REGULATORY GENE EFFECTS ON RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *Bacillus subtilis*

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ABSTRACT

REGULATORY GENE EFFECTS ON RECOMBINANT HUMAN GROWTH HORMONE PRODUCTION BY *Bacillus subtilis*

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In this study, regulatory gene effects on recombinant human growth hormone (rhGH) production by *Bacillus subtilis* were investigated. For this purpose, firstly *Bacillus* strains, which are deficient in *abrB*, *aprE*, *degQ*, *degS*, *degU*, *scoC*, *sinI*, *sinR*, and *spo0A* genes, were selected according to the regulatory gene network of *aprE* gene (serine alkaline protease gene of *B. subtilis*) since due to the *degQ* promoter and the pre-signal sequence of *subC* gene cloned in front of the hGH gene, hGH is produced by mimicking the serine alkaline protease synthesis. R-*Bacillus* strains were constructed by transformation of pMK4::*pre*(*subC*)::*hGH* plasmid to the selected strains. Thereafter, by the laboratory scale experiments, strains having the highest hGH production capacity were determined as *scoC*, *aprE*, *sinR*, and *degU* knockout strains. Using these strains, fermentation experiments were carried out in pilot-scale bioreactor in defined medium. Effect of pH control was also investigated and the highest cell and hGH concentration was obtained by *scoC* knockout strain in pH controlled

operation as 1.62 kg m^{-3} and 126 g m^{-3}, respectively. By this strain, the overall product and cell yield on total substrate were found as 16.12 g kg^{-1} and 0.15 g g^{-1}, respectively. Furthermore, the highest total protease activity was attained by *degU* knockout strain as 65 U cm^{-3}. On the other hand, maximum total organic acid secretion was determined as 1.31 kg m^{-3} in *aprE* knockout strain.

Keywords: Recombinant human growth hormone, *Bacillus subtilis*, regulatory genes, pH

ÖZ

REKOMBİNANT İNSAN BÜYÜME HORMONUNUN *Bacillus subtilis* İLE ÜRETİMİNDE REGÜLASYON GENLERİNİN ETKİLERİ

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Bu çalışmada, regülasyon genlerinin *Bacillus subtilis* ile rekombinant insan büyüme hormonu üretimi üzerindeki etkileri incelenmiştir. Bu amaçla ilk olarak ekspresyon vektör üzerindeki *subC* promoter ve sinyal sekansı nedeniyle, *aprE* geninin (*B. subtilis*'deki serin alkalin proteaz geni) regülasyon ağındaki genler belirlenmiş ve *abrB, aprE, degQ, degS, degU, scoC, sinI, sinR,* ve *spo0A* genleri silinmiş olan *Bacillus* suşları konak hücre olarak seçilmiştir. Daha sonra, pMK4::*pre*(*subC*)::*hGH* plazmidinin bu suşlara transformasyonu ile recombinant *Bacillus* suşları oluşturulmuştur. Bu suşlarla yapılan laboratuvar ölçekli biyoreaktör deneyleri sonucunda en yüksek insan büyüme hormonu üretim kapasitesine sahip suşlar *scoC, aprE, sinR* ve *degU* genleri silinmiş suşlar olduğu bulunmuştur. Bu suşlar ile tanımlı ortam kullanarak yarı kesikli biyoreaktörlerde fermentasyon deneyleri yapılmış, pH kontrolünün üretim üzerinde etkisi araştırılmıştır. En yüksek hücre ve hGH derişimine kontrol pH koşulunda *scoC* geni silinmiş *Bacillus* suşu ile 1.62 kg m^{-3} ve 126 g m^{-3} olarak ulaşılmıştır. Bu suş için toplam substrat üzerinden ürün ve hücre verimi 16.12 g kg^{-1} ve 0.15 kg kg^{-1}

olarak bulunmuştur. En yüksek toplam proteaz aktivitesi ise *degU* geni silinmiş suş ile 65 U cm^{-3} olarak ulaşılmıştır. Diğer taraftan, en yüksek toplam organik asit derişimi *aprE* geni silinmiş suş ile 1.31 kg m^{-3} olarak elde edilmiştir.

Anahtar kelimeler: Rekombinant insan büyüme hormonu, *Bacillus subtilis*, regülasyon genleri, pH

To my grandparents

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NOMENCLATURE

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

Greek Letters

ρ	Density	$g\ L^{-1}$
η	Effectiveness factor (=OUR/OD)	
μ	Specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Initial condition
O	Oxygen
p	Protein
pro	Protease
R	Bioreaction medium
rp	Recombinant protein
S	Substrate
X	Cell

Abbreviations

CDW	Cell dry weight
DNA	Deoxyribonucleic acid
hGH	Human growth hormone
HPCE	High pressure capillary electrophoresis
HPLC	High pressure liquid chromatography
rhGH	Recombinant Human growth hormone
SAP	Serine Alkaline Protease
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
Ala	Alanine

Cysteine	Cys
Aspartic Acid	Asp
Glutamic Acid	Glu
Phenyalanine	Phe
Glycine	Gly
Histidine	His
Isoleucine	Ile
Lysine	Lys
Leucine	Leu
Methionine	Met
Asparagine	Asn
Proline	Pro
Glutamine	Gln
Arginine	Arg
Serine	Ser
Threonine	Thr
Valine	Val
Tryptophan	Trp
Tyrosine	Tyr

CHAPTER 1

INTRODUCTION

Biotechnology is defined as ‘the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or nonliving materials for the production of knowledge, goods and services’ (Zechendorf, 2002). This definition is made by considering both traditional and modern biotechnology processes. Traditional processes such as fermentation date back to around 1500 B.C. On the other hand, developments in modern biotechnological processes such as DNA, protein or cell-based technologies have been made over the last two decades.

Modern biotechnology is one of the key enabling technologies of the 21st century owing to its wide range of applications in health care, agriculture and industry. Major application in health is the production of therapeutic products. Human genome sequence data is used for the identification of these products. Then, by the advances in genetic engineering, living organisms such as plant and animal cells, bacteria, viruses and yeasts are altered to produce therapeutic products for human use. These organisms in which the bioconversion processes occur can be regarded as mini-production plants based on the metabolism of the organism. By the advances in biochemical engineering, fermentation processes have become a fully controlled and highly efficient modern process group. Therefore, the research related with therapeutic biomolecule production is focused on enhancement of the production process by considering the metabolic reactions. That leads to evolution of metabolic engineering, ‘the targeted and purposeful alteration of metabolic pathways found in an

organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly' (Lessard, 1996).

Since the genomes of most of the industrial organisms have already been sequenced, these organisms can be optimized in order to achieve more efficient, cheaper production processes with higher yields by using genetic, protein and metabolic pathway engineering tools (Lessard, 1996).

Currently, owing to its wide range of applications, the biotechnology is playing an important role in economic development. According to a report released by Ernst&Young, revenues of the world's publicly-traded biotech companies topped $70 billion in 2006 growing 14% compared to 2005 (http://www.reuters.com/article/idUS L1534079120070415). Big pharmaceutical companies forecast about 60% of this revenue growth to come from biologic products such as antibodies, hormones and enzymes (http://about.datamonitor.com/media/pr/biotechnology).

Hormones are an important group of industrial biotechnological products. They are substances, usually peptide or steroid, produced by one tissue and conveyed by the bloodstream to another to affect physiological activity, such as growth and metabolism (King, 2006). Human growth hormone (hGH), a 191-amino acid poly-peptide hormone, is secreted by the anterior pituitary gland. It has many important functions on the human body, namely stimulation of body growth, and immune system, the strengthening bones and tendons, repair of damaged cells, induction of protein synthesis, decrease in protein degradation, and increase in glucose transport (King, 2006). hGH is secreted at its highest level during the youth. After this period, its secretion starts to decline. In children, its deficiency causes short height and growth, while in adults it leads to lack of energy, strength, and bone mass (Molitch, 2006). Deficiency can be overcome by the hGH administration. hGH is also used in treatments of some diseases unrelated to its deficiency. It can be used to treat diseases causing shortness, such as chronic renal failure, idiopathic short stature, intrauterine growth retardation, Prader-Willi syndrome, and Turner syndrome; to maintain muscle

mass wasted in AIDS patients; to reverse the effects of aging; to enhance weight loss in obesity (Liu, 2007; King, 2006). Therefore, producing pure hGH is a very vital process.

Prior to development of recombinant DNA technology, hGH was extracted from the pituitary glands of cadavers. However, by this way, to achieve a wholly synthetic hGH and unlimited supply of it were not possible (Maybe, 1984). Furthermore, some unusual cases were observed in people received this cadaver-derived hGH (Gardner, 2007). Then, human growth hormone was produced using the recombinant DNA technology by Genentech in 1981. In 1985, recombinant hGH (rhGH) replaced cadaver-derived hGH for therapeutic use in the world. As of 2010, manufacturers and brands of rhGH approved by the U.S. Food and Drug Administration (FDA) are shown in the Table 1.1. Global sales of hGH reached $2.7 billion in 2008 (Competitor Analysis Series).

In hormone production by bioprocesses, there are some crucial factors that should be taken into account to increase the yield and to ensure the product quality. These are mainly the selection of host microorganism and the bioreactor operation conditions, i.e., medium composition, pH, temperature, oxygen transfer rate, operation mode. The host microorganism is selected considering its potential to efficiently produce the product of interest. Generally, microorganisms that are well characterized, and have the known genetic structure are preferred (Rumbold, 2000). Thus, since its genome was sequenced before the others, *Escherichia coli* (bacteria) become the most widely used microorganism in recombinant protein production. However, it has important disadvantages; for instance, lack of glycosylation, and other post-translational modifications, insoluble and unstable protein secretion and lack of avility to secrete large amount of protein to the extracellular medium, as well as its advantages such as plasmid stability, high yield of vector DNA (Rumbold, 2000). Hence, bacteria of the genus *Bacillus*, which has the ability to secrete large quantities of protein directly into the medium, has started to be more pronounced for industrial fermentation processes (Arbige et al., 1993).

Table 1 The recombinant hGH brands and manufacturers(http://www.somatropin.net)

Manufacturers	Brands
Genentech	Nutropin, Genotropin
Eli Lilly	Humatrope
Novo Nordisk	Norditropin
Serono	Saizen, Serostim, Zorbtive
NeoGenica	Hypertropin
GeneScience	Jintropin

Bacillus subtilis is the mostly used species of the genus *Bacillus* as host for a variety of reasons, including its high growth rates; short fermentation times; its capacity to secrete proteins into extracellular medium; the GRAS (generally regarded as safe) status with the FDA; and its known biochemistry, physiology, and genetics (Debabov, 1982). The major problem in the recombinant protein production by *B. subtilis* is the synthesis of large amount of extracellular proteases degrading proteins in the medium (Doi et al., 1984; Wang et al., 1988). Hence, various protease-knockout strains were constructed. Another problem is the lack of strong and controllable promoters. Therefore, replacement of the foreign gene promoter with naturally expressed *B. subtilis* promoters is very important (Yoneda, 1980).

In the first studies on rhGH production, *E. coli* was used (Gray et al., 1985; Becker and Hsiung, 1986; Chang et al., 1987; Kato et al., 1987). Owing to the advantages of *Bacillus* species for recombinant protein production, the number of studies in which the genus *Bacillus* is preferred has increased (Franchi et al., 1991; Kajino et al., 1997).

In the last study on rhGH production by *B. subtilis*, a structurally stable expression vector has been developed (Özdamar et al., 2009). They cloned the hybrid-gene of two DNA fragments, i.e., signal (pre-) DNA sequence of *B. licheniformis*

serine alkaline protease (SAP) gene (*sub*C) and cDNA encoding the hGH into pMK4 plasmid and made it expressed under the deg-promoter in *B. subtilis* host. Thereafter, the fermentation characteristics were investigated using *B.subtilis* BGSC-1A751 (npr^-, apr^-) carrying pMK4*::pre(subC)::hGH* in a defined medium. In this expression system, rhGH was produced mimicking the extracellular serine alkaline protease (subtilisin) synthesis.

The *aprE* gene of *B. subtilis* codes for the serine alkaline protease and its expression is regulated by a complex network of activator and repressor proteins such as AbrB, DegQ, DegS, DegU, ScoC, SinI, SinR, and Spo0A (Yang et al., 1986; Kunst et al., 1988; Gaur et al., 1991; Bai et al., 1992; Mandic-Mulec et al., 1992; Bai et al., 1993; Olmos et al., 1996). In order to understand the effect of the corresponding genes, i.e., *abrB, aprE, degQ, degS, degU, scoC, sinI, sinR* and *spo0A*, on the rhGH production (via subtilisin expression), recombinant strains lacking of these genes of *B. subtilis* should be constructed. Then, expression system of the vector can be better understood and developed to get higher yields of rhGH.

Hence, in this study, the effects of the regulation genes of *aprE* on the rhGH production were investigated by performing fermentation experiments with nine selected *B. subtilis* knockout strains, i.e., *degQ*-, *degU*-, *degS*-, *sinI*-, *sinR*-, *scoC*-, *abrB*-, *spo0A*-, *aprE*-, carrying pMK4::*pre*(*sub*C)::*hGH* plasmid. Thereafter, with recombinant *Bacillus* (r-*Bacillus*) strains having the highest rhGH production (*degU*-, *scoC*-, *sinR*-, *aprE*-), pilot-scale bioreactor experiments were performed in order to determine fermentation and oxygen transfer characteristics.

CHAPTER 2

LITERATURE SURVEY

2.1 Hormones

2.1.1 General Characteristics

Hormones are the chemical messengers of the body in the form of complex nature. They are produced in one part or organ of the body in order to stimulate or regulate the activity of an organ or a part of the body (Nussey and Whitehead, 2001).

The majority of the hormones are synthesized by glands of the endocrine system such as adrenal, pituitary, pancreas, pineal, thyroid, thymus glands, and the ovaries or testes. These glands secrete hormones directly into the bloodstream to be carried to the target organ. However, not all hormones are produced by endocrine glands. For instance, the mucous membranes of the small intestine secrete hormones that stimulate secretion of digestive juices from the pancreas (Pincus et al., 1956). Other hormones are produced in the placenta to regulate fetal development.

All hormones have some general characteristics as follows: They are secreted as the body needs them and not stored. Their secretion is regulated by other hormones, neurotransmitters, and a negative feedback system. They are transported by blood with small transmission rate, but their duration of action is mostly long-lasting. They can either be antagonistic or synergistic to another hormone (Schwyzer, 1964).

2.1.2 The Effects of Hormones

Although the function of hormones is generally related to homeostasis such as regulation of blood sugar, they also control growth and development, reproduction, influence behavior, metabolism, apoptosis (programmed cell death), immune system and hunger cravings. Also, they can regulate secretion of other hormones (Becker et al., 2001; Nussey and Whitehead, 2001)

2.1.3 Classification

According to their chemical nature, hormones are divided into three groups; namely, amino acid derivatives, peptide hormones, and lipid derivatives. The first class of hormones (amino acid derivatives) is derivatives of tyrosine and tryptophan amino acids while the second one, peptide hormones, consists of chains of amino acids. If peptide hormone is composed of hundreds of amino acids, they are called protein hormones; on the other hand, if they contain carbohydrate side chains, they are named as glycoprotein hormones. Hormones of this group regulate sleep and sugar concentration in the blood. Lipid derived hormones contain lipids and the main classes of it is steroid hormones. Steroids are secreted by the adrenal cortex and gonads. Most of them are sex hormones having functions on maturation and fertility. They derive from cholesterol and have the basic ring structure. Examples for each class of hormones, amino acid derivatives, peptide hormones and lipid derivatives, are epinephrine, dopamine, melatonin; insulin, growth hormones; and calcitriol, prostaglandins, respectively (Harrow and Sherwin, 1934; Jameson and De Groot, 2010).

2.2 Human Growth Hormone (hGH)

2.2.1 Chemical and Physical Properties

hGH, which is also known as somatotropin, is synthesized by the somatotropic cells within the pituitary glands on the frontal lobe of brain. It is a 191-amino acid, polypeptide hormone with an isoelectric point (pI) of 5.2 (Hummel et al., 1975). The empirical formula of the hGH is $C_{990}H_{1529}N_{262}O_{300}S_7$.

The gene encoding the DNA is localized on the chromosome 17 (Harper et al., 1982). Four cysteine molecules, which are found within the locations enumerated as 53, 165, 182 and 189 on the polymeric structure of hGH, form two disulfide bond within the active molecule (Cys^{53}-Cys^{165}-*large loop*- and Cys^{182}-Cys^{189}-*small loop*-) (Brems et al., 1990). This structure is shown on the Figure 2.1. The secondary structure of the hGH, single-chain polypeptide, is 55% α-helical (Farmer et al., 1976). Tertiary structure of the hGH is given in Figure 2.2.

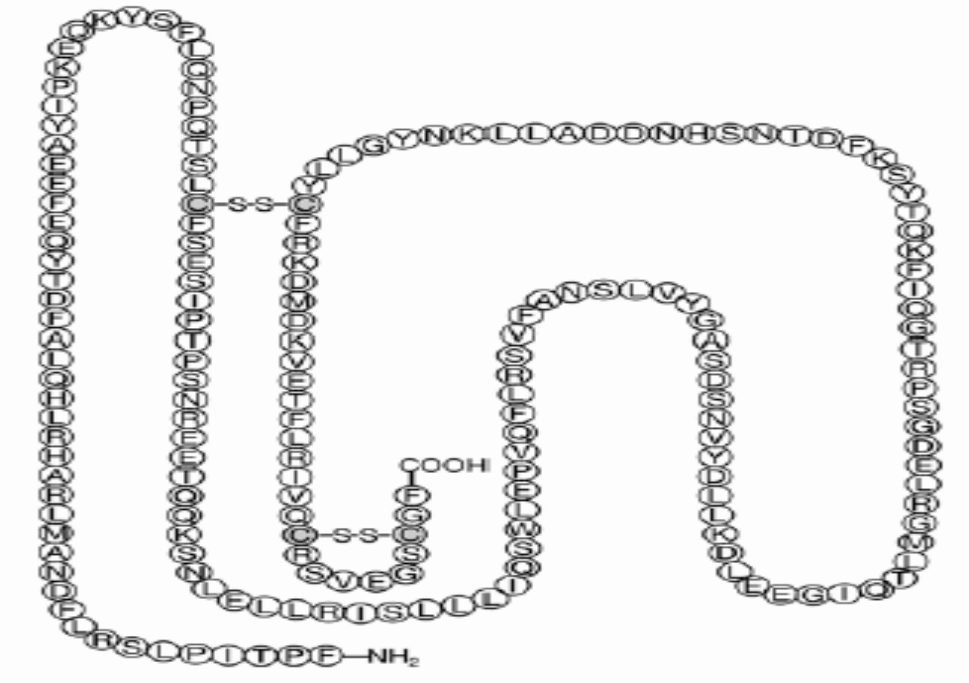


Figure 2.1 Amino acid sequence of 22 kDa-human growth hormone (http://dailymed. nlm.nih.gov)

There are five growth hormone genes on the chromosome 17. Two of them, namely *hGH*-N (normal) and *hGH*-V (variant) have the same structure with only difference in the location of the 13 different amino acids on the poly-peptide chain (Chen et al., 1989). *hGH*-N gene is expressed on the pituitary glands, while *hGH*-V gene was expressed in the placenta (DeNoto et al., 1981). There are three forms of *hGH*-V gene having molecular weights of 22 kDa, 25 kDa, and 26 kDa; on the other hand, *hGH*-N gene has two isomers with molecular weights of 22kDa, and 20kDa. 90% of the total hGH secreted by the pituitary glands is the larger isomer having the molecular weight of 22kDa, and the rest is the smaller one (Hattori et al., 1999). The *hGH*-N with 22 kDa weight is needed in the body for growth and development;

whereas the effect of the other isomer on the body is not known. Secretion of hGH is regulated by two hypothalamic hormones: growth hormone-releasing hormone (GHRH) and somatostatin. While the former stimulate the hGH secretion, the latter inhibits it (DeNoto et al., 1981).

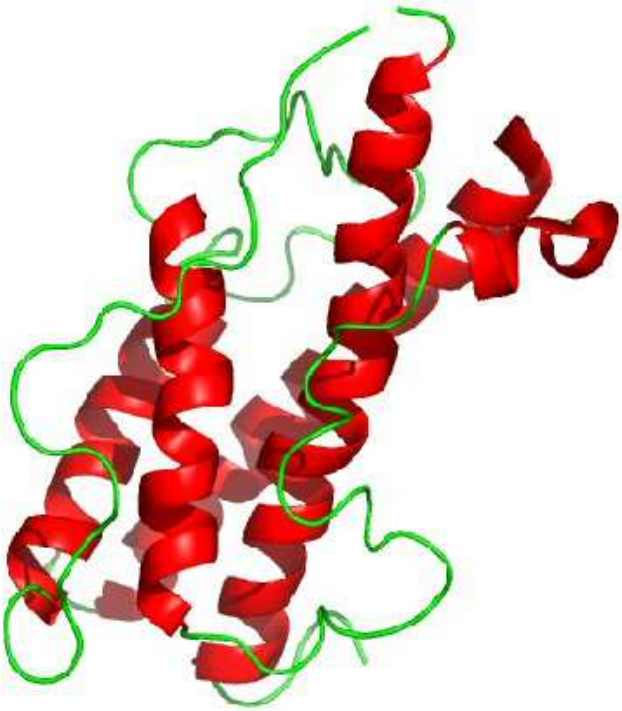


Figure 2.2 Tertiary structure of hGH (http://chemistry.umeche.maine.edu)

2.2.2 Biological Properties and Therapeutic Use

The main biological function of the hGH is signaling the growth of skeleton and cartilage. hGH can affect the target tissue directly or indirectly.

hGH stimulate protein metabolism by delaying the amino acid catabolism; lipid metabolism by accelerating the fatty acid oxidation; carbohydrate metabolism by its insulin-antagonistic effect (Chawla et al., 1983).

hGH secretion increases until youth, then it starts to decrease. Both deficiency and over-secretion of hGH result in serious diseases. Lack of hGH secretion in children leads to short height and slow growth, and in adults results in reduction in energy, strength and bone mass (Molitch, 2006). This deficiency can be overcome by therapeutic use of hGH. It has also healing effects on the bone fractures, skin burns, ulcer, wounds, chronic renal failure, Prader-Willi syndrome, Turner syndrome, obesity and muscle mass reduction (Davidson, 1987; King, 2006; Liu et al., 2007).

Most of the indication of aging such as heart and vascular diseases, overweight, the hair whitening, and wrinkling of the skin is caused by the reduction in secretion of hGH in the body. Hence, hGH is considered as anti-aging medicine, also (Liu et al., 2007).

2.2.3 Manufacture

Owing to its wide range of effects on the body, manufacture of pure hGH is a vital process. Firstly, hGH was supplied from the bovine pituitary glands. Then, it was found that hGH isolated from animals is ineffective in human body. Hence, in 1956, first cadaver-derived hGH was prepared by isolating the hGH from the pituitary glands of the cadavers (Li and Papkoff, 1956). To achieve high yield and quality, several methods have been developed for isolation process. However, not only cadaver-derived hGH has limited supply but also it has vital side effects on the body. Unusual cases such as fatal Creutfeldt Jacob disease, neoplasia was observed in

people treated with it (Croes et al., 2002). Therefore, by the invention of genetic engineering techniques, studies on the production of recombinant hGH gathered pace.

The gene encoding the hGH was cloned firstly by Goeddel et al. (1979). Then, by using the biochemical engineering techniques Genentech produced the recombinant hGH under the brand name Protropin by *E.coli* in 1985. This hGH has 192 amino acids with methionyl at the N-terminus although the natural hGH consists of 191 amino acids. This hGH become the first recombinant hGH approved by FDA. Then, in 1986, Eli Lilly produced Humatrope, biosynthetic hGH containing 191 amino acids without methionyl initiation. This hGH was approved by FDA, also since it has different biological effect as well as a different structure from Protropin.

E. coli was used in the first studies on the recombinant hGH production (Gray et al., 1985; Becker and Hsiung, 1986; Chang et al., 1987; Kato et al., 1987). However, expression of recombinant proteins by *E. coli* has some disadvantages such as inability to secrete proteins into extracellular medium. Since the hGH excreted to the extracellular medium has the same characteristics with the natural one, the number of studies in which the genus *Bacillus* is preferred for the hGH production has increased (Franchi et al., 1991; Kajino et al., 1997).

2.3 Bioprocess Characteristics

Biosynthetic production is achieved by metabolic reactions in microorganism driven by addition of suitable carbon source (methanol, glucose, sorbitol, etc.) to the medium. To increase the production capacity, microorganisms that will be used as host for the production, are mutated or modified genetically. Another bioprocess characteristic is bioreactor operating parameters. For biochemical production, fermenter systems that are not complex in structure are used and bioreactor parameters are easy to change and control. During fermentation, by-products can be produced in large amounts as well as the desired product. Hence, to decrease the by-product formation and increase the selectivity and yield of the recombinant product,

first of all, bottlenecks of the process should be investigated by using metabolic engineering techniques. Then, with the microorganisms that are constructed considering the genetic and metabolic engineering principles, fermenter operating parameters are optimized.

2.3.1 Microorganism

In order to produce the protein product at high quality and selectivity in biotechnological processes, the first factor that must be considered is selection of host microorganism. A microorganism can produce different products, and different products can be produced by one microorganism. Therefore, a potential host should produce the desired product efficiently; secrete large amount of product; produce small amount of side products; be regarded as safe; and be genetically well characterized (Rumbold, 2009).

In fermentation processes, medium compositions and operating conditions can be modified to increase the selectivity and yield; however production is limited by the capacity of microorganism. Nowadays, with genetic engineering, mutation, and selection techniques, microorganisms having high yield and selectivity can be constructed.

Since hGH is a non-glycosylated protein, it has been mostly produced by prokaryotic expression systems. Since *E. coli* and *B. subtilis* are the microorganisms that have fulfilled all the above criteria, they are preferred in hGH production. Using *E. coli* in hGH production has some disadvantages. One of them is the synthesis of methionyl initiation at the start of the hGH (Glasbrenner, 1986). This disadvantage is overcome by enzymatic cleavage of the initiation (Becker and Hsiung, 1986; Hsiung et al., 1986). The other disadvantage is the intracellular expression of the hGH leading to difference from nature form of hGH. Therefore, periplasmic production of hGH was achieved (Chang et al., 1987; Kato et al., 1987; Castan et al., 2002; Soares et al., 2003).

On the other hand, by using *Bacillus* species for hGH production, high level extracellular production (40-240 g m^{-3}) was achieved (Nakayama et al., 1988; Franchi et al., 1991; Kajino et al., 1997; Şentürk, 2006; Yılmaz, 2008).

2.3.1.1 The Genus *Bacillus*

Bacillus is a gram-positive bacterium that is endospore-forming, obligate aerobe and rod-shaped (Claus and Berkelery, 1986). It has nontoxic cell wall, and the ability to secrete proteins to the extracellular medium. Guanine + Cytosine (GC) ratio of *Bacillus* is 32-62% showing the genetic heterogeneity of the genus even within the strains of a species (Holt, 1984). Some species can move with the flagella on their lateral side while some species cannot move. *Bacilli* are unicellular bacteria and proliferate by dividing into two. At harsh conditions, they form endospores that regain activity at suitable medium and conditions. They dissimilate the organic substrates by intensive respiration, intensive fermentation or both fermentation and respiration. Molecular oxygen acts as the terminal electron acceptor in respiration metabolism (Buchanan and Gibbson, 1974). Some of them are pathogenic and cause diseases producing toxins while some of them are listed as GRAS (generally regarded as safe) by FDA (Çalık, 1998). By the help of the genetic and biochemical data of *Bacillus* species, optimization for production could be easier. For instance, recently, the sequencing of 22 *Bacillus* strains has been completed and 32 sequence projects continue (http://www.ncbi.nlm.nih.gov/). This makes genome scale metabolic flux analysis possible for *Bacillus* strains.

The genus has the species that can be fermented in the acidic, neutral and alkaline conditions and extreme temperatures leading to production of various proteins with the desired properties such as pH, temperature (Schallmey et al., 2004). As compared to other microorganisms, they can be easily manipulated genetically, easily grow. Main products fermented by the species of *Bacillus* are hydrolytic

enzymes, antibiotics, insecticides, nucleosides, vitamins and other molecules (Arbige et al., 1993).

2.3.1.1.1 *Bacillus subtilis*

For research on *Bacillus*, *Bacillus subtilis* is used since its genetic, biochemical and physiologic properties are well-defined. It is regarded as a priority organism for gram-positive bacteria. It is used for expression of various foreign genes owing to its non-pathogenecity (Law et al., 2003). Despite having the potential to vie with *E. coli*, *B. subtilis* is not as popular as *E. coli* since change of microorganism in industrial processes takes a lot of time due to optimization problems (Westers et al., 2004).

As well as its advantages in terms of recombinant protein production, using *Bacillus* has four main disadvantages: lack of stable expression vectors, protease secretion (mostly alkaline and neutral), plasmid instability, and existence of malfolded proteins (Westers et al. 2004). However, recent efforts have overcome these problems for the specific products.

The first two drawbacks can be overcome by constructing foreign strong and controllable promoters and so expression vectors using genetic engineering techniques (Law et al., 2004; Morimoto et al., 2008). To decrease the protease activity degrading proteins in the medium, protease gene-deficient *Bacillus* strains are used. Also, protease inhibitors are added to the growth medium (Zweers et al., 2008). For example, by using *B. subtilis* WB800 that lacks eight extracellular protease genes, staphylokinase production was increased 8-fold (Ye et al., 1999). However, this also depends on the metabolic reactions, that is, not for all proteins, this protease gene-deficient strain increase production. Yılmaz (2008) performed hGH production by using *B. subtilis* WB700 (seven protease genes-deficient) and BGSC1A751 (*aprE* and *npr* genes-deficient) and with BGSC1A751 two fold-higher hGH was obtained than that with WB700.

Until the investigation of the rolling-circle mechanism of replication of *B. subtilis* plasmids, plasmids isolated from *S. aureus* were cloned to *B. subtilis* resulting in low transformation efficiency, and plasmid instability (Bron, 1990). In this mechanism, single stranded (ss) DNA intermediates are generated, and they are converted to duplex DNA molecules. If ssDNA molecules are not converted efficiently and accumulate, then plasmid instability is the case. However, single strand origins (SSOs) of *B. subtilis* plasmids are converted to duplex molecules owing to their potential to form stem-loop structure (Del Solar et al., 1987; Gruss et al., 1987; Boe et al., 1989; Meijer et al., 1995). Hence, with selection and characterization of functional plasmid-located SSOs, stable expression vectors can be constructed for *B. subtilis*. The lack of strong promoters in *B. subtilis* can be overcome by inserting foreign gene promoters to the expression vectors.

In the literature concerning hGH production, Nakayama et al. (1988), Franchi et al. (1991), Şentürk (2006), Yılmaz (2008) and Özdamar et al. (2009) used *B. subtilis*; whereas Kajino et al. (1997) used *B. brevis*.

