HUMAN GROWTH HORMONE PRODUCTION BY METABOLICALLY ENGINEERED *B. subtilis*: FEEDING STRATEGY DEVELOPMENT

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ABSTRACT

HUMAN GROWTH HORMONE PRODUCTION BY METABOLICALLY ENGINEERED *B. subtilis*: FEEDING STRATEGY DEVELOPMENT

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In this study, the aim is to develop an effective feeding strategy for therapeutically important protein, recombinant human growth hormone (rhGH) production by *Bacillus subtilis scoC* knockout (*scoC*$^-$) strain carrying pMK4::*pre*(*subC*)::*hGH* plasmid. In the first part of the research program, laboratory scale air filtered shake bioreactor experiments were conducted to optimize the production conditions. Thereafter, semi-batch production processes at pilot scale bioreactor system were performed by designing exponential feeding strategies in order to enhance the rhGH production capacity. In this context, the effects of glutamine and medium composition on the cell concentration and rhGH production were separately investigated in batch bioreactor experiments at laboratory scale. The maximum rhGH concentration was obtained in the defined medium containing 0.05 g L^{-1} glutamine (G-0.05) as 76 mg L^{-1} at t=28 h. In the second part of the air filtered shake bioreactor experiments, the effects of medium composition were investigated in seven different media in order to achieve high cell cultivations leading to rhGH productions at high levels by semi-batch operation. The maximum cell and rhGH concentrations were obtained as 2.47 g L^{-1} and 75 mg L^{-1} in the medium P-6 which basically contained 8 g L^{-1} peptone and 5 mL L^{-1} trace salt solutions different than that of the defined medium, respectively. Based on these results, the medium containing peptone and trace salt solutions in the presence of glucose and $(NH_4)_2HPO_4$ was used as the new feeding substrate medium for the semi-batch experiments. In the pilot scale bioreactor experiments, eight different feeding strategies were designed for rhGH production in semi-batch processes. The effects of exponential feeding strategies with the pre-determined specific growth rates chosen as 0.04, 0.10, 0.17 and 0.26 h^{-1} on rhGH production were investigated. The highest cell and rhGH concentrations were achieved as 4.43 g L^{-1} and 366 mg L^{-1}, respectively, by the glucose based semi-defined medium feeding strategy conducted at a pre-determined specific growth rate μ_0=0.17 h^{-1} at t=28 h. By this feeding strategy, the overall product and cell yield on total substrate were acquired as 62.92 g kg^{-1} and 0.73 g g^{-1}, respectively.

Keywords: Recombinant human growth hormone, *Bacillus subtilis*, semi-batch, feeding strategy

ÖZ

METABOLİK MÜHENDİSLİK *B. subtilis* İLE İNSAN BÜYÜME HORMONU ÜRETİMİ: BESLEME STRATEJİSİ GELİŞTİRİLMESİ

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Bu çalışmada, terapatik öneme sahip proteinlerden rekombinant insan büyüme hormonunun (rhGH) pMK4::*pre*(*subC*)::*hGH* plazmidi taşıyan *Bacillus subtilis scoC* geni silinmiş (*scoC*$^-$) suş ile yarı-kesikli üretimi için etkin bir besleme stratejisi geliştirilmesi amaçlanmıştır. Birinci alt-araştırma programında, rhGH üretim koşullarını optimize etmek üzere ilk olarak laboratuvar ölçek hava filtreli biyoreaktör deneyleri yapılmış; ikinci alt-araştırma programında da pilot ölçek biyoreaktör sisteminde yarı-kesikli üretim prosesleri rhGH üretim kapasitesini arttırmak için üstel besleme stratejileri tasarlanarak gerçekleştirilmiştir. Glutamin ve ortam bileşiminin hücre derişimi ve rhGH üretimi üzerine etkileri ayrı ayrı laboratuvar ölçek kesikli proseslerde araştırılmış; en yüksek rhGH derişimi 0.05 g L^{-1} glutamin içeren glukoz temelli tanımlı ortamda (G-0.05) t=28 st'de 76 mg L^{-1} olarak elde edilmiştir. Laboratuvar ölçek hava filtreli biyoreaktör deneylerinin ikinci aşamasında, ortam bileşiminin etkisi yarı-kesikli proseslerle yüksek derişimlerde rhGH üretimleri için gerekli yüksek hücre derişimi elde etmek amacıyla yedi farklı ortamın etkisi araştırılmış; en yüksek hücre ve rhGH derişimleri, tanımlı ortama ek olarak 8 g L^{-1} pepton ve 5 mL L^{-1} eser miktarda tuz çözeltileri içeren ortamda (P-6) sırasıyla 2.47 g L^{-1} ve 75 mg L^{-1} olarak elde edilmiştir. Bu sonuçlara bağlı olarak, glukoz ve $(NH_4)_2HPO_4$ varlığında pepton ve eser miktarda tuz çözeltileri içeren ortam, yarı-kesikli deneyler için besleme substrat ortamı olarak seçilmiştir. Pilot ölçek biyoreaktör deneylerinde, yarı kesikli proseslerde rhGH üretimi için sekiz farklı besleme stratejisi tasarlanmıştır. Üstel besleme stratejilerinin 0.04, 0.10, 0.17 ve 0.26 st^{-1} olarak seçilen öngörülmüş özgül çoğalma hızları ile rhGH üretimi üzerine etkileri araştırılmıştır. En yüksek hücre ve rhGH derişimlerine μ_0=0.17 st^{-1}'de glukoz temelli yarı-tanımlı ortam beslemesi ile t=28 st'te sırasıyla 4.43 g L^{-1} ve 366 mg L^{-1} olarak ulaşılmıştır. Bu besleme stratejisi ile toplam substrat üzerinden toplam ürün ve hücre verimi sırasıyla 62.92 g kg^{-1} ve 0.73 g g^{-1} olarak elde edilmiştir.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, *Bacillus subtilis*, yarı-kesikli, besleme stratejisi

To my family

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NOMENCLATURE

C	Concentration	g L^{-1} or mol m^{-3}
C_O*	Saturated dissolved oxygen concentration	mol m^{-3}
Da	Damköhler number (OD/OTR_{max})	
DO	Dissolved oxygen	%
E	Enhancement factor (K_La/K_La_0)	
k	Reaction rate constant	
K_La	Overall liquid phase mass transfer coefficient	s^{-1}
K_La_0	Physical overall liquid phase mass transfer coefficient	s^{-1}
N	Agitation rate	min^{-1}
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	Volumetric 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	Process time	h
T	Medium temperature	^oC
U	One unit of an enzyme	
V	Volume	L
Y	Yield	g g^{-1}
$\overline{Y}$	Overall yield	g g^{-1}

Greek Letters

ρ	Density	$g\ L^{-1}$
η	Effectiveness factor (OUR/OD)	
μ	Specific growth rate	h^{-1}
μ_0	Pre-determined specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Initial condition
O	Oxygen
p	Protein
pro	Protease
S	Substrate
X	Cell

Abbreviations

AOX 1	Alcohol oxidase 1
ATP	Adenosine triphosphate
CAGR	Compound annual growth rate
cDNA	Complementary DNA
CER	CO_2 evaluation rate
CJD	Creutzfeldt-Jakob disease
CM	Cytoplasmic reticulum
DNA	Deoxyribonucleic acid

EMP	Embden-Meyerhof-Parnas
ER	Endoplasmic reticulum
FDA	Food and drug administration
GHD	Growth hormone deficiency
GRAS	Generally recognized/regarded as safe
SAP	Serine alkaline protease
hGH	Human growth hormone
HPCE	High performance capillary electrophoresis
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor-1
IR	Inoculation ratio
LPS	Lipopolysaccharides
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PDH	Pyruvate dehydrogenase
PI	Protease inhibitor
PP	Pentose phosphate
PYR	Pyruvate
R5P	Ribose-5-phosphate
rhGH	Recombinant human growth hormone
RIA	Radio immune assay
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
Trp	Tryptophane

CHAPTER 1

INTRODUCTION

The history of fermentation processes beyond traditional biotechnology was based on a number of ancient ways of using living organisms to produce novel products or modify existing ones. In other words, the history of biotechnology started with the human activity on the practices of breadmaking and fermented products such as that obtained from the transformation of milk into cheeses and yogurts about thousands of years ago (Ratledge and Kristiansen, 2006). Although Louis Pasteur in the nineteenth century called attention to the living organisms in that processes, the actual role of those microorganisms on biochemical mechanisms was unknown till the advances in microbiology and biochemistry. After the discovery of the structure of DNA by the scientists (R. Franklin, M. Wilkins, J. Watson, and F. Crick), also awarded with the Nobel Prize in 1962, scientists and engineers worked on biotechnology focused on the genetic studies. Later through the developments in technology, people discovered to control those processes and began to produce valuable biotechnological products in large quantities. The term "industrial biotechnology", also called modern biotechnology, first emerged in the literature in the early 1980s when the science of genetic engineering was searching through the fields of recombinant DNA technology for medical applications. Industrial biotechnology involves the applications of enzymes and whole cells for the production of chemical products, including pharmaceuticals, fine chemicals related to human health and environment (Soetaert and Vandamme, 2010). It generates feasible changes in an organism through the manipulation of its genomic model in a controlled and less time-consuming manner in comparison to traditional biotechnology techniques. Thereby, these industrial microorganisms have been technically changed to yield fine products in cost-efficient and environmentally adequate ways (Nielsen *et al*., 2003).

By the current trends in biotechnology, biopharmaceuticals which are very high-value, very small-volume biomolecules are becoming an important part of the total global market (http://www.bccresearch.com/report/biologic/therapeutic-drugs-bio079a.html). Revenues of the biotech industry were nearly half of the market, or exactly 48.8 %, in 2009 with the largest segment. Through progress in the techniques of gene splicing and recombination in biotechnology, the market value of biopharmaceutical sector reached the $72 billion mark in 2010. Authorities in global economy are forecasting to increase at an 8.2% a compound annual growth rate (CAGR) to reach $107 billion by research and innovation by 2015 (Walsh, 2010).

Therapeutics arising from biotechnological products is a branch of science and technology dealing with the treatment of different diseases which results with deleterious

effects in human health. Recombinant therapeutic products have represented the fastest growing segment of the pharmaceutical industry. Anticoagulants, blood factors, bone proteins, enzymes, antibodies, growth factors, interferons, interleukins, thrombolytics and hormones are the major therapeutic proteins used in diseases.

Hormones are chemical signals released from living cells that are transported in the blood to the target cells and tissues to have vital biological effects. They regulate biochemical reactions through specific receptors by affecting gene expression in the body (Norman et al., 1997). The human growth hormone, hGH, consisting of 191 amino acid residues, is secreted from the anterior lobe of pituitary gland. It is a non-glycosylated polypeptide with a wide range of biological functions including protein synthesis, stimulation of the immune system and metabolism. It directly not only controls and stimulate the growth in children, but also regulates muscle mass and bone mass in adults for a healthy body composition.

Growth hormone deficiency emerges when the pituitary gland does not secrete enough growth hormone to the body. Due to its variety of biological activities, the growth hormone has therapeutic applications in the treatment of chronic renal insufficiency, injuries, bone fractures, Turner syndrome, Prader-Willi syndrome and dwarfism (Özdamar *et al*., 2009). The treatment of growth hormone deficiency is taking the hormone, engineered by recombinant DNA technology, mature and biologically active, externally in required quantity. Human growth hormone, hGH, is a significant component of the industrial biomolecules that is used as a therapeutic drug in growth hormone deficiency (GHD). hGH was firstly produced by the isolation of pituitary gland which was extracted from human cadavers. Nonetheless, the drug produced by that method failed and caused to the deaths of some patients by triggering the Creutzfeldt-Jakob disease (CJD). Later on, by the developments on DNA technology, the first recombinant human growth hormone by the trade name of Protropin was produced by Genentech in 1985. Unlike its mature form found in human, Protropin had an additional methionine at its N-terminus and contained 192 amino acids leading to side effects in some users. Hence, a novel recombinant human growth hormone, Humatrope, in mature form with 191 amino acid residues was developed by Eli Lilly and approved by U.S. Food and Drug Administration (FDA) in 1987. Brands and manufacturers of recombinant human growth hormone (rhGH) are given in Table 1.1 (http://accessdata.fda.gov).

A bioprocess is a series of biotechnological production operations taking place via controlled parameters. Therefore, for recombinant hormone production, it is necessary to control some bioreactor operation conditions together such as medium design, temperature, pH, oxygen transfer characteristics related to agitation rate and concentration of dissolved oxygen, operation mode. Furthermore, host selection is the other important factor affecting production level of the product. New forms of organisms, namely genetically modified organisms, obtained through recombinant DNA techniques have been the result of the use of restriction enzymes and ligases with the functions of cutting DNA and joining foreign genes with the DNA of the host cell, respectively (Moiser and Ladisch, 2009). Bacteria, yeast, insect and mammalian cells are the most commonly used hosts for heterologous protein expression. Bacterial hosts are one of the most favored production systems for recombinant protein production due to having properties of fast growth, high efficiency and relatively inexpensive production (Ratledge and Kristiansen, 2006).

New biological derived therapeutics have supported to improve production systems leading to fine pharmaceutical proteins. *Escherichia coli* and *Bacillus* species are the most commonly used cell factories for the production of industrial proteins recombinantly. Because they easily reach to high cell density in the cultivation medium, they are favourable for the large scale production systems inexpensively (Westers *et al*., 2004). Formation of inclusion bodies is the main problem for *E. coli* in the protein expression. This causes inefficient refolding of the protein and expensive downstream processing which relatively affects the yield of the product. On the other hand, the genus *Bacillus* is one of the most widely used expression system for the production of heterologous proteins. When it is compared with *E. coli*, *B. subtilis* is non-pathogenic and free of endotoxins and that is approved by U.S. FDA by the status of GRAS (generally recognized/regarded as safe). Moreover, it has the ability to secrete large amounts of protein directly into the production medium in short process time (Zweers *et al*., 2008). As well as its advantages, secretion of proteases and plasmid instability are the main disadvantages of it for recombinant protein production. However, these problems can be overcome by using protease inhibitors or mutants and integration of plasmid into the chromosome, respectively.

There are three host microorganisms that have been used for recombinant human growth hormone production, i.e., *E. coli*, *B. subtilis*, *P. pastoris* (Trevino *et al*., 2000; Çalık *et al*., 2008). First, genetic studies related to hGH gene encoding human growth hormone and intracellular hGH production began in 1979 by *E.coli* (Goeddel *et al*., 1979). Becker and Hsiung (1986) achieved the secretion of hGH in high amounts to the periplasmic medium by using a new vector system. Jensen and Carlsen (1990) indicated that presence of high concentration of acetate and salt affecting the productivity negatively was toxic. In addition, they emphasized that glucose-limited semi-batch operations were more desirable than the batch fermentations. Due to the ability of *Bacillus* species in secretion of the proteins directly to the culture medium, use of these hosts in recombinant hormone production has gained importance from past to today (Nakayama *et al*., 1988; Franchi *et al*., 1991; Kajino *et al*., 1997).

Table 1.1 Brands and manufacturers of recombinant human growth hormone

Manufacturers	**Brands**
Eli Lilly	Humatrope* 1987
Genentech	Nutropin* 1994
Pharmacia and UpJohn	Genotropin * 1995
Ferring Pharmaceuticals	Tev-Tropin* 1995
EMD Serono	Saizen & Serostim* 1996, Zorbtive* 2003
Novo Nordisk Inc.	Norditropin* 2000
Sandoz	Omnitrope * 2006

* U.S. FDA approval date

Özdamar *et al*. (2009) developed a novel expression system mimicking the serine alkaline protease (SAP) to produce human growth hormone extracellularly. To achive this, a hybrid gene of two DNA fragments containing signal (pre-) sequence of *Bacillus licheniformis* SAP gene (*subC*) and chromosomal DNA encoding hGH were cloned into pMK4 plasmid. Thus, a new plasmid called pMK4::*pre(subC)*::*hGH* was constructed and expressed in *B. subtilis* (*npr*$^-$ *apr*$^-$) and *B. subtilis* (*spo*$^-$) strains (Özdamar *et al*., 2009). Şahin (2010) investigated the regulatory gene effects on recombinant human growth hormone production by using *B. subtilis* knockout strains, i.e., *degQ*$^-$, *degU*$^-$, *degS*$^-$, *sinI*$^-$, *sinR*$^-$, *abrB*$^-$, *spo0A*$^-$, *aprE*$^-$ and *scoC*$^-$ and obtained the highest rhGH production by *B. subtilis* (*scoC*$^-$).

In this context, the objective of this study is to develop an effective feeding strategy leading to higher recombinant human growth hormone (rhGH) production by *Bacillus subtilis* (*scoC*$^-$). Therefore, pilot-scale bioreactor experiments with exponential feeding of carbon and carbon-nitrogen sources at variable specific growth rates were carried out. Cell growth, substrate consumption, fermentation and oxygen transfer characteristics as well as the formation of the product, rhGH, and by-products were investigated in the semi-batch operations without feedback control.

CHAPTER 2

LITERATURE SURVEY

2.1 Hormones

2.1.1 General Properties

Hormones are the messages creating a chemical and biochemical network between the cells in the body. These well-defined quantities are secreted or produced by particular glands or cells (Norman and Litwack, 1997). Hormones that are prepared by special endocrine glands are secreted by being released continuously or periodically through the blood to the transfer points (Pekin, 1979). They regulate growth, development and metabolic functions of the body. A hormone is synthesized and stored within the cell maintained by endocrine gland. Endocrine system is regulated by hypothalamus using motor neurons to send electrical messages and hormones to the target cells in the body. A chemical change related to the concentration of some components, e.g., potassium, calcium, glucose in the bloodstream occurs by releasing a hormone.

2.1.2 Structures and Functions

Hormones, primary messengers, activate the cellular responses in the body by three main events: reception, signal transduction and response. In the reception step, hormones generally bind to receptors on the cell surface by passing through the plasma membrane. After reception, hormones make a change on receptors and this induces variations on another molecule called the secondary messenger. Finally, this cellular molecule causes a main change in the cell. At the end of the response, second messenger may activate a gene to make a new protein or stop making a new protein, or it may interact with an enzyme and may affect directly its activity (Norman and Litwack, 1997).

A hormone can be a member of one of the three classes: proteins and peptides, amines derived from amino acids, and steroids. They are usually classified according to their structures. The first structure is emerged over steroids such as androgens, estrogens, progestins and corticoids. They regulate physiological functions of the body especially on sexual organization. Furthermore, amino acid derivatives such as epinephrine (adrenaline)

related to heart rate are composed of short chains of amino acid. Steroids hormones are hydrophobic and made of cholesterol so that they can easily pass from bloodstream to the hydrophobic layer of plasma membrane. Proteins and peptides, e.g., insulin, growth hormone, prolactin is a polypeptide hormone consisting of long chains of amino acids and they are in hydrophilic structure. Therefore, the transport of them through the cell membranes is more difficult than the steroids. Amino acid derivatives such as epinephrine are water-soluble hormones like peptides. Because of this, they generally bind to receptors on the outside of the cell (Binkley, 1995).

2.2 Human Growth Hormone (hGH)

2.2.1 Chemical and Biological Properties

One of the most significant hormones secreted from the anterior lobus (lobe) of pituitary gland is somatotropin known as growth hormone. This hormone consisting of 191 amino-acid has a molecular mass of 22,125 daltons (Da) (Glick *et al*., 2010) (Figure 2.2). It contains four helices and two disulfide bridges that give the tertiary structure of it (Figure 2.1). After synthesized, it is stored and secreted by somatotroph cells (Delgadillo *et al*., 2011).

Having molecular differences in mRNA bonding and post-translational modifications, growth hormone is a heterogeneous protein comprising of various isoforms. Main human growth hormone genes (GH1 or GH-N, GH2 or GH-V, CS1 or CSA and CS2 or CSB) are gathered on the chromosomal locus of 17q24.2. Only the expression of GH1 is performed by somatotrophic cells of the pituitary, while the others are under control of placenta. The main source of the growth is GH1 gene and its product is a single chain protein having 191 amino acids and a molecular mass of 22,129 Da with two disulfide bonds as stated before. By the other possibility in mRNA splicing, GH has an isoform of having 176 amino acids with a molecular mass of 20,274 Da (Baumann, 2009).

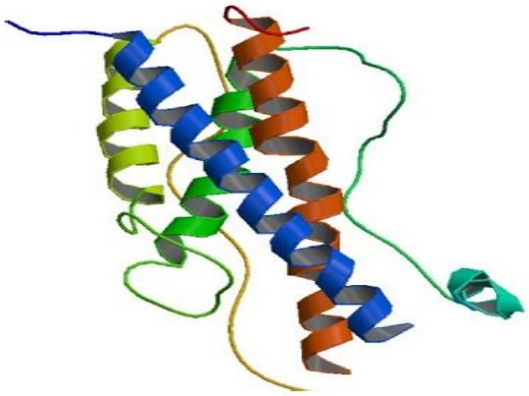

Figure 2.1 Tertiary structure of human growth hormone (Delgadillo *et al*., 2011)

Primary biological functions of hGH as follows,

- Stimulation of somatic (bodily) growth
- IGF-1 generation
- N (nitrogen), P (phosphorus) and Na (sodium) retention
- Lipolysis
- Amino acid transport into muscle
- Stimulation of immune function

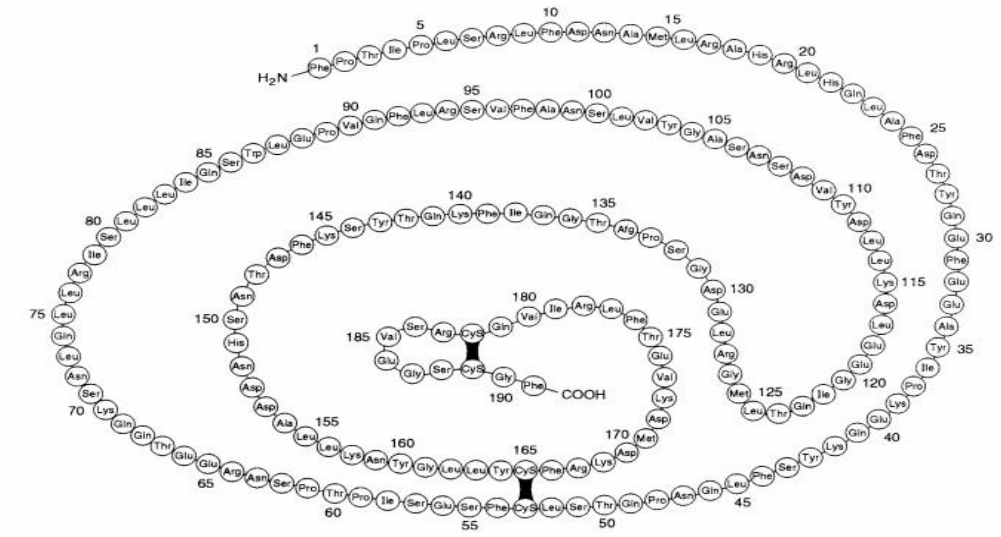

Figure 2.2 Covalent structure of human growth hormone (Norman and Litwack, 1997)

2.2.2 The Importance of hGH

The human growth hormone directly affects the protein metabolism by delaying amino acid catabolism in order to convert amino acids into body proteins. Moreover, lipid metabolism and carbohydrate metabolism are also stimulated by human growth hormone by accelerating the oxidation of triglycerides and affecting insulin-antagonistic activity, respectively (Chawla *et al*., 1983). The effects of growth hormone can be seen either directly or through the functions of other hormones. The most critical one is insulin-like growth factor-1 (IGF-1). Growth hormone has a crucial role in the production of insulin-like growth factor-1. While IGF-1 is an elementary factor in the encouragement of the growth in childhood, it has also anabolic effects such as regulation of metabolism, cellular synthesis of DNA in adults (Glick *et al*., 2010). The level of IGF-1 gradually increases by age in children. By the end of the childhood, it begins to decrease by age, as do the concentration of human growth hormone in the body.

However, certain anomalies due to the secretion of growth hormone can be observed in children and adults. These problems are related to either deficiency or superabundance of the human growth hormone. Growth hormone deficiency is mainly emerged with short stature and dwarfism resulting from either mutations in genes or damaged hypothalamus/pituitary gland. A number of disorders observed in children like chronic renal insufficiency, Turner syndrome, Prader-Willi syndrome, abnormal growth and idiopathic short stature can be treated with injections of growth hormone. In chronic renal (kidney) insufficiency, toxins are accumulated in blood stream of children due to less functions of kidney. Turner syndrome is related to chromosomal abnormality and mostly seen in the girls whose entire or critical part of the X chromosome is lost. Prader-Willi syndrome is a rare genetic condition related to insufficient muscle form, small organs e.g., hands, feet, mouth and obesity (Kappelgaard and Laursen, 2011; Delgadillo *et al*., 2011).

Adults also suffer from the deficiency of growth hormone like children. They do not have enough growth hormone. Therefore, health problems such as skin burns, wounds, osteopenia (bone loss), decreased muscle strength, cardiovascular disease e.g., low density lipoprotein cholesterol (LDL), hyperinsulinemia can be healed by growth hormone. Furthermore, benefits of anti-aging effects of human growth hormone are also investigated for cancer treatment (Liu et al., 2007).

2.2.3 Recombinant Human Growth Hormone (rhGH) Production

Early on decades, availability of human growth hormone was constricted because it was only supplied from human cadaver pituitaries. Nonetheless, intake of natural human growth hormone was halted and withdrawn from the market in 1985. Treatments on some patients caused deaths by a disease called Creutzfeldt-Jakob disease (CJD). CJD is a fatal disease and comes up with a pathogenic agent, a prion that contaminates the hormone. By the same year, scientists were able to produce biosynthetic form of human growth hormone by using the techniques in genetic engineering and recombinant DNA technology.

First studies to clone the hGH gene encoding human growth hormone were undertaken by Goeddel *et al*. (1979). General approach in this study was to use the hybrid-gene technique on hGH gene creating by the combination of clones of chemically synthesized DNA and cDNA produced by using mRNA of pituitary gland in enzymatic reactions. By the treatment of cDNA of hGH gene with a specific endonuclease, HaeIII, a DNA sequence of hGH consisting of 551 base pairs and encoding the amino acids of 24-191 was obtained. In addition to that, a chemically DNA adaptor fragment having ATG initiation codon and coding the amino acid residues 1-23 of hGH. By integration of these two DNA fragments jointly, a synthetic-natural hybrid gene was formed in this strategy. A new plasmid known as pHGH107 was also constructed in order to synthesize fMet-hGH. This polypeptide consisting of 191 amino acids and containing N-terminal methionine residues was produced in *E. coli* with identical immunological characteristics of natural hGH.

Ikehara *et al*. (1984) chemically synthesized the gene encoding hGH comprising of 191 amino acids and methionine and this hGH gene was expressed in *E. coli* under the control of tryptophane (trp) promoter. The efficiency in met-hGH production with the

plasmid of pGH-L9 was higher than the other promoters. According to the radio immune assay (RIA) analysis, the concentration of met-hGH was measured as 169 mg L^{-1} or $2.9x10^6$ molecules per cell of human growth hormone.

Gray *et al*. (1985) provided the secretion of rhGH to the periplasmic space by constructing two plasmids. These transformed plasmids to *E. coli* were pPreHGH207-2 and pAPH-1 participated in the expression of rhGH under the control of *E. coli trp* promoter and pre-hGH signal peptide; phoA promoter and pho-hGH signal peptide, respectively. Concentration of rhGH was measured by RIA analysis method. 76 % of 450 ng/mL/A_{550} hGH expressed with the plasmid of pPreHGH207-2 and 82 % of 230 ng/mL/A_{550} hGH expressed with the plasmid of pAPH-1 were achieved to secrete into the periplasmic space under osmotic pressure. Moreover, structural characteristics of rhGH such as monomeric structure, the number of disulfide bonds were observed as the same as natural hGH in the N-terminal sequence analysis.

Extracellular secretion of mammalian proteins in cellular microorganisms is very important. The first extracellular/periplasmic secretion of rhGH was observed in *E. coli* carrying *omp*A signal peptide in 1986 (Becker and Hsiung, 1986). 10-15 μg/A_{600} hGH was produced by using the secretion vectors of pOmpA-hGH1 and pOmpA-hGH2. Even though 78 % portion of produced human growth hormone was secreted into the periplasmic space, only 72 % of it in the same structure with the natural form. As a result of the previous studies, the problems coming up with the formation of methionine in the N-terminal of the protein and the formation precipitate due to low solubility were prevented by the periplasmic production of rhGH (Hsiung *et al*., 1986).

Chang *et al*. (1987) aimed to secrete human growth hormone extracellular medium in high amounts. In order to achieve this, they expressed a hybrid gene comprising the gene encoding hGH and signal peptide of enterotoxin II (STII) under the control of alkaline phosphatase promoter (*pho*A) in *E. coli*. *E. coli* having phGH4R and phGH4L produced about 15 to 25 μg/A_{550} hGH counting nearly 10 % of total protein. *E. coli* 294 and *E. coli* W3110 were compared according to the hGH production capacity in a variety of media containing several concentrations of phosphate and it was observed that *E. coli* W3110 was less sensitive than *E. coli* 294 in phosphate regulation. To sum up, the highest hGH production was achieved with the plasmid of phGH4L in low phosphate concentration by *E. coli* W3110.

Kato *et al*. (1987) constructed a novel plasmid, pEAP8 carrying penicillinase promoter of *Bacillus sp*., *kil* gene of plasmid pMB9 and *hGH* gene for *E. coli* to secrete hGH directly to the culture medium. After transformation of this plasmid to *E. coli*, the transport process of produced hGH was began with inner membrane of *E. coli* by the help of penicillinase signal sequence and was proceeded with permeable outer membrane provided by the *kil* gene and then culture medium. Accordingly, production of 80 % of total protein (6.7 mg hGH/mL culture) was provided by *E. coli* carrying pPSHhGH11 plasmid with the secretion of periplasmic space. On the other hand, 55 % of total hGH (11.2 mg hGH/mL culture) produced by *E. coli* with the plasmid of p8hGH1 and 42 % of it (8.6 mg hGH/mL culture) were secreted to the culture medium and the periplasmic space, respectively. Produced hGH was specified in biologically active and authentic by the analyses of SDS-PAGE and immunoblot.

