RECOMBINANT THERAPEUTIC PROTEASE PRODUCTION BY *Bacillus* sp.

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ABSTRACT

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The first aim of this study is the development of extracellular recombinant therapeutic protease streptokinase producing *Bacillus sp*., and the second aim is to determine fermentation characteristics for streptokinase production. In this context, the signal (*pre*-) DNA sequence of *B.licheniformis* (DSM1969) extracellular serine alkaline protease enzyme gene (*sub*C: Acc. No. X03341) was ligated to 5' end of the streptokinase gene (*skc:* Acc. No. S46536) by SOE (Gene Splicing by Overlap Extension) method through PCR. The resulting hybrid gene *pre(sub*C*)*::*skc* was cloned into the pUC19 plasmid. Then, the hybrid gene was sub-cloned to pMK4 plasmid which is an *E. coli-Bacillus* shuttle vector with high copy number and high stability. Recombinant plasmid pMK4::*pre(sub*C*)*::*skc* was finally transferred into *B. subtilis* (*npr-apr-)* and *B. licheniformis* 749/C (ATCC 25972) species. Streptokinase production capacities of these two recombinant *Bacillus* species were compared. The highest production was observed in recombinant *B. lichenifomis* 749/C (ATCC 25972) strain in a defined medium which was

optimized in terms of carbon and nitrogen sources by a statistical approach, namely Response Surface Methodology (RSM). RSM evaluated the streptokinase concentration as the response and the medium components as the independent variables. The highest recombinant streptokinase concentration was found as 0.0237 kgm^{-3} at glucose and $(NH_4)_2HPO_4$ concentrations of 4.530 and 4.838 kgm^{-3} respectively. The fermentation and oxygen transfer characteristics of the streptokinase production were investigated in a 3 dm^3 pilot scale batch bioreactor (Braun CT2-2) equipped with temperature, pH, foam, air inlet and agitation rate controls having a working volume of V_R=1.65 dm^3 using the production medium optimized for the recombinant *B. lichenifomis* 749/C (ATCC 25972) strain. Streptokinase and β-lactamase activities, cell, glucose and organic acid concentrations, dissolved oxygen, pH, oxygen uptake rate, overall liquid phase mass transfer coefficient for oxygen, maintenance coefficient for oxygen, specific cell growth rate and yield coefficients were determined through the bioprocess. The bioprocess of recombinant streptokinase production was performed at uncontrolled pH of these bioreactor operation conditions: air inlet rate of Q_0/V_R=0.5 vvm, and the agitation rate of N=400min^{-1}. The resulting streptokinase volumetric activity reached its maximum as 1.16 $PUml^{-1}$ (0.0026 g/l streptokinase) at t=20 h.

Keywords: Streptokinase, β-lactamase, production, recombinant *Bacillus* sp, recombinant *E. coli* sp, pUC19, pMK4, response surface methodology (RSM), bioprocess operation parameters

ÖZ

TERAPATİK REKOMBİNANT PROTEAZ STREPTOKİNAZIN *Bacillus* TÜRLERİYLE ÜRETİMİ

Korkmaz, Nuriye

Yüksek Lisans, Kimya Mühendisliği Bölümü

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Bu çalışmanın ilk amacı hücre dışı rekombinant streptokinaz üreten *Bacillus* türlerini geliştirmek, ikinci amacı streptokinaz üretimi için fermantasyon ve oksijen aktarım karakteristiklerinin belirlenmesidir. Bu kapsamda, *B.licheniformis* (DSM1969) hücre dışı serin alkali proteaz enzim geninin (*sub*C: Acc. No. X03341) sinyal (*pre-*) DNA dizini streptokinaz geninin (*skc:* Acc. No. S46536) 5' ucuna entegre edilmiştir. Elde edilen *pre(sub*C*)*::*skc* hibrid geni ilk olarak pUC19 vektörüne klonlanmıştır. İkinci basamakta, hibrid gen *E. coli-Bacillus* vectorü olan pMK4 plasmidine alt-klonlama ile klonlanmıştır. Rekombinant plasmid pMK4::*pre(sub*C*)*::*skc B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) hücrelerine aktarılmış ve streptokinaz üretim kapasiteleri kıyaslanmıştır. Rekombinant *B. licheniformis* 749/C (ATCC 25972) hücrelerinin ilgili proteini hücre dışı daha iyi ürettiği saptanmıştır. Rekombinant *B. licheniformis* 749/C (ATCC 25972) hücrelerinde karbon ve azot kaynaklarının streptokinaz üretimine etkisi istatiksel bir metod olan Yüzey Cevap Metodu (RSM) ile optimize edilmiştir. Glukoz ve $(NH_4)_2HPO_4$ konsantrasyonları sırasıyla 4.530 ve 4.838 kgm^{-3} olacak şekilde belirlenen tanımlı ortamda üretilen en yüksek

teorik streptokinaz konsantrasyonu 0.0237 kgm^{-3} olarak belirlenmiştir. Streptokinaz üretiminin fermentasyon ve oksijen aktarım koşulları; sıcaklık, pH, köpük, hava giriş, agitasyon kontrollü ve 3.0 dm^3 hacimli pilot ölçekli biyoreaktörde (Braun CT2-2), rekombinant *B. lichenifomis* 749/C (ATCC 25972) hücreleri için önceden optimize edilmiş olan ortam bileşenleri kullanılarak incelenmiştir. Biyoproses süresince, streptokinaz ve β-laktamaz aktivitelerinin, hücre, glukoz ve organik asit konsantrasyonlarının zamanla değişimi; dinamik yöntem kullanılarak oksijen tüketim hızı ve sıvı faz kütle aktarım katsayısı; verim ve yaşam katsayıları belirlenmiştir. Rekombinant streptokinaz üretim biyoprosesi kontrolsüz pH' da Q_0/V_R=0.5 vvm ve N=400 dk^{-1} biyoreaktör işletim koşulları uygulanarak yürütülmüştür. Maksimum streptokinaz hacimsel aktivitesi biyoprosesin t=20 st' de 1.16 $PUml^{-1}$ olarak gözlenmiştir.

Anahtar Kelimeler: Streptokinaz, β-laktamaz, üretim, rekombinant *Bacillus* türleri, rekombinant *E. coli* türleri, pUC19, pMK4, yüzey cevap metodu (RSM), biyoproses işletim koşulları

To my Family

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NOMENCLATURE

a	The gas liquid interfacial area per unit liquid volume; $m^2\ m^{-3}$
PU	Streptokinase volumetric activity; PU L^{-1}
A_λ	Absorbance
C_{OA}	Organic acid concentration; kg m^{-3}
C_O	Dissolved oxygen concentration; mol m^{-3}; kg m^{-3}
C_{O0}	Initial dissolved oxygen concentration; mol m^{-3}; kg m^{-3}
C_O^*	Oxygen saturation concentration; mol m^{-3}; kg m^{-3}
C_X	Cell concentration; kg dry cell m^{-3}
C_G	Glucose concentration; kgm^{-3}
C_{YE}	Yeast extract concentration; kgm^{-3}
$C_{(NH4)2HPO4}$	$(NH_4)_2HPO_4$ concentration; kgm^{-3}
Da	Damköhler number (=OD / OTR_{max}; Maximum possible oxygen utilization rate per maximum mass transfer rate)
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
K_La_0	Physical overall liquid phase mass transfer coefficient; s^{-1}
K_La	Overall liquid phase mass transfer coefficient; s^{-1}
K_m	Michealis constant for the substrate
N	Agitation or shaking rate; min^{-1}
pH_0	Initial pH
Q_0	Volumetric air feed rate; $m^3\ min^{-1}$
q_o	Specific oxygen uptake rate; kg kg^{-1} DW h^{-1}
q_p	Specific product formation rate; U L^{-1} h^{-1}/(kg m^{-3} cell)
r	Volumetric rate of reaction; mol $m^{-3}\ s^{-1}$
Vmax	Maximum rate of reaction at infinite reactant concentration
r_0	Oxygen uptake rate, mol $m^{-3}\ s^{-1}$; kg $m^{-3}\ h^{-1}$
r_P	Product formation rate; PU $L^{-1}\ h^{-1}$
t	Bioreactor cultivation time; h
T	Bioreaction medium temperature; °C
C_{OA}	Total organic acid concentration; kg m^{-3}

U	One unit of an enzyme
V	Volume of the bioreactor; m^3
V_R	Volume of the bioreaction medium; m^3
$Y_{X/O}$	Yield of cell on oxygen; kg kg^{-1}
$Y_{P/O}$	Yield of product on oxygen; PU L^{-1} /(kg O_2)
$Y_{P/X}$	Yield of product on cell; PU L^{-1} /(kg m^{-3} cell)

Greek Letters

η	Effectiveness factor (=OUR/OD; the oxygen uptake rate per maximum possible oxygen utilization rate)
μ	Specific cell growth rate; h^{-1}
μ_{max}	Maximum specific cell growth rate; h^{-1}
λ	Wavelength; nm

Abbreviations

Ac	Acetic acid
Asp	Aspartic acid
Cit	Citric acid
Form	Formic acid
Fum	Fumaric acid
Gluc	Gluconic acid
Mal	Malic acid
Male	Maleic acid
OD	Oxygen demand (=μ_{max} C_X / $Y_{X/O}$; mol m^{-3} s^{-1})
OTR	Oxygen transfer rate, mol m^{-3} s^{-1}
OTR_{max}	Maximum possible mass transfer rate (=$K_L a C_O$*; mol m^{-3} s^{-1})
RSM	Response Surface Methodology
Ox	Oxaloacetic acid
Suc	Succinic acid

CHAPTER 1

INTRODUCTION

The discovery of DNA was a tremendous stimulus to genetic research, and many biologists contributed to the great age of genetics. In the 14 years between 1952 and 1966, the structure of DNA was determined, the genetic code cracked, and the processes of transcription and translation described. Then in the years 1971-1973, a whole new methodology referred to as recombinant DNA (rDNA) technology or genetic engineering was developed, enabling previously impossible experiments to be planned and carried out. The techniques spawned modern biotechnology, which is the use of biological and biochemical materials and processes by putting genes to work in production of rDNA products such as hormones of therapeutic interest, growth factors, thrombolytic agents, blood coagulation products, anticoagulants, interferons, interleukins and therapeutic enzymes needed in medicine and industrial processes. (Brown, 2001 and Bhopale et al., 2005).

Enzymes are protein catalysts speeding up and co-ordinating the multitude of chemical reactions necessary to develop and maintain life. Enzymes alter the rate in which a thermodynamic equilibrium is reached, but do not change that equilibrium implying that enzymes work reversibly (Drauz, 1995). There are two important features of enzymes used as drugs that distinguish them from all other types of drugs. Firstly, with great affinity and specificity, enzymes often bind and act on their targets. Secondly, enzymes are catalytic species converting multiple target molecules to the desired products. These two characteristics make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecules cannot. These features lead to the development of many enzyme drugs for a wide range of disorders (Vellard et al., 2003). Moreover, since enzymes act in a

temperature range of 20-40 ^{0}C and in a pH range of 5-8, undesired side-reactions such as isomerization, racemization and decomposition are minimized (Faber, 2000).

Enzymes can show different ranges of specificity either in the nature of the substrate(s) they act on or in the reaction they catalyze. Some enzymes which will use a wide range of substrates having the required suitable chemical bond have low specificities (*bond specificity*). Low specificity is generally seen in degradative enzymes, e.g. esterases and certain peptidases. An intermediate group of enzymes show *group specificity*, e.g. hexokinase, which catalyzes the phosphorylation of sugars that are aldohexoses. On the other hand, there are many enzymes showing *absolute* or *near-absolute specificity*. They catalyze their reactions with only a single substrate, e.g. urease catalyzes the reaction with only urea (Price and Stevens, 1999).

According to the types of reactions they catalyze, enzymes are divided into six major classes (Table 2.1). Streptokinase (E.C 3.4.99.22 – a hydrolase) is a bacterial protein not found naturally in human circulation (Banerjee et al., 2004). It is a single chain protein containing 415 amino acids, and having a molecular weight of 45,000-50,000 Da (Kirk and Othmer, 1994). It shows maximum activity at pH 7.5 and its isoelectric pH (the isoelectric pH is the pH at which a protein will not migrate in an electric field and is determined by the charged groups in the protein) is 4.7. It does not contain phosphorous, conjugated carbohydrates and lipids (Banerjee et al., 2004).

Cardiovascular diseases include Coronary Heart Disease or diseases of arteries (Arteriosclerosis, including hardening of the arteries, or Atherosclerosis), Heart Attack (Myocardial Infarction), Arrhythmias, Congestive Heart Disease, Stroke, High Blood Pressure (Hypertension), Rheumatic Heart Disease and other malfunctioning of the cardiovascular system. Among these cardiovascular diseases, the major cause of Acute Myocardial Infarction is coronary arterial thrombosis (thrombus formation) which occurs as the blood clot ages, and the polymerization of fibrin cross-linking and other blood materials increses so that it becomes more

resistant to lysis. The faster the coronary arterial thrombus is lyzed, the less irreversible damage done on the myocardium (Kirk and Othmer, 1994).

In the therapy of heart attack, streptokinase, urokinase and t-PA are used as the plasminogen activators one of which is needed for the process fibrinolysis, a two step biochemical process underlying thrombolysis depending on the degradation of the fibrin network holding the blood clot together in a coronary or celebral artery in the case of myocardial infarction. The main components in plasminogen activation forming the fibrinolytic system include plasma zymogen, plasminogen; its activated product, the proteolytic enzyme, plasmin; plasminogen activators; inhibitors for the plasmin, plasminogen activators, fibrin and fibrinogen (Vaheri et al., 2004 & Kunamneni et al., 2007). Streptokinase complexes with circulatory plasminogen. This 1:1 stoichiometric complex is a high-specific activity protease, activating plasminogen to plasmin (Banerjee et al., 2004). The resulting complex is an active protease degrading fibrin in the blood clot (Figure 2.1).

In the literature, streptokinase is studied in various respects. Scientific papers that are reviewed and investigated in order to develop a background understanding about the streptokinase can be collected in three groups according to the host organisms used in the production of the streptokinase: papers on *Bacillus subtilis, Escherichia coli* and yeasts:

Determination of whether a *B. subtilis* promoter and signal sequence (levansucrase (*sac*B) promoter and signal sequence) would be better than the native streptokinase (*skc*) promoter and signal sequence is aimed and the corresponding scientific research is conducted by Wong et al., (1993) and finally, a 2.5 fold increase in the activity is reported for the extracellular production of streptokinase in *B. subtilis* WB600 (6 extracellular protease deficient strain). In Wu et al., (1998), the short in vivo half-life of streptokinase is prolonged by site-directed mutagenesis using pSK3 plasmid carrying *sac*B promoter and signal sequence (the sucrose-inducible

regulatory region encoding levansucrase) and 2.2-2.5 fold increase in the activity is observed in *B. subtilis* WB600 (6 extracellular protease deficient strain). Staphylokinase is a promising blood-clot dissolving agent for the treatment of patients suffering from a heart attack similar to streptokinase. To determine whether a *B. subtilis* promoter and signal sequence (*sac*B signal sequence and promoters P43, P*amy*, P*sac*B) would be better than the native staphylokinase promoter and signal sequence is aimed in the study of Ye et al., (1998) by using *B. subtilis* WB700 (7-extracellular protease deficient strain). Finally, it is observed that the resulting purified staphylokinase protein has a specific activity of 2840 units/mg.

By the removal of 13 N-terminal amino acids, the production and secretion of streptokinase using *omp*A signal sequence in *E. coli* JM109 strain is enhanced by Lee et al., (1997). The secretion level of the resulting recombinant protein into the extracellular medium appears to be twice higher than that of wild-type streptokinase. Approximately 4500 IU of protein per 1 ml LB-ampicillin medium is secreted into extracellular medium in 12 hours after induction. In the study of Zhang et al., (1999), a process for the pilot production of recombinant streptokinase is established. As the host organism, *E. coli* K802 strain is used, and pST plasmid is constructed to produce recombinant streptokinase by fed-batch cultivation of *E. coli*. After renaturation and purification steps, 12.9 g of recombinant streptokinase with 97.8% of purity and about 10^5 IU/mg of specific activity is obtained.

Secretion of streptokinase is performed by replacing the native secretion signal codons of streptokinase encoding gene with those from α-factor leader peptide and expressing the fusion construct under the control of the methanol-inducible alcohol oxidase (ox) promoter of *Pichia pastoris* GS115 is performed in the study of Pratap et al., (2000). The constructed recombinant plasmid is integrated into the *P. pastoris* genome. The extracellular streptokinase activities obtained for *Pichia pastoris*, *Escherichia coli* and *Streptococcus equisimilis* are reported as 3200 IU/ml, 1500 IU/ml and 150 IU/ml respectively. A chimeric gene consisting of the signal

sequence of the Plus pheromone of *Schizosaccharomyces pombe* fused in-frame with the mature streptokinase from *Streptococcus* sp. is constructed and inserted into an expression vector containing the thiamine-regulated promoter in order to enhance the expression level of streptokinase without glycosylation and degradation in *Sz. Pombe* by Kumar et al., (2004).

Strong promoters are those that can sustain a high rate of transcription; strong promoters usually control genes whose translation products are required in large amounts by the cell. In contrast, weak promoters, which are relatively inefficient, direct transcription of genes whose products are needed in only small amounts. Clearly, an expression vector should carry a strong promoter, so that the cloned gene is transcribed at the highest possible rate (Brown, 2001).

In previous studies**,** streptokinase was produced extracellularly from the gene on modified plasmids under the control of various promoters and signal sequences: *sac*B promoter and signal sequence of the sucrose-inducible regulatory region encoding levansucrase gene (Wong et al., (1993), Wu et al., (1998), Ye et al., (1998)), OmpA signal sequence of the outer membrane protein OmpA encoding gene (Lee et al., (1997)), original promoter and signal sequence of streptokinase encoding gene (Zhang et al., (1999)), α-factor leader peptide sequence and methanol-inducible alcohol oxidase (ox) promoter sequence of *Pichia pastoris* (Pratap et al., (2000)) and signal sequence of the Plus pheromone of *Schizosaccharomyces pombe* and the thiamine-regulated promoter (Kumar et al., (2004)). A high level of expression of streptokinase with 50–100% processing of the signal sequence and secretion of the mature streptokinase into the periplasmic fraction is observed. Moreover, an activity value of 4133 units/ml is reported.

In this study, as the promoter and signal sequence to be used, the gene encoding subtilisin Carlsberg (s*ub*C gene), which is an alkaline serine protease produced by *Bacillus licheniformis* was used. These promoter and signal peptide

regions are effective in that they are all functional which can be depicted from the various studies published in the literature:

It is aimed to develop *Bacillus* species carrying s*ub*C gene encoding SAP enzyme in order to increase the yield and selectivity for extracellular SAP production in the study of Çalık et al., (2003-a). The work, Çalık et al., (2003-d), reports on the design of a complex medium based on simple and complex carbon sources, i.e. glucose, sucrose, molasses, and defatted-soybean, and simple and complex nitrogen sources, i.e. $(NH_4)_2HPO_4$, casein, and defatted-soybean, for SAP production by recombinant *Bacillus subtilis* carrying pHV1431::*subC* gene. SAP production on a complex medium with various recombinant *Bacillus* species and the effects of oxygen transfer on SAP production were performed by Çalık et al., (2003-e). The work, Çalık et al., (2004-a), describes SAP production in a complex medium based on physically pretreated molasses by recombinant *Bacillus subtilis* carrying pHV1431::*subC* gene. The effects of oxygen transfer were investigated in 3.5 dm^3 bioreactor systems.

The effects of oxygen transfer on SAP production by *Bacillus licheniformis* on a defined medium with the single carbon source citric acid as sole carbon source were investigated in 3.5 dm^3 batch bioreactor systems (Çalık et al., 1998). A metabolic flux-based stoichiometric model based on the proposed metabolic network was applied to metabolic fluxes of the central carbon pathways in the SAP bioprocess by *Bacillus licheniformis* in a defined medium where citrate was used as the only carbon source in Çalık et al., (1999). The effects of the controlled and uncontrolled pH conditions on SAP production by *Bacillus licheniformis* were investigated on a defined medium with the single carbon source glucose in batch bioreactors (Çalık et al., 2002-a).

The choice of host cell must fit into a bioprocess strategy. That strategy includes not only plans for efficient production, but also how a product is to be recovered and purified. The development of processes for making products from

genetically engineered organisms requires that a large number of choices be made. Important characteristics of selected host system for any protein production from recombinant DNA includes: high growth rate, availability of genetic systems, high expression level, low cost media, low levels of proteolytic degradation, level of secretion, and safety (Schuler and Kargi, 2002).

The demand for foreign gene expression systems is rapidly increasing. Production of heterologous proteins at high levels by bacteria is commonly achieved using *Escherichia coli* as the host (Li et al., 2004). The main reason for the popularity of *E. coli* is the broad knowledge base for *E. coli. E. coli* physiology and genetics are probably far better understood than for any other living organism (Schuler and Kargi, 2002). However, the *E. coli* expression system still has disadvantages. For example, it is a pathogenic bacterium and has endotoxins (lipopolysaccharide); it secretes protein into the periplasm and often into inclusion bodies. *Bacillus* sp. is an occasional alternative host for expression of heterologous secretory proteins (Li et al., 2004).

In this study, since as the promoter and signal sequence to be used, the gene encoding subtilisin Carlsberg, which is an alkaline serine protease produced by *Bacillus licheniformis* is used, in order to produce the recombinant protein streptokinase extracellularly, it is advantageous to employ *Bacillus* sp. as an expression host. First, it has a huge capacity for secreting proteins directly into the growth medium, which greatly facilitates their downstream processing. It is widely used for the production of industrial enzymes, so it is well known with respect to fermentation technology. It is a genetically highly amenable host organism from which a large variety of genetic tools have been developed. Some strains show natural competence. Some have a transparent genome, because their complete sequence is known. *B. subtilis* and *B. licheniformis* were the two chosen possible expression hosts because they are non-pathogenic, and free of endotoxins, and bears "generally recognized as safe" (GRAS) status. Finally, they are of non-biased codon usage (Li et al., 2004). The absence of lipopolysaccharides (endotoxins) from the cell

wall is another positive aspect of them (Westers et al., 2006). Moreover, as stated above, SAP production is commonly studied with these *Bacillus* species and the promoter and signal peptide regions of SAP encoding gene which were also used in this study, are observed to be effective in that they are all functional which can be depicted from the various studies given above.

Streptokinase production capacities of these two recombinant *Bacillus* species were compared. The highest production was observed in recombinant *B. lichenifomis* 749/C (ATCC 25972) strain in a defined medium which was optimized in terms of carbon and nitrogen sources. Finally, fermentation characteristics of recombinant streptokinase production were studied. In the literature, bioprocess parameters and oxygen transfer characteristics in *β*-lactamase production by *Bacillus* species were studied by Çelik et al., (2004). *Bacillus licheniformis* 749/C ATCC 25972 strain was used in this study as the production host. The effects of oxygen transfer on the *β*-lactamase production by *Bacillus licheniformis* were investigated in a glucose-based defined medium in 3.0 dm3 batch bioreactor systems by Çalık et al., (2005). The effects of pH on *endo*- and *exo*-metabolome profiling of *β*-lactamase producing *Bacillus licheniformis* 749/C ATCC 25972 strain were investigated at controlled-pH and uncontrolled-pH values using a glucose-based defined medium in the work, İleri et al., (2006).

In conclusion, in this study, the promoter and signal (*pre*-) DNA sequence of *B. licheniformis* (DSM1969) extracellular serine alkaline protease (SAP) enzyme gene (*sub*C: Acc. No. X03341) was ligated to 5' end of the streptokinase gene (*skc:* Acc. No. S46536) by SOE (Gene Splicing by Overlap Extension) method through PCR. In order to perform this ligation, fistly proper forward and reverse primers were designed for the cloning of the fusion product of *skc* and *pre(sub*C) genes into the selected plasmids after determining the suitable restriction recognition sites (*Eco*RI and *Bam*HI) for the hybrid gene. Initially, each gene was amplified by PCR with *pre(subC)* forward/reverse and *skc* forward/reverse primers seperately. These

amplified DNA fragments were associated with each other by SOE method resulting in the hybrid gene '*pre(sub*C*)*::*skc'*. Next, the chimeric gene was cloned into pUC19 and then sub-cloned into pMK4 *E. coli-Bacillus* shuttle vector from the restriction recognition sites of *Eco*RI and *Bam*HI. Recombinant plasmid pMK4::*pre(sub*C*)*::*skc* was firstly transferred into *E. coli* TG1 strain by induced competence, and then into *B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) by electroporation and expressed in these *Bacillus* species under the control of the promoter region of *pre(sub*C*)* gene. Streptokinase production capacities of these two recombinant *Bacillus* species were compared. The highest production was observed in recombinant *B. lichenifomis* 749/C (ATCC 25972) strain in a defined medium which was optimized in terms of carbon and nitrogen sources. Finally, fermentation characteristics of streptokinase production were studied.

CHAPTER 2

LITERATURE SURVEY

2.1 Enzymes

2.1.1 General Characteristics

Enzymes are catalysts evolved in nature for speeding up and co-ordinating the multitude of chemical reactions necessary to develop and maintain life. All enzymes are proteins with the exception of the recently discovered ribozymes. As catalysts, enzymes alter the rate in which a thermodynamic equilibrium is reached, but do not change that equilibrium implying that enzymes work reversibly (Drauz, 1995). An enzyme could not be consumed or could not be changed irreversibly during the chemical reaction. There are thousands of seperate chemical reactions carried out by the cells of all living organisms. Without enzymes, majority of these chemical reactions would continue very slowly (Scheve, 1984). This acceleration of the reaction rate is achieved by lowering the activation energy of the corresponding process (Drauz, 1995).

Specificity is a term of high importance for enzymes in that, a selection between enzymes of high or low selectivity can be performed to achieve the desired function (Bailey, 1986). The most important meaning for the enzymologist refers to an enzyme's discrimination between several substrates competing for an active site: for example, the specificity of a particular aminoacyl-tRNA synthetase for a particular amino acid and a particular tRNA in a mixture of all the amino acids and all the tRNA s. This is the definition of specificity that is relevant to biological systems (Fersht, 1984). It is thought that enzyme specificity arises from the three dimensional

conformation of an enzyme that leads to the formation of the active site responsible for the catalytic ability of an enzyme (Bailey, 1986). This specificity characteristic of all enzymes decreases interference by unwanted substrates; moreover it minimizes the problems that can be arisen by undesired by-products. By-product formation is also unwanted in any industrial process, since for getting rid of this by-product, an additional process step is needed which is economically undesirable (Drauz, 1995).

Another property of enzymes is that they frequently need cofactors for their functioning. These non-protein compounds combine with an inactive enzyme to give a catalytically active enzyme-cofactor complex. Metal ions are the simplest cofactors such as Co^{2+}, Ca^{2+}, Zn^{2+}, etc (Bailey, 1986). Besides metal ions, cofactors or coenzymes serve to activate groups and participate in the catalytic process. They may act by nucleophilic or electrophilic attack on the substrate to initiate a reaction (Drauz, 1995).

The active sites of enzymes generally contain important acidic or basic groups (Fersht, 1984). An enzyme can be affected by the pH change in that this variation in pH can change the conformation of protein structure or can ionize the substrate or the active site (Atkinson and Mavituna, 1991). Enzymes contain many polar amino acids at the surface which may be protonated or unprotonated depending on the pH of the surrounding medium (Drauz, 1995). Along with an optimum pH value, an optimum temperature is also necessary for an enzyme to work fastest. Usually, at quite low temperatures enzymes lose their activity (Bailey, 1986). The optimal temperature of operation has to be lowered if long reaction times or long service life of an enzyme are required (Drauz, 1995). The optimum temperature for all enzymes generally lies between 37-47^{0}C, and the optimum pH is ranked from acidic, i.e., 1.0, to alkaline, i.e., 10.5 for different enzymes (Kirk and Othmer, 1994).

Engineering of enzymes is now becoming a reality through the use of recombinant DNA technology and nucleic acid chemistry. These methods should

eventually lead to the ultimate achievement of design and synthesis of novel enzymes (Fersht, 1984). Mostly, enzymes used in industrial processes are isolated from microorganisms. These enzymes are the catalysts for the synthesis of industrial chemicals. Some advantages of using microbial sources for the production of catalysts are: the enzyme stability is increased; the production time for catalysts is reduced; the catalyst cost can be decreased; multi-catalytic processes can be performed (Moses and Cape, 1991). Additionally, the main criteria for the choice of biological source and for the design of the process are: availability of the starting material in constant quality, reproducibility and reliability of the final product, the overall cost of the process (Drauz, 1995).

2.1.2 Classification of Enzymes

The systematic name of an enzyme is based on the equation of the chemical reaction taking place and the type of reaction, followed by the suffix –ase (Drauz, 1995). A system was developed for classifying enzymes by the Commission on Enzymes of the International Union of Biochemistry (Atkinson and Mavituna, 1991). By international agreement, each enzyme is assigned with a recommended name (Table 2.1) and four groups of numbers by the enzyme commission (EC) (Chaplin, 1990). Moreover, according to the types of reactions they catalyze enzymes are divided into six major classes. Each of these major classes is then divided into numerical subclasses and sub-subclasses with respect to individual reactions concerned. The forth number in the classification indicates the serial number of the enzyme in that subclass (Atkinson and Mavituna, 1991).

For instance, E.C 3.4.99.22 is the EC number of streptokinase, which is a hydrolase acting as a plasminogen activator complexing with circulatory plasminogen. This 1:1 stoichiometric complex is a high-specificity protease, activating plasminogen to plasmin (A. Banerjee et al., 2004).

Table 2.1 International classification of enzymes (Drauz, 1995)

Number	Class	Type of reaction catalyzed
1	Oxidoreductases	Catalyze oxidation/reduction reactions transferring hydrogen, oxygen, and/or electrons, between molecules
2	Transferases	Catalyze group-transfer reactions
3	Hydrolases	Catalyze the hydrolytic cleavage of bonds
4	Lyases	Catalyze the non-hydrolytic cleavage of bonds by elimination reactions leaving double bonds or adding groups to a double bond
5	Isomerases	Transfer functional groups within molecules to give isomeric forms
6	Ligases	Catalyze the covalent joining of two molecules coupled with the hydrolysis of an energy rich bond in ATP or similar triphosphates

2.1.3 Enzyme Activity

The catalytic activity term is used frequently for the characterization of an enzyme (Drauz, 1995). When an enzyme is going to be used in a chemical process, the major concerning point is the activity of the enzyme as a measure of its content. It is hard to determine exact amount of an enzyme present or used in a process in absolute terms (e.g. grams), since there is frequently a purity problem and since a portion of that enzyme may be found in inactive form. Thus, the parameter that is commonly applied is the activity term. Thus, enzymes are marketed in terms of

activity (Chaplin, 1990). In other words, enzymes are quantified by measuring their catalytic activity and sold on the basis of activity (Drauz, 1995).

The unit for the catalytic activity is the Katal (kat), as defined by the International Union of Biochemistry (IUB), 1 kat corresponds to the amount of enzyme catalyzing the conversion of one mole of substrate per second at 30 ^{o}C under specified conditions. In the biochemical literature, another quantity is often used, the international unit (IU); one unit (U) of enzyme activity is described as the amount that catalyzes the conversion of one micromole of substrate per minute under specific defined conditions (Drauz, 1995). Another term, specific activity is defined as units per mg of protein (Gutfreund, 1965).

It is only possible to make a comparison between the activities of different enzymes when the procedures of assay are done definetely in the same way (Faber, 2000). To ensure reproducible and meaningful results measuring enzyme activity several points have to be taken into consideration (Drauz, 1995). On the other hand, it is obvious that the optimal operation conditions for laboratories and suppliers vary (Chaplin, 1990). As a result, if it is desired to achieve reproducible results for activities, parameters such as temperature, pH, substrate concentration which affect reaction rates should be controlled carefully (Fersht, 1984). Parameters determining the enzyme properties are substrates, cosubstrates, coenzymes, effectors, inhibitors, pH, temperature, buffer, organic solvents/cosolvents, ionic strength, viscosity of the medium, redox potential (oxygen sensitivity), heavy metal ions, influences resulting from reactor conditions (Drauz, 1995).

2.2 Streptokinase

A fast growth of biotechnology companies has occurred after the advent of recombinant DNA (rDNA) technology. This technology has also lead to the development of the pharmaceutical industry resulting in production of therapeutic rDNA products, mostly hormones of therapeutic interest, growth factors, thrombolytic agents, blood coagulation products, anticoagulants, interferons, interleukins and therapeutic enzymes (Bhopale et al., 2005). There are two important features of enzymes used as drugs that distinguish them from all other types of drugs. Firstly, with great affinity and specificity, enzymes often bind and act on their targets. Secondly, enzymes are catalytic species converting multiple target molecules to the desired products. These two characteristics make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecules cannot. These features lead to the development of many enzyme drugs for a wide range of disorders (Vellard et al., 2003).

Cardiovascular diseases include Coronary Heart Disease or diseases of arteries (Arteriosclerosis, including hardening of the arteries, or Atherosclerosis), Heart Attack (Myocardial Infarction), Arrhythmias, Congestive Heart Disease, Stroke, High Blood Pressure (Hypertension), Rheumatic Heart Disease and other dysfunctions of the cardiovascular system (Kirk and Othmer, 1994).

Heart attack results from the restricted blood flow in the coronary arteries, thus from insufficient delivery of oxygen to heart muscles, then the muscles will infarct or die. Heart muscle (myocardium) is formed up from muscle cells (myocardial cells). Myocardial cells receive nutrient from coronary arteries branched from aorta. Blockage of these coronary arteries occurs during Coronary Artery Disease (CAD) (Kirk and Othmer, 1994).

Collagen is the major extracellular matrix protein in the heart and represents a crucial target for antiremodeling and cardioprotective therapy. Excessive deposition of collagen, leading to cardiac fibrosis, is a major determinant of cardiac disfunction, myocardial infarction and arrhythmogenecity associated with sudden death (Zannad et al., 2005).

The major cause of Acute Myocardial Infarction is coronary arterial thrombosis (thrombus formation). As the blood clot ages, the polymerization of fibrin cross-linking and other blood materials increses and it becomes more resistant to lysis. The faster the coronary arterial thrombus is lyzed, the less irreversible damage done on the myocardium (Kirk and Othmer, 1994).

A healthy hemostatic system suppresses the development of blood clots in normal circulation. A blood clot (thrombus) consists of blood cells in a matrix of protein fibrin. Enzyme mediated dissolution of the fibrin clot is known as thrombolysis or fibrinolysis (Banerjee et al., 2004). Fibrinolysis is the biochemical process underlying thrombolysis since both processes depend on the degradation of the fibrin network that holds the blood clot together in a coronary or celebral artery in the case of myocardial infarction. Myocardial Infarction and stroke can be treated with thrombolytics (clot busters) which are plasminogen activators (Longstaff et al., 2005).

Thrombolytic therapy, 1980s first introduced, is essentially the delivery of plasminogen activators into the circulation (Longstaff et al., 2005). This therapy is based on fibrinolysis (Figure 2.1). Thrombolytic therapy saves about 30 lives in 1000 patients presenting within six hours of symptom onset (Schofield et al., 2004).

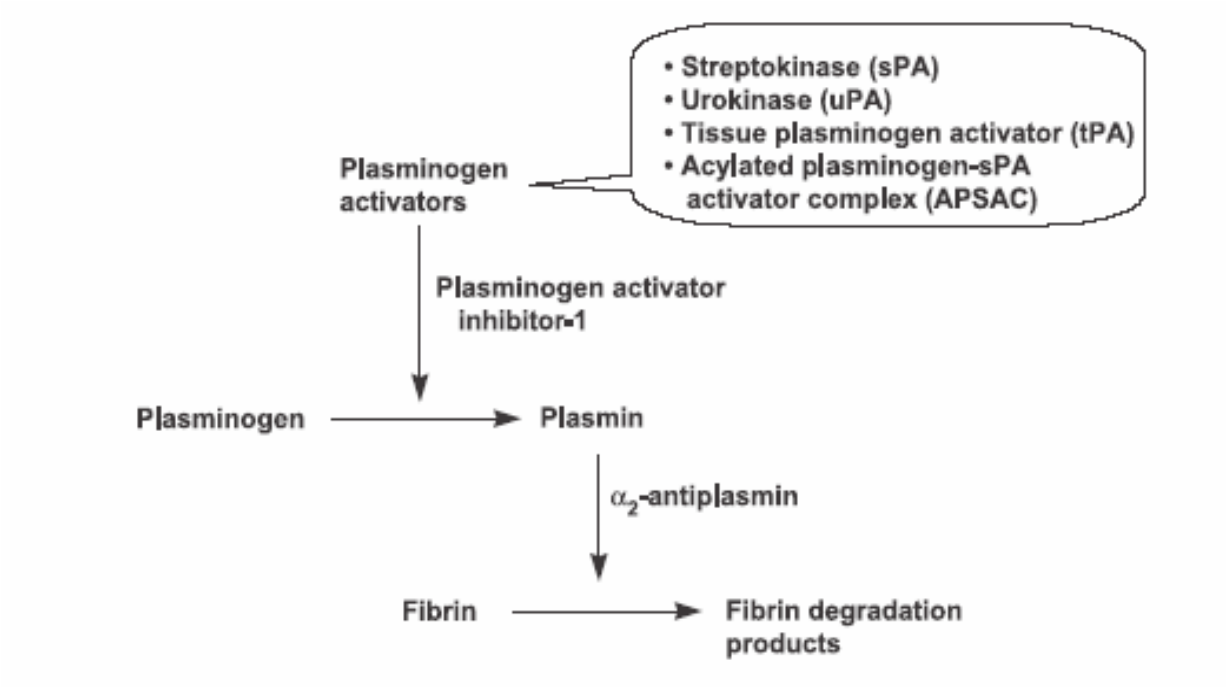

Figure 2.1 Schematic representation of fibrinolysis (Banerjee et al., 2004).

The main components in plasminogen activation forming the fibrinolytic system include plasma zymogen, plasminogen; its activated product, the proteolytic enzyme, plasmin; plasminogen activators; inhibitors for the plasmin, plasminogen activators, fibrin and fibrinogen (Vaheri et al., 2004 & Kunamneni et al., 2007). More commonly speaking, various known thrombolytic agents can be listed as: tissue plasminogen activator (tPA), streptokinase, alteplase, tenecteplase, urokinase, acylated plasminogen-streptokinase activator complex and prourokinase (Kirk and Othmer, 1994).

The first generation of thrombolytics (streptokinase and urokinase) are plasminogen activators resulting in a large scale plasmin production in the circulation. Streptokinase is secreted by invasive bacteria. Urokinase is a naturally occuring protease that can be isolated from human urine and found in inactive form in circulation as pro-enzyme (Longstaff et al., 2005).

Second generation thrombolytics with fibrin binding domains were produced by genetic engineering with the aim of targetting the plasminogen activator to a fibrin clot. Tissue plasminogen activator (tPA) is developed. Following from tPA, third generation thrombolytics were engineered. The goal was to improve properties in terms of longer plasma half-life, resistance to natural inhibitors or improved fibrin binding making them more fibrin specific (Longstaff et al., 2005).

Protein engineering of second and third generation thrombolytics may be interpreted as a failure. Basic scientific considerations, in vitro data and animal studies have not been translated into significant improvements in outcomes in clinical trials. This lack of clear improvement and the much increased cost of second and third generation thrombolytics means that streptokinase is still the most widely used thrombolytic for the treatment of acute myocardial infarction in many parts of the world (Longstaff et al., 2005 & Kunamneni et al., 2007).

Streptokinase is a protein produced by a group of C β-hemolytic streptococci (Kirk and Othmer, 1994). Streptokinase (E.C 3.4.99.22) is a bacterial protein that does not occur naturally in human circulation (Banerjee et al., 2004). It is a single chain protein containing 415 amino acids, molecular weight of 45,000-50,000 Da (Kirk and Othmer, 1994). It shows maximum activity at pH 7.5 and its isoelectric pH (the isoelectric pH is the pH at which a protein will not migrate in an electric field and is determined by the charged groups in the protein) is 4.7. It does not contain phosphorous, conjugated carbohydrates and lipids (Banerjee et al., 2004). It is available as a lypholized powder and freely soluble in water.

In contrast to other anticoagulants, streptokinase promotes thrombolysis (Kirk and Othmer, 1994). Unlike tPA and uPA, proteases, streptokinase possesses no enzymatic actibity of its own. It gets this property by complexing with circulatory plasminogen (Banerjee et al., 2004). But, only after streptokinase combines with plasminogen on a 1:1 basis to form a streptokinase-plasminogen complex, to expose

the active site of the plasminogen portion, it becomes an active species (Kirk and Othmer, 1994). The resulting complex converts plasminogen to plasmin which is an active protease degrading fibrin in the blood clot (Wu et al., 1998).

Thrombolytic activity of streptokinase lasts for 3-4 h in the circulation system (Kirk and Othmer, 1994). It is a clinically important and cost effective plasminogen activator (Banerjee et al., 2004). It is used as a thrombolytic agent in acute Myocardial Infarction, Coronary Artery Thrombosis, Deep Vein Thrombosis and Pulmonary Embolism. Side effects are bleeding, hemorrhage, fever, and allergy (Kirk and Othmer, 1994).

Streptokinase activates plasminogen by both fibrin dependent and independent mechanisms. The C-terminal domain recognizes plasminogen substrate. Similarly the Asp_{41}-His_{48} region of streptokinase is important in binding the substrate plasminogen. The first 59 amino acids are important. Without these N-terminal amino acids, streptokinase has an unstable secondary structure (Banerjee et al., 2004).

A more detailed expression of steps involved in plasmin generation is shown in Figure 2.2. Reaction 1 is important for streptokinase and urokinase. The second generation thrombolytics such as tPA require fibrin as a cofactor (Longstaff et al., 2005).

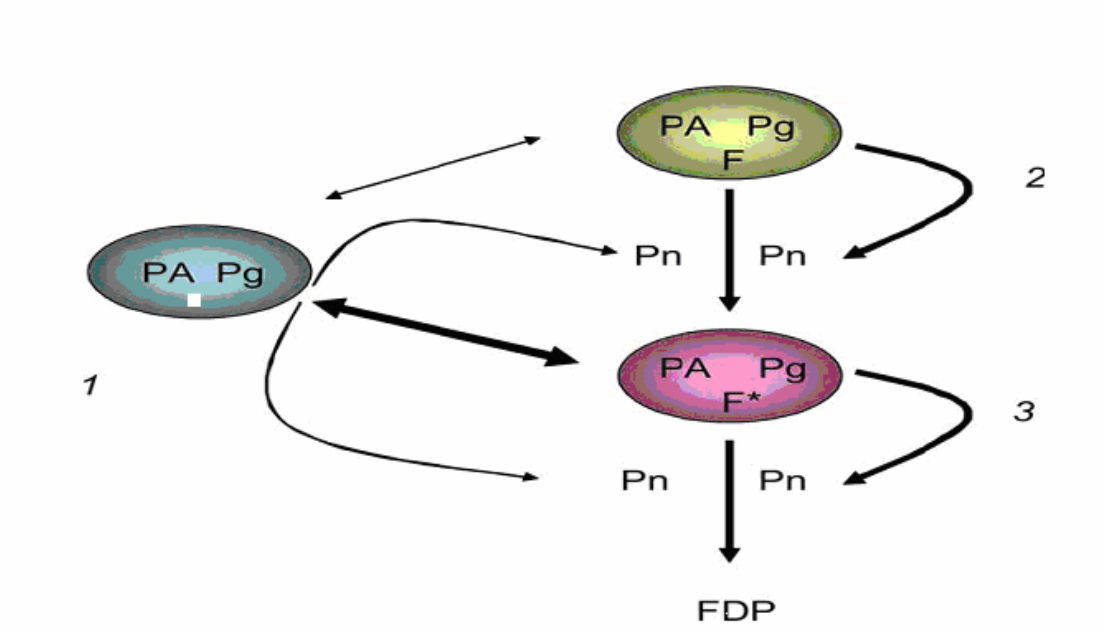

Figure 2.2 Scheme showing plasminogen activation reactions taking place in a fibrin clot. Reaction (1) is the generation of plasmin (Pn) from plasminogen (Pg) catalysed by plasminogen activator (PA) taking place in solution in the absence of fibrin (F). Reaction (2) shows the formation of a ternary complex of PA–Pg–F leading to generation of Pn at the fibrin surface. Plasmin digests fibrin initially generating C terminal lysines in partially degraded fibrin (F\) which presents additional binding sites for PA and Pg. Eventually, fibrin is degraded to soluble fibrin degradation products (FDP) as the clot is lysed (Longstaff et al., 2005).

Plasminogen is a single chain glycoprotein with a molecular mass of ~92 kDa. It is synthesized mainly in liver and this inactive form circulates in the vasculature but, it is also found in other body fluids. Secreted plasminogen is a precursor that is cleaved by plasminogen activators at a single site, Agr_{561} – Val_{562} bond, to yield a two-chain plasmin held together by two disulfide bonds (Vaheri et al., 2004).

In mammalian circulation the enzyme responsible for fibrinolysis is plasmin (a tripsin like serine protease). Inactive protein plasminogen is converted to active plasmin by plasminogen activators (Banerjee et al., 2004). The end product of plasminogen activation cascade, plasmin, can leave extracellular matrix proteins, activate proteinases and deliver growth factors (Vaheri et al., 2004).

Scientific papers that are reviewed and investigated in order to develop a background understanding about the streptokinase can be collected in three groups according to the host organisms used in the production of the streptokinase: papers on *Bacillus subtilis, Escherichia coli* and yeasts.

Determination of whether a *B. subtilis* promoter and signal sequence would be better than the native streptokinase (*skc*) promoter and signal sequence is aimed and the corresponding scientific research is conducted by Wong et al., (1993). In this study, with the available cloned *skc* gene, production of the secreted streptokinase from *B. subtilis* WB600 (6 extracellular protease deficient strain) is studied. A modified *skc* which has the original *skc* promoter and signal sequence replaced with the *B. subtilis* levansucrase promoter and signal sequence is also constructed. The structural gene *skc* of streptokinase from *Streptococcus equisimilis* H46A has been cloned and sequenced. To determine whether a *B. Subtilis* promoter and signal sequence would be better than the native promoter and signal sequence, two vectors are designed. pSK1 caries the intact *skc* gene including its promoter region. pSK3 carries the sequence encoding streptokinase, levansucrase (*sac*B) promoter and signal sequence. Finally, 2.5 fold increase in the activity is reported.

