การผลิตฟิวชันโปรตีน IL-2/FU-MK-1-scFv โดย *Pichia pastoris* ในถังหมัก และลักษณะสมบัติของฟิวชันโปรตีนที่บริสุทธิ์บางส่วน

นายสาคร อนุลีจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PRODUCTION OF FUSION PROTEIN IL-2/FU-MK-1-scFv BY *Pichia pastoris* IN FERMENTER AND CHARACTERIZATION OF PARTIALLY PURIFIED FUSION PROTEIN

Mr. Sakorn Anuleejun

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Thesis Title	PRODUCTION OF FUSION PROTEIN IL-2/FU-MK-1-scFv BY *Pichia pastoris* IN FERMENTER AND CHARACTERIZATION OF PARTIALLY PURIFIED FUSION PROTEIN
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Field of Study	Biotechnology
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สาคร อนุลีจันทร์: การผลิตฟิวชันโปรตีน IL-2/FU-MK-1-scFv โดย *Pichia pastoris* ในถังหมักและลักษณะสมบัติของฟิวชันโปรตีนที่บริสุทธิ์บางส่วน (PRODUCTION OF FUSION PROTEIN IL-2/FU-MK-1-scFv BY *Pichia pastoris* IN FERMENTER AND CHARACTERIZATION OF PARTIALLY PURIFIED FUSION PROTEIN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. สุชาดา จันทร์ประทีป, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. ธนาภัทร ปาลกะ, 96 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตฟิวชันโปรตีนของ IL-2 และชิ้นส่วนแอนติบอดีสายเดี่ยว humanized FU-MK-1-scFv โดย *Pichia pastoris* แอนติบอดีสายเดี่ยว humanized FU-MK-1-scFv ดังกล่าวมีความจำเพาะต่อแอนติเจน MK-1 ซึ่งเป็นไกลโคโปรตีนที่แสดงออกบนผิวเซลล์มะเร็งหลายชนิด จึงสามารถใช้สำหรับภูมิคุ้มกันบำบัดในโรคมะเร็ง จากการเพิ่มปริมาณและเชื่อมต่อยีนที่ประมวลรหัส IL-2/FU-MK-1-scFv เข้าสู่เวคเตอร์ pPICZαA เพื่อการแสดงออก และทรานส์ฟอร์มรีคอมบิแนนท์พลาสมิด pPICZαA-IL-2/FU-MK-1-scFv เข้าสู่เซลล์เจ้าบ้าน *Pichia pastoris* สายพันธุ์ GS115 คัดเลือกโคลนที่แสดงออกและหลั่งฟิวชันโปรตีน จากนั้นหาภาวะที่เหมาะสมในการผลิตฟิวชันโปรตีนโดยแปรผันภาวะดังนี้ ค่า pH ในช่วง 3 ถึง 10 อุณหภูมิ 20 25 30 และ 37 องศาเซลเซียส และความเข้มข้นของเมทานอล 0.01 0.1 และ 0.5 เปอร์เซ็นต์โดยปริมาตร ผลการทดลองแสดงให้เห็นว่า ภาวะที่เหมาะสมคือ pH 3 อุณหภูมิ 30 องศาเซลเซียส และเหนี่ยวนำการแสดงออกด้วยความเข้มข้นของเมทานอล 0.1 เปอร์เซ็นต์ สามารถผลิตฟิวชันโปรตีนได้สูงสุด 258±13 มิลลิกรัมต่อลิตร เมื่อขยายการผลิตแบบเฟด-แบชในถังหมักขนาด 5 ลิตร พบว่าสามารถผลิตฟิวชันโปรตีนได้ 109±5 และ 425±2 มิลลิกรัมต่อลิตร เมื่อใช้อาหาร modified basal salts และ BMMY ตามลำดับ ผลการทดสอบแอคติวิตีด้านความจำเพาะของฟิวชันโปรตีนที่บริสุทธิ์บางสวนต่อแอนติเจน MK-1 ที่แสดงออกบนผิวของเซลล์สัตว์เลี้ยงลูกด้วยนม Chinese hamster ovary (CHO) cells โดยวิธี cell lysate ELISA และเปรียบเทียบกับการทดสอบของฟิวชันโปรตีนต่อ CHO cells ที่ไม่มีการแสดงออกของ MK-1 บนผิวเซลล์ พบว่า ฟิวชันโปรตีน IL-2/FU-MK-1-scFv มีความจำเพาะอย่างมีนัยสำคัญในทางสถิติต่อแอนติเจน MK-1 ($p<0.05$, Student' s *t* test)

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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The aim of this study was to express a fusion protein of IL-2 and humanized single chain variable fragment antibody FU-MK-1-scFv in *Pichia pastoris*. The FU-MK-1-scFv recognizes a cell surface glycoprotein (designated MK-1) that is overexpressed in a majority of human carcinomas. Thus, it can be used for immunotherapy of cancer. The fusion gene encoding IL-2/FU-MK-1-scFv was amplified and ligated into the expression vector pPICZαA. The expression vector pPICZαA-IL-2/FU-MK-1-scFv was successfully transformed into *P. pastoris* strain GS115. Next, *P. pastoris* strain GS115 harboring pPICZαA-IL2/FUscFv(V_H-V_κ) and capable of secreting fusion protein was chosen for optimizing the production of fusion protein production by examining the effect of pH, temperature, and methanol concentrations. The highest production of the fusion protein at 258±13 mg/L was obtained under pH 3 and 30 °C with a methanol concentration of 0.1% for 96 hours induction. Fed-batch cultivation in 5 L fermenter, it was found that the amount of secreted fusion protein was 109±5 mg/L when the modified basal salt medium was used whereas the amount of secreted fusion protein was increased up to 425±2 mg/L when BMMY medium was used. To investigate biding activity of the partial purified fusion protein, cell lysate ELISA method was applied in this study. Our result demonstrated that the produced fusion protein retained specific binding activity to MK-1 antigen due to it significantly bound to MK-1 expressing Chinese hamster ovary (CHO) cell, but not MK-1 non-expressing CHO cell. The results were compared using Student's *t* test (p<0.05).