In the study of Nakayama *et al*. (1988), a secretion vector in *Bacillus subtilis*, called pNPA225, which was based on *Bacillus amyloliquefaciens* neutral protease gene was constructed and, after that recombinant plasmids phGH324 and phGH526 containing a mature human growth hormone gene were constructed by using this secretion vector. However, phGH526 could permit *B. subtilis* to secrete a 10-fold higher level of hGH production into the medium compared with phGH324. The secreted hGH was the same as the authentic one in size and N-terminal amino acid sequence. On the other hand, this secreted hGH was biologically active and had the same specific activity as the authentic hGH on the growth stimulation of a rat lymphoma cell. It was also stated that the larger hGH was present in the cell membrane as a major species. The obtained secretion level from a high cell density culture of *B. subtilis* transformed with phGH526 was 40 mg L^{-1}. It was also suggested in this study that high-density culture of transformed *B. subtilis* was important in developing a highly efficient production process for the heterologous protein.

Hsiung *et al*. (1989) investigated the high level secretion of human growth hormone to the extracellular medium of *E. coli*. In order to achieve this, they transferred pOmpAhGH2 plasmid containing pOmpA signal sequence and *hGH* gene and pJL3 plasmid having the gene of bacteriocin released protein (BRP) into *E. coli*. *lpp-lac* promoter-operon system were found in each plasmid. BRP protein was used to increase the permeability of the outer-membrane of *E. coli*. Hence, 4.5 μg/mL/A_{550} hGH was secreted into the culture medium in the induced conditions by IPTG (isopropylthio-beta-D-galactoside). Whereas production conditions were the same as pJL3 plasmid, recombinant hGH production level with plasmid pOmpAhGH2 was 10-15 μg/mL/A_{550} hGH. Furthermore, purification of excreted hGH was carried out in 98 % purity with column chromatography operated by the principle of reversed-phase.

Jensen and Carlsen (1990) were aimed to investigate the effects of several medium conditions on the cytoplasmic production of hGH carrying different N-terminal amino acid residues in *E. coli*. Thus, the highest hGH production performance was provided by MAE-hGH which was one of the alternatives. They indicated that presence of high concentration of acetate and salt affecting the productivity negatively was toxic. Besides, they emphasized that glucose-limited fed-batch operations were more desirable than the batch fermentations. Effect of carbon source was examined and high glucose concentration affected the hGH production negatively. However, constant feeding of glucose in fed-batch operation increased the yield in 3-fold. The highest yield was reached by the exponential feeding of glucose in consistence with cell growth profile. By improving the feeding strategy on glucose, hGH was produced as 2000 mg/L.

Franchi *et al*. (1991) formed a series of hybrid plasmids to be transformed into *Bacillus subtilis* for hGH production. In this study, increasing the solubility of polypeptide, purification of produced hormone in an easy method and efficient removal of unnatural residues of the hormone to produce hGH in an authentic form were aimed. Hence, the residues in different sizes in N-terminal region of hGH were examined to the solubility. Long extensions decreased the solubility and the maximum solubility values were taken as 70 % and 80 % in the host organisms carrying the plasmids pSM291 and pSM274, respectively. Purification step eased with the charge differences between the hybrid polypeptide and the bulk proteins which were more basic. After the purification, 90 % of

produced polypeptide was recovered and 98 % portion of it gained in pure hGH form by applying the method of gel filtration chromatography.

Kajino *et al*. (1997) concentrated on the production of natural and biologically active form of hGH by preventing the produced heterologous proteins from the degradation of extracellular protease enzymes. Although there were a wide range of manufacturing procedures, it was the first time to study with a *Bacillus* species instead of *Bacillus subtilis* on the extracellular secretion mechanism. Researchers reviewing this gap in the literature carried out rhGH production by using the mutant microorganisms having lower protease activity and adding EDTA into the culture medium to inhibit the degradation activity. hGH production capacity was increased from 148 mg/L to 240 mg/L by the optimization of production medium. After all, *B. brevis* was proven to be a potential host microorganism in the secretion and production of hGH.

Shin *et al*. (1998) developed a new protein production system. N-terminal-5 sequences of human tumor necrosis, enterokinase and histidine regions were used in this system. Scaling up to the large systems was also possible due to easy separation processes with the efficient expression system. Cell concentration of *E.coli* containing pT2GH vector was increased with any gene expression until to reach high cell density in fed-batch fermentations. After all, promoter T7 was induced to synthesize. Culture medium was in pH-controlled at growth phase and amount of IPTG added to the medium for induction was $3.0x10^{-2}$ mmol g^{-1}. This increased the gene expression in the ratio of 23 %. Nearly about 9 g/L hGH was produced in this study. They determined the purity of produced hGH as 99 % by utilizing SDS-PAGE, HPLC and RP-HPLC.

High-cell density fermentation process in *E. coli* on recombinant hGH production was investigated by Zhang *et al*. (1998). This method had the advantages of low volume and cost by increasing the productivity. However, formation of acetate as by-product was a disadvantage, as well. Acetate production could be decreased by the modification of growth medium. Therefore, the purpose of this article was to investigate the effect of fermentation conditions on production. They used *E. coli* k802 as host microorganism and carried out the experiments in both batch and fed-batch operations. Only difference in fed-batch fermentation was feeding glycerol gradually in increased amount to the medium. Glycerol was more preferable than glucose as a carbon source. Hence, high cell density (38.6-118.8 g/L) was obtained by using glycerol. By this strategy, carbon flow in glycolysis pathway was gradually slowed down, and thus the formation of by-products was reduced. When cell density doubled, concentration of hGH also doubled. Degradation of product was also decreased in high cell density culture when it was compared with the other fermentation processes. Short period of fermentation process decreasing from 16 h to 10 h could be considered to cause this effect since protease could not find enough time to degrade the produced proteins.

Bylund *et al*. (2000) investigated the production of recombinant hGH in *E. coli* in aerobic conditions by fed-batch fermentation. They were aimed to determine the models of production and protein degradation and understand the role of scaling up in this model and the gradients due to glucose feeding on product quality. Three different scale reactors (laboratory, scale-down and pilot-scale, 3 m^3) were compared. Not only glucose feeding but

also oxygen limitation was important parameter on the production of hGH. 80 % more total yield was obtained by scale-down reactor.

In the study of Patra *et al*. (2000), human growth hormone was expressed with insoluble form in *E. coli*. Several buffer solutions were tested to dissolve the produced hormone and the best result was obtained in pH 12.5 by 100 mM tris buffer containing urea (2M) as 1.6 g hGH/mL. hGH which was soluble in buffer containing low concentration of urea preserved its secondary structure and efficiency of recovery for proteins was increased. 1.6 g/L hGH was produced with the cell concentration of 25 g/L in fed-batch operation undergoing 10 hours. Purification and separation of hGH was obtained by chromatographic methods of ion exchange and gel filtration, respectively.

Trevino *et al*. (2000) first used *Pichia pastoris* to produce hGH under the control of alcohol oxidase 1 (AOX1) promoter and alpha-factor signal sequence of *Saccharomyces cerevisiae.* While 11 mg/L hGH was produced in the induction of oxygen limitation, this number was increased to 49 mg/L secreted directly into the production medium in high cell cultivation. 40 % of total protein was identified as hGH.

Roytrakul *et al*. (2001) studied hGH production in *E. coli*. They worked on the production of synthetic gene by using various oligonucleotides bound to hGH nucleotide sequence. In order to minimize the translation trouble initially, a 624 bp (base pairs) cassette containing start codon and ribosome binding region. *hGH* gene was expressed under the control of T7 promoter. After all, high efficiency was obtained as 20 % of total cell protein.

Castan *et al*. (2002) investigated the effects of oxygen-enriched fermentation on cell concentration and hGH production in *E. coli*. Two processes by fed-batch fermentations including biomass formation and rhGH production were examined separately. In the biomass formation process, exponential growth phase lasted longer and $Y_{O/S}$ increased by increasing the oxygen concentration in the medium. Formation of acetic acid (acetate) and formic acid (formate) were very low until increasing the oxygen concentration and then the values rose to 0.35 g/L and 0.43 g/L, respectively. Cell concentration was increasing by the enriched-oxygen supply and it suddenly began to decrease at the end of the process due to the cell lysis leading to accumulation of acetate and formate. In the recombinant hGH production process with 40 % enriched-oxygen supply even though feed rate of glucose and the concentration of production medium were increased to double, cell concentration was only increased to 77 % and the total product was decreased to 50 % despite the non-degraded formations.

Tabandeh *et al*. (2004) aimed to increase the production capacity of rhGH in *E. coli* by utilizing heat shock. Profiles of cell growth and hGH production were compared for various media containing different carbon sources. Production by heat-induced meant that firstly heating the culture media to 42 ^{0}C for 30 minutes and then keeping at 37 ^{0}C for 4 hours. Consequently, rhGH production started at 37 ^{0}C. If production media kept at 42 ^{0}C for more than 30 minutes, degradation of hGH was observed. Thus, it was understood that proteases were secreted overly at higher temperatures. Besides, defined, semi-defined and complex media were used to compare the hGH production. According to the results, defined medium was appropriate not only for cell growth but also for recombinant protein production. On the other hand, effects of glucose and glycerol were investigated as carbon sources. Owing to

slow transport of glycerol into the cell, accumulation of acetate decreased as to use glucose. When glycerol was used instead of glucose, cell concentration and hGH concentration were rose to 100 g/L and 2.7 g/L, respectively as well.

Çalık *et al*. (2008) constructed a novel expression system in *Pichia pastoris* as a host microorganism to produce recombinant human growth hormone. For this purpose, designed plasmid called pPICZαA::*hGH* controlled by AOX1 promoter was cloned into *Pichia pastoris* in order to investigate rhGH production yield. The highest hGH concentration was achieved by colony-15 as in constant value for three days. Although, higher expression levels were obtained with colonies 3 and 9, produced hGH was undergone to degradation after 24 hours due to activities of proteases. Therefore, colony-15 was selected for large scale productions and this new species was denominated as *Pichia pastoris* HGH-15. After the fermentation process for 24 hours, 115 mg/L hGH concentration was reached by this new designed expression system. Produced hormone carried his-tag sequence and it was purified by a special affinity resin with the ratio of 85 % by utilizing this property. MALDI-ToF MS analysis revealed that purified human growth hormone was 1573 Da greater than the standard hGH. This difference was thought to be arisen from 12 amino acids added to the N-terminal site of hGH. Hence, purified hGH digested by Factor Xa having protease recognition site and its purity increased to 88 %. After all, the structure of the purified hormone was proven as authentic one by MALDI-ToF MS analysis.

Shang *et al*. (2009) targeted to avoid the problem of oxygen limitation in large scale production of human growth hormone in *E. coli*. They carried out the experiments on the bioreactors having volumes of 5 L and 30 L. Production of rhGH, cell growth, formation of by-product were compared on various scale fermentation processes by using air or oxygen in high purity as oxygen sources by *E. coli* carrying pEHUB-hGH plasmid. 63.6 g/L and 4.8 g/L were obtained as cell and rhGH concentration respectively in the 5 L bioreactor while feeding the high pure oxygen to the system. On the other hand, cell concentration and rhGH concentration were determined as 51.6 g/L and 4.0 g/L respectively in 30 L bioreactor production system. While 2-fold increase in productivity was observed in 5 L fermentation system by feeding of high purity oxygen, 4-fold increase was obtained for the production system of 30 L. Oxygen limitation and acetic acid formation were also prevented by adding oxygen in high purity to the medium.

Özdamar *et al*. (2009) researched constructing a new recombinant plasmid mimicking the secretion and production system of serine alkaline protease (SAP) in *Bacillus* species required for the synthesis of recombinant human growth hormone. Therefore, a hybrid gene was constituted by joining the DNA fragments of SAP gene (*subC*) of *B. lincheniformis* and *hGH* gene. And then, this hybrid gene was cloned into pMK4 plasmid and expressed in the host organisms of *Bacillus subtilis* (*npr*$^-$ *apr*$^-$) and *Bacillus subtilis* (*spo*$^-$) under the control of *deg*Q promoter. r-hGH production with fermentation characteristics were examined in defined production media by a comparative way with the cell harboring only pMK4 plasmid. Introduction of new biochemical reactions to the intracellular reaction network with the production of recombinant protein caused to obtain different results as expected only from the experiments conducted by the host carrying only pMK4 plasmid. According to the results, higher concentrations of organic acids were acquired by the cell carrying *pMK4*::sub(C)::hGH and contrary higher concentrations of amino acids were obtained by *B. subtilis* harboring solely plasmid. The highest recombinant human growth hormone

concentration was achieved as 70 mg/L at 32 h of the process. Fermentation and oxygen transfer characteristics showed that intracellular reaction rates obtained by SAP production were different from the new rates related to hGH production. Consequently, it was understood that rhGH production with this novel expression system was applicable to the metabolic flux analysis.

Orman *et al*. (2009) investigated the impacts of carbon sources on the production of rhGH by *Pichia pastoris Mut*$^{+}$ and *Pichia pastoris Mut*^{s} in batch processes. When methanol was used as the only carbon source, growth rate of *Mut*^{s} was slow. In the same conditions with *Mut*$^{+}$ strain, 6.0 g dry cell/dm^3 and 0.032 g/dm^3 were obtained as cell concentration and rhGH concentration, respectively. Although cell concentration obtained by strain *Mut*$^{+}$ was higher 2-fold than *Mut*^{s}, 0.16 g/dm^3 rhGH was produced by *Mut*^{s} in complex medium without glycerol. In a glycerol-methanol feeding, consumption of methanol was started with no detection of glycerol in production medium. Hence, it was indicated that glycerol repressed the *AOX1* promoter. The highest hGH concentration was obtained as 0.11 g/dm^3 by *Mut*^{s} strain. Thus, *Mut*^{s} strain was an efficient host for the production of hGH in glycerol-methanol complex medium.

Çalık *et al*. (2010-a) aimed to investigate the effects of the feed rates of methanol (μ_0=0.02, 0.03 and 0.04 h^{-1}) and oxygen transfer characteristics on the production of hGH, protease and the yield of the process in the presence of sorbitol in fed-batch process by *P. pastoris*. According to the results, the highest rhGH production was accomplished by μ_0=0.03 h^{-1} as 270 mg L^{-1} which was approximately 2-fold higher than the other feeding rates of methanol. Moreover, cell growth rate was increased by increasing the feeding rate of methanol, but there was no relationship with the consumption of sorbitol. The yield of human growth hormone on substrate was 2.09 g kg^{-1} as the highest value at the specific growth rate of 0.03 h^{-1}. Contrary to this, the yield of cell on substrate was 0.15 g g^{-1} as the lowest value. These results indicated that biochemical reactions were carried out in the direction of product formation instead of biomass. Alterations in the feeding rate also changed oxygen transfer characteristics and affected in direct proportion. Moreover, the lowest protease activity was obtained with μ_0=0.03 h^{-1} and this indicated that recombinant protein production was high in the low levels of proteases as expected.

Çalık *et al*. (2010-b) investigated the effect of pH on the production of hGH in *P. pastoris*. At the same time, the parameters affected the biochemical process such as cell growth, oxygen transfer characteristics and protease activity were examined. In the experiments, pH values were maintained at the values between 3 and 7 with the increment of 0.5. While the highest hGH concentration was obtained as 0.27 g L^{-1} at pH=5.0, the highest cell concentration was obtained as 53 g L^{-1} at pH=6.0. On the other hand, oxygen consumption rate increased with the increasing pH values up to pH=6.0. When all the results were considered, pH was a deterministic factor on the production of rhGH. Therefore, it was stated that pH=5.0 was the optimum condition for the production.

Özdamar *et al*. (2010) set up a metabolic model based on the mathematical model in genome-scale on the gene of recombinant human growth hormone of *Bacillus subtilis*. Experimental data used in the model was taken from the scientific study elsewhere (Çalık *et al*., 2008). Intracellular biochemical reaction fluxes were determined by solving the mass balances based on the genome-scale model. Intracellular reaction network was developed for

rhGH production. Then, intracellular and extracellular transfer rates were used to solve this model at pseudo-state condition. This metabolic model was consisting of 990 metabolites and 1340 reactions. After all, according to the profiles of cell and rhGH concentrations bioprocess was divided into 5 periods. Reaction network used in the model starting with 328 reactions decreased to 313 reactions in the second period and it increased to 314 reactions by initiation of rhGH production in the third period. And then, reaction system decreased to 296 reactions by progressing the cell growth and rhGH production in the fourth period. Finally, model was completed by 135 reactions in the fifth period.

Çalık *et al*. (2011) aimed to investigate the effect of feeding rate of methanol (0.02, 0.03, 0.04 h^{-1}) on the intracellular reaction rates during the production of hGH in *Pichia pastoris* and to develop new feeding strategies in order to develop hGH production. Fed-batch production process was carried out in 3 L-bioreactor with the host organism, *P. pastoris hGH-Mut*$^{+}$. According to the data obtained from the experimental results, process was divided into four periods. These were Period I ($33 \leq t < 42$ h) which was the exponential phase increased the hGH production, Period II ($42 \leq t < 48$ h) decreasing the specific growth rate, Period III ($48 \leq t < 51$ h) reaching the concentration of hGH to the highest value, Period IV ($t > 51$h) decreasing the cell growth and hGH production. Extracellular concentrations related to the rates obtained at specific points of each period were determined for metabolic flux analysis. When all metabolism was taken into account, Period I was required to start with methanol feeding at the specific growth rate of $\mu_0=0.03$ h^{-1}. After that, decreasing the feeding rate to $\mu_0=0.02$ h^{-1} in the middle of the Period II was considered to increase the hGH production when all the biomass and hGH rates were evaluated in the cellular metabolism.

2.3 Bioprocess Development

2.3.1 Host Microorganism

Effective production systems for biopharmaceutical products which are desired to obtain in high quality and quantity are very critical in biotechnological processes. The selection of a suitable host microorganism and appropriate conditions are crucial factors for the production and downstream operations of bio-products.

Bacterial production systems as host microorganisms are preferable alternatives comparing to other living organisms owing to the conditions of rapid growth, high efficiency, easy to cultivate and inexpensive production in high cell densities at small process times. *Bacillus* species and *Escherichia coli* are the most commonly utilized prokaryotes for the large scale production of recombinant proteins. Post-translational modifications of proteins including formation of disulphide bond and glycosylation are not seen in bacterial hosts in comparison to eukaryotic cells. Recombinant proteins can be produced intracellularly or secreted either into the periplasmic medium like in *E. coli* or into the fermentation broth like in *Bacillus* species (Ratledge and Kristiansen, 2006).

Escherichia coli is a useful host for protein production in large-scale fermentations due to its ability to be grown easily. Therefore, this advantage of *E. coli* makes it attractive for the production of heterologous proteins industrially. However, major problem emerges

with *E. coli* in the production of proteins. Formation of inclusion bodies usually can be occurr via aggregation of protein molecules within the cell. The separation and purification of the secreted proteins from the inclusion bodies is essential to obtain the desired product as pure (Zweers *et al*., 2008). When *B. subtilis* and *E. coli* are compared, there are some advantages of *B. subtilis*, which is Gram-positive bacteria in the production of pharmaceutical proteins. *B. subtilis* is considered as a generally recognized as a safe organism, GRAS defined in abbreviation form. Thus, it is accepted as for the use of food and drug products by Food and Drug Administration (FDA). Almost all Gram-negative bacteria like *E. coli* have lipopolysaccharides (LPS) usually considered as endotoxins in their outer cell membrane structure. Endotoxin substances that are pyrogenic damage to the mammalians especially humans. Moreover, these endotoxins affect the purification of the desired product negatively. Extracellular secretion of the proteins in high capacity by *B. subtilis* facilitates the downstream process as being a natural separation way of the product from the cell (Westers *et al*., 2004).

As human growth hormone is a non-glycosylated protein, it has been most frequently synthesized in these expression systems. *B. subtilis* is a potential cell factory over *E. coli* on the production of recombinant human growth hormone when some factors are deliberated. One of them is the formation of methionine at the initiation of the hGH tending to artificial structure of the hormone unlike the natural form. It is not functional on the secretion of hGH into the extracellular medium due to the protein degradation leading to protein and cell aggregates in the cell structure (Özdamar *et al*., 2009). Therefore, *Bacillus* species, especially *B. subtilis*, are potential hosts to achieve the production of recombinant hGH in high levels (Nakayama et al., 1988; Franchi et al., 1991; Kajino et al., 1997; Şentürk, 2006; Yılmaz, 2008; Özdamar et al., 2009; Şahin, 2010).

2.3.1.1 Genus *Bacillus*

Bacillus is a prokaryotic and aerobic microorganism that is a member of Gram-positive bacteria. This genus has more than 60 species and represents a great genetic diversity based on the ratio of chromosomal base composition, Guanine + Cytosine (G + C), in the range of 36-60 %. The Genus *Bacillus* has had an important role in the field of medicine and fermentation processes since 1970s. There are several reasons to carry researches out with this kind of bacteria. Firstly, they consist of a number of chemoorganotrophs using organic compounds to oxidize chemical bonds for energy can be easily cultivated. Moreover, a wide variety of *Bacillus* species such as psychrophiles, mesophiles, thermophiles, alkalophilics, neutrophilics, and acidophilies are successfully defined (Table 2.1) (Priest, 1977).

Bacillus species have been used in biotechnological areas as effective production hosts. Their ability to secrete of endogenous and heterologous proteins with high capacity makes them important for obtaining information about their fermentation technology (Simonen and Palva, 1993). Several special products produced by *Bacilli* family in the field of food and industry as generally recognized as safe (GRAS). It is approved by U.S. Food and Drug Administration (Arbige *et al*., 1993). The genus *Bacillus* is also one of the most commonly used strain in protein engineering manipulations by the techniques of

mutagenesis. The total value of the enzyme market was nearly $800 million of which two-thirds were produced by the genus *Bacillus* at the end of the 20th century (Ferrari *et al*., 1993).

Formation of endospore is a characteristic feature of the genus *Bacillus*. These endospores that contain dipicolinic acid are very important for the bacteria to resist of many physical and chemical conditions in the environment. Spore formation in bacteria is also related to several biotechnological products such as insect toxins, antibiotics and extracellular enzymes (Priest, 1993). In the endospore forming bacteria, the maximum production of extracellular enzymes and proteins commonly takes place in the late logarithmic (exponential) or early stationary phases of growth before sporulation. The transition from logarithmic phase to stationary phase of the growth is very important. The sudden consumption of the carbon source from the medium and the variations leading to derepress many catabolite-repressed genes in the culture environment happen at this transition time (Priest, 1977).

Bacillus species are commonly used as host organisms for the production of homologous and heterologous proteins. The genes of proteins that are extracellularly synthesized are generally expressed in the gram-positive bacteria of *Bacillus* with their own promoters. They also use their own secretion signals, without need to use specific secretion vectors for the secretion of their products to the medium. On the other hand, exoenzymes produced in this way are not directly affected by the degradation functions of proteases secreted by *Bacillus* host. As a result of that, the yield of the secreted product is high.

Table 2.1 Allocation of some *Bacillus* species to groups on the basis of phenotypic similarities (Priest, 1993)

Species	**Characteristics of Group**
Group I	
B. alvei *B. amylolyticus* *B. apiarius* *B. azotofixans* *B. circulans* *B. glucanolyticus* *B. larvae* *B. lautus* *B. lentimorbus* *B. macerans* *B. macquariensis* *B. pabuli* *B. polymyxa* *B. popilliae* *B. psychrosaccharolyticus* *B. pulvifaciens*	All species are facultative anaerobes and grow strongly in absence of oxygen. Acid is produced from variety of sugars. Endospores are ellipsoidal and swell the mother cell.

Table 2.1 (Continued)

Group II	
B. alcalophilus *B. amyloliquefaciens* *B. anthracis* *B. atrophaeus* *B. carotarum* *B. firmus* *B. flexus* *B. laterosporus* *B. lentus* *B. licheniformis* *B. megaterium* *B. mycoides* *B. niacini* *B. pantothenticus* *B. pumilus* *B. simplex* ***B. subtilis*** *B. thuringiensis*	All species produce acid from variety of sugars including glucose. Most are able to grow at least weakly in absence of oxygen, particularly if nitrate is present. Spores are ellipsoidal and do not swell the mother cell.
Group III	
(*B. alginolyticus*) *B. aneurinolyticus* *B. azatofornans* *B. badius* *B. brevis* (*B. chondroitinus*) *B. freudenreichii* *B. gordonae*	The strict aerobes do not produce acid from sugars; names in brackets are exceptions. They produce ellipsoidal spores that swell the mother cell.
Group IV	
(*B. aminovorans*) *B. fusiformis* *B. globisporus* *B. insolitus* *B. marinus* *B. pasteurii* (*B. psychrophilus*) *B. sphaericus*	All species produce spherical spores that may swell the mother cell and contain L-lysine or ornithine in cell wall. All species are strictly aerobic, but some have limited ability to produce acid from sugars.

Table 2.1 (Continued)

Group V	
B. coagulans *B. flavothermus* *B. kaustophilus* *B. pallidus* *B. schlegelii* *B. smithii* *B. stearothermophilus* *B. thermocatenulatus* *B. thermocloacae* *B. thermodenitrificans* *B. thermoglucosidasius* *B. thermoleovorans* *B. thermoruber* *B. tusciae*	These thermophilic species all grow optimally at > 50 ^{0}C. Physiologically and morphologically, they are heterogeneous, but most produce oval spores that swell the mother cell.
Group VI	
B. acidocaldarius *B. acidoterrestris* *B. cycloheptanicus* *B. pallidus* *B. schlegelii* *B. smithii* *B. stearothermophilus* *B. thermocatenulatus*	Thermophilic, acidophilic species with membraneous ω-alicyclic fatty acids.

2.3.1.2 Gram-Positive Bacteria: *Bacillus subtilis*

Growing soil-based *Bacillus subtilis* is a gram-positive bacterium in rod-shaped. *B. subtilis* is non-pathogenic and this is approved by U.S. Food and Drug Administration with the status of generally recognized as safe.

The first known product produced by *B. subtilis* is natto which is a Japanese fermented food made of soybeans. At present, *B. subtilis* is a well-known producer host for several biochemicals, enzymes and also heterologous proteins. Another important ability of *B. subtilis* is that it secretes the proteins directly to the culture medium. Consequently, this bacterium is known as an attractive cell factory for pharmaceutical proteins and industrial enzymes (Zweers *et al*., 2008).

Gram-positive cell wall is a very simple structure. A thick cell wall containing peptidoglycan and teichoic acid encloses the cytoplasmic membrane of the *Bacillus* cell. Protein synthesis occurs in cytoplasm, and then the *Bacillus* cell exports the products in order to the cytoplasmic membrane, the cell wall, and the external medium. The cell wall of

the *B. subtilis* contains autolytic enzymes degrading the cell wall components and necessary for normal growth and cell division (Simonen and Palva, 1993).

In *B. subtilis*, cytoplasmic protein production is very successful. Several proteins in active forms can be produced commercially by *B. subtilis* secretion system. Because *B. subtilis* does not have an outer cell membrane, it can secrete the proteins directly into the process medium. This impressive future facilitates the downstream processing and provides large quantities of proteins produced by *B. subtilis* (Zweers *et al*., 2008). Due to the developed studies in recombinant DNA technology with *Bacillus subtilis*, it has clearly had more interest than the other *Bacillus* species on protein secretion and production (Simonen and Palva, 1993).

On the other hand, some shortcomings belong to *B. subtilis* for the production of recombinant proteins are: lack of satisfactory expression vectors, existence of proteases, plasmid instability and presence of unfolded proteins (Westers *et al*., 2004). However, these problems can be overcome by using a number of methods such as using chaperons and/or catalysts for folding and protease knockout strains and/or protease inhibitors for production (Zweers *et al*., 2008). Protease activity of *B. subtilis* is not only decreased by protease deficient strains but also affects the yield of the protein mediately with metabolic reactions. Yılmaz (2008) increased hGH production two fold by *B. subtilis* (*apr*$^{-}$ *npr*$^{-}$) in comparison with *B. subtilis* WB700 (seven protease deficient strain). By using engineering techniques in genetic science, expression vectors can be stable by constructing of controllable promoters. Integration of the replicative plasmid into the chromosome removes the plasmid instability (Heap *et al*., 2012).

Producing U.S. FDA approved pharmaceutical proteins by *B. subtilis* can be possible by choosing an effective host with all knowledge on promoters, plasmids, signal peptides, secretion mechanism and fermentations, proteases and mutant strains (Westers *et al*., 2004). Owing to the advantages, hGH as a pharmaceutical hormone was produced by Nakayama *et al*. (1988), Franchi *et al*. (1991), Şentürk (2006), Yılmaz (2008), Özdamar *et al*. (2009) and Şahin (2010).

2.3.1.3 Expression and Secretion of Proteins in *Bacillus* Species

Cellular growth is the conversion of substrates that is essential for growth and production into the biomass and bio-products. During the cellular growth, also some metabolic products called by-products are formed as a result of catabolic reactions within the cell. The other bio-products are the proteins, e.g., acting as hormones or antibiotics which have significant value at industry. The essential elements for the cell growth are carbon source, nitrogen source, energy source, minerals and vitamins. Commonly carbon and energy sources to provide the Gibbs free energy for cell growth are alike (Nielsen *et al*., 2003).

After the cell growth, production of the macromolecules namely proteins are occurred by the cell. Secreting a protein is transferring it from the place that it is synthesized, generally the cytoplasm, to the other places in the cell along the secretion pathway. Secretory proteins are released out of the cell.

Secreted proteins firstly appear as preproteins. Preproteins have an amino-terminal extension in their structure called signal peptide. This signal peptide is important for the transportation and the separation of secreted proteins from the cytoplasmic proteins. It binds to the membrane by the help of soluble cytoplasmic protein components called as chaperones. In bacteria the secreted proteins must pass through the cytoplasmic membrane (CM) for targeting. In contrast to bacteria, endoplasmic reticulum (ER) membrane is functional in eukaryotic cells. Chaperones are effective for the tertiary structure of the protein in the folding processes. On the other hand, if the protein gets its tertiary structure before translocation, a step at which the transferring of the protein from cytoplasm either to or through a membrane occurs, it cannot further be secreted. A specific protease, a signal (leader) peptidase, removes the signal peptide during the translocation process. After the processes of translocation and signal peptide cleavage, the exported proteins stay as protein-membrane complexes until their folding is complete (Figure 2.3) (Simonen and Palva, 1993).