2.3.1.2 Expression Vector for hGH Production

Expression and secretion of proteins in *B. subtilis* necessitate the N-terminal signal sequence precursors and strong promoters. Expression vectors are plasmids that introduce these precursors and promoters into the host strain by the ability to be replicated, inherited, and transcribed in it (Schallmey et al., 2004).

Plasmids are the DNA molecules containing the gene encoding the desired product, and a strong promoter enhancing the transcription of the gene of interest (Lipps, 2008). Therefore, selection of promoter and sequence precursors cloned to plasmids is very critical for efficient recombinant protein production by *B. subtilis*.

By the invention of recombinant DNA technology, creation of plasmids enhancing replication in *B. subtilis* gained importance. The first generation shuttle vectors are pMK3 and pMK4 (Sullivan et al., 1984). Creation of these plasmids is

regarded as the milestone in terms of recombinant protein production by gram positive bacteria. pMK4 become the most widely used plasmid for recombinant protein expression in *B. subtilis* and other gram positive bacteria. This plasmid is replicated by the rolling-circle mechanism without any stability problem proving the fact that pMK4 successfully supports replication of the desired product (Bron, 1990). The genetic and physical maps of these plasmids are given Figure 2.3.

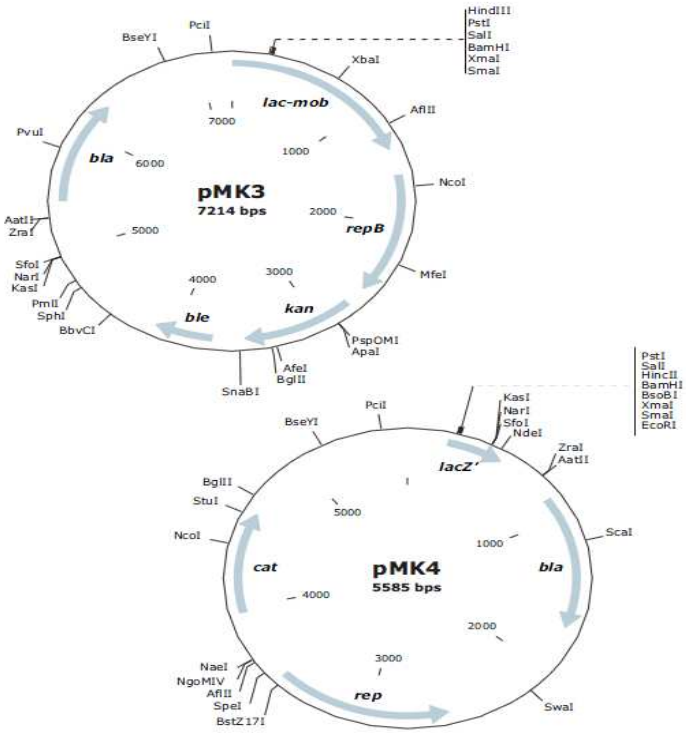


Figure 2.3 Genetic and physical maps of pMK3 and pMK4 (Sullivan et al., 1984)

Özdamar et al. (2009) constructed a highly stable expression vector for hGH production by *B. subtilis*. To make hGH secrete extracellularly, they fused the signal - *pre*(*subC*) sequence of extracellular serine alkaline protease (SAP) gene, which is isolated from *Bacillus licheniformis*, into pMK4 plasmid. After signal sequence, cDNA sequence of hGH gene was cloned to the plasmid. These genes on the plasmid are expressed under the deg promoter. Therefore, hGH is produced mimicking the SAP (subtilisin) synthesis.

2.3.1.3 Protein Expression and Secretion in *Bacillus*

Genes have to be expressed for protein synthesis. First step is transcription in which mRNA molecules complementary to DNA sequence of gene are produced. Then, these mRNA molecules are decoded in ribosome to form amino acid chains. In this step, amino acids are carried by the tRNAs. Peptide chain (protein) produced gains activity later. The basic mechanism of gene expression is given in Figure 2.4.

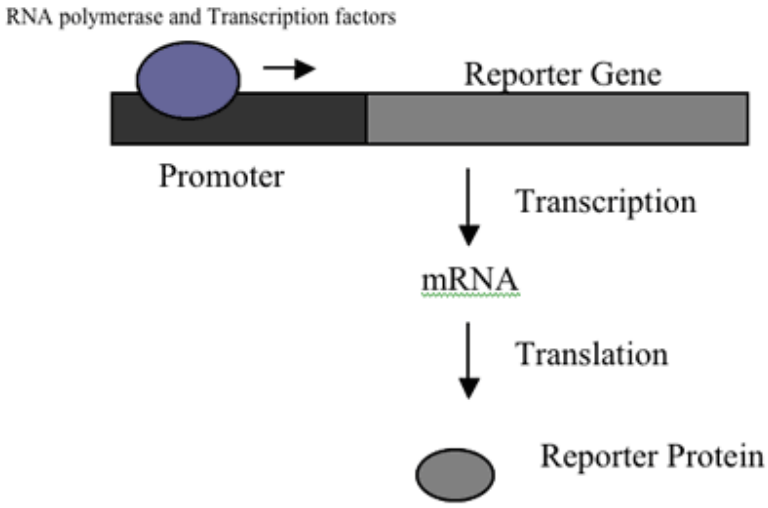


Figure 2.4 Gene expression mechanism (www.clas.ufl.edu/jur/200611/)

Proteins are synthesized firstly with signal sequences as preproteins. This signal peptide helps the differentiation of extracellular proteins from cytoplasmic proteins. That is, signal sequence directs the proteins to the secretion pathway. This is achieved by binding of signal peptide to membrane (Harwood et al., 1990).

The steps in protein secretion affecting the yield of production can be seen in Figure 2.5. Proteins do not gain 3-D form in the cytoplasm generally because chaperons that are soluble proteins prevent preproteins from folding to maintain them in suitable form for translocation. Translocation is the step in which protein is transported to the membrane or inside the membrane. To prevent folding of proteins is critical for secretion since if protein gains 3-D structure before translocation, protein is not secreted much then. Precursor is directed to its specific membrane by signal peptide or chaperons or both of them. Style of translocation to the other side of membrane is not known. Translocase which are membrane-protein complex, help the transfer of proteins through the endoplasmic reticulum membrane and cytoplasmic

membrane. Signal peptide is removed by a specific protease, or signal peptidase during the translocation process. Secreted proteins stay on the membrane until they fold. This folding process is carried out by specific chaperons (Harwood et al., 1990; Simonen and Palva, 1993). Thereafter, extracellular proteases attack the protein to degrade. Hence, protease deficient strains or inhibitors are used to decrease protease activity.

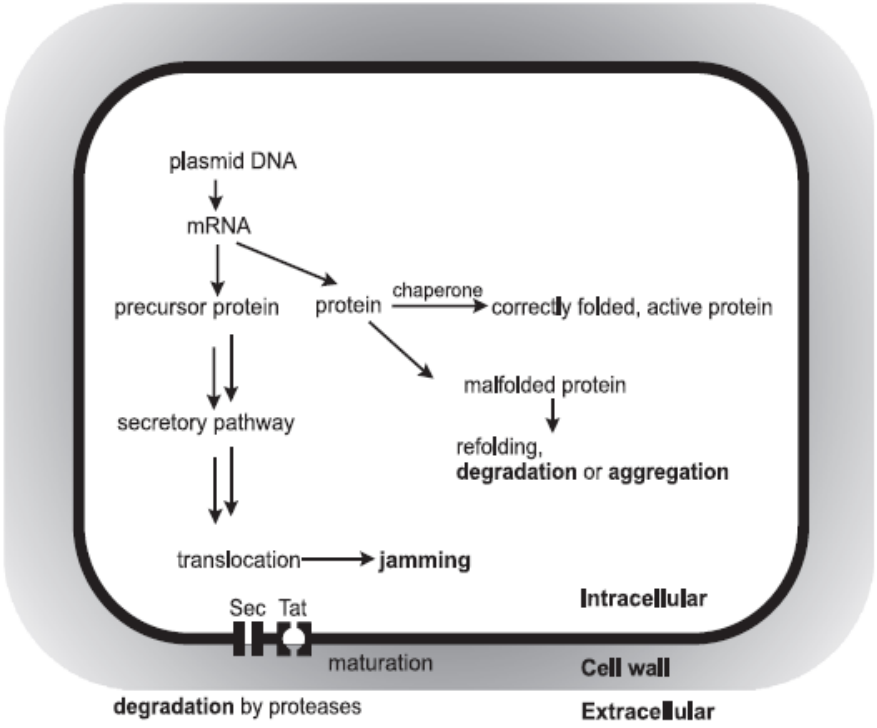


Figure 2.5 Schematic of steps that affect the yield of heterologous proteins (Westers et al., 2004)

2.3.1.4 Regulatory Gene Network of *aprE* Gene

Signal sequence of *subC* gene on the recombinant pMK4::*pre*(*subC*)::*hGH* plasmid mimics the extracellular subtilisin production in *B. subtilis*, and the subtilisin gene of *B. subtilis* is *aprE*. *aprE* has a complex regulation network with repressors and activators such as AbrB, ScoC, SinI; and DegQ, DegS, DegU, SinR, Spo0A, respectively (Gaur et al., 1991; Kallio et al., 1991; Smith, 1993; Ogura et al., 1994; Strauch, 1995; Olmos et al., 1997; Koide et al., 1999; Hata et al., 2001; Ogura et al., 2003; Ogura et al., 2004). These regulators are the product of the activated genes; for example, AbrB is the product of the *abr*B gene.

As can be seen from the Figure 2.6, *aprE* expression is controlled mainly by major routes of AbrB, DegU, ScoC, and SinR. These proteins affect the *aprE* by directly binding to it (Ogura et al., 2003). When DegU is phosphorylated by phosphate transfer from the DegS, it stimulates the expression of the *aprE* gene (Kunst et al., 1994). DegU-DegS regulation system affects the expression of *degQ* positively (Msadek et al., 1991). Other regulator is Spo0A, which has effects on the other regulators also, become active by phosphorylation when the cell enters stationary phase. This phosphorylated Spo0A represses the *abrB* leading to the activation of *aprE* and repression of *scoC* (Ferrari et al., 1986; Perego and Hoch, 1988; Strauch et al., 1989; Strauch, 1995).

Other regulator, ScoC regulates the *sinI* and *sinR* as well as the *aprE*. When *sinI* activates, protein encoded by it represses *sinR* by binding it (Gaur et al., 1988; Gaur et al., 1991; Bai et al., 1993). *sinI* is controlled by AbrB, ScoC, and Spo0A, and the SinR is expressed by inhibition of *sinI* through the activation of ScoC (Gaur et al., 1988; Bai et al., 1993; Shafikhani et al., 2002).

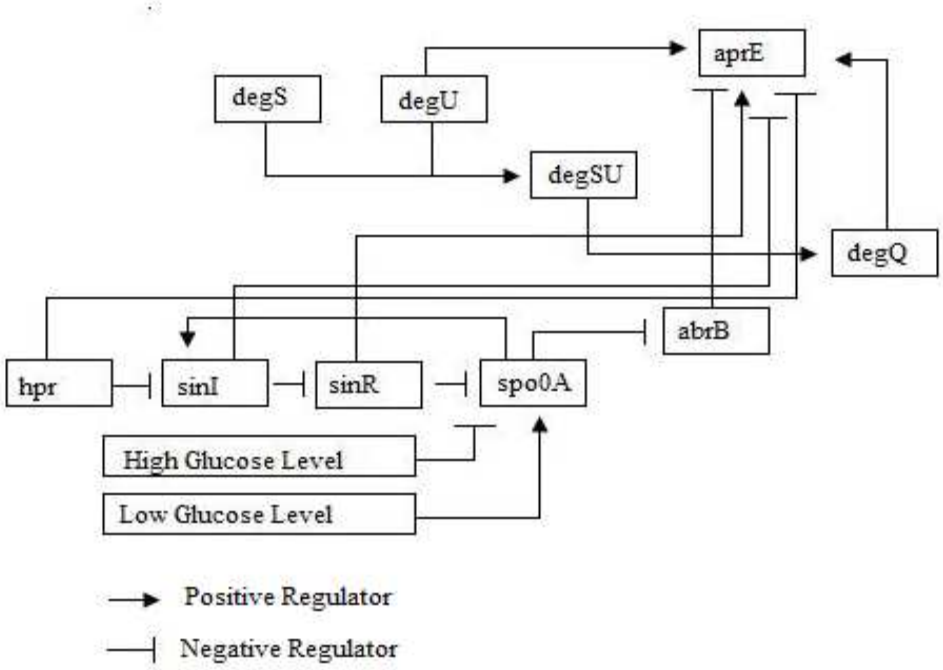


Figure 2.6 Regulatory gene network of *aprE* gene

Protein products of these regulatory genes (AbrB, DegQ, DegS, DegU, ScoC, SinI, SinR and Spo0A) are functional agents of the late-growth development (Table 2.1). Among these proteins, the ones whose function and control mechanism are well-characterized are AbrB, DegU, ScoC, SinR and Spo0A.

The presence of *degQ* gene results in a 70-fold increase of alkaline protease amount. The overproduction of DegQ causes elevation of *aprE* transcription (Shimotsu and Henner, 1986). Sites of DegQ action are in the -141 to -164 region of the *aprE* promoter (Msadek et al., 1991). Overproduction of DegQ necessitates DegS-DegU system. The data on DegQ shows that it may be a transcription factor. It is not sure since deletion of degQ gene has no apparent phenotype (Yang et al., 1986). *degQ*

gene is repressed by glucose in the medium, and activated by growth in poor carbon sources, by amino acid deprivation, and by phosphate starvation.

ScoC inactivates the protease production and sporulation. This protein product binds to the upstream regions of *nprE* and *aprE* (Smith, 1993). There are four binding sites on *aprE* promoter for ScoC binding. Absence of ScoC does not change the temporal expression of the genes that it represses.

AbrB represses *aprE*, *spoVG*, *spo0E*, and *spo0H* expressions. *abrB* also is repressed by itself (Strauch et al., 1989). AbrB protein binds to *aprE* promoter. AbrB activates *scoC* expression.

DegU protein has a major role in extracellular enzyme production control. Deletion of *degU* gene results in activation of *amyE*, *nprE*, and *aprE* genes. For DegS-DegU action, there are sites on *aprE* but these proteins do not interact directly with its target genes (Smith, 1993).

Spo0A is a late-growth function regulator. It inactivates abrB when phosphorylated, while it activates *spo0H*, *spo0E*, and *spoVG*. *spo0A* is essential for sporulation process (Hoch et al., 1985; Olmedo et al., 1990).

SinI protein controls the activity of SinR at a posttranslational level. It was shown that SinI has greatest similarity to SinR. SinI interacts with SinR before the SinR binds to *aprE*. SinR is a repressor of sporulation (Smith, 1993).

Direct regulators of *aprE* expression are ScoC, SinR, and AbrB. Respression of these proteins does not alter temporal regulation of *aprE*. Also, DegQ and DegU do not change the timing of *aprE* transcription (Smith, 1993).

Since these genes affect the *aprE* expression, they can also affect recombinant hGH (rhGH) production prominently. Due to this complex regulation system, not only the repressor of the *aprE*, but also the activators of it may cause enhancement in rhGH production. In order to understand the effect of these genes on the rhGH production (via vector mimicking subtilisin expression), recombinant strains lacking of these genes (knockout strains) of *B. subtilis* should be constructed. Then, expression system of the vector can be better understood and further developed to get higher yields of

rhGH by metabolic flux analysis (MFA). Also, by MFA, bottlenecks of the process can be determined and then multiple gene deletions can be performed from the selected knockout strains in different combinations.

Table 2.1 Functions of the regulatory genes of *aprE* (Kunst et al., 1974; Klier et al., 1987; Gaur et al., 1988; Henner[a,b] et al., 1988; Dubnau et al., 1991)

Gene	**Gene Products (Proteins)**	**Functions of the Protein Produced**
abrB	AbrB	essential for competence, control of growth development
degQ	DegQ	expression of intracellular protease enzymes and extracellular enzymes
degS	DegS	control of extracellular enzyme synthesis, phosphorylation of *degU*
degU	DegU	essential for competence, and flagellar formation, degradation enzyme regulation
scoC	ScoC	control of proteases synthesis, motility and sporulation, regulation of late-growth function
sinI	SinI	antagonizes SinR function at level of protein
sinR	SinR	essential for competence, motility, and autolysin production
spo0A	Spo0A	sporulation, transcription regulator

2.3.1.5 Transformation of Plasmids to *B. subtilis* Strains

Recombinant plasmids constructed from the nature plasmids can be introduced to host strain via transformation. Transformation to *B. subtilis* strains was discovered in 1958 by Spizizen. There are several methods to transfer the gene to the bacteria; namely, natural transformation, and artificial transformation such as chemical transformation, protoplast fusion and electroporation (Ferrari and Hoch, 1989).

In natural transformation, bacteria take the foreign plasmid when they are mixed. If the plasmid contains the homolog regions to host chromosome, transformation frequency increases (Lopez et al., 1982). Plasmids can be transformed to *Bacillus* species by this method.

In chemical transformation, cells are processed to be competent before the introduction of plasmid. There are several protocols for this such as $CaCl_2$ method. In this method, the exact mechanism of how cell takes the plasmid has not known, yet.

In protoplast transformation, cell wall is hydrolyzed partially by lysozyme enzyme. Osmotic stabilizers in the medium prevent the cell lyzing (Chung et al., 1989). The frequency of this transformation is quite high (up to more than 10^7 transformants per μg of DNA).

The last method is the electroporation at which DNA is fused to the cell by applying electric voltage. The exact mechanism of how cell takes the plasmid has not known for this method, also (Glazer and Nikaido, 1995).

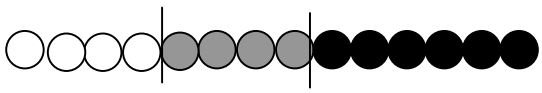
Selection of the method for transformation depends on several factors such as the host cell, and the plasmid size. For example, if the plasmid is large, electroporation could be suitable. Besides, electroporation is used in otherwise untransformable *Bacilli* and competence-deficient strains of *B. subtilis* (Masson et al., 1989).

After the transformation process, cells carrying the gene of interest should be selected by the appropriate methods. Since there would be many colonies after transformation, selective markers are required. The most common method for this

purpose is using the plasmid containing the antibiotic resistant gene. By this way, cells are grown on the solid medium with the antibiotic to which plasmid has resistant. At colonies that grow on this medium, transformation is completed successfully.

2.3.1.6 Extracellular Secretion of Serine Alkaline Protease in *B. subtilis*

Transport of extracellular proteins to the extracellular medium is a complex process. In the intracellular medium, serine alkaline protease consists of three parts: signal peptide, pro-peptide, and protein (SAP) as can be seen in the Figure 2.7. The critical functions of the signal peptide found on the N terminal of the extracellular enzymes are to achieve transfer of enzyme from cell membrane to the extracellular medium. Signal peptide is made up of three portions; namely, N terminal part, hydrophobic core, and C terminal part (Nielsen et al., 1997). At the N terminal, 2-7 amino acids are found, and the net average charge of these amino acids is 2-3.1. Positively charged N terminal interacts with the negatively charged cell wall. Hydrophobic core ends with either glycine or proline amino acids, and consists of neutral amino acids such as leucine, isoleucine, valine, alanine, and phenylalanine. The function of this part is interaction with the cell membrane and initiation of translocation. The last part, C terminal, contains the polar amino acids (Deane et al., 1989; Milton et al., 1992).



Signal peptide *Pro- peptide* *SAP*

Figure 2.7 Schematic representation of SAP inside the cell

Signal peptide is separated from the enzyme during the translocation or just after the translocation. This process is shown in the Figure 2.8.

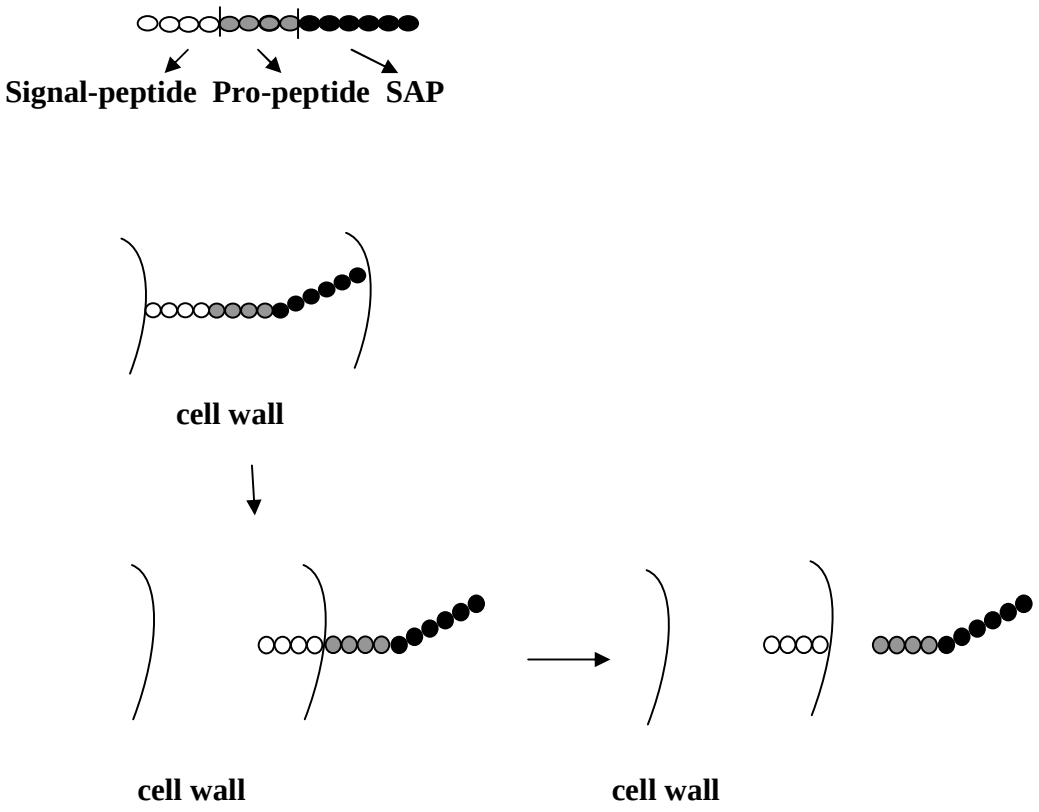


Figure 2.8 Transport of SAP to outside the cell

At the structure of SAP, there is also propeptide between the signal peptide and enzyme. This part is found only in gram positive bacteria and contains mostly charged amino acids. It has many functions on the protein structure such as to increase solubility of protein by interaction with cations on the cell wall, to form the 3-D

structure of proteins and to prevent the proteolytic hydrolysis of N terminal of the protein (Nagarajan, 1993). Signal peptide is cleaved from SAP by signal peptidase, while the cleavage of propeptide from enzyme is a much more complex mechanism (Markanyan et al., 1996).

2.3.2 Metabolic Engineering

Production in biotechnological processes is achieved by consecutive and parallel reactions within the host cell. Bioreactions took place inside the cell and the bioreaction pathways showing the interaction between these reactions are called the *metabolic pathways*. On the other hand, metabolic engineering is defined as the modification of the bioreactions by genetic engineering techniques to increase the yield and selectivity of the desired product or the alteration of the cell properties by addition of new reactions (Stephanopoulos, 1998). Mainly, it aims to enhance biomolecule production by analyzing and designing the metabolic reaction pathways. By the developments in the molecular biology techniques, analytical methods and mathematical tools in the last decade, metabolic engineering gains importance. It is used for the enhancement of the biochemical product, new chemicals, and chiral components production; and the metabolic analysis of tissue and organs for medical purposes (Stephanopoulos et al., 1998).

To apply metabolic engineering tools to the bioprocess, functions and the structure of the host cell should be known. Growth and protein production are also the consequences of these reactions like many other metabolites and intermediates. These reactions include reactions forming metabolites by breakdown of the substrate into smaller molecules; reactions in which big molecules are formed by polymerization of small molecules; reactions leading to organelle formation by assembling of macromolecules; and reactions resulting in transfer of substrate and metabolites from the cell into the outside medium, or vice versa. The required Gibbs free energy for these reactions is supplied again by these reactions (Nielsen and Villadsen, 1994).

Cells can be regarded as “micro-bioreactors” owing to more than 1000 reactions catalyzed by many different enzymes (Çalık et al., 1999). All these reactions took place inside the cell are called metabolism (Bailey and Ollis, 1986). There are four main functions of the cell metabolism (Lehninger, 1979):

- Intracellular energy formation by using inputs with high chemical energy or photosynthesis, and conversion of energy from different types to the required type,
- Conversion of input molecules to intermediates of the macromolecules,
- Synthesis of macromolecules by combining monomers,
- Synthesis and breakdown of biomolecules which are vital for cell functions.

Metabolism divides into two parts; catabolism, and anabolism. The phase of the cell in which conversion of carbohydrates, proteins and lipids found in the surrounding of cell to amino acids, organic acids, CO_2, ammonia, or urea; and the production of free energy needed for anabolism take place is called catabolism. On the other hand, anabolism is biosynthetic phase of the cell in which macromolecules such as polysaccharides, nucleic acids, lipids are synthesized from the monomers by using energy formed during the catabolism. Compounds that involve in these reactions are called *metabolites*, and the pathways that they form are named *metabolic pathways*. Catabolism and anabolism go hand-in-hand; even they can occur at the same time. The main metabolic pathways are glycolysis pathway, glyconeogenesis pathway, TCA (tricarboxylic acid) cycle, and pentose phosphate pathway (PPP) (Lehninger, 1979; Yang et al., 1998). Schematic of the main reactions in a bacterial cell is given in Figure 2.9.

a. Catabolic reactions

Energy source used mostly in cell growth is provided from sugars. These sugars turn into metabolic substances such as lactic acid, CO_2, and ethanol, and during this transformation, ATP, NADPH, and NADH are formed. NADH and NADPH are both cofactors; however, NADH is used in catabolic reactions like oxidative phosphorylation, whereas NADPH is used in anabolic reactions. When sugars enter the cell, they form G6P or F6P, and these intermediates are catabolized by entering glycolysis pathway, PPP, and TCA cycle, in sequence (Nielsen and Villadsen, 1994).

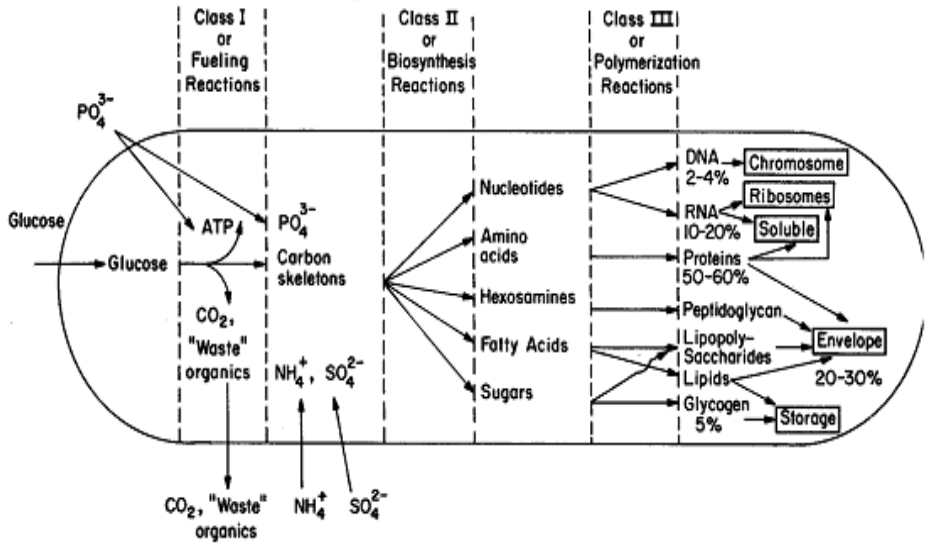


Figure 2.9 Schematic diagram of reactions in a bacterial cell (Lehninger, 1979)

Glycolysis pathway (Figure 2.10) is the first reaction system of the carbon catabolism in which glucose is turned into pyruvate and ATP. Besides, in this pathway, metabolites that are the first inputs of TCA, some amino acids and PPP are formed (Lehninger et al., 1993).

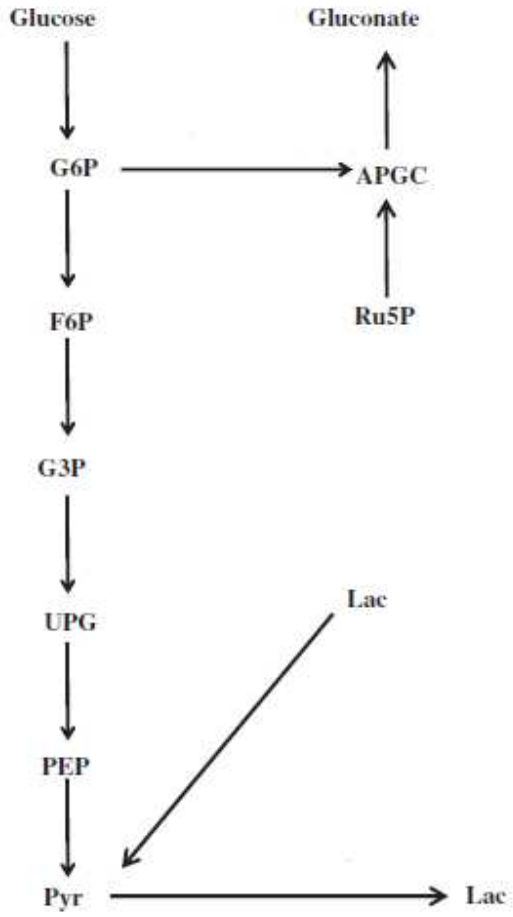


Figure 2.10 Schematic of glycolysis pathway

In aerobic processes, step after glycolysis pathway is acetyl coenzyme A (Acetyl-CoA) formation reaction by oxidative decarboxylation of pyruvate, and then this Acetyl-CoA enters TCA cycle (Figure 2.11), one of steps of the carbon catabolism, and could turn into CO_2 and H_2O completely. In TCA cycle, one mole ATP, four moles NADH and one mole $FADH_2$ are produced per mole of oxidized pyruvate. NAD^+ and FAD^+ used in pyruvate oxidation are regenerated from NADH and $FADH_2$. NADH and $FADH_2$, electron carriers, are formed in respiration cycle; therefore, this oxidation process containing free oxygen takes place only in aerobic microorganisms (Nielsen and Villadsen, 1994). During TCA cycle, also metabolites required for the synthesis of the aspartic acid, and glutaric acid group amino acids are produced.

During another reaction network of the carbon catabolism, PPP, NADPH (electron carrier of biochemical reactions) is formed. Furthermore, ribose 5-phosphate and erythrose 4-phosphate, which are needed for the synthesis of purine and pyrimidine nucleotides, are produced in this pathway (Nielsen and Villadsen, 1994).