In Wu et al., (1998), the short in vivo half-life of streptokinase is prolonged by site-directed mutagenesis. Plasmin is a trypsin-like serine protease cleaving the peptide bond after lysine or arginine. In this study, site-directed mutagenesis based on inverse PCR method is applied to change Lys59 to glutamine or glutamic acid and a six-extracellular-protease-deficient *B. subtilis* strain, WB600 (*trpC2 nprA apr epr bfp*

mpr::ble nprB::ery) is used as the host organism. *skc* without Lys59 or Lys59+Lys386 is cloned by pSK3 carrying *sac*B promoter and signal sequence (the sucrose-inducible regulatory region encoding levansucrase) and 2.2-2.5 fold increase in the activity is observed.

Staphylokinase is a promising blood-clot dissolving agent for the treatment of patients suffering from a heart attack similar to streptokinase. To determine whether a *B. subtilis* promoter and signal sequence would be better than the native staphylokinase promoter and signal sequence is aimed in the study of Ye et al., (1998). It would be desirable to produce staphylokinase in large quantities for biochemical characterization and clinical trials. By using a 7-extracellular protease deficient *B. subtilis* WB700 (*trpC2nprE aprE epr bpf* D*mpr::ble* D*nprB::bsr* D*vpr::ery)*, intact staphylokinase can be produced via secretion. To optimize the production and stability of the vectors, both the promoter and signal sequence of staphylokinase are replaced by *B. subtilis sac*B signal sequence and promoters P43, P*amy*, P*sac*B. pSAKN, pSAKB, pSAKB, pSAKP, pSAKA expression vectors are developed. The final purified staphylokinase protein has a specific activity of 2840 units/mg.

By the removal of 13 N-terminal amino acids, the production and secretion of streptokinase using OmpA signal sequence in *E. coli* is enhanced by Lee et al., (1997). The secretion level of the resulting SKDN13 protein into the extracellular medium appears to be two times higher than that of wild-type streptokinase. Approximately 4500 IU of SKDN13 protein per 1 ml LB-ampicillin medium is secreted into extracellular medium in 12 hours after induction. In the study reported, it is stated that *E. coli* JM109 strain as the main host microorganism is used; pSK100, pSKΔNB plasmids, to which *ompA* signal sequence is inserted, are constructed.

In the study of Zhang et al., (1999), a process for the pilot production of recombinant streptokinase is established. As the host organism, *E. coli* K802 strain is

used, and pST plasmid is constructed to produce recombinant streptokinase by fed-batch cultivation of *E. coli.* After renaturation and purification steps, 12.9 g of recombinant streptokinase with 97.8% of purity and about 10^5 IU/mg of specific activity is observed, the yield of protein and the recovery of activity are 44.9% and 51%, respectively.

Secretion of the protein streptokinase is performed by replacing the native secretion signal codons of streptokinase encoding gene with those from α-factor leader peptide and expressing the fusion construct under the control of the methanol-inducible alcohol oxidase (ox) promoter of *Pichia pastoris* in the study of Pratap et al., (2000). The results presented show that N-linked glycosylation of streptokinase leads to 30±40% enhancement of the protein stability and resistance towards degradation but does not interfere with its fibrinolytic function. *Pichia pastoris* GS115 and *Escherichia coli* A61 are the host microorganisms. pPICK20 is the corresponding integration plasmid used. The resulting recombinant plasmid pPICK20 is integrated into the *P. pastoris* genome. The extracellular streptokinase activities obtained for *Pichia pastoris*, *Escherichia coli* and *Streptococcus equisimilis* are reported as 3200 IU/ml, 1500 IU/ml and 150 IU/ml respectively.

A chimeric gene consisting of the signal sequence of the Plus pheromone of *Schizosaccharomyces pombe* fused in-frame with the mature streptokinase from *Streptococcus* sp. is constructed and inserted into the expression vector, pJRK1, containing the thiamine-regulated promoter in order to enhance the expression level of streptokinase without glycosylation and degradation in *Sz. Pombe* by Kumar et al., (2004). A high level of expression of streptokinase with 50–100% processing of the signal sequence and secretion of the mature streptokinase into the periplasmic fraction is observed. Moreover, an activity value of 4133 units/ml is reported.

2.3 Genetic Engineering Techniques

The discovery of the role of DNA was a tremendous stimulus to genetic research, and many biologists contributed to the great age of genetics. In the 14 years between 1952 and 1966, the structure of DNA was determined, the genetic code cracked, and the processes of transcription and translation described. Then the years 1971-1973 was described as a revolution in experimental biology. A whole new methodology was developed, enabling previously impossible experiments to be planned and carried out. These methods, referred to as recombinant DNA technology or genetic engineering, and having at their core the process of gene cloning, lead another great age of genetics. The techniques spawned modern biotechnology, which puts genes to work in production of proteins and other compounds needed in medicine and industrial processes. According to DNA folklore, Kary Mullis invented the polymerase chain reaction (PCR) in 1985 (Brown, 2001). Selective amplication of any specific DNA region by PCR, association of two different DNA fragments side by side by gene splicing by overlap extension (SOE) method, DNA concentration determination, analysis of secreted proteins by SDS-Polyacrylamide gel electrophoresis, and restriction digestion are found among the basic principles applied in genetic engineering.

2.3.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction results in the selective amplication of a region of DNA molecule. Any region of any DNA molecule can be chosen, so long as the sequences at the borders of the region are known. The border sequences must be known because in order to carry out a PCR, two short oligonucleotides must hybridize to the target DNA molecule, one to each strand. These oligonucleotides, which act as primers for the DNA synthesis reactions, delimit the region that will be amplified. PCR is carried out in a single test tube simply by mixing DNA with a set of reagents and placing the tube in a thermal cycler, a piece of equipment that enables

the mixture to be incubated at a series of temperatures that are varied in a preprogrammed manner. The basic steps in a PCR experiment are as follows (Figure 2.3):

1) The mixture is heated to 94^{O}C, at which the hydrogen bonds holding together the two strands of the double-stranded DNA are broken, causing the molecule to denature.

2) The mixture is cooled down to 50-60 ^{O}C. The two strands of each molecule could join back together at this temperature, but most do not because the mixture contains a large excess of short oligonucleotides or primers, which anneal to the DNA molecules at specific positions.

3) The temperature is raised to 74 ^{O}C. This is the optimum working temperature for the *Taq* DNA polymerase that is present in the mixture. At this stage, the enzyme attaches to one end of each primer and synthesizes new strands of DNA, complementary to the template DNA molecules.

4) The temperature is increased back to 94 ^{O}C. The double-stranded molecules denature into single strands. This begins a second cycle of denaturation-annealing-synthesis, at the end of which there are eight DNA strands. By repeating the cycles 25 times, the template double-stranded molecule is converted into over 50 million new double-stranded molecules.

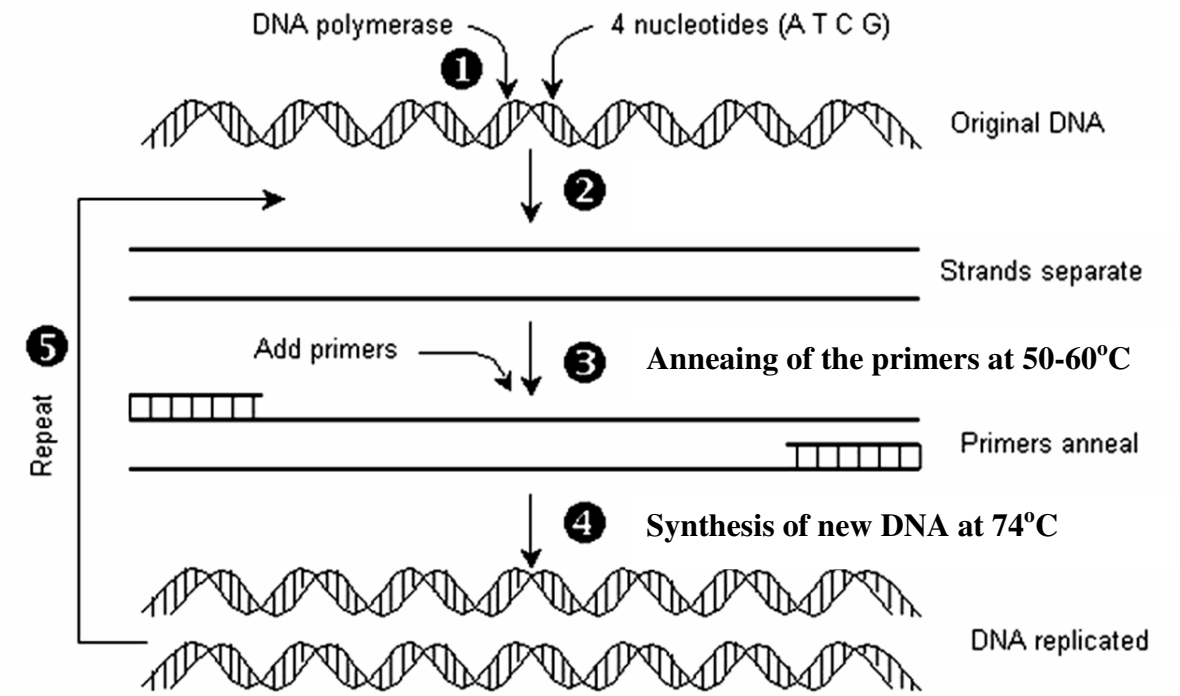

Figure 2.3 The basic steps in the polymerase chain reaction (Brown, 2001 & http://www.biologymad.com)

The optimum temperature for annealing depends on the primers used. The pH of the buffer used decreases with increasing temperature. The actual pH varies between about 6.8 and 7.8 during the reaction. The time taken for each cycle is considerably longer than 3 min, depending upon the rates of heating and cooling between steps. The PCR does not efficiently amplify sequences longer than about 3 kb (Old and Primrose, 1994).

By repeated cycles of heat denaturation, primer hybridization, and extension, there follows a rapid exponential accumulation of the specific target fragment of DNA. After 22 cycles, an amplification of about 10^6-fold is expected, and amplifications of this order are actually attained in practice (Old and Primrose, 1994).

Amplification is usually carried out by the enzyme DNA polymerase I from *Thermus aquaticus.* This organism lives in hot springs, and many of its enzymes, including *Taq* polymerase, are thermostable, meaning that they are resistant to denaturation by heat treatment. The thermostability of *Taq* polymerase is an essential requirement in PCR methodology (Brown, 2001). The *Taq* polymerase lacks a 3'-5' proofreading exonuclease activity. This lack appears to contribute to errors during PCR amplification due to misincorporation of nucleotides. Partly to overcome this problem, other thermostable DNA polymerases with improved fidelity have been sought, although the *Taq* polymerase remains the most widely used enzyme for PCR. In certain applications, especially where amplified DNA is cloned, it is important to check the nucleotide sequence of the cloned product to reveal any mutations that may have occurred during the PCR (Old and Primrose, 1994).

2.3.2 Gene Splicing by Overlap Extension (SOE) Method

It was initially reported by Horton et al., (1989) and Ho et al., (1989) that the gene splicing by overlap extension method is based on the association or recombination of fragments from the genes that are developed in seperate polymerase chain reactions. Fragments from the genes that are to be recombined are generated in seperate PCRs. The primers are designed so that the ends of the PCR products contain complementary sequences. When these PCR products are mixed, denaturated and reannealed strands having the matching sequences at their 3' ends overlap and act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are spliced together.

As shown in Figure 2.4, reaction 1 is a PCR reaction using primers a and b to generate AB product. Product CD is produced in a seperate PCR reaction by the primers c and d. Oligos b and c match their template genes in their 3' ends, but their 5' portions are designed so that the two oligos are complementary. Then, the

segments are mixed along with excess primers a and d, denatured, reannealed, and primer-extended by DNA polymerase (the intermediates of this reaction are shown in the box). The end of one strand from each product is capable of hybridizing with the complementary end from the other product. The strands having this overlap at their 3' ends can act as primers for one another and can be extended by PCR to form the full length recombinant product comprising a sequence corresponding the sequences of the first and second DNA. The recombinant product is PCR-amplified in the presence of a and b.

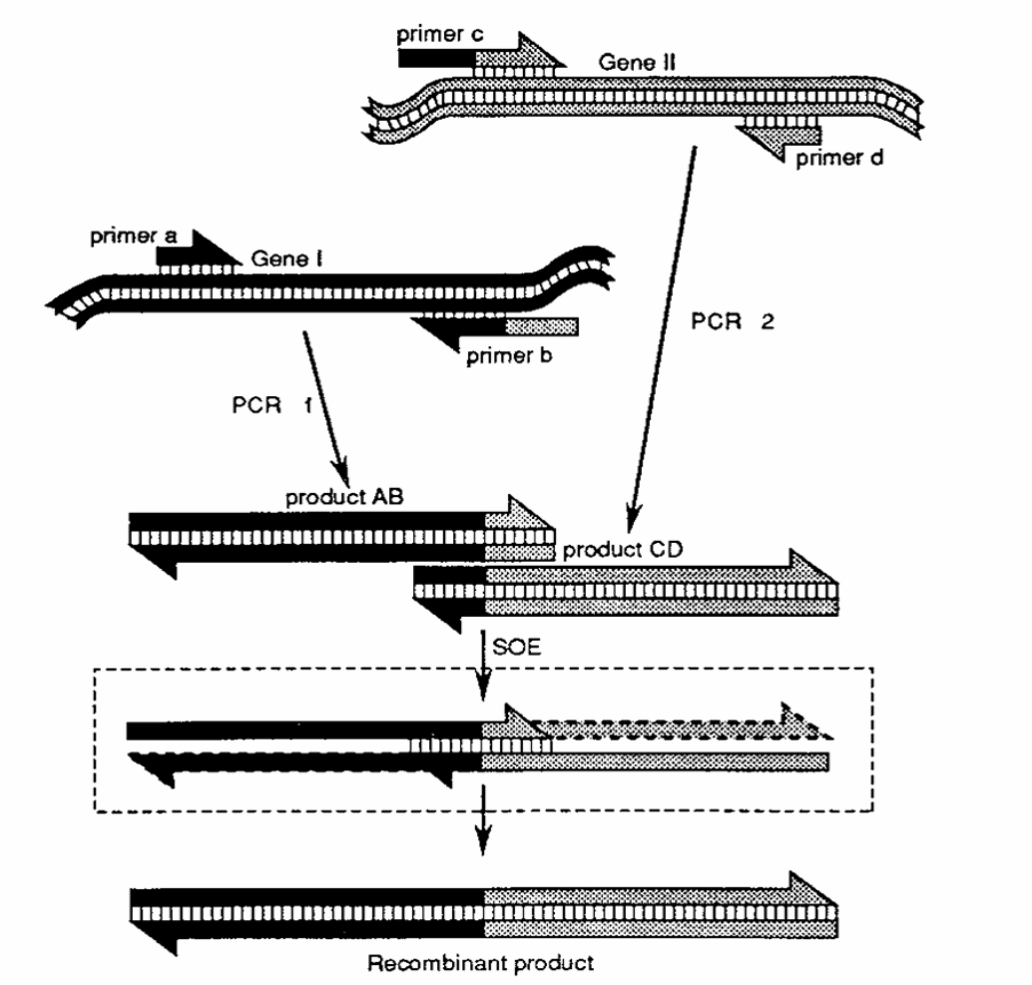

Figure 2.4 The mechanism of gene splicing by overlap extension (SOE) method (Horton et al., 1993)

2.3.3 Determination of DNA Concentration

It is crucial to know exactly how much DNA is present in a solution when carrying out a gene cloning experiment. Fortunately, DNA concentrations can be accurately measured by ultraviolet (UV) absorbance spectrophotometry. The amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Usually absorbance is measured at 260 nm, at which wavelength an absorbance (A_{260}) of 1.0 corresponds to 50µg of double-stranded DNA per ml. Ultraviolet absorbance can also be used to check the purity of a DNA preparation. With a pure sample of DNA the ratio of the absorbances at 260 nm and 280 nm (A_{260}/A_{280}) is 1.8. Ratios of less than 1.8 indicate that the preparation is contaminated, either with protein or with phenol (Brown, 2001).

Gel electrophoresis is not only used as an analytical method, it is routinely used preparatively for the purification of specific DNA fragments. The gel is composed of polyacrylamide or agarose. Agarose is suitable for separating DNA fragments ranging in size from a few hundred to about 20 kb. Polyacrylamide is preferred for smaller DNA fragments. An agarose gel is a complex network of polymeric molecules whose average pore size depends on the buffer composition and the type and concentration of agarose used. The larger the pore size, the greater the DNA which can pass through and hence the larger the molecules which can be separated. Aaij & Borst (1972) showed that the migration rates of the DNA molecules were inversely proportional to the logarithms of the molecular weights. In any event, gel electrophoresis is frequently performed with marker DNA fragments of known size which allow accurate size determination of an unknown DNA molecule by interpolation. A particular advantage of gel electrophoresis is that the DNA bands can be easily detected at high sensitivity. The bands of DNA in the gel are stained with the intercalating dye ethidium bromide, and as little as 0.05µg of DNA in one band can be detected as visible fluorescence when the gel is illuminated with ultraviolet light (Old and Primrose, 1994).

In practice the composition of the gel determines the sizes of the DNA molecules that can be separated. A 0.5 cm thick slab of 0.5% agarose, which has relatively large pores, would be used for molecules in the size range 1-30 kb, allowing, for example, molecules of 10 and 12 kb to be clearly distinguished. At the other end of the scale, a very thin (0.3 mm) 40% polyacrylamide gel, with extremely small pores, would be used to separate much smaller DNA molecules, in the range 1-300bp, and could distinguish molecules differing in length by just a single nucleotide (Brown, 2001).

The rate of migration of linear DNA fragments is proportional to the applied voltage at low rates of voltages. However, when the voltage is larger than 125 volts, electrophoresis buffer will evaporate and the gel structure can be damaged (Smith and Wood, 1991).

2.3.4 SDS-Polyacrylamide Gel Electrophoreis of Proteins

Under conditions that ensure dissociation of proteins into their individual polypeptide subunits and that minimize aggregation, nearly all analytical electrophoreses of proteins are carried out in polyacrylamide gels. Most commonly, before the proteins are loaded into the gel, SDS, the strong anionic detergent, is used in combination with a reducing agent and heat in order to dissociate the proteins. Denaturated polypeptides bind to SDS and then they become negatively charged. The amount of the SDS that is bound is almost usually proportional to the molecular weight of the polypeptide. Moreover, it is independent of the sequence of that polypeptide. Then, SDS-polypeptide complex migrates through the gel according to the size of the protein. Detection of the unlabeled proteins seperated by polyacrylamide gel electrophoresis is typically performed by staining, either with Coomassie Brilliant Blue or with silver salts. Coomassie Brilliant Blue allows the visualization of proteins as blue bands inside the translucent matrix of the gel by its

nonspecific binding to proteins but not the gel in a relatively rapid and straightforward reaction. Silver staining method is more sensitive, but more difficult to perform. It is more adventageous in that it enables the detection of proteins 100 fold lower than those detected by Coomassie Brilliant Blue staining method (Sambrook, 2001).

2.3.5 Restriction Enzymes

Restriction endonucleases, commonly called restriction enzymes, are able to cut DNA molecules with a high degree of specificity for particular sequences (Dale, 1994). These degradative enzymes are synthesized by many species of bacteria; over 1200 different ones have been characterized. Three different classes of restriction endonucleases are recognized, each distinguished by a slightly different mode of action. Type I and III are rather complex and have only a limited role in genetic engineering. Type II restriction endonucleases, on the other hand, are the cutting enzymes that are so important in gene cloning (Brown, 2001).

Most of those in common use are type II restriction enzymes which can cut the DNA within (or immediately adjacent to) the sequence that they recognize. Such enzymes will therefore yield a characteristic and predictable pattern of fragments when they cut a specific piece of DNA (Dale, 1994). For example, the restriction endonuclease called *Pvu*I (isolated from *Proteus vulgaris*) cuts DNA only at the hexanucleotide CGATCG (Brown, 2001).

The exact nature of the cut produced by a restriction endonuclease is of considerable importance in the design of a gene cloning experiment. Many restriction endonucleases make a simple double-stranded cut in the middle of the recognition sequence, resulting in a blunt end or flush end. *Pvu* II and *Alu* I are examples of blunt end cutters. However, quite a large number of restriction endonucleases cut DNA in a

slightly different way. With these enzymes the two DNA strands are not cut exactly at the same position. Instead, the cleavage is staggered, usually by two or four nucleotides, so that the resulting DNA fragments have short single-stranded overhangs at each end. These are called sticky or cohesive ends an example of which can be seen in Figure 2.5 (Brown, 2001).

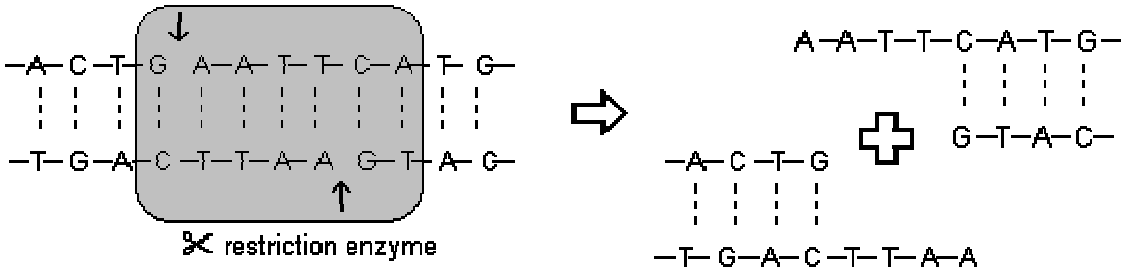

Figure 2.5 A schematic representation of a sticky end formation after the restriction enzyme digest of a DNA sequence by *Eco*RI (http://www.biologymad.com)

The restriction enzymes used in this study and their recognition sequences are listed in Table 2.2.

Table 2.2 The recognition sequences and cleavage points for the restriction enzymes used in this study (Brown, 2001)

Enzyme	Organism	Recognition sequence	Blunt or sticky end
*Eco*RI	*Escherichia coli*	G^AATTC	Sticky
*Bam*HI	*Bacillus amyloliquefaciens*	G^GATCC	Sticky

2.3.6 Cloning and Expression Vectors

A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a vector, to produce a chimera or recombinant DNA molecule. Plasmids are circular molecules of DNA having an independent existence in the bacterial cell. They carry one or more genes, and often these genes are responsible for a useful characteristic displayed by the host bacterium (Figure 2.6). For example, the ability to survive in normally toxic concentrations of antibiotics such as chloramphenicol or ampicillin is often due to the presence in the bacterium of a plasmid carrying antibiotic resistance genes. In the laboratory, antibiotic resistance is often used as a selectable marker to ensure that bacteria in a culture contain a particular plasmid. All plasmids possess at least one DNA sequence that can act as an origin of replication, so they are able to multiply within the cell quite independently of the main bacterial chromosome. A few types of plasmids are also able to replicate by inserting themselves into the bacterial chromosome. These integrative plasmids or episomes may be stably maintained in this form through numerous cell divisions, but will at some stage exist as independent elements (Brown, 2001).

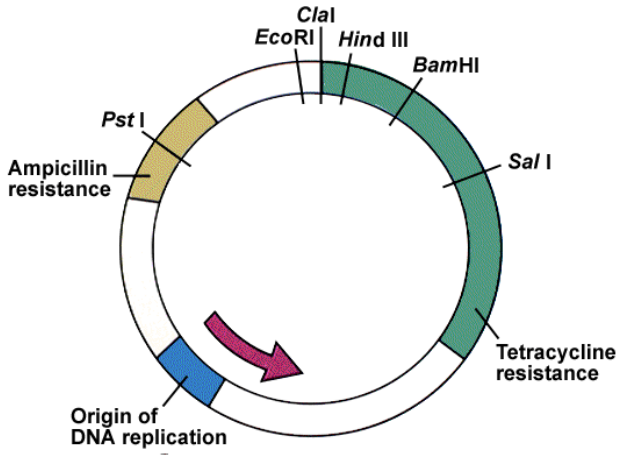

Figure 2.6 A schematic representation of a plasmid cloning vector (http://faculty.abe.ufl.edu)

Most expression vectors carry a selectable marker and a strong promoter. Some vectors carry terminators, antiterminators, a repressor gene, translation signals, secretion signals, reporter genes, or the potential for producing a hybrid protein product (Smith, 1994).

An ideal cloning vehicle would have the following three properties: low molecular weight, ability to confer readily selectable phenotypic traits on host cells, single sites for a large number of restriction endonucleases, preferably in genes with a readily scorable phenotype (Old and Primrose).

Vectors designed primarily for general cloning and sequencing, such as the pUC series, are also used for expression purposes (Smith, 1994).

pUC18 and pUC19 vectors are small, high copy number, *E.coli* plasmids, 2686 bp in length. They are identical except that they contain multiple cloning sites (MCS) arranged in opposite orientations. pUC18/19 plasmids contain: (1) the pMB1 replicon *rep* (source - plasmid pBR322). The high copy number of pUC plasmids is due to the lack of the *rop* gene and due to a point mutation in *rep* of pMB1; (2) *bla* gene, coding for beta-lactamase responsible for the resistance to ampicillin (source - plasmid pBR322); (3) region of *E.coli* operon *lac* containing CAP protein binding site, promoter P_{lac}, *lac* repressor binding site and 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of beta-galactosidase (source - M13mp18/19). This fragment, which can be synthesized by IPTG induction, is capable of intra-allelic (alfa) complementation with a defective form of beta-galactosidase encoded by host (mutation *lacZ*DM15). In the presence of IPTG, bacteria synthesise both fragments of the enzyme and form blue colonies on media containing X-Gal. Insertion of DNA into the MCS located within the *lacZ* gene (codons 6-7 of *lacZ* are replaced by MCS) inactivates the N-terminal fragment of beta-galactosidase and removes alfa-complementation. Therefor, bacteria carrying recombinant plasmids give rise to white

colonies on the solid media. The map shows restriction enzymes cutting pUC18/19 DNA once (Figure 2.7) (http://www.fermentas.com).

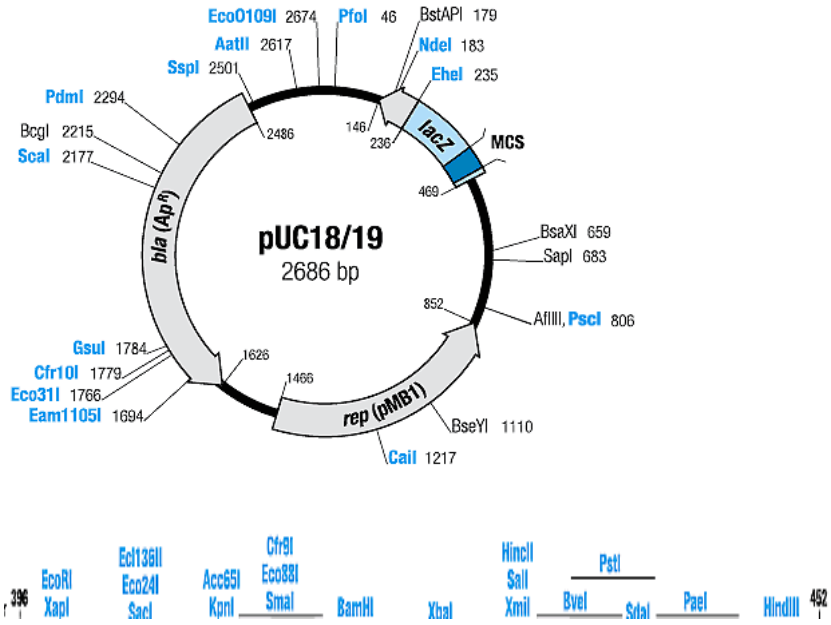

Figure 2.7 pUC18/19 cloning vector and retriction enzyme list of MCS (http://www.fermentas.com)

For the gene cloning in *Bacillus* species, among the constructed vectors, *E. coli / Bacillus* shuttle vector pMK4 was selected (Figure 2.8(A)). It is constructed from partial *Sau*3A digests of pUC9 and pC194. Insertion of foreign DNA into the multiple cloning sites within a part of the *lacZ* gene results in a Lac^+ phenotype in the appropriate *Escherichia coli* host strain and can easily be detected.

In Çalık et al., (2004-b), a*ro*H gene cloning onto pUC19 and sub-cloning onto pMK4 is studied (Figure 2.8(B)). Similarly, in this study the hybrid streptokinase gene is also cloned firstly onto pUC19 and then sub-cloned onto pMK4. It carries *E. coli* beta-galactosidase gene, ampicillin resistance gene and chloramphenicol resistance gene.

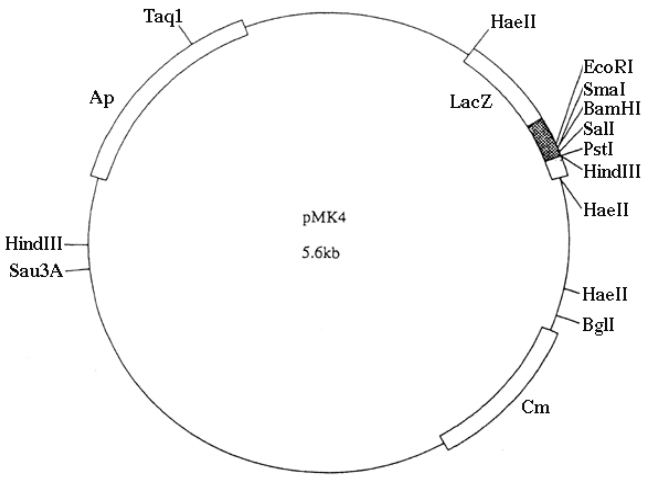

Figure 2.8 (A) pMK4 expression vector. (Sullivan et al. 1984)

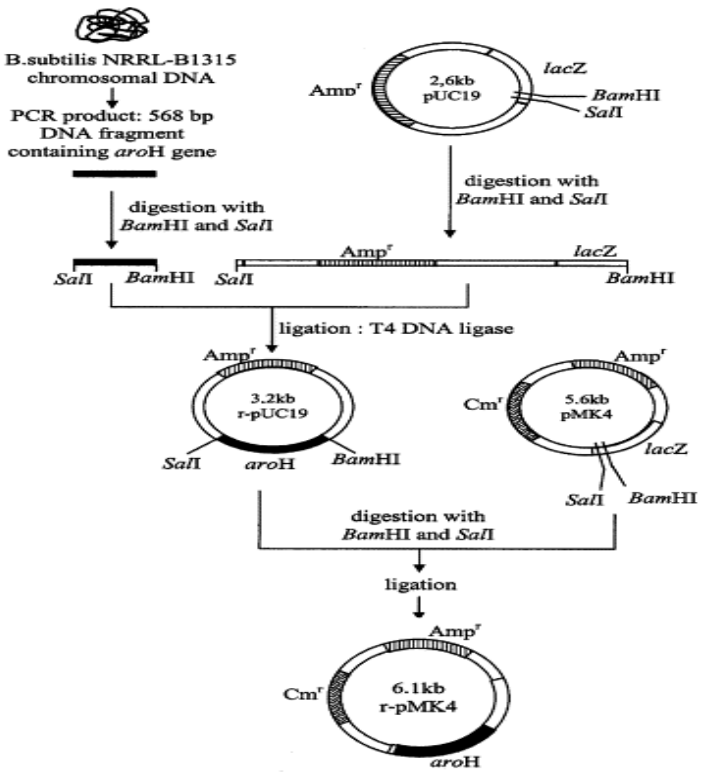

Figure 2.8 (B) The schematic diagram of a*ro*H gene cloning onto pUC19 and sub-cloning onto pMK4 (Çalık et al., 2004-b)

2.3.7 Ligation Reaction and Transformation

The final step in construction of a recombinant DNA molecule is the joining together of the vector molecule and the DNA to be cloned. This process is called as ligation, and the enzyme that catalyses the reaction is called DNA ligase. In the cell the function of DNA ligase is to repair single-stranded breaks arisen in double-stranded DNA molecules during, for example, DNA replication. DNA ligases from most organisms can also join two individual fragments of double-stranded DNA together (Brown, 2001).

All living cells produce DNA ligases, but the enzyme used in genetic engineering is usually purified from *E. coli* that have been infected with T4 phage. Within the cell the enzyme carries out the very important fuction of repairing any discontinuities that may arise in one of the strands of a double-stranded molecule. In the test tube, purified DNA ligases, as well as repairing single-strand discontinuities, can also join together individual DNA molecules or the two ends of the same molecule. The chemical reaction involved in ligating two molecules is exactly the same as discontinuity repair, except that two phosphodiester bonds must be made, one for each strand (Brown, 2001).

Ligation of complementary sticky ends is much more efficient (Figure 2.9). This is because compatible sticky ends can base pair with one another by hydrogen bonding, forming a relatively stable structure for the enzyme to work on. If the phosphodiester bonds are not synthesized fairly quickly the sticky ends will fall apart again. These transient, base-paired structures increase the efficiency of ligation by increasing the length of time that the ends are in contact with another (Brown, 2001).

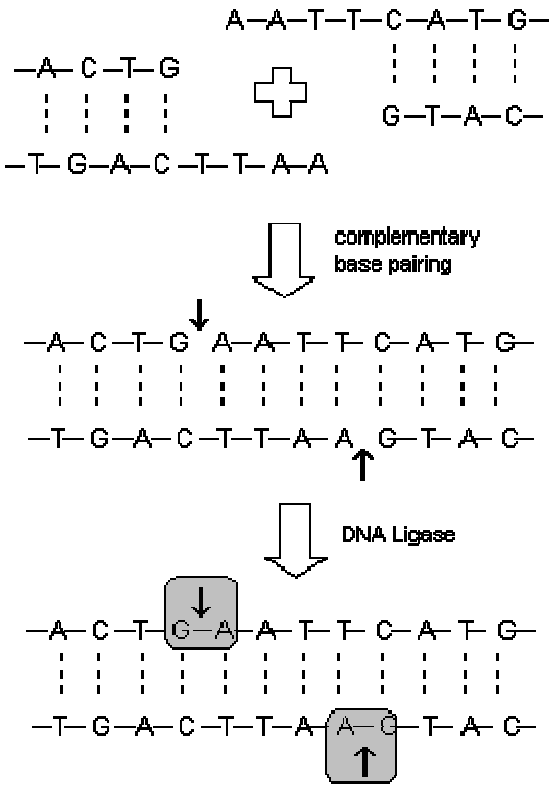

Figure 2.9 A schematic representation of ligation reaction (http://www.biologymad.com)

By ligation, a DNA fragment can be inserted into a plasmid which then acts as a vector, allowing the inserted fragment to be replicated after introduction into a bacterial cell by transformation. In a sense, it was the discovery of transformation, the uptake of DNA by a bacterial cell, that initiated the study of bacterial genetics and molecular biology. Four types of transformation which are in many ways quite distinct can be considered: natural competence, induced competence, protoplast transformation, and electroporation (Dale, 1994).

Natural competence is the mostly studied and used type of transformation. The competent cells are able to take up linear double-stranded DNA and in order for this DNA to be replicated and inherited; it must undergo recombination with a

homologous region of the recipient chromosome. Although some organisms are able to take up totally unrelated DNA, the absence of homology prevents recombination occurring, the DNA taken up is therefore degraded and lost. This process is therefore limited to introduction of DNA from closely related organisms, usually different genetically marked strains of the same species (Dale, 1994).

In many organisms (including *E. coli*), competent cells do not appear to occur naturally. For these bacteria, a second type of transformation is used, in which a competent state is induced artificially. There are numerous variations on the procedure for doing this, the simplest of which involves washing the cells repeatedly with cold calcium chloride solution. The competent cells are mixed with the DNA solution and subjected to a heat treatment, such as heating them at 42^{o}C for 1-2 minutes and then transferring them back onto ice. They are then diluted into broth and incubated at 37^{o}C to allow expression of the newly acquired DNA before plating out onto an appropriate medium. This procedure is commonly used for introducing genetically manipulated plasmid DNA into *E. coli* (Dale, 1994).

Enzymatic removal of the cell wall, in the presence of an osmotic stabiliser such as sucrose, generates protoplasts in which large areas of the cytoplasmic membrane are exposed. Addition of the DNA solution, together with polyethylene glycol (PEG) causes the cells to take up the DNA. The PEG is then removed by centrifugation and the cells allowed to regenerate on an osmotically stabilised medium. If the correct conditions are used, a very high proportion of the resultant colonies will be transformed (Dale, 1994).

A recent development is the introduction of the technique of electroporation. In this procedure, the bacterial cells are mixed with plasmid DNA and are subjected to a brief pulse of high-voltage electricity. This causes the DNA to enter the cell. The transformants can then be selected on the basis of the antibiotic resistance conferred by the plasmid (Dale, 1994).

2.3.8 Selection and Screening of Recombinant Plasmids

Transformation of competent cells is an inefficient procedure, however carefully the cells have been prepared. Although 1 ng of the plasmid vector can yield 1000-10 000 transformants, this represents the uptake of only 0.01% of all the available molecules. Furthermore, 10 000 transformants is only a very small portion of the total number of cells that are present in a competent culture. This last fact means that some way must be found to distinguish a cell that has taken up a plasmid from many thousands that have not been transformed. The answer is to make use of a selectable marker carried by the plasmid. A selectable marker is simply a gene that provides a transformed cell with a new characteristic, one that is not possessed by a non-transformant (Brown, 2001).

Plasmid cloning vectors are therefore constructed so as to carry one or more antibiotic resistance genes. pUC18/19 carries a gene coding for the enzyme β-lactamase, which confers resistance to ampicillin by hydrolysis of the antibiotic. There is a second marker, namely a β-galactosidase gene. Transformation of a suitable host strain with this plasmid will therefore yield cells that produce a functional β-galactosidase. These can be detected using a chromogenic substrate known as X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactosidase) which gives rise to a blue product when hydrolyzed by β-galactosidase. Thus, the colonies containin pUC18/19 will be blue, while those without the plasmid will be white. Yet, this is not normally used as a selectable marker - both types of cell will grow- but it does provide an additional and useful form of discrimination (Dale, 1994).

Resistance to the antibiotic is not due to merely the presence of the plasmid in the transformed cells. The resistance gene on the plasmid must also be expressed, so that the enzyme that detoxifies the antibiotic is synthesized. Expression of the resistance gene begins immediately after transformation, but it will be a few minutes before the cell contains enough of the enzyme to be able to withstand the toxic effects

of the antibiotic. For this reason the trasnformed bacteria should not be plated onto the selective medium immediately after the heat shock treatment, but first placed in a small volume of liquid medium, in the absence of antibiotic, and incubated for a short time (Brown, 2001).

The expression plasmid pMK4 carries *E. coli* beta-galactosidase gene, ampicillin resistance gene and chloramphenicol resistance gene for the selection of correct transformants in *E. coli.*

2.3.9 Gene Expression and Control Mechanisms

All genes have to be expressed in order to function. The first step in expression is transcription of the gene into a complementary RNA strand (Figure 2.10). For some genes, for example those coding for transfer RNA (tRNA) and ribosomal RNA (rRNA), the transcript itself is the functionally important molecule. For other genes the transcript is translated into a protein molecule (Brown, 2001).

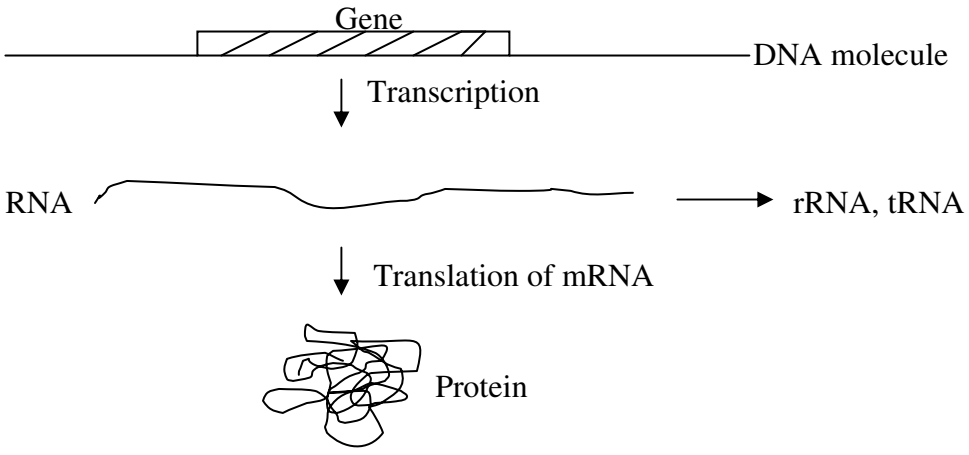

Figure 2.10 Fundamentals of gene expression (Brown, 2001)

If the flow of information from the structure of the gene to the functioning of the protein is analyzed, series of factors can be identified that can influence the overall regulation of that functioning (Figure 2.11). These are:

1) The number of copies of the gene. In general, if there are several copies of a gene the level of product is likely to be higher.
2) The efficiency with which the gene is transcribed. The major factor at this level is the initiation of transcription by RNA polymerase, although there are other mechanisms for influencing the amount of mRNA produced.
3) The stability of the mRNA. It is important to recognize that the amount of specific mRNA will be determined by the combined effect of the rate at which it is produced and the length of time each molecule persists in an active state in the cell.
4) The efficiency with which the mRNA is translated into protein. This will be influenced by the efficiency of initiation, and also by factors that affect the rate at which the ribosomes travel along the mRNA.
5) The stability of the protein product. As with the mRNA, the amount of protein reflects both its rate of production and stability. Different proteins vary in their stability to a very marked degree, as might be expected from their different functions: a protein that forms part of a cellular structure is likely to be more stable than one that transmits a signal for switching on a transient cellular event.
6) Post-translational effects. This includes a wide variety of events such as protein folding which is necessary for conversion of polypeptide chains into a biologically active conformation, as well as covalent modifications that can influence the activity of the protein. Phosphorylation is especially an important mechanism for regulating the function of specific proteins (Dale, 1994).

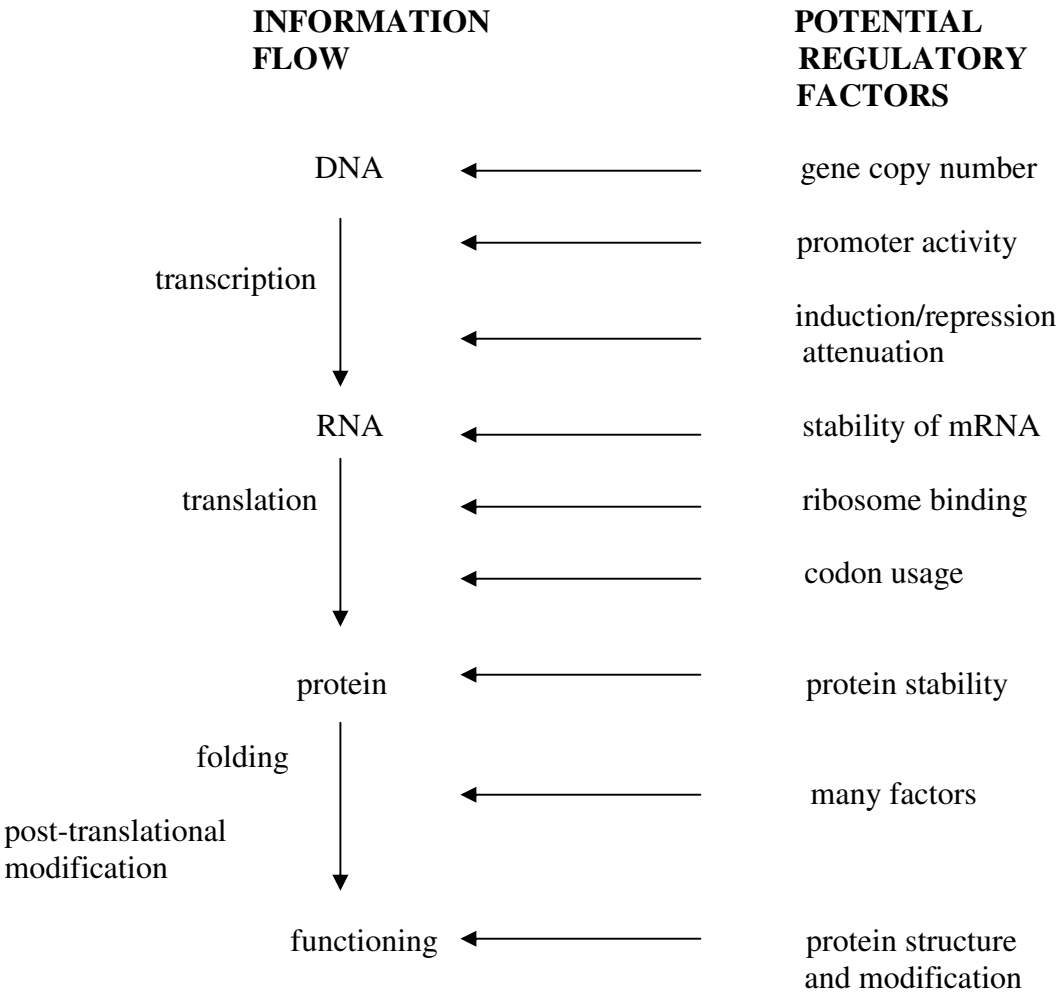

Figure 2.11 Information flow and regulatory factors (Dale, 1994)

2.3.9.1 Transcriptional Control

In transcriptional control, both the promoter activity and induction/repression attenuation should be considered.

2.3.9.1.1 Promoters

The principal method of control of gene expression in bacteria is by regulating the amount of mRNA produced from that gene, and the primary influence on that process is the nature of the promoter. This is the site, adjacent to the 5' end of the gene, to which RNA polymerase will bind to initiate transcription. In general then, it can be stated that each gene must have a promoter attached to it (Dale, 1994).

Strong promoters are those that can sustain a high rate of transcription; strong promoters usually control genes whose translation products are required in large amounts by the cell. In contrast, weak promoters, which are relatively inefficient, direct transcription of genes whose products are needed in only small amounts. Clearly, an expression vector should carry a strong promoter, so that the cloned gene is transcribed at the highest possible rate (Brown, 2001).

A second factor to be considered when constructing an expression vector is whether it will be possible to regulate the promoter in any way. Two major types of gene regulation are recognized –induction and repression. An inducible gene is one whose transcription is switched on by addition of a chemical to the growth medium; often this chemical is one of the substrates for the enzyme coded by the inducible gene. In contrast, a repressible gene is switched off by addition of the regulatory chemical. It may be therefore possible to extend the regulation to the expression vector, so that the chemical that induces or represses the gene normally controlled by the promoter is also able to regulate expression of the cloned gene (Brown, 2001).

The most highly utilized promoters for overexpression have two features in common: they are efficient and repressible. Repressible promoters are superior to unregulated promoters because continuous expression of plasmid-encoded proteins generally puts the host at a selective disadvantage in a population where some cells have lost the plasmid or acquired deletions and other mutations (Smith, 1994).

In this study, the vector pUC19, not carrying a specific promoter in its structure was selected for the cloning of the hybrid streptokinase gene firstly into pUC19 in *E.coli* and then, for the sub-cloning step into pMK4 in host microorganisms *E. coli* and *Bacillus* species.

