-	-	-	-	0.161	0.001	1.091
	18	0.231	2.052	1.219	-	-	-	-	0.338	0.003	1.843
									0.249	0.004	1.420
Total											
1.64											
1.133											
1.716											
2.508											
5.495											
4.705											
5.175											

Table 4.7 The variations in organic acid concentrations with the cultivation time in semi-batch bioreactor operation strategies
(Continued)

t, h	Concentration, g L ⁻¹										
	Oxalic acid	Gluconic acid	Formic acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Fumaric acid	Succinic acid
BR5	0	0.091	0.217	0.010	0.021	0.021	0.607	-	0.022	0.003	-
	3	0.122	0.214	0.017	0.050	0.106	0.332	-	0.039	0.006	-
	6	0.132	0.296	0.020	0.009	0.196	0.430	-	0.052	0.005	-
	9	0.135	0.271	0.024	0.011	0.246	0.619	-	0.057	0.005	-
	12	0.479	2.005	0.244	1.109	0.037	0.211	-	0.080	0.015	-
	15	0.696	1.072	0.380	1.740	-	-	0.001	0.125	0.016	1.695
	18	0.783	2.347	0.834	-	-	-	0.002	0.221	0.027	1.695
t, h	Concentration, g L ⁻¹										
	Oxalic acid	Gluconic acid	Formic acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Fumaric acid	Succinic acid
BR6	0	0.022	2.480	0.046	0.003	0.044	0.690	-	0.030	0.002	-
	3	0.014	0.758	0.039	0.003	0.031	1.269	-	0.026	0.001	-
	6	0.017	0.314	0.205	0.014	0.033	1.483	-	0.042	0.002	-
	9	0.037	0.453	0.099	0.010	0.126	1.513	-	0.060	0.003	-
	12	0.074	1.061	0.106	0.011	0.288	-	0.002	1.604	0.006	-
	15	0.130	2.333	0.531	0.154	-	-	0.004	1.020	0.004	1.152
	18	0.069	2.869	0.135	0.042	-	-	0.002	0.359	0.002	-
t, h	Concentration, g L ⁻¹										
	Oxalic acid	Gluconic acid	Formic acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Fumaric acid	Succinic acid
BR7	0	0.245	1.074	0.107	0.003	3.753	0.981	-	0.511	0.001	-
	3	0.240	0.233	0.056	0.005	0.064	0.725	-	-	0.001	-
	6	0.184	1.198	0.047	0.003	0.023	0.752	-	0.031	0.001	-
	9	0.576	0.942	0.121	0.066	-	0.985	0.002	0.168	0.002	-
	12	1.847	3.272	0.144	0.070	-	-	0.002	0.370	0.002	-
	15	1.230	3.230	0.263	0.100	-	-	0.001	0.497	0.003	1.523
	18	0.431	3.934	0.603	0.148	-	-	0.002	0.427	0.010	-

CHAPTER 5

CONCLUSION

In this study, the effect of synchronization combined with aging on r-protein production characteristic of yeast population was investigated to enhance rhGH production owing to insufficient outcomes on synchronization effects in *P. pastoris*. The synchronization via starvation allowed us to elucidate the shift in cell metabolism in terms of the behavior of cell growth, r-protein production and carbon source consumption at distinct cell ages. For this purpose, four sub-research studies were conducted. In the first part, the possibly alternative *P. pastoris* strain called as *PDC* knockout *P. pastoris* was developed and then characterized for both of growth and r-protein production characteristics on different carbon sources in batch operation followed by semi-batch operation where the two different substrates-fed to the system with an exponential profile with pre-determined specific growth rate of $\mu_0 = 0.10 \text{ h}^{-1}$. Since the lower concentration levels of rhGH produced by recombinant *P. pastoris pdcΔ* under the control of *GAP* promoter compared to the rhGH concentrations produced by recombinant *P. pastoris* under the control of *GAP* promoter (Zerze, 2012; Keskin, 2014), the experiments related rhGH production was maintained with previously developed strain by Zerze (2012). In the second stage, the optimized conditions of starvation being one of the synchronization methods was determined by confocal microscopy imaging with evaluation of intracellular expressed eGFP by *P. pastoris GAP::eGFP* according to vacuole formation and intracellular distribution of eGFP. As a result of visualization, the conditions were improved when considering the population age, two types of pre-cultivation media and cultivation time in pre-

cultivation phase. In the following stage, the effects of synchronous populations on rhGH production were taken into consideration with different carbon sources in production phase in air-filtered shake bioreactors. The rhGH concentrations at $t = 24$ h at which the highest cell concentration reached were judged to illuminate how starvation as a synchronization method influenced the rhGH production and secretion. In last stage, two experiment sets of semi-batch operation by using exponential glucose feeding with pre-determined specific growth rate of $\mu_0 = 0.10 \text{ h}^{-1}$ were performed in laboratory scale bioreactor that excluded pH change and poor mass transfer of oxygen. Hence, the effects of synchronization, aging, and their combination on rhGH production were solely assessed. The first set of experiments included the differently aged asynchronous yeast populations which were also negative controls of synchronized ones while the second set of experiments was comprised of synchronous populations at same ages. The negative control of both experiment sets was asynchronous yeast population at age of $\text{OD}_{600} = 4$ that was recommended by Invitrogen. In all laboratory scale bioreactor experiments, the batch and semi-batch operations were implemented at $T = 30^\circ\text{C}$, $\text{pH} = 5.0$, $N = 900 \text{ rpm}$ and $C_{\text{DO}} = 20\%$ within the working volume of $V_R = 0.9\text{-}2.2 \text{ L}$ after an initial batch operation while all batch operations in air-filtered laboratory scale bioreactors were carried out at $T = 30^\circ\text{C}$, $N = 200 \text{ rpm}$, $\text{pH}_0 = 5.7$ within the working volume of $V_0 = 50 \text{ mL}$.

Within the scope of characterization of the possibly alternative *PDC* knockout *P. pastoris* strain, five strategies were designed. Three of them named as B1, B2 and B3, were performed in batch operation containing 20 g L^{-1} glucose, 20 g L^{-1} glycerol and mixing of 20 g L^{-1} glycerol and 7.8 g L^{-1} acetate as in form of sodium salts at $t = 0 \text{ h}$, respectively. Two of them were exponential feeding strategies, namely, B4 and B5, for glucose and glycerol, respectively. The major findings obtained from these strategies can be summarized as:

- In batch operations, the maximum specific growth rates (μ_{max}) on glucose and glycerol were 0.19 h^{-1} and 0.24 h^{-1} , respectively. However, the specific growth rate on the mixing of glycerol and acetate showed gradually decreased synovial wave in the range between 0.16 h^{-1} being μ_{max} on acetate and 0.20 h^{-1} being μ_{max} on glycerol and in the range between 0.13 h^{-1} and 0.19 h^{-1} . Thereby, this

synovial wave of specific growth rate demonstrated that carbon sources were sequentially taken. Hence, the values of the specific growth rate ensured ones in the literature.

- In batch operation, the highest rhGH concentration was obtained as $C_{rhGH,max}=3.6 \text{ mg L}^{-1}$ at $t_{max} = 16 \text{ h}$ in B2 containing glycerol whereas the lowest $C_{rhGH,max}=1.7 \text{ mg L}^{-1}$ in B3 containing mixing of glycerol and acetate. However, the highest cell concentration was attained as $C_{X,max}=15.5 \text{ g DCW L}^{-1}$ in B3 owing to ability of growth on two type of carbon sources.
- As a result of rhGH concentrations in batch operations, glucose and glycerol were chosen as two different carbon sources in semi-batch operation. Unlike batch operation, the highest $C_{rhGH,max}=21.62 \text{ mg L}^{-1}$ at $t = 21 \text{ h}$ was attained in B4 where glucose was introduced to the system in semi-batch operation.
- The highest $C_{X,max}$ was obtained as 111.4 g L^{-1} at $t = 21 \text{ h}$ in B5 in which glycerol was exponentially fed.
- Unlike hSOD production by Nocon et al. (2014), the rhGH production by *PDC* gene deficient *P. pastoris* strain was resulted in low concentrations since the lower specific rate of product formation.
- To improve rhGH production, the oxygen conditions should be shifted to lower oxygen conditions below 20% defined as oxygen limited one and hypoxic one, and so the results obtained from both *P. pastoris* strain *GAP::hGH* and *PDC* knockout *GAP::hGH* should be compared. Accordingly, it should be decided whether or not *Pdc⁻* strain is potential producer for r-protein production

In the optimization of starvation as a synchronization method, ten strategies were developed in air-filtered shake bioreactors. In first six strategies, YPD and BMGY as pre-cultivation media were combined with three different population ages of $OD_{600} = 4, 8, \text{ and } 16$. The pre-cultivation time was adjusted 24 h that was recommended by Manukyan et. al (2011) prior to starvation phase for 6 h. Because the presence of heterogeneous cell size, the absence of completely round cell shape, the availability of intracellular inhomogeneous distribution of eGFP, and vacuole

formation in differently aged synchronous cell populations in BMGY, the pre-cultivation time was decreased to 15-18 h in other four strategies for synchronous cell populations aging to $OD_{600} = 4$ and 16 in two pre-cultivation media. The most highlighted results achieved in this stage can be mentioned as follows:

- In all strategies carried out in YPD, the desired characteristics of cells that was mentioned above were achieved irrespective of pre-cultivation time and aging. Conversely, the pre-cultivation time required in BMGY was changed according to population ages.
- In BMGY, vacuole formation in synchronous populations aging to $OD_{600} = 16$ could not be totally prevented even though pre-cultivation time was reduced to 15-18 h that was proposed by Invitrogen. Additionally, the better physiological state of cell population might have been fulfilled if the cultivation time had been below 15-18 h but the bioprocess required the growth dynamic of yeast population grown for 15-18 h. Thereby, it was optimized condition even if it was not best one.
- Accordingly, YPD as a pre-cultivation medium is a good choice to increase the number of healthy cells after starvation process.
- During starvation phase, exponential growth mentioned in previous studies was observed, and the increase fold in cell concentration varied according to the cell age
- The main inference resulted from this study stage was that starvation as a synchronization procedure was not only dependent on the type of organism but also the age of cell, the type of pre-cultivation media chosen, and physiological state of cell changed with respect to time in that medium.
- Further, the age of cell before starvation determined the cultivation time in that medium to decrease budding index for accomplishing synchronization.

The air-filtered shake bioreactor experiments as third stage of study were conducted for the assessment of rhGH production under the effect of synchronization.

The twelve strategies comprised of combinations of two pre-cultivation media mentioned above, two production media (glucose and glycerol) and three cell ages of were accomplished. Four strategies among them were negative controls including asynchronous populations at age of $OD_{600} = 4$ while the others had differently aged synchronized populations. The main results obtained from batch operation in the air-filtered shake bioreactors can be summarized as follows:

- Cell concentrations were similar and nearly 12 g DCW L^{-1} in all strategies excepting synchronous cell populations aging to $OD_{600} = 16$.
- Use of glycerol as a sole carbon source with combination of aging and synchronization in production phase following starvation process improved r-protein production whereas synchronization had adverse influence on the amount of rhGH produced by the aged populations grown on glucose.

The laboratory scale bioreactor conditions changed the fate of synchronization compared to that in small scale shake bioreactor experiments. Thereby, a real performance of the cells should not be evaluated in shake flasks bioreactors due to oxygen limited conditions, the inadequate control of pH, and control and regulation of the promoter used in r-protein expression. Among two sets of experiments, first one was consisted of BR2, BR3 and BR4 strategies that included asynchronous populations aging to $OD_{600} = 4, 8$, and 16 , respectively, while the other one contained similarly aged synchronized populations included in BR5, BR6 and BR7. The worthwhile findings can be listed as follows:

- Among all strategies, the highest cell concentration was attained as $C_X = 142 \text{ g DCW L}^{-1}$ in asynchronous populations aging to $OD_{600} = 4$ in BR2 while the highest cell concentration in all of them were attained at $t = 15 \text{ h}$. On the other hand, the lowest one was obtained as $C_X = 100 \text{ g DCW L}^{-1}$ in synchronous populations aging to $OD_{600} = 16$ in BR7. The values of the highest cell concentrations decreased from younger cell populations to older ones with and/or without starvation even if the synchronization in pre-cultivation phase did not alter the batch phase duration.

- In all strategies, the predetermined specific growth rate of $\mu_0 = 0.10$ kept for first 9 h decreased and then a greater deviation from μ_0 was occurred during late-exponential phase prior to stationary phase.
- The highest rhGH concentration was achieved as 570 mg L⁻¹ in mostly aged synchronous yeast population in BR7 and also 2.72-fold higher than that of asynchronous population at same age in BR6 and 1.6-fold higher than the maximum concentration obtained by asynchronous populations in BR2.
- The highest one among the specific rhGH production rates (q_{rhGH}) of differently aged synchronous and asynchronous cell populations was 0.77 mg g⁻¹ h⁻¹ in BR7. In all strategies excluding BR7, the values of specific rhGH production rates were maximum at $t = 3$ h and then decreased with respect to time. In BR7, there was deviation at $t = 15$ h at which maximum rhGH concentrations was reached.
- In all semi-batch operations, glucose was not detected in the fermentation medium until $t = 15$ h. The highest amount of accumulated glucose reached to 17.1 g L⁻¹ in BR7 and was same as that in BR6. The value of accumulated glucose concentration increased from younger populations to older ones and the difference between glucose concentrations in synchronous and asynchronous populations at same age reduced from older ones to younger ones.
- The specific substrate consumption rates (q_s) were mainly determined by cell concentration since the volumetric glucose consumption rates were similar until the time at which the glucose was accumulated. The highest q_s was acquired as 0.32 g g⁻¹ h⁻¹ in BR7 owing to low cell concentration. The values of q_s in BR2 and BR3 were linear but the others had an inflection point at $t = 9$ h.
- The overall yield coefficients define system behavior. The overall biomass yields in BR3, BR4, BR5 and BR6 were in similar range while the values in B2 and BR7 were close to each other and also lower than others. On the other

hand, the overall product yield on substrate and the overall product yield on cell did not have common range for all strategies. Most of all, the highest overall product yield with the lowest overall biomass yield were acquired as 15.32 g g^{-1} and 0.13 g g^{-1} in BR7, respectively.