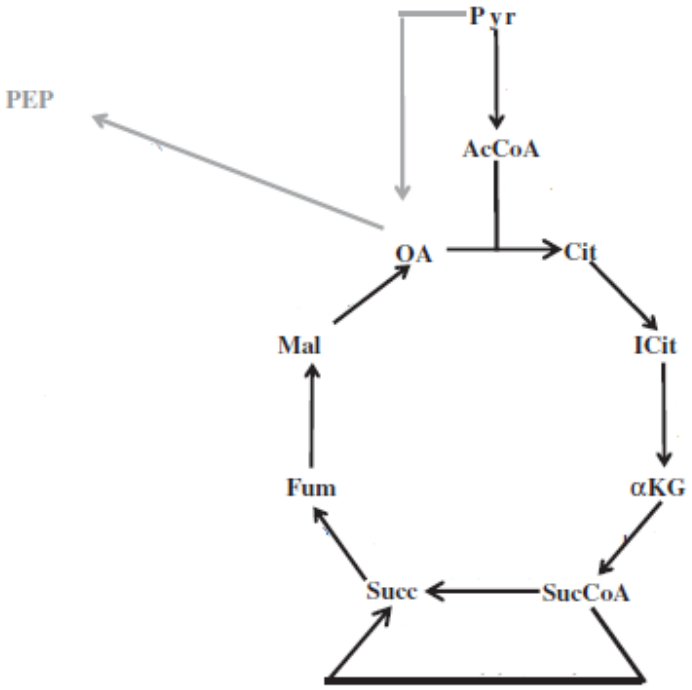


Figure 2.11 Schematic of TCA cycle

b. Anabolic reactions

90% of the cell consists of the macromolecules such as proteins, lipids, carbohydrates, RNA. These molecules are synthesized by consecutive polymerization reactions, and these reactions are known as anabolic reactions (Yang et al., 1998).

Almost 70% of the energy produced in the cell is used in the protein synthesis stating that protein synthesis needs large amount of energy (Nelson and Cox, 2005). This energy is supplied by the breakage of chemical bonds during carbon catabolism. Redox reactions of energy carrier molecules, ATP and NADPH, are shown in Figure 2.12.

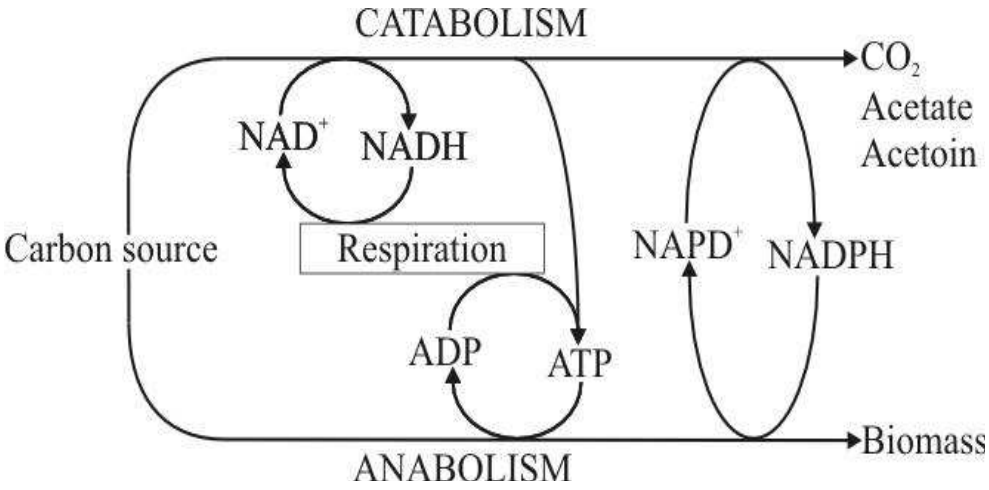


Figure 2.12 Link of redox cofactors NADH and NADPH with catabolism and anabolism.

2.3.2.1 Metabolic Flux Analysis (MFA)

In order to investigate the product distribution during growth, product and side-product formation in bioreactor and to develop the bioprocess, reactions that take place within the cell and the rates of these reactions have to be known. For this purpose, rate limiting reactions should be determined. Knowing the rate of the reactions is important for determination of pathway splits and alternative pathways; calculation of extracellular fluxes and maximum theoretical yield; development of growth medium and bioreactor operation conditions (Çalık, 1998; Çalık and Özdamar, 2002). The reaction map of *B. subtilis* is shown in the Figure 2.13 (Özdamar et al., 2010).

After determination of metabolic reactions, for all substances mass balance equations are written. The accumulation rate of the i-th substance is equalized to

algebraic summation of rate of all reactions in which this compound is consumed or produced, and transportation rate of this compound:

$$r_{Ri}V + r_{Ti}V = \frac{d(C_iV)}{dt} \tag{2.1}$$

where r_{Ri} is the net reaction rate with respect to i-th substance and r_{Ti} is the net transportation rate of i-th compound. This differential equation system can be defined by vector differential equations:

$$A * r(t) = c(t) \tag{2.2}$$

where A is the stoichiometric coefficient matrix of mxn (m=number of reactions, n=number of substances) reaction system, r(t) is the nx1 reaction rate vector and c(t) is the nx1 accumulation rate vector.

$$c(t) = c_1(t) + c_2(t) \tag{2.3}$$

$$c(t) = c_1(t) \tag{2.4}$$

$c_1(t)$ and $c_2(t)$ are extracellular and intracellular metabolite accumulation rate vectors, respectively. The accumulation and consumption rates of the extracellular metabolites are found by the slope of the concentration vs. time profiles of the metabolites. The phase at which metabolic flux analysis is performed is assumed as steady-state condition; therefore, equation 2.3 is simplified to 2.4 by taking $c_2(t)$ as 0.

There are three options for the solution of this vector system (Çalık and Özdamar, 2002):

- If m is equal to n, then there is only one solution.
- If m is greater than n, there is one solution and almost exact solution is attained.
- If m is less than n, mathematical model can be solved by optimization. For this purpose, an objective function (Z) is formed.

$$Z = \sum \alpha_i r_i \tag{2.5}$$

where α_i is the stoichiometic coefficient for i-th component.

By minimizing or maximizing this objective function, equations system can be solved. Variables of this mathematical model are metabolic fluxes in the unit of mmol g^{-1} CDW h^{-1}. Fluxes are found by dividing the slope of metabolite concentration vs. time graph to cell concentration. Cell growth rate, r_X (g CDW g^{-1} CDW h^{-1}) is found by dividing the cell growth rate per unit volume of bioreactor to cell concentration (C_X) (Çalık, 1998). For MFA, process is divided into periods considering the drastic changes in cell, product or side-product concentrations. Then, at times when these changes are observed, fluxes are calculated and so analysis is performed.

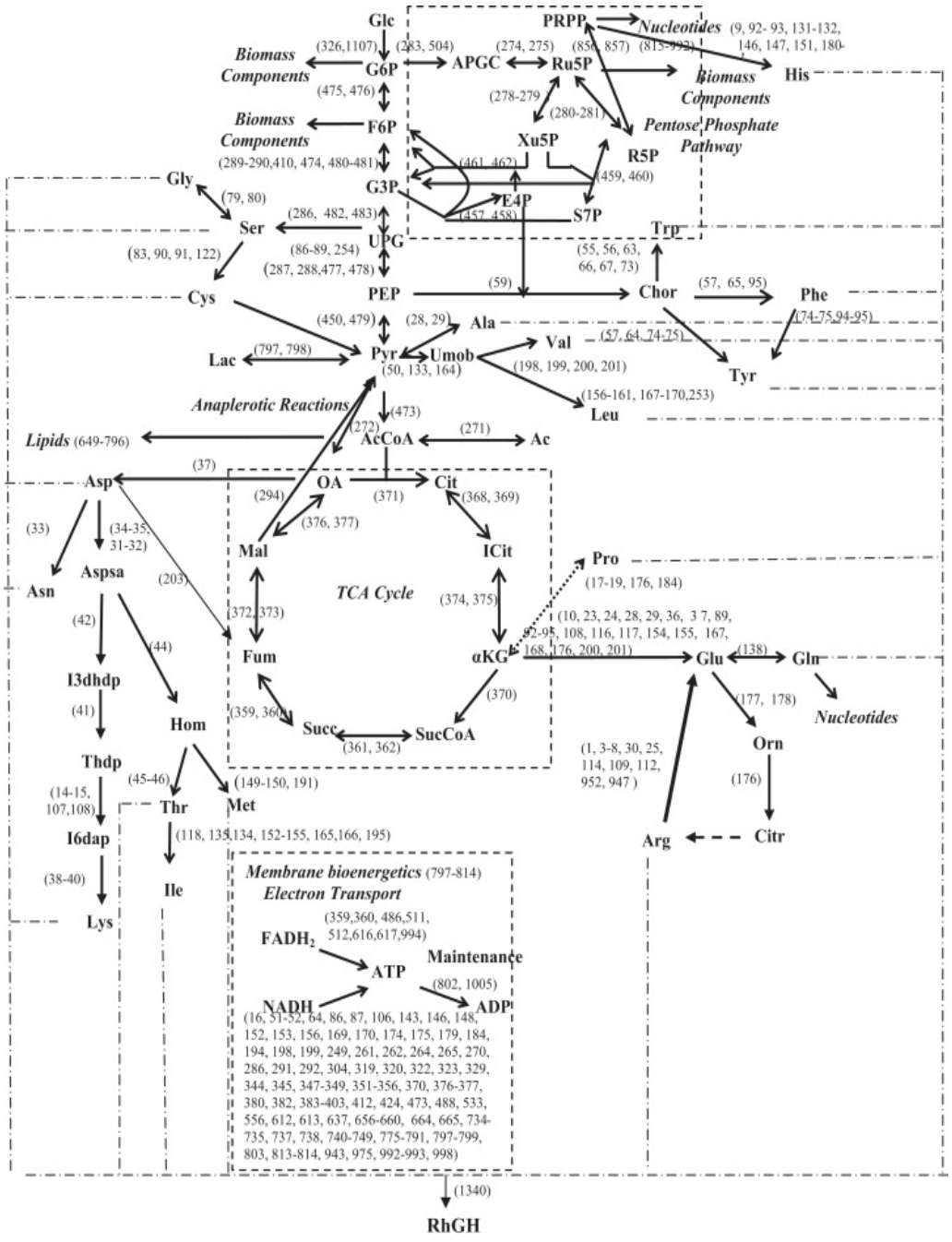


Figure 2.13 The metabolic pathway map of recombinant *B. subtilis* (Özdamar et al., 2010)

2.3.3 Medium Design

Medium selection is vital not only for growth but also for the desired protein production affecting the synthesis reactions that take place within the cell. All nutrients have different effects on the pathways according to their dosage; therefore, amount of each nutrient added to the medium should be optimized, also. According to the required amount for the cell, nutrients divided into two categories; namely, macronutrients, and micronutrients. Macronutrients are nutrients required in concentrations higher than 10^{-4} M such as carbon, hydrogen, Mg^{2+}, nitrogen, K^{+}, sulphur, oxygen and phosphorus, while micronutrients are those required in concentrations less than 10^{-4} M such as growth hormones, trace elements (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mo^{2+}, Na^{+1}, Zn^{2+}, Co^{2+}, Ni^{2+}), metabolic precursors, and vitamins (Shuler and Kargi, 2002). Functions of some major nutrients and their sources are given in Table 2.2.

Nutrients needed for cell to maintain its physiological functions are supplied inside the medium to host cells. This medium can be divided into two as defined and undefined (complex) medium. Defined medium is a medium all of whose constituents are known with their amounts. This medium generally contains salts and glucose as a carbon source; however, amino acids, and vitamins can be added according to the need of the host microorganism. On the other hand, in complex medium, exact amount of the constituents is not known. Complex medium is composed of growth factors, trace elements, vitamins and hormones. Complex medium is used for a wide range of microorganisms due to having nutrients greater than the required values, while the defined medium is used mostly for heterotrophic microorganisms that use organic compounds as carbon sources (Bailey and Ollis, 1986; Nealson, 1999).

Table 2.2 Function and sources of some nutrients (Todar, 2000; Madigan and Martinko, 2005)

Elements	Source	Physiological Function
Macronutrients		
Carbon	CO_2, organic compounds	Component of cellular material
Hydrogen	CO_2, H_2O, organic compounds, O_2	Main component of organic compounds and cell water
Magnesium	Magnesium salts ($MgSO_47H_2O$, $MgCl_2$)	Cofactor of certain enzymes, cellular cation
Nitrogen	NH_3, NO_3, N_2, proteins, amino acids	Component of amino acids, nucleic acis, coenzymes and nucleotides
Oxygen	H_2O, CO_2, O_2	Component of cellular material, and cell water; electron acceptor in respiration
Phosphorus	KH_2PO_4, K_2HPO_4, K_3PO_4	Component of certain coenzymes, nucleotides, nucleic acids, phospholipids
Potassium	Potassium salts	Cofactor of certain enzymes, cellular cation
Sulphur	SO_4, H_2S, organic sulphur compounds	Component of certain coenzymes and proteins
Micronutrients		
Calcium	Calcium salts	Cofactor of certain enzymes, cellular cation, component of endospores
Copper		Involved in carbohydrate and nitrogen metabolism
Iron	Iron salts	Cofactor of certain enzymes, component of cytochromes, and some nonheme proteins
Manganese		Involved in nitrogen metabolism
Nickel		Activation of urease enzyme
Zinc		Involved in protein synthesis and growth regulation

In the literature related to the hGH production, Nakayama et al. (1988) and Kajino et al. (1997) used complex mediums containing glucose as carbon source for the rhGH production by the *Bacillus* species. In the study of Kajino et al. (1997), the complex medium containing 20 kg m^{-3} polypeptone P1, 2 kg m^{-3} yeast extract, 30 kg m^{-3} glucose, 0.1 kg m^{-3} $CaCl_2.2H_2O$, 0.1 kg m^{-3} $MgSO_4.7H_2O$, 0.01 kg m^{-3} $FeSO_4.7H_2O$, 0.01 kg m^{-3} $MnSO_4.4H_2O$ and 0.01 kg m^{-3} $ZnSO_4.7H_2O$ was found to be optimum for rhGH production by *B. brevis* 31-OK whose proteases activity was decreased. On the other hand, in the study of Nakayama et al. (1988), the complex medium composed of 10 kg m^{-3} tryptone soy broth, 5 kg m^{-3} yeast extract, and 10 kg m^{-3} NaCl gave the maximum hGH production in *B. subtilis* MT500.

In the study of Şentürk (2006), the effect of glucose on the hGH production by *B. subtilis* BGSC-1A197 (*spo*$^-$) and *B. subtilis* BGSC-1A751 (*apr*$^-$ *npr*$^-$) carrying pMK4::*pre*(*subC*)::*hGH* was studied. Growth medium used in SAP production by recombinant *B. licheniformis* having the same signal peptide was used in that study (Çalık et al., 2004). However, without adding protease inhibitor to this medium, hGH production was not observed. Maximum hGH was produced with the medium containing 8 kg m^{-3} glucose, 4.71 kg m^{-3} $(NH_4)_2HPO_4$, 2.0 kg m^{-3} KH_2PO_4, 0.043 kg m^{-3} Na_2HPO_4 and 5.63 kg m^{-3} NaH_2PO_4 with addition of 7.44 μM protease inhibitor (Sigma, P-2714).

2.3.4 Bioreactor Operation Parameters

2.3.4.1 Temperature

Medium temperature affects not only the growth rate but also the consumption of the carbon and energy sources. It can change the growth and production yields. Furthermore, as temperature decreases, yield of substrate and energy sources also decreases due to more energy requirement caused by the decrease in temperature. Temperature decrease leads to the fall in the magnesium, potassium and phosphate

yields. Also, at low temperatures, diffusional limitations will be the case. On the other hand, increase of temperature above optimum growth temperature causes cell deaths due to denaturation of proteins. In some conditions, it was observed that temperature affects the metabolic pathways, also (Pekin, 1980; Scragg, 1988; Shuler and Kargi, 2002).

Hence, for every microorganism, there is an optimum temperature, or range in which the maximum growth/product formation is attained. In order to increase the yield and selectivity of the desired product, bioprocess should be conducted at this optimum temperature value or range.

In the studies on hGH production by *E. coli*, Jensen and Carlsen (1990), Bylund et al. (2000), and Castan et al. (2002) used 30°C, while Shin et al. (1998a-b), and Tabandeh et al. (2004) preferred 37°C as operating temperature without investigating the temperature effect. Furthermore, in the literature related to the hGH production by different *B. subtilis* strains, Nakayama et al. (1988) conducted hGH production at 30°C, whereas Şentürk (2006), and Yılmaz (2008) performed hGH production at 37°C without studying the effect of temperature.

2.3.4.2 pH

pH is another important parameter for the cell growth and so the desired protein production. Every microorganism has an optimal pH range to grow; some has a broad range while others have a narrower range. For many bacteria, this range is 6.5-8.0 (Scragg, 1988).

During the fermentation process, hydrogen ion concentration in the medium changes due to the metabolic activity of the cell (organic acid formation, amino acid consumption, etc.) and in spite of these changes cell keeps internal pH constant. Proton gradient across the cell membrane is needed for mass transport, energy production, and many metabolic functions (Nielsen and Villadsen, 1994). Therefore, external pH value alters metabolic activity and so production yield since cell try to

maintain the proton gradient. In order to enhance the yield and selectivity of the desired product, external pH could need to be kept constant at a value or a range for some bioprocesses, while other bioprocesses could require uncontrolled medium pH (Pekin, 1980).

Throughout the bioprocess, change in pH is an important indicator of the changes and deviations in metabolic pathways. Decrease in medium pH is observed when carbohydrates, amino acids, and the organic acids that are produced by the intracellular reactions are secreted to the medium; while medium pH increases when the metabolites secreted to the medium are transferred back to the cell and are used inside the cell. Since the intracellular reactions and their rates change depending on the medium pH, host microorganisms can have different optimum pH range for growth and the desired product production (Çalık et al., 2002; Shuler and Kargi, 2002; Hornbæk et al., 2004).

In the literature related to hGH production by *E. coli* and *Bacillus* species, pH and the operation strategy used is given in Table 2.3. The first study investigating the pH effect on hGH production by *B. subtilis* is Yılmaz (2008). Maximum hGH was obtained as 46 g m^{-3} at pH_0=7.5 in uncontrolled operation in this study. Also, they investigate the effect of initial pH on the glucose consumption, and oxygen transfer characteristics throughout the bioprocess. However, there is no work in the literature concerning the effect of pH control on the hGH production.

Table 2.3 pH and operation strategy used in studies related to hGH production

Source	Host microorganism	pH	Operation strategy
Jensen and Carlsen, 1990	*E. coli*	7.20	Controlled
Zhang et al., 1998	*E. coli*	7.20	Controlled
Shin et al., 1998a,b	*E. coli*	6.75	Controlled
Patra et al., 2000	*E. coli*	7.00	Controlled
Bylund et al., 2000	*E. coli*	7.00	Controlled
Castan et al., 2002	*E. coli*	7.00	Controlled
Tabandah et al., 2004	*E. coli*	7.00	Controlled
Nakayama et al., 1988	*B. subtilis*	6.00-7.00	Uncontrolled
Şentürk, 2006	*B. subtilis*	7.25	Uncontrolled
Yılmaz, 2008	*B. subtilis*	7.50	Uncontrolled

In the literature, there are three studies investigating the effect of pH on the product, side-product, oxygen transfer characteristics, and metabolic fluxes (Çalık et al., 2002; Çalık et al., 2003; İleri and Çalık, 2006). In the study of Çalık et al. (2002), controlled and uncontrolled pH strategies were studied for the SAP production by *B. licheniformis* at pH=7.0-7.5. They found that changing medium pH affects the glucose consumption rate, cell concentration profiles, dissolved oxygen concentration, SAP activity and side product distribution. In another study of Çalık et al. (2003), maximum SAP activity was achieved at pH=7.1 in uncontrolled operation as 900 U

cm^{-3}, and at the same pH when process is shifted to controlled pH operation SAP activity decreased to 720 U cm^{-3}. Also, when fermentation is conducted at pH=7.0 in uncontrolled operation, maximum activity attained was 700 U cm^{-3}. These studies show that even small changes of the initial pH value create a significant difference in yield and selectivity of the desired product, and the importance of the operation strategy in terms of product yield.

The first study in the literature investigating the effect of pH on the intracellular and extracellular metabolites in *Bacillus* species is İleri and Çalık (2006). The maximum β-lactamase activity was obtained at pH_0=7.5 in uncontrolled operation as 57 U. In this study, by drawing N^+, K^+, and H^+ profiles, it was found that there is also N^+-H^+ pomp as well as N^+-K^+ pomp that keeps electropotential gradient constant across the cell membrane. Besides, it was found that amino acids and organic acids inside the cell are 580-, and 20-fold higher than those secreted to extracellular medium, respectively. They investigated that increase in the medium pH leads to increase in acetic acid secretion, and decrease in lactic acid secretion.

These studies related to pH effect on production by *Bacillus* species show that even for same microorganism optimum pH value and the operation strategy could be different for different processes. Therefore, as well as initial pH effect (Yılmaz, 2008), also operation strategy should be investigated for hGH production by *B. subtilis* in order to enhance the yield and selectivity of product.

2.3.4.3 Oxygen Transfer Characteristics

In aerobic processes, cells need oxygen to perform their metabolic functions such as respiration, growth and protein synthesis. Therefore, dissolved oxygen concentration and its transfer rate are important factors. Deficiency or excess of oxygen transfer affect the yield and selectivity of the desired product. The oxygen requirement and transfer in a bioprocess depend on the microorganism, physical properties of the growth medium, bioreactor and its impeller configuration. In stirred-

tank bioreactors, inlet oxygen/air rate (aeration) and impeller speed (agitation) are important parameters for oxygen transfer. Oxygen transfer rate to the cells is a limiting factor in determination of the bioconversion rate since biochemical reaction rates are generally higher oxygen transfer rate to the medium (Bailey and Ollis, 1986).

According to the two film theory, oxygen in the growth medium is transferred to the cell at nine steps as shown in the Figure 2.14.

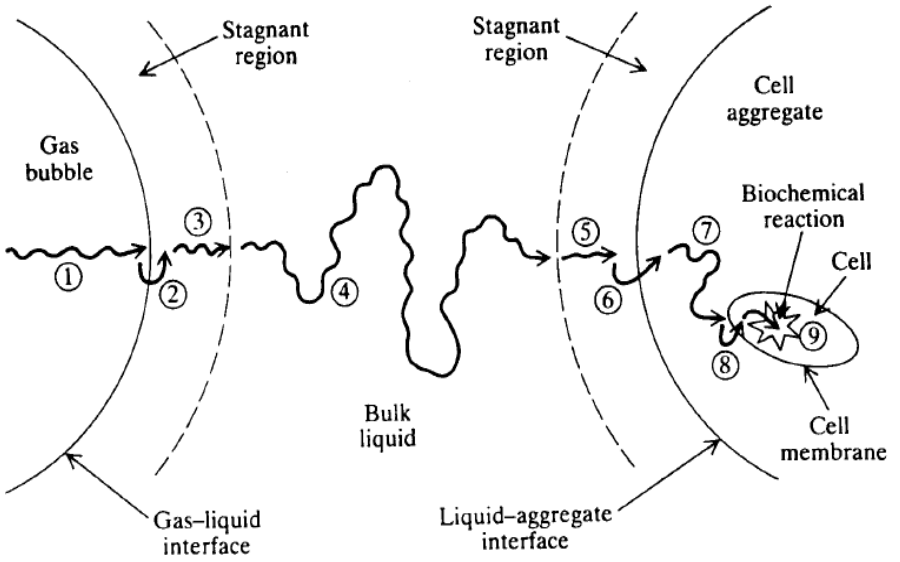


Figure 2.14 Schematic of two-film theory of oxygen transport mechanism from a gas bubble into the inside of cell (Bailey and Ollis, 1986)

1. Transfer of oxygen from gas bubble to the gas-liquid interface
2. Movement across the gas-liquid interface
3. Diffusion of oxygen through the stagnant liquid region

4. Transport of oxygen through the bulk liquid
5. Diffusion of oxygen through the stagnant liquid
6. Movement across the liquid-cell interface
7. Diffusion through the solid to the individual cell
8. Transport across the cell envelope
9. Transport from the cell envelope to the reaction site

The solubility of oxygen in the water is low. For instance, glucose solubility in water is almost 6000-fold higher than oxygen solubility in water (Stanbury et al., 1995). Therefore, in oxygen transfer mechanism, gas film resistance is negligible when compared to liquid film transfer. By increasing aeration agitation rate, film resistances surrounding the gas bubbles decreased, and hereby oxygen transfer rate can be enhanced. For example, OTR increased 4-fold by changing agitation rate from 300 rpm to 600 rpm in the study of Ahmad et al. (1994). If cells do not clump, then 6^{th} step will be eliminated. Since the cells have a tendency to be adsorbed by the interfaces, dissolved oxygen could have to overcome only liquid film resistance.

Rate of solution of oxygen is controlled by the formation of new film layers surrounding the gas bubbles. If the bioprocess medium does not mix well, and concentration profiles in liquid phase (due to cell dispersion in liquid) is important, oxygen transfer resistance at the bulk liquid limits this rate of solution. Hence, to decrease all the oxygen transfer resistances in liquid phase, medium should be stirred mechanically. By this way, oxygen is transferred to the cells by both molecular and convective transport (Bailey and Ollis, 1986).

If diffusion inside the cell clump, and transport through the interfaces (gas-liquid, liquid-cell, cell membrane) do not pose resistance, oxygen transfer rate (OTR) from gas to liquid per unit volume can be defined in terms of the volumetric liquid phase mass transfer coefficient:

$$OTR = N_A a = K_L a(C_O^* - C_O) \tag{2.6}$$

where N_A is the molar mass transfer flux of molecule A (mol m^{-3} s^{-1}), a is the gas-liquid interfacial area per unit volume (m^2 m^{-3}), K_La is the volumetric liquid phase mass transfer coefficient (s^{-1}), C_O^* is saturated dissolved oxygen concentration (mol m^{-3}) and C_O is the actual dissolved oxygen concentration(mol m^{-3}). Since the solubility of oxygen in water is low, K_La will be nearly equal to liquid side volumetric mass transfer coefficient (k_La) (Nielsen and Villadsen, 1994).

In aerobic fermentation processes, oxygen transfer to the cell affects recombinant protein production by changing the metabolic fluxes. OTR defined by equation 2.6 depends on the aeration rate, agitation speed and oxygen uptake rate (OUR) of microorganism (Richard, 1961). Therefore, OUR and K_La are called "oxygen transfer characteristics".

Aerobic bioprocesses are designed by determining the rate-limiting factor. This could be OTR, OUR or consumption rate of another substrate in the medium. By deciding the maximum possible oxygen utilization rate and maximum OTR, rate limiting parameter can be found. The maximum OTR is N_A value at $C_O=0$ ($N_{Amax}=k_La\ C_O^*$), while the maximum oxygen utilization rate is found by $-r_{Omax}=C_X\ \mu_{max} / Y_{X/O}$ where C_X is cell concentration, $Y_{X/O}$ is the cell produced per unit mass of oxygen consumed (Shuler and Kargi, 2002).

If maximum OTR is greater than maximum oxygen utilization rate, main resistance for the increased oxygen concentration is microbial metabolism and bioprocess becomes reaction-limited. Otherwise, C_O is almost zero and so bioprocess becomes mass transfer limited. In borderline cases, both steps can control the process. Actually, the situation is more complex than this. If oxygen concentration in liquid phase is above the critical value, cells are saturated with oxygen, and correlation between respiration rate and the dissolved oxygen concentration is not observed anymore (Bailey and Ollis, 1986; Pumphrey, 1996).

Many factors can affect the oxygen requirement of the microorganism. Oxygen uptake rate (OUR) depends on the physiological properties of host microorganism, and carbon source in the medium. Glucose, one of the carbon sources, enters the

metabolism more quickly than the other carbohydrates. For example, OUR values of Penicillin production by *P. chrysogenum* are 3.4, 4.7, 9.4 mmol m^{-3} s^{-1} for lactose, sucrose, and glucose, respectively (Koffler et al., 1945). Oxygen is mostly used for the cell growth; however it is also required for oxidation reactions in metabolic functions, biosynthesis and product formation. Thus, by the help of the metabolic stoichiometry, oxygen utilization rate for the growth can be related to the substrate consumption rate (Bailey and Ollis, 1986; Doran, 2000).

During the exponential growth phase of the host cell, oxygen uptake rate increases and oxygen transfer rate is not at enough level, dissolved oxygen concentration in the medium decreases due to the oxygen demand of the cells. With the increase in medium viscosity caused by the growth and product formation by the time, oxygen transfer rate and so the oxygen uptake rate decreases. During the stationary growth phase, oxygen demand of the cell decreases and most of this oxygen demand is required for metabolic activities. Dissolved oxygen concentration in the medium increases also when oxygen uptake rate decreases. (Çalık et al., 1998).

K_La is an important parameter for gas-liquid mass transfer in the bioreactor. It depends on the microorganism, rheological properties of the fermentation broth, design of bioreactor and impeller, inlet gas flow rate and agitation speed (Alba et al., 1973). To know whether the dissolved oxygen in the medium is transferred to the cell at the enough level is possible by determining the oxygen transfer coefficients. Rainer (1990) classified the methods for experimental determination of K_La value as:

- Direct measurement methods
  - Dynamic method
  - Gas mass balance method
- Indirect measurement methods
  - Gassing- out method
  - Electrote-momentum method
  - Sulfite oxidation method

- Glucose-oxidase method
- CO_2 method

Dynamic method is a widely used method for determination the value of K_La in bioreactors. Some of the advantages of this method is being easy to apply and that it does not require analysis of the gas mixture. At the unsteady-state condition, when the mass balance equation is applied to the liquid phase:

$$K_L a(C_O^* - C_O) + r_O^{\cdots\cdots} C_X = \frac{dC_O}{dt} \tag{2.7}$$

This method comprises the examining of the dissolved oxygen (DO) concentration decrease by closing the gas inlet for a while, then DO concentration increase by reopening the gas inlet (Rainer, 1990) (Figure 2.15). Until $t=t_0$ when the gas inlet is closed, DO concentration is at a value of C and at $t=t_1$, gas inlet is opened. From $t=t_0$ till $t=t_1$, decrease in DO concentration is observed. At this time interval, since there is no oxygen transfer, $K_La(C_O^*-C_O)$ term is equal to zero and equation 2.7 is simplified to equation 2.8:

$$r_O = \frac{dC_O}{dt} \tag{2.8}$$

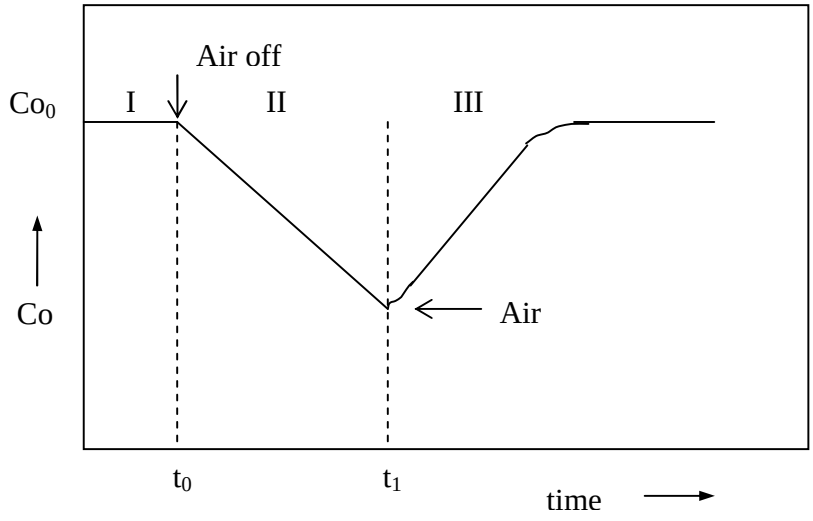


Figure 2.15 Changes in dissolved oxygen concentration with time in dynamic method implementation

From the equation 2.8, OUR ($-r_O$) and from the equation 2.9, oxygen uptake rate per unit cell dry weight ($-r_O^{```}$) can be found (Doran, 2000).

$$r_O = r_O^{```} C_X \tag{2.9}$$

When gas inlet is opened at $t=t_1$, increase in DO concentration is observed and the equation 2.7 become valid. Then, by using the equation 2.7 and 2.8, ($dC_O/dt-r_O$) vs C_O graph is plotted (Figure 2.16). Slope of this plot gives K_La.

