FED-BATCH MEDIUM MODIFICATION FOR SECRETED SINGLE CHAIN VARIABLE FRAGMENT AGAINST HIV-1 P17 PROTEIN PRODUCTION USING COMBINED TWO FACTORIAL EXPERIMENTS IN *Escherichia coli* BIOREACTOR

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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTSFOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL TECHNOLOGY

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Porntip Paopang

หัวข้อวิทยานิพนธ์	การปรับปรุงอาหารเลี้ยงเชื้อแบบกึ่งกะเพื่อผลิตซิงเกิลเชน แวริเอเบิลแฟรกเมนต์ แบบคัดหลั่งต่อโปรตีน p17 ของเชื้อเอชไอวี-1 จากเชื้อเอชเชอริเชีย โคไล ในถังชีวปฏิกรณ์โดยใช้การทดลองแบบแฟคทอเรียลสองวิธีร่วมกัน
ผู้เขียน	นางสาวพรทิพย์ เผ่าผาง
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บทคัดย่อ

การนำวิธีเลี้ยงเชื้อแบบ fed-batch cultivation มาใช้ใน Biopharmaceutical production programs สามารถเพิ่มปริมาณการผลิตโปรตีนขณะที่มีวิธีการที่ไม่ซับซ้อน ในระดับอุตสาหกรรมคำนึงถึงการพัฒนากระบวนการเลี้ยงเชื้อที่ได้ปริมาณเชื้อจำนวนมาก เพื่อให้เพียงพอกับความต้องการของตลาดที่เพิ่มขึ้นและลดต้นทุนการผลิต แม้จะมีความก้าวหน้าทางด้านวิศวกรรมของเซลล์แบคทีเรียที่จะทำให้ได้เซลล์ที่มีประสิทธิภาพสูง มีการพัฒนาอาหารเลี้ยงเชื้อและ process parameter settings ที่จำเป็นในการผลิตที่สูงสุดของเซลล์ ในการศึกษาครั้งนี้ได้ทำการทดสอบการหาความเข้มข้นของอาหารเลี้ยงเชื้อชนิดเติมที่เหมาะสมโดยการใช้วิธีการหาสภาวะที่เหมาะสมสองวิธีร่วมกันได้แก่วิธี Packlett-Burman design และ Sequential Simplex Optimization จากการศึกษาพบว่า การใช้วิธีการหาสภาวะที่เหมาะสมสองวิธีร่วมกันสามารถหาความเข้มข้นของอาหารเลี้ยงเชื้อชนิดเติมที่เหมาะสมโดยลดจำนวนการทดลอง 40.7% และปริมาณน้ำหนักเซลล์แห้งมากกว่า 1.24 เท่า เมื่อเปรียบเทียบกับการใช้ Sequential Simplex Optimization เพียงวิธีเดียว เมื่อนำอาหารเลี้ยงเชื้อชนิดเติมที่เหมาะสมไปใช้ในการเลี้ยงเชื้อด้วยวิธี fed-batch cultivation พบว่าการเติมอาหารเลี้ยงเชื้อด้วยอัตรา 20 ml/h ทำให้มีการเพิ่มการเจริญของเชื้อการสร้างโปรตีนทั้งหมดจากเชื้อ และ scFv anti-p17 activity เพิ่มขึ้น 4.43, 1.48 และ 6.5 เท่า ตามลำดับเมื่อเปรียบเทียบกับการเลี้ยงเชื้อด้วยวิธีปกติคือ batch cultivation

Thesis Title	Fed-batch Medium Modification for Secreted Single Chain Variable Fragment Against HIV-1 p17 Protein Production Using Combined Two Factorial Experiments in *Escherichia coli* Bioreactor	
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ABSTRACT

Biopharmaceutical production programs have been established using fed-batch cell culture protocols, which can support high volumetric productivity while providing low operational complexity. The industry is concerned with developing high-titer cell culture processes to meet increasing market demands and reduce manufacturing costs. Although advancements in bacterial cell engineering have enabled the development of high-performing recombinant cell lines, developments in cell culture media and process parameter settings are required to realize the maximum production of those cells. We investigated two combined optimization methods, Packlett-Burman design and Sequential Simplex Optimization, for optimization of feed medium. The new combined optimization method decreased the time required by 40.7% compared to the experiment trial. Dry cell weight was 1.24 times higher than in the individual method. The fed-batch cultivation with an optimal feeding rate of 20 ml/h increased cell growth, total protein production and scFv anti-p17 activity by 4.43, 1.48 and 6.5 times compared to batch cultivation respectively.

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LIST OF ABBREVIATIONS

%	percent
°C	degree Celsius
Ab	antibody
PBD	Packlett-Burman design
SS	Sequential Simplex Optimization
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electropheresis
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding fragment
Fv	variable fragment
g	gravity
h	hour
HRP	horseradish peroxidase
kDa	kilodalton
M	Molar
mAb	monoclonal antibody
min	minute
mg	milligram
ml	milliliter
OD	optical density
Ig	immunoglobulin
rpm	round per minute
scFv	single chain variable fragment
µl	microliter
µg	microgram

CHAPTER 1

Introduction

1.1 Statement and significant of the problem

P17 is a structural protein that critically involved in the most stages of life cycle of HIV-1. It is also active extracellulary in deregulating biological activity of many different immune cells that involved in HIV pathogenesis. Therefore, p17 protein represents a target for developing the HIV treatment and diagnosis [1]. Since, the recombinant antibodies are widely use, the single chain variable fragment against HIV-1 p17 protein (scFv anti-p17) was developed to use for diagnosis and the therapeutics treatments in HIV-1 patient. It was developed as a recombinant protein which expressed in *Escherichia coli* (*E. coli*). However, the scFv production defect is the low products and incomplete folding processes that usually result in protein accumulation known as inclusion bodies. To provide the large amount of scFvs for for diagnostic and therapeutic research, the recombinant engineering and large scale expression in prokaryotic systems should be developed [2].

Recombinant DNA technology and large-scale culture techniques have been combined to produce various recombinant proteins. High cell density cultivation (HCDC) has been developed to increase production of recombinant proteins in appropriate amounts for research, clinical and industrial approaches [3,4]. Fed-batch cultivation is an effective method which widely used to achieve high cell density of a culture [5]. The addition of concentrated medium in this process can prevent the nutrient limitations and prolong the growth phase leading to higher biomass and product concentrations [6].

To achieve the high cell density by using fed-bath cultivation, the considerable factor that affect growth of *E. coli* is feed medium. The feed medium composes of the concentrated components that required for cell growth besides can inhibit when added

above critical concentrations [7]. Excess carbon sources can cause metabolic by-products and the precipitation of mineral in medium due to difficulty in measurement of cell density and purification. Another point is high ion concentration may affect membrane potential and activate different stress mechanisms [8]. The appropriate amount of each medium component is needed to achieve successful high cell density growth of *E. coli* and high protein productions. In term of industrial approach, the high concentration of medium components increase the cost of production such as complex nitrogen sources which has been reported to help in prolonging the cell growth and increase levels of recombinant protein expression [3, 9]. In order to develop a well-design feed medium which compose of the optimized amount of components. The optimization methods are required to provide feed medium which can be used for higher cell growth and protein production whereas the cost of production was reduced.

The common method of medium optimization is one factor at a time (OF method). It performs by changing one factor at a time while keeping the other at fixed levels. This method is time consuming, laborious and unable to study the interactions between the components. Moreover, it is unpredictable to study each component since the medium contain many components because of the large experiment numbers. For overcoming OF method, the Sequential Simplex Optimization method was used. This method involves changing all factor levels and adjust a large number of factors simultaneously to achieve optimal response of system within few experiments and not requiring any difficult calculation due to reduce time consuming[10]. Sequential Simplex Optimization has been applied in optimization for the process of chemistry, biochemistry and also microbiology including recombinant protein productions [11]. In the multiple factor experiment, the Plackett-Burman design (PBD) has been used to screen for critical factor. PBD presents a fast screening procedure andmathematically computes the significance of large numberof factors in one experiment to save time and maintain reliable information on each component [12,13]. The key factors which necessary to be optimized are evaluated by subsequent experiment. This method can reduce the experiment which conducted by a large number of insignificant factors.

In previous study, we developed the fermentation process to produce secreted single chain variable fragment against HIV-1 p 17 protein (scFv anti-p17) expressed in *E. coli* HB2151 by using batch cultivation. Herein, we continued our work by combine two factorial experiments to modify feed medium using Plackett-Burman design for screening the critical components, then subsequently optimized by Sequential Simplex Optimization and compare to the individual Sequential Simplex Optimization method. The fed-batch process was performed using the optimized medium. This study is the first report demonstrating the medium optimization by combination of Plackett-Burman design and Sequential Simplex Optimization.

1.2 Literature review

1.2.1 Human Immunodeficiency Virus (HIV)

HIV-1 is a lentivirus which infects cells of the human immune system, an infection that cause the disease state known as Acquired Immuno Deficiency Syndrome (AIDS). As an enveloped retrovirus, HIV-1 infection starts with the interaction of the viral envelope proteins and cell surface proteins on the surface of T-cells and macrophages, lead to the fusion of host and viral membranes. After the deposition of the viral core in the host cell cytoplasm, the viral RNA genome is reverse transcribed into DNA. DNA is then transported into the nucleus where it is integrated into chromosomal DNA and replicated by host factors. This formation of proviral infection leads to the expression of viral accessory and structural proteins, which must assemble within the target cell to form functional progeny virions capable of initiating a new round of infection as shown in figure 1.1[14].

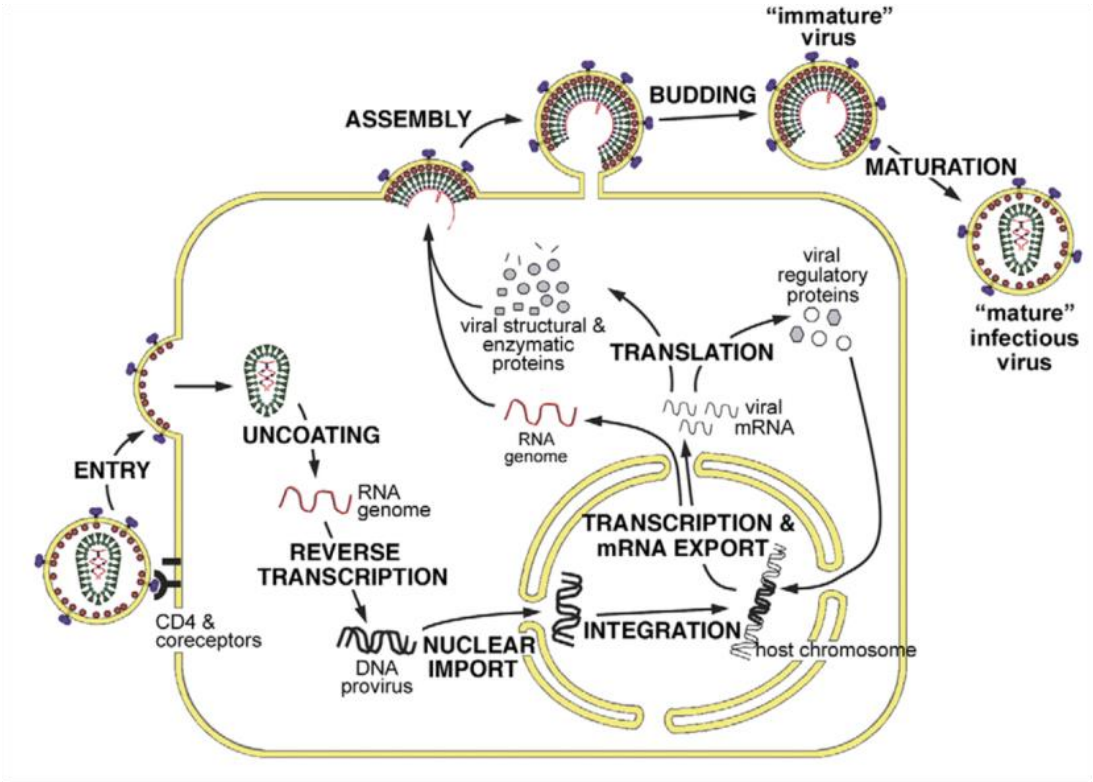

Figure 1.1 Summary of the HIV-1 replication cycle. The HIV-1 enter to the T-cell by binding at the receptor and then uncoating in the cytoplasm. The RNA of the virus is trancripted to DNA by reverse transcriptase. Then the viral DNA is imported to nucleous of the host cell for integrating into the host chromosome. The viral DNA is then trascripted to mRNA which composes of RNA genome and the gene that translated to accessory protein. The virus protein and genome are assembled at the plasma membrane then budding out of the cell and maturation to infectious virus [15].

The HIV-1 genome comprises nine genes. The three major coding regions are 5'-*gag-pol-env*-3' which are synthesized as a single precursor, whilst the other six genes encode the accessory proteins that are multifunctional molecules playing an important role in the pathogenesis of HIV-1 infection. The HIV-1 Gag protein is initially synthesized as a polyprotein precursor encoded by the HIV-1 *gag* gene. During budding of the virus, the HIV-1 protease cleaves the Gag pre-cursor into the mature proteins p17 matrix, p24 capsid, p7 nucleocapsid, p6 and two smaller spacer peptides p1 and p2 [1].

The matrix protein p17 is a important structure of HIV. It is the 132-amino acid polypeptide that forms a protective shell attached to the inner surface of the plasma

membrane of the virus. The matrix protein plays a role in many steps during virus replication. The major function of the matrix protein is to direct binding to and assembly at the plasma membrane [1]. During assembly, the HIV-1 matrix protein interacts to cytoplasmic domain of gp41 directly. p17 acts as a scaffold that brings together Env and Gag proteins in infected cells. It associates with the gp41 cytoplasmic tail during assembly and is responsible for incorporation of Envelope into virus particle [15]. Yu *et al*. reported that the deletions and amino acids substitutions in p17 prevent the incorporation of Envelope into viral particles [16]. The role of the matrix protein in post entry events of viral replication has been established. Mutation of the highly conserved amino acid 20, causes defect in viral DNA synthesis and endogeneous reverse transcription and reduces infectivity of virus. It is suggested that this mutation can cause the unstable of the viral core or reverse transcription complex so that reverse transcription is impaired or viral DNA is degraded [17]. There was a report implied that p17 also act as a chemokine which induce a functional program in monocyte. This study demonstrated that CXCR1 receptor is the receptor which responsible for p17 chemokine-like activity. After binding to CXCR1, p17 involve intracellular signaling pathway which play a role in monocyte migration. The triggering of monocyte and chemotaxis of monocyte leading to increase these cells at the HIV-1 infection sites. This process promotes inflammation which is major events in AIDS pathogenesis [18]. According to the studies suggested the important of p17 protein, this structural protein should be concern as a target of HIV treatment and diagnosis.

The scFv anti-p17 was developed for the aims of diagnosis and therapeutic applications. The scFv anti-p17 was capable of binding to C-terminal epitope of the p17 domain specifically. In previous study, the scFv anti-p17 was constructed in baculoviruses and expressed in BV-infected sf9 cells. The two versions of scfv anti-p17 wrer scFvG2/p17 and scFvE2/p17. The scFvG2/p17 was insoluble while scFvE2/p17 showed the soluble form. They also found that the scFvE2/p17 was secreted to extracellular medium and was able to react with the C-terminal epitope of the p17 [19]. The scFv anti-p17 DNA was cloned to into a pcomb3x vector and transform to E.coli HB2151. It was produced and secreted to the culture medium. The investigation of binding efficiency by ELISA show that scFv anti-p17 was able to bind to the target

protein and the mutant peptide. The result of the binding activity from the peptide based ELISA was correlated to the molecular docking program [20]. There were the evidences that p17 is active extracellularly and can be detected in plasma [1]. Therefore, the diagnosis of HIV was probably developed by detection of p17 protein. The scFv anti-p17 can be used in diagnostic kits. Moreover, the scFv anti-p17 can be developed to use as therapeutic treatments in HIV patient by blocking the p17 protein to interfere the viral replication and prevent AIDS pathogenesis. The scFv anti-p17 should have been used for diagnostic and therapeutic research. According to the expression and production problem of scFv, the recombinant engineering and large scale expression in prokaryotic systems should be developed to provide the large amount of active scFv anti-p17 for the applications.