As the promoter and signal sequence to be used, the gene encoding subtilisin Carlsberg, which is an alkaline serine protease produced by *Bacillus licheniformis* was used. From this gene, promoter and signal sequence were amplified through a PCR by specific pre-designed primers, and then this amplified portion was associated with the gene encoding streptokinase by SOE method. This hybrid gene was inserted upstream of the structural gene of plasmid pUC19 and then it was sub-cloned onto pMK4 which is a *E. coli / Bacillus* shuttle vector. In the gene encoding for subtilisin Carlsberg, there are two possible promoter regions which is reported by Jacobs et al., (1985). The first one is an attractive sigma-43 promoter found at nucleotides 34 to 39 (-35) and 57 to 61 (-10), respectively. In addition, there is a sigma-37 promoter at position 48 to 67 in which 8 out of 10 nucleotides are identical to the -10 consensus sequence of GGAATTGTTT (Jacobs et al., 1985). These promoter regions are effective in that they are all functional which can be depicted from the study of Çalık et al., (2003-a) where a performance analysis is performed for the overexpression of alkaline serine protease encoding gene in different *Bacillus* species.

2.3.9.1.2 Terminators

As well as the signal for the start of transcription (the promoter) a gene, or an operon, will also carry a sequence that stops transcription at the end of the operon. These sequences are known as terminators. A characteristic feature of a terminator is the presence of a region of an inverted repeat: this means a short sequence that is complementary to, and in the reverse direction from, a region just preceding it. The way in which such a structure can lead to termination of transcription is very different from the promoter mechanism, primarily in that the terminator is actually transcribed,

with termination occurring subsequently, whereas the promoter sequence is not transcribed (Dale, 1994).

Many examples in the literature describe expression systems that utilize strong promoters without terminators behind the expressed gene. However, it has been shown that placement of a strong terminator at the 3' end of the gene results in greater stability of the plasmid and greater expression of the recombinant gene. In the absence of a terminator, transcription can proceed into the plasmid, causing overexpression of other genes carried on the vector, potentially causing harm to the cell (Smith, 1994).

2.3.9.2 Translational Control

It is at the level of translation that some of the most irksome problems arise in attempting to overexpress heterologous proteins. Translation involves multiple components and steps and the resulting implication is that each step is a potential target for regulation. These are initiation, elongation, and termination.
Initiation step includes ribosome binding site (RBS), Shine-Dalgarno sequence (SD), spacer region between the SD sequence and the initiation codon, initiation codon and the second codon (Smith, 1994).

The ribosome binding site (RBS) is functionally defined as the portion of mRNA that is protected from Rnase digestion by ribosomes that have bound but not initiated translation. The purpose of the SD sequence is to allow the proper alignment of the ribosome with the initiation codon. The distance between the SD and the initiation codon ranges from 5 to 13 nucleotides and depends on the SD sequence and perhaps other elements in the mRNA. The most commonly used initiation codon is AUG, but occasionally, UUG and GUG are employed in *E. coli*. (Smith, 1994).

Little is known about the existence of regulatory mechanisms acting on translation termination. It is conceivable that even a slight stalling at the stop codon could affect protein levels dramatically by causing subsequent ribosomes to back up (Smith, 1994).

2.3.9.3 Protein Secretion in Bacillus Species

The proteins secreted are synthesized as precursors with an amino terminal extension, which contains the signal sequence or the signal peptide. They are translocated across the cytoplasmic membrane of the bacterial cell vectorially with the concomitant removal of the signal sequence (Smith, 1994).

With regard to the feasibility of the secretion of heterologous proteins in *Bacillus*, the role of the signal sequence is crucial. The presence of a functional signal sequence is an absolute requirement for secretion to take place. It contributes to the folding of the nascent polypeptide chain, it is the target of the binding and recognition by the translocator machinery, and it interacts with the components of the translocator in the initiation of the translocation of the polypeptide across the membrane. On the contrary, the mature part of the exported protein, the part released from the cell after the cleavage of the signal sequence, has no known active function in the process (Smith, 1994).

Bacillus is an alternative host for expression and secretion of heterologous proteins. However, low yields of protein production limit its use on a wide scale. The secretory pathway of proteins can be divided into three functional stages: the early stage, involving the synthesis of secretory pre-proteins, their interaction with chaperones and binding to the secretory translocase; the second stage, translocation across the cytoplasmic membrane; and the last stage, including removal of the signal peptide, protein refolding and passage through the cell wall. One of the major limitations when using *Bacillus* as an expression host is its secretion of high levels of

proteases. At the onset of the stationary phase, *Bacillus* secretes at least seven extracellular proteases, including alkaline protease, neutral proteases, metalloprotease E, and three serine proteases. Although native *Bacillus* proteins are generally resistant to these proteases, heterologous proteins are often rapidly degraded in their presence. As a result, strains of *Bacillus* that are multiply deficient in extracellular proteases have been developed. Currently, protease-deficient strains WB600 and WB700 of *Bacillus subtilis* have been constructed. Recent studies have suggested that not only the secreted proteases but also cell-associated proteases are responsible for the degradation of secreted heterologous proteins. Cell-wall-bound proteins CWBP52 and CWBP23 are the processed products of the *wprA* gene. WB800 was generated by inactivation of the chromosomal *wprA* gene in WB700 (Li et al., 2004).

Although the primary structures of different amino-terminal signal peptides show little similarity, three distinct domains can nevertheless be recognized. The aminoterminal N-domain of signal peptides contains at least one arginine or lysine residue, although this positively charged residue does not seem to be strictly required for protein export. The H-domain, following the N-domain, is formed by a stretch of hydrophobic residues that seem to adopt an a-helical conformation in the membrane. Helix-breaking glycine or proline residues are frequently present in the middle of this hydrophobic core. The latter residues might allow the signal peptide to form a hairpin-like structure that can insert into the membrane. In one model for signal peptide function, it was proposed that unlooping of this hairpin results in insertion of the complete signal peptide in the membrane (Figure 2.12) (Tjalsma et al., 2000).

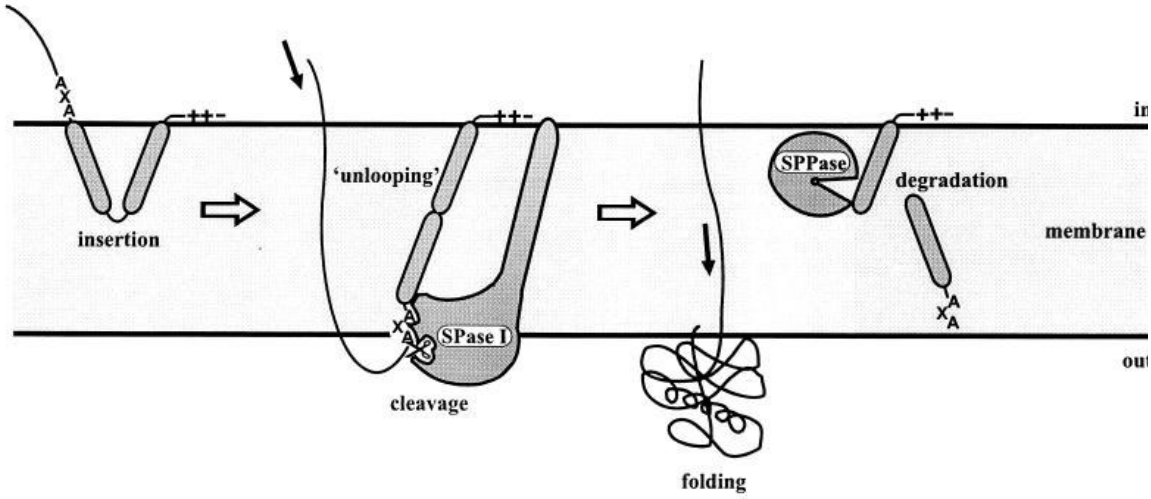

Figure 2.12 Model for signal peptide insertion into the cytoplasmic membrane and cleavage by signal peptidase I (SpaseI) (Tjalsma et al., 2000)

The C-region is located adjacent to the H-region, usually with a helix-breaking proline or a glycine residue at position -4 to -6 relative to the cleavage site, and ends with the -1, -3 or ''Ala-X-Ala'' SPase recognition sequence (Figure 2.13). A typical bacterial signal peptide consists of a positively charged N-terminus (N-region), a central hydrophobic region (H-region), and a polar Cterminal region (C-region). Helix-breaking proline or glycine residues are often found in the middle of the H-region and between the H- and C-regions at the -6 position relative to the cleavage site. The SPase recognition sequence consists of small aliphatic residues at positions -1 and -3, relative to the cleavage site. The most common residue at these positions is Ala (Van Roosmalen et al., 2004).

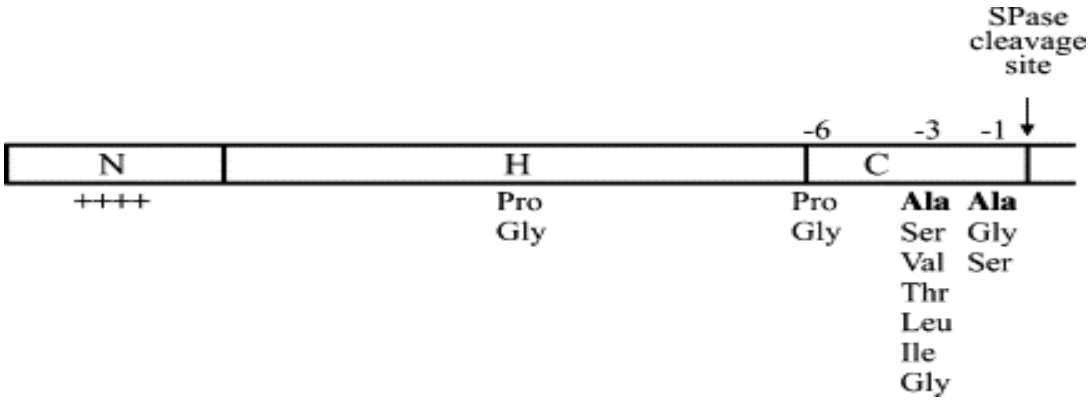

Figure 2.13 Secretory signal peptides (Van Roosmalen et al., 2004)

2.3.9.3.1 Signal Peptide of *Bacillus licheniformis*, *pre(sub*C)

In Jacobs et al., (1985), the cloning and sequencing of the entire coding sequence as well as the 5' and 3' flanking sequences for subtilisin Carlsberg which is an alkaline serine protease produced by *Bacillus licheniformis* are described. The sequence reveals an overall structure includes a 29 residue signal peptide and a 76 residue pro-region preceeding the C-terminal protein consisting of 274 residues. The putative signal sequence has similar properties to other signal sequences from Gram positive bacteria. This includes a basic N-terminal segment followed by a strech of uncharged residues. The cleavage site mostly follows the (-3,-1) rule, giving cleavage preferentially after the residues Ala-X-Ala. Based on this rule and other structural considerations, it is predicted that the signal peptidase processing occurs after the residues Ala-Ser-Ala. In Table 2.3, some signal peptides of *Bacillus* species are demonstrated.

Table 2.3 Signal peptides of Bacillus species (Signal peptide cleavage site is indicated by arrows) (Tjalsma et al., 2000; Simonen et al., 1993)

Protein	Species of origin	Signal peptide
α-Amylase	*B. licheniformis*	MKQHKRLYARLLPLLFALIFLLPHSA**AAA** ↓
α-Amylase	*B. subtilis*	MFAKRFKTSLLPLFAGFLLLFHLVLEGPAA**ASA** ↓
α-Amylase	*B. amyloliquefaciens*	MIQKRKRTVSFRLVLMCTLLFVSLPITK**TSA** ↓
Subtilisin	*B. amyloliquefaciens*	MRGKKVWISLLFALALIFTMAFGSTSS**AQA** ↓
Subtilisin Carlsberg	*B. licheniformis*	MMRKKSFWLGMLTAFMLVFTMAFSDS**ASA** ↓

Determination of whether a *B. subtilis* promoter and signal sequence would be better than the native streptokinase (*skc*) promoter and signal sequence is aimed and the corresponding scientific research is conducted by Wong et al., (1993). In this study, with the available cloned *skc* gene, production of the secreted streptokinase from *B. subtilis* WB600 (6 extracellular protease deficient strain) is studied. A modified *skc* which has the original *skc* promoter and signal sequence replaced with the *B. subtilis* levansucrase promoter and signal sequence is also constructed. The structural gene *skc* of streptokinase from *Streptococcus equisimilis* H46A has been cloned and sequenced. To determine whether a *B. Subtilis* promoter and signal sequence would be better than the native promoter and signal sequence, two vectors are designed. pSK1 caries the intact *skc* gene including its promoter region. pSK3 carries the sequence encoding streptokinase, levansucrase (*sac*B) promoter and signal sequence. Additionally, 2.5 fold increase in the activity is reported .

Staphylokinase is a promising blood-clot dissolving agent for the treatment of patients suffering from a heart attack similar to streptokinase. To determine whether a *B. subtilis* promoter and signal sequence would be better than the native staphylokinase promoter and signal sequence is aimed in the study of Ye et al., (1998). It would be desirable to produce staphylokinase in large quantities for biochemical characterization and clinical trials. By using a 7-extracellular protease deficient *B. subtilis* WB700, intact staphylokinase can be produced via secretion. To optimize the production and stability of the vectors, both the promoter and signal sequence of staphylokinase are replaced by *B. subtilis sac*B signal sequence and promoters P43, P*amy*, P*sac*B. pSAKN, pSAKB, pSAKB, pSAKP, pSAKA expression vectors are developed. The final purified staphylokinase protein has a specific activity of 2840 units/mg.

There is no strong evidence for the function of pro-peptide sequence. In the studies of Wong et al., (1986) and Wang et al., (1988), only the pre-peptide region is used for the secretion of heterologous protein secretion in *B. subtilis*. Also, in this study, the signal peptide (*pre(sub*C)) with its native ribosomal binding site or namely, promotor region was utilized for the extracellular production of streptokinase.

2.4 Bioprocess Parameters in Enzyme Production

Bioprocess is defined as the operation including the transformation of some raw material into some other product by microorganisms, plant or animal cell cultures or by chemical products derived from these organisms (Moses and Cape, 1991).

In the industrial view, it is aimed to optimize a bioprocess system in order to maximize both the yield and the productivity of a product. In aerobic bioprocesses, to improve the expression of a product, these important factors shoud be concerned:

1) Microorganism
2) Medium composition
3) Bioreactor operation parameters
    i) Temperatıre
    ii) pH
    iii) Oxygen transfer rate
        a) Air inlet rate (Q_0/V_R)
        b) Agitation rate (N)

2.4.1 Microorganism

The choice of host cell must fit into a bioprocess strategy. That strategy includes not only plans for efficient production, but also how a product is to be recovered and purified. The development of processes for making products from genetically engineered organisms requires that a large number of choices be made. Important characteristics of selected host system for any protein production from recombinant DNA includes: high growth rate, availability of genetic systems, high expression level, low cost media, low levels of proteolytic degradation, level of secretion, and safety. Moreover, when posttranslational modifications of a product are necessary, then an eukaryotic host system must be chosen (Schuler and Kargi, 2002).

The streptokinase gene (*skc*) cloned from *Streptococcus equisimilis* H 46A has been expressed in several gram positive and negative bacteria as *B. subtilis* and *E. coli.* Moreover, production in yeast also exists. In Wong et al., (1993), with the available cloned *skc* gene, production of the secreted streptokinase from *B. subtilis* WB600 (6 extracellular protease deficient strain) is studied. The production and secretion of streptokinase using *E. coli* JM109 strain is enhanced by Lee et al., (1997). *B. subtilis* WB600 (6 extracellular protease deficient strain) is also used as the host organism by Wu et al., (1998). In the study of Zhang et al., (1999), a process for the pilot production of recombinant streptokinase is established. As the host

organism, *E. coli* K802 strain is used. Secretion of the protein streptokinase is performed under the control of the methanol-inducible alcohol oxidase (ox) promoter of *Pichia pastoris* in the study of Pratap et al., (2000). *Pichia pastoris* GS115 and *Escherichia coli* A61 are the host microorganisms. A chimeric gene consisting of the signal sequence of the Plus pheromone of *Schizosaccharomyces pombe* fused in-frame with the mature streptokinase from *Streptococcus* sp. is constructed and streptokinase is produced in *Sz. Pombe* by Kumar et al., (2004).

2.4.1.1 *Escherichia coli*

If posttranslational modifications are unnecessary, *E. coli* is most often chosen as the initial host. The main reason for the popularity of *E. coli* is the broad knowledge base for *E. coli. E. coli* physiology and genetics are probably far better understood than for any other living organism. A wide range of host background is available, as well as vectors and promoters. This large knowledge greatly facilitates sophisticated genetic manipulations. The well-defined vectors and promoters greatly speed the development of an appropriate biological catalyst. The relatively high growth rates for *E. coli* coupled with the ability to grow *E. coli* to high cell concentrations (50 g dry wt/L) and with the high expression levels possible from specific vector–promoter combinations (about 25% to 50% or more of total protein) can lead to extremely high volumetric productivities. Also, *E. coli* will grow on simple and inexpensive media. These factors give *E. coli* many economic advantages (Schuler and Kargi, 2002).

However, *E. coli* is not a perfect host. The major problems result from the fact that *E. coli* does not normally secrete proteins. When proteins are retained intracellularly and produced at high levels, the amount of soluble active protein present is usually limited due to either proteolytic degradation or insolubilization into inclusion bodies (Schuler and Kargi, 2002).

A typical gram-negative cell is *E. coli*. It has an outer membrane supported by a thin peptidoglycan layer. Peptidoglycan is a complex polysaccharide with amino acids and forms a structure somewhat analogous to a chain-link fence. A second membrane (the inner or cytoplasmic membrane) exists and is separated from the outer membrane by the periplasmic space. The cytoplasmic membrane contains about 50% protein, 30% lipid, and 20% carbohydrate. The cell envelope serves to retain important cellular compounds and to preferentially exclude undesirable compounds in the environment. Loss of membrane integrity leads to cell lysis and cell death. The cell envelope is crucial to the transport of selected material in and out of the cell (Schuler and Kargi, 2002).

In this study the *E. coli* strains of XL1 and TG1 were used as the initial host microorganisms, whose general characteristics are listed in Table 2.4.

Table 2.4 Bacterial strains and plasmids used in this study
(Jeong and Lee et al., 2003)

***E. coli* strains**	**Relevant characteristics**
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac* F′ [*proAB*$^{+}$*lac1*^{q}*lacZ*Δ M15 Tn*10*(Tet)]
TG1	*F′ traD36 lacI*$_{q}$ Δ(*lacZ*)M15 *proA*$^{+}$*B*$^{+}$/*supE*Δ (*hsdM-mcrB*) *5* (r_k^- m_k^- *mcrB*$^{-}$) thi Δ(*lac-proAB*)

In this study, the vector pUC19, not carrying a specific promoter in its structure was selected for the cloning of the hybrid streptokinase gene firstly onto pUC19 in *E.coli* and then, for the sub-cloning step onto pMK4 in *E. coli* and *Bacillus* species hosts.

The promoter region of the gene encoding subtilisin Carlsberg, which is an alkaline serine protease produced by *Bacillus licheniformis* was utilized. From this gene, promoter and signal sequence were amplified through a PCR by specific pre-designed primers, and then this amplified portion was associated with the gene encoding streptokinase by SOE method. This hybrid gene was inserted upstream of the structural gene of plasmid pUC19 and then it was sub-cloned onto pMK4 which is a *E. coli / Bacillus* shuttle vector.

2.4.1.2 The Genus *Bacillus*

The genus, *Bacillus*, are rod shaped, straight cells. They are very resistant to many adverse conditions and they can form endospores. Sporulation is not repressed by exposure to air. Generally, they are gram positive, or some species are gram positive only in early stages of growth, or some are gram negative. They have peritrichous flagella and they are aerobic or facultatively anaerobic. Certain pigments are produced on certain media (Bergey, 1989).

The endospore is the most important aspect of *Bacillus* ecology for several reasons. First, because heat treatment is the most common selective isolation procedure for the recovery of *Bacillus* sp. from the environment, most studies concentrate on the endospore to the exclusion of vegetative cells. Second, because the spore is a dormant structure of great longevity, ecological studies are often simply estimates of the accumulation of spores in an environment rather than an assessment of the contribution of the bacterium to the environment. Nevertheless, a large number of spores of a particular species in a habitat is strongly indicative of previous or continuous growth and metabolism in that niche (Sonenshein, 1993).

The morphological division of *Bacillus* sp. into i) species that produce oval endospores that do not distend the mother cell, ii) species that produce oval endospores that distend the mother cell, and iii) species that produce spherical endospores is a beneficial, informal classification of the genus. Initial indications of

how *Bacillus* sp. might be subdivided came from numerical analysis of phenotypic features. Three comprehensive and independent studies in this area have provided essentially congruous results, and the main findings are summarized in Table 2.5 (Sonenshein, 1993).

Bacillus subtilis and *Bacillus licheniformis* species are gram positive cells. Gram positive cells do not have an outer membrane. Rather they have a very thick, rigid cell wall with multiple layers of peptidoglycan. Gram positive cells also contain teichoic acids covalently bonded to the peptidoglycan. Because, gram positive bacteria have only a cytoplasmic membrane, they are often much better suited to excretion of proteins. Such excretion can be technologically advantageous hence it simplifies the downstream processing of the desired protein product (Schuler and Kargi, 2002 & Sriraman et al., 2006). Moreover, it is reported that the predominant peptidoglycan type of the strains of *Bacillus* sp. is of the direct linked *meso*-diaminopimelic acid type (Bergey, 1989).

Table 2.5 Classification of some *Bacillus* sp. according to their phenotypic similarities (Sonenshein, 1993)

Species	Characteristics of the species
Group I	
B. alvei *B. amylolyticus* *B. apiarius* *B. azotofixans* *B. circulans* *B. glucanolyticus* *B. larvae* *B. lautus* *B. lentimorbus* *B. macerans* *B. macquariensis* *B. pabuli* *B. polymyxa* *B. popilliae* *B. psychrosaccharolyticus* *B. pulvifaciens* *B. thiaminolyticus* *B. validus*	All species are facultative anaerobes and grow strongly in the absence of oxygen. Acid is produced from variety of sugars. Endospores are ellipsoidal and swell the mother cell.
Group II	
B. alcolophilus *B. amyloliquefaciens* *B. anthracis* *B. atrophaeus* *B. carotorum* *B. firmus* *B. flexus* *B. laterosporus* *B. lentus* *B. licheniformis* *B. niacini* *B. pantothenicus* *B. pumilus* *B. simplex* *B. subtilis* *B. thuringiensis*	All species produce acid from variety of sugars including glucose. Most are able to grow at least weakly in absence of oxygen, particularly if nitrate is present. Spores are ellipsoidal and do not swell the mother cell.

Table 2.4 - Continued

Species	Characteristics of the species
Group III *B. alginolyticus* *B. aneurinolyticus* *B. azotoformans* *B. badius* *B. brevis* *B. chondroitinus* *B. frudenreichii* *B. gordonae*	This strict aerobes do not produce acid from sugars; names in bracket are exceptions. They produce ellipsoidal spores that swell the mother cell. Most species have an oxidative metabolism and produce alkaline reactions in peptone media.
Group IV *B. aminovorans* *B. fusiformis* *B. globisporus* *B. insolitus* *B. marinus* *B. pasteurii* *B. psychrophilus*	All species produce spherical spores that may swell the mother cell and contain L-lysine or ornithine in cell wall. These bacteria are strictly oxidative and in most cases will not use sugars as a source of carbon or energy, preferring acetate or amino acids.
Group V *B. coagulans* *B. flovothermus* *B. kaustophilus* *B. pallidus* *B. schlegelii* *B. smithii* *B. stearothermophilus* *B. thermodenitrificans* *B. thermoglucusidasius* *B. thermoleovrans* *B. thermoruber*	These thermophilic species all grow optimally at >50^{o}C. Physiologically and morphologically, they are heterogeneous, but most produce oval spores that swell the mother cell.
Group VI *A. acidocaldarius* *A. acidoterrestiris* *A. sikloheptanius*	Thermophilic, cacidophilic species with membraneous ω-alicyclic fatty acids.
Unassisgned species *B. benzoervorans* *B. fastidiosus* *B. nagonoensis*	It is not clear how can these species be allocated with conviction to one of these groups.

It is aimed to develop *Bacillus* species carrying s*ub*C gene encoding serine alkaline protease (SAP) enzyme in order to increase the yield and selectivity for SAP production in the study of Çalık et al., (2003-a). For this aim, nine host *Bacillus* species were used: *B. alvei*, *B. amyloliquefaciens*, *B. badius*, *B. cereus*, *B. coagulans*, *B. firmus*, *B.licheniformis*, *B. sphaericus* and *B. subtilis*. The effect of the host *Bacillus* species on SAP production on a defined medium with glucose together with the by-product formation was investigated. Among the recombinant *Bacillus* species, the highest activity increase compared to the wild types was obtained with r-*B. sphaericus.* In the corresponding study, a term of enhancement factor is defined as the maximum SPA activity of recombinant species per maximum activity of wild types. According to this definition, it can be depicted that *B. firmus, B. sphaericus* and *B. subtilis* are likely to be the most suitable hosts for heterologous protein production.

In Wong et al., (1993), with the available cloned *skc* gene, production of the secreted streptokinase from *B. subtilis* WB600 (6 extracellular protease deficient strain) is studied. The 6-extracellular-protease-deficient *B. subtilis* strain, WB600 (*trpC2 nprA apr epr bfp mpr::ble nprB::ery*) is also used as the host organism in producing extracellular streptokinase by Wu et al., (1998). By using a 7-extracellular protease deficient *B. subtilis* WB700 (*trpC2nprE aprE epr bpf* D*mpr::ble* D*nprB::bsr* D*vpr::ery)*, intact staphylokinase is produced via secretion in the study of Ye et al., (1998).

Important characteristics of selected host system for any protein production from recombinant DNA includes: high growth rate, availability of genetic systems, high expression level, low cost media, low levels of proteolytic degradation, level of secretion, and safety (Schuler and Kargi, 2002). When all of these conditions were concerned, in this study *B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) species were selected as the potential hosts for the the extracellular production of streptokinase.

2.4.1.3 *E.coli* or the Genus *Bacillus*

The demand for foreign gene expression systems is rapidly increasing. Production of heterologous proteins at high levels by bacteria is commonly achieved using *Escherichia coli* as the host (Li et al., 2004). The main reason for the popularity of *E. coli* is the broad knowledge base for *E. coli. E. coli* physiology and genetics are probably far better understood than for any other living organism (Schuler and Kargi, 2002). However, the *E. coli* expression system still has disadvantages. For example, it is a pathogenic bacterium and has endotoxins (lipopolysaccharide); it secretes proteins into the periplasm and often into inclusion bodies. *Bacillus* sp. is an occasional alternative host for expression of heterologous secretory proteins (Li et al., 2004).

It is advantageous to employ *Bacillus* sp. as an expression host. First, it has a huge capacity for secreting proteins directly into the growth medium, which greatly facilitates their downstream processing. It is widely used for the production of industrial enzymes, so it is well known with respect to fermentation technology.
It is a genetically highly amenable host organism from which a large variety of genetic tools have been developed. Some strains show natural competence. *B. subtilis* has a transparent genome, because its complete sequence is known. *B. subtilis* and *B. licheniformis* are non-pathogenic, and free of endotoxins, and bears "generally recognized as safe" (GRAS) status. Finally, it is of non-biased codon usage (Li et al., 2004). The absence of lipopolysaccharides (endotoxins) from the cell wall is another positive aspect of it (Westers et al., 2006). It is now evident that the main problem with secretion of several heterologous proteins in *Bacillus* species is in the export process itself, and therefore the use of the various methods to reduce proteolysis can substantially improve the yields of only proteins that are efficiently synthesized and secreted (Simonen et al., 1993). To overcome this problem, protease deficient strains of *B.subtilis* such as WB600 (*trpC2 nprA apr epr bfp mpr::ble nprB::ery*) and

WB700 (*trpC2nprE aprE epr bpf* D*mpr::ble* D*nprB::bsr* D*vpr::ery)* were developed (Wong et al., 1993, Wu et al., 1998 , Ye et al., 1998).

2.4.1.4 Cell Growth, Kinetics and Yield Factors

When a small quantity of living cells is added to a liquid solution of essential nutrients at a suitable temperature and pH, the cells will grow. For unicellular organisms which divide as they grow, increases in biomass are accompanied by increases in the number of cells present. Associated with the cell growth are two other processes: uptake of some material from the cell's environment and release of metabolic end products into the surroundings. The rates of these processes vary widely as growth occurs (Bailey and Ollis, 1986).

Microbial growth is a good example of an autocatalytic reaction. The rate of growth is directly related to cell concentration, and cellular reproduction is the normal outcome of this reaction. The rate of microbial growth is characterized by the specific growth rate, μ, which is defined as

$$\mu = \frac{1}{C_X} \cdot \frac{dC_X}{dt} \qquad (2.1)$$

where C_X is the cell mass concentration (kg m^{-3}), t is time (h), and μ is the specific growth rate (h^{-1}).

Batch growth refers to culturing cells in a vessel with an initial charge of medium that is not altered by further nutrient addition or removal. This form of cultivation is simple and widely used both in the laboratory and in indusrty. When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. A

typical batch growth curve includes the following phases: 1) lag phase, 2) logarithmic or exponential phase, 3) deceleration phase, 4) stationary phase, and 5) death phase.

The lag phase occurs immediately after inoculation and is a period of adaptation of cells to a new environment. Microorganisms reorganize their molecular constituents when they are transferred to a new medium. During this phase, cell mass may increase a little, without an increase in cell number density.

The exponential growth phase is also known as the logarithmic growth phase. In this phase, the cells have adjusted to their new environment. After this adaptation period, cells can multiply rapidly, and cell mass and cell number density increase exponentially with time. This is a period of balanced growth in which all components of a cell grow with the same rate. That is, average composition of a single cell remains approximately constant during this phase of growth. During this balanced growth, the specific growth rate determined from either cell number or cell mass would be the same.

The deceleration growth phase follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. For a typical bacterial culture, these changes occur over a very short period of time. The rapidly changing environment results in unbalanced growth. In the exponential phase, the cellular metabolic control system is set to achieve maximum rates of reproduction.

The stationary phase starts at the end of deceleration phase, when the net growth rate is zero or when the growth rate is equal to the death rate. Even though the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. Primary metabolites are growth-related products and secondary metabolites are nongrowth-related. In fact, the production of certain

metabolites is enhanced during the stationary phase due to the metabolite deregulation.

The death phase follows the stationary phase. However, some cell death may start during the stationary phase, and clear demarcation between these two phases is not always possible. Often, dead cells lyze, and intracellular nutrients released into the medium are used by the living organisms during stationary phase. At the end of the stationary phase, either because of nutrient depletion or toxic product accumulation, the death phase begins (Schuler and Kargi, 2002).

In batch cell growth cycle, the growth rate is first order:

$$r_X = \frac{dC_X}{dt} = \mu C_X \qquad C_X = C_{X0} \qquad \text{at } t = 0 \tag{2.2}$$

where C_X and C_{X0} are cell concentrations at time t and t = 0 (Schuler and Kargi, 2002).

Similarly, the substrate consumption and product formation rates are defined as:

$$r_S = -\frac{dC_S}{dt} \tag{2.3}$$

$$r_P = \frac{dC_P}{dt} \tag{2.4}$$

The biomass and product yields, $Y_{X/S}$ and $Y_{P/S}$, respectively, are extremely important parameters since they represent the efficiency of conversion of the substrate into biomass and products. They are defined as the mass of biomass or product formed per unit mass of substrate consumed (Table 2.6) (Scragg, 1988):

$$Y_{X/S} = - \frac{dC_X}{dC_S} = \frac{dC_X/dt}{-dC_S/dt} = \frac{r_X}{r_S} \qquad (2.5)$$

$$Y_{P/S} = - \frac{dC_P}{dC_S} = \frac{dC_p/dt}{-dC_S/dt} = \frac{r_p}{r_S} \qquad (2.6)$$

The usual method of measuring yields is to measure the amount of biomass or product formed and substrate consumed over some time period and calculating from equations (2.5) and (2.6):

$$\overline{Y}_{X/S} = \frac{\Delta C_X}{\Delta C_S} \qquad (2.7)$$

$$\overline{Y}_{P/S} = \frac{\Delta C_P}{\Delta C_S} \qquad (2.8)$$

where C_X, C_p and C_s are mass of cell, product and substrate respectively. These are observed yield coefficients and may represent the global yield, that is me

total biomass or product formed compared with the total substrate consumed over the whole growth cycle, or they can be measured at any time during the growth cycle (Scragg, 1988).

Table 2.6 Definition of yield coefficients

Symbol	Definition	Unit
$Y_{X/S}$	Mass of cells produced per unit mass of substrate consumed	kg cell kg^{-1} substrate
$Y_{X/O}$	Mass of cells produced per unit mass of oxygen consumed	kg cell kg^{-1} oxygen
$Y_{S/O}$	Mass of substrate consumed per unit mass of oxygen consumed	kg substrate kg^{-1} oxygen
$Y_{P/X}$	Mass of product formed per unit mass of cell produced	kg product kg^{-1} cell
$Y_{P/S}$	Mass of product formed per unit mass of substrate consumed	kg product kg^{-1} substrate
$Y_{P/O}$	Mass of product formed per unit mass of oxygen consumed	kg product kg^{-1} oxygen

It is important to realize that the yield coefficients are not constant throughout the growth phase since they change with growth rate due to the maintenance energy (m) requirement (Scragg, 1988). The cell must always expend energy to maintain an energized membrane and transport of nutrients and for essential metabolic functions

such as motility and repair of damage to cellular structures. This energy expenditure is called maintenance energy (Schuler and Kargi, 2002).

Microbial growth, product formation, and substrate utilization rates are usually expressed in the form of specific rates since bioreactions are autocatalytic. The specific rates are used to compare the effectiveness of various fermentation schemes and biocatalysts. The specific rate of product formation and substrate utilization are proportional to the specific rate of growth (Schuler and Kargi, 2002):

$$q_p = \frac{1}{C_X} \cdot \frac{dC_p}{dt} \tag{2.9}$$

$$q_s = \frac{1}{C_X} \cdot \frac{dC_s}{dt} \tag{2.10}$$

The maintenance coefficient of oxygen, m_o, is defined as the amount of oxygen required for maintenance. Oxygen is consumed for cell growth, product and by-product formations, and for maintenance purposes in an aerobic process. The oxygen consumption rate for cell growth is defined as:

$$r_{O1} = - \frac{dC_X/dt}{Y_{X/O}} \tag{2.11}$$

Oxygen consumption rate for cell maintenance is defined as:

$$r_{O2} = m_o C_X \tag{2.12}$$

If the oxygen consumption rate for product formation is omitted, then, the total oxygen consumption rate becomes:

$$r_O = r_{O1} + r_{O2} \tag{2.13}$$

Substituting equations (2.11) and (2.12) into (2.13) gives:

$$-r_O = \frac{dC_X/dt}{\overline{Y}_{X/O}} - m_o C_X \tag{2.14}$$

By using the definition of specific growth rate, μ, equation (2.14) can be rewritten as:

$$-r_O = \frac{\mu C_X}{\overline{Y}_{X/O}} - m_o C_X \tag{2.15}$$

When divided by μC_x, and substituted into the equation (2.15), a new equation can be obtained as such:

$$\frac{-r_O}{\mu C_X} = \frac{1}{Y_{X/O}} = \frac{1}{\overline{Y}_{X/O}} - \frac{m_o}{\mu} \tag{2.16}$$

Then, the slope of the plot $1/ Y_{X/O}$ versus $1/\mu$ gives the value of oxygen consumption rate for maintenance, m_o; and from the intercept, cell yield on oxygen uptake where product formation is omitted, $Y_{X/O,}$ could be determined.

Similarly, from the slopes of $1/\mu$ versus $1/ Y_{X/O}$ plots, m_s, maintenance coefficient for substrate, values can be gathered. m_0 and m_s may differ with the

change in bioprocess parameters such as: type of microorganism, type of substrate, pH, and temperature.

2.4.2 Medium Design

In a suitable growth-supporting medium, unicellular microorganisms increase in size and ultimately divide into two by a process of binary fission or budding. It is important to realize that a cell which is apparently not growing may still be viable, but the environment is unable to support growth due the depletion of an essential nutrient, the presence or production of toxic materials or a change in the physical environment, such as oxygen deoletion, pH or temperature (Scragg, 1988). Thus, medium design is an important point that must be considered carefully in bioprocesses.

Nutrients required by cells can be classified in two categories (Schuler and Kargi, 2002):

1) *Macronutrients* are needed in concentrations larger than 10^{-4} M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} are major macronutrients.

2) *Micronutrients* are needed in concentrations of less than 10^{-4} M. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Na^{+}, vitamins, growth hormones, and metabolic precursors are micronutrients.

A synthetic medium is one in which chemical composition is well defined. Such media can be constructed by supplementing a mineral base with the necessary carbon, nitrogen, and energy sources as well as any necessary vitamins. Complex

media, on the other hand, contain materials of undefined composition. Common complex media include yeast extract, beef broth, blood-infusion broth, corn-steep liquor, and sewage (Bailey, 1986).

In the literature, reported studies on streptokinase production by *Bacillus* sp. are observed to utilize complex media rather than defined media. Firstly, Wong et al., (1993) aims to determine whether a *B. subtilis* promoter and signal sequence (levansucrase (*sac*B) promoter and signal sequence) would be better than the native streptokinase (*skc*) promoter and signal sequence and finally, a 2.5 fold increase in the activity is reported for the extracellular production of streptokinase in *B. subtilis* WB600 (6 extracellular protease deficient strain) containing pre-constructed expression vectors in superrich medium, composed of 2.0% yeast extract, 2.5% tryptose, 3.0% K_2HPO_4 and 3.0% glucose, containing 10 μg of kanamycin per ml. In Wu et al., (1998), it is aimed to prolong the short in vivo half-life of streptokinase by site-directed mutagenesis using pSK3 plasmid carrying *sac*B promoter and signal sequence (the sucrose-inducible regulatory region encoding levansucrase). In this study, *B. subtilis* WB600 (6 extracellular protease deficient strain) is used as the expression host with the same production medium as Wong et al., (1993), and 2.2-2.5 fold increase in the activity is observed. Staphylokinase is a promising blood-clot dissolving agent for the treatment of patients suffering from a heart attack similar to streptokinase. To determine whether a *B. subtilis* promoter and signal sequence (*sac*B signal sequence and promoters P43, P*amy*, P*sac*B) would be better than the native staphylokinase promoter and signal sequence is aimed in the study of Ye et al., (1998) by using *B. subtilis* WB700 (7-extracellular protease deficient strain) containing pre-constructed expression vectors in modified superrich medium, composed of 2.5% yeast extract,1.5% bactotrypton, 0.3% K_2HPO_4 and 1.0% glucose, containing 10 μg of kanamycin per ml. Finally, it is observed that the resulting purified staphylokinase protein has a specific activity of 2840 units/mg and more than 2.0 fold increase in staphylokinase production is observed.

2.4.3 Bioreactor Operation Parameters

Any state of a bioprocess depends on many parameters and variables; but especially on the state of the microorganism. Temperature, pH, foam, stirring rate, and dissolved oxygen concentration are some of these parameters influencing the patterns of microbial growth and product formation (Çalık et al., 1999).

2.4.3.1 Temperature and pH

The reaction temperature and pH of the growth medium are the process conditions with a bearing on growth kinetics. It is normally desired to keep both of these variables constant and especially at their optimal values throughout the fermentation process, hence they are often called culture parameters to distinguish them from other variables such as reactant concentrations, stirring rate, oxygen supply rate, etc., which can change dramatically from the start to the end of the fermentation (Nielsen and Villadsen, 1994).

The increase of reaction rates does not occur indefinitely as temperature increases (Johnson, 1998). Increasing temperature up to a certain point starts protein denaturation (Nielsen and Villadsen, 1994). When the temperature climbs too high, the enzymes used to facilitate biochemical reactions fail to retain their actions. They denature, meaning that they change irreversibly. The human body temperature of 37^0C offers the advantage of a high rate of metabolism and a high degree of alertness (Johnson, 1998).

Enzyme activity is reduced by a factor of 2-3 for each 10^0C reduction in temperature (Johnson, 1998).

In this study, the effect of temperature on streptokinase activity was not investigated. Instead, the *E. coli* and *Bacillus* sp. strains were grown at 37^0C which is the optimum temperature value of their growth.

Hydrogen ion concentration (pH) affects the activity of enzymes and therefore the microbial growth rate. The optimal pH for growth may be different from that for product formation. Commonly, the acceptable pH range varies about the optimum by ±1 to 2 pH units. Different organisms have different pH optima: the pH optimum for many bacteria ranges from pH = 3 to 8; for yeast, pH = 3 or 6 (Schuler and Kargi, 2002).

The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in the pH. Enzymes are normally active only within a certain pH interval, and the total enzyme activity of the cell is therefore a complex function of the environmental pH (Nielsen and Villadsen, 1994).

From the profiles of the maximum specific growth rate as a function of pH, it is observed that the optimum is relatively broad. Thus, the cell activity does not change much when one moves one pH unit away from the optimum pH leads to drastic reductions in the microbial activity (Nielsen and Villadsen, 1994).

Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level with large variations in the pH of the extracellular medium, but only at the expense of a significant increase in the maintenance demands, since Gibbs free energy has to be used for maintaining the proton gradient across the cell membrane (Nielsen and Villadsen, 1994). Intracellular pH is affected by the extracellular pH which can influence the intracellular metabolic fluxes due to the changes in the enzyme activities, the transport kinetics of metabolites, proton-driven transportes, cellular energetics and buffering capacity of the cytoplasm (İleri and Çalık et al., 2006).

Oxygen transfer and pH conditions are some of the bioreactor operation conditions affecting product and by-product formations in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes. Some bioprocesses require controlled pH conditions; on the contrary, others may require uncontrolled pH operations, for the aim of increasing the product yield and selectivity. The effects of the controlled and uncontrolled pH conditions on serine alkaline protease (SAP) production by *Bacillus licheniformis* were investigated on a defined medium with the single carbon source glucose in batch bioreactors (Çalık et al., 2002-a). The effect of pH on endo- and exo-metabolome profiling of β-lactamase producer *Bacillus licheniformis* 749/C (ATCC 25972) was studied at controlled and uncontrolled pH conditions (İleri and Çalık et al., 2006). The influences of the controlled- uncontrolled pH conditions and initial pH on the product and by-product profiles and oxygen transfer characteristics along with the intracellular reaction rates in SAP production by recombinant *Bacillus licheniformis* were investigated in batch bioreactors in the study of Çalık et al., (2003-c).

Lowering of pH due to accumulation of lactic acid and the concomitant induction of acid tolerance response (ATR) may affect the recombinant protein produced. In Sriraman et al., (2006), the effect of culture pH and the associated ATR was investigated on production of recombinant streptokinase. Streptokinase gene was cloned and expressed as a secretory protein in *Lactococcus lactis* under the control of P170 promoter. It was found to undergo degradation to form inactive products leading to low productivity. It was found that high pH and high initial phosphate concentration leads to suppression of ATR and this results in at least 2.5-fold increase in streptokinase productivity and significant decrease in degradation of streptokinase.

Many organisms have mechanisms to maintain intracellular pH at a relatively constant value in the presence of fluctuations in environmental pH. In many fermentations, pH can vary substantially. Often the nature of the nitrogen source can be important. Moreover, pH can vary because of the production of organic acids, the

utilization of acids, or the production of bases. The supply or evolution of CO_2 can alter the pH greatly in some systems. Therefore, pH control by means of a buffer or an active pH control system is important (Schuler and Kargi, 2002).

In the literature, neither the working temperature nor the corresponding pH values are specified for the production of recombinant streptokinase protein in *Bacillus* sp. (Wong et al., (1993), Wu et al., (1998), Ye et. al., (1998)). In this study, *Bacillus licheniformis* 749/C (ATCC 25972) was selected as the production host, and the host was cultured at 37^0C.

2.4.3.2 Oxygen Transfer and K_La Measurement

The transfer of oxygen into the microbial cell in aerobic fermentation processes strongly influences product formation by affecting metabolic pathways and altering metabolic fluxes; thus, to fine-tune bioreactor performance in relation with the physiology of the microorganism, the extent of oxygen-transfer requirement should be clarified (Çalık et al., 2000). Some bioprocesses require high oxygen transfer rates, while others require controlled oxygen transfer rates to regulate oxygen uptake rates (Çalık et al., 2003-c). In order to design, scale-up, and operate the bioreactor with enough mass transfer, the oxygen consumption rates and oxygen transfer coefficients which are the indicators of the mass transfer characteristics of a fermentation process are required; however, due to the complex composition of the fermentation liquid, it can be difficult to predict these parameters with reasonable accuracy (Çalık et al., 1998).