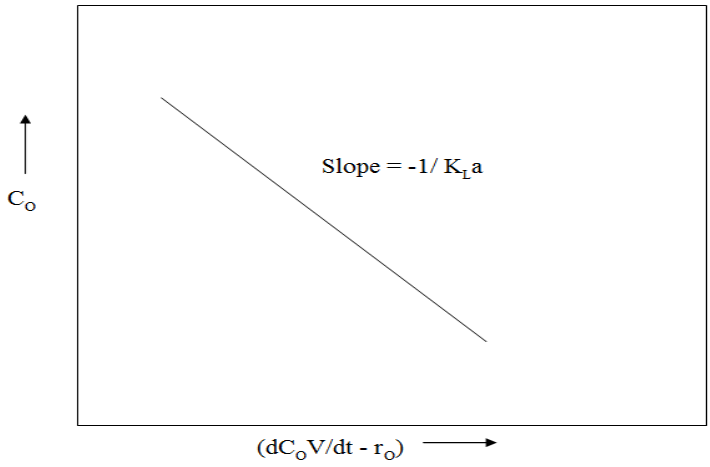


Figure 2.16 Determination of $K_L a$ by dynamic method

DO concentration does not decrease quickly when gas inlet is closed. Removing of gas bubbles from the bioreactor takes time due to the gas hold-up. At high agitation rates, oxygen transfer from interface on the liquid phase would occur. To reduce this effect, when the gas inlet is closed, impeller speed is also decreased (Rushton and Oldshue, 1953).

$K_L a$ changes with agitation rate and gas flow rate, and increases with agitation rate. At conditions in which rheological properties of the bioprocess medium do not change much, $K_L a$ does not be affected by the Schmidt number which is the ratio of momentum diffusivity to mass diffusivity. Intracellular reaction rates and particles that are found in the mass transfer region affect mass transfer. Hence, $K_L a$ of the fermentation broth is expected to change with OUR, size of the microorganism and cell concentration (Çalık et al., 1997; Doran, 2000).

Agitation and gas charged to the bioreactor cause to foam formation. Addition of chemical anti-foams to the medium affects mass transfer resistances at liquid-gas interfaces. They cause the decrease in surface tension, and so increase in bubble

diameter. Since large bubbles have less interfacial area, low gas hold-up (limited time for oxygen to dissolve), anti-foams leads to decrease in K_La (Alba et al., 1973; Motarjemi and Jameson, 1978; Onodera et al., 1993).

In the literature concerning the hGH production, dissolved oxygen concentration is kept constant at 20% in the studies of Jensen and Carlsen (1990), and Castan et al. (2002), and at 40% in the studies of Shin et al. (1998[a,b]), and Tabandeh et al. (2004). However, in none of these studies, effect of agitation rate and inlet gas flow rate on the hGH production was investigated. The first study investigating the effect of oxygen transfer on hGH production by *B. subtilis* is Şentürk (2006). In this study, the effect of oxygen transfer is examined at agitation rates of N=500, 700, 800 min^{-1} and aeration rates of Q_0/V_R=0.5, 0.7 vvm in a 1 dm^3 bioreactor by using the defined medium and maximum hGH production is obtained at N=800 min^{-1} and Q_0/V_R=0.5 vvm.

Oxygen transfer rate affects not only the yield and selectivity of the product but also the side-product distribution and metabolic fluxes. Çalık et al. (1998) is the first study examining the effect of oxygen transfer on the product and side-product formation by *B. licheniformis*. In this study, SAP production by *B. licheniformis* is carried at agitation rates of N=150, 500, 750 min^{-1}, at aeration rate of Q_0/V_R=1 vvm, in a 3.5 dm^3 bioreactor using the defined medium. The maximum SAP activity was obtained at MOT condition (N=500 min^{-1}) as 441 U cm^{-3}. Besides, it was observed that cell concentration and amino acid concentrations at LOT (N=150 min^{-1}) and HOT (N=750 min^{-1}) conditions are higher than those at MOT condition (N=500 min^{-1}). This result shows that one of the amino acids that are not found in the medium has a regulative effect on cell metabolism. Bottlenecks in the SAP production by *B. licheniformis* are determined by investigating the effect of oxygen transfer on the metabolic fluxes (Çalık and Özdamar, 1999; Çalık et al., 1999).

In the study of Çalık et al. (2000), effect of oxygen transfer on the SAP production by *B. lichenisformis* is investigated by performing bioreactor experiments at N=150, 500, 750 min^{-1}, and Q_0/V_R=0.2, 0.5, 1.0 vvm. The maximum SAP activity

was obtained at Q_0/V_R=0.5 vvm and N=750 min^{-1} as 500 U cm^{-3}. Also, two-stage oxygen transfer strategy was implemented in this study. Until the production phase, oxygen transfer condition (Q_0/V_R=0.2, N=750 min^{-1}) in which the growth is maximum is applied. Then, oxygen transfer condition is shifted to the conditions (Q_0/V_R=0.5, N=750 min^{-1}) in which maximum SAP activity was attained. By this way, SAP production is increased 0.36-fold.

These studies show that oxygen transfer has an important effect on the cell metabolism and process yield. Studies carried out for the oxygen transfer strategy for SAP production by *Bacillus* species enlightens the hGH production by *B. subtilis* also. That is, Şentürk (2006) used the same oxygen transfer conditions to determine the optimum transfer rates, and MOT condition is found as optimum for protein production as in these studies. By MFA at optimum oxygen transfer condition, bottlenecks can be determined and hGH production can be enhanced by constructing a new microorganism using metabolic engineering techniques.

2.3.5 Evaluation of Kinetic Data from Bioreactor

Cells, micro-bioreactors, take substrates in the medium by different transport mechanisms, and then consume them by intracellular reactions to grow and produce metabolic products. Cell kinetic, that is substrate consumption, cell product and side-product production kinetics can be defined by structured and unstructured models. Heterogeneous cell clumps with different properties is called as segregated, while cell clumps with common properties are named as unsegregated. Therefore, models considering segregated cells describe the real situation whereas models in which cell clumps with same properties are regarded as one component describe the ideal case. Generally, cell kinetics should be defined by mathematical models depending on the key parameters of the process (Bailey and Ollis, 1986).

Cell concentration profiles with respect to time have sigmoid shape consisting of lag, exponential and stationary phases. Cell growth rate, r_X can be related to C_X by Monod Model:

$$r_X = \frac{dC_X}{dt} = \mu C_X \tag{2.10}$$

where μ is the specific growth rate. Besides, there are models relating μ to substrate concentration, or considering product or substrate inhibitory effect (Shuler and Kargi, 2002).

Consumption rate of the carbon source, $-r_S$, and production rate of product, r_P can be defined as, respectively (Shuler and Kargi, 2002):

$$-r_S = -\frac{dC_S}{dt} \tag{2.11}$$

$$r_P = \frac{dC_P}{dt} \tag{2.12}$$

The specific production and consumption rates are found as:

$$-q_S = -\frac{1}{C_X}\frac{dC_S}{dt} \tag{2.13}$$

$$q_P = \frac{1}{C_X}\frac{dC_P}{dt} \tag{2.14}$$

Specific rates are used to compare different fermentation condtions since they provide the relative data.

Cell or product formed per substrate consumed can be defined by yield coefficients:

$$Y_{X/S} = \frac{r_X}{-r_S} = \frac{dC_X}{-dC_S} \tag{2.15}$$

$$Y_{P/S} = \frac{r_p}{-r_S} = \frac{dC_p}{-dC_S} \tag{2.16}$$

Similarly, cell formed per unit mass of oxygen consumed is defined as:

$$Y_{X/O} = \frac{r_X}{-r_O} \tag{2.17}$$

In order to calculate the yields over a period of time, overall yield definitions are used:

$$\bar{Y}\, x/s = \frac{\Delta X}{\Delta S} \tag{2.18}$$

$$\bar{Y}\, p/s = \frac{\Delta P}{\Delta S} \tag{2.19}$$

Definitions of different yield coefficients are given in Table 2.4. $Y_{X/S}$ and $Y_{X/O}$ depend not only on the microorganism but also on the carbon source. Yield coefficients are constant during the process. They change depending on growth rate and metabolic activities (Bailey and Ollis, 1986).

Table 2.4 Definitions of different yield coefficients (Blanch and Clark, 1997)

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

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals were supplied from Difco Laboratories, Fluka Ltd., Merck Ltd. and Sigma Chemical Company. These chemicals were analytical grade.

3.2 The Microorganism

B. subtilis strains that are given with their accession numbers in Table 3.1 were obtained from Bacillus Genetic Stock Center (BGSC) as spore dots on the filter disks within the foil packets. These cells were revived by dropping the disks on a suitable solid medium (given in section 3.4.2), hydrating them with a few drops of LB broth and incubating the plates at 37°C overnight. Single colonies were streaked.

To prevent microorganisms get old and lose activity by the time, microorganisms were inoculated to microbanks and stored at -80°C.

Table 3.1 Strains and plasmid used in the study

Microorganism	Description	Source (Reference)
B. subtilis 1A751	*aprE*, *npr*-deficient strain	BGSC
B. subtilis 1A53	*degQ*-deficient strain	BGSC
B. subtilis MT42	*degS*-deficient strain	Tanaka et al. (1991)
B. subtilis 1A165	*degU*-deficient strain	BGSC
B. subtilis 1A68	*abrB*-deficient strain	BGSC
B. subtilis 1A178	*scoC*-deficient strain	BGSC
B. subtilis 1S97	*sinI*-deficient strain	BGSC
B. subtilis 1S97	*sinR*-deficient strain	BGSC
B. subtilis 1A197	*spo0A*-deficient strain	BGSC

Plasmid	Source (Reference)
pMK4::*pre*(*subC*)::*hGH*	Şentürk (2006); Özdamar et al. (2009)

3.3 Genetic Engineering Methods

For hGH production by selected *B. subtilis* strains as alternative hosts, recombinant strains were constructed by transformation of plasmid (pMK4::*pre*(*subC*)::*hGH*) to those strains. For this purpose, this plasmid is isolated from *E. coli*, firstly.

3.3.1 Plasmid Isolation from *E. coli*

pMK4::*pre*(*subC*)::*hGH* plasmid is isolated from *E. coli*. For plasmid DNA isolation from *E. coli*, the GeneJET^{TM} Plasmid Miniprep Kit (Fermentas AB) was used. The contents of this kit are given in Appendix A. The protocol followed is:

1. Inoculate 5 ml of Luria-Bertani (LB) medium (containing the selective antibiotic) with single colony picked from a freshly streaked plate and incubate it at 37°C, and 250 rpm overnight.
2. Harvest the culture by centrifugation at 8000 rpm, 25°C for 2 min and discard all supernatant.

   Note: All centrifugations after this step are carried out at 13200 rpm, and all steps are performed at room temperature.
3. Add 250 μL of the Resuspension Solution to resuspend the pelleted cells completely and take this suspension into a microcentrifuge tube.
4. Add 250 μL of the Lysis Solution and mix it by inverting tube 4-6 times until the solution becomes viscous.
5. Add 350 μL of the Neutralization Solution and then mix thoroughly by inverting tube 4-6 times.
6. Centrifuge solution for 5 min to form pelleted cell debris and chromosomal DNA.
7. Take the supernatant into the supplied GeneJET^{TM} spin column by pipetting. Be careful not to disturb the white precipitate.
8. Centrifuge the solution for 1 min and poor off the flow-through. Put the column back into the same tube.
9. Add 500 μL of the Wash Solution and centrifuge again for 30-60 minutes. After discarding the flow-through, place the column into tube.
10. Repeat step 9.

11. Poor off the flow-through and to get rid of the residual Wash Solution centrifuge for 1 min.
12. Place the GeneJET^{TM} spin column into a 1.5 mL microcentrifuge tube. With the aim of eluting the plasmid DNA, add 50 μL of the Elution Buffer to the center of the membrane found on the column. Be careful not to disturb membrane with pipet. Incubate the solution for 2 min, then centrifuge for 2 min.
13. Remove the column. Solution that remains in tube is the purified plasmid DNA. Store it at -20°C.

3.3.2 Determination of DNA Length by Agarose Gel Electrophoresis

For separation of DNA fragments by gel electrophoresis, as gel constituent, agarose and as gel solution, 1X TBE buffer whose components are given in Appendix B were used. Concentration of agarose gel used was 1% (w/v), and 3 μL EtBr (Ethidium bromide, 10 kg m^{-3}), which is a fluorescent tag was also added to the gel. DNA samples were prepared by adding 1/6 volume of 6X DNA Loading Dye (Fermentas AB). Thereafter, firstly λ DNA Hind III Marker (Fermentas AB) (Appendix C) or λ DNA/Eco91I (BstEII) Marker (Fermentas AB**)** (Appendix C), then samples were loaded to agarose gel. Gel was run in the gel electrophoresis tank filled with 1X TBE buffer at constant voltage at 80 V until the dyes complete their run through the agarose gel. Then, using UV Transilluminator (UVP), gel was visualized and compared with marker in order to determine molecular weight of the purified plasmid DNA.

3.3.3 Transformation of Plasmid DNA to *B. subtilis* strains by Electroporation Method

The shuttle vector carrying the gene of interest (pMK4::*pre*(*subC*)::*hGH*) was transformed to into the selected *B. subtilis* by electroporation method as follows:

Preparation of Competent Cells :

1. Inoculate 5 mL of LB medium with a freshly grown *B. subtilis* colony. Then, incubate at 37°C and 180 rpm overnight.
2. Inoculate 250 μL of cells grown in step 1 into the 50 mL of LB medium and incubate at 37°C and 180 rpm for 3 h.
3. Harvest cells by centrifugation at 4000 rpm for 15 min.
4. Wash the harvested cells with 1 mM HEPES buffer (Appendix B) two times and then with cold electroporation buffer (Appendix B) one time. Keep cells cold during this step.
5. Resuspend the cells by adding 250 μL cold electroporation buffer and keep the suspension at 4°C for 10 min.

Transformation of Plasmid DNA into B. subtilis Strains by Electroporation :

1. Adjust voltage of the electroporation system (Bio-Rad) as 16 kV cm^{-1}.
2. Mix 40 μL of competent cell and plasmid DNA less than 5 μL (100-500 ng).
3. Put the cell/DNA mixture into the electroporation cuvettes that was cold before and place the cuvette into the holder of the system.
4. Apply electric-pulse, and after keeping cells 2-3 min at room temperature, add 0.5 mL LB medium and incubate at 37°C for 3 h.
5. Spread cells over LB agar supplemented with antibiotic and incubate at 37°C overnight.

3.3.4 Plasmid DNA Isolation from *B. subtilis* by Alkaline Lysis Method

To control whether the transformation was successful, plasmid DNA was isolated from *B. subtilis* strains and the length of the isolated plasmid was checked (by gel electrophoresis) and compared with recombinant pMK4 plasmid size. The alkaline lysis method below was used for isolation.

1. Inoculate 2.5 mL LB medium containing the selective antibiotic with a single *B. subtilis* colony.
2. Incubate the inoculated medium at 37°C and 200 rpm overnight.
3. Centrifuge 1.2 mL of the grown cells at 10000 rpm and 25°C for 1 min and discard the all supernatant by the help of a pipet.
4. Resuspend the cells by adding 1.2 mL of TSE buffer (Appendix B).
5. Repeat the step 3.
6. Resuspend the cells by adding 500 µL Solution I (Appendix B) containing 2 g L^{-1} egg-white lysozyme and homogenize the suspension vortexing for 10 s.
7. Incubate the cells at 37°C until cells are lysed (usually 10-20 min).
8. Put the mixture to ice and add 400 µL of Solution II (Appendix B). Keep the mixture on ice for 4 min after homogenizing by vortex.
9. Add 300 µL of Solution III (Appendix B) and keep it on ice for 5 min.
10. Centrifuge the mixture at 16000 rpm and 4°C for 6 min.
11. Take ~600 µL of the supernatant into a new eppendorf tube.
12. Add 600 µL of phenol/chloroform/isoamyl alcohol (25/24/1), and vortex for 1 s. repeat the vortexing process after 1 min.
13. Centrifuge at 16000 rpm and 4°C for 6 min.
14. Take ~500 µL of the upper phase into a new eppendorf tube. Be careful not to disturb other phases.
15. Add 500 µL of chloroform/isoamyl alcohol (24/1) and centrifuge at 16000 rpm and 4°C for 2 min after vortexing for 5 s.

16. Take ~450 μL of the upper phase into a new eppendorf tube.
17. Add 1 mL of 96% ethanol to the tube, mix, and keep on ice for 10 min.
18. To form pelleted DNA, centrifuge 16000 rpm and 4°C for 10 min.
19. Remove the supernatant by pipetting carefully.
20. Add 1 mL of 80% ethanol. Centrifuge for 1 min and remove supernatant as that in step 19.
21. Repeat step 20.
22. Wait for the pellet to dry. Store plasmid DNA at -20°C.

3.4 hGH Production by Recombinant *B. subtilis* Strains

3.4.1 Microbank

Vials which contain porous ceramic beads having the ability to bound cells, and cryopreservative liquid making cells bound beads homogeneously are called Microbank^{TM} (Pro-Lab). Cells were incubated into this vial, and excess of the cryopreservative liquid was removed. Then, these vials were stored at -80°C. Microbank^{TM} technology offer long-term storage microorganism..

3.4.2 The Solid Medium

Components of the solid medium used in hGH production by *B. subtilis* are given in Table 3.2 (Çalık, 1998). After the sterilizing (with saturated steam at 121°C and 1.2 atm for 20 min) the medium, as antibiotic chloramphenical was added to the medium (to be 7 μg cm^{-3} in the medium) to conserve the plasmid. Then, ~ 30 mL of medium was poured into the petri plate. Then, bead taken from the Microbank^{TM} vial was directly streaked on to the solid medium and cells were incubated at 37°C for 24 h. Cells were stored at 4°C after they were grown.

Table 3.2 The solid medium for recombinant *Bacillus* species

Nutrients	**Concentration, kg m^{-3}**
Meat extract	3
Peptone	5
Agar	15

3.4.3 The Precultivation Medium

Components of the precultivation medium used in hGH production by *B. subtilis* were given in Table 3.3 (Çalık, 1998). Again, chloramphenical was added to the medium to be 7 μg cm^{-3} in the medium. Cells grown on the solid medium were inoculated into 30 mL of the precultivation medium inside the air-filtered Erlenmeyer flasks having a volume of 150 cm^3. Then, these flasks were placed to orbital shakers (B. Braun, Certomat) to incubate cells at 37°C and 200 rpm until cell concentration of OD_{600}= 0.28-0.34 was attained. This takes approximately 3 h.

Table 3.3 The precultivation medium for recombinant *Bacillus* species

Nutrients	**Concentration, kg m^{-3}**
Soytone	15.0
Peptone	5.0
$CaCl_2$	0.1
Na_2HPO_4	0.25
$MnSO_4.2H_2O$	0.01

3.4.4 The Production Medium

Components of the production medium are given in Table 3.4. 10 cm^3 of the cells grown in the precultivation medium was inoculated into 100 cm^3 of production medium (containing 7 μg cm^{-3} chloramphenicol) inside 500-cm^3 air-filtered Erlenmeyer flask with an inoculation ratio of 1:10. Cells were incubated at 37°C and 200 rpm in orbital shakers (B. Braun, Certomat). When cell concentration reached OD_{600}=0.28-0.39, protease inhibitor cocktail (Sigma P-2714) was added to the medium, also.

Table 3.4 The production medium for recombinant *Bacillus* species

Nutrients	**Concentration, kg m^{-3}**
Glucose	8.000
$(NH_4)_2HPO_4$	4.710
KH_2PO_4	2.000
Na_2HPO_4	0.043
NaH_2PO_4	5.630

3.4.5 Scale-up Steps and Bioreactor System

Pilot-scale bioreactor used in hGH production is given in Figure 3.1. As the first step, cells were streaked on to the solid medium from Microbank^{TM}; then inoculation was carried out from solid medium to the precultivation medium at the second step, from precultivation medium to the precultivation medium with a ratio of 1:10 at the third step, from the precultivation medium to production medium inside the pilot-scale bioreactor with a ratio of 1:10.

hGH production experiments were performed at two different scales; namely laboratory-scale and pilot-scale. At laboratory-scale, hGH production was studied by using the 110 cm^3 production medium given in Table 3.4 in 500-cm^3 air-filtered Erlenmeyer flasks at 37°C and 200 rpm for 26-32 h.

On the other hand, at pilot-scale hGH production, inoculation was performed into 1 dm^3 production medium (V_R=1 dm^3) with a pH_0=7.5 inside the 3.0-dm^3 pilot-scale bioreactor with a inoculation ratio of 1:10. The pilot-scale bioreactor (Braun CT2-2) used in this study has a working volume of V_R=0.5-2.0 dm^3, and consists of stirring rate, pH, temperature, dissolved oxygen, aeration rate control systems. There

are two four-blade Rushton turbines in the bioreactor. Temperature control was achieved by jacket around the bioreactor, steam generator and external cooler; and oxygen was supplied to the bioreactor by using compressor and pure oxygen tube. Oxygen concentration was adjusted by mass flow controller, also.

3.4.5.1 Pilot-Scale hGH Production

Steps followed in preparation of pilot-scale bioreactor (Braun CT2-2) for hGH production are :

1. Pure water inside the bioreactor was drained.
2. pH probe was disconnected from the bioreactor. By using standard buffers at pH=4.0 and pH=7.0 (Mettler Toledo), pH calibration was carried out. Thereafter, probe place back into the bioreactor.
3. Bioreactor is filled with 1-1.4 dm^3 of ultra pure water before the sterilization process. Gas inlet filter was switched to sterilization position. The bioreactor was covered by protective coating. Then sterilization at 121°C and 1.2 atm for 20 min was started.
4. Glucose was sterilized separately, that is, other production medium components were sterilized together at the same sterilization conditions above. Salts were prepared as stock solutions and sterilized by filtering 0.45 μm filters (Sartorius AG).
5. The sterilized production medium was transferred to the bioreactor via the sterilized by silicon hoses.
6. Operation conditions were adjusted as pH_0=7.5, Q_0/V_R=0.5 vvm, T=37°C, N=750 min^{-1}.
7. Calibration of pO_2 was performed at cultivation temperature and impeller speed. For this purpose, medium was deoxygenated by feeding pure nitrogen into the bioreactor, and dissolved oxygen concentration was set to 0% at this

condition. Then, medium was oxygenated by supplying air to the bioreactor from compressor, and dissolved oxygen concentration was set to 100%.

8. Cells grown in precultivation medium were inoculated to the bioreactor via sterilized silicon hoses (with an inoculation ratio of 1:10).
9. When the cell concentration reached to OD_{600}=0.28-0.39, 0.744 M protease inhibitor cocktail (Sigma P-2714) was added to the medium.
10. When needed during the bioprocess, sterilized 30% Antifoam A (Sigma 5758) was injected to the system.

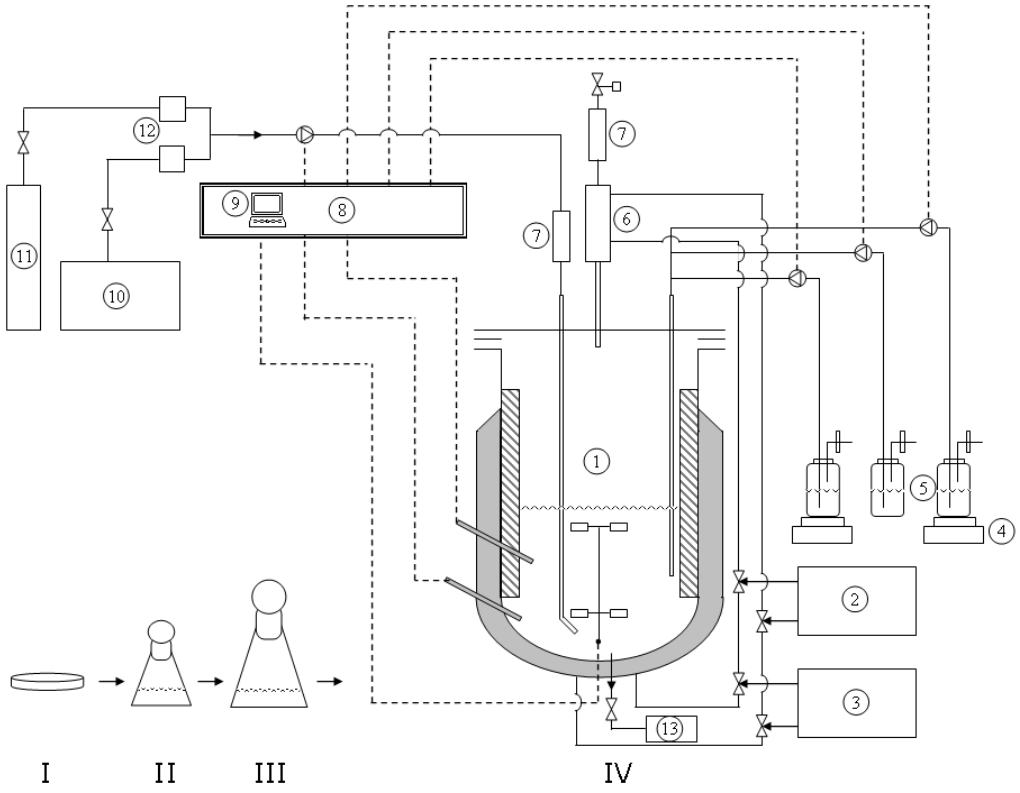


Figure 3.1 The schematic diagram of the pilot-scale bioreactor system

3.5 Analytical Methods

Samples were taken from the bioreactor at different times of the bioprocess. Firstly, cell concentration was measured, then sample was centrifuged at 12000 g and 4°C for 10 min. Supernatant was filtered using 0.45 μm filters (Sartorius, AG), and glucose, hGH, organic acids analysis were performed on this filtered sample.

3.5.1 Cell Concentration

Cell concentration was determined by absorbance measurement with UV- Vis spectrophotometer (Thermo Spectronic, Heλios α). As wavelength 600 nm was used since it was found that calibration curve draw at 600 nm was more sensitive than those obtained at 400-600 nm. Calibration curve was given in Appendix D.

3.5.2 Glucose Concentration

Reduced glucose concentration was measured with UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) at λ=505 nm using Glucose Analysis Kit (Biyozim) (Boyacı et al., 2005). The contents of the kits are given in Appendix A.

Principle of the Analysis :

Peroxide formation by the oxidation reaction of D-glucose catalyzed by glucoseoxydase enzyme was given in equation 3.1. On the other hand, equation 3.2 shows the reaction of peroxide formed in equation 3.1 with 4-aminoantipyrine and phenol in the catalysis of peroxidase. As a result of these reactions, mixture became red due to presence of iminoquinone. The concentration of this substance is directly proportional to glucose concentration.

$$D-glu\cos e + H_2O \xrightarrow{glu\cos e\ oxidase} Gluconate + H_2O_2 \qquad (3.1)$$

$$H_2O + 4-A\min oantipyrine + Phenol \xrightarrow{peroxidase} \mathrm{Im}inoquinone + H_2O \qquad (3.2)$$

Firstly, calibration curve was obtained with standard glucose concentrations. Calibration curve and preparation of standard glucose solutions were given in

Appendix D and A, respectively. The method given below was used for glucose analysis of samples:

1. Samples were diluted to a final concentration less than or equal to 2 kg m^{-3}.
2. 0.05 mL of sample or standard solution was added to the test tubes.
3. Be careful to keep temperature of analysis solutions and tubes at analysis temperature. Add 0.05 mL of glucose analysis reagent to the tubes.
4. 0.40 mL of analysis buffer was added into the tubes.
5. Mixture in the tube was diluted with pure water to 2.50 mL.
6. Mixture was incubated at 25°C for 40 min or at 37°C for 20 min.
7. As blank, samples that do not contain any sugar were used for absorbance measurement.
8. Absorbance values of all samples were measured at 505 nm using UV-Vis spectrophotometer (Thermo Spectronic, Heλios α).

3.5.3 hGH Concentration

hGH concentration was measured with High Performance Capillary Electrophoresis (Agilent). Schematic representation of HPCE is given in Figure 3.2. In inlet and outlet vials, electrolyte solution and buffer exist, respectively. Firstly, sample is injected by applying pressure or voltage into the column, then, electrolyte solution is injected. Ions of electrolyte solution supplies the current needed in HPCE. Then, by the application of voltage across the capillary, different zones of sample are formed due to the different electrophoretic mobilities of ions or charged colloids. As the sample migrates towards the end of the capillary due to electic field, zones formed are detected by the detector (Kuhr and Monnig, 1992).

For hGH analysis, fused silica capillary column with a length of 60 cm and a radius of 75 μm was used. As the electrolyte solution 50 mM borate buffer (pH=10) containing AccuPure Z1-Methyl reagent (Waters), and as the buffer 50 mM borate

buffer at pH=10 were used (Appendix B). The reason why Z1-Methyl reagent was used is to make the protein adsorption on to the capillary surface decrease. Samples inside vials were introduced into the column by applying pressure. Analysis was performed at 12 kV and 15 °C for 40 min using UV detection at λ=214 nm and electropherograms were obtained at the end of the analysis (Çalık et al., 1998). The sample electropherogram is given in Appendix E.