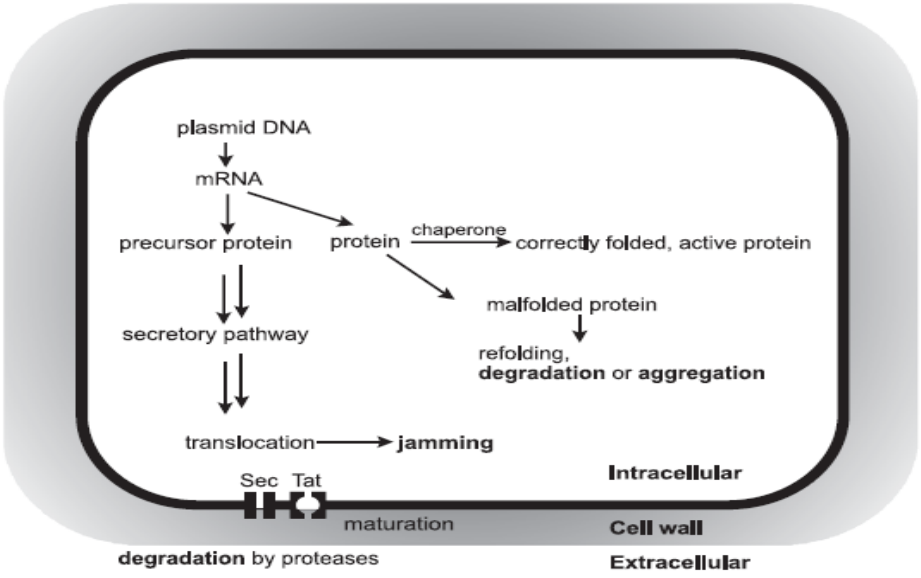

Figure 2.3 Schematic processes of the secretion and the production steps of heterologous proteins (Westers *et al*., 2004)

The *Bacillus* secretion system is the first studied system in the bacteria. However, the knowledge about its secretion mechanism is not enough. Therefore, translocation assay is needed to clarify the knowledge about the protein export mechanism in gram-positive bacteria. Due to the developed studies in recombinant DNA technology with *B. subtilis*, it has clearly had more interest than the other *Bacillus* species on protein export studies (Studer and Karamata, 1988).

Degradation function of the proteases is a major problem for *Bacillus* species in the production and secretion of foreign proteins. Various mutants of which genes are deleted related to protease activity are used to solve this problem. Also protease inhibitors have been

used to deactive their functions. Thus, proteins can be produced without any degradation in their structure (Şentürk, 2006; Yılmaz, 2008; Özdamar *et al*., 2009; Şahin, 2010). However, industrial large-scale production may be risky owing to their toxicity, expense and their opposite effects on the growth of the bacteria. One of the major enzymes of *B. subtilis* is serine alkaline protease (SAP) having three parts inside the cell: signal peptide, pro-peptide and the main protein fragment (SAP) (Figure 2.4).

Lipoproteins are a kind of secretion proteins found in cytoplasmic or outer membrane with lipid particles and synthesized by *E. coli* and *Bacillus* species. For instance, β-lactamase, a lipoprotein, is found on the outer surface of the cytoplasmic membrane of the several gram-positive bacteria. The signal peptides of bacterial lipoproteins are corresponding to the other signal peptides by the following features; a tripartitate structure with a positively charged NH_2 terminus, a hydrophobic core and a cleavage region.

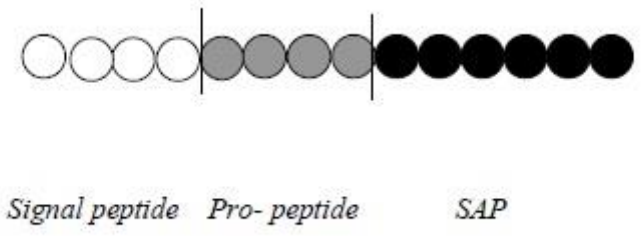

Figure 2.4 Structure of secreted SAP inside the cell

Pro-peptides known as protein precursors are found in *Bacillus* secretory proteins between the signal peptide and the developed fragment of the protein. Secreted *Bacillus* proteins remove from their long and short pro-peptides (signal peptides) after the process of translocation as seen in Figure 2.5. Long pro-peptides are the main part in the synthesis of proteases such as serine alkaline proteases and neutral proteases. It is also known that pro-peptides are responsible for folding and activation of the proteases after the translocation.

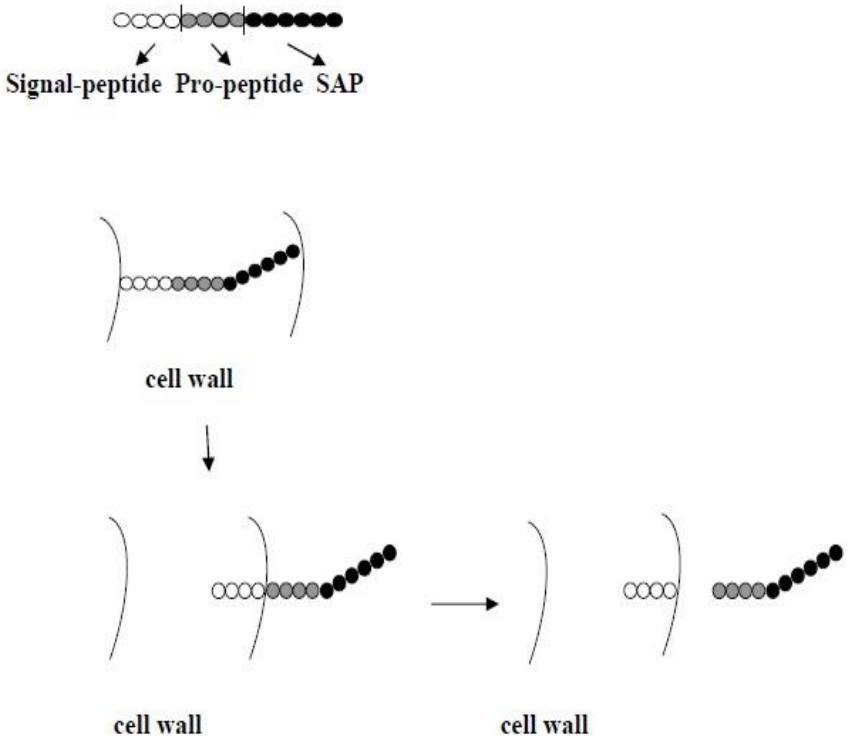

Figure 2.5 Transport process of SAP to the extracellular medium

Proteases are generally secreted in the late-growth phase. The most common and studied proteases in the Bacillus species are subtilisin (alkaline serine protease) and neutral protease (metallo-protease) having 77 and 194 amino acid residues in their pro-peptides, respectively (Simonen and Palva, 1993).

2.3.1.4 Regulatory Gene Network of the Expression of *aprE* Gene

Scarcity of nutrients directs the microorganisms to secrete its degradative enzymes, produce target proteins, develop motility and competence especially in *Bacillus subtilis* and initiate the spore formation. Development of competence needs glucose whereas sporulation process is repressed by carbon source. This proves the existence of interrelated regulatory controls in a microorganism physiology.

Signal sequence of *subC* gene on the target plasmid, pMK4::*pre*(*subC*)::*hGH* imitates the extracellular serine alkaline protease (subtilisin) production in *B. subtilis*. The subtilisin gene of *B. subtilis* is *aprE*. There are many protein regulators had functions as being inducers or repressors that control the secretion of *aprE* gene. This protein network consists of AbrB, DegQ, DegS, DegU, SinI, SinR, Spo0A and ScoC (Hpr) (Smith, 1993; Kallio *et al*., 1991; Ogura *et al*., 1994; Strauch, 1995). Expression of *aprE* gene and also late-growth development are regulated and controlled by the primary pathways of the proteins such as degS, degU, degQ, sinR, abrB, scoC (Hpr), etc. (Figure 2.6).

DegU indicates a major effect in regulating of extracellular enzyme synthesis. DegU and its connate, DegS are responsible for the expression of *degQ* and *sacB*. Enhanced production of alkaline protease, neutral protease, xylanase, β-glucanase and α-amylase, loss of motility and lowered competence are observed by the functions of both degU and degQ. Transcription of *aprE* increases by the overproduction of DegQ. DegS-DegU system is required for the over expression of degQ and has positive effect on the expression of subtilisin. The overproduction of DegR, 60 amino acids protein coded by *degR*, causes to be an increase in the production of alkaline and neutral proteases and levansucrase. On the other hand, initial transcription levels of *aprE* are enhanced by DegR. ComA activates the *degQ* expression.

TenA coding a protein of 236 amino acids is related to the enhancement of extracellular enzymes. Secretion levels of levansucrase, alkaline and neutral proteases are increased nearly 10 fold over by a multi-copy plasmid with both tenA and tenI of a strain. Moreover, an additional enhancement of 5 fold for alkaline protease and 3 fold for levansucrase activity was observed in the use of a plasmid containing only tenA. Therefore, tenI, 205 amino acid protein, could be an inhibitor of tenA function.

SenS is a DNA fragment from *Bacillus nato*. When it is cloned into the plasmid of *B. subtilis*, production of alkaline and neutral proteases, α-amylase and alkaline phosphatase increase two to three fold. Studies on the reporter gene assays for the expression of aprE indicate that senS affects the transcription of the target gene, *aprE* positively in vivo. However, there is no certain information about the direct relation between senS protein and aprE promoter (Strauch, 1995).

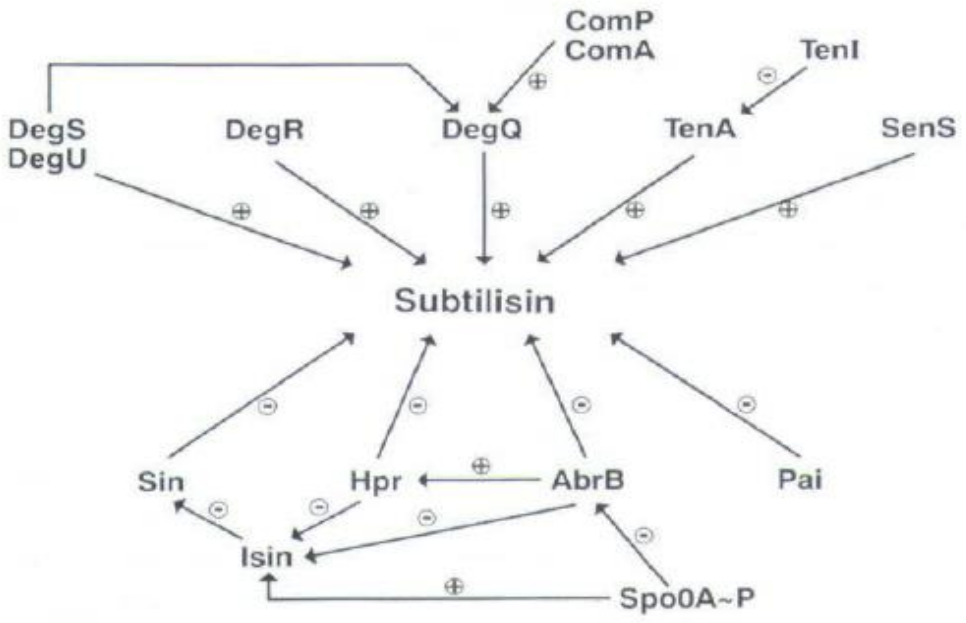

Figure 2.6 Regulatory gene network of *aprE* gene on subtilisin expression (Strauch and Hoch, 1993)

The gene fragment of Pai in *B. subtilis* is repressed the production of extracellular proteases. It inhibits the sporulation and expression of *aprE*, *nprE* and *sacB*, when it is overproduced.

Spo0A is a critical regulator in the late-growth functions. It behaves both as a positive and a negative regulatory key component. While AbrB is repressed when spo0A is phosphorylated, spo0P, sinI is activated by spo0A. Thus, early initiation of sporulation starts by derepression of spo0H, spo0E and spoVG.

Activity of SinR is controlled by the contrast function of SinI. SinR inhibits the sporulation process by presence on a multi-copy plasmid. Extracellular production of alkaline and neutral proteases is also inhibited with the result of 10 % less protease activity (Smith, 1993).

AbrB is a DNA binding protein and has an effective role in the transition phase. AbrB represses *aprE* and early sporulation genes such as *spo0H*, *spo0E* and *spoVG*. It induces the expression of *hpr* and suppresses the competence when it is overproduced.

The mutations on the *hpr* gene are responsible for the overproduction (16 to 37 fold) of alkaline and neutral proteases. The other genetic lesions are *catA* and *scoC*. Sporulation occurs by glucose insensitively leading to enhanced exo-protease production. *catA* and *scoC* are also mapped in the same locus with the *hpr* gene in *Bacillus subtilis* chromosome. The functions of protein Hpr are closely related to work of these genes. Hpr or scoC inhibits the production of proteases and sporulation. Therefore, *hpr*, *catA* and *scoC* mutations eliminate this repressive effect of hpr protein. The hpr or scoC protein consisting of 119 amino acids and having a molecular weight of 23.7 kDa has been obtained as pure and proven that it binds to the regions having regulatory functions of nprE and aprE. Therefore, *hpr* or *scoC* mutations (hpr^- or $scoC^-$) increase the transcriptional levels of *aprE* (Smith, 1993).

All in all, recombinant human growth hormone production is conspicuously affected by these regulatory genes of the expression of *aprE* gene since designed vector of the cell mimicks the subtilisin production. Hence, recombinant null strains of *B. subtilis* were constructed in order to recognize the effects of these genes on the production of rhGH. The highest rhGH yield was achieved by the *scoC* knockout strain of *B. subtilis* (Şahin, 2010).

2.3.2 Intracellular Reaction Network

Metabolism is the series of biochemical reactions in order to maintain an organism's vital activities. A living microorganism consists of a great number of compounds and metabolites. Biochemical synthesis of these macromolecules is basically derived from carbon and energy sources. Metabolism consists of consecutive enzyme catalyzed reaction steps which is also called as metabolic pathway. The metabolic pathway of recombinant *Bacillus subtilis* is given in Figure 2.7. There are specific biochemical pathways interconnected the sequences of reactions including primary and secondary metabolites for each producible microorganism (Stephanopoulos *et al.*, 1998). Basic metabolic pathways are glycolysis, glikoneogenesis, TCA (tricarboxylic acid) cycle and pentose phosphate.

Cellular metabolism can be divided into two groups with its general definition: Primary metabolism and secondary metabolism. While primary metabolism is connected with the cellular metabolism including growth processes of the cell, secondary metabolism refers to many reaction steps leading to antibiotics, hormones, etc. Reactions of catabolism and anabolism, as stated in the following sections, take place simultaneously (Lehninger *et al*., 1993).

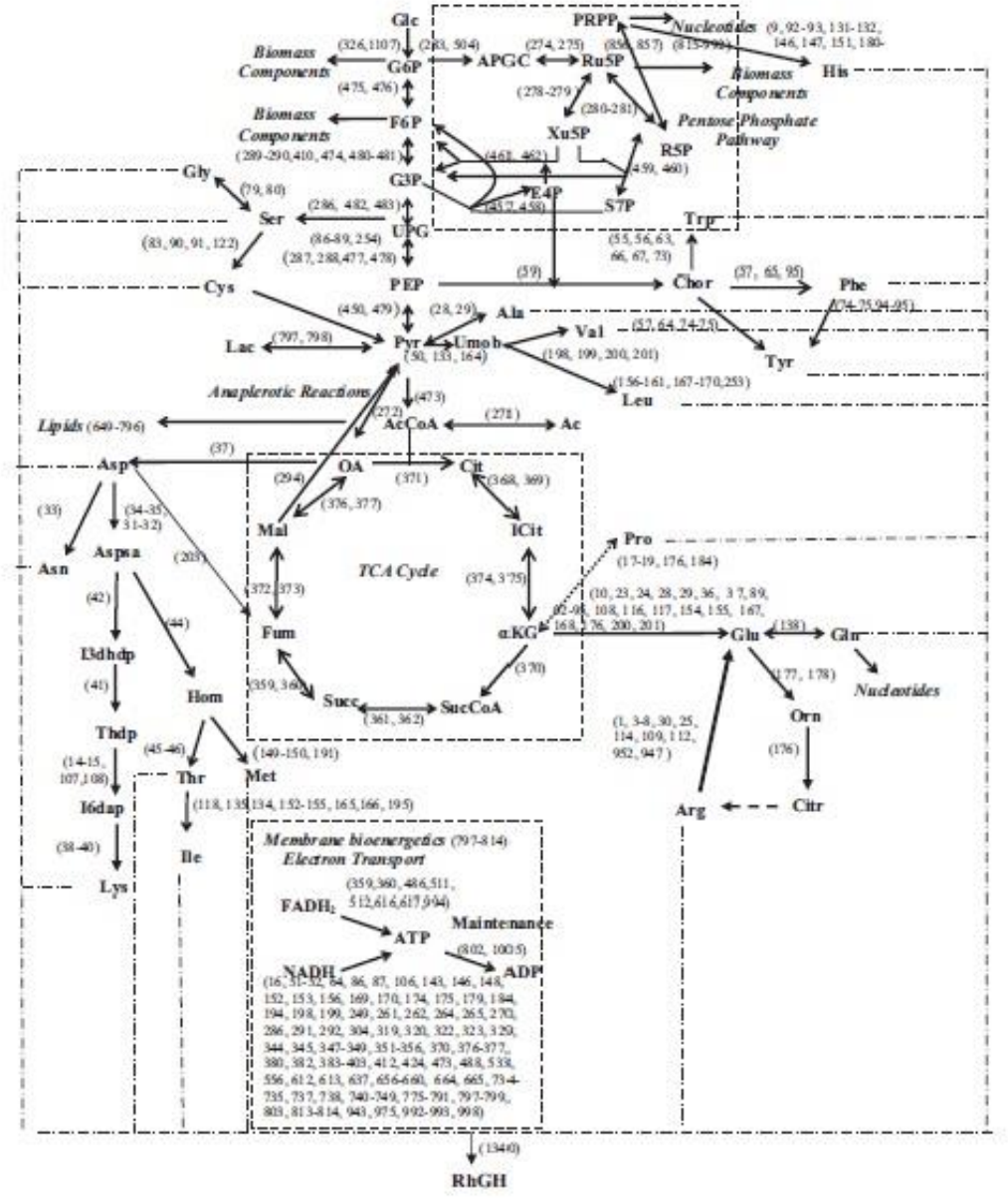

Figure 2.7 The metabolic pathway map of recombinant *B. subtilis* (Özdamar *et al*., 2010)

a) Catabolic Reactions

Fueling process of substrate molecules (carbohydrates, proteins, fats) into the precursor substances having smaller molecular weights (pyruvate, CO_2, i.e.) is defined as catabolism. In catabolic pathways, ATP or free energy that is converted to reduced electron carriers (NADH and NADPH) is released.

Substrate molecules are firstly taken into cytoplasm of the cell and then converted into precursor metabolites and thus, Gibbs free energy is released. This released Gibbs free energy is stored as chemical energy either by ATP in high energy phosphate bonds or by NADPH/NADP+ and NADH/NAD+ in reduced electron carrier molecules (Nielsen *et al*., 2003). NADH is used in catabolic reactions whereas NADPH is used in anabolic reactions.

The Gibbs free energy released by the hydrolysis of high energy phosphate bonds in ATP:

$$ADP+ \approx P - ATP - H_2O = 0 \quad ; \quad \Delta G^0 = -30.5\,kJ/mole \qquad (2.1)$$

The critical pathways of catabolism are glycolysis, TCA cycle and oxidative phosphorylation.

In most prokaryotic or eukaryotic cells, glucose is catabolized via the glycolytic pathway, glycolysis (Figure 2.8). Glycolysis pathway is a converting mechanism of glucose or glucose-6-phosphate into the pyruvate by Embden-Meyerhof-Parnas (EMP) pathway. Pyruvate (PYR) is then oxidized via the citric acid cycle (TCA) to generate ATP. There is also another metabolic pathway, pentose phosphate (PP) pathway, utilizing glucose to produce NADPH for reductive biosynthesis and specialized products such as ribose-5-phosphate (R5P) and erythrose-4-phosphate needed by the cell (Lehninger *et al*., 1993). The overall reactions of EMP and PP pathway are given in the equations 2.2 and 2.3, respectively.

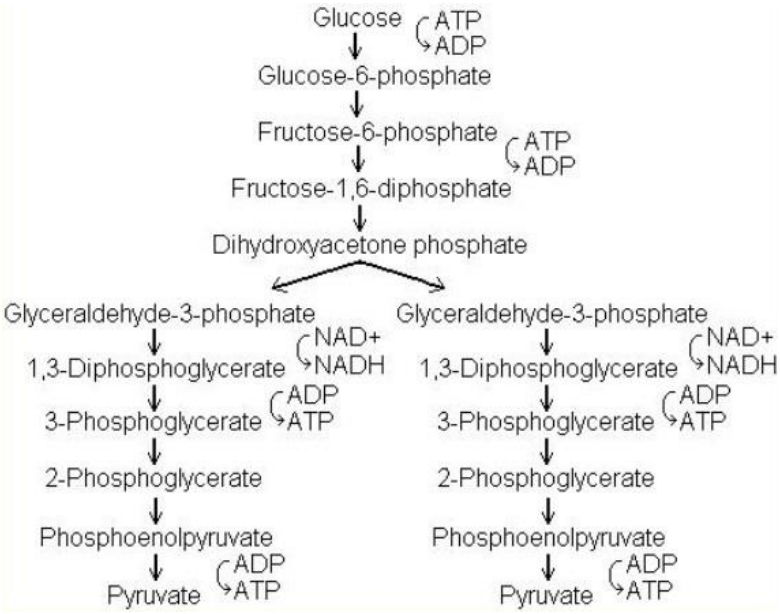

Figure 2.8 Schematic representation of glycolysis pathway

$$2\,PYR + 2\,ATP + 2\,H_2O + 2\,NADH + 2\,H^+ \rightarrow$$
$$Glu\cos e + 2\,ADP + 2 \approx P + 2\,NAD^+ \qquad (2.2)$$

$$3\,G6P + 6\,NADP^+ + 3\,H_2O \leftrightarrow$$
$$2\,F6P + G3P + 3\,CO_2 + 6\,NADPH + 6\,H^+ \qquad (2.3)$$

Oxidation of glucose is completed by aerobic respiration in TCA cycle (Figure 2.9). Pyruvate formed in glycolysis pathway is oxidized to Acetyl-CoA and CO_2 by the pyruvate dehydrogenase (PDH). As a result of the oxidation of 1 mole pyruvate, 1 mole ATP, 4 moles NADH and 1 mole $FADH_2$ are formed in TCA cycle.

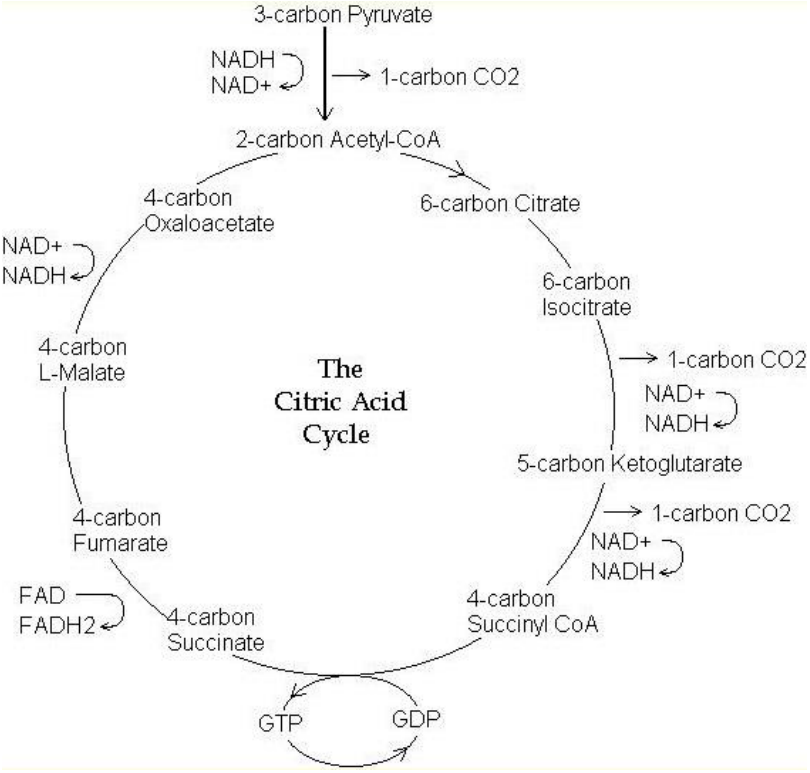

Figure 2.9 Schematic representation of TCA cycle

b) Anabolic Reactions

Anabolism is the synthesis reaction series of macromolecules in complex structure such as nucleic acids, proteins from the simpler metabolites having small molecular weights. ATP utilized in anabolic reactions is obtained by the hydrolysis of NADPH.

The macromolecules which are formed larger part of the cell are required to synthesize by the polymerization reactions of the amino acids and nucleic acids. Approximately 70 % of the energy produced in the cell by the catabolic reactions is used for protein synthesis (Nielsen *et al*., 2003).

2.3.3 Medium Design

Requirement of nutrients for fermentation processes varies with not only the types of the microorganisms but also their species and strains. Organisms can be divided into four categories: photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic organisms. Photoautotrophic organisms use CO_2 as the main carbon source and depend on light as an energy source. In contrast to photoautotrophics, photoheterotrophic organisms utilize organic compounds for carbon source. Chemoautotrophic organisms employ CO_2 as a primary carbon source and also utilize reduced inorganic compounds for energy

requirements. On the other hand, carbon and energy sources are both provided by organic compounds in chemoheterotrophic organisms. Great majority of bacteria are included in this category. The biological importance of major chemical elements in microorganisms is stated in Table 2.2 (Vogel and Todaro, 1997).

Medium design affecting productivity of fermentation processes is very important as well as the host organisms which are designed to produce the desired product. Fermentation medium is generally classified into two groups: chemically defined (synthetic or defined) or undefined (natural or complex) (Zhang and Greasham, 1999). A defined or synthetic medium contains totally pure chemical compounds in known ratios. Conversely, undefined medium components are in natural origin having substantial inorganic materials and the proportions of compounds within are not chemically defined. Moreover, semi-defined medium is an alternative medium needed for some processes in literature and means that only one or two nutrients of its composition are in complex origin (Kerovuo *et al*., 2000; Vuolanto *et al*., 2001; Oh *et al*., 2002). A term "minimal" is also used for a media that contains only necessary components with defined compositions. Nutrients contained in these media are generally classified as: sources of carbon, nitrogen and sulfur, trace and essential elements.

Table 2.2 Physiological functions of essential elements in microorganisms (Stainer et al., 1976; Bennett and Frieden, 1967)

Element	Symbol	Physiological Function
Hydrogen	H	Component of cellular water and organic cell materials
Carbon	C	Component of organic cell materials
Nitrogen	N	Component of proteins, nucleic acids and coenzymes
Oxygen	O	Component of cellular water and organic materials, as O_2 electron acceptor in respiration of aerobes
Sodium	Na	Principal extracellular cation
Magnesium	Mg	Important divalent cellular cation, inorganic cofactor for many enzymatic reactions, functions in binding enzymes to substrates
Phosphorus	P	Component of phospholipids, coenzymes and nucleic acids
Sulfur	S	Component of cysteine, cystine, methionine and proteins as well as some coenzymes as CoA and cocarboxylase
Chlorine	Cl	Principal intracellular and extracellular anion
Potassium	K	Principal intracellular cation, cofactor for some enzymes
Calcium	Ca	Important cellular cation, cofactor for enzymes as proteinases
Manganese	Mn	Inorganic cofactor cation, cofactor for enzymes as proteinases
Iron	Fe	Component of cytochromes, cofactor for a number of enzymes

Table 2.2 (Continued)		
Cobalt	Co	Component of vitamin B_{12}
Copper	Cu	Inorganic components of special enzymes
Zinc	Zn	
Molybdenum	Mo	

2.3.3.1 The Carbon Source

Generally carbon ratio in biomass is based on 50 % carbon on a dry weight. In order to provide carbon and energy needs, a number of microorganisms can utilize a sole compound. Carbohydrates are major sources carbon, hydrogen, oxygen and metabolic energy. They are often present in the production medium in amounts greater than other nutrients (0.2-25 %). Carbohydrates are responsible for the production of ATP in biological energetics. For instance, glucose is the most preferable carbohydrate and it is changed over carbon dioxide, water and energy during catabolism. Carbohydrate metabolism is controlled by the Embden-Meyerhof pathway (EMP), the Krebs or tricarboxylic acid cycle (TCA), the pentose-phosphate pathway (PPP) in catabolism process.

Glucose is converted to pyruvate by anaerobic process in EMP. At this point, glucose, fructose and galactose may also choose the other aerobic pathway to the TCA cycle. 2 moles of ATP per mole of glucose broken down in the EMP is obtained. If the cells contain the additional oxidative pathways, the $NADH_2$ goes into the oxidative phosphorylation to generate ATP. Ethanol, lactic acid, butanol, isopropanol, acetone, etc. may be the precursor to many products resulting from pyruvate in fermentative organisms. Pyruvic and lactic acids are converted to CO_2 and H_2O in TCA cycle. Oxidation process of all fatty acids and carbon structures of numerous amino acids is also carried out in TCA cycle. When pyruvic acid is the initial material, the overall reaction is:

$$2C_3H_4O_3 + 5O_2 + 30\,ADP + 30\,P_i \rightarrow 6CO_2 + 4H_2O + 30\,ATP \qquad (2.4)$$

Apparently, the EMP and TCA cycle are principal sources of ATP for energy and some intermediates for amino acid and lipid synthesis (Vogel and Todaro, 1997).

The PPP functions pentoses and is critical for nucleotide (ribose-5-phosphate) and fatty acid ($NADPH_2$) biosynthesis.

2.3.3.2 The Nitrogen and Sulfur Source

Nitrogen source is as essential as the carbon source in fermentation processes. Reduced forms of nitrogen and sulfur such as amino and sulfhydryl groups are found in the cell as organic compounds. Many photosynthetic organisms fulfill the needs of these elements as nitrates and sulfates. Most non-photosynthetic organisms such as bacteria and fungi take in nitrogen and sulfur from nitrates and sulfates. Some organisms cannot provide the reduction of these elements. Hence, reduced forms of them such as ammonia salts for

nitrogen and either sulfide or cysteine containing a sulfhydryl group for sulfur must be contributed. Amino acids and peptones are also alternatives to be used for complex nitrogen and sulfur sources. Contrary to eukaryotes, various prokaryotic microorganisms can also use natural nitrogen, N_2 by the process of nitrogen fixation involving the reduction of N_2 to ammonia. Cellular materials such as DNA, RNA and amino acids are synthesized by the addition of nitrogen in anabolic reactions. Urea is also used for nitrogen as an organic source and increases the pH value of the fermentation broth. Corn steep liquor, yeast extracts, peptones, hydrolysates and digests from casein, milk proteins, etc. are the other nitrogen sources. These complex nitrogen sources originating from the by-products of the industries of agricultural and food can be also used to achieve commercially more rapid and efficient production system in industrial fermentations (Vogel and Todaro, 1997).

