SEMI-DEFINED MEDIUM BASED FEEDING STRATEGY DEVELOPMENT FOR HUMAN GROWTH HORMONE PRODUCTION BY RECOMBINANT *Bacillus subtilis*

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

SEMI-DEFINED MEDIUM BASED FEEDING STRATEGY DEVELOPMENT FOR HUMAN GROWTH HORMONE PRODUCTION BY RECOMBINANT *Bacillus subtilis*

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In this study, the aim was to develop an efficient semi-defined medium based continuous feeding strategy for recombinant human growth hormone (rhGH) production by *Bacillus subtilis* 1A178 *scoC* knockout (*scoC*$^-$) strain carrying pMK4*::pre(subC)::hGH* plasmid. In this context, firstly laboratory-scale air-filtered shake bioreactor experiments were carried out in order to investigate the effects of peptone as the complex carbon and nitrogen source on the cell growth and rhGH production both in the absence and the presence of $MgSO_4$. The highest cell and rhGH concentrations were achieved as C_X=1.92 g L^{-1} and C_{rhGH}=140 mg L^{-1}, respectively, at t=24 h in the semi-defined medium PM-8, containing 8 g L^{-1} peptone and 0.122 g L^{-1} $MgSO_4$ in addition to the defined production medium consisting of 8 g L^{-1} glucose, 4.71 g L^{-1} $(NH_4)_2HPO_4$, 2 g L^{-1} KH_2PO_4, 0.043 g L^{-1} Na_2HPO_4, 5.63 g L^{-1} NaH_2PO_4, and 5 ml L^{-1} trace salt solution (PTM1). Based on these results, in the second phase of the research, $MgSO_4$-containing production medium was used for pilot-scale bioreactor experiments together with the feed substrate stock solutions containing peptone at two different concentrations. Six continuous feeding strategies were designed for semi-batch rhGH production process based on the pre-determined

specific growth rates, μ_0=0.10, 0.17 and 0.25 h^{-1}, and the transition cultivation times from batch to semi-batch operation, t_T=2 h and t_T=4 h, were investigated. The highest rhGH concentration was obtained as C_{rhGH}=497 mg L^{-1} in the semi-defined medium based feeding strategy conducted at μ_0=0.25 h^{-1} where the transition cultivation time was t_T=4 h by using the feed substrate stock solution comprising of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 100 g L^{-1} peptone and 5 mL L^{-1} PTM1 at t=22 h when the cell concentration was reached to C_X=8.29 g L^{-1}. The overall product and cell yield on total substrate were attained as $\overline{Y}_{P/S}$=7.21 g kg^{-1} and $\overline{Y}_{X/S}$=0.12 g g^{-1}, respectively.

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

ÖZ

REKOMBİNANT *Bacillus subtilis* İLE İNSAN BÜYÜME HORMONU ÜRETİMİ İÇİN YARI-TANIMLI ORTAM TEMELLİ BESLEME STRATEJİSİ GELİŞTİRİLMESİ

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Bu çalışmada, rekombinant insan büyüme hormonunun (rhGH), pMK4*::pre(subC)::hGH* plazmidi taşıyan, *scoC* geni silinmiş *Bacillus subtilis* 1A178 (*scoC*$^-$) suşu ile, yarı-tanımlı üretim ortamında yarı-kesikli işletimle üretim verimini artırmak için sürekli besleme stratejisi geliştirilmesi amaçlanmıştır. Önce, laboratuvar-ölçek mikrobiyolojik-hava filtreli orbital-karıştırmalı kesikli-biyoreaktör deneyleri ile kompleks karbon ve azot kaynağı peptonun, $MgSO_4$ varlığında ve yokluğunda, hücre çoğalması ve rhGH üretimi üzerine etkisi araştırılmış; en yüksek hücre ve rhGH derişimleri, 8 g L^{-1} glukoz, 4.71 g L^{-1} $(NH_4)_2HPO_4$, 2 g L^{-1} KH_2PO_4, 0.043 g L^{-1} Na_2HPO_4, 5.63 g L^{-1} NaH_2PO_4, ve 5 ml L^{-1} eser miktarda tuz çözeltisinden (PTM1) oluşan tanımlı üretim ortamına ek olarak 8 g L^{-1} pepton ve 0.122 g L^{-1} $MgSO_4$ içeren yarı-tanımlı üretim ortamında (PM-8), t=24 st'te, sırasıyla, C_X=1.92 g L^{-1} ve C_{rhGH}=140 mg L^{-1} olarak elde edilmiştir. Bu sonuçlara bağlı olarak, ikinci alt-araştırma programında, pilot-ölçek biyoreaktör deneyleri için $MgSO_4$ içeren üretim ortamı, iki farklı derişimde pepton içeren besleme substrat stok çözeltisi ile birlikte kullanılmıştır. Yarı-kesikli rhGH üretim prosesleri için altı besleme stratejisi tasarlanmış; kesikli işletimden yarı-kesikli işletime geçiş kalma

süresi t_T=2 st ya da t_T=4 st iken μ_0=0.10, 0.17, ve 0.25 st^{-1} olarak öngörülmüş özgül çoğalma hızlarında gerçekleştirilen üstel besleme stratejilerinin rhGH üretimi üzerine etkileri araştırılmıştır. En yüksek rhGH derişimi, 200 g L^{-1} glukoz, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 100 g L^{-1} pepton ve 5 mL L^{-1} PTM1 içeren substrat stok çözeltisinin t≤4 st'ten itibaren μ_0=0.25 st^{-1}'te beslendiği yarı-tanımlı ortam temelli besleme strateji ile t=22 st'te C_{rhGH}=497 mg L^{-1} olarak elde edilmiştir. Aynı kalma süresinde (t=22 st) hücre derişimi C_X=8.29 g L^{-1}; toplam substrat tüketimi üzerinden ürün ve hücre verimi ise, sırasıyla, $\bar{Y}_{P/S}$=7.21 g kg^{-1} ve $\bar{Y}_{X/S}$=0.12 g g^{-1}'dir.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, *Bacillus subtilis*, yarı-tanımlı ortam, 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_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	Recombinant	
t	Process time	h
t_T	Transition cultivation time from batch to semi-batch	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	Product
pro	Protease
S	Substrate
X	Cell

Abbreviations

ATP	Adenosine triphosphates
cDNA	Complementary DNA
CJD	Creutzfeldt-Jakob disease
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
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	Lipopolysaccharide
mRNA	Messenger RNA
PPP	Pentose phosphate pathway
rhGH	Recombinant human growth hormone
RNA	Ribonucleic acid
TCA	Tricarboxylic acid

CHAPTER 1

INTRODUCTION

Biotechnology is defined as the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production or the modification of knowledge, goods and services (http://www.oecd.org). In this sense, biotechnology includes various traditional fermentation processes originated thousands years ago such as bread making, brewing and yoghurt or cheese manufacturing from milk. However, at present, the term, industrial biotechnology, which is also known as modern biotechnology, implies particularly the use of genetically altered organisms for industrial or commercial applications as a result of developments in molecular biology and recombinant DNA (rDNA) technology over the last few decades (Nielsen et al., 2003; Nelson and Cox, 2005; Madigan and Martinko, 2010).

Many recombinant proteins (r-proteins) are commercially produced as well as numerous native ones by using: metabolic engineering and genetic engineering principles and methods, respectively, to design and construct new recombinant systems; protein engineering and biochemical engineering principles to design the production media and then the bioreactor operation conditions and strategies, for production of biomolecules, to use in the areas of medicine, diagnostics, agriculture, food, nutrition, detergents, leather, textiles, pulp and paper, further in novel polymers and plastics (Demain and Vaishnav, 2009). Among these sectors, pharmaceutical industry has a major importance since the market share of recombinant therapeutics gradually increased in recent years. Nearly 30 compounds, which have been introduced into therapy previously, constitute a market size of U.S. $ 50-60 billion while approximately 300 preparations are estimated to be in development in all over

the world (Schmidt, 2004). Thus, gene recombinant pharmaceuticals are continually enhancing their portion and are expected to reach U. S. $ 107 billion-size until 2015 (Walsh, 2010).

In this respect, today one of the most economically robust fields of biotechnology is the production of human proteins, particularly hormones, in large scale. Even though pharmaceutically important mammalian proteins can be extracted from the native tissues in order to use for the treatment of several clinical conditions, separation and purification processes are extremely difficult and costly due to the low amounts of the biomolecules. Alternatively these proteins can be produced in cell culture although this option is also much more expensive and time-consuming than cultivating microorganisms which have gained the ability to produce the desired metabolite in high yield as a result of the genetic engineering procedures applied (Swartz, 1996). Hence, it is concluded that recombinant microbial strains are usually more favorable for highly efficient production of commonly used human proteins such as insulin, human growth hormone (hGH), interferon, erythropoietin (EPO), follicle stimulating hormone (FSH) and blood coagulation factors.

Hormones are biochemical messengers found in all multicellular organisms. These regulatory substances are secreted by special glands and transported to target site of action by the circulatory system in order to coordinate various physiological activities (Nelson and Cox, 2005). Although insulin was the first human protein obtained by using rDNA technology, several unusual complications appeared during this practice since the hormone contains a number of disulphide bonds between two short polypeptides (Madigan and Martinko, 2010).

On the other hand, human growth hormone (hGH), also known as somatotrophin or somatotropin, consists of 191 amino acid residues on a single polypeptide and does not need any post-translational modifications such as glycosylation and disulphide bond formation. This hormone is excreted from the anterior pituitary in order to promote a wide variety of metabolic activities including normal body growth, protein synthesis, immune responses, lactation and ovulation. Owing to the vital effects of hGH in both children and adults, severe diseases such as pituitary dwarfism, acromegaly and gigantism, emerge as a result of either deficiency or overproduction of the hormone. Besides these two indications, a great number of

other cases like Turner's syndrome, Prader-Willi syndrome, idiopathic short stature, chronic renal failure, bone fractures, bleeding ulcers, skin burns, wounds, cardiovascular diseases, AIDS and some cancer types can be partially or completely treated by the exogenous hGH administration (Walsh, 2007). Therefore highly-purified somatotrophin is produced in industrial scale for general medical use.

Before the development of rDNA technology, first GH isolated from the bovine pituitary glands was used on humans for therapeutic purposes. However, this ineffective source was not sufficient to fulfill the demand. Hence, administration of hGH extracted from the pituitaries of deceased human donors became an issue from 1956 until the death of 13 people who had received prion infected hGH therapy. After the cloning of hGH gene by Goeddel et al. (1979), recombinant human growth hormone (rhGH) was produced for the first time by Genentech under the trade name Protropin in 1985. Unfortunately this initial preparation could not promote identical responses to the native hormone since the protein had an extra methionine residue at the N-terminus. Therefore, the terminal methionyl was removed by a different cloning strategy and the novel rhGH composed of 191 amino acid residues was manufactured by Eli Lilly under the trade name Humatrope in 1986. This newly developed rhGH was approved by U.S. Food and Drug Administration (FDA) in 1987. Today all hGH preparations (Table 1.1) used clinically by more than 20000 people are provided from recombinant microbial sources; mainly by *Escherichia coli*, *Bacillus subtilis* or *Pichia pastoris* as the host organism (Çalık et al., 2008).

Among these microorganisms, bacterial hosts are more favorable for the production of heterologous proteins which do not require any post-translational modifications for activation as these expression systems generally provide higher biomass and/or product yields in a shorter process time on inexpensive nutrients (Terpe, 2006).

Thus, the most commonly preferred host for rhGH production has been *E. coli* since the first genome that was completely sequenced belonged to this Gram-negative bacterium (Goeddel et al., 1979; Ikehara et al., 1984 ; Gray et al., 1985; Becker and Hsiung, 1986; Hsiung et al., 1986; Chang et al., 1987; Kato et al., 1987; Hsiung et al., 1989; Jensen and Carlsen, 1990; Uchida et al., 1997; Shin et al., 1998b; Zhang et al., 1998; Bylund et al., 2000; Patra et al., 2000; Castan et al., 2002; Soares

et al., 2003; Tabandeh et al., 2004). In spite of various advantages of using *E. coli* in rhGH production process such as easy accessibility, high plasmid stability and simple promoter control, there are also some disadvantages like the formation of inclusion bodies containing inactive form of the desired metabolite, the lack of an efficient secretion system and the presence of a pyrogenic lipopolysaccharide (LPS) layer in the cell wall (Jana and Deb, 2005).

Table 1.1 hGH preparations provided from recombinant microbial sources and approved by FDA for general medical use (Walsh, 2007)

Trade Name	Manufacturer	Approval Date
Humatrope	Eli Lilly	1987
BioTropin	Biotechnology General	1991
Nutropin	Genentech	1993
Nutropin AQ	Schwartz Pharma AG	1995
Genotropin	Pharmacia & Upjohn	1995
Saizen	Serono Laboratories	1996
Serostim	Serono Laboratories	1996
Norditropin	Novo Nordisk	2000
Somavert	Pharmacia	2003
Omnitrope	Sandoz	2006
Valtropin	Biopartners	2007

Hence, *B. subtilis*, Gram-positive equivalent of *E. coli*, has been also investigated for rhGH production as this genetically well-characterized bacterium has the ability to secrete large amounts of heterologous proteins to the fermentation medium directly as well as other *Bacillus* species (Nakayama et al., 1988; Franchi et

al., 1991; Şentürk, 2006; Yılmaz, 2008; Özdamar et al., 2009; Şahin, 2010; Gökbudak, 2013). Besides the absence of strong promoters and stable expression vectors, the presence of unfolded proteins and the plasmid instability, the main drawback of *B. subtilis* is the degradation of protein products due to proteolytic enzyme secretion (He et al., 1991). This problem can be overcome by using either genetically modified protease deficient strains or protease inhibitor cocktails (Wu et al., 1991; Murashima et al., 2002).

In the study of Özdamar et al. (2009), a novel expression system imitating the synthesis and secretion of serine alkaline protease (SAP) was constructed for extracellular rhGH production. For this purpose, firstly a hybrid gene composed of two DNA fragments; signal peptide coding region (pre-) of *Bacillus licheniformis* SAP gene (*subC*), and complementary DNA (cDNA) coding hGH, was cloned into pMK4 plasmid. After the constructed plasmid, named as pMK4*::pre(subC)*::*hGH*, was expressed under *degQ* promoter in two knockout strains; *B. subtilis (npr$^-$ apr$^-$)* and *B. subtilis (spo$^-$)*, the mature *hGH* sequence was verified together with the proper signal peptide processing by N-terminal sequence and mass spectrometry analyses. Then, Şahin (2010) used several knockout strains (*degQ$^-$, degU$^-$, degS$^-$, sinI$^-$, sinR$^-$, abrB$^-$, spo0A$^-$, aprE$^-$* and *scoC$^-$*) in order to investigate the regulatory gene effects on rhGH production and the maximum rhGH production was achieved by *B. subtilis (scoC$^-$)*. Accordingly, Gökbudak (2013) carried out a series exponentially semi-batch operations at pilot-scale with various pre-determined specific growth rates by using *B. subtilis (scoC$^-$)* in order to develop an efficient feeding strategy for higher rhGH production. However, the use of defined medium for feeding limited both the cell growth and rhGH production.

In this regard, the aim of this study was to develop an efficient semi-defined medium based feeding strategy in order to enhance recombinant human growth hormone (rhGH) production by *Bacillus subtilis* 1A178 (*scoC$^-$*). For this purpose, six continuous feeding strategies were designed: the glucose-based feed medium containing peptone at two different concentrations was exponentially fed to the bioreactor at three different pre-determined specific growth rates in pilot-scale semi-batch bioreactor experiments. Afterwards, the cell growth, substrate consumption and rhGH production profiles were comparatively evaluated as well as the protease

activities, oxygen transfer and fermentation characteristics in order to determine the efficiency of feeding strategies developed.

CHAPTER 2

LITERATURE SURVEY

2.1 Hormones

Hormones, biochemical messengers in multicellular organisms, are produced by special glands and transported by the circulatory system, mainly by the bloodstream, in order to control and regulate target cell activities (Campbell and Reece, 2001).

2.1.1 General Features

Hormones mediate selective communications between certain glands and target tissues or organs. Their activities are usually long-lasting despite the low transportation rates (Nelson and Cox, 2005). Production and secretion of these physiological regulators in consequence of chemical changes in the body such as the change of the calcium, potassium or glucose concentration in the blood are regulated by neurotransmitters, other hormones and negative or positive feedback control (Finch and Rose, 1995). A hormone that cannot be stored when not required, may have a synergistic or an antagonistic effect on another hormone (Schwyzer, 1964). In general, low amounts of hormones are required for various metabolic activities such as growth and development, digestion, respiration, excretion, movement, reproduction, lactation, sensory perception and mood control (Gibson, 2010).

Endocrine glands consisting of hormone producing cells, are primarily responsible for continuous or periodic hormone secretion in animals (Pekin, 1979).

Major endocrine glands, which constitute the endocrine system, are adrenal, pituitary, pineal, thymus, thyroid glands, pancreas and ovaries or testes (Campbell and Reece, 2001). The hypothalamus serves a link between the nervous system and the endocrine system via pituitary gland since this portion of the brain produces and secretes particular neurotransmitters called as releasing hormones or hypothalamic factors for the stimulation or the inhibition of pituitary hormone secretion.

2.1.2 Structural and Functional Classification

Mammalian hormones are generally classified into three groups according to their chemical structures and mechanisms of action (Nelson and Cox, 2005):

i. Amino acid-derived hormones, also known as monoamines, are short chains of aromatic amino acids, such as tyrosine, tryptophan and phenylalanine. These water-soluble biochemicals are formed by the activity of aromatic amino acid decarboxylase enzymes.
ii. Peptide or protein hormones are long chains of amino acids. They are named as protein hormones when the number of amino acid residues in the chain is more than one hundred. On the other hand, protein hormones having carbohydrate side-chains are called glycoprotein hormones. Nonpeptide hormones are also included by this group even though there are no peptide connections in their structures.
iii. Lipid- or phospholipid-derived hormones consist of lipids or phospholipids and the major classes are steroid hormones containing cholesterol and eicosanoids.

Amino acid-derived, peptide and protein hormones display their activities via binding to the surface receptors of the target cell whereas steroids bind to the nuclear receptors within the cell since transportation of the hydrophobic steroids through the cell membrane with the same characteristic is easier than that of these hydrophilic hormones (Campbell and Reece, 2001).

On the other hand, hormones can alternatively be classified into three groups; namely, endocrine hormones, paracrine hormones and autocrine hormones according

to the modes of their transport from the certain glands to the target tissues or organs. Endocrine hormones are released into the bloodstream so they are transferred to the target cells via blood. Paracrine hormones are secreted to the extracellular medium by the hormone-producing cells and are diffused by the contiguous target cells while autocrine hormones bind to the own surface receptors of the producer cells (Campbell and Reece, 2001).

2.2 Human Growth Hormone (hGH)

hGH, also known as somatotrophin or somatotropin, is a non-glycosylated polypeptide hormone produced by the somatotropic cells in the anterior pituitary. hGH is not completely specific to the species so growth hormones (GH) of other primates are also biologically active in humans.

The synthesis and release of hGH are regulated by two peptide hypothalamic factors, growth hormone releasing hormone (GHRH), also known as growth hormone releasing factor (GHRF) or somatorelin, and growth hormone release inhibiting hormone (GHRIH), also known as somatostatin. Additionally, insulin-like growth factor 1 (IGF-1) secreted from the liver, mediates many growth promoting activities of hGH on body growth. Therefore, there is a hormonal axis between hGH, GHRH, GHRIH and IGF-1 as shown in Figure 2.1 (Walsh, 2007).

2.2.1 Chemical and Physical Properties

Mature hGH, containing 191 amino acid residues, displays a molecular mass of 22 kiloDalton (kDa) and this anionic, four helix-bundle protein with the empiric formula of $C_{990}H_{1529}N_{262}O_{300}S_7$ has an isoelectric point (pI) of 5.1 (Goeddel et al., 1979). The nucleotide sequence of the gene encoding hGH is given in Figure 2.2.

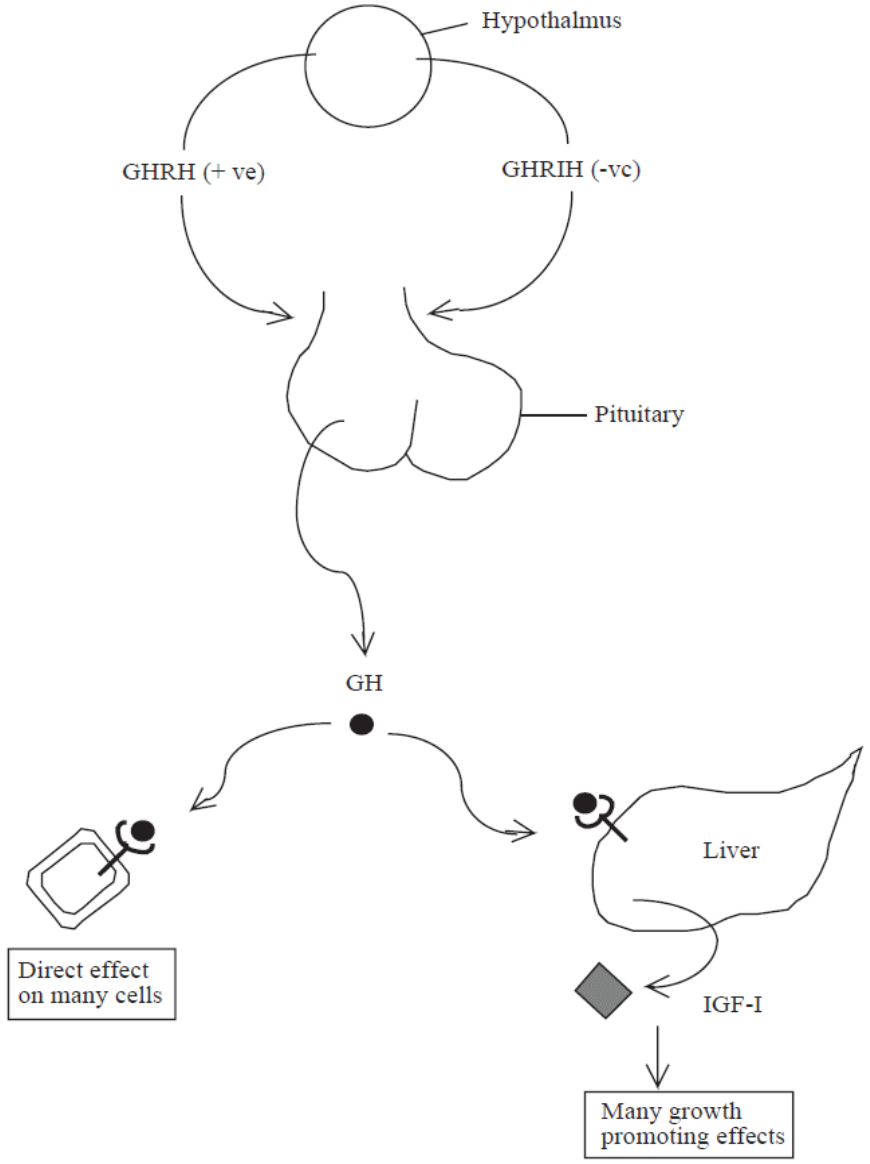

Figure 2.1 Overview of the regulation of hGH secretion from the pituitary gland by the hormonal axis between GH, GHRH, GHRIH and IGF-1 (Walsh, 2007)

55 % of the hormone's secondary structure consists of α-helices which are essential for the specific protein binding to hGH receptors (Farmer et al., 1976; Binkley, 1995). As demonstrated in Figure 2.3, the main four helices are located between the amino acids of 9-34, 72-92, 106-128 and 155-184 while there are also three shorter α-helices in the polypeptide chain. Nearly 20 hydrophobic amino acid residues are found in the hydrophobic central core of hGH (Kopchick, 2003). Furthermore, four cysteine residues which mediate formation of the hormone's

tertiary structure (Figure 2.4) owing to two characteristic intrachain disulfide linkages are on the 53rd, 165th, 182nd and 189th positions of the amino acid sequence (Cys_{53}-Cys_{165}; large loop and Cys_{182}-Cys_{189}; small loop) (Binkley, 1995).

```
ttc cca act ata cca cta tct cgt cta ttc gat aac gct atg ctt cgt gct
cat cgt ctt cat cag ctg gcc ttt gac acc tac cag gag ttt gaa gaa gcc
tat atc cca aag gaa cag aag tat tca ttc ctg cag aac ccc cag acc tcc
ctc tgt ttc tca gag tct att ccg aca ccc tcc aac agg gag gaa aca caa
cag aaa tcc aac cta gag ctg ctc cgc atc tcc ctg ctg ctc atc cag tcg
tgg ctg gag ccc gtg cag ttc ctc agg agt gtc ttc gcc aac agc cta gtg
tac ggc gcc tct gac agc aac gtc tat gac ctc cta aag gac cta gag gaa
ggc atc caa acg ctg atg ggg agg ctg gaa gat ggc agc ccc cgg act ggg
cag atc ttc aag cag acc tac agc aag ttc gac aca aac tca cac aac gat
gac gca cta ctc aag aac tac ggg ctg ctc tac tgc ttc agg aag gac atg
gac aag gtc gag aca ttc ctg cgc atc gtg cag tgc cgc tct gtg gag ggc
agc tgt ggc ttc tag ctg ccc ggg tgg cat ccc tgt gac ccc tcc cca gtg
cct ctc ctg gcc
```

Figure 2.2 Nucleotide sequence of hGH (Binkley, 1995)

In humans the gene encoding hGH is located in the q22-24 region of chromosome 17 (Owerbach et al., 1980). Amongst these five genes, *hGH*-N (normal), also known as GH1, and *hGH*-V (variant), also known as GH2, have the similar structure although there is a difference between the two polypeptide chains in the position of 13 dissimilar amino acid residues (Chen et al., 1989). The pituitary gland is only responsible for the expression of *hGH*-N gene, whereas all others are expressed in placenta (DeNoto et al., 1981).

There are two hGH isoforms considering *hGH*-N gene since messenger RNA (mRNA) of the polypeptide may undergo an alternative splicing which yields a shorter hGH molecule comprising 176 amino acid residues with a molecular mass of 20 kDa (Walsh, 2007). This smaller isoform with unknown function is only 10 % of the total hGH secreted whereas the majority is the larger one (Leung et al., 2002). There are also three hGH isoforms with molecular weights of 22 kDa, 25 kDa and 26 kDa encoded by *hGH*-V gene in humans.

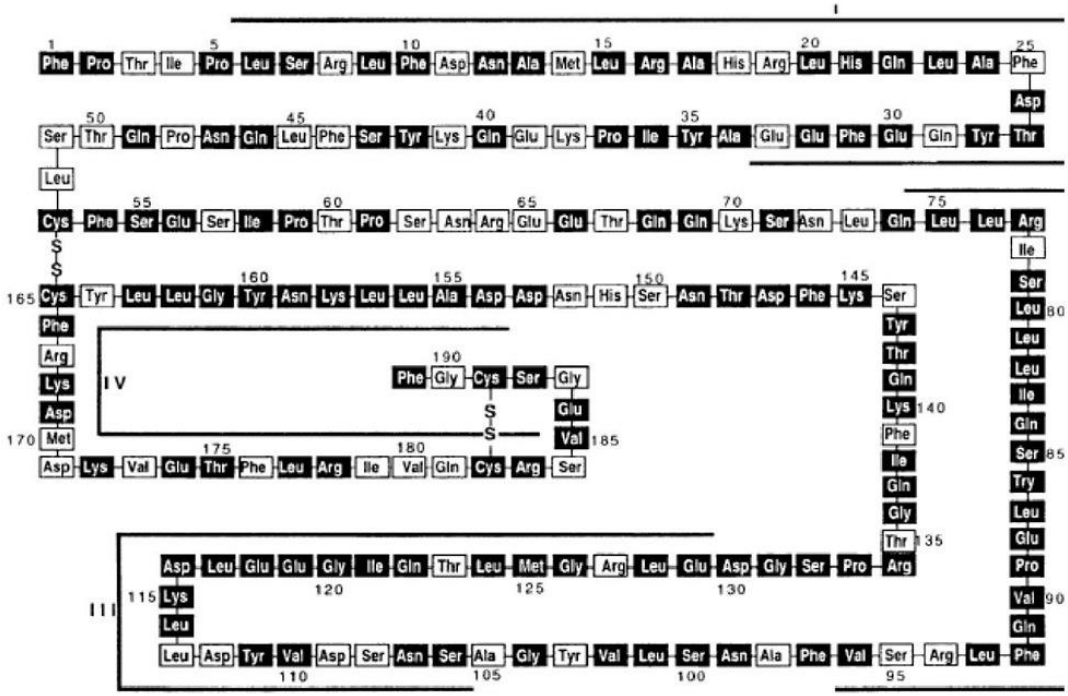

Figure 2.3 Amino acid sequence of hGH: shaded boxes represent conserved amino acids in mammalian GHs and straight lines around the peptide chain represent the amino acid residues locating in helices I, II, III, and IV (Goodman et al., 1996)

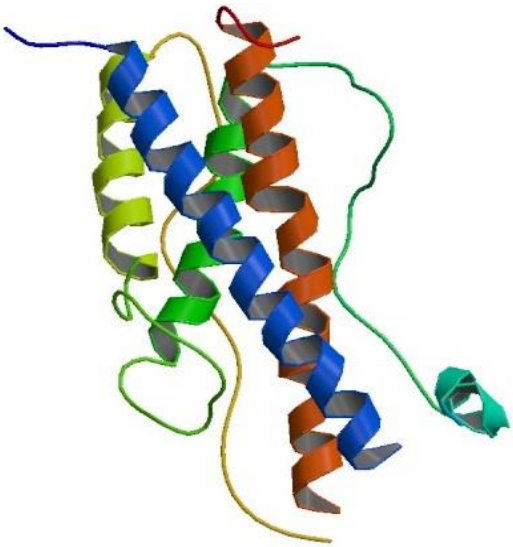

Figure 2.4 Three-dimensional structure of hGH (http://www.rcsb.org/pdb)

2.2.2 Biological Properties and Physiological Effects

hGH mainly displays an anabolic activity on the target cells having hGH specific surface receptors. The hormone directly stimulates the growth of bone, muscle and cartilage cells via the MAPK/ERK pathway. Besides this direct effect, binding of hGH to its hepatic receptors results in the production of IGF-1 via the JAK-STAT signaling pathway (Binder et al., 2007). At the end of childhood, the amounts of hGH and IGF-1 in the body start to decrease whereas both of them gradually increase up to this time. Some of major physiological effects promoted by hGH are summarized below; many of these are direct even though others are mediated by the activity of IGF-1 (King, 2006; Walsh, 2007):

- Stimulation of growth in all internal organs excluding the brain
- Increase of calcium retention and bone mineralization
- Increment of muscle mass via sarcomere hypertrophy
- Stimulation of protein synthesis in many tissues
- Mobilization of depot lipids from adipose tissues: lipolytic effect
- Elevation of blood glucose levels: anti-insulin effect
- Increase of muscle and cardiac glycogen stores
- Reduction of liver glucose uptake
- Stimulation of gluconeogenesis in the liver
- Increment of T4 to T3 deiodination
- Increase of kidney size and renal function
- Contribution for the maintenance and function of the pancreatic islets
- Stimulation of reticulocytosis in the bone marrow
- Promoting the immune system

2.2.3 Therapeutic Uses

In adults and children, both overproduction and deficiency of hGH cause severe diseases. Gigantism is derived from overproduction of the hormone during the years of active body growth whereas acromegaly, a condition characterized by enlarged hands and feet as well as other coarse features, is occurred by hGH overproduction after the primary body growth. On the other hand, a lack of the

hormone secretion during active body growth caused by gene mutation or damage in hypothalamus or pituitary gland results in pituitary dwarfism (Walsh, 2007). Short stature is also observed due to a great number of other conditions, such as Turner's syndrome, Prader-Willi syndrome, idiopathic short stature and chronic renal failure, particularly in children (Bolar et al., 2008; Cázares-Delgadillo et al., 2011).

Although until now treatment of short stature or defective growth caused by GH deficiency, various diseases or medical conditions has been the major application, hGH has a wide variety of therapeutic uses, including induction of lactation, stimulation of ovulation, treatment of obesity, counteracting ageing and body building (Walsh, 2007). Furthermore, owing to its superior healing activity, the hormone is beneficial for the treatment of bleeding ulcers, bone fractures, skin burns, wounds and cardiovascular diseases. Cancer treatment by exogenous hGH administration has also been investigated (Liu et al., 2007).

2.2.4 Production of Recombinant Human Growth Hormone (rhGH)

Owing to the partial species specificity of hGH, GH extracted from the bovine pituitaries was used on humans for therapeutic purposes listed above, prior to the development of rDNA technology. However, subsequently it was understood that this GH was not as effective as the native form of hGH. Hence, the use of somatotrophin isolated from the pituitary glands of deceased human donors was started in 1956 (Li and Papkoff, 1956). Nevertheless, this treatment also came to an instantaneous end in 1985 when at least 13 people, who had received hGH therapy previously, died because of Creutzfeld–Jacob disease (CJD) which was defined as a rare but fatal neurological disorder derived from a prion infection in pituitary extracts (Walsh, 2007).

Therefore, as of the same year, several rhGH preparations were coming on stream by using recombinant DNA technology and other genetic engineering tools. After the gene of hGH was cloned by Goeddel et al. (1979), rhGH was produced for the first time by Genentech under the trade name Protropin in 1985 with the fermentation process of *E. coli*. In this initial recombinant preparation, there was an extra methionine residue at the N-terminal of the protein as a result of the AUG start

codon attached to hGH gene. Even though the first rhGH containing 192 amino acid residues differed from the native hGH, it was approved as a therapeutic protein by FDA.

On the other hand, in following years the terminal methionyl was eliminated by using an alternative cloning strategy so the second rhGH composed of 191 amino acid residues as well as the native form of hGH was produced by Eli Lilly under the trade name Humatrope in 1986 with *E. coli* fermentation again. Besides the completely same structures proved by *in vitro* analysis, clinical experiments conducted in humans confirmed that the recombinant form promoted identical responses to the native hormone. Thus, this newly developed rhGH preparation was also approved for general medical use.