- According to these relative values, among the all systems, the best system of cell populations as producer was obtained in BR7, that has highest overall product yield with the lowest overall biomass yield on cell, being as 15.32 g g^{-1} and 0.13 g g^{-1} , respectively.
- In all semi-batch operations, gluconic acid, succinic acid, acetic acid and pyruvic acid were detected in fermentation medium. Amongst all these organic acids in all bioreactor experiments, the highest one was gluconic acid accumulated after $t = 9 \text{ h}$ at which the oxygen limitation had begun. The other ones were accumulated for same reason. The maximum amount of total organic acids in the fermentation medium was obtained as 7.6 g L^{-1} in BR7. The value of total organic acids demonstrated that the aging rather than synchronization caused higher organic acid production.
- Accordingly, the synchronization in pre-cultivation phase before glucose fed-batch operation promoted r-protein production in cell-aged populations while lower rhGH production was obtained in asynchronous populations with aging. Furthermore, the cell metabolism and carbon source were directed to other metabolic pathways rather than to reproduce itself when cell populations were aged.
- Metabolic flux analysis (MFA) should be performed for BR2 and BR7 to elucidate how synchronization with aging influenced and directed metabolic pathway and also forced cells to promote rhGH production.
- Considering the specific glucose consumption rates and accumulated amount of glucose and total organic acids in BR7, the need for redesigning of the feeding strategy was concluded in the further hours to improve r-protein production.

- Glycerol as a sole carbon source should be exponentially fed to laboratory scale bioreactor to determine the influence of carbon sources with synchronization on r-protein production since glycerol forced yeast population to lower multi-bud formation in both of glycerol batch and glycerol fed-bath operation of *P. pastoris* contrary to methanol fed-batch operation (Jin and Yuan, 2007).
- Starvation as a synchronization method should be applied in semi-batch operation.
- Fed-batch operation with periodic starvation and the use of glycerol in fed-batch phase should combine with glycerol batch operation initiated with the synchronous yeast population mentioned in this study in order to understand clearly the effect of synchronization on r-protein production.

REFERENCES

- Abbott, D.A., Zelle, R.M., Pronk, J.T., van Maris, A.J.A. 2009. "Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges." *FEMS Yeast Research* 9 (8): 1123–1136. doi:10.1111/j.1567-1364.2009.00537.x.
- Agarwal, P.K., Uppada, V., Noronha, S.B. 2013. "Comparison of pyruvate decarboxylases from *Saccharomyces cerevisiae* and *Komagataella pastoris* (*Pichia pastoris*)." *Applied Microbiology and Biotechnology* 97 (21): 9439–9449. doi:10.1007/s00253-013-4758-4.
- Aggarwal, P., Padmanabhan, B., Bhat, A., Sarvepalli, K., Sadhale, P.P., Nath, U. 2011. "The TCP4 transcription factor of *Arabidopsis* blocks cell division in yeast at G1 → S transition." *Biochemical and Biophysical Research Communications* 410 (2): 276–281. doi:10.1016/j.bbrc.2011.05.132.
- Ahmad, M., Hirz, M., Pichler, H., Schwab, H. 2014. "Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production." *Applied Microbiology and Biotechnology* 98 (12): 5301–5317. doi:10.1007/s00253-014-5732-5.
- Alberghina, L., Mavelli, G., Drovandi, G., Palumbo, P., Pessina, S., Tripodi, F., Coccetti, P., Vanoni, M. 2012. "Cell growth and cell cycle in *Saccharomyces cerevisiae*: basic regulatory design and protein-protein interaction network." *Biotechnology Advances* 30 (1): 52–72. doi:10.1016/j.biotechadv.2011.07.010.
- Alexpoulou, O., Abs, R., Maiter, D. 2010. "Treatment of adult growth hormone deficiency: who, why and how? A review." *Acta Clinica Belgica* 65 (1): 13–22. doi:10.1179/acb.2010.002.

- Amon, A. 1990. "Synchronization Procedures." In C. Guthrie and G.R. Fink (Eds.), *Guide to Yeast Genetics and Molecular and Cell Biology*, San Diego: Academic Press.
- Ayyar, V.S. 2011. "History of growth hormone therapy." *Indian Journal of Pediatrics* 15 (7): 162–165. doi:10.1007/s12098-011-0609-4.
- Bae, C.S., Yang, D.S., Lee, J., Park, Y.H. 1999. "Improved process for production of recombinant yeast-derived monomeric human G-CSF." *Applied Microbiology and Biotechnology* 52 (3): 338–344. doi:10.1007/s002530051529.
- Baumann, G.P. 2009. "Growth hormone isoforms." *Growth Hormone & IGF Research* 19 (4): 333–340. doi:10.1016/j.ghir.2009.04.011.
- Bauman, R.W. 2007. *Microbiology with Diseases by Taxonomy, 2nd Ed.*, Pearson Benjamin Cummings.
- Benedini, S., Terruzzi, I., Lazzarin, A., Luzi, L. 2008. "Recombinant human growth hormone: rationale for use in the treatment of HIV-associated lipodystrophy." *Biodrugs* 22 (2): 101–112. doi:10.2165/00063030-200822020-00003
- Berlec, A., Strukelj, B. 2013. "Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells." *Journal of Industrial Microbiology and Biotechnology* 40 (3–4): 257–274. doi:10.1007/s10295-013-1235-0.
- Beuse, M., Bartling, R., Kopmann, A., Diekmann, H., Thoma, M. 1998. "Effect of the dilution rate on the mode of oscillation in continuous cultures of *Saccharomyces cerevisiae*." *Journal of Biotechnology* 61 (1): 15–31. doi:10.1016/S0168-1656(98)00016-9.
- Bhavagan, N.V. 2002a. "Endocrine Metabolism 1: Introduction." In *Medical Biochemistry, 4th Ed.*, San Diego: Harcourt Academic Press.
- Bhavagan, N.V. 2002b. "Endocrine Metabolism II: Hypothalamus and Pituitary." In *Medical Biochemistry, 4th Ed.*, San Diego: Harcourt Academic Press.

- Bigelis, R., Keesey, J.K., Fink, G.R. 1981. "The yeast his4 multifunctional protein. Immunochemistry of the wild type protein and altered forms." *The Journal of Biological Chemistry* 256 (10): 5144–5152.
- BioPlan Associates. 2015. *Report and Survey of Biopharmaceutical Manufacturing Capacity and Production*.
- Black, J.G., Black, L.J. 2015. *Microbiology: Principles and Explorations. 9th Ed.*, New Jersey: Wiley.
- Brady, C.P., Shimp, R.L., Miles, A.P., Whitmore, M., Stowers, A.W. 2001. "High-level production and purification of P30P2MSP1(19), an important vaccine antigen for malaria, expressed in the methylotropic yeast *Pichia pastoris*." *Protein Expression and Purification* 23 (3): 468–475. doi:10.1006/prep.2001.1526.
- Brandt. 2016. "Chapter 1. Steroid Chemistry and Steroid Hormone Action." *Endocrine*. https://www.rose-hulman.edu/~brandt/Chem330/EndocrineNotes/Chapter_1_Steroids.pdf.
- Breeden, L.L. 1997. "Alpha-factor synchronization of budding yeast." *Methods in Enzymology* 283: 332–341. doi: 10.1016/S0076-6879(97)83027- 3.
- Breunig, K.D., Bolotin-Fukuhara, M., Bianchi, M.M., Bourgarel, D., Falcone, C., Ferrero, I., Frontali, L., Goffrini, P., Krijger, J.J., Mazzoni, C., Milkowski, C., Steensma, H.Y., Wesolowski–Louvel, M., Zeeman, A.M. 2000. "Regulation of primary carbon metabolism in *Kluyveromyces lactis*." *Enzyme and Microbial Technology* 26 (9–10): 771–780. doi:http://dx.doi.org/10.1016/S0141-0229(00)00170-8.
- Campbell, N.A., Reece, J.B. 2001. *Biology. 6th Ed.*, New York: Benhamin Cummings.
- Cázares-Delgadillo, J., Ganem-Rondero, A., Kalia, Y.N. 2011. "Human growth hormone: new delivery systems, alternative routes of administration, and their pharmacological relevance." *European Journal of Pharmaceutics and Biopharmaceutics* 78 (2): 278-288. doi:10.1016/j.ejpb.2011.01.006.

- Cereghino, G.P., Cereghino, J.L., Ilgen, C., Cregg, J.M. 2002. "Production of Recombinant Proteins in Fermenter Cultures of the Yeast *Pichia Pastoris*." *Current Opinion in Biotechnology* 13(4):329-332. doi: 10.1016/S0958166902003300.
- Cereghino, G.P.L., Cereghino, J.L., Sunga, A.J., Johnson, M.A., Lima, M., Gleesonb, M.A.G., Cregg, J.M. 2001. "New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of *Pichia pastoris*." *Gene* 263 (1–2): 159–169. doi: 10.1016/S0378-1119(00)00576-X.
- Cereghino, G.P.L., Cregg, J.M. 1999. "Applications of yeast in biotechnology: protein production and genetic analysis." *Current Opinion in Biotechnology* 10 (5): 422–427. doi:10.1016/S0958-1669(99)00004-X.
- Cereghino, G.P.L., Sunga, A.J., Cereghino, J.L., Cregg, J.M. 2002. "Expression of foreign genes in the yeast *Pichia pastoris*". *Genetic Engineering: Principles and Methods* 23: 157-169. doi:10.1007/0-306-47572-3_9
- Cereghino, J.L., Cregg, J.M. 2000. "Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*." *FEMS Microbiology Reviews* 24: 45–66. doi:10.1111/j.1574-6976.2000.tb00532.x.
- Chiruvolu, V., Eskridge, K., Cregg, J., Meagher, M. 1998. "Effects of glycerol concentration and pH on growth of recombinant *Pichia pastoris* yeast." *Applied Biochemistry and Biotechnology* 75 (2): 163–173. doi:10.1007/Bf02787771.
- Choi, B.K., Bobrowicz, P., Davidson, R.C., Hamilton, S.R., Kung, D.H., Li, H., Miele, R.G., Nett, J.H., Wildt, S., Gerngross, T.U. 2003. "Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*." *PNAS* 100 (9): 5022–5027. doi: 10.1073/pnas.0931263100.
- Choi, D.B., Park, E.Y. 2006. "Enhanced production of mouse α -amylase by feeding combined nitrogen and carbon sources in fed-batch culture of recombinant *Pichia pastoris*." *Process Biochemistry* 41 (2): 390–397. doi:10.1016/j.procbio.2005.06.020.

- Choi, S., Song, S., Choi, C., Park, J.K. 2009. "Microfluidic self-sorting of mammalian cells to achieve cell cycle synchrony by hydrophoresis." *Analytical Chemistry* 81 (5): 1964–1968. doi:10.1021/ac8024575.
- Clare, J.J., Romanes, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Payne, M.M., Sreekrishna, K., Henwood, C.A. 1991. "Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies." *Gene* 105 (2): 205–212. doi:10.1016/0378-1119(91)90152-2.
- Clarke, K. G. 2013. *Bioprocess Engineering: An Introductory Engineering and Life Science Approach. 1st Ed.*, Woodhead Publishing.
- Clemmons, D.R. 2004. "The relative roles of growth hormone and IGF-1 in controlling insulin sensitivity." *Journal of Clinical Investigation* 113 (1): 25–27. doi:10.1172/JCI200420660.
- Connors, N.C. 2002. "Culture Medium Optimization and Scale-Up for Microbial Fermentations." In V.A. Vinci and S.R. Parekh (Eds.), *Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells*. Springer Science and Business Media.
- Cooper, S. 2004. "Rejoinder: whole-culture synchronization cannot, and does not, synchronize cells." *Trends in Biotechnology* 22 (6): 274–276. doi:10.1016/j.tibtech.2004.04.011.
- Cos, O., Ramón, R., Montesinos, J.L., Valero, F. 2006. "Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review." *Microbial Cell Factories* 5 (1): 17. doi:10.1186/1475-2859-5-17.
- Craigie, E., Mullins, J.J., Bailey, M.A. 2009. "Glucocorticoids and Mineralocorticoids." In M. Bader (Eds.), *Cardiovascular Hormone Systems: From Molecular Mechanisms to Novel Therapeutics*. doi:10.1002/9783527626236.ch1.

- Crane D.I., Gould, S.J. 1994. "The *Pichia pastoris* *HIS4* gene: nucleotide sequence, creation of a non-reverting *his4* deletion mutant, and development of *HIS4*-based replicating and integrating plasmids." *Current Genetics* 26 (5): 443–450. doi:10.1007/BF00309932
- Cregg, J.M., Cereghino, J.L., Shi, J., Higgins, D.R. 2000. "Recombinant protein expression in *Pichia pastoris*." *Molecular Biotechnology* 16 (1): 23-52. doi:10.1385/MB:16:1:23.
- Cregg, J.M., Vedvick, T.S., Raschke, W.C. 1993. "Recent advances in the expression of foreign genes in *Pichia pastoris*." *Nature Biotechnology* 11: 905–910. doi:10.1038/nbt0893-905
- Çalık, P., Ata, Ö., Güneş, H., Massahi, A., Boy, E., Keskin, A., Öztürk, S., Zerze, G.H., Özdamar, T.H. 2015. "Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: from carbon source metabolism to bioreactor operation parameters." *Biochemical Engineering Journal* 95: 20–36. doi:10.1016/j.bej.2014.12.003.
- Çalık, P., Bayraktar, E., İnankur, B., Soyaslan, E.Ş., Şahin, M., Taşpınar, H., Açık, E., Yılmaz, R., Özdamar, T.H. 2010. "Influence of pH on recombinant human growth hormone production by *Pichia pastoris*." *Journal of Chemical Technology and Biotechnology* 85 (12): 1628–1635. doi:10.1002/jctb.2474.
- Çalık, P., Orman, M.A., Çelik, E., Halloran, M., Çalık, G., Özdamar, T.H. 2008. "Expression system for synthesis and purification of recombinant human growth hormone in *Pichia pastoris* and structural analysis by MALDI-ToF mass spectrometry." *Biotechnology Progress* 24(1):221-226.
- Çalık, P., Çalık, G., Takaç, S., Ozdamar, T.H. 1999. "Metabolic flux analysis for serine alkaline protease fermentation by *Bacillus licheniformis* in a defined medium: effects of the oxygen transfer rate." *Biotechnology and Bioengineering* 64 (2): 151–167. doi:10.1002/(SICI)1097-0290(19990720)64:2<151::AID-BIT4>3.0.CO;2-U.