2.3.3.3 The Source of Trace and Essential Elements

Minerals are essential elements for cells during the cultivation. Iron (Fe^{2+} and Fe^{3+}), zinc (Zn^{2+}), manganese (Mn^{2+}), molybdenum (Mo^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}) and calcium (Ca^{2+}) are the trace elements required in low levels having various functions of vitamin synthesis, cell wall transport and coenzyme cooperation to catalyze intracellular reactions of the network. Trace elements might supply to both primary and secondary metabolite production. While manganese can affect the production of enzymes, zinc and iron may induce antibiotic production in case they are used in essential amount. Longevity can be enhanced by manganese in *Bacillus* species, by iron in *Escherichia* species and by zinc in *Torulopsis* species. All necessary inorganic cations are provided by inorganic salts (Vogel and Todaro, 1997). Vitamins are the other growth sources that have to be also supplied in limited amounts in order to function as coenzymes to many reactions.

2.3.4 Bioreactor Operation Modes and Parameters

A medium design with the oxygen transfer characteristics and pH conditions for the functions of an aerobic microorganism in a bioprocess is very critical on the biomolecule synthesis. Getting information about genetic mechanism and identifying intracellular metabolic reaction network is a prerequisite to improve the product capacity in a bioprocess (Çalık *et al*., 2003). Following, the bioreactor operation conditions and parameters affecting metabolic pathways should be investigated and evaluated with strategic approaches in order to increase the product yield and selectivity.

2.3.4.1 Batch Operation

In batch operation, suitable microorganisms for recombinant production are inoculated into the bioreactor in which there is already sterile production medium in it. Generally stirred tank bioreactors are used for batch fermentation. All nutrients are loaded to the bioreactor before the cultivation, except oxygen and base or acid are supplied during the fermentation

process. Oxygen and base or acid are added for the aerobic fermentation and pH adjustment, respectively (Vogel and Todaro, 1997).

During a batch fermentation process, the composition of the culture medium, the concentration of the biomass, the yield of the desired protein and the consumption of the substrate are all related to the phases of the microorganism growth. Lag phase is the initial phase for the adaptation of the cell into the new environmental conditions. There is no growth in this phase. After the adaptation, the cell mass in the production medium begin to increase with the constant, maximum specific growth rate. This intermediate period is called log or exponential phase. In this case, the rate of increase of the biomass with time is given in the equation-2.5 where μ is the specific growth rate and C_X is the cell concentration (Glick *et al*., 2010).

$$\frac{dC_X}{dt} = \mu C_X \tag{2.5}$$

Available substrate in the cultivation medium is depleted by the end of the log phase and the cell quickly enters on the deceleration phase. After the exhaustion of the essential nutrients such as main carbon source or the accumulation of some metabolites that inhibit the cell growth, stationary phase begins. In this phase, generally target products are synthesized even if the cell concentration remains constant. Death phase related to the rapid decreasing the amount of biomass is then seen after this period. However, the production is halted commercially and the cells are harvested before this period. Representation of batch growth is illustrated in Figure 2.10.

Batch operation is usually effective, with high productivity due to the autocatalytic nature of metabolic reactions inside the microorganism. Entire conversion of substrate can also be feasible in batch fermentation process.

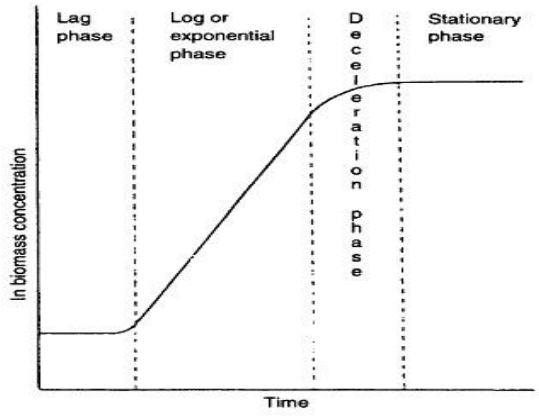

Figure 2.10 Typical phases of microbial growth (Stanbury and Whitaker, 1984)

In the literature, recombinant human growth hormone production by *Bacillus subtilis* has been conducted by batch operation mode to date by the studies of Şentürk (2006), Yılmaz (2008), Özdamar *et al*. (2009) and Şahin (2010). They all used defined medium for recombinant production and Şahin (2010) achieved the highest rhGH concentration and the highest cell concentration as 126 mg L^{-1} and 1.62 g L^{-1}, respectively.

2.3.4.2 Continuous Operation (Chemo-Stat)

Steady-state operation conditions are maintained in continuous fermentation. Fresh production medium is supplied continuously during the fermentation process. At the same time, an identical volume of spent medium is simultaneously removed from the bioreactor. The main assumptions of this operation are the constant total number of cells and the constant total volume of bioreactor. The growth of the cells is controlled by the growth-limiting substrate, thus this operation is also described as chemo-stat. Loss of cells via the outflow is balanced by the new cell divisions as given in equation-2.6. The flow of production medium through the bioreactor is described by dilution rate (D) which is defined as the volumetric flow rate divided by constant working volume of the bioreactor (Stanbury and Whitaker, 1984).

$$\frac{dC_X}{dt} = \mu C_X - D C_X \qquad (2.6)$$

Under steady-state conditions, the term dC_X/dt is equal to zero. Therefore, $\mu C_X = DC_X$ and $\mu=D$. Thus, dilution rate is used to control the growth rate of the microorganism in continuous operation.

High productivity and constant product quality can be attained by continuous fermentation process. Physiological state of the biomass is uniform. Nonetheless, some cells may lose their specific recombinant plasmids due to the long duration of operation (500 to 1000 hours). These cells divide faster than the cells containing plasmids hence, yield of the production might be decreased. In order to avoid this handicap, cloned gene can be integrated into the genome of the host microorganism (Glick *et al*., 2010). Moreover, maintenance of the sterile conditions for sustained process is the other problem of continuous fermentation and increase the cost of the production.

2.3.4.3 Semi-Batch (Fed-Batch) Operation

Semi-batch (fed-batch) operation is an intermediate mode that between batch and continuous operations. Unlike the other two bioreactor operations, one or more nutrients within the fresh medium are fed to the bioreactor continuously or consecutively during the fermentation without the outflow of the culture medium in semi-batch operation. Therefore, the working volume of the bioreactor increases with time (Stanbury and Whitaker, 1984). The generally established term “fed-batch” in biochemical engineering is used instead of the identical term “semi-batch” in chemical engineering. The primary feature of semi-batch

processes is that the concentrations of the substrates fed into the production medium can be controlled by altering the feed rate. This directly affects the productivity of the target metabolite.

Carrying out the microbial processes by semi-batch technique offers various advantages on substrate inhibition, catabolite repression, effect of glucose, high cell concentrations, etc. Substrates such as alcoholic and organic compounds can inhibit the cell growth during the cultivation. This problem can be overcome by adding such substrates suitably to the culture broth by semi-batch operation for instance, as applied for methanol in the study of Orman *et al*. (2009). When the glucose concentration in the medium gets higher for microbial processes, the resulting higher concentration of ATP represses the biosynthesis. Therefore, semi-batch technique is worked to keep the glucose concentration in the medium low level. Furthermore, this excess amount of glucose is also caused to ethanol formation as by product in aerobic processes. High concentration of substrates is needed to supply to the batch culture to attain the high cell cultivation. However, this kind of high capacity batch system has inhibitory effects on the production. Also much more oxygen is needed for more nutrients and it is not optimal for the batch system (Yamanè and Shimizu, 1984).

Controlling the substrate concentration within the culture broth is the main objective of semi-batch operation. The kind of the nutrient and its feeding mode can be effectively selected for microbial production. Not only the high cell concentration which directly affects the cell productivity, but also the high product formation are related to the feeding the essential nutrients by various strategies. Although trial and error method is sometimes used to identify the semi-batch technique that is the most usable, genetics and biochemical engineering make the decisions simple on choosing the optimal feeding strategy. Classification of semi-batch operations as seen in Table 2.3 is organized according to the feeding strategies.

The semi-batch operations without feedback control are subdivided into intermittent, constant, exponential and optimized according to feed rates (Yamanè and Shimizu, 1984). In the intermittent addition, nutrient/nutrients is fed to the system in pulses provided the substrate concentration in it the same until used up. If the substrate is fed gradually at increased rate, high cell concentration is achieved by providing more nutrients with time.

In constant feed rate strategy, nutrients are supplied to the bioreactor with a constant pre-determined rate. The value of the specific growth rate progressively decreases and the raise on the cell concentration gets slower over time due to the higher volume in the production medium and high cell cultivation (Lee, 1996). Lee and Parulekar (1993) investigated the synthesis of α-amylase by *B. subtilis* TN106 in a defined medium containing glucose or starch as the carbon source. They found out that in the semi-batch operations with constant feeding strategy, the higher the volumetric feed rate, the higher was the concentration of recombinant cells and the lower was the specific α-amylase activity.

Exponential feeding strategy allows the microorganisms to grow at a constant specific growth rates. Nutrients are exponentially fed to the bioreactor with a pre-determined specific growth rate (μ). Under non-ideal conditions in exponential feeding, this constant specific growth rate may deviate from the targeted value. In such circumstances, extra feedback control mechanisms may be needed. Exponential feeding strategies have been successfully

utilized for *B. subtilis* fermentations. For instance, Oh *et al*. (2002) studied on the exponential feeding at variable specific growth rates for subtilisin production. They observed that maximum subtilisin concentration was achieved at the specific growth rate of 0.35 h^{-1} after the co-feeding of glucose with peptone ended. On the other hand, Huang *et al*. (2004) researched into α-amylase production with dual feeding of carbon and nitrogen sources and amino acid supplements at μ=0.047 h^{-1} to obtain a high cell density (17.6 g L^{-1}) and a final α-amylase activity (41.4 U mL^{-1}).

Table 2.3 Classification of semi-batch operations (Yamanè and Shimizu, 1984)

Without Feedback Control	
Intermittent addition	Pulse nutrient feeding
Constant rate	Feeding nutrient at a pre-determined constant rate
Exponentially increased rate	Feeding nutrient at an exponential rate with a pre-determined constant rate
Optimized	Feeding nutrient with combined strategy
With Feedback Control	
Indirect feedback control	DO, pH, cell concentration, etc.
Direct feedback control	Substrate concentration control (constant or optimal)

Optimized feeding is based on the combined feeding strategies. Christiansen *et al*. (2003) investigated savinase formation in high cell density semi-batch cultivations with both linear and exponential feeding. Then, the highest overall yield of savinase was obtained with an exponential feeding of glucose at μ=0.047 h^{-1}.

The semi-batch operations with feedback control are controlled directly or indirectly. Indirect feedback control is based on the feeding nutrients and simultaneously measuring the some parameters such as dissolved oxygen (DO), pH, cell concentration, CO_2 evaluation rate (CER), etc. For example, when DO raises sharply, the substrate in the medium is consumed. Therefore, essential amount of substrate is required by adding pre-determined feeding rate to maintain the desired limit. On the other hand, concentration of the substrate is the main parameter that considered in direct feedback control (Yamanè and Shimizu, 1984; Lee, 1996).

In the current study, exponential feeding strategies at various specific growth rates were developed for recombinant human growth hormone production by *B. subtilis*.

2.3.4.4 Temperature

Biochemical pathways that are active during cell growth and product formation are very temperature sensitive. To maintain the temperature at an optimal constant value is very critical for the bioprocess (Nielsen *et al*., 2003). Since the growth metabolism is directly affected by the process temperature, enzymatic activities in the intracellular reaction network are also affected.

Utilization of the carbon and energy sources is also associated with the medium temperature. Yield of the product varies with it. Moreover, as temperature lowers from its optimum value, the yields of the substrate and the product reduce as a result of more energy necessity. At low temperatures, enzyme-catalyzed reactions work less efficiently. Moreover, the fluidity of the membrane decreases due to the tendency of the lipids to be hardened. This may be a problem for the secretion of the proteins (Hogg, 2005). On the contrary to the low temperature, denaturation of proteins leading to thermal cell deaths takes place at high temperatures.

Generally, microorganisms can grow and maintain their biological activities over a broad range of temperatures, from nearly the freezing point of water to the boiling point. Therefore, there is an ideal temperature or interval for every microorganism to achieve the highest growth and the highest product synthesis. Hence, optimum temperature for a bioprocess should be chosen carefully to carry out the processes for biological production efficiently.

In the literature concerning human growth hormone production, Jensen and Carlsen (1990), Bylund *et al*. (2000), Tabandeh *et al*. (2004) studied by *E. coli* at 30 ^{o}C whereas Shin *et al*. (1998) conducted the experiments at 37 ^{o}C as working temperature without controlling the optimum temperature. When considering the studies related to rhGH production in the literature by *Bacillus subtilis*, Nakayama *et al*. (1988) carried out the bioreactor experiments at 30 ^{o}C while Şentürk (2006), Yılmaz (2008), Şahin (2010) accomplished their studies at 37 ^{o}C as the operating temperature without focusing on the temperature effect.

2.3.4.5 pH

Hydrogen ion concentration of the medium varies with the metabolic functions of the cell. Microorganisms intend to keep the intracellular pH value constant though the presence of extracellular differences. Mass and energy transfer and the other some metabolic activities occur by the proton gradient due to the pH change of the inner and outer of the cell (Nielsen *et al*., 2003). Due to the energy consumption or production related to the internal and external pH consistency, production yield can change according to the pH profile of the bioprocess.

For many microorganisms, the optimum operating pH value is generally around pH=7 (pH=6.5-8). Type of the microorganism affects the pH conditions to grow. For instance, pH range in bacteria is larger than in fungi (Hogg, 2005). Alkaline conditions are more suitable for bacteria.

Formation and secretion of organic acids and amino acids through the reaction network in the cell cause to decrease in the medium pH. On the contrary, medium pH is increased via the metabolites that are transferred and used by the cell.

Owing to having several optimum pH values for cell growth and product formation, microorganisms should be carefully selected that are the most suitable for the medium pH. Optimum medium pH for cell growth may differ from the medium pH required for the product formation. The interactions between the pH operating conditions and the intracellular reaction network directly affect the performance of the bioreactor. In order to increase the product yield and selectivity, some processes should be operated by controlled pH conditions as distinct from the uncontrolled pH conditions (Çalık *et al*., 2002).

In the literature associated with rhGH production by *E. coli* and *Bacillus* species, pH values of the operation are generally chosen for *E. coli* by controlled strategy changing in the range of pH=6.75-7.20 (Shin *et al*., 1998; Bylund *et al*., 2000; Castan *et al*., 2002; Jensen and Carlsen, 1990). On the other hand, Nakayama *et al*. (1988), Şentürk (2006), Yılmaz (2008) carried out their studies at the uncontrolled (initial) pH values, 6.00-7.00, 7.25, 7.50, respectively. Moreover, Şahin (2010) investigated the pH control on rhGH production by *Bacillus subtilis* (*scoC*$^-$), the strain used in the current study, and she obtained 1.41 fold higher product production in controlled pH=7.50 strategy.

2.3.4.6 Oxygen Transfer Characteristics

Oxygen transfer to be considered in aerobic processes is a critical parameter that affects yield and selectivity of the product. In biotechnological processes, required oxygen transfer is directly related to the type of the microorganism, the physical property of production medium, configuration of the bioreactor and the agitator. Stirred-tank and bubble column bioreactors are preferred types of bioreactors in microbial processes. Owing to good mixing, heat and mass transfer with high efficiency can be provided in stirred-tank bioreactors. In stirred-tank bioreactors, oxygen transfer is a function of agitation rate and oxygen/air inlet rate as stirrer speed and gas flow rate, respectively. In general, transfer of oxygen from a gas bubble (air bubble) to the solid/liquid particle (microorganism) is slower compared to the other biochemical reactions taking place during an aerobic process. Thus, transport rate can be the step that controls the overall process rate.

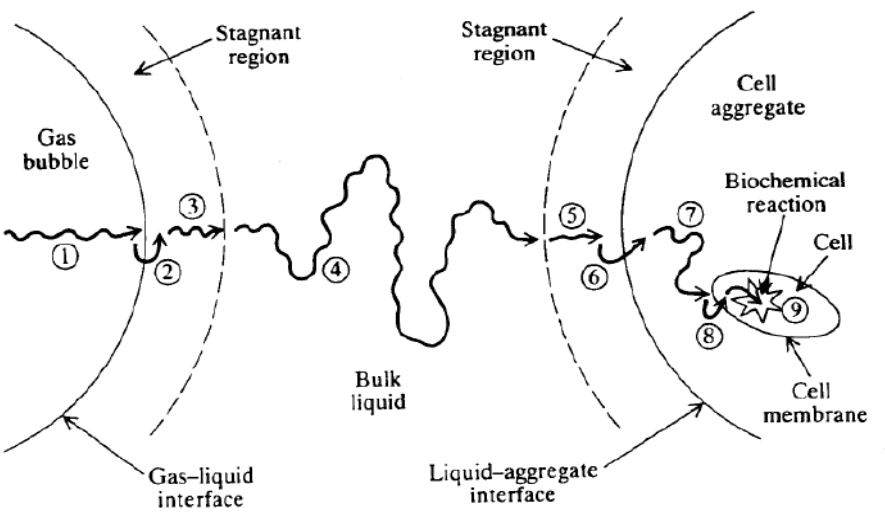

Figure 2.11 Schematic diagram of gas-liquid and liquid-solid mass transfer in aerobic bioprocesses (Bailey and Ollis, 1986)

Gas-liquid mass transfer is modeled by the two-film theory as oxygen transfer from a gas bubble to the cell in culture medium. The steps (Figure 2.11) involved in this mechanism are:

1. First step on the transport of oxygen from gas bubble to the gas-liquid interface
2. Transport across the gas-liquid interface
3. Transfer of oxygen along the stagnant part of bulk liquid by diffusion
4. Movement through the bulk liquid
5. Transfer of oxygen along the stagnant part of bulk liquid surrounding the cell
6. Transport across the liquid-cell mass interface
7. Diffusion of oxygen from the cell mass to the single cell
8. Movement across the cell wall and cell membrane
9. Transport from the cytoplasm of the cell to the site of the biochemical reaction

Generally in laboratory-scale bioreactors, the fourth step is very fast due to the homogeneity provided by well-mixing in culture media. Because the size of the most cells is relatively small, the resistance occurring by intracellular transport is usually negligible (Nielsen *et al*., 2003). Transport mechanisms across the cell envelope can be divided into three: free diffusion, facilitated diffusion and active transport. The first two mechanisms are passive and there is no need for Gibbs free energy. Whereas free diffusion occurs through a concentration gradient, facilitated one is a faster transport provided by a specific carrier protein. Active transport is the transport with input of free energy addition to the facilitated diffusion (Stephanopoulos *et al*., 1998; Ratledge and Kristiansen, 2006). Since the solubility of oxygen is very low in aqueous solutions, an uninterrupted feed is needed during the aerobic bioprocesses.

Oxygen transfer rate (OTR) from gas to liquid is defined as,

$$OTR = N_O a = K_L a\left(C_O^* - C_O\right) \tag{2.7}$$

Where N_O is the overall mass transfer flux of oxygen (mol m^{-3} s^{-1}), a is the specific gas-liquid interfacial area (m^2 m^{-3}), K_La is the overall volumetric liquid phase mass transfer coefficient (s^{-1}), C_O^* is the saturated dissolved oxygen concentration (mol m^{-3}) and C_O is the actual dissolved oxygen concentration (mol m^{-3}). Now that gas phase film resistance is often ignorable when it is compared with the liquid film resistance, K_La is almost identical with the liquid phase mass transfer coefficient (k_La) (Nielsen *et al.*, 2003). Since K_L and a cannot be easily measured separately, generally the product K_La representing the oxygen transfer is measured.

Metabolic activity and solubility affect the concentration gradient that are driving forces in oxygen transfer rate. Gas solubility varies with the medium composition (presence of salts or not), concentration, pressure, temperature and biochemical reactions (Ochoa-Garcia and Gomez, 2009). Saturated concentration of oxygen for water under the conditions that are at 25 ^oC and 1 atm is 7 mg L^{-1} (Bailey and Ollis, 1986). In comparison with the liquid phase film resistance, gas phase film resistance is negligible in oxygen transfer due to the low solubility of oxygen in aqueous medium. Therefore, the overall volumetric liquid phase mass transfer coefficient is almost equal to the liquid phase mass transfer coefficient.

The maximum possible mass transfer rate (OTR_{max}) can be specified as,

$$OTR_{\max} = \left(N_O\right)_{\max} a = K_L a\, C_O^* \tag{2.8}$$

Dissolved oxygen concentration in aerobic respiration is directly related to oxygen transfer rate from the gas bubble to the liquid phase and then the consumption rate of oxygen by the microorganism which is also known as the oxygen uptake rate (OUR) given in equation-2.9 in which q_O is the specific oxygen consumption rate, $-r_O'''$ is the oxygen uptake rate per unit cell dry weight and C_X is the cell concentration. Hence, oxygen transfer characteristics are originated from the major parameters: K_La and OUR. In many aerobic biotechnological processes, oxygen transfer is the rate limiting step controlling the bioprocesses with the cell growth.

$$OUR = -r_O = -r_O''' C_X = q_O C_X \tag{2.9}$$

There are various methods to determine the value of K_La such as the direct method, the sulphite method, the hydrogen peroxide method, tracer methods and the dynamic method (Nielsen *et al.*, 2003). The dynamic method is frequently used in fermentation processes to evaluate the oxygen transfer characteristics of the system. It is based on the measurement of the dissolved oxygen related to the respiratory activity of the microorganism through the cultivation.

Material balance for oxygen in a liquid phase of a batch-wise bioreactor at unsteady state conditions is written as:

$$K_L a\left(C_O^* - C_O\right) + r_O''' C_X = \frac{dC_O}{dt} \tag{2.10}$$

In the dynamic method, the gas (oxygen or air) inlet to the bioreactor is closed down with minimum agitation rate for a while (a few minutes) and decrease in the concentration of the dissolved oxygen is followed until the gas inlet is reopened (Region-II in Figure 2.12). During this period oxygen transfer rate is zero, hence at that time all dissolved oxygen within the broth is consumed by the microorganisms. It is simplified in the equation-2.11:

$$r_O = \frac{dC_O}{dt} \tag{2.11}$$

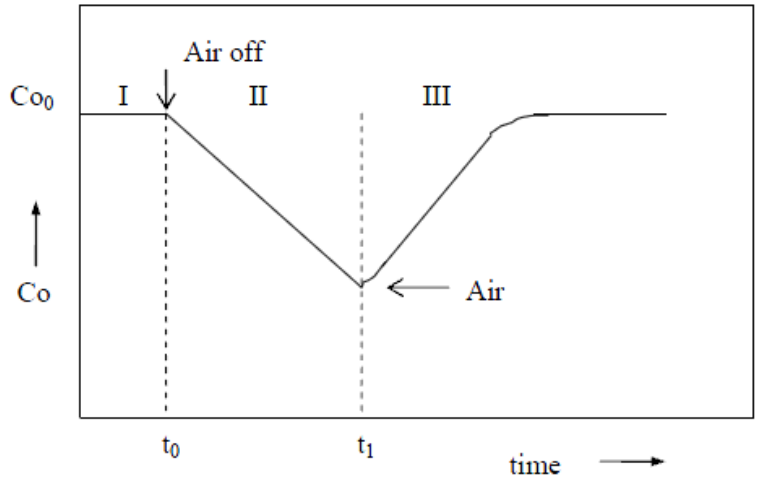

Figure 2.12 Variations in dissolved oxygen concentration with respect to time in the experimental approach of the dynamic method

Afterwards, the gas flow is reopened and the flow rate of air or oxygen is set to its operation value. Henceforward, equation-2.10 is valid for Region-III as seen in Figure 2.12. This procedure can be repeated at the specific time intervals chosen according to the bioprocess conditions (i.e. every 4 hours). Then, K_La can be determined by a slope of the graph which is plotted as (dC_O/dt-r_O) versus C_O (Figure 2.13).

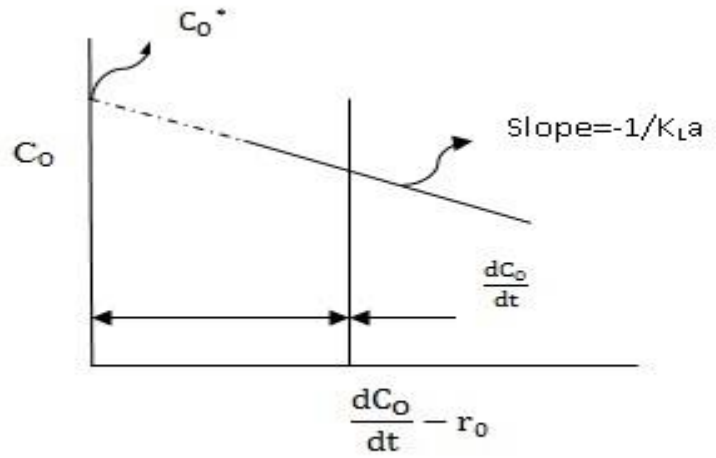

Figure 2.13 Computation of $K_L a$ via dynamic method

The term, K_La_0 is also an important parameter as well as K_La to understand the further mass transfer resistances due to the presence of microorganism. The same procedure is applied to calculate K_La_0 as in calculating the K_La. Only difference comes up with the de-oxygenated process which is conducted by nitrogen flowing. Since the oxygen uptake rate is zero, equation-2.10 can be simplified as:

$$K_L a_0 \left(C_O^* - C_O\right) = \frac{dC_O}{dt} \tag{2.12}$$

And then, K_La_0 can be determined by a slope of the graph which is plotted as (dC_O/dt) versus C_O. Enhancement factor ($E= K_La/K_La_0$) is also be determined to investigate the efficiency of the oxygen transfer and utilization.

In order to determine the rate limiting step in a bioprocess, followings described by Çalık *et al*. (2003) such as maximum possible oxygen utilization rate, Damköhler number, efficiency factor etc. need to be also evaluated.

The maximum possible oxygen utilization rate which is also known as oxygen demand, OD:

$$OD = \frac{\mu_{\max} C_X}{Y_{X/O}} \tag{2.13}$$

The Damköhler number (Da) is the ratio of the maximum possible oxygen utilization rate to the mass transfer rate (Equation-2.14).

$$Da = \frac{OD}{OTR_{max}} \tag{2.14}$$

The other term efficiency factor (η) is the ratio of the oxygen uptake rate to the oxygen demand as given in the equation-2.15:

$$\eta = \frac{OUR}{OD} \tag{2.15}$$

When the studies on the fermentation and oxygen transfer characteristics by *Bacillus* species, it is obvious that oxygen transfer rate not only affects the product yield and the selectivity but also it controls metabolic fluxes and the distribution of by-products. Çalık *et al*. (1998) focused on the effects of oxygen transfer on the production of SAP by *Bacillus licheniformis*. They worked on the different agitation rates, 150 min^{-1} to 750 min^{-1} in the bioreactor experiments at the air inlet rate as Q_0/V_R=1 vvm. They achieved the maximum SAP activity as 441 U mL^{-1} by MOT condition (N=500 min^{-1}). Afterwards, Çalık *et al*. (2000) researched into the various air inlet rates, Q_0/V_R=0.2 to 1 vvm together with the agitation rates as under the same conditions in the previous study. They observed the maximum SAP activity as 500 U mL^{-1} by MOT_2 condition (N=750 min^{-1} and Q_0/V_R=0.5 vvm). Finally, Çalık *et al*. (2004) investigated the effects of oxygen transfer on the production of SAP by using complex medium containing molasses. They understood that rich medium does not affect the optimal oxygen transfer condition for *Bacilli*. The results related to the oxygen transfer obtained from the SAP production by *Bacillus* is also flashed on the rhGH production. Optimum oxygen transfer condition (MOT_2) is also verified for rhGH production (Şentürk, 2006; Yılmaz, 2008; Şahin, 2010).

2.3.5 Bioprocess Kinetics in Semi-Batch Operation

2.3.5.1 Mass Balance Equation for the Cell

Recombinant cells utilized in microbial processes act as micro-bioreactors. Specific formation or consumption rates of substrate, biomass and product affect the process yield and productivity which are fermentation characteristics of the bioprocess.

The increase in the number of the cells thereby the population refers to microbial growth. Specific growth rate (μ) is the measuring term related to the cell growth. When the material balance is constructed for the cell, either for batch or semi-batch operation, the mass balance equation for the cell is constructed as follows:

$$r_X V = \frac{d(C_X V)}{dt} \tag{2.16}$$

where r_X is the cell growth rate, C_X is the cell concentration, (g L^{-1}), V is the bioreactor liquid-phase volume, and t is the cultivation time. The main assumption in equation-2.16 is

that the cells are generated batch-wise and not lost through sampling. The cell formation rate r_X is defined as:

$$r_X = \mu C_X \tag{2.17}$$

where μ (h^{-1}) is the specific growth rate. By inserting equation-2.17 into equation-2.16 one can derive equation-2.18, as follows:

$$\mu C_X V = \frac{d(C_X V)}{dt} \tag{2.18}$$

Since the operation mode is semi-batch, the volume change due to the feed of substrate(s) should be defined. Therefore, the second assumption is that the bioreactor liquid medium has a constant density, which can be obtained from the continuity equation for the semi-batch bioreactor, as follows:

$$\frac{dV}{dt} = Q_{in} - Q_{out} \tag{2.19}$$

where, Q is the volumetric flow rate ($m^3\ h^{-1}$). Since volume change is due to substrate(s) feeding $Q_{in} = Q > 0$, but $Q_{out} = 0$; thus, inserting the equation-2.19 into equation-2.18:

$$\frac{dC_X}{dt} = \left(\mu - \frac{Q}{V}\right) C_X \tag{2.20}$$

Rearranging the equation-2.20, the specific growth rate for semi-batch bioreactor is derived as follows:

$$\mu = \frac{d C_X}{dt}\frac{1}{C_X} + \frac{Q}{V} \tag{2.21}$$

2.3.5.2 Mass Balance Equation for the Substrate(s)

For each of the substrate which fed to the semi-batch bioreactor, the mass-balance equation is constructed as follows:

$$Q_S\, C_{S_0} + r_S V = \frac{d(C_S V)}{dt} \tag{2.22}$$

The substrate consumption rate, r_S is defined with a first order kinetic equation which is the function of C_X, where the kinetic coefficient q_S is the specific substrate consumption rate (h^{-1}):

$$r_S = q_S C_X \tag{2.23}$$

By inserting the equation-2.23 into equation-2.22 one can derive equation-2.24, consequently q_S is derived as follows:

$$Q_S C_{S_0} + q_S C_X V = C_S \frac{dV}{dt} + V \frac{dC_s}{dt} \tag{2.24}$$

$$q_S = \frac{1}{C_X}\left(\frac{C_S}{V} Q + \frac{dC_S}{dt} - \frac{Q_S}{V} C_{S_0}\right) \tag{2.25}$$

To calculate the volumetric feed rate of the substrate(s) as a function of the cultivation time, assuming quasi-steady state for the substrate C_S ($dC_S/dt = 0$), based on the equation-2.22, the substrate consumption rate (r_S) is defined as in terms of the cell formation rate (r_X) and the yield (selectivity) coefficient $Y_{X/S}$ as follows:

$$-r_S = \left(r_X / Y_{X/S}\right) \tag{2.26}$$

and, inserted in equation-2.22:

$$Q_S C_{S_0} - \frac{r_X V}{Y_{X/S}} = C_S \frac{dV}{dt} \tag{2.27}$$

By inserting the terms, $r_X = \mu C_X$ and $dV/dt = Q_S$ into equation-2.27;

$$Q_S C_{S_0} - \frac{\mu C_X V}{Y_{X/S}} = C_S Q_S \tag{2.28}$$

The solution of the differential equation-2.16 together with the kinetic equation-2.17 is:

$$C_X V = C_{X_0} V_0 e^{\mu t} \tag{2.29}$$

By inserting equation-2.29 into equation-2.28, the volumetric feed rate of the substrate(s) is derived as follows:

$$Q_S = \frac{\mu C_{X_0} V_0}{Y_{X/S}(C_{S_0} - C_S)} \exp(\mu t) \tag{2.30}$$

2.3.5.3 Mass Balance Equation for the rhGH Production

The product recombinant human growth hormone is produced batch-wise in the semi-batch bioreactor. Therefore, the material balance for the product is written as:

$$r_P V = \frac{d(C_P V)}{dt} \tag{2.31}$$

where,

$$r_P = q_P C_X \tag{2.32}$$

where q_P is the specific formation rate of the product ($g_{product}\ g_{cell}^{-1}\ h^{-1}$) and C_P is the product concentration (g L^{-1}). The recombinant product formation rate (r_P) is defined with a first-order kinetic equation which is the function of C_X and q_P.