The studies in the literature related to recombinant hGH production by bacterial sources are listed in Table 2.1 according to the host microorganism selected. From the initial studies up to the present, *E. coli* has been preferred more frequently for the production of rhGH since this bacterium is the first organism whose genome is completely identified (Table 2.1). However, this microorganism is not able to secrete the hormone to the fermentation medium directly whereas extracellular production of rhGH decreases the number of separation and purification steps and reduces the cost of downstream processes which are carried out in order to obtain highly purified therapeutic protein. Therefore, *B. subtilis* having well-characterized genetic features also have been used in rhGH production process owing to their efficient extracellular secretion mechanism (Table 2.1).

Table 2.1 The studies in the literature related to hGH production by recombinant bacterial sources

Microorganism	Plasmid(s)	Promoter	Signal Peptide	Medium	Type of hGH Production	Results	Reference
E. coli 294	pHGH107	*lac*	-	-	Intracellular	2.4 µL mL^{-1} methylated hGH was produced.	Goeddel et al., 1979
E. coli HB101	phGH-1 pGH-L8 pGH-L9 pGH-L11 pGH-L13 pCT-1	*trp*	-	-	Intracellular	168.7 µL mL^{-1} methylated hGH was achieved by using pGH-L9 plasmid.	Ikehara et al., 1984
E. coli 294	pPreHGH207-2 pAPH-1	*trp* *pho*A	pre-hGH pho-hGH	Complex	Periplasmic Intracellular	450 ng mL^{-1} A_{550}^{-1} hGH (% 76 secretion) and 230 ng mL^{-1} A_{550}^{-1} hGH (82 % secretion) were obtained by using pPreHGH207-2 and pAPH-1 plasmids, respectively.	Gray et al., 1985
E. coli K-12 RV308	pOmpA-hGH1 pOmpA-hGH2	*lpp-lac*	*omp*A	Complex	Periplasmic Intracellular	10-15 µg A_{600}^{-1} hGH (78 % secretion) was acquired but only 72 % of the secreted r-protein was similar to the native hGH.	Becker and Hsiung 1986; Hsiung et al., 1986
E. coli 294 *E. coli* W3110	phGH4R phGH4L	*pho*A	Signal peptide of enterotoxin II; STII	Complex	Periplasmic Intracellular	15.4 µg ml^{-1} A_{550}^{-1} hGH (90 % secretion) was attained by using *E. coli* W3110 with phGH4L plasmid in low phosphate concentration.	Chang et al., 1987

Table 2.1 The studies in the literature related to hGH production by recombinant bacterial sources (Continued)

Microorganism	Plasmid(s)	Promoter	Signal Peptide	Medium	Type of hGH Production	Results	Reference
E. coli HB101	p8hGH1 pPShGH11	*Ex*-K *Ex*	Signal peptide of penicillinase	Complex	Extracellular Periplasmic Intracellular	55 % and 42 % of 20.5 mg mL^{-1} hGH produced were secreted to the medium and the periplasmic space, respectively when p8hGH1 plasmid carrying *kil* gene and *Ex* promoter was used.	Kato et al., 1987
E.coli K-12 RV308	pOmpA-hGH2 pJL3	*lpp-lac*	*omp*A	Complex	Extracellular	10-15 µg mL^{-1} A_{550}^{-1} hGH was produced while 4.5 µg mL^{-1} A_{550}^{-1} hGH was secreted to the medium in the presence of 20 µM isopropyl-1-thio-β-D-galactopy ranoside (IPTG) as an inducer.	Hsiung et al., 1989
E. coli MC1061	pAT153	Synthetic promoter	-	Complex	Intracellular	2000 mg L^{-1} hGH was achieved by using exponential glucose feeding strategy in semi-batch operation as a result of substrate inhibition prevented.	Jensen and Carlsen, 1990
E. coli 294 *E. coli* W3110	pGHV40 pGHV42 pGHV45 pGHR10 pGHR12 pGHR13	*tac*	ompA *npr* *npr* *npr* *npr* *npr*	Complex	Periplasmic	76.1 mg L^{-1} hGH was acquired by using *E. coli* W3110 together with pGHR10 plasmid. However, the molecular weight of hGH produced was 20 kDa.	Uchida et al., 1997
E. coli BL21	pT2GH	*lac*UV5	-	Complex	Intracellular	9 g L^{-1} hGH was obtained in semi-batch operation by the addition of 0.03 mmol g^{-1} IPTG although 90 % of the r-protein was initially insoluble in the cytoplasm.	Shin et al.,1998b

Table 2.1 The studies in the literature related to hGH production by recombinant bacterial sources (Continued)

Microorganism	Plasmid(s)	Promoter	Signal Peptide	Medium	Type of hGH Production	Results	Reference
E. coli k802	-	-	-	Complex	Intracellular	Not only cell concentration but also hGH production level was doubled and process time was reduced from 16 h to 10 h by the use of glycerol instead of glucose for feeding in semi-batch operation when DO % = 20-25 %.	Zhang et al., 1998
E. coli W3110	pRB322 derivative	-	-	Defined	Intracellular	The highest hGH level was attained as 75.8 au in pilot-scale bioreactor whereas 80 % increase in hGH yield and 30 % decrease in hGH degradation were determined in scale-down reactor.	Bylund et al., 2000
E. coli M15	pQE 60-hGH	T5	-	Complex	Intracellular	1.6 g L^{-1} hGH produced within 10 h in semi-batch operation were soluble in 100 mM Tris buffer containing 2M urea (pH=12.5).	Patra et al., 2000
E. coli W3110	pRB322 derivative	-	-	Defined	Periplasmic	100 au hGH was obtained by the use of reference medium. However, the production level decreased to 37 au hGH in (% 40) oxygen-enriched medium.	Castan et al., 2002

Table 2.1 The studies in the literature related to hGH production by recombinant bacterial sources (Continued)

Microorganism	Plasmid(s)	Promoter	Signal Peptide	Medium	Type of hGH Production	Results	Reference
E. coli RRI *E. coli* W3110 *E. coli* HB2151 *E. coli* RB791 *E. coli* TP2339 *E. coli* BMH7118	pRK248cIts	λP_L	DsbA npr STII Natural hGH signal peptide (reference)	Complex	Periplasmic	The highest hGH concentration was achieved as 420 µg ml^{-1} by *E. coli* RB791 carrying a vector with the DsbA signal sequence. Additionally, the production level was increased 2.5-fold (3.9 µg mL^{-1} A_{600}^{-1} hGH) by the use of this vector instead of the reference vector in *E. coli* W3110.	Soares et al., 2003
E. coli A6-5	pET21-*hgh* pGP1-2	T7 λP_L	-	Defined Complex	Intracellular	Operation time is decreased from 41 h to 32 h by the use of complex medium and hGH concentration increased from 2 g L^{-1} to 2.7 g L^{-1} when glycerol was preferred instead of glucose.	Tabandeh et al., 2004.
B. subtilis MT500	phGH324 phGH526	Promoter of *B. amyloliquefaciens* neutral protease gene	*Pre*-signal of *B. amyloliquefaciens* neutral protease gene	Complex	Extracellular	When phGH526 plasmid containing transcription terminator of *B. amyloliquefaciens* neutral protease gene was used, 40 mg L^{-1} hGH (10-fold lower than that of phGH324) was secreted to the fermentation medium in high cell density culture of *B. subtilis* MT500.	Nakayama et al., 1988
B. subtilis SMS118	pSM214 pSM215 pSM274 pSM250 pSM291	-	pSM212	Complex	Intracellular	The highest hGH solubilities were acquired as 70 % and 80 % by the use of pSM291 and pSM274 plasmids, respectively.	Franchi et al., 1991

Table 2.1 The studies in the literature related to hGH production by recombinant bacterial sources (Continued)

Microorganism	Plasmid(s)	Promoter	Signal Peptide	Medium	Type of hGH Production	Results	Reference
B. subtilis 1A751 (*npr⁻ apr⁻*) *B. subtilis* 1A197 (*spo⁻*)	pMK4:: *pre(subC)::hGH*	*degQ*	*Pre*-signal of serine alkaline protease (SAP) gene (*subC*)	Defined	Extracellular	70 mg L^{-1} hGH was produced within 32 h by *B. subtilis* 1A751 (*npr⁻ apr⁻*) carrying pMK4::*pre(subC)::hGH* plasmid in the fermentation process conducted at Q_O/V_R=0.5 vvm and N=800 rpm.	Şentürk, 2006; Özdamar et al., 2009
B. subtilis WB700 (seven protease gene deleted) *B. subtilis* 1A751 (*npr⁻ apr⁻*)	pRB373:: *pre(subC)::hGH* pMK4:: *pre(subC)::hGH*	*degQ*	*Pre*-signal of serine alkaline protease (SAP) gene (*subC*)	Defined	Extracellular	46 mg L^{-1} hGH was achieved at t=32 h by the use of *B. subtilis* 1A751 (*npr⁻ apr⁻*) carrying pMK4::*pre(subC)::hGH* plasmid when the initial pH was pH_0=7.5 (uncontrolled).	Yılmaz, 2008
B. subtilis 1A751 (*npr⁻ apr⁻*) *B. subtilis* 1A53 (*degQ⁻*) *B. subtilis* MT42 (*degS⁻*) *B. subtilis* 1A165 (*degU⁻*) *B. subtilis* 1A68 (*abrB⁻*) *B. subtilis* 1A178 (*scoC⁻*) *B. subtilis* 1S97 (*sinI⁻*) *B. subtilis* 1S97 (*sinR⁻*) *B. subtilis* 1A197 (*spo⁻*)	pMK4:: *pre(subC)::hGH*	*degQ*	*Pre*-signal of serine alkaline protease (SAP) gene (*subC*)	Defined	Extracellular	126 mg L^{-1} hGH was attained at t=24 h by the use of *scoC* gene knockout strain, *B. subtilis* 1A178 (*scoC⁻*) when pH was controlled at pH_C=7.5.	Şahin, 2010
B. subtilis 1A178 (*scoC⁻*)	pMK4:: *pre(subC)::hGH*	*degQ*	*Pre*-signal of serine alkaline protease (SAP) gene (*subC*)	Complex Defined	Extracellular	366 mg L^{-1} hGH was achieved at t=28 h by exponential feeding of semi-defined substrate stock solution consisting of glucose, $(NH_4)_2HPO_4$, peptone and PTM1 at μ_0=0.17 h^{-1} in semi-batch operation.	Gökbudak, 2013

2.3 Host Organism

In recent years, the number of recombinant protein products used for therapeutic applications has increased drastically. Therefore, the rapid and economical production of highly purified and well-characterized recombinant proteins in large scale has a major importance in pharmaceutical industry (Demain and Vaishnav, 2009). In order to obtain such products, the first step is the selection of an appropriate host organism being in generally recognized as safe (GRAS) status with well-known genetic characteristics. Additionally, this potential host should be able to produce the desired metabolite efficiently and secrete a large portion of the product to the fermentation medium directly while the formation of by-products during the bioprocess is negligible (Rumbold, 2000).

In this respect, bacterial expression systems are generally favorable for heterologous protein production since the majority of bacteria are able to grow rapidly on inexpensive nutrients and high cell densities and/or production levels can be obtained in a shorter process time by using these easily cultivated microorganisms. Moreover, a great number of cloning vectors and mutant host strains are available today since there is a lot of information about their genetic characteristics (Schmidt, 2004).

On the other hand, bacteria are not able to make post-translational modifications such as glycosylation and disulphide bond formation. Therefore, when any post-translational modification is necessary for the activity of the heterologous protein, alternative host organisms such as baculovirus systems, yeasts, filamentous fungi, insects and mammalian cells should be preferred instead of bacterial expression systems (Demain and Vaishnav, 2009). Hence, today larger therapeutic proteins are generally produced in mammalian cell cultures, particularly with Chinese hamster ovary cells whereas the expression of smaller pharmaceuticals is carried out in bacterial cell cultures, especially with *E. coli* and *B. subtilis* cells (Jana and Deb, 2005).

E. coli is the most commonly used bacterial expression system for recombinant protein production in pharmaceutical industry since the cheapest,

simplest and fastest expression can be achieved with this microorganism (Andersen and Krummen, 2002). In addition, there are a large number of studies in the literature about the fermentation characteristics of this easily accessible bacterium while its genetics are much more understood than those of any other microorganism (Swartz, 2001). *E. coli* genome can be rapidly and precisely altered, promoter control is not so difficult, and plasmid copy number can be easily changed. At the same time heterologous proteins are accumulated by this organism up to 80 % of its dry weight (Demain and Vaishnav, 2009).

However, besides the general disadvantages of bacterial expression systems, there are also some other drawbacks of using *E. coli* in heterologous protein production such as the lack of a secretion system which provides the efficient release of the desired product into the fermentation medium (Jana and Deb, 2005). In addition to this, the number of separation and/or purification operations conducted after the production process should be increased in order to obtain endotoxin-free therapeutic proteins since the lipopolysaccharide (LPS) layer in the cell wall of the Gram-negative bacterium is pyrogenic for humans and other mammalians (Terpe, 2006). Moreover, proteins which are produced by *E. coli* are usually inactive and insoluble due to the formation of inclusion bodies so they require refolding at the end of the bioprocess. Unfortunately if the desired protein has many disulphide bonds between the peptide chains, refolding is not always possible (Fischer et al., 1993). Finally, acetate formed as a result of high cell densities displays a toxic effect on cells although this problem can be overcome by controlling the oxygen level in the system (Demain and Vaishnav, 2009).

hGH does not need any post-translational modifications so prokaryotic expression systems, mainly *E. coli* and *B. subtilis*, have been frequently used for its recombinant production. Even though *E. coli* is more frequently chosen due to the advantages mentioned above, there are also some disadvantages of using this bacterium in this bioprocess. The major problem is the presence of an extra methionine residue at the N-terminal of the protein in consequence of the AUG start codon inserted at the beginning of the gene. This terminal methionine may be eliminated either by a different cloning strategy or by enzymatic cleavage (Walsh, 2007). The other drawback is the intracellular production of hGH by *E. coli*. Hence,

Bacillus species, particularly *B. subtilis*, have been preferred for highly efficient extracellular hGH production (Nakayama et al., 1988; Franchi et al., 1991; Kajino et al., 1997; Şentürk, 2006; Yılmaz, 2008; Özdamar et al., 2009; Şahin, 2010; Gökbudak, 2013).

2.3.1 The Genus *Bacillus*

Bacillus is a genus of rod-shaped, endospore-forming, obligately aerobic or facultatively anaerobic, Gram-positive bacteria (Claus and Berkelery, 1986; Turnbull, 1996). These unicellular prokaryotes reproduce by binary fission. The Guanine+Cytosine (G+C) content of bacterial DNA represents 32-62 % genetic heterogeneity so this genus includes a great diversity of strains as indicated in Table 2.2 (Holt, 1984; Madigan and Martinko, 2010).

While some strains are motile owing to the peritrichous flagella, others cannot move. In some species, cultures are Gram-positive only in the early stages of growth so they may turn Gram-negative within the time (Turnbull, 1996). In order to obtain useful energy for cell metabolism they assimilate a wide variety of nutrients by fermentation, aerobic respiration or both fermentation and aerobic respiration. Whereas a large proportion of the genus is mesophilic, there are also some extremophilic *Bacillus* species including thermophiles, psycrophiles, acidophiles, alkalophiles and halophiles (Madigan and Martinko, 2010). The majority of the genus is characterized as GRAS by FDA due to absence of endotoxins from the cell wall (Arbige et al., 1993). On the other hand, there are also a few pathogenic bacilli which produce disease causing toxins (Çalık, 1998a).

As a result of this diversity, the genus *Bacillus* has been extensively used in food and pharmaceutical industries for many years, mainly in order to produce enzymes, food additives, vitamins, antibiotics and insecticides (Arbige et al., 1993).

Table 2.2 General properties of *Bacillus* species (Madigan and Martinko, 2010)

Characteristics	**Genus/Species**
I. Oval or cylindrical endospores, facultative aerobes, casein and starch hydrolyzed	
▪ Sporangia not swollen, endospore wall thin	
Thermophiles and acidophiles	*Bacillus coagulans*
	Alicyclobacillus acidocaldarius
Mesophiles	*Bacillus licheniformis*
	Bacillus cereus
	Bacillus anthracis
	Bacillus megaterium
	Bacillus subtilis
Insect pathogen	*Bacillus thuringiensis*
▪ Sporangia distinctly swollen, spore wall thick	
Thermophiles	*Geobacillus stearothermophilus*
Mesophiles	*Paenibacillus polymyxa*
	Bacillus macerans
	Bacillus circulans
Insect pathogens	*Paenibacillus larvae*
	Paenibacillus popilliae
II. Spherical endospores, obligate aerobes, casein and starch not hydrolyzed	
▪ Sporangia swollen	*Bacillus sphaericus*
▪ Sporangia not swollen	*Sporosarcina pasteurii*

The most remarkable feature of the bacteria that allows its widespread use is the ability to excrete high level of functional proteins to the fermentation medium immediately (Simonen and Palva, 1993). The Gram-positive bacteria are already more advantageous in this aspect due to the lack of an outer membrane outside the cytoplasmic membrane and this facilitates downstream processes.

Besides highly efficient homologous protein production, *Bacillus* species are also potential hosts for production of heterologous nonglycosylated proteins since genetic manipulations can be readily applied on these genetically well-characterized and metabolically robust microorganisms. There is no need to design alternative signal peptide coding regions because their own signal sequences and promoters can be used for exoprotein production. Moreover, proteases secreted by bacilli cannot directly degrade extracellular enzymes produced with this technique (Demain and Vaishnav, 2009).

Bacillus species are able to survive under extreme environmental conditions, sometimes even for centuries since their endospores including dipicolinic acid, are resistant to heat, cold, radiation, desiccation and disinfectants. Priest (1993) reported that when endospore-forming bacteria such as bacilli are used in bioprocesses, the highest production level of extracellular enzymes and proteins is obtained before sporulation. Namely in the late logarithmic phase or in the early stationary phase, derepression of many catabolite-repressed genes, due to the consumption of carbon source, is the key factor for protein production (Priest, 1977).

2.3.1.1 *Bacillus subtilis*

A member of the genus *Bacillus*, *B. subtilis*, is a rod-shaped, endospore-forming and catalase-positive microorganism as shown in Figure 2.5 (Madigan and Martinko, 2010). This Gram-positive bacterium is a facultative anaerobe in contrast to previously known (Nakano and Zuber, 1998). Although *B. subtilis* is generally found in soil, this species can also live in the intestine as a normal gut commensal (Hong et al., 2009). Having a flagellar structure provides the ability to move quickly in liquid media. In the case of non-pathogenic and non-toxicogenic strains, the prokaryotic microorganism and many substances derived from this microbial strain

including carbohydrases and proteolytic enzymes are regarded as safe by FDA (Madigan and Martinko, 2010).

In laboratory studies, *B. subtilis* is frequently used as the Gram-positive equivalent of the extensively studied Gram-negative bacterium, *E. coli* since it is considered as a model organism in order to study biochemistry, genetics and physiology of Gram-positive bacteria. Research on its sporulation process, that is a simplified example of cellular differentiation, are principally prominent in this respect (Piggot and Hilbert, 2004). Furthermore, due to its effective secretion system, *B. subtilis* is commercially used for extracellular protein production in large-scale (Simonen and Palva, 1993). A variety of industrial enzymes and pharmaceutical proteins obtained in this way may be heterologous because this species is highly amenable to genetic manipulations (Zweers et al., 2008).

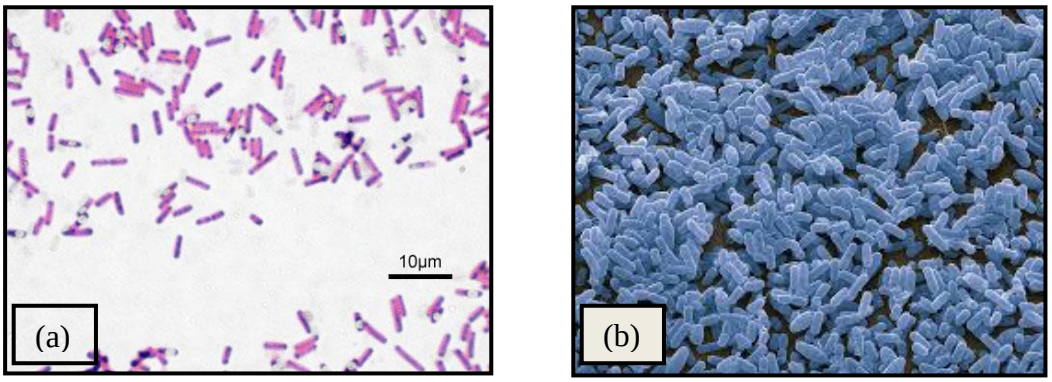

Figure 2.5 (a) Gram-stained sporulating *B. subtilis* (b) Scanning electron microscopy (SEM) image of *B. subtilis*

Nevertheless, there are also some drawbacks of using *B. subtilis* for recombinant protein production processes such as absence of strong promoters and stable expression vectors, instability of plasmids, existence of unfolded proteins and secretion of proteolytic enzymes (Westers et al., 2004). Fortunately it is possible to solve these problems by using various strategies developed; foreign strong and controllable promoters can be constructed by using genetic engineering approaches (Morimoto et al., 2008). Integration of replicative plasmid into host genomic DNA provides the stable plasmid (Heap et al., 2012). Use of chaperons and/or catalysts in production medium increases the possibility of correct protein folding. Genetically

manipulated protease deficient strains or protease inhibitor cocktails can be used in order to prevent proteolytic enzyme secretion for more efficient production processes (Ye et al., 1999; Zweers et al., 2008). On the other hand, the production level of the recombinant protein also depends on metabolic network of microorganism. For instance, in the study of Yılmaz (2008) *B. subtilis* 1A751 (*apr* encoding alkaline proteases, and *npr* encoding neutral proteases, genes deficient) produced 2-fold higher hGH than *B. subtilis* WB700 (seven protease genes deficient). Therefore, it was concluded that the knockout of these seven genes had an inhibitory effect on hGH production process in the metabolic network of *B. subtilis* WB700.

As a result of its advantages, Nakayama et al. (1988), Franchi et al. (1991), at Ankara University: Şentürk (2006), Yılmaz (2008), and Özdamar et al. (2009); and at Middle East Technical University: Şahin (2010) and recently Gökbudak (2013) preferred *B. subtilis* for the production of rhGH, since its genome has been sequenced in 1997, and then re-sequenced in 2009, and lastly in 2013 (Belda et al., 2013); whereupon, the information about its promoters, plasmids, and signal sequences, and annotations of the gene functions have been in the focus of interest of research groups and presently sufficient enough to design new recombinant systems for new recombinant protein productions, and to analyze the intracellular reaction pathways.

2.3.1.2 Secretory System of *Bacillus* Species

Proteins are transferred by the producer cell from their sites of synthesis to their sites of function along the cytoplasm or cellular membranes, in other words through the export or secretion pathways and this process is called protein export. Exported proteins which are transported to the destinations outside of the cell are named secretory proteins.

A secreted protein is initially synthesized as a precursor having an amino-terminal extension, namely a signal peptide, which is required for targeting the secretory protein to the secretion pathway. In the second step, soluble cytoplasmic targeting factors specifically bind to the preprotein in order to transfer it to the translocation machinery in the cell membrane or the signal peptide of the preprotein

directly binds to the cytoplasmic membrane. In eukaryotic cells, the endoplasmic reticulum (ER) membrane is functionally equivalent to the bacterial cell membrane (Simonen and Palva, 1993).

In the translocation machinery, in order to fulfill the energy requirement of the export, nucleotide triphosphates are hydrolyzed by a translocation motor which binds to the polypeptide after it is transported across a proteinacious membrane channel by a membrane protein complex, called as translocase. Then, the signal peptide is removed by the activity of a specific proteolytic enzyme, named as signal peptide peptidase, and the mature protein obtained remains membrane associated until the folding process (Tjalsma et al., 2004; Fu et al., 2007).

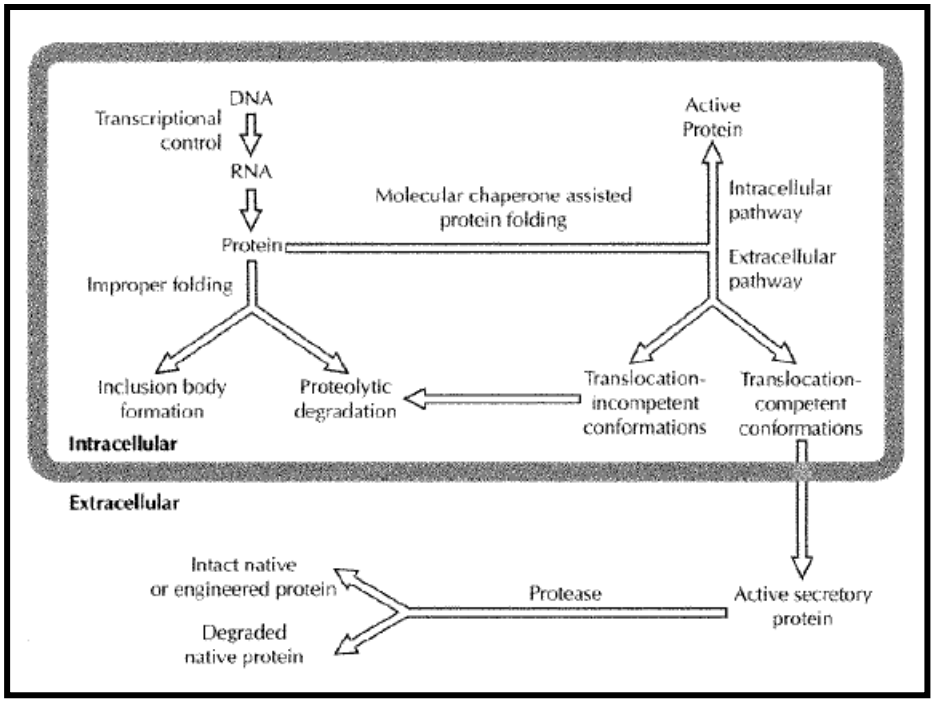

Figure 2.6 Cellular processes affecting the final production levels of heterologous proteins produced by *Bacillus* species (Wong, 1995)

Specific soluble proteins in the cytoplasm known as chaperones play a pivotal role in promoting the proper protein folding. The improperly folded proteins may form inclusion bodies if they are not degraded by proteases. Hence, molecular chaperons also minimize the formation of inclusion bodies (Wong, 1995). In addition to these, some chaperons are essential for the accomplished translocation process

since the partially unfolded and translocation-competent conformation of the preproteins is maintained by these types of proteins. If folding is not avoided before the translocation, the tertiary structure of the protein inhibits the export (Simonen and Palva, 1993).

The first studied bacterial secretion system belongs to *Bacillus* species since they are able to excrete several proteins to the fermentation medium efficiently (Harwood et al., 2008). However, the secretion of heterologous proteins by these types of microorganisms may be inefficient in spite of the high capacity in homologous protein secretion (Bolhuis et al., 1999). In order to improve this, all key parameters in gene expression, proteins synthesis, folding, secretion and stability as outlined in Figure 2.6 should be well-characterized because they can directly affect the final production level of heterologous protein (Wong, 1995).

Since *Bacillus* species excrete proteases into the fermentation medium at the end of the exponential growth phase, degradation of the extracellular protein product is another major problem that decreases the final production level after the secretion process (Simonen and Palva, 1993). Some heterologous proteins are naturally resistant to these proteases so others can also be made more resistant via protein engineering (Wong, 1995). Additionally, knockout strains developed by the deletion of genes related to protease activity can be preferred or protease inhibitors may be used in order to overcome this drawback (Şentürk, 2006; Yılmaz, 2008; Özdamar et al., 2009; Şahin, 2010; Gökbudak 2013). However, in large scale, there is an additional cost as well as the toxicity due to the use of these inhibitors and sometimes the growth rate of the microorganism or the formation rate of the product reduces as a result of the gene deletion.

In contrast to the negative effect of proteases, the most studied *Bacillus* secretory proteins are serine alkaline protease (SAP or subtilisin) and neutral protease (metalloprotease) because of their high secretion levels. Inside the cell, subtilisin is composed of three parts; a typical gram-positive signal peptide, a highly charged pro-peptide and the mature substrate protein (SAP) as demonstrated in Figure 2.7 whereas just the two parts remain outside of the cell as a result of the separation of the signal peptide during the translocation or shortly after the

translocation as shown in Figure 2.8 (Simonen and Palva, 1993; Harwood et al., 2008).

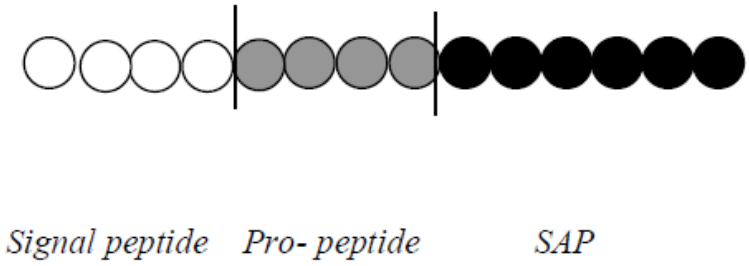

Figure 2.7 Three parts of subtilisin inside the cell

Signal peptide is composed of three distinct regions; namely N terminal region (N), hydrophobic core (H) and C terminal region (C), respectively. The N terminal region, containing at least one basic, in other words positively charged amino acid residue such as arginine or lysine, interacts with the translocation machinery and the negatively charged phospholipids in the membrane. On the other hand, the H region includes hydrophobic, in other words neutral amino acid residues such as leucine, isoleucine, alanine and phenylalanine while in the middle of this domain there are some helix-breaking amino acids such as glycine and proline. These residues facilitate the insertion of the peptide into the membrane in the translocation process via the formation of hairpin-like structure. However, locating glycine and proline at the end of the hydrophobic region is prominent for the cleavage process. Finally the C terminal region formed by polar amino acid residues contains the cleavage site for the specific signal peptide peptidase (Tjalsma et al., 2004).

Pro-peptides, consisting of 70 to 200 amino acid residues, are located between the signal peptide and the primary protein fragment. These peptides are not directly required for the translocation process whereas they are necessary for the post-translocational folding since the stable and functional forms of the secreted proteins are just provided by their activity. Even though pro-peptides are defined as intramolecular chaperons, their purified forms can also be used to catalyze the folding of their mature proteins *in vitro* (Harwood et al., 2008).

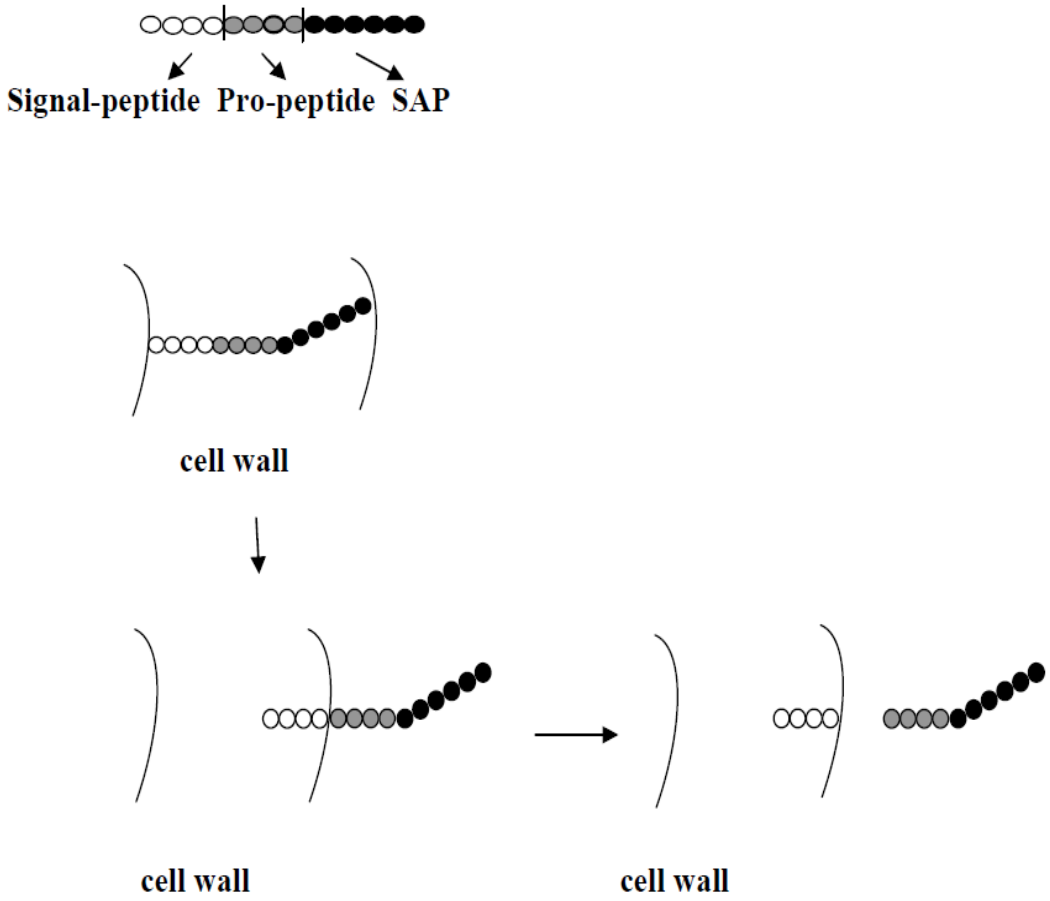

Figure 2.8 Steps in the transfer of subtilisin to the outside of the cell

In the case of subtilisin, since the propeptide remains mature protein associated after folding, the enzymatic activity of subtilisin is limited. Therefore, the maximum proteolytic activity is obtained as a result of the self cleavage and degradation of the propeptide (Yabuta et al., 2001). Besides, enabling proper protein folding, another major activity of the SAP pro-peptide is making up the competitive inhibition on the active enzyme.