- Daly, R., Hearn, M.T.W. 2005. "Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production." *Journal of Molecular Recognition* 18 (2): 119–138. doi:10.1002/jmr.687.
- Damasceno, L.M., Huang, C.J., Batt, C.A. 2012. "Protein secretion in *Pichia pastoris* and advances in protein production." *Applied Microbiology and Biotechnology* 93 (1): 31–39. doi:10.1007/s00253-011-3654-z.
- Day, A., Schneider, J., Schneider, B.L. 2004. "Yeast Cell Synchronization." In H.B. Lieberman (Eds.), *Cell Cycle Checkpoint Control Protocols*. New Jersey: Humana Press.
- De Schutter, K., Lin, Y.C., Tiels, P., van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouzé, P., De Peer, Y.V., Callewaert, N. 2009. "Genome sequence of the recombinant protein production host *Pichia pastoris*." *Nature Biotechnology* 27 (6): 561–566. doi:10.1038/nbt.1544.
- Dechant, R., Peter, M. 2008. "Nutrient signals driving cell growth." *Current Opinion in Cell Biology* 20 (6): 678–687. doi:10.1016/j.ceb.2008.09.009.
- Demain, A.L., Vaishnav, P. 2009. "Production of recombinant proteins by microbes and higher organisms." *Biotechnology Advances* 27: 297–306. doi:10.1016/j.biotechadv.2009.01.008.
- Dikicioglu, D., Wood, V., Rutherford, K.M., McDowall, M.D., Oliver, S.G. 2014. "Improving functional annotation for industrial microbes: a case study with *Pichia pastoris*." *Trends in Biotechnology* 32 (8): 396–399. doi:10.1016/j.tibtech.2014.05.003.
- Doran, P.M. 2012. *Bioprocess Engineering Principles. 2nd Ed.*, Oxford: Academic Press. <http://www.amazon.com/Bioprocess-Engineering-Principles-Second-Edition/dp/012220851X>.

- Dragosits, M., Frascotti, G., Bernard-Granger, L., Vázquez, F., Giuliani, M., Baumann, K., Rodríguez-Carmona, E., Tokkanen, J., Parrilli, E., Wiebe, M.G., Kunert, R., Maurer, M., Gasser, B., Sauer, M., Branduardi, P., Pakula, T., Saloheimo, M., Penttilä, M., Ferrer, P., Tutino, M.L., Villaverde, A., Porro, D., Mattanovich, D. 2011. "Influence of growth temperature on the production of antibody Fab fragments in different microbes: a host comparative analysis." *Biotechnology Progress* 27 (1): 38–46. doi:10.1002/btpr.524.
- Ecamilla-Treviño, L.L., Viader-Salvadó, J.M., Barrera-Saldaña, H.A., Guerrero-Olazarán, M. 2000. "Biosynthesis and secretion of recombinant human growth hormone in *Pichia pastoris*." *Biotechnology Letters* 22 (2): 109–114. doi:10.1023/A:1005675920451.
- Ertola, R.J., Giulietti, A.M., Castillo, F.J. 1994. "Design, Formulation, and Optimization of Media." In J.A. Asenjo and J.C. Merchuk (Eds.), *Bioreactor System Design*. New York: CRC Press.
- Eurwilaichitr, L., Roytrakul, S., Suprasongsin, C., Manitchotpisit, P., Panyim, S. 2002. "Glutamic acid and alanine spacer is not necessary for removal of MF α -1 signal sequence fused to the human growth hormone produced from *Pichia pastoris*." *World Journal of Microbiology and Biotechnology* 18 (6): 493–98. doi:10.1023/A:1016383326627.
- Ferrer-Miralles, N., Domingo-Espín, J., Corchero, J.L., Vázquez, E., Villaverde, A. 2009. "Microbial factories for recombinant pharmaceuticals." *Microbial Cell Factories* 8: 17. doi:10.1186/1475-2859-8-17.
- Fidan, O., Zhan., J. 2015. "Recent advances in engineering yeast for pharmaceutical protein production." *Royal Society Of Chemistry Advances* 5: 86665–86674. doi:10.1039/c5ra13003d.

- Flikweert, M.T., Kuyper, M., van Maris, A.J., Kötter, P., van Dijken, J.P., Pronk, J.T. 1999. "Steady-state and transient-state analysis of growth and metabolite production in a *Saccharomyces cerevisiae* strain with reduced pyruvate-decarboxylase activity." *Biotechnology and Bioengineering* 66 (1): 42–50. doi: 10.1002/(SICI)1097-0290(1999)66:1<42::AID-BIT4>3.0.CO;2-L.
- Fox, M.H. 2004. "Methods for synchronizing mammalian cells." *Methods in Molecular Biology* 241 (6): 11–16. doi: 10.1385/1-59259-646-0:11.
- Frykman, S., Sreenc., F. 2001. "Cell cycle-dependent protein secretion by *Saccharomyces cerevisiae*." *Biotechnology and Bioengineering* 76 (3): 259–268. doi:10.1002/bit.10003.
- Gao, M.J., Li, Z., Yu, R.S., Wu, J.R., Zheng, Z.Y., Shi, Z.P., Zhan, X.B., Lin, C.C. 2012. "Methanol/sorbitol co-feeding induction enhanced porcine interferon- α production by *P. pastoris* associated with energy metabolism shift." *Bioprocess and Biosystems Engineering* 35 (7): 1125–1136. doi:10.1007/s00449-012-0697-1.
- Garcia-Ochoa, F., Gomez, E. 2009. "Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview." *Biotechnology Advances* 27 (2): 153–176. doi:10.1016/j.biotechadv.2008.10.006.
- Garcia-Ochoa, F., Gomez, E., Santos, V.E., Merchuk, J.C. 2010. "Oxygen uptake rate in microbial processes: an overview." *Biochemical Engineering Journal* 49 (3): 289–307. doi:10.1016/j.bej.2010.01.011.
- Garcia-Ortega, X., Ferrer, P., Montesinos, J.L., Valero, F. 2013. "Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive *GAP* promoter." *Biochemical Engineering Journal* 79: 172–181. doi:10.1016/j.bej.2013.07.013.

- Gasser, B., Prielhofer, R., Marx, H., Maurer, M., Nocon, J., Steiger, M., Puxbaum, V., Sauer, M., Mattanovich, D. 2013. “*Pichia pastoris*: protein production host and model organism for biomedical research.” *Future Microbiology* 8 (2): 191–208. doi: 10.2217/fmb.12.133.
- Gellissen, G. 2000. “Heterologous protein production in methylotrophic yeasts.” *Applied Microbiology and Biotechnology* 54 (6): 741–750. doi:10.1007/s002530000464.
- “Gerngross, T.U. 2004. “Advances in the Production of Human Therapeutic Proteins in Yeasts and Filamentous Fungi.” *Nature Biotechnology* 22(11):1409–14. doi: 10.1038/nbt1028
- Ghoms, P.G., Kakmeni, F.M.M., Kofane, T.C., Tchawoua, C. 2014. “Synchronization dynamics of chemically coupled cells with activator-inhibitor pathways.” *Physics Letters A* 378 (38–39): 2813–2823. doi:10.1016/j.physleta.2014.05.057.
- Gleeson, M.A.G., White, C.E., Meininger, D.P., Komives, E.A. 1998. “Generation of protease-deficient strains and their use in heterologous protein expression.” *Methods in Molecular Biology* 103: 81–94. doi:10.1385/0-89603-421-6:81.
- Gonçalves, A.M., Pedro, A.Q., Maia, C., Sousa, F., Queiroz, J.A., Passarinha, L.A. 2013. “*Pichia pastoris*: a recombinant microfactory for antibodies and human membrane proteins.” *Journal of Microbiology and Biotechnology* 23 (5): 587–601. doi:10.4014/jmb.1210.10063.
- Grinna, L.S., Tschopp, J.F. 1989. “Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*.” *Yeast* 5 (2): 107–115. doi:10.1002/yea.320050206.
- Gustavsson, A.K., van Niekerk, D.D., Adiels, C.B., Goksör, M., Snoep, J.L. 2014. “Heterogeneity of glycolytic oscillatory behaviour in individual yeast cells.” *FEBS Letters* 588 (1): 3–7. doi:10.1016/j.febslet.2013.11.028.

- Gustavsson, M., Lehtiö, J., Denman, S., Teeri, T.T., Hult, K., Martinelle, M. 2001. "Stable linker peptides for a cellulose-binding domain-lipase fusion protein expressed in *Pichia pastoris*." *Protein Engineering* 14 (9): 711–715. doi:10.1093/protein/14.9.711.
- Güneş, H., Boy, E., Ata, Ö., Zerze G.H., Çalık, P., Özdamar, T.H. 2015. "Methanol feeding strategy design enhances recombinant human growth hormone production by *Pichia pastoris*." *Journal of Chemical Technology and Biotechnology* 91 (3): 664–671. doi: 10.1002/jctb.4619
- Hamilton, S.R., Davidson, R.C., Sethuraman, N., Nett, J.H., Jiang, Y., Rios, S., Bobrowicz, P., Stadheim, T.A., Li, H., Choi, B.K., Hopkins, D., Wischnewski, H., Roser, J., Mitchell, T., Strawbridge, R.R., Hoopes, J., Wildt, S., Gerngross, T.U. 2006. "Humanization of yeast to produce complex terminally sialylated glycoproteins." *Science* 313 (5792): 1441–1443. doi: 10.1126/science.1130256.
- Heijnen, J.J. 2009. "Growth Nutrients and Diversity." In C. Smolke (Eds.), *The Metabolic Pathway Engineering Handbook: Fundamentals*. CRC Press.
- Hélène, B., Céline, L., Patrick, C., Fabien, R., Christine, V., Yves, C., Guy, M. 2001. "High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*." *Process Biochemistry* 36 (8–9): 907–913. doi:10.1016/S0032-9592(00)00296-X.
- Henley, D.V., Lindzey, J., Korach, K.S. 2005. "Steroid Hormones." In P.M. Conn and S. Melmed (Eds.), *Endocrinology: Basic and Clinical Principles*. Totowa: Humana Press.
- Henry, H.L. 2014. "Chapter 8. Eicosanoids." *Hormones*, 171–88. doi:10.1016/B978-0-12-369444-7.00008-5.
- Higgins, D.R., Cregg, J.M. 1998. "Introduction to *Pichia pastoris*." *Methods in Molecular Biology* 103: 1–15. doi:10.1385/0-89603-421-6:1.

- Hiller-Sturmhöfel, S., Bartke, A. 1998. "The endocrine system an overview". *Alcohol Health & Research World* 22 (3): 153–164.
- Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D. 2004. "Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*." *Biotechnology and Bioengineering* 85 (4): 367-375. doi:10.1002/bit.10904.
- Huhtaniemi, I., Martini, L. 2014. "Endocrinology - Study of the Hormonal Regulation of the Body." In M. Caplan (Eds.), *Reference Module in Biomedical Research*. doi:10.1016/B978-0-12-801238-3.07829-6.
- Humphrey, T., Pearce, A. 2005. "Cell Cycle Molecules and Mechanisms of the Budding and Fission Yeast." In E. Noguchi and M.C. Gadaleta (Eds.), *Cell Cycle Control: Mechanisms and Protocols*. New Jersey: Humana Press.
- Jahic, M., Gustavsson, M., Jansen, A.K., Martinelle, M., Enfors, S.O. 2003. "Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes." *Journal of Biotechnology* 102 (1): 45–53. doi:10.1016/S0168-1656(03)00003-8.
- Jahic, M., Veide, A., Charoenrat, T., Teeri, T., Enfors, S.O. 2006. "Process technology for production and recovery of heterologous proteins with *Pichia pastoris*." *Biotechnology Progress* 22 (6): 1465–1473. doi:10.1021/bp060171t.
- Jia, L., Yuan, J.Q. 2007. "Cell cycle model for recombinant *Pichia pastoris* during glycerol fed-batch cultivation." *Process Biochemistry* 42 (5): 828–833. doi:10.1016/j.procbio.2007.02.002.
- Jones, E.W. 1991. "Tackling the protease problem in *Saccharomyces cerevisiae*." *Methods in Enzymology* 194: 428–453. doi:10.1016/0076-6879(91)94034-A.
- Jordà, J., Jouhten, P., Cámara, E., Maaheimo, H., Albiol, J., Ferrer, P. 2012. "Metabolic flux profiling of recombinant protein secreting *Pichia pastoris* growing on glucose: methanol mixtures." *Microbial Cell Factories* 11 (57): 1–14. doi: 10.1186/1475-2859-11-57.