1.2.2 Recombinant scFv antibody

Recombinant antibody is increasingly being used in medical diagnostic and therapeutic applications. A variety of recombinant antibody formats have been designed for specific applications including engineered modifications to antigen binding and molecular weight (MW). One of the most popular types are scFvs as they have been successfully modified into a number of different Ab formats and are easily expressed by several expression systems. scFvs contain the complete antigen binding site, which includes the variable heavy (V_H) and variable light (V_L) domains of an Ab. The V_H domain is linked to a V_L domain by the polypeptide linker which usually vary from 10 to 25 amino acids in length and typically include hydrophilic amino acids (figure 1.2) [21].

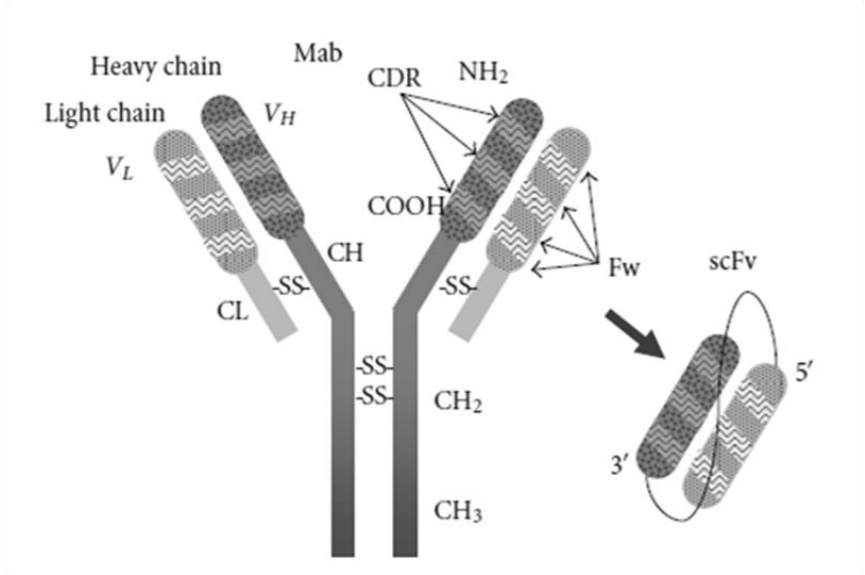

Figure 1.2 Antibody model showing subunit composition and domain distribution along the polypeptide chains. Single-chain fragment variable (scFv) antibody composes of variable heavy (V_H) and variable light (V_L) domains which linked by the polypeptide linker [22].

scFv antibodies have been constructed mainly from hybridoma, spleen cells from immunized mice and B lymphocytes from human. To attain scFv, mRNA is isolated from hybridoma cell followed by reverse transcribed into DNA to serve as a template for antibody genes amplification. This process can create large libraries with a diverse range of antibody V_H and V_L genes. The libraries are screened for target antigen binding and amplified after selection with the target antigen. The difference types of libraries such as phage-display, ribosome-display and microbial cell surface can be used. Each type of the library is suit for difference use due to its limitation. The selection depends on the type of antigen and the amount of scFv which can provide [21].

scFv have been isolated and expressed in various expression systems such as mammalian cell, yeast, plant and also insect cells. The microbial expression system is most often applied for the production of scFv [21]. Miller *et al*. investigated the expression host of the scFv including *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Escherichia coli* which can provide the highest yield of scFv. They reported that the

expression in *E. coli* was the quickest and most consistent way to obtain and characterize scFv for downstream applications. This study suggested that among the microbial hosts, *E.coli* is able to rapidly, efficiently and consistently produce scFv antibodies [23]. *E.coli* was used to produce scFv in many studies. A single chain variable fragment (scFv) specific towards *B. pseudomallei* exotoxin was express in *E.coli* ER2537. The scFv exhibited high specificity towards the *B. pseudomallei* exotoxin. Thus, these scFv could be used for suitable reagents for future affinity purification of the exotoxin [24]. *E. coli* system was used to produce ScFv antibody fragment against the hepatitis B surface antigen (HBsAg) [25] and scFv specific for acidic isoferritin (AIF) for tumor marker of primary hepatic carcinoma [26].

Although *E.coli* is a convenient production system, scFv production in cytoplasm leads to irregular or incomplete folding processes that usually result in their accumulation as insoluble, and usually refractile, aggregates known as inclusion bodies (IBs). Many studies developed the techniques to overcome this problem. One of the process is production of the extracellular scFv which secreted to the culture medium. The scFv fusion with maltose binding protein from *E. coli* can export to periplasmic space and the coexpression of bacteriocin release protein allow the hybrid protein to released into the culture medium [27]. The *E. coli* was used to produce the soluble of a mouse anti-bovine ribonuclease A scFv fused with Fc region of human Ig1 in oxidizing cytoplasm and co-expression of molecular chaperones. The result delicate that the *E. coli* cytoplasmic production system with oxidizing cytoplasm and molecular chaperones might offer a new approach for the soluble production of scFv-Fc and other Fc fusion protein [28].

scFvs are recombinant antibodies that have been used for diagnostic research. The latex agglutination test (LAT) using recombinant scFv for the detection of K99+ enterotoxigenic *E. coli* strains was developed. The assay showed the similar to conventional method with the simple and rapid detection [29]. The recombinant anti-intimin scFv was studied. It was able to recognize the conserved region of purified intimin. Moreover, the investigated showed that anti-intimin scFv can detect the intimin that is the virulence factor in enteropathogenic *Escherichia coli* (EPEC) isolated [30].

The scFv was also used for the therapeutic research. Cattepoel *et al.* developed 22C4 scFv antibody for the treatment of Alzhimer's disease. They characterized a single chain from the complementary determining region (CDRs) of the V_H and V_L domain of 22C4 IgG and expressed in *E.coli*. The 22C4 scFv was entered the brains of APP transgenic mice brain by intranasal application. The result showed that the 22C4 scFv can inhibit Amyloid-beta peptide (Aβ) aggregation and prevent Aβ induced neurotoxicity [31].

1.2.3 Fed-batch cultivation

Fed-batch cultivation has been used to achieve the high cell density of microorganism. This culture method is the fermentation method which add feed medium in the base culture medium. Therefore, the quantity of medium are increased along the cultivation time. Fed-batch cultivation is started with batch cultivation, when the microorganism grows properly, the feed medium is then added to the process continuously. This method is used to overcome the growth limitation of microorganism. The comparison of growth characteristic in batch and fed-batch cultivation is shown in Figure 1.1. In the general batch culture, when microorganisms are grown, the phases of growth that can be detected are lag phase, log phase, stationary phase and dead phase. The growth of microorganism decreases due to nutrition limitation in culture medium. In fed-batch cultivation, the addition of feed medium can provide the necessary nutrients along culture time and prolong the growth phase lead to higher growth and product concentrations [32].

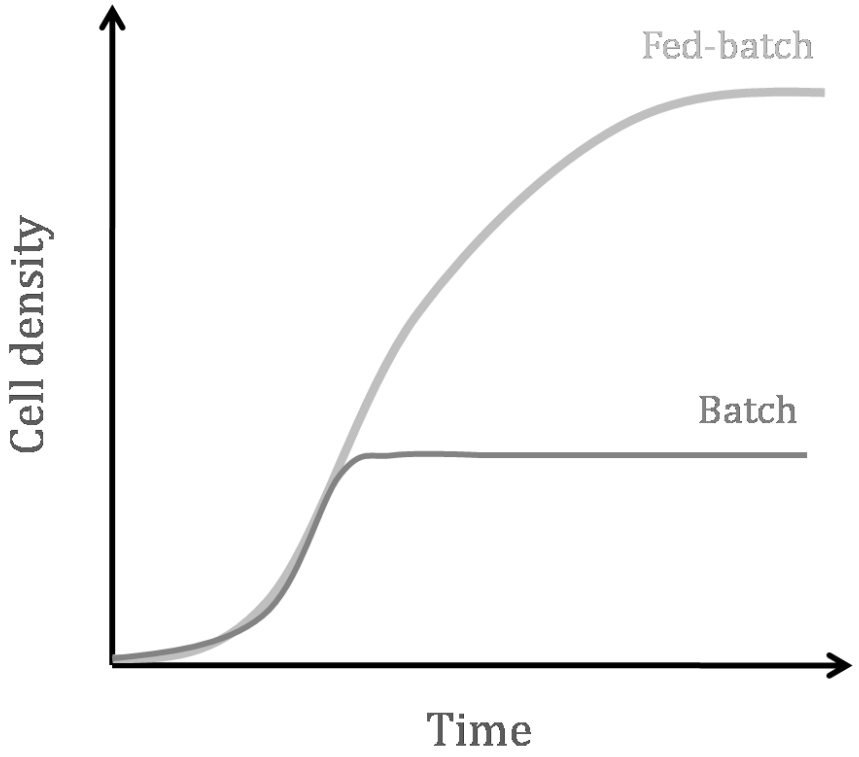

Figure 1.3 The growth characteristic of microorganism in batch and fed-batch cultivation.

The variety of expression hosts can be used for recombinant protein production compose of prokaryotes such as *Escherichia coli* and *Bacillus subtilis* and eukaryotes such as *Saccharomyces cerevisiae* and *Pichia pastoris*. *E. coli* is an appropriate host cell for recombinant protein productions due to high level of expression and it can be grown in simple media so it is easy to be conducted in large scale culture [9, 33]. The recombinant protein productions in *E.coli* by using fed-batch production have been reported. They developed the feed medium which composes of glucose and ammonia as carbon and nitrogen sources to produce recombinant interferon alpha 1 using fed-batch cultivation. They reported that fed-batch cultivation can increase the growth of *E.coli* TG1, the production of recombinant interferon alpha 1 and reduce the accumulation of acetate and other metabolite products [34]. The potato carboxypeptidase inhibitor (PCI) was expressed in *E. coli* BW25113 (pIMAM3) and produced by using fed-batch cultivation. The glycerol feed medium was added in the process. The result

demonstrated that the growth of *E. coli* BW25113 (pIMAM3) was increased and PCI was produced 50% of the total protein [35]. The *E. coli* fed-batch cultivation is widely used for production of recombinant proteins such as recombinant human leptin [36], recombinant 6-deoxyerythronolide B (6-dEB) [37], recombinant human epidermal growth factor (hEGF) [38], human interferon-gamma (hIFN-γ) [39], 5-aminolevulinate (ALA) [40] and TATm-survivin (T34A) [3]. The all results reported that fed-batch cultivation increased the growth of *E.coli* lead to the maximum recombinant protein productions.

Recently, the fed-batch cultivation has been used to produce the recombinant antibodies. The scFv-albumin fusions expressed in *Saccharomyces cecrvisiae* was produced by using fed-batch cultivation. This system increased the levels of secreted scFv-albumin fusions into the supernatant. The pharmacokinetic studies showed that the product improved circulatory half-life [41]. The fed-batch production of M18 single chain antibody (M18 scAb) which is the antibody against anthrax toxin PA was developed. The cell densities of induction were examined. The low density induction increased the cell growth and M18 scAb production yield. The scAb from fed-batch cultivation showed biological activity equivalent to scAb from shake flask cultivation [42]. According to the studies, the strategy of large-scale production of antibody would be useful for clinical and research use. The fed-batch cultivation should be developed for production of the other recombinant proteins.

1.2.4 Medium optimization method

Feed medium is the important factor that affect to the success of fed-batch cultivation. It composes of high concentration of carbon source, nitrogen source and minerals to enhance growth of recombinant cells and protein production. The concentration of medium composition should be concerned. It was reported that the growth of *E. coli* can be limited by other nutritional requirements including carbon, nitrogen, phosphorus, sulfur, magnesium potassium iron, manganese, zinc, copper and some growth factors [43]. The required media ingredients can inhibit growth when add at high concentrations. Resenberg *et.al.* established that nutrients such as glucose at a concentration of 50 g/l, ammonium at 3 g/l, iron at 1.15 g/l, magnesium at 8.7 g/l,

phosphorus at 10 g/l and zinc at 0.038 g/l inhibit *E. coli* growth [44]. Moreover, the high concentration of medium compositions lead to precipitation which affect purification operations and monitoring devices. Therefore, the well-design feed medium should be optimized.

The optimization method which generally used is one factor at a time method (OF method). The classical method of changing one variable at a time while keeping others at a constant level was found inefficient. It does not explain interaction effects among the variables and their effect on the fermentation process. Moreover it is a time consuming laborious practice because of the large number of experiments. To overcome the limitation of OF method, the factorial experiment is used for medium optimization [12, 13].

1.2.4.1 Plackett-Burman design (PBD)

Plackett-Burman design is a factorial experiment that allows efficient screening of key variables for further optimization. For the given number of observation, the linear effect of all factors can be screened with maximum accuracy. This design is practical when investigating large number of factors to produce optimal or near optimal response. Plackett-Burman design (PBD) is a fraction of a two-level factorial design and allows the investigation of n variables (medium components) with at least $n+1$ experiments. Each variable is represented at two levels, high and low, which are denoted by (+) and (−), respectively. This method performs by selection of the most appropriate design matrix which determined by the number of factors selected for the experimentation. Then the experiment is run and the samples is measured, the data from the experiment are used to calculate the effects and to determine the statistical significance of those effects [12].

PBD was used to screen the significant factor that affect the production of alkaline protease expressed in *Bacillus* sp. RKY3. The significant factors were corn starch, yeast extract, corn steep liquor and inoculum size. Those factors were further optimized by response surface methodology. The production of alkaline protease in optimized medium presented 2-3 fold increased and higher enzyme activity [45]. The

screening of significant medium composition which had effect on flavin mononucleotide (FMN) production by *Candida famata* 13-76 demonstrated that the significant factor were KH_2PO_4, $CaCl_2$, $(NH_4)_6Mo_7O_{24}$, $CuSO_4$ and yeast extract. These five factors were optimized by central composite design (CCD). In optimized medium, the growth of *Candida famata* 13-76 and FMN production were increased [46]. PBD was used to obtain the optimized concentration of glycerol in medium for ethanol production by *E. coli*. The cells can increase ethanol production in optimized glycerol medium [47]. The medium optimization by PBD followed by response surface methodology showed that the significant factor that affected riboflavin production in *Bacillus subtilis* were glucose, $NaNO_3$, K_2HPO_4, $ZnSO_4$ and $MnCl_2$. After the optimization, the optimized medium was used for fed-batch cultivation. The growth of *Bacillus subtilis* and riboflavin production were improved [48]. Moreover, PBD was used in the secreted renilla luciferase production in *Bacillus subtilis* using fed-batch cultivation. The feed medium composition was screened by PBD to examine the significant factor and further optimized by Box-Behnken design. The production using the optimized medium showed the increment growth of *Bacillus subtilis* and renilla luciferase production [49]. According to the studies, the PBD is useful for screening the key factor that affect to the experiment. However, a key factor has to be optimized by subsequently experiment such as response surface methodology which widely used. In this study, the PBD will be used to screen the significant factor then the optimization will be performed by Sequential Simplex Optimization method.