2.3.1.3 Regulatory Gene Network in *aprE* Gene Expression

Microorganisms have interrelated regulation networks which provide simultaneous control on most of their metabolic activities in response to the current growth phase and environmental conditions. For instance, in the case of *B. subtilis*,

growth takes place in the presence of glucose while this carbon source represses the sporulation process.

Signal sequence of *subC* gene on the recombinant plasmid, pMK4::*pre(subC)*::*hGH* stimulates the extracellular production of SAP, subtilisin, in *B. subtilis* where subtilisin is encoded by the gene of *aprE*. In order to control the expression of *aprE* gene, there is a complex regulatory network containing numerous inducers and repressors; AbrB, DegQ, DegS, DegU, ScoC, SinI, SinR and Spo0A as represented in Figure 2.9 (Henner et al., 1988; Gaur et al., 1991; Kallio et al., 1991; Bai et al., 1992; Strauch, 1995; Olmos et al., 1997; Hata et al., 2001; Ogura et al., 2004). These proteins, produced by the expression of *abrB, degQ, degS, degU, scoC, sinI, sinR and spo0A* genes, respectively, are principally essential for the late-growth activities (Kunst et al., 1975; Ferrari et al., 1988).

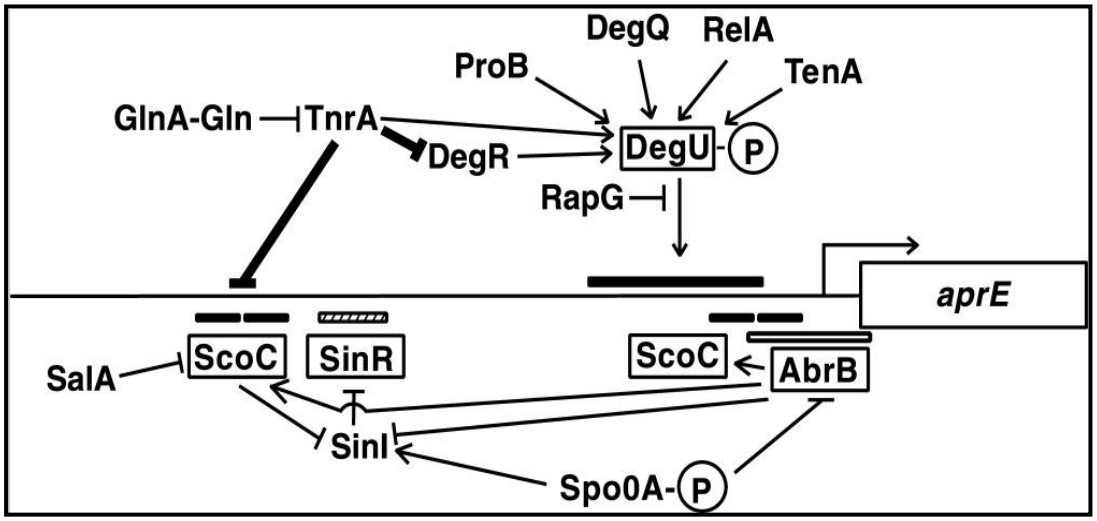

Figure 2.9 Regulatory gene network in *aprE* gene encoding SAP (Abe et al., 2009)

Extracellular subtilisin synthesis, namely the expression of *aprE*, is regulated mainly by AbrB, DegU, ScoC and SinR which are enclosed by rectangles in Figure 2.9 when these regulators bind upstream regions of the gene (Ogura et al., 2003; Abe et al., 2009).

The expression of *aprE* is enhanced by the overproduction of DegQ or DegR. As a result of *degQ* overexpression, there is a phosphate transfer from DegS to DegU and in this case the transcription of *aprE* gene is also stimulated by the activated

DegU since this regulatory protein has a major effect on the extracellular SAP production (Kunst et al., 1994; Ogura et al., 1994; Ogura et al., 2003). Moreover, DegS, DegU and ComA positively affect the expression of *deqQ*, while the gene expression is repressed in the presence of glucose (Msadek et al., 1990). Conversely, *deqQ* is activated by the absence of carbon, nitrogen or phosphate source in the growth medium.

According to the literature, the protein products of *tenA*, *tenI* and *senS* genes are also effective on the expression of *aprE* gene. TenA increases the extracellular enzyme production significantly whereas TenI generally displays an inhibitory effect on *tenA* gene. On the other hand, *senS* actually belongs to *Bacillus nato*. However, if the recombinant plasmid containing *senS* gene is transferred to *B. subtilis*, the expression of *aprE* gene is also increased by the activity of SenS (Strauch, 1995). Conversely, the transcription of *aprE, sacB* or *nprE* genes in *B. subtilis* is inhibited by the overexpression of *pai* gene. The protein product of *nprE* gene, NprE, is particularly responsible for neutral protease production (Bolhuis et al., 1999).

Besides Pai, another regulatory protein, ScoC also inhibits the subtilisin production and the sporulation by controlling the expression of *aprE* as well as the synthesis of SinI and SinR (Koide et al., 1999). However, the lack of *scoC* gene does not affect the temporary expression of the target genes to be repressed. On the promoter of *aprE* gene, there are four ScoC binding sides and the proteins, SinI and SinR, have very similar structures. When ScoC is activated via phosphorylation, the transcription of *sinR* is induced by the inactivation of *sinI*. In addition to ScoC, the expression of *sinI* is also regulated by AbrB and Spo0A (Gaur et al., 1988; Gaur et al., 1991; Bai et al., 1992).

The product of *spo0A* gene which is required for sporulation is able to affect other regulatory proteins positively or negatively. Spo0A is phosphorylated in the stationary phase and the expression of *abrB* is repressed by the activated regulator, Spo0P. In this wise, *aprE* gene is activated while *scoC* gene is inactivated (Ferrari et al., 1986; Perego and Hoch, 1988). On the other hand, nonphosphorylated form of the protein, Spo0A, induces *sinI* gene and the production of the sporulation inhibitory protein, SinR, is repressed when the activated SinI binds to the gene of *sinR* (Gaur et al., 1988; Gaur et al., 1991; Bai et al., 1992; Smith, 1993).

Derepression of *spo0E*, *spo0H* and *spoVG* by Spo0A initiate the sporulation process in the late-growth phase while the expressions of these three genes as well as *aprE* transcription are repressed by the product of *abrB* gene in the transition phase. AbrB also suppresses the gene encoding itself, *abrB* (Strauch et al., 1989). The expression of *scoC* is induced by this regulatory protein while the overexpression of *abrB* activates *hpr* gene.

Since the genes of *catA*, *scoC* and *hpr* are located in the same locus in *B. subtilis* genome, the activities of the proteins encoded by these three genes, namely Hpr, CatA and ScoC, are so similar. Hpr inhibits the subtilisin production and the sporulation as well as ScoC. For instance, if there is a mutation on *hpr* gene, the overexpression of *aprE* takes place. Hence, the extracellular production of SAP is increased by using *hpr* or *scoC* knockout strains (Smith, 1993).

Consequently, hGH production process by recombinant *B. subtilis* is also influenced by this complex regulatory network since it directly affects the simulated subtilisin synthesis through the expression of *aprE* gene. The production of rhGH is normally enhanced by the activity of the repressors. However, the inducers may also have a positive impact on the rhGH production due to the interrelated characteristic of the regulation. Therefore, the rhGH production capacities of various knockout strains of *B. subtilis* were investigated by Şahin (2010) in order to determine the effect of these regulatory genes and the highest rhGH production was obtained by using the *scoC* knockout (*scoC*$^-$) strain.

2.4 Medium Design

A large proportion of bacteria use organic compounds as both carbon and energy sources so they are called as chemoorganotrophs or chemoheterotrophs (Nelson and Cox, 2005). When working with such microorganisms, the composition of the fermentation medium is effective on not only cell growth but also the desired metabolite production since these processes are results of the interactions between intracellular and extracellular effectors.

Table 2.3 Macronutrients required by microorganisms
(Madigan and Martinko, 2010)

Element	Common form/forms found in nature
Carbon (C)	CO_2, organic compounds
Hydrogen (H)	H_2O, organic compounds
Oxygen (O)	H_2O, O_2, organic compounds
Nitrogen (N)	NH_3, N_2, organic nitrogen compounds
Phosphorus (P)	PO_4^{3-}
Sulphur (S)	H_2S, SO_4^{2-}, organic S compounds
Potassium (K)	K^+ in aqueous solution or as various K salts
Magnesium (Mg)	Mg^{2+} in aqueous solution or as various Mg salts
Sodium (Na)	Na^+ in aqueous solution or as NaCl or other Na salts
Calcium (Ca)	Ca^{2+} in aqueous solution or as $CaSO_4$ or other Ca salts

Besides water, cells mainly consist of macromolecules which are polymers of smaller units, known as monomers. Hence, in a microbial process, these required components, are named as nutrients, should be provided to microorganisms through the culture medium. Although types of nutrients and their appropriate concentrations in the medium may vary according to the type of the organism, they are generally classified into two groups; macronutrients and micronutrients. The required amounts of macronutrients are higher than 0.1 mM whereas the concentrations of micronutrients used in the medium are less than 0.1 mM. As shown in Table 2.3 and Table 2.4, the major macronutrients can be listed as carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur, potassium, magnesium, sodium and calcium while the micronutrients are primarily vitamins, growth factors and trace elements such as Cl^-, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mo^{2+}, Ni^{2+} and Zn^{2+} .

Microorganisms are able to metabolize at least fifty of the chemical elements. Therefore, all these microbial nutrients originate from the chemical elements but just a few of them are dominant in biological systems.

Table 2.4 Micronutrients (trace elements, vitamins and growth factors) generally required by microorganisms (Madigan and Martinko, 2010)

Element	**Physiological Location and/or Function**
Cobalt (Co)	Vitamin B_{12}; transcarboxylase of propionic acid bacteria
Copper (Cu)	Respiration, cytochrome c oxidase; photosynthesis, plastocyanin, some superoxide dismutases
Iron (Fe)	Cytochromes; catalases; peroxidases; iron-sulfur proteins; oxygenases; some nitrogenases
Manganese (Mn)	Certain superoxide dismutases, water-splitting enzyme of oxygenic phototrophs; activator of various enzymes
Molybdenum (Mo)	Certain flavin-containing enzymes; some nitrogenases; nitrate reductases, sulfite oxidases; some formate dehydrogenases
Nickel (Ni)	Most hydrogenases; coenzyme F_{430} of methanogens; carbon monoxide dehydrogenase; urease
Zinc (Zn)	Carbonic anhydrase; alcohol dehydrogenase; RNA and DNA polymerases; and many DNA-binding proteins

Table 2.4 Micronutrients (trace elements, vitamins and growth factors) generally required by microorganisms (Continued) (Madigan and Martinko, 2010)

Element	Physiological Location and/or Function
Folic acid	One-carbon metabolism; methyl group transfer
Biotin	Fatty acid biosynthesis; β-decarboxylations; some CO_2 fixation reactions
Cobalamin (B_{12})	Reduction of and transfer of single carbon fragments; synthesis of deoxyribose
Lipoic acid	Transport of acyl groups in pyruvate and α-ketoglutarate decarboxylations
Nicotinic acid (niacin)	Precursor of NAD^+; electron transport in oxidation-reduction reactions
Pantothenic acid	Precursor of coenzyme A; activation of acetyl and other acyl derivatives
Riboflavin	Precursor of FMN, FAD in flavoproteins involved in electron transport
Thiamine (B_1)	α-Decarboxylations; transketolase
Vitamins B6	Amino acid and keto acid transformations

The culture medium, which is described as the nutrient solution used to grow microorganisms in the laboratory, is divided into two broad classes; defined medium and complex medium. Defined or synthetic medium is prepared by using exact amounts of highly purified inorganic or organic substances. Thus, the composition of the medium is known precisely (Zhang and Greasham, 1999). On the other hand, knowledge of the exact content is not essential for growing many organisms. In this case, complex or undefined medium containing impure compounds, namely digests of animal, plant or microbial products such as casein, beef extract, tryptic soy broth and yeast extract, are favorable (Nielsen et al., 2003). Even though higher cell and product yields are generally achieved by using complex medium, separation and purification of the desired product from defined medium are easier and cheaper. In addition to this, when a complex medium is preferred, in spite of practical use of the powdered digests, there may be problems about the repeatability of the bioprocess due to variable nutrient composition. Therefore complex media are frequently utilized just for precultivation steps whereas defined media are used through production processes. Besides defined and complex media, there is also an intermediary form; semi-defined medium containing only one or two complex constituents in the composition.

In the literature concerning rhGH production by *Bacillus* species, Nakayama et al. (1988) and Kajino et al. (1997) preferred complex medium containing glucose as the sole carbon source in their studies conducted by using recombinant *B. subtilis* and *B. brevis* strains, respectively whereas recombinant *B. subtilis* culture was grown in liquid VY complex medium for the same goal by Franchi et al. (1991). On the other hand, Şentürk (2006) examined the effect of glucose concentration on rhGH production by *B. subtilis* BGSC-1A197 (*spo*$^-$) and *B. subtilis* BGSC-1A751 (*apr*$^-$ *npr*$^-$) carrying pMK4*::pre(subC)::hGH* plasmid in defined medium and the highest hGH production was obtained in the presence of 8 g L^{-1} glucose, 4.71 g L^{-1} $(NH_4)_2HPO_4$, 2.0 g L^{-1} KH_2PO_4, 5.63 g L^{-1} NaH_2PO_4, 0.043 g L^{-1} Na_2HPO_4 and 7.44 μM protease inhibitor cocktail (Sigma, P-2714). Therefore, Yılmaz (2008) and Şahin (2010) also preferred this defined medium in their studies. However, Gökbudak (2013) investigated the effect of peptone and trace elements solution, PTM1, in the previously developed medium on rhGH production by *B. subtilis* BGSC-1A178

(*scoC*$^-$). For this purpose, firstly air-filtered shake bioreactor experiments were conducted at laboratory-scale. Since the maximum hGH production was achieved when these two components were together, semi-defined medium containing 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 as the stock solution was used for feeding in pilot-scale semi-batch bioreactor experiments. Additionally, 0.122 g L^{-1} $MgSO_4$ was also started to be utilized in the production medium as well as 5 mL L^{-1} PTM1 (Gökbudak, 2013).

2.4.1 Carbon Source

In a typical cell, nearly 50 % of the dry weight consists of carbon. Therefore, the major importance in any fermentation medium belongs to the carbon source since all cells require relatively larger amounts of carbon in order to make new cell materials. Hence, bacteria, as well as most of other microorganisms, are able to use a wide variety of organic substances such as sugars, aromatic compounds, amino acids, fatty acids and organic acids as their carbon sources. However, generally only one carbon source is preferred for a simple defined medium and according to the bacterium that will be cultured, the type of carbon source and its concentration may vary. In this respect, carbohydrates are extensively used carbon and energy sources. Their concentrations are usually higher than that of other components in culture medium (0.2-25 %) and glucose is the most preferred carbohydrate since this monosaccharide is assimilated easily by cells (Nelson and Cox, 2005; Campbell and Reece, 2001).

2.4.2 Nitrogen Source

In a bacterial cell, almost 12 % of the dry weight consists of nitrogen so this element can be also defined as one of the most abundant components in the cell as well as carbon. Microorganisms require nitrogen primarily in order to construct nucleic acids and proteins since the monomeric units of these polymers, nucleic acids and amino acids, respectively, are partially composed of this element. Therefore, using sufficient amount of a suitable nitrogen source in culture medium is essential

for fermentation processes. Similar to the carbon source, the type of the nitrogen source and its concentration needed may change according to the type of the organism to be cultured.

Although there are both inorganic and organic forms of nitrogen in nature, the majority of available nitrogen is in inorganic form, *i.e.*, as molecular nitrogen (N_2) in air, and as industrially produced ammonia (NH_3). Practically all bacteria are able to use ammonia as their nitrogen source (Nielsen et al., 2003). However, molecular nitrogen can satisfy only the nitrogen requirements of nitrogen-fixing bacteria since these microorganisms are able to carry out the reduction of molecular nitrogen to ammonia. Hence, using reduced forms of the element such as ammonium salts is inevitable when the reduction of nitrogen cannot be provided by the cell (Madigan and Martinko, 2010).

On the other hand, culture medium can contain an organic nitrogen source such as urea. Generally there is a significant increase in pH of the fermentation medium as a result of using this compound. Amino acids, peptones and digests of animal, plant or microbial products such as casein, meat extract and yeast extract are also alternative complex nitrogen sources. These industrial by-products usually ensure more efficient microbial fermentation processes (Vogel and Todaro, 1997).

2.4.3 Other Macronutrients

Even though required amounts of other macronutrients are relatively lower than that of carbon and nitrogen, their physiological functions in the cell are crucial.

Cells need sulphur mainly in order to construct two types of amino acids, cysteine and methionine. In addition to this, its structural role in various vitamins such as thiamine, biotin and lipoic acid as well as in coenzyme A is vital (Nelson and Cox, 2005). In nature most of sulphur is in inorganic form such as sulfate (SO_4^{2-}) and sulfide (HS^-) whereas cells have organic forms of the element, namely sulfhydryl groups. Generally sulfate can satisfy the sulphur requirements of bacteria which are able to carry out the reduction of this compound. However, similar to the nitrogen source, reduced forms of the element such as cysteine containing sulfhydryl group or

sulfide should be used directly in the fermentation medium when the reduction of sulphur cannot be supplied by the cell (Schiff, 1979).

Phosphorus, which is present in both inorganic and organic forms in nature is used by microorganisms primarily in order to construct phospholipids and nucleic acids. Potassium is responsible for the regulation of enzyme activity, particularly in protein synthesis. Similarly magnesium is effective on the activity of many enzymes and the stabilization of nucleic acids, ribosomes, and membranes. Calcium is able to stabilize cell walls in most organisms as well as its contribution to heat stability of endospores. On the other hand, sodium is essential for just some microorganisms as a reflection to the habitat. Therefore, this element is considered as a micronutrient in other cases (Madigan and Martinko, 2010).

2.5 Bioreactor Operation Conditions

Besides the medium composition, bioreactor operation conditions such as pH, temperature and oxygen transfer rate are also influence the cell growth and product formation via altering the metabolic fluxes, thus they should be optimized first in order to improve the product selectivity and the production capacity of the bioprocess (Çalık et al., 1999).

2.5.1 Temperature

Temperature, one of the most critical bioreactor operation parameters, should be kept constant at its optimum value throughout the fermentation process since it is able to affect cellular metabolism including microbial growth, substrate consumption and product formation pathways due to the lack of internal temperature regulation in microorganisms (Nielsen and Villadsen, 1994). When temperature is below the optimum value, enzymatic activity is typically decreased by a factor of 2-3 as a result of 10°C reduction so cell and product yields are lower due to increased energy requirement (Johnson, 1999). In addition to this, there are some mass transfer limitations through hardened cell membrane at lower temperatures so this may

adversely affect extracellular protein production (Hogg, 2005). Nevertheless when a significantly higher temperature is preferred for the bioprocess, growth rate decreases and protein denaturation causes thermal cell death (Georgiou and Valax, 1996).

Although there is usually a broad temperature range for survival of microbial strains, each microorganism has an optimal temperature that gives maximum cell and product yields on substrate whereby yield coefficients are also affected by temperature. However, sometimes optimum temperatures required for the highest microbial growth and the highest product formation may be different. Therefore, the ideal temperature range for a higher efficiency should be determined according to the aim of the bioprocess, such as production of biomass, a primary metabolite or a secondary metabolite.

In the literature in respect to rhGH production, Jensen and Carlsen (1990), Bylund et al. (2000), Castan et al. (2002) and Tabandeh et al. (2004) carried out their studies at 30°C by using genetically engineered *E. coli* strains. On the other hand, Shin et al. (1998a-b) preferred 37°C for the same bioprocess. Similarly hGH production by recombinant *B. subtilis* was conducted at at 30°C by Nakayama et al. (1988) while Şentürk (2006), Yılmaz (2008), Şahin (2010) and Gökbudak (2013) performed this operation at 37°C without any investigation to determine the optimum fermentation temperature.

2.5.2 pH

pH of the fermentation medium is another important bioreactor operation parameter since this physical property is able to affect the energy metabolism, enzyme activities and transport processes of microorganisms. While intracellular pH is kept constant during the bioprocess, hydrogen ion concentration of the micro-environment changes as a result of metabolic activities of the cell (Bailey and Ollis, 1986). pH of the medium decreases with the secretion of amino acids and organic acids whereas it starts to increase with the metabolite transport into the cell or with the cell death. Moreover, cell and product yields on substrate are affected by pH change since the hydrogen ion gradient formed across the membrane is essential for energy generation (Campbell and Reece, 2001).

Therefore, there is an optimum pH range for each microorganism with ± 1-2 pH units. Optimal hydrogen ion concentrations required for the highest microbial growth and the highest product generation may be different like temperature. It is usually around pH=7.0 even though bacteria can survive in a broad range, particularly under alkaline conditions such as pH=6.5-8.0 (Padan et al., 2005).

Hence, the ideal pH range should be identified via considering the main objective of the fermentation or the microorganism is chosen according to pH of the selected medium. In some bioprocesses, controlled pH conditions are required in order to enhance the product yield and selectivity while working at uncontrolled pH might be more advantageous for others (Çalık et al., 2003a).

In the literature regarding to rhGH production, Bylund et al. (2000), Patra et al. (2000), Castan et al. (2002) and Tabandeh et al. (2004) conducted their studies at pH=7.00 by using recombinant *E. coli* strains whereas the same bioprocess was carried out at pH=6.75 by Shin et al. (1998b) and at pH=7.20 by Jensen and Carlsen (1990) and Zhang et al. (1998). Hydrogen ion concentration in the medium was controlled and kept constant in all of these studies without any investigation on the effect of pH control. Nevertheless, Nakayama et al. (1988), Şentürk (2006) and Yılmaz (2008) performed hGH production by genetically engineered *B. subtilis* under uncontrolled pH conditions at the initial pH values of 6.00-7.00, 7.25 and 7.50, respectively. The effect of pH control during this fermentation process was firstly examined by Şahin (2010) and controlled pH=7.50 was determined as the most suitable condition for higher rhGH production so it was also used in the study of Gökbudak (2013).

2.5.3 Oxygen Transfer

Aerobic organisms require oxygen for growth, cell maintenance and product generation so the oxygen transfer into the microbial cell is one of the most critical bioreactor operation parameters in aerobic fermentations since it directly affects metabolic pathways and changes metabolic fluxes towards product formation (Çalık et al., 1999). Therefore, the oxygen transfer characteristics, are associated with the physiology of the microorganism through the design of the fermentation medium

based on its qualitative and quantitative chemical composition, towards the metabolism and intracellular reaction network. The intracellular reaction network need to be directed to the synthesis of the biomolecule in the "biomolecule synthesis domain" whose limits can be clarified by the bioreactor operation conditions; where, to fine-tune bioreactor performance creative strategies are needed to be designed and investigated (Çalık et al., 2000). In this context, high oxygen transfer conditions are more suitable for some bioprocesses, whereas others are conducted at low oxygen transfer rates (Çalık et al., 1998b). The most crucial parameters affecting oxygen transfer rate and dissolved oxygen concentration in bioreactor are the air inlet (Q_O/V_R) and agitation rates (N) (Çalık et al., 1999; Çalık et al., 2000).

The saturated dissolved oxygen concentration in water is 7 mg L^{-1} at 25°C when the pressure is equal to 1 atm. As a result of the low solubility of oxygen in aqueous solutions (nearly 10 parts per million, ppm), the oxygen transfer to the microorganism is generally considered as the limiting step of aerobic bioprocesses that determines the overall reaction rate (Bailey and Ollis, 1986). Although various conditions such as medium composition, temperature and pressure are able to change the solubility, oxygen limitation occurs especially at high cell densities because in this case the oxygen consumption rate and the oxygen feed rate are almost equal. The transfer of oxygen from gas phase (air bubble) to solid phase (cell) is explained in nine steps by the two film theory as demonstrated in Figure 2.10 (Garcia-Ochoa et al., 2010):

1. Transport from the interior region of the gas bubble to the gas-liquid interface
2. Movement along the gas-liquid interface
3. Diffusion through the stagnant region of the bulk liquid surrounding the gas bubble
4. Transfer throughout the bulk liquid
5. Diffusion across the stagnant region of the bulk liquid surrounding the cell
6. Movement along the liquid-cell interface. If there is a solid particle such as a clump or a flock including cells, then diffusion from the cell aggregate to the individual cell

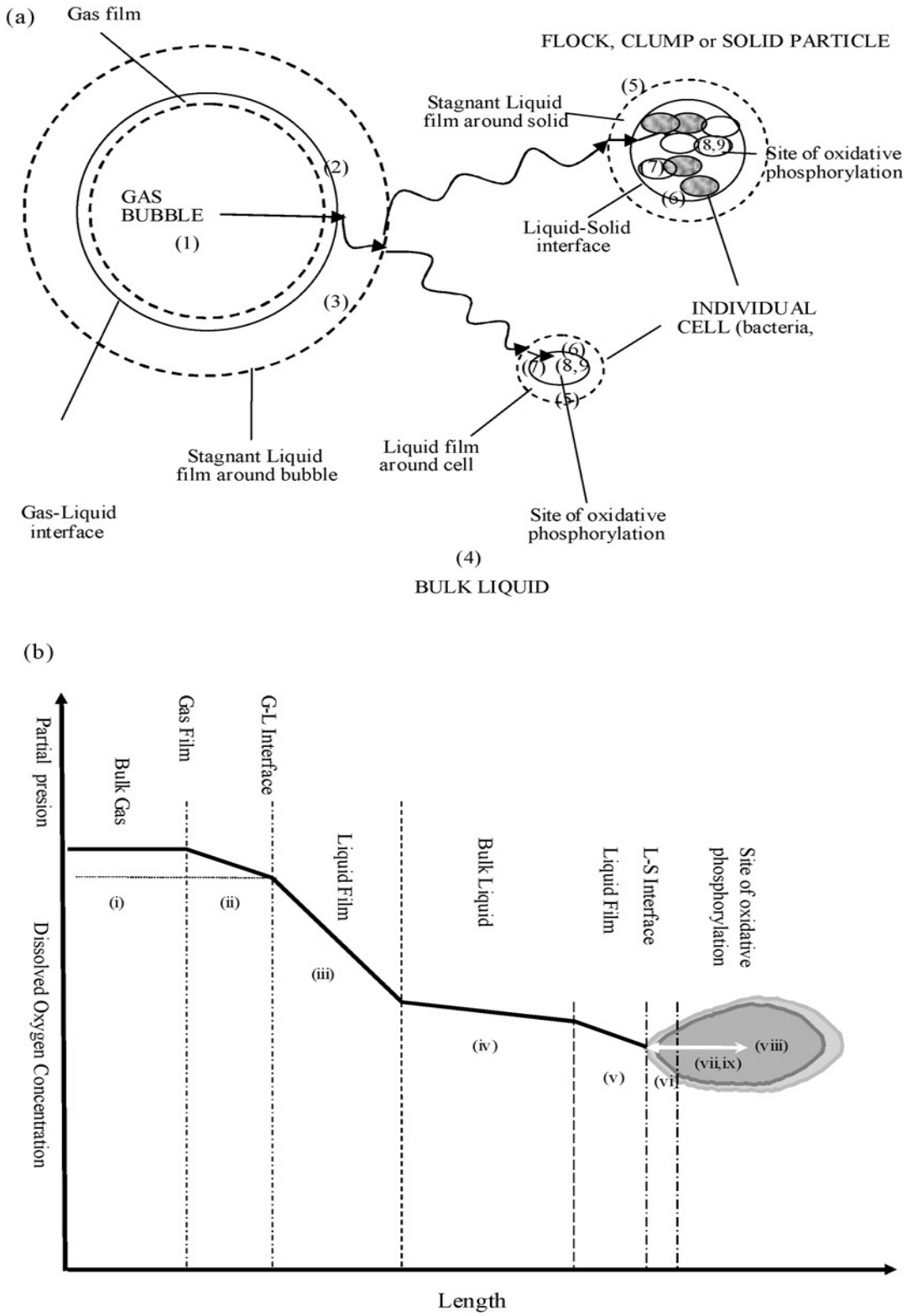

Figure 2.10 (a) Steps of oxygen transport from gas phase (air bubble) to solid phase (cell) (b) Oxygen concentration profile from gas phase to solid phase (Garcia-Ochoa et al., 2010)

7. Transfer towards the biochemical reaction site in the cytoplasm
8. Biochemical reactions which involve either oxygen consumption or CO_2 production
9. Transport of the produced gases in the reverse direction

When the cells are homogenously dispersed in the well-mixed fermentation medium, the transfer of oxygen through the bulk liquid is very fast. If the cell aggregates are not formed, the second part of the sixth step is not active so the small non-polar molecule is readily transported across the cell membrane by passive diffusion (Campbell and Reece, 2001). As a result of the small size of the microorganism, the resistance against the movement within the cell is generally neglected (Nielsen and Villadsen, 1994). Hence, the main limitation for the transfer of oxygen in aerobic fermentation processes is derived from the stagnant region of bulk liquid surrounding the gas bubble and oxygen transfer rate (OTR) from gas to liquid is represented as:

$$OTR = N_O a = K_L a \left(C_O^* - C_O \right) \tag{2.1}$$

where N_O is the molar mass transfer flux of oxygen (mol m^{-2} s^{-1}), a is the gas-liquid interfacial area per unit volume (m^2 m^{-3}), K_La is the overall 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}). In Equation 2.1, the overall liquid phase mass transfer coefficient, K_La and the overall driving force, (C_O^*-C_O) are used since the interfacial concentrations cannot be measured. Due to the low solubility of oxygen in aqueous solutions, the liquid phase film resistance has a major effect when it is compared to the gas phase film resistance. Therefore, K_La is approximately equal to the liquid phase mass transfer coefficient, k_La.

The maximum possible oxygen transfer rate (OTR_{max}) can be indicated as:

$$OTR_{\max} = \left(N_O \right)_{\max} a = K_L a \, C_O^* \tag{2.2}$$

OTR is affected by physical, chemical and biological factors such as rheology of the medium, type of the microorganism, aeration rate, agitation rate, temperature and the oxygen uptake rate (OUR) of the cell. The OUR, one of the most important oxygen transfer characteristics in aerobic processes as well as K_La, influenced mainly by the carbon source in the medium and the growth phase of the cell, is defined as:

$$OUR = -r_O = -r_O^{'''} C_X = q_O C_X \tag{2.3}$$

where $-r_o$ is the oxygen consumption rate (mol m^{-3} s^{-1}), $-r_o^{'''}$ is the oxygen consumption rate per unit cell dry weight (mol kg^{-1} s^{-1}), q_o is the specific oxygen consumption rate (mol kg^{-1} s^{-1}) and Cx is the cell concentration (kg m^{-3}). Hence, the maximum possible oxygen uptake rate (OUR_{max}) is given by the following equation:

$$OUR_{\max} = -(r_o)_{\max} = C_X \mu_{\max} / Y_{X/O} \tag{2.4}$$

where $Y_{X/O}$ is the mass of cell generated per unit mass of oxygen consumed, called as the oxygen yield coefficient (kg kg^{-1}).

Dissolved oxygen concentration in the fermentation broth is the primary result of the balance between OUR and OTR so its mass balance in the well-mixed liquid phase at unsteady-state conditions can be written as (Garcia-Ochoa et al., 2010):

$$K_L a \left(C_O^* - C_O\right) + r_O^{'''} C_X = \frac{dC_O}{dt} \tag{2.5}$$

where dC_O/dt is the oxygen accumulation in the liquid phase, the first term on the left side is OTR and the second term is OUR.

The increased medium viscosity in consequence of the cell growth, product and by-product formation reduces OTR value in exponential growth phase. Since it is generally lower than OUR value in this period, dissolved oxygen concentration in the fermentation broth gradually decreases with the cultivation time. However, in stationary phase, oxygen demand of the microorganism is less so dissolved oxygen concentration increases due to the decreased OUR (Çalık, 1998b). In some microbial processes, dissolved oxygen concentration reduces to zero since oxygen demand is

too high and cannot increase even in the stationary phase, despite the improvement in OTR by increasing air flow rate, stirrer speed or oxygen concentration in air supplied (Gomez et al., 2006).

Although OUR, OTR and K_La have been usually measured by different techniques in the same experiment, a single procedure can be used in order to obtain these experimental values simultaneously (Garcia-Ochoa et al., 2010). In aerobic fermentation processes, the identification of K_L and a values separately is difficult so several methods have been developed for direct or indirect K_La determination such as the dynamic gassing-out method, the static gassing-out method, the oxygen balance method, the sulphite oxidation method and the glucose oxidase method (Rainer, 1990).