- Jorgensen, J.O.L. 1997. "Growth Hormone." In *Molecular And Cellular Endocrinology*, 451–66. JAI Press Inc.
- Kato, Y., Murakami, Y., Sohmiya, M., Nishiki, M. 2002. "Regulation of human growth hormone secretion and its disorders." *Internal Medicine* 41 (1): 7–13. doi:10.2169/internalmedicine.41.7
- Keskin, A., 2014. "Glucose-Based Feeding Strategy Development in Recombinant Human Growth Hormone Production by *Pichia pastoris* for Semi-Batch Bioreactor Operation." Thesis of Master of Science, Middle East Technical University, Ankara
- Kim, H., Yoo, S.J., Kang, H.A. 2014. "Yeast synthetic biology for the production of recombinant therapeutic proteins." *FEMS Yeast Research* 15: 1–16. doi:10.1111/1567-1364.12195.
- Kleine, B., Rossmanith, W.G. 2016. *Hormones and the Endocrine System*. Heidelberg: Springer.
- Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Tomomitsu, K. 2000. "High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, *Pichia pastoris*, based on optimal methanol feeding strategy." *Journal of Bioscience and Bioengineering* 90 (3): 280–288. doi:10.1016/S1389-1723(00)80082-1.
- Kochar, I.P.S. 2010. "Recent trends in use of growth hormone therapy." *Apollo Medicine* 7 (1): 13–20. doi:10.1016/S0976-0016(12)60002-7.
- Kopchick, J.J., Woodley, F.W. 1997. "Regulation of Growth Hormone Gene Expression." In D. Leroith (Eds.), *Advances in Molecular and Cellular Endocrinology* 1:51–82. JAI Press Inc.
- Koser, S.A. 1968. *Vitamin Requirements of Bacteria and Yeast*. Springfield.

- Krause, W., Dubois, R.N. 2000. "Eicosanoids and the large intestine." *Prostaglandins and Other Lipid Mediators* 61 (3-4): 145–161. doi:10.1016/S0090-6980(00)00069-1.
- Kurtzman, C.P. 2012. "*Komagataella populi* sp. nov. and *Komagataella ulmi* sp. nov., two new methanol assimilating yeasts from exudates of deciduous trees." *Antonie van Leeuwenhoek* 101 (4): 859–868. doi:10.1007/s10482-012-9702-6.
- Lee, W. C., Bhagat, A.A.S., Huang, S., van Vliet, K.J., Han, J., Lim, C.T. 2011. "High-throughput cell cycle synchronization using inertial forces in spiral microchannels." *Lab on a Chip* 11 (7): 1359–167. doi:10.1039/c0lc00579g.
- Li, P., Anumanthan, A., Gao, X.G., Ilangoan, K., Suzara, V.V., Düzgüneş, N., Renugopalakrishnan, V. 2007. "Expression of recombinant proteins in *Pichia pastoris*." *Applied Biochemistry and Biotechnology* 142 (2): 105–124. doi:10.1007/s12010-007-0003-x.
- Li, Z., Moy, A., Sohal, K., Dam, C., Kuo, P., Ulrich, B., Whittaker, J., Whittaker, M., Düzgüneş, N., Konopka, K., Franz, A.H., Cereghino, J.L., Cereghino, G.P.L. 2009. "Expression and characterization of recombinant human secretory leukocyte protease inhibitor (SLPI) protein from *Pichia pastoris*." *Protein Expression and Purification* 67 (2): 175–181. doi:10.1016/j.pep.2009.06.001.
- Liu, H., Bravata, D.M., Olkin, I., Nayak, S., Roberts, B., Garber A.M., Hoffman, A.R. 2007. "Systematic review: the safety and efficacy of growth hormone in therapy." *Annals of Internal Medicine* 146 (2): 104–115. doi:10.7326/0003-4819-146-2-200701160-00005.
- Love, K. R., Panagiotou, V., Jiang, B., Stadheim, T.A., Love, J.C. 2010. "Integrated single-cell analysis shows *Pichia pastoris* secretes protein stochastically." *Biotechnology and Bioengineering* 106 (2): 319–325. doi: 10.1002/bit.22688.

- Löffelholz, C., Husemann, U., Greller, G., Meusel, W., Kauling, J., Ay, P., Kraume, M., Eibl, R., Eibl, D. 2013. "Bioengineering parameters for single-use bioreactors: overview and evaluation of suitable methods." *Chemie-Ingenieur-Technik* 85 (1–2): 40–56. doi:10.1002/cite.201200125.
- Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M. 2005. "Heterologous protein production using the *Pichia pastoris* expression system." *Yeast* 22 (4): 249-270. doi:10.1002/yea.1208.
- Madigan, M.T., Martinko, J.M. 2010. *Brock Biology of Microorganisms. 13th Ed.*, New Jersey: Prentice Hall.
- Mandenius, C.F. 2016. *Bioreactors: Design, Operation and Novel Applications*. John Wiley & Sons.
- Manukyan A, Abraham L, Dungrawala H, Schneider BL. 2011. Synchronization of yeast. In: Banfalvi G, editor. *Cell Cycle Synchronization: Methods and Protocols*. London: Humana Press. p 173–200.
- Massahi, A., Çalık, P. 2015. "In-Silico Determination of *Pichia Pastoris* Signal Peptides for Extracellular Recombinant Protein Production." *Journal of Theoretical Biology* 364: 179–88. doi:10.1016/j.jtbi.2014.08.048.
- Mattanovich, D., Graf, A., Stadlmann, J., Dragosits M., Redl, A., Maurer, M., Kleinheinz, M., Sauer, M., Altmann, F., Gasser, B. 2009. "Genome, secretome and glucose transport highlight unique features of the protein production host *Psishia pastoris*." *Microbial Cell Factories* 8:29. doi:10.1186/1475-2859-8-29.
- Meehl, M.A., Stadheim, T.A. 2014. "Biopharmaceutical discovery and production in yeast." *Current Opinion in Biotechnology* 30:120-127. doi:10.1016/j.copbio.2014.06.007.
- Møller, N., Gjedsted, J., Gormsen, L., Fuglsang, J., Djurhuus, C. 2003. "Effects of growth hormone on lipid metabolism in humans." *Growth Hormone & IGF Research* 13: 18–21. doi:10.1016/S1096-6374(03)00048-0.

- Morowitz, H.J., Kostelnik, J.D., Yang, J., Cody, G.D. 2000. "The origin of intermediary metabolism." PNAS 97 (14): 7704–7708. doi:10.1073/pnas.110153997.
- Navarro, F.J., Weston, L., Nurse, P. 2012. "Global control of cell growth in fission yeast and its coordination with the cell cycle." Current Opinion in Cell Biology 24 (6): 833–837. doi:10.1016/j.ceb.2012.10.015.
- Nelson, D.L., Cox, M.M. 2005. Lehninger Principles of Biochemistry. 4th Ed., New York: Freeman Publishers.
- Neymann, T.C., Wegerhoff, S., Engell, S. 2011. "Synchronization of budding yeast cultures." IFAC Proceedings Volumes 44 (1): 8384–8390. doi:10.3182/20110828-6-IT-1002.01712.
- Nielsen, J., Villadsen, J., Liden, G. 1994. Bioreaction Engineering Principles. 2nd Ed., New York: Plenum.
- Norman, A.W., Litwack, G. 1997. "General Considerations of Hormones." In Hormones, 2nd Ed., San Diego: Academic Press.
- O'Callaghan, J., O'Brien, M.M., McClean, K., Dobson, A.D. 2002. "Optimisation of the expression of a trametes versicolor laccase gene in Pichia pastoris." Journal of Industrial Microbiology & Biotechnology 29 (2): 55–59. doi:10.1038/sj.jim.7000268.
- Okafor, N. 2016. "Industrial Media and the Nutrition of Industrial Organisms." In Modern Industrial Microbiology and Biotechnology, 54–77. CRC Press.
- Paez-Pereda, M., Stalla, G.K. 2005. "Hormones of the Pituitary." In T. Steckler, N.H. Kalin, and J.M.H.M. Reul (Eds.), *Handbook of Stress and the Brain*. Elsevier B.V. doi:10.1016/S0921-0709(05)80007-0.
- Palomares, L.A., Kuri-Breña, F., Ramirez, O.T. 2007. "Industrial recombinant protein production." *Biotechnology* 5.

- Pavlovic, M., Girardin, E., Kapetanovic, L., Ho, K., Trouvin, J.H. 2008. "Similar biological medicinal products containing recombinant human growth hormone: European regulation." *Hormone Research in Pediatrics* 69 (1): 14–21. doi:10.1159/000111790.
- Perez-Ibave, D.C., Rodriguez-Sanchez, I.P., Garza-Rodriguez, M.L., Barrera-Saldana, H.A. 2014. "Extrapituitary growth hormone synthesis in humans." *Growth Hormone & IGF Research* 24 (2–3): 47–53. doi:10.1016/j.ghir.2014.01.005.
- Pikovsky, A., Rosenblum, M., Kurths, J., Chirikov, B., Budker, N., Cvitanovic, P., et al. 2003. "Synchronization: A Universal Concept in Nonlinear Sciences." *Cambridge Nonlinear Science Series* 12, 432. doi:10.1063/1.1554136.
- Pittoors, E., Guo, Y., van Hulle, S.W.H. 2014. "Modeling dissolved oxygen concentration for optimizing aeration systems and reducing oxygen consumption in activated sludge processes: a review." *Chemical Engineering Communications* 201 (8): 983–1002. doi:10.1080/00986445.2014.883974.
- Porro, D., Sauer, M., Branduardi, P., Mattanovich, D. 2005. "Recombinant protein production in yeasts." *Molecular Biotechnology* 31 (3): 245–259. doi:10.1385/MB:31:3:245.
- Rai, B. 2016. "Concept of Fermentation Technology." *Essentials of Industrial Microbiology*.
https://books.google.com.tr/books?id=xzMHBAQAQBAJ&source=gbs_navlink_s_s.
- Richard, P. 2003. "The rhythm of yeast." *FEMS Microbiology Reviews* 27 (4): 547–557. doi:10.1016/S0168-6445(03)00065-2.
- Romagnoli, G., Cundari, E., Negri, R., Crescenzi, M., Farina, L., Giuliani, A., Bianchi, M.M. 2011. "Synchronous protein cycling in batch cultures of the yeast *Saccharomyces cerevisiae* at log growth phase." *Experimental Cell Research* 317 (20): 2958–2968. doi:10.1016/j.yexcr.2011.09.007.

- Rupes, I. 2002. "Checking cell size in yeast." *Trends in Genetics* 18 (9): 479–485. doi:10.1016/S0168-9525(02)02745-2.
- Sethuraman, N., Stadheim, T.A. 2006. "Challenges in therapeutic glycoprotein production." *Current Opinion in Biotechnology* 17 (4): 341–346. doi:10.1016/j.copbio.2006.06.010.
- Shaw, J., Payer, K., Son, S., Grover, W.H., Manalis, S.R. 2012. "A microfluidic 'baby machine' for cell synchronization." *Lab on a Chip* 12 (15): 2656. doi:10.1039/c2lc40277g.
- Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S.M., Hegedus, D. 2003. "Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*." *Protein Expression and Purification* 28 (2): 321–330. doi:10.1016/S1046-5928(02)00706-4.
- Smith, W.L., Murphy, R.C. 2016. "Chapter 9 – The Eicosanoids: Cyclooxygenase, Lipoxygenase and Epoxygenase Pathways." *Biochemistry of Lipids, Lipoproteins and Membranes*, 259–296. doi:10.1016/B978-0-444-63438-2.00009-2.
- Sonntag, W.E., Ramsey, M., Carter, C.S. 2005. "Growth hormone and insulin-like growth factor-1 (IGF-1) and their influence on cognitive aging." *Ageing Research Reviews* 4 (2): 195–212. doi:10.1016/j.arr.2005.02.001.
- Sperling, M.A. 2016. "Traditional and novel aspects of the metabolic actions of growth hormone." *Growth Hormone & IGF Research* 28: 69–75. doi:10.1016/j.ghir.2015.06.005.
- Stanbury, P.F., Whitaker, A., Hall, S.J. 2016. "Media for Industrial Fermentation." In *Principles of Fermentation Technology, 3rd Ed.*, Butterworth-Heinemann.
- Stryjewska, A., Kiepusa, K., Librowski, T., Lochynski, S. 2013. "Biotechnology and genetic engineering in the new drug development. Part III. Biocatalysis, metabolic engineering and molecular modelling." *Pharmacological Reports* 65 (5): 1102–1111.

- Tolman, K.G. 2000. "Eicosanoids and the liver." *Prostaglandins & Other Lipid Mediators* 61 (3-4): 163-174. doi:10.1016/S0090-6980(00)00070-8.
- Turner, J.J., Ewald, J.C., Skotheim, J.M. 2012. "Cell size control in yeast." *Current Biology* 22 (9): R350-R359. doi:10.1016/j.cub.2012.02.041.
- Uchiyama, K, Morimoto, M., Yokoyama, Y., Shioya, S. 1997. "Cell cycle dependency of rice alpha-amylase production in a recombinant yeast." *Biotechnology and Bioengineering* 54 (3): 262–271. doi:10.1002/(SICI)1097-0290(19970505)54:3<262::AID-BIT7>3.0.CO;2-D.
- van Maris, A.J.A., Geertman, J.M.A., Vermeulen, A., Groothuizen, M.K., Winkler, A.A., Piper, M.D.W., van Dijken, J.P., Pronk, J.T. 2004. "Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C₂-independent, glucose-tolerant, and pyruvate-hyperproducing yeast." *Applied and Environmental Microbiology* 70 (1): 159–166. doi:10.1128/AEM.70.1.159.
- van Urk, H., Voll, W.S.L., Scheffers, W.A., van Dijken, J.P. 1990. "Transient-state analysis of metabolic fluxes in crabtree-positive and crabtree-negative yeasts." *Applied and Environmental Microbiology* 56 (1): 281–287.
- Verma, A.S., Agrahari, S., Rastogi, S., Singh, A. 2011. "Biotechnology in the realm of history." *Journal of Pharmacy And Bioallied Sciences* 3 (3): 321–323. doi:10.4103/0975-7406.84430.
- Vijayakumar, A., Novosyadlyy, R., Wu, Y., Yakar, S., LeRoith, D. 2009. "Biological effects of growth hormone on carbohydrate and lipid metabolism." *Growth Hormone & IGF Research* 20 (1): 1–7. doi:10.1016/j.ghir.2009.09.002.
- Vogl, T., Hartner, F.S., Glieder, A. 2013. "New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*." *Current Opinion in Biotechnology* 24 (6): 1094-1101. doi:10.1016/j.copbio.2013.02.024.
- Walker, G.M. 1999. "Synchronization of yeast cell populations." *Methods in Cell Science* 21 (2): 87–93. doi:10.1023/A:1009824520278.