1.2.4.2 Sequential simplex optimization

Sequential Simplex Optimization (SS) is a multivariate self-seeking chemometric technique for locating the optimum on a response surface. It will be originally developed for computer curve fitting of functions and has been appropriated for use in chemical applications. A simplex is defined as a geometrical figure that has a number of vertexes equal to one more than the number of dimensions in factor space. For example, for 2 factors, the simplex is a triangle. The innitial simplex is composed of k + 1 vertex which k is the number of factors [10, 11]. Each movement involves changing all factor levels, therefore factor interactions are accommodated in this

scheme. It can inform possible factor interactions whereby the optimum for one variable may depend on the particular level of other factors. There are two major types of sequential simplex algorithm which are fixed-size simplex and variable-size simplex which used in this study [50]. The sequential simplex algorithm was calculated by worksheet (Table 1.1). The rules of variable-size simplex were 3 main rules. There were as following:

1. Rank the vertexes of the first simplexon a worksheet in decreasing order of response from best to worst. Put the worst vertex into the row labeled W

2. Calculate and evaluate R:
   A. If $N \leq R \leq B$,use simplex BNR and go to 3
   B. If $R > B$, calculate and evaluate E
      If $E \geq B$, use simplex BNE and go to 3
      If $E < B$, use simplex BNR and go to 3
   C. If $R < N$
      If $R \geq W$, calculate and evaluate C_R,use simplex BN C_R and go to 3
      If $R < W$, calculate and evaluate C_W,use simplex BN$C_W$and go to 3

3. Never transfer the current row labeled W to the next worksheet. Always transfer the current row labeled N to the row labeled W on the next worksheet. Rank the remaining retained vertexes in the order of decreasing response on the new worksheet, and go to 2 [51].

Table 1.1 Worksheet for two factor variable size simplex calculation [51].

Simplex No.____→____	Factor					
	X_1	X_2	Response	Rank	Vertex number	Times Retained
Coordinates of Retained vertexes				B		
				N		
Σ						
$\overline{P} = \Sigma/k$						
W				W		
$\overline{P} - W$						
$R = \overline{P} + (\overline{P} - W)$				R		0
$(\overline{P} - W)/2$						
$C_w = \overline{P} - (\overline{P} - W)/2$				C_w		0
$C_r = \overline{P} + (\overline{P} - W)/2$				C_r		0
$E = R + (\overline{P} - W)$				E		0

Sequential Simplex Optimization has been applied in optimization for the field of chemistry. For example, the SS method was used to optimize the ion-pair reversed phase high performance liquid chromatographic (IP-RPHPLC) analysis of 4-(2-pyridylazo) resorcinol (PAR) chelates of Co(II), Ni(II) and Cr(III). It took only 19 experiments to optimize three-variable system with good resolution and short analysis time [52]. The optimization condition for separation of a mixture of 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs) by high performance liquid chromatography (HPLC) using the SS method showed good resolution in reasonable elution times and high sensitivity for the full range of PAH compounds [53]. Moreover, SS method was used to develop Cremophor-free lipid-based paclitaxel (PX) nanoparticle. The optimized nanoparticles was successfully entrapped paclitaxel and were stable at 4 ºC over five months in PBS at 37 ºC over 102 h [54]. The SS method was applied to use for environment resolution. This method was used to optimize coagulants for phosphorus removal in wastewater. It can determine the optimum conditions with a small number of

experiments (18–25) and improve phosphorus removal efficiencies by at least 30% for each coagulant [55]. In the food industrials, SS method was used to optimize the separation of biogenic amine in fish with ion exchange high performance liquid chromatography. This application is useful for determination of the quality of fish [56].

The SS method was applied in biochemistry and also microbiology including recombinant protein productions. SS method was employed for the optimization of growth and carotenoid production in *Rhodotorula glutinis* using a substrate containing hydrolyzed mung bean waste flour. The result showed that cell dry weight and carotenoid content were respectively 43 and 20% higher than optimized by one factor at a time method with less experiment number [10]. Butr-Indr *et.al.* used SS method to optimized the recombinant biotinylated SVV-BCCP production by *E. coli* in mineral ion supplemented medium. Comparison to one factor at a time method, the cell density and biotinylated SVV-BCCP production were higher with minimum number of experiments [11]. The optimization of growth conditions for single cell protein production by yeast cells with SS method resulted that the number of experiments was decreased to 28 runs from 540. When a factorial method was applied, the cost of yeast biomass production operating was decreased to 5% [57]. Furthermore, SS method was emploted to investigate the effect of aeration and agitation rates of protease production by *Staphylococcus aureus* mutant RC128. The modified rotating simplex method had been successfully used to examine the best combination of agitation and aeration rates for maximum production of extracellular proteases in a stirred tank bioreactor operating with the batch system [58]. The SS method is useful for optimization of the system with a large number of factors. This method has been demonstrated as the rapid optimization strategy for chemistry, biochemistry and microbiology processes.

1.3 Objectives

1. To apply the combined Packlett-Burman design and Sequential Simplex Optimization for modification of feed medium.
2. To use the modified feed medium for single chain variable fragment against HIV-1 p17 protein by using fed-batch cultivation.

CHAPTER 2

Materials and methods

2.1 Chemicals and equipment

Chemicals and equipments used in this study were shown in Appendix A and Appendix B

2.2 Bacterial strain and plasmid vector

Bacterial strain *E. coli* HB2151 containing pComb3X-scFvp17 expression vector were kindly provided by Prof. Dr. Chatchai Tayapiwatana, Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Science, Chiang Mai University.

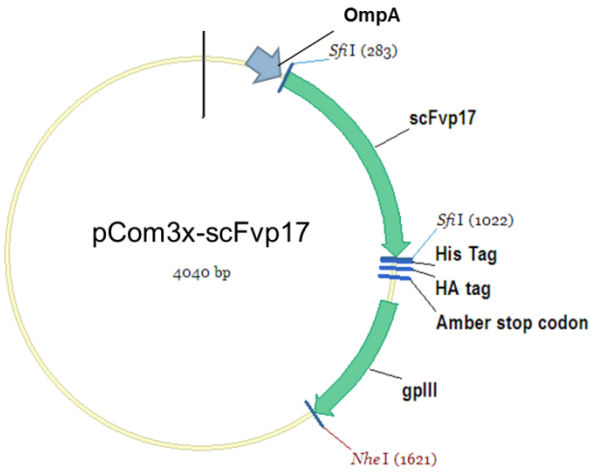

Figure 2.1 Schematic figure of pComb3X-scFvp17 phagemid vector. The scFv anti-p17 was cloned into pComb3X-SS phagemid vector *via Sfi*I restriction site. The scFv anti-p17 was fused with OmpA signal sequence at the N-terminal and the C-terminus with His_6 and HA tag, amber stop codon and gpIII, respectively.

2.3 Cultivation of *E. coli* HB2151 containing pComb3X-scFvp17

2.3.1 Batch fermentation

Batch fermentation was carried out using a 5-L fermenter (Minifor, Infors-ht, Switzerland). Precultures of strain *E. coli* HB2151 containing pComb3X-scFvp17 were prepared in Terrific broth containing 120 µg/ml ampicillin for 35°C, shaking at 200 rpm overnight. The overnight cultures were then inoculated into 100 ml of terrific broth to achieve the initial cell density of 0.5 at OD_{600}. The seed culture was transferred into the fermenter with a broth volume of 1 L. The operational condition was set at 35°C. The cells were induced with 58 µM of Isopropyl-beta-*D* thiogalactopyranoside (IPTG) at density (OD_{600}) of 4 and decrease temperature after induction to 25°C. The inoculation was incubated for 18 h at agitation rate of 400 rpm.

2.3.2 Fed-batch fermentation

Fed-batch fermentation in flask scale were carried out in 500 ml Erlenmeyer flasks containing 100 ml of terrific broth supplemented with 120 µg/ml ampicillin and 1% (w/v) glucose. The culture condition was 35°C and shaking rate at 200 rpm. When cell density reach to 4 at OD_{600}, the optimized feed medium was added and incubated at 25°C for 24 h to determine final dry cell weight.

Fed-batch fermentation in fermenter scale was started with batch fermentation as described above. When the cell density reach to 4 at OD_{600}, the 500 ml of optimized feed medium was added at 3 different feeding rates of 10, 20 and 50 ml/h. The cells were continuously induced with 58 µM of IPTG by mixing with feed medium and incubation at 25ºC at agitation rate 400 rpm.

2.4 Cell growth determination

Cell growth was monitored by measuring the optical density at 600 nm. Dry cell weight was also determined. The 15 ml of culture medium were centrifuged at 4000xg for 15 min. The pellets were washed with 15 ml of PBS and dried at 80°C for 72 h before weighing.

2.5 Cell extraction and protein precipitation of recombinant extracellular scFv anti-p17 protein

2.5.1 Cell extraction by subcellular fractionation method

The bacterial cultures were harvested by centrifugation at 4500xg, 4°C for 30 min. The supernatant was precipitated with 70% $(NH_4)_2SO_4$ saturation and centrifuged at 8000xg, 4°C for 30 min. Supernatant was removed. The precipitated protein was reconstituted in 1 ml of Tris-buffered saline (TBS) pH 8.4. Protein suspension was dialyzed overnight against TBS buffer pH 8.4 at 4°C. The precipitated protein containing 10% (v/v) glycerol was kept at -70ºC. The cell pellets were disrupted by hypotonic solution method. The cell pellets were resuspended in 5 ml of ice-cold PBS and transferred to 1.5 ml microcentrifuge tube. The resuspended cells were centrifuged at 3500xg, 4ºC for 10 min and the supernatant was removed. The pellet was resuspended in 5 ml of fractionation buffer (1M Tris-HCl pH8.0, 0.5 M Na_2EDTA, 20% sucrose (w/v)) and incubated at room temperature for 10 min. After centrifugation at 10000xg, 4ºC for 10 min, the supernatant was removed. The pellet was resuspended in 2.5 ml of ice-cold 5mM $MgSO_4$, incubated on ice for 10 min and centrifuged at 10000xg, 4ºC for 10 min. The periplasmic fraction was collected and stored in 10% (v/v) glycerol at -70ºC. The pellet was resuspended in 5 ml of ice-cold PBS and centrifuged at 10000 ×g, 4ºC for 10 min. The supernatant was removed. The pellet was resuspended in 2.5 ml of ice-cold PBS and sonicated on ice for 30s by sonicator. The sonication process was done 3 times. The solution was centrifuged at 10000xg, 4ºC for 10 min. The cytoplasmic fraction was collected and stored in 10% (v/v) glycerol at -70ºC.

2.5.2 Protein precipitation by 70% ammonium sulfate ($(NH_4)_2SO_4$) saturation

The 100 ml of culture broth was harvested by centrifugation at 4500xg, 4°C for 15 min. The supernatant was precipitated with 70 % $(NH_4)_2SO_4$ saturation in an ice bath and centrifuged at 8000xg, 4°C for 30 min. Supernatant was removed and the precipitated protein was reconstituted with 1 ml of TBS buffer, pH 8.4. Protein

suspension was dialyzed overnight in TBS buffer at 4°C. The precipitated protein containing 10% (v/v) glycerol was kept at -70ºC.

2.6 Detection of scFv anti-p17

2.6.1 Determination of secreted total protein by BCA protein assay

The secreted total protein from protein precipitation method was determined by BCA protein assay reagent kit (Pierce, USA) with microplate procedure. The BCA working solution was prepared by mixing 50:1 (BCA Reagent A: BCA Reagent B). The albumin (BSA) standard was diluted for a set of protein standard including 2000, 1500, 1000, 750, 500, 250, 125 and 25 µg/ml of final BSA concentration. The 25 µl of secreted protein and BSA standard were pipetted into a microplate well. The 200 µl of working solution was added to each well and mixed plate thoroughly on a plate shaker for 30s. The plate was covered and incubated at 37°C for 30 min. The plate was measured the absorbance at 562 nm on a plate reader.

2.6.2 Detection of scFv anti-p17 by Enzyme-linked immunosorbent assay (ELISA)

A microtiter plate was coated with 50 µg of p17 peptide (DTGHSSQVSQNY) (Genscript, USA) in 50 µl of carbonate/bicarbonate buffer, pH 9.6 at 4°C for 18 h. The coated wells were blocked with 200 µl of 2% (w/v) bovine serum albumin (BSA) in TBS for 1 h at room temperature. The wells were washed for 3 times with washing buffer (0.05% (v/v) Tween 20 in phosphate buffer saline (PBS, pH 7.2)). The 50 µl of precipitated protein (200 µg/ml) diluted with 2% (w/v) BSA in TBS was added into each p17 peptide well. After incubation at room temperature for 1 h, the plate was washed 3 times with washing buffer. The unbound antigen was removed. The bound scFv anti-p17 was traced by adding 50 µl of 50 µg/ml mouse monoclonal anti-HA-HRP conjugated antibodies. After 1 h incubation and washing step, 50 µl of TMB color substrate (Pierce, USA) was applied to each well. The plate was incubated at room temperature for 30 min for color development. The reaction was stopped by adding 50

µl of 1 N HCl and the optical density (OD) was measured at 450 nm. The mAb-p17 was used as an antibody control in the ELISA system.

2.6.3 Detection of scFv anti-p17 by SDS-PAGE and western immunoblotting

The scFv anti-p17 was separated on a 12.5% SDS-PAGE. The samples were boiled for 5 min and loaded onto the well of SDS-PAGE. Electrophoresis was carried out by applying constant voltage at 100 volts using 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 as an electrophoretic buffer. After electrophoresis, the separated proteins were stained with coomassie blue and transferred to a polyvinylidene fluoride (PVDF) membrane. The blotting was carried out by applying constant current at 58 mA using 0.025 M Tris, 0.192 M glycine, 0.5% (w/v) SDS and 20% (v/v) methanol, pH 8.3 as blotting buffer. The membrane was blocked at 4°C for 18 h by 5% (w/v) skim milk in PBS pH 7.2. After blocking step, the membrane was incubated with mouse anti-HA monoclonal antibodies (Sigma,USA) 1:3000 diluted in 5% (w/v) skim milk in PBS pH 7.2 for 1 h at room temperature on shaking platform. The membrane was washed for 5 times with 0.05% (v/v) Tween 20 in PBS pH 7.2. The membrane was incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin antibodies (KPL, USA) 1:3000 diluted in 5% skim milk in PBS pH 7.2 for 1 h at room temperature on a shaking platform. Unbound conjugated was washed out for 4 times with 0.05% (v/v) Tween 20 in PBS pH 7.2. The membrane was washed with PBS, pH 7.2 and added with chemiluminescent substrates for the last step. The immunoreactive bands were visualized by a chemiluminescent detection system (Pierce, USA).

2.6.4 Quantitative determination of scFv anti-p17

The 8 µg of crude protein sample that contained scFv anti-p17 was mixed with 1.8 µg standard BSA. The scFv anti-p17 protein was separated by 12.5% SDS-PAGE. Measurements of scFv anti-p17 protein bands intensity on gels were carried out by ImageQuant TL programme of Image Scanner III (GE healthcare, USA). The presence of the particular proteins and the peak area were measured and calculated into the known quantity of the internal standard (standard BSA) according to the formula:

$$X = \frac{P_{protein\ peak}}{P_{int.\ standard}} \times \frac{S_{int.\ standard}}{S_{total\ protein}} \times 100[\%]$$

Where: X, protein content in the total quantity of scFv anti-p17 (%); $P_{protein\ peak}$, peak surface area corresponding to the examined protein; $P_{int.\ standard}$, peak surface area corresponding to the internal standard (standard BSA); $S_{totalprotein}$, crude protein concentration that loaded onto gel; $S_{int.\ standard}$, internal standard concentration in the crude sample loaded onto gel. The method was modified from Tomaszewska-Gras *et al*. [59]

2.7 Feed Medium optimization

2.7.1 Medium optimization by individual Sequential Simplex Optimization

The medium formulation consisting of the factors was optimized by Sequential Simplex Optimization**.** The total of six factors composed of glycerol, yeast extract, tryptone, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 7H_2O$. The initial simplex was selected by choosing n+1 experimental designs that covered a wide range of values in the factor space. The responses of the experiments were ranked according to the definitions: best response (B), next to the worst response (NW), concentration on the worst side (Cw) and reflection (R). The vertex condition calculated by the SS method that produced the worst response in the initial simplex was replaced by a new set of factors for the next experiment in n-space by reflecting through the centroid of the remaining plane in hyperspace (formed by the n remaining vertices). Reflections, expansions and contractions were accomplished by following the variable-sized simplex rules. Boundary violations were handled by assigning the worst response to the results of that experiment and proceeding to calculate the next set of factor levels to be tried as a Cw contraction.