Amongst these techniques, the most widely used one is the dynamic method which is mainly based on the respiratory activity of growing organisms in the bioreactor. This simple procedure can be repeated for several times during the production process at certain time intervals. For the implementation of the method, firstly the gas inlet to the system is interrupted for a few minutes and the agitation rate is decreased to a minimum level in order to prevent surface aeration. As shown in Figure 2.11, decrease in dissolved oxygen concentration (at t_0-t_1) is monitored by using an oxygen probe since the depletion of DO in the fermentation broth gives OUR. In this case, K_La is equal to zero due to the lack of oxygen transfer in the medium so Equation 2.5 can be simplified as (Garcia-Ochoa et al., 2010):

$$r_O = \frac{dC_O}{dt} \tag{2.6}$$

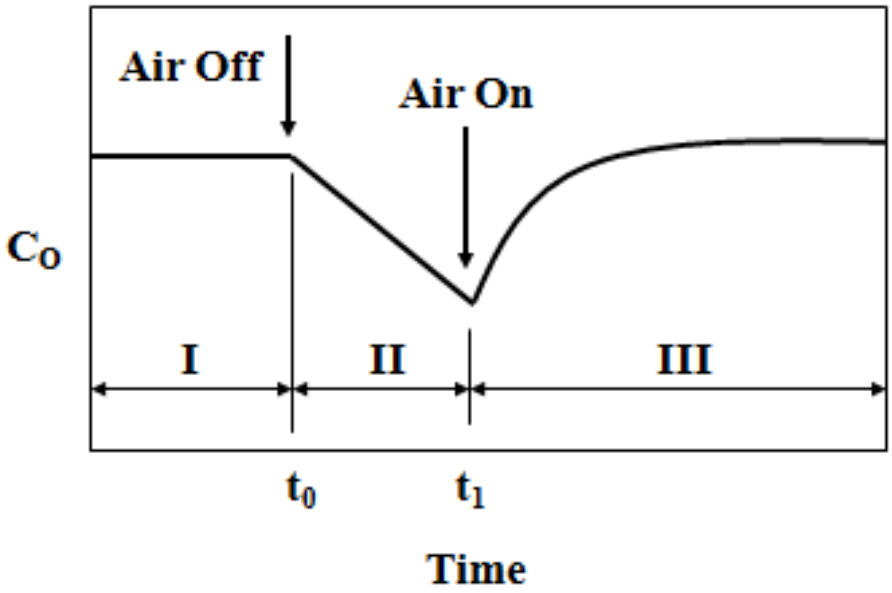

Figure 2.11 Changes in dissolved oxygen concentration with respect to time in the dynamic method

Afterward, the gas flow is reintroduced under the previous operational conditions in order to ensure the same OTR in the system (Taguchi and Humphrey, 1966). For Region-III in Figure 2.11, Equation 2.5 can be used again after K_La is calculated from the slope of the graph plotted as ($dC_O/dt-r_o$) versus C_O in Figure 2.12.

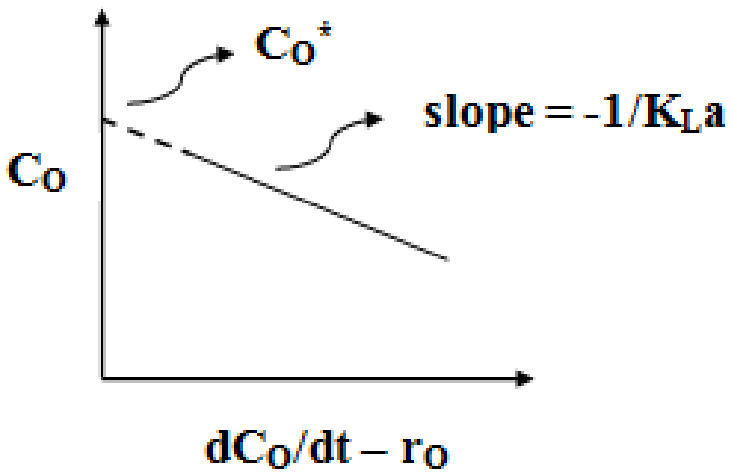

Figure 2.12 Calculation of K_La by using the dynamic method

The existence of microorganisms in the medium increases the resistances to oxygen transfer so in order to analyze this effect, K_La_0 is also determined by using the same technique before the inoculation. However, nitrogen flow is supplied to the bioreactor for the first step and Equation 2.5 can be simplified to Equation 2.7 since OUR is equal to zero in this case.

$$K_L a_0 \left(C_O^* - C_O\right) = \frac{dC_O}{dt} \tag{2.7}$$

Thereafter, K_La_0 is calculated from the slope of the graph in Figure 2.12 which is plotted without $-r_o$ term in the x-axis and the ratio of K_La to K_La_0 gives the enhancement factor (E) that indicates the efficiency of the oxygen transfer and uptake.

Some other experimental values should also be calculated for the determination of the rate limiting step in an aerobic bioprocess (Çalık et al., 2003b). For instance, the oxygen demand (OD) which is also known as the maximum possible oxygen utilization rate is demonstrated by the following equation:

$$OD = \frac{\mu_{\max} C_X}{Y_{X/O}} \tag{2.8}$$

In addition to OD, the Damköhler number (Da) is defined as the ratio of the oxygen demand to the maximum oxygen transfer rate (Equation 2.9),

$$Da = \frac{OD}{OTR_{\max}} \tag{2.9}$$

while the ratio of the oxygen consumption rate to the maximum possible oxygen utilization rate is called as the efficiency factor (η) (Equation 2.10).

$$\eta = \frac{OUR}{OD} \tag{2.10}$$

In the literature concerning the determination of oxygen transfer characteristics in bacilli fermentation, SAP production process was carried out at various agitation rates (150 min^{-1} to 750 min^{-1}) together with several aeration rates

(0.2 vvm to 1 vvm) in order to examine the effect of oxygen transfer on the production of subtilisin by *B. licheniformis* (Çalık et al., 1998b; Çalık et al., 2000). Since the highest SAP activity was obtained when the agitation rate was equal to 750 min^{-1} as the air inlet rate was maintained at 0.5 vvm, Şentürk (2006), Yılmaz (2008), Şahin (2010) and Gökbudak (2013) were also used these oxygen transfer conditions for hGH production by recombinant *B. subtilis* strains.

2.6 Bioreactor Operation Modes

There are several bioreactor operation modes used for microbial processes mainly in order to provide uniform cell dispersion in the culture fluid. These modes are basically divided into two, batch and continuous operations, whereas modifications of batch operation; namely fed-batch, repeated batch and repeated fed-batch modes, can be also preferred for a particular bioprocess. The differences among these operation modes are well understood when change in the volume of the fermentation medium with respect to time as shown in Figure 2.13 is examined (Asenjo and Merchuk, 1994). Based on the multi-phase “gas (air) - liquid (aqueous medium) - solid (micro-organism)/catalytic” nature of the bio-reaction, bioprocesses are conducted in batch- or semi-batch bioreactors.

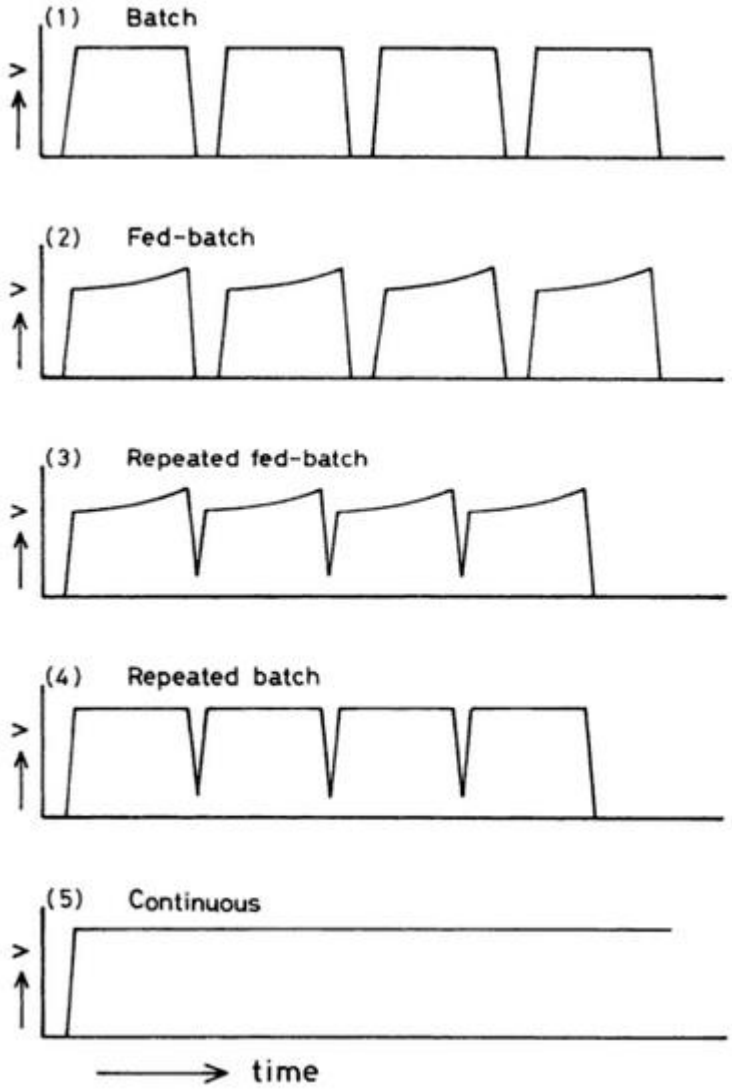

Figure 2.13 Variations in volume of the fermentation medium with respect to time for various bioreactor operation modes (Asenjo and Merchuk, 1994)

2.6.1 Batch Operation

In the batch bioprocess, bioreactor is filled with sterile medium containing all nutrients required for one run of cultivation, before the inoculation of living cells. Then nothing (except pure oxygen/air for aeration and acid/base for pH adjustment) is added to the culture or removed from it as growth proceeds. Therefore, the concentrations of cells, substrates and products vary with time in the system (Bailey and Ollis, 1986).

Microbial growth in batch operation is generally modeled with four successive phases, lag phase, log/exponential phase, stationary phase and death phase as indicated in Figure 2.14.

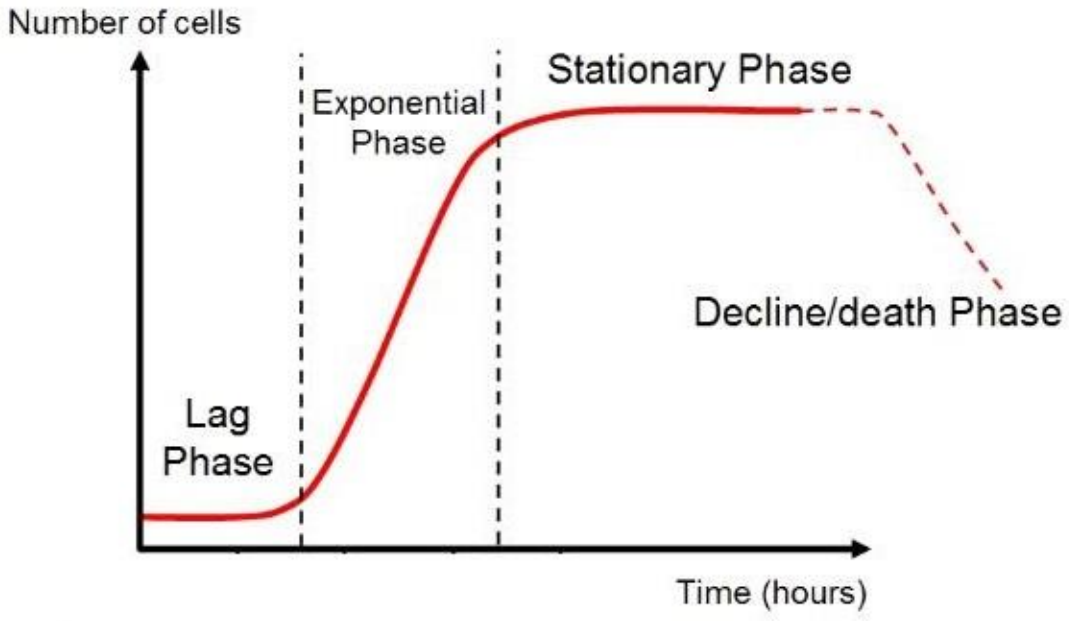

Figure 2.14 Microbial growth curve basically composed of four distinct regions

In the lag phase, the microorganisms adapt themselves to the new culture conditions and synthesize some essential biomolecules for the growth. During this period the individual cells just mature since they are not able to divide yet. This phase must be kept as short as possible for an efficient bioprocess; the composition of the production medium should be fairly similar to that of the precultivation medium in order to prevent the microbial shock and the appropriate dilution ratio should be determined in order to shorten the adaptation period.

After the lag phase, throughout the exponential phase characterized by the cell growth with a constant specific growth rate (μ), the number of new microorganisms appearing per unit time, the growth rate, is proportional to the present population (Bailey and Ollis, 1986):

$$\frac{dC_X}{dt} = \mu C_X \tag{2.11}$$

During this period the number of cells and the rate of its increase double with each consecutive time period. Therefore, the slope of the graph plotted as time versus the natural logarithm of cell number gives the maximum number of divisions per cell per unit time, μ_{max}, as demonstrated in Figure 2.15.

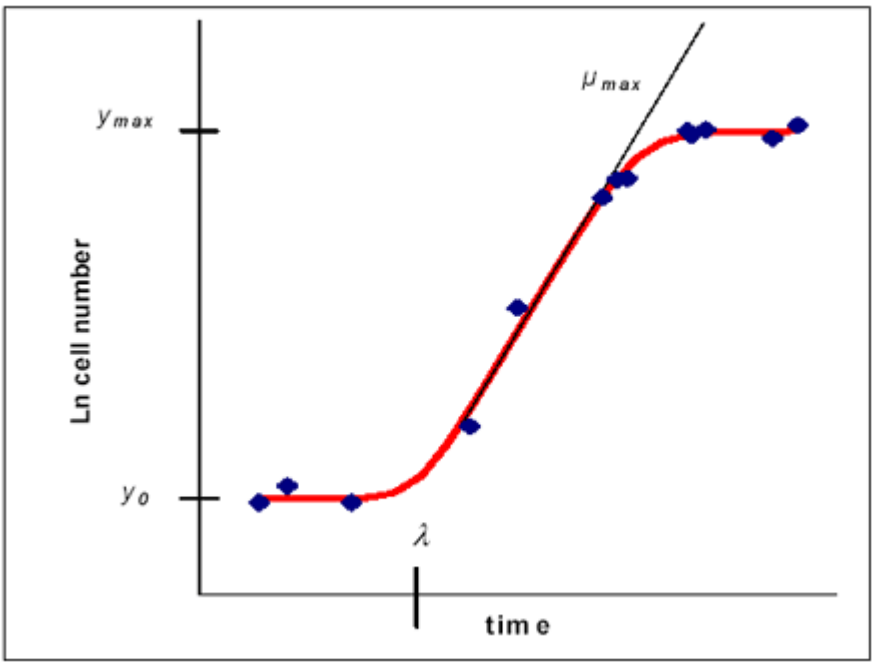

Figure 2.15 A sigmoidal bacterial growth curve: y_0 is the natural logarithm of the initial cell concentration, y_{max} is the natural logarithm of the maximum cell concentration, μ_{max} is the maximum specific growth rate and λ is the time in the lag phase (http://www.ifr.ac.uk/bacanova/project_backg.html)

Exponential growth cannot continue indefinitely due to the depletion of nutrients and/or the accumulation of inhibitory products in the fermentation medium. Thus, in the stationary phase the growth slows down and the cell concentration remains constant since the growth rate is equal to the death rate (Bailey and Ollis, 1986). This growth phase is particularly desired for industrial secondary metabolite production processes whereas the log phase is usually preferred for the production of biomass or primary metabolites.

If the microorganisms are not harvested from the broth anymore, their number rapidly decreases in the logarithmic decline phase because of the higher death rate.

In batch operation, complete conversion of the substrate in a shorter process time is possible so high productivity can be obtained with little risk of contamination or cell mutation. When this technique is compared to continuous operation for the same bioreactor volume, the capital investment is relatively low. On the other hand, this operation mode has also some disadvantages such as lower productivity levels due to the time required for filling, sterilizing, emptying and cleaning the bioreactor, increased stress on instruments because of frequent sterilization, higher cost for labor and/or process control for this non-stationary system and more hygiene problems during filling, emptying and cleaning (Nielsen and Villadsen, 1994). Therefore, batch operation is preferred when there is a minimal risk of contamination or strain mutation or if only small amounts of the product are desired (Lim and Shin, 2013).

Şentürk (2006), Yılmaz (2008), Özdamar et al. (2009) and Şahin (2010) used batch bioreactor operation for rhGH production by recombinant *B. subtilis* and the highest rhGH level obtained by Şahin (2010) was 126 mg L^{-1} while the highest cell concentration achieved in the same study was 1.62 g L^{-1}.

2.6.2 Continuous Operation

Based on the multi-phase “gas (air) - liquid (aqueous medium) - solid (micro-organism)/catalytic” nature of the bio-reaction, industrial bioprocesses can be conducted in batch- or semi-batch bioreactors. As the micro-organism which has a short life time behaves as a semi-batch micro-bioreactor in the bioreactor system; moreover, if a tubular bioreactor is used the axial dispersion caused by the air creates axial-mixing and disturbs plug flow (or turbulent flow) hydrodynamic character; on the other hand, if a back-mixed flow bioreactors (chemo-stat) is used because of aging of micro-organism the cell should be supplied also in the continuous feed; therefore continuous bioreactors are only exercises for some academic researchers, not for production of biomolecules.

2.6.3 Semi-Batch (Fed-Batch) Operation

In semi-batch bioprocess, a fresh medium containing one or more substrates is fed into the bioreactor during the cultivation whereas cells and product/products remain in the system until the end of the process (Parulekar and Lim, 1985). Semi-batch operation in chemical engineering is generally known as fed-batch cultivation in biochemical engineering and this intermediary operation mode is frequently used for industrial microbial processes owing to its various benefits. The main advantage of semi-batch bioprocess is that the concentrations of the fed nutrients in the fermentation medium can be controlled by changing the feed rate. This feature has a positive impact on the primary fermentation characteristics, namely on the yield and the productivity of the desired product (Lim and Shin, 2013).

In industrial microbial processes, the semi-batch operation is favorable in many cases such as (Yamanè and Shimizu, 1984):

- Some components used in the production medium such as alcohols and aromatic compounds adversely affect the growth of microorganism. Feeding such nutrients for a particular time period decreases the substrate inhibition on cells.
- In order to achieve very high cell densities in batch culture, high initial substrate concentrations are required. These high concentrations of the nutrients become inhibitory for the microbial growth so this problem can be overcome in semi-batch operation mode by feeding such substrates to the bioreactor continuously with lower concentrations.
- When there is an excess amount of sugar in the culture liquid, ethanol is produced by baker's yeasts despite the presence of sufficient dissolved oxygen in the fermentation medium. This phenomenon, which causes a significant decrease in biomass yield, is called as the Crabtree effect. Similarly in aerobic culture of *E. coli* and *B. subtilis* because of the bacterial Crabtree-effect, organic acids (particularly acetic, lactic and formic acid) are formed at high glucose concentrations in the culture medium. In this case, the organic acids produced deteriorate physiology of the bacteria as well as

inhibit the growth of cells. Hence, semi-batch operation mode can be preferred in order to ensure an intermediate sugar concentration in the broth.

- If the carbon-energy source in the medium is rapidly metabolized by the microorganisms, the increase in the intracellular concentration of adenosine triphosphates (ATP) leads to the repression on the biosynthesis of enzymes particularly involved in catabolic pathways. Therefore, this phenomenon is named as catabolite-repression. When the concentration of the carbon-energy source in the medium is kept at low levels, the enzyme biosynthesis is derepressed so the energy metabolism is accelerated.
- An auxotrophic mutant cannot survive in the absence of a particular nutrient. However, if the concentration of the required substrate in the production medium is more than necessary, the productivity decreases due to the feedback inhibition and/or the end product repression unless the abundant microbial growth. Hence, in order to obtain the maximum production capacity with less biomass, the limited concentration of the required nutrient is provided by the controlled feed rate in semi-batch operation.
- If a foreign gene on a plasmid has a regulable promoter, the gene expression in recombinant cells is controlled by the absence of a compound (responsible gene) or by the presence of a nutrient (inducible gene) in the medium. Early expression of the regulable gene generally has a negative impact on the cell growth and the plasmid stability. Thus, controlled feeding of such substrates and keeping their concentrations as low as possible is critical in order to increase the production level.
- The early stationary phase of the microbial growth is particularly desired for the production of secondary metabolites. Unfortunately this period is very short in a common batch process due to starvation since the microorganisms consume the carbon-energy source rapidly at the beginning of the bioprocess. Therefore, in non-growth-associated microbial processes, the carbon-energy source should be fed into the bioreactor at a controlled rate in order to prolong this production phase.
- In some microbial bioprocesses, the increase in viscosity of the fermentation medium due to the cell growth and product formation causes severe problems

related to the mass transfer. In this case, the viscosity can be reduced by water supply in order to extend operation time in semi-batch cultivation.

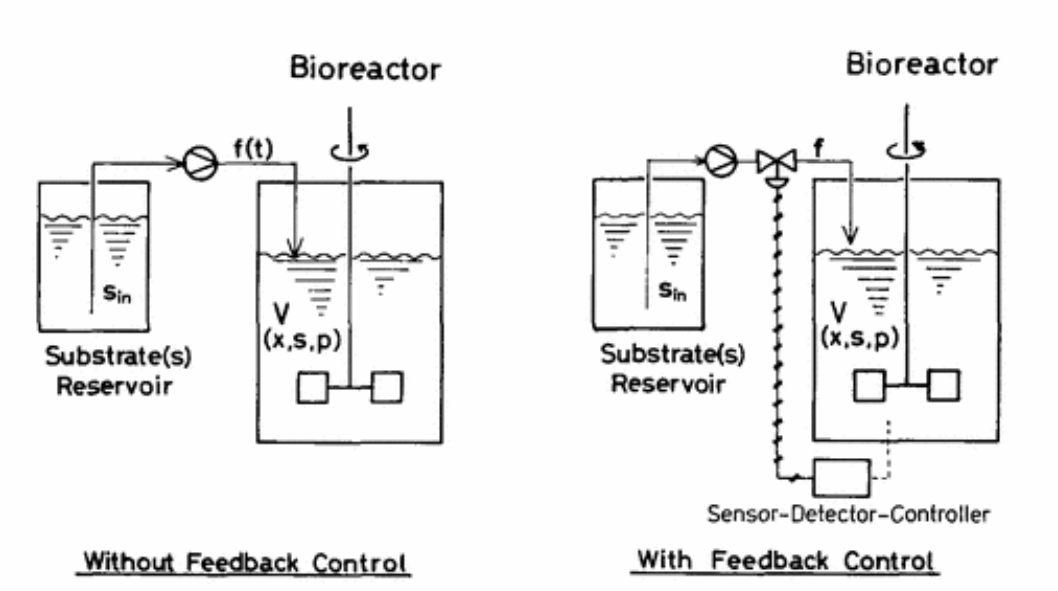

Figure 2.16 Schematic representations of different feeding modes in semi-batch operation (Yamanè and Shimizu, 1984)

In order to perform an effective semi-batch bioprocess, firstly the type of the substrate/substrates which will be fed into the bioreactor and then, the feeding mode which will be the optimum for the given microbial process should be determined carefully. In the first step, the most appropriate fed nutrient/nutrients are identified by using various disciplines, such as microbiology, biochemistry, physiology and genetics, simultaneously. However, in some cases, work with the trial and error method is unavoidable. On the other hand, determination of the optimum feeding mode in the second step is directly related to biochemical engineering principles (Bailey and Ollis, 1986). As shown in Figure 2.16, semi-batch operations mainly classified into two groups according to the feeding mode; processes without feedback control and processes with feedback control (Asenjo and Merchuk, 1994).

The semi-batch bioprocesses without feedback control in the first group are further subdivided based on feed rates (Table 2.5). In the case of intermittent addition, the substrate concentration in the bioreactor is kept almost constant since

pulses are implemented in order to supply this nutrient before it is completely consumed in the system. Nevertheless, when a fresh medium containing one or more substrates is fed into the bioreactor with a constant pre-determined rate, the operation is defined as constantly semi-batch process. However, the microbial growth with a constant specific growth rate is just provided by using exponential feeding strategy. In this case, first an appropriate pre-determined specific growth rate (μ) is selected and then, feeding is exponentially increased due to the time-dependent exponential term in the related equation. Unfortunately if the specific growth rate does not remain constant because of non-ideal conditions in the microbial process, additional feedback control strategies should be developed. Finally, optimized semi-batch operation is described as a combination of these feeding strategies since change in the feed rate of the substrate may be effective on the yield and the productivity (Yamanè and Shimizu, 1984).

Table 2.5 Classification of semi-batch cultures (Asenjo and Merchuk, 1994)

Without feedback control	**With feedback control**
Intermittent addition	Indirect feedback control
Constantly semi-batch	Direct feedback control
Exponentially semi-batch	▪ Constant control
Optimized semi-batch	▪ Optimum control
Others	

On the other hand, the semi-batch bioprocesses with feedback control in the second group are further subdivided based on the type of control (Table 2.5). When the direct feedback control strategy is used, the concentration of the fed substrate in the bioreactor is followed either continuously in situ or intermittently with a sensor-detector system (Asenjo and Merchuk, 1994). However, in the case of indirect feedback control some other operational parameters instead of the concentration of the fed substrate, such as dissolved oxygen concentration, cell concentration or pH, are monitored in order to evaluate the necessity of feeding.

In the literature regarding recombinant hGH production by *B. subtilis*, the exponentially semi-batch operation mode with various pre-determined specific growth rates was implemented for the first time by Gökbudak (2013) where the highest cell and rhGH concentrations achieved were 366 mg L^{-1} and 4.43 g L^{-1}, respectively. However in this study direct feedback control was also used since feeding of the semi-defined medium containing glucose was stopped as a result of the glucose accumulation in the production medium.

2.6.3.1 Kinetic Data Evaluation in Semi-Batch Operation

In semi-batch operation as in any type of bioreactor operation, the main fermentation characteristics, namely the productivity and the yield, are affected by some key parameters such as specific growth rate of microorganism, specific utilization rate of substrate and specific formation rate of product. Therefore, mathematical models should be derived using these parameters in order to understand microbial process kinetics (Bailey and Ollis, 1986).

2.6.3.1.1 Mass Balance Equation for the Cell

Microbial growth is defined as the increase in the number of microorganisms. Thus, if it is assumed that during the bioprocess there is no cell loss due to sampling or any other reason, the mass balance equation for the cell can be written as:

$$r_X V = \frac{d(C_X V)}{dt} \tag{2.13}$$

where C_x is the concentration of the cells, V is the working volume of the bioreactor and t is the cultivation time. The growth rate of the cell, r_X is demonstrated as:

$$r_X = \mu C_X \tag{2.14}$$

where μ is the specific growth rate of the microorganism as mentioned before. Hence, when Equation 2.14 is inserted in Equation 2.13, the derived equation is:

$$\mu C_X V = \frac{d(C_X V)}{dt} \tag{2.15}$$

In semi-batch operation the working volume of the bioreactor changes with time. If it is assumed that there is no change in the density of the fermentation medium, this alteration can be described as:

$$\frac{dV}{dt} = Q_{in} - Q_{out} \tag{2.16}$$

where Q_{in} is the volumetric flow rate of the input and Q_{out} is the volumetric flow rate of the output. There is no output in semi-batch operation so Equation 2.16 is rewritten as:

$$\frac{dV}{dt} = Q_{in} \tag{2.17}$$

When Equation 2.17 is inserted into Equation 2.15 in order to express the effect of the feed, the derived equation is denoted as:

$$\frac{dC_X}{dt} = \left(\mu - \frac{Q}{V}\right) C_X \tag{2.18}$$

Therefore, in semi-batch operation the specific growth rate of the microorganism can be calculated by using the rearranged form of Equation 2.18 as follows:

$$\mu = \frac{d\,C_X}{dt}\frac{1}{C_X} + \frac{Q}{V} \tag{2.19}$$

2.6.3.1.2 Mass Balance Equation for the Substrate

In semi-batch operation the basic mass balance equation for the substrate that is fed into the bioreactor can be given as:

$$Q_S\, C_{S_0} + r_S\, V = \frac{d(C_S\, V)}{dt} \tag{2.20}$$

where Qs is the volumetric feed rate of the substrate, C_{S0} is the concentration of the substrate in the feed medium and C_S is the concentration of the substrate in the bioreactor. The first order kinetic equation is written for the substrate utilization rate, r_S, depending on the cell concentration as follows:

$$-r_s = q_s C_x \tag{2.21}$$

where q_S is the specific substrate utilization rate. If Equation 2.21 is inserted into Equation 2.20, the derived equation is represented as:

$$Q_S C_{S_0} - q_s C_X V = C_S \frac{dV}{dt} + V \frac{dC_S}{dt} \tag{2.22}$$

Therefore, q_S can be expressed by the rearranged form of Equation 2.22 as follows:

$$q_s = \frac{1}{C_X}\left(\frac{Q_S}{V} C_{S_0} - \frac{C_S}{V}\frac{dV}{dt} - \frac{dC_S}{dt}\right) \tag{2.23}$$

Under quasi-steady state conditions it is assumed that the concentration of the substrate in the bioreactor does not change with time ($dC_S/dt = 0$). In this case, the rate of substrate utilization which is only related to the cell formation, is demonstrated as:

$$-r_S = (r_X / Y_{X/S}) \tag{2.24}$$

where $Y_{X/S}$ is the cell yield on the substrate. Therefore, when Equation 2.24 is inserted into Equation 2.20, the derived equation is denoted as:

$$Q_S C_{S_0} - \frac{r_X V}{Y_{X/S}} = C_S \frac{dV}{dt} \tag{2.25}$$

Since Q_S can be also defined as the change in the working volume of the bioreactor (dV/dt), Equation 2.25 can be rewritten as:

$$Q_S C_{S_O} - \frac{r_X V}{Y_{X/S}} = C_S Q_S \tag{2.26}$$

Then μC_X term is used instead of r_X as shown in Equation 2.14 for the reorganized form of Equation 2.26 as follows:

$$Q_S C_{S_0} - \frac{\mu C_X V}{Y_{X/S}} = C_S Q_S \tag{2.27}$$

When Equation 2.13 is solved differentially by using Equation 2.14, the final expression obtained can be indicated as:

$$C_X V = C_{X_0} V_0 e^{\mu t} \tag{2.28}$$

Consequently if Equation 2.28 is inserted into Equation 2.27, the volumetric feed rate of the substrate as a function of the cultivation time is given as:

$$Q_S = \frac{\mu C_{X_0} V_0}{Y_{X/S}(C_{S_0} - C_S)} \exp(\mu t) \tag{2.29}$$

2.6.3.1.3 Mass Balance Equation for the Product

In semi-batch operation the mass balance equation for the product is denoted as:

$$r_P V = \frac{d(C_P V)}{dt} \tag{2.30}$$

where C_P is the product concentration in the bioreactor. In this case, the first order kinetic equation is written for the product formation rate, r_P, depending on the cell concentration as follows:

$$r_P = q_P C_X \tag{2.31}$$

where q_P is the specific product formation rate. If Equation 2.31 is inserted into Equation 2.30 before the derived equation is rearranged, the specific product formation rate can be represented as:

$$q_P = \frac{1}{C_X}\left(\frac{C_P}{V}Q + \frac{dC_P}{dt}\right) \tag{2.32}$$

2.6.3.1.4 Yield Coefficients

In microbial process, production of biomass or product by microorganisms can be described quantitatively by the yield coefficients expressed as the mass of cell or product formed per unit mass of substrate consumed as follows (Hong, 1989):

$$Y_{X/S} = \frac{r_X}{-r_S} = \frac{dC_X/dt}{-dC_S/dt} \tag{2.33}$$

$$Y_{P/S} = \frac{r_P}{-r_S} = \frac{dC_P/dt}{-dC_S/dt} \tag{2.34}$$

Additionally when Equation 2.33 and Equation 2.34 are written for a finite period of time, the overall yield coefficients for cell and product are obtained as:

$$\overline{Y}_{X/S} = \frac{\Delta C_X/\Delta t}{-\Delta C_S/\Delta t} \tag{2.35}$$

$$\overline{Y}_{P/S} = \frac{\Delta C_P/\Delta t}{-\Delta C_S/\Delta t} \tag{2.36}$$

In aerobic bioprocess, since oxygen is continuously consumed by the microorganisms for their metabolic activities and the product is formed by using oxygen, the cell and product yields on oxygen can be also calculated as:

$$Y_{X/O} = \frac{r_X}{-r_O} \tag{2.37}$$

$$Y_{P/O} = \frac{r_P}{-r_O}$$

(2.38)

The yield coefficients which are commonly used for the derivation of mathematical models in microbial fermentation process are listed in Table 2.6.

Table 2.6 Definitions of commonly used yield coefficients

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

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals

All chemicals used in this study were analytical grade and purchased from Difco Laboratories, Fluka Co., Merck & Co. Inc. and Sigma Aldrich Co.

3.2. The Microorganism

The microorganism which was utilized for extracellular human growth hormone production was *Bacillus subtilis* 1A178 (*scoC*$^-$) strain carrying pMK4::*pre*(*subC*)::*hGH* plasmid (Şentürk, 2006; Özdamar et al., 2009). The hybrid-gene composed of two DNA fragments, i.e., signal peptide coding region (*pre*-) of *B. licheniformis* SAP gene (*subC*) and complementary DNA (cDNA) encoding hGH, behind *degQ* promoter was cloned into the pMK4 plasmid (Özdamar et al., 2009). The designed and constructed recombinant system was transferred into *B. subtilis* 1A178 (*scoC*$^-$). In *B. subtilis* 1A178 (*scoC*$^-$), *scoC*, responsible from the production of alkaline protease and inhibitory of sporulation, was deleted from the host organism genome.

To conserve both the viability and the productivity of the cell in the long term, the recombinant microbial culture was maintained on porous beads in a vial named Microbank^{TM} (Pro-Lab Diagnostics) and stored at -80°C.

3.3. Recombinant Human Growth Hormone Production by *B. subtilis* (*scoC*$^-$)

In the present study some main steps were conducted successively for extracellular human growth hormone production by recombinant *B. subtilis*. The media/systems used in these steps were as follows.

3.3.1. Microbank

Microbank^{TM} (Pro-Lab Diagnostics) is a sterile vial composed of chemically treated porous beads which serve as improved carriers to support microorganisms and specially formulated cryopreservative liquid which ensures longer survival and higher recoveries for both bacterial and fungal cultures. At the beginning of the study young colonial cells from the pure culture were inoculated into this vial and it was inverted 4-5 times to emulsify microorganisms. After waiting for 1-2 minutes to adsorb the cells onto the surface of porous beads, the excess cryopreservative fluid was removed to leave the inoculated beads as free of liquid as possible before the long term storage at -80°C.

3.3.2. Solid Medium

The solid medium which was used for the growth of recombinant *B. subtilis* in hGH production process was prepared according to the composition in Table 3.1 (Çalık, 1998a).