By rearranging equation-2.31, the specific formation rate of the product can be expressed as:

$$q_P = \frac{1}{C_X}\left(\frac{C_P}{V}Q + \frac{dC_P}{dt}\right) \tag{2.33}$$

The ratio of the quantity of a molecule produced to the quantity of a molecule consumed in the reaction can be defined as yield (selectivity) coefficient. The cell yield on substrate and the product yield on substrate are given in the following equations, respectively, as:

$$Y_{X/S} = \frac{r_X}{-r_S} = \frac{dC_X/dt}{-dC_S/dt} \tag{2.34}$$

$$Y_{P/S} = \frac{r_P}{-r_S} = \frac{dC_P/dt}{-dC_S/dt} \tag{2.35}$$

Accordingly, the cell yield on oxygen consumed is described as:

$$Y_{X/O} = \frac{r_X}{-r_O} \tag{2.36}$$

The yield coefficients can be also calculated for a finite period of time defining as the overall yield coefficients as stated in equations 2.37 and 2.38.

$$\overline{Y}_{X/S} = \frac{\Delta C_X/\Delta t}{-\Delta C_S/\Delta t} \tag{2.37}$$

$$\overline{Y}_{P/S} = \frac{\Delta C_P / \Delta t}{-\Delta C_S / \Delta t} \tag{2.38}$$

Definitions of the several yield coefficients often used to evaluate the microbial processes are given in Table 2.4.

Table 2.4 Definitions of several yield coefficients

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

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals utilized in the present study were in analytical grade and provided from the following chemical companies and the laboratories: Sigma Aldrich Co., Merck & Co. Inc., Fluka Co. and Difco Laboratories.

3.2 The Microorganism

Bacillus subtilis 1A178 (*scoC*$^-$) strain carrying pMK4::*pre*(*subC*)::*hGH* plasmid (Şentürk, 2006; Özdamar *et al*., 2009) was used for human growth hormone production. This recombinant microorganism has been stored in the microbanks (Pro-Lab Diagnostics) at -80 ^oC to maintain its viability and activity.

3.3 Recombinant hGH Production by *B. subtilis* (*scoC*$^-$)

3.3.1 Microbank

Microbank^{TM} is a storage technology developed for bacterial and fungal system. Microbank method for storage in microorganisms is more convenient than customary methods such as glycerol medium or lyophilization. It is based on a sterile vial comprising of chemically treated porous beads and cryopreservative solution. Young colonial cells are inoculated with this cryopreservetative solution and then the porous beads are washed to adsorb the cells onto the surface. The final step is to aspirate the excess cryopreservative solution and keep at -80 ^oC for prolonged storage.

3.3.2 Solid Medium

Composition of the solid medium of recombinant *B. subtilis* for growth and hGH production is given in Table 3.1 (Çalık, 1998). After preparing the solution, sterilization was performed with the saturated steam at 121 ^{o}C and 1.2 atm for 20 minutes. Antibiotic was used to conserve the plasmid of recombinant microorganism so that chloramphenicol was added as an antibiotic in the concentration of 7.14 μg mL^{-1} in the medium. Then ~ 30 mL of medium was poured into the petri dish and the solidification of agar was expected. After that, transfer of microorganisms from Microbank^{TM} via the porous beads was carried out under sterile conditions. Then, cells were incubated at 37 ^{o}C for 24 hours and stored at 4 ^{o}C after they were grown in the solid medium.

Table 3.1 The composition of the solid medium for recombinant *B. subtilis*

Compound	**Concentration, g L^{-1}**
Meat Extract	3
Peptone	5
Agar	15

3.3.3 Precultivation Medium

Composition of the precultivation medium of recombinant *B. subtilis* for hGH production is given in Table 3.2 (Çalık, 1998). After sterilization of the medium, chloramphenicol was once more added (to be in 7.14 μg mL^{-1} in the medium) into the medium. Then, the cells grown in the solid medium were inoculated into the precultivation medium having a volume of 33 mL in which the laboratory scale air-filtered Erlenmeyer flasks with the total volume of 150 mL. After that, cells were incubated at 37 ^{o}C and 200 min^{-1} via the orbital heating controlled shakers (B. Braun, Certomat BS-T) until the cells reach to OD_{600}=0.28-0.34 corresponding to the cell concentration of C_X = 0.6 – 0.7 g L^{-1}. This lasts nearly 3 hours.

Table 3.2 The composition of the precultivation medium for recombinant *B. subtilis*

Compound	Concentration, g L^{-1}
Soytone	15.00
Peptone	5.00
$CaCl_2$	0.10
Na_2HPO_4	0.25
$MnSO_4.2H_2O$	0.01

3.3.4 Production Medium

Composition of the production medium of recombinant *B. subtilis* for hGH production is given in Table 3.3 (Çalık, 1998). After the sterilization step, chloramphenicol was again added to the production medium to be in the concentration of 7.14 μg mL^{-1}. The cells grown in the precultivation medium in desired concentration were inoculated into the production medium having a volume of 100 mL in which the laboratory scale air-filtered Erlenmeyer flasks with the total volume of 500 mL. Cells were transferred into the production medium with an inoculation ratio (IR) of 1:10. After that, the cells were incubated at 37 ^{o}C and 200 min^{-1} via the orbital heating controlled shakers (B. Braun, Certomat BS-T). Moreover, protease inhibitor cocktail (Sigma P-2714) was supplied after the required cell concentration (OD_{600}=0.28-0.34) was attained.

Table 3.3 The composition of the production medium for recombinant *B. subtilis*

Compound	Concentration, g L^{-1}
Glucose	8.000
$(NH_4)_2HPO_4$	4.710
KH_2PO_4	2.000
Na_2HPO_4	0.043
NaH_2PO_4	5.630

3.3.5 Scale-up Steps Directed to Pilot Scale Production

In the batch and semi-batch experiments, a pilot scale bioreactor was used for hGH production (Figure 3.1). In the first step, cell culture was transferred on to the solid medium by using Microbank^{TM}. After that, inoculation was performed from the first precultivation medium to the second one with the ratio of 1:10. The same procedure was repeated from second precultivation medium to the production medium where the medium inside the pilot scale bioreactor.

There were air filtered shake bioreactor experiments at laboratory scale and the bioreactor experiments at pilot scale which were carried out to specify the hGH production in different conditions.

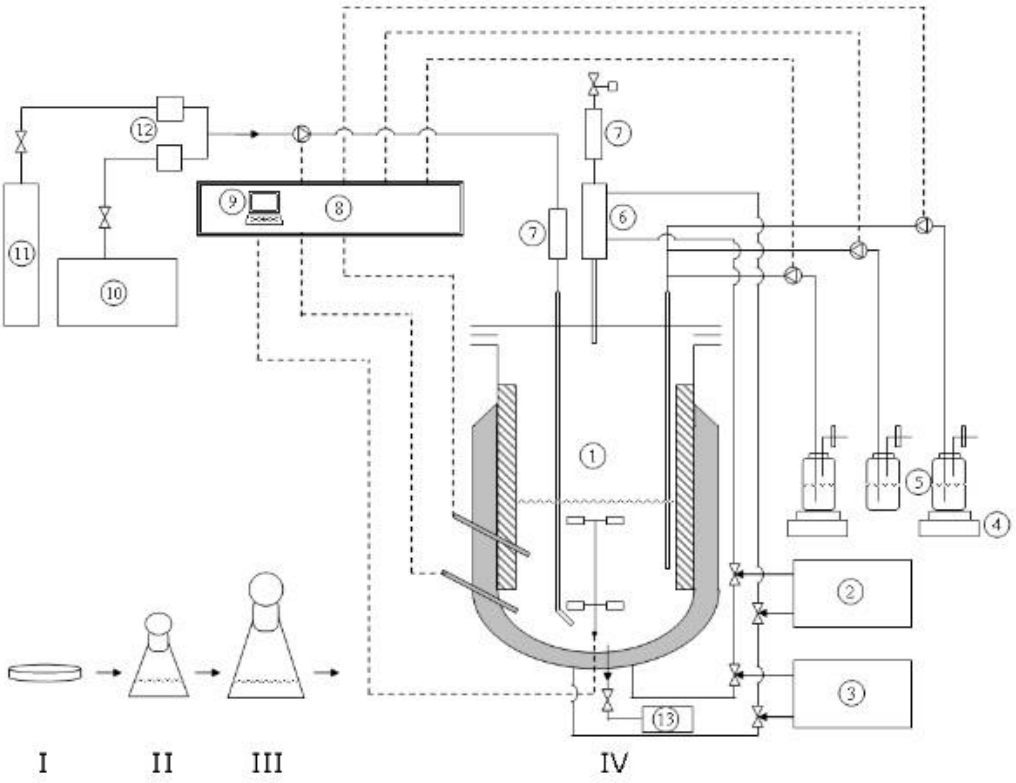

Figure 3.1 Schematic setup of pilot scale bioreactor system. I: Solid medium; II: Precultivation medium-I, V = 33 mL; III: Precultivation medium-II, V = 100 mL; IV: Pilot scale bioreactor system, V = 1 L which is composed of (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2/N_2 tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008)

3.3.5.1 Air Filtered Shake Bioreactor Experiments at Laboratory Scale

In the air filtered shake bioreactor experiments, while the precultivation medium, was the same, production medium could be different in terms of additional compounds to investigate the production performance of the microorganism. Therefore, two different air filtered shake bioreactor experiments were conducted. The cells were inoculated into the production media by applying the steps in Sections 3.3.2 and 3.3.3. The production media used in the laboratory scale experiments except the original one, indicated as P-1, (Table 3.3) are given in Table 3.4.

Table 3.4 Production media used in laboratory scale air filtered shake bioreactor experiments

	SH-1		**SH-2**					
Media	**G-0.1**	**G-0.05**	**P-2**	**P-3**	**P-4**	**P-5**	**P-6**	**P-7**
Compound	**Concentration, g L^{-1}**							
Glucose	8.000	8.000	8.000	8.000	8.000	8.000	8.000	8.000
$(NH_4)_2HPO_4$	4.710	4.710	4.710	4.710	4.710	-	4.710	4.710
KH_2PO_4	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000
Na_2HPO_4	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043
NaH_2PO_4	5.630	5.630	5.630	5.630	5.630	5.630	5.630	5.630
Glutamine	0.100	0.050	-	-	-	-	-	-
PTM1* (mL)	-	-	0.500	-	-	0.500	0.500	0.500
$MgSO_4.7H_2O$	-	-	0.250	-	-	0.250	0.250	0.250
Peptone	-	-	-	8.000	-	-	8.000	-
Yeast Extract	-	-	-	-	8.000	-	-	8.000
$(NH_4)_2SO_4$	-	-	-	-	-	4.760	-	-
H_3PO_4 (85 %) (mL)	-	-	-	-	-	2.090	-	-

* The composition of PTM1 is given in Table 3.5.

* SH-1: Shake Bioreactor-1 and SH-2: Shake Bioreactor-2.

Table 3.5 The composition of the trace salt solutions PTM1 (Sibirny *et al*., 1990)

Compound	Concentration, g mL^{-1}
$CuSO_4.5H_2O$	0.600
NaI	0.008
$MnSO_4.H_2O$	0.300
$Na_2MoO_4.2H_2O$	0.020
H_3BO_3	0.002
$ZnCl_2$	2.000
$FeSO_4.7H_2O$	6.500
$CoCl_2.6H_2O$	0.090
H_2SO_4	0.500
Biotin	0.020

3.3.5.2 Batch Experiments in Pilot Scale Bioreactor System

In the batch experiments at the pilot scale bioreactor for hGH production, the cell culture was transferred into the production medium (Table 3.3) inside the bioreactor having the working volume of V_R = 1.1 L. The pilot scale bioreactor (B. Braun CT2-2) was used in the batch and the semi-batch bioreactor operations having a working volume of V_R = 0.5-2.0 L. Moreover, the control units such as pH, temperature, dissolved oxygen, anti-foam, stirring and aeration rate are also used to control the bioprocess steps. Sterilization of the bioreactor vessel was also performed before loading the production medium by the superheated steam. Temperature control was acquired by jacket via circulation of hot and cold water. There are also two stirrers in the Rushton turbine type with four blades in the bioreactor system.

The steps of the bioreactor to be followed in the study for recombinant hGH production were:

a) System was opened before six hours so that oxygen electrode system could be polarized.
b) Pure water inside the bioreaction vessel was drained.
c) pH electrode was removed from the bioreactor connections in order to calibrate. Standard buffer solutions (Mettler Toledo) at the specific pH values of pH=4.0 and pH=7.0 were used for the pH calibration. After the calibration, pH electrode was connected again to the bioreactor.
d) Bioreaction vessel was filled by distilled water up to the pH and O_2 electrodes. Filter at the gas inlet moved into the sterilization position. Protective jacket of

bioreactor in steel was attached to the outer glass surface. Then, reactor mode was shifted to sterilization (121 ^{o}C and 1.2 atm) from the control unit for 20 minutes.

e) Production medium of which components were sterilized before was fed to the bioreactor by using silicon hosepipes. Glucose (main carbon source in the production medium) was sterilized independently apart from the other components. The stock solutions including salts were used after filtering by 0.45 μm filters (Sartorius).

f) Bioreactor operation conditions were set to the optimum values as T=37 ^{0}C, pH_0=7.5, Q_0/V_R=0.5 vvm and N=750 min^{-1}.

g) By adjusting the temperature and agitation rate, O_2 calibration was carried out. In order to do that, air inlet was disconnected and N_2 gas was feeded to the bioreactor for deoxygenating of medium. Concentration of dissolved oxygen (pO_2) was adjusted to 0 %. Then, similar procedure was applied for oxygenating of medium by feeding air to the reactor via compressor. After that, concentration of dissolved oxygen was adjusted to 100 %.

h) Cell culture grown in the precultivation medium-II was transferred into the bioreactor with the inoculation ratio of 1:10 by using sterilized hosepipe.

i) Protease inhibitor cocktail (Sigma P-2714) was supplied after the required cell concentration (OD_{600}=0.28-0.34) was attained.

j) Sterilized 30 % Antifoam A (Sigma 5758) was feeded in droplets in case of the foaming. Moreover, 5 M KOH solution (25 % NH_3 solution for BR-1) was added to the bioreactor under control in order to keep the medium pH constant (pH=7.5).

3.3.5.3 Semi-Batch Experiments in Pilot Scale Bioreactor System

In the semi-batch bioreactor experiments, the hGH production steps for the batch process were used. However, in every semi-batch bioreactor operation, a continuous feed stream containing substrate or substrates with a pre-determined feed-rate were designed and applied. Glucose was used as the major feeding substrate in the semi-batch experiments. Moreover, peptone, $(NH_4)_2HPO_4$ and PTM1 were also fed in addition to glucose in some strategies (BR-7 and BR-8). To develop the feeding strategy in hGH production under pH=7.5 controlled operation, the effects of specific growth rate values changed in the range of 0.04-0.26 h^{-1} with the initial reactor volume of 1.1 L were studied. Using the equation-2.30 derived in section 2.3.5.2, the volumetric feed rate of the substrate (Q_S) can be simplified by assuming:

$C_{So} >> C_S$

in the bioreactor (Yamanè and Shimizu, 1984; Çelik *et al*., 2009; Çalık *et al*., 2010-a):

$$Q(t) = \frac{\mu_0 V_0 C_{X_0}}{C_{S_0} Y_{X/S}} \exp(\mu_0 t) \qquad (3.1)$$

is used for the calculation of the volumetric feed rate by pre-determining $\mu_0 Y_{X/S}$ values. In equation-3.1: $Q_S(t)$ (L h^{-1}) is the volumetric flow rate, μ_0 (h^{-1}) is the pre-determined specific growth rate, V_0 (L) is the initial bioreactor volume, C_{X0} (g L^{-1}) is the initial cell concentration, C_{S0} (g L^{-1}) is the initial feed substrate concentration, $Y_{X/S}$ (g g^{-1}) is the cell yield on substrate and t (h) is the cultivation time. The value of $Y_{X/S}$ was selected as 0.27 g g^{-1} according to Şahin's (2010) study and the stock concentration of feed substrate was specified at 200 g L^{-1}.

3.4 Analysis

During the laboratory and pilot scale experiments conducted for hGH production, cell concentration was firstly measured from the samples taken from the bioreactor at certain times. Then, the sample was centrifuged at 12000 g and +4 ^{o}C for 10 minutes to separate the filtrate containing the cell mass from the supernatant. After filtering the supernatant from the 0.45 μm porous filters, substrate (glucose), hGH, organic acid concentrations and protease activity were determined by analytical methods.

3.4.1 Cell Concentration

Cell concentration was determined by measuring the absorbance values via UV-Vis Spectrophotometer (Thermo Spectronic, Heλios-α) at 600 nm. This specific wavelength was selected as optimum after scanning the wavelengths in the range of 200-800 nm. Calibration curve for *B. subtilis* to determine the cell concentration was given at Appendix C.

3.4.2 Glucose Concentration

Glucose oxidation method was used to determine the glucose concentration in the production medium. In order to achieve this, samples were prepared by using a Glucose Analysis Kit (Biyozim-Biasis) and then glucose concentration was measured via UV-Vis Spectrophotometer (Thermo Spectronic, Heλios-α) at λ=505 nm. Equipments required for the analysis and the ingredients of the analysis kit are given in Appendix A.

In the first step, D-glucose was oxidized to gluconate in the presence of H_2O and O_2 by glucose oxidase enzyme that catalyzed the reaction (equation-3.2). As a result of this rection, produced hydrogen peroxide reacted with phenol and 4-aminoantipyrine by peroxidase enzyme and formed iminoquinone compound (equation-3.3) in red colour given maximum absorbance at 505 nm.

$$D-Glucose+O_2+H_2O \xrightarrow{Glucose\ oxidase} Gluconate+H_2O_2 \quad (3.2)$$

$$H_2O_2+4-Aminoantipyrine+Phenol \xrightarrow{Peroxidase} Iminoquinone+H_2O \quad (3.3)$$

Calibration curve was prepared according to the standard glucose solutions (Appendix C). Analysis procedure was followed as:

a) Analysis solutions in Glucose Analysis Kit were prepared.
b) Test tubes were numbered as blank, standards and samples.
c) 0.05 mL sample or standard glucose solution was added to the test tubes.
d) Test tubes and analysis solutions were kept at analysis temperature (25 ^{o}C or 37 ^{o}C).
e) 0.05 mL glucose analysis reagent was added into the test tubes.
f) Then, 0.40 mL analysis buffer was added.
g) Final volume in the test tubes was completed to 2.50 mL by pure water.
h) Mixture in the test tubes were incubated either at 25 ^{o}C for 40 min or at 37 ^{o}C for 20 min.
i) Spectrophotometer was set to autozero by using blank solution.
j) Absorbance values were determined via UV-Vis Spectrophotometer (Thermo Spectronic, Heλios-α) at λ=505 nm.

3.4.3 hGH Concentration

hGH concentration was determined by High Performance Capillary Electrophoresis, HPCE (Agilent Technologies). In general, HPCE system is comprised of sample introduction system, inlet and outlet buffer reservoirs, high-voltage power supply, a fused silica capillary tube (column) with an optical viewing window, a detector (UV detector) and an integrator (computer) as seen in Figure 3.2. This instrumental analysis method is provided to perform efficiently separations of anions, cations, amino acids, organic acids, polynucleotides, peptides, dyes, carbohydrates, biopolymers and macromolecular proteins (Çalık, 1998).

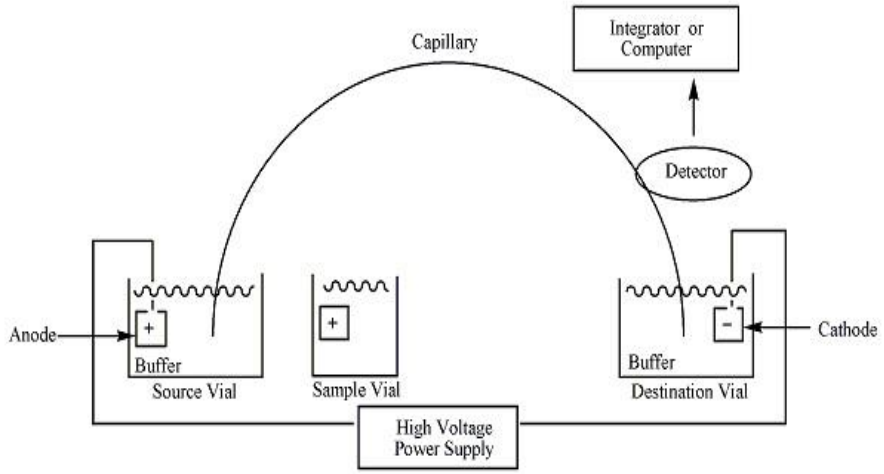

Figure 3.2 Schematic diagram of the generic HPCE system (http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/Capillary_Elecrophoresis)

High-voltage power supply is attached to the electrodes from both sides for inducing the electric field. Sample is migrated from anode (electrolyic solution) to cathode (aqueous buffer) throughout the capillary column by electroosmatic and electophoretic flow of the buffer solutions. Optical detection is made at the cathodic end of the capillary tube and absorbance of the sample is measured via UV-Vis light. According to the charged ionic species, the mass/concentration of the sample is obtained from the electropherogram (Appendix D). Calibration curve was also prepared for hGH concentration (Appendix C).

In the analysis to determine the hGH concentration, fused silica capillary column composed of 72 cm-length and 75 μm-inner radius was used. 50 mM borate buffers were used as electrolyte and buffer solutions (Appendix B). Electrolyte solution contained also AccuPure Z1-Methyl reagent (Waters) apart from the buffer solution.

HPCE analysis conditions used in the hGH production were given as follows:

- Column : Capillary column coated with SiO_2
- Column dimensions : 720 mm x 75 μm
- Power supply : Positive
- Operating voltage : 12 kV
- Injection type : Hydrostatic pressure
- Injection volume : 10 μL
- Analysis temperature : 15 ^{o}C
- Electrolyte solution : Modified borate buffer (pH=10)
- Detector : UV (λ = 214 nm)
- Duration of analysis : 40 min

3.4.4 Organic Acid Concentration

Concentrations of organic acids secreted into the production medium by *B. subtilis* were measured by using High Performance Liquid Chromatography, HPLC (Waters, Alliance 2695). Standard organic acid solutions were prepared at certain concentrations to constitute the calibration curves (Appendix C). Samples and standards were filtered by 0.45 μm filters and diluted not to damage the column before loading to HPLC.

Operation conditions and utilized method for determination of organic acid concentration were specified below (İleri and Çalık, 2006):

- Column : Capillary Optimal ODS, 5 μm
- Column dimensions : 4.6 mm x 250 mm
- System : Reversed phase chromatography
- Mobil Phase : 3.12 % (w/v) NaH_2PO_4, $0.62x10^{-3}$ % (v/v) H_3PO_4
- Mobil Phase Flow Rate : 0.8 mL min^{-1}
- Column Temperature : 30 ^{o}C
- Detector : Waters 2487-Dual absorbance (λ = 210 nm)
- Injection Volume : 5 μL
- Duration of analysis : 15 min + 5 min (delay)

3.4.5 Protease Activity Assay

Protease activity during hGH production was determined via the method including the hydrolysis of casein. One unite (1 U) of proteolytic activity was described as the activity which releases 4 nmol tyrosine per unit time (Moon and Parulekar, 1991). Total protease activity was measured from the supernatants of the samples. Mainly three types of proteases that are acidic, neutral and alkali proteases consist of the total protease activity. Therefore, three different buffer solutions were used to evaluate the protease activity (Appendix B). 2 mL of Hammerstein casein (0.5 % w/v) was dissolved separately in 0.05 M borate buffer for alkali proteases, 0.05 M sodium acetate buffer for acidic proteases and 0.05 M sodium phosphate buffer for neutral proteases and mixed with 1 mL of diluted supernatant, and then hydrolyzed at 30 ^{o}C for 20 minutes. After the hydrolyzing time, reaction was interrupted by the addition of tricloroacetic acid, TCA (10 % w/v) and the storage on ice for 20 minutes. After that, mixture was centrifugated at 19000 g and + 4 ^{o}C for 10 minutes following by keeping at room temperature for 5 minutes. Then, the absorbance was measured by UV-Vis Spectrophotometer (Thermo Spectronic, Heλios-α) at 275 nm in quartz cuvettes. Protease activity in U cm^{-3} was determined from the absorbance data by the following equation:

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

3.4.6 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

K_La and OUR related to the oxygen transfer characteristics on hGH production were determined by the dynamic method explained in detail in Section 2.3.4.6. K_La_0 was also determined by same procedure before the inoculation of the microorganisms to the bioreactor. Dynamic method was applied at every four hours before taking the samples during the bioprocess in order to determine the K_La values.

CHAPTER 4

RESULTS AND DISCUSSION

In this study, semi batch (fed-batch) operations were conducted in order to enhance recombinant human growth hormone production by *B. subtilis* (*scoC*$^-$) strain carrying pMK4::*pre*(*subC*)::*hGH* plasmid (Şentürk, 2006; Özdamar *et al*., 2009; Şahin, 2010) for the first time. In this frame, production processes at both laboratory scale and pilot scale were carried out interdependently to enhance rhGH production capacity in accordance with batch operation. In the laboratory scale production processes, the influence of the amino acid glutamine in the presence of glucose and effect of medium composition were investigated to increase the rhGH production and cell concentration. Exponential feeding strategies were designed and performed according to the outcomes of the productions at laboratory scale in the pilot scale bioreactor experiments. Thereafter, the effects of the feeding strategies on the cell growth, substrate consumption, rhGH production, and protease activities were determined and compared. Moreover, the fermentation characteristics, the specific consumption and production rates with the yield (selectivity) coefficients, organic acid profiles and oxygen transfer characteristics were calculated to determine the effects of the designed feeding strategies on the rhGH production.

4.1 Production of Recombinant Human Growth Hormone by Air Filtered Shake Bioreactor Experiments at Laboratory Scale

Based on the literature survey on the pleiotropic regulatory gene *scoC* and protein production by semi-batch processes in *B. subtilis*, effects of glutamine and medium composition were investigated in air filtered shake bioreactor experiments at laboratory scale.

4.1.1 Effect of Glutamine on rhGH Production by *B. subtilis* (*scoC*$^-$)

Since recombinant hGH is produced by *B. subtilis* (*scoC*$^-$) strain carrying pMK4::*pre*(*subC*)::*hGH* plasmid via mimicking the serine alkaline protease (subtilisin) production, activators or repressors of *aprE* gene (responsible gene for subtilisin production) are figured on hGH production either directly or indirectly. *scoC* is one of the regulatory genes that has a direct function in the initiation of sporulation at the transition phase of the growth in *B. subtilis*. In *scoC* knockout *B. subtilis* strains, sporulation process occurs in the

presence of glucose thereby inhibiting the protein production. However, these kinds of strains do not permit sporulation if both glucose and glutamine are present in the production medium. Therefore, effect of glutamine on rhGH production was investigated at two concentrations in air filtered shake bioreactors with respect to batch process. Therefore, rhGH production capacity was compared in the media containing 8 g L^{-1} glucose + 0.1 g L^{-1} glutamine, 8 g L^{-1} glucose + 0.05 g L^{-1} glutamine, and only 8 g L^{-1} glucose which are represented as G-0.1, G-0.05 and P-1, respectively. Air filtered shake bioreactor experiments having working volume of V_R=110 mL were performed at operating conditions that were T=37 ^oC, N=200 min^{-1} and pH_0=7.5 optimized elsewhere (Yılmaz, 2008). Moreover, 150 µL of protease inhibitor was added to the production media to inhibite the synthesis of proteases at t=8 h. The cell growth profiles were obtained as can be seen in Figure 4.1.