The six factors were optimized by this method. The initial conditions were 7 experiments (table 2.1). Each experiment was carried out in triplicate trials. The *E. coli* HB2151 containing pComb3X-scFvp17 expression vector was culture in flask scale. The culture was started in 100 ml of terrific broth supplemented with 1% (w/v) glucose

and 120 µg/ml ampicillin. When the cell density reach OD_{600} = 4, The 50 ml of feed medium from each experiment was added to the cultures. After incubation for 24 h, the culture broth was collected to determine dry cell weight.

Table 2.1 The initial experiment of Sequential Simplex Optimization method

Vertex	Glycerol (%w/v)	Yeast extract (g/L)	Tryptone (g/L)	$MgSO_4•7H_2O$ (g/L)	$FeSO_4•7H_2O$ (g/L)	$CaCl_2•7H_2O$ (g/L)
1	1.5	47.3	6.0	39.0	0.4	0.4
2	1.4	43.0	5.5	36.0	0.7	0.4
3	1.2	38.7	5.0	33.0	0.6	0.7
4	1.0	34.4	4.5	30.0	0.6	0.6
5	1.9	30.1	4.0	27.0	0.5	0.6
6	1.8	55.9	3.5	24.0	0.5	0.5
7	1.7	51.6	6.5	21.0	0.4	0.5

Table 2.2 Sequential Simplex Optimization method worksheet for experiment design

	factor									
	Glycerol (%w/v)	Yeast extract (g/L)	Tryptone (g/L)	$MgSO_4•7H_2O$ (g/L)	$FeSO_4•7H_2O$ (g/L)	$CaCl2•7H2O$ (g/L)	Response	Rank	Vertex number	Times retaimed
Coordinates of retained vertexes							B			
							N			
							N			
							N			
							N			
							N			
							W			
ε										
P = ε/k										
w										
(P-W)										
R = P + (P-W)										
(P-W)/2										
Cw= P - (P-W)/2										
Cr = P + (P-W)/2										
E = R + (P-W)										

2.7.2 Medium optimization by combined Plackett–Burman design and Sequential Simplex optimization.

The six factors were screened to determine the significant factor which affected the growth of *E. coli* HB2151 containing pComb3X-scFvp17 by using Plackett-Burman design. The factors were set the high (+1) and low (-1) level as presented in the table 2.3

Table 2.3 The level of selected factors for the Plackett–Burman design

Factors	Factor setting	
	Low (-1)	High (+1)
A. Glycerol (%)	0.1	5
B. Yeast extract (g/L)	0.5	20
C. Tryptone (g/L)	0.5	25
D. $MgSO_4 \cdot 7H_2O$ (g/L)	0.05	10
E. $FeSO_4 \cdot 7H_2O$ (g/L)	0.05	1
F. $CaCl_2 \cdot 7H_2O$ (g/L)	0.05	1

For six factors, the experimental design matrix of Plackett–Burman design was selected. The experimental designs were shown in the table 2.4

Table 2.4 The experimental designs of Plackett–Burman design

Experiments	Factors						
	A	B	C	D	E	F	G
1	+1	+1	+1	-1	+1	-1	-1
2	-1	+1	+1	+1	-1	+1	-1
3	-1	-1	+1	+1	+1	-1	+1
4	+1	-1	-1	+1	+1	+1	-1
5	-1	+1	-1	-1	+1	+1	+1
6	+1	-1	+1	-1	-1	+1	+1
7	+1	+1	-1	+1	-1	-1	+1
8	+1	+1	+1	-1	+1	-1	-1

The *E. coli* HB2151 containing pComb3X-scFvp17 expression vector was cultured in flask scale. The culture was started in 100 ml of terrific broth supplemented with 1% (w/v) glucose and 120 µg/ml ampicillin. When the cell density reach OD_{600} = 4, The 50 ml of feed medium from each experiment was added to the cultures. After incubation for 24 h, the culture broth was collected to determine dry cell weight. Each experiment was carried out in triplicate trials. Then the dry cell weight was analyzed to obtain the significant factor by statistical software SPSS. The medium formulation consisting of the significant factor was subsequently optimized by Sequential Simplex Optimization as describe in 2.7.1**.**

CHAPTER 3

Results

3.1 Medium optimization by individual Sequential simplex optimization

The SS method was used to optimize the concentration of feed medium composition for fed-batch production of extracellular recombinant scFv anti-p17 protein. The experiment was investigated by varying six parameters including glycerol, yeast extract, tryptone, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 7H_2O$. The experiment was started with 7 conditions for an initial simplex vertex of SS analysis and calculated for next condition (vertex) by worksheet of SS method. In worksheet, response values were requiring result from the experiment. Response values were listed for the row that corresponded to simplex vertex. The vertex giving the best response (B) was listed on the top row. The vertex giving the next to the worst response (N) was listed on the bottom row of the coordinates of the retained vertex. The worst vertex was rejected, but was listed in the middle of the worksheet in the wastebasket vertex (W) row. The next vertex was calculated by SS formula in the worksheet. Each vertex performed in a triplicate trial. In this study, the dry cell weight of *E.coli* HB2151 was used as the response function values. All six factors were calculated in different levels in each vertex. The feed medium from each vertex was added into the bacterial flask culture and detected dry cell weight after feeding for 24 h as the response. The first seven experiments constituted the initial simplex (for 6 factors). The feed medium optimization using SS method took 27 trials. The constant of response presented in vertex 13-17. Vertex 14 was selected as the optimum because it achieved the highest dry cell weight of 2.62 g/L as demonstrated in the table 3.1.

Table 3.1 Results of the movement of the simplex during the experiment

Vertex	Rank[a]	Glycerol (%w/v)	Yeast extract (g/L)	Tryptone (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	$FeSO_4 \cdot 7H_2O$ (g/L)	$CaCl_2 \cdot 7H_2O$ (g/L)	Response[b] DCW (g/L)
1	NW	1.5	47.3	6.0	39.0	0.4	0.4	2.24±0.30
2	B	1.4	43.0	5.5	36.0	0.7	0.4	2.37±0.10
3	NW	1.2	38.7	5.0	33.0	0.6	0.7	2.00±0.10
4	NW	1.0	34.4	4.5	30.0	0.6	0.6	2.12±0.26
5	NW	1.9	30.1	4.0	27.0	0.5	0.6	2.00±0.30
6	NW	1.8	55.9	3.5	24.0	0.5	0.5	2.06±0.08
7	W	1.7	51.6	6.5	21.0	0.4	0.5	1.96±0.22
8	R	1.3	31.3	3.0	42.0	0.6	0.6	1.62±0.09
9	Cw	1.6	46.6	5.6	26.3	0.5	0.5	1.52±0.10
10	R	1.4	36.6	3.9	36.8	0.6	0.5	1.65±0.08
11	Cw	1.4	39.1	4.3	34.1	0.5	0.5	1.09±0.06
12	R	1.5	44.1	5.2	28.9	0.5	0.5	1.74±0.30
13	Cw	1.5	42.8	5.0	30.2	0.5	0.5	2.60±0.00
14	R	1.9	45.8	4.5	29.1	0.4	0.3	2.62±0.06
15	E	2.2	49.4	4.2	27.1	0.3	0.1	2.53±0.06
16	R	1.3	31.7	6.4	39.8	0.5	0.3	2.58±0.04
17	R	2.2	52.2	6.0	37.0	0.3	0.1	2.63±0.09
18	R	2.7	61.2	6.8	40.1	0.2	0.1	2.05±0.15
19	Cr	1.9	41.0	1.5	27.4	0.5	0.1	2.85±0.20
20	R	2.2	37.8	3.8	21.6	0.6	0.1	2.07±0.18
21	Cr	2.3	44.6	4.7	27.5	0.2	0.1	2.09±0.30
22	R	1.6	43.4	5.3	33.9	0.5	0.3	3.07±0.30
23	E	1.3	36.3	6.3	38.7	0.6	0.4	2.87±0.02
24	R	1.5	39.6	5.8	35.8	0.5	0.3	2.05±0.15
25	Cw	2.1	55.5	4.2	25.6	0.5	0.3	2.16±0.10
26	R	1.5	37.6	5.8	36.2	0.5	0.3	2.47±0.08
27	R	1.9	49.6	4.7	29.2	0.5	0.3	2.57±0.02

[a] In sequential simplex terminology, B is best response, NW is next-to-the-worst response, Cw is contraction on the worst side, Cr is contraction on the reflection side, R is reflection and E is expansion.

[b] Values are mean and standard deviation of triplicate determinations.

The movement of the simplex toward the optimum was summarized in Figure 3.1 in which the response function (dry cell weight) was plotted as a function of vertex number as the simplex moves through factor space. The result showed that the response values decreased continually in vertex 1-11. Then the response value increased in vertex 12-13 and appeared to being constant in vertex 13-14. Besides the response values still varied in vertex 15-26 and backed to constant level at vertex 27.

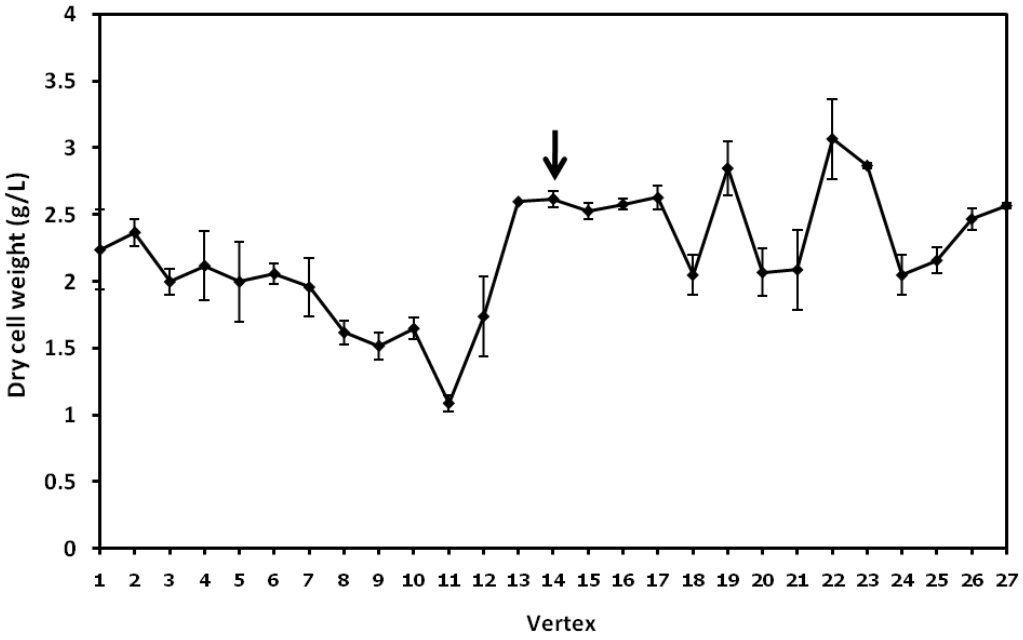

Figure 3.1 Response function for the Simplex Optimization of *E.coli* HB2151 growth indicated in dry cell weight and optimum condition was shown by the arrow.

The levels of each factor as a function of vertex number were illustrated in Figure3.2. Concentration of glycerol, yeast extract, tryptone and $MgSO_4 \cdot 7H_2O$ showed considerable variation. They were varied prosperously until in the response were constant near the optimum point. This suggested that appropriate concentration of glycerol, yeast extract, tryptone and $MgSO_4 \cdot 7H_2O$ were available at vertex 13-17 where the response of dry cell weight is high (Figure3.1). The concentrationof $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 7H_2O$ was decreased at the optimal condition. The optimal concentration of feed medium optimized by individual SS method composed of glycerol 1.8 % (w/v),

yeast extract 45.8 g/l, tryptone 4.5 g/l, $MgSO_4 \cdot 7H_2O$ 29.0 g/l, $FeSO_4 \cdot 7H_2O$ 0.4 g/l and $CaCl_2 \cdot 7H_2O$ 0.3 g/l.

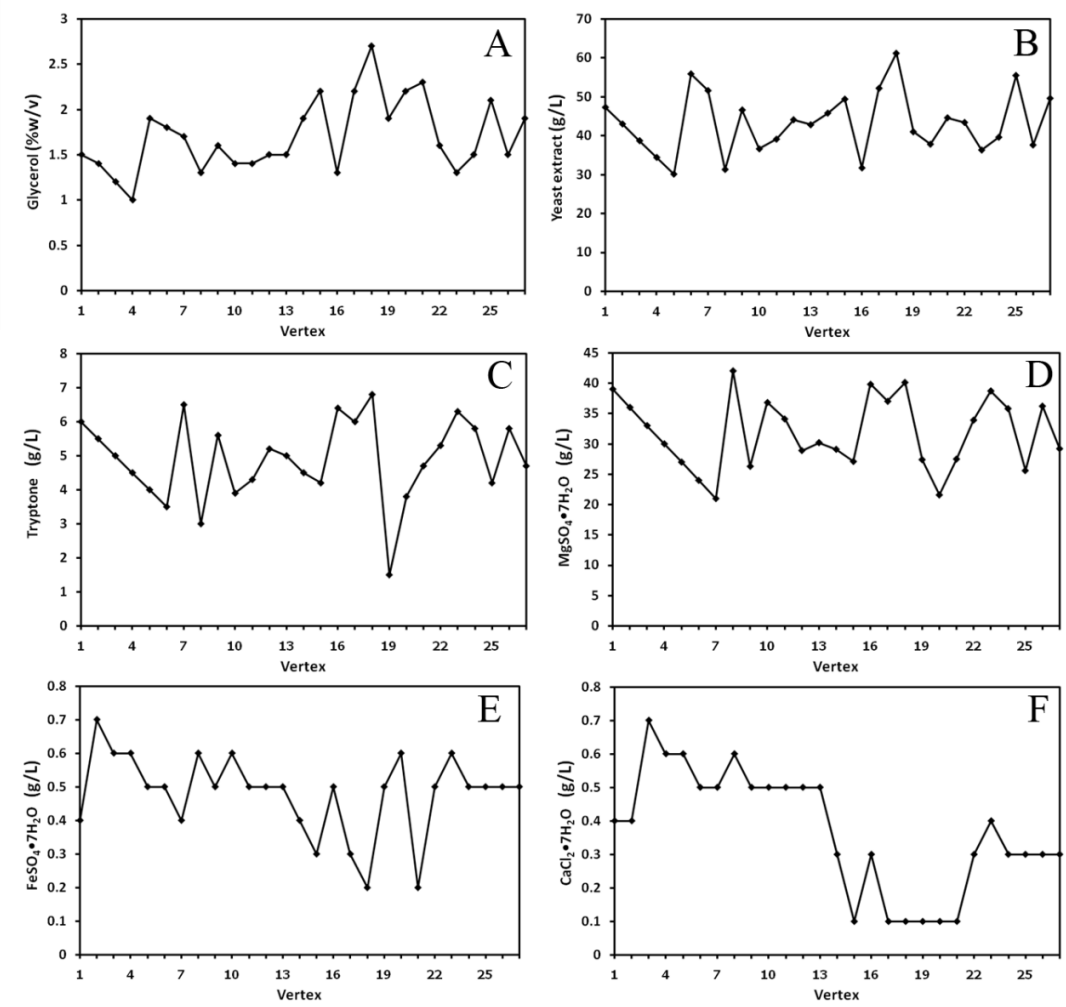

Figure 3.2 Variation of factors and the response function throughout the Simplex Optimization. A: % Glycerol (w/v), B: Yeast extract (g/L), C: Tryptone (g/L), D: $MgSO_4 \cdot 7H_2O$ (g/L), E: $FeSO_4 \cdot 7H_2O$ (g/L) and F: $CaCl_2 \cdot 7H_2O$ (g/L).