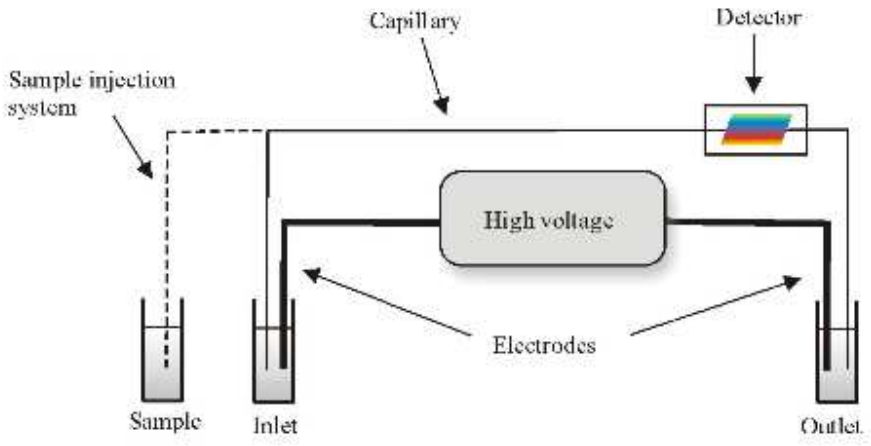


Figure 3.2 Schematic of a typical CE instrumentation (http://www.scitopics.com/Capillary_Electrophoresis.html)

Other operating conditions used in HPCE during protein analysis were as follows:

Conditions used for protein analysis in HPCE (Agilent) :

- Column : Capillary column packed with SiO_2
- Column dimensions : 60 cm x 75 μm
- Power supply : Positive
- Operating voltage : 12 kV
- Injection type : Hydrodynamic injection (applying pressure)
- Injection volume : 10 μL
- Analysis temperature : 15°C
- Electrolyte solution : Modified borate buffer (pH=10)
- Detector, wavelength : UV, 214 nm
- Duration of analysis : 40 min

3.5.3.1 Ultrafiltration

Before SDS-PAGE analysis, samples were concentrated to observe thicker bands on the gel. For this purpose, by nitrogen tube, a pressure of 3.8 bar was applied to the stirred cells (Amicon) containing the sample and 10 kDa regenerated cellulose ultrafitration membrane (Millipore). This process was continued until 10-fold higher concentration was attained.

3.5.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is the most widely used electrophoresis type. It can be used for DNA and RNA as well as proteins. The difference of the SDS-PAGE from the PAGE Electrophoresis is usage of SDS (Sodium Dodecyl Sulfate). SDS molecules that have anionic structure make molecules have primer structure, and make proteins be

negatively charged. By this way, charged protein molecules are separated according to their molecular weights. As matrix, polyacrylamide gel is used. Polyacrylamide gel is formed by polymerization of acrylamide and N-N'-Methylenebisacrylamide. This polymerization reaction occurs by cross-connecting of bisacrylamide molecules to linear chains of acrylamide formed by connection of adjacent molecules. As catalyst, TEMED (N, N, N', N'-Tetramethylethylenediamine); and as the free-radical source of this catalyst, APS (Ammonium persulfate) are used. Amount of acrylamide and ratio of acrylamide/ bisacrylamide determine the separation capacity of the gel. As the ratio of acrylamide/ bisacrylamide increases, gel warms much, and becomes more fragile (Leammli, 1970).

Preparation of Gel and Samples, and Running the Gel:

1. Gel cast were prepared. After adding APS and TEMED to the resolving gel (Appendix B), and resolving gel was pipeted into the gel cast till the bottom of the green line. Then, ButOH/dH_2O was poured by pipeting. After waiting 45 min for gel to polymerize, stacking gel (after APS and TEMED were added) (Appendix B) was pipeted into the gel cast. At this step, Teflon combs were inserted to the gel cast. Schematic of the gel cast is given in the Figure 3.3.

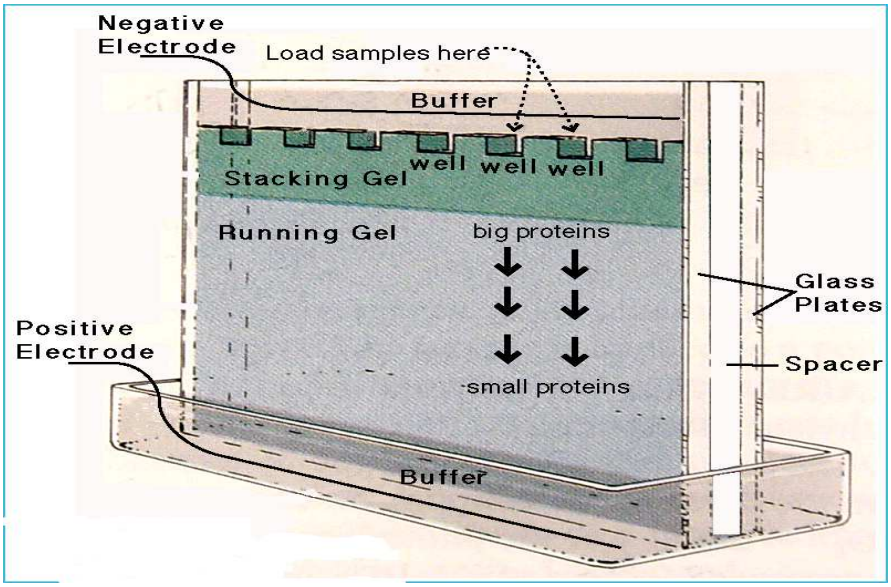


Figure 3.3 Schematic of SDS-PAGE (http://dolly.biochem.arizona.edu/Bioc462b_Honors_Spring_2009/ssenef/methods.html)

2. After the polymerization of the gel for 20 min, combs were removed, and gel cast were placed into the electrophoresis unit. Electrophoresis tank was filled with 1X SDS-PAGE running buffer (Appendix B).
3. Samples were mixed with the sample loading buffer (Appendix B) at a ratio of 1:2. Then, they were incubated at 95°C for 4 min. 2-Mercaptoethanol in the sample loading buffer breaks the disulfide bonds found in 3-D structure of the proteins. Other bonds found in 3-D structure of proteins are broken by heating, and protein molecules take the linear shape. On the other hand, glycerol in sample loading buffer concentrates the sample and makes sample sink to the bottom of the gel well. After heating step, samples were kept on ice for 5 min, then centrifuged and vortexed.

4. 15 μL of sample and 3 μL PageRuler^{TM} Prestained Protein Ladder (Fermentas AB) (Appendix C) were loaded into the wells. Gels were run at 40 mA of constant current until blue lane reached the bottom. This step takes approximately 45 min. After running step, silver staining procedure was followed. By comparing sample bands with marker bands, molecular weight of the protein product was determined. Silver staining protocol is given in Appendix F.

3.5.4 Amino Acid Concentration

Amino acid concentrations were measured using an amino acid analysis system (HPLC, Waters). For amino acid analysis, Pico-Tag method was used for derivatization of amino acids since free amino acids cannot be detected by HPLC (Cohen, 1983). Analysis was performed by using New-Pico Gradient Method which was developed by modification of Waters Picofree Gradient Method (Çalık et al., 1998). In this method, at the first step, phenylthiocarbamyl amino acid derivatives (PTC-AA) were produced by the reaction of free amino acids with phenylisothiocyanate (PITC). At the second step, PTC-AA produced was injected to the system. During the analysis, two different solvents pass through the column by forming gradient. Gradient formed by these solvents is given in Table 3.5.

Table 3.5 Gradient program of New-Pico method

t (min)	Flow rate (cm^3 min^{-1})	A%	B%
0.0	1.0	100.0	0.0
10.0	1.0	54.0	46.0
10.5	1.0	0.0	100.0
11.5	1.0	0.0	100.0
12.0	1.0	0.0	100.0
12.5	1.0	100.0	0.0
18.0	1.0	100.0	0.0

From the chromatograms obtained, concentrations of amino acids were determined by comparing areas with those of the standard amino acid solutions. Analysis was performed using the conditions below :

Conditions used for amino acid analysis :

- Column : Amino acid analysis column (Nova-Pak C18, Millipore)
- Column dimensions : 3.9 mm x 30 cm
- System : Reversed phase chromatography
- Mobile phase flow rate : 1 cm^3 min^{-1}
- Column temperature : 38°C
- Detector, wavelength and sensitivity : UV/VIS, 254 nm, 0.05 AUFS
- Injection volume : 4 μL
- Duration of analysis : 20 min
- Components of mobile phase A (v/v): 6% acetonitrile, 94% deionized water, 1.79% (w/v) sodium acetate trihydrate, 0.05% triethylamine
- Components of mobile phase B (v/v): 66.6% acetonitrile, 33.3% deionized water

3.5.5 Organic Acid Concentration

HPLC (Waters, Alliance 2695) was used for organic acid analysis. Concentrations of organic acids were calculated by using the calibration curves obtained by the analysis of standard organic acid solutions. Calibration curves of the organic acids observed in the system are given in Appendix D. The conditions below were used for the analysis (İleri and Çalık, 2006).

Conditions used for organic acid analysis in HPLC :

- Column : Capital Optimal ODS, 5 μm
- Column dimensions : 4.6 mm x 250 mm
- System : Reversed phase chromatography
- Mobile phase flow rate : 0.8 cm^3 min^{-1}
- Column temperature : 30°C
- Detector, and wavelength : Waters 2487 dual absorbance detector, 210 nm
- Injection volume : 5 μL
- Duration of analysis : 15 min+5 min delay
- Components of mobile phase : 3.12% (w/v) NaH_2PO_4, 0.62 x 10^{-3} (v/v) H_3PO_4

3.5.6 Protease Activity Assay

Activity of the protease was determined by absorbance measurement of hydrolysate formed by enzymatic hydrolysis of casein. The unit of the enzymatic activity is U cm^{-3} and 1 U (1 unit) protease activity was defined as the activity that releases 4 nmol tyrosine per unit time by Moon and Parulekar (1991). For protease analysis, supernatant of samples taken throughout the bioprocess were used.

Three types of proteases were analyzed; namely, alkali, neutral and basic proteases. Hence, three different buffers were used for the analysis of these proteases. These are 0.05 M borate buffer (pH=10.0), 0.05 M sodium phosphate buffer (pH=7.0) and 0.05 M sodium acetate buffer (pH=5.0), respectively. Firstly, casein solutions having 0.5% (w/v) were prepared by adding casein to analysis buffers. Then, samples were diluted with the buffer of interest. 2 mL of the casein solution was mixed with 1 mL of the diluted sample and the resulting mixture was incubated at 37°C, 105 min^{-1} for 20 min. Thereafter, reaction was stopped by addition of 2 mL of 10% (w/v) trichloroacetic acid (TCA) solution. After keeping the reaction mixture on ice for 15 min, mixture was centrifuged at 4°C, 10500 rpm for 10 min. During this step, casein that was not reacted collapsed. Absorbance of the supernatant was measured at 275

nm in UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) using quartz cuvette. As blank, mixtures that contain buffer instead of sample were used. Finally, protease activity was determined by using the calibration equation below (equation 3.3) as U cm^{-3} (Çalık, 1998) :

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

3.5.7 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

K_La and OUR were determined by the method explained in 2.3.4.4. Before inoculation into the production medium, without microorganism, physical liquid pahse mass transfer coefficient (K_La_0) was determined by applying dynamic method. Then, K_La values were determined at every four hours throughout the process. The detailed explanation of the dynamic method was given in section 2.3.4.4.

CHAPTER 4

RESULTS AND DISCUSSION

Effects of regulatory genes on recombinant human growth hormone production by *B. subtilis* were investigated to enhance hGH production. For this purpose, firstly *B. subtilis* strains whose regulatory genes are deleted were selected; then protease inhibitor addition time was determined by carrying out fermentation experiments at air-filtered Erlenmeyer flasks using the medium optimized by Şentürk (2006); thereafter pilot-scale bioreactor experiments with the strains, which have the highest hGH production capacity at air-filtered Erlenmeyer flasks, were perfomed at operating conditions optimized by Yılmaz (2008); and finally effect of controlled pH strategy on hGH production was investigated.

4.1 Selection of *B. subtilis* Strains

Since hGH production mimics the subtilisin production in *B. subtilis* due to the promoter and signal sequence of *sub*C gene on the recombinant pMK4::*pre*(*subC*):: *hGH* plasmid, expression or repression of regulatory genes of *aprE* (subtilisin gene of *B. subtilis*) is expected to affect the hGH production positively or negatively. Hence, by using the *B. subtilis* strains in which *aprE* regulatory genes are deleted, effect of these genes on rhGH production can be investigated.

aprE gene has a complex regulatory gene network with many repressors and activators. Among these genes, those that are well-characterized and controlled by nutrient deprivation in the medium were chosen to be studied since glucose concentration changes during the fermentation process resulting in activation or repression of the genes. The selected genes and their functions are given in Table 4.1.

The *B. subtilis* strains lacking of these genes were supplied from BGSC (*Bacillus* Genetic Stock Center).

The effect of these genes of the *aprE* expression is given in Table 4.1.

Table 4.1 Effect of the regulatory genes on *aprE* expression

Genes	***aprE* expression**
abrB	Decrease
degQ	Increase
degS	no direct effect
degU	Increase
scoC	Decrease
sinI	Decrease
sinR	Increase
spo0A	no direct effect

4.2 Construction of Recombinant *B. subtilis* strains

To be able to produce rhGH by *B. subtilis*, recombinant strains were constructed by plasmid transformation to these strains. First of all, pMK4::*pre*(*subC*)::*hGH* plasmid were isolated from *E. coli* (Özdamar et al., 2009), then were transformed into the selected *B. subtilis* strains.

4.2.1 Isolation of pMK4::*pre*(*subC*)::*hGH* Plasmid from *E. coli*

The plasmid size of pMK4::*pre*(*subC*)::*hGH* plasmid isolated from *E. coli* was verified by agarose gel electrophoresis (Figure 4.1).

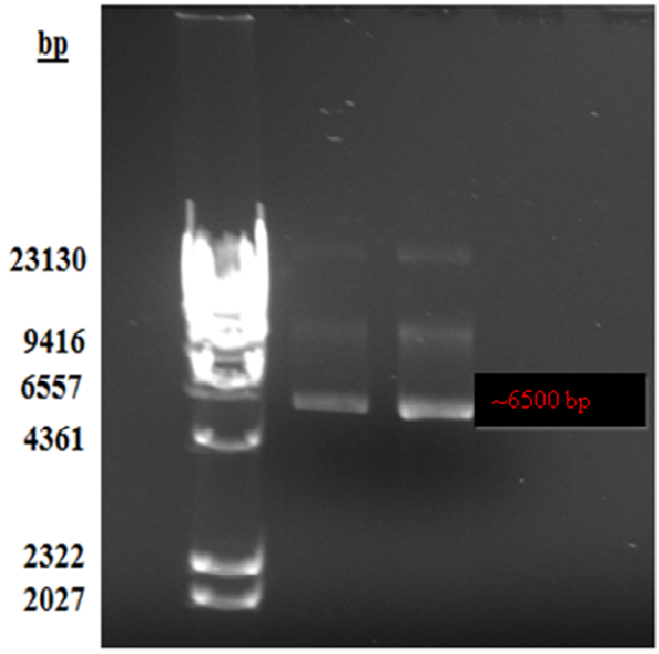


Figure 4.1 Agarose gel electrophoresis image of pMK4::*pre*(*subC*)::*hGH* plasmid after isolation from *E. Coli*. 1. well: λDNA/HindIII Marker (Fermentas AB**)**; 2. well: pMK4::*pre*(*subC*)::*hGH* plasmid isolated; 3. well: pMK4::*pre*(*subC*)::*hGH* plasmid isolated.

4.2.2 Transformation of pMK4::*pre*(*subC*)::*hGH* Plasmid to *B. subtilis* strains

pMK4::*pre*(*subC*)::*hGH* plasmid was transformed to *B. subtilis* strains by electroporation at C=25 μF, R=200 Ω, and V/L=10 kV cm^{-1}. The *Bacillus* colonies that grew on solid medium with chloramphenicol (selective antibiotic) were selected and to ensure that the transformation was successful, plasmid isolation from these selected colonies were performed. Then, plasmid size of the isolated pMK4::*pre*(*subC*)::*hGH* was verified by agarose gel electrophoresis image (Figure 4.2).

~5600 bp

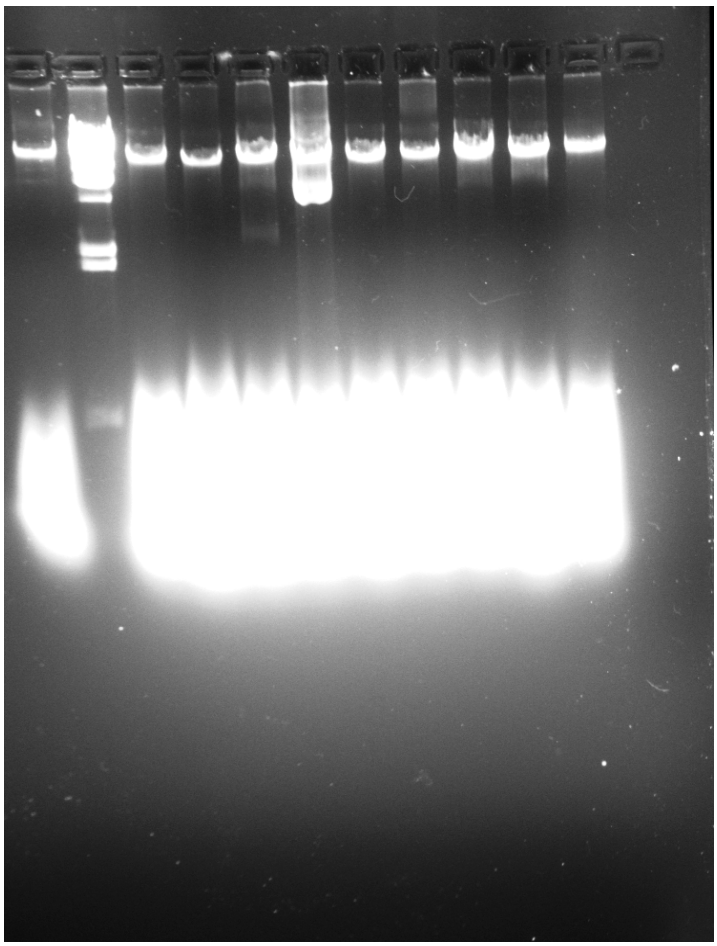


Figure 4.2 Agarose gel electrophoresis image of pMK4::pre(*subC*)::*hGH* plasmid after isolation from *B. subtilis*. 1. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *abrB*-; 2. well: λ DNA/Eco91I (BstEII) Marker (Fermentas AB**)**; 3. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *aprE*-; 4. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *degQ*-; 5. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *degS*-; 7. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *degU*-; 8. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *scoC*-; 9. well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *sinI*-; 10. well:pMK4::pre(*subC*)::*hGH* plasmid isolated from *sinR*-; 11.well: pMK4::pre(*subC*)::*hGH* plasmid isolated from *spo0A*- knockout *Bacillus* strain.

4.3 Comparison of hGH Production Capacities of Recombinant *Bacillus* Strains

With the recombinant *Bacillus* strains constructed, fermentation experiments were carried out at air-filtered Erlenmeyer flasks at V_R=110 cm^3, T=37°C, N=200 min^{-1} in the medium given in Table 3.4 to determine the addition time of protease inhibitor. Initial pH (pH_0) was adjusted to 7.5 with addition of sufficient amount of 10 M KOH. Another set of fermentation experiments were performed in order to examine the rhGH production capacity of the strains. hGH in the medium was analyzed by SDS-PAGE.

4.3.1 Determination of Protease Inhibitor Addition Time

Activity of proteases is induced just after the exponential growth phase (Westers et al., 2004). Hence, protease inhibitor should be added to the medium through the end of the exponential growth phase (when cell concentration reaches OD_{600}=0.28-0.39). Growth curve for each strain was developed by fermentation experiments performed at air-filtered Erlenmeyer flasks at V_R=110 cm^3, T=37°C, N=200 min^{-1} (Figure 4.3). According to the growth curves, protease inhibitor addition time for each strain is given in Table 4.2.

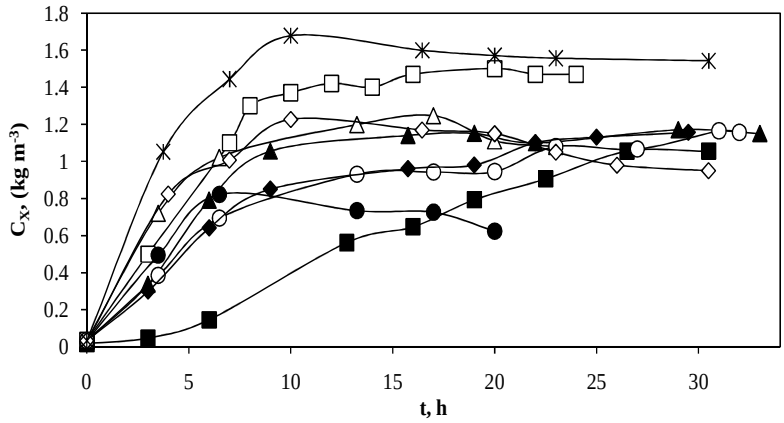


Figure 4.3 Cell growth diagrams for recombinant *Bacillus* strains: *abrB*- (■), *aprE*- (□), *degQ*- (▲), degS- (Δ), *degU*- (●), *scoC*- (○), *sinI*- (♦), *sinR*- (◊), *spo0A*- (ж)

Table 4.2 Protease addition time of r-*Bacillus* strains

Strains	**Inhibitor Addition Time (h)**
abrB-	12
aprE-	7
degQ-	9
degS-	6
degU-	6
scoC-	7
sinI-	8
sinR-	9
spo0A-	7

4.3.2 Comparison of hGH Production Capacity of *Bacillus* Strains

After determination of protease inhibitor amount that should be added to the medium, rhGH production of r-*Bacillus* strains was examined in defined medium given at Table 3.4 at air-filtered Erlenmeyer flasks 500 cm^3 in size that had working volume capacities of 110 cm^3. 150 µL of protease inhibitor was added to the medium in order to inhibit synthesis of alkaline proteases.

At time when hGH concentration reached its maximum value, cells were harvested by centrifugation and then the supernatant was concentrated 10-fold by ultrafiltration. Samples were loaded to SDS-PAGE system. hGH production capacities of r-*Bacillus* strains were compared according to the thickness of the band in SDS-PAGE gel which have molecular weight of 22 kDa (Figure 4.4).

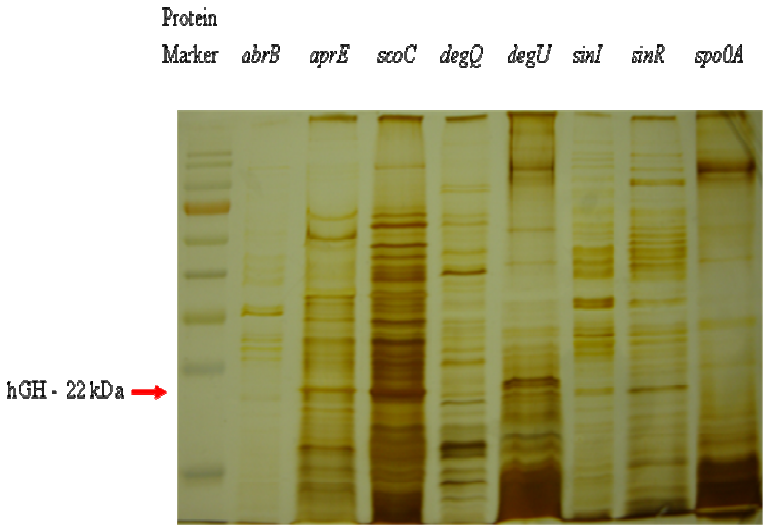


Figure 4.4 SDS-PAGE image of rhGH produced by *B. subtilis* strains

When protease inhibitor added to the growth medium, there was no growth observed in r-*Bacillus* strain deficient in *degS* gene. This might be related to the effect of proteases on the cell wall. Being one of the activators of *aprE* gene and strong promoter, *degQ* gene is expressed by the phosphorylation of DegU by DegS. Therefore, deletion of *degS* results in decrease in protease secretion. It is known that proteases in the medium affect the cell wall tolerance positively and cause the loss of density-dependent growth control (Chou et al., 1974; Joflle et al., 1980). Hence, owing to the low protease activity caused by addition of the protease inhibitor and repression of *aprE* gene, growth inhibition and autolysin might have occurred.

As can be seen from Figure 4.4, the highest rhGH was produced by *scoC* knockout strain as 64 mg L^{-1}, 3.2- fold higher than that produced by *aprE* knockout strain (20 mg L^{-1}). Amount of rhGH produced by *aprE* and *sinR* knockout strains were close to each other (Figure 4.5).

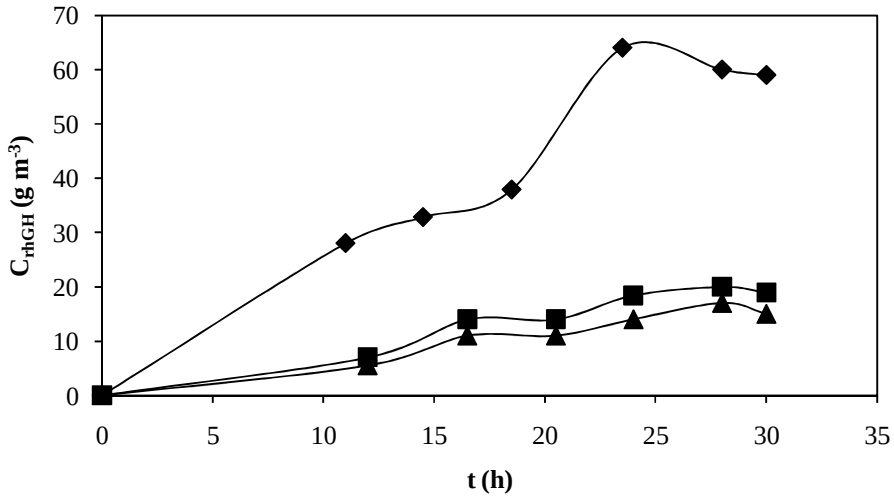


Figure 4.5 rhGH concentration profiles of r-*Bacillus* strains: *scoC*- (♦); *aprE*- (■); *sinR*- (▲)

When a sharp increase was observed in hGH production (t=18-23), glucose consumption also increased sharply at that time interval (t=15-26 h) (Figure 4.6). First drastic increase in glucose consumption at t=0-7 h occurred due to the high cell growth rate. Cell concentration profiles of the strains are given in Figure 4.7. When protease inhibitor was added to the medium (t=7-9 h), growth and glucose consumption remained stationary for a while, then continued to increase. Maximum cell concentrations were attained as 0.9, 1.14, and 1.47 kg m^{-3} at t=26, 30, and 32 h by *aprE, sinR* and *scoC* knockout strains, respectively.

Between the hGH and cell concentrations, there was no direct proportionality. That is, although *sinR* knockout strain attained higher cell concentration than *aprE* knockout strain, *aprE* strain produced higher amount of hGH. Maximum specific hGH production rates (q_{hGH}) for *scoC, aprE,* and *sinR* knockout strains are 1.72, 1.54, and 0.72 kg kg^{-1} CDW h^{-1}, respectively.

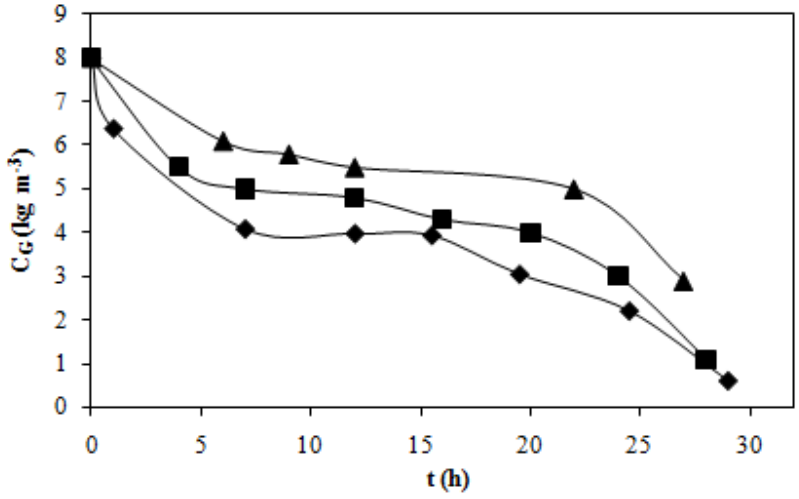


Figure 4.6 Glucose consumption profiles of r-*Bacillus* strains: *scoC*- (♦); *aprE*- (■); *sinR*- (▲)

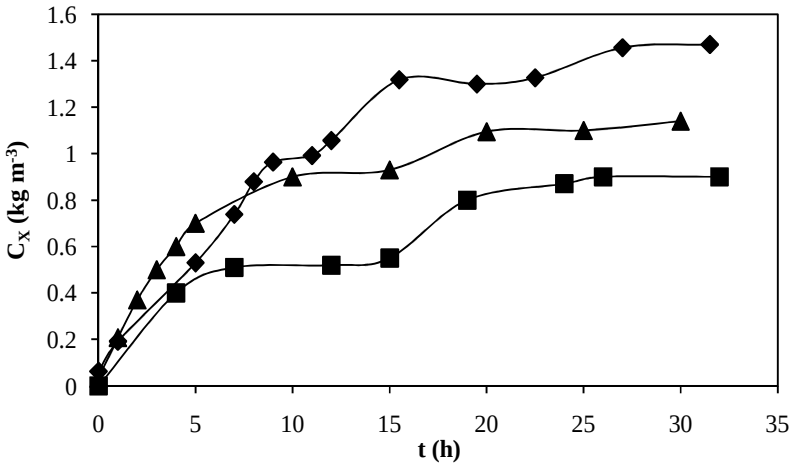


Figure 4.7 Growth curve diagrams for r-*Bacillus* strains: *scoC*- (♦); *aprE*- (■); *sinR*- (▲)

Actually the fact that these r-*Bacillus* strains (*aprE*, *scoC*, *sinR* knockout strains) have the high hGH production capacity is an expected result in terms of the protease secretion, which leads to degradation of proteins. All these strains have low *aprE* expression. That is, *aprE* is already deficient in *aprE* gene; *sinR* knockout strain *aprE* gene is repressed; and in *scoC* knockout strain, *aprE* is repressed by *sinI*, whose repressor is *scoC*. *degU* knockout strain was chosen since it was thought that signal sequence was not cleaved considering the band above the one having molecular weight of hGH.

4.4 Comparison of Fermentation and Oxygen Transfer Characteristics of r-*Bacillus* Strains

After determination of the most favorable r-*Bacillus* strains for hGH production, pilot scale bioreactor experiments were carried out. Hence, effect of regulatory genes on the pH and dissolved oxygen profiles; cell, glucose, rhGH, amino acid and organic acid concentrations; and oxygen transfer characteristics were investigated in pilot scale bioreactor 3 dm^3 in size that had working volume capacities of 1.1 dm^3 at 37°C, 750 min^{-1}, V_R/Q_0=0.5 vvm, C_{G0}=8 kg m^{-3} using the medium in Table 3.4. Initial pH was adjusted to 7.5 by addition of 10 KOH. The effects of controlled and uncontrolled pH strategy on the process were investigated for *scoC* knockout strain. For this strain, bioreactor experiment in controlled pH condition was denoted as *scoC*-pH_C, while that in uncontrolled pH condition was denoted as *scoC*-pH_{UC}. For other strains only controlled pH strategy was implemented and experiments were denoted as the knockout gene names. pH was controlled throughout the bioprocess manually with 10 M KOH. Abbreviations used for fermentation experiments are given in Table 4.3.