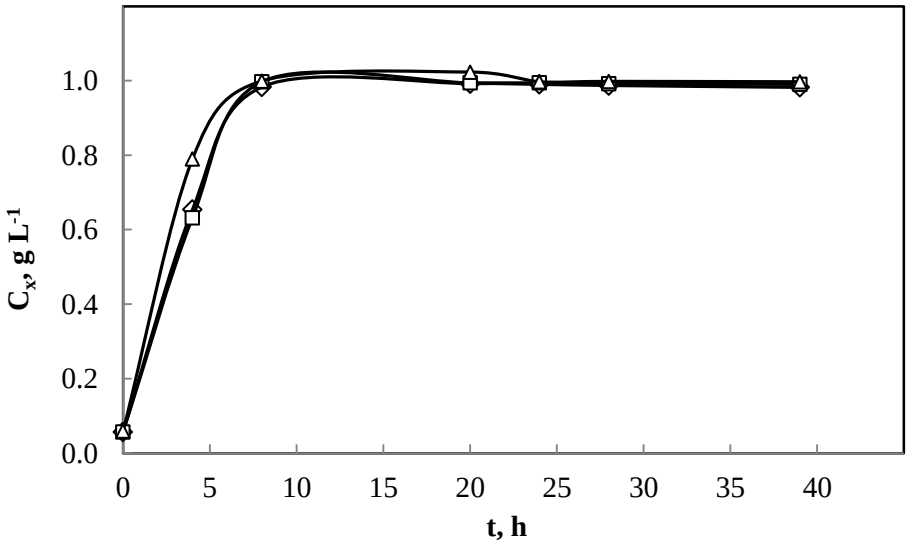

Figure 4.1 The variations in cell concentration with the cultivation time in the production of rhGH by *B. subtilis* (*scoC*$^-$): G-0.1 (◊), G-0.05 (□), P-1 (Δ)

The cell concentration increased rapidly until the protease inhibitor was added, and then stationary phase of the growth started. Although the cell concentration profiles were close to each other, the highest cell concentration was achieved as C_X=1.02 g L^{-1} at t=20 h in the production medium containing glucose at C_{G0}=8 g L^{-1}. The maximum cell concentrations for the media containing both glucose and glutamine of $C_{Glutamine}$=0.1 g L^{-1} and $C_{Glutamine}$=0.05 g L^{-1} were 0.99 g L^{-1} and 1.00 g L^{-1} at t=20 h, respectively.

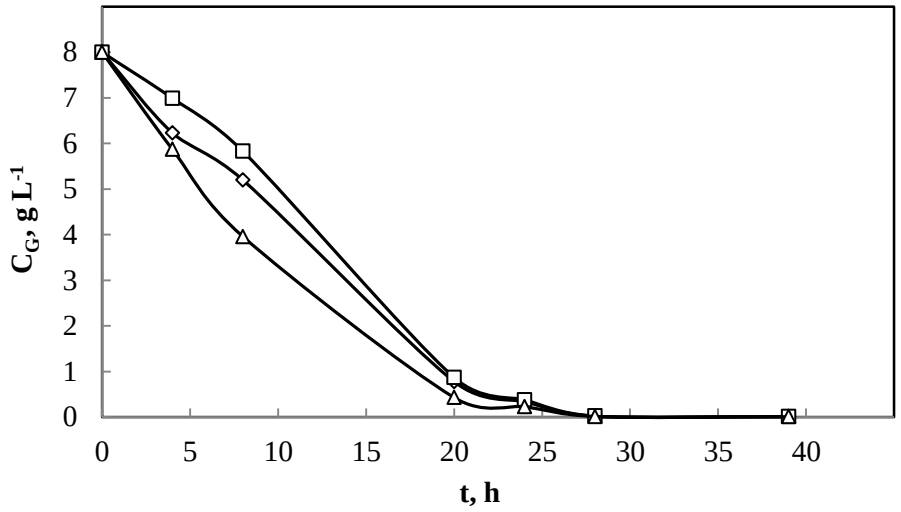

Figure 4.2 The variations in glucose concentration with the cultivation time in the production of rhGH by *B. subtilis* (*scoC*): G-0.1 (◊), G-0.05 (□), P-1 (Δ)

Glucose concentration profiles with respect to the cultivation time were given in Figure 4.2. While there are some differences in the glucose consumption rates in the early stages of the process, glucose concentration decreased to the limiting values after t=20 h at each three cases. Maximum hGH concentrations were reached when more than 95 % of glucose in the media were observed to be exhausted. rhGH production profiles with the fermentation time were given in Figure 4.3.

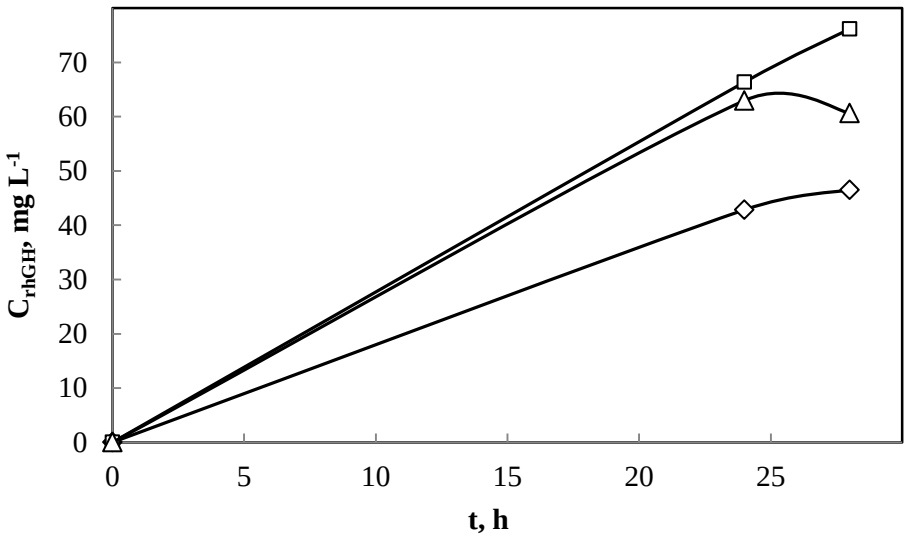

Figure 4.3 The variations in rhGH concentration with the cultivation time in the production of rhGH by *B. subtilis* (*scoC*): G-0.1 (◊), G-0.05 (□), P-1 (Δ)

The highest rhGH was produced in the medium containing 0.05 g L^{-1} glutamine as 76 mg L^{-1} at t =28 h, 1.2-fold higher than that produced by P-1 medium. On the other hand, maximum rhGH concentration for G-0.1 and P-1 were attained as C_{rhGH}=47 mg L^{-1} at t =28 h and C_{rhGH}=63 mg L^{-1} at t =24 h.

4.1.2 Effect of Medium Composition on rhGH Production by *B. subtilis* (*scoC*⁻)

Semi-batch operation technique is characteristically used in bioprocesses to reach a high cell cultivation to enhance the desired product formation in the bioreactor system. After conducting a number of bioreactor experiments by semi-batch strategies, it is revealed that to achieve a higher cell concentration, than that of C_X=1.02 g L^{-1} in defined medium is not possible. Consequently, a semi-defined medium comprising glucose, trace elements, mineral salts and a complex nitrogen source such as peptone or yeast extract should be achieved. Six different media were designed as stated in Table 3.4 and used together with the original defined medium (P-1) in air filtered shake bioreactor experiments. Laboratory scale experiments with these production media were performed to obtain high cell density fermentations for the bioreactor experiments using semi-batch operations.

In these shake bioreactor experiments with V_R=110 mL, agitation rate and temperature was controlled with initial pH_0=7.5 by optimum operating values at 200 min^{-1} and 37 ^oC, respectively. Since proteases are active just after the logarithmic growth phase, protease inhibitor was added as 150 µL into the production media at t=12 h to prevent the cell growth inhibition. Variations in the cell concentrations for each media can be seen in Figure 4.4.

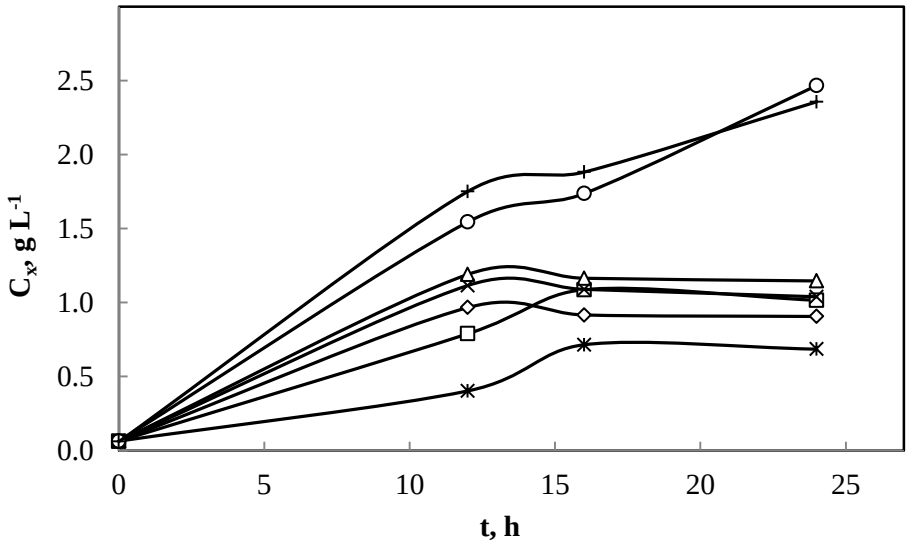

Figure 4.4 The variations in cell concentration with the cultivation time in the production of rhGH by *B. subtilis* (*scoC*⁻) for seven different media: P-1 (◊), P-2 (□), P-3 (Δ), P-4 (x), P-5 (*), P-6 (○), P-7 (+)

As can be seen from Figure 4.4, the highest cell concentrations were achieved as C_X=2.47 g L^{-1} and C_X=2.36 g L^{-1} by the production media, P-6 and P-7 containing peptone and yeast extract with the trace elements of PTM1 (Table 3.5), respectively. Although several cell concentration profiles were obtained, the change in the protease addition time delayed the process time to pass into the stationary phase at all the conditions.

Variations in glucose concentration with the cultivation time were given in Figure 4.5. Glucose consumption was greatest between t=0 and 12 h in the media of P-3, P-4, P-5 and P-6 containing 8 g L^{-1} peptone or yeast extract.

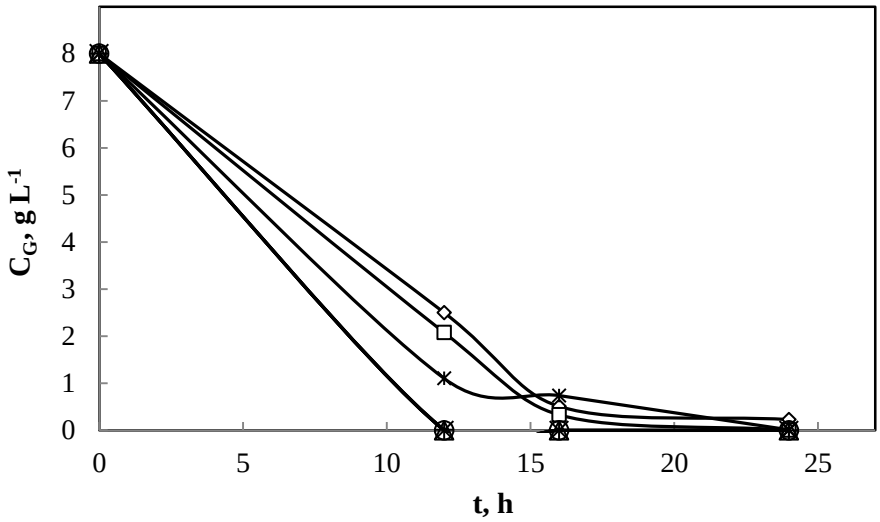

Figure 4.5 The variations in glucose concentration with the cultivation time in the production of rhGH by *B. subtilis* (*scoC*$^-$) for seven different media: P-1 (◊), P-2 (□), P-3 (Δ), P-4 (x), P-5 (*), P-6 (○), P-7 (+)

Recombinant hGH concentrations at t=24 h were given in Figure 4.6. Maximum rhGH concentration was determined as 76 mg L^{-1} in the medium (P-3) containing 8 g L^{-1} peptone as a complex nitrogen source in addition to 4.71 g L^{-1} $(NH_4)_2HPO_4$. On the other hand, recombinant hGH concentration in P-6 medium was obtained as C_{rhGH}=75 mg L^{-1} which was close to the value of P-3 medium. Presence of PTM1 and $MgSO_4$.7 H_2O in glucose defined medium did not affect rhGH production. Furthermore, yeast extract repressed the rhGH production as in the media P-4 and P-7, respectively as C_{rhGH}=36 mg L^{-1} and C_{rhGH}=41 mg L^{-1} were produced. On the other hand, utilization of H_3PO_4 (85 %) with $(NH_4)_2SO_4$ as a nitrogen source affected neither cell growth nor rhGH production. The lowest rhGH concentration was obtained as 10 mg L^{-1} in P-5 medium. Based on these results, the medium comprising of peptone and PTM1 was chosen for the further semi-batch studies in order to enhance the cell density and rhGH production capacity.

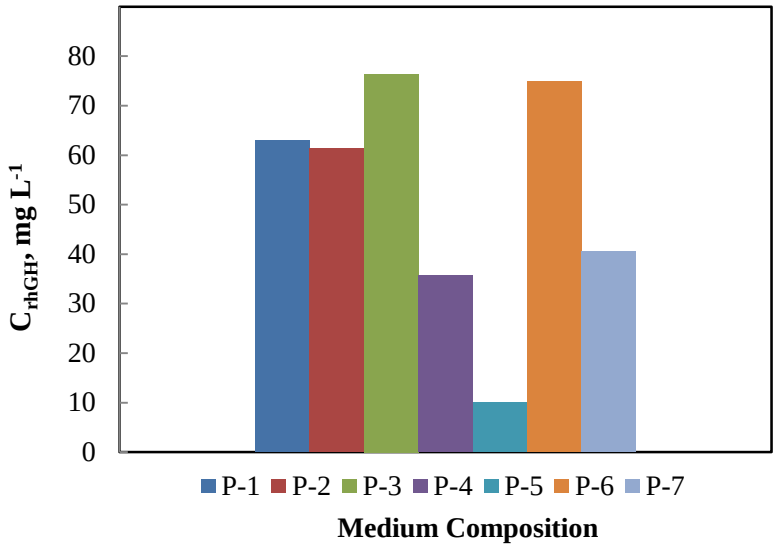

Figure 4.6 The rhGH concentration at t=24 h in the production of rhGH by *B. subtilis* (*scoC*⁻) for seven different media

4.2 Production of Recombinant Human Growth Hormone by Bioreactor Experiments at Pilot Scale

In order to increase the cell density and recombinant hormone production, semi-batch experiments were performed and compared together with the batch process at pilot scale. The effects of glucose and glucose based semi-defined medium feeding rates on recombinant hGH production by *B. subtilis* (*scoC*⁻) were investigated.

In the first set of bioreactor experiments, the amount of rhGH produced was compared with that of the batch operation. Thereafter, air filtered shake bioreactor experiments that related to the medium composition were conducted in order to increase the cell concentration during the bioprocess. In addition, optimization studies of the exponential feeding strategy with this new feeding medium were carried out.

Based on these aims, pilot scale bioreactor experiments were conducted in a bioreactor system having temperature, pH, dissolved oxygen, agitation rate and foam controls. Bioreactor experiments were performed under controlled-pH strategy reported elsewhere with a working volume of V_R=1.1-1.5 L (Şahin, 2010). The operating conditions were selected according to the previous studies on rhGH production by *B. subtilis* (Yılmaz, 2008). These operating parameters were adjusted as T=37 ^oC, pH=7.5, N=750 $min^{-1}$and Q_O/V_R=0.5 vvm.

4.2.1 Feeding Strategy Development for rhGH Production

Eight different feeding strategies were designed (Table 4.1). The semi-batch experiments with exponential feeding strategies were implemented at four different pre-determined specific growth rates which were 0.04, 0.10, 0.17 and 0.26 h^{-1}. All of the specific growth rates for feeding the main carbon source of glucose or semi-defined medium as required substrate in rhGH production were used separately in the bioreactor experiments. For all bioreactor conditions including feeding step, operating parameters for semi-batch cultivation were set to the specific parameters mentioned in section 3.3.5.3. The pre-determined specific growth rate as μ_0=0.04 h^{-1} for BR-1 strategy was chosen according to the previous studies in *Bacillus* species (Christiansen *et al*., 2003).

The abbreviations of the strategies carried out in the semi-batch experiments for rhGH production were given in Table 4.1. In the first feeding strategy (BR-1), glucose was fed to the bioreactor system with the specific growth rate of μ_0=0.04 h^{-1} during 20 h of the process. Substrate feeding was started at t=10 h and halted at t=30 h of the bioprocess. Hydrogen ion (H^+) concentration inside the bioreactor was controlled by 25 % NH_3 solution at its optimum value, pH=7.5. 25 % NH_3 solution was used instead of KOH solution because it may also be a nitrogen source for the cell. Protease inhibitor was added to the production medium at t=8 h to inhibite the degradative functions of the proteases.

In the second strategy abbreviated as BR-2, glucose was added to the production medium with the pre-determined specific growth rate of μ_0=0.10 h^{-1}. Feeding was started at t=10 h as in BR-1 but maintained for 3 hours due to any change in the cell concentration. Thereafter, specific growth rate was increased to μ_0=0.26 h^{-1} to observe the variations in cell and rhGH concentrations at the process time, t=14-15 h. Hydrogen ion concentration was kept constant at pH=7.5 by adding 5 M KOH solution in a controlled manner. Protease inhibitor was added at an optimum value determined by the working volume of the bioreactor at t=8 h.

The third and fourth feeding strategies were repeats to test the reproducibility. In the third and fourth feeding strategies (BR-3 and BR-4), feeding time for substrate was shifted and brought forward. After feeding ended, product formation was expected to begin. Therefore, glucose was fed to the bioreactor system at the beginning of the exponential phase with the specific growth rate of μ_0=0.17 h^{-1} at t=4-12 h. pH was kept at constant pH=7.5 during the bioprocess by utilizing 5 M KOH solution. On the other hand, protease inhibitor adding time was also postponed to t=12 h to prevent cell growth inhibition.

In the fifth production strategy (BR-5), exponential specific growth rate was decreased to μ_0=0.10 h^{-1} as compared with the strategies of BR-3 and BR-4.

In the sixth experiment abbreviated as BR-6, exponential glucose feeding was started at t=4-10:43 h with μ_0=0.17 h^{-1} following by volumetric feed rate of F(t)=6.084x10^{-3} L h^{-1} (or 6.63 g h^{-1}) during the process time of t=16:47-20:56. pH inside the bioreactor was kept at pH=7.5 and protease inhibitor was added to the system at t=12 h.

Only glucose as the substrate was thought to be insufficient for cell growth. Therefore, glucose based semi-defined medium for semi-batch experiments was designed according to the results of the laboratory shake bioreactor experiments. In the seventh strategy (BR-7), this semi-defined medium was fed to the system exponentially at the specific growth rate of μ_0=0.17 h^{-1} during t=4-25 h. The composition of this new feeding substrate stock solution was comprising of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 50 g L^{-1} peptone and 5 mL L^{-1} PTM1. Moreover, antibiotic was added as 2-fold higher than before. pH and the protease inhibitor addition time were set as identical to the sixth strategy.

In the last production strategy (BR-8), exponential pre-determined specific growth rate was decreased to μ_0=0.10 h^{-1} when this rhGH production strategy was compared with the strategy of BR-7.

Table 4.1 Explanations for semi-batch strategies used in bioreactor experiments

Strategies Carried Out	**Strategy Definition (Exponential feeding at constant specific growth rates)**
BR-1	Exponential glucose feeding at μ_0=0.04 h^{-1} during 20 h (t=10-30 h) (pH=7.5 controlled with 25 % NH_3, t_{PI}=8 h)
BR-2	Exponential glucose feeding at μ_0=0.10 h^{-1} at t=10-13 h followed by with μ_0=0.26 h^{-1} at t=14-15 h (pH=7.5 controlled with 5 M KOH, t_{PI}=8 h)
BR-3 and BR-4	Exponential glucose feeding at μ_0=0.17 h^{-1} at t=4-12 h (pH=7.5 controlled with 5 M KOH, t_{PI}=12 h)
BR-5	Exponential glucose feeding at μ_0=0.10 h^{-1} at t=4-12 h (pH=7.5 controlled with 5 M KOH, t_{PI}=12 h)
BR-6	Exponential glucose feeding at μ_0=0.17 h^{-1} at t=4-10:43 h followed by at t=16:47-20:56 h with F(t)=6.084x10^{-3} L h^{-1} (or F(t)=6.63 g h^{-1}) (pH=7.5 controlled with 5 M KOH, t_{PI}=12 h)
BR-7	Exponential semi-defined medium (glucose+peptone+$(NH_4)_2HPO_4$+PTM1) feeding at μ_0=0.17 h^{-1} at t=4-25 h (pH=7.5 controlled with 5 M KOH, t_{PI}=12 h)
BR-8	Exponential semi-defined medium (glucose+peptone+$(NH_4)_2HPO_4$+PTM1) feeding at μ_0=0.10 h^{-1} at t=4-25 h (pH=7.5 controlled with 5 M KOH, t_{PI}=12 h)

4.2.1.1 Effects of Feeding Strategy on Cell Growth

The variations in cell concentration with the cultivation time for different feeding strategies and batch process were given in Figure 4.7. As seen from Figure 4.7, cell concentrations were close to each other except for the feeding strategies of BR-7 and BR-8. By the time that protease inhibitor was added to the production medium (t=8 h or 12 h), cell passed to the stationary growth phase in batch production and some feeding strategies from BR-1 to BR-6. Although cell growth was observed as stable for BR-8 at t=12-16 h, cell growth rates were increasingly high for the feeding strategies of BR-7 and BR-8 during the bioprocess.

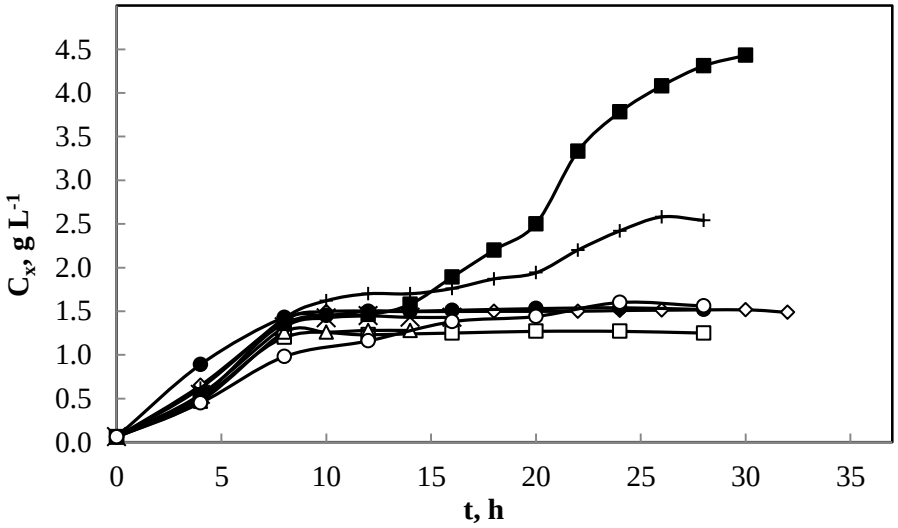

Figure 4.7 The variations in cell concentration with the cultivation time for different feeding strategies in the production of rhGH by recombinant *B. subtilis* (*scoC*$^-$) at bioreactor operating conditions, C_{G0}=8.0 g L^{-1}, T=37 ^oC, pH_C=7.5, N=750 min^{-1}, Q_O/V_R=0.5 vvm: Batch (○), BR-1 (◊), BR-2 (x), BR-3 and BR-4 (□), BR-5 (Δ), BR-6 (●), BR-7 (■), BR-8 (+)

The highest cell concentration was obtained by the seventh feeding strategy as C_X=4.43 g L^{-1} at t=30 h. This value was 2.77-, 2.91-, 3.06-, 3.49-, 3.46-, 2.88-, and 1.72-fold higher than the maximum cell concentration acquired by the bioprocesses of batch production, BR-1, BR-2, BR-3 and BR-4, BR-5, BR-6 and BR-8, respectively. Şahin (2010) reported the maximum cell concentration as 1.62 g L^{-1} which is lower than the cell concentrations attained in this study by the feeding strategies of BR-7 and BR-8. As distinct from others in BR-5, cells degraded after 16 h of the process owing to the limiting substrate conditions. High cell cultivations were achieved in the strategies BR-7 and BR-8 in which semi-defined substrate medium was utilized in exponential feeding. The difference in the maximum cell concentrations between BR-7 and BR-8 came from the higher feeding amounts of substrate medium containing glucose, peptone, $(NH_4)_2HPO_4$ and PTM1 in BR-7.

4.2.1.2 Effects of Feeding Strategy on Substrate Consumption

Not only cell growth but also product and by-product formation entirely depend upon the consumption rate of the carbon source in the production medium. Therefore, excess or limited amount of glucose in the medium directly affects the bioproces. The variations in glucose concentration with the cultivation time for several feeding strategies and batch process were represented in Figure 4.8. Almost 50 % or higher fraction of glucose was consumed at the beginning of batch and semi-batch fermentations in all of the conditions. Glucose is never detected in BR-1 after t=16 h. All the substrate fed to the bioreactor was used by the cell for the cell functions. Therefore, the value of specific growth rate (μ_0=0.04 h^{-1}) was not enough to provide a high product yield. Due to the same consumption profile in BR-2 which was controlled at higher specific growth rate, it was obvious that feeding time was critical for the product formation. Because rhGH is secreted in the late-growth phase, feeding needs to be started and ended at the exponential growth phase. Consequently, substrate feeding was started between t=4-12 h in other bioreactor conditions conducted with semi-batch operations that were BR-3 and BR-4 and BR-5.

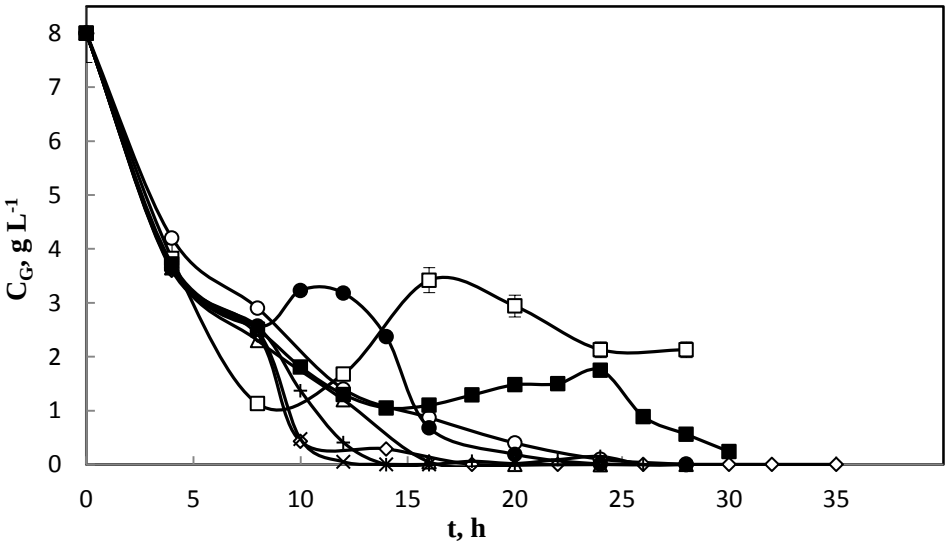

Figure 4.8 The variations in glucose concentration with the cultivation time for different feeding strategies in the production of rhGH by recombinant *B. subtilis* (*scoC*$^-$) at bioreactor operating conditions, C_{G0}=8.0 g L^{-1}, T=37 ^oC, pH_C=7.5, N=750 min^{-1}, Q_O/V_R=0.5 vvm: Batch (○), BR-1 (◊), BR-2 (x), BR-3 and BR-4 (□), BR-5 (Δ), BR-6 (●), BR-7 (■), BR-8 (+)

Although glucose accumulated in BR-3 and BR-4 strategies, product formation was rather high in the other glucose feeding strategies (BR-1, BR-2, BR-5 and BR-6). There was no glucose in the medium in BR-5 at t=16-30 h. rhGH production was performed by limited substrate in the medium. In the sixth feeding strategy (BR-6), glucose feeding was halted for

a while after t=10 h due to the accumulation of glucose in the production medium. After t=16 h, substrate was fed to the bioreactor with a constant volumetric feed rate (Table 4.1). Again glucose accumulated starting from t=18 h in BR-7, and then it was consumed by the end of the process. Finally, the pre-determined specific growth rate for feeding substrate was decreased to μ_0=0.10 h^{-1} in BR-8 to prevent the glucose accumulation in the broth. However, the glucose amount fed to the bioreactor was not enough to obtain the cell and product formation, similar to the results obtained in BR-7. On the other hand, semi-batch bioreactor operation characteristics based on the substrate glucose are presented in Table 4.2.

Table 4.2 Semi-batch bioreactor operation characteristics based on the substrate glucose

Strategies	Batch operation cultivation time prior to semi-batch operation (t), h	Initial glucose for batch operation at t=0 (m_{G0}), g	Maximum rhGH		Overall values within t=0 – t=t_{max}		
			Cultivation time (t_{max}), h	Concentration C_{rhGH}, mg L^{-1}	Total glucose fed (m_G), g	Total glucose consumed, Δm_G, g	Consumption ratio of glucose fed, %
BR-1	10	8	24	78	6.64	11.27	100
BR-2	10	8	16	50	12.15	20.15	100
BR-3	4	8	16	195	12.66	15.91	62.45
BR-5	4	8	16	156	4.84	12.78	98.84
BR-6	4	8	16	185	16.27	23.49	95.24
BR-7	4	8	28	366	67.08	74.31	98.85
BR-8	4	8	24	265	17.01	24.87	99.16

4.2.1.3 Effects of Feeding Strategy on rhGH Production

The variations in recombinant hGH concentration with the cultivation time for batch and semi-batch operations were presented in Figure 4.9. As seen from Figure 4.9, cell growth profiles and rhGH concentration profiles resemble each other in general manner. rhGH production increased crucially after the substrate feeding was started in all production conditions.

Throughout the semi-batch processes, the maximum rhGH concentration was attained by BR-7 at t=28 h as 366 mg L^{-1}. This value was 2.93-, 4.69-, 7.32-, 1.88-, 2.35-, 1.98-, and 1.38-fold higher than the maximum hGH concentration obtained by the other productions of batch, BR-1, BR-2, BR-3 and BR-4, BR-5, BR-6 and BR-8, respectively. Pre-determined feeding profile leading to the highest rhGH production for BR-7 strategy was given in Appendix-E.