3.2 Medium optimization by combined Plackett–Burman design and Sequential Simplex optimization

3.2.1 The screening of significant factor by Plackett–Burman design

Plackett–Burman design was used for initial screening of significant feed medium components. Six components were set with two levels (e.g. the high and low level). Glycerol was recruited for study as the carbon source. Tryptone and yeast extract were chosen as the nitrogen source. The $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 7H_2O$ were used for mineral supplementation. Plackett–Burman experimental design for 8 trials with two levels of each factors were carried out for fed-batch fermentation in flask cultures of *E.coli* HB2151. The growth of bacterial strain as the dry cell weight was determined after feeding of the various medium as dedicated in the table 3.2.

Table 3.2 Plackett–Burman experimental design matrix with the observed response (dry cell weight)

Trials	A	B	C	D	E	F	Dry cell weight[a] (g/L)
1	+1	+1	+1	-1	+1	-1	2.43±0.03
2	-1	+1	+1	+1	-1	+1	1.90±0.09
3	-1	-1	+1	+1	+1	-1	1.96±0.19
4	+1	-1	-1	+1	+1	+1	1.96±0.06
5	-1	+1	-1	-1	+1	+1	1.87±0.06
6	+1	-1	+1	-1	-1	+1	2.24±0.06
7	+1	+1	-1	+1	-1	-1	1.99±0.12
8	+1	+1	+1	-1	+1	-1	2.12±0.08

[a]Mean and standard deviation values of triplicate determinations.

After the experiment trials, the results of dry cell weight from each experiment were analyzed by statistical program SPSS to examine the significant factor which affect the growth of *E.coli* HB2151. The result from the analysis of variance (ANOVA) and parameter estimates using SPSS was summarized in Table 3.3. The *P* value indicates a statistical confidence of a factor estimate. A *P* value of < 0.2 is used as a

cutoff point for selecting the significant factor. The result indicated that glycerol, tryptone and $MgSO_4 \cdot 7H_2O$ were significance for growth of bacteria. Among them, glycerol and tryptone have the positive estimate, indicating their conducive effect but $MgSO_4 \cdot 7H_2O$ with the negative estimate would be adversed to growth of bacteria.

Table 3.3 Result of ANOVA for the Plackett–Burman design.

Factor	**Estimate**	***P* value**
A. Glycerol (%)	0.643	0.086
B. Yeast extract (g/L)	0.017	0.969
C. Tryptone (g/L)	0.509	0.198
D. $MgSO_4 \cdot 7H_2O$ (g/L)	-0.584	0.128
E. $FeSO_4 \cdot 7H_2O$ (g/L)	0.061	0.885
F. $CaCl_2 \cdot 7H_2O$ (g/L)	-0.368	0.370

3.2.2 The significant feed medium component optimization by Sequential Simplex optimization

The significant feed medium components including glycerol, tryptone and $MgSO_4 \cdot 7H_2O$ were selected for further optimized by Sequential simplex optimization. The feed medium from each vertex was added into the bacterial flask culture and dry cell weight was detected after feeding for 24 h as the response. Table 3.4 demonstrates all of the results for the individual vertexes during the movement of the simplex. The first four experiments constitute the initial simplex (for 3 factors). Near the optimum the simplex contracted as expected and the response function plateaued. Vertex 14 was selected as the optimum in addition of the result achieved the highest cell dry weight of 3.86 g/L.

Table 3.4 Results of the movement of the simplex during the experiment

Vertex	Rank[a]	Glycerol (%w/v)	$MgSO_4 \cdot 7H_2O$ (g/L)	Tryptone (g/L)	Response[b] (CDW) (g/L)
1	B	2.1	9.6	5	3.20±0.00
2	NW	1.8	12	8	2.80±0.00
3	NW	2.4	7.2	6	2.80±0.08
4	W	1.5	4.8	7	2.70±0.08
5	R	2.7	14.4	5.6	1.40±0.19
6	Cw	1.8	7.2	6.65	1.47±0.08
7	R	2.4	12	5.95	1.31±0.15
8	Cw	1.95	8.4	6.48	2.45±0.20
9	R	2.25	9.6	6.66	2.15±0.18
10	Cw	2.02	9	7.2	2.34±0.08
11	R	2.18	10.2	5.4	2.00±0.30
12	Cw	2.06	9.3	6.75	3.46±0.30
13	R	2.59	5.4	3.8	3.85±0.20
14	E	2.99	2.1	1.7	3.86±0.10
15	R	2.6	1.6	3.16	3.79±0.30
16	Cr	2.77	3.6	3.62	3.76±0.26

[a] In sequential simplex terminology, B is best response, NW is next-to-the-worst response, Cw is contraction on the worst side, Cr is contraction on the reflection side, R is reflection and E is expansion.

[b] Value is mean and standard deviation of triplicate determinations.

The movement of the simplex toward the optimum was summarized in Figure 3.3 in which the response function (dry cell weight) was plotted as a function of vertex number as the simplex moves through factor space. The result showed that the response values increased in vertex 1-3. Then the response value continually decreased in vertex 4-11 and increased with being constant in vertex 13-16.

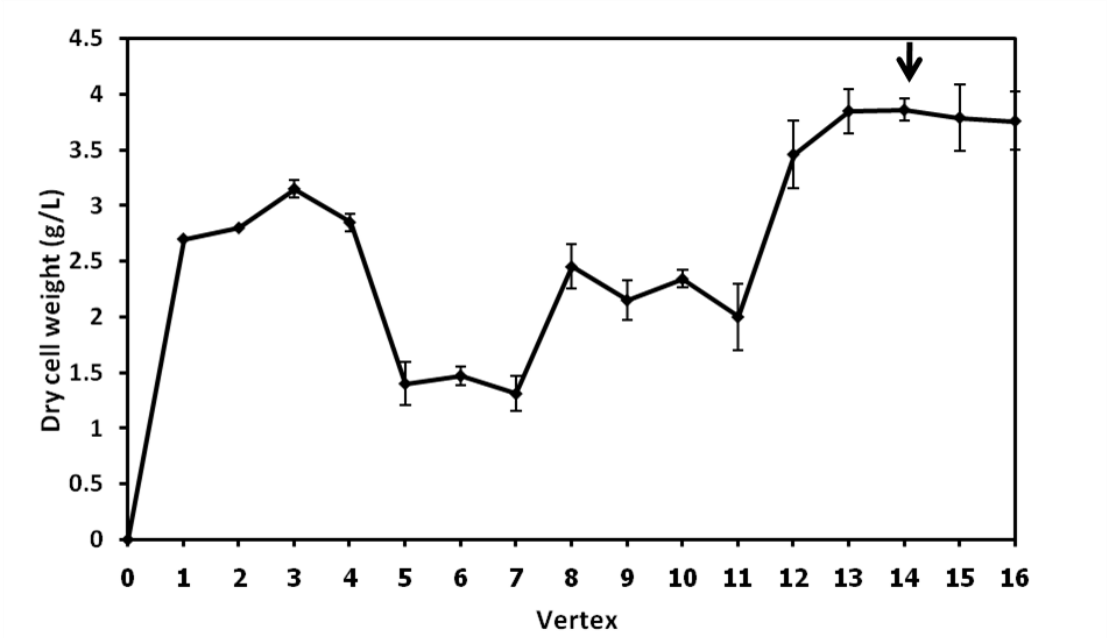

Figure 3.3 Response function for the Simplex Optimization of *E.coli* HB2151 growth indicated in dry cell weight and optimum condition was shown by the arrow.

The level of each factor as a function of vertex number was illustrated in Figure 3.4. Concentration of tryptone and $MgSO_4 \bullet 7H_2O$ showed considerable variation. They decreased until near the optimum. This suggested that appropriate concentration of nitrogen and mineral are available at vertex 11- 16 where the response of cell dry weight was high (Figure 3.3). The lowest responses (vertices 5–11) presented the upper level of the broth nutrients. In contrast, the concentration of glycerol was increased while the tryptone concentration was decreased at theoptimum point.

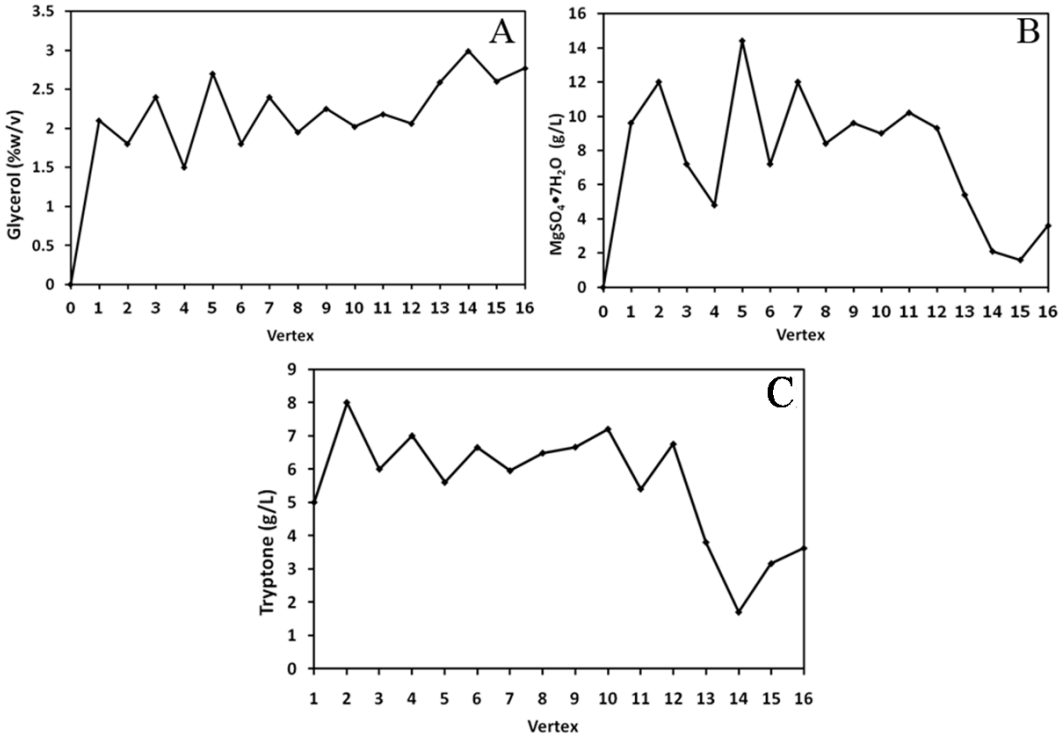

Figure 3.4 Variation of factors, variables and the response function throughout the Simplex Optimization. A: Glycerol (%w/v), B: $MgSO_4 \bullet 7H_2O$ (g/L) and C: Tryptone (g/L).

The comparison of optimized condition was summarized in Table 3.5. The result demonstrated that feed medium optimization using Packlett-Burman design followed by sequential simplex optimization produced more dry cell weight and decreased experiment trial. The number of trials in individual sequential simplex optimization were 27 times whereas the factors which were selected using Packlett-Burman design and optimized by sequential simplex optimization took 16 times of trials. The experiments were reduced for 40.7 %. The dry cell weight of combined methods was increase more than the individual method for 1.24 times.

Table 3.5 The comparisons of the optimization method

Method	Glycerol (%)	Yeast extract (g/l)	Tryptone (g/l)	$MgSO_4 \cdot 7H_2O$ (g/l)	$FeSO_4 \cdot 7H_2O$ (g/l)	$CaCl_2 \cdot 7H_2O$ (g/l)	Response[a] (DCW) (g/L)
SS	1.8	45.8	4.5	29.0	0.4	0.3	2.62±0.30
PBD-SS	2.99	20.0	1.7	2.1	0.5	0.5	3.86±0.10

[a] Value is mean and standard deviation of triplicate determinations.

3.3 Fed-batch cultivation and production of extracellular recombinant scFv anti-p17 in fermenter

The optimized feed medium using Packlett-Burman design combined with Sequential simplex optimization was used in this experiment for fed-batch culture of *E.coli* HB2151 containing pComb3X-scFvp17 expression vector. The optimized medium was feed with three different feeding rates compose of 10, 20 and 50 ml/h. The feeding was start at OD_{600} of 4 or 6 h after inoculation. The feeding was ended when feed medium was added in fermenter for 500 ml and IPTG was added continuously with the feeding. figure 3.5 presented the time profiles of cell dry weight, feed volume in the culture with feeding rate of 10 ml/h. The feeding rate showed dramatic increase of the cell growth until the culture time of 27 h then remained stable. The production of scFv anti-p17 was detected at the 30, 36 and 42 h of the culture. The results were demonstrated in figure 3.6 and figure 3.7. The maximum dry cell weight was 13.0 g/l. The highest scFv anti-p17 (OD_{450}) was 0.147 at culture time of 36 h. The results were presented in table 3.6.

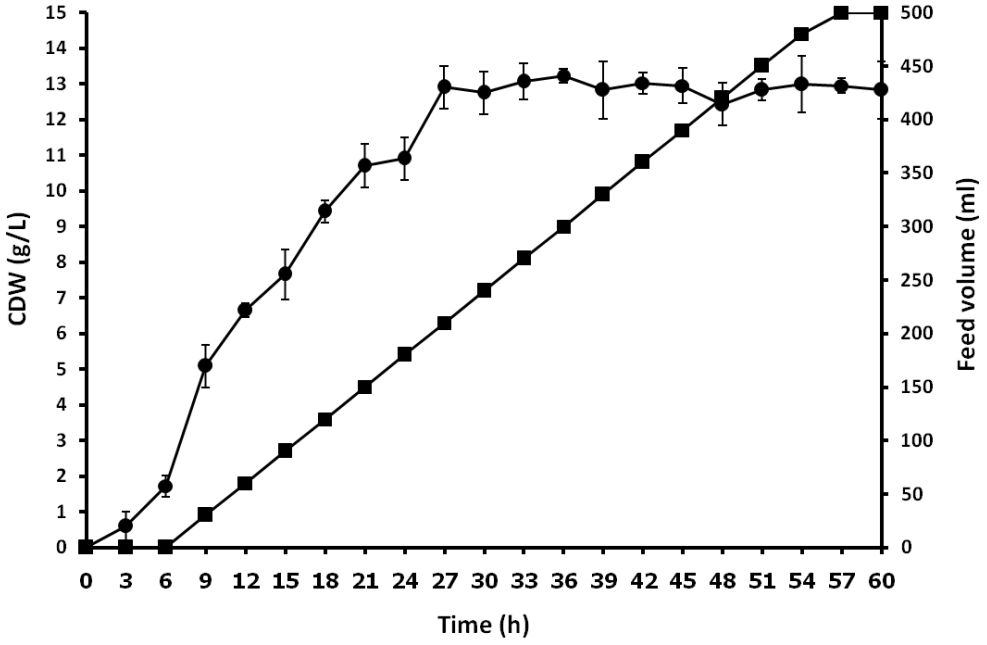

Figure 3.5 Time profiles for dry cell weight (●) and feed volume (■) during fed-batch cultivation with feeding rate of 10 ml/h.