Throughout the bioprocess, cell, glucose, amino acid, organic acid and hGH concentrations as well as protease activity were measured and dynamic method was applied for determination of K_La. hGH, and organic acid analysis were performed with the samples that were centrifuged and filtered by 0.45 μm filters (Sartorius AG) using HPCE (Agilent). For the determination of glucose concentration and protease activity, supernatant of these samples were used. To be able to compare the strains in terms of production capacity, specific consumption and production rates, and yields were calculated.

Table 4.3 Abbreviations used for bioreactor experiments

Knockout Strain	pH Strategy	Abbreviations
aprE	Uncontrolled	*aprE*-pH_{UC}
aprE	Controlled	*aprE*-
scoC	Controlled	*scoC*-pH_{C}
scoC	Uncontrolled	*scoC*-pH_{UC}
sinR	Controlled	*sinR*-
degU	Controlled	*degU*-

4.4.1 pH and Dissolved Oxygen Concentration Profiles

pH and dissolved oxygen concentration profiles with time are given in Figure 4.8 and Figure 4.9. Initial pH of the medium (pH_0) at all experiments was 7.5. Except *scoC*-pH_{UC} case, pH was kept at 7.5.

At all experiments, until t=4 h medium pH decreased sharply due to cell growth. This fall continued until t=23-29 h when the cells entered stationary growth phase and sharp increase in hGH production was observed. Intracellular pH of *Bacillus* species is 7.5 and it does not change with medium pH (Frankena et al., 1986). Therefore, protons are pumped in or out of the cell to maintain constant intracellular pH.

At *scoC*-pH_{UC} case, maximum pH difference was obtained as $\Delta pH=0.47$. Yılmaz (2008) studied the hGH production by *Bacillus subtilis* carrying pMK4::*pre*(*subC*)::*hGH* plasmid and deficient in *aprE* gene at the same bioreactor conditions (N=750 min^{-1}, Q_0/V_R=0.5 vvm, pH_0=7.5, T=37°C) but in uncontrolled pH

condition and reported the maximum pH difference (ΔpH) as 0.81. The reason for this difference in ΔpH could be difference in organic acid, and other metabolites secretion. For instance, until t=4 h, cell concentration for *aprE* knockout strain was 1.44-fold higher than that for *scoC*-pH_{UC} knockout strain. Also, *aprE* knockout strain secreted 1.88-fold higher organic acids than that secreted by *scoC*-pH_{UC} knockout strain. At t=31 h, medium pH became constant for *aprE* and *scoC*-pH_{UC} knockout strains as 6.97 and 7.28, respectively.

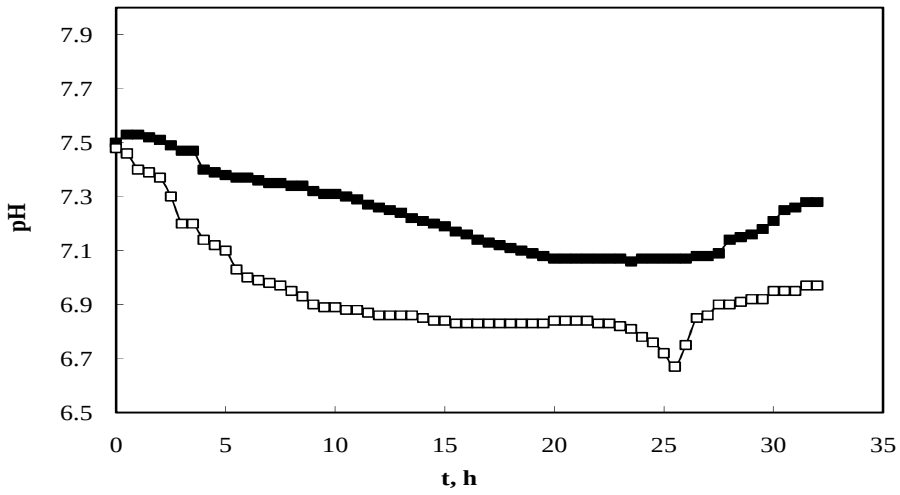


Figure 4.8 The variations in medium pH with the cultivation time. C_{G0} = 8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *scoC*-pH_{UC}: (■); *aprE*-pH_{UC} (Yılmaz, 2008).

Until t=6 h when the cell concentration increased sharply, C_O decreased for all cases, then started to increase. The sharpest increase in C_O was obtained with *sinR* knockout strain. The reason for this behavior might be the fact that the sharpest increase in cell concentration at the early stages of the bioprocess was achieved by the *sinR* knockout strain. The cell concentration obtained by *sinR* knockout strain at t=6 h was 1.16-, 1.28-, 1.42-, and 1.89- fold higher than those obtained by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, respectively. Besides, at these hours, maximum hGH production was attained by *sinR* knockout strain.

From t=6 h until t=20-26 h, dissolved oxygen concentration deviated around the same value. After t=20-26 h when the cells entered stationary growth phase, C_O started to increase. At the end of the bioprocess, the lowest C_O was obtained as 0.181 mol m^{-3} at the *scoC*-pH_{UC} case, while the highest C_O was obtained as 0.193 mol m^{-3} by the *sinR* knockout strain. Generally, C_O values reported by the study of Yılmaz (2008), the hGH production by *Bacillus subtilis* carrying pMK4::*pre*(*subC*)::*hGH* plasmid and deficient in *aprE* gene at the same bioreactor conditions (N=750 min^{-1}, Q_0/V_R=0.5 vvm, pH_0=7.5, T=37°C) but in uncontrolled pH condition, was higher than those obtained by this study owing to the higher cell and hGH concentrations obtained by the strains used in this study.

The variation in C_O due to hGH production shows the oxygen requirement for the hGH synthesis. The fact that higher amount of hGH was obtained in pilot-scale bioreactor than that in air-filtered Erlenmeyer flasks supported this finding since the oxygen transfer is more effective in bioreactor.

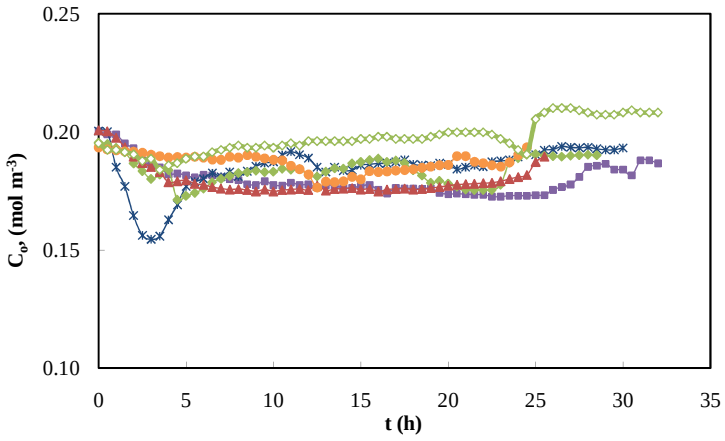


Figure 4.9 The variations in dissolved oxygen concentration with the cultivation time. C_{G0} = 8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *aprE*-pH$_{UC}$: (◊) (Yılmaz, 2008); *degU*-: (●); *scoC*-pH$_C$: (▲); *scoC*-pH$_{UC}$: (■); *sinR*-: (ж).

4.4.2 Cell and Glucose Concentration Profiles

The variations in cell concentration for all r-*Bacillus* strains are given in Figure 4.10. Until t=6-8 h when the protease inhibitor was added to the medium, cell growth rate was high, then cell concentration became stationary for a while, and again started to increase. The maximum cell concentration was obtained at *scoC*-pH$_C$ as 1.62 kg m^{-3} which is 1.34-, 1.57-, 2.15-, 2.17- fold higher than the maximum cell concentration attained by *sinR*, *scoC*-pH$_{UC}$, *degU*, and *aprE*, respectively. Yılmaz (2008) reported the maximum cell concentration as 0.90 kg m^{-3}, which is generally lower than the cell concentrations obtained in this study, in the study of hGH production by *Bacillus subtilis* carrying pMK4::*pre*(*subC*)::*hGH* plasmid and deficient in *aprE* gene at the

same bioreactor conditions (N=750 min^{-1}, Q_0/V_R=0.5 vvm, pH_0=7.5, T=37°C) but in uncontrolled pH condition.

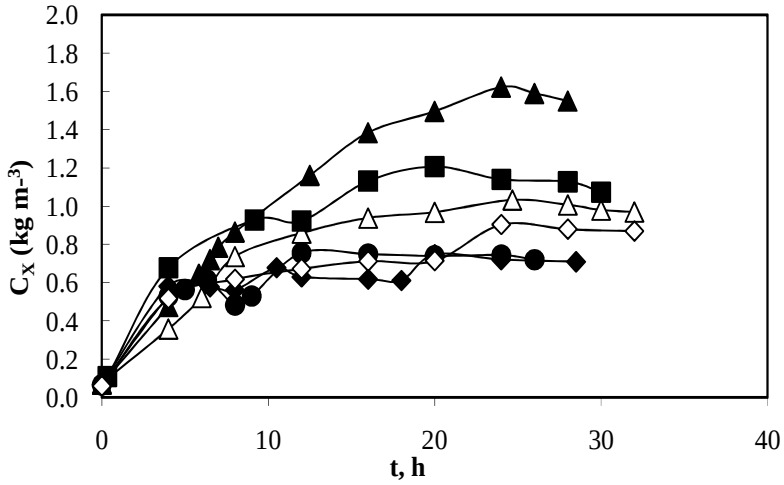


Figure 4.10 The variations in cell concentration with the cultivation time. C_{G0} = 8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *aprE*-pH_{UC}: (◊) (Yılmaz, 2008); *degU*-: (●); *scoC*-pH_C: (▲); *scoC*-pH_{UC}: (Δ); *sinR*-: (■).

The variations in glucose concentration are given in Figure 4.11. At the early stages of the bioprocess when the cell growth rate was high (t=0-6 h), almost 50% of glucose in the medium was consumed by all the strains. During the first four hours of the bioprocess, glucose consumption was lower for *sinR* knockout strains whereas the cell growth rate was higher than those obtained by the other strains. The reason of this behavior could be the fact that *sinR* knockout strain has higher yield ($Y_{X/S}$). The lowest glucose concentration was attained at t=28 h by scoC-pH_{UC} as 0 kg m^{-3}

corresponding to consumption of 100% of the initial glucose in the medium. 94, 95, 98 and 99% of the initial glucose in the medium was consumed by the *sinR*, *degU*, *scoC*-pH_C, and *aprE* knockout strains, respectively. By the addition of protease inhibitor glucose consumption rate decreased as well as cell growth rate. However, after t=12-16 h when the sharp increase in hGH production was observed, glucose consumption rate increased for all strains.

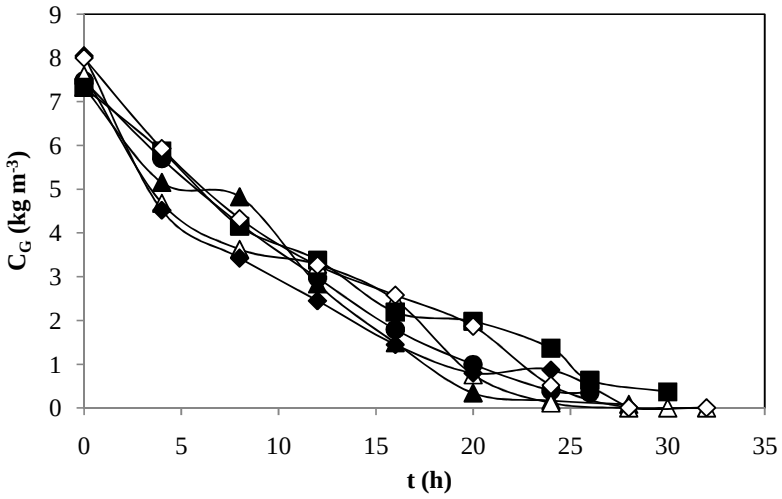


Figure 4.11 The variations in glucose concentration with the cultivation time. C_{G0} = 8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *aprE*-pH_{UC}: (◊) (Yılmaz, 2008); *degU*-: (●); *scoC*-pH_C: (▲); *scoC*-pH_{UC}: (Δ); *sinR*-: (■).

4.4.3 hGH Concentration Profiles

The variations in rhGH concentration with cultivation time for all strains are given in Figure 4.12. The cultivation time where the highest hGH production was attained in laboratory scale bioreactors shifted 4 and 2 hours forth for *aprE*, and *sinR* knockout strains in the pilot-scale bioreactor experiments, respectively. For *scoC* knockout strain, this time did not change for *scoC*-pH_C case, while it shifted 4 hours ahead for *scoC*-pH_{UC} case. As compared to laboratory scale experiments, in bioreactor hGH production capacity increased at least 1.15-fold. For example, for *scoC* knockout strain, it was increased from 64 g m^{-3} to 74 g m^{-3} (in uncontrolled pH condition).

Throughout the bioprocess, the maximum hGH concentration was obtained by *scoC*-pH_C at t=24 h as 126 g m^{-3} which is 1.59-, 1.62-, 1.70-, and 1.71- fold higher than those obtained by *aprE*, *sinR*, *degU* strains and *scoC*-pH_{UC} case, respectively. When hGH concentration reached its maximum value, cells entered stationary phase and glucose consumption rate approached to zero.

The sharpest increase in hGH production rate was observed at t=8, 12, 8, 12, and 8 h as 6.33, 5.26, 5.21, 9.77, and 3.30 g m^{-3} h^{-1} for *aprE*, *degU*, *sinR* knockout strains, and *scoC*-pH_C, *scoC*-pH_{UC} cases, respectively (Figure 4.13). Then, production rate decreased and after t=23-29 h it started to increase for all strains, again.

The reason why *scoC* strain has the highest hGH production capacity could be repression of *aprE*, serine alkaline protease gene. Actually, in *scoC* knockout strain regulatory network will be deficient in one repressor, *scoC*; however, the fact that sinI, repressor of *aprE* will not be repressed by *scoC* might result in repression of *aprE*.

In the literature, the maximum hGH production by *Bacillus* species was reported as 70 g m^{-3} by the study of Yılmaz (2008). In this study, this amount was increased at least 1.06-fold and utmost 1.82-fold.

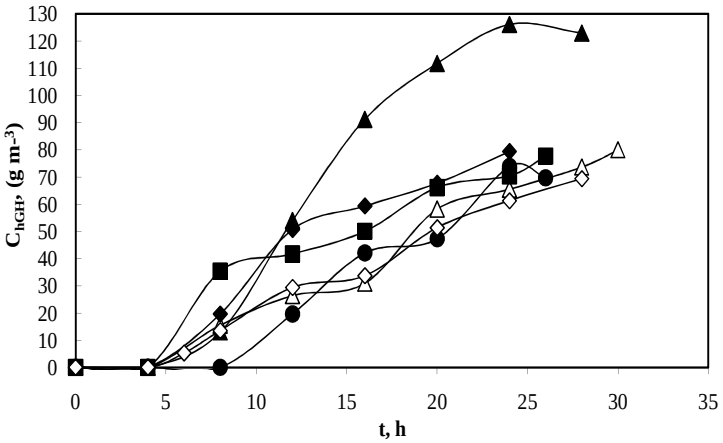


Figure 4.12 The variations in rhGH concentration with the cultivation time. C_{G0} = 8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *aprE*-pH_{UC}: (◊) (Yılmaz, 2008); *degU*-: (●); *scoC*-pH_C-: (▲); *scoC*-pH_{UC}-: (Δ); *sinR*-: (■).

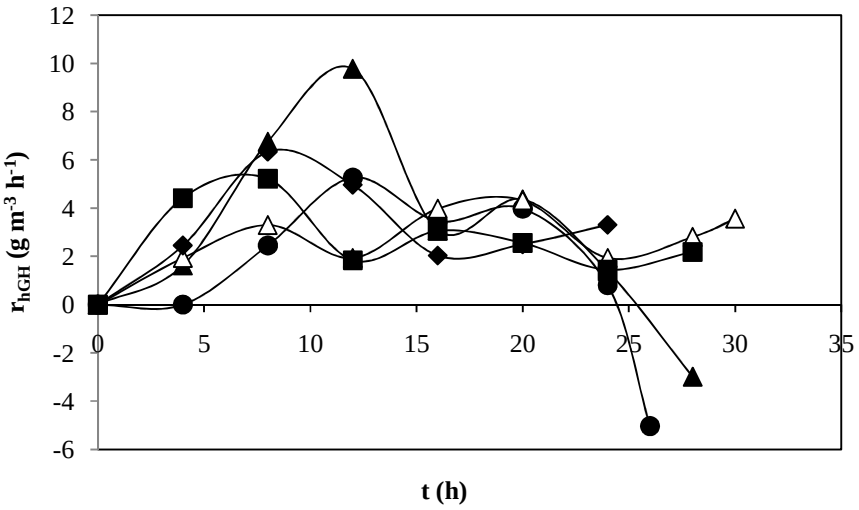


Figure 4.13 The variations in production rate of hGH (r_{hGH}) with the cultivation time. C_{G0} = 8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *degU*-: (●); *scoC*-pH_C-: (▲); *scoC*-pH_{UC}-: (Δ); *sinR*-: (■).

4.4.4 Protease Activity Profiles

Protease secretion is one of the important bottlenecks in recombinant protein production by *Bacillus* species. Therefore, total protease activity was also determined for all strains and total protease concentrations for all strains are given in Figure 4.14. Total protease concentrations are the sum of the acidic, alkali and neutral proteases.

Neutral proteases for all strains were almost in the same range as 4-6 U cm^{-3}. Generally, neutral protease concentrations were lower than other protease concentrations for all strains. Alkali proteases also had the similar profiles for all strains; however, acidic proteases for different strains did not have same or close activity values. Lowest alkali protease concentration was obtained at *scoC*-pH_C not by

aprE knockout strain confirming the existence of the other alkali genes than *aprE* gene.

The highest total protease concentration was attained at t=20 h by *degU* knockout strain as 65 U cm^{-3} which was 1.15-, 2.06-, 2.25-, and 2.62- fold higher than those obtained by *aprE*, *sinR*, *scoC*-pH_C, and *scoC*-pH_{UC} knockout strains. hGH concentrations are consistent with these results. That is, the lowest hGH concentration was obtained by *degU* strain which had the highest protease activity. Also, when the protease activity reached its maximum, hGH concentration started to decrease for all strains. Specific protease production rate (q_{pro}) for *degU* knockout strain is also the highest as 0.16 kg kg^{-1} CDW h^{-1}. Although the total protease activity is high for *aprE* knockout strain, the fact that q_{pro} is low as 0.09 kg kg^{-1} CDW made high hGH production possible for this strain. *scoC*-pH_C and *scoC*-pH_{UC} had the same level of protease activity with average q_{pro} as 0.018 kg kg^{-1} CDW, and *sinR* knockout strain secreted the lowest amount of proteases having an average value as 14.8 U cm^{-3}.

Total protease concentration increased sharply during the first eight hours of the bioprocess, then by the addition of the protease inhibitor to the medium production rate of proteases decreased. At t=16-20 h, protease production rate increased again.

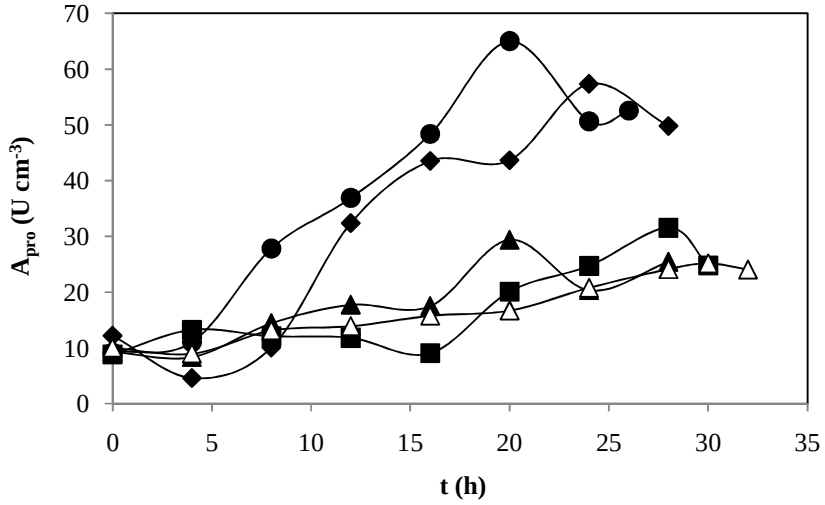


Figure 4.14 The variations in total proteases activity with the cultivation time. C_{G0}=8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *degU*-: (●); *scoC*-pH_C: (▲); *scoC*-pH_{UC}: (Δ); *sinR*-: (■).

4.4.5 Organic Acid Concentration Profiles

Organic acids detected in the medium for all strains are given in Table 4.4.a and Table 4.4.b. The organic acids detected in the medium were citric acid, gluconic acid, α-ketoglutaric acid, lactic acid, and fumaric acid. Those that have the highest concentrations for all strains were gluconic acid, and that has the lowest concentrations was fumaric acid. These are the TCA cycle organic acids. Since oxygen is regarded as main driving force in the TCA cycle and the it is utilized during the hGH synthesis, determination of these organic acids concentrations are needed to perform MFA.

By *degU* knockout strain, the highest lactic acid concentration was attained as 0.283 kg m^{-3}. Lactic acid accumulation in the medium results in growth inhibition. By

sinR knockout strain, the lowest lactic acid excretion was observed. The low lactic acid values confirm that adequate level of oxygen was supplied to the TCA cycle.

Gluconic acid synthesis requires extremely high amount of oxygen. Then, it enters PPP. However, if the glucose concentration in the medium is low, PPP is repressed and so the gluconic acid accumulates. Hence, the reason of the high gluconic acid concentration could be incomplete oxidation of glucose. The highest gluconic concentration was achieved by *aprE* knockout strain as 1.22 kg m^{-3}.

α-ketoglutaric acid, a vital organic acid in TCA cycle, was produced at the highest level by *aprE* and *scoC*-pH_C knockout strains. On the other hand, for *aprE*-pH_{UC} and *scoC*-pH_{UC}, α-ketoglutaric acid concentration was lower indicating that α-ketoglutaric acid was excreted more in controlled pH operation.

Citric acid is also an important organic acid in TCA cycle. Citric acid excretion was started at t=8, 12, 16, 20, and 24 h for *sinR*, *aprE*, *scoC*-pH_C, *scoC*-pH_{UC}, and *degU* knockout strains, respectively. It reached its maximum by *sinR* knockout strain.

Fumaric acid, isomer of malic acid, enters TCA cycle; hence, accumulation of it means that cycle does work sufficiently. It was in the negligible level for all strains confirming there is no bottleneck in the fumaric acid step of TCA cycle.

The variations in total organic acid concentrations can be seen in Figure 4.15. The highest total organic production was observed at *aprE* knockout strain as 1.31 kg m^{-3}. The lowest total organic acid concentration was obtained by *scoC*-pH_{UC} as 0.46 kg m^{-3}, respectively.

Table 4.4.a The variations in organic acid concentration in the fermentation broth with the cultivation time for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains

Knockout Strains	Time (h)	Citric Acid (kg m^{-3})	Fumaric Acid (kg m^{-3})	Gluconic Acid (kg m^{-3})	Glutaric Acid (kg m^{-3})	Lactic Acid (kg m^{-3})
	0.5	0.0000	0.0000	0.0000	0.0000	0.0000
	4	0.0000	0.0006	0.3315	0.0010	0.0000
	8	0.0000	0.0016	0.3296	0.0025	0.2285
degU	12	0.0000	0.0012	0.5672	0.0050	0.2830
	16	0.0000	0.0012	0.3895	0.0054	0.1829
	20	0.0000	0.0008	0.4646	0.0048	0.1330
	24	0.0184	0.0002	0.5130	0.0041	0.1302
	0.5	0.0000	0.0000	0.0000	0.0005	0.0000
	4	0.0000	0.0000	0.1446	0.0020	0.0000
	8	0.0000	0.0000	0.1788	0.0011	0.0000
aprE	12	0.0325	0.0026	0.2420	0.0037	0.3087
	16	0.0451	0.0003	0.3440	0.0017	0.1364
	20	0.0177	0.0009	0.5513	0.0024	0.0000
	24	0.0288	0.0000	0.6492	0.0045	0.0000
	28	0.0518	0.0000	1.2239	0.0155	0.0000
	0.5	0.0000	0.0000	0.0000	0.0000	0.0000
	4	0.0000	0.0003	0.0646	0.0007	0.0105
	8	0.0000	0.0004	0.0924	0.0007	0.0167
***scoC*-pH_C**	12	0.0000	0.0006	0.3212	0.0021	0.0217
	16	0.0243	0.0009	0.3652	0.0051	0.0357
	20	0.0286	0.0008	0.4061	0.0087	0.0288
	24	0.0322	0.0007	0.5448	0.0146	0.0292

Table 4.4.b The variations in organic acid concentration in the fermentation broth with the cultivation time for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains

Knockout Strains	Time (h)	Citric Acid (kg m^{-3})	Fumaric Acid (kg m^{-3})	Gluconic Acid (kg m^{-3})	Glutaric Acid (kg m^{-3})	Lactic Acid (kg m^{-3})
	0.5	0.0000	0.0000	0.0000	0.0000	0.0000
	4	0.0000	0.0006	0.1185	0.0000	0.0127
	8	0.0000	0.0009	0.1176	0.0000	0.0433
	12	0.0000	0.0008	0.1259	0.0000	0.0469
***scoC*-pH_{UC}**	16	0.0000	0.0008	0.2348	0.0000	0.0148
	20	0.0254	0.0000	0.0000	0.0003	0.0136
	24	0.0287	0.0006	0.3794	0.0003	0.0499
	28	0.0416	0.0008	0.2680	0.0004	0.0385
	30	0.0309	0.0006	0.4118	0.0003	0.0410
	0.5	0.0000	0.0009	0.0000	0.0000	
	4	0.0000	0.0001	0.1411	0.0024	
	8	0.0496	0.0003	0.2665	0.0038	
	12	0.0804	0.0004	0.3392	0.0047	
sinR	16	0.0568	0.0004	0.2228	0.0060	
	20	0.0752	0.0006	0.7710	0.0083	
	24	0.0612	0.0004	0.7227	0.0076	
	26	0.0572	0.0003	0.4514	0.0061	
	30	0.0673	0.0004	0.4673	0.0091	

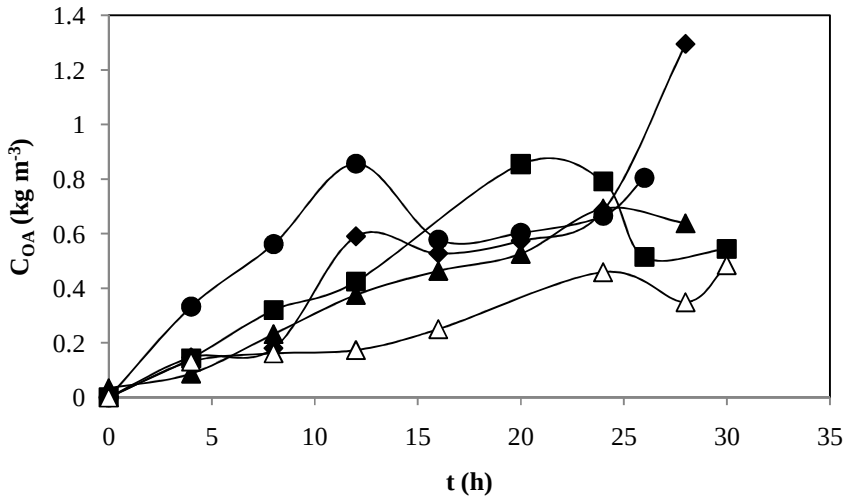


Figure 4.15 The variations in total organic acid concentration with the cultivation time. C_{G0}=8.0 kg m^{-3}, T=37°C, V_R= 1.1 dm^3, N=750 min^{-1}, Q_0/V_R=0.5 vvm. *aprE*-: (♦); *degU*-: (●); *scoC*-pH_C: (▲); *scoC*-pH_{UC}: (Δ); *sinR*-: (■).

4.4.6 Amino Acid Concentration Profiles

For cell to perform its metabolic functions and so the biomolecule production, amino acids which are intermediates of the PPP, TCA cycle, etc., and are in primary sequence of hGH should be produced. The variations in amino acid concentrations in the fermentation broth with the cultivation time for all knockout strains were given in Table 4.5.a, 4.5.b, 4.6.a, 4.6.b, 4.7.a, 4.7.b, 4.8.a., 4.8.b, 4.9.a, and 4.9.b.

The reasons why the amino acids were found in the extracellular medium are attack of proteases to the proteins in the extracellular medium (leading to degradation of proteins) and excretion of excess amino acids into the extracellular medium. The amino acids that have the highest concentration for all strains were alanine (Ala), aspartic acid (Asp), lysine (Lys), phenylalanine (Phe), and glycine (Gly). For all strains, leucine (Leu) had a negligible amount and also glutamic (Glu) was not

detected. This can be resulted from the fact that 13.6% and 7.3% of hGH were Leu and Glu, respectively.

At the early stages of the bioprocess, Leu, valine (Val), Lys, arginine (Arg), and Gly were detected in the medium. These amino acids could have been provided with the yeast extract or the medium.

The highest total amino acid concentration was measured as 0.36 kg m^{-3} by *sinR* knockout strain while the lowest one was obtained as 0.10 kg m^{-3} by *scoC*-pH_C.