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

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

After sterilization with saturated steam at 121°C and 1.2 atm for 20 minutes in autoclave, chloramphenicol was added to the medium (to be 7.14 µg mL^{-1} in the medium) as an antibiotic to preserve the plasmid of the recombinant bacterium. The petri dish containing nearly 25 mL of the medium was kept for a while for the solidification of agar before microorganisms were transferred onto the solid medium via streaking of an inoculated porous bead from Microbank^{TM} under aseptic conditions. Cells were incubated at 37°C for 24 h for the growth and then, stored at 4°C.

3.3.3. Precultivation Medium

The precultivation medium which was used for the growth of recombinant *B. subtilis* in hGH production process was prepared according to the composition in Table 3.2 (Çalık, 1998a). After sterilization with saturated steam, the required amount of filtered $CaCI_2$ solution and chloramphenicol were added to the medium (to be 7.14 µg mL^{-1} in the medium).

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

Component	**Concentration, g L^{-1}**
Soytone	15.00
Peptone	5.00
$CaCI_2$	0.10
Na_2HPO_4	0.25
$MnSO_4.2H_2O$	0.01

Thereafter, the laboratory scale air-filtered Erlenmeyer flask containing 33 mL of the precultivation medium in a total volume of 150 mL was inoculated with the cells grown on the solid medium under aseptic conditions, this flask was placed

to the orbital shaker (B. Braun, Certomat BS-T) to incubate the cells at 37°C and 200 min^{-1} until the cell concentration of C_x = 0.6-0.7 g L^{-1} (OD_{600} = 0.28-0.34 where DF = 6) was obtained in approximately 3 hours.

3.3.4 Production Medium

The production medium which was used for hGH production by recombinant *B. subtilis* was prepared according to the composition in Table 3.3 (Çalık, 1998a). Based on the study of Gökbudak (2013), this reference medium contained 5 mL L^{-1} trace salt solution PTM1 (Table 3.4), as well. After sterilization with saturated steam, again chloramphenicol was added to the medium (to be 7.14 µg mL^{-1} in the medium). Then, the cells grown in the precultivation medium were transferred into the laboratory scale air-filtered Erlenmeyer flask containing 100 mL of the production medium in a total volume of 500 mL with an inoculation ratio (IR) of 1:10 under aseptic conditions. Afterwards, the flask was placed to the orbital shaker (B. Braun, Certomat BS-T) to incubate the cells at 37°C and 200 min^{-1} until the cell concentration of C_x = 0.6-0.7 g L^{-1} (OD_{600} = 0.28-0.34 where DF = 6) was obtained. At that time 150 µl protease inhibitor cocktail (Sigma P-2714) was added to the culture medium.

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

Component	**Concentration, g L^{-1}**
Glucose	8.000
$(NH_4)_2HPO_4$	4.710
KH_2PO_4	2.000
$MgSO_4$	0.122
Na_2HPO_4	0.043
NaH_2PO_4	5.630
PTM1 (mL)	5.000

Table 3.4 The compound of trace salt solution PTM1 (Sibirny et al., 1990)

Component	Concentration, g mL^{-1}
$CuSO_4 \cdot 5H_2O$	0.600
NaI	0.008
$MnSO_4 \cdot H_2O$	0.300
$Na_2MoO_4 \cdot 2H_2O$	0.020
H_3BO_3	0.002
$ZnCI_2$	2.000
$FeSO_4 \cdot 7H_2O$	6.500
$CoCI_2 \cdot 6H_2O$	0.090
H_2SO_4	0.500
Biotin	0.020

3.3.5 Scale-up Steps for Pilot-Scale Production

The pilot-scale bioreactor system used for both batch and semi-batch rhGH production experiments in the present study is given in Figure 3.1. For the first step, cells from Microbank™ were streaked onto the solid medium. Then, cells grown on this medium were inoculated into the precultivation medium with an inoculation ratio of 1:10 as the second step. Thereafter, inoculation was carried out from the first precultivation medium to the second one again with an inoculation ratio of 1:10 in the third step. Finally, cell culture was transferred from the second precultivation medium to the production medium in the pilot-scale bioreactor with the same inoculation ratio.

Experiments to enhance hGH production by recombinant *B. subtilis* were conducted at two different scales, namely laboratory-scale carried out in air-filtered shake bioreactors and pilot-scale performed in the pilot-scale bioreactor.

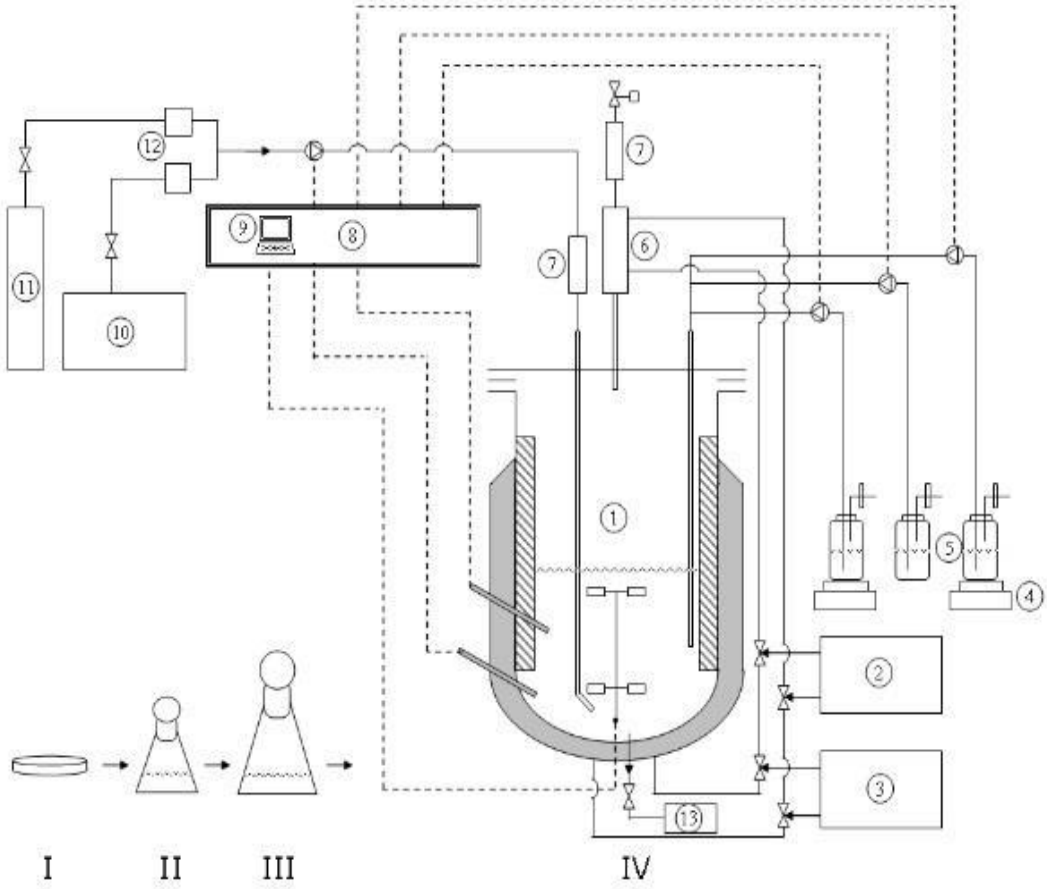

Figure 3.1 Schematic setup of pilot scale bioreactor system. I: Solid medium; II: Precultivation medium-I, V = 33 mL; III: Precultivation medium-II, V = 100 mL; IV: Pilot scale bioreactor system, V = 1 L which is composed of (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (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 O2/N2 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, the solid medium and the precultivation medium used were not changed. However, the composition of the production medium was altered by the addition of peptone at different concentrations

to investigate the effects of this complex carbon and nitrogen source on the cell growth and rhGH production both in the absence and the presence of $MgSO_4$. The production media used in the laboratory-scale rhGH production processes except the reference one, named as the control medium (Table 3.3), are given in Table 3.5. The microorganisms were inoculated into these production media following the steps mentioned in Sections 3.3.2 and 3.3.3.

Table 3.5 The compounds of the production media used in laboratory-scale air-filtered shake bioreactor experiments

Medium	**P-2**	**P-4**	**P-6**	**P-8**	**PM-2**	**PM-4**	**PM-6**	**PM-8**
Component	**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	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
PTM1 (mL)	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Peptone	2.000	4.000	6.000	8.000	2.000	4.000	6.000	8.000
$MgSO_4 \cdot 7H_2O$	-	-	-	-	0.250	0.250	0.250	0.250

3.3.5.2 Batch Experiments in Pilot-Scale Bioreactor

In this study a 3.0 L pilot-scale bioreactor (B. Braun CT2-2) having a working volume of V_R = 0.5-2.0 L was used for both batch and semi-batch pilot-scale hGH production experiments. The system contains some basic operation units to control different bioprocess parameters such as temperature, pH, foam, dissolved oxygen, stirring rate, aeration rate and feed inlet rate. In addition to these, there is an

external cooler and a heat generator connected to the system for in-situ sterilization and temperature control. In-situ sterilization of the bioreactor is performed by saturated steam at 121°C and 1.2 atm for 20 minutes before loading of 1 L sterilized production medium into the vessel. Moreover, circulation of cold and hot water in jacket around the bioreactor is essential for temperature control. In this system, stirring is supplied by two Rushton type impellers with four blades, aeration is provided by an air compressor and a pure oxygen tube and homogenous oxygen distribution is maintained by the help of four baffles and a sparger. Dissolved oxygen concentration is adjusted by a mass flow controller. Addition of antifoam, feed, acid or base solutions to the system are carried out by the way of inlet ports.

The steps applied for rhGH production in the pilot-scale bioreactor were:

1. The system was run a few hours earlier for polarization of the oxygen probe.
2. Pure water in the bioreactor was discharged completely.
3. pH probe was disconnected from the bioreactor to calibrate. For pH calibration standard buffer solutions (Mettler Toledo) at pH = 4.0 and pH = 7.0 were utilized. Then, the probe was placed to the bioreactor again.
4. Before starting the sterilization the bioreactor was filled with pure water above the upper impeller.
5. The gas inlet filter was checked to be sterilization position.
6. The glass part of the bioreactor was covered with the protective jacket made by stainless steel.
7. Afterwards, sterilization with saturated steam at 121°C and 1.2 atm for 20 minutes was started from the control unit.
8. At the same time glucose and salt solutions in the production medium were sterilized separately with saturated steam at 121°C and 1.2 atm for 20 minutes in autoclave.
9. After cooling to an adequate temperature, the sterilized production medium with a volume of 1 L was loaded to the bioreactor by a sterilized silicon hose.
10. Operation conditions of the bioreactor were adjusted to optimal values; T = 37°C; pH_0 = 7.5, N = 750 min^{-1} and Q_0/V_R = 0.5 vvm.
11. pO_2 calibration was performed after adjustment of temperature and agitation speed. For this aim, firstly the production medium was deoxygenated

completely (adjusted to pO_2 = 0 %) with pure N_2 feeding while there was no air inlet. And then the production medium was oxygenated totally (adjusted to pO_2 = 100 %) by air fed to the bioreactor from the compressor.

12. Thereafter, the production medium was inoculated with cells grown sufficiently in the precultivation medium-II by a sterilized silicon hose with an inoculation ratio of 1:10.
13. At 12th hours of the production process 1500 µl protease inhibitor cocktail (Sigma P-2714) was added to the system.
14. Throughout this bioprocess 5 M KOH solution was used in order to ensure the constant pH value (pHc = 7.5) and sterilized Antifoam A solution (30 %, v/v) (Sigma 5758) was dropped into the medium in the case of foaming.

3.3.5.3 Semi-Batch Experiments in Pilot-Scale Bioreactor

The steps applied for rhGH production in batch operation were also used for semi-batch rhGH production processes. Nevertheless, for each feeding strategy developed, a semi-defined feed medium consisting of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 50 or 100 g L^{-1} peptone and 5 mL L^{-1} PTM1 was prepared as the substrate stock solution and then, exponentially fed to the bioreactor according to a pre-determined specific growth rate, μ_0=0.10, 0.17 or 0.25 h^{-1}, starting from t_T=2 or 4 h. Glucose was the main feeding substrate in all semi-batch bioreactor experiments so the volumetric feed rate of this substrate was calculated by using the Equation 2.29 which was derived in Section 2.6.3.1.2 and simplified by the assumption of C_{S0}>>C_S (Yamanè and Shimizu, 1984; Çelik et al., 2009; Çalık et al., 2010):

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

In Equation 3.1, $Q_S(t)$ is the volumetric feed rate of the substrate (L h^{-1}), μ_0 is the pre-determined specific growth rate (h^{-1}), V_0 is the initial bioreactor volume (L), C_{X0} is the initial cell concentration (g L^{-1}), C_{S0} is the initial feed substrate concentration (g L^{-1}), $Y_{X/S}$ is the cell yield on substrate (g g^{-1}) and t is the feeding time (h). The pre-fixed cell yield on substrate was $Y_{X/S}$=0.27 g g^{-1} according to the

study of Şahin (2010), the initial feed substrate concentration in the substrate stock solution was C_{S0}=200 g L^{-1} and the initial bioreactor volume was V_0=1.1 L.

3.4 Analysis

In all hGH production experiments samples taken from the bioreactor at certain time intervals were firstly analyzed to determine the cell concentration. After centrifugation at +4°C and 12000 rpm for 10 minutes in order to precipitate biomass, supernatant was filtered using 0.45 μm porous filters (Sartorius, AG) and filtrate was used to measure substrate (glucose), product (hGH), by-product (organic acids) concentrations and protease activity subsequently.

3.4.1 Cell Concentration

Cell concentration was measured using UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) at 600 nm where was selected as optimum in the range of 200-800 nm on the basis of cell dry weights. The calibration curve drawn for *B.subtilis* at this wavelength is given in Appendix C. Samples taken from the bioreactor were diluted with distilled water to obtain an absorbance value in the range of 0.1-0.8.

3.4.2 Glucose Concentration

In order to measure the glucose concentration in the production medium Glucose Analysis Kit (Biyozim-Biasis) and UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) at 505 nm were used, respectively. The components of the analysis kit and other equipments needed during this measurement are given in Appendix A.

In the assay, gluconate and hydrogen peroxide are produced as a result of the oxidation reaction of D-glucose catalyzed by glucose oxidase in the presence of H_2O and O_2 (Equation 3.2). Then, hydrogen peroxide formed in the first step reacted with 4-aminoantipyrine and phenol in the catalysis of peroxidase and iminoquinone was

synthesized (Equation 3.3). The glucose concentration in the medium is proportional to the concentration of iminoquinone and red color of this compound gives the maximum absorbance at 505 nm.

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

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

First of all standard glucose solutions at certain concentrations (Appendix A) were prepared in order to obtain the calibration curve (Appendix C) required to calculate glucose concentration in the sample from the absorbance value. And then, glucose analysis procedure given below was applied:

1. In the first step glucose analysis reagent in Glucose Analysis Kit (Biyozim-Biasis) was dissolved in 2.50 mL glucose analysis buffer via shaking vigorously for one minute.
2. Then, samples were diluted with distilled water in order to obtain a glucose concentration less than or equal to 2 g L^{-1} at the end of the analysis.
3. Afterwards, test tubes were called as blank, standards or samples. Meanwhile analysis solutions and test tubes were strictly kept at analysis temperature (25^oC or 37^oC).
4. 0.05 mL standard solution or sample was first put in the test tubes.
5. Then, 0.05 mL glucose analysis reagent and 0.40 mL glucose analysis buffer were added into the test tubes, respectively.
6. Final volume of mixture was completed to 2.50 mL with distilled water subsequently.
7. Mixture in the test tubes was kept at 25^oC for 40 minutes or at 37^oC for 20 minutes.
8. For zero base in UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) blank solution prepared without glucose was used.
9. Absorbance values were eventually obtained by using UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) at 505 nm.

3.4.3 hGH Concentration

During the experiments hGH concentration was analyzed by using High Performance Capillary Electrophoresis, HPCE (Agilent Technologies). As demonstrated in Figure 3.2, the system is mainly composed of a sample injection system, a high-voltage power supply connected to the electrodes (anode and cathode) from both sides, a source and a destination vials filled with an electrolytic solution and an aqueous buffer, respectively, a capillary column with a small window near its cathodic end, a UV detector and a computer as an output device. Çalık (1998a) reported that a wide variety of biomolecules as carbohydrates, amino acids, peptides, macromolecular proteins, organic acids, polynucleotides and many other biopolymers can be separated efficiently based on their electrophoretic mobility by this instrumental technique.

At the beginning of the analysis sample is introduced to the capillary tube by the effect of pressure or voltage. Then, it migrates from anode to cathode through the column due to the electrophoretic flow of the buffer solutions under applied electric field. The optical viewing window on the tube allows UV-VIS light to pass through the analyte and measure the absorbance. At the end of the assay the composition of the sample is identified in terms of concentrations of ionic species from the electropherogram.

In this study a fused silica capillary tube with a length of 72 cm and an inner radius of 75 μm was utilized in HPCE system. Firstly 50 mM borate buffer (pH=10) was prepared as the aqueous buffer and then AccuPure Z1-Methyl reagent (Waters) was added in order to obtain the electrolyte solution (Appendix B). This reagent was used to minimize protein adsorption on the capillary surface. The hGH analysis was conducted at $15^{o}C$ and 12 kV for 40 minutes and the wavelength used for UV detection was λ=214 nm (Çalık et al., 1998a). A calibration curve (Appendix C) was drawn by using standard hGH solutions (0.050, 0.075 and 0.1 g L^{-1}) which were prepared from a commercial hGH preparation (Humatrope, Eli Lilly and Company) to calculate hGH concentration from the sample electropherogram (Appendix D).

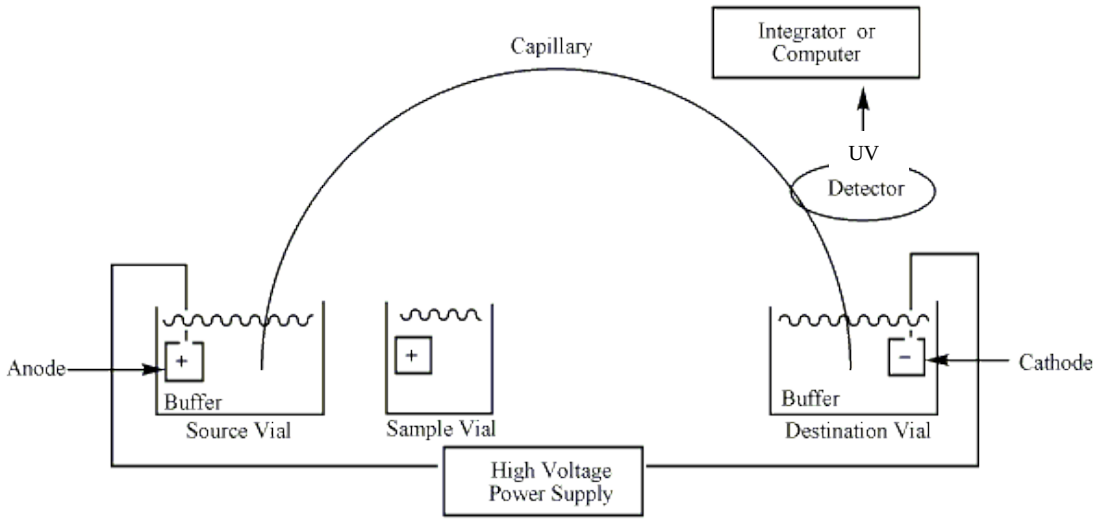

Figure 3.2 Instrumental setup of a common HPCE system (http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/Capillary_Electrophoresis)

The operational conditions and the procedure used for hGH analysis in HPCE were as given below (Çalık et al., 1998a):

- Column: Capillary column packed with SiO_2
- Column dimensions: 72 cm x 75 µm
- Power supply: Positive
- Operating voltage: 12 kV
- Injection type: Hydrostatic pressure
- Injection volume: 10 µL
- Analysis temperature : 15°C
- Electrolyte solution: Modified borate buffer (pH=10)
- Detector: UV (λ=214 nm)
- Duration of analysis: 40 min

3.4.4 Organic Acid Concentration

During hGH production experiments High Performance Liquid Chromatography, HPLC (Waters, Alliance 2695) was utilized in order to determine concentrations of organic acids excreted into the production medium. The calibration

curves given in Appendix C were obtained using standard organic acid solutions at certain concentrations. Via these calibration curves organic acid concentrations in samples were calculated from the related peak areas in the chromatogram. Samples filtered by 0.45 µm porous filters (Sartorius, AG) were diluted with a ratio of 1:3 with distilled water not to damage the column before the analysis.

The operational conditions and the procedure used for organic acid analysis in HPLC were as listed below (İleri and Çalık, 2006):

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

3.4.5 Protease Activity Assay

The method based on the hydrolysis of casein as a protein substrate was used in order to determine protease activity spectrophotometrically in the supernatants of the samples taken from the bioreactor throughout hGH production process. Moon and Parulekar (1991) defined one unit (1 U) protease activity as the activity that is responsible for 4 nmol tyrosine release per unit time. Three different buffer solutions (Appendix B) were utilized to identify total protease activity since three different types of proteases, acidic, neutral and alkali, were analyzed separately. First of all, 0.5 % (w/v) casein solutions were prepared by the addition of casein into these buffer solutions (0.05 M sodium acetate buffer, pH=5.0; 0.05 M sodium phosphate buffer, pH=7.0; 0.05 M borate buffer, pH=10.0). Then, 2 mL of the casein solution was added to 1 mL of the supernatant diluted with the buffer of interest previously and the mixture was incubated at 30^oC and 200 min^{-1} for 20 minutes in the orbital shaker (B. Braun, Certomat BS-T) for hydrolysis of the protein. After all, 2 mL of 10 %

(w/v) trichloroacetic acid (TCA) solution was supplemented to the medium which was kept on ice for 20 minutes in order to stop the reaction. Finally, centrifugation followed by incubation at room temperature for 5 minutes was conducted at 4°C, 10500 rpm for 10 minutes and the absorbance of the supernatant was measured at 275 nm with UV-Vis Spectrophotometer (Thermo Spectronic, Heλios-α) by using quartz cuvettes. For the blank solution the buffer of interest was used instead of sample and protease activity (U mL^{-1}) was calculated from the absorbance value by using the following equation (Çalık, 1998a):

$$A = \left(\frac{Absorbance}{0.8 \times 1/\mu mol.cm^{-3}} \right) \left(\frac{1U}{4nmol/\min} \right) \left(\frac{1}{20\min} \right) \left(\frac{1000nm}{1\mu mol} \right) (Dilutionratio)$$

(3.4)

3.4.6 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

Dynamic method explained in Section 2.5.3 was applied in order to determine oxygen transfer characteristics (K_La and OUR) at every four hours of hGH production process. Before the inoculation of the cells into the bioreactor, physical liquid phase mass transfer coefficient (K_La_0) was also calculated by using this method.

CHAPTER 4

RESULTS AND DISCUSSION

In this study, effects of batch and semi-batch operations were investigated in order to increase recombinant human growth hormone (rhGH) production by *B. subtilis* 1A178 (*scoC*$^-$) strain carrying pMK4*::pre(subC)::hGH* plasmid (Özdamar et al., 2009; Şahin, 2010; Gökbudak, 2013). For this purpose, first air-filtered shake bioreactor experiments at laboratory-scale were conducted to determine the influences of peptone as the complex carbon and nitrogen source on the cell growth and rhGH production. The effects of the inorganic salt $MgSO_4$, in the presence of peptone were also investigated. In the second phase of the research, a batch operation was carried out in the pilot-scale bioreactor by using the designed production medium; thereafter, various exponential feeding strategies were developed. Semi-batch operations were implemented by using the glucose-based semi-defined medium containing peptone at two different concentrations. The transition cultivation time (t_T) indicating the time of shift from batch operation to semi-batch operation was also investigated in these pilot-scale rhGH production processes. Throughout the operations, the cell, glucose, rhGH, organic acid concentrations and protease activities were calculated as well as fermentation characteristics such as specific rates, yield coefficients and oxygen transfer characteristics and evaluated in order to determine the efficiency of the feeding strategies designed.

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

The use of semi-defined medium containing only one or two complex components generally increases both the cell growth and protein production in microbial processes. In addition to this, magnesium (Mg), one of the most required elements by microorganisms, is essential for the activity of various enzymes and the stabilization of nucleic acids, ribosomes and membranes while another macronutrient, sulphur (S), is necessary for the construction of some amino acids and vitamins. Therefore, firstly the effect of peptone which is an enzymatic digest of animal proteins on the cell formation and rhGH production both in the absence and presence of $MgSO_4$ was investigated in air-filtered shake bioreactors.

4.1.1 Effects of Peptone and $MgSO_4$ on the Cell Growth, Substrate Consumption and rhGH Production

In the laboratory-scale experiments, the production medium which was previously developed for rhGH production by recombinant *B. subtilis* strains (Şentürk, 2006) was used as a reference medium and the influences of peptone was investigated at four different concentrations. The reference medium containing 2 g L^{-1} peptone, 4 g L^{-1} peptone, 6 g L^{-1} peptone, and 8 g L^{-1} peptone were named as P-2, P-4, P-6 and P-8, respectively; whereas the media containing 0.122 g L^{-1} $MgSO_4$ in addition to peptone were called as PM-2, PM-4, PM-6 and PM-8, respectively. In air-filtered shake bioreactor experiments, the working volume was V_R=110 mL and the operating conditions were T=37°C, N=200 min^{-1} and pH_0=7.5 (Yılmaz, 2008). Furthermore, at the beginning of the production process, 500 µL PTM1 and at t=12 h, 150 µL protease inhibitor cocktail were added to the reference medium based on the results of Gökbudak (2013).

The cell concentration profiles obtained by the use of the designed media were shown in Figure 4.1. In each case, the concentration of cells increased linearly until t=9 h and then, the stationary phase started following to the exponential growth phase. The increase in peptone concentration significantly enhanced the cell

formation. Additionally, the medium having $MgSO_4$ provided better cell growth than the $MgSO_4$-free medium. Hence, the highest cell concentration was obtained as C_X=1.92 g L^{-1} at t=24 h in the medium PM-8 containing 8 g L^{-1} peptone and 0.122 g L^{-1} $MgSO_4$ whereas the maximum cell concentrations achieved at the same time by using the control medium, P-2, PM-2, P-4, PM-4, P-6, PM-6, and P-8 were 0.96, 1.05, 1.15, 1.25, 1.33, 1.45, 1.59, and 1.86 g L^{-1}, respectively.

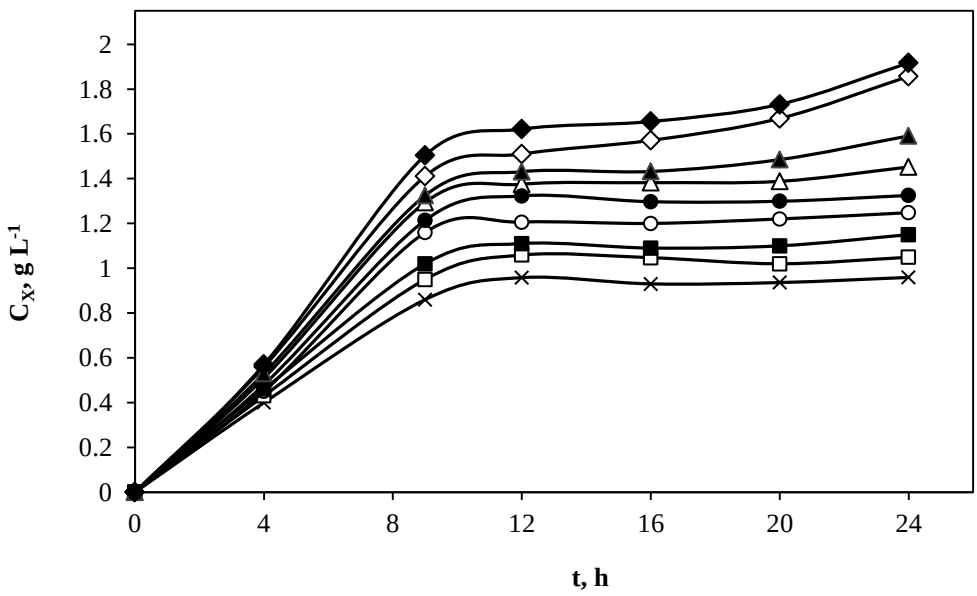

Figure 4.1 The variations in cell concentration with the cultivation time in nine designed media used for laboratory-scale rhGH production by *B. subtilis* 1A178 (*scoC*⁻): Control (x), P-2 (□), PM-2 (■), P-4 (○), PM-4 (●), P-6 (Δ), PM-6 (▲), P-8 (◊), PM-8 (♦)

The variations in glucose concentration with respect to the cultivation time in the air-filtered shake bioreactors containing nine designed media were given in Figure 4.2. Although there were some differences in glucose consumption rates in the early stages of rhGH production process, the concentration of glucose rapidly decreased until t=12 h and then the carbon source was completely consumed in P-2, PM-2, P-4, PM-4, P-6, PM-6, P-8 and PM-8 media. Thus, the initiation of the stationary phase was directly related to the depletion of glucose in the media containing peptone. The decrease in peptone concentration and the absence of

$MgSO_4$ in the culture broth reduced the glucose consumption rate so glucose was utilized more slowly in the control medium and its concentration fell below the limiting value barely after t=16 h.

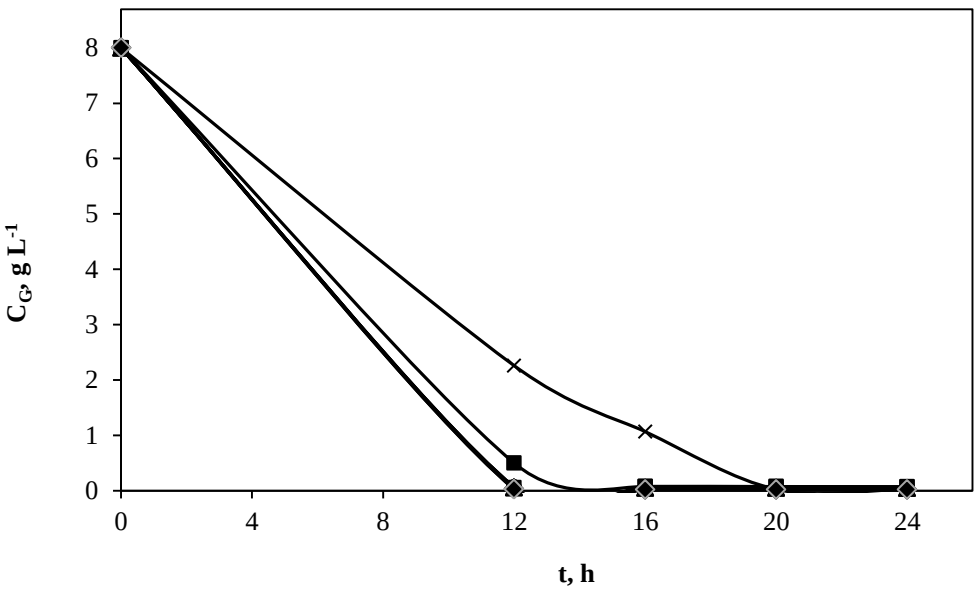

Figure 4.2 The variations in glucose concentration with the cultivation time in nine designed media used for laboratory-scale rhGH production by *B. subtilis* 1A178 (*scoC*$^-$): Control (x), P-2 (□), PM-2 (■), P-4 (○), PM-4 (●), P-6 (Δ), PM-6 (▲), P-8 (◊), PM-8 (♦)

The changes in rhGH concentration with the cultivation time in each medium were presented in Figure 4.3. Similar to the cell concentration profiles, the increase in peptone concentration and the use of $MgSO_4$ in the production medium enhanced the rhGH production. The highest rhGH concentration was attained as C_{rhGH}=140 mg L^{-1} at t=24 h in the medium PM-8 containing 8 g L^{-1} peptone and 0.122 g L^{-1} $MgSO_4$. Nevertheless, the maximum rhGH produced at the same time in the control medium, P-2, PM-2, P-4, PM-4, P-6, PM-6 and P-8 were 62, 74, 77, 81, 98, 111, 120 and 133 mg L^{-1}, respectively. Air-filtered shake bioreactor experiments were ended at t=24 h, before a decrease in rhGH concentration since the highest rhGH production by *B. subtilis* 1A178 (*scoC*$^-$) in PTM1-free reference medium was achieved at=24 h (Şahin, 2010; Gökbudak, 2013).

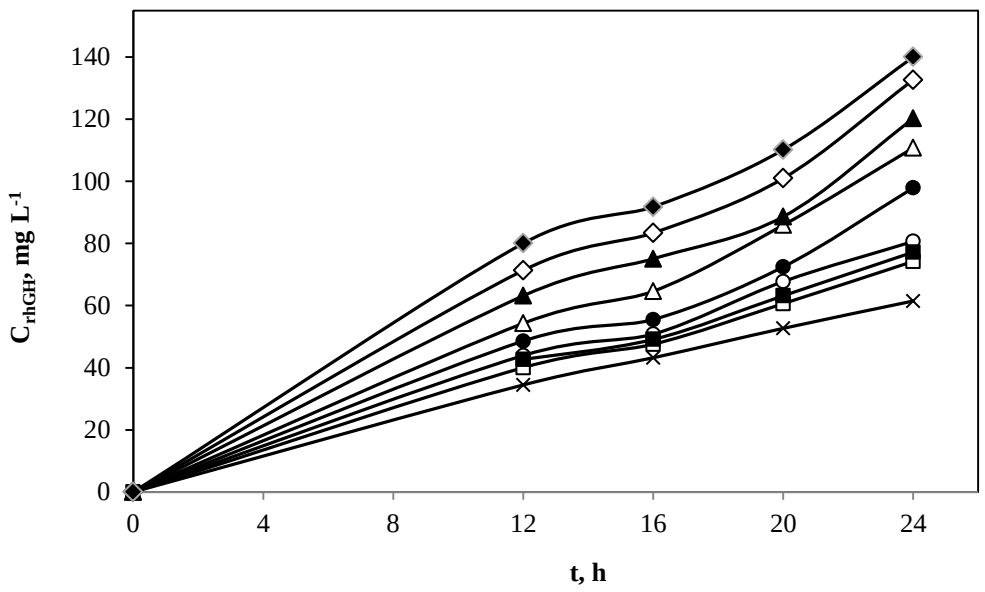

Figure 4.3 The variations in rhGH concentration with the cultivation time in nine designed media used for laboratory-scale rhGH production by *B. subtilis* 1A178 (*scoC*⁻): Control (x), P-2 (□), PM-2 (■), P-4 (○), PM-4 (●), P-6 (Δ), PM-6 (▲), P-8 (◊), PM-8 (♦)

In consequence of these results, the complex carbon and nitrogen source peptone was employed together with the inorganic salt $MgSO_4$ in the design of the production media for the batch and semi-batch bioreactor experiments at pilot-scale in order to improve the microbial growth and rhGH production simultaneously. However, the concentration of peptone in the semi-defined continuous feed medium was investigated in order to avoid its accumulation in the fermentation medium. Moreover, since the solubility of $MgSO_4$ was relatively low, this component was used batch-wise and added only to the initial production medium which was well-mixed throughout the bioprocess. Thus, the precipitation of the inorganic salt was prevented.