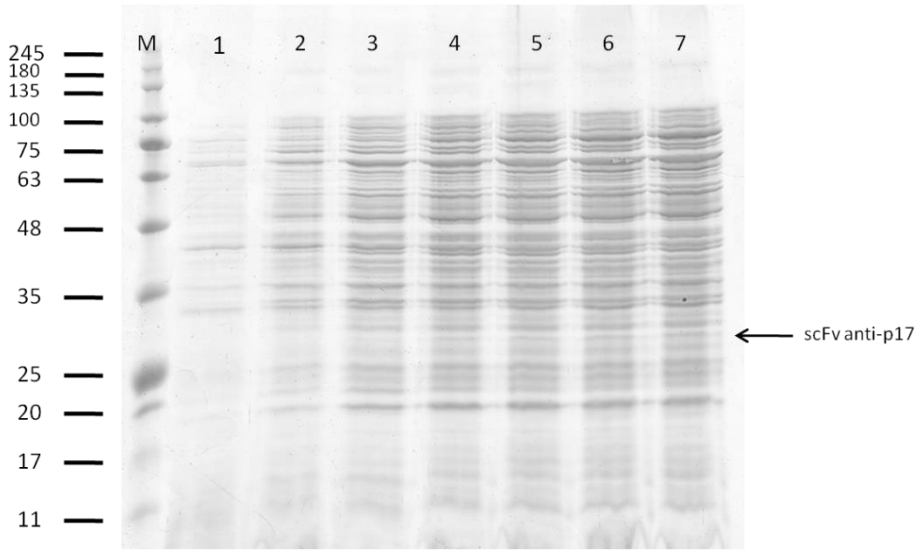

Figure 3.6 SDS-PAGE and analysis of the expression of scFv anti-p17 by *E.coli* HB2151 in fed-batch cultivation with feeding rate of 10 ml/h; Lane M: Prestained protein ladder, Lane 1: crude protein that cultured at 6 h, Lane 2: 12 h, Lane 3: 18 h, Lane 4: 24 h, Lane 5: 30 h, Lane 6: 36 h and Lane 7: 42 h. The feeding and induction started at 6 h of the culture. (Arrows were pointed to scFv anti-p17.)

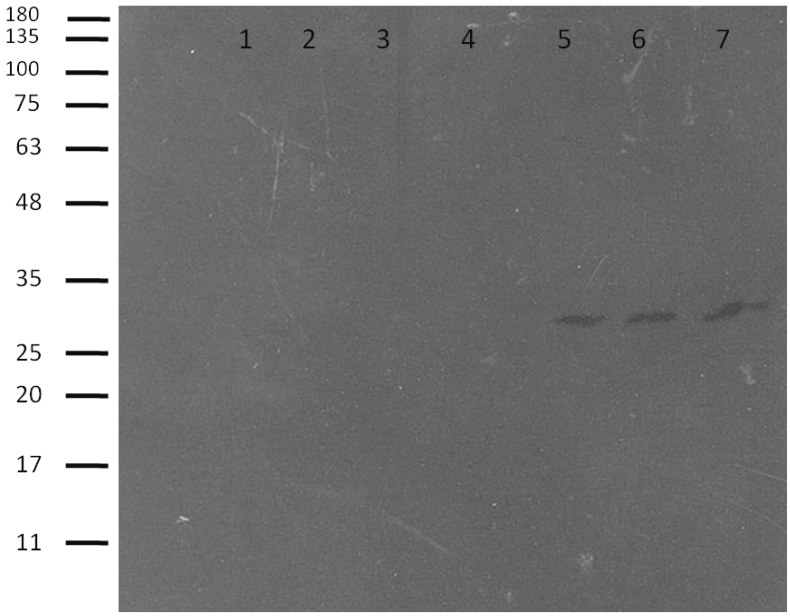

Figure 3.7 Western immunoblotting analysis of the expression of scFv anti-p17 by *E.coli* HB2151 in fed-batch cultivation with feeding rate of 10 ml/h; Lane 1: crude protein that cultured at 6 h, Lane 2: 12 h, Lane 3: 18 h, Lane 4: 24 h, Lane 5: 30 h, Lane 6: 36 h and Lane 7: 42 h. The feeding and induction started at 6 h of the culture.

Table 3.6 the dry cell weight total protein and scFv anti-p17 activity during the time of fed-batch cultivation with feeding rate of 10 ml/h

Time (h)	**DCW (g/L)[a]**	**scFv anti-p17[a,b] (OD_{450})**
6	1.7±0.3	0.051±0.12
12	6.6±0.2	0.059±0.05
18	9.4±0.3	0.071±0.03
24	10.9±0.6	0.084±0.03
30	12.7±0.6	0.097±0.04
36	13.0±0.2	0.147±0.04
42	13.0±0.3	0.138±0.03
48	12.4±0.6	0.127±0.04
54	13.0±0.8	0.145±0.09
60	12.8±0.8	0.139±0.03

[a] Value is mean and standard deviation of triplicate determinations.

[b] The optical density was detected by ELISA. The crude protein concentration of 50 µg/ml was tested.

The time profiles of cell dry weight and feed volume in the culture with feeding rate of 20 ml/h presented in figure 3.8. The feeding rate showed dramatic increase of the cell growth along feeding period. The production of scFv anti-p17 was detected along the time of the culture. The results were demonstrated in figure 3.9 and figure 3.10. Besides the 20 ml/h feeding rate presented scFv anti-p17 (OD_{450}) decreased after 24 h of culture which can observed by ELISA. The maximum dry cell weight and of feeding rate 20 ml/h were 8.7 g/l. The highest scFv anti-p17 (OD_{450}) was 1.610 at culture time of 24 h. The results were presented in table 3.7.

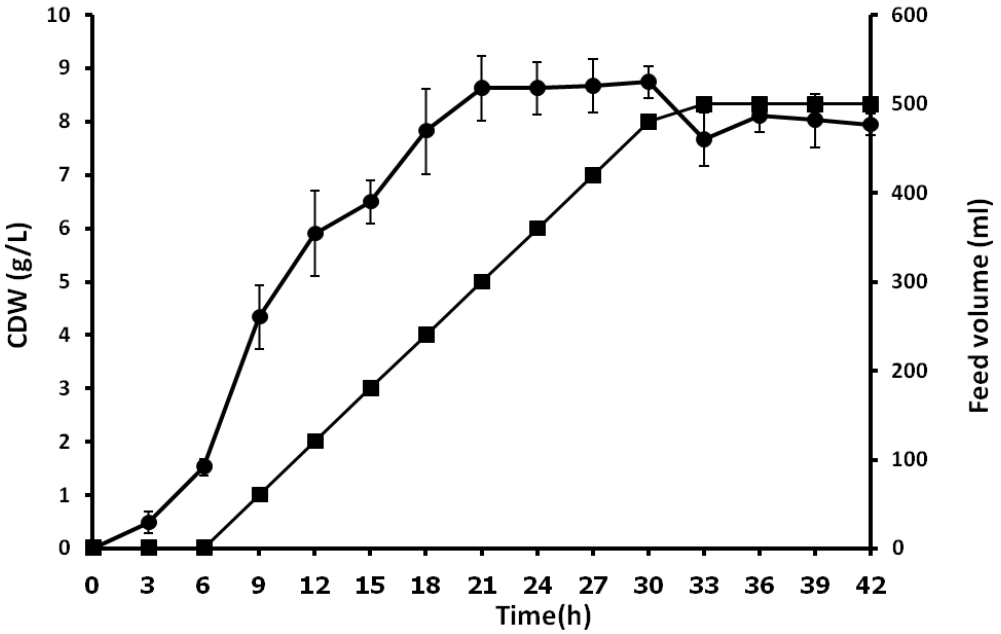

Figure 3.8 Time profiles for dry cell weight (●), and feed volume (■) during fed-batch cultivation with feeding rate of 20 ml/h.

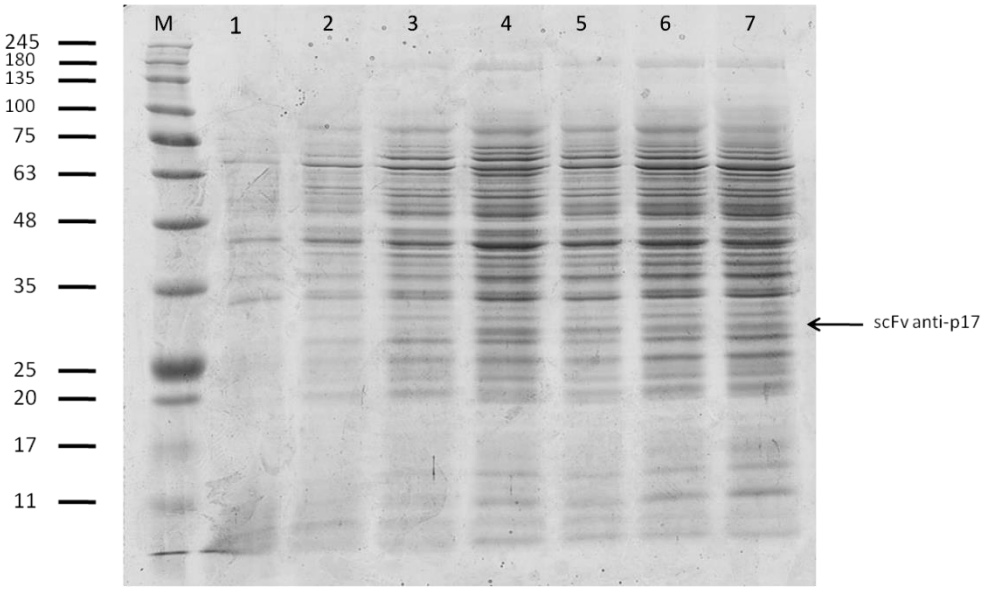

Figure 3.9 SDS-PAGE and analysis of the expression of scFv anti-p17 by *E.coli* HB2151 in fed-batch cultivation with feeding rate of 20 ml/h; Lane M: Prestained protein ladder, Lane 1: crude protein that cultured at 6 h, Lane 2: 12 h, Lane 3: 18 h, Lane 4: 24 h, Lane 5: 30 h, Lane 6: 36 h and Lane 7: 42 h. The feeding and induction started at 6 h of the culture. (Arrows were pointed to scFv anti-p17)

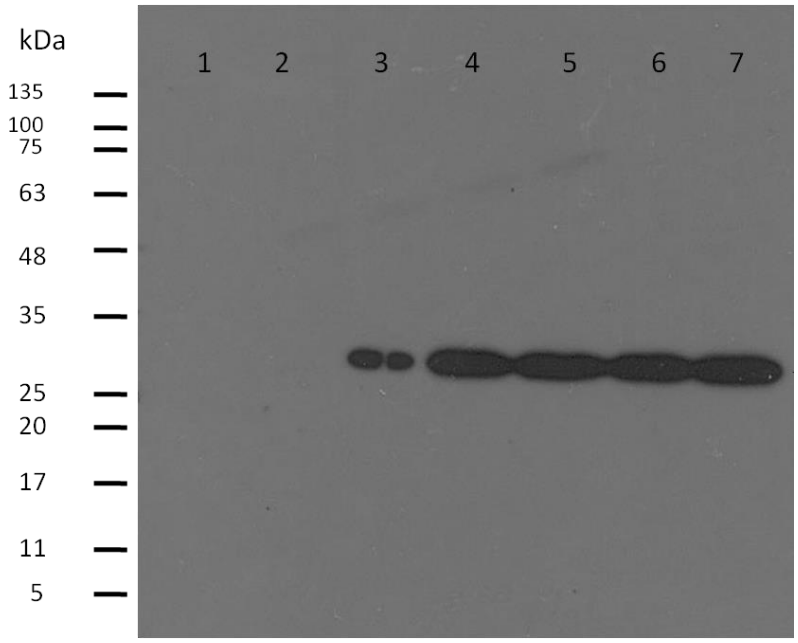

Figure 3.10 Western immunoblotting analysis of the expression of scFv anti-p17 by *E.coli* HB2151 in fed-batch cultivation with feeding rate of 20 ml/h; Lane 1: crude protein that cultured at 6 h, Lane 2: 12 h, Lane 3: 18 h, Lane 4: 24 h, Lane 5: 30 h, Lane 6: 36 h and Lane 7: 42 h. The feeding and induction started at 6 h of the culture.

Table 3.7 the dry cell weight and scFv anti-p17 during the time of fed-batch cultivation with feeding rate of 20 ml/h

Time (h)	**DCW (g/l)[a]**	**scFv anti-p17[a,b] (OD_{450})**
6	1.5±0.15	0.058±0.03
12	5.9±0.8	0.142±0.06
18	7.8±0.8	1.526±0.09
24	8.6±0.5	1.610±0.10
30	8.7±0.3	1.397±0.12
36	8.0±0.3	0.976±0.06
42	7.9±0.2	0.868±0.04

[a] Value is mean and standard deviation of triplicate determinations.

[b] The optical density was detected by ELISA. The crude protein concentration of 50 µg/ml was tested.

The time profiles of cell dry weight and feed volume scFv in the culture with feeding rate of 50 ml/h presented in figure 3.11. The feeding rate showed dramatic increase of the cell growth along feeding period. The production of scFv anti-p17 was detected along the time of the culture. The results were demonstrated in figure 3.12 and figure 3.13. Besides the 50 ml/h feeding rate presented scFv anti-p17activity decreased after 24 h of culture which can observed by ELISA. The maximum dry cell weight and of feeding rate 20 ml/h were 6.6 g/l. The highest scFv anti-p17 (OD_{450}) was 0.289 at culture time of 24 h. The results were presented in table 3.8.

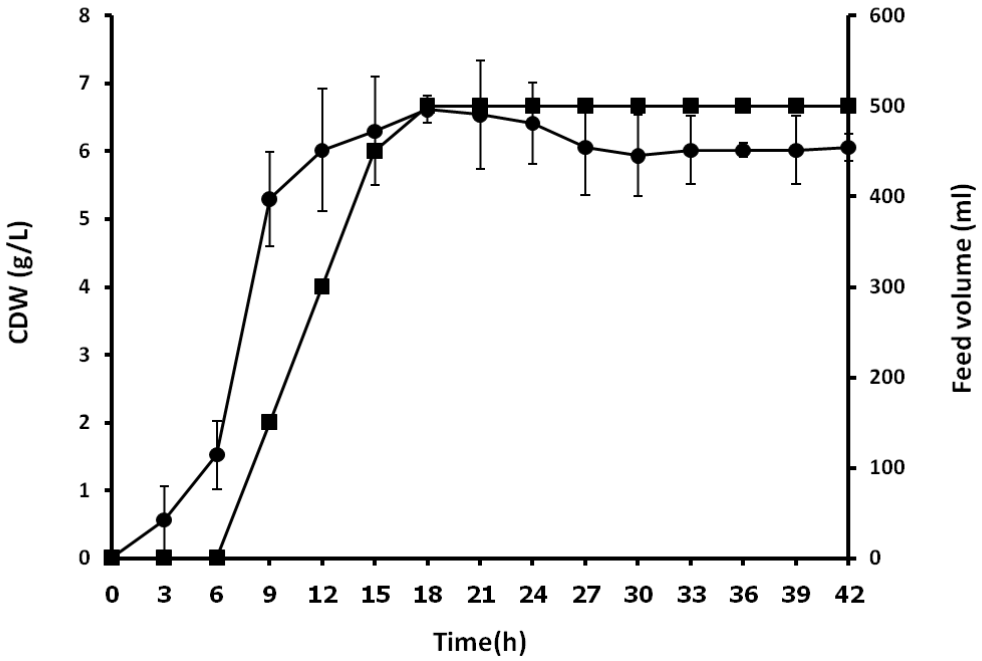

Figure 3.11 Time profiles for dry cell weight (●) and feed volume (■) during fed-batch cultivation with feeding rate of 50 ml/h.