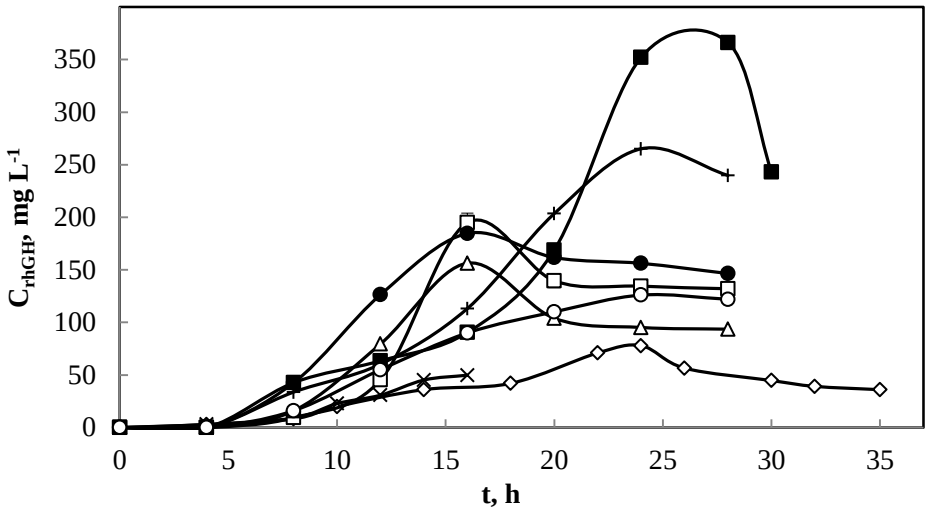

Figure 4.9 The variations in rhGH concentration with the cultivation time for different feeding strategies in the production of rhGH by recombinant *B. subtilis* (*scoC*$^-$) at bioreactor operating conditions, C_{G0}=8.0 g L^{-1}, T=37 ^oC, pH_C=7.5, N=750 min^{-1}, Q_O/V_R=0.5 vvm: Batch (○), BR-1 (◊), BR-2 (x), BR-3 and BR-4 (□), BR-5 (Δ), BR-6 (●), BR-7 (■), BR-8 (+)

Based on rhGH production capacity results, batch process was better than the semi-batch processes with the feeding strategies of BR-1 and BR-2. The rhGH concentrations obtained in BR-1 and BR-2 were 1.62-, and 2.52-fold lower than the rhGH concentration attained by batch bioprocess, respectively. BR-3 and BR-4 and BR-5 had the identical production profiles with BR-6. Feeding rate of glucose was decreased by shifting the pre-determined specific growth rate as μ_0=0.10 h^{-1} in BR-5 due to the glucose accumulation in BR-3 and BR-4. However, this amount of glucose was not enough to achieve and exceed the rhGH concentration value obtained by BR-3 and BR-4. After that, glucose accumulation tried to be under control in the sixth strategy (BR-6) by halting the glucose feeding at t=10:43 h when it was compared with BR-3 and BR-4. Then, glucose feeding started again at t=16:47-20:56 h with the optimum utilized volumetric feed rate of Q(t)= 6.63 g h^{-1} due to the low glucose concentration in the medium. Although the exponential feeding strategy was changed in BR-6 according to BR-3 and BR-4, the rhGH concentration value was 1.05-fold lower than that obtained in BR-3 and BR-4. In the last production strategy (BR-8), the highest rhGH concentration was obtained as 265 mg L^{-1} at t=24 h. When it was compared with BR-7, the only difference affecting rhGH production was in the pre-determined specific growth rate which was decreased from μ_0=0.17 h^{-1} to μ_0=0.10 h^{-1}.Generally rhGH concentrations decreased significantly in the ratio of 3-34 % in all operations after the maximum rhGH concentration was achieved.

In the literature, the highest recombinant hGH production by *Bacillus subtilis* (*scoC*$^-$) strain was reported as 126 mg L^{-1} acquired in the study of Şahin (2010) by batch bioprocess. In this current study, this amount was increased to 2.90-fold with the exponential feeding strategy of BR-7.

4.2.1.4 Effects of Feeding Strategy on Total Protease Activity

Proteases are the main problem in the recombinant protein production by *Bacillus* species. Presence of the proteases in the culture broth causes the reduction on the protein yield due to the degradation function on the desired product. Therefore, extracellular protease activity was determined according to the method given in section 3.4.5 and the total protease activity profiles with respect to time for the semi-batch strategies were represented in Figure 4.10. Total protease activity was determined according to the summation of the acidic, neutral and alkaline protease activities.

The maximum total protease activity was obtained at t=8 h by BR-1 strategy as 29 U cm^{-3} that was 1.07-, 1.38-, 1.26-, 1.32-, 1.81-, and 1.60-fold higher than the activities determined by BR-2, BR-3, BR-5, BR-6, BR-7 and BR-8, respectively. Recombinant hGH profiles were coherent with the total protease profiles. The lowest protease activity was detected at time when the highest rhGH concentration was determined in all cases. When protease inhibitor was added to the bioreaction medium, proteolytic activities of the proteases decreased. This reduction happened at t=8 h for BR-1 and BR-2 and at t=12 h for the other semi-batch strategies.

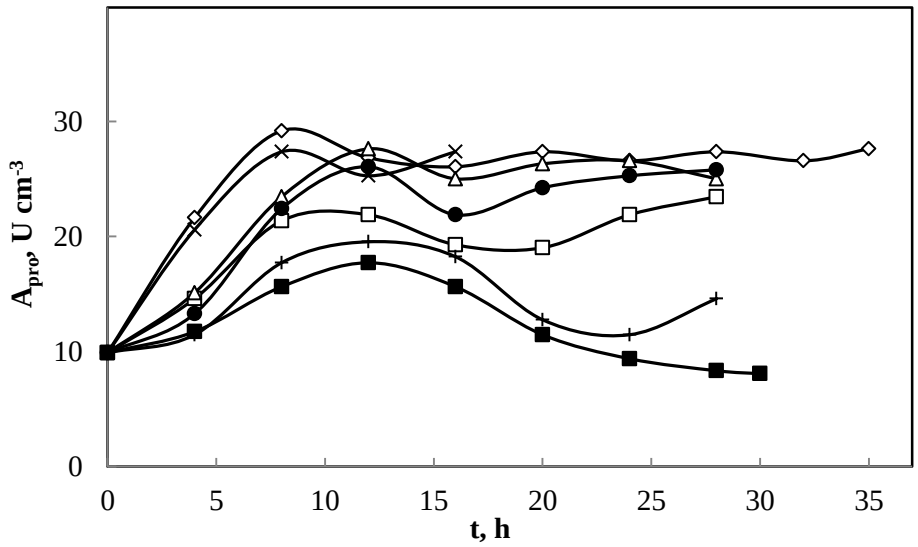

Figure 4.10 The variations in total protease activity with the cultivation time for different feeding strategies in the production of rhGH by recombinant *B. subtilis* (*scoC*$^-$) at bioreactor operating conditions, C_{G0}=8.0 g L^{-1}, T=37 ^oC, pH_C=7.5, N=750 min^{-1}, Q_O/V_R=0.5 vvm: BR-1 (◊), BR-2 (x), BR-3 (□), BR-5 (Δ), BR-6 (●), BR-7 (■), BR-8 (+)

4.2.1.5 Effects of Feeding Strategy on Organic Acid Concentration Profiles

Organic acids are essential materials functioning either through bioreaction pathways or cycles in the intracellular reaction network of the living organisms. The variations in the profiles of organic acids detected in the bioreaction medium were given for all the feeding strategies in Table 4.3. These organic acids were mainly α-ketoglutaric acid, formic acid, fumaric acid, gluconic acid, lactic acid, citric acid and succinic acid.

α-Ketoglutaric acid is one of the integral components of TCA cycle. In the early hours of the production processes, concentrations of the α-ketoglutaric acid were vey low. However, these values sligthly increased by with respect to time in the strategies, i.e., BR-3, BR-6, BR-7 and BR-8. This was because TCA cycle cannot work effectively.

Lactic acid was mainly observed in high concentrations in the all of the feeding strategies except BR-7 and BR-8. Accumulation of lactic acid in the production medium was related to the growth inhibition. This result was consistent with the cell growth profiles.

Gluconic acid synthesizes and enters the pentose phosphate pathway in the presence of oxygen. In order to be used in the PPP, substrate concentration in the medium should be sufficient. When glucose accumulation was observed in BR-3, BR-6 and BR-7, gluconic acid concentrations were at low levels.

Table 4.3 Variations in the organic acid concentrations with the cultivation time for semi-batch strategies

	Concentration, g L^{-1}						
t, h	**4**	**8**	**12**	**16**	**20**	**24**	**28**
			BR-1				
α-Ketoglutaric acid	0.001	0.002	0.000	0.001	0.001	0.001	0.000
Formic acid	0.073	0.037	0.007	0.037	0.024	0.054	0.095
Fumaric acid	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Gluconic acid	0.092	0.151	0.201	0.222	0.284	0.387	0.793
Lactic acid	0.077	0.148	0.247	0.233	0.260	0.254	0.203
Citric acid	0.000	0.009	0.000	0.004	0.000	0.001	0.004
Succinic acid	0.003	0.019	0.036	0.010	0.018	0.089	0.092
Total	0.246	0.366	0.491	0.507	0.587	0.786	1.187

Table 4.3 Variations in the organic acid concentrations with the cultivation time for semi-batch strategies (Continued)

	Concentration, g L^{-1}						
t, h	**4**	**8**	**12**	**16**	**20**	**24**	**28**
			BR-2				
α-Ketoglutaric acid	0.001	0.002	0.000	0.065			
Formic acid	0.109	0.037	0.030	0.173			
Fumaric acid	0.000	0.000	0.000	0.000			
Gluconic acid	0.108	0.158	0.198	0.197			
Lactic acid	0.006	0.053	0.117	0.195			
Citric acid	0.000	0.029	0.000	0.000			
Succinic acid	0.007	0.065	0.043	0.084			
Total	0.231	0.344	0.388	0.714			
			BR-3				
α-Ketoglutaric acid	0.001	0.003	0.000	0.057	0.076	0.055	0.068
Formic acid	0.065	0.015	0.048	0.192	0.164	0.140	0.100
Fumaric acid	0.000	0.000	0.001	0.000	0.000	0.000	0.000
Gluconic acid	0.014	0.097	0.086	0.107	0.198	0.289	0.212
Lactic acid	0.057	0.113	0.209	0.220	0.375	0.258	0.256
Citric acid	0.000	0.042	0.004	0.000	0.000	0.000	0.000
Succinic acid	0.005	0.017	0.065	0.087	0.054	0.000	0.000
Total	0.142	0.287	0.413	0.663	0.867	0.742	0.636
			BR-5				
α-Ketoglutaric acid	0.001	0.003	0.001	0.000	0.000	0.000	0.000
Formic acid	0.061	0.019	0.056	0.114	0.119	0.049	0.006
Fumaric acid	0.000	0.000	0.000	0.000	0.001	0.001	0.001
Gluconic acid	0.071	0.095	0.302	0.202	0.351	0.307	0.279
Lactic acid	0.010	0.044	0.213	0.319	0.302	0.250	0.321
Citric acid	0.000	0.031	0.000	0.000	0.000	0.000	0.020
Succinic acid	0.006	0.029	0.095	0.034	0.020	0.010	0.000
Total	0.149	0.221	0.667	0.669	0.813	0.617	0.627

Table 4.3 Variations in the organic acid concentrations with the cultivation time for semi-batch strategies (Continued)

Concentration, g L^{-1}							
t, h	**4**	**8**	**12**	**16**	**20**	**24**	**28**
			BR-6				
α-Ketoglutaric acid	0.002	0.004	0.045	0.059	0.091	0.096	0.099
Formic acid	0.043	0.004	0.044	0.154	0.323	0.175	0.098
Fumaric acid	0.000	0.000	0.001	0.000	0.001	0.001	0.001
Gluconic acid	0.025	0.056	0.050	0.071	0.347	0.501	0.549
Lactic acid	0.096	0.145	0.262	0.243	0.226	0.290	0.282
Citric acid	0.002	0.054	0.078	0.057	0.042	0.004	0.007
Succinic acid	0.022	0.059	0.085	0.066	0.074	0.090	0.022
Total	0.190	0.322	0.527	0.650	1.104	1.157	1.058
			BR-7				
α-Ketoglutaric acid	0.003	0.010	0.008	0.028	0.024	0.030	0.054
Formic acid	0.103	0.074	0.037	0.187	0.191	0.155	0.168
Fumaric acid	0.000	0.001	0.000	0.001	0.001	0.001	0.001
Gluconic acid	0.036	0.162	0.178	0.063	0.054	0.119	0.165
Lactic acid	0.014	0.055	0.151	0.144	0.019	0.018	0.003
Citric acid	0.000	0.040	0.083	0.026	0.006	0.005	0.000
Succinic acid	0.000	0.008	0.034	0.043	0.033	0.028	0.000
Total	0.156	0.350	0.491	0.492	0.328	0.356	0.391
			BR-8				
α-Ketoglutaric acid	0.002	0.016	0.029	0.024	0.036	0.041	0.044
Formic acid	0.015	0.012	0.150	0.119	0.141	0.253	0.170
Fumaric acid	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Gluconic acid	0.053	0.124	0.098	0.076	0.170	0.220	0.262
Lactic acid	0.009	0.016	0.023	0.020	0.016	0.019	0.015
Citric acid	0.027	0.020	0.061	0.014	0.034	0.015	0.022
Succinic acid	0.000	0.018	0.045	0.028	0.049	0.072	0.043
Total	0.107	0.207	0.407	0.282	0.447	0.621	0.557

Fumaric and succinic acids are the other important organic acids in TCA cycle. There was no accumulation of fumaric acid in all the conditions. Moreover, succinic acid concentrations were generally at low levels. Therefore, there was not any constriction in these steps of TCA cycle. On the other hand, formic acid amount was nonignorable for all strategies. The highest formic acid concentration was obtained as 0.253 g L^{-1} in BR-8.

Furthermore, citric acid synthesis started generally at t=8 h and decreased at the end of the process time. Citric acid levels in the strategies except BR-6, BR-7 and BR-8 were regarded as negligible.

4.2.1.6 Oxygen Transfer Characteristics

Throughout the fermentation liquid phase mass transfer coefficient (K_La), enhancement factor ($E= K_La/K_La_0$), oxygen transfer rate (OTR), oxygen uptake rate (OUR), maximum possible mass transfer rate (OTR_{max}), maximum possible oxygen utilization rate or oxygen demand ($OD=\mu_{max}C_X/Y_{X/O}$), Damköhler number ($Da=OD/OTR_{max}$) and efficiency factor (η=OUR/OD) were determined. Variations in these parameters for the feeding strategies were given in Table 4.4. Oxygen transfer characteristics for seventh feeding strategy (BR-7) were not able to be determined due the malfunction of oxygen probe during the cultivation.

Table 4.4 Variations in oxygen transfer characteristics for semi-batch strategies

Feeding Strategies	t (h)	K_La (s^{-1})	E (K_La/K_La_0)	OTR*10^3 (mol m^{-3} s^{-1})	OTR_{max}*10^3 (mol m^{-3} s^{-1})	OUR*10^3 (mol m^{-3} s^{-1})	OD*10^3 (mol m^{-3} s^{-1})	Da	η
BR-1	0	0.023	1.00	-	-	-	-	-	-
	4	0.051	1.59	7.91	18.87	0.91	0.92	0.05	8.58
	8	0.042	1.31	8.10	15.54	0.93	3.20	0.21	2.53
	12	0.027	0.84	5.49	9.99	0.63	19.13	1.92	0.29
	16	0.041	1.28	11.30	15.17	1.30	1014.24	66.86	0.01
	20	0.043	1.34	9.71	15.91	1.12	348.75	21.92	0.03
	24	0.032	1.00	6.96	11.84	0.80	179.69	15.18	0.04
	28	0.046	1.44	10.48	17.02	1.21	-	-	-
	32	0.041	1.28	11.86	15.17	1.37	-	-	-
BR-2	0	0.023	1.00	-	-	-	-	-	-
	4	0.032	1.00	7.54	11.84	0.87	10.74	0.91	0.70
	8	0.040	1.25	7.95	14.80	0.92	38.18	2.58	0.21
	12	0.011	0.34	3.46	4.07	0.40	147.76	36.31	0.02
	16	0.038	1.19	9.76	14.06	1.12	47.42	3.37	0.21
BR-3	0	0.023	1.00	-	-	-	-	-	-
	4	0.051	2.22	2.54	17.21	0.29	4.42	0.25	0.73
	8	0.039	1.70	3.61	16.65	0.42	111.89	7.70	0.26
	12	0.034	1.48	3.21	13.51	0.37	258.31	19.43	0.15
	16	0.040	1.74	3.03	13.69	0.35	310.11	22.74	0.10
	20	0.057	2.48	3.04	17.95	0.35	-	-	-
	24	0.073	3.17	2.76	24.05	0.32	263.42	9.75	0.00

Table 4.4 Variations in oxygen transfer characteristics for semi-batch strategies (Continued)

Feeding Strategies	t (h)	K_La (s^{-1})	E (K_La/K_La_0)	OTR*10^3 (mol m^{-3} s^{-1})	OTR_{max}*10^3 (mol m^{-3} s^{-1})	OUR*10^3 (mol m^{-3} s^{-1})	OD*10^3 (mol m^{-3} s^{-1})	Da	η
BR-5	0	0.023	1.00	-	-	-	-	-	-
	4	0.075	3.26	4.36	27.75	0.50	5.59	0.20	0.78
	8	0.032	1.39	3.05	11.84	0.35	15.58	1.32	0.20
	12	0.034	1.48	2.97	12.58	0.34	9.85	0.78	0.30
BR-6	0	0.023	1.00	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
	12	0.037	1.61	3.81	13.69	0.44	233.80	17.08	0.02
	16	0.043	1.87	3.93	15.91	0.45	674.94	40.73	0.01
	20	0.028	1.22	1.90	10.36	0.22	316.50	30.55	0.01
	24	0.015	0.65	0.52	5.55	0.06	-	-	-
	28	0.022	0.96	0.63	8.14	0.07	-	-	-
BR-8	0	0.020	1.00	-	-	-	-	-	-
	4	0.041	2.05	3.06	15.17	0.35	4.58	0.30	0.67
	8	0.042	2.10	4.46	15.54	0.51	19.22	1.24	0.23
	12	0.016	0.80	1.66	5.92	0.19	27.26	4.60	0.06
	16	0.019	0.95	2.02	7.03	0.23	48.65	6.92	0.04
	20	0.030	1.50	2.91	11.1	0.34	28.01	2.52	0.10
	24	0.011	0.55	1.29	4.07	0.15	17.09	4.20	0.08
	28	0.034	1.70	1.94	12.58	0.22	19.96	1.59	0.10

Liquid phase mass transfer coefficient depends upon the agitation rate, characteristics of impeller related to bioreactor design and the rheological properties of the production medium. First two items remained unchanged. The only parameter influencing K_La could be the medium characteristics. High K_La values were observed in the early hours of the bioprocess for the feeding strategies. Viscosity of the medium may increase by the secretion of products. Therefore, reductions in the K_La values were observed at the production times. Average K_La values for the feeding strategies varied in the range of 0.027-0.045 s^{-1}.

Enhancement factor indicates the presence of microorganism in the biochemical reactions. In order to calculate the enhancement factor, K_La_0 was determined as 0.023 s^{-1} almost for all conditions (K_La_0=0.020 s^{-1} for BR-8). In general, E values changed in the range of 0.34-3.26. Low E values point to that biochemical reactions take place gradually with effective mass transfer.

According to the values of oxygen transfer and oxygen uptake rates, oxygen transfer into the production broth was sufficient in all conditions. OUR values were high at the early stages of the bioprocess due to the high cell growth and at the rhGH production times, i.e, t=12-20 h. The highest OTR values attained by the feeding strategies; BR-1, BR-2, BR-3, BR-5, BR-6 and BR-8 were 11.86, 9.76, 3.61, 4.36, 3.93 and 4.46 mmol m^{-3} s^{-1}, respectively.

In order to find the rate limiting step in the rhGH production, Damköhler number (Da) and efficiency factor (η) need to be determined. OD and OTR_{max} values are critical to specify these dimensionless numbers. Da values which are higher than 1 state mass transfer limited condition. On the contrary, Da numbers which are lower than 1 state bioreaction limited condition in bioprocesses. Until t=4 h, the bioprocess was biochemical reaction limited, afterwards it turned to the mass transfer limited.

Efficiency factors were very low related to high OD values for all the conditions. η values lower than 1 were observed throughout the bioprocesses since the microorganism utilized less oxygen than the oxygen demand. It was also remarkable that when Da increased, η decreased or vice versa.

4.2.1.7 Specific Rate and Selectivity (Yield) Coefficients

The variations in fermentation characteristic related to the bioprocess efficiency were represented in Table 4.5 for the feeding strategies carried out at pilot scale experiments. These fermentation characteristics are mainly composed of specific rates, i.e., the specific growth rate (μ), specific substrate consumption rate (q_S), the specific product formation rate (q_P), the specific oxygen uptake rate (q_O) and yield coefficients (selectivity values), i.e., the cell yield on substrate consumption ($Y_{X/S}$), the product yield on substrate consumption ($Y_{P/S}$) and the cell yield on oxygen consumption ($Y_{X/O}$).

The specific growth rates in all feeding strategies were very low except that at the first hours of the bioprocess. The highest μvalues for BR-1, BR-2, BR-3, BR-5, BR-6, BR-7 and BR-8 were determined as 0.26, 0.29, 0.29, 0.32, 0.19, 0.31 and 0.27 h^{-1}, respectively.

Table 4.5 Variations in fermantation characteristics for semi-batch strategies

Feeding Strategies	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})
	4	0.26	1.40	1.59	1.08	0.13	0.63	0.18
	8	0.10	0.67	2.05	0.38	0.62	4.58	0.11
	12	0.01	0.42	2.49	0.25	0.03	6.18	0.02
BR-1	16	0.00	0.87	2.41	0.21	0.00	3.99	0.00
	20	0.00	0.75	3.49	0.28	0.00	3.54	0.00
	24	0.00	0.53	-	0.37	0.00	2.01	0.00
	28	0.00	0.80	-	0.55	0.00	-	-
	32	-	0.92	-	0.00	-	-	-
	4	0.29	1.59	2.08	1.26	0.11	0.69	0.18
	8	0.11	0.69	2.50	0.40	0.66	5.21	0.12
BR-2	12	0.00	0.28	3.92	0.45	0.07	19.20	0.03
	16	0.14	0.79	4.63	1.16	1.40	145.71	0.11
	4	0.29	0.53	2.25	2.79	0.11	0.00	0.57
	8	0.10	0.42	4.84	3.16	0.09	1.09	0.21
	12	0.03	0.30	19.83	12.24	0.01	1.54	0.02
BR-3	16	0.01	0.28	10.89	0.15	0.01	12.37	0.01
	20	0.00	0.28	-	0.13	0.04	-	0.00
	24	-	0.25	-	0.08	0.00	-	0.00
	28	-	-	-	0.00	-	-	-
	4	0.32	1.07	0.42	0.93	0.09	0.00	0.30
	8	0.08	0.28	0.79	0.46	0.17	0.35	0.29
	12	-	0.27	15.13	1.01	0.00	0.52	0.46
BR-5	16	-	-	10.52	0.01	-	22.37	-
	20	-	-	-	0.00	-	-	-
	24	-	-	-	0.00	-	-	-
	28	0.00	-	-	0.00	-	-	-
	4	0.19	-	6.04	1.38	0.19	0.00	-
	8	0.06	-	11.14	0.80	0.14	10.94	-
BR-6	12	0.01	0.29	12.03	0.15	0.03	26.37	0.02
	16	0.00	0.30	3.14	0.95	0.00	16.53	0.01
	20	0.00	0.14	-	0.75	0.01	-	0.02
	24	-	0.04	-	0.01	0.10	-	-
	28	-	0.05	-	0.00	-	-	-
	4	0.31	-	10.33	1.94	0.11	0.00	-
	8	0.09	-	5.89	0.69	0.72	36.49	-
	12	0.05	-	4.29	0.12	0.10	17.73	-
BR-7	16	0.08	-	7.57	1.24	-	182.52	-
	20	0.12	-	14.58	1.72	-	-	-
	24	0.07	-	7.73	0.02	-	-	-
	28	0.03	-	0.81	0.07	-	-	-
	4	0.27	0.57	6.85	1.46	0.13	0.00	0.48
	8	0.10	0.36	5.32	0.52	0.76	32.36	0.26
	12	0.03	0.11	5.90	0.19	0.14	12.33	0.22
BR-8	16	0.01	0.13	9.65	0.41	0.20	132.52	0.13
	20	0.02	0.17	7.57	0.50	-	-	0.25
	24	0.02	0.06	0.71	0.00	-	-	0.50
	28	0.01	0.09	-	0.01	-	-	0.45

The specific oxygen uptake rates were generally low in the bioprocesses during the cultivation time. The higher q_O values were observed at the beginning of the logarithmic growth phase. The highest q_O values for BR-1, BR-2, BR-3, BR-5, BR-6 and BR-8 were determined as 1.40, 1.59, 0.53, 1.07, 0.30 and 0.57 g g^{-1} h^{-1}, respectively.

The trend in the variations of q_S and q_P was similar to each other as one of them increased the other one also increased in general. The highest q_P values for the strategies stated as BR-1, BR-2, BR-3, BR-5, BR-6, BR-7 and BR-8 were determined as 3.49, 4.63, 19.83, 15.13, 12.03, 14.58 and 9.65 g g^{-1} h^{-1}, respectively. The variation in r_{rhGH} and q_{rhGH} with the cultivation time is presented in Figure 4.11 for BR-7, the feeding strategy where the highest rhGH production was obtained. According to Figure 4.11, trends in the profiles of the rhGH production rates and the specific rhGH formation rates were close to each other. When recombinant hGH production rate was the highest, q_{rhGH} was the highest, too. The maximum r_{rhGH} and q_{rhGH} values attained by BR-7 were 32.75 mg L^{-1} h^{-1} and 14.58 g kg^{-1} h^{-1} at t=20 h, respectively.

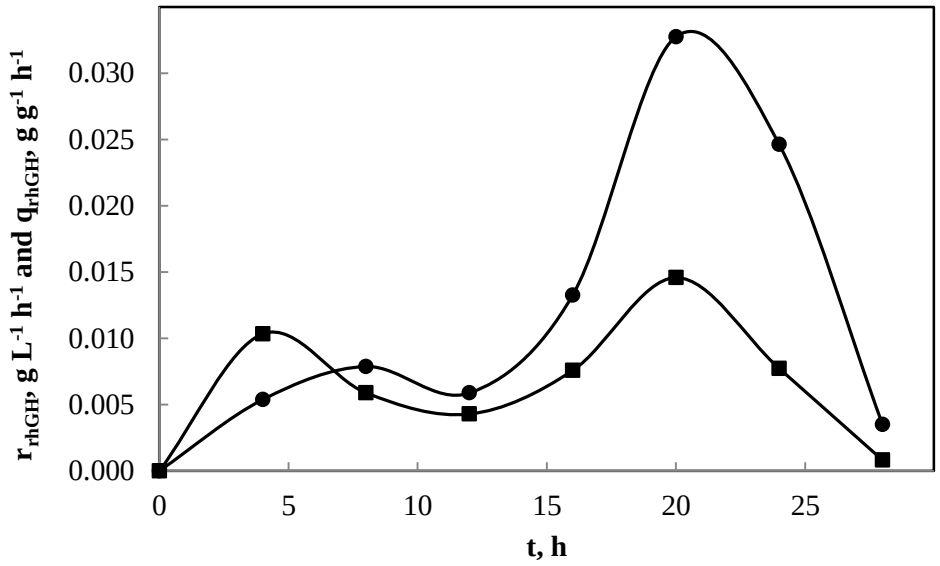

Figure 4.11 The relationship of r_{rhGH} and q_{rhGH} versus time for the feeding strategy, BR-7 conducted at pilot scale bioreactor system with the operating conditions of C_{G0}=8.0 g L^{-1}, T=37 ^oC, pH_C=7.5, N=750 min^{-1}, Q_O/V_R=0.5 vvm: r_{rhGH} (●), q_{rhGH} (■)

Due to the low cell concentrations except in BR-7 and BR-8, $Y_{X/S}$ values were generally low except only for the early hours throughout the bioprocesses. In all the production conditions, the maximum $Y_{P/S}$ value was attained by BR-7 as 182.52 g g^{-1} at t=16 h. On the other hand, $Y_{X/O}$ values were very limited since oxygen could provide at insufficient levels.

The overall product yield on total substrate ($\overline{Y}_{P/S}$) for BR-7 was determined as 62.92 g kg^{-1} that was 6.38-, 9.53-, 1.50-, 3.15-, 2.39-, and 2.16-fold higher than the values obtained by the feeding strategies of BR-1, BR-2, BR-3, BR-5, BR-6 and BR-8, respectively. On the

other hand, The overall cell yield on total substrate ($\overline{Y}_{X/S}$) for BR-7 was determined as 0.73 g g^{-1} that was 4.29-, 4.06-, 2.52-, 4.06-, 3.84-, and 2.70-fold higher than the values obtained by the feeding strategies of BR-1, BR-2, BR-3, BR-5, BR-6 and BR-8, respectively. These results indicate that substrate utilization occurred more effectively in BR-7 feeding strategy.

CHAPTER 5

CONCLUSIONS

In this study, the aim is to develop an effective feeding strategy for the recombinant human growth hormone (rhGH) production by *Bacillus subtilis* (*scoC*$^-$) strain carrying pMK4::*pre*(*subC*)::*hGH* plasmid. In this context, the research program for rhGH production was conducted in both laboratory scale air filtered shake bioreactor and pilot scale bioreactors. According to the outcomes of the laboratory scale air filtered shake bioreactor experiments, exponential feeding strategies were designed to enhance the rhGH production with respect to batch production by pilot scale bioreactor fermentation. The effects of the designed feeding strategies on the cell growth, substrate consumption, rhGH production and protease activities were investigated. Moreover, the fermentation characteristics, the specific substrate consumption and rhGH production rates together with the yield (selectivity) coefficients, organic acid profiles, and oxygen transfer characteristics were calculated for every strategy designed to understand the response of *B. subtilis* cells to the feeding strategies.