- Walsh, G. 2002. "Biopharmaceuticals and biotechnology medicines: an issue of nomenclature." *European Journal of Pharmaceutical Sciences* 15 (2): 135–138. doi:10.1016/S0928-0987(01)00222-6.
- Walsh, G. 2005. "Biopharmaceuticals: Recent Approvals and Likely Directions." *Trends in Biotechnology* 23 (11): 553–58. doi:10.1016/j.tibtech.2005.07.005.
- Wang, S.H., Yang, T.S., Lin, S.M., Tsai, M.S., Wu, S.C., Mao, S.J.T. 2002. "Expression, characterization, and purification of recombinant porcine lactoferrin in *Pichia pastoris*." *Protein Expression and Purification* 25 (1): 41–49. doi:10.1006/prep.2001.1607.
- Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V., Cregg, J.M. 1997. "Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter." *Gene* 186 (1): 37–44. doi:10.1016/S0378-1119(96)00675-0.
- Watson, S.E., Rogol, A.D. 2013. "Recent updates on recombinant human growth hormone outcomes and adverse events." *Current Opinion in Endocrinology Diabetes and Obesity* 20 (1): 39–43. doi:10.1097/MED.0b013e32835b7ea8.
- Weigent, D.A. 1996. "Immunoregulatory properties of growth hormone and prolactin." *Pharmacology & Therapeutics* 69 (693): 237–257. doi:10.1016/0163-7258(96)00001-0.
- Wells, J.A., de Vos, A.M. 1993. "Structure and function of human growth hormone: implications for the hematopoietins." *Annual Review of Biophysics and Biomolecular Structures* 22: 329–351. doi:10.1146/annurev.bb.22.060193.001553.
- Wuttke, W., Jarry, H., Seidlová-Wuttke, D. 2010. "Definition, classification and mechanism of action of endocrine disrupting chemicals." *Hormones* 9 (1): 9–15.
- Zerze, G.H., 2012. "Comparison of human growth hormone production performance of two different metabolically engineered *Pichia pastoris*." Thesis of Master of Science, Middle East Technical University, Ankara

APPENDIX A

CHEMICALS AND SUPPLIERS

Table A. 1 List of chemicals and commercially available solutions required in medium recipes, their molecular formulas and their suppliers

Chemical	Molecular Formula	Supplier
Ammonium sulfate	$(\text{NH}_4)_2\text{SO}_4$	Sigma
Antifoam Y-30 emulsion	-	Sigma
Bacto agar	-	Sigma
Bacto peptone	-	Sigma
Boric acid		Sigma
D-Biotin	$\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$	Sigma
Calcium chloride dehydrate	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	Sigma
Chloramphenicol	$\text{C}_{11}\text{H}_{12}\text{Cl}_2\text{N}_2\text{O}_5$	Sigma
Cobalt chloride	CoCl_2	Sigma
Cobalt chloride hexahydrate	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	Sigma
Copper sulfate	CuO_4S	Sigma
D-Glucose	$\text{C}_6\text{H}_{12}\text{O}_6$	Merck
D-Glucose monohydrate	$\text{C}_6\text{H}_{12}\text{O}_6 \cdot \text{H}_2\text{O}$	Sigma
Glycerol (87%)	$\text{C}_3\text{H}_8\text{O}_3$	Sigma
Iron sulfate heptahydrate	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Sigma
LB-agar	-	Sigma

Table A.1 List of chemicals and commercially available solutions required in medium recipes, their molecular formulas and their suppliers (*continued*).

Chemical	Molecular Formula	Supplier
LB-broth	-	Sigma
Magnesium chloride hexahydrate	MgSO ₄ .6H ₂ O	Merck
Manganese chloride	MnCl ₂	Sigma
Manganese sulfate monohydrate	MnSO ₄ .H ₂ O	Sigma
Peptone (bacteriological)	-	Sigma
Potassium chloride	KCl	Sigma
Potassium dihydrogen monobasic	KH ₂ PO ₄	Sigma
di-Potassium hydrogen dibasic	K ₂ HPO ₄	Sigma
Potassium hydroxide (85%)	KOH	Sigma
Potassium sulfate	K ₂ SO ₄	Sigma
Sodium acetate	C ₂ H ₃ O ₂ Na	Sigma
Sodium chloride	NaCl	Sigma
Sodium iodide	NaI	Sigma
Sodium molybdate dehydrate	Na ₂ MoO ₄ .2H ₂ O	Sigma
Sulfuric acid	H ₂ SO ₄	Sigma
Yeast extract	-	Sigma
Yeast nitrogen base without amino acids	-	Sigma
Zeocin	C ₅₅ H ₈₆ N ₂₀ O ₂₁ S ₂	Invivogen
Zinc chloride	ZnCl ₂	Sigma

Table A. 2 List of chemicals and commercially available solutions required in genetic techniques, their molecular formulas and their suppliers

Chemical	Molecular Formula	Supplier
Agarose	-	Sigma
DNA ladder_Lambda DNA HindIII	-	Thermo-Fischer
DNA ladder_Gene Ruler Express	-	Thermo-Fischer
DNA loading buffer (6X)	-	Thermo-Fischer
dNTPs	-	Thermo-Fischer
Ethanol (100%)	$C_{10}H_{16}N_2O_3S$	Merck
Ethidium bromide	$C_{21}H_{20}BrN_3$	Sigma
Ethylenediaminetetraacetic acid disodium salt hydrate	$C_{11}H_{12}C_{12}N_2O_5$	Sigma
Glass beads	-	-
Glycine	$C_2H_5NO_2$	Sigma
Hydrochloric acid	HCl	Sigma
Phenol chloroform isoamyl alcohol (25:24:1)	-	Fluka
Polyethylene glycol, 3350	$C_{2n}H_{4n+2}O_{n+1}$	Sigma
Polyethylene glycol, 8000	$C_{2n}H_{4n+2}O_{n+1}$	Sigma
Primers	-	Sentromer
Restriction enzymes (PagI)	-	Thermo-Fischer
RNase-A	-	Thermo-Fischer
Sodium chloride	NaCl	Sigma
Sodium dodecyl sulfate	$C_{12}H_{25}O_4S.Na$	Sigma
TAE buffer (50X)	-	Sigma
Taq DNA polymerase	-	Thermo-Fischer
TBE buffer (50X)	-	Sigma
Tris base/ THAM/ TRIZMA	$C_4H_{11}NO_3$	Sigma
Triton-X	$C_{14}H_{22}O(C_2H_4O)_n$	Sigma
Tween 20	$C_{58}H_{114}O_{26}$	Sigma

Table A. 3 List of chemicals and commercially available solutions required in analysis (SDS-PAGE, Dot-Blot, HPLC)

Chemical	Molecular Formula	Supplier
Ammonium persulfate	$(\text{NH}_4)_2\text{S}_2\text{O}_8$	Bio-Rad
Bromophenol blue	-	Sigma
Calcium carbonate	-	Sigma
DAB substrate kit	-	Abcam
Ethanol (95%)	$\text{C}_2\text{H}_6\text{O}$	Sigma
Formaldehyde	$\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$	Sigma
Glacial acetic acid	CH_3COOH	Sigma
Glycine	$\text{C}_2\text{H}_5\text{NO}_2$	Sigma
Glycerol	$\text{C}_{11}\text{H}_{12}\text{C}_{12}\text{N}_2\text{O}_5$	Sigma
His-taq antibody	-	Abcam
Horseradish peroxidase	-	Abcam
Hydrochloric acid	HCl	Sigma
2-Mercaptoethanol	$\text{C}_2\text{H}_6\text{OS}$	Fluka
Methanol	CH_3OH	Merck
Milk powder	-	Sigma
Silver nitrate	AgNO_3	Sigma
Sodium chloride	NaCl	Sigma
Sodium thiosulfate pentahydrate	$\text{Na}_2\text{O}_3\text{S}_2 \cdot 5\text{H}_2\text{O}$	Sigma
Sodium dodecyl sulfate	$\text{C}_{12}\text{H}_{25}\text{O}_4\text{S} \cdot \text{Na}$	Sigma
Tris base/ THAM/ TRIZMA	$\text{C}_4\text{H}_{11}\text{NO}_3$	Sigma
Tween 20	$\text{C}_{58}\text{H}_{114}\text{O}_{26}$	Sigma

APPENDIX B

BUFFERS, STOCK SOLUTIONS and KITS

Table B. 1 SDS-PAGE Solutions (Fastcast© Gel-Biorad Kit)

Solution	Recipe
10% (w/v) APS (Ammonium persulfate)	0.1 g of APS can be weighed and then added to 1 ml dH ₂ O. Solution should be well mixed and freshley prepared
Resolving Gel	3 ml resolver A and 3 ml resolver B of FastCast© gel solution, 30 µl of APS solution and 3 µl of TEMED can be mixed and poured into
Stacking Gel	1 ml stackerA and 1 ml stacker B of FastCast© gel solution, 10 µl of APS solution and 2 µl of TEMED can be mixed and poured into.

Table B.1 SDS-PAGE Solutions (Fastcast© Gel-Biorad Kit) (*Continued*)

Solution	Recipe
Note:	APS and TEMED should be mixed with Resolving and stacking gel prior to pouring
<i>4X SDS-PAGE Sample Loading Buffer</i>	200 mM Tris-HCl at pH 6.8; 40% glycerol; 6% SDS; 0.013% Bromophenol blue ; 10% 2-mercaptoethanol can be mixed and stored at -20°C
<i>5X SDS-PAGE Running Buffer</i>	15 g of Tris Base, 72 g glycine, 5 g sodium dodecyl sulfate can be mixed and volume should be solven in 1 L of dH ₂ O

Table B. 2 Silver Staining Solutions

Solution	Recipe
<i>Fixer</i>	Mixing of 100 ml methanol, 24 ml acetic acid, 100 µl 37% formaldehyde and then adding of dH ₂ O up to 200 ml
<i>Ethanol Washing</i>	Mixing of 75 ml ethanol and 75 ml dH ₂ O
<i>Pretreatment</i>	Dissolve 0.05 g of Na ₂ O ₃ S ₂ .5H ₂ O (sodium thiosulfate pentahydrate) in 250 ml dH ₂ O and then 2 ml of this solution must be removed to add in developing solution
<i>Silver Nitrate</i>	Dissolve 0.2 g of silver nitrate (AgNO ₃) in 100 ml dH ₂ O and add 75 µl of 37% formaldehyde
<i>Developing</i>	Dissolve 2.25 g of potassium carbonate in 100 ml dH ₂ O and add both 75 µl of 37% formaldehyde and 2 ml of pretreatment solution
<i>Stop</i>	Mixing of 50 ml methanol, 12 ml acetic acid and then adding of dH ₂ O up to 200 ml