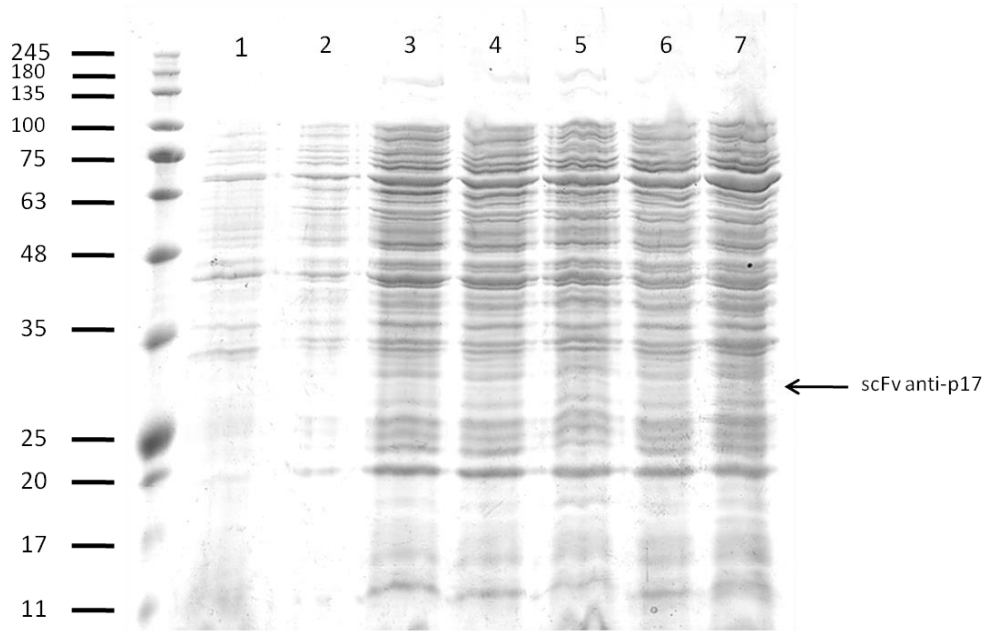

Figure 3.12 SDS-PAGE and analysis of the expression of scFv anti-p17 by *E.coli* HB2151 in fed-batch cultivation with feeding rate of 50 ml/h; Lane M: Prestained protein ladder, Lane 1: crude protein that cultured at 6 h, Lane 2: 12 h, Lane 3: 18 h, Lane 4: 24 h, Lane 5: 30 h, Lane 6: 36 h and Lane 7: 42 h. The feeding and induction started at 6 h of the culture. (Arrows were pointed to scFv anti-p17.)

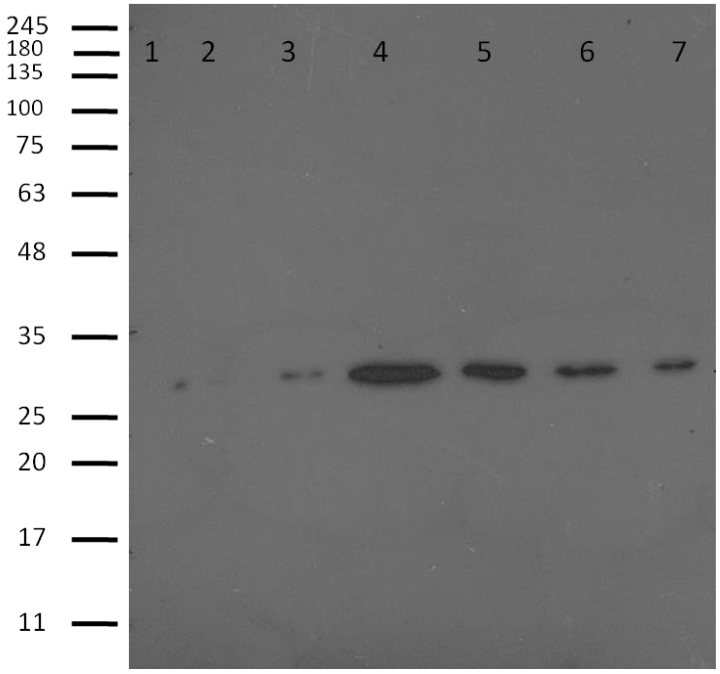

Figure 3.13 Western immunoblotting analysis of the expression of scFv anti-p17 by *E.coli* HB2151 in fed-batch cultivation with feeding rate of 50 ml/h; Lane 1: crude protein that cultured at 6 h, Lane 2: 12 h, Lane 3: 18 h, Lane 4: 24 h, Lane 5: 30 h, Lane 6: 36 h and Lane 7: 42 h. The feeding and induction started at 6 h of the culture.

Table 3.8 the dry cell weight and scFv anti-p17 during the time of fed-batch cultivation with feeding rate of 50 ml/h

Time (h)	**DCW (g/L)[a]**	**scFv anti-p17[a,b] (OD_{450})**
6	1.5±0.5	0.058±0.03
12	6.0±0.9	0.075±0.04
18	6.6±0.2	0.131±0.03
24	6.4±0.6	0.289±0.05
30	5.9±0.6	0.163±0.09
36	6.0±0.1	0.166±0.09
42	6.0±0.2	0.158±0.03

[a] Value is mean and standard deviation of triplicate determinations.

[b] The optical density was detected by ELISA. The crude protein concentration of 50 µg/ml was tested.

The figure 3.14 demonstrated the time profile of scFv anti-p17 (OD_{450}) increasing compared with the non-induced condition. The maximum scFv anti-p17 was produced by 20 ml/h feed rate at 24 h of the culture. The scFv anti-p17 was higher 26.8 times over non-induce condition.

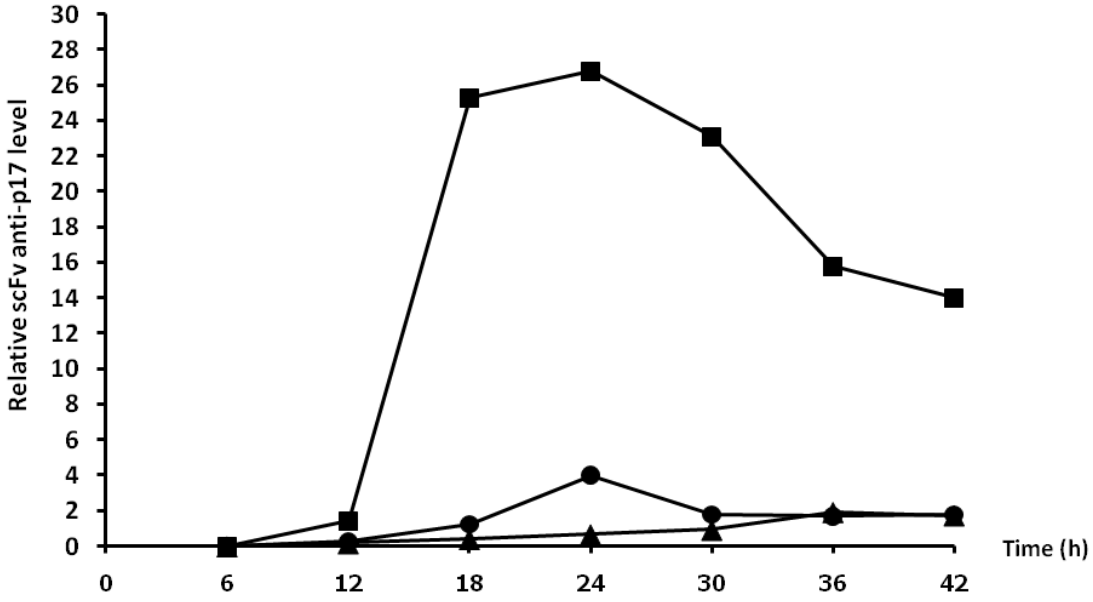

Figure 3.14 Time profiles for relative scFv anti-p17 level during fed-batch cultivation with feeding rate of 10 (▲), 20 (■) and 50 (●) ml/h. The relative scFv anti-p17 level was calculated by OD_{450} of scFv anti-p17 in induced condition to non-induced condition. The crude protein concentration 50 µg/ml was tested.

In comparison of all feeding rates, feeding rate 10 ml/h presented the maximum growth of *E.coli* HB2151of 13.0 g/l. The maximum increasing of scFv anti-p17 production demonstrated at the feeding rate of 20 ml/h. The production was 26.8 times over the non-induce condition. The induction was started at 6 h of the culture process. The scFv anti-p17 was started to produce at 12 h and increased along the fed-batch culture. The maximum scFv anti-p17 (OD_{450}) was presented at culture time of 24 h which observed by ELISA. The feeding rate at 20 ml/h was appropriate for the production of extracellular scFv anti-p17 by fed-batch cultivation and the culture time should be performed at 24 h.

In this study, the production comparing between batch and fed-batch cultivation was done. The results were summarized in table 3.9. Fed-batch cultivation at feeding rate 20 ml/h increased higher cell growth, total protein and scFv anti-p17 activity than batch cultivation for 4.43, 1.48 and 6.50 times respectively.

Table 3.9 Comparison of the batch and fed-batch cultivation

Cultivation method	**Maximum dry cell weight (g/L)**	**Total protein (mg/mL)**	**scFv anti-p17 activity[a] (OD_{450})**
Batch	1.60±0.03	2.50±0.60	0.22±0.20
Fed-batch	8.70±0.10	6.20±0.10	1.65±0.10

[a] The crude protein concentration of 50 µg/mL were tested

3.4. Quantitative determination of scFv anti-p17

The 8 µg of crude proteins that contained scFv anti-p17 protein was mixed with 1.8µg of standard BSA as internal control. The scFv anti-p17 proteins was determined by SDS-PAGE method. The scFv anti-p17 protein bands intensity on SDS-PAGE (Figure 3.15) was determined by ImageQuant TL programme of Image Scanner III (GE healthcare, USA).

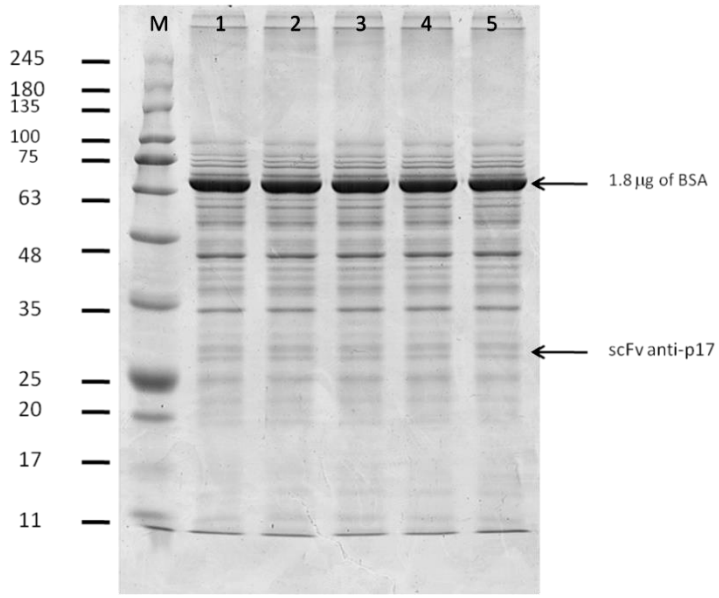

Figure 3.15 SDS-PAGE analysis for quantitative determination of scFv anti-p17 using 1.8 µg of standard BSA as internal control. Lane 1-5 showed crude protein that mixed with 1.8 µg of standard BSA. Lane M showed prestained protein ladder.

The scFv anti-p17 wasalso detected by westernimmunoblotting (Figure 3.16). scFv anti-p17 were found in all fraction.

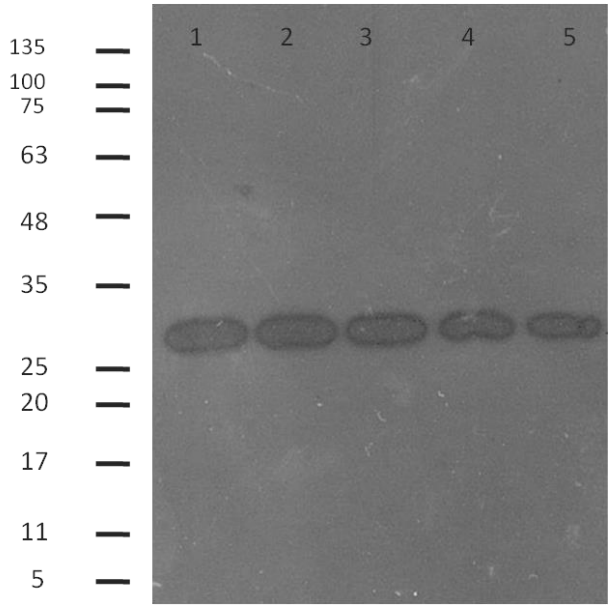

Figure 3.16 Western immunoblotting analysis of scFv anti-p17. Lane 1-5 showed crude protein that mixed with 1.8 µg of standard BSA.

The result presented the peaks area of scFv anti-p17 protein in Table 3.11.The mean of peak area was 3764. The peak area was calculated in SDS-PAGE quantitative formula to estimate amount of scFv anti-p17. The amount of scFv anti-p17 that was calculated from the formula was 0.532 mg/l of culture medium.

Table 3.10 The peaks area of scFv anti-p17 bands on SDS-PAGE analyzed by ImageQuant TL programme of Image Scanner III.

Experiment	Peak area	Mw
1	3703	28.3
2	4493	28.0
3	3823	27.6
4	3251	28.6
5	3548	28.6
Mean	3764	28.2
SD	460	0.43

3.5 Protein Secretion of the recombinant extracellular scFv anti-p17 protein (scFv anti-p17) in *E. coli*

After the production of scFv anti-p17 using fed-batch cultivation with optimized feed medium in fermenter scale, the appropriate feed rate of 20 ml/h at culture time 24 h was demonstrated the highest scFv anti-p17 production. The *E. coli* cells from this condition were extracted by subcellular fractionation method. The three fractions from this method were cytoplasmic fraction, periplasmic fraction and extracellular fraction. Batch and fed-batch cultivation were compared.

The protein expression comparison between Batch and fed-batch cultivation was presented in figure 3.16 and figure 3.17. The result presented that the scFv anti-p17 protein band in periplasm was absence in fed-batch cultivation. The scFv anti-p17 from batch cultivation was accumulated in the periplasmic space. The result of relative scFv anti-p17 (OD_{450}) showed that the soluble scFv anti-p17 derived from fed-batch

cultivation was secreted at a high level into the culture medium. The relative scFv anti-p17 (OD_{450}) of the extracellular crude protein from fed-batch and batch cultivation were 100% and 31% respectively. The relative scFv anti-p17 (OD_{450}) of the crude protein in periplasm from fed-batch and batch cultivation were 4% and 16% respectively.The relative scFv anti-p17 (OD_{450}) of soluble scFv anti-p17 in cytoplasmic fraction of the *E.coli* from fed-batch and batch cultivation were 52% and 24%. This study demonstrated that the fed-batch cultivation provided the high level of secreted protein production. The accumulation of protein in periplasm was decreased while the secreated protein was elevated in the culture medium. The medium and method designated in this study offered the archiving extracellular production of recombinant scFv anti-p17 protein in *E. coli*.