Dissolved oxygen is an important substrate in aerobic fermentations and may be a limiting substrate, since oxygen is a sparingly soluble gas in water. At high cell concentrations, the rate of oxygen consumption may exceed the rate of oxygen supply, leading to oxygen limitations (Schuler and Kargi, 2002). The transfer of oxygen from the gas to the liquid microorganism takes place in several steps. Firstly,

the oxygen must travel through the gas to the gas-liquid interface then through the interface, through the bulk liquid, and finally into the organism (Scragg, 1988). These are described in Figure 2.14 as:

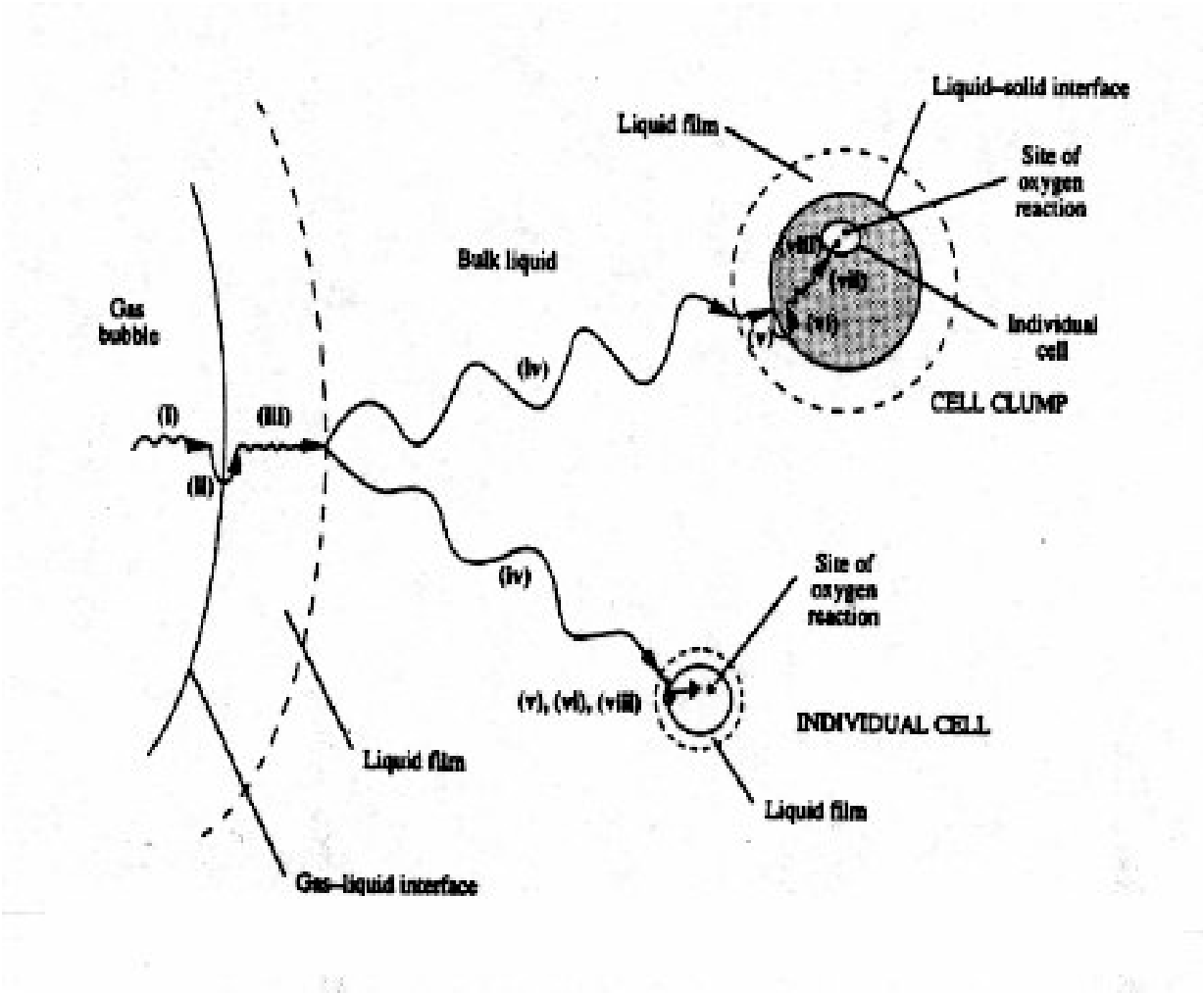

Figure 2.14 Steps for transfer of oxygen from gas bubble to cell. (i) transfer from the interior region of the bubble to the gas-liquid interface; (ii) movement through the gas-liquid interface; (iii) diffusion through the relatively stagnant liquid film surrounding the bubble; (iv) transport through the bulk liquid; (v) diffusion through the relatively stagnant liquid film surrounding the cells; (vi) movement across the liquid-cell interface; (vii) if the cells are in a floc, clump or solid particle, diffusion through the solid to the individual cell; and (viii) transport through the cytoplasm to the site of reaction (Doran, 1995).

Oxygen transfer from gas bubbles to cells is usually limited by oxygen transfer through the liquid film surrounding the gas bubbles. The rate of oxygen transfer (OTR) from the gas to liquid medium is demonstrated by:

$$OTR = K_L a(C_O^* - C_O) \tag{2.17}$$

where K_L is the oxygen transfer coefficient (m/h), a is the gas-liquid interfacial area (m^2/m^3), $K_L a$ is the volumetric oxygen transfer coefficient (h^{-1}), C_O^* is saturated dissolved oxygen concentration (kg/m^3), C_O is the actual dissolved oxygen concentration in the broth (kg/m^3) (Schuler and Kargi, 2002).

Many factors influence the total microbial oxygen demand. The more important of these are cell species, culture growth phase, carbon nutrients, pH, and the nature of the desired microbial process, i.e., substrate utilization, biomass production, or product yield (Bailey, 1986).

The rate of oxygen uptake (OUR) is defined as:

$$OUR = -q_O C_X = r_O = \frac{\mu C_X}{Y_{X/O}} \tag{2.18}$$

where q_O is the specific rate of oxygen consumption (kg O_2/ kg dry weight cells.h), $Y_{X/O}$ is the oxygen yield coefficient (kg dry weight cells/kg O_2), and C_X is the cell concentration (kg dry weight cells/m^3) (Schuler and Kargi, 2002).

The term $K_L a$ is dependent on the physico-chemical properties of the bioreactor media, and on the physical properties and operating conditions of the vessel. It can be controlled by the agitation conditions and the air flow rate. Oxygen is a substrate which limits growth, but, above a certain concentration, growth will become independent of oxygen concentration. Knowledge of $K_L a$ behaviour allows

the operation of bioreactors at conditions where oxygen is not a limiting factor for growth. Many methods have been developed for the experimental determination of K_La values. The most direct is the unsteady-state method based on a material balance on the oxygen in the liquid phase as (Scragg, 1988):

$$\frac{dC_O}{dt} = K_La\,(C_O^* - C_O) + r_O \qquad (2.19)$$

A typical response curve of the dynamic method is shown in Figure 2.15.

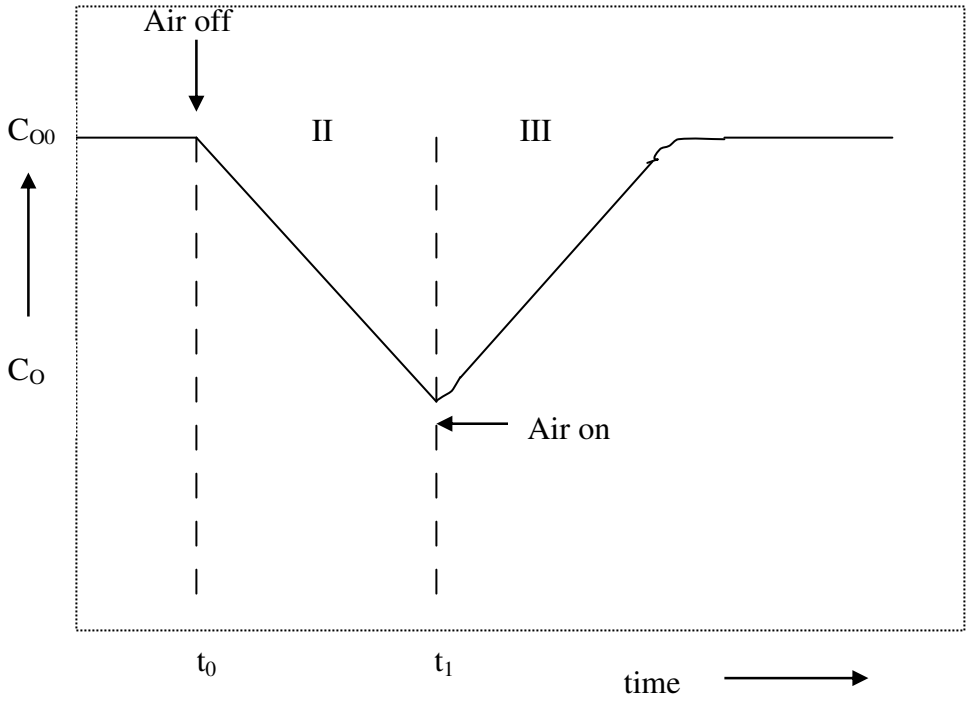

Figure 2.15 Variation of dissolved oxygen concentration with respect to time in dynamic method for K_La measurement

At time t_0, as can be seen in Figure 2.15, when the air flow is stopped in the broth, dissolved oxygen concentration, C_O, decreases, and because there is no oxygen transfer occuring in region-II, equation (2.19) becomes:

$$\frac{-dC_O}{dt} = r_O \qquad (2.20)$$

Using equation (2.20) in the region-II, it is possible to determine the oxygen uptake rate, r_O. When the air inlet flow is turned on, an increase in C_O is observed as a function of time in the region-III. Then, equation (2.19) is again valid for this region. Combining equations (2.18) and (2.19) gives:

$$C_O = -\frac{1}{K_La}\left(\frac{dC_O}{dt} - r_O\right) + C_O^* \qquad (2.21)$$

K_La can be determined from the slope of C_O vs $(dC_O/dt - r_O)$ plot.

The dynamic method can also be applied to conditions under which there is no reaction, i.e., $r_O = 0$. This is interesting when studying the effect of operating parameters, e.g., the stirring speed and the gas flow rate, on the volumetric mass transfer coefficient in model media (Nielsen and Villadsen, 1994). Moreover, air inlet is turned back on making C_O increase as a function of time. Equation (2.21) then becomes:

$$C_O = -\frac{1}{K_La_0}\frac{dC_O}{dt} + C_O^* \qquad (2.22)$$

The mass transfer coefficient, K_La_0, can be determined from the slope of C_O versus dC_O/dt plot.

A large number of different empirical correlations for the volumetric mass transfer coefficient K_La have been presented in the literature. Most of these correlations can be written in the form:

$$K_La = k\left(\frac{P}{V_R}\right)^{\beta} u_S^{\alpha} \qquad (2.23)$$

Where u_S is the superficial gas velocity (m s^{-1}), P/V_R is the power dissipation per unit volume (W m^{-3}) and k is the empirical constant. β and α parameters are specific for the considered system, i.e. for the bioreactor design (Nielsen and Villadsen, 1994).

As the promoter and signal sequence to be used, the gene encoding subtilisin Carlsberg, which is an alkaline serine protease (SAP) produced by *Bacillus licheniformis* was inserted in the upstream region of the gene encoding streptokinase. Therfore, a literature survey was conducted, and various papers investigating SAP production were studied.

In the study of Çalık et al., (2000), the effects of oxygen transfer on the production and product distribution in serine alkaline protease (SAP) fermentation by *Bacillus licheniformis* and oxygen-transfer strategy in relation to the physiology of the bacilli were investigated on a defined medium with citric acid as sole carbon source in 3.5-dm^3 batch bioreactor systems. By forming a 3 × 3 matrix with the parameters air-inlet rates of Q_O/V_R = 0.2, 0.5, 1.0 vvm, and agitation rates of N = 150, 500, 750 min^{-1}, the effects of oxygen transfer were investigated at nine different conditions. Among the constant air-flow and agitation-rate fermentations, Q_O/V_R = 0.5 vvm, N = 750 min^{-1}oxygen-transfer conditions produced maximum SAP activity that was 500 U cm^{-3}, at t = 37 h.

The work, Çalık et al., (2003-d), reports on the design of a complex medium based on simple and complex carbon sources, i.e. glucose, sucrose, molasses, and defatted-soybean, and simple and complex nitrogen sources, i.e. $(NH_4)_2HPO_4$, casein, and defatted-soybean, for serine alkaline protease (SAP) production by recombinant *Bacillus subtilis* carrying pHV1431::*subC* gene. The highest SAP activity was obtained as 5350Ucm^{-3} in the medium that contained (kgm^{-3}): $C^o_{soybean}$ = 20,

$C^{o}_{sucrose}$ = 15, $C^{o}_{Na2HPO4}$ = 0.021, and $C^{o}_{NaH2PO4}$ = 2.82, that was 6.5-fold higher than that of the SAP produced in the defined medium. By using the designed complex medium, oxygen transfer characteristics of the bioprocess were investigated; and, Damköhler number that is the oxygen transfer limitation increases with the cultivation time until t = 14 h; and, at t > 20 h both mass transfer and biochemical reaction resistances were effective. Overall oxygen transfer coefficient varied between 0.010 and 0.044 s^{-1}; volumetric oxygen uptake rate varied between 0.001 and 0.006 $molm^{-3}$ s^{-1}; and specific oxygen uptake rate varied between 0.0001 and 0.0022 mol kg^{-1} DWs^{-1} throughout the bioprocess.

In the study of Çalık et al., (2003-c), the influence of controlled- and uncontrolled-pH conditions together with the initial pH on the product and by-product distributions and oxygen transfer characteristics, whereupon the process rate limitations in relation to the intracellular reaction rates were investigated in serine alkaline protease (SAP) fermentation process by recombinant *Bacillus licheniformis* carrying pHV1431::*subC* on a defined medium with the only carbon source glucose in the pH range of 6.80–7.25 in batch bioreactors. According to the biomass and SAP production process, bioprocess was divided into two periods; Period I ($0<t\leq10$ h) covers the cell growth phase and Period II ($10<t\leq24$ h) covers the SAP production phase. In Period I in both operations, while the oxygen uptake rate (OUR) increased with the increase in initial pH the oxygen transfer rate (OTR) decreased. In Period II, among uncontrolled-pH operations OUR was the lowest at pH = 7.10 while OTR decreased with the increase in initial pH. At the oxygen transfer condition applied, the bioprocess is biochemical reaction limited at all the conditions; nevertheless, with the decrease in initial pH, Damköhler number that is the oxygen transfer limitation decreases.

The work, Çalık et al., (2004-a), describes Serine alkaline protease (SAP) production in a complex medium based on physically pretreated molasses by recombinant *Bacillus subtilis* carrying pHV1431::*subC* gene. The effects of oxygen

transfer were investigated in 3.5 dm^3 bioreactor systems with controls for agitation rate, dissolved oxygen, pH, temperature, and foam formation under two different agitation rates, ie N = 500 and 750 min^{-1}, and four different air flow rates, ie Q/V_R = 0.2, 0.5, 0.7, and 1.0 vvm, at a molasses concentration equivalent to initial sucrose concentration (C_{So}) of 20 kgm^{-3}. At Q/V_R = 0.5vvm and N = 750 min^{-1}, SAP activity reached 2250U cm^{-3} at t = 36 h. The oxygen transfer coefficient (K_La) and oxygen uptake rate $(-r_O)$ were measured throughout the fermentations and their variation with the oxygen transfer conditions were determined.

In the literature, among the referred papers where *Bacillus* sp. were used as the host microorganism (Wong et al., (1993), Wu et al., (1998), Ye et. al., (1998)), the effects of oxygen transfer on recombinant streptokinase protein are observed not to be investigated. In this study, the effects of oxygen transfer were investigated in 3.5 dm^3 batch bioreactor (Braun CT2-2) having a working volume of 0.5-2.0 dm^3 applying an uncontrolled pH condition with controls of temperature, agitation rate, dissolved oxygen, and foam formation. The utilized bioreactor operation conditions are as follows: T = 37^0C, Q/V_R = 0.5vvm and N = 400 min^{-1}. The oxygen transfer coefficient (K_La) and oxygen uptake rate $(-r_O)$ were measured throughout the fermentation and their variation with the oxygen transfer conditions were identified.

2.5 Response Surface Methodology (RSM)

RSM is a collection of mathematical and statistical techniques for devoloping and optimizing processes (Raymond, 1995). The greatest application of RSM has been in industrial research, especially where a large number of variables in a system influence some feature of the system. This feature (i.e., reaction yield, production cost, etc.) is called the response. In the system, there are input variables or independent variables affecting the response (Myers, 1971).

RSM encompasses setting up a series of experiments yielding enough and reliable measurements of the response of interest, determining the optimal settings for experimental factors producing the maximum (or minimum) response value (Anderson, 1987).

There are many published papers showing the successful application of known RSM techniques in many areas such as biology, chemistry, engineering, food industry, education and others.

When it is said that the value of the response Y depends on X_1, X_2, ..., X_k of k quantitative factors, then there exists a response function of X_1, X_2, ..., X_k supplying the corresponding value of Y, that is,

$$Y = f(X_1, X_2, ..., X_k) \tag{2.29}$$

Consider the response function $Y = f(X_1)$ for a single factor. With the Taylor Series expansion about some arbitrary point X_0, Y can be written as:

$$Y = f(X_0) + (X_1 - X_0)f`(X_0) + \frac{1}{2}(X_1 - X_0)^2 f``(X_0) + ... \tag{2.30}$$

where f `(X_0) and f```(X_0) are the first and second derivatives of f (X_1) evaluated at X_0. The expansion (Equation 2.30) reduces to a polynomial of the form

$$Y = f(X_1) = B_0 + B_1X_1 + B_{11}X_1^2 + ... \quad (2.31)$$

where the coefficients B_0, B_1 and B_{11} are parameters depending on X_0 and the derivatives of f (X_1) evaluated at X_0.

By taking the terms up to degree 1, an equation of a straight line is obtained which is refered as a first-order model in X_1.

$$Y = f(X_1) = B_0 + B_1X_1 \quad (2.32)$$

By taking to degree 2, an equation of a parabola which is the second-order model in X_1 is obtained

$$Y = f(X_1) = B_0 + B_1X_1 + B_2X_1^2 \quad (2.33)$$

For two factors, X_1 and X_2, a polynomial equation is obtained

$$Y = f(X_1, X_2) = B_0 + B_1X_1 + B_2X_2 + B_{11}X_1^2 + B_{22}X_2^2 + B_{12}X_1X_2 + ... \quad (2.34)$$

This model is called the "second-order response surface" and the parameters B_0, B_1, B_2, ..., B_{12}, ... are called "regression coefficients or parameters". X_1 and X_2 are input variables in the regression function (Anderson, 1987).

Using a computer program, f (X_1, X_2) can be plotted either as a solid surface in a three-dimensional space or as a contour plot showing the surface to be mound shaped.

In the industrial world, optimizing the conditions for a process is very important problem. In such cases, a second-order model can be used to approximate the yield response in a selected narrow region, performed in this study also, and from examination of this approximate response surface, the optimal levels, i.e. carbon and nitrogen source concentrations in a production medium for a bioprocess, could be chosen (Myers, 1971).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals were analytical grade, and obtained from Sigma Ltd., Difco Laboratories, Fluka Ltd. and Merck Ltd. Standard streptokinase was puchased as the drug Streptase from ZLB Behring.

3.2 The Microorganism and Plasmids

In Table 3.1, all the microorganisms and plasmids used in this study for the extracellular production of recombinant protein streptokinase are listed.

All microorganisms are stored in the microbanks (PRO-LAB), by inoculating young colonial growth into cyropreservative fluid present in the vial. After providing the adsorption of microorganisms into the porous beads, excess cyropreservative was aspirated and inoculated cyrovial stored at -55^0C.

3.3 The Solid Medium

Under sterile conditions, all *E.coli* strains and *Bacillus* species, which were stored at 4^0C on agar slants, were inoculated on freshly prepared agar slants, and they are incubated at 37^0C overnight. The composition of the solid medium is shown in Table 3.2. Suitable antibiotics (according to the antibiotic resistances of the microorganisms, i.e., ampicillin resistance in pUC19 vector and chloramphenicol resistance in pMK4 vector) are added to each agar slant after the steam sterilization

step conducted at 121^0C for 20 minutes. The concentration of each antibiotic is listed in Table 3.3.

Table 3.1 Microorganisms and plasmids used in this study (NRRL: Northern Regional Research Center; ATTC: American Type Culture Collection)

Strains	**Source/Reference**
E.coli TG1 (*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F[*traD*36 *proAB*$^+$ *lacl*^{q} *lac* ZΔM15])	. Prof. Dr. Semra Kocabıyık
B. subtilis (*apr*$^-$, *npr*$^-$)	Calık et al., 2003-a
B. licheniformis 749/C (ATCC 25972)	Çelik et al.,2004

Plasmids	**Source/Reference**
pUC19	Yanisch-Peron et al., 1985
pMK4	Sullivan et al., 1984
pUC19::*pre(subC)::skc*	This work
pMK4::*pre(subC)::skc*	This work

Table 3.2 The composition of the solid medium

Compound	Concentration, kg m^{-3}
Soytryptone	10.0
Yeast Extract	5.0
NaCl	10.0
Agar	15.0
pH	7.5

Table 3.3 The final concentration of antibiotics in the solid and liquid media

Antibiotic	Concentration, kg m^{-3}
Ampicillin	0.01
Chloramphenicol	0.035

3.4 The Precultivation Medium

During the bioprocess experiments, the recombinant *Bacillus* species, previously grown on agar slants, were inoculated into the precultivation medium and then, incubated at 37^0C and at N = 200 min^{-1} overnight in heating rate and agitation controlled orbital shakers (B.Braun, Certomat BS-T) by using air-filtered Erlenmeyer flasks of 150 mL in size having volume capacities of 33 mL. The compositions of precultivation medium are demonstrated in Table 3.4. In order to increase the stability of the plasmid, selective antibiotics were inserted into the precultivation medium in specific amounts as listed in Table 3.2.

Table 3.4 The composition of the precultivation medium for r-*Bacillus* species (Çalık et al., 2003-a)

Compound	Concentration, kg m^{-3}
Soytryptone	15.0
Peptone	5.0
$MnSO_4.2H_2O$	1.0×10^{-2}
Na_2HPO_4	0.25
$CaCl_2$	0.10

3.5 The Production Medium

The recombinant *Bacillus* species, grown in preculture medium until the exponential growth phase was reached, were next inoculated in the production medium. As the starting point for the medium design, reference production media were selected as given in İleri and Çalık et al., (2006). The compositions were listed in Table 3.5.

Table 3.5 The composition of the production medium for r- *Bacillus* species (İleri and Çalık et al., (2006))

Compound	Concentration, kg m^{-3}
Glucose	7.0
$(NH_4)_2HPO_4$	7.1
Salt solution:	
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	1.0×10^{-3}
$ZnSO_4.7H_2O$	1.0×10^{-3}
$MnSO_4.H_2O$	7.5×10^{-5}
$CuSO_4.5H_2O$	1.0×10^{-5}

In order to obtain a high level of expression of the desired recombinant protein, streptokinase, a defined medium design was done. Afterwards, the effect of controlled pH on biomass and streptokinase production capacity of the recombinant microorganisms which is one of the bioprocess operation parameters was investigated. The investigated parameters are demonstrated in Table 3.6.

Table 3.6 The investigated medium components and bioprocess parameters for recombinant streptokinase production in *Bacillus* species

Medium Component & Aim		**Bioreactor Operation Parameters**
Glucose $(NH_4)_2HPO_4$	Concerted effect of initial carbon and nitrogen source	Medium composition and pH

All components of the medium were steam sterilized at 121 ^oC for 20 min. Glucose solution was sterilized separately. The medium design laboratory experiments were conducted in 150 ml Erlenmeyer flasks having 33 ml working volume capacities. *Bacillus* species were cultured at a cultivation temperature of 37^oC and at an agitation rate of 200 min^{-1} in heating rate and agitation controlled orbital shakers (B.Braun, Certomat BS-T).

To investigate the effect of controlled pH on biomass and streptokinase production capacity of the recombinant *Bacillus* species, the pilot scale 3.0 dm^3 batch bioreactor (Braun CT2-2), having a working volume of 0.5-2.0 dm^3, and consisting of temperature, pH, foam and stirring rate controls, was used. The bioreactor has a steam generator, external cooler, and a jacket surrounding the bioreactor for sterilization and temperature control purposes. It has two four-blade Rushton turbines consisted of four baffles and a sparger.

3.6 Analyses

Samples were collected at various cultivation times during the bioprocess. After the cell concentration was determined, in order to precipitate the recombinant *Bacillus* cells and the cell debris, the production medium was centrifuged at 13200 min^{-1} , 4^{o}C for 10 min. Thereafter, supernatant was used for the determination of cell concentration, streptokinase concentration, streptokinase activity, glucose concentration, organic acid concentration and for the SDS-PAGE analysis.

3.6.1 Cell Concentration

A UV-vis spectrophotometer (Thermo Spectronic, Heλios α) was used in order to determine cell concentrations based on cell dry weights using the calibration curve (Appendix H) obtained at 600 nm.

3.6.2 Streptokinase Concentration

Streptokinase concentrations were determined with a high performance capillary electrophoresis at 254 nm (Waters HPCE, Quanta 4000E) based on the calibration curve prepared by using the standard streptokinase solutions at different concentrations (Appendix K). The samples were analyzed at 20kV and 15°C with a negative power supply by hydrostatic pressure injection, using an electrolyte solution at pH 10.0.

3.6.3 Streptokinase Activity

The procedure for streptokinase activity quantitation was similar to that used by Castellino et al., (1976) with small modifications. In this procedure a synthetic chromogenic substrate for plasmin, N-(p-tosyl)-glycyl-prolyl-lysine-4-nitroanilide

acetate salt was used. Streptokinase activity was determined by measuring the rate of cleavage of 4-nitroaniline from the substrate monitored at 421 nm by the UV-vis spectrophotometer.

As the reagents, plasminogen (10 mg/ml in 0.05 M Tris-HCl-0.1 M L-lysine, pH 7.5), stock streptokinase solution (0.7 mg/ml in 0.001 M Tris-HCl, pH 7.5), substrate, N-(p-tosyl)-gly-pro-lys 4-nitroaniline acetate salt solution, (0.5 mM in the final assay solution, pH 7.5) and the activity assay solution (0.1 M NaCl-0.1 M L-lysine, pH 7.5) were prepared fresh (Castellino et al., 1976).

A streptokinase solution was prepared with a concentration of 0.7 mg/ml in 0.001 M Tris-HCl (pH 7.5). Firstly, 1 ml of assay solution was added to the plasminogen (0.015 ml) at 37°C. Then, 0.02 ml of the desired dilution of streptokinase was added. After waiting for exactly 5 min at 37°C, 0.1 ml stock substrate solution was added, and the cleavage rate of 4-nitroaniline from the substrate per min was measured at 421 nm. Then, the calibration, initial rates versus substrate concentration and the Lineweaver-Burk curves were plotted (Figures 4.15-4.16).

In the work, Ramalingam et al., (2007), streptokinase activity is defined as plasmin units/ml of sample solution. Plasmin activity was calculated by this formula:

$$\frac{\text{PU}}{\text{ml of sample solution}} = \frac{V}{v\varepsilon d}\frac{\Delta A}{\text{min}}$$

where V is the final assay volume (ml); v sample volume (ml); ε (=9.75 mmol/cm) molar absorption coefficient for 4-nitroaniline; d light path in the cuvette (1 cm); ΔA/min is the change in absorbance/min. Streptokinase activity is measured in plasmin units/ml. Activity is expressed in IU/ml with Streptase (ZLB Behring) as standard streptokinase.

3.6.4 GlucoseConcentration

A method called glucose oxidation method was used for the determination of glucose concentration (Boyacı et al., 2005). In this method, D-glucose is oxidized in the presence of the enzyme glucose oxidase (equation 3.1) and peroxide, product of this reaction, is further reacted with 4-aminoantipyrine and phenol in the presence of peroxidase enzyme to form iminoquinone (equation 3.2) giving the red color which can be observed spectrophotometrically and which is proportional to glucose concentration.

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2 \qquad (3.1)$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{Iminoquinone} + H_2O \qquad (3.2)$$

A glucose analysis kit (Biyozim D-glukoz, Biasis) was employed in order to determine glucose concentrations according to manufacturer's instructions, and the calibration curve for the standard glucose solution is demonstrated in Appendix L .

In this method, samples having glucose concentrations more than 1 g/l were dilluted to those less than or equal to 1 g/l. Then, the analysis solution of 0.4 ml, deionized water of 2 ml and the glucose analysis reactant solution of 0.05 ml were added to standard glucose solutions and also to 0.05 ml samples. These treated samples were either incubated at 37 ^{o}C for 20 min. One sample not containing any reducing sugar was also treated with the analysis solution in order to be used as the blank for the spectrophotometric analysis conducted at 505 nm.

3.6.5 Organic Acid Concentrations

An organic acid analysis system (Waters, HPLC, Alliance 2695) was used to measure the organic acid concentrations. In this method, the calculation of concentrations is based on the chromotogram of standard organic acid solutions which is conducted in the analysis system based on reversed phase HPLC. Before being loaded to the analysis system, samples were filtered with 45 μm filters (ACRODISC CR PTFE). As the mobile phase, 0.312% (w/v) NaH_2PO_4 and $0.62x10^{-3}$ % (v/v) H_3PO_4 were utilized. Needle and seal wash were done with pure water and 20 % (v/v) acetonitrile respectively. Conditions of the analysis were as follows:

Column	: Capital Optimal ODS, 5 μm
Column dimensions	: 4.6 mm x 250 mm
System	: Reversed phase chromatography
Mobile phase flow rate	: 0.8 ml/min
Column temperature	: 30 °C
Detector and wavelength	: Waters 2487 Dual absorbance detector, 254 nm
Injection volume	: 10 μl
Analysis period	: 20 min

3.6.6 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

The Dynamic Method (Rainer, 1990) was used in order to determine the liquid phase mass transfer coefficient and oxygen uptake rate throughout the streptokinase production process as demonstrated in section 2.4.3.2.

The physical mass transfer coefficient (K_La_0) was determined prior to inoculating the microorganisms to the bioreactor. After the bioprocess was started, dynamic oxygen transfer experiments were done at certain cultivation times for a

short period of time, by this way biological activities of the microorganisms are not affected. During this period, air inlet was completely stopped and meanwhile the agitation rate was lowered to N=50 min^{-1} in order to minimize the surface aeration.

3.6.7 Response Surface Methodology

In order to determine the optimum compositions of medium components that maximize the streptokinase production capacity, the statistical approach, Response Surface Methodology (RSM) was applied. The statistical software MINITAB ® Release 14 was used for the medium design experiments performed by RSM.

There are mainly 3 commands employed while doing response surface designs. Each command line applied in MINITAB ® Release 14 is displayed at the beginning and explained in detail.

1. Stat » DOE » Response Surface » Create Response Surface Design: This command is used to create the design:

- The appropriate response surface design is chosen by concerning the experimental region of interest, number of runs that can be performed and other considerations such as cost and time
- The number of factors should be specified.
- The names, the minimum and the maximum values of factors are defined.
- The value of α (alpha) is specified such that if α ‹ 1, the axial points will be placed inside the cube; if α ›, 1, the axial points will be placed outside the cube. Placing α=1 gives a face-centered design.
- After performing all of these steps, a response surface design is created by Minitab.

2. Stat » DOE » Response Surface » Analyze Response Surface Design: After performing the experiment, the responses were inserted into the design table, and the results were analyzed by this command.

- Analysis is performed where coding is performed as −1 for the low level, +1 for the high level, and 0 for the center points. Minitab displays the output based on this coded form. However, uncoding performs the analysis using the values that is assigned in the factors subdialog box. Minitab displays the output using the assigned values.
- After performing the analysis with Minitab, a session window output was displayed and the results are interpreted in terms of the lack of fit of the model (p), R^2 and the estimated regression coefficients. Lack of fit and R^2 are important for checking the fitted model in that an incorrect or under-specified model can result in misleading conclusions. The estimated regression coefficients are used to determine the second order polynomial giving the relation between variables and the response.

3. Stat » DOE » Response Surface » Analyze Response Surface Design » Graphs: This command is used to create graphs giving a relation between the response and the variables. Generally, two types of graphs namely, contour and surface plots are used by the experimenters performing response surface designs. A contour plot provides a two-dimensional view where all points having the same response are connected in order to produce contour lines of constant responses. A surface plot gives a three-dimensional view that may present a clearer demonstration of the response surface.

3.7 Genetic Engineering Techniques

3.7.1 Materials

3.7.1.1 Enzymes, Kits, and Molecular Size Markers

Ethidium bromide, agarose low temperature gelling, ampicillin, chloramphenicol, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), isopropyl β-D-1-thiogalactopyranoside (IPTG), egg-white lyzozyme were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ethylenediaminetetraacetic acid (EDTA), α-D-glucose, sucrose, Tris-base, tryptone, yeast extract, sodium chloride (NaCl), sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) were purchased from Merck, Darmstadt, Germany. Agarose was from Boehringer Mannheim, Germany. Agar was from Difco, Detroit, USA. Ethanol was from Reidel-de Häen, Seelze, Germany. N,N dimethyl formamide was from Fluka AG, Bucks SG, Switzerland.

Taq DNA polymerase was purchased from Sigma Chemical Co., St. Louis, MO, USA. *Pfu* DNA polymerase was from Promega Co., Madison, WI, USA. Ribonuclease A (Dnase and protease free), T4 DNA Ligase, restriction endonucleases *Eco*RI, *Bam*HI and their buffers were from MBI Fermentas AB, Vilnius, Lithuania. Lambda DNA/Hind III Marker, Lambda DNA/EcoRI+HindIII Marker, Gene Ruler 50 bp DNA ladder and 6X Loading Dye were obtained from MBI Fermentas.

Wizard® Plus SV Miniprep DNA Purification System was purchased from Promega Co, WI, USA. DNA Extraction Kit was from MBI Fermentas AB, Vilnius, Lithuania. QIAprep Spin Miniprep Kit and QIAquick PCR Purification Kit were from Qiagen Inc., Valencia, USA. Gene Elution Kit was purchased from Gene Mark Molecular Biology Tools. All of the primers were synthesized by Thermo Hybaid GmbH (Germany) laboratories.

3.7.1.2 Buffers and Solutions

Buffers and solutions utilized in this study and their compositions are all stated in Appendix A. Autoclaving at 121^0C for 20 minutes or filter sterilization through 0.20µm filters (Sartorius) were applied for the sterilization of solutions.

3.7.1.3 Plasmid Vector, Molecular Size Markers and Genomic DNA Sequence Data

The map of the vectors pUC19 and pMK4 are illustrated in Table 2.8. DNA molecular size markers are given in Appendix G. Nucleotide sequences of promoter and signal peptide sequences of serine alkaline protease encoding gene from *B. licheniformis* (DSM1969) (*sub*C: Acc. No. X03341) and the nucleotide sequence of streptokinase encoding gene from *Streptococcus equisimilis* streptokinase gene (*skc:* Acc. No. S46536) with marked primer sequences are given in Appendix B. The source of streptokinase gene is pMF1*::skc* plasmid isolated from *E. coli* (ATCC 39613) purchased from LGC Promochem.

3.7.2 Agarose Gel Electrophoresis

Plasmid samples, restriction enzyme digested DNA fragments, and PCR products were analyzed by using Submarine agarose gel apparatus (Mini SubTM DNA Cell, Bio Rad, Richmond, CA, U.S.A) with 1% (w/v) agarose gel (Boehringer Mannheim, Germany) and 1x TAE as the running buffer. DNA samples of 10-20 µl were mixed with 1/10 volume of tracking dye and loaded on the gel. Electrophoresis was carried out at 60 volts. The bands were visualized with a UV transilluminator (Vilber Lourmat, Marne La Vallée Cedex, France) and gel photographs were taken by using a gel imaging and documentation system (Vilber Lourmat Gel Imaging and Analysis System, Marne La Vallée Cedex, France). The molecular sizes of DNA fragments were determined by referring to calibration curves, which were obtained by plotting the log molecular weights of known marker fragments against migration

distance on the gel. Figure 3.1 shows calibration curves for Gene Ruler 1 kb DNA Ladder (MBI Fermentas AB, Vilnius, Lithuania) and Lambda DNA/*Eco*RI+*Hin*dIII, molecular size marker (MBI Fermentas AB, Vilnius, Lithuania).

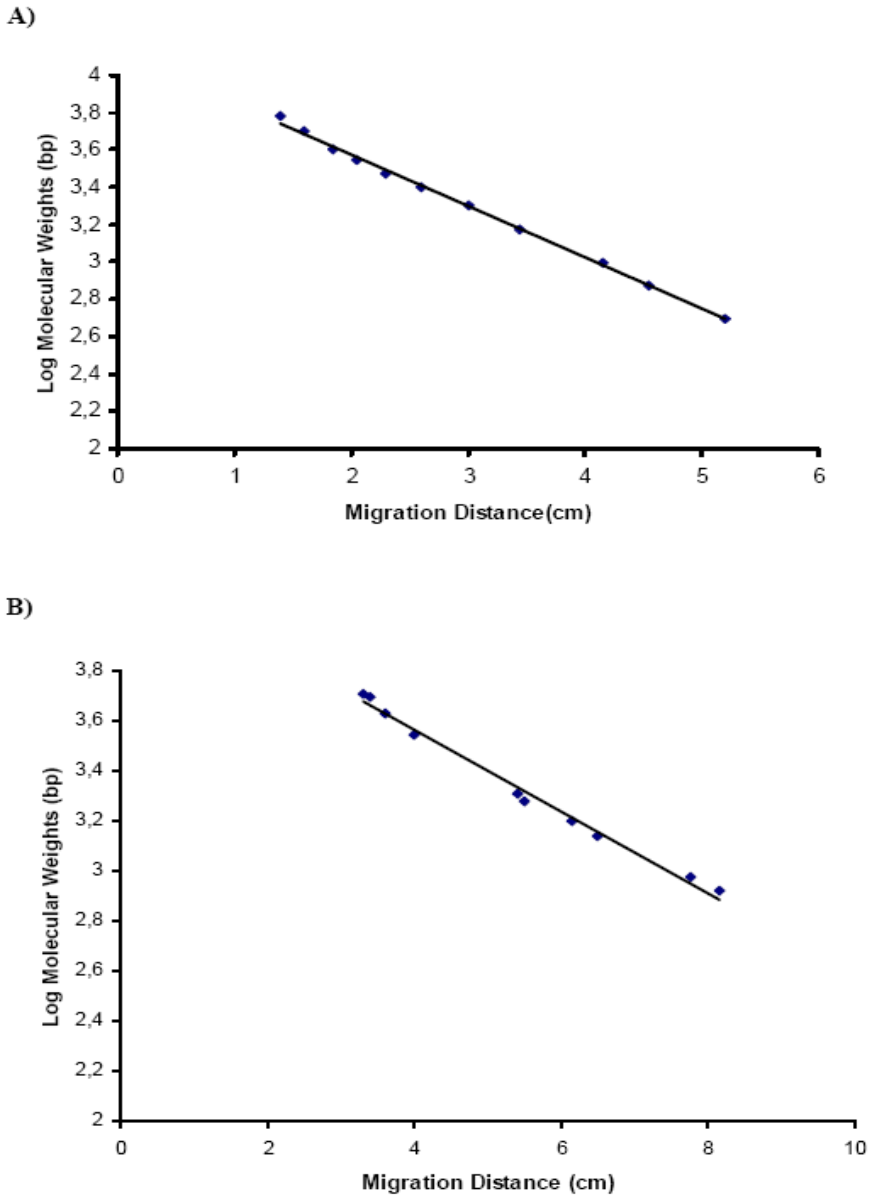

Figure 3.1 Calibration curves for **A)** Gene Ruler 1 kb DNA Ladder (MBI Fermentas AB, Vilnius, Lithuania) and **B)** Lambda DNA/*Eco*RI+*Hin*dIII, molecular size marker (MBI Fermentas AB, Vilnius, Lithuania).

3.7.3 PCR Amplification of Target Genes

Design of primers was performed by referring to the sequences of *skc* gene and *pre(sub*C) gene (Appendix B). Nucleotide sequences of *skc* gene (Accession no: S46536) and serine alkaline protease gene (*sub*C) were gathered from National Institutes of Health (ABD), National Library of Medicine, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Restriction mapper web-page of ABD Molecular Biology Resources (http://www.restriction mapper.org) was used for the determination of restriction enzyme sites. Proper primers were designed for the cloning of the fusion product of *skc* and *pre(sub*C) genes into the selected plasmids after determining the suitable restriction recognition sites (*Eco*RI and *Bam*HI) for the hybrid gene according to these principles:

1. The nucleotide sequence of each primer should be compatible with the template region of the target DNA to be amplified.
2. Primers should contain 18 to 45 nucleotides in length.
3. Forward and reverse primers should have closer melting temperatures.
4. Primer self-complementarity must be avoided.
5. Primer dimer formation should be avoided such that 3' ends of the primers should not be complementary.
6. It is important to end the 3' ends of each primer with an appropriate number of G or C nucleotides in order to increase the annealing efficiency.
7. Each primer should have at least 43% of G+C nucleotide content (Özçelik, 2003).

A computer program (NAR) was used in designing the primers by investigating the possibility of dimer formation, primer self-complementarity, melting temperature, ΔG and ΔH (all are demonstrated in Appendix C). Designed primers

were synthesized in Thermo Hybaid GmbH (Germany) laboratories. The abbreviations and the nucleotide sequences of the primers used in this study are illustrated in Table 3.7.

Table 3.7 Primers designed for amplification of the desired gene fragments

Name	**Sequence**
Forward Primer for *pre(sub*C)	5' GCGCGAATTCGCGGTCTATTCATACTTTCG 3'
Reverse Primer for *pre(sub*C)	5' AGGTCCAGCAATAGCAGAAGCGGAATCG 3'
Forward Primer for *skc*	5' GCGATTCCGCTTCTGCTATTGCTGGACCTGAGTGG 3'
Reverse Primer for *skc*	5' GCCCGGATCCTTATTTGTCGTTAGGGTTATCA 3'

The Techgene Thermal Cycler (Techgene, Techne Inc. NJ. USA) was used for PCR amplification processes. PCR process parameters and the contents of the reaction mixture of 50 µl volume are shown in Tables 3.8 and 3.9.

Table 3.8 PCR process parameters

1 cycle	T = 94 ^{O}C	2-3 min
30 cycles	T = 94 ^{O}C	1 min
	T = 55-60 ^{O}C	1 min
	T = 72 ^{O}C	34 s-3 min
1 cycle	T = 72 ^{O}C	10 min
	T = 4 ^{O}C	5 min

Table 3.9 Components of PCR reaction mixture

Components	**Amounts**
10X PCR Buffer (with Mg^{++})	5 µl
dNTPs (1 mM)	10 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Template DNA (0.01-1 µg)	1-5 µl
Sterile dH_2O	up to 49 µl
DNA polymerase	2.5 U

3.7.4 Purification of PCR Products

QIAquick PCR Purification Kit was used for the purification of PCR products according to manufacturer's recommendations. 1 volume of PCR reaction sample was mixed with 5 volume of PB buffer and the mixture was transfered into the QIAquick spin column. The column was washed with PE buffer twice after centrifugation, and DNA molecules were eluted with proper amount of sterile water.

3.7.5 Plasmid Isolation

Plasmids were isolated using Wizard® Plus SV Miniprep DNA Purification System Kit according to the manufacturers' instructions (Promega Co., Madison, WI, USA). *E. coli* cells harboring the recombinant plasmids were inoculated into a 10 ml LB broth supplemented with 50 µg/ml ampicillin at a final concentration, and incubated overnight at 37°C with vigorous shaking at 160 rpm (Heidolph UNIMAX1010, Heidolph Instruments GmbH, Kelheim, Germany). The cells were harvested by centrifugation at 4 000 rpm for 15 min (Herause Sepatech, GmbH, Osterode, Germany) and the pellet was resuspended with 250 µl Cell Resuspension Solution. The cells were lysed by adding 250 µl Cell Lysis Solution and the proteins were digested by adding 10 µl Alkaline Protease Solution with the incubation at room temperature for 5 minutes. After the addition of 350 µl Neutralization Solution and incubation for 5 minutes on ice, the cell debris was centrifuged at 13 000 rpm for 10 minutes (Herause Sepatech, GmbH, Osterode, Germany). The supernatant was transferred to Spin Column of the Kit, and centrifuged at 13 000 rpm for 1 minute in order to bind the DNA to the column. The bound DNA was washed twice, first with 750 µl and then with 250 µl Wash Solution, and eluted by adding 100 µl of Nuclease-Free Water. Out of 100 µl of plasmid sample approximately 15 µl was run to the

agarose gel to check the efficiency of purification. The remaining sample was stored at -20ºC for further use.

3.7.6 Restriction Enzyme Digestion

Restriction enzyme digestion of the genes of interest were performed by incubating DNA samples with the appropriate restriction enzymes (REs) and buffers in 20 µl final reaction volume at 37°C for 3 h. A double RE digestion was performed in two parts, each continuing for 1.5 h. Firstly, the RE *Eco*RI was utilized in 19 µl final reaction volume. After 1.5 h incubation, *Bam*HI was added and another 1.5 h incubation at 37°C was performed. The composition of the restriction enzyme digestion reaction mixtures was illustrated in Table 3.10.

Table 3.10 Components of reaction mixture of restriction digestion with *Eco*RI and *Bam*HI REs

Components	**Amounts**
Buffer Y^+ Tango	2 µl
DNA sample	~ 45-200 ng
*Eco*RI RE (10 U/µl)	1 µl
*Bam*HI RE (10 U/µl)	1 µl
Sterile dH_2O	up to 19 µl

3.7.7 DNA Extraction from Agarose Gel

MBI Fermentas DNA Extraction Kit (MBI Fermentas AB, Vilnius, Lithuania) was used according to the manufacturers' instructions for the isolation of the DNA fragments. The agarose gel slice containing the DNA band was excised and placed

into an eppendorf tube, weighed and dissolved in Binding Solution (3 volumes of buffer/1 volume of gel) with the incubation at 55ºC for 5 minutes. Then, 2 µl of Silica Powder Suspension was added per 1µg of DNA and incubated at 55ºC for additional 10 minutes. To keep the silica powder in suspension, the mixture was mixed gently for every 2 minutes. Silica powder/DNA complex was pelleted by centrifugation at 13 000 rpm (Herause Sepatech, GmbH, Osterode, Germany) for 1 minute and the supernatant was removed . The silica resin was washed three times with 500 µl ice cold Wash Buffer as the pellet was resuspended completely and the supernatant was removed during each washing step. Then, the silica was air dried for 10-15 minutes. For elution of DNA, the resin was suspended into water and incubated at 55ºC for 5 minutes then centrifugation was performed at 13 000 rpm (Herause Sepatech, GmbH Osterode, Germany) for 1 minute.

3.7.8 Ligation Reaction

After the restriction digestion, sticky ends occur in both PCR amplified genes and isolated vectors. The amplified genes were cloned into suitable vectors with the ligation reaction composed of the mixture containing 1 volume of plasmid sample/5 volume of DNA fragment sample, 2 µl T4 DNA Ligase (5 U/µl) and 2 µl T4 DNA Ligase Buffer. The mixture was incubated overnight at 4°C.

3.7.9 Preparation of Competent *E. coli* Cells

The overnight culture of *E. coli* TG1 cells (1/100 volume) was inoculated into 20 ml LB broth. They were grown at 37°C with vigorous shaking at 171 rpm (Heidolph UNIMAX1010, Heidolph Instruments GmbH, Kelheim, Germany). The growth was screened by measuring the optical density of cell culture at 600 nm by the Shimadzu UV-160A double beam spectrophotometer (Shimadzu Analytical Co., Kyoto, Japan). When the logarithmic growth phase was reached, the cells were

collected by centrifugation at 4°C, 4 000 rpm for 10 minutes (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, MO, USA). The supernatant was discarded and the pellet was dissolved in 1/10 volume TSS solution. Then, the prepared solution was distributed as 100 µl to eppendorf tubes and stored at -80°C until use.

3.7.10 Transformation and Screening

3.7.10.1 Transformation of Plasmid DNA to *E. coli*

Competent *E. coli* cells were taken from -80°C deep freezer and melted on ice in order to be used in transformation. An aliquot (20 µl) of overnight ligation mixture was added to the melted cells and mixed gently. Then, an incubation was performed on ice for 30 minutes. Cells were transferred to glass tubes containing 0.6 ml LB broth supplemented with glucose and incubated at 37°C with vigorous shaking at 230 rpm for 1.5 hours (Heidolph UNIMAX1010, Heidolph Instruments GmbH, Kelheim, Germany). Appropriate dilutions of competent cells were spread onto selective LB agar plates containing ampicillin, IPTG and X-Gal and incubated at 37°C for overnight.