APPENDIX C

CALIBRATION CURVES

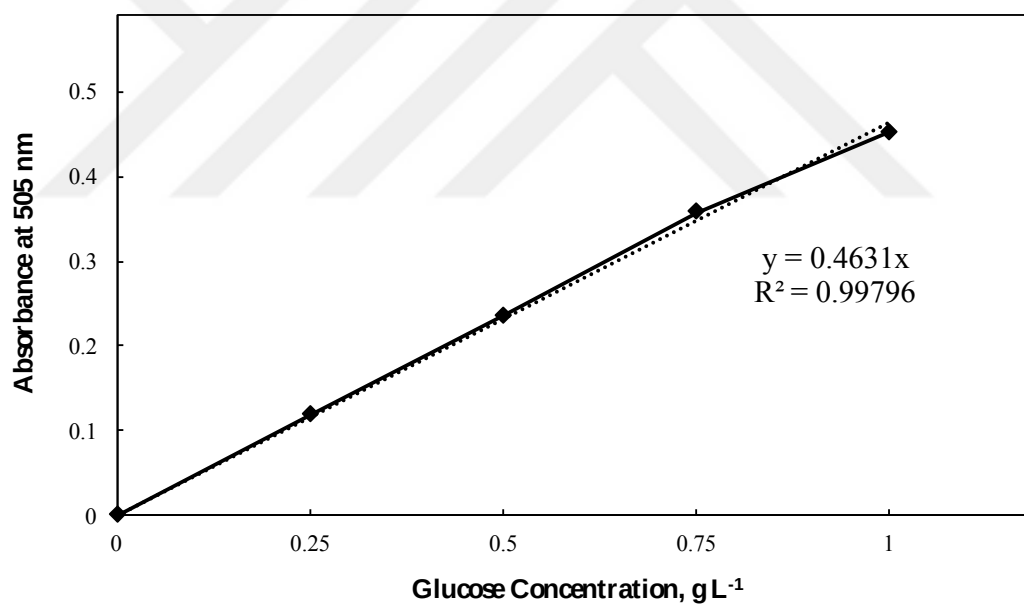


Figure C. 1 Calibration Curve for Glucose Concentration

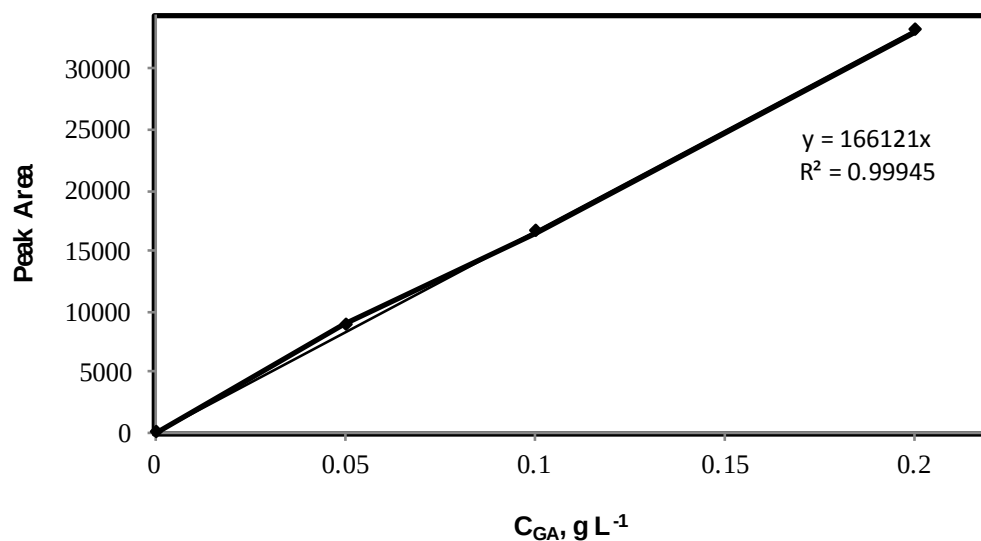


Figure C. 2 Calibration Curve for Gluconic Acid Concentration

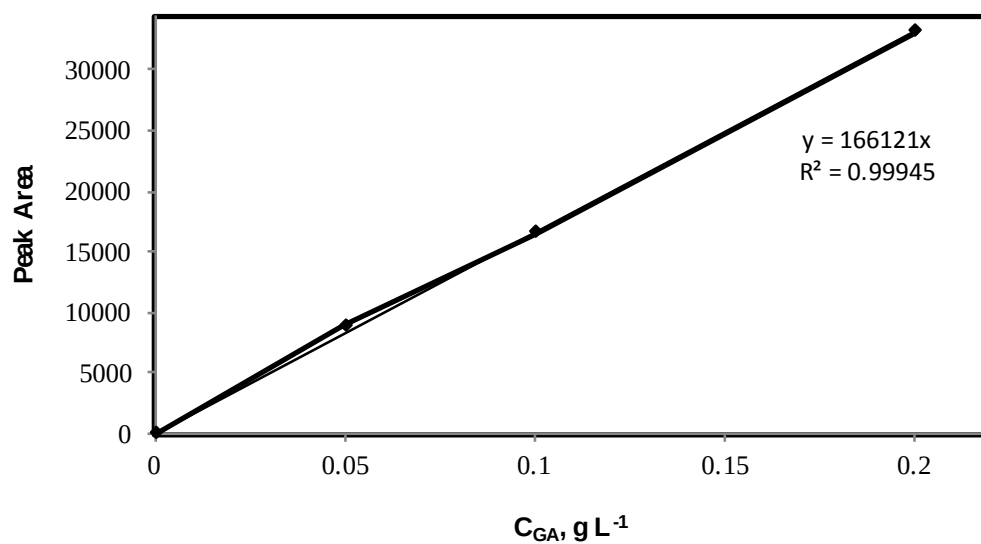


Figure C. 3 Calibration Curve for Pyruvic Acid Concentration

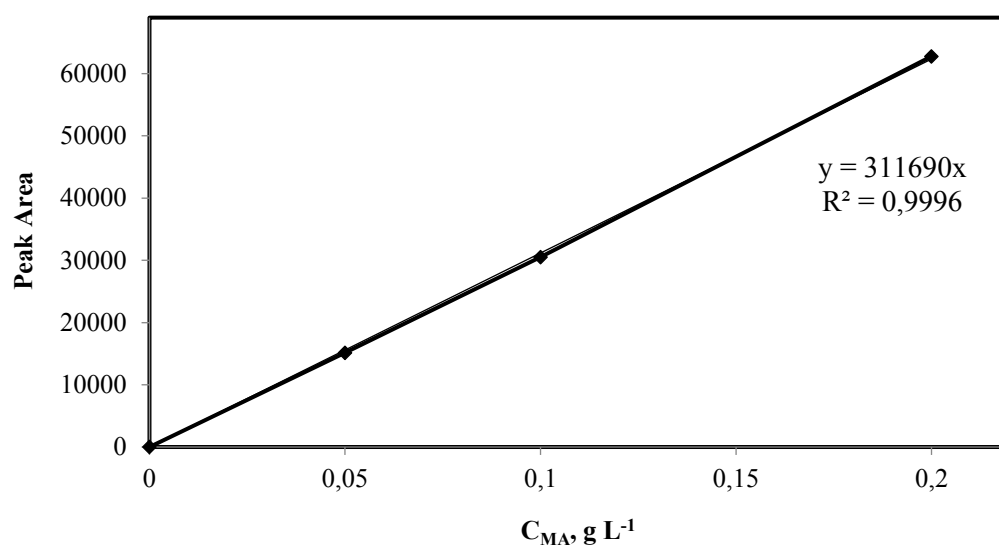


Figure C. 4 Calibration Curve for Malic Acid Concentration

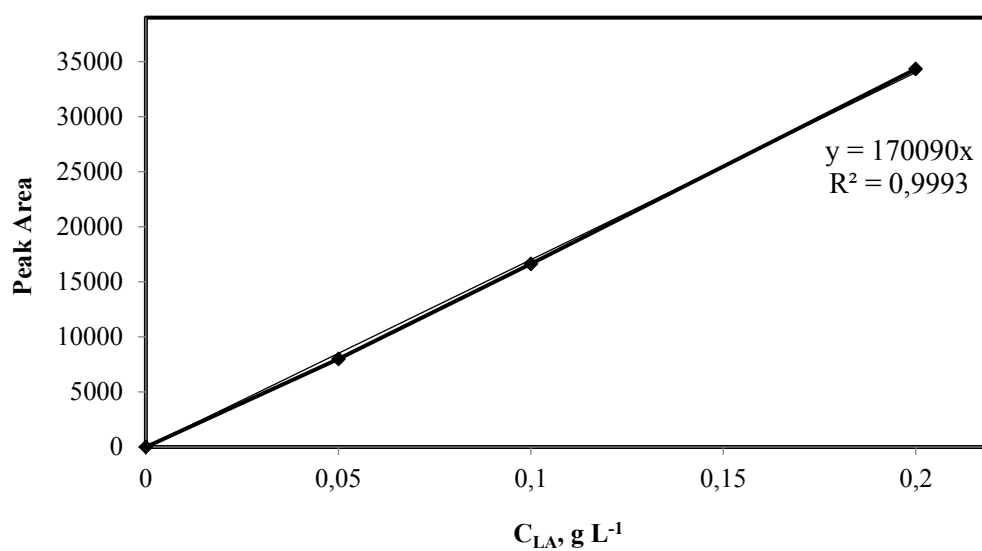


Figure C. 5 Calibration Curve for Lactic Acid Concentration

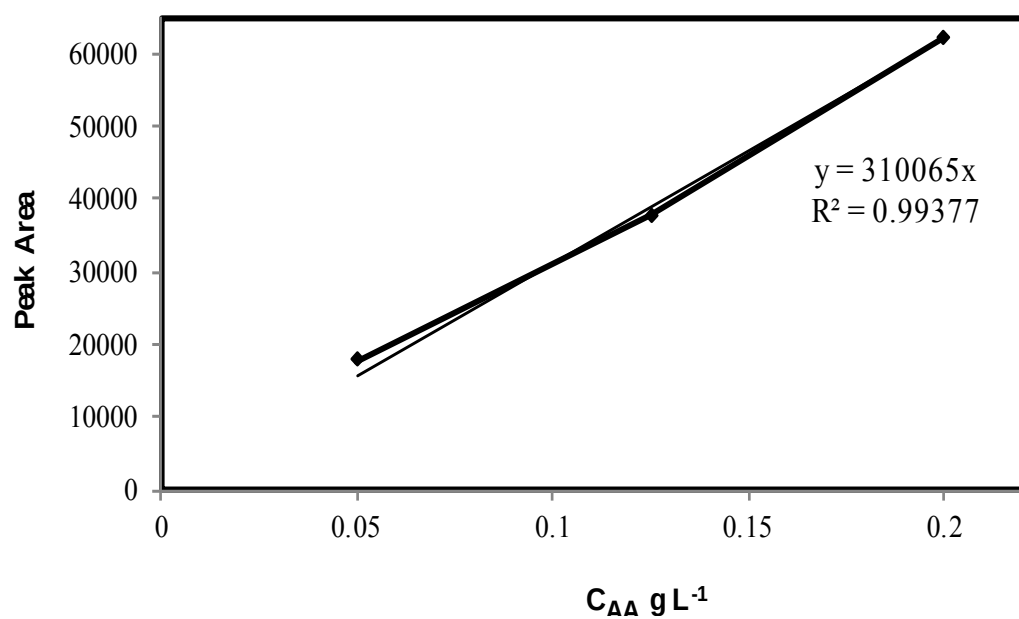


Figure C. 6 Calibration Curve for Acetic Acid Concentration

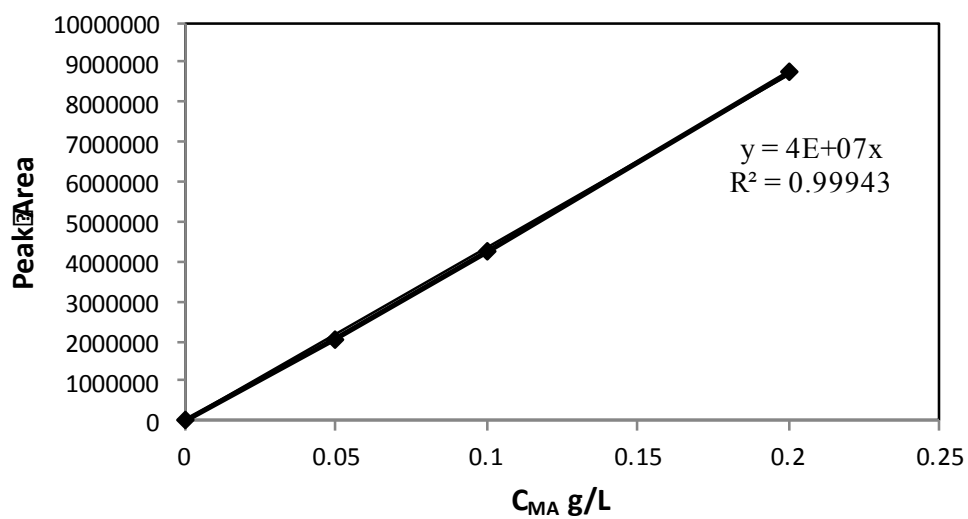


Figure C. 7 Calibration Curve for Maleic Acid Concentration

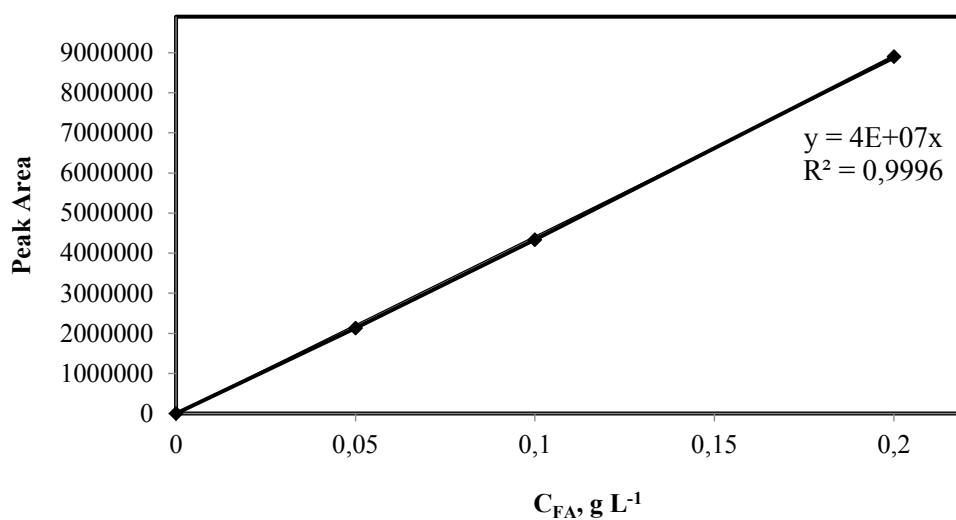


Figure C. 8 Calibration Curve for Fumaric Acid Concentration

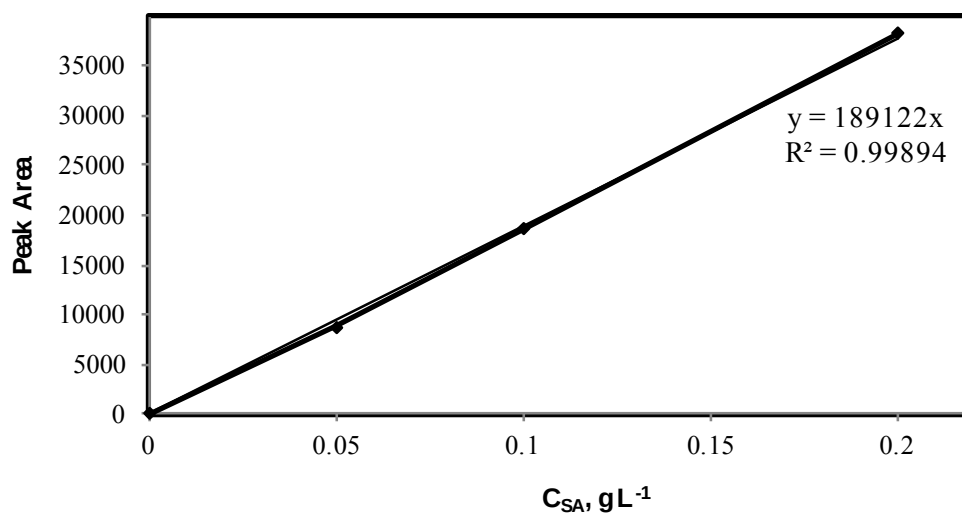


Figure C. 9 Calibration Curve for Succinic Acid Concentration

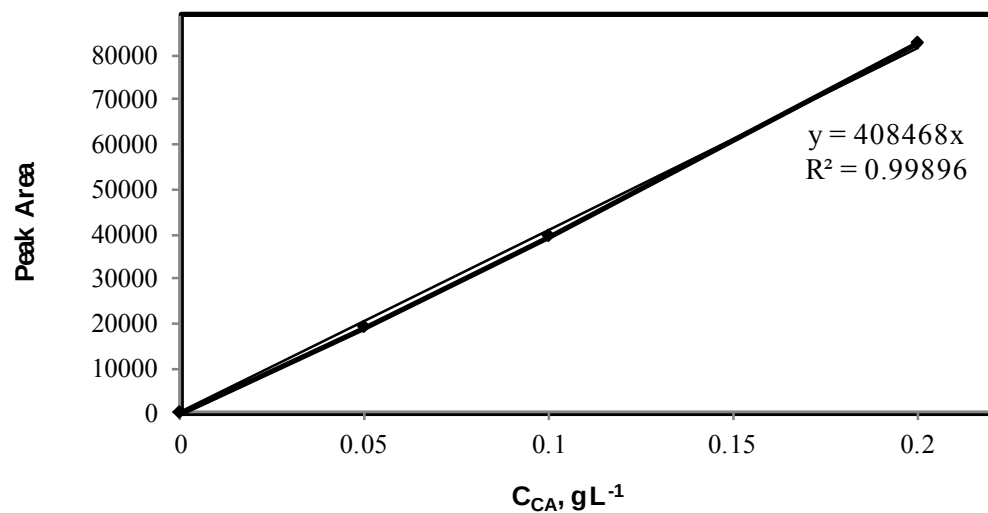