4.2 Recombinant Human Growth Hormone Production by Batch and Semi-Batch Bioreactor Experiments at Pilot-Scale

Following the air-filtered shake bioreactor experiments at laboratory-scale, a series of batch and semi-batch operations were designed by evaluating the outcomes of the preliminary research programme and the previous studies in the literature on rhGH production by *Bacillus* species. Then, they were consecutively performed at pilot-scale in order to enhance the recombinant protein production basically by achieving an increase in cell density.

When the desired metabolite production fairly depends on the cell concentration in the culture broth, semi-batch technique is favorable since this intermediary operation mode directly triggers the cell growth in microbial processes. Even though several semi-batch bioreactor experiments were carried out in order to improve rhGH production by *B. subtilis* 1A178 (*scoC*$^-$) in our research group (Gökbudak, 2013), the use of defined medium as the continuous feed limited both the bacterial growth and rhGH formation. For this reason, the substrate stock solution consisting 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 was designed for feeding (Gökbudak, 2013); and, the maximum cell density and the highest rhGH concentration were achieved by the use of this semi-defined feed medium together with an effective exponential feeding strategy.

Hence, in this study the semi-defined production medium containing peptone as the glucose-based semi-defined feed medium was used in the batch operation and the first semi-batch bioreactor experiment which were conducted in order to verify the efficiency of the previous work mentioned above. Thereafter, the following semi-batch bioprocesses were implemented to investigate the effects of the increased peptone concentration in the feed medium and the feeding strategies designed on both the cell growth and rhGH production.

For both batch and semi-batch bioreactor experiments, a 3 L pilot-scale bioreactor was used. The working volume varied in the range of V_R=1.1-2.0 L while the operating conditions were adjusted to T=37°C, pH_0=7.5, N=750 min^{-1} and Q_O/V_R=0.5 vvm as previously optimized by Yılmaz (2008). However, in the last four

semi-batch operations, after the agitation rate was gradually increased from 750 min^{-1} to 900 min^{-1}, oxygen enriched air was supplied to the medium in order to keep the dissolved oxygen concentration above 40 %. In all cases, 5 M KOH solution was used to maintain the hydrogen ion concentration in the fermentation broth at its initial value pH_0=7.5, since the controlled pH strategy was preferred in accordance with the results of Şahin (2010). Furthermore, at the beginning of the production process, 5 mL L^{-1} PTM1 and at t=12 h, 1500 µL protease inhibitor cocktail were added to the production medium according to the results of Gökbudak (2013).

4.2.1 Batch rhGH Production

The first bioreactor experiment at pilot-scale was a batch operation and conducted as a reference experiment in order to determine the behaviour of peptone when this complex carbon and nitrogen source was used in the production medium at the concentration of 2 g L^{-1}. Based on the conclusions of the air-filtered shake bioreactor experiments at laboratory-scale, 0.122 g L^{-1} $MgSO_4$ was also added to the reference medium containing PTM1 (Şentürk, 2006; Gökbudak, 2013).

In the batch operation strategy, the cell concentration rapidly increased until t=8 h, having a value of C_X=1.67 g L^{-1} (Figure 4.4). Then, the stationary phase began as a result of the complete consumption of glucose in the fermentation medium (Figure 4.5). After the highest rhGH concentration was obtained as C_{rhGH}=79 mg L^{-1} at t=16 h (Figure 4.6), the concentration of the recombinant protein started to decrease by the activity of proteases (Figure 4.7).

Based on these results, the use of peptone in the reference medium containing $MgSO_4$ and PTM1 initially improved the cell growth. However, 8 g L^{-1} glucose was not sufficient for the rapidly increased biomass. Since rhGH was produced in the late-growth phase, this short operation enhanced neither the cell formation nor the recombinant protein production.

4.2.2 Feeding Strategy Development for Semi-Batch rhGH Production

Besides the batch operation, six continuous feeding strategies were designed for semi-batch rhGH production process at pilot-scale as summarized in Table 4.1. In all these bioreactor experiments, the production medium was prepared by the addition of 0.122 g L^{-1} $MgSO_4$ to the reference medium containing PTM1 batch-wise (Şentürk, 2006; Gökbudak, 2013) as in the batch operation.

In all semi-batch operations, the substrate stock solution consisting of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 50 or 100 g L^{-1} peptone, 5 mL L^{-1} PTM1 and chloramphenicol at 2-fold higher concentration than that of the production medium was exponentially fed to the bioreactor according to one of three pre-determined specific growth rates of μ_0=0.10, 0.17 and 0.25 h^{-1}. Feeding of the semi-defined designed media were initiated at either t_T=2 h or t_T=4 h and it was smoothly continued the end of rhGH production process.

For the first strategy (BR-1) the pre-determined specific growth rate was selected as μ_0=0.17 h^{-1} and exponential feeding of the semi-defined medium 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 was started at t_T=4 h as in the study of Gökbudak (2013). The main difference of this operation was the addition of 2 g L^{-1} peptone to the initial production medium batch-wise. However, peptone was not used in the production medium in other designed semi-batch operations because of its influence on the rhGH production as a batch component, in BR-1.

In the second strategy (BR-2) the semi-defined medium containing 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 100 g L^{-1} peptone and 5 mL L^{-1} PTM1 was fed to the bioreactor with the pre-determined specific growth rate of μ_0=0.17 h^{-1} within t=4-28 h in order to investigate the effects of 2-fold higher peptone concentration in the feed stream on the cell growth and recombinant protein production.

Table 4.1 Definitions of the feeding strategies designed for semi-batch rhGH production by *B. subtilis* 1A178 (*scoC*$^-$) at pilot-scale

Strategy	Strategy Description
BR-1	Semi-defined medium (200 g L^{-1} glucose + 117.65 g L^{-1} $(NH_4)_2HPO_4$ + 50 g L^{-1} peptone + 5 mL L^{-1} PTM1) exponentially fed with μ_0=0.17 h^{-1} within t=4-26 h to the production medium containing 2 g L^{-1} peptone at t=0 h
BR-2	Semi-defined medium (200 g L^{-1} glucose + 117.65 g L^{-1} $(NH_4)_2HPO_4$ + 100 g L^{-1} peptone + 5 mL L^{-1} PTM1) exponentially fed with μ_0=0.17 h-1 within t=4-28 h
BR-3	Semi-defined medium (200 g L^{-1} glucose + 117.65 g L^{-1} $(NH_4)_2HPO_4$ + 100 g L^{-1} peptone + 5 mL L^{-1} PTM1) exponentially fed with μ_0=0.25 h^{-1} within t=4-24 h
BR-4	Semi-defined medium (200 g L^{-1} glucose + 117.65 g L^{-1} $(NH_4)_2HPO_4$ + 100 g L^{-1} peptone + 5 mL L^{-1} PTM1) exponentially fed with μ_0=0.25 h^{-1} within t=2-24 h
BR-5	Semi-defined medium (200 g L^{-1} glucose + 117.65 g L^{-1} $(NH_4)_2HPO_4$ + 100 g L^{-1} peptone + 5 mL L^{-1} PTM1) exponentially fed with μ_0=0.25 h^{-1} within t=4-18 h and constantly fed according to q_S = 1.6 g g^{-1} h^{-1} within t=18-26 h
BR-6	Semi-defined medium (200 g L^{-1} glucose + 117.65 g L^{-1} $(NH_4)_2HPO_4$ + 100 g L^{-1} peptone + 5 mL L^{-1} PTM1) exponentially fed with μ_0=0.25 h^{-1} within t=4-14 h and with μ_0=0.10 h^{-1} within t=14-28 h

In the third rhGH production strategy (BR-3), the pre-determined specific growth rate was increased to μ_0=0.25 h^{-1} in order to prevent the depletion of glucose in the system while the composition of the substrate stock solution which was exponentially fed to the bioreactor during t=4-24 h was completely same with the feed composition used in the previous strategy (BR-2).

In the fourth semi-batch bioreactor experiment (BR-4), exponential feeding was initiated at t_T=2 h instead of t_T=4 h in order to investigate the effects of the transition cultivation time on the microbial growth and rhGH production. Therefore, neither the composition of the glucose-based semi-defined feed medium nor the pre-determined specific growth rate was same as in the third strategy (BR-3).

For the fifth rhGH production strategy (BR-5), the semi-defined medium was fed to the bioreactor with the pre-determined specific growth rate, μ_0=0.25 h^{-1}, within t=4-18 h and then according to the pre-calculated specific substrate utilization rate q_S=1.6 g g^{-1} h^{-1}, within t=18-26 in order to avoid glucose accumulation in the bioreactor. This value was calculated by using the data of the third semi-batch bioreactor experiment (BR-3).

In the last feeding strategy (BR-6), the semi-defined medium was fed to the bioreactor with the pre-determined specific growth rate, μ_0=0.25 h^{-1}, within t=4-14 h. Later, this value was decreased to μ_0=0.10 h^{-1} for t=14-28 h to prevent the increase in glucose concentration in the culture broth with the cultivation time.

4.2.2.1 Effects of Feeding Strategy on Cell Growth Profiles

The variations in the cell concentration with the cultivation time in the designed semi-batch bioreactor operation strategies together with batch operation strategy were demonstrated in Figure 4.4. When considering the maximum cell densities attained, the microbial growth profiles for the semi-batch bioreactor experiments were similar. Nevertheless, there was a conspicuous difference in the batch process since the cell concentration exponentially increased until t=8 h, only.

The cell growth rate was significantly enhanced by implementing the first feeding strategy (BR-1) which was based on the use of peptone in the initial

production medium as well as in the glucose-based semi-defined feed medium. The following semi-batch bioreactor experiments were also effective in terms of microbial growth compared to the previous studies in the literature related to rhGH production by *B. subtilis* 1A178 (*scoC*$^-$). In contrast to the results reported by Şahin (2010) and Gökbudak (2013), the addition of protease inhibitor cocktail did not adversely affect bacterial growth in the semi-batch operations. While the cell formation continued with a nearly constant rate throughout the production process in BR-1, BR-2 and BR-6, the increase in the cell concentration stopped at t=22 h in the feeding strategies of BR-3, BR-4 and BR-5.

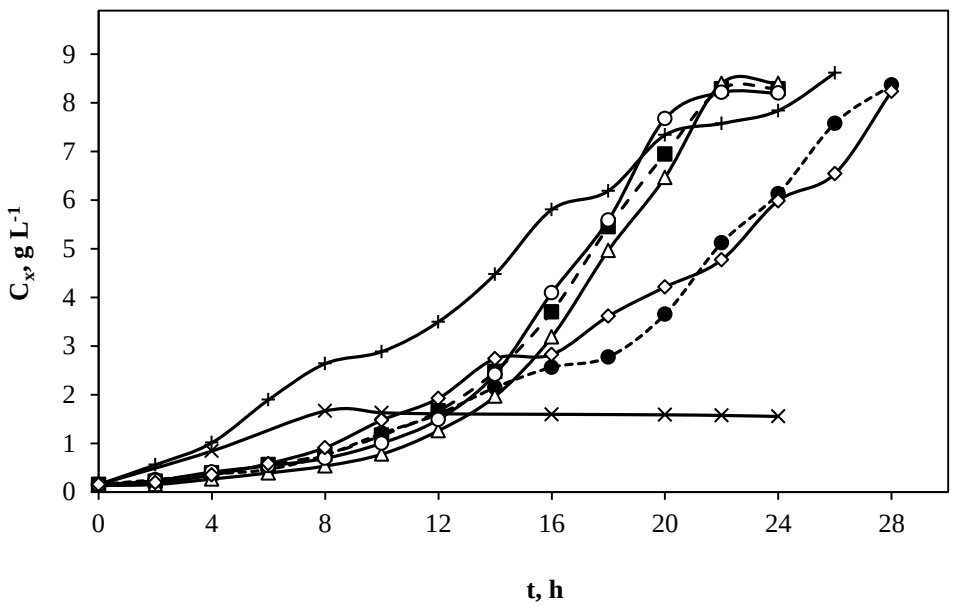

Figure 4.4 The variations in cell concentration with the cultivation time in semi-batch bioreactor operation strategies together with batch operation strategy. C_{G0}=8 g L^{-1}, T=37°C and pH_C=7.5. Batch (x), BR-1 (+), BR-2 (●), BR-3 (■), BR-4 (Δ), BR-5 (o), BR-6 (◊)

Even though the highest cell concentration was achieved by the first feeding strategy (BR-1) as 8.62 g L^{-1} at t=26 h, the maximum cell densities acquired in the other semi-batch bioreactor experiments were also quite high and close to each other; 8.37 g L^{-1} for BR-2, 8.29 g L^{-1} for BR-3, 8.40 g L^{-1} for BR-4, 8.22 g L^{-1} for BR-5 and 8.24 g L^{-1} for BR-6. The highest cell concentration for this bioprocess was previously

reported as C_X=4.43 g L^{-1} by Gökbudak (2013); however, by the use of 2 g L^{-1} peptone batch-wise together with 50 g L^{-1} peptone-containing feed solution in BR-1, 1.95-fold higher cell concentration was obtained. In addition to this, 2-fold higher peptone concentration in the substrate stock solution which was fed to the system with the same pre-determined specific growth rate, μ_0=0.17 h^{-1}, also significantly increased the cell growth but with a lower rate in the second feeding strategy (BR-2). Nevertheless, when the transition cultivation time was changed from t_T=4 h (BR-3) to t_T=2 h (BR-4), the cell growth profile did not change significantly as can be seen in Figure 4.4. Contrarily, feeding higher amounts of the semi-defined medium owing to the increased pre-determined specific growth rate, μ_0=0.25 h^{-1}, shortened the operation time for the cell formation as in BR-3 while lower feed amounts based on the pre-calculated specific substrate utilization rate, q_S = 1.6 g g^{-1} h^{-1}, or the decreased pre-determined specific growth rate, μ_0=0.10 h^{-1}, caused the prolonged the process as in the semi-batch bioreactor experiments of BR-5 and BR-6, respectively.

4.2.2.2 Effects of Feeding Strategy on Substrate Consumption Profiles

In microbial processes the production rate of the desired metabolite is primarily dependent on the cell concentration in the medium. Since the cell formation rate is directly affected by the substrate concentration, not only the depletion of the main carbon and energy source but also the accumulation of any substrate in the fermentation broth should be controlled throughout the operation in order to make use of the production capacity of microorganisms as much as possible.

The main carbon and energy source used in this study was glucose. Hence, the changes in glucose concentration with the cultivation time in semi-batch bioreactor operation strategies together with batch operation strategy were presented in Figure 4.5. In addition to this, semi-batch bioreactor operation parameters based on the carbon source glucose, were given in Table 4.2.

The glucose consumption profiles of the batch operation and the first feeding strategy (BR-1) were almost the same until t=4 h due to the use of identical production media. Although glucose was depleted at t=8 h in both cases, the cell growth continued until the end of the bioprocess, t=28 h, in the semi-batch operation

of BR-1 owing to the exponential feeding of the glucose-based semi-defined medium according to the pre-determined specific growth rate, μ_0=0.17 h^{-1}. In this experiment, glucose started to accumulate at t=20 h. However, feeding was not interrupted since this process was carried out without feedback control as well as all other semi-batch bioreactor experiments in this study.

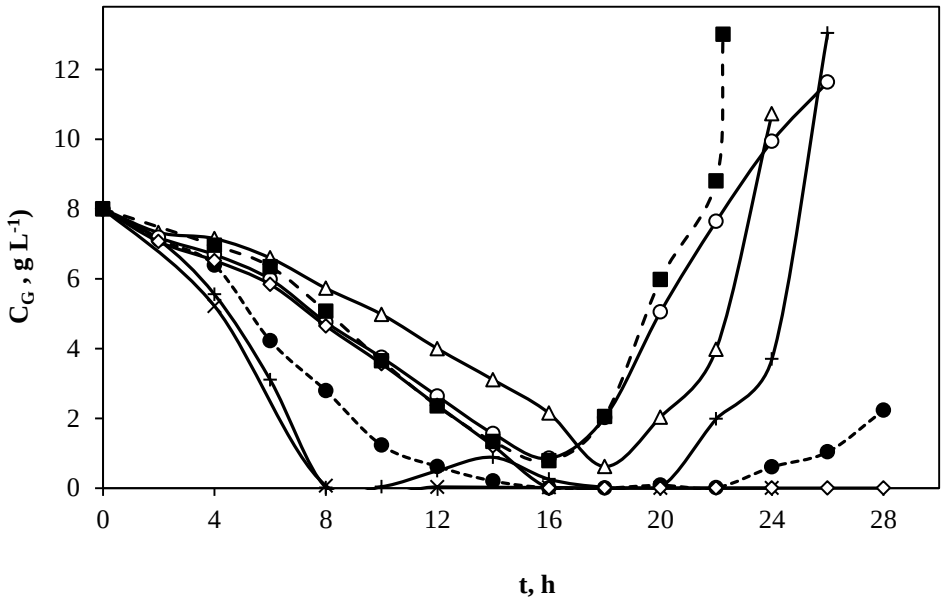

Figure 4.5 The variations in glucose concentration with the cultivation time in semi-batch bioreactor operation strategies together with batch operation strategy. C_{G0}=8 g L^{-1}, T=37°C and pH_C=7.5. Batch (x), BR-1 (+), BR-2 (●), BR-3 (■), BR-4 (Δ), BR-5 (o), BR-6 (◊)

On the other hand, the glucose utilization profiles for the semi-batch operations of BR-2, BR-3, BR-5 and BR-6 were close to each other until t=4 h as expected. In the feeding strategy of BR-2, glucose concentration decreased until t=16 h, then it increased again beginning from t=22 h. Due to the complete consumption of glucose between t=16 h and t=22 h, efficient rhGH production was not achieved in this experiment since this period was critical for the recombinant protein production. Hence for the following feeding strategy (BR-3), the pre-determined specific growth rate was increased from μ_0=0.17 h^{-1} to μ_0=0.25 h^{-1}. Even though glucose was never

exhausted in this case, it was started to accumulate at t=16 h and its concentration reached to C_G=42 g L^{-1} at t=24 h.

Table 4.2 Semi-batch bioreactor operation parameters based on the carbon source

Strategy	Initial glucose for batch operation (g)	Maximum rhGH		Overall values within t=0-t_{max}		
		Cultivation time (h)	Concentration of rhGH, (mg L^{-1})	Total glucose fed (g)	Total glucose consumed (g)	Consumption ratio of glucose (%)
BR-1	8	22	187	153.61	148.57	91.9
BR-2	8	18	154	79.98	85.75	97.5
BR-3	8	22	497	198.31	164.31	79.6
BR-4	8	22	430	140.61	137.89	92.8
BR-5	8	26	462	139.75	136.12	92.1
BR-6	8	28	361	44.30	52.30	100

Since relatively high glucose concentrations in the medium inhibited many metabolic pathways in the cell, glucose accumulation was tried to be avoided in order to enhance rhGH production capacity of microorganisms. In the fifth bioreactor experiment (BR-5), after the substrate stock solution was exponentially fed to the system with μ_0=0.25 h^{-1} within t=4-18 h, feeding was continued within t=18-26 h according to the pre-calculated specific substrate utilization rate, q_s= 1.6 g g^{-1} h^{-1}, in order to keep the glucose concentration in the range of C_G=1-2 g L^{-1}. However, glucose also accumulated in BR-5 starting from t=16 h. Therefore, in the last feeding strategy (BR-6) the pre-determined specific growth rate was decreased from μ_0=0.25 h^{-1} to μ_0=0.10 h^{-1} at t=14 h as an earlier response while glucose was never detected in the fermentation medium after t=16 h in this case.

In addition to these, in the semi-batch bioreactor experiment of BR-4, the glucose concentration decreased much more slowly since the feeding of the glucose-based semi-defined medium was initiated two hours earlier. Although the glucose consumption profile of this operation was very similar to that of BR-3 and BR-5, the

process shifted forwards two hours, namely the accumulation of glucose in the culture broth started at t=18 h instead of at t=16 h.

4.2.2.3 Effects of Feeding Strategy on rhGH Production Profiles

The variations in rhGH concentration with the cultivation time in the designed semi-batch bioreactor operation strategies together with batch operation strategy were presented in Figure 4.6. In general, r-protein concentration increased as parallel to the cell growth and decreased after a maximum because of its hydrolysis by the synthesized proteases.

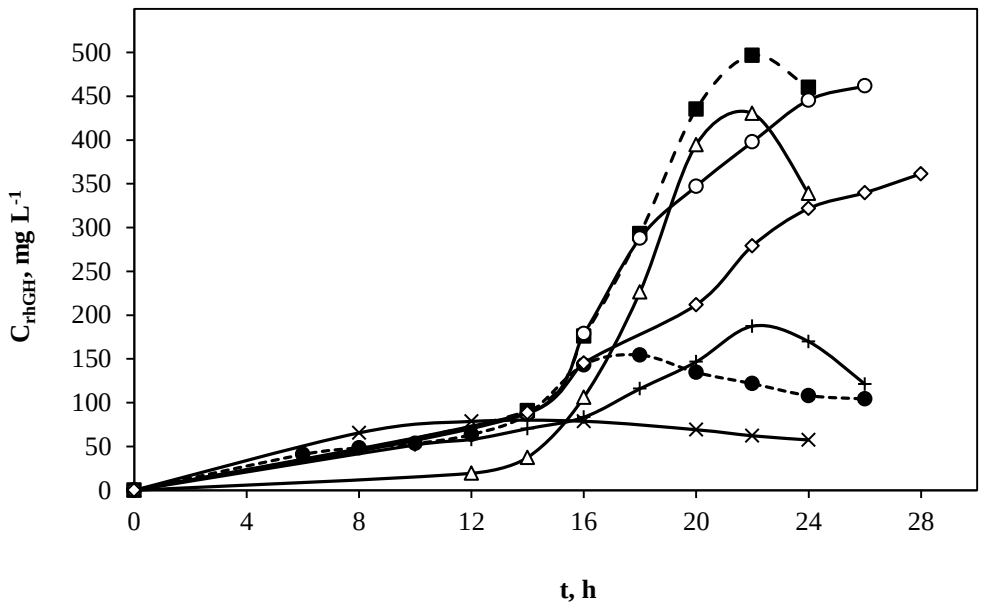

Figure 4.6 The variations in rhGH concentration with the cultivation time in semi batch bioreactor operation strategies together with batch operation strategy. C_{G0}=8 g L^{-1}, T=37°C and pH_C=7.5: Batch (x), BR-1 (+), BR-2 (●), BR-3 (■), BR-4 (Δ), BR-5 (o), BR-6 (◊)

As can be seen in Figure 4.6, the highest rhGH concentration was acquired as C_{rhGH}=497 mg L^{-1} at t=22 h in BR-3 strategy. This production level was 6.29-, 2.66-, 3.23-, 1.16-, 1.08- and 1.38-fold higher than the maximum rhGH concentration obtained in batch, BR-1, BR-2, BR-4, BR-5 and BR-6 operations, respectively. The

pre-calculated $Q_S(t)$ curve resulted the highest recombinant protein production in the third feeding strategy (BR-3) was given in Appendix E.

In the previous studies, the highest rhGH production by *B. subtilis (scoC⁻)* was reported as 366 mg L^{-1} (Gökbudak, 2013). This value was achieved at t=28 h by the exponential feeding of the semi-defined medium 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 with μ_0=0.17 h^{-1} within t=4-25 h. On the other hand, in the current study, 1.36-fold higher rhGH concentration was attained at t=22 h by the exponential feeding of the substrate stock solution consisting of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 100 g L^{-1} peptone and 5 ml L^{-1} PTM1with μ_0=0.25 h^{-1} within t=4-24 h in the feeding strategy of BR-3. As a result, the productivity was significantly increased in this work since the cultivation time required to achieve the maximum rhGH concentration was shortened from t=28 h to t=22 h.

The lowest rhGH production was determined in the batch operation due to the lowest cell growth. On the other hand, even though the highest cell concentration was attained in the first feeding strategy (BR-1), rhGH production level did not increase as much as expected. In this case, the presence of peptone in the initial production medium triggered the microbial growth in the early stages of the bioprocess. However, since the glucose concentration was limited in the late-growth phase, the cell metabolism did not shift to rhGH production instead of biomass formation and/or cell maintenance as in the following bioreactor experiment (BR-2).

The recombinant protein production profiles for the feeding strategies of BR-3 and BR-4 were close to each other. In both cases, the concentration of rhGH rapidly increased starting from t=14 h, gave the maximum at t=22 h and then began to decrease. Nevertheless, the highest rhGH concentration acquired in the semi-batch operation BR-4 was lower due to the lower feed amount. The decreased production level and the prolonged process time in BR-5 and BR-6 were also related to the reduced feeding after t=18 h and t=14 h, respectively.

Hence, the accumulation of glucose in the fermentation medium was more favorable than the depletion of this carbon and energy source for rhGH production by *B. subtilis* 1A178 (*scoC⁻*).

4.2.2.4 Effects of Feeding Strategy on Protease Activity Profiles

High level of protease secretion is one of the major problems in recombinant protein production by *Bacillus* species since the final product yield is significantly decreased by the proteolytic activity of these enzymes. In order to investigate the effects of feeding strategy on extracellular protease production by *B. subtilis* 1A178 (*scoC*$^-$), the protease activity assay given in Section 3.4.5 was implemented for all batch and semi-batch bioreactor experiments. The changes in protease activity with the cultivation time in each operation were shown in Figure 4.7.

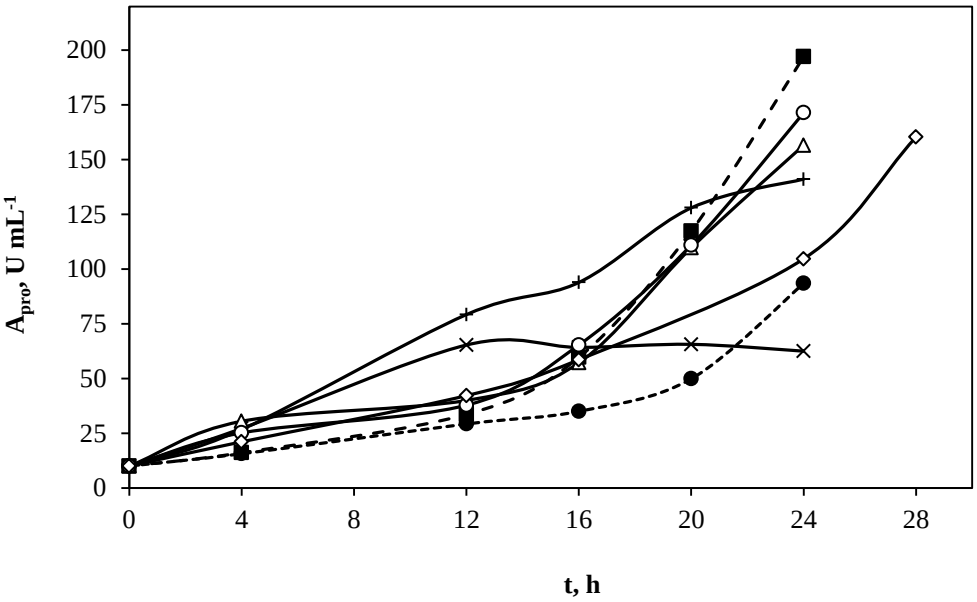

Figure 4.7 The variations in protease activity with the cultivation time in semi-batch bioreactor operation strategies together with batch operation strategy. C_{G0}=8 g L^{-1}, T=37°C and pH$_C$=7.5: Batch (x), BR-1 (+), BR-2 (●), BR-3 (■), BR-4 (Δ), BR-5 (o), BR-6 (◊)

The highest proteolytic activity was attained as A_{pro}=197 U mL^{-1} at t=24 h in the third feeding strategy (BR-3). This value was 2.99-, 1.40-, 2.12-, 1.26-, 1.15- and 1.23-fold higher than the maximum protease activity determined for the batch operation and the semi-batch bioreactor experiments of BR-1, BR-2, BR-4, BR-5 and BR-6, respectively.

In the previous studies regarding to rhGH production by *B. subtilis* 1A178 (*sco*C$^-$), the highest rhGH production was achieved when the total protease activity was minimum. However, the result was different in this study, since 1500 µL protease inhibitor cocktail was insufficiently used for each bioreactor experiment irrespective of the working volume and/or the cell concentration obtained at t=12 h. Moreover, protease inhibitor cocktail addition time and mode (pulse or continuous) were not optimized for the newly developed batch or semi-batch operations.

In the batch bioreactor experiment, the protease activity was slightly reduced by the addition of the protease inhibitor cocktail at t=12. Nevertheless, non-increasing proteolytic activity from t=8 h was mainly related to the interrupted microbial growth in this case. In the feeding strategies of BR-1 and BR-2, the activity increase was also slowed down by the addition of the protease inhibitor cocktail just for a while.

On the other hand, the linear increase in proteolytic activity was not prevented by the addition of protease inhibitor cocktail in the bioreactor experiments of BR-3, BR-4, BR-5 and BR-6. Accordingly, since the protease activity exponentially increased until the end of the bioprocess in these four semi-batch operations, the concentration of rhGH in the medium either decreased or remained constant from a certain moment.

4.2.2.5 Effects of Feeding Strategy on Organic Acid Concentration Profiles

The availability of four-, five- and six-carbon organic acids directly affects the activity of metabolic pathways in the cell since these substances are essential for some certain biochemical reactions particularly in the Krebs (tricarboxylic acid; TCA) cycle. However, in microbial processes if the desired metabolite is a recombinant protein as in this study, the formation of these by-products, should be kept under control throughout the operation. In this context, the variations in the concentrations of the organic acids with respect to time were analyzed for semi-batch bioreactor operation strategies together with batch operation strategy as listed in Table 4.3. The organic acids detected in rhGH production medium were mainly gluconic acid, formic acid, lactic acid, citric acid, α-ketoglutaric acid, succinic acid,

fumaric acid and malic acid. Amongst all these organic acids, the concentration of lactic acid was generally highest while the lowest concentration belonged to fumaric acid in all batch and semi-batch bioreactor experiments.

Gluconic acid is synthesized and enters the pentose phosphate pathway (PPP) when the dissolved oxygen concentration in the medium is fairly high. However if the substrate concentration is insufficient, this organic acid starts to accumulate since it cannot be used in PPP. Therefore, the gluconic acid concentrations in the batch operation and the feeding strategies of BR-1 and BR-6 were relatively high due to the complete consumption of glucose whereas the accumulation of the main carbon and energy source resulted in lower gluconic acid concentrations in the bioreactor experiments of BR-3, BR-4 and BR-5. On the other hand, gluconic acid was not effectively synthesized in the semi-batch operation of BR-2 since the dissolved oxygen concentration in the system was very low after t=20 h.

Formic acid is usually found in nature in the form of formate and this conjugate base is produced via the reduction of carbondioxide by the action of formate dehydrogenase enzyme. In all pilot-scale rhGH production processes, formic acid started to accumulate from the beginning of the operation and the highest formic acid concentration was acquired as 0.180 g L^{-1} at t=24 h in the forth feeding strategy (BR-4). Nevertheless, the maximum formic acid concentrations obtained by the bioreactor operations of BR-3 and BR-5 were also quite high.

Lactic acid is synthesized from pyruvate by the activity of lactate dehydrogenase enzyme in animals, plants and prokaryotes. In the case of facultative anaerobic microorganisms, this process is normally conducted when the dissolved oxygen concentration in the medium is insufficient for aerobic respiration. Thus, relatively high lactic acid concentration was attained by the second semi-batch operation (BR-2). However, if pyruvate is formed faster than it can be metabolized in Krebs cycle, this organic acid is also produced in the presence of oxygen. Since the accumulation of lactic acid in the fermentation medium inhibits microbial growth, the exponential increase in the cell concentration stopped at t=22 h in the feeding strategies of BR-3, BR-4 and BR-5.