3.7.10.2 Transformation of Plasmid DNA to *Bacillus* Species by Electroporation

Recombinant shuttle vector pMK4 was transformed into *Bacillus* species according to the method described by Özçelik, 2003.

A singe freshly grown *Bacillus* colony was taken from the plate and inoculated into 5 ml LB medium to be incubated at 37°C with vigorous shaking at 180 rpm for 3 hours (B. Braun Biotech Certomat BS-1, Sartorius Group). The cells were harvested by centrifugation at 4°C, 4 000 rpm for 15 minutes (Santrifüj /

Eppendorf – centrifuge 5415-R). Separated cells were washed with 3 ml 1mM HEPES (pH = 7.0) buffer and with cold electroporation buffer (25% PEG and 0.1 M mannitol) twice. During washing processes, cells were kept cold. Thereafter, washed cells were resuspended in 1/200 volume of cold electrophoration buffer (˜250 µl) and kept on ice for 10 minutes. 3 µl (˜100 ng) plasmid DNA was added into 40 µl cells and the mixture was transferred to an electroporation cuvette. After the cuvette was placed into the Electroporation System (Bio-Rad), 2.5 kV of voltage were given to the sample. Then, 500 µl of LB medium without any antibiotic was added to the cuvette and the mixture was transferred to a sterile ependorf tube to be incubated at 37°C with vigorous shaking at 180 rpm for 3 hours (B. Braun Biotech Certomat BS-1, Sartorius Group) to recover the cells. Next, cells were spreaded over the LB agar containing plates supplemented with the proper antibiotic using a sterile, bent glass rod. Finally, the plates were incubated at 37°C for onernight (Nüve Incubator, EN 055). Corresponding recombinant colonies would be visible in 14-24 hours.

3.7.11 Plasmid DNA Isolation from *Bacillus* Species by Alkaline Lysis Method

A singe freshly grown *Bacillus* colony was taken from the plate and inoculated into 15 ml LB medium (150 ml flask) to be incubated at 37°C with vigorous shaking at 200 rpm for overnight (B. Braun Biotech Certomat BS-1, Sartorius Group). 1.5 ml of the cells were harvested by centrifugation at room temperature, 10 000 rpm for 1 minute (Santrifüj / Eppendorf – centrifuge 5415-R). Separated cells were washed with 1.5 ml SET Buffer and again, cells were harvested by centrifugation at room temperature, 10 000 rpm for 1 minute (Santrifüj / Eppendorf – centrifuge 5415-R). Thereafter, washed cells were resuspended in a freshly prepared 500 µl Solution I (containing 2 mg/ml egg-white lyzozyme). After homogenization by vortexing for 10 seconds, cells were incubated at 37°C for 10-20 minutes. Then, the samples were put on ice, and 400 µl Solution II was added. After homogenization by vortexing for 10 seconds, cells were kept on ice for 10 minutes.

300 µl Solution III was added. After homogenization by vortexing for 10 seconds, cells were kept on ice for 10 minutes. Centrifugation was performed at 4°C, 13 500 rpm for 10 minutes. The supetnatant was transferred into sterile eppendofs such that each containing 600 µl. Then, 600 µl phenol/chloroform/isoamylalcohol (25/24/1) mixture was added, vortexed for 10 seconds, after waiting 1 minute again vortexed for another 10 seconds. In order to seperate the phases, centrifugation was performed at 4°C, 13 500 rpm for 5 minutes. The upper phase was taken into new ependorf tube. A same volume of chloroform/isoamylalcohol (24/1) mixture was added and vortexed for 10 seconds. In order to seperate the phases, centrifugation was performed at 4°C, 13 500 rpm for 3.5 minutes. The upper phase was taken into new ependorf tube. After adding 1 ml absolute ethanol, the sample was kept on ice for 1 hour in order to precipitate the plasmid DNA. Centrifugation was performed at 4°C, 13 500 rpm for 10 minutes, and the plasmid DNA observed as a pellet was elluted by a proper volume of sterile water. After the addition of 1 µl Rnase enzyme, it was incubated at 37 °C for 15-20 minutes and then stored at -20°C until use.

3.7.12 DNA Sequencing

The DNA sequencing was performed by automatic DNA sequencers (Microsynth GmbH, Switzerland) using the appropriate primers designed to control the presence of the target genes.

3.7.13 SDS-Polyacrylamide Gel Electrophoreis of Proteins

Under conditions that ensure dissociation of proteins into their individual polypeptide subunits and that minimize aggregation, nearly all analytical electrophoreses of proteins are carried out in polyacrylamide gels. Most commonly, before the proteins are loaded into the gel, SDS, the strong anionic detergent, is used in combination with a reducing agent and heat in order to dissociate the proteins.

Denaturated polypeptides bind to SDS and then they become negatively charged. The amount of the SDS that is bound is almost usually proportional to the molecular weight of the polypeptide. Moreover, it is independent of the sequence of that polypeptide. Then, SDS-polypeptide complex migrates through the gel according to the size of the protein. Detection of the unlabeled proteins seperated by polyacrylamide gel electrophoresis is typically performed by staining, either with Coomassie Brilliant Blue or with silver salts. Coomassie Brilliant Blue allows the visualization of proteins as blue bands inside the translucent matrix of the gel by its nonspecific binding to proteins but not the gel in a relatively rapid and straightforward reaction. Silver staining method is more sensitive, but more difficult to perform. It is more adventageous in that it enables the detection of proteins 100 fold lower than those detected by Coomassie Brilliant Blue staining method (Sambrook, 2001).

3.7.13.1 Pouring SDS-polyacrlamide Gels

The glasses are cleaned with ethanol and the glass plates are assembled according to the manufacturer's instructions. Distilled water is pored between glasses in order to check whether the glasses are properly sealed or not. If there is no decrease in water level, glasses are properly sealed. Then, the water can be pured out and the glasses can be left to dry. Meanwhile, appropriate volume of solutions containing the desired concentration of monomer solution for 12% resolving gel are prepared, using the values given in Appendix E. Polymerization would begin as soon as the NNN'N'-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) solutions are added. At this point, the mixture should be poured into the gap between the glass plates. It is important to leave sufficient space for the stacking gel. Then, some water is added to overlay the monomer solution and leave the gel in a vertical position until polymerization is completed. After 30 min, the water is poured out and the area above the resolving gel is dried with a filter paper before pouring the stacking gel. A comb is placed in the gel sandwich and it should

be tilt so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth while pouring the monomer solutions. Finally, the gel is left to polymerize for 30-45 minutes.

3.7.13.2 Preparation of Samples and the Running the Gel

Samples are prepared by diluting at least 1:1 with sample buffer and heated at 95°C for 5 min, while the stacking gel still polymerizes. When the polymerization finishes (after 30 min), the gel is placed in electrophoresis apparatus and the reservoir is filled with running buffer. 20 µl of each sample are loaded into the wells and the running is conducted at 40 mA. The usual expected run time is approximately 45 minutes.

3.7.13.3 Staining SDS-Polyacrylamide Gels with Silver Salts

The gels were silver stained using the procedure of Blum *et* al. (1987) as shown in Table 3.11.

Table 3.11 Silver staining Procedure

	STEP	SOLUTION	TIME OF TREATMENT	COMMENTS
1	Fixing	Fixer	≥ 1 hr	Overnight incubation is all right
2	Washing	50% Ethanol	3 x 20 min	Should be fresh
3	Pre-treatment	Pretreatment Solution	1 min	Should be fresh
4	Rinse	Distilled water	3 x 20 sec	Time should be exact
5	Impregnate	Silver Nitrate Solution	20 min	
6	Rinse	Distilled water	2 x 20 sec	Time should be exact
7	Developing	Developing Solution	~ 5 min	After a few minutes add some distilled water to proceed the reaction slowly. Time should be determined by observation of color development
8	Wash	Distilled water	2 x 2 min	
9	Stop	Stop Solution	≥ 10 min	The gels can be kept in this solution overnight

3.7.14 Western Blotting of Proteins

Western blot is a method of detecting a protein in a sample by using a specific antibody of the protein. It also gives information about the relative amounts of that protein in different samples and the size of the protein.

3.7.14.1 Blotting

After running the protein sample in SDS-polyacrlamide gel electrophoresis system, the stacking gel is cut off and the top left-hand corner of the resolving gel is nicked. 2 blotting papers and the membrane are cut, and the membrane is wetted with methanol. Then, it is transfered into the tray to equilibrate with the transfer buffer for at least 10 min before starting the blotting. 2 sponges and 2 blotting papers are placed into the tray to be wetted. The cassette is then opened and placed into the tray filled with the transfer buffer. The gel is transfered into the glass tray containing at least 3 cm of 1XTransfer buffer, and peeled off gently from the glass plate. It is important to make sure that there are no bubbles between the gel and the membrane. Since proteins will move from negatively charged pole to the positive, the positive electrode should be above the membrane and the negative one should be below the gel. The arrangement of the components used in the cassette formation from down to up is as follows: anode (+)[white side]-sponge-blotting paper-membrane (Millipore, Immobilon™ , Trandfer Membranes)-gel-blotting paper-sponge-cathode (-)[black side]. Then, the cassette is closed and locked. After closing the cassette, if the stack seems to be loose, sheets of blotting paper should be added; but if it seems to be tight, the top sponge should be replaced with a sheet of blotting paper. The cassette is placed inside the tank such that the black side of each cassette faces the black cathode panel. Finally, this assembly is put inside the SDS-running tank filled with 1XTransfer buffer. An ice pack is placed to the empty side of the running tank. The gel is run at 50 V for 3 hours in such an orientation that the whole assembly is put over a magnetic stirrer in order to provide a homogenized medium inside.

3.7.14.2 Blocking, Antibody (Ab) Incubation and Detection Using ECL Western Blotting Kit

The membrane is removed from the cassette and is put into a box in a fashion that the protein side is facing up. The membrane is then washed with 50 ml TBS-T for 3 times (3x10 min, 70 rpm, room temperature) in order to prevent non-specific binding of Ab with the protein. The membrane is further immersed in TBS-T-Milk for 1 h at room temperature with shaking at 70 rpm. Then, it is washed with 50 ml TBS-T(3x10 min). The membrane is now transfered to a petri plate. The primary Ab (Rabit Anti-Streptokinase Polyclonal Antibody, Unconjugated, AbD Serotec) is diluted in TBS-T-Milk as 1:200 (10 ml TBS-T + 0.3 g skim milk + 50 µl Ab). The membrane is incubated in the diluted Ab for 1 h at 4 ^{0}C for over night. The membrane is transfered into the gel box and it is washed with TBS-T for 3 times as before. Meanwhile, the secondary Ab is diluted in TBS-T-Milk as 1:10 000 (10 ml TBS-T + 0.3 g skim milk + 2 µl Ab). Then, the membrane is placed into a small container for incubating it with the diluted secondary Ab for 1 h at room temperature on the shaker platform. The membrane is transfered into the gel box and it is washed with TBS-T. Then, by using BiØmeda Corp, Diaminobenzidine Chromogen Kit, the hybridized protein bands are made visible by following the manufacturer's instructions as such: 5 ml dH_2O, 5 drops of Solution [A] and 1 pill of [B] are mixed and vortexed. After waiting 5 min, 1 drop of Solution [C] is added to this mixture. Finally, this preperation is poured over the membrane placed inside a new petri plate. Then, it is waited to observe the protein bands.

CHAPTER 4

RESULTS AND DISCUSSION

The first aim of this study is the development of extracellular recombinant streptokinase producing *Bacillus sp*., and the second aim is to determine fermentation characteristics for streptokinase production. In this context, the signal (*pre*-) DNA sequence of *B.licheniformis* (DSM1969) extracellular serine alkaline protease enzyme gene (*sub*C: Acc. No. X03341) was ligated to 5' end of the streptokinase gene (*skc:* Acc. No. S46536) by SOE (Gene Splicing by Overlap Extension) method through PCR. The resulting hybrid gene *pre(sub*C*)*::*skc* was cloned into the pUC19 plasmid. Then, the hybrid gene was sub-cloned to pMK4 plasmid which is an *E. coli-Bacillus* shuttle vector with high copy number and high stability. Recombinant plasmid pMK4::*pre(sub*C*)*::*skc* was finally transferred into *B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) species. Streptokinase production capacities of these two recombinant *Bacillus* species were compared. The highest production was observed in recombinant *B. lichenifomis* 749/C (ATCC 25972) strain in a defined medium which was optimized in terms of carbon and nitrogen sources. Finally, fermentation characteristics of streptokinase production were studied.

4.1 Recombinant *Bacillus* Species Development for Extracellular Streptokinase Production by Genetic Engineering Techniques

In previous studies**,** streptokinase was produced extracellularly from the gene on modified plasmids under the control of various promoters and signal sequences in *Bacillu*s sp. *sac*B promoter and signal sequence of the sucrose-inducible regulatory region encoding levansucrase gene (Wong et al., (1993), Wu et al., (1998) and Ye et al., (1998)).

In this study, the promoter and signal (*pre*-) DNA sequence of *B. licheniformis* (DSM1969) extracellular serine alkaline protease enzyme gene (*sub*C: Acc. No. X03341) was ligated to 5' end of the streptokinase gene (*skc:* Acc. No. S46536) by SOE (Gene Splicing by Overlap Extension) method through PCR. In order to perform this ligation, fistly proper forward and reverse primers were designed for the cloning of the fusion product of *skc* and *pre(sub*C) genes into the selected plasmids after determining the suitable restriction recognition sites (*Eco*RI and *Bam*HI) for the hybrid gene. Initially, each gene was amplified by PCR with *pre(subC)* forward/reverse and *skc* forward/reverse primers seperately. These amplified DNA fragments were associated with each other by SOE method resulting in the hybrid gene '*pre(sub*C*)*::*skc'*. Next, the chimeric gene was cloned into pUC19 and then sub-cloned into pMK4 *E. coli-Bacillus* shuttle vector from the restriction recognition sites of *Eco*RI and *Bam*HI. Recombinant plasmid pMK4::*pre(sub*C*)*::*skc* was firstly transferred into *E. coli* TG1 strain by induced competence, and then into *B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) by electroporation and expressed in these *Bacillus* species under the control of the promoter region of *pre(sub*C*)* gene.

4.1.1 Primer Design for the Amplification of *skc*, *pre(subC)* and the Hybrid Genes

It is Jacobs et al., (1985 - Acc. No: X03341), who firstly reported the gene (*sub*C) encoding subtilisin Carlsberg from *Bacillus licheniformis* NCBI 6816. The signal sequence of extracellular serine alkaline protease enzyme gene, *pre(sub*C*)* gene, was amplified from the of *B. licheniformis* (DSM1969) chromosomal DNA (Çalık et al., 2003-b) and the recombinant pMF1::*skc* plasmid isolated from *E. coli* (ATCC 39613) was used as the template for amplifying the *skc* gene (Acc. No: S46536).

Nucleotide sequences of *skc* gene (Acc. No: S46536) and *sub*C (Acc. No: X03341) were gathered from National Institutes of Health (ABD), National Library of Medicine, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and all sequences were illustrated in Appendix B. Four primers (forward and reverse), two for *pre(sub*C*)* and two for *skc* gene were designed and utilized in order to develop the hybrid gene '*pre(sub*C*)*::*skc'*. The *Eco*RI restriction enzyme recognition sequence was fused at 5' end of the *pre(sub*C*)* forward primer. As it can be seen in Figure 4.1, extra sequences complementary to *pre(sub*C*)* and *skc* genes were inserted at 5' ends of primers 2 and 3 in order to obtain the overlapping ends for the amplified segments. So that, during the further denaturation and annealing processes of PCR, these overlapping fragments would act as primers of each other. Finally, the recombinant hybrid gene will be obtained by the extension of these overlaps by DNA polymerase. The *Bam*HI restriction enzyme recognition sequence was integrated at 3' end of the *skc* reverse primer.

All primers were designed by the use of a computer program called NAR, in which the possibility of dimer and self-complementary formation affinities and thermodynamic properties of the primers were checked. The results are presented in Appendix C.

1) restriction enzyme region + pre(*sub*C) forward primer

***Eco*RI (5' GAATTC 3')**

Sequence: 5' GCGCGAATTCGCGGTCTATTCATACTTTCG 3'

2) *pre*(*sub*C) reverse primer + complementary strand to *skc*

Sequence: 5' AGGTCCAGCAATAGCAGAAGCGGAATCG 3'

3) complementary strand to *pre(subC)* + *skc* forward primer

Sequence: 5' GCGATTCCGCTTCTGCTATTGCTGGACCTGAGTGG 3'

4) restriction enzyme region + *skc* reverse primer

***Bam*HI (5' GGATCC 3')**

Sequence: 5' GCCCGGATCCTTATTTGTCGTTAGGGTTATCA 3'

Figure 4.1 Schematic illustration of primer design in this study. 1) *Eco*RI RE recognition sequence was fused at 5' end of the *pre(sub*C*)* forward primer as a single stranded tail; 2) Overlapping extension complementary to *skc* template strand was associated to 5' end of the *pre(sub*C*)* reverse primer, which is complementary to the sense strand of *pre(sub*C*)* gene; 3) Overlapping extension complementary to *pre(sub*C*)* template strand was associated to 5' end of the *skc* forward primer, which is complementary to the sense strand of *skc* gene; 4) *Bam*HI RE recognition sequence was fused at 3' end of the *skc* reverse primer, which is complementary to the sense strand of the *skc* gene, as a single stranded tail.

4.1.2 Amplification of *pre(subC), skc* and Hybrid Genes by Polymerase Chain Reaction (PCR) and SOE Method

Under the scope of genetic engineering techniques, the signal peptide sequence of subtilisin Carlsberg gene, *pre(subC)*, was amplified from the *B. licheniformis* (DSM1969) chromosomal DNA by the first PCR with the appropriate primers (*Eco*RI RE recognition sequence was fused at 5' end of the *pre(sub*C*)* forward primer, and overlapping extension complementary to *skc* template strand was associated to 5' end of the *pre(sub*C*)* reverse primer). The resulting amplified *pre(subC)* gene (final length of 285 bp) is demonstrated in Figure 4.2-a. In the second PCR, the mature protein gene encoding streptokinase was amplified from the recombinant pMF1::*skc* plasmid isolated from *E. coli* (ATCC 39613) with the designed primers (overlapping extension complementary to *pre(sub*C*)* template strand was associated to 5' end of the *skc* forward primer, and *Bam*HI RE recognition sequence was fused at 3' end of the *skc* reverse primer, which is complementary to the sense strand of the *skc* gene). The resulting amplified *skc* gene (final length of 1245 bp) is demonstrated in Figure 4.2-b. These two sequences having overlapping ends complementary to each other were associated via gene splicing by overlap extension method (SOE) and amplified by the third PCR. Theoretically speaking, these fragments are combined together in a further fusion reaction where the overlapping ends anneal to the other strand in a fashion that 3' overlap of each strand serves as a primer for the 3' extension of the complementary strand (Ho et al., 1989). The resulting amplified hybrid gene, *pre(sub*C*)*::*skc*, (final length of 1530 bp) is presented in Figure 4.2-c.

The annealing temperatures and the corresponding time values were determined according to the length of each DNA fragment. In Tables 4.1, 4.2 and 4.3, all PCR components and operation parameters are listed. The *pre(sub*C*)* and *skc* genes were amplified with proofreading *Pfu* DNA polymerase instead of *Taq* DNA polymerase, which has an ability to insert adenine (A) overhangs to 3' ends of each

PCR product, in order not to allow any foreign nucleotide association between the signal peptide and the mature protein sequences. The reason is that, a frameshift mutation would occur due to these additional residues, and this would result in a 3-D conformational change of the desired protein product or this would decrease the efficiency of signal peptidase in releasing the mature protein to the extracellular region (Tjalsma et al., 2000). Thus, it is not advisable to use *Taq* DNA polymerase in the amplification of DNA fragments that are to be combined together further. However, these amplified fragments were associated via the gene splicing by overlap extension method (SOE) and amplified by the third PCR with *Taq* DNA polymerase, because it was previously proven that this polymerase has a higher yield and, the resulting 3' A-overhangs do not cause any frameshift mutation in the hybrid gene structure.

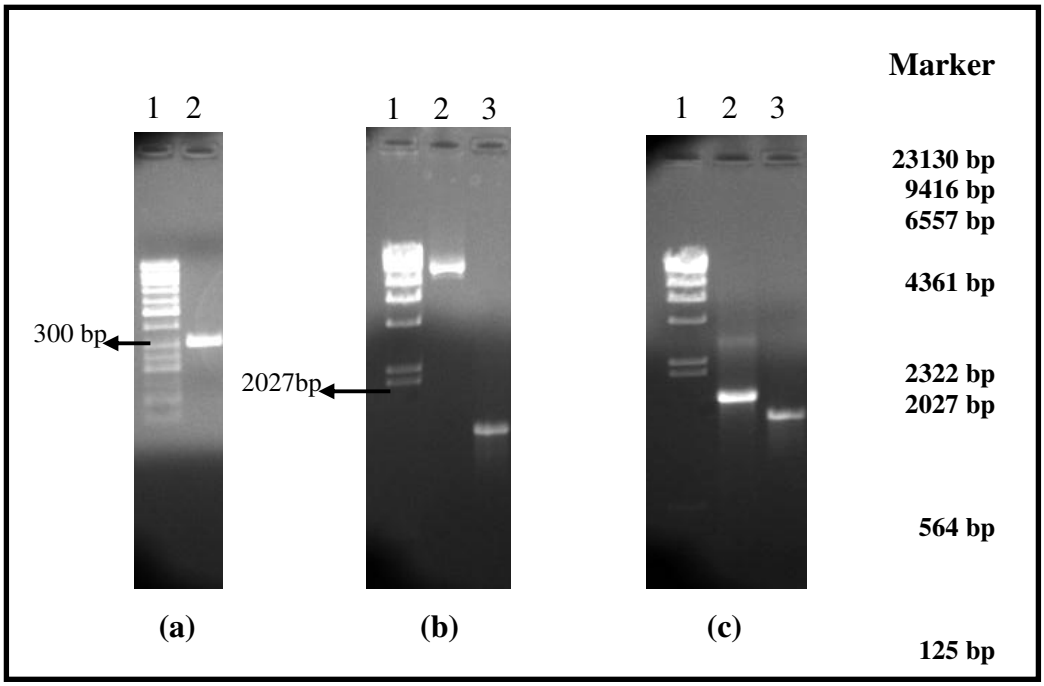

Figure 4.2 Agarose gel electrophoresis images of *pre(subC), skc* and the hybrid genes. **(a)** Lane 1: Low range marker DNA ladder; Lane 2: *pre(subC)* gene amplified with *Pfu* DNA polymerase. **(b)** Lane1: λDNA/*Hind*III marker; Lane 2: Isolated pMF1::*skc* plasmid; Lane 3: *skc* gene amplified with *Taq* DNA polymerase. **(c)** Lane 1: λDNA/*Hind*III marker; Lane 2: hybrid gene amplified with *Taq* DNA polymerase; Lane 3: *skc* gene amplified with *Taq* DNA polymerase.

Table 4.1 PCR process parameters for amplifying *pre(subC)* gene

1 cycle	T = 94 ^{O}C	3 min
30 cycles	T = 94 ^{O}C	1 min
	T = 59 ^{O}C	1 min
	T = 72 ^{O}C	34 s
1 cycle	T = 72 ^{O}C	10 min
	T = 4 ^{O}C	5 min

Table 4.2 PCR process parameters for amplifying *skc* and the hybrid genes

		Genes to be amplified	
		skc	**hybrid**
1 cycle	T = 94 ^{O}C	3 min	3 min
30 cycles	T = 94 ^{O}C	1 min	1 min
	T = 60 ^{O}C	1 min	1 min
	T = 72 ^{O}C	2 min	3 min
1 cycle	T = 72 ^{O}C	10 min	10 min
	T = 4 ^{O}C	5 min	5 min

Table 4.3 Components of PCR reaction mixture for amplifying *pre(subC), skc* and the hybrid genes

	Genes to be amplified		
	pre(subC)	***skc***	**hybrid**
Components	**Amounts**		
10X PCR Buffer (with Mg^{++})	5 µl	5 µl	5 µl
dNTPs (1 mM)	10 µl	10 µl	10 µl
Forward primer (10 µM)	1 µl	1 µl	1 µl
Reverse primer (10 µM)	1 µl	1 µl	1 µl
Template DNA	~3 µg cDNA	~60 ng pMF1::*skc*	~ 22 ng *pre(subC)* + ~ 48 ng *skc*
Sterile dH_2O	up to 49 µl	up to 49 µl	up to 49 µl
DNA polymerase	2.5 U *Pfu*	2.5 U *Pfu*	2.5 U *Taq*

In PCR, instead of using fresh PCR products as the template DNA it is preferable to start with pure templates in order to eliminate any possible contamination since the PCR reaction mixture contains many compounds like proteins, organic solvents, salts and metal ions which can inhibit the polymerase activity and thus reduce the yield of PCR. Therefore, before used as templates, the *pre(subC)*and *skc* genes were purified with the PCR Purification Kit (QIAquick).

4.1.3 Ligation of the Hybrid Gene into pUC19 Cloning Vector and Transformation in *E. coli* TG1 Strain

The hybrid gene which was amplified through PCR and then purified (C_{hybrid} = 8.8 ng μl^{-1}) and pUC19 vector (C_{pUC19} = 0.5 μg μl^{-1}, Fermentas) were digested with, firstly *Eco*RI restriction enzyme for 1.5 h at 37 ^{o}C then with *Bam*HI restriction enzyme for another 1.5 h at 37 ^{o}C in a reaction mixture of 20 µl final volume whose components are listed in Table 4.4. Digested DNA samples were purified via an extraction process from the agarose gel by MBI Fermentas DNA Extraction Kit. Thereafter, digested and purified hybrid gene (C_{hybrid} = 5.7 ng μl^{-1}) was cloned into purified linear pUC19 vector (C_{pUC19} = 24.3 ng μl^{-1}) from the multiple cloning site located within the *lacZ* gene inactivating the N-terminal fragment of beta-galactosidase and abolishes alfa-complementation. Bacteria carrying recombinant plasmids therefore give rise to white colonies. Components of the ligation reaction which was conducted at 4^{o}C overnight are demonstrated in Table 4.5.

After transformation of the ligation product into *E. coli* TG1 strain, transformants were spread on LB-agar medium supplemented with ampicillin (0.5 ml/100 ml LB from 10 mg/ml stock), Xgal (0.504 ml/200 ml LB from 20 mg/ml stock) and IPTG (0.2 ml/200 ml LB from 20 mg/ml stock) and incubated at 37^{o}C for 12-18 h. According to the blue-white screening method, putative white colonies were selected and further incubated overnight in LB-broth medium supplemented with ampicillin (0.5 ml/100 ml LB from 10 mg/ml stock) at 37^{o}C for plasmid DNA isolation. The assembly of the recombinant molecule, pUC19:: *pre(sub*C*)*::*skc*, is schematically illustrated in Figure 4.3.

Table 4.4 Components of reaction mixture of restriction digestion of the hybrid gene and pUC19 cloning vector with *Eco*RI and *Bam*HI REs

	Hybrid gene	**pUC19 plasmid**
Components	**Amounts**	**Amounts**
Buffer Y^+ Tango	2 µl	2 µl
DNA sample	~ 88 ng	1.5 µg
*Eco*RI RE (10 U/µl)	1 µl	1 µl
*Bam*HI RE (10 U/µl)	1 µl	1 µl
Sterile dH_2O	up to 19 µl	up to 19 µl

Table 4.5 pUC19 and hybrid gene, *pre(sub*C*)*::*skc*, ligation reaction conditions

Components	**Amounts**
Insert DNA	~ 80 ng
Vector DNA	~ 48 ng
Ligation Buffer (with ATP)	2 µl
T4 DNA ligase (5u/µl)	2 µl
dH_2O	up to 19 µl
TOTAL	20 µl

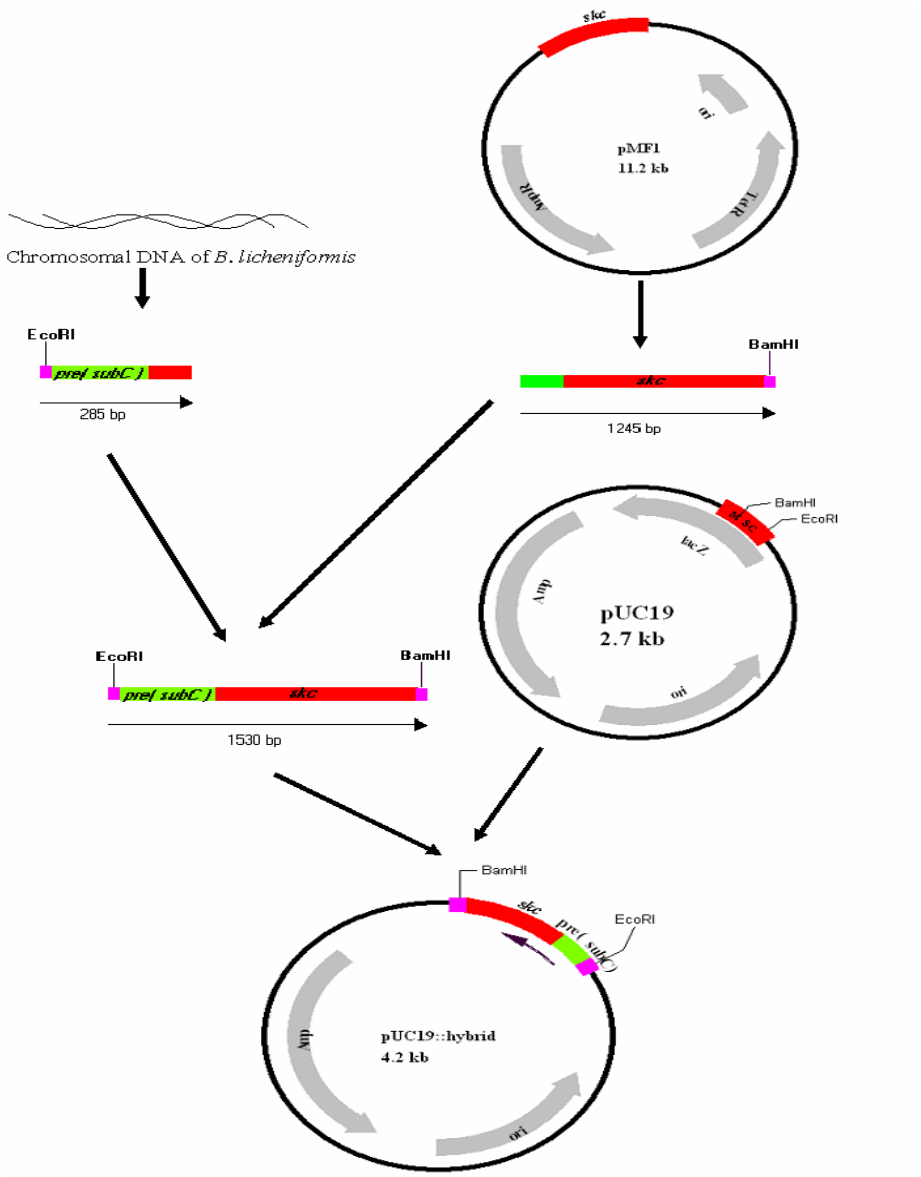

Figure 4.3 Schematic representation of the construction of recombinant pUC19::hybrid vector. 285 bp DNA fragment of the signal peptide sequence of subtilisin Carlsberg gene(with the promoter region also), *pre(subC)*, with *Eco*RI RE recognition region at its 5' end (shown as pink) and a region of complementary sequence to *skc* gene at its 3' end (shown as red) was associated with to 1245 bp *skc* gene having *Bam*HI RE recognition region at its 3' end (shown as pink) and a region of complementary sequence to *pre(sub*C*)* gene at its 5' end (shown as green) leading to 1530 bp DNA fragment of the hybrid gene. The hybrid gene, *pre(sub*C*)*::*skc*, having *Eco*RI and *Bam*HI endings was cloned into the vector pUC19 from *Eco*RI and *Bam*HI restriction sites. The insertion and transcription directions were demonstrated by arrows.

The isolated putative recombinant pUC19:: *pre(sub*C*)*::*skc* plasmids were digested with *Eco*RI and *Bam*HI REs in order to confirm the integration of hybrid gene into the cloning vector pUC19. After this RE digestion, a gene portion having the same length of the hybrid gene was observed as verified by the agarose gel electrophoresis results demonstrated in Figure 4.4.

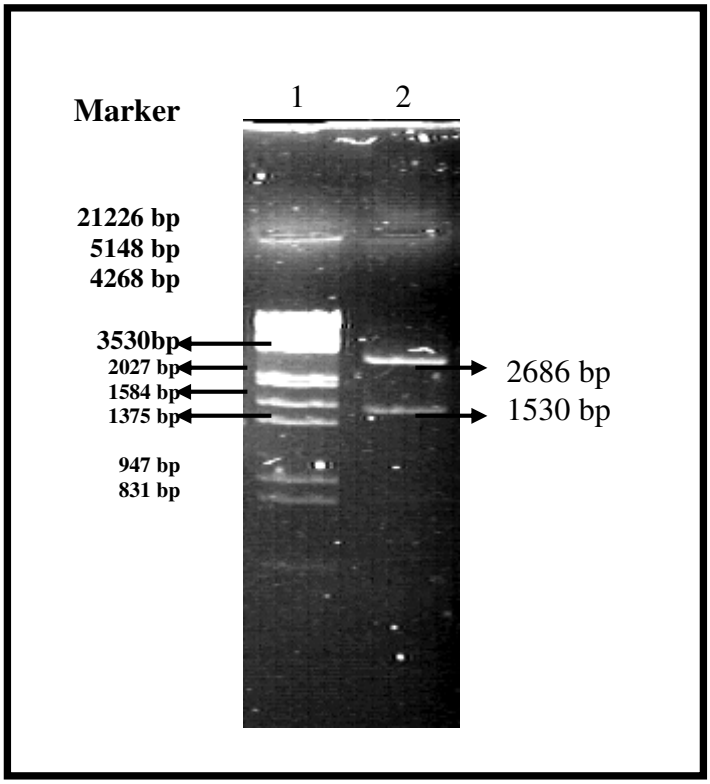

Figure 4.4 Agarose gel electrophoresis image of the RE digestion analysis result of the putative recombinant pUC19 plasmid. Lane 1: λDNA/*Eco*RI+*Hind*III molecular size marker; Lane2: *Eco*RI/*Bam*HI double digestion of recombinant pUC19::*pre(sub*C*)*::*skc* plasmid excised 2686 bp (pUC19) and 1530 bp (*pre(sub*C*)*::*skc*) fragments

4.1.4 Ligation of the Hybrid Gene into pMK4 Expression Vector and Transformation in *Bacillus* Species

The hybrid gene was purified from agarose gel after digesting the recombinant vector, pUC19:: *pre(sub*C*)*::*skc*, with *Eco*RI and *Bam*HI (Table 4.6). Likewise, *E. coli*/*Bacillus* shuttle vector pMK4 was digested with the same REs and then purified from agarose gel by Bio 101 Gene Clean Kit (Figure 4.6).

The digested hybrid gene (C_{hybrid} = 9.3 ng μl^{-1}) was ligated with the purified and linearized pMK4 plasmid (C_{pMK4} = 14.0 ng μl^{-1}) from the sticky ends by T4 DNA ligase. The ligation reaction was performed at 4^{o}C overnight in a reaction volume of 20 µl (Table 4.7). The resulting recombinant plasmid, pMK4:: *pre(sub*C*)*::*skc,* was transformed into *E. coli* TG1 strain by induced competence, transformants were spread on LB-agar medium supplemented with ampicillin (0.5 ml/100 ml LB from 10 mg/ml stock), Xgal (0.504 ml/200 ml LB from 20 mg/ml stock) and IPTG (0.2 ml/200 ml LB from 20 mg/ml stock) and incubated at 37^{o}C for 12-18 h. According to the blue-white screening method, putative white colonies were selected and further incubated overnight in LB-broth medium supplemented with ampicillin (0.5 ml/100 ml LB from 10 mg/ml stock) at 37^{o}C for plasmid DNA isolation. The assembly of the recombinant molecule pMK4::*pre(sub*C*)*::*skc*, is schematically illustrated in Figure 4.5.

Table 4.6 Components of reaction mixture of restriction digestion of the pUC19::*pre(sub*C*)*::*skc* and pMK4 cloning vector with *Eco*RI and *Bam*HI REs

	pUC19:: *pre(sub*C*)*::*skc*	**pMK4 plasmid**
Components	**Amounts**	**Amounts**
Buffer Y^+ Tango	2 µl	2 µl
DNA sample	~ 280 ng	~ 210 ng
*Eco*RI RE (10 U/µl)	1 µl	1 µl
*Bam*HI RE (10 U/µl)	1 µl	1 µl
Sterile dH_2O	up to 19 µl	up to 19 µl

Table 4.7 pMK4 and hybrid gene, *pre(sub*C*)*::*skc*, ligation reaction conditions

Components	**Amounts**
Insert DNA	~ 93 ng
Vector DNA	~ 42 ng
Ligation Buffer (with ATP)	2 µl
T4 DNA ligase (5u/µl)	2 µl
dH_2O	up to 19 µl
TOTAL	20 µl

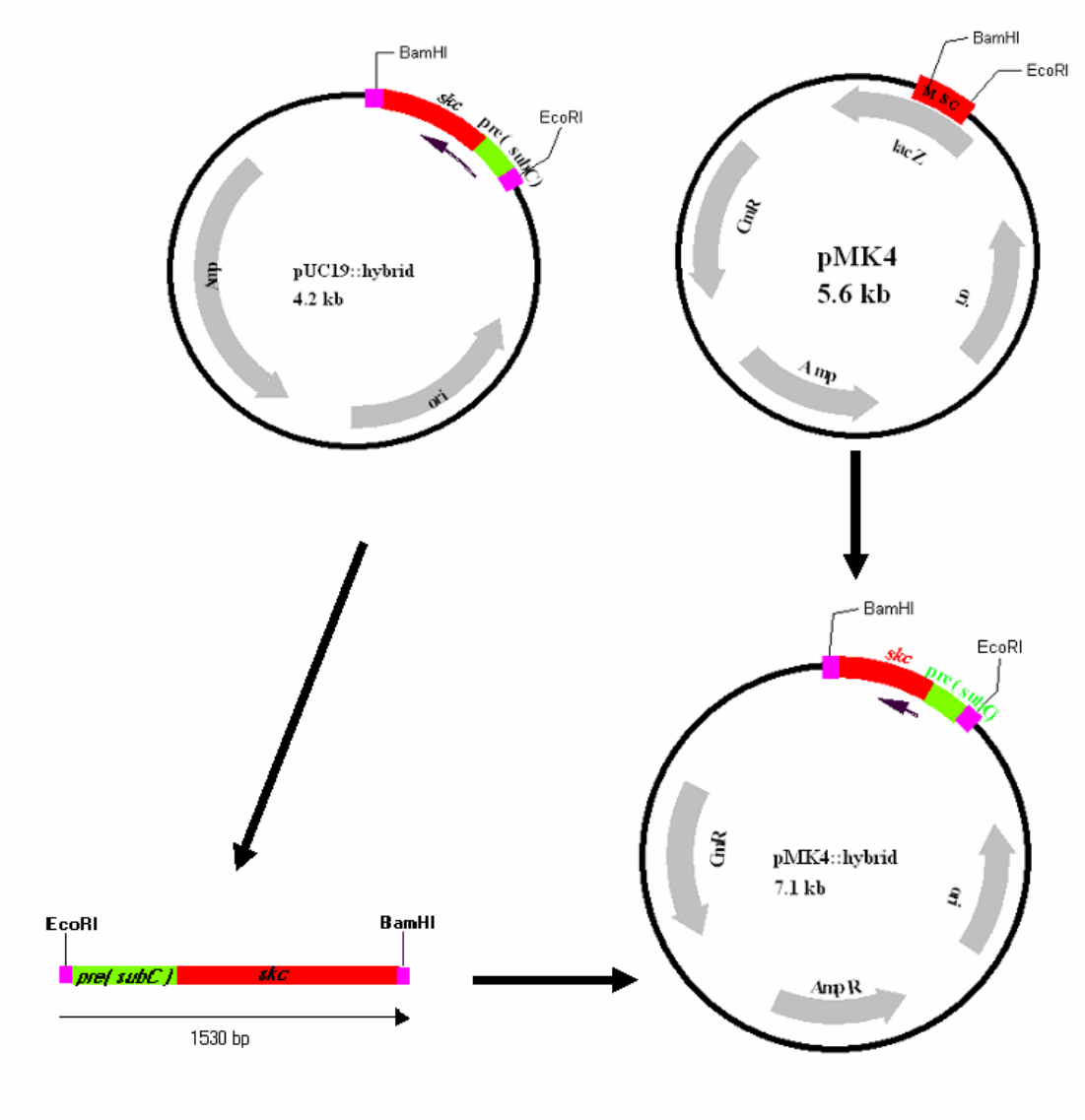

Figure 4.5 Schematic representation of the construction of recombinant pMK4::hybrid vector. 1530 bp DNA fragment of the hybrid gene, *pre(sub*C*)*::*skc*, having *Eco*RI and *Bam*HI endings was cloned into the vector pMK4 from *Eco*RI and *Bam*HI restriction sites. The insertion and transcription directions were demonstrated by arrows.

The isolated putative recombinant pMK4::*pre(sub*C*)*::*skc* plasmids were digested with *Eco*RI and *Bam*HI REs in order to confirm the integration of hybrid gene into the cloning vector pMK4. After this RE digestion, a gene portion having the same length of the hybrid gene was observed as verified by the agarose gel electrophoresis results demonstrated in Figure 4.7.

The DNA sequencing was performed by automatic DNA sequencers (Microsynth GmbH, Switzerland) using the appropriate pre-designed primers proving that the cloning was successful.

The recombinant pMK4::*pre(sub*C*)*::*skc* plasmids were further transformed into *Bacillus* species of *B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) by electroporation at 2.5 kV. Transformants were spread on LB-agar medium supplemented with chloramphenicol (7µg/ml LB from 35 mg/ml stock), and incubated at 37^{o}C for 12-18 h. Putative recombinant colonies were selected and further incubated overnight in LB-broth medium supplemented with chloramphenicol (7µg/ml LB from 35 mg/ml stock) at 37^{o}C and 200 rpm for plasmid DNA isolation.

The isolated putative recombinant pMK4::*pre(sub*C*)*::*skc* plasmids were subjected to PCR by using the forward and reverse primers of the hybrid gene in order to confirm the integration of hybrid gene into the cloning vector pMK4. After this PCR, a gene portion having the same length of the hybrid gene was observed as verified by the agarose gel electrophoresis results demonstrated in Figures 4.8 and 4.9.