Table 4.5.a The variations in amino acid concentrations in the fermentation broth with the cultivation time for *degU* knockout strain

Time (h)	Ala	Arg	Asp	Glu	Gly	His	Ile	Leu
0	0.0327	0.0014	0.0040	0	0.0043	0.002	0	0.0025
4	0	0.0050	0	0	0.0022	0	0.0029	0.0112
8	0.0084	0	0.0017	0	0.0232	0.0037	0	0.0067
12	0.0033	0.002	0.0014	0.0075	0.0176	0.0017	0	0
16	0	0	0	0	0.0093	0	0	0
20	0.0046	0	0.0013	0.0071	0.0094	0	0	0

Table 4.5.b The variations in amino acid concentrations in the fermentation broth with the cultivation time for *degU* knockout strain

Time (h)	Lys	Met	Phe	Pro	Thr	Tyr	Val	Ser+Gln
0	0.0529	0	0.0644	0	0.0024	0	0.0103	0.0146
4	0.0450	0.0192	0.0610	0	0.0002	0.0175	0.0088	0.0043
8	0.0621	0.0216	0.0651	0	0	0	0.0088	0.0181
12	0.0285	0	0.0602	0.0002	0	0	0	0.0299
16	0.0558	0	0.0657	0.0011	0	0	0.0066	0.0042
20	0.0863	0	0	0.0004	0	0	0	0.0048

Table 4.6.a The variations in amino acid concentrations in the fermentation broth with the cultivation time for *sinR* knockout strain

Time (h)	Ala	Arg	Asn	Asp	Glu	Gly	His	Ile
0	0.0022	0.0061	0	0	0	0.0073	0.0027	0.0093
4	0	0	0	0.0011	0	0.0037	0	0
8	0	0	0	0	0	0.0017	0	0
12	0	0	0	0.0019	0	0.0032	0	0
16	0	0	0.0023	0.0027	0	0.0033	0	0
20	0	0	0	0.0019	0	0.0015	0	0
24	0	0	0.003	0.0037	0	0.0012	0	0.0019
26	0	0	0	0	0	0.0018	0	0.0016

Table 4.6.b The variations in amino acid concentrations in the fermentation broth with the cultivation time for *sinR* knockout strain

Time (h)	Leu	Lys	Met	Phe	Pro	Thr	Trp	Val	Ser+Gln
0	0.0302	0.0830	0.0094	0.1027	0.0007	0.0033	0.004	0.0453	0.0091
4	0.0015	0.0380	0	0.0630	0	0	0	0	0
8	0.1033	0	0	0.0591	0	0	0	0	0.0150
12	0.0021	0.0439	0	0.0602	0	0	0	0	0.0403
16	0.0021	0.0328	0	0.0597	0	0	0	0	0.0289
20	0.0019	0.0326	0	0.0587	0	0	0	0	0
24	0	0.0396	0	0.0583	0	0.0015	0	0	0.0062
26	0.0015	0	0.02	0.0584	0.0287	0.0009	0	0.0105	0.0077

Table 4.7.a The variations in amino acid concentrations in the fermentation broth with the cultivation time for *aprE* knockout strain

Time (h)	Ala	Arg	Asn	Asp	Cys	Glu	Gly
0	0.0008	0.0011	0	0	0.0034	0	0.0116
4	0.0010	0	0	0	0	0	0.0074
8	0.0036	0	0	0	0	0.0097	0.0132
12	0.0037	0	0	0.0012	0.0032	0.0082	0.0197
16	0.0030	0	0	0.0016	0	0.0126	0.0148
20	0	0.0008	0	0.0015	0	0.0034	0.0031
24	0	0	0	0	0	0.0031	0.0032
26	0	0	0	0	0	0.0036	0

Table 4.7.b The variations in amino acid concentrations in the fermentation broth with the cultivation time for *aprE* knockout strain

Time (h)	Ile	Leu	Lys	Phe	Val	Ser+Gln
0	0.0025	0.0093	0.0516	0.0608	0.0078	0
4	0.0336	0.0063	0.0539	0.0639	0.0079	0.0028
8	0	0	0.0567	0.0640	0	0
12	0	0	0.0557	0.0659	0	0
16	0	0	0.0507	0.0608	0	0
20	0	0.0024	0.0381	0.0610	0.0089	0
24	0	0	0.0366	0.0602	0.0077	0
26	0	0	0.0405	0.0616	0.0089	0

Table 4.8.a The variations in amino acid concentrations in the fermentation broth with the cultivation time for *scoC*-pH_C

Time (h)	Ala	Arg	Asn	Asp	Glu	Gly	His
0	0.0024	0.014	0	0	0	0.0042	0.0052
4	0.0008	0	0	0	0.0038	0.0016	0.0021
8	0.0008	0	0	0.0050	0	0.0011	0.0017
12	0.0011	0	0.0042	0.0070	0	0.0030	0.0018
16	0.0040	0	0.0033	0.0063	0	0.0020	0
20	0.0096	0	0	0.0060	0.0019	0.0065	0.0031
24	0.0121	0	0	0	0.0021	0.0042	0.0043
28	0.0011	0	0	0	0	0.0048	0

Table 4.8.b The variations in amino acid concentrations in the fermentation broth with the cultivation time for *scoC*-pH_C

Time (h)	Ile	Leu	Lys	Phe	Thr	Val	Ser+Gln
0	0.0083	0.0291	0.0438	0.0657	0.00139	0.0266	0
4	0	0	0.0481	0.0653	0	0	0.0045
8	0	0	0.0378	0.0581	0	0	0
12	0	0	0.0364	0.0582	0	0	0.0115
16	0	0	0.0365	0.0576	0	0	0.0050
20	0	0	0.0448	0.0582	0	0.0126	0.0240
24	0	0	0.0459	0.0576	0	0.0088	0.0075
28	0	0	0.0466	0.0580	0	0	0.0230

Table 4.9.a The variations in amino acid concentrations in the fermentation broth with the cultivation time for *scoC*-pH_{UC}

Time (h)	Ala	Arg	Asn	Asp	Cys	Glu	Gly
0	0.0012	0.0026	0.0025	0	0	0	0.0017
4	0.0012	0.0024	0	0	0	0.0080	0.0018
8	0.0012	0.0022	0	0.0048	0	0.0035	0.0088
12	0.0020	0	0.0039	0.0161	0	0.0029	0.0015
16	0.0036	0	0.0483	0.0089	0.0179	0.0023	0.0163
20	0.0077	0	0.0029	0.0422	0	0	0
24	0.0021	0	0.0048	0.0378	0.0147	0	0.0016
28	0.0011	0	0.0056	0.0046	0	0	0.0020
32	0.0010	0	0.0079	0	0.0036	0	0.0109

Table 4.9.b The variations in amino acid concentrations in the fermentation broth with the cultivation time for *scoC*-pH_{UC}

Time (h)	Ile	Leu	Lys	Met	Phe	Val	Ser+Gln
0	0.0042	0.0129	0.0536	0	0.0626	0.015872	0.0062
4	0	0	0.0745	0	0.0600	0	0.0073
8	0	0	0.0567	0	0.0590	0	0.0673
12	0	0	0.0542	0	0.0589	0	0.0084
16	0	0	0.0522	0	0.0581	0	0
20	0	0	0.0543	0	0.0577	0	0.0095
24	0	0	0.0065	0	0.0586	0.0079	0.0078
28	0	0	0.0540	0	0.0594	0.0083	0.0155
32	0	0	0.0596	0	0.0585	0	0

4.4.7 Oxygen Transfer Characteristics

Throughout the bioprocess, several oxygen transfer parameters were determined to investigate the efficiency of the oxygen transfer and utilization. These parameters are the volumetric liquid mass transfer coefficient (K_La), enhancement factor ($E=K_La/K_La_0$), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate ($OD=\mu_{max}C_X/Y_{X/O}$), maximum possible mass transfer rate ($OTR_{max}=K_La\ C_O^*$), Damköhler number ($Da=OD/OTR_{max}$), and effectiveness factor (η= OUR/OD). The variations in these parameters for all strains are given in Table 4.10.a and 4.10.b.

K_La was calculated using the dynamic method. K_La depends on the agitation rate, bioreactor design, and rheological properties of the fermentation medium (viscosity of the medium, etc.). Since agitation rate and impeller speed did not change during the bioprocess, only factor having influence on K_La was the medium properties. High K_La values are the indicator of the high oxygen transfer meaning that growth rate and yield are high. Generally, K_La increased until t=12, 16, 16, 16, and 24 h for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains, respectively; then

decreased for all strains. Until these cultivation times, the highest growth rate and the sharpest increase in hGH concentration were observed for all strains. Average K_La values obtained at all strains were very close to each other having a range of 0.022-0.029 s^{-1}. The maximum K_La values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 0.035, 0.040, 0.043, 0.045, and 0.043 s^{-1}, respectively. By the secretion of proteins, metabolites and intermediate molecules into the fermentation broth, viscosity of the medium increased resulting in a decrease in K_La. Hence, low K_La obtained by the *aprE* knockout strain could be explained by its highest organic acid secretion.

To calculate E values, K_La_0 was determined with the medium deficient in microorganism as 0.009 s^{-1}. The trend in E profile was the same with that in K_La values, as expected. Generally, E values were slightly higher than 1.0 in the range of E=1.0-5.0. Low E values indicate that the biochemical reactions occur slowly with efficient mass transfer.

Metabolic activities of the cell, such as growth and protein and metabolite synthesis increase the OUR. At the early stages of the bioprocess, the OUR was nearly equal to the OTR since there was no oxygen accumulation. Until t=16-24 h, the OUR increased for all strains due to the increase in the cell growth and hGH production rates. Thereafter, due to the decrease in glucose consumption and hGH production rates, the OUR started to decrease. The highest OUR values obtained for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 1.50, 1.34, 2.23, 2.49, and 1.68 mmol m^{-3} s^{-1}, respectively. High OUR observed by scoC-pH_{UC} knockout strain at t=16 h could be the result of the sharp increase in the glucose consumption and hGH production rates at t=16 h. The lowest OUR values were observed at the beginning and end of the bioprocess.

During the fermentation process, the OTR changes depending on the level of OUR and K_La. As the cell growth rate increased, the OTR also increased. The maximum OTR values obtained by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 1.90, 1.84, 2.33, 2.97, and 1.98 mmol m^{-3} s^{-1}, respectively.

To determine the rate limiting steps in the bioprocess, the dimensionless numbers, Da and η are calculated. In order to determine these numbers, the maximum possible oxygen utilization rate (OD) and the maximum possible mass transfer rate (OTR_{max}) were found. OD values generally increased with the cultivation time; whereas OTR_{max} firstly increased and then decreased. The reason why OD values is so high is the fact that it is calculated assuming cells grow at the highest rate throughout the bioprocess. Thus, at the early stages of the bioprocess when the growth rate was at its maximum, OD and so the Da values are meaningful for this process. Da is the ratio of the case in which OUR is maximum, to the case in which the OTR is maximum. As Da is directly proportional to OD, Da values are also high. Da values, higher than 1, indicate mass transfer limited case whereas Da values, lower than 1, indicates the reaction limited case. Until t=4 h, process was reaction limited and then it shifted to reaction limited case. The highest Da value was obtained as 45.62 by *aprE* knockout strain, as expected since by this strain the highest organic acid concentration was obtained as well as high hGH production. These molecules in the medium create a mass transfer resistance zone.

Due to the high OD values, η values throughout the bioprocess were very low. During the first hours of the process, η values were higher than 1, indicating that cells consumed oxygen at a high rate. As the bioprocess proceeds, η values are lower than 1 indicating that the cells consumed less oxygen than the oxygen demand. The lowest η value was obtained by *aprE* knockout strain as 0.005.

Besides, there is an inverse relation between Da and η. As one of them increases, the other decreases, or vice versa.

Table 4.10.a The variations in oxygen transfer characteristics for all knockout strains

Knockout Strains	t (h)	K_La (s^{-1})	E k_La/k_La_0	$OTRx10^3$ ($molm^{-3}s^{-1}$)	$OTR_{max}x10^3$ ($molm^{-3}s^{-1}$)	$OURx10^3$ ($molm^{-3}s^{-1}$)	$OD\ x10^3$ ($molm^{-3}s^{-1}$)	Da	η
	0.5	0.009	1.00	0.95	2.03	0.85	0.80	0.39	1.069
	4	0.016	1.78	0.74	3.62	0.54	15.89	4.39	0.034
	8	0.020	2.21	0.92	4.50	0.62	23.44	5.21	0.026
degU	12	0.024	2.67	1.34	5.42	0.94	44.75	8.25	0.021
	16	0.040	4.44	1.84	9.04	1.34			
	20	0.024	2.67	0.86	5.42	0.66			
	24	0.020	2.22	0.72	4.52	0.58			
	0.5	0.016	1.72	1.02	3.50	0.92	0.40	0.11	2.312
	4	0.022	2.44	1.45	4.97	1.35	17.71	3.56	0.076
	8	0.017	1.89	0.95	3.84	0.85	175.26	45.62	0.005
	12	0.035	3.89	1.26	7.91	1.16	159.42	20.15	0.007
aprE	16	0.026	2.89	1.20	5.88	1.10	73.15	12.45	0.015
	20	0.021	2.36	1.82	4.79	1.22	103.76	21.66	0.012
	24	0.025	2.78	1.90	5.65	1.50			
	28	0.022	2.44	1.01	4.97	0.71			
	0.5	0.015	1.67	1.29	3.39	1.19	0.50	0.15	2.375
	4	0.022	2.44	2.33	4.97	2.23	6.90	1.39	0.323
	9	0.030	3.33	1.68	6.78	1.38	14.56	2.15	0.095
***scoC*-pH_C**	13	0.031	3.44	1.36	7.01	0.96	23.63	3.37	0.041
	16	0.043	4.78	2.28	9.72	1.80	57.90	5.96	0.031
	20	0.034	3.78	2.28	7.68	1.98	70.20	9.14	0.028
	26	0.022	2.44	1.01	4.97	0.91			

Table 4.10.b The variations in oxygen transfer characteristics for all knockout strains

Knockout Strains	t (h)	K_La (s^{-1})	E k_La/k_La_0	OTRx10^3 ($molm^{-3}s^{-1}$)	OTR_{max}x10^3 ($molm^{-3}s^{-1}$)	OURx10^3 ($molm^{-3}s^{-1}$)	OD x10^3 ($molm^{-3}s^{-1}$)	Da	η
	0.5	0.012	1.33	0.79	2.71	0.692	0.40	0.15	1.736
	4	0.024	2.67	1.10	5.42	0.904	6.97	1.28	0.130
	8	0.029	3.22	2.20	6.55	1.904	20.16	3.08	0.094
	12	0.034	3.78	2.24	7.68	1.844	42.81	5.57	0.043
	16	0.045	5.00	2.97	10.17	2.49	83.36	8.20	0.030
***scoC*-pH_{UC}**	20	0.029	3.22	1.62	6.55	1.324	99.08	15.12	0.013
	24	0.022	2.44	1.01	4.97	0.612	140.18	28.19	0.004
	28	0.022	2.44	1.45	4.97	0.952			
	30	0.017	1.89	0.95	3.84	0.852			
	32	0.013	1.44	0.99	2.94	0.788			
	0.5	0.013	1.44	0.99	2.94	0.89	0.71	0.24	1.257
	4	0.017	1.89	1.12	3.84	0.92	8.63	2.25	0.107
	8	0.025	2.78	1.40	5.65	1.20	32.25	5.71	0.037
	12	0.021	2.33	1.18	4.75	0.88	45.76	9.64	0.019
sinR	16	0.030	3.33	1.38	6.78	1.08	43.23	6.38	0.025
	20	0.041	4.56	1.89	9.27	1.49			
	24	0.043	4.78	1.98	9.72	1.68			
	28	0.026	2.89	0.94	5.88	0.74			
	30	0.024	2.67	1.10	5.42	0.90			

4.4.8 Specific Rates, and Yield Coefficients

The variations in the fermentation characteristics in terms of the specific rates are given in Table 4.11.a and 4.11.b. These are the specific growth rate (μ), specific glucose (substrate) consumption rate (q_S) and the specific hGH production rate (q_P).

μ for all strains decreased throughout the bioprocess. The maximum μ values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 1.56, 1.74, 1.45, 0.95, and 1.31 h^{-1}, respectively. These values are attained at the first hours of the bioprocess.

q_O generally decreased throughout the bioprocess, as expected, since cells need more oxygen at the beginning of exponential growth phase. The maximum q_O values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 0.67, 1.39, 0.84, 0.62, and 0.74 g g^{-1} h^{-1}, respectively.

q_S firstly decreased with time, however, then by the sharp increase in q_P at t=12-24 h, q_S started to increase again. The highest values were obtained at the first hour of process as 17.34, 7.62, 12.34, 13.61, and 3.32 g g^{-1} h^{-1} by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains.

The trend in q_P with time is first increase, then decrease and again increase. The highest values were obtained as 11.28, 7.06, 8.42, 5.39, and 6.52 g kg^{-1} h^{-1} by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains.

The maximum $Y_{X/S}$ values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were attained as 0.20, 0.23, 0.29, 0.34, and 0.39 g g^{-1}. On the other hand, maximum $Y_{X/O}$ values were obtained at the beginning of the process as 2.32, 1.26, 1.72, 1.34, and 1.74 g g^{-1} for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains. The highest $Y_{P/S}$ values were 34.41, 21.35, 101.07, 19.26, and 22.37 g kg^{-1} for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains. Shuler and Kargi (1992) reported the typical $Y_{X/S}$, and $Y_{X/O}$ values range for the bacteria as 0.4-0.6 g g^{-1} and 0.9-1.4 g g^{-1}, respectively. Except the *scoC*-pH_C knockout strain, strains used glucose inefficiently according to $Y_{X/S}$ after the early stages of process. $Y_{X/O}$

values decreased and were very low. This could be the result of insufficient oxygen supply.

The overall product yield on total substrate ($Y_{P/S}$') for *scoC*-pH_C was determined as 16.12 g kg^{-1} which was 1.45-, 1.76-, 1.61-, and 1.58- fold higher than those of *aprE, degU, scoC*-pH_{UC}, and *sinR* knockout strains. On the other hand, the overall cell yield on total substrate ($Y_{X/S}$') for *scoC*-pH_C was determined as 0.15 g g^{-1} which was 1.82-, 2.98-, 1.47-, and 1.06-fold higher than those of *aprE, degU, scoC*-pH_{UC}, and *sinR* knockout strains. These findings show that glucose was used more efficiently in *scoC*-pH_C case.

Table 4.11.a The variations in fermentation characteristics in terms of specific rates and yields for all knockout strains

Knockout Strains	t (h)	μ (h^{-1})	q_O (g g^{-1} h^{-1})	q_P*10^{-3} (g g^{-1} h^{-1})	q_S (g g^{-1} h^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/S}*10^{-3}$ (g g^{-1})	$Y_{X/O}$ (g g^{-1})
	0.5	1.74	1.39		7.62	0.23		1.26
	4	0.10	0.20		0.84	0.12		0.50
	8	0.06	0.19	5.07	0.78	0.08	6.48	0.31
degU	12	0.04	0.15	6.96	0.42	0.09	16.73	0.26
	16	-0.03	0.15	4.87	0.35	0.10	13.83	
	20	-0.07	0.12	7.06	0.33	0.21	21.35	
	24	-1.09	0.17	1.98	0.30	3.64	6.61	
	0.5	1.56	0.67		17.34	0.09		2.32
	4	0.11	0.24	4.21	0.99	0.11	4.24	0.44
	8	0.01	0.27	11.28	0.46	0.03	24.51	0.04
	12	0.01	0.22	7.88	0.39	0.03	20.11	0.05
aprE	16	0.02	0.21	3.29	0.33	0.07	9.85	0.11
	20	0.02	0.20	3.35	0.10	0.20	34.41	0.10
	24	-0.02	0.16	4.58	0.14	0.12	33.48	
	28	-0.03	0.19		0.51			
	0.5	1.45	0.84		12.34	0.12		1.72
	4	0.21	0.24	3.42	0.72	0.29	4.76	0.87
	9	0.10	0.13	7.81	0.37	0.27	21.35	0.75
***scoC*-pH_C**	13	0.06	0.09	8.42	0.39	0.14	21.47	0.62
	16	0.03	0.10	2.22	0.25	0.12	9.02	0.30
	20	0.02	0.08	2.92	0.10	0.21	29.28	0.27
	24	-0.03	0.06	0.86	0.01	3.19	101.07	

Table 4.11.b The variations in fermentation characteristics in terms of specific rates and yields for all knockout strains

Knockout Strains	t (h)	μ (h^{-1})	q_O ($g\ g^{-1}\ h^{-1}$)	q_P*10^{-3} ($g\ g^{-1}\ h^{-1}$)	q_S ($g\ g^{-1}\ h^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	$Y_{P/S}*10^{-3}$ ($g\ g^{-1}$)	$Y_{X/O}$ ($g\ g^{-1}$)
scoC-pH_{UC}	0.5	0.95	0.62	0.00	13.61	0.07	0.00	1.54
	4	0.23	0.55	5.39	1.46	0.16	3.70	0.42
	8	0.08	0.28	4.47	0.25	0.34	17.88	0.30
	12	0.03	0.17	2.25	0.18	0.16	12.77	0.17
	16	0.01	0.16	4.24	0.35	0.04	11.96	0.09
	20	0.01	0.17	4.46	0.32	0.04	13.96	0.08
	24	0.01	0.10	1.87	0.10	0.06	19.26	0.06
	28	-0.06	0.10	2.78	0.01		250.30	
	30	-0.05	0.15	4.24	0.00			
	32	0.03	0.16					
sinR	0.5	1.31	0.74	0.00	3.32	0.39	0.00	1.74
	4	0.15	0.17	6.52	0.64	0.24	10.19	0.89
	8	0.03	0.10	5.62	0.37	0.09	15.34	0.32
	12	0.03	0.12	1.99	0.29	0.10	6.82	0.23
	16	0.03	0.10	2.70	0.17	0.18	16.00	0.30
	20	0.00	0.10	2.12	0.09	0.02	22.37	0.02
	24	-0.01	0.11	1.27	0.29	0.03	4.44	
	28	-0.02	0.08	1.94	0.14	0.11	13.57	
	30	-0.03	0.10		0.11			

4.4.9 The Effect of Controlled-pH Strategy on hGH Production

To investigate the effect of pH control on the hGH production, both uncontrolled (pH_{UC}) and controlled (pH_C) pH strategy were applied by using *scoC* knockout strain. In the literature, hGH was produced by *E. coli* at controlled pH condition; contrarily, by *B. subtilis* at uncontrolled pH condition, without investigation of the favorable pH strategy.

According to the findings, in controlled pH operation, higher cell and hGH concentration were achieved with higher yields. Maximum cell concentration obtained at *scoC*-pH_C was 1.57-fold higher than that at *scoC*-pH_{UC}. Furthermore, the highest hGH concentration achieved at *scoC*-pH_C case was 1.71-fold higher than those obtained at *scoC*-pH_{UC}. More organic acid secretion was observed at *scoC*-pH_C also. This can be explained by H^+ transfer outside the cell to keep the intracellular pH at 7.5. at uncontrolled pH operation, pH of the medium decreased below 7.5 therefore organic acid secretion is less than that of pH controlled operation.

Furthermore, results of hGH production study by *aprE* knockout strain in controlled pH condition was compared with those obtained by Yılmaz (2008) at the same conditions but in uncontrolled pH condition. While the maximum cell concentration obtained at uncontrolled pH operation was 1.21-fold higher than that at controlled pH operation, 1.14- fold higher hGH production was achieved in controlled pH operation. Again, in controlled pH operation, higher amount of organic acids was secreted into the medium.

Considering these findings, it can be said that controlled pH strategy was favored for hGH production by *B. subtilis*.

CHAPTER 5

CONCLUSIONS

In this study, the regulatory gene effects on the rhGH production by *B. subtilis* were investigated considering the effect of the pH control. Firstly, *Bacillus* strains whose certain genes are deleted were chosen according to the regulatory network of *aprE* gene. Then, with the recombinant *Bacillus* strains constructed by transformation of pMK4::pre(*subC*)::*hGH* plasmid, strains having the highest hGH production capacity were determined by the fermentation experiments at the laboratory-scale air-filtered stirred bioreactors. Thereafter, by the selected strains, hGH production experiments were performed in pilot-scale bioreactors using the glucose-based defined medium. Also, for the *scoC* knockout strain, the effect of pH control was investigated by considering the cell, hGH, organic acid, glucose and protease concentrations as well as the oxygen transfer characteristics.

In order to determine the performance of the gene-deficient strains having the highest hGH production capacity, and the protease inhibitor addition time, the fermentation experiments were carried out in the defined medium (8 kg m^{-3} glucose, 4.71 kg m^{-3} $(NH_4)_2HPO_4$, 2.0 kg m^{-3} KH_2PO_4, 0.043 kg m^{-3} Na_2HPO_4 and 5.63 kg m^{-3} NaH_2PO_4) at 37°C, and 200 min^{-1} at the laboratory-scale air-filtered stirred-bioreactors 500 cm^3 in size that had working volume capacities of 110 cm^3. Initial pH (pH_0) was adjusted to 7.5 with the addition of 10 M KOH. Protease inhibitor addition times were determined as t=12, 7, 9, 6, 6, 7, 8, 9, and 7 for *abrB*, *aprE*, *degQ*, *degS*, *degU*, *scoC*, *sinI*, *sinR*, and *spo0A* knockout strains, respectively. Protease inhibitor were added to the medium at the determined cultivation times and the highest cell concentration was obtained by the *scoC* knockout strain as 64 g m^{-3} which was 3.2-fold higher than that produced by *aprE* knockout strain (20 g m^{-3}). The concentrations of the rhGH produced by *aprE* and *sinR* knockout strains were close to each other.

The maximum cell concentrations were attained as 0.9, 1.14, and 1.47 kg m^{-3} at t=26, 30, and 32 h by *aprE*, *sinR* and *scoC* knockout strains, respectively.

Between the hGH and cell concentrations, there was no direct proportionality. That is, although *sinR* knockout strain attained higher cell concentration than *aprE* knockout strain, *aprE* strain produced higher amount of hGH. Maximum specific hGH production rates (q_{hGH}) for *scoC*, *aprE*, and *sinR* knockout strains are 1.72, 1.54, and 0.72 kg kg^{-1} CDW h^{-1}, respectively. In addition to those strains, *degU* knockout strain was chosen also, since it was thought that signal sequence was not cleaved considering the band above the one having molecular weight of hGH.

After selection of the host *B. subtilis* strains, the effect of the regulatory genes on hGH production was investigated considering the protein production, cell, glucose, amino acid and organic acid concentrations with protease activity at the pilot-scale bioreactor which makes pH and oxygen control possible. The temperature, aeration and agitation rates were kept constant at T=37°C, Q_0/V_R=0.5 vmm, and N=750 min^{-1}. In the controlled pH condition, four sets of batch bioreactor experiments were carried out for the selected strains. For *scoC* knockout strain, both controlled pH and uncontrolled pH strategies were applied. pH control was achieved manually. Initial pH (pH_0) was adjusted to 7.5 by 10 M KOH.

Effect of pH control on the hGH production was investigated by *scoC* knockout strain. In the controlled pH operation, higher cell and hGH concentrations were achieved with higher yields. The maximum cell concentration obtained at *scoC*-pH_C was 1.57-fold higher than that at *scoC*-pH_{UC}. Moreoever, the highest hGH concentration achieved at *scoC*-pH_C case was 1.71-fold higher than those obtained at *scoC*-pH_{UC}. More organic acid secretion was observed at *scoC*-pH_C also. Furthermore, the results of hGH production study by *aprE* knockout strain in the controlled pH condition was compared with those obtained by Yılmaz (2008) at the same operation conditions but at uncontrolled pH condition. While the maximum cell concentration obtained at uncontrolled pH operation was 1.21-fold higher than that at controlled pH operation, 1.14- fold higher hGH production was achieved in the controlled pH

operation. Again, in the controlled pH operation, higher amount of organic acids was excreted into the bioreactor production medium. These findings show that controlled pH strategy was favored for hGH production by *B. subtilis*. Hence, for the other strains, controlled pH strategy was applied.

At all the experiments, until t=4 h where pH decreased due to the cell growth; and this trend continued until t=23-29 h where the cells enter stationary growth phase and a sharp increase in hGH production was observed. At *scoC*-pH_{UC} case, maximum pH difference was determined as ΔpH=0.47. By *aprE*-pH_{UC} (Yılmaz, 2008), the maximum pH difference (ΔpH) was reported as 0.81. At t=31 h, medium pH became constant for *aprE* and *scoC*-pH_{UC} knockout strains as 6.97 and 7.28, as respectively.

Until t=6 h where the cell concentration increases sharply, C_O decreased at all cases. The sharpest decrease in C_O was obtained by *sinR* knockout strain. The cell concentration obtained by *sinR* knockout strain at t=6 h was 1.16-, 1.28-, 1.42-, and 1.89- fold higher than those obtained by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC} knockout strains, respectively. Besides, at these early cultivation times, the maximum hGH production was attained by *sinR* knockout strain. From t=6 h until t=20-26 h, the dissolved oxygen concentration deviated around the same value. After t=20-26 h when the cells entered stationary growth phase, C_O started to increase. At the end of the bioprocess, the lowest C_O was obtained as 0.181 mol m^{-3} at the *scoC*-pH_{UC} case, while the highest C_O was obtained as 0.193 mol m^{-3} by the *sinR* knockout strain. Generally, C_O values obtained by *aprE*-pH_{UC} (Yılmaz, 2008) was lower than those obtained by this study owing to the higher cell and hGH concentrations obtained by the gene knockout strains used in this study.

Until t=6-8 h when the protease inhibitor was added to the medium, the cell growth rate was high, then cell concentration became stationary for a while, and then started to increase. The maximum cell concentration was obtained at *scoC*-pH_C as 1.62 kg m^{-3} which is 1.34-, 1.57-, 2.15-, 2.17- fold higher than the maximum cell concentration attained by *sinR*, *scoC*-pH_{UC}, *degU*, and *aprE*, respectively. Yılmaz

(2008) reported the maximum cell concentration as 0.90 kg m^{-3} for *aprE*-pH_{UC}, which is generally lower than the cell concentrations obtained in this study.

At the early stages of the bioprocess when the cell growth rate was high (t=0-6 h), almost 50% of glucose in the medium was consumed for all the strains. During the first four hours of the bioprocess, the glucose consumption was lower for *sinR* knockout strains whereas the cell growth rate was higher than those obtained by the other strains. The lowest glucose concentration was attained at t=28 h by scoC-pH_{UC} as 0 kg m^{-3} corresponding to consumption of 100% of the initial glucose in the medium. 94, 95, 98 and 99% of the initial glucose in the medium was consumed by the *sinR*, *degU*, *scoC*-pH_C, and *aprE* knockout strains, respectively. By the addition of protease inhibitor glucose consumption rate decreased as well as the cell growth rate. The glucose consumption profiles of the strains were not similar to each other since cell concentrations attained at the same time were different for different strains.

The time when the highest hGH production was attained in the laboratory scale experiments shifted 4 and 2 hours forth for *aprE*, and *sinR* knockout strains in pilot-scale bioreactor experiments, respectively. For *scoC* knockout strain, this time did not change for *scoC*-pH_C case, while it shifted 4 hour ahead for *scoC*-pH_{UC} case. As compared with the laboratory scale bioreactor results, in bioreactors the hGH production capacity increased at least 1.15-fold. The maximum hGH concentration was obtained by *scoC*-pH_C at t=24 h as 126 g m^{-3} which is 1.59-, 1.62-, 1.70-, and 1.71- fold higher than those obtained by *aprE*, *sinR*, *degU* strains and *scoC*-pH_{UC} case, respectively. When the hGH concentration reached its maximum value, the cells entered the stationary phase and the glucose consumption rate approaches to zero. The sharpest increase in hGH production rate was observed at t=8, 12, 8, 12, and 8 h as 6.33, 5.26, 5.21, 9.77, and 3.30 g m^{-3} h^{-1} for *aprE*, *degU*, *sinR* knockout strains, and *scoC*-pH_C, *scoC*-pH_{UC} cases, respectively. The production rate first decreased and after t=23-29 h it started to increase by all the strains. In the literature, the maximum hGH production by *Bacillus* species was reported as 70 g m^{-3} by the study of Yılmaz

(2008). In this study, however hGH production was increased at least 1.06-fold and utmost 1.82-fold.