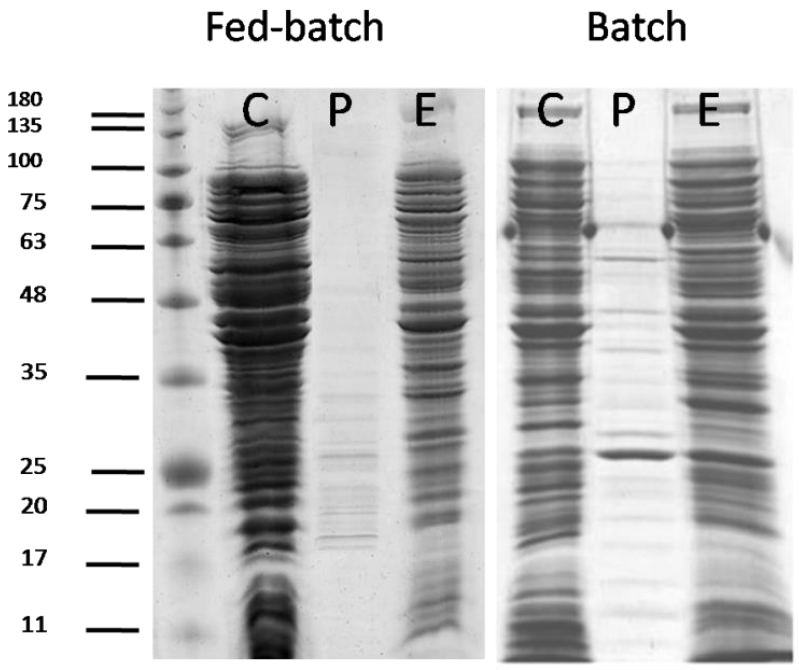

Figure 3.17 SDS-PAGE analysis of the expression of scFv anti-p17 in cytoplasm (C), periplasm (P) and extracellular space (E) by batch and fed-batch cultivation.

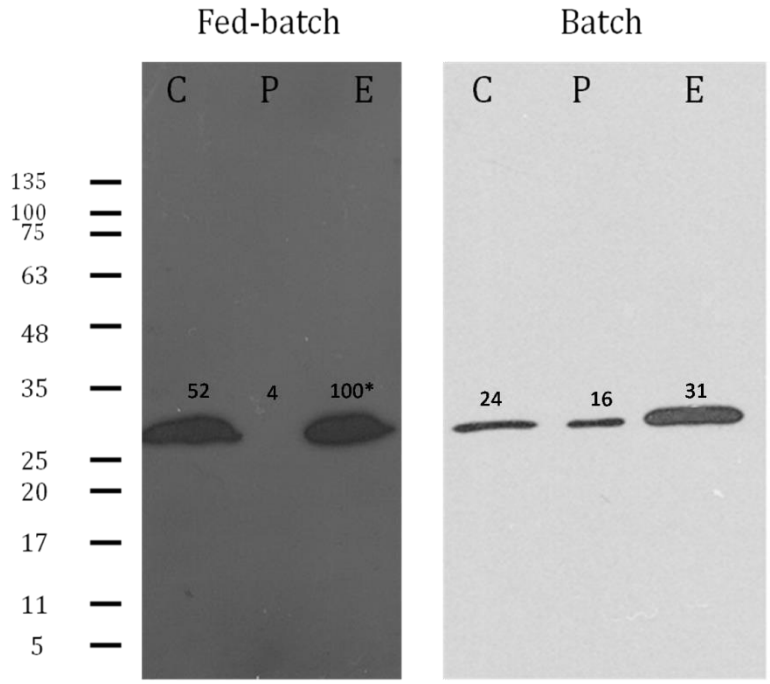

Figure 3.18 Western immunoblotting analysis of the expression of scFv anti-p17 in cytoplasm (C), periplasm (P) and extracellular space (E) by batch and fed-batch cultivation. * The result of relative scFv anti-p17 (OD_{450}).

CHAPTER 4

Discussion

The feed medium is the one of factor that affects successful fed-batch cultivation. Feed medium composition should be considered in appropriate concentration to enhance the growth of microorganism for reaching high cell density and protein production. Therefore, the medium optimization by using optimization method was used to optimize the medium concentration. The one factor at a time (OF) method was previously used to obtain the optimum since it was time and cost consuming method. The optimize method using statistical approach is applied to reach the optimized condition rapidly.

The Sequential Simplex Optimization method has the advantage of taking into account factor interaction. This means that the global optimum may be found at a point different from that which would be obtained by optimizing each factor independently. This phenomenon is typical of most multifactor chemical and biochemical systems. Changing all factor levels with each new experiment allows movement toward the global optimum. The Sequential Simplex Optimization method can be applied and usually involves a relatively small number of experiments to quickly reach the optimum [10]. In this study, the feed medium optimization by individual Sequential Simplex Optimization method was done. All six factors were optimized and took 27 experiments of trial.

The optimization by combined two methods was investigated in this study. The result presented that screening of critical medium compositions using Packlett-

Burman design, glycerol, tryptone and $MgSO_4 \cdot 7H_2O$ were the significant factors for the growth of *E. coli* HB2151. Glycerol and tryptone play a role as carbon and nitrogen sources which effect on cell growth. Excess glycerol leads to metabolic by-products such as acetate, formate and ethanol [7]. Durnin *et al.* reported that the most abundant product of glycerol was ethanol. It was produced more than 60% of the final production mixture [60]. Addition of inappropriate concentration of nitrogen sources can decrease the biomass and yield due to feed back inhibition of amino acid [8]. There was a work demonstrated that tryptone was an important nitrogen source for *E. coli* which involved medium-dependent oxidative stress. Medium containing tryptone can affect the ability of *E.coli* to resist exposure to various from of oxidative stress [61]. The concentration of $MgSO_4 \cdot 7H_2O$ involves the cell growth, associated with DNA synthesis and maintains the permeability barrier and integrity of the bacterial cells [62]. The free magnesium is required by many intracellular enzymes. For example, *E. coli* DNA polymerase and amino acid activating enzymes [63]. By this combined method, the significant factors from Packlett-Burman design were subsequently optimized by Sequential Simplex Optimization. The experiment number of trials took 16 experiments.

The comparisons of individual and combined method suggest that the screening of significant factor was crucial to reduce the number of trials in optimization process since unnecessary variation of insignificant factor was eliminated. Among the optimized concentration of each medium composition, the individual Sequential Simplex Optimization resulted in higher yeast extract, tryptone and all minerals than combined optimization method. Yeast extract and tryptone are expensive ingredients leading to higher cost of production [60]. Remarkably, the concentration of $MgSO_4 \cdot 7H_2O$ of individual Sequential Simplex Optimization was very high. The high ion concentration in growth medium affect membrane potential and activate different stress mechanism that reduce growth rate and terminate growth cycle [64]. Thus, the *E. coli* HB2151 favor growth in feed medium which optimized by combined methods.

The mass of recombinant protein production, fed-batch cultivation is often employed to obtain a high production yield. The addition of feed medium prevents nutrient depletion during log phase and prolongs the cell growth [6]. Feeding is one of

important factors for successful process. The study of different feeding rates indicated that the high feeding rate (50 ml/h) led to reduce the growth of *E. coli* HB2151. The fed-batch process in this study was continuous induction by feeding and induction occurred simultaneously. The inducer concentration depended on feeding rate. The low feeding rate (10 ml/h) may not be able to supply the IPTG levels required for the recombinant protein production. The feeding rate of 20 ml/h was appropriated for fed-batch cultivation with this optimized medium. This feed rate was able to enhance the growth of *E. coli* to 8.7 g/l dry cell weight and presented the highest scFv anti-p17 (OD_{450}) of 1.610. In previous study, Ramalingam *et al.* investigated the effect of feeding strategies on recombinant streptokinase production in *E.coli* BL21 (DE3). The results showed that the specific growth rate of *E. coli* and streptokinase production was decreased at the low and high feeding rate. The feeding rate that lower than the substance uptake rate may not be able to supply the nutrient level required for maintaining the growth of *E.coli* and recombinant protein production. Moreover, the maximum feeding production throughout the culture, nutrient and metabolite products are observed. It caused toxic to the cells and inhibited the growth and protein production [5].

Pinsach *et al*. used the continuous IPTG induction to produce rhamnulose 1-phosphate aldolase (RhuA). They demonstrated that the continuous IPTG induction minimized metabolic overload of host cell and prolonged the induction phase. The inducer feeding allowed the turning of transcription rate below the critical values, extending the induction phase and obtaining good RhuA yields [65]. In this work, we also applied the continuous induction by mixing the IPTG with feed medium to improve productivity yield of scFv anti-p17. Thus, the feeding rate was also very important to the scFv anti-p17 production. The low feeding rate was not able to add the appropriated concentration of inducer and the high feeding rate of inducer was toxic and cause the metabolic burden in the host cells [66].

Comparative of two culture methods compose of fed-batch and batch cultivation. The fed-batch cultivation at feeding rate of 20 ml/h increased the cell growth, total extracellular protein and scFv anti-p17 activity than batch cultivation 4.43,

1.48, and 6.50 times, respectively. Associated with the previous study, Fu *et al.* enhanced the 5-aminolevulinate (ALA) production by *E.coli* using batch and fed-batch cultivation. The result presented that fed-batch cultivation is an improvement of batch cultivation for either higher cell density or better product yield. The feeding of substrate was able to maintain the substrate concentration in more steady and suitable level for longer period and resulted in the higher ALA productivity [40].

The result of protein fractionation of the cell from fed-batch cultivation presented less retention of scFv anti-p17 in periplasm than batch cultivation. The scFv anti-p17 secreted to the culture broth extremely high level. The ELISA result presented more active scFv anti-p17 produced in cytoplasmic section. Moreover, the accumulation of the protein presented less level in the periplasmic space. The ELISA of the soluble active scFv anti-p17 was demonstrated the new formular of feed medium and feed strategy could increase production secretion and stability of the scFv production process. In previous study, Backlund *et al*. investigated the effect of feed rate of periplasmic retention for the production of antibody fragment (Fab). For the fed-batch cultivation, the product retention depended on the post induction feed rate. There was increased leakage of Fab to the medium and a higher specific product yield at the appropriated feed rate [67]. In this study, the feed medium was optimized to the optimum concentration by Packlett-Burman design and Sequential Simplex Optimization. Feeding was varied to provide the rate that appropriates to feed and induces the protein production. This designated fed-batch process was able to enhance the protein secretion from periplasm to the culture medium.

CHAPTER 5

Conclusion

This study has demonstrated the utility of the Packlett-Burman design combined with Sequential Simplex Optimization for medium optimization and successful design of fed-batch cultivation for protein production of extracellular recombinant scFv anti-p17. The combined method reduced the experiment trial and provided the favor growth of *E.coli* HB2151in optimized feed medium. By developed fed-batch cultivation, the extracellular recombinant scFv anti-p17 activity was increased by 6.5 times compared to batch cultivation. The essential outcome of our study has overcome the complexity in obtaining a suitable feed medium for large scale production of scFv anti-p17. The combined methods are applicable to the optimization of other recombinant protein production schemes.

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APPENDICES

APPENDIX A

Chemicals and equipments

1. CHEMICALS AND ANTIBODIES

All chemicals used in this study were analytical grade reagents

- Albumin from bovine serum (Sigma-Aldrich , USA)
- Ammonium persulfate (Bio Basic INC, Canada)
- Ammonium sulfate (Bio Basic INC, Canada)
- Ampicillin (NIDA Pharma incorporation, Thailand)
- Anti-HA affinity matrix (Roche, Switzerland)
- Anti-HA monoclonal antibody (Sigma-Aldrich,USA)
- BCA protein assay reagent kit (Pierce, Rockford, USA)
- Dextrose (Difco Laboratories, USA)
- Ethanol (Merck, Germany)
- Methanol (Merck, Germany)
- Non-fat powdered milk (Bio Basic INC, Canada)
- Isopropyl-beta-*D*-thiogalactopyranoside (Sigma-Aldrich , USA)
- Glycerol (Merck, Germany)
- p17 peptide (Genscript,USA)
- HRP-conjugated monoclonal anti-HA antibody (Genscript,USA)
- HRP-conjugated goat anti-mouse immunoglobulin antibodies (KPL, USA)
- HCl (Merck, Germany)
- PageRuler^{TM} Prestained Protein Ladder (Fermentus, USA)
- Sodium dodecyl sulfate (Sigma-Aldrich ,USA)
- Chemiluminescent substrate (Pierce, USA)
- TEMED (BioRad, USA)
- TMB color substrate(KPL, USA)

- Tryptone (Merck, Germany)
- Tween 20 (BioRad, USA)
- Yeast Extract (Merck, Germany)

2. MICROORGANISM

- *Escherichia coli* HB2151 (provided from Prof. Dr. Chatchai Tayapiwatana)

3. EQUIPMENTS

- Bioreactor (Minifor, Infors-ht, Switzerland)
- ELISA reader (TECEN, Austria)
- Centrifuge 5S10R (Eppendorf, Germany)
- Hoefer SE 260 mini-vertical gel electrophoresis (Amersham pharmacia biotech, USA)
- Image scanner III (GE healthcare, USA)
- QuixStand™ benchtop system (GE healthcare, USA)
- Semi-Dry Electrophoretic Transfer unit (GelmanSciences, Denmark)
- Ultrasonic processor (Hielscher ultrasound technology,Germany)
  Peristaltic pump (Atto, Japan)

APPENDIX B

Reagent preparation

1.	Terrific broth		1	L
	Yeast extracts		24	gm
	Tryptone		12	gm
	Glycerol 100%		4	ml
	dH_2O	to	900	ml
	Adding Phosphate buffer		100	ml
2.	Feed medium			
	Glycerol		2.99	% (w/v)
	Yeast extract		20	gm
	Tryptone		1.7	gm
	$MgSO_4 \cdot 7H_2O$		2.1	gm
	$FeSO_4 \cdot 7H_2O$		0.5	gm
	$CaCl_2 \cdot 7H_2O$		0.5	gm
	dH_2O	to	1	L
3.	Phosphate buffer		500	ml
	KH_2PO_4		11.57	gm
	K_2HPO_4		62.71	gm
	Adding dH_2O	to	500	ml

4. Phosphate buffer saline (PBS), pH 7.2 — 1 L

NaCl		8	gm
KCl		0.2	gm
Na_2PO_4		1.15	gm
KH_2PO_4		0.2	gm
dH_2O	to	1	L

5. Tris buffer saline (TBS)

Tris		78.82	gm
NaCl		54.75	gm
adjust to pH 7.5 by 5N HCl			
dH_2O	to	1000	ml

6. Reagent for SDS-PAGE

6.1	Acrylamide solution		200	ml
	Acrylamide		60	gm
	Bisacrylamide		1.6	gm
	dH_2O	to	200	ml
6.2	4x Resolving gel buffer pH 8.8			
	Tris-HCl		36.3	gm
	dH_2O	to	150	ml
	adjust to pH 8.8 by 5N HCl			
	dH_2O	to	200	ml
6.3	4x Stacking gel buffer pH 6.8		50	ml
	Tris-HCl		3.0	gm
	dH_2O		40	ml

	adjust to pH 6.8 by 5N HCl			
	dH_2O	to	50	ml
6.4	10% SDS		100	ml
	SDS		10	gm
	dH_2O	to	100	ml
6.5	10% Ammonium persulfate			
	Ammonium persulfate		0.1	gm
	dH_2O	to	1.0	ml
6.6	Tank buffer		10	L
	Tris-HCl		30.28	gm
	Glycine		144.13	gm
	SDS		10	gm
	dH_2O	to	10	L

7. Reagent for western immunoblotting

7.1	Blotting buffer		1	L
	Tris-base		3.03	gm
	Glycine		14.41	gm
	SDS		0.5	gm
	dH_2O	to	800	ml
	Added methanol		200	ml
7.2	0.05% tween20/PBS buffer		500	ml
	Tween 20		250	µl
	PBS buffer	to	500	ml

8. Reagent for ELISA

8.1	0.05% tween20/TBS buffer		500	ml
	Tween 20		250	µl
	TBS buffer	to	500	ml

8.2	0.05 M carbonate buffer, pH 9.6		100	ml
	Na_2CO_3		0.159	gm
	$NaHCO_3$		0.293	gm
	Na_3N_3		0.02	gm
	dH_2O	to	90	ml
	adjust pH to 9.6			
	dH_2O	to	100	ml

CURRICULUM VITAE

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Education

1994-1999	Primary school, Jakarnboon school, Phitsanulok, Thailand
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