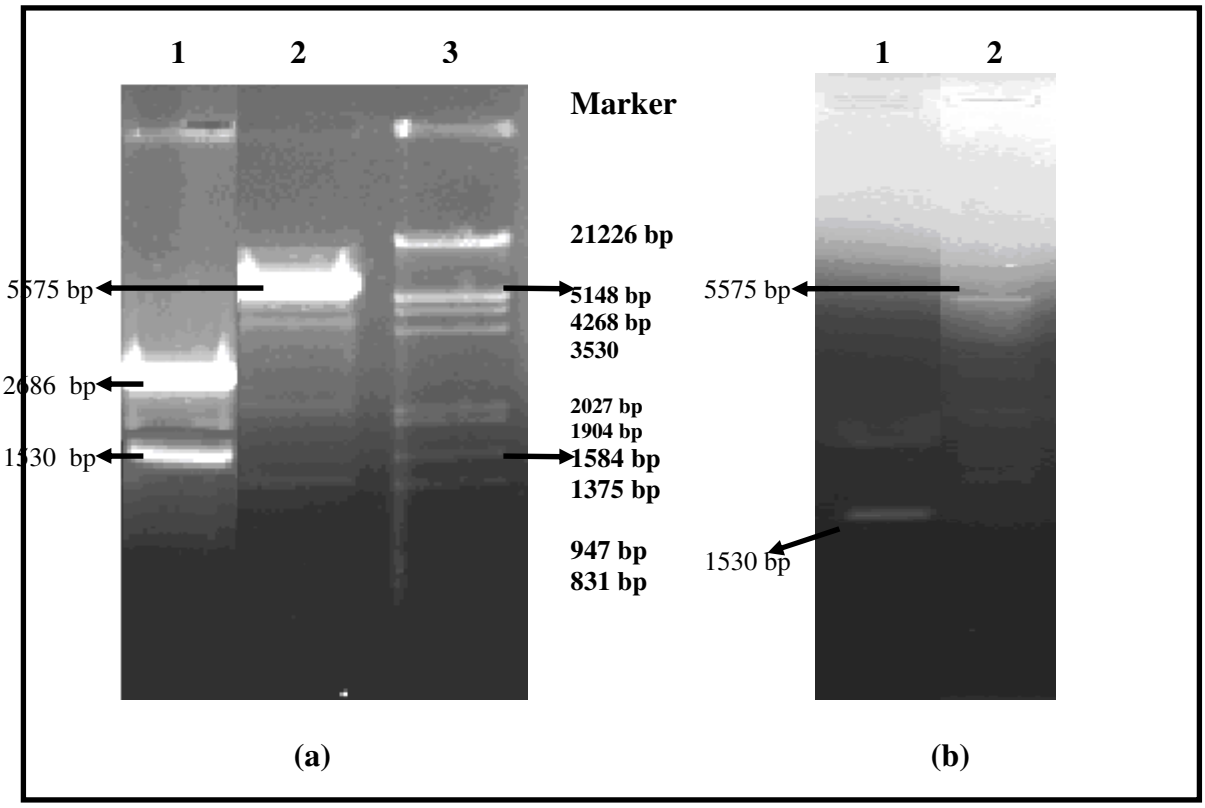

Figure 4.6 Agarose gel electrophoresis images of digested r-pUC19 and pMK4 plasmids before and after purification **(a)** Lane 1: r-pUC19 vector cut with *Eco*RI and *Bam*HI produced 2686 bp (pUC19) linear fragment and 1530 bp (*pre(sub*C*)*::*skc*) fragment; Lane 2: pMK4 plasmid cut with *Eco*RI and *Bam*HI excised 5575 bp (pMK4) fragment ; Lane 3: λDNA/*Eco*RI+*Hind*III molecular size marker **(b)** Lane1: Purified *pre(sub*C*)*::*skc* hybrid gene from agarose gel (a); Lane 2: Purified pMK4 plasmid from agarose gel (a)

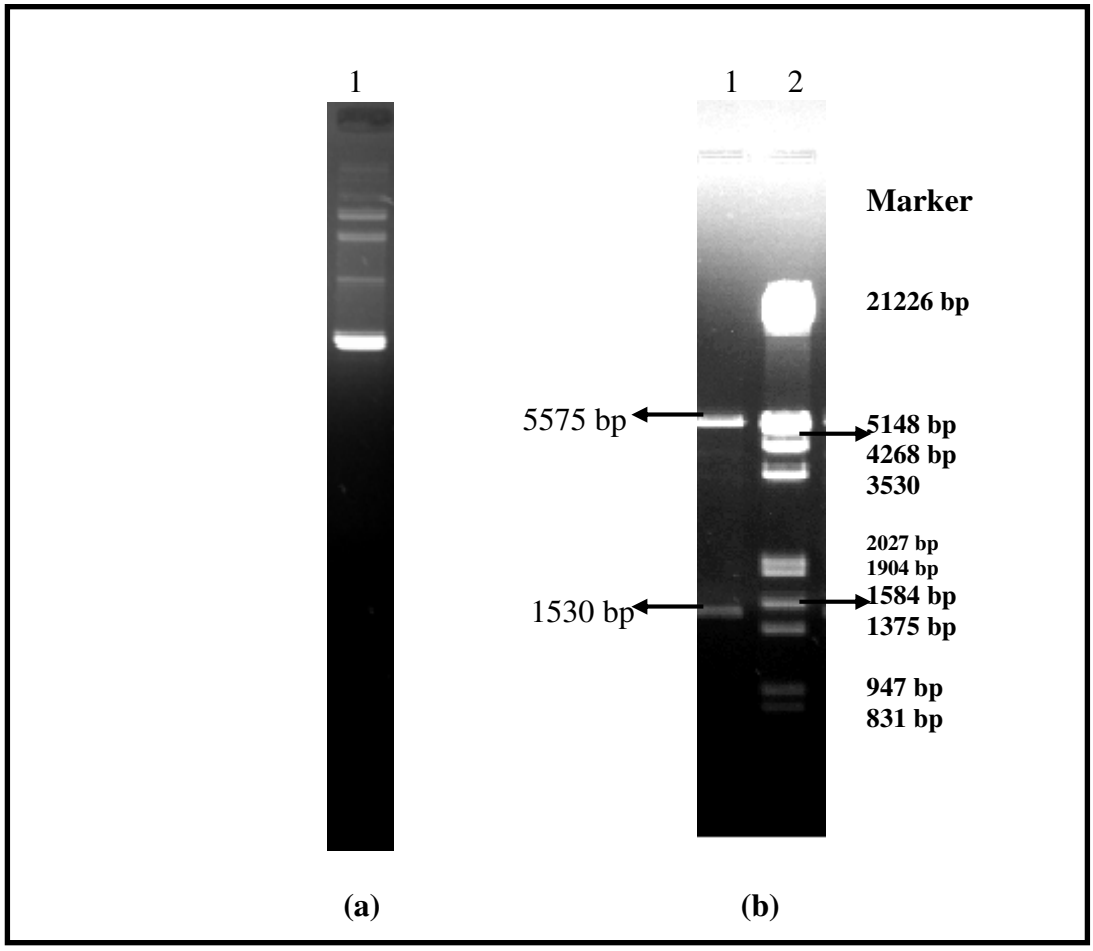

Figure 4.7 Agarose gel electrophoresis image of the RE digestion analysis result of the putative recombinant pMK4 plasmid. **(a)** Lane 1: Isolated r-pMK4 plasmid; **(b)** Lane1: *Eco*RI/*Bam*HI double digestion of recombinant pMK4:: *pre(sub*C*)*::*skc* plasmid excised 5575 bp (pMK4) and 1530 bp (*pre(sub*C*)*::*skc*) fragments; Lane 2: λDNA/*Eco*RI+*Hind*III molecular size marker

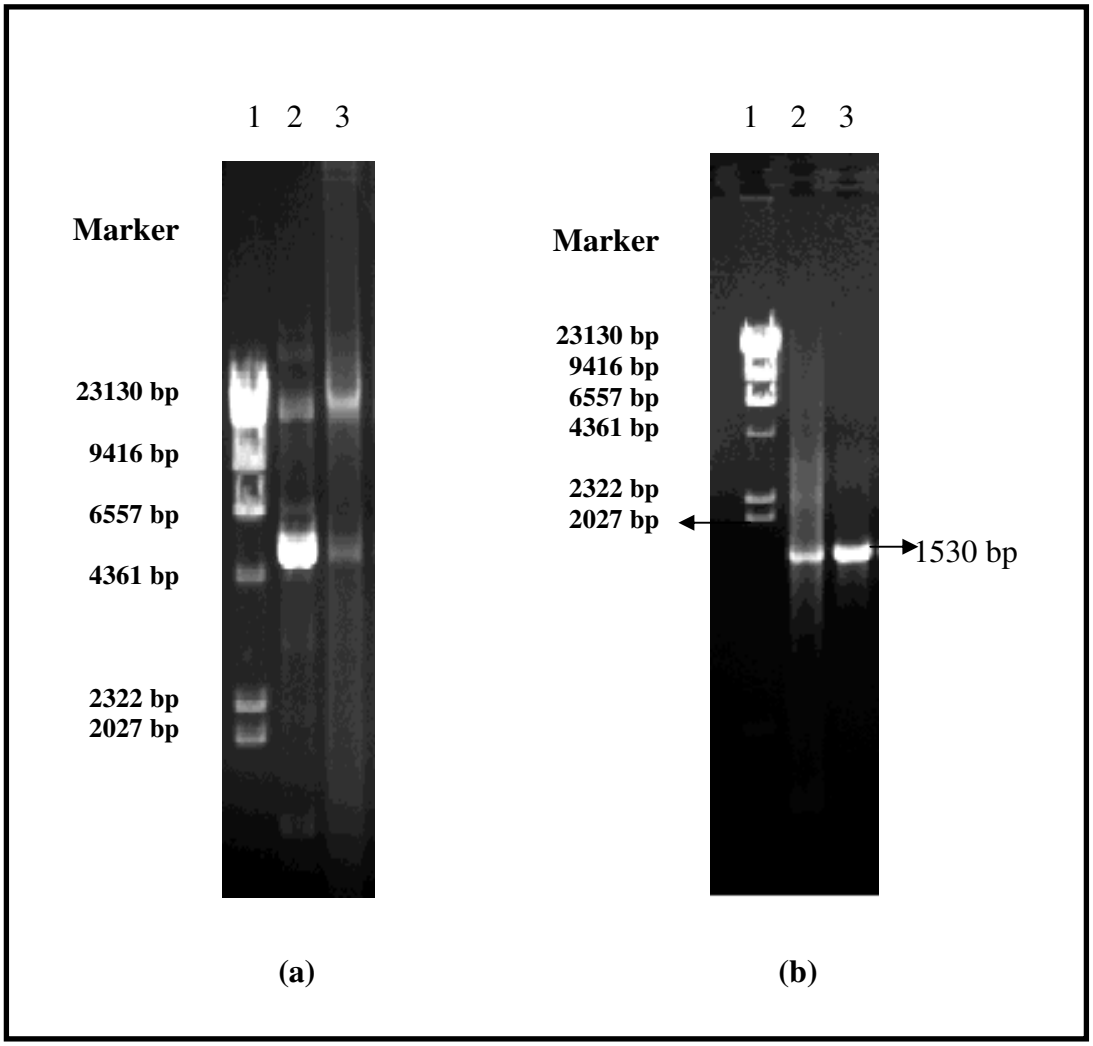

Figure 4.8 Agarose gel electrophoresis image of the plasmid isolation and control PCR results for r-*B. subtilis* (*npr- apr-).* **(a)** Lane 1: λDNA/*Hind*III marker; Lane 2: Isolated r-pMK4 plasmid from *E. coli* TG1 strain; Lane 3: Isolated r-pMK4 plasmid from putative r-*B. subtilis* (*npr- apr-)* strain. **(b)** Lane 1: λDNA/*Hind*III marker; Lane 2: Hybrid gene amplified with *Taq* DNA polymerase; Lane 3: 1530 bp (*pre(sub*C*)*::*skc*) fragment obtained from the control PCR of isolated r-pMK4 plasmid

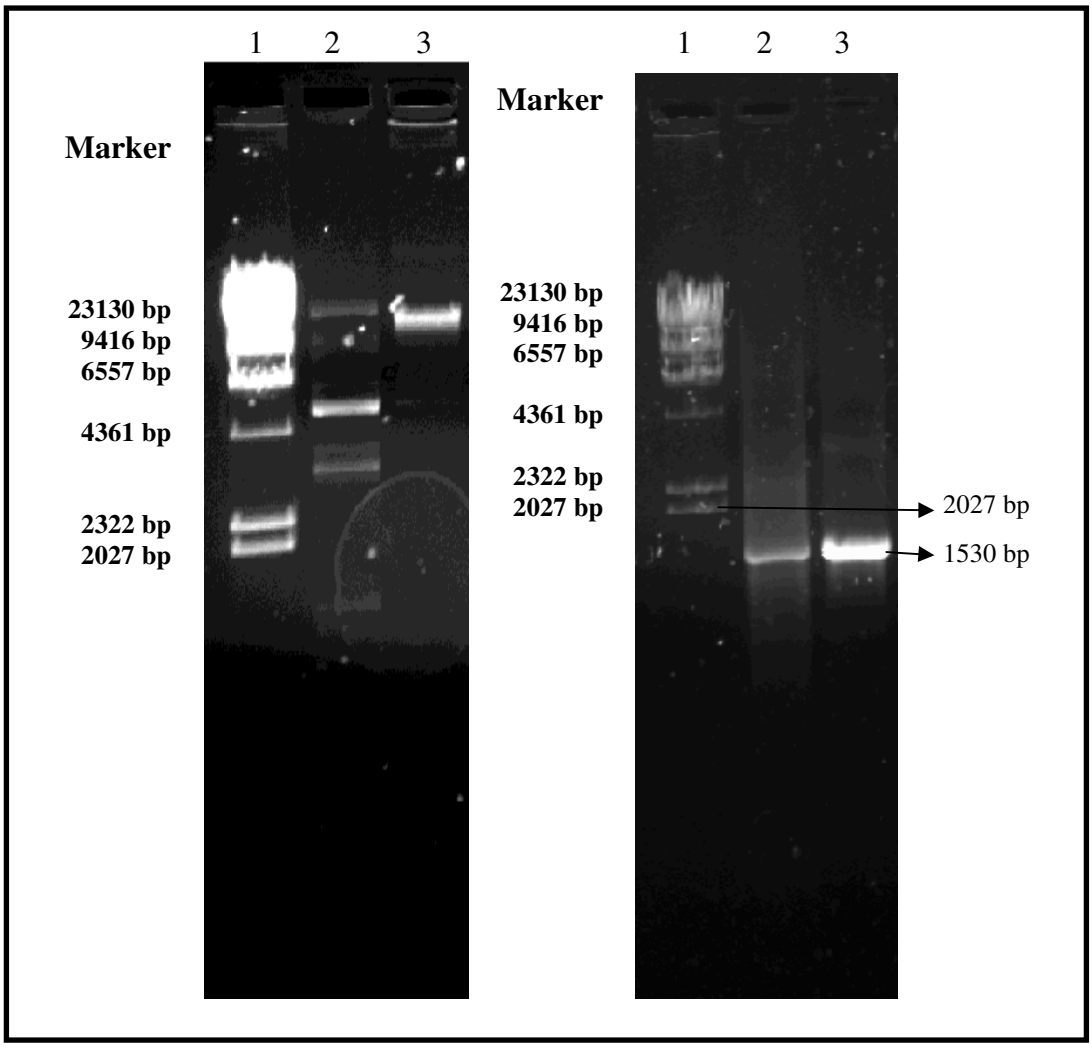

Figure 4.9 Agarose gel electrophoresis image of the plasmid isolation and control PCR results for r-*B. licheniformis* 749/C (ATCC 25972). **(a)** Lane 1: λDNA/*Hind*III marker; Lane 2: Isolated r-pMK4 plasmid from recombinant *E. coli* TG1 strain; Lane 3: Isolated r-pMK4 plasmid from putative *B. licheniformis* 749/C (ATCC 25972) strain. **(b)** Lane 1: λDNA/*Hind*III marker; Lane 2: Hybrid gene amplified with *Taq* DNA polymerase; Lane 3: 1530 bp (*pre(sub*C*)*::*skc*) fragment obtained from the control PCR of isolated r-pMK4 plasmid

4.2 Enzyme Production and Activity Analysis

Two recombinant species: *B. licheniformis* 749/C (ATCC 25972) and *B. subtilis* (*npr- apr-)* were firstly compared in terms of streptokinase production capacities. Supernatants of both species were harvested at different time points from the reference production medium (section 3.5) at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}. The samples were analyzed by High Performance Capillary Electrophoresis System. As a result, it was observed that r-*B. subtilis* (*npr- apr-)* did not produce any streptokinase; however it was seen that r-*B. licheniformis* 749/C (ATCC 25972) produced streptokinase accordingly giving a maximum at t=22 h.

4.2.1 SDS-PAGE Analysis of Recombinant Streptokinase Product

The extracellular streptokinase produced by the recombinant *B. licheniformis* 749/C (ATCC 25972) harvested at t=22 h from the reference production medium (section 3.5) at conditions: V=33 cm^3, T=37°C, N=200 min^{-1} was analyzed by SDS-PAGE after concentrating the supernatant through the spin column.

Electrophoresis was carried out in polyacrylamide gel by treating the samples with a reducing agent called SDS followed by heat exposure. A commercial drug Streptase was used as the standard streptokinase puchased from ZLB Behring. This commercial drug has BSA and glutamic acid in its structure as stated in its prospectus in addition to pure streptokinase as verified through the analyses carried out in Capillary Electrophoresis system. After the electrophoresis, separated proteins were stained with silver salts. As can be seen in Figure 4.10, the standard streptokinase sample gave 2 bands having 47 and 43 kDa molecular weights implying that the streptokinase was found in 2 active forms: 47 kDa intact mature streptokinase and 43 kDa degradation product that is also mentioned in Wong et al., (1993) and Wu et al., (1998). SDS-PAGE results illustrated that streptokinase enzyme was cloned into *B. licheniformis* and the recombinant *B. licheniformis* produced the enzyme extracellularly.

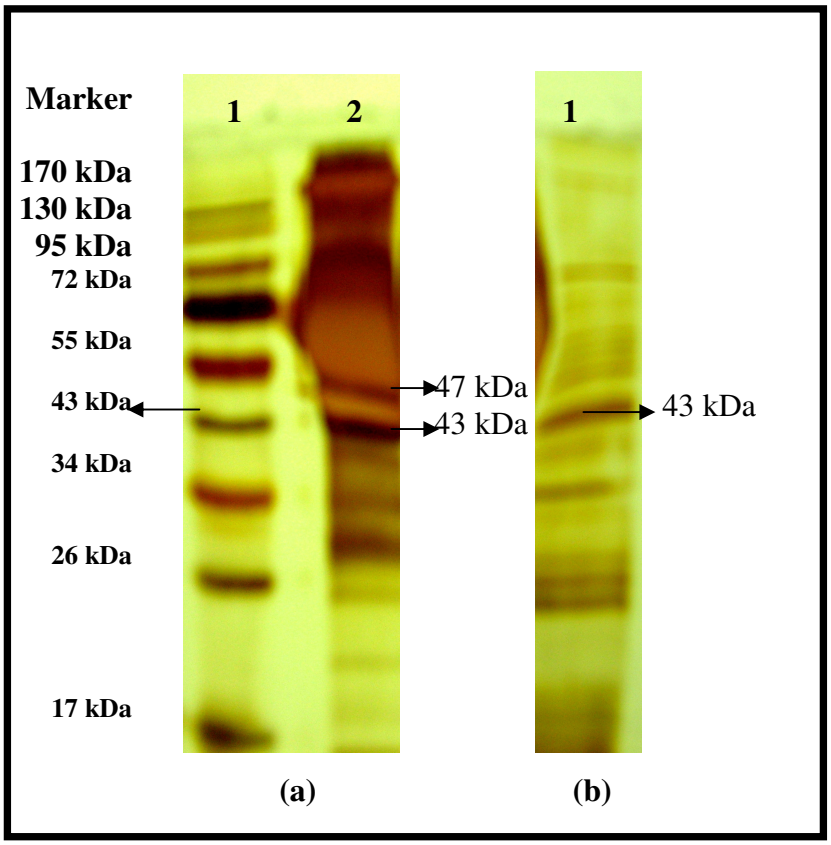

Figure 4.10 SDS-PAGE image of putative recombinant streptokinase product of the recombinant *B. licheniformis* 749/C (ATCC 25972) harvested at t=22 h from the reference production medium (section 3.5) at conditions: V=33 cm^3, T=37°C, N=200 min^{-1} **(a)** Lane 1: Prestained protein ladder; Lane 2: Standard streptokinase solution containing BSA and glutamic acid; **(b)** Lane 1: Concentrated supernatant

4.2.2 Dot Blot Analysis of Transformed *B. licheniformis* colonies

19 transformed recombinant *B. licheniformis* colonies grown in LB medium were applied to dot blot analysis with rabbit anti-streptokinase polyclonal Ab in order to determine the colony producing more streptokinase. As can be deduced from Figure 4.11, the 10th colony was observed to produce streptokinase more efficiently in LB-medium.

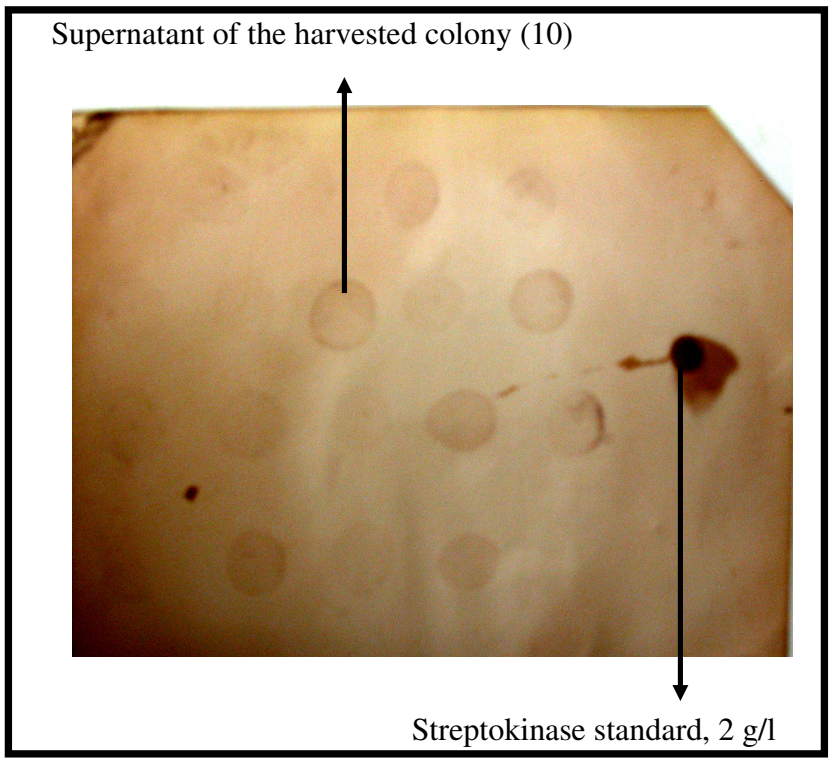

Figure 4.11 Dot blot image of recombinant *B. licheniformis* colonies

4.2.3 Response Surface Analysis

A face-centered, central composite design with two factors was used in the medium design process (Table 4.8). The main advantage of this method is that it is possible to investigate the effects of two variables at the same time in a single experiment performed by growing the selected colony of *B. licheniformis* in laboratory scale bioreactors at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, and harvesting the recombinant cells at t=22 h from each medium.

4.2.3.1 Preliminary Experiments (Selection of factor levels)

The individual effect of each parameter on recombinant streptokinase production was investigated in order to narrow down the range of values of each factor before an experimental design with RSM was applied to the system. The experiments were performed in laboratory scale bioreactors at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}.

Firstly, the effects of three glucose concentration values of 5, 7 and 10 kgm^{-3} (İleri, 2005) were investigated on recombinant streptokinase production in *B. licheniformis* 749/C (ATCC 25972) at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}. Supernatants of the harvested recombinant cells at t=5, 7, 18, 22, 26 h from three different production media where $(NH_4)_2HPO_4$ concentration was 7.1 kgm^{-3} were applied to High Performance Capillary Electrophoresis System. Consequently, no streptokinase peak was observed in the samples collected from the production medium having a glucose concentration of 10 kgm^{-3}. Moreover, the highest peak for streptokinase was observed for the medium having a glucose concentration of 5 kgm^{-3} at t=22 h.

Then, the effects of three glucose concentration values of 3, 5 and 7 kgm^{-3} were investigated on recombinant streptokinase production in *B. licheniformis* 749/C

(ATCC 25972) at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}. Supernatants of the harvested recombinant cells at t= 7, 11, 15, 18, 21, 22, 25 h from three different production media where $(NH_4)_2HPO_4$ concentration was 7.1 kgm^{-3} were applied to High Performance Capillary Electrophoresis System. Consequently, the highest peaks for streptokinase were observed for the medium having a glucose concentration of 5 kgm^{-3} at each time point among which at t= 22h the highest peak for streptokinase was seen.

Finally, the upper and lower limits of production medium components were determined. The lower limit for glucose concentration was decided to be 2 kgm^{-3} and the upper limit was 6.5 kgm^{-3}. By keeping the C/N values constant as 2.17 (İleri, 2005), the lower limit for $(NH_4)_2HPO_4$ concentration was calculated as 2.03 kgm^{-3} and the upper limit as 6.59 kgm^{-3}.

4.2.3.2 Response Surface Analysis Results

A face-centered, central composite design with two factors (glucose and $(NH_4)_2HPO_4$ concentrations) was used in the medium design process. Table 4.8 is the output of a computer program ANOVA. The lower limit for glucose concentration was 2 kgm^{-3} and the upper limit was 6.5 kgm^{-3}; the lower limit for $(NH_4)_2HPO_4$ concentration was 2.03 kgm^{-3} and the upper limit was 6.59 kgm^{-3}. When these values were input to the program, it gave 13 experimental data points. The main advantage of this method is that it is possible to investigate the effects of these two variables at the same time in a single experiment performed by growing the selected colony of *B. licheniformis* in laboratory scale bioreactors at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, and harvesting the recombinant cells at t=22 h from each medium.

Table 4.8 Face centered cube design arrangement and responses

RUNS	INDEPEPENDENT VARIABLES		RESPONSE
	C_G kgm^{-3}	$C_{(NH4)2HPO4}$ kgm^{-3}	C_{SK} kgm^{-3}
1	2.00	2.03	0.00200
2	6.50	2.03	0.00733
3	2.00	6.59	0.00400
4	6.50	6.59	0.01460
5	2.00	4.31	0.02130
6	6.50	4.31	0.01070
7	4.25	2.03	0.01020
8	4.25	6.59	0.02000
9	4.25	4.31	0.02330
10	4.25	4.31	0.02090
11	3.00	3.04	0.01220
12	5.00	5.07	0.03030
13	4.25	4.31	0.02090

13 experimental data points were obtained for recombinant streptokinase production in order to perform a statistical analysis for optimizing the medium components. The design matrix and the fitness of the each term were analyzed by means of ANOVA (Table 4.9). The regression coefficients for each term are demonstrated in Table 4.10. The resulting second-order polynomial equation for the recombinant streptokinase production was found to be:

$$C_{SK}\,(kgm^{-3}) = -0.04020 + 0.01243C_G + 0.01478\,C_{(NH4)2HPO4} - 0.00150\,C_G^{\,2} - 0.00164\,C_{(NH4)2HPO4}^{\,2} + 0.00024\,C_G\,C_{(NH4)2HPO4} \quad (4.1)$$

Table 4.9 Analysis of Variance (ANOVA) for the recombinant streptokinase production

	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F	p < 0.05
Regression	5	0.000635	0.000127	4.44	0.038
Linear	2	0.000335	0.000167	5.85	0.032
Square	2	0.000503	0.000252	8.80	0.012
Interaction	1	0.000006	0.000006	0.22	0.652
Residual Error	7	0.000200	0.000029		
Lack-of-Fit	5	0.000196	0.000039	20.46	0.047
Pure Error	2	0.000004	0.000002		
Total	12				

Table 4.10 Regression coefficients of predicted polynomial model

Term	Coefficient	t	p < 0.05
Constant	-0.04020	-2.482	0.042
Linear			
C_G (kgm^{-3})	0.01243	2.050	0.080
$C_{(NH4)2HPO4}$ (kgm^{-3})	0.01478	2.468	0.043
Quadratic			
C_G (kgm^{-3})* C_G (kgm^{-3})	-0.00150	-2.310	0.054
$C_{(NH4)2HPO4}$ (kgm^{-3})*$C_{(NH4)2HPO4}$(kgm^{-3})	-0.00164	-2.584	0.036
Interactions			
C_G (kgm^{-3}) * $C_{(NH4)2HPO4}$ (kgm^{-3})	0.00024	0.471	0.652

The plot of experimental values of recombinant streptokinase concentration versus those calculated from the equation (4.1) indicated a considerable fit with a correlation coefficient of 0.91 as demonstrated in Figure 4.12.

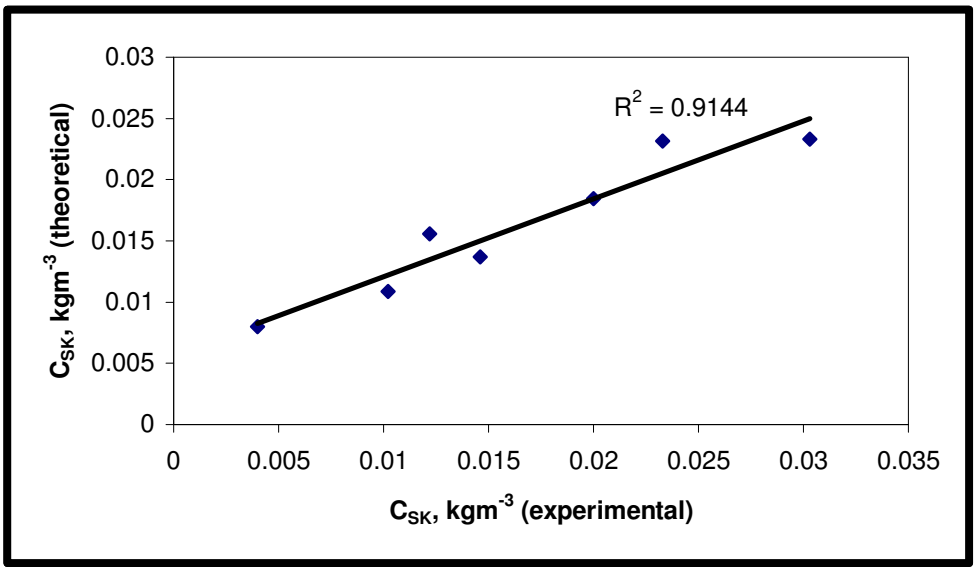

Figure 4.12 Correlation of experimental values of recombinant streptokinase concentration versus calculated ones.

Response surface and contour plots (Figure 4.13 and 4.14) show the effects of the independent variables on recombinant streptokinase production.

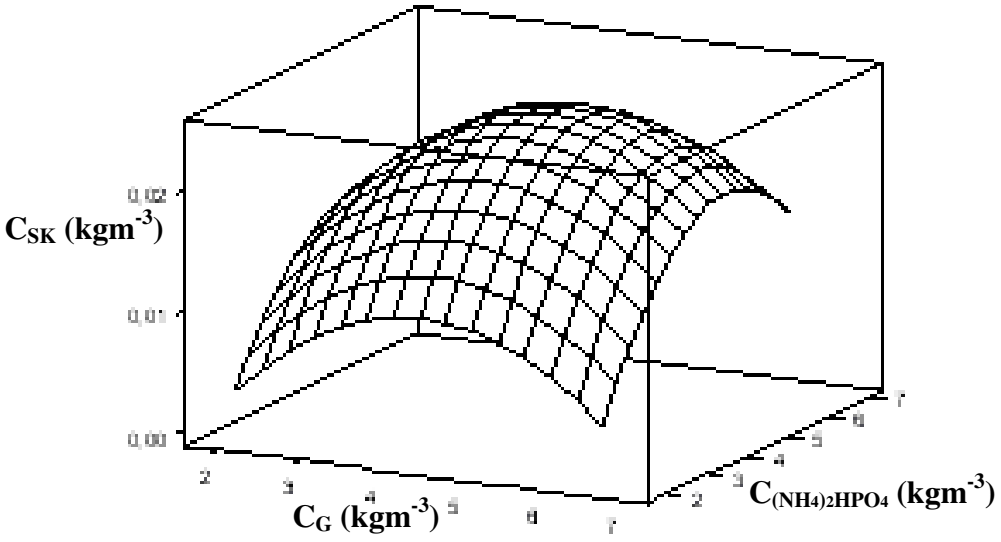

Figure 4.13 Response surface plot for carbon and nitrogen source interaction for recombinant *B. licheniformis* 749/C (ATCC 25972) harvested at t=22 h at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}.

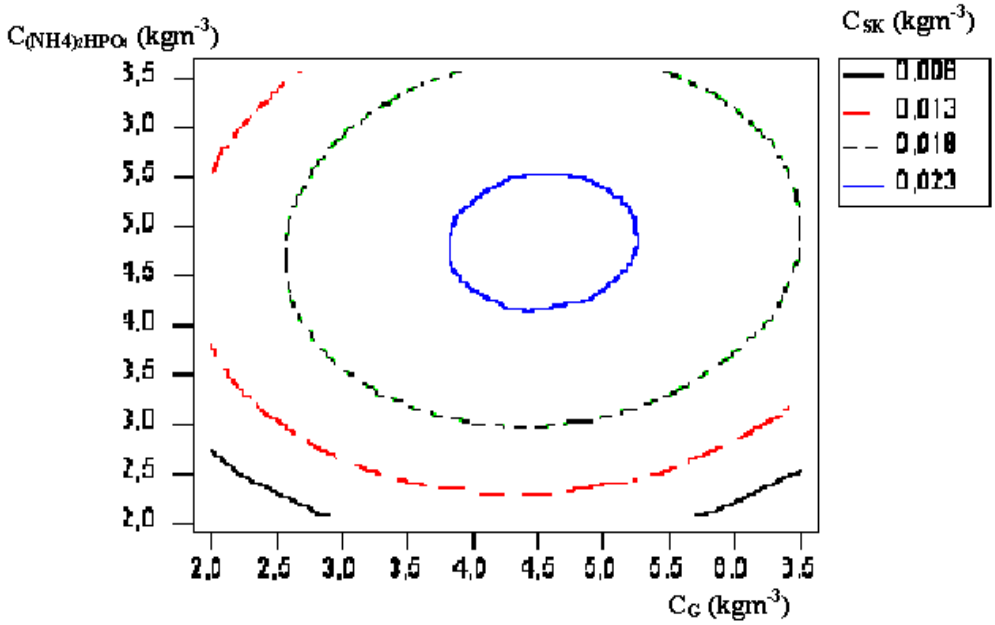

Figure 4.14 Counter plot for carbon and nitrogen source interaction for recombinant *B. licheniformis* 749/C (ATCC 25972) harvested at t=22 h at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}.

4.2.4 Optimized medium

The optimized carbon and nitrogen source concentrations were calculated by using the second-order polynomial equation (4.1). After performing the calculations, the resulting optimized theoretical recombinant streptokinase concentration was found as 0.0237 kgm^{-3} at glucose and $(NH_4)_2HPO_4$ concentrations of 4.530 and 4.838 kgm^{-3} respectively.

4.2.5 Enzyme Kinetics

Enzyme kinetics of the commercial standard streptokinase drug was studied in order to determine the minimum concentration of substrate to be used in the following activity analyses. The importance of this concentration is that, after that point the observed reaction rate catalyzed by the enzyme would be independent of the substrate concentration and only it depends on the initial enzyme concentration. As a result, observed activity of the enzyme can be correlated with its concentration. For this aim:

Firstly, the Michealis-Menten plot (Figure 4.15) for the commercial drug Streptase as the standard streptokinase puchased from ZLB Behring was plotted at T=37°C. It was concluded that after the substrate (a synthetic chromogenic substrate for plasmin, N-(p-tosyl)-glycyl-prolyl-lysine-4-nitroanilide acetate salt) concentration of 0.5 mM in 1 ml of assay solution, observed reaction rate did not depend on the substrate concentration. In the region where $C_{S0} \geq 0.5$ mM, observed reaction rate is only a function of enzyme production. Thus, in further enzyme activity experiments this substrate concentration was used in order to relate the reaction rate to directly the streptokinase concentration produced by recombinant *B. licheniformis.*

Further, Km and v_{max} values for streptokinase were evaluated using the commercial drug Streptase as the standard streptokinase puchased from ZLB Behring. In this procedure a synthetic chromogenic substrate for plasmin, N-(p-tosyl)-glycyl-

prolyl-lysine-4-nitroanilide acetate salt was used. From the Lineweaver-Burk plot (Figure 4.16), v_{max} and Km values were determined, at T=37°C, as $2.8x10^{-4}$ mMs^{-1} and 0.42 mM, respectively.

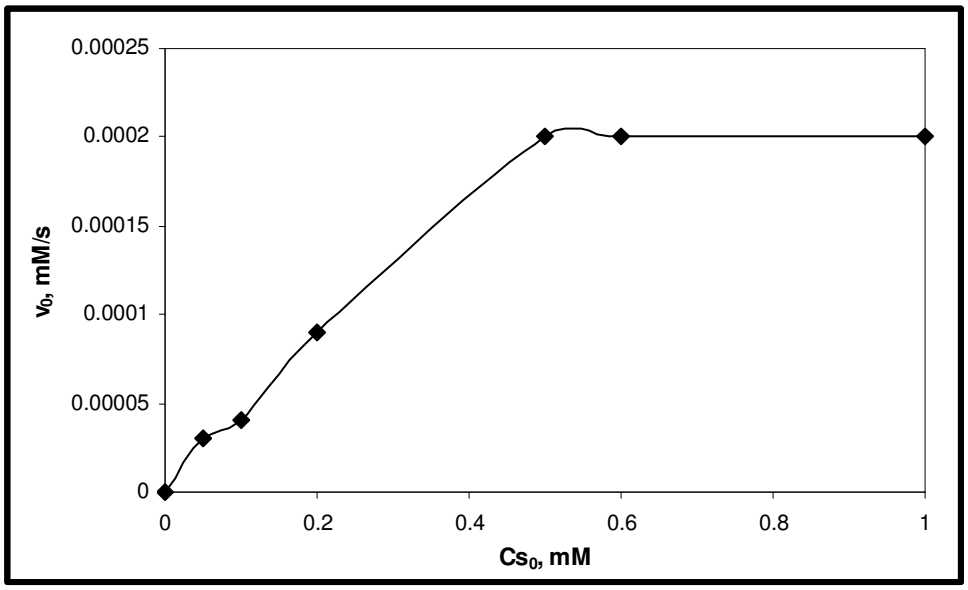

Figure 4.15 Michealis-Menten plot of standard streptokinase.

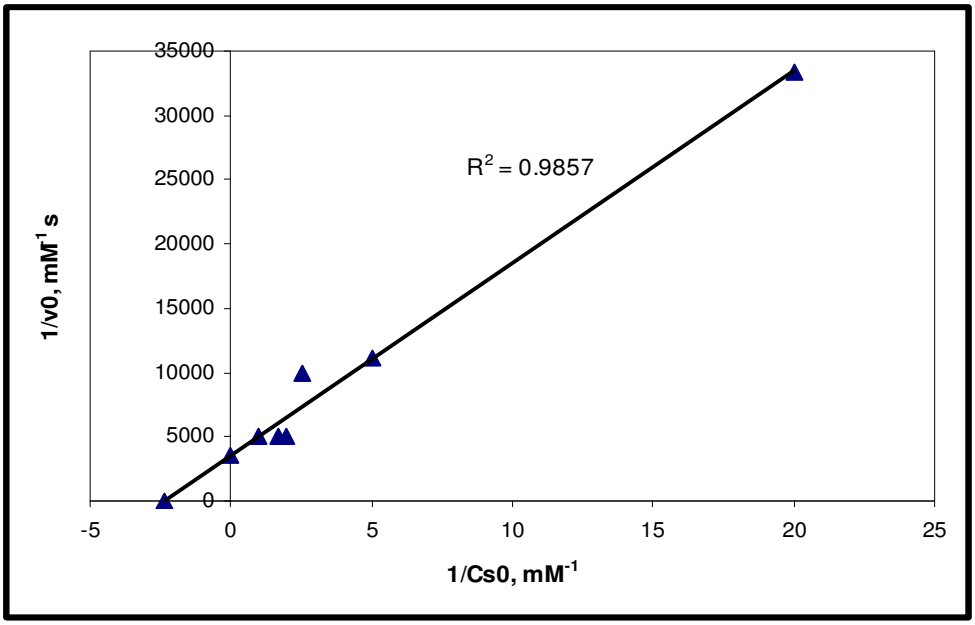

Figure 4.16 Lineweaver-Burk plot of standard streptokinase.

4.2.5.1 Correlation Between Enzyme Activity and Concentration

Using the standard streptokinase puchased as the drug Streptase from ZLB Behring, enzyme activity was determined in order to obtain a relationship between the streptokinase concentration and activity.

Volumetric activity for streptokinase is defined as plasmin units/ml of the sample solution. 0.7 g/l standard streptokinase solution was observed to give an activity of 206 PU/ml. The supernatant of the selected recombinant producer of streptokinase *B. licheniformis* colony grown in the pre-optimized medium in laboratory scale bioreactors at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, harvested at t=22 h of the bioprocess was subjected to Capillary Electrophoresis analysis. The resulting streptokinase concentration was determined as 0.0037 g/l. The volumetric activity of this supernatant sample was found as 1.62 PU/ml.

4.2.6 Dot Blot Analysis

Using the optimized medium, the selected recombinant producer of streptokinase *B. licheniformis* colony was grown in this pre-optimized production medium in laboratory scale bioreactors at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}. Then, supernatants of harvested colonies at different time points: 4h, 6h, 8h, 13h, 14h, 16h, 17h, 18h, 20h, 21h, 22h, 24h; were applied to dot blot analysis with rabbit anti-streptokinase polyclonal Ab in order to determine at which time point the selected colony produced the most streptokinase. At the end of the analysis, the brightest halo was observed at t=21 h of the bioprocess as can be seen in Figure 4.17.

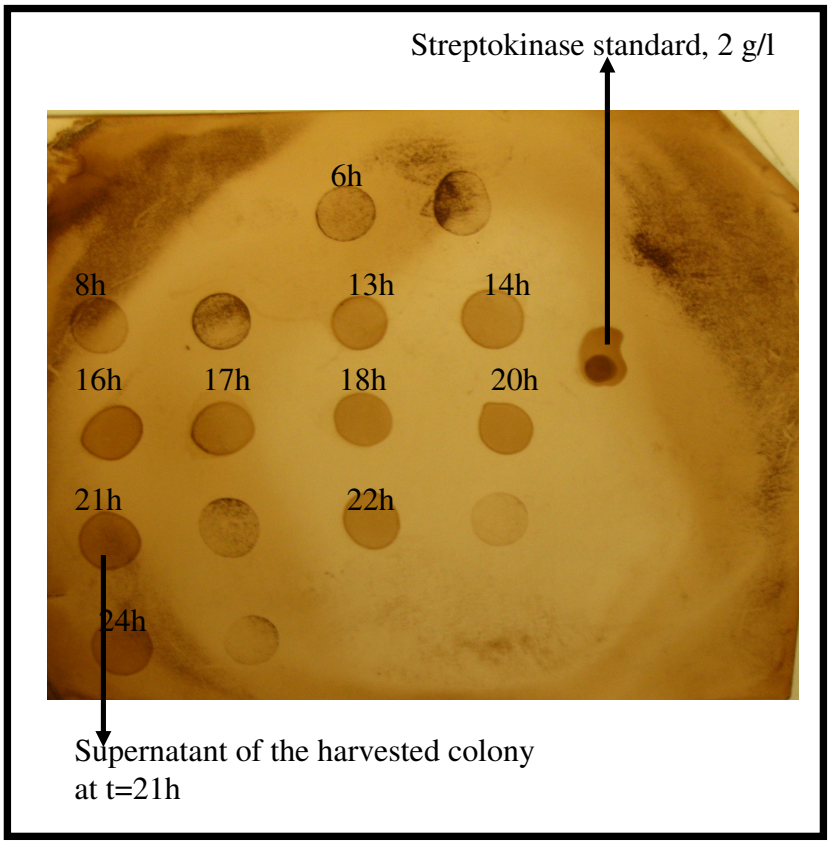

Figure 4.17 Dot blot image of recombinant *B. licheniformis* colony (10) harvested at different time points

4.2.7 The Pilot Scale Bioreactor Experiment

The fermentation and oxygen transfer characteristics of the streptokinase production were investigated in a 3 dm^3 pilot scale batch bioreactor (Braun CT2-2) equipped with temperature, pH, foam, air inlet and agitation rate controls having a working volume of V_R=1.65 dm^3 using the production medium optimized for the recombinant *B. lichenifomis* 749/C (ATCC 25972) strain. Streptokinase and β-lactamase activities, cell, glucose and organic acid concentrations, dissolved oxygen, ph, oxygen uptake rate, overall liquid phase mass transfer coefficient for oxygen, maintenance coefficient for oxygen, specific cell growth rate and yield coefficients were determined through the bioprocess. The bioprocess of recombinant

streptokinase production was performed at uncontrolled pH of these bioreactor operation conditions: air inlet rate of Q_0/V_R=0.5 vvm, and the agitation rate of N=400min^{-1}.

A laboratory scale bioreactor experiment was also conducted in parallel with the bioreactor at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}. Streptokinase and β-lactamase activity together with the cell concentration profiles were investigated throughout the bioprocess in order to compare the results with the bioreactor.

In literature, there is no study reporting the changes in pH and the dissolved oxygen profiles throughout the streptokinase production by any recombinant *Bacillus* sp. in a pilot scale bioreactor system. On the other hand, in the thesis study of İleri (2005), the effects of pH and different modes of feeding on β-lactamase production and the cell metabolism were investigated with *B. licheniformis* 749/C (ATCC 25972) strain. In the following sections, several comparisons were made with this study where these conditions: V_R= 1.65x10^{-3} dm^3, Qo/V_R=0.5 vvm, N=500 min^{-1}, had been utilized for the β-lactamase production in a medium of uncontrolled pH.

4.2.7.1 pH and Dissolved Oxygen Profiles

The variation of pH with the cultivation time is demonstrated in Figure 4.18. The initial pH of the production medium was pH_0=7.39. Throughout the bioprocess, pH decreased continuously to pH=6.86. Finally, pH of the culture medium reached a constant value at t=16 h, indicating that the proton transport of the cell stopped signalling the end of any bioproduct production process. A cell pumps H^+ ions in or out through the membrane under the influence of the operational conditions, resulting in a natural tendency in the change of pH of the environment during the bioprocess despite the fact that the cell's intracellular pH is kept constant (Çalık et al., 2003-c). For instance, the intracellular cytoplasmic pH of *B. licheniformis* is 7.5. This decrease could be an outcome of the consumption of the diammonium hydrogenphosphate

present in the optimized medium of recombinant *B. lichenifomis.* As ammonia was consumed, hydrogen ions were excreted into the culture medium, resulting in a decrease in pH of the medium (Schuler and Kargı, 2002). Also, amino acid and organic acid production throughout the bioprocess could contribute to this decrease.

Dissolved oxygen amount in the fermentation broth depends on the extent of the oxygen transfer rate to the media and the oxygen uptake rate by the cells. According to the growth status of the cells, it changes with the cultivation time. The variation of dissolved oxygen concentration with the cultivation time is shown in Figure 4.18.

Dissolved oxygen decreased to 21.7 % of saturation value (C_{DO}=0.0434 mol/m^3) due to the high oxygen demand at the beginning of the process till t=4 h. This sharp decrease was the result of a rapid dissolved oxygen uptake by the fast growing cells. Then, dissolved oxygen gradually increased and reached almost a stationary value at the end of the process. In the thesis study of İleri (2005), the effects of pH and different modes of feeding on β-lactamase production and the cell metabolism were investigated with *B. licheniformis* 749/C (ATCC 25972), and a similar dissolved oxygen profile was observed for an uncontrolled pH operation mode such that a decrease was reported at the initial hours of the bioprocess with 50-65% of the saturation value (C_{DO}=0.1-0.13 $molm^{-3}$) till the end of t=6h. Afterwards, the profile showed a gradual increase until the end of the bioprocess.

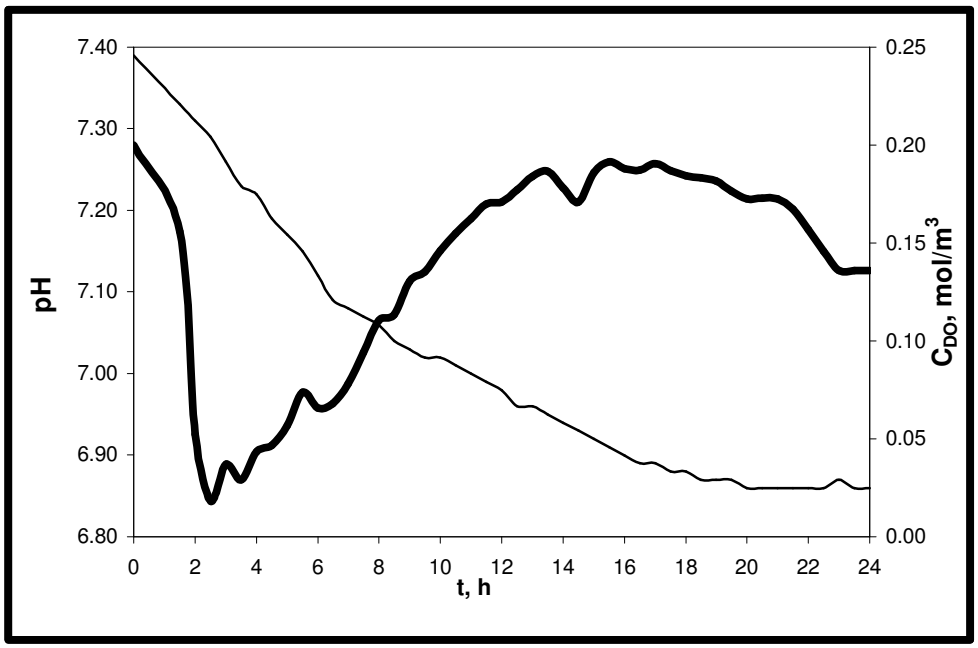

Figure 4.18 The variations of pH (thin curve) and dissolved oxygen concentration (thick curve) with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm, C_{DO}*=0.20 $molm^{-3}$.