Total protease concentrations are the sum of the concentrations of the acidic, alkali and neutral proteases. The highest total protease concentration was attained at t=20 h by *degU* knockout strain as 65 U cm^{-3} which was 1.15-, 2.06-, 2.25-, and 2.62-fold higher than those obtained by *aprE, sinR, scoC*-pH_C, and *scoC*-pH_{UC} knockout strains. The lowest hGH concentration was obtained by *degU* strain which has the highest protease activity. Specific protease production rate (q_{pro}) for *degU* knockout strain is also the highest as 0.16 kg kg^{-1} CDW h^{-1}. Although the total protease activity is high for *aprE* knockout strain, the fact that q_{pro} is low as 0.09 kg kg^{-1} CDW made high hGH production possible for this strain. Total protease concentration increased sharply during the first eight hours of the bioprocess, then by the addition of the protease production rate of proteases decreased. At t=16-20 h, protease production rate again, increases.

The organic acids detected were gluconic acid, α-ketoglutaric acid, lactic acid, citric acid, and fumaric acid. Those that have the highest concentrations for all strains were gluconic acid, and that has the lowest concentrations was α-ketoglutaric acid. These are the TCA cycle organic acids. By *degU* knockout strain, the highest lactic acid concentration was attained as 0.283 kg m^{-3}. By sinR knockout strain, the lowest lactic acid excretion was observed. The low lactic acid values confirm that adequate level of oxygen was supplied to the TCA cycle. The highest gluconic acid concentration was achieved by *aprE* knockout strain as 1.22 kg m^{-3}. α-ketoglutaric acid, a vital organic acid in TCA cycle, was produced at the highest level by *aprE* and *scoC*-pH_C knockout strains. On the other hand, for *aprE*-pH_{UC} and *scoC*-pH_{UC}, α-ketoglutaric acid concentration was lower indicating that α-ketoglutaric acid was excreted more in controlled pH operation. Citric acid excretion was started at t=8, 12, 16, 20, and 24 h for *sinR*, *aprE*, *scoC*-pH_C, *scoC*-pH_{UC}, and *degU* knockout strains, respectively. It reached its maximum by *sinR* knockout strain. Fumaric acid was in the negligible level for all strains confirming there is no bottleneck in the fumaric acid

step of TCA cycle. The highest total organic production was observed at *aprE* knockout strain as 1.31 kg m^{-3}. The lowest total organic acid concentration was obtained by *scoC*-pH_{UC} as 0.46 kg m^{-3}, respectively.

Generally, K_La increased until t=12, 16, 16, 16, and 24 h for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains, then decreased for all strains. Average K_La values obtained at all strains were very close to each other having a range of 0.022-0.029 s^{-1}. The maximum K_La values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 0.035, 0.040, 0.043, 0.045, and 0.043 s^{-1}, respectively.

The trend in E value profile was the same with that in K_La values, as expected. Generally, E values were slightly higher than 1.0 in the range of E=1.0-5.0.

At the early stages of the bioprocess, the OUR was nearly equal to the OTR since there was no oxygen accumulation. Until t=16-24 h, OUR increased for all strains due to the increase in cell growth and hGH production rates. Thereafter, due to decrease in the glucose consumption and hGH production rates, the OUR started to decrease. The highest OUR values obtained for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 1.50, 1.34, 2.23, 2.49, and 1.68 mmol m^{-3} s^{-1}, respectively. The lowest OUR values were observed at the beginning and end of the bioprocess.

The maximum OTR values obtained by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 1.90, 1.84, 2.33, 2.97, and 1.98 mmol m^{-3} s^{-1}, respectively.

OD values generally increased with the cultivation time; whereas OTR_{max} firstly increased and then decreased. Until t=4 h, the process was oxygen transfer limited and then shifted to reaction limited condition. The highest Da value was obtained as 45.62 by *aprE* knockout strain.

Due to the high OD values, η values throughout the bioprocess were very low. During the first hours of the process, η values were higher than 1, indicating that cells consumed oxygen at a high rate. At the later times of the bioprocess, η values are

quite lower than 1 indicating that the cells consumed less oxygen than oxygen demand. The lowest η value was obtained by *aprE* knockout strain as 0.005.

μ for all strains decreased throughout the bioprocess. The maximum μ values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 1.56, 1.74, 1.45, 0.95, and 1.31 h^{-1}, respectively.

q_O generally decreased throughout the bioprocess. The maximum q_O values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were 0.67, 1.39, 0.84, 0.62, and 0.74 g g^{-1} h^{-1}, respectively.

q_S firstly decreases with the cultivation time, however, then by a sharp increase in q_P at t=12-24 h, q_S started to increase again. The highest values were obtained at the first hours of process as 17.34, 7.62, 12.34, 13.61, and 3.32 g g^{-1} h^{-1} by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains.

Througout the cultivation time, q_P first increases, then decreases and again increases. The highest values were obtained as 11.28, 7.06, 8.42, 5.39, and 6.52 g kg^{-1} h^{-1} by *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains.

The maximum $Y_{X/S}$ values for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains were attained as 0.20, 0.23, 0.29, 0.34, and 0.39 g g^{-1}. On the other hand, maximum $Y_{X/O}$ values were obtained at the beginning of the process as 2.32, 1.26, 1.72, 1.34, and 1.74 g g^{-1} for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains. The highest $Y_{P/S}$ values were 34.41, 21.35, 101.07, 19.26, and 22.37 g kg^{-1} for *aprE*, *degU*, *scoC*-pH_C, *scoC*-pH_{UC}, and *sinR* knockout strains. Shuler and Kargi (1992) reported the typical $Y_{X/S}$, and $Y_{X/O}$ values range for the bacteria as 0.4-0.6 g g^{-1} and 0.9-1.4 g g^{-1}, respectively. Except the *scoC*-pH_C knockout strain, strains used glucose inefficiently according to $Y_{X/S}$ after the early stages of the process. $Y_{X/O}$ values are very low as this could be the result of insufficient oxygen supply.

In this study, the influences of the regulatory genes on the recombinant human growth hormone production by *Bacillus subtilis* carrying pMK4::*pre*(*subC*)::*hGH* plasmid were investigated together with the bioreactor operation conditions. *scoC*

knockout *B. subtilis* at controlled pH condition was concluded, respectively, as the favorable *B. subtilis* strain and the bioreactor operation condition.

To be able determine the relation of expression of regulation genes with hGH production, microarray studies could be performed for all strains. Also, by MFA reaction rates took place within the cell can be found and which reactions rates increased or decreased can be known when the specific genes are expressed by considering the gene expression data. According to these findings, further gene deletions can be performed. Furthermore, to enhance hGH production, effect of initial glucose concentration could be investigated on the process by trying values higher than C_{G0}=8 kg m^{-3} since most of the glucose in the medium was consumed at C_{G0}=8 kg m^{-3} for all strains.

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APPENDIX A

CONTENTS OF THE KITS

Fermentas GeneJET™ Plasmid Miniprep Kit :

- Resuspension solution
- Lysis solution
- Neutralization solution
- Wash solution
- RNase A
- Elution buffer
- GeneJET™ spin columns
- Collection tubes (2 mL)

Glucose Analysis Kit (Biyozim) :

- Glucose analysis reagent
- Glucose analysis buffer
- 0.5 g glucose

Preparation of standard glucose solutions :

1. Stock glucose solution with concentration of 10 kg m^{-3} was prepared by diluting 0.1 g glucose to 10 ml with dH_2O.
2. Then, by diluting this stock solution, glucose solutions with concentration of 0.25, 0.50, 0.75, and 1.00 kg m^{-3} were prepared.

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

10X TBE	
Tris base	108 kg m^{-3}
Boric acid	55 kg m-3
EDTA	9.3 kg m-3
Autoclave for 20 min	

1 mM HEPES	
HEPES	0.024 g
dH_2O	100 mL
Adjust pH to 7.0 with NaOH	
Filter	

Cold Electroporation Buffer	
PEG	25%
D-Mannitol	0.1 M

TSE

Tris-HCl, pH=8.0	10 mM
NaCl	300 mM
EDTA	10 mM

Solution I

Tris-HCl, pH=8.0	10 mM
EDTA	10 mM
NaCl	50 mM
Sucrose	8% (w/v)

Solution II

SDS	1% (w/v)
NaOH	0.2 M

Solution III

Potassium acetate	5 M
Acetic acid	60 mL
dH_2O	Complete up to 100 mL

50 mM Borate Buffer (pH=10)

$Na_2B_4O_7.10H_2O$	0.4763 g
Milli Q water	25 mL

pH is adjusted to 10 by 1 M NaOH

Filter

Electrolyte Solution

$Na_2B_4O_7.10H_2O$	0.4763 g
Z-1 Methyl reagent	4.531 g
Milli Q water	25 ml

pH is adjusted to 10 by 1 M NaOH

Filter

Resolving Gel (12%)	
Water	3.4 mL
30% Acrylamide/ bis	4.0 mL
1.5 M Tris-HCl, pH=8.8	2.5 mL
10% SDS	0.1 mL
10% APS	50 µL
TEMED	5 µL

Stacking Gel (5%)	
Water	3.4 mL
30% Acrylamide/ bis	2.8 mL
0.5 M Tris-HCl, pH=6.8	0.85 mL
10% SDS	50 µL
10% APS	25 µL
TEMED	5 µL

4X Sample Loading Buffer

1M Tris-HCl, pH=6.8 (200mM)	8 mL
Glycerol (40%)	16 mL
SDS (6%)	2.4 g
1% Bromophenol blue(0.013%)	0.5 mL
dH_2O	12 mL
TEMED	5 µL

Before using, add 10% 2-mercaptoethanol

5X SDS-PAGE Running Buffer

Tris Base (25 mM)	15 g
Glycine (192 mM)	72 g
SDS (0.1 %)	5 g
Water to	1000 mL

Fixer Solution

Methanol	150 mL
Acetic acid	36 mL
Formaldehyde	150 µL
dH_20 up to	300 mL

Pretreatment Solution

Sodium thiosulphate	0.08 g
dH_20 up to	400 mL

Silver Nitrate Solution

Silver nitrate	0.8 g
37% formaldehyde	300 µL
dH_2O	400 mL

Developing Solution

Potassium carbonate	9 g
Pretreatment solution	8 mL
37% formaldehyde	300 µL
dH_2O	400 mL

Stop Solution

Methanol	200 mL
Acetic acid	48 mL
dH_2O up to	400 mL

APPENDIX C

MOLECULAR WEIGHT MARKERS

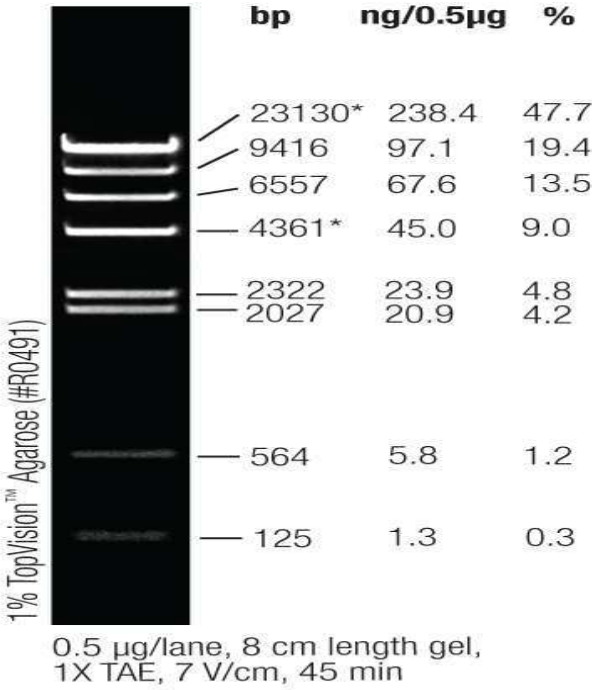

Figure C.1 λ DNA Hind III Marker (Fermentas AB)

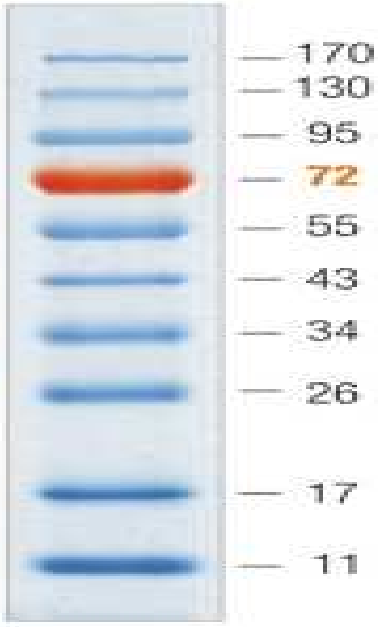

Figure C.2 PageRuler™ Prestained Protein Ladder (Fermentas AB)

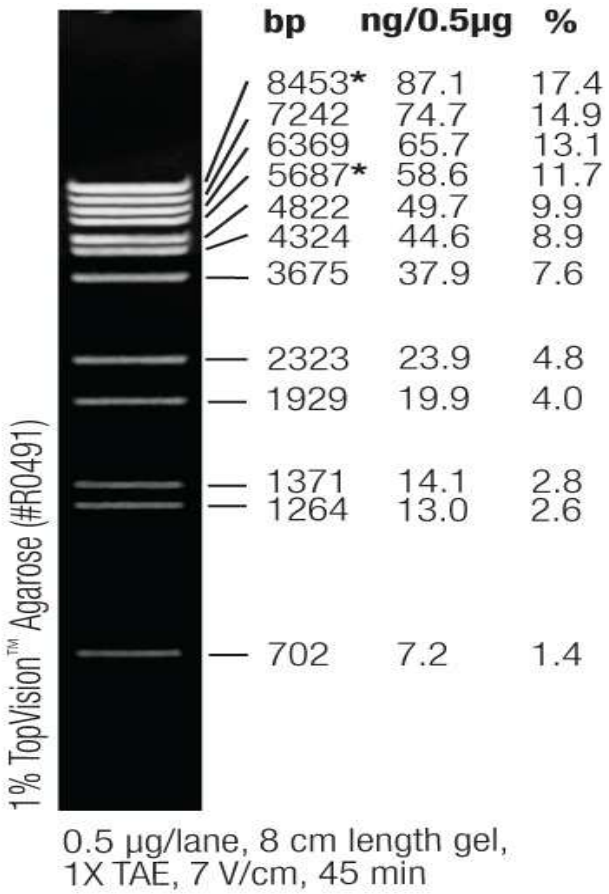

Figure C.3 λ DNA/Eco91I (BstEII) Marker (Fermentas AB**)**

APPENDIX D

CALIBRATION CURVES

Calibration Curve for Cell Concentration :

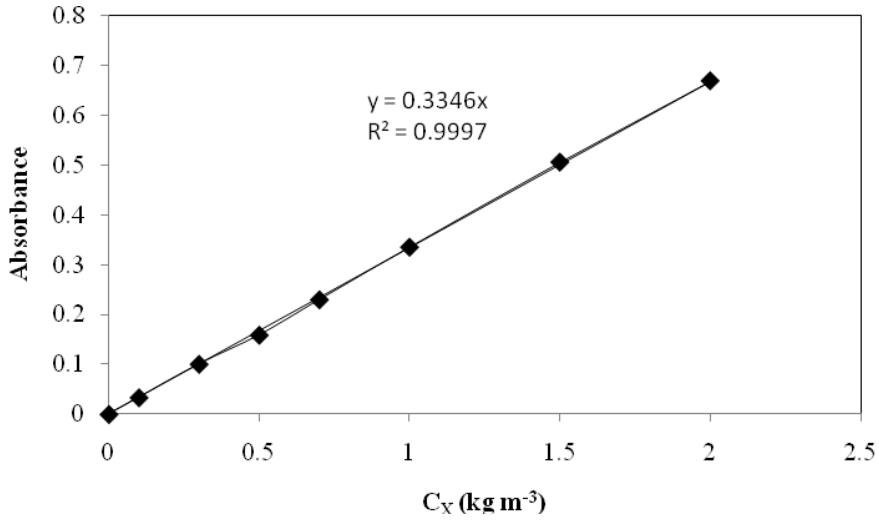

Figure D.1 Calibration curve obtained for cell concentration

Calibration Curve for Maleic Acid Concentration :

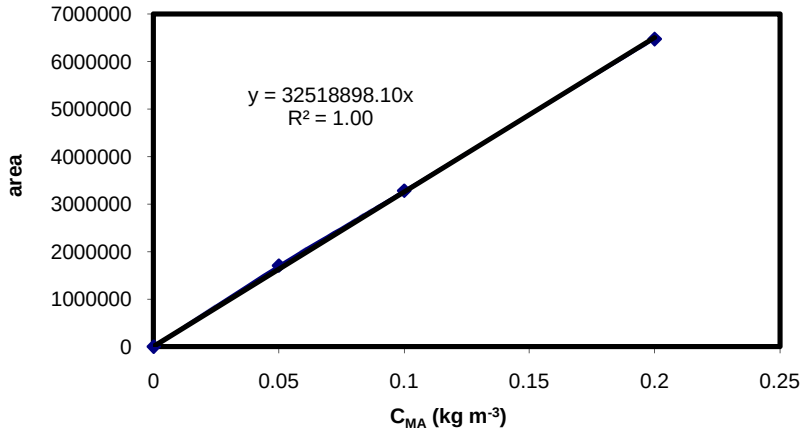

Figure D.2 Calibration curve obtained for maleic acid concentration

Calibration Curve for Gluconic Acid Concentration :

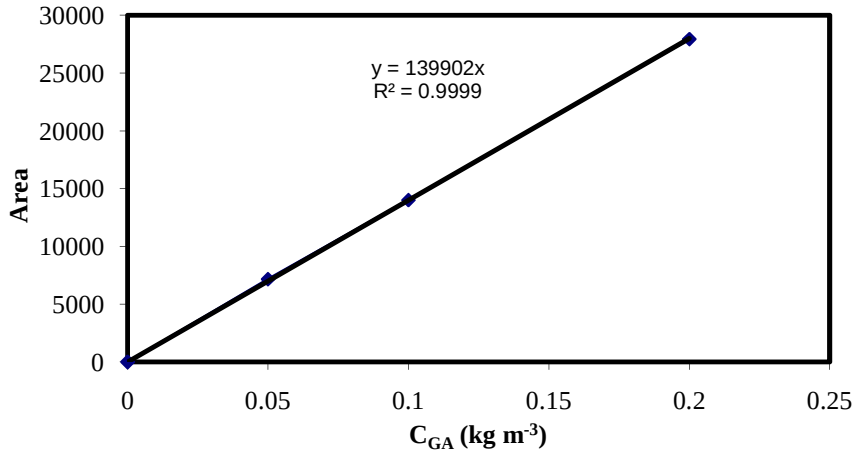

Figure D.3 Calibration curve obtained for gluconic acid concentration

Calibration Curve for Acetic Acid Concentration :

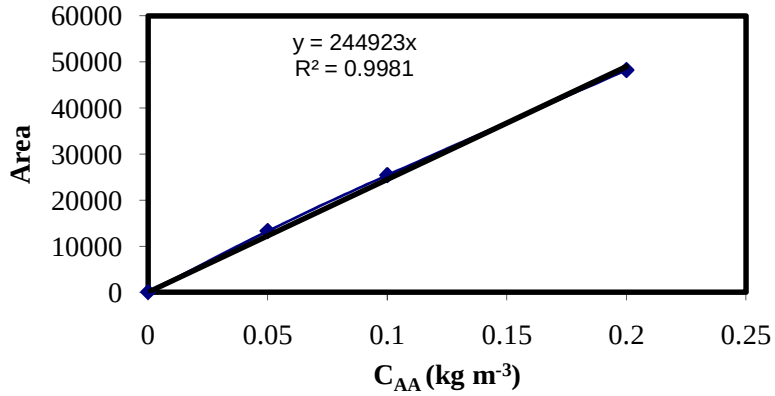

Figure D.4 Calibration curve obtained for acetic acid concentration

Calibration Curve for Pyruvic Acid Concentration :

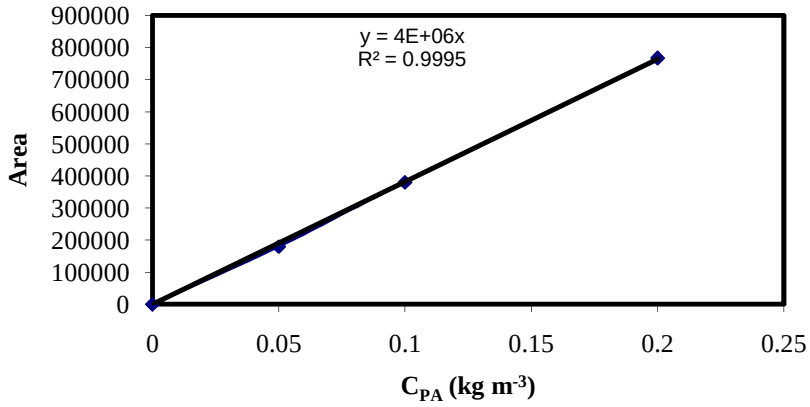

Figure D.5 Calibration curve obtained for pyruvic acid concentration

Calibration Curve for Formic Acid Concentration :

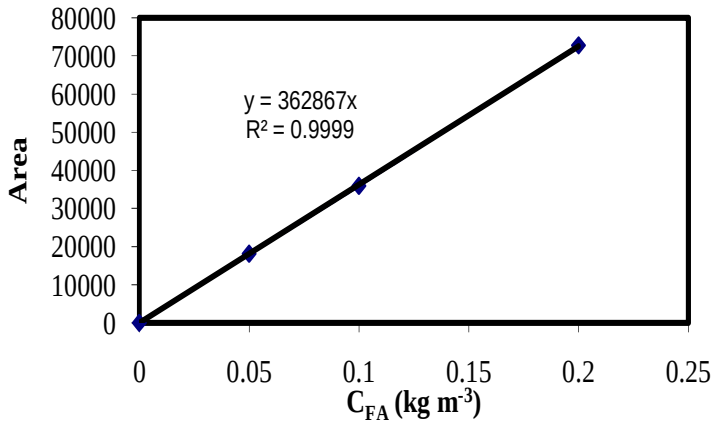

Figure D.6 Calibration curve obtained for formic acid concentration

Calibration Curve for Oxalic Acid Concentration :

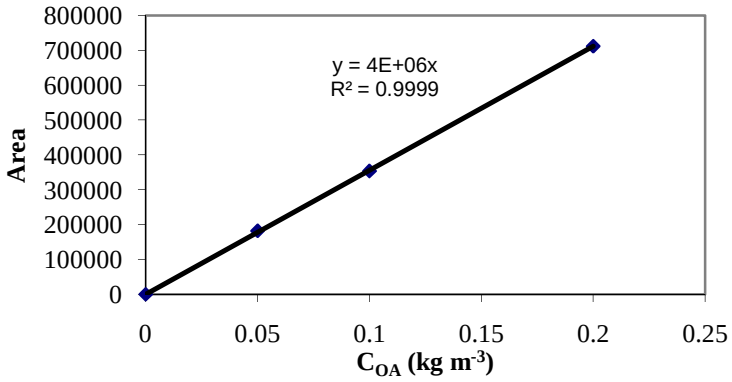

Figure D.7 Calibration curve obtained for oxalic acid concentration

Calibration Curve for Fumaric Acid Concentration :

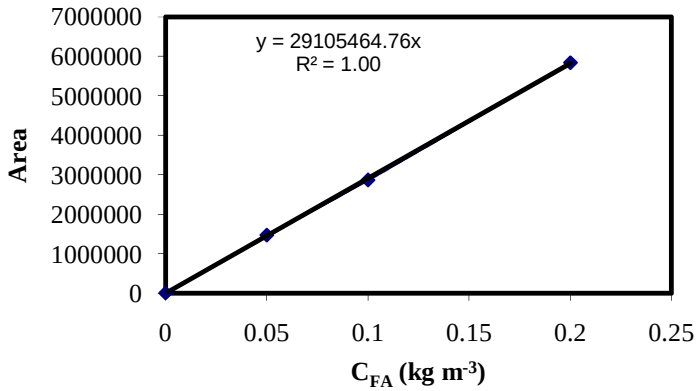

Figure D.8 Calibration curve obtained for fumaric acid concentration

Calibration Curve for Lactic Acid Concentration :

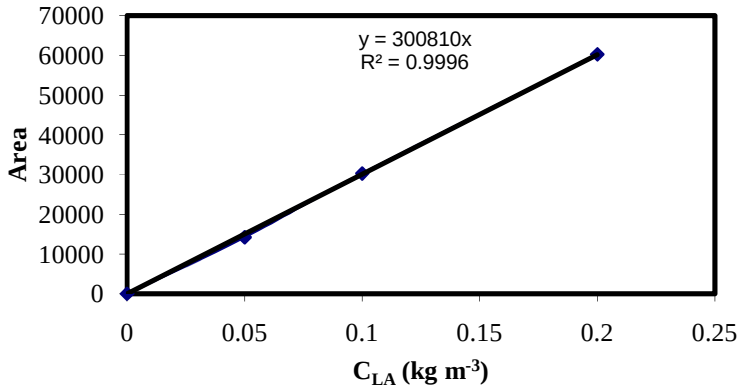

Figure D.9 Calibration curve obtained for lactic acid concentration

Calibration Curve for Citric Acid Concentration :

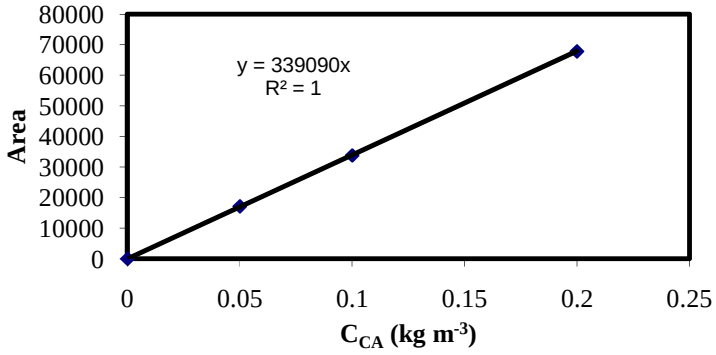

Figure D.10 Calibration curve obtained for citric acid concentration

Calibration Curve for Glucose Concentration :

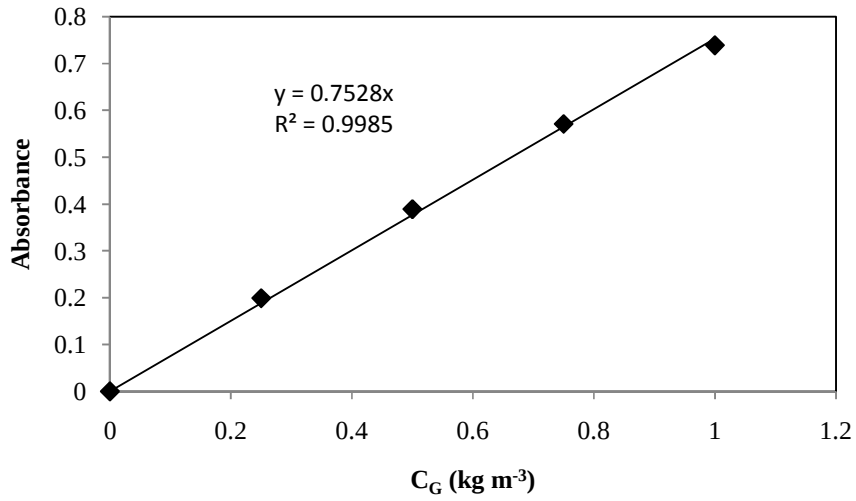

Figure D.11 Calibration curve obtained for glucose concentration

APPENDIX E

ELECTROPHEROGRAM OF hGH STANDARD

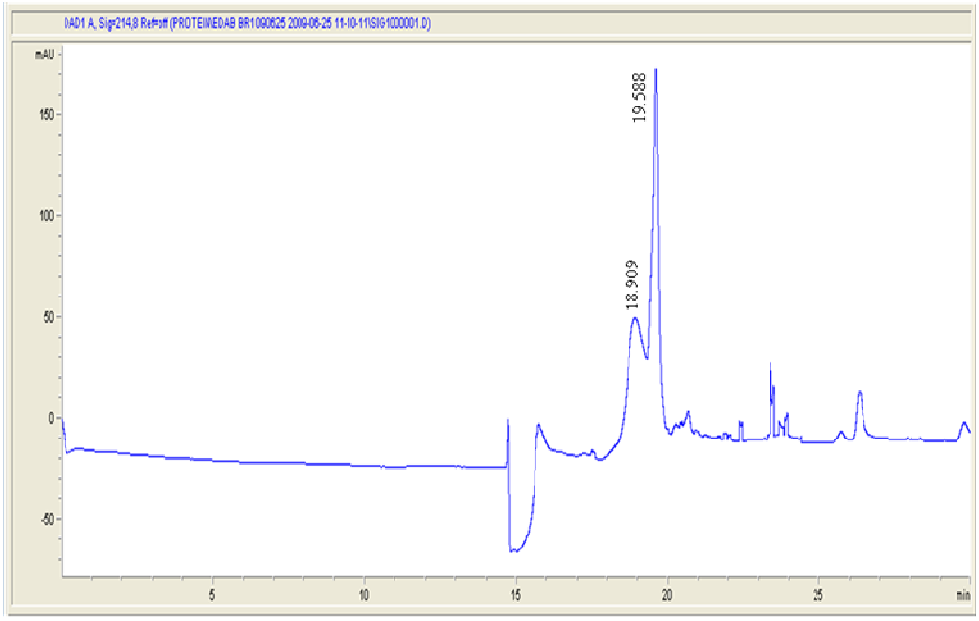

Figure E.1 Electropherogram of 0.05 g L^{-1} standard hGH

APPENDIX F

SILVER STAINING PROTOCOL

Staining SDS-Polyacrylamide Gels with Silver Salts :

After the running was completed, the gels were silver stained using the procedure of Blum et al. (1987) which is given below. Preparation of solutions used was given in Appendix B. These solutions should be fresh.

- *Fixing step* : When the running process was complete, gel was taken into the fixer solution to incubate for at least 1 h. Gel can remain in fixer overnight.
- *Washing step* **:** Gel was washed with 50% ethanol solution for 20 min three times. After each 20 min, ethanol solution was replenished.
- *Pretreatment step* **:** At this step, gel was treated with pretreatment solution for 1 min.
- *Rinse step* **:** Gel was rinsed by the distilled water for 1 min. At every 20 s, water was replenished.
- *Impregnate step* **:** Gel was treated with silver nitrate solution for 20 min.
- *Rinse step* **:** Gel was rinsed with water for 20 s. Then, water is replenished and gel was rinsed for another 20 s.
- *Developing step :* Development solution was poured on to gel, and color development were observed.
- *Wash :* As the color developed on the gel, distilled water was added.
- *Stop :* After observation of clear bands, reaction was stopped by addition of stop solution. Gel can be kept in this solution overnight.