Table 4.3 The variations in organic acid concentrations with the cultivation time in batch and semi-batch bioreactor operation strategies

	Concentration, g L^{-1}					
t, h	**4**	**8**	**12**	**16**	**20**	**24**
			BATCH			
Gluconic Acid	0.063	0.144	0.175	0.212	0.244	0.278
Formic Acid	0.025	0.029	0.025	0.035	0.028	0.025
Lactic Acid	0.087	0.120	0.169	0.181	0.259	0.296
Citric Acid	0.002	0.006	0.049	0.057	0.040	0.002
Glutaric Acid	0.001	0.002	0.006	0.007	0.007	0.005
Succinic Acid	0.000	0.000	0.000	0.000	0.000	0.000
Fumaric Acid	0.000	0.001	0.003	0.003	0.002	0.002
Malic Acid	0.010	0.018	0.042	0.044	0.042	0.029
			BR-1			
Gluconic Acid	0.061	0.077	0.096	0.148	0.215	0.377
Formic Acid	0.060	0.046	0.054	0.043	0.058	0.099
Lactic Acid	0.071	0.146	0.203	0.209	0.256	0.328
Citric Acid	0.000	0.001	0.002	0.001	0.043	0.081
Glutaric Acid	0.001	0.001	0.004	0.004	0.008	0.019
Succinic Acid	0.000	0.000	0.033	0.067	0.079	0.070
Fumaric Acid	0.000	0.000	0.001	0.001	0.001	0.001
Malic Acid	0.008	0.018	0.049	0.072	0.169	0.102

Table 4.3 The variations in organic acid concentrations with the cultivation time in batch and semi-batch bioreactor operation strategies (Continued)

	Concentration, g L^{-1}					
t, h	**4**	**8**	**12**	**16**	**20**	**24**
			BR-2			
Gluconic Acid	0.034	0.045	0.064	0.092	0.103	0.144
Formic Acid	0.017	0.010	0.014	0.027	0.062	0.079
Lactic Acid	0.015	0.057	0.175	0.245	0.340	0.388
Citric Acid	0.002	0.004	0.006	0.004	0.004	0.023
Glutaric Acid	0.000	0.000	0.001	0.002	0.005	0.013
Succinic Acid	0.000	0.000	0.000	0.000	0.054	0.079
Fumaric Acid	0.000	0.000	0.000	0.000	0.001	0.001
Malic Acid	0.006	0.019	0.056	0.104	0.172	0.208
			BR-3			
Gluconic Acid	0.033	0.051	0.084	0.112	0.123	0.187
Formic Acid	0.024	0.010	0.033	0.057	0.140	0.173
Lactic Acid	0.015	0.100	0.284	0.419	0.453	0.465
Citric Acid	0.000	0.001	0.005	0.009	0.063	0.079
Glutaric Acid	0.000	0.001	0.002	0.005	0.015	0.032
Succinic Acid	0.000	0.000	0.000	0.065	0.075	0.082
Fumaric Acid	0.000	0.000	0.001	0.001	0.001	0.001
Malic Acid	0.007	0.013	0.034	0.038	0.070	0.103

Table 4.3 The variations in organic acid concentrations with the cultivation time in batch and semi-batch bioreactor operation strategies (Continued)

	Concentration, g L^{-1}					
t, h	**4**	**8**	**12**	**16**	**20**	**24**
			BR-4			
Gluconic Acid	0.027	0.053	0.085	0.101	0.174	0.152
Formic Acid	0.000	0.005	0.018	0.042	0.116	0.180
Lactic Acid	0.021	0.065	0.151	0.292	0.419	0.498
Citric Acid	0.002	0.004	0.008	0.013	0.034	0.066
Glutaric Acid	0.000	0.001	0.001	0.004	0.012	0.022
Succinic Acid	0.000	0.000	0.000	0.048	0.063	0.067
Fumaric Acid	0.000	0.001	0.001	0.001	0.001	0.001
Malic Acid	0.004	0.008	0.018	0.030	0.065	0.085
			BR-5			
Gluconic Acid	0.034	0.056	0.079	0.104	0.106	0.182
Formic Acid	0.023	0.018	0.029	0.076	0.116	0.179
Lactic Acid	0.019	0.077	0.180	0.421	0.449	0.507
Citric Acid	0.000	0.001	0.002	0.022	0.057	0.078
Glutaric Acid	0.000	0.000	0.002	0.007	0.017	0.039
Succinic Acid	0.000	0.000	0.000	0.000	0.076	0.087
Fumaric Acid	0.000	0.000	0.000	0.000	0.001	0.001
Malic Acid	0.009	0.009	0.026	0.049	0.075	0.106

Table 4.3 The variations in organic acid concentrations with the cultivation time in batch and semi-batch bioreactor operation strategies (Continued)

	Concentration, g L^{-1}					
t, h	**4**	**8**	**12**	**16**	**20**	**24**
			BR-6			
Gluconic Acid	0.032	0.058	0.078	0.129	0.173	0.314
Formic Acid	0.026	0.008	0.016	0.043	0.038	0.061
Lactic Acid	0.015	0.056	0.193	0.250	0.321	0.317
Citric Acid	0.002	0.001	0.005	0.017	0.051	0.027
Glutaric Acid	0.000	0.000	0.001	0.005	0.004	0.008
Succinic Acid	0.000	0.000	0.000	0.073	0.022	0.056
Fumaric Acid	0.000	0.000	0.000	0.001	0.001	0.001
Malic Acid	0.004	0.005	0.014	0.045	0.042	0.080

The other organic acids detected in the fermentation medium; namely citric acid, α-ketoglutaric acid, succinic acid, fumaric acid and malic acid are involved in TCA cycle. Therefore, the accumulation of one or more of these intermediates with the cultivation time indicates the low efficiency in aerobic respiration.

Citric acid is a vital organic acid for Krebs cycle since its conjugate base, citrate, is required for the physiological oxidation of various organic compounds including fats, proteins and carbohydrates to carbondioxide and water. In all batch and semi-batch bioreactor operations, the accumulation of this substance started in the early stages of the bioprocess and the highest citric acid concentration was obtained as 0.081 g L^{-1} at t=24 h in the first feeding strategy (BR-1) since dissolved oxygen concentration in the fermentation medium fell below the limited values after t=16 h.

α-Ketoglutaric acid is also a key intermediate in aerobic respiration since TCA cycle can be replenished by anaplerotic reactions at this junction via producing the conjugate base of α-ketoglutaric acid, α-ketoglutarate, from the transamination of glutamate or through the activity of glutamate dehydrogenase on glutamate. In this study, the α-ketoglutaric acid concentrations attained were generally at low levels. However, these amounts slightly increased within the time in the semi-batch bioreactor operations of BR-3, BR-4 and BR-5.

Succinic acid is another important organic acid in citric acid cycle because its conjugate base, succinate, is able to donate electrons to the electron transport chain via flavin adenine dinucleotide (FAD). The concentrations of this organic acid began to increase after t=12 h or t=16 h in the semi-batch fermentation processes while it was never detected in the batch operation.

Fumaric acid is formed by the oxidation of succinate through the activity of succinate dehydrogenase. Then, this intermediate is converted to malate via the addition of a hydroxyl group to fumarate by the enzyme, fumarase. As a result of the high efficiency of this step, the accumulation of fumaric acid was negligible in the batch operation and six continuous feeding strategies performed.

Malic acid can be also produced from pyruvate via anaplerotic reactions. In all cases, this intermediate accumulated more than the other organic acids involved in TCA cycle and the highest malic acid concentration was obtained as 0.208 g L^{-1} at t=24 h in the second feeding strategy (BR-2) as a result of the oxygen deficiency in the system after t=20 h.

4.2.2.6 Effects of Feeding Strategy on Oxygen Transfer Characteristics

During rhGH production processes, oxygen transfer characteristics, namely volumetric 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 oxygen transfer rate ($OTR_{max}= K_La\ C_O^*$), maximum possible oxygen utilization rate ($OD=\mu_{max}C_X/Y_{X/O}$), Damköhler number ($Da=OD/OTR_{max}$) and efficiency factor (η=OUR/OD) were calculated in order to evaluate the impact of the

feeding strategy on oxygen transfer characteristics. The variations in these parameters with the cultivation time in semi-batch bioreactor operation strategies together with batch operation strategy were given in Table 4.4 and the on-line measured dissolved oxygen concentration profiles were presented in Figure 4.8.

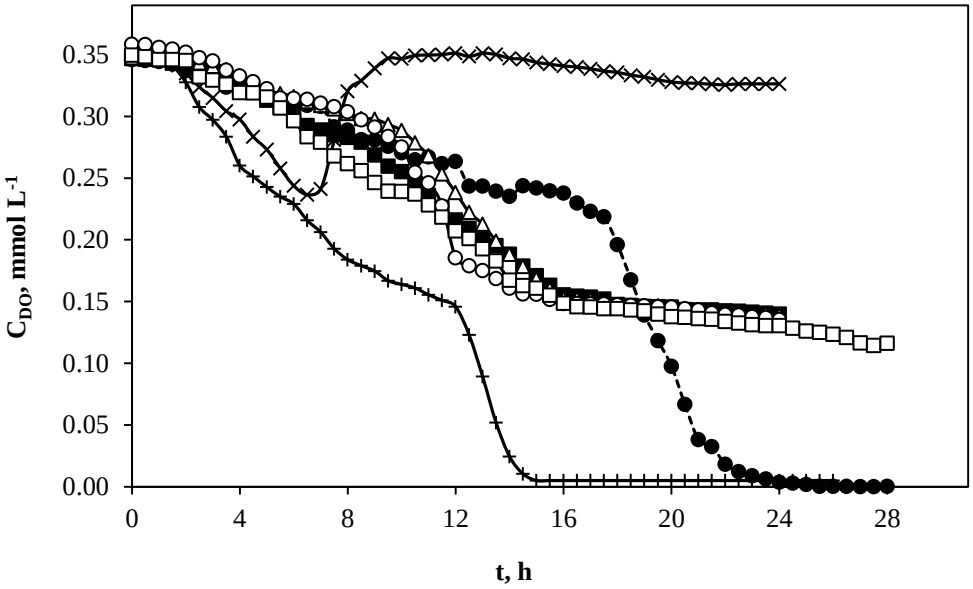

Figure 4.8 The variations in dissolved oxygen concentration with the cultivation time in semi-batch bioreactor operation strategies together with batch operation strategy. C_{G0}=8 g L^{-1}, T=37°C and pH_C=7.5. Batch (x), BR-1 (+), BR-2 (●), BR-3 (■), BR-4 (Δ), BR-5 (o), BR-6 (◊)

Liquid phase mass transfer coefficient mainly depends on the air flow rate (Q_O/V_R), agitation rate (N), configuration of the bioreactor and rheological properties of the fermentation medium. In the batch operation and the feeding strategies of BR-1 and BR-2, first two parameters did not change throughout the bioprocess so the only factor affecting K_La values was the characteristics of the designed production medium. On the other hand, gradually increased agitation rate also had an influence on liquid phase mass transfer coefficient in the semi-batch operations of BR-3, BR-4, BR-5 and BR-6. In all bioreactor experiments, the efficiency of oxygen transfer was high in the early stages of the process as a result of high K_La values. However, then these coefficients started to decrease since the viscosity of the fermentation broth

increased with the cultivation time due to the cell growth, product and by-product formation. Nevertheless, the efficient oxygen transfer period was prolonged; in other words the decrease in K_La values started at t=16 h instead of t=12 h, in BR-3, BR-4, BR-5 and BR-6. Even though relatively lower K_La values were obtained at t=20 h in the last four semi-batch operations, there was a significant increase in these numbers at t=24 h in consequence of the enhanced agitation rate. The volumetric liquid phase oxygen transfer coefficients for the pilot-scale bioreactor experiments were in the range of 0.016-0.054 s^{-1} while the highest K_La values were generally close to each other.

Oxygen uptake rate increases as a result of the cell growth and protein production in microbial processes. Therefore, higher OUR values were obtained at the early stages of the bioprocess in all bioreactor experiments as expected. The highest OUR value was determined as 16.24 mmol m^{-3} s^{-1} at t=16 h in the first feeding strategy (BR-1) due to the highest cell concentration attained. Hence, oxygen was consumed faster in this semi-batch bioreactor experiment. On the other hand, the maximum OUR values acquired in the semi-batch operations of BR-3, BR-4 and BR-5 were also high in compliance with the enhanced rhGH production levels. In these bioreactor experiments both OUR and OTR started to decrease after t=16 h due to the reduced glucose consumption and rhGH production rates. Since OUR values were generally equal to OTR values or higher than those, oxygen did not accumulate during the bioprocess while it was never detected in the fermentation medium after t=16 h in the first semi-batch operation (BR-1) and after t=20 h in the second semi-batch operation (BR-2) as shown in Figure 4.8. The highest oxygen transfer rates acquired in the batch operation and the feeding strategies of BR-1, BR-2, BR-3, BR-4, BR-5 and BR-6 were 2.83, 16.23, 5.75, 9.23, 10.01, 9.09 and 8.85 mmol m^{-3} s^{-1}, respectively.

Table 4.4 The variations in oxygen transfer characteristics with the cultivation time in batch and semi-batch bioreactor operation strategies

Strategy	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
	0	0.015	1.00	0.22	5.55	-	-	-	-
	4	0.039	2.60	2.83	14.43	2.83	5.05	0.35	0.56
	8	0.041	2.73	2.03	15.17	2.03	14.14	0.93	0.14
BATCH	12	0.044	2.93	0.83	16.28	0.83	-	-	-
	16	0.038	2.53	1.11	14.06	1.11	-	-	-
	20	0.033	2.20	1.39	12.21	1.39	-	-	-
	24	0.021	1.40	0.92	7.77	0.92	-	-	-
	0	0.015	1.00	0.21	5.55	-	-	-	-
	4	0.039	2.60	4.29	14.43	4.29	5.60	0.39	0.77
	8	0.043	2.87	8.00	15.91	8.01	27.18	1.71	0.29
BR-1	12	0.045	3.00	10.09	16.65	10.10	35.68	2.14	0.28
	16	0.044	2.93	16.23	16.28	16.24	78.54	4.82	0.21
	20	0.038	2.53	14.03	14.06	14.03	161.78	11.51	0.09
	24	0.024	1.60	8.87	8.88	8.87	221.14	24.90	0.04

Table 4.3 The variations in oxygen transfer characteristics with the cultivation time in the batch and semi-batch bioreactor experiments (Continued)

Strategy	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-2	0	0.015	1.00	0.36	5.55	-	-	-	-
	4	0.044	2.93	1.99	16.28	1.99	3.90	0.24	0.51
	8	0.048	3.20	3.89	17.76	3.89	7.65	0.43	0.51
	12	0.054	3.60	5.75	19.98	5.76	16.32	0.82	0.35
	16	0.027	1.80	3.49	9.99	3.49	13.96	1.40	0.25
	20	0.016	1.07	4.36	5.92	4.37	14.30	2.42	0.31
	24	-	-	-	-	-	-	-	-
	28	-	-	-	-	-	-	-	-
BR-3	0	0.015	1.00	0.37	5.55	-	-	-	-
	4	0.043	2.87	1.99	15.91	1.99	4.07	0.26	0.49
	8	0.044	2.93	3.83	16.28	3.83	7.41	0.45	0.52
	12	0.046	3.07	7.08	17.02	7.08	12.88	0.76	0.55
	16	0.043	-	9.23	15.91	9.23	20.69	1.30	0.45
	20	0.021	-	4.72	7.77	4.72	22.84	2.94	0.21
	24	0.038	-	8.75	14.06	8.75	86.33	6.14	0.10

Table 4.3 The variations in oxygen transfer characteristics with the cultivation time in batch and semi-batch bioreactor experiments (Continued)

Strategy	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-4	0	0.015	1.00	0.34	5.55	-	-	-	-
	4	0.043	2.87	1.61	15.91	1.61	3.40	0.21	0.47
	8	0.045	3.00	3.05	16.65	3.05	5.24	0.31	0.58
	12	0.048	3.20	6.32	17.76	6.33	9.62	0.54	0.66
	16	0.046	-	10.01	17.02	10.01	19.60	1.15	0.51
	20	0.019	-	4.26	7.03	4.26	16.91	2.41	0.25
	24	0.036	-	8.31	13.32	8.31	57.83	4.34	0.14
BR-5	0	0.015	1.00	0.37	5.55	-	-	-	-
	4	0.045	3.00	1.68	16.65	1.68	4.05	0.24	0.42
	8	0.044	2.93	2.91	16.28	2.92	5.96	0.37	0.49
	12	0.042	2.80	7.75	15.54	7.76	10.89	0.70	0.71
	16	0.041	-	9.09	15.17	9.09	19.24	1.27	0.47
	20	0.020	-	4.49	7.40	4.49	26.86	3.63	0.17
	24	0.037	-	8.68	13.69	8.68	217.41	15.88	0.04

Table 4.4 The variations in oxygen transfer characteristics with the cultivation time in batch and semi-batch bioreactor operation strategies (Continued)

Strategy	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-6	0	0.015	1.00	0.31	5.55	-	-	-	-
	4	0.042	2.80	2.13	15.54	2.13	3.67	0.24	0.58
	8	0.045	3.00	4.88	16.65	4.88	7.95	0.48	0.61
	12	0.043	2.87	7.00	15.91	7.00	21.32	1.34	0.33
	16	0.040	-	8.85	14.8	8.85	34.98	2.36	0.25
	20	0.017	-	3.95	6.29	3.95	16.85	2.68	0.23
	24	0.034	-	8.14	12.58	8.14	38.74	3.08	0.21
	28	0.023	-	5.84	8.51	5.84	34.18	4.02	0.17

Damköhler number and efficiency factor are required in order to determine the rate limiting step in the bioprocess. These dimensionless numbers are calculated by using maximum possible oxygen utilization rate, maximum possible oxygen transfer rate and oxygen uptake rate.

Da number which is lower than 1 (Da<<1) indicates biochemical reaction limited condition. However, if this value is higher than 1, mass transfer requirement becomes dominant in the bioreactor. The Da values, in BR-1, BR-2, BR-3, BR-4, BR-5, and BR-6 were, respectively, changes within 0.39 to 11.51, 0.24 to 2.42, 0.26 to 2.94, 0.21 to 4.34, 0.24 to 3.63, and 0.24 to 4.02. In the early cultivation times as Da<1, but not very low, the bioprocess was not biochemical reaction limited; however, later at higher cultivation times the mass (oxygen) transfer requirement increased, but the bioprocess was not oxygen transfer limited, except after t>20 h in BR-1 and BR-5. This analysis reveals that oxygen transfer conditions were designed appropriately.

The efficiency factors decreased with the cultivation time in contrast to the Damköhler numbers in all rhGH production processes. The oxygen demand was very high and the amount of oxygen consumed by microorganisms was less than this OD value. Therefore η values calculated for the batch and semi-batch bioreactor experiments were lower than 1 and changed in the range of 0.04-0.75.

4.2.2.7 Effects of Feeding Strategy on Specific Rates and Yield Coefficients

Fermentation characteristics primarily consisting of specific rates such as specific growth rate (μ), specific oxygen uptake rate (q_O), specific substrate utilization rate (q_S) and specific product formation rate (q_P), and yield coefficients such as cell yield on oxygen ($Y_{X/O}$), cell yield on substrate ($Y_{X/S}$), product yield on substrate ($Y_{P/S}$) and product yield on cell ($Y_{P/X}$) directly determine the bioprocess performance. Therefore, all these fermentation characteristics were calculated for semi-batch bioreactor operation strategies together with batch operation strategy (Table 4.5).

In most of the semi-batch bioreactor experiments, the specific growth rates (μ) were close to the pre-determined ones (μ_0) until the end of the bioprocess. In the batch operation and the feeding strategies of BR-1 and BR-2, the highest specific growth rates were obtained as 0.22, 0.31 and 0.21 h^{-1}, respectively at t=4-8 h, then μ values gradually decreased with the cultivation time. On the other hand, in the last four bioreactor experiments, namely for BR-3, BR-4, BR-5 and BR-6, the maximum μ values were acquired as 0.23, 0.27, 0.30 and 0.25 h^{-1}, respectively at t=8-12 h. Feeding higher amounts of the substrate stock solution as a result of the increased pre-determined specific growth rate, μ_0=0.25 h^{-1}, starting from t=4 h could be the main reason of this delay.

In general, the specific oxygen uptake rates decreased with the time since the microorganisms required more oxygen at the beginning of the logarithmic growth phase. This result was consistent with the decrease in OUR value after t=12-16 h in all semi-batch rhGH production processes. The highest q_O values attained for the batch operation and the feeding strategies of BR-1, BR-2, BR-3, BR-4, BR-5 and BR-6 were 0.39, 0.49, 0.62, 0.58, 0.70, 0.60 and 0.71 g g^{-1} h^{-1}, respectively. Based on these results, oxygen was more efficiently used in the semi-batch bioreactor experiments where rhGH production levels were relatively higher as well as cell concentrations achieved.

In most cases the specific substrate utilization rate- and the specific product formation rate- profiles were parallel to each other from the beginning of rhGH production. Since q_S decreased throughout the bioprocess in general, the highest values for the batch operation and the semi-batch bioreactor experiments; BR-1, BR-2, BR-3, BR-4, BR-5 and BR-6 were determined as 1.18, 1.38, 2.16, 2.03, 1.99, 2.22 and 1.88 g g^{-1} h^{-1}, respectively at the early stages of the bioprocess while the maximum q_P values were achieved as 9.74, 6.05, 16.41, 18.47, 16.38, 17.43 and 20.46 g kg^{-1} h^{-1} in the same order at t=4-8 h.

Table 4.5 The variations in fermentation characteristics with the cultivation time in batch and semi-batch bioreactor operation strategies

Strategy	t (h)	μ (h^{-1})	q_O ($g\ g^{-1}\ h^{-1}$)	q_S ($g\ g^{-1}\ h^{-1}$)	q_P ($mg\ g^{-1}\ h^{-1}$)	$Y_{X/O}$ ($g\ g^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	$Y_{P/S}$ ($mg\ g^{-1}$)	$Y_{P/X}$ ($mg\ g^{-1}$)
Batch	4	0.22	0.39	1.18	9.74	0.58	0.25	0.00	0.00
	8	0.06	0.14	0.39	5.90	0.41	0.16	12.73	79.60
	12	0.00	0.06	0.00	1.03	-	-	384.79	-
	16	0.00	0.08	0.00	-	-	-	7.59	-
	20	0.00	0.10	0.00	-	-	-	-	-
	24	0.00	0.07	0.00	-	-	-	-	-
BR-1	4	0.31	0.49	1.38	6.05	0.63	0.35	0.00	0.00
	8	0.12	0.35	0.70	2.86	0.34	0.19	5.63	29.84
	12	0.12	0.33	0.65	1.78	0.34	0.16	1.82	11.08
	16	0.10	0.32	0.00	2.26	0.26	0.21	3.29	15.48
	20	0.07	0.22	0.98	2.06	0.16	0.10	3.04	29.55
	24	0.06	0.13	0.94	1.65	0.12	0.06	1.59	27.48

Table 4.5 The variations in fermentation characteristics with the cultivation time in the batch and semi-batch bioreactor experiments (continued)

Strategy	t (h)	μ (h^{-1})	q_O ($g\ g^{-1}\ h^{-1}$)	q_S ($g\ g^{-1}\ h^{-1}$)	q_P ($mg\ g^{-1}\ h^{-1}$)	$Y_{X/O}$ ($g\ g^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	$Y_{P/S}$ ($mg\ g^{-1}$)	$Y_{P/X}$ ($mg\ g^{-1}$)
BR-2	4	0.20	0.62	2.16	16.41	0.33	0.13	0.00	0.00
	8	0.21	0.59	1.54	10.64	0.34	0.08	10.15	124.13
	12	0.15	0.41	0.76	7.57	0.34	0.19	3.49	18.41
	16	0.11	0.16	0.66	3.94	0.64	0.19	15.75	81.11
	20	0.14	0.14	0.85	-	0.89	0.14	-	-
	24	0.13	-	0.90	-	-	0.18	-	-
	28	0.10	-	0.74	0.31	-	0.12	0.43	3.52
BR-3	4	0.20	0.58	1.61	18.47	0.33	0.23	0.00	0.00
	8	0.21	0.57	2.03	13.22	0.36	0.10	14.52	152.32
	12	0.23	0.49	1.91	9.49	0.45	0.11	2.72	24.69
	16	0.21	0.29	1.69	13.64	0.62	0.14	6.46	47.67
	20	0.16	0.08	1.39	9.91	1.06	0.14	10.45	74.74
	24	0.12	0.12	0.37	5.04	0.33	0.15	6.77	45.67

Table 4.5 The variations in fermentation characteristics with the cultivation time in the batch and semi-batch bioreactor experiments (continued)

Strategy	t (h)	μ (h^{-1})	q_O ($g\ g^{-1}\ h^{-1}$)	q_S ($g\ g^{-1}\ h^{-1}$)	q_P ($mg\ g^{-1}\ h^{-1}$)	$Y_{X/O}$ ($g\ g^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	$Y_{P/S}$ ($mg\ g^{-1}$)	$Y_{P/X}$ ($mg\ g^{-1}$)
BR-4	4	0.19	0.70	1.75	8.94	0.27	0.16	0.00	0.00
	8	0.24	0.66	1.99	16.38	0.35	0.09	6.66	70.44
	12	0.27	0.58	1.78	9.16	0.45	0.13	9.14	68.86
	16	0.23	0.36	1.54	13.55	0.56	0.17	3.42	20.28
	20	0.16	0.08	1.56	7.94	1.33	0.15	12.49	82.15
	24	0.12	0.11	1.14	0.84	0.50	0.09	1.04	11.90
BR-5	4	0.17	0.47	1.67	17.43	0.35	0.20	0.00	0.00
	8	0.20	0.49	2.22	14.55	0.40	0.07	13.80	198.95
	12	0.30	0.60	2.16	10.93	0.48	0.10	2.86	27.62
	16	0.22	0.26	1.43	9.57	0.74	0.16	6.43	39.34
	20	0.11	0.07	0.83	6.08	0.99	0.17	7.80	46.50
	24	0.05	0.12	0.66	4.89	0.13	0.07	6.60	92.19

Table 4.5 The variations in fermentation characteristics with the cultivation time in batch and semi-batch bioreactor operation strategies (continued)

Strategy	t (h)	μ (h^{-1})	q_O ($g\ g^{-1}\ h^{-1}$)	q_S ($g\ g^{-1}\ h^{-1}$)	q_P ($mg\ g^{-1}\ h^{-1}$)	$Y_{X/O}$ ($g\ g^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	$Y_{P/S}$ ($mg\ g^{-1}$)	$Y_{P/X}$ ($mg\ g^{-1}$)
BR-6	4	0.23	0.71	1.88	20.46	0.33	0.13	0.00	0.00
	8	0.25	0.70	1.74	12.47	0.35	0.12	15.23	124.69
	12	0.13	0.42	1.10	6.74	0.31	0.16	3.09	19.64
	16	0.11	0.36	0.66	6.35	0.28	0.10	8.65	83.37
	20	0.10	0.11	0.39	5.33	0.87	0.28	11.41	41.04
	24	0.10	0.16	0.39	3.75	0.54	0.26	15.62	61.01
	28	0.08	0.08	0.32	1.79	0.84	0.24	5.33	22.09

The maximum $Y_{X/O}$ values for the batch process and the semi-batch operations of BR-1, BR-2, BR-3, BR-4, BR-5 and BR-6 were 0.58, 0.63, 0.89, 1.06, 1.33, 0.99 and 0.87 g g^{-1}, respectively. In the last four bioreactor experiments, the highest cell yield on oxygen was obtained at t=20 h as a result of the increased agitation rate and oxygen enriched air supply. On the other hand, the maximum $Y_{X/S}$ values for the pilot-scale bioreactor experiments; batch, BR-1, BR-2, BR-3, BR-4, BR-5 and BR-6 were determined as 0.25, 0.35, 0.19, 0.23, 0.17, 0.20 and 0.28 g g^{-1}, respectively. Relatively low $Y_{X/S}$ values acquired throughout the bioprocess were related to high feed amounts in the semi-batch operations. In addition to this, since the microorganism had a certain capacity in terms of recombinant protein production, the maximum $Y_{P/S}$ and $Y_{P/X}$ values attained for the feeding strategies of BR-2, BR-3, BR-4, BR-5 and BR-6 were close to each other.

The overall product yields on total substrate ($\bar{Y}_{P/S}$) were 9.87, 3.38, 8.07, 7.21, 7.48, 6.51, 8.72 g kg^{-1} and the overall cell yields on total substrate ($\bar{Y}_{X/S}$) were 0.19, 0.12, 0.14, 0.12, 0.14, 0.14, 0.20 g g^{-1} for the operation strategies; batch, BR-1, BR-2, BR-3, BR-4, BR-5 and BR-6, respectively. Based on these results, the main carbon source, glucose, was efficiently used in the pilot-scale bioreactor experiments except the first feeding strategy (BR-1).

CHAPTER 5

CONCLUSIONS

In this study, semi-defined medium-based continuous feeding strategies were designed for semi-batch operations in order to enhance recombinant human growth hormone (rhGH) production by *B. subtilis* 1A178 (*scoC*$^-$) strain carrying pMK4*::pre(subC)::hGH* plasmid. In this context, firstly air-filtered shake bioreactor experiments were performed at laboratory-scale to investigate the influences of peptone as the complex carbon and nitrogen source on the cell growth and recombinant protein production. Thereafter, semi-batch rhGH production processes were conducted in pilot-scale bioreactors following an initial batch operation by using the glucose-based semi-defined medium according to the result of the laboratory-scale experiments. The effects of six feeding strategies designed on the cell growth, glucose consumption and rhGH production were investigated. Additionally, the fermentation characteristics, namely specific rates and yield coefficients, protease activities, organic acid concentrations and oxygen transfer characteristics were calculated for each bioreactor experiment in order to determine the microbial response to the feeding strategy applied.

In air-filtered shake bioreactor experiments, the effects of peptone concentration on the cell formation and rhGH production were investigated both in the absence and the presence of $MgSO_4$. For this purpose, eight semi-defined media containing peptone at four different concentrations were designed based on the reference medium (control). Then, the laboratory-scale operations were carried out at T=37°C, N=200 min^{-1} and pH_0=7.5 within the working volume of 110 mL. At the beginning of the production process, 500 µL PTM1 and at t=12 h, 150 µL protease inhibitor cocktail were also added to the medium in each case. The highest cell and

rhGH concentrations were obtained as C_X=1.92 g L^{-1} and C_{rhGH}=140 mg L^{-1} at t=24 h in the semi-defined medium (PM-8) containing 8 g L^{-1} peptone and 0.122 g L^{-1} $MgSO_4$. Since primarily the increase in peptone concentration and secondarily the presence of $MgSO_4$ in the medium significantly improved the cell growth and rhGH production, these two components were used for batch and semi-batch bioreactor experiments at pilot-scale. Nevertheless, the concentration of peptone in the semi-defined feed medium was investigated to prevent its accumulation in the fermentation medium and $MgSO_4$ was only used in the production medium batch-wise to avoid the precipitation of this low-solubility inorganic salt in the substrate stock solution used for feeding.

In pilot-scale bioreactor experiments, six semi-defined medium based continuous feeding strategies were designed to increase rhGH production by *B. subtilis* 1A178 (*scoC*$^-$). In this context, the semi-batch rhGH production processes were implemented at T=37°C, pH_0=7.5, N=750 min^{-1} and Q_O/V_R=0.5 vvm within the working volume of V_R=1.1-2.0 L after an initial batch operation. In the last four semi-batch bioreactor experiments, the agitation rate was gradually increased from 750 min^{-1} to 900 min^{-1} and oxygen enriched air was supplied in order to maintain the dissolved oxygen concentration in the fermentation medium above 40 %. The pH was controlled during the bioprocess by 5 M KOH solution in all cases. Moreover, at the beginning of the production process, 5 mL L^{-1} PTM1 and at t=12 h, 1500 μL protease inhibitor cocktail were added to the medium.

The first bioreactor experiment was performed as a batch operation and the effects of peptone-containing production medium on the cell growth and rhGH production was investigated at pilot-scale. However, in the semi-batch rhGH production processes, except in the first feeding strategy (BR-1), the original defined medium was used as the production medium. In general, the semi-defined medium consisting of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, either 50 or 100 g L^{-1} peptone and 5 mL L^{-1} PTM1 was exponentially fed to the bioreactor at three different pre-determined specific growth rates of 0.10, 0.17 and 0.25 h^{-1}. In the first strategy (BR-1), the batch-wise peptone-containing production medium was used together with the semi-defined feed medium having 50 g L^{-1} peptone and the substrate stock solution was fed to the system with μ_0=0.17 h^{-1} within t=4-26 h. In the second

feeding strategy (BR-2), the semi-defined medium containing 100 g L^{-1} peptone was fed to the bioreactor with μ_0=0.17 h^{-1} within t=4-28 h. In the third semi-batch bioreactor experiment (BR-3), the pre-determined specific growth rate was increased to μ_0=0.25 h^{-1} in order to avoid the depletion of glucose and the semi-defined medium was fed to the bioreactor within t=4-24 h. In the forth strategy (BR-4), exponential feeding according to μ_0=0.25 h^{-1} was started at t_T=2 h instead of t_T=4 h and was continued until the end of the bioprocess, t=24 h. In the fifth semi-batch bioreactor experiment (BR-5) the semi-defined medium was fed to the bioreactor with μ_0=0.25 h^{-1} within t=4-18 h and according to q_S=1.6 g g^{-1} h^{-1} within t=18-26 h to prevent the accumulation of glucose in the fermentation medium. In the last feeding strategy (BR-6), the pre-determined specific growth rate was μ_0=0.25 h^{-1} within t=4-14 h whereas it was decreased to μ_0=0.10 h^{-1} for t=14-28 h in order to keep the glucose concentration in the range of C_G=1-2 g L^{-1}. The main results obtained for batch and semi-batch bioreactor operation strategies can be summarized as follows:

- The highest cell concentration was attained as C_X=8.62 g L^{-1} at t=26 h in the first semi-batch bioreactor experiment (BR-1). However, the maximum cell densities achieved in the other feeding strategies were also greater than C_X=8.20 g L^{-1} and nearly same cell concentrations were acquired in shorter process times in the semi-batch operations of BR-3, BR-4 and BR-5. Therefore, the use of peptone in the glucose-based feed medium is more effective on the cell growth than the use of this complex carbon and nitrogen source in the initial production medium. Furthermore, the addition of protease inhibitor cocktail at t=12 h does not adversely affect the increase in cell concentration in any case while the growth inhibition after t=22 h in BR-3, BR-4 and BR-5 strategies, is indeed noteworthy.
- The glucose consumption rates were generally higher at the beginning of the logarithmic growth phase. The main carbon source was totally consumed until t=8 h in the batch operation. For this reason, when the peptone-containing production medium is used for the batch process, the initial glucose concentration should be more than C_{G0}=8 g L^{-1} for an efficient rhGH production together with the increased cell growth. On the other hand, since

q_S decreased throughout the bioprocess, glucose accumulation started at t=16-22 h in most of the semi-batch bioreactor experiments. Nevertheless, feeding of the glucose-based semi-defined medium was never halted, since rhGH production processes were carried out without feedback control in this study.