4.2.7.2 Cell Growth and Glucose Concentration Profiles

The cell concentration variation profile with cultivation time is given in Figure 4.19. Between t = 0-8h of the bioprocess, the cell concentration was observed to increase continuously, and the highest value was reached at t = 8h which was the onset of stationary growth phase. The highest cell concentration was gathered at t = 8h as Cx = 0.52 kgm^{-3}. İleri reported that the cell concentration increased between t = 0-6 h reaching their stationary phase between t=6-8h with the highest cell concentration of Cx = 0.60 kgm^{-3}. This difference could be resulted from the fact that in this study, the *Bacillus* sp. used was recombinant and was carrying a plasmid. Moreover, the compositions of the growth media are different together with the bioreactor operation conditions applied.

For the laboratory scale bioreactor having these conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, the cell concentration change with cultivation time is shown in Figure 4.20. Between t = 0-6 h of the bioprocess, the cell concentration was observed to increase continuously, and the highest value was reached at t = 6h which was the onset of stationary growth phase. The highest cell concentration was gathered at t = 6h as Cx = 0.67 kgm^{-3}. The differences in cell concentration values between the bioreactor and shaker could have been resulted from the different bioreactor operation conditions and thus the different oxygen transfer characteristics.

The change in cell concentration with the cultivation time can be related to the dissolved oxygen concentration of the cultivation medium. The dissolved oxygen concentration decreases with the growth time, until the microorganism reaches the stationary phase after when the dissolved oxygen concentration starts to increase.

Between t=0-8 h of the bioprocess, the cell concentration increased up to Cx = 0.52 kgm^{-3}. Between t=0-8 h the dissolved oxygen concentration decreased from 79 % of the saturation value (C_{DO} = 0.16 $molm^{-3}$) to 21.7 % of saturation value (C_{DO} = 0.0434 $molm^{-3}$) due to the high oxygen demand at the beginning of the process till t=4 h. However, when cells reached their stationary growth phase, the dissolved oxygen concentration started to increase significantly showing that cells did not use oxygen as much as they consumed in their exponential growth phase.

Glucose concentration of the production medium was observed to decrease throughout the bioprocess from its initial value of C_G^0 = 4.53 kgm^{-3} as expected (Figure 4.19). This declining profile was also reported by İleri (2005). The highest glucose consumption rate was attained between t=0-4 h which is the period for early exponential phase where the cells require more energy for the continuation of their growth. In contrast, after t=18 h, glucose was consumed with a considerably slower rate, and finally reached a concentration of 0.1 kgm^{-3} in the fermentation medium indicating that almost all glucose was used up by the cells throughout the bioprocess.

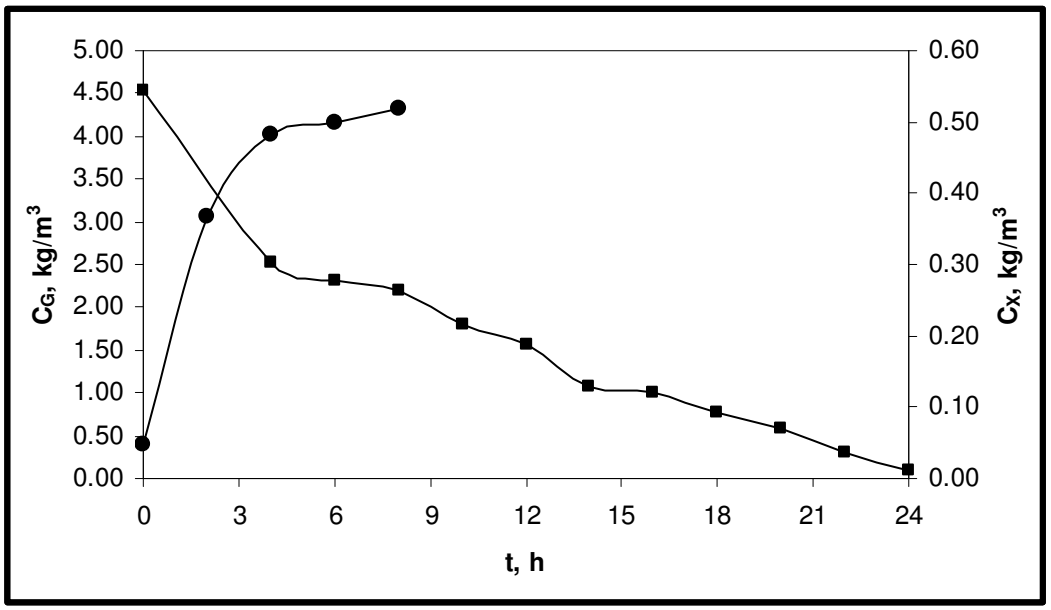

Figure 4.19 The variations of cell (●) and glucose (■) concentrations with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

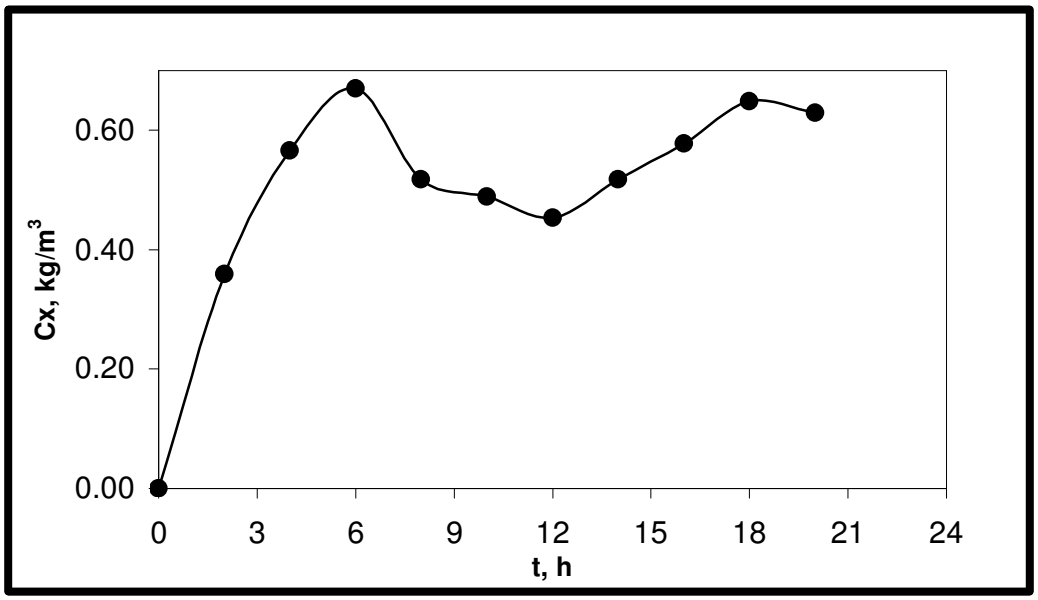

Figure 4.20 The variation of cell concentration with the cultivation time; T=37°C, N=200 min^{-1}, V=33 cm^3.

4.2.7.3 Streptokinase and β-lactamase Activity Profiles

Streptokinase and β-lactamase volumetric activity profiles throughout the cultivation time are demonstrated in Figure 4.21 for the bioreactor and in Figure 4.22 for the laboratory scale bioreactor.

Volumetric activity for streptokinase is defined as plasmin units/ml of the sample solution. No streptokinase volumetric activity was observed including t=8h, then it increased with the cultivation time until t=16 h. Meanwhile, β-lactamase volumetric activity was observed to increase steadily reaching the highest value of 134 Ucm^{-3} at t=16 h. In the thesis study of İleri (2005), it is stated that β-lactamase showed its maximum volumetric activity at t=14 h as 57 Ucm^{-3}. More than 2 fold increase in β-lactamase activity was obtained in the present study than the thesis study of İleri (2005). The variaties in bioreactor operation conditions together with the differences in two media components' concentrations could have lead to this result. Additionally, since in this study, *B. licheniformis* was in recombinant state, not native, this could have increased their production capacity of β-lactamase in that the recombinant plasmid pMK4::*pre(sub*C*)*::*skc* carries β-lactamase gene naturally. In the present study, after the time point t=16 h, activity of streptokinase started to increase after the stationary path it attained between t=12-16h. Most probably, since β-lactamase reached its highest concentration implying that other proteases were also higly expressed at this stage, streptokinase could have been degraded by these proteases leading to a stationary condition in its volumetric activity. Starting from t=16 h, while the β-lactamase volumetric activity followed a decreasing path, the streptokinase volumetric activity climbed to higher values reaching its maximum as 1.16 $PUml^{-1}$ (0.0026 g/l streptokinase) at t=20 h.

Among the reported studies investigating recombinant streptokinase prodyction, it can be stated that Wong et al., (1993) and Wu et al., (1998) use the same *Bacillus* sp., which is *B. subtilis* WB600 (6 extracellular protease deficient

strain) containing pre-constructed expression vectors. These species are cultivated in superrich medium, composed of 2.0% yeast extract, 2.5% tryptose, 3.0% K_2HPO_4 and 3.0% glucose, containing 10 μg of kanamycin per ml. Altough in both studies the same activity measurement method is employed with the same synthetic substrate as in the present study, only relative activities are presented in both studies as such: a 2.5 fold increase in the activity is reported for the extracellular production of streptokinase in *B. subtilis* WB600 (6 extracellular protease deficient strain) in Wong et al. (1993); and 2.2-2.5 fold increase in the activity is observed in Wu et al. (1998). Consequently, a comparison of activity values of the present work with the literature employing *Bacillus sp*. could not be performed.

In the laboratory scale bioreactor at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, no streptokinase volumetric activity was observed including t=8h likewise the bioreactor, then it increased with the cultivation time until t=12 h. Meanwhile, β-lactamase volumetric activity was observed to increase steadily reaching the highest value of 99 Ucm^{-3} at t=12 h. After the time point t=12 h, activity of streptokinase followed a declining path until t=16h. The similar trend was observed for the β-lactamase volumetric activity profile. Starting from t=16 h, both β-lactamase and streptokinase volumetric activities climbed to higher values. Streptokinase volumetric activity reached its maximum as 2.76 $PUml^{-1}$ (0.0063 g/l streptokinase) at t=22 h.

It can be concluded that, in the laboratory scale bioreactor, while the oxygen transfer conditions were more favourable for streptokinase production than in the bioreactor, they were less effective for β-lactamase production.

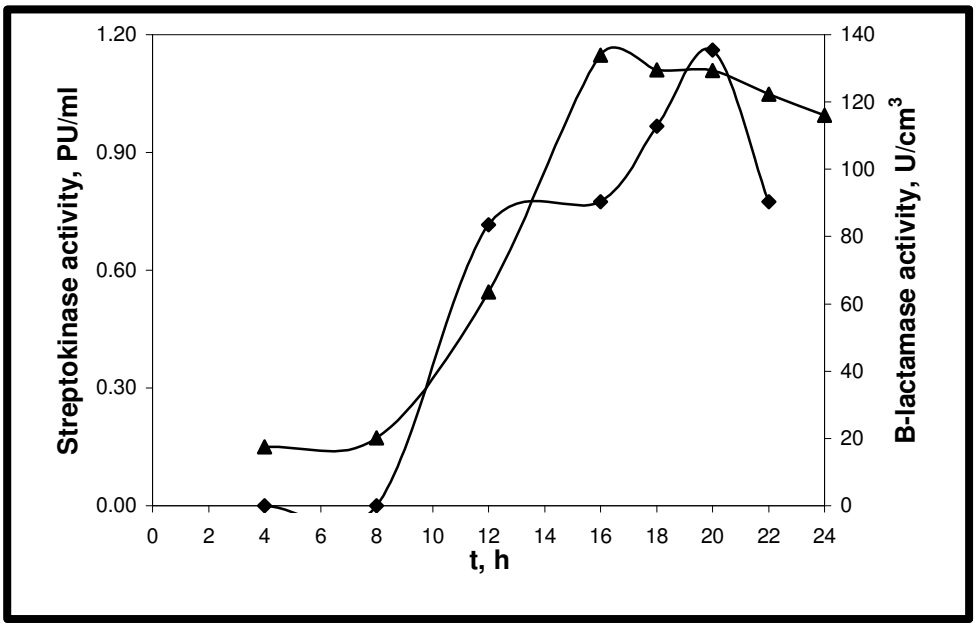

Figure 4.21 The variations in the streptokinase (■) and β-lactamase (▲) volumetric activities with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

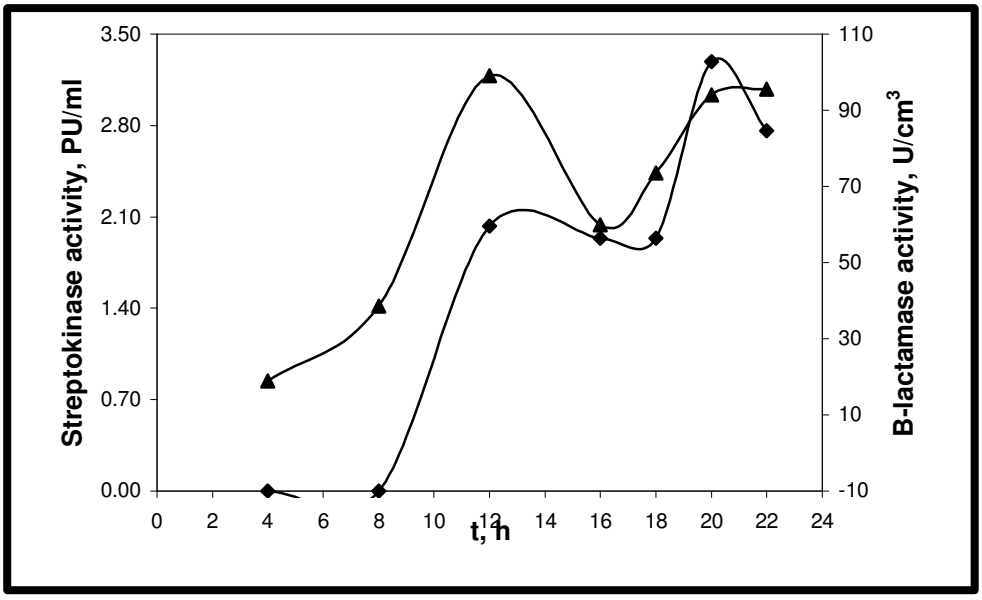

Figure 4.22 The variations in the streptokinase (■) and β-lactamase (▲) volumetric activities with the cultivation time; T=37°C, N=200 min^{-1}, V=33 cm^3.

4.2.7.4 Oxygen Transfer Characteristics

The Dynamic Method was used in finding the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r0, and oxygen transfer coefficient, K_La. At t<0 h, the physical oxygen transfer coefficient K_La0 was quantified as 0.00527 s^{-1} in the production medium in the absence of cells. The variations in K_La, the enhancement factor E (=K_La/K_La0), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor (η) throughout the bioprocesses are given in Table 4.11.

Volumetric mass transfer coefficient, K_La, is a physical parameter, depending on operational conditions such as geometrical characteristics of the bioreactor, properties of the cultivation medium and the presence of cells. Figure 4.23 shows the variation of K_La with cultivation time. K_La is a strong parameter affecting the oxygen transfer rate during an aerobic bioprocess. When K_La values were evaluated in terms of their change with respect to time, the highest K_La was determined as 0.02952 s^{-1}at t=2 h. E, the biological enhancement factor can be calculated by the ratio of experimental oxygen mass transfer coefficient in the presence of microorganisms and that measured in the absence of microorganisms. E values (Figure 4.24) were observed to decrease sharply between hours t=2-8 h and rather slowly between t=8-14 h taking values between 0.54-5.60 throughout the bioprocess. K_La and consequently the biological enhancement factor, E were higher in the early growth phase of the bioprocess than in the production phase.

In the study of İleri (2005), it is observed that K_La and consequently the enhancement factor E are higher in β-lactamase production phase than in growth phase of the bioprocesses, varing between 0.0100-0.0122 and 0.98-1.06, respectively. The reason for low E values in the growth phase was explained because of low actual C_{DO}* values compared to C_{DO}*= 0.20 $molm^{-3}$ assumption.

In addition, low enhancement factor, E, values imply that the mass transfer rate is much higher than the reaction rate for oxygen. It was observed that E values were higher in the early growth phase of the bioprocess than in the production phase implying that OUR by the cells was higher then intracellular biochemical reaction rates were much more higher in this stage. As E values started to decrease, it can be stated that the biochemical reactions started to slow down compared to the early growth phase.

As stated above, K_La depends on temperature, agitation rate, rheological properties of the fermentation medium and presence of fine particles in the mass transfer area. The observed increase in K_La, could be resulted from the decrease in viscosity of the cultivation medium due to metabolite secretion, as temperature and agitation rate were maintained constant throughout the bioproces (İleri, 2005). Similarly, the decrease in K_La values, could be resulted from the increase in viscosity of the cultivation medium due to metabolite utilization.

OUR is affected by the growth phases of the microorganisms. Table 4.11 and Figure 4.25 demonstrate the variation of OUR with cultivation time and cell concentration respectively. OUR increases during the lag phase and exponential phases of the microbial growth and takes a maximum at the very beginning of the exponential phase as it is the case in this analysis. OUR increased during the exponential growth phase of the microorganism, attained the maximum value for a cell concentration of 0.48 kgm^{-3} and afterwards remained constant during the stationary phase of the growth. The highest OUR was observed as 0.0006 $molm^{-3}s^{-1}$ at t=4 h of cell growth phase. After the cells reached the end of the stationary phase at t=10 h, OUR began to decrease taking 0.0005 mol $m^{-3}s^{-1}$. In İleri (2005), it is observed that OUR was higher in cell growth phase where the growth rates and metabolic activities of the cells are high rather than the enzyme production phase, and took its maximum value as 0.0009 mol $m^{-3}s^{-1}$. The variation of oxygen transfer rate (OTR) with cultivation time is given in Table 4.11. OTR, which is proportional to the

difference between the equilibrium concentration, C_{DO}^{*} and the dynamic dissolved oxygen concentration in the medium, took its highest value at the beginning of the bioprocess at t=2 h as 0,0044 $molm^{-3}s^{-1}$ and decreased to an extent depending on the oxygen uptake rate.

Damköhler (Da) number is defined in order to express the oxygen limitation in an aerobic bioprocess. Da is a dimensionless number, relating the maximum oxygen utilization and maximum oxygen transfer rates. At the beginning of the bioprocess, biochemical reaction limitations were effective (Da<1); however after t=2 h of the bioprocess, mass transfer resistances became more effective (Da>>1). Da versus the cell concentration profile for the bioprocess was demonstrated in Figure 4.26. For all operations of İleri (2005), mass transfer resistances were reported to be more effective throughout the bioprocesses.

The change in effectiveness factor along a bioprocess should be taken into consideration (Figure 4.27 and 4.28). At the beginning of the bioprocess, the effectiveness factor, η, took considerably higher values, indicating that the cells were consuming oxygen with a higher rate approaching the maximum oxygen uptake rate (OD). For both conditions, η decreased with the increase of Da. This reveals that there is a reverse proportionality between the effectiveness factor and the Damköhler number in a bioprocess. This approach can be easily seen in Figure 4.28.

The change of maximum oxygen uptake rate, oxygen demand (OD), with cultivation time is given in Table 4.11. As seen, OD increased with the increase in cell concentration implying that the maximum possible oxygen utilization rate (OD) depends on the level of microbial growth.

Table 4.11 The variations in oxygen transfer parameters with cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

t	K_La (s^{-1})	E K_La/K_La_0	OTRx10^3 ($molm^{-3}s^{-1}$)	OTR_{max}x10^3 ($molm^{-3}s^{-1}$)	OURx10^3 ($molm^{-3}s^{-1}$)	ODx10^3 ($molm^{-3}s^{-1}$)	Da OD/OTR_{max}	η OUR/OD
0			0.13	0.61	0.00			
2	0.02952	5.60	4.36	5.90	0.40	3.04	0.51	0.13
4	0.01647	3.13	2.58	3.29	0.60	19.64	5.96	0.03
6	0.01489	2.83	2.00	2.98	0.60	74.52	25.00	0.01
8	0.00303	0.57	0.27	0.61	0.60			
10	0.00352	0.67	0.19	0.70	0.60			
12	0.00309	0.59	0.09	0.62	0.50			
14	0.00595	1.13	0.13	1.19	0.50			
16	0.00562	1.07	0.07	1.12	0.40			
18	0.00522	0.99	0.08	1.04	0.50			
20	0.00286	0.54	0.08	0.57	0.60			
22	0.0062	1.18	0.27	1.24	0.70			
24	0.00355	0.67	0.23	0.71	0.90			

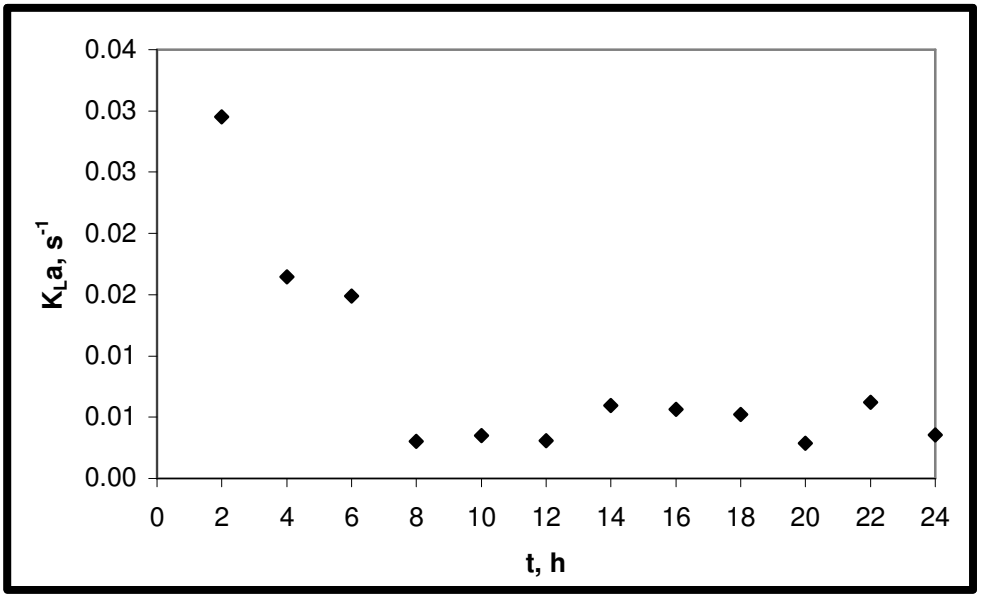

Figure 4.23 The variation of K_La with cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

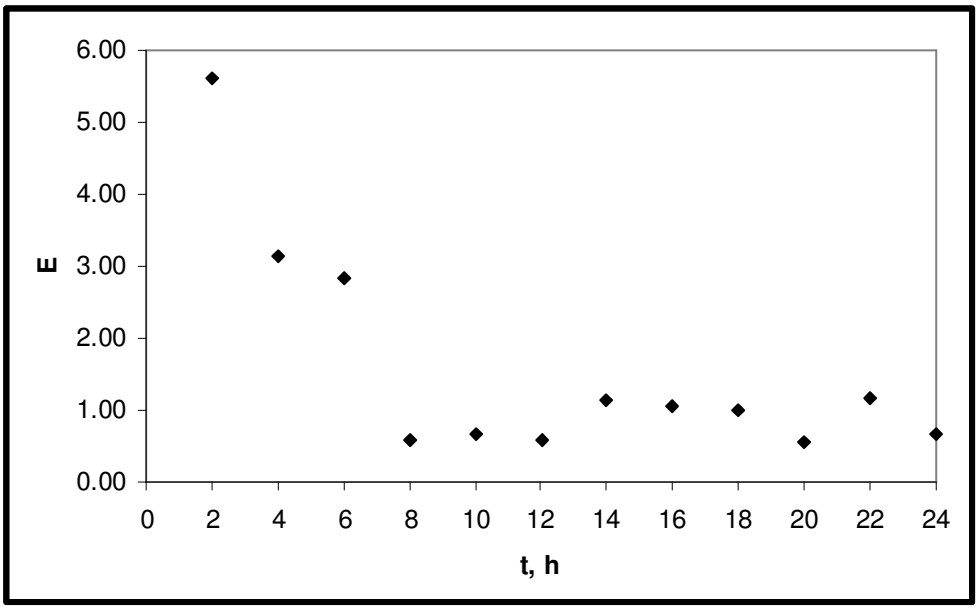

Figure 4.24 The variation of E with cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

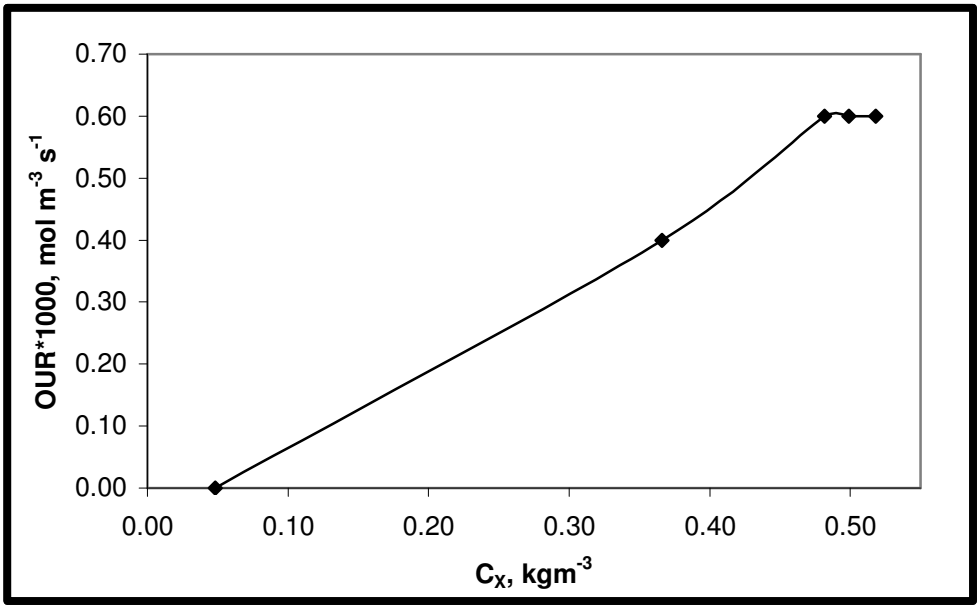

Figure 4.25 The variation of OUR with cell concentration; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

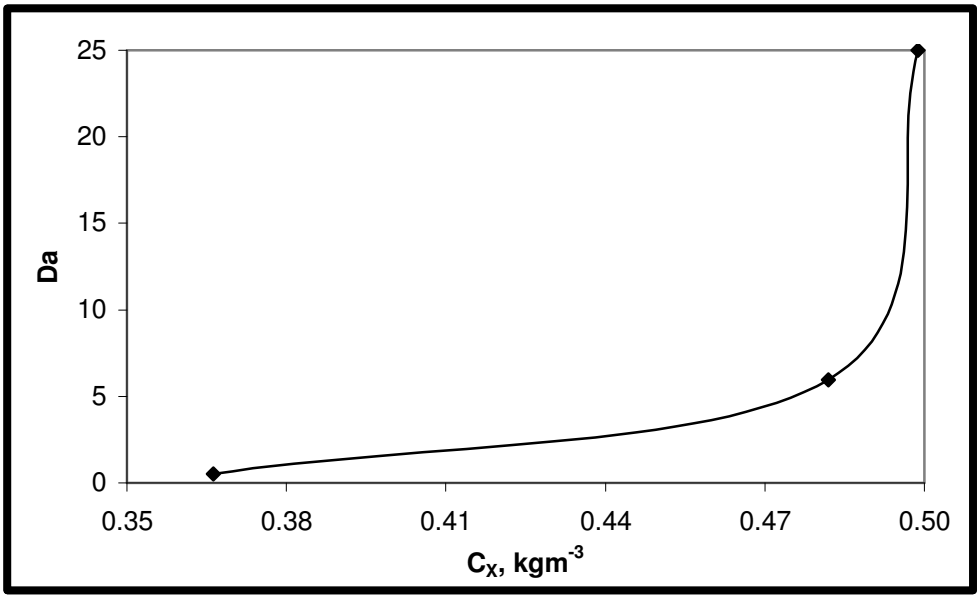

Figure 4.26 The variation of Da (OD/OTR_{max}) with cell concentration; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

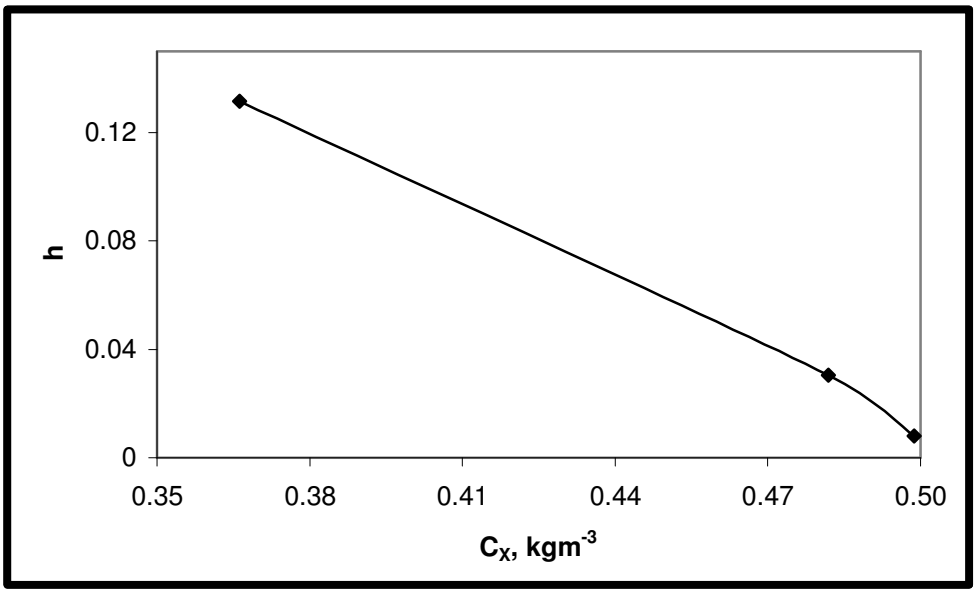

Figure 4.27 The variation of η (OUR/OD) with cell concentration; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

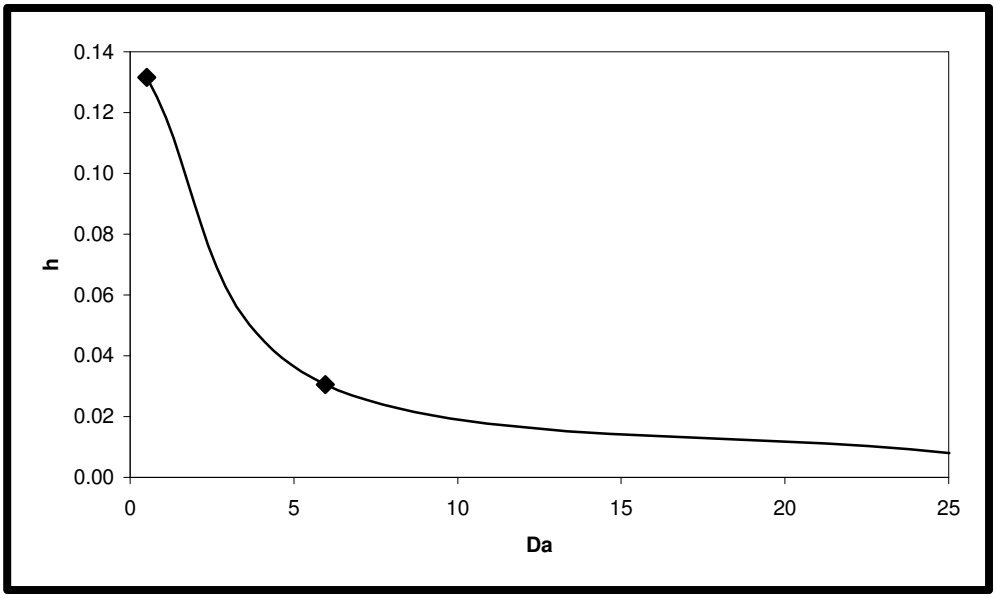

Figure 4.28 The relationship between η and Da; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

4.2.7.5 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_0, maintenance coefficient for oxygen, m_0, and the yield coefficients with the cultivation time are listed in Table 4.12.

The specific growth rate (μ) decreased with cultivation time as can be seen in Figure 4.29 similar to the results of İleri (2005). The maximum value of μ was quantified as 2.25 h^{-1} at t=0.1 h. It was 4.18 h^{-1} at t=0.5 h in İleri (2005). At the beginning of the bioprocesses, a considerably sharp decrease in specific growth rate was reported. After t=6 h, cell concentration did not change remarkably and specific growth rate remained almost constant approximately as $\mu=0$ h^{-1} througout the bioprocess.

The variation of specific oxygen uptake rate, q_0, with cultivation time is shown in Figure 4.30. Specific oxygen uptake rate was observed to decrease with the cultivation time, and the highest value of q_0 was gathered as 0.143 kg kg^{-1} h^{-1} at t=4 h. A maximum q_0 of 0.439 kg kg^{-1} h^{-1} was detected by İleri (2005) during the growth phase. As seen in Figure 4.30, the highest $Y_{X/O}$ was obtained as 2.353 kg cell/kg O_2 at t=2 h indicating that highest cell yield on oxygen consumption was obtained. The highest $Y_{X/O}$ was noted as 1.189 kg cell/kg O_2 by İleri (2005). $Y_{X/O}$ was seen to decrease with cultivation time. In general, at the beginning of the bioprocesses (t=2 or 3 h), a sharp decrease in $Y_{X/O}$ was observed due to the increase of OUR as seen in Figure 4.31.

The change of r_p (PUL^{-1} h^{-1}) with cultivation time is given in Figure 4.31. r_p is calculated in terms of streptokinase volumetric activity. As seen in Table 4.12, the highest $Y_{P/O}$ was obtained as 1679.725 PUL^{-1} / (kg m^{-3} O_2) t=12 h indicating that highest product yield in terms of streptokinase volumetric activity on oxygen consumption was obtained.

From the slope of the plot of $1/Y_{X/O}$ versus $1/\mu$, the rate of oxygen consumption for maintenance was determined as given in Table 4.12. m_0 is the oxygen consumption for maintenance calculated as 0,139 kg kg^{-1} h^{-1} by neglecting the oxygen consumption for by-product formation.

Table 4.12 The variations in specific growth rate and yield coefficients; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

t (h)	μ (h^{-1})	r_o (kg O_2/m^3h)	q_o (kg O_2 /kg h)	q_s (kg glucose / kg h)	r_p ($PUL^{-1}h^{-1}$)	$Y_{S/o}$ (kg glucose/kg O_2)	$Y_{x/o}$ (kg cell/kg O_2)	$Y_{p/o}$ (PUL^{-1}/ (kg m^{-3} O_2))	m_0 (kg kg^{-1} h^{-1})
0.1	2.250	0.000	0.000	10.359					
2	0.296	0.046	0.126	1.363		10.834	2.353		
4	0.069	0.069	0.143	0.769		5.367	0.479		
6	0.018	0.069	0.139	0.173		1.245	0.131		
8		0.069	0.133	0.242	89.391	1.815		1293.266	
10		0.069				2.279			
12		0.058			96.752	3.203		1679.725	0.139
14		0.058				2.425			
16		0.046			14.391	1.575		312.295	
18		0.058			48.376	1.836		839.862	
20		0.069			21.752	1.694		314.701	
22		0.081			96.752	1.470		1199.803	
24		0.104				0.737			

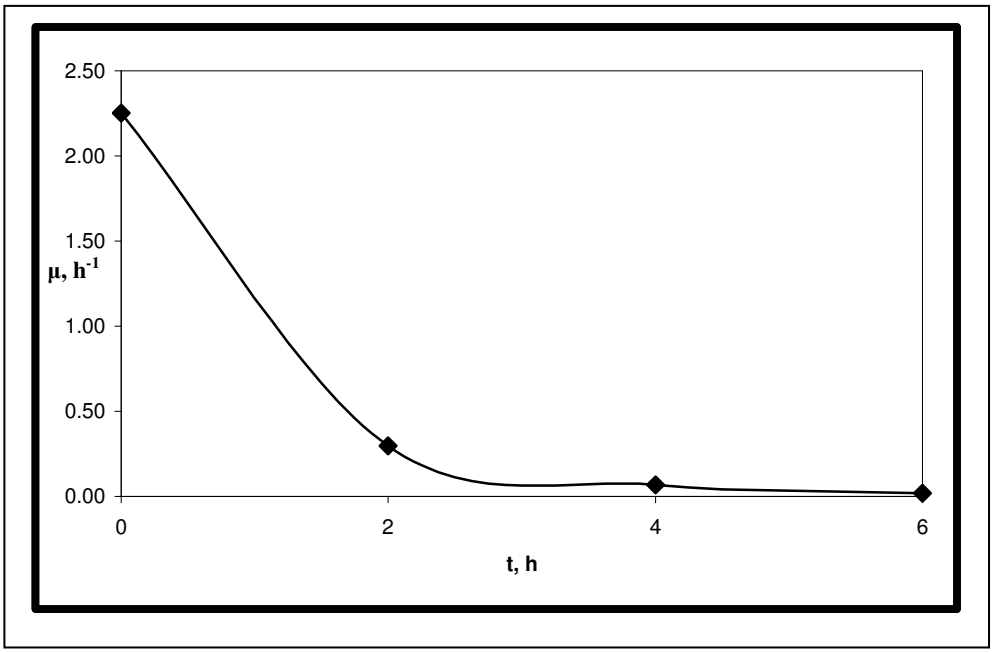

Figure 4.29 The variation in the specific growth rate with the cultivation time, agitation, air inlet rates and pH strategy at conditions; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

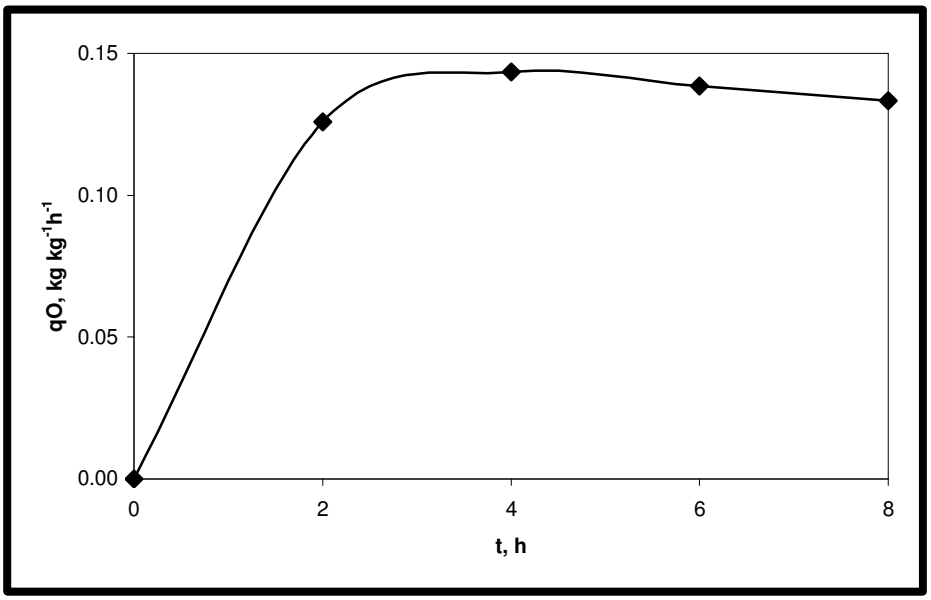

Figure 4.30 The variation in the specific oxygen uptake rates with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

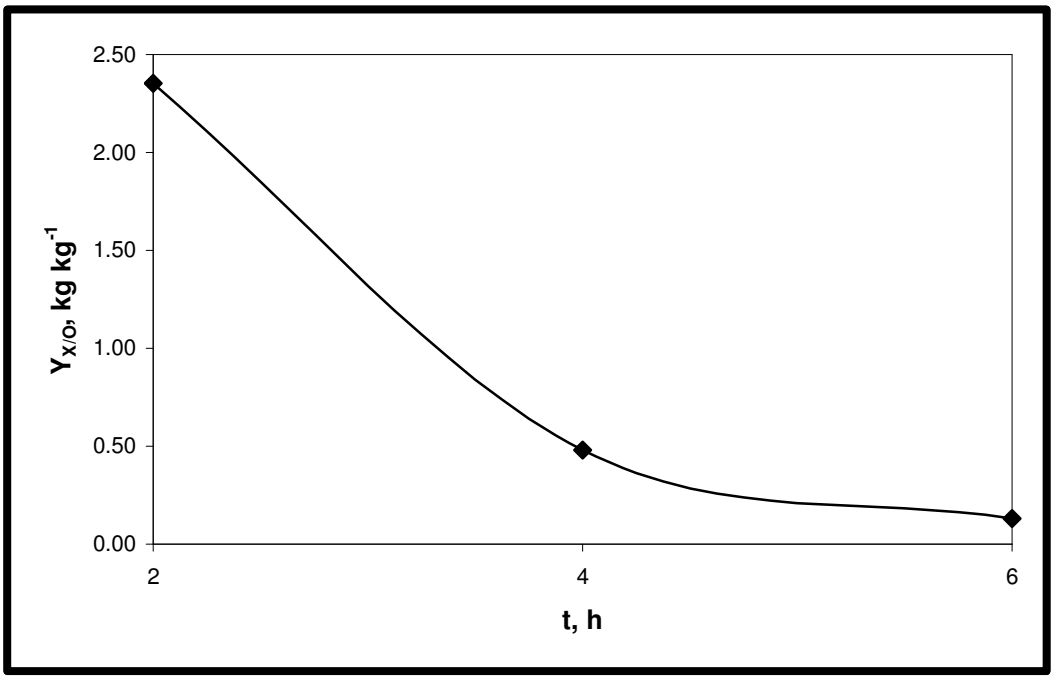

Figure 4.31 The variation in the yield coefficient, $Y_{X/O}$, with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

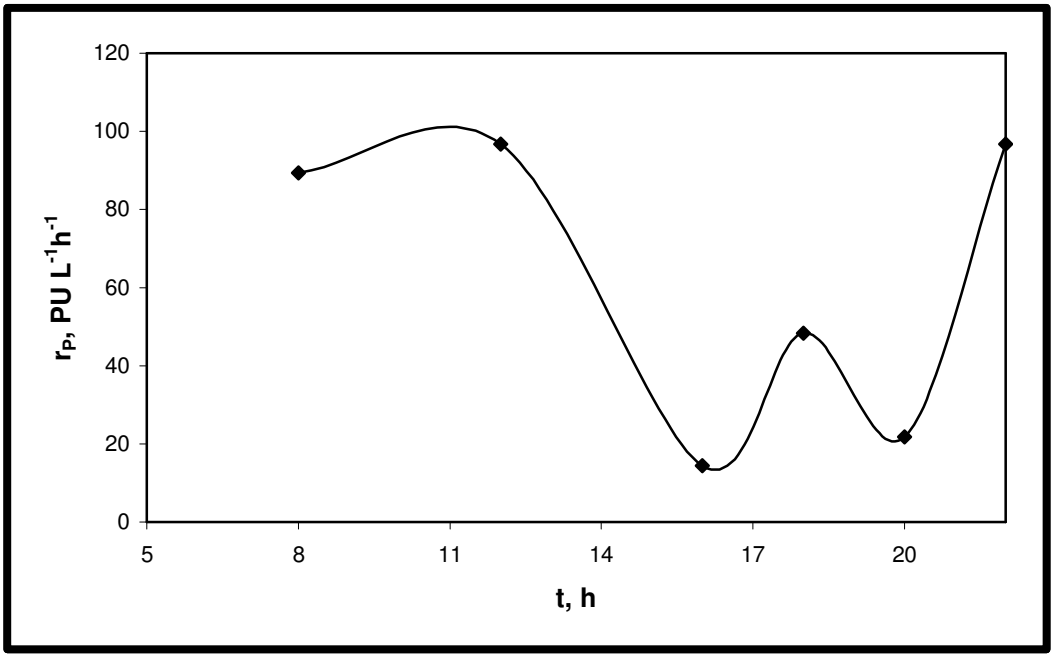

Figure 4.32 The variation in the r_p with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

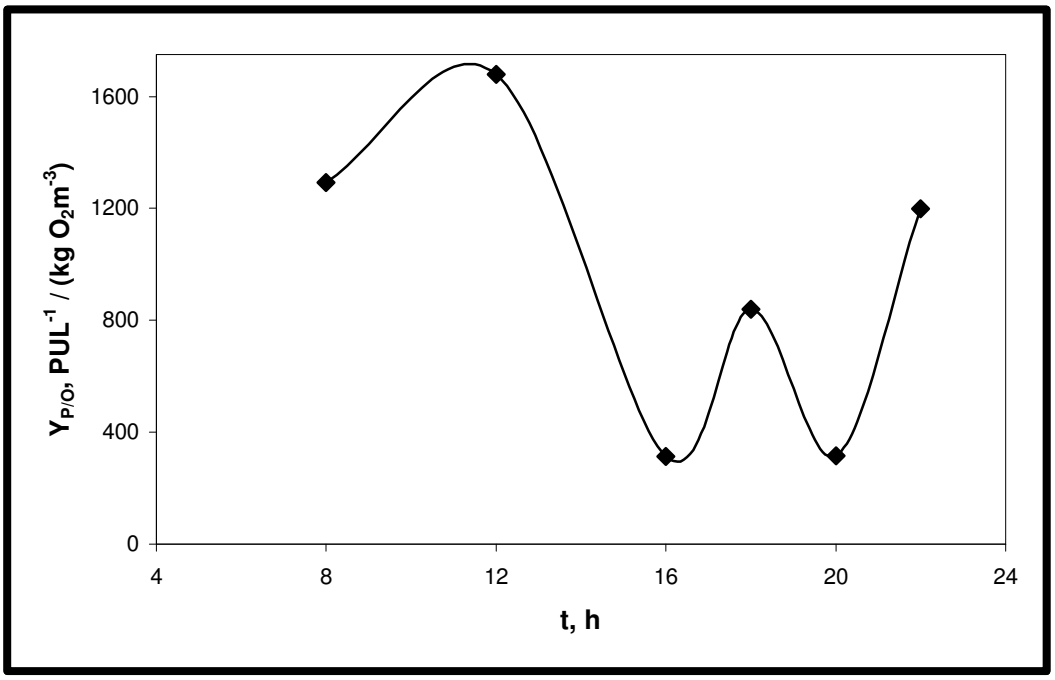

Figure 4.33 The variation in the yield coefficient, $Y_{P/X}$, with the cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

4.2.7.6 Organic Acid Concentration Profiles

The variation of various organic acid concentrations in the fermentation broth with cultivation time is demonstrated in Table 4.13. The major organic acids secreted to the fermentation medium were gluconic, glutaric and malic acids as can be seen in Table 4.13. Moreover, the highest total organic acid concentration was observed to be 1.82 kg m^{-3} at t=16 h (Figure 4.34). On the other hand, the highest total organic acid concentration was determined as 0.783 kgm^{-3} at t=8 h by İleri (2005).