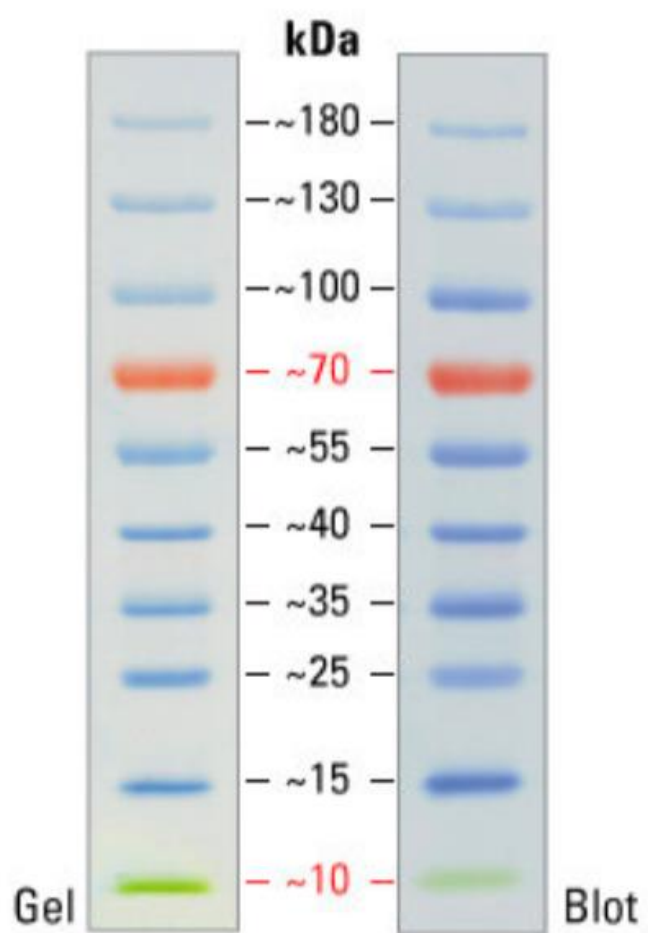


Figure C. 10 Calibration Curve for Citric Acid Concentration

APPENDIX D

SDS-PAGE PROTEIN LADDER





APPENDIX E

SDS-PAGE PROTEIN ANALYSES

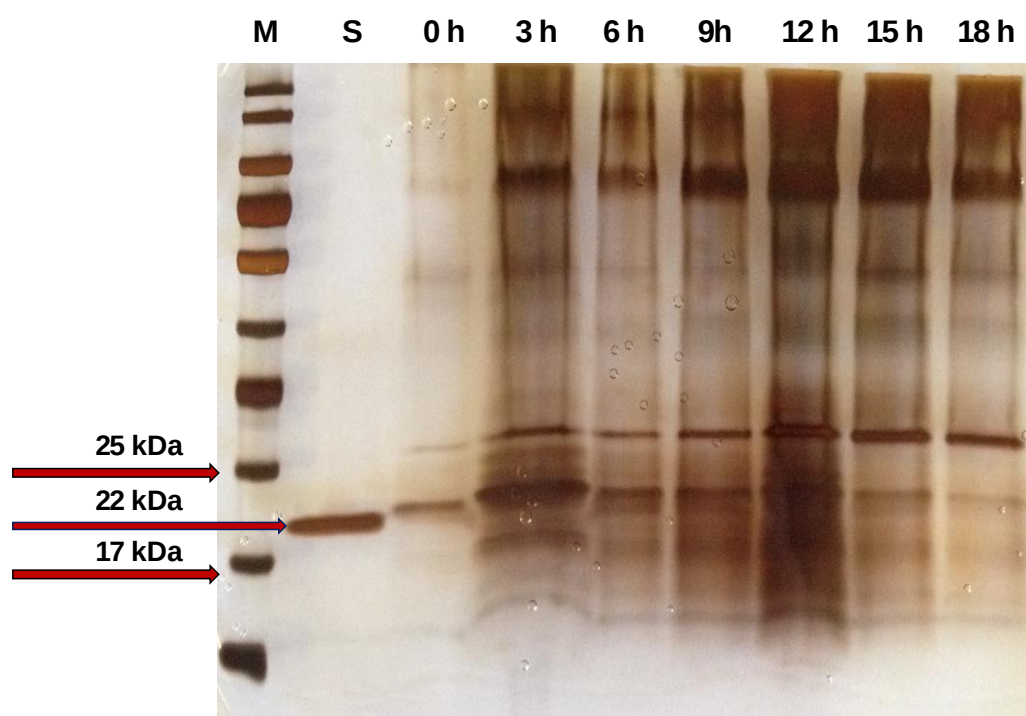


Figure E. 1 Silver stained SDS-PAGE gel images of protein produced in BR1, 1:10 diluted samples, M: Marker of PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

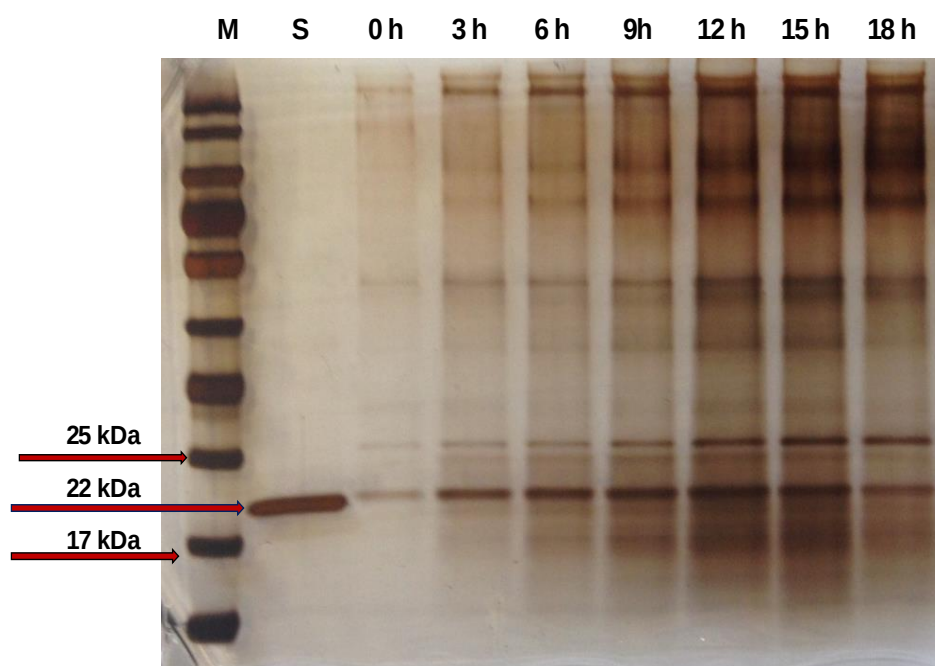


Figure E. 2 Silver stained SDS-PAGE gel images of protein produced in BR2, 1:10 diluted samples, M: PageRuler protein ladder, S: 0.05 g L^{-1} hGH standard

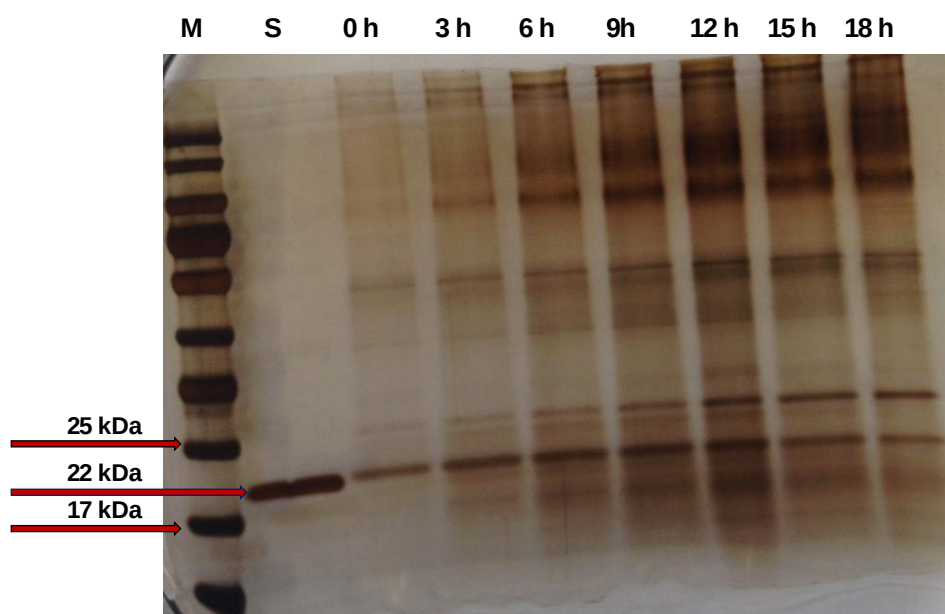


Figure E. 3 Silver stained SDS-PAGE gel images of protein produced in BR3, 1:10 diluted samples, , M: PageRuler protein ladder, S: 0.05 g L^{-1} hGH standard

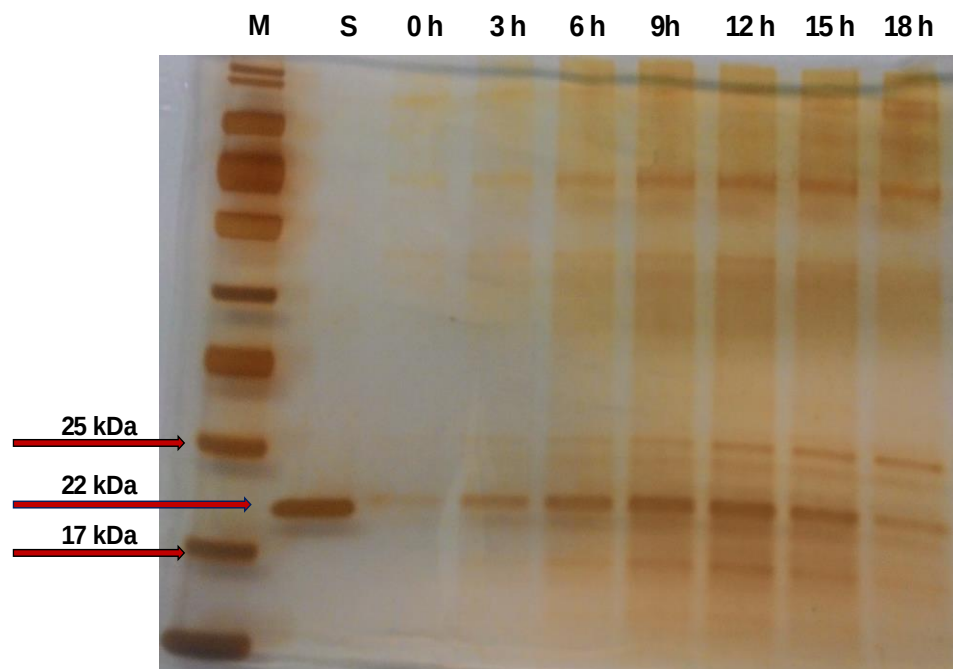


Figure E. 4 Silver stained SDS-PAGE gel images of protein produced in BR4, 1:10 diluted samples, M: PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