In air filtered shake bioreactor experiments at laboratory scale, effect of glutamine on rhGH production was firstly determined at the operating conditions of T=37 ^oC, N=200 min^{-1}, pH_0=7.5, C_{G0}=8 g L^{-1} within the working volume of V_R=110 mL. Moreover, protease inhibitor was added to the media at t=8 h. The maximum cell concentration for the medium (G-0.05) containing 8 g L^{-1} glucose + 0.05 g L^{-1} glutamine was achieved as 1.00 g L^{-1} at t=20 h. The highest rhGH concentration was also obtained by G-0.05 production medium as 76 mg L^{-1} at t=28 h. This value was 1.2-fold higher than that obtained by the medium containing only glucose as compared with G-0.05. Due to the low difference in rhGH production, adding of glutamine into the production medium was not tried on pilot scale production. However, it can be more effective with an optimized semi-batch strategy. In the second set of the laboratory scale shake bioreactor bioreactor experiments, effect of medium composition on cell concentration and so on the rhGH production was investigated to achieve high cell cultivations in semi-batch experiments. Six different media were designed in accordance with literature and performed for rhGH production with the original defined medium (P-1). As distinct from the operating conditions at laboratory scale, protease inhibitor addition time was shifted to t=12 h in order to prevent the cell growth inhibition. The highest cell concentration was achieved as C_X=2.47 g L^{-1} by the semi-defined production medium, P-6 containing 8 g L^{-1} glucose together with 8 g L^{-1} peptone and the trace elements of 5 mL L^{-1} PTM1. Moreover, new protease inhibitor addtion time worked on the cell to pass into the stationary phase later. Maximum rhGH concentration was achieved as 76 mg L^{-1} in the medium (P-3) containing 8 g L^{-1} glucose as a carbon source and 8 g L^{-1}

peptone as a complex nitrogen source in addition to 4.71 g L^{-1} $(NH_4)_2HPO_4$. A close rhGH concentration to that was obtained as well in P-6 medium as C_{rhGH}=75 mg L^{-1}. Presence of only PTM1 or $MgSO_4.7\ H_2O$ in glucose defined medium did not affect rhGH production. Although yeast extract was effective to obtain high cell concentration, it repressed the rhGH production. On the other hand, utilization of H_3PO_4 (85 %) with $(NH_4)_2SO_4$ as a nitrogen source affected neither cell growth nor rhGH production. The lowest rhGH concentration was obtained as 10 mg L^{-1} in P-5 medium. According to these results, the production medium containing peptone and PTM1 was chosen for the further semi-batch studies in order to enhance the cell density and rhGH production capacity.

In pilot scale bioreactor experiments, the designed semi-batch bioreactor operations were conducted after an initial batch operation, at the operating conditions of T=37 ^oC, N=750 min^{-1}, pH=7.5, Q_O/V_R=0.5 vvm and C_{G0}=8 g L^{-1} in a bioreactor working volume of V_R=1.1-1.5 L; and, the effects of exponential feeding strategies on rhGH production by *B. subtilis* (*scoC*$^-$) were investigated. In general, the semi-batch rhGH production processes were performed at four different pre-determined specific growth rates of 0.04, 0.10, 0.17, and 0.26 h^{-1}. In the designed semi-batch processes, the continuously fed substrate(s) was either the sole carbon source glucose or the semi-defined medium. The initial pH in the bioreactor was pH=7.5, and was controlled by 5 M KOH solution in all bioreactor experiments except in BR-1, in which pH was controlled by using 25 % NH_3 solution which is also used a nitrogen source, as well. Furthermore, protease inhibitor was added to the production media at t=12 h; however, in BR-1 and BR-2 protease inhibitor was added at t_{PI}=8 h. In the first feeding strategy abbreviated as BR-1, glucose was fed to the bioreactor system with the pre-determined specific growth rate of μ_0=0.04 h^{-1} at t=10-30 h. In the second feeding strategy (BR-2), glucose feeding was started with μ_0=0.10 h^{-1} at t=10 h and maintained for 3 hours owing to low cell concentration. Then, it was increased to μ_0=0.26 h^{-1} at t=14-15 h to observe the variations in cell and rhGH concentrations. BR-3 and BR-4 were the exponential feeding strategies that were identical to each other. Reproducibility of BR-3 was provided by BR-4. Since feeding was started and ended before the product formation, feeding time for substrate was shifted as compared with BR-1 and BR-2. In BR-3 and BR-4, glucose was added to the production medium with the specific growth rate of μ_0=0.17 h^{-1} at t=4-12 h. In the fifth exponential feeding strategy (BR-5), pre-determined specific growth rate was decreased to μ_0=0.10 h^{-1} by comparison with the strategies of BR-3 and BR-4. In the other feeding strategy named as BR-6, glucose feeding was started at t=4-10:43 h with the specific growth rate of μ_0=0.17 h^{-1} and followed by the volumetric feed rate of F(t)=6.084x10^{-3} L h^{-1} (or 6.63 g h^{-1}) at t=16:47-20:56. Based on the outcomes of the laboratory shake bioreactor experiments to acquire high cell density and high rhGH production, glucose based semi-defined medium as substrate feeding was tried on the exponential feeding strategies of BR-7 and BR-8. The composition of this new feeding substrate as stock solution was included 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 50 g L^{-1} peptone and 5 mL L^{-1} PTM1. In BR-7, this semi-defined medium was fed to the bioreactor system at μ_0=0.17 h^{-1} during t=4-25 h. As distinct from BR-7, the pre-determined specific growth rate was decreased to to μ_0=0.10 h^{-1} in BR-8. The major results attained for the feeding strategies are summarised as follows:

1) The highest cell concentration was achieved in BR-7 as C_X=4.43 g L^{-1} at t=30 h, and then second highest value was obtained in BR-8 (C_X=2.58 g L^{-1}), with the semi-defined

medium. The results reveal that high cell cultivation is provided by using the semi-defined feeding medium. In addition, protease inhibitor does not affect the cell growth in BR-7 and BR-8, contrary to the results obtained with the sole carbon source glucose in the other semi-batch feeding strategies BR-1 to BR-6 and batch operation. It is indeed noteheworthy the cell degradation observed in BR-5 after t=16 h.

2) In general, the glucose consumption rates were higher at the beginning of the exponential growth phase. Moreover, substrate feeding time was crucial for the protein production secreted in the late-growth phase as in the case valid for rhGH production by *B. subtilis*. Therefore, the batch-operation shifted to semi-batch operation at t=4 h, by starting substrate feeding continuously as a function of the cultivation time calculated by the equation-2.30, except in BR-1 and BR-2. In the semi-batch process, glucose limitation was more influential than glucose accumulation on the rhGH production. Glucose concentration in the production medium can be kept within C_G=1-2 g L^{-1} before and during the rhGH production period.

3) The highest rhGH production was achieved in BR-7 at t=28 h as 366 mg L^{-1} with the semi defined medium, which was 1.38-fold higher than that of obtained in BR-8, and ca. 7.3-fold higher than that of obtained in the semi-bach processes with the sole carbon source glucose (BR-1 to BR-6) and by the batch production. As expected, the highest q_{rhGH} was calculated in BR-7 as 14.58 mg g^{-1} h^{-1} at t=20 h. However, due to protease synthesis in the cells, the protein rhGH was hydrolysed and its concentrations were decreased after the maximum rhGH concentration was obtained.

4) The variations in total protease activities were coherent with rhGH concentration profiles. When protease inhibitor was added to the bioreactor system (t=8 or 12 h), proteolytic activities of the proteases decreased and the lowest protease activity was determined at the time the highest rhGH concentration was achieved in all the production conditions. The maximum total protease activity was obtained at t=8 h by BR-1 strategy as 29 U cm^{-3}.

5) Overall, in all processes α-ketoglutaric acid, formic acid, fumaric acid, gluconic acid, lactic acid, citric acid and succinic acid were detected in the medium. Fumaric, succinic and citric acids levels were lower or negligible when they were compared with the other TCA cycle integral component of α-ketoglutaric acid. Due to the increasing concentrations of α-ketoglutaric acid, TCA cycle did not work effectively in the strategies, i.e., BR-3, BR-6, BR-7 and BR-8. Gluconic acid which is responsible on PPP pathway with the sufficient amount of substrate was detected at low levels in BR-3, BR-6 and BR-7. This was because of the glucose accumulation detecting in these strategies. Lactic acid was generally observed in all feeding strategies except BR-7 and BR-8 related to the growth inhibiton. On the other hand, formic acid concentrations were nonignorable for all the strategies and the highest formic acid concentration was determined in BR-8 as 0.253 g L^{-1}.

6) For all the feeding strategies until t=4 h, the bioprocess was biochemical reaction limited, thereafter it shifted to the mass transfer limited condition. On the other hand, the highest overall product and cell yield on total substrate were obtained in the most efficient exponential feeding strategy (BR-7) as 62.92 g kg^{-1} and 0.73 g g^{-1}, respectively. This result reveals that the substrate utilization occurred more efficiently in the feeding strategy BR-7.

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APPENDIX A

CONTENTS OF THE KITS

Glucose Analysis Kit (Biyozim-Biasis)

Equipments Required for the Analysis

- Micro pipette (1000 µL, 20-200 µL)
- Volumetric flask (100-250 mL)
- Macro cuvette (3 mL)
- Vortex
- Water bath (37 ^{o}C)
- Spectrophotometer (λ=505 nm)

Ingredients of the Analysis Kit

- Glucose analysis reagent
  Glucose oxidase ≥ 10000 IU/bottle
  Peroxidase ≥ 1000 IU/bottle
  4-Aminoantipyrine (0.2 mM)

- Glucose analysis buffer (Concentrated, 50 mL)
  Potassium dehydrogen phosphate (0.4 M)
  Phenol (0.2 mM)

- Glucose standard
  Glucose (0.5 g)

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

HPCE Analysis Solutions

- **Electrolyte Solution**

$Na_2B_4O_7.10\ H_2O$	(0.4763 g)
Z-1 Methyl Reagent	(4.5310 g)
dH_2O	(25 mL)
NaOH*	(1M)

*pH is adjusted to 10.0 by 1 M NaOH before degassing and filtering the solution.

- **Borate Buffer Solution**

$Na_2B_4O_7.10\ H_2O$	(0.4763 g)
dH_2O	(25 mL)
NaOH*	(1M)

*pH is adjusted to 10.0 by 1 M NaOH before degassing and filtering the solution.

Protease Activity Assay Solutions

- **0.05 M Sodium Acetate Buffer (for acidic proteases)**

CH_3COOH	(0.713 mL dissolved in 25 mL dH_2O)
CH_3COONa	(2.052 g dissolved in 50 mL dH_2O)

Titrate CH_3COONa solution with CH_3COOH solution to adjust pH to 5.0. Then, dilute the solution to 500 mL before putting into the autoclave and storing at +4 ^oC.

- **0.05 M Sodium Phosphate Buffer (for neutral proteases)**

$Na_2HPO_4.7\ H_2O$	(6.70 g dissolved in 50 mL dH_2O)
$NaH_2PO_4.2\ H_2O$	(3.90 g dissolved in 50 mL dH_2O)

Titrate $Na_2HPO_4.7\ H_2O$ solution with $NaH_2PO_4.2\ H_2O$ solution to adjust pH to 7.0. Then, dilute the solution to 500 mL before putting into the autoclave and storing at room temperature.

- **0.05 M Borate Buffer (for alkaline proteases)**

$Na_2B_4O_7.10\ H_2O$	(2.381 g)
dH_2O	(250 mL)
NaOH*	(1M)

*pH is adjusted to 10.0 by 1 M NaOH before adding dH_2O till 500 mL, filtering and storing the solution at + 4 ^{o}C.

APPENDIX C

CALIBRATION CURVES

Calibration Curve for Cell Concentration

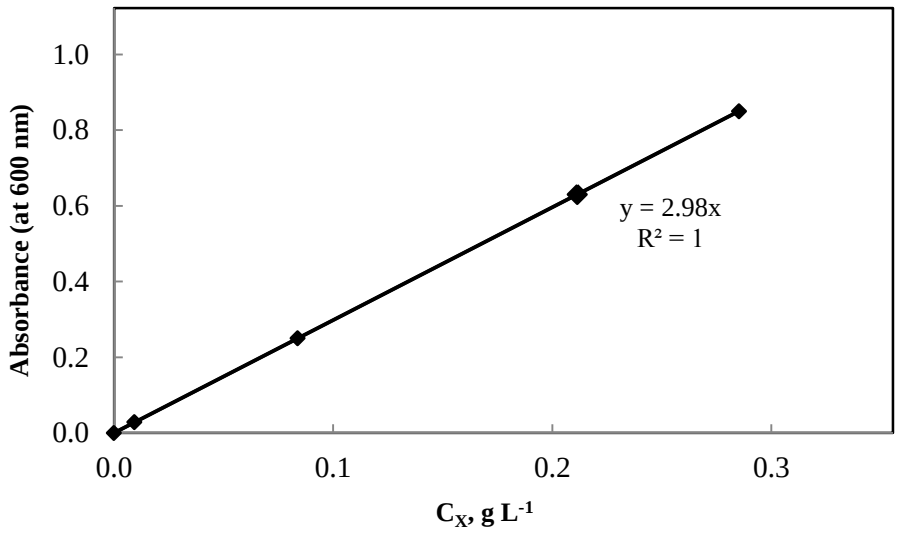

Figure C.1 Calibration curve for cell concentration

According to the equation obtained from the plot:

$$C_x = \frac{Absorbance}{2.98} x\left(Dilution\ Ratio\right) \quad \text{(C.1)}$$

Calibration Curve for Glucose Concentration

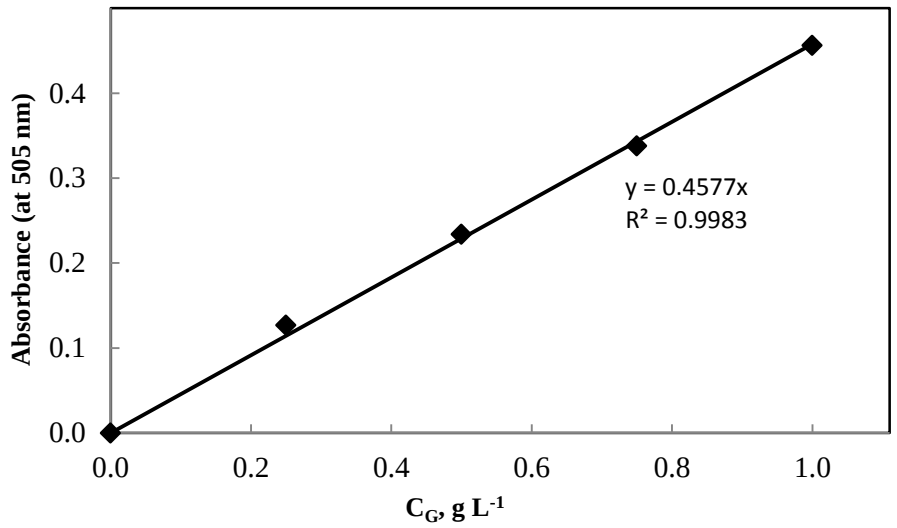

Figure C.2 Calibration curve for glucose concentration

According to the equation obtained from the plot:

$$C_G = \frac{Absorbance}{0.4577} x\left(Dilution\ Ratio\right) \qquad \text{(C.2)}$$

Calibration Curve for rhGH Concentration

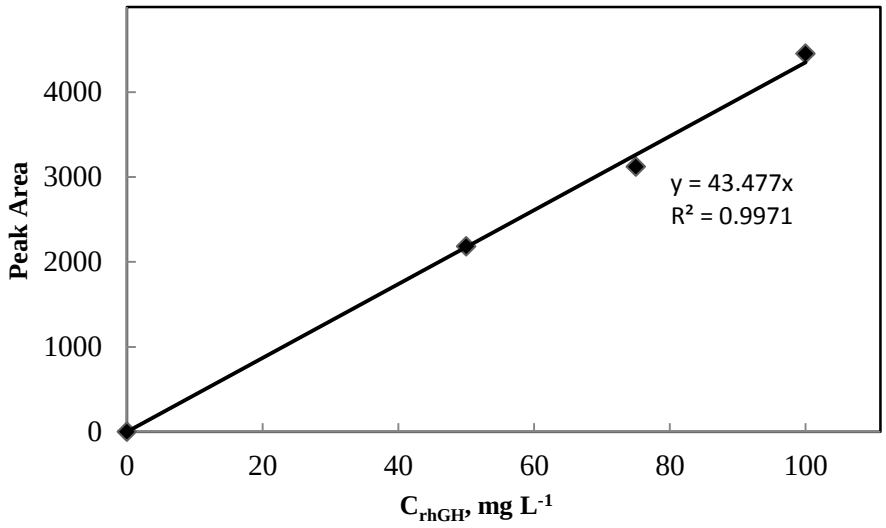

Figure C.3 Calibration curve for rhGH concentration

According to the equation obtained from the plot:

$$C_{rhGH} = \frac{Peak\ Area}{43.477} \qquad \text{(C.3)}$$

Calibration Curves for Organic Acid Concentrations

- **α-Ketoglutaric Acid**

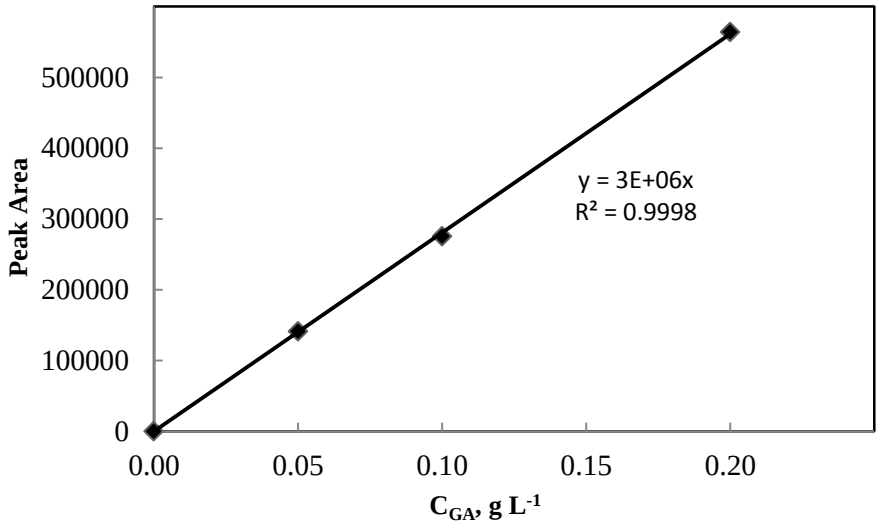

Figure C.4 Standard calibration curve for α-ketoglutaric acid concentration

- **Formic Acid**

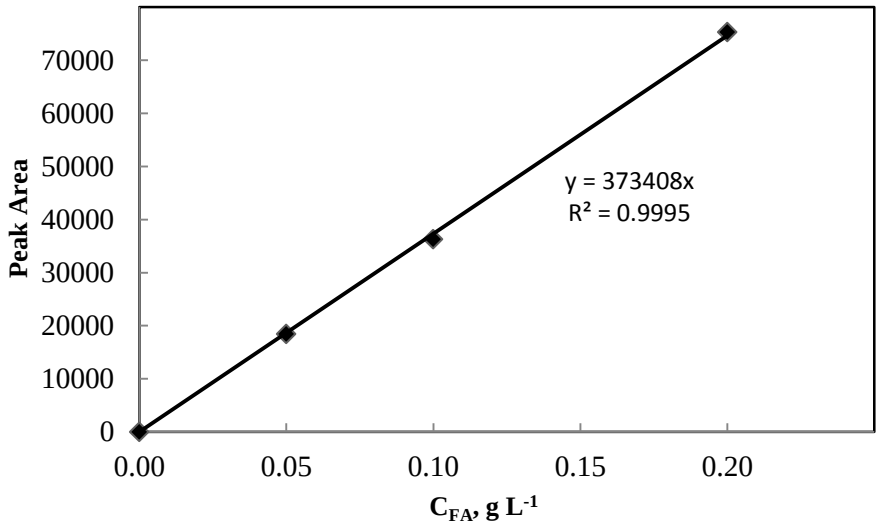

Figure C.5 Standard calibration curve for formic acid concentration

- **Fumaric Acid**

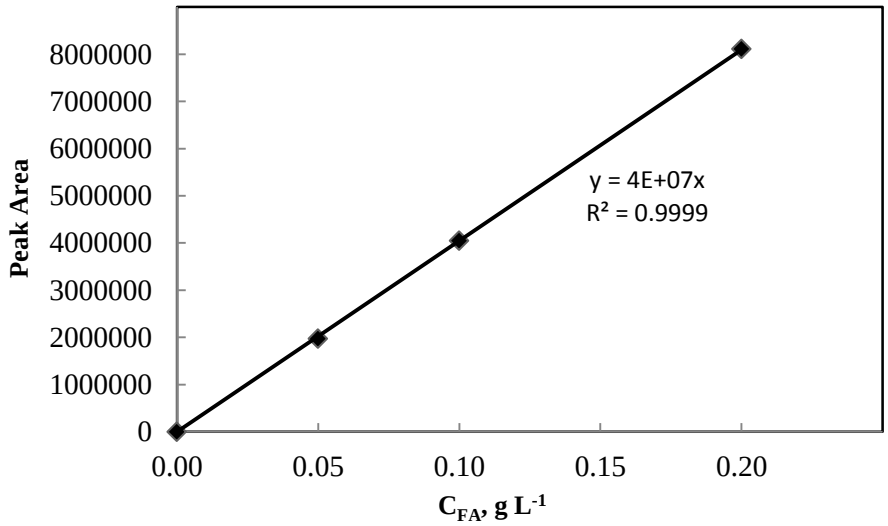

Figure C.6 Standard calibration curve for fumaric acid concentration

- **Gluconic Acid**

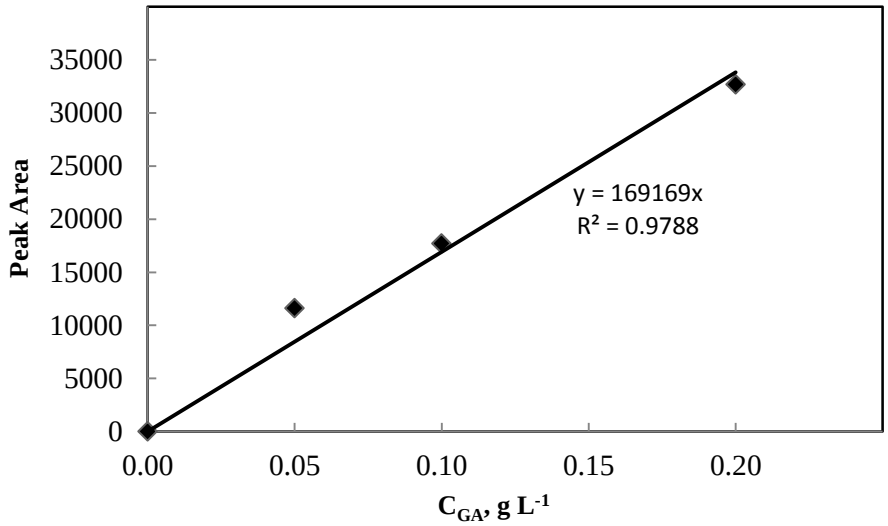

Figure C.7 Standard calibration curve for gluconic acid concentration

- **Lactic Acid**

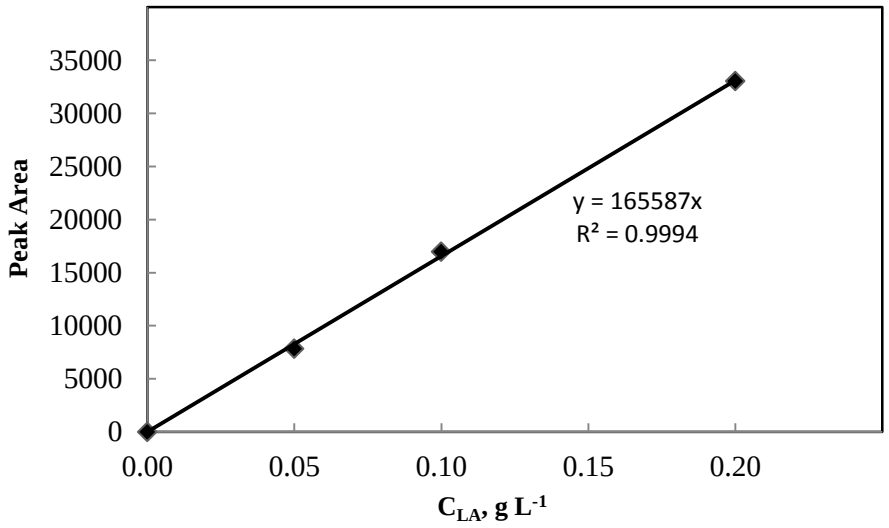

Figure C.8 Standard calibration curve for lactic acid concentration

- **Citric Acid**

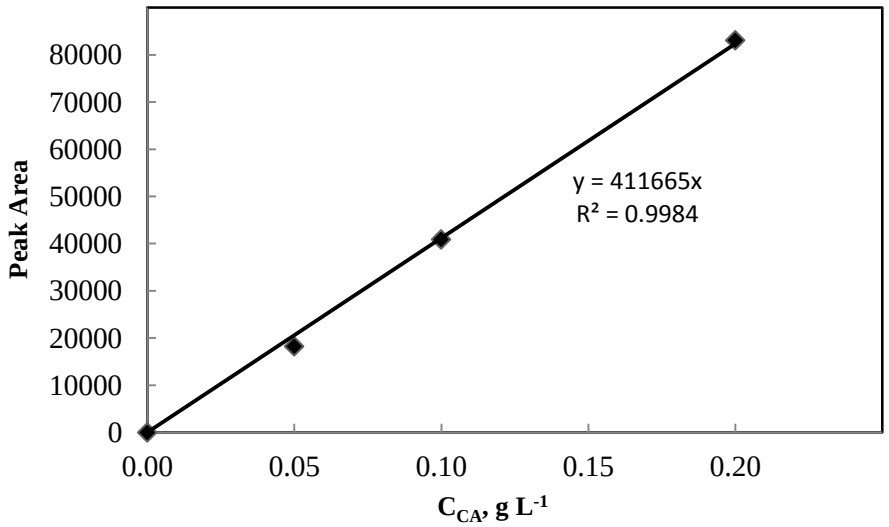

Figure C.9 Standard calibration curve for citric acid concentration

- **Succinic Acid**

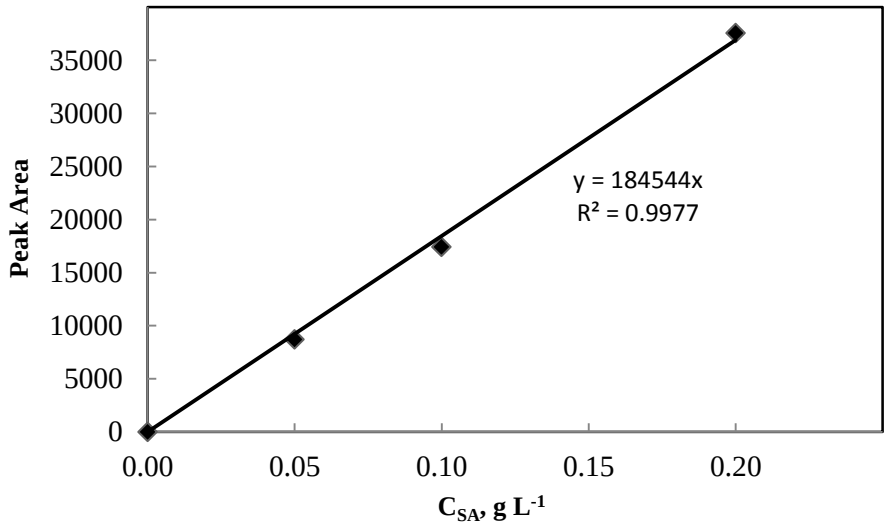

Figure C.10 Standard calibration curve for succinic acid concentration

APPENDIX D

ELECTROPHEROGRAM OF hGH STANDARD

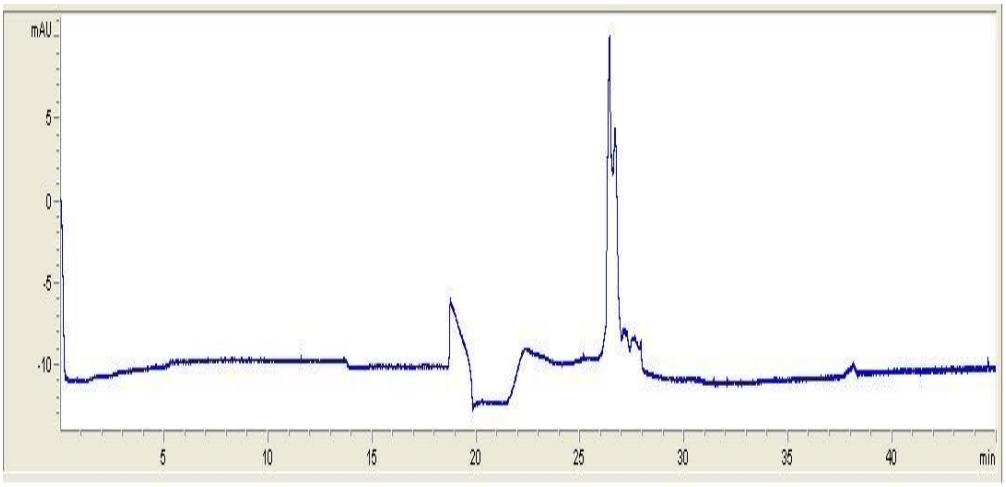

Figure D.1 Electropherogram of 0.1 g L^{-1} standard hGH

APPENDIX E

PRE-DETERMINED FEEDING PROFILE

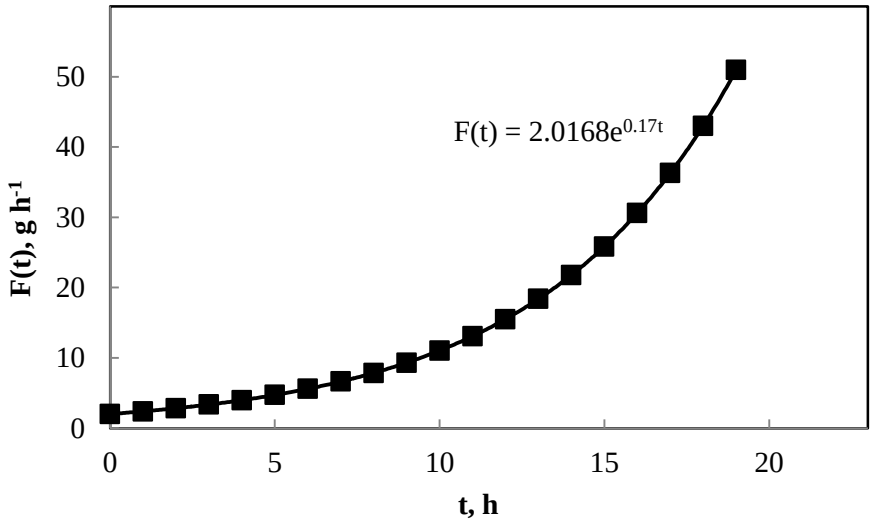

Figure E.1 Pre-determined feeding profile for optimum feeding strategy obtained in BR-7, μ_0=0.17 h^{-1}: t=0 is the time that feeding was started