In addition to gluconic, glutaric and malic acids, moderate concentrations of succinic, formic and acetic acids were excreted during bioprocess. Acetic and oxalic acids were started to be secreted into the cultivation medium after t=4 h, aspartic acid after t=8 h, maleic and fumaric acids after t=14 h, and citric acid after t=16 h of the

bioprocess. In the thesis study of İleri (2005), it was said that acetic, fumaric, and succinic acids were excreted in trace amounts as observed similarly in the present work.

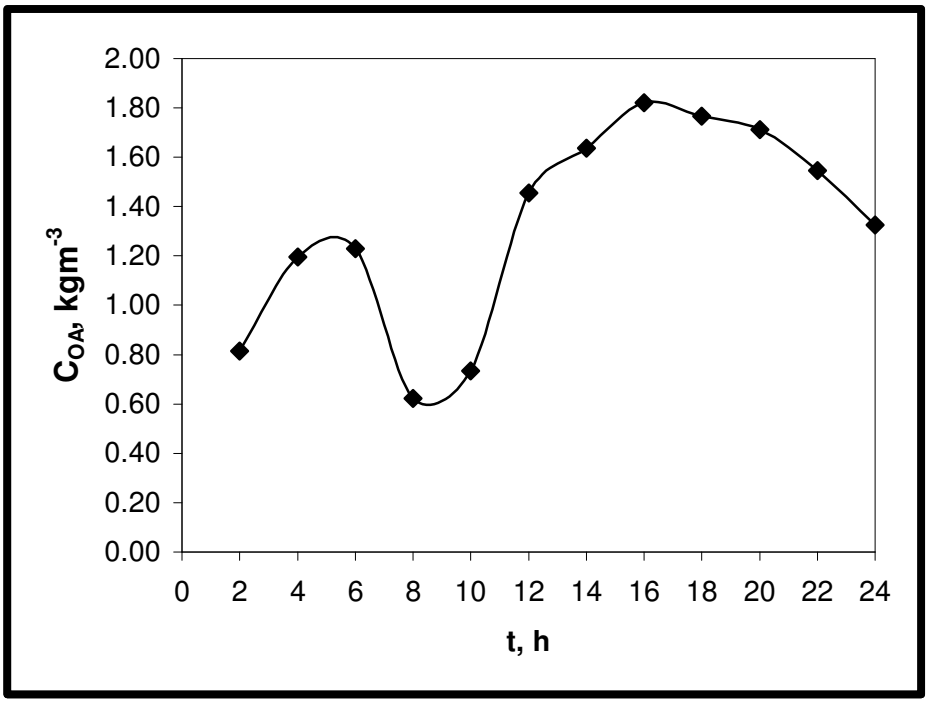

Figure 4.34 The variation of total organic acid concentration with cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm.

Table 4.13 The variations in organic acid concentrations with cultivation time; T=37°C, N=400 min^{-1}, Q_o/V_R=0.5 vvm

Time (h)	Suc	Gluc	Ac	αKG	Cit	Asp	Mal	Male	Form	Fum	Ox	ΣC_{OA}, kgm^{-3}
2	0.010	0.364	-	0.236	0.007	-	0.191	-	0.0057	-	-	0.8137
4	0.072	0.473	0.315	0.051	-	-	0.239	-	0.030	-	0.0170	1.1970
6	0.035	0.489	0.221	0.162	-	-	0.253	-	0.060	-	0.0100	1.2300
8	0.055	0.366	0.039	0.020	-	0.033	0.060	-	0.039	-	0.0110	0.6230
10	0.045	0.377	0.090	0.089	-	0.067	0.027	-	0.032	-	0.0065	0.7335
12	0.024	0.674	0.187	0.167	-	0.111	0.229	-	0.053	-	0.0090	1.4540
14	0.015	0.850	0.209	0.154	-	0.136	0.193	0.00028	0.065	0.00075	0.0147	1.6377
16	0.064	0.980	0.224	0.044	-	0.154	0.267	0.00025	0.055	0.00016	0.0314	1.8198
18	0.053	0.854	0.089	0.189	0.051	0.144	0.322	0.00100	0.055	-	0.0087	1.7667
20	0.050	0.749	0.095	0.223	0.047	0.152	0.329	0.00110	0.057	-	0.0089	1.7120
22	0.038	0.592	0.066	0.260	0.051	0.159	0.313	0.00120	0.059	-	0.0080	1.5472
24	0.044	0.471	0.018	0.301	0.020	0.172	0.227	0.00056	0.064	-	0.0067	1.3243

CHAPTER 5

CONCLUSIONS

The first aim of this study is the development of extracellular recombinant streptokinase producing *Bacillus sp*., and the second aim is to determine fermentation characteristics for streptokinase production. In this context, the following conclusions were drawn:

1. In this study, the promoter and signal (*pre*-) DNA sequence of *B. licheniformis* (DSM1969) extracellular serine alkaline protease enzyme gene (*sub*C: Acc. No. X03341) was ligated to 5' end of the streptokinase gene (*skc:* Acc. No. S46536) by SOE (Gene Splicing by Overlap Extension) method through PCR. In order to perform this ligation, fistly proper forward and reverse primers were designed for the cloning of the fusion product of *skc* and *pre(sub*C) genes into the selected plasmids after determining the suitable restriction recognition sites (*Eco*RI and *Bam*HI) for the hybrid gene.
2. The hybrid gene, *pre(sub*C*)*::*skc*, which was amplified through PCR and then purified, and pUC19 vector were digested with *Eco*RI and *Bam*HI restriction enzymes. This hybrid gene was cloned into purified linear pUC19 vector from the multiple cloning site located within the *lacZ* gene inactivating the N-terminal fragment of beta-galactosidase and abolishes alfa-complementation.
3. After transformation of the ligation product into *E. coli* TG1 strain, transformants were spread on LB-agar medium supplemented with ampicillin, Xgal and IPTG; and incubated at 37^{o}C for 12-18 h. According to the blue-white screening method, putative white colonies were selected and transformed *E. coli* TG1 colony was determined.
4. The hybrid gene was purified from agarose gel after digesting the recombinant vector, pUC19:: *pre(sub*C*)*::*skc*, isolated from recombinant

E.coli TG1 with *Eco*RI and *Bam*HI. Likewise, *E. coli/Bacillus* shuttle vector pMK4 was digested with the same restriction enzymes and then purified from agarose gel. The digested hybrid gene was ligated with the purified and linearized pMK4 plasmid from the sticky ends by T4 DNA ligase. The resulting recombinant plasmid, pMK4:: *pre(sub*C*)*::*skc,* was transformed into *E. coli* TG1 strain by induced competence

5. The recombinant pMK4::*pre(sub*C*)*::*skc* plasmids were further transformed into *Bacillus* species of *B. subtilis* (*npr- apr-)* and *B. licheniformis* 749/C (ATCC 25972) by electroporation at 2.5 kV. Transformants were spread on LB-agar medium supplemented with chloramphenicol and incubated at 37^{o}C for 12-18 h. Putative recombinant colonies were selected in order to determine transformed *Bacillus* sp.
6. Two recombinant species: *B. licheniformis* 749/C (ATCC 25972) and *B. subtilis* (*npr- apr-)* were firstly compared in terms of streptokinase production capacities. Supernatants of both species were harvested at different time points from the reference production medium (section 3.5) at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}. The samples were analyzed by High Performance Capillary Electrophoresis System. As a result, it was observed that r-*B. subtilis* (*npr- apr-)* did not produce any streptokinase; however it was seen that r-*B. licheniformis* 749/C (ATCC 25972) produced streptokinase accordingly.
7. The extracellular streptokinase produced by the recombinant *B.licheniformis* 749/C (ATCC 25972) harvested at t=22 h from the reference production medium (section 3.5) was analyzed by SDS-PAGE after concentrating the supernatant through the spin column. SDS-PAGE results illustrated that streptokinase enzyme was cloned into *B. licheniformis* and the recombinant *B. licheniformis* produced the enzyme extracellularly.
8. A statistical approach, namely Response Surface Methodology (RSM) was used in the medium design process. 13 experimental data points were obtained for recombinant streptokinase production in order to perform a

statistical analysis for optimizing the medium components. The design matrix and the fitness of the each term were analyzed by means of ANOVA. After performing the calculations, the resulting optimized recombinant streptokinase concentration was found as 0.0237 kgm^{-3} at glucose and $(NH_4)_2HPO_4$ concentrations of 4.530 and 4.838 kgm^{-3} respectively.

9. The Km and v_{max} values for streptokinase were evaluated using the standard streptokinase puchased as the drug Streptase from ZLB Behring. The standard streptokinase showed typical Michealis-Menten kinetics. From the Lineweaver-Burk plot, v_{max} and Km values were determined, at T=37°C, as $2.8x10^{-4}$ mMs^{-1} and 0.42 mM, respectively.
10. The fermentation and oxygen transfer characteristics of the streptokinase production were investigated in a 3 dm^3 pilot scale batch bioreactor (Braun CT2-2) equipped with temperature, pH, foam, air inlet and agitation rate controls having a working volume of V_R=1.65 dm^3 using the production medium optimized for the recombinant *B. lichenifomis* 749/C (ATCC 25972) strain. Streptokinase and β-lactamase activities, cell, glucose and organic acid concentrations, dissolved oxygen, pH, oxygen uptake rate, overall liquid phase mass transfer coefficient for oxygen, maintenance coefficient for oxygen, specific cell growth rate and yield coefficients were determined through the bioprocess. The bioprocess of recombinant streptokinase production was performed at uncontrolled pH of these bioreactor operation conditions: air inlet rate of Q_0/V_R=0.5 vvm, and the agitation rate of N=400min^{-1}.
11. The initial pH of the production medium was pH_0=7.39. Throughout the bioprocess, pH decreased continuously to pH=6.86. This decrease could be an outcome of the consumption of the ammonium sulfate present in the optimized medium of recombinant *B.lichenifomis.* As ammonia was consumed, hydrogen ions were excreted into the culture medium, resulting in a decrease in pH of the medium. Also, amino acid and organic acid production throughout the bioprocess could contribute to this decrease.

Finally, pH of the culture medium reached a constant value at t=16 h, indicating that the proton transport of the cell stopped signalling the end of any bioproduct production process.

12. Dissolved oxygen decreased to 21.7 % of saturation value (C_{DO}=0.0434 mol/m^3) due to the high oxygen demand at the beginning of the process till t=4 h. This sharp decrease was the result of a rapid dissolved oxygen uptake by the fast growing cells. Then, dissolved oxygen gradually increased and reached almost a stationary value at the end of the process.
13. Between t = 0-8h of the bioprocess, the cell concentration was observed to increase continuously, and the maximum value was reached at t = 8h which is the onset of stationary growth phase. The highest cell concentration was gathered at t = 8h as Cx = 0.52 kgm^{-3}. Between t=0-8 h of the bioprocess, the cell concentration increased upto Cx = 0.52 kgm^{-3}. Between t=0-8 h the dissolved oxygen concentration decreased from 79 % of the saturation value (C_{DO} = 0.16 $molm^{-3}$) to 21.7 % of saturation value (C_{DO} = 0.0434 $molm^{-3}$) due to the high oxygen demand at the beginning of the process till t=4 h. However, when cells reached their stationary growth phase, the dissolved oxygen concentration started to increase significantly showing that cells did not use oxygen as much as they consumed in their exponential growth phase.
14. In the laboratory scale bioreactor at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, between t = 0-6 h of the bioprocess, the cell concentration was observed to increase continuously, and the maximum value was reached at t = 6h which was the onset of stationary growth phase. The highest cell concentration was gathered at t = 6h as Cx = 0.67 kgm^{-3}. The differences in cell concentration values between the bioreactor and shaker could have been resulted from the different bioreactor operation conditions and thus the different oxygen transfer characteristics.
15. Glucose concentration of the production medium was observed to decrease throughout the bioprocess from its initial value of C_G^0 = 4.53 kgm^{-3} as

expected. The highest glucose consumption rate was attained between t=0-4 h which is the period for early exponential phase where the cells require more energy for the continuation of their growth. In contrast, after t=18 h, glucose was consumed with a considerably slower rate, and finally reached a concentration of 0.1 kgm^{-3} in the fermentation medium indicating that almost all glucose was used up by the cells throughout the bioprocess.

16. Volumetric activity for streptokinase is defined as plasmin units/ml of the sample solution. No streptokinase volumetric activity was observed including t=8h, then it increased with the cultivation time until t=16 h. Meanwhile, β-lactamase volumetric activity was observed to increase steadily reaching the highest value of 134 Ucm^{-3} at t=16 h. In the present study, after the time point t=16 h, activity of streptokinase started to increase after the stationary path it attained between t=12-16h. Most probably, since β-lactamase reached its highest concentration implying that other proteases were also higly expressed at this stage, streptokinase could have been degraded by these proteases leading to a stationary condition in its volumetric activity. Starting from t=16 h, while the β-lactamase volumetric activity followed a decreasing path, the streptokinase volumetric activity climbed to higher values reaching its maximum as 1.16 $PUml^{-1}$ (0.0026 g/l streptokinase) at t=20 h.
17. In the laboratory scale bioreactor at conditions: V=33 cm^3, T=37°C, N=200 min^{-1}, no streptokinase volumetric activity was observed including t=8h likewise the bioreactor, then it increased with the cultivation time until t=12 h. Meanwhile, β-lactamase volumetric activity was observed to increase steadily reaching the highest value of 99 Ucm^{-3} at t=12 h. After the time point t=12 h, activity of streptokinase followed a declining path until t=16h. The similar trend was observed for the β-lactamase volumetric activity profile. Starting from t=16 h, both β-lactamase and streptokinase volumetric activities climbed to higher values. Streptokinase volumetric

activity reached its maximum as 2.76 $PUml^{-1}$ (0.0063 g/l streptokinase) at t=22 h.

18. The Dynamic Method was used in finding the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r0, and oxygen transfer coefficients, K_La. The variations in K_La, oxygen uptake rate, oxygen transfer rate, the enhancement factor E ($=K_La/K_La_0$), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor (η) throughout the bioprocesses were determined.
19. When K_La values were evaluated in terms of their change with respect to time, the highest K_La was determined as 0.02952 s^{-1}at t=2 h. E, the biological enhancement factor can be calculated by the ratio of experimental oxygen mass transfer coefficient in the presence of microorganisms and that measured in the absence of microorganisms. E values were observed to decrease sharply between hours t=2-8 h and rather slowly between t=8-14 h taking values between 0.54-5.60 throughout the bioprocess. K_La and consequently the biological enhancement factor, E were higher in the early growth phase of the bioprocess than in the production phase implying that OUR by the cells was higher then intracellular biochemical reaction rates were much more higher in this stage. As E values started to decrease, it can be stated that the biochemical reactions started to slow down compared to the early growth phase. K_La depends on temperature, agitation rate, rheological properties of the fermentation medium and presence of fine particles in the mass transfer area. The observed increase in K_La, could be resulted from the decrease in viscosity of the cultivation medium due to metabolite secretion, as temperature and agitation rate were maintained constant throughout the bioprocess. Similarly, the decrease in K_La values, could be resulted from the increase in viscosity of the cultivation medium due to metabolite utilization.

20. OUR increases during the lag phase and exponential phases of the microbial growth and takes a maximum at the very beginning of the exponential phase as it is the case in this analysis. OUR increased during the exponential growth phase of the microorganism, attained the maximum value for a cell concentration of 0.48 kgm^{-3} and afterwards remained constant during the stationary phase of the growth. The highest OUR was observed as 0.0006 $molm^{-3}s^{-1}$ at t=4 h of cell growth phase. After the cells reached the end of the stationary phase at t=10 h, OUR began to decrease taking 0.0005 $molm^{-3}s^{-1}$.

21. OTR, which is proportional to the difference between the equilibrium concentration, C_{DO}^{*} and the dynamic dissolved oxygen concentration in the medium, took its highest value at the beginning of the bioprocess at t=2 h as 0,0044 $molm^{-3}s^{-1}$ and decreased to an extent depending on the oxygen uptake rate.
22. Da is a dimensionless number, relating the maximum oxygen utilization and maximum oxygen transfer rates. At the beginning of the bioprocess, biochemical reaction limitations were effective (Da<1); however after t=2 h of the bioprocess, mass transfer resistances became more effective (Da>>1).
23. At the beginning of the bioprocess, the effectiveness factor, η, took considerably higher values, indicating that the cells were consuming oxygen with a higher rate approaching the maximum oxygen uptake rate (OD). For both conditions, η decreased with the increase of Da. This reveals that there is a reverse proportionality between the effectiveness factor and the Damköhler number in a bioprocess.
24. The change of maximum oxygen uptake rate, oxygen demand (OD), with cultivation time was investigated. OD increased with the increase in cell concentration implying that the maximum possible oxygen utilization rate (OD) depends on the level of microbial growth.

25. The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_0, maintenance coefficient for oxygen, m_0, and the yield coefficients with the cultivation time were studied. The maximum value of μ was quantified as 2.25 h^{-1} at t=0.1 h. After t=6 h, cell concentration did not change remarkably and specific growth rate remained almost constant approximately as μ=0 h^{-1} througout the bioprocess. Specific oxygen uptake rate was observed to decrease with the cultivation time, and the highest value of q_0 was gathered as 0.143 kg kg^{-1} h^{-1} at t=4 h. The highest $Y_{X/O}$ was obtained as 2.353 kg cell/kg O_2 at t=2 h indicating that highest cell yield on oxygen consumption was obtained. $Y_{X/O}$ was seen to decrease with cultivation time. In general, at the beginning of the bioprocesses (t=2 or 3 h), a sharp decrease in $Y_{X/O}$ was observed due to the increase of OUR.
26. The change of r_p (PUL^{-1} h^{-1}) with cultivation time was determined in terms of streptokinase volumetric activity. The highest $Y_{P/O}$ was obtained as 1679.725 PUL^{-1} / (kg m^{-3} O_2) t=12 h indicating that highest product yield in terms of streptokinase volumetric activity on oxygen consumption was obtained.
27. From the slope of the plot of $1/Y_{X/O}$ versus 1/μ, the rate of oxygen consumption for maintenance was determined as 0,139 kg kg^{-1} h^{-1} by neglecting the oxygen consumption for by-product formation.
28. The major organic acids secreted to the fermentation medium were gluconic, glutaric and malic acids. Moreover, the highest total organic acid concentration was observed to be 1.82 kg m^{-3} at t=16 h. In addition to gluconic, glutaric and malic acids, moderate concentrations of succinic, formic and acetic acids were excreted during bioprocess. Acetic and oxalic acids were started to be secreted into the cultivation medium after t=4 h, aspartic acid after t=8 h, maleic and fumaric acids after t=14 h, and citric acid after t=16 h of the bioprocess.

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APPENDIX A

BUFFERS and SOLUTIONS

LB

Soytryptone	10 kg m^{-3}
Yeast exract	5 kg m^{-3}
NaCl	10 kg m^{-3}

LBA

Soytryptone	10 kg m^{-3}
Yeast exract	5 kg m^{-3}
NaCl	10 kg m^{-3}
Agar	15 kg m^{-3}

SET Buffer

NaCI	75 mM
EDTA	25 mM

10X TBE Buffer

Tris	108 kg m^{-3}
Boric Acid	55 kg m^{-3}
EDTA	9.3 kg m^{-3}

TAE Buffer

Tris-acetate	0.04 M
EDTA	0.001 M, pH 8.0

TSE Buffer

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

SOLUTION I

Tris HCl, pH= 8.1	10 mM
EDTA	10 mM
NaCl	50 mM
Sacchorose	8% (w/v)

SOLUTION II

SDS	1% (w/v)
NaOH	0.2 M

SOLUTION III

Potassium Acetate	5 M
Acetate Acid	60 ml
dH_2O	Up to 100 ml

Antibiotics	Stock Solutions	
	Concentration	Storage
Ampcillin	100 mg/ml in dH_2O	-20 ^oC
Chloramphenicol	35 mg/ml in ethanol	-20^oC

APPENDIX B

GENE SEQUENCE OF *pre(sub*C) GENE:

(Accession No:X03341)

gcggtctattcatactttcgaactgaacatttttctaaaacagttattaataaccaaaaaattttaaa
ttggtcctccaaaaaaataggcctaccatataattcatttttttttctataataaattaacagaataat
tggaatagattatattatccttctatttaaattattctgaataaagaggaggagagtgagtaatg*****at
gaggaaaaagagtttttggcttgggatgctgacggccttcatgctcgtgttcacgatggcattcagcg
attccgcttctgctgctcaaccggcgaaaaatgttgaaaaggattatattgtcggatttaagtcagga
gtgaaaaccgcatctgtcaaaaaggacatcatcaaagagagcggcggaaaagtggacaagcagtttag
aatcatcaacgcggcaaaagcgaagctagacaaagaagcgcttaaggaagtcaaaaatgatccggatg
tcgcttatgtggaagaggatcatgtggcccatgccttg

***** Indicates the start codon

GENE SEQUENCE OF SKC GENE INCLUDING *pre(sub*C) GENE EXTENSION AT 5' END:

(Accession No: S46536)

gcggtctattcatactttcgaact*gaacatttttctaaaacagttattaataaccaaaaaattttaa
attggtcctccaaaaaaataggcctaccatataattcatttttttttctataataaattaacagaataa
ttggaatagattatattatccttctatttaaattattctgaataaagaggaggagagtgagtaatgat
gaggaaaaagagtttttggcttgggatgctgacggccttcatgctcgtgttcacgatggcattcagcg
attccgcttctgctattgctggacctgagtggctgctagaccgtccatctgtcaacaacagccaatta
gttgttagcgttgctggtactgttgaggggacgaatcaagacattagtcttaaatttttgaaattga
cctaacatcacgacctgctcatggaggaaagacagagcaaggcttaagtccaaaatcaaaaccatttg
ctactgatagtggcgcgatgccacataaacttgaaaaagctgacttactaaaggctattcaagaacaa
ttgatcgctaacgtccacagtaacgacgactactttgaggtcattgattttgcaagcgatgcaaccat
tactgatcgaaacggcaaggtctactttgctgacaaagatggttcggtaaccttgccgacccaacctg
tccaagaatttttgctaagcggacatgtgcgcgttagaccatataaagaaaaaccaatacaaaatcaa
gcgaaatctgttgatgtggaatatactgtacagtttactcccttaaaccctgatgacgatttcagacc
aggtctcaaagatactaagctattgaaaacactagctatcggtgacaccatcacatctcaagaattac
tagctcaagcacaaagcattttaaacaaaacccacccaggctatacgatttatgaacgtgactcctca
atcgtcactcatgacaatgacattttccgtacgattttaccaatggatcaagagtttacttaccatgt
caaaaatcgggaacaagcttatgagatcaataaaaaatctggtctgaatgaagaaataaacaacactg
acctgatctctgagaaatattacgtccttaaaaaaggggaaaagccgtatgatccctttgatcgcagt
cacttgaaactgttcaccatcaaatacgttgatgtcaacaccaacgaattgctaaaaagcgagcagct
cttaacagctagcgaacgtaacttagacttcagagatttatacgatcctcgtgataaggctaaactac
tctacaacaatctcgatgcttttggtattatggactataccttaactggaaaagtagaggataatcac
gatgacaccaaccgtatcataaccgtttatatgggcaagcgacccgaaggagagaatgctagctatca
tttagcctatgataaagatcgttataccgaagaagaacgagaagtttacagctacctgcgttatacag
ggacacctatacc**tgataaccctaacgacaaataa***

* Indicates the primer binding sequences

APPENDIX C

THERMODYNAMIC PROPERTIES OF DESIGNED PRIMERS TOGETHER WITH DIMER AND SELF-COMPLEMENTARY FORMATION AFFINITIES

Table A.1 Thermodynamic properties of designed primers.

Name	Length	GC%	T_m (^oC)	ΔG kcal/mol	ΔH kcal/mol
Forward Primer for *pre(sub*C)	30	50	59.9	-56.9	-249.5
Reverse Primer for *pre(sub*C)	28	54	58.8	-51.8	-225.9
Forward Primer for *skc*	35	57	60.2	-66.3	-279.5
Reverse Primer for *skc*	32	47	59.7	-57.9	-259.3

APPENDIX C

SELF COMPLEMENTARY and DIMER FORMATION AFFINITIES of PRIMERS

1) *pre(subC)* Forward Primer:

Sequence: 5' GCGCGAATTCGCGGTCTATTCATACTTTCG 3'

*Eco*RI RE Sequence

Dimer formation:

```
                    5' GCGCGAATTCGCGGTCTATTCATACTTTC 3'
                       | | | | | | | | | | | | | |
3' GCTTTCATACTTATCTGGCGCTTAAGCGCG 5'
```

Self-complementarity:

```
                    5'  GCGCGAA┐
                       | | | |  |
3' GCTTTCATACTTATCTGGCGCTT┘
```

2) *pre(subC)* Reverse Primer:

Sequence: 5' AGGTCCAGCAATAGCAGAAGCGGAATCG 3'

Dimer formation:

```
                    5' AGGTCCAGCAATAGCAGAAGCGGAATCG 3'
                       | | |       |    |       | | |
                  3' GCTAAGGCGAAGACGATAACGACCTGGA 5'
```

Self-complementarity:

```
                    5'  AGGTCCAGCAATA┐
                       | | |         G
                    3' GCTAAGGCGAAGAC┘
```

3) *skc* Forward Primer:

Sequence: 5' GCGATTCCGCTTCTGCTATTGCTGGACCTGAGTGG 3'

Dimer formation:

```
                    5' GCGATTCCGCTTCTGCTATTGCTGGACCTGAGTGG 3'
                       |    | | |       |    |       | | |    |
                 3' GGTGAGTCCAGGTCGTTATCGTCTTCGCCTTAGCG 5'
```

Self-complementarity:

```
                          5'  GCGAT┐
                             | | |  |
3' GGTGAGTCCAGGTCGTTATCGTCTTCGCCT┘
```

4) *skc* Reverse Primer:

Sequence: 5' GCCCGGATCCTTATTTGTCGTTAGGGTTATCA 3'

*Bam*HI RE Sequence

Dimer formation:

```
                    5' GCCCGGATCCTTATTTGTCGTTAGG 3'
                       | | | | | |
3' ACTATTGGGATTGCTGTTTATTCCTAGGCCCG 5'
```

Self-complementarity:

```
                    5'  GCCCGGATCCTTA┐
                       | | |   |      T
                    ATTGGGATTGCTGTT┘
```

APPENDIX D

MAP of SITES for RESTRICTION ENDONUCLEASES

Restriction enzymes that are cutting the *skc* gene fragment are listed in Table A.2; whereas that of those noncutting the *skc* gene as follows:

Noncutter restriction enzymes of *skc* gene: AarI, AatII, AclI, AflII, AgeI, AloI, AlwNI, ApaI, ApaLI, AscI, AsuII, AvrII, BalI, BamHI, BbvCI, BciVI, BfiI, BplI, Bpu10I, BsaAI, BsaXI, BseRI, BsmI, Bsp1407I, BspLU11I, BstEII, BstXI, BtsI, DraII, DrdI, Eam1105I, Eco31I, Eco57I, Eco57MI, EcoRI, EcoRII, EcoRV, Esp3I, FalI, FseI, FspAI, GsuI, Hin4I, HindIII, HpaI, KpnI, MluI, MmeI, NaeI, NdeI, NheI, NotI, NruI, PI-PspI, PI-SceI, PacI, PfoI, PleI, PmaCI, PmeI, PpiI, PpuMI, PshAI, PsiI, PsrI, PvuI, PvuII, RsrII, SacI, SacII, SanDI, ScaI, SexAI, SfiI, SgfI, SmaI, SmlI, SnaBI, SpeI, SrfI, SspI, StuI, SwaI, TaqII, TatI, TfiI, Tth111I, VspI, XbaI, XhoI

Table A.2 Restriction enzymes cutting the *skc* gene.

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BsaBI	GATNNNNATC	6	blunt	1	1442
PshAI	GACNNNNGTC	6	blunt	1	383
SspI	AATATT	6	blunt	1	1105
StuI	AGGCCT	6	blunt	1	89
SwaI	ATTTAAAT	8	blunt	1	164
XmnI	GAANNNNTTC	6	blunt	1	1165
AccI	GTMKAC	6	five_prime	1	632
AflII	CTTAAG	6	five_prime	1	450
AflIII	ACRYGT	6	five_prime	1	702
AsuII	TTCGAA	6	five_prime	1	18
BsmAI	GTCTC	5	five_prime	1	823

Table A.2, continued

Bsp1407I	TGTACA	6	five_prime	1	774
BspHI	TCATGA	6	five_prime	1	960
BspLU11I	ACATGT	6	five_prime	1	702
BstEII	GGTNACC	6	five_prime	1	657
Eco31I	GGTCTC	6	five_prime	1	823
FokI	GGATG	5	five_prime	1	240
HindIII	AAGCTT	6	five_prime	1	1034
MfeI	CAATTG	6	five_prime	1	541
PleI	GAGTC	5	five_prime	1	937
SexAI	ACCWGGT	7	five_prime	1	813
TatI	WGTACW	6	five_prime	1	774
Tth111I	GACNNNGTC	6	five_prime	1	815
VspI	ATTAAT	6	five_prime	1	47
BsrI	ACTGG	5	three_prime	1	1341
Eco57I	CTGAAG	6	three_prime	1	1236
Eco57MI	CTGRAG	6	three_prime	1	1236
Hpy99I	CGWCG	5	three_prime	1	571
NspI	RCATGY	6	three_prime	1	706
TspGWI	ACGGA	5	three_prime	1	967
TspRI	CASTG	5	three_prime	1	1089
HindII	GTYRAC	6	blunt	2	324, 1191
MslI	CAYNNNNRTG	6	blunt	2	247, 965
AvaII	GGWCC	5	five_prime	2	71, 292
BbvI	GCAGC	5	five_prime	2	290, 1230
BspMI	ACCTGC	6	five_prime	2	429, 1490
EcoRII	CCWGG	5	five_prime	2	813, 918
NheI	GCTAGC	6	five_prime	2	1231, 1417

Table A.2, continued

TfiI	GAWTC	5	five_prime	2	271, 372
TseI	GCWGC	5	five_prime	2	303, 1218
BsaXI	ACNNNNNCTCC	6	three_prime	2	179, 209
BseMII	CTCAG	5	three_prime	2	287, 1088
BsmI	GAATGC	6	three_prime	2	262, 1419
FalI	AAGNNNNNCTT	6	three_prime	2	619, 651
HphI	GGTGA	5	three_prime	2	868, 1161
ApoI	RAATTY	6	five_prime	3	59, 391, 685
SmlI	CTYRAG	6	five_prime	3	450, 872, 887
BseRI	GAGGAG	6	three_prime	3	197, 200, 936
MboII	GAAGA	5	three_prime	3	1081, 1468, 1471
SfaNI	GCATC	5	five_prime	4	218, 482, 591, 1296
Tsp45I	GTSAC	5	five_prime	4	856, 940, 954, 1153
TspDTI	ATGAA	5	three_prime	4	92, 231, 950, 1082
BccI	CCATC	5	five_prime	5	252, 324, 643, 871, 1180
Hin4I	GAYNNNNNVTC	6	three_prime	8	301, 333, 397, 429, 803, 835, 1078, 1110

APPENDIX E

PREPARATION OF RESOLVING AND STACKING GELS AND RELATED BUFFERS FOR SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Table A.3 Resolving and stacking gel compositions

Component	Stacking Gel	Resolving Gel		
	5%	7.5%	10%	12%
30% acrylamide mix	1.67 ml	2.5 ml	3.33 ml	4 ml
dH_2O	5.68 ml	4.85 ml	4.05 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5 ml	2.5 ml	2.5 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml	-	-	-
10% (w/v) SDS	100 µl	100 µl	100 µl	100 µl
10% (w/v) ammonium persulfate	60 µl	50 µl	50 µl	50 µl
TEMED	15 µl	10 µl	10 µl	10 µl

PREPARATION of COMPONENTS USED for SILVER STAINING of SDS-POLYACRYLAMIDE GELS

A. Fixer

150 ml methanol + 36 ml acetic acid + 150 µl of 37% formaldehyde are mixed and completed to 300 ml with distilled water. This solution can be used several times.

B. 50% Ethanol

600 ml pure ethanol + 600 ml distilled water are mixed and this solution should be prepared fresh.

C. Pretreatment Solution

0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) is dissolved in 400 ml distilled water. 8 ml of this solution should be put aside for further use in the preparation of developing solution.

D. Silver Nitrate Solution

0.8 g silver nitrate is dissolved in 400 ml distilled water and then 300 µl of 37% formaldehyde is added to this solution.

E. Developing Solution

9 g potassium carbonate are dissolved in 400 ml distilled water and 8 ml of the pretreatment solution and 300 µl of 37% formaldehyde are then added to this solution.

F. Stop Solution

200 ml methanol + 48 ml acetic acid are mixed and completed to 400 ml with distilled water.

APPENDIX F

WESTERN BLOTTING SOLUTIONS

10XTransfer Buffer Stock

Tris base 30.3 g

Glycine 144.1 g

Water to 1 l

Store at 4 ^{0}C

1XTransfer Buffer

10XTransfer Buffer stock 100 ml

Methanol 200 ml

Water 700 ml

Prepare fresh and chill to 4 ^{0}C

10XTBS

Tris-base 12.11 g

NaCl 87.66 g

Water 1 l

Adjust pH= 7.6 using 3M NaOH or HCl and store at room temperature.

TBS-T

10XTBS 60 ml

Tween-20 0.6 ml

Water 540 ml

Prepare fresh

TBS-T-Milk

5% Milk powder (low fat) in TBS

Prepare fresh

APPENDIX G

DNA AND PROTEIN MARKERS

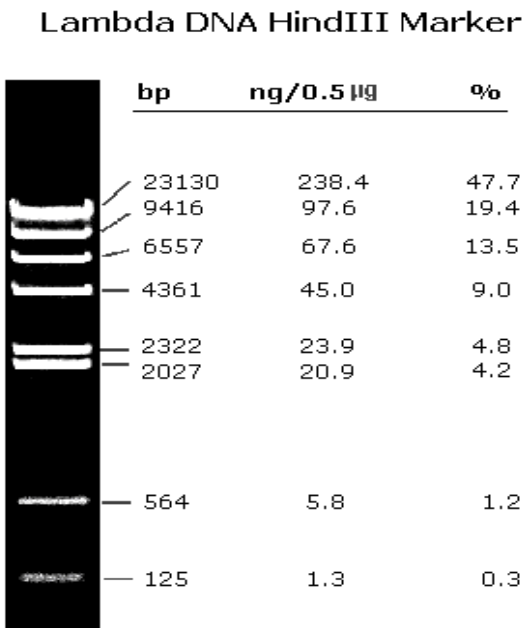


Figure A.1 Discrete DNA fragments of Lambda DNA/*Hind*III Marker in bp

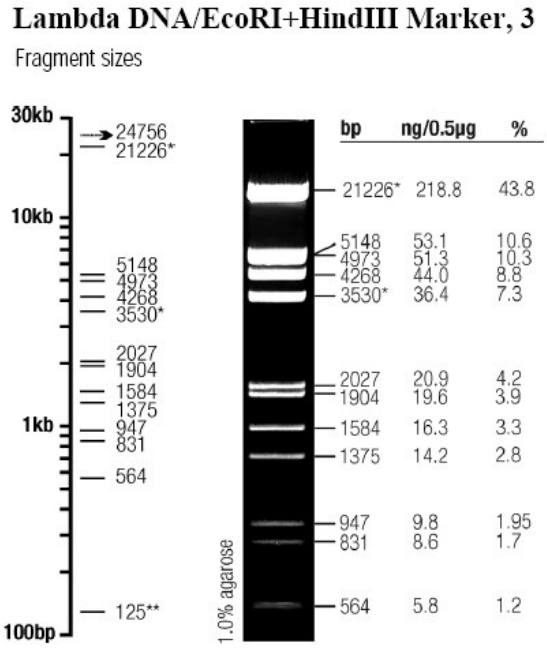


Figure A.2 Discrete DNA fragments of Lambda DNA/*Eco*RI+*Hind*III Marker in bp

APPENDIX G

Protein	Source	Approx. MW (kDa)
β-galactosidase	*E.coli*	116.0
Bovine serum albumin	Bovine plasma	66.2
Ovalbumin	Chicken egg white	45.0
Lactate dehydrogenase	Porcine muscle	35.0
Restriction endonuclease *Bsp*981	*E.coli*	25.0
β-lactoglobulin	Bovine milk	18.4
Lysozyme	Chicken egg white	14.4

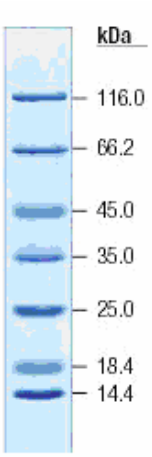


Figure A.3 Molecular weights of discrete proteins of Protein Molecular Marker in kDa

APPENDIX H

CALIBRATION of *Bacillus licheniformis* CONCENTRATION

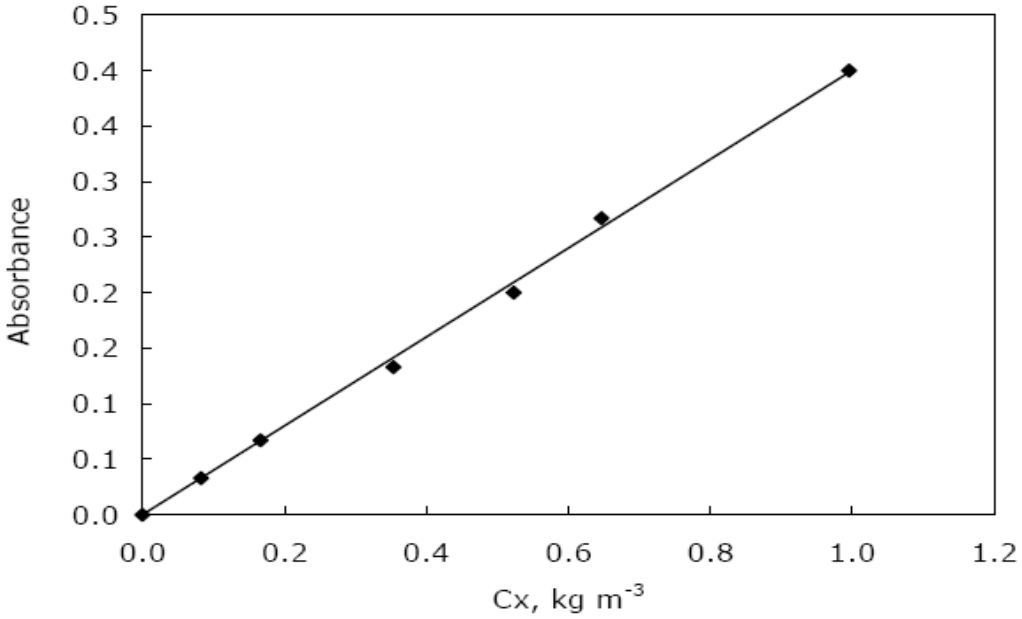


Figure A.1 Calibration curve for *Bacillus licheniformis* concentration

Slope of the calibration curve, m=2.49 1/kg m^{-3} (λ=600 nm)

$$Cx = \frac{Absorbance}{2.49} \times DilutionRatio$$

APPENDIX I

CALIBRATION OF BETA-LACTAMASE ACTIVITY

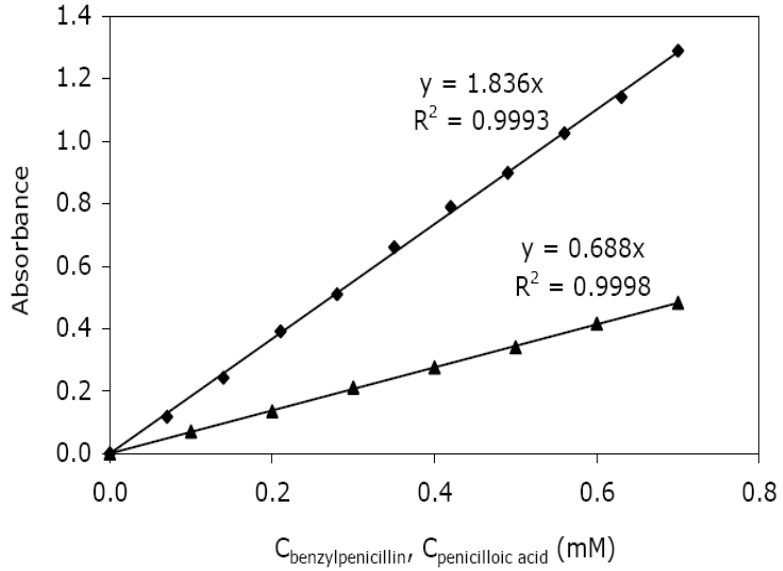


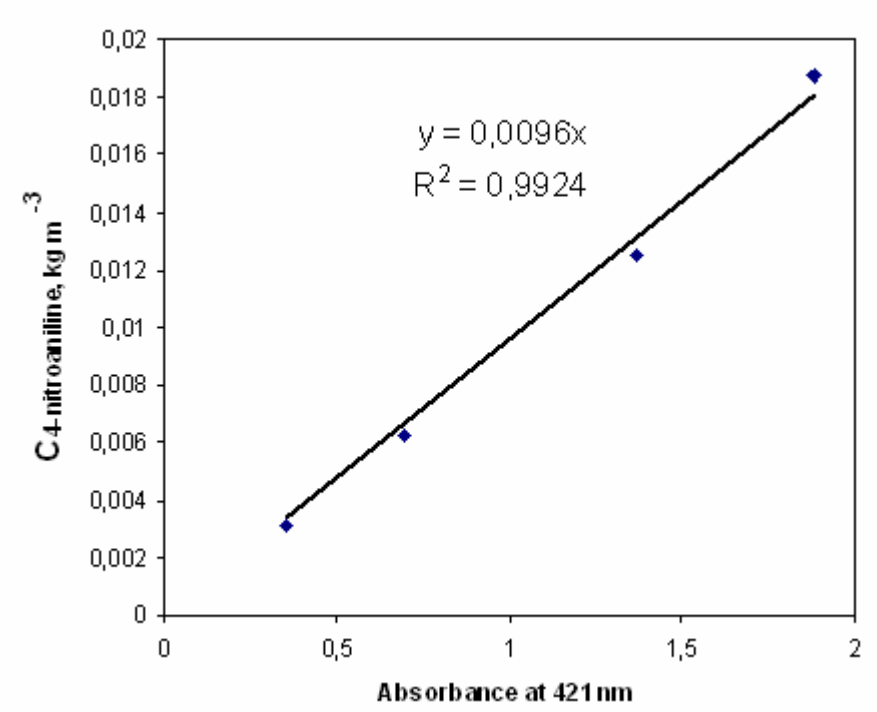
Figure A.2 Calibration Curve of the benzylpenicillin and penicilloic acid in 0.1 M phosphate buffer, pH=7.0, T=30°C, λ=232nm. Benzylpenicillin, (♦); Penicilloic acid, (▲).

One unit of β-lactamase activity was defined as the amount of enzyme that could hydrolyze 1μmol of benzylpenicillin at 30°C and pH 7.0 in one minute. The product of the hydrolysis reaction, penicilloic acid, also gives an absorbance at 232nm, therefore, the difference of the slopes is taken, m_1-m_2=1.148 mM^{-1}. The activity, U cm^{-3} is given by (Çelik, 2003),

$$A = \frac{C_{A0} m_1 - A_\lambda}{(m_1 - m_2) mM^{-1}} x \frac{1U}{10^{-3} mmol} x \frac{1l}{10^3 cm^3} x DilutionRatio$$

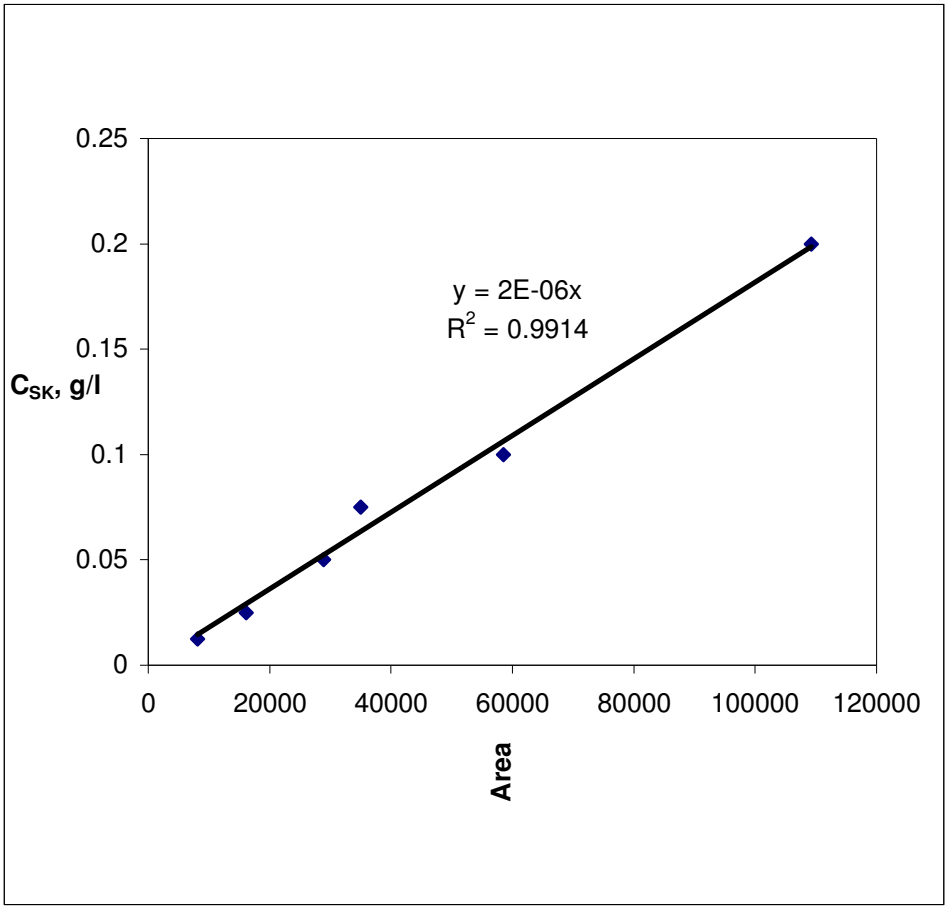
APPENDIX J

CALIBRATION OF 4-nitroaniline



APPENDIX K

CALIBRATION OF STREPTOKINASE



APPENDIX L

CALIBRATION OF GLUCOSE