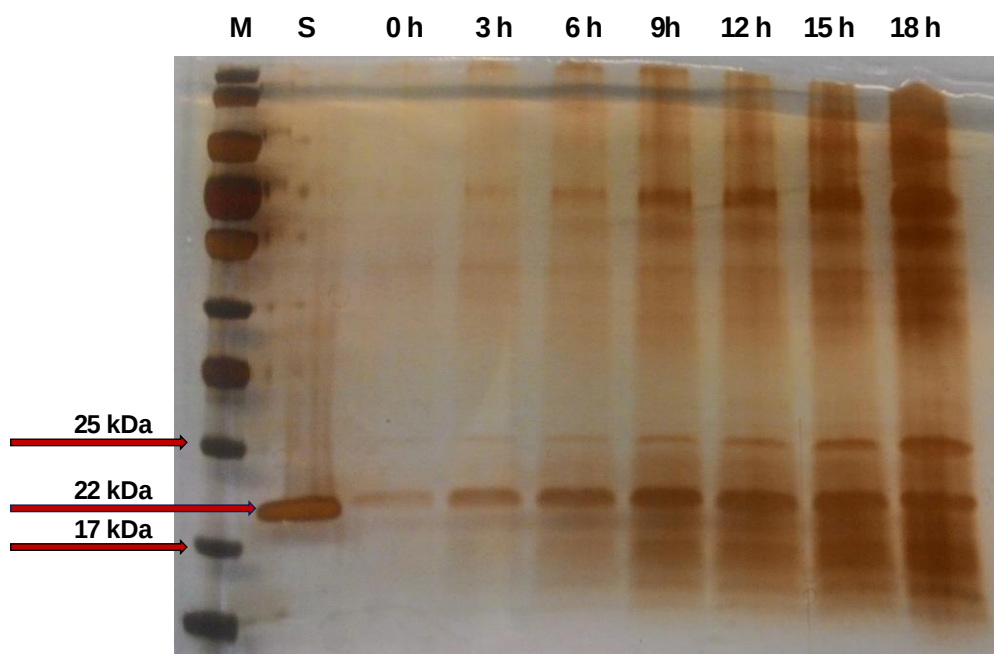


Figure E. 5 Silver stained SDS-PAGE gel images of protein produced in BR5, 1:10 diluted samples, M: PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

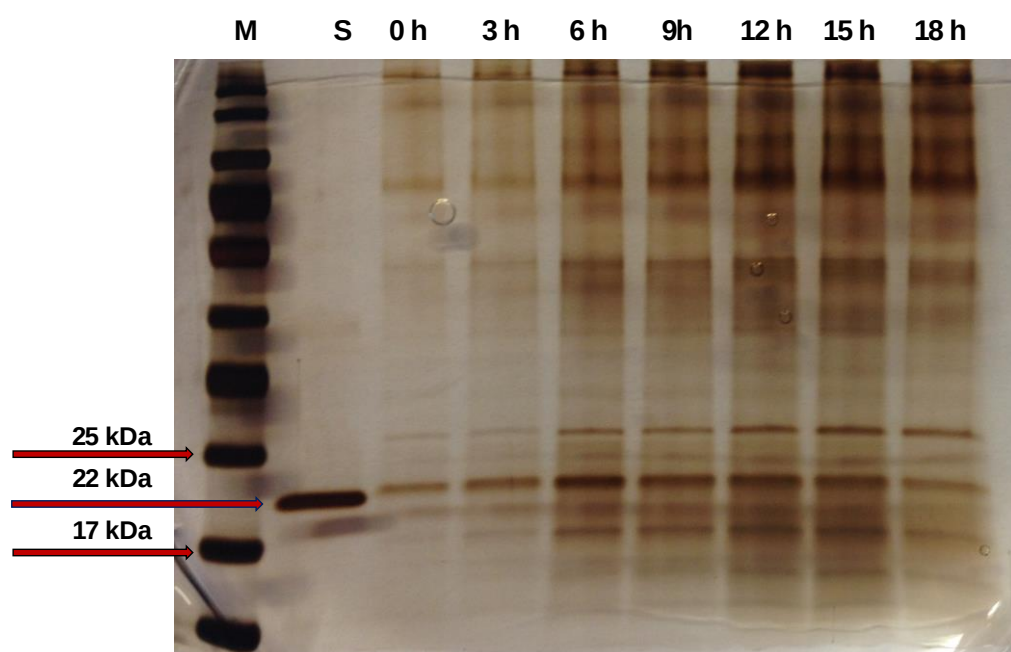


Figure E. 6 Silver stained SDS-PAGE gel images of protein produced in BR5, 1:10 diluted samples, M: PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

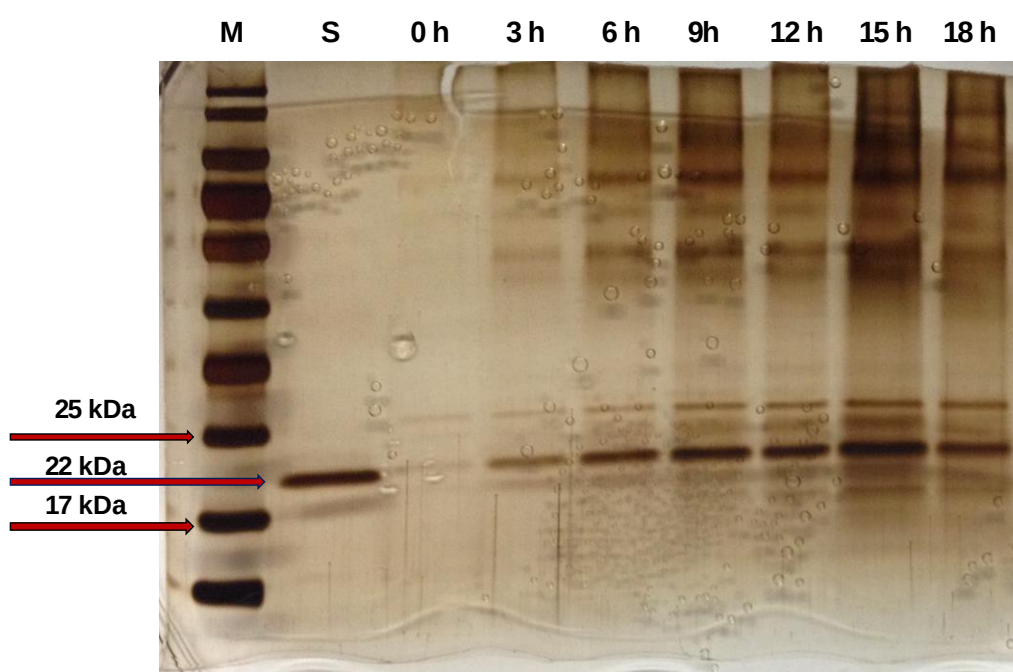


Figure E. 7 Silver stained SDS-PAGE gel images of protein produced in BR7, 1:10 diluted samples, M: PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

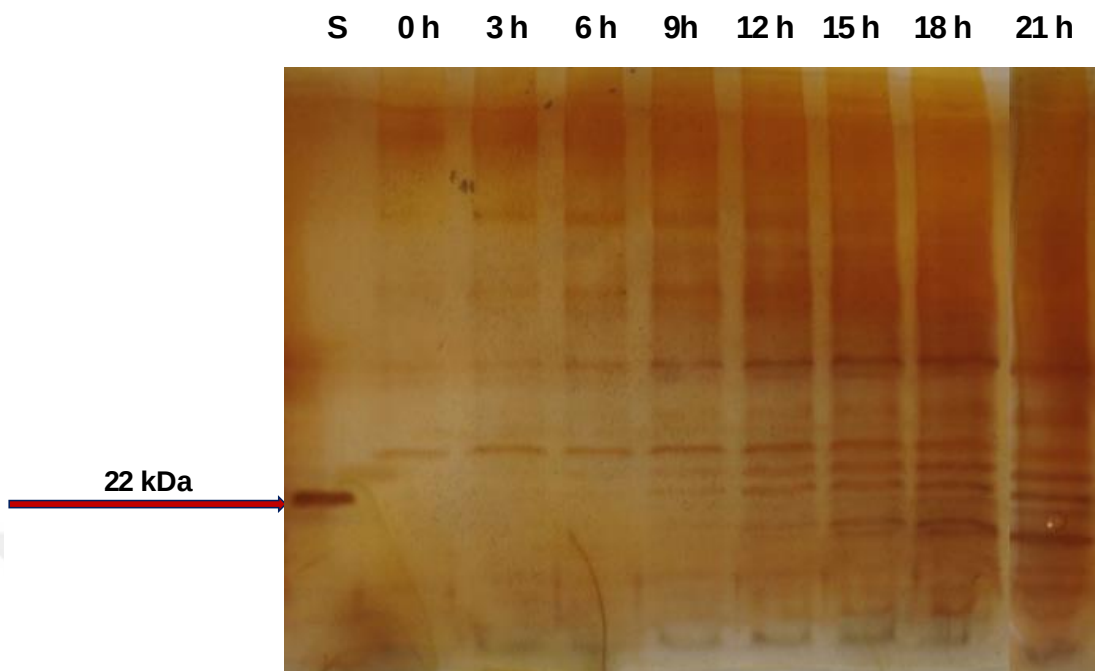


Figure E. 8 Silver stained SDS-PAGE gel images of protein produced in B4, no dilution, M: Marker of PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

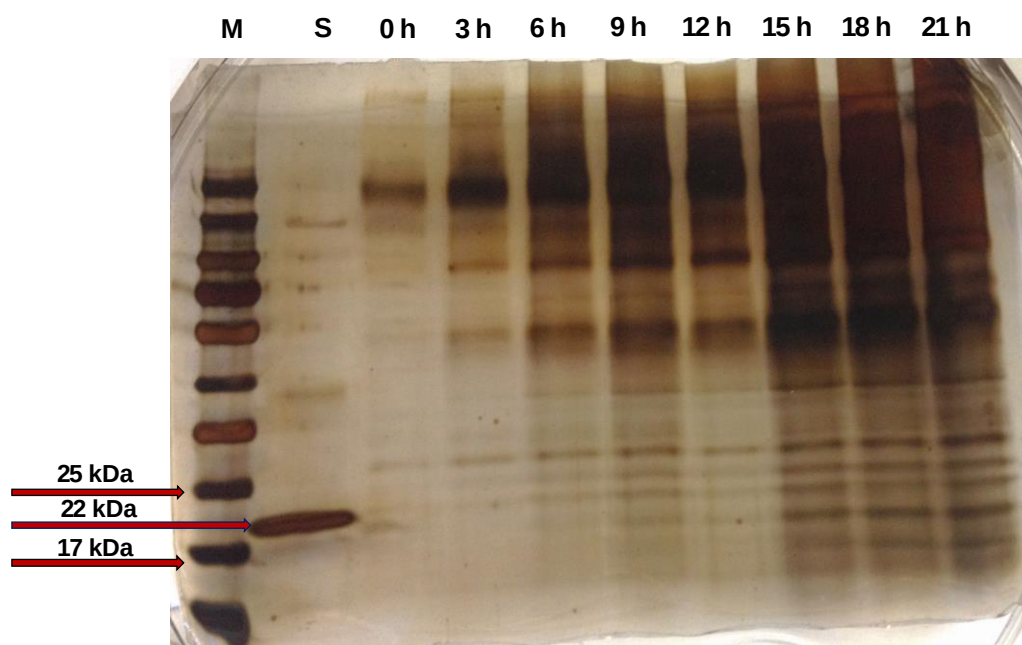


Figure E. 9 Silver stained SDS-PAGE gel images of protein produced in B5, 1:2 diluted samples, M: PageRuler protein ladder, S: 0.05 g L⁻¹ hGH standard

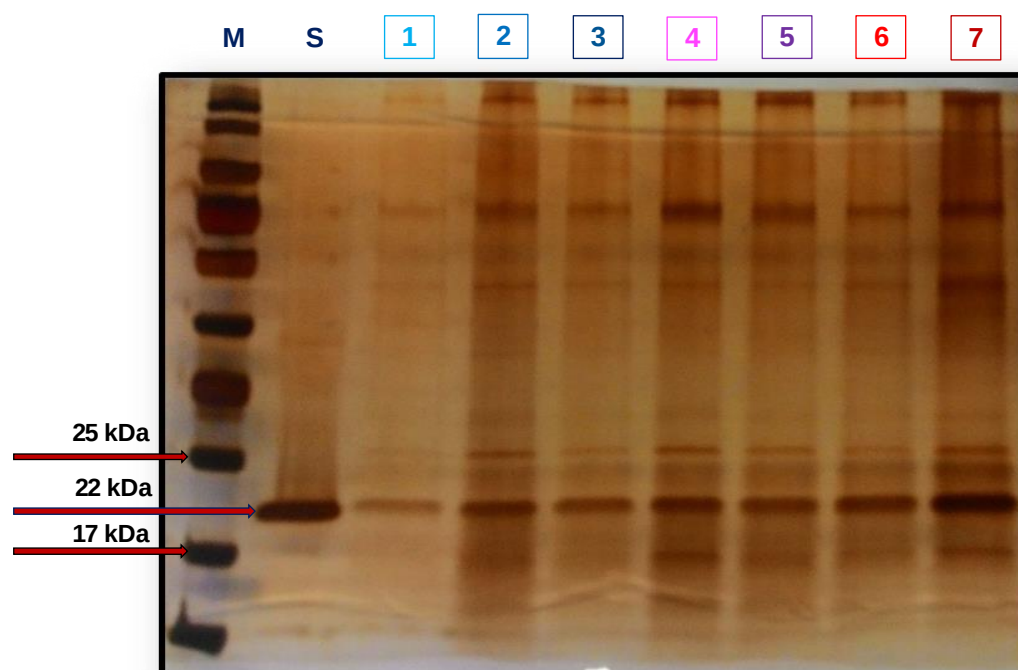


Figure E. 10 Silver stained SDS-PAGE gel images of protein produced that was the highest concentration obtained of all operations applied in laboratory scale bioreactor experiments. M: PageRuler protein ladder; S: 0.05 g L^{-1} hGH standard, Lane 1: $t = 6 \text{ h}$ in BR1, Lane 2: $t = 12 \text{ h}$ in BR2, Lane 3: $t = 6 \text{ h}$ in BR3, Lane 4: $t = 12 \text{ h}$ in BR4, Lane 5: $t = 9 \text{ h}$ in BR5, Lane 6: $t = 6 \text{ h}$ in BR6, and Lane 7: $t = 15 \text{ h}$ in BR7