- The highest rhGH concentration was achieved as 497 mg L^{-1} at t=22 h in the semi-batch bioreactor experiment of BR-3. Although the concentration of the recombinant protein usually increased as parallel to the cell growth, the maximum rhGH concentration obtained in the first feeding strategy (BR-1) was 2.66-fold lower than this value. Since the use of peptone in the initial production medium batch-wise did not increase the rhGH production as much as the cell growth, this complex carbon and nitrogen source was used only in the semi-defined continuous feed substrate stock solution in other semi-batch operations. In this case, the increase in peptone concentration in the glucose-based feed medium enhanced the rhGH production and shortened the process time when the transition cultivation time was t_T=4 h as in BR-3. Additionally, the microorganisms did not produce rhGH efficiently when the glucose concentration in the fermentation medium was limited in the late-growth phase. These results reveal that the accumulation of glucose in the fermentation medium is more favorable than its depletion for rhGH production by *B. subtilis* 1A178 *(scoC*$^-$).
- Since the expression system designed for rhGH production mimics serine alkaline protease (SAP) production due to the SAP gene (*subC*) promoter (*degQ*) cloned in front of the hGH gene, protease activity profiles were parallel to rhGH concentration profiles. The highest protease activity was obtained as 197 U cm^{-3} at t=24 h in the third feeding strategy (BR-3) so the decrease in rhGH concentration at the last hours of the bioprocess was related to the increased proteolytic activity with the cultivation time. The maximum protease activities determined for the other feeding strategies except BR-2 were also greater than 140 U cm^{-3}. This result was not surprising since 1500 μL protease inhibitor cocktail was added to the medium in all bioreactor experiments irrespective of the working volume and/or the cell concentration attained at t=12 h.

- In general, the organic acids detected in rhGH production medium were gluconic acid, formic acid, lactic acid, citric acid, α-ketoglutaric acid, succinic acid, fumaric acid and malic acid. Amongst all these organic acids, that had the highest concentration was lactic acid in all bioreactor experiments. Since the dissolved oxygen concentration fell below the limited value at t=20-24 h in the second feeding strategy (BR-2), relatively high lactic acid concentration was obtained in this operation. However, the same organic acid was produced in the semi-batch bioreactor experiments of BR-3, BR-4 and BR-5 in the presence of oxygen probably since pyruvate was formed faster than it could be metabolized in TCA cycle. The exponential increase in the cell concentration stopped at t=22 h in these three designed strategies because the accumulation of lactic acid in the fermentation medium inhibited the cell growth. On the other hand, the accumulation of one or more organic acids involved in Krebs cycle indicates the low efficiency in aerobic respiration. In all cases, malic acid accumulated more than the other intermediates in TCA cycle. This could be the result of anaplerotic reactions providing conversion of pyruvate to malate. The highest malic acid concentration was attained as 0.208 g L^{-1} at t=24 h by the second feeding strategy (BR-2) due to the oxygen deficiency in the system after t=20 h.
- In the early cultivation times as Da<1, but not very low, the bioprocess was not biochemical reaction limited; however, then the mass (oxygen) transfer requirement increased, but the bioprocess was not oxygen transfer limited, except after t>20 h in BR-1 and BR-5. This analysis reveals that oxygen transfer conditions were designed appropriately.
- rhGH is a high-value product. In general, $\bar{Y}_{X/S}$ and $\bar{Y}_{P/S}$ values calculated for the semi-batch bioreactor experiments were close to each other and lower than the overall cell and product yields attained in batch bioreactor. Nevertheless, since the maximum rhGH concentrations achieved in semi-batch bioreactor experiments were higher than that of batch bioreactor, the semi-batch operation is more advantageous than batch operation and the best semi-defined medium based feeding strategy designed is BR-3 due to the highest rhGH concentration acquired.

- In the study of Gökbudak (2013), the highest rhGH production by *B. subtilis* 1A178 *(scoC⁻)* was achieved as 366 mg L^{-1} at t=28 h by the exponential feeding of the semi-defined feed medium 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 at μ_0=0.17 h^{-1} within t=4-25 h. In this work in BR-3, 1.36-fold higher rhGH concentration was obtained at t=22 h by the exponential feeding of the substrate stock solution comprising of 200 g L^{-1} glucose, 117.65 g L^{-1} $(NH_4)_2HPO_4$, 100 g L^{-1} peptone and 5 ml L^{-1} PTM1 at μ_0=0.25 h^{-1} within t=4-24 h. As a result, the productivity was significantly increased in the current study since the operation time needed to achieve the maximum rhGH concentration was markedly shortened.
- Not only the amount of protease inhibitor cocktail required but also its addition time and mode (pulse or continuous) should be optimized as in the study of Şahin (2010) or protease deficient strains should be used for the newly developed semi-defined medium based continuous feeding strategies.
- Metabolic flux analysis (MFA) should be performed for each bioreactor operation strategy developed in order to investigate the product distribution during the cell growth, product and by-product formation.

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APPENDIX A

CONTENTS OF THE KITS

Glucose Analysis Kit (Biyozim-Biasis)

Components of the Analysis Kit

- Glucose analysis buffer (concentrated, 50 mL)
  Potassium dehydrogen phosphate (0.4 M)
  Phenol (0.2 mM)
- Glucose analysis reagent
  4-Aminoantipyrine (0.2 mM)
  Glucose oxidase ≥ 10000 IU/bottle
  Peroxidase ≥ 1000 IU/bottle
- Glucose standard
  Glucose (0.5 g)

Other Equipments Used for the Analysis

- Distilled water
- Test tube (glass or plastic)
- Micro pipette (20-200 μL and 100-1000 μL)
- Micro cuvette (1mL)
- Vortex
- Incubator (T = 37°C)
- Spectrophotometer (λ = 505 nm)

Preparation of Standard Glucose Solutions

- Firstly 0.1 g glucose was dissolved in 10 mL distilled water in order to obtain the glucose stock solution with a concentration of 10 g L^{-1}.
- Then the standard glucose solutions (0.25, 0.50, 0.75, 1.00 g L^{-1}) were prepared from this stock solution with the consecutive dilutions.

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

Solutions for HPCE Analysis

Borate Buffer Solution

- $Na_2B_4O_7.10H_2O$ (0.4763 g)
- Distilled water (25 mL)
- NaOH (1 M)

  1 M NaOH was used to adjust pH = 10.0 before degassing and filtering the solution.

Electrolytic Solution

- $Na_2B_4O_7.10H_2O$ (0.4763 g)
- Z-1 Methyl Reagent (4.5310 g)
- Distilled water (25 mL)
- NaOH (1 M)

  1 M NaOH was used to adjust pH = 10.0 before degassing and filtering the solution.

Buffer Solutions for Protease Activity Assay

0.05 M Sodium Acetate Buffer (pH=5.0) for Acidic Proteases

- CH_3COOH (0.713 mL dissolved in 25 mL distilled water)
- CH_3COONa (2.052 g dissolved in 50 mL distilled water)

  After CH_3COONa solution was titrated with CH_3COOH solution to adjust pH = 5.0, the solution was diluted to 500 mL before autoclaving and storing it at +4°C.

0.05 M Sodium Phosphate Buffer (pH=7.0) for Neutral Proteases

- $Na_2HPO_4.7\ H_2O$ (6.70 g dissolved in 50 mL dH2O)
- $NaH_2PO4.2\ H_2O$ (3.90 g dissolved in 50 mL dH2O)

  After$Na_2HPO_4.7\ H_2O$ solution was titrated with $NaH_2PO_4.2\ H_2O$ solution to adjust pH = 7.0, the solution was diluted to 500 mL before autoclaving and storing it at +4°C.

0.05 M Borate Buffer (pH=10.0) for Alkaline Proteases

- $Na_2B_4O_7.10\ H_2O$ (2.381 g)
- Distilled water (250 mL)
- NaOH (1M)

  1 M NaOH was used to adjust pH = 10.0 before adding distilled water till 500 mL, filtering (0.45 µm porous filters; Sartorius, AG) and storing the solution at + 4°C.

APPENDIX C

CALIBRATION CURVES

Calibration Curve for Cell Concentration

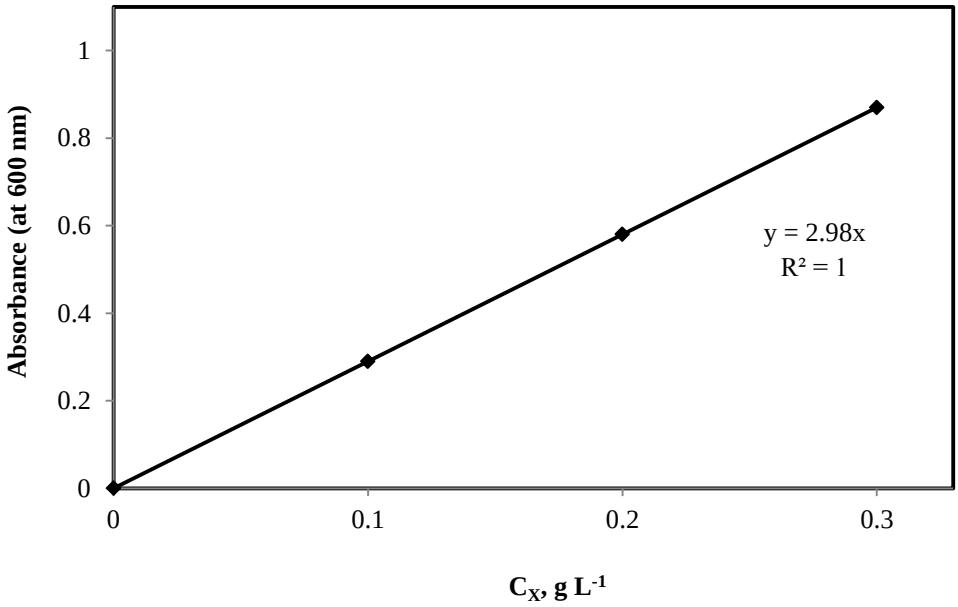

Figure C.1 Calibration curve for cell concentration

Based on the equation obtained from the plot:

$$C_X = \frac{Absorbance}{2.98} \times (Dilution\ ratio) \qquad (C.1)$$

Calibration Curve for Glucose Concentration

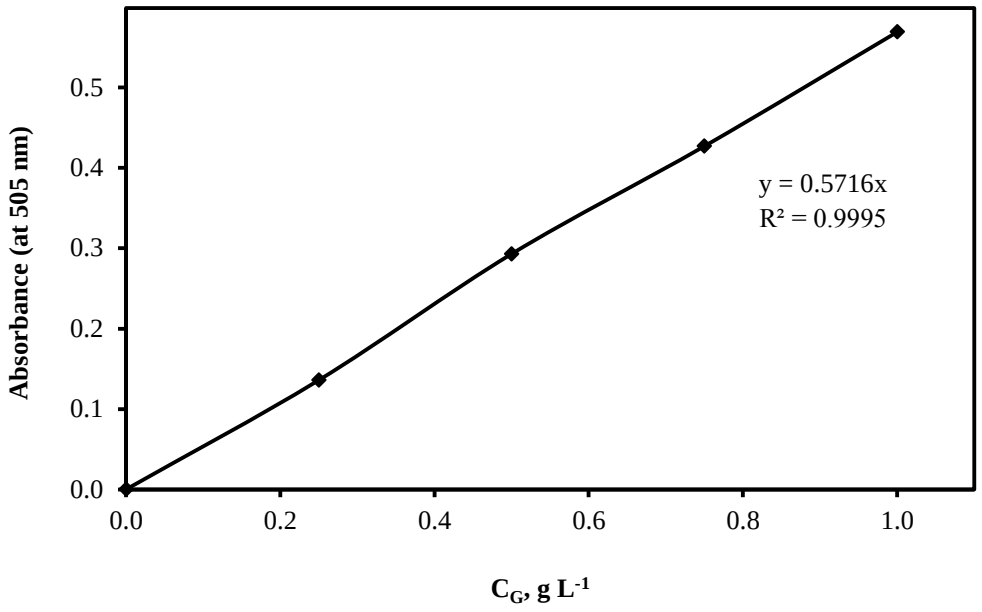

Figure C.2 Calibration curve for glucose concentration

Based on the equation obtained from the plot:

$$C_G = \frac{Absorbance}{0.5716} \times (Dilution\ ratio) \qquad (C.2)$$

Calibration Curve for rhGH Concentration

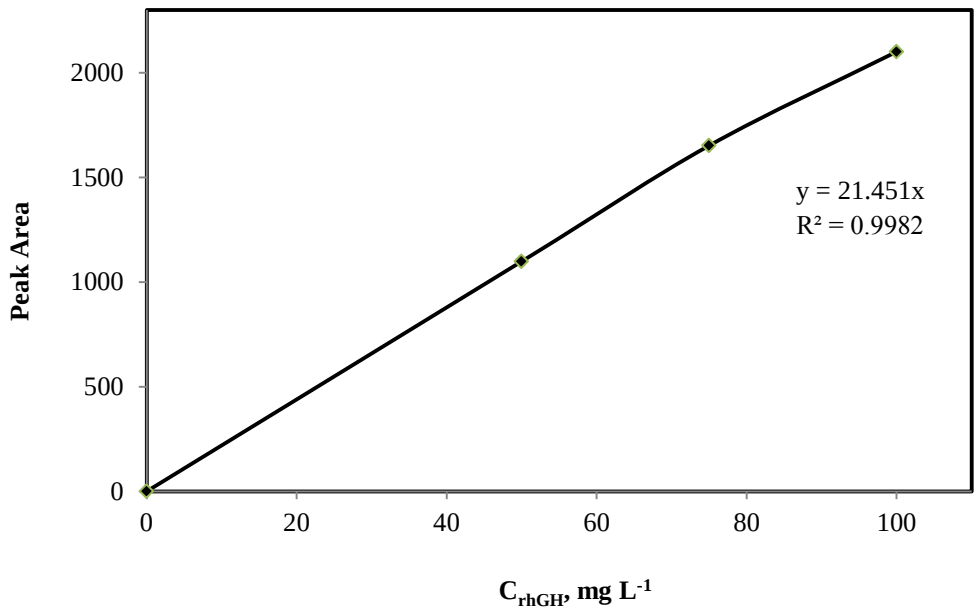

Figure C.3 Calibration curve for rhGH concentration

Based on the equation obtained from the plot:

$$C_{rhGH} = \frac{Peak\ Area}{21.451} \qquad (C.3)$$

Calibration Curve for Organic Acid Concentrations

- **Gluconic Acid**

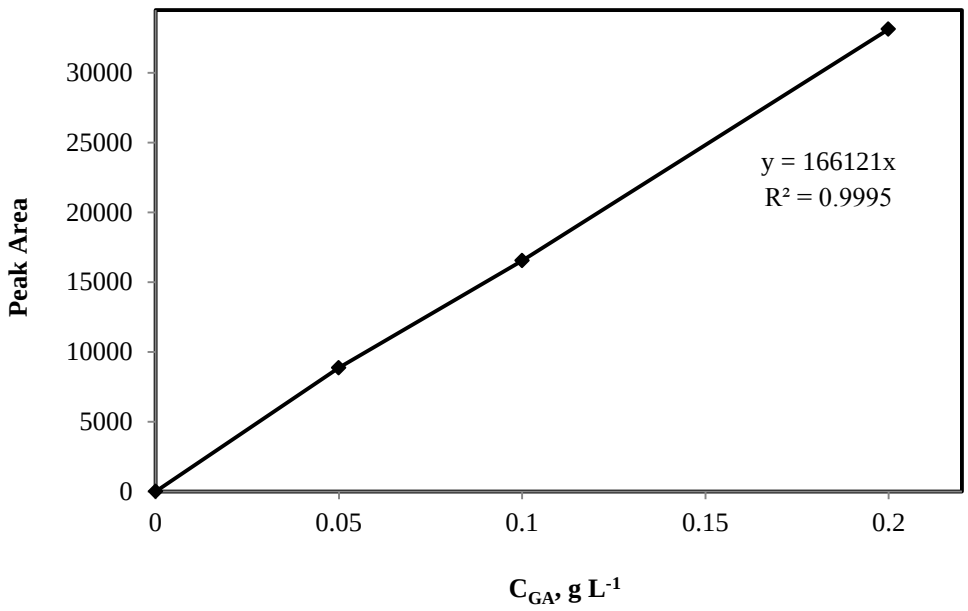

Figure C.4 Calibration curve for gluconic acid concentration

Based on the equation obtained from the plot:

$$C_{GA} = \frac{Peak\ Area}{166121} \qquad (C.4)$$

- **Formic Acid**

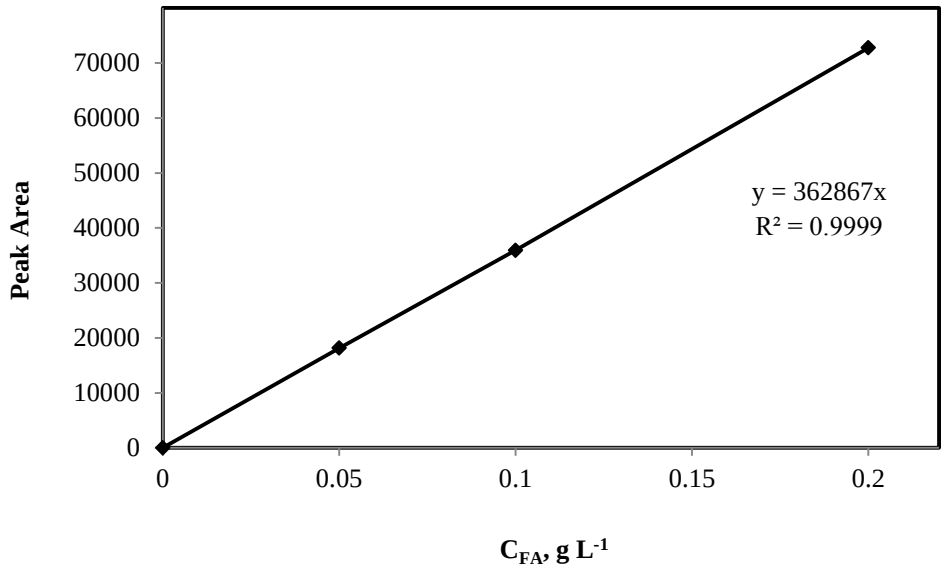

Figure C.5 Calibration curve for formic acid concentration

Based on the equation obtained from the plot:

$$C_{FA} = \frac{Peak\ Area}{362867} \qquad (C.5)$$

- **Lactic Acid**

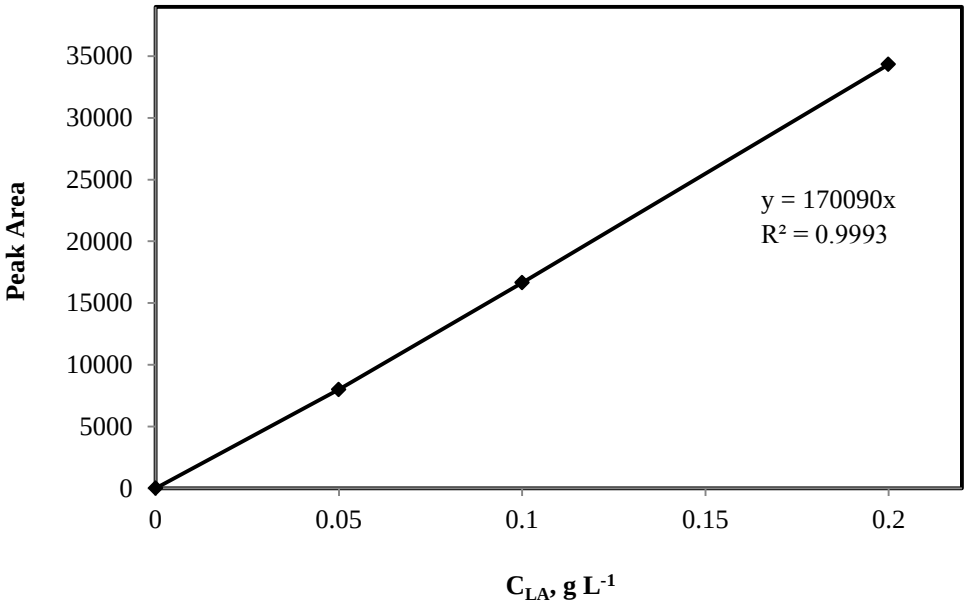

Figure C.6 Calibration curve for lactic acid concentration

Based on the equation obtained from the plot:

$$C_{LA} = \frac{Peak\ Area}{170090} \qquad (C.6)$$

- **Citric Acid**

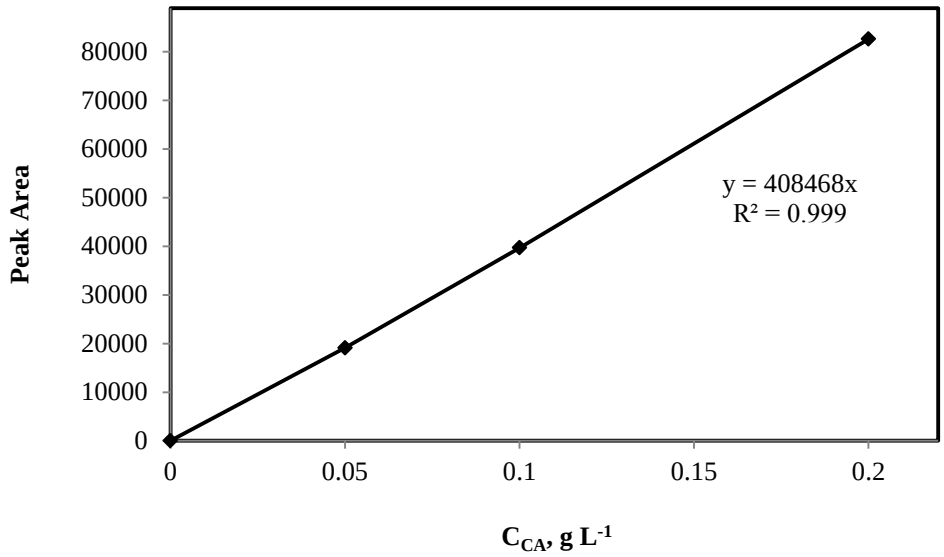

Figure C.7 Calibration curve for citric acid concentration

Based on the equation obtained from the plot:

$$C_{CA} = \frac{Peak\ Area}{408468} \qquad (C.7)$$

- **α-Ketoglutaric Acid**

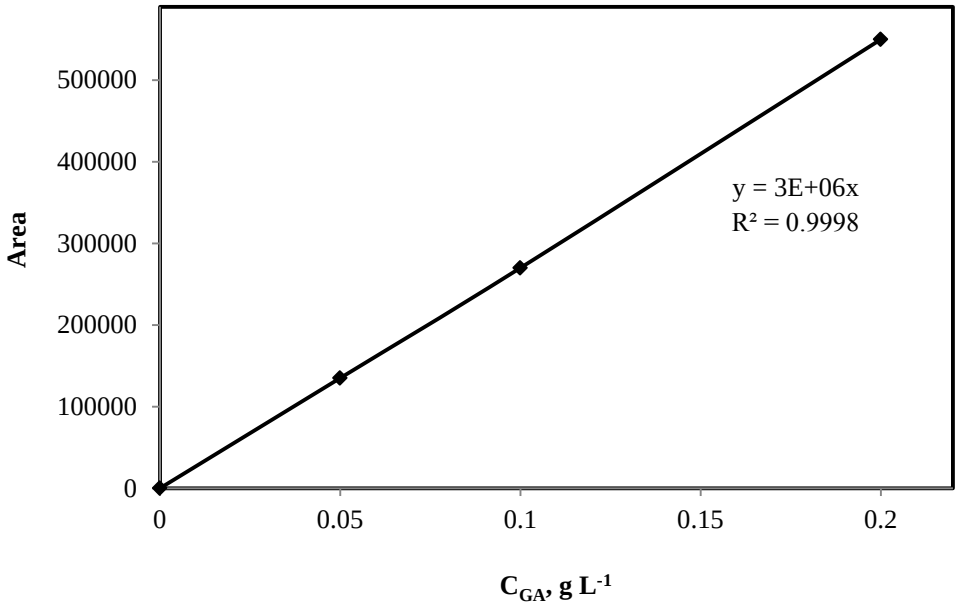

Figure C.8 Calibration curve for α-ketoglutaric acid concentration

Based on the equation obtained from the plot:

$$C_{GA} = \frac{Peak\ Area}{3 \times 10^6} \qquad (C.8)$$

- **Succinic Acid**

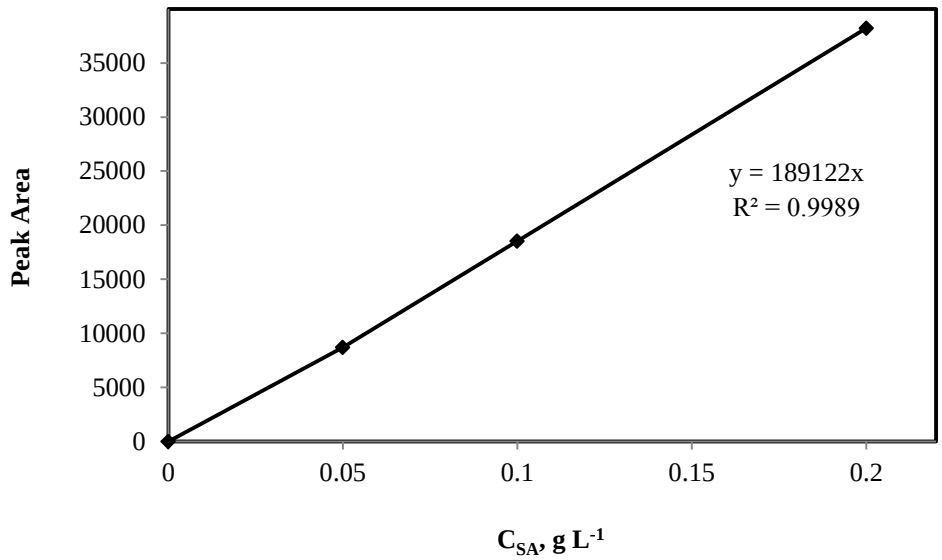

Figure C.9 Calibration curve for succinic acid concentration

Based on the equation obtained from the plot:

$$C_{SA} = \frac{Peak\ Area}{189122} \qquad (C.9)$$

- **Fumaric Acid**

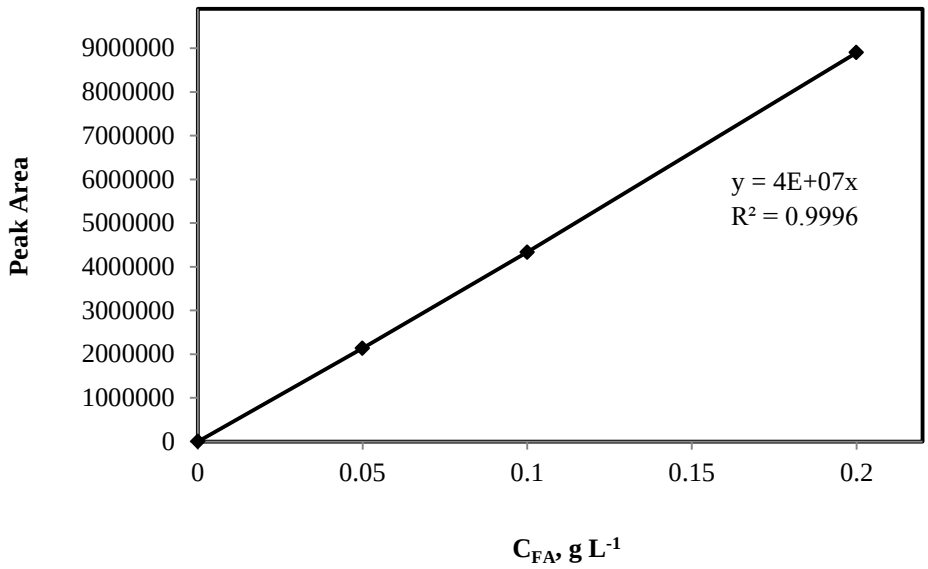

Figure C.10 Calibration curve for fumaric acid concentration

Based on the equation obtained from the plot:

$$C_{FA} = \frac{Peak\ Area}{4 \times 10^7} \qquad (C.10)$$

- **Malic Acid**

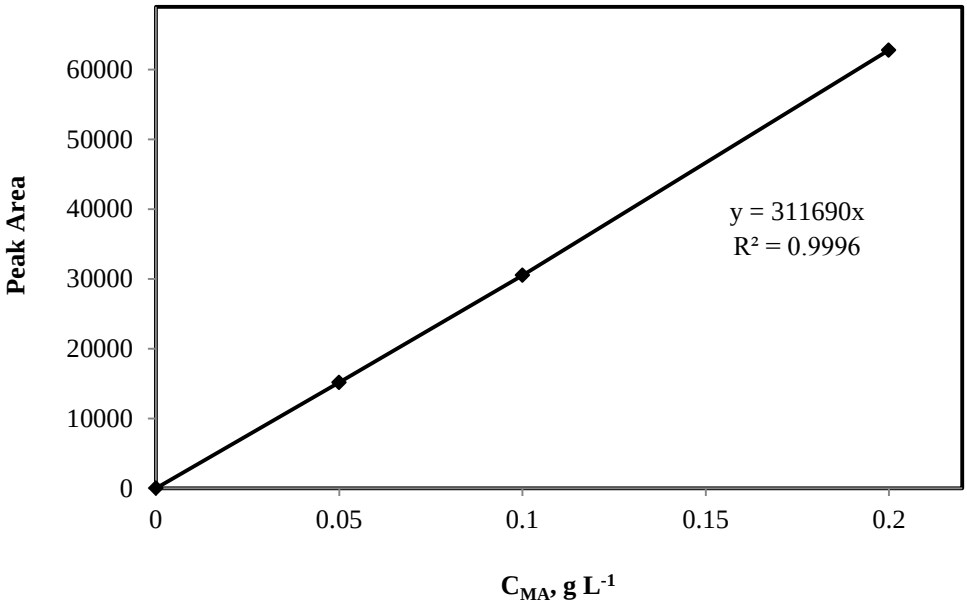

Figure C.11 Calibration curve for malic acid concentration

Based on the equation obtained from the plot:

$$C_{MA} = \frac{Peak\ Area}{311690} \qquad (C.11)$$

APPENDIX D

ELECTROPHEROGRAM OF hGH STANDARD

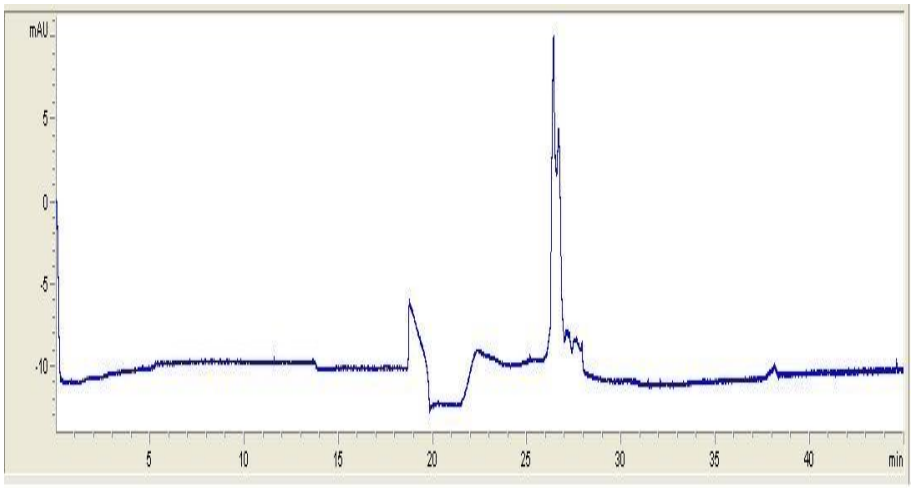

Figure D.1 Electropherogram of 0.1 g L^{-1} standard hGH

APPENDIX E

PRE-DETERMINED FEEDING PROFILE

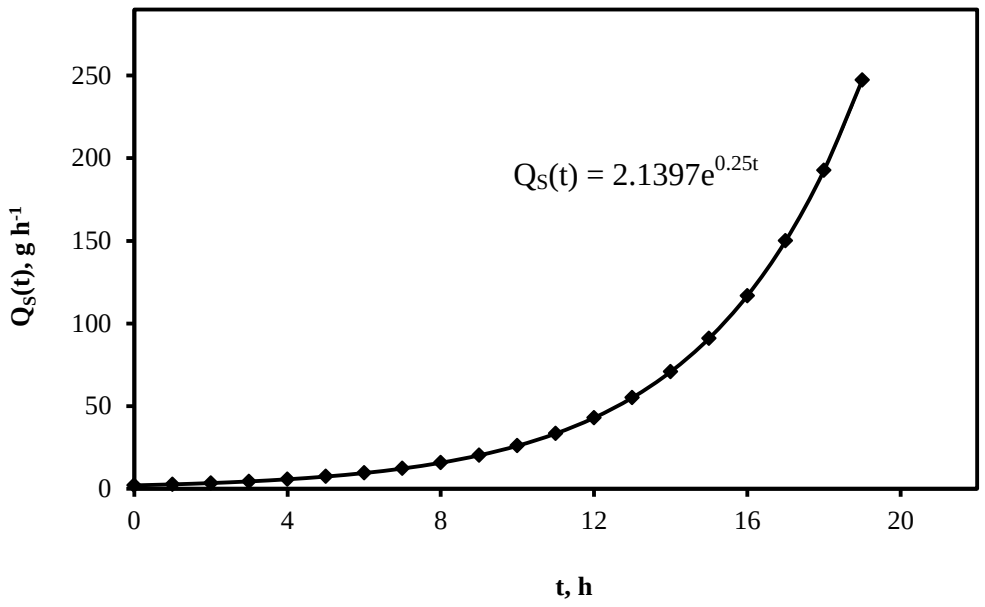

Figure E.1 Pre-determined feeding profile for the optimum semi-defined medium based feeding strategy (BR-3), μ_0=0.25 h^{-1}: t=0 is the time feeding started