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ELISA	enzyme-linked immunosorbent assay
MAb	monoclonal antibody
Ab	antibody
Ag	antigen
BSA	bovine serum albumin
scFv	single chain variable fragment
Fab	fragment antigen-binding
IgG	immunoglobulin G
IgM	immunoglobulin M
^{o}C	degree Celsius
PBS	phosphate buffer saline
PEG	polyethylene glycol
PVDF	polyvinylidine fluoride membrane
h	hour
min	minute
M	molar
mM	millimolar
g	gram
ng	nanogram
pg	picogram
L	liter
mL	milliliter
µL	microliter
nm	nanometer
v/v	volume per volume

w/v	weight per volume
rpm	round per minute
IL	interluekin
SCID	Severe Combined Immunodeficiency
kDa	kilodalton
FDA	Food and Drug Administration
NK	natural killing
TNF	tumor necrosis factor
IFN	interferon
AOX	alcohol oxidase
YPD	yeast extract peptone dextrose
YPDS	yeast extract peptone dextrose sorbitol
BMGY	buffer glycerol-complex medium
BMMY	buffer methanol-complex medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
OD	optical density
WCW	wet cell weight
DCW	dry cell weight
EDTA	ethylenediaminetetraacetic acid
MEM	minimum essential media
CHO	Chinese hamster ovary
CEA	carcinoma embryonic antigen

CHAPTER I

INTRODUCTION

1.1 Introduction

MK-1 antigen, GA733-2 antigen, 17-1A antigen, KS1/4 antigen or C215 antigen are transmembrane glycoprotein and show promise as targets in approaches to active and passive immunotherapy of colorectal cancer. The MK-1 antigen is recognized by monoclonal antibody FU-MK-1 and widely expressed on the surface of a majority of carcinomas, including carcinoma of colon, pancreas, gall bladder, bile duct, breast, and lung (Tomita et al., 2000). Because MK-1 antigen is released from carcinoma cells into the blood circulation system under certain conditions so it can be measured in human serum. The MK-1 level in serum from healthy human is lower than 2 ng/mL whereas the MK-1 level in patients with malignant tumors of various tissue organs rises up to 2-78 ng/mL (Abe et al., 2002).

Interleukin 2 (IL-2), a pleiotropic glycoprotein in immune system, has been accepted for use in cancer immunotherapy of human. The interaction of IL-2 with its receptor on immunological cells stimulates proliferation and activation of natural killer cells, thus generating lymphokine-activated killer (LAK) cell. However, given a high dose of IL-2 can cause the severe toxicity in patients and suboptimal concentration at the tumor site have limited its efficiency *in vivo*. The selective delivery of IL-2 at the tumor site would be avoided the toxicity associated with systemic administration (Matsumoto et al., 2002).

Single chain variable fragment (scFv) antibody is a fusion protein of variable region of antibody heavy chain (V_H) and light chain (V_L) fused together into a single chain polypeptide chain by a short peptide linker. It carries the complete-binding site in a single poly-peptide chain of small size that remains stable biological activity even at low concentration. Because of its small molecular size, scFv could be cleaned clear more rapidly from the blood in patients and has better tumor penetration property when compared with full-length antibody. It may also elicit little or no immune response after administration due to short duration in the circulatory system of patients. These advantages of scFv over full-length antibody can enhance the immune

response to the tumor site while reducing systemic side-effect (Damasceno et al., 2004).

Some researchers have attempted to engineer the scFv-IL-2 fusion protein that retains both scFv and IL-2 associated function. So scFv-IL-2 is able to target to tumor sites and activate of antitumor response that in some cases results in a complete elimination of the tumor (Lio, 2001; Zang, 2006; Shen, 2006). Kuroki et al.(2002) have expressed the fusion protein FU-MK-1-scFv/IL-2 in *P. pastoris* in flask cultivation and reported the amount of the scFv antibody approximately 2 mg/L in the culture medium supernatant. They purified the fusion protein by Ni-affinity chromatography and characterized for its biological activity in SCID mice model. The fusion protein showed a marked suppress tumor growth in SCID mice model and also enhanced the immune response to human MK-1 expressing tumor while significantly reducing systemic toxicity. However, the fusion protein production has never been optimized and produced in fermenter (Kuroki et al., 2002).

In this study, we reported the optimization in flask cultivation for high expression of IL-2/FU-MK-1-scFv using *P. pastoris* strain GS115 by examining the effect of pH value from 3 to 10, temperature at 20, 25, 30, and 37 ^{o}C and methanol concentration of 0.01, 0.1, and 0.5%. Next, the optimal condition obtained from flask cultivation was applied in the fed-batch cultivation in 5 L fermenter. Finally, the fusion protein was purified by Ni-affinity chromatography and tested the specific binding antigen against the MK-1 expressing CHO cell.

1.2 The objectives of the research

1.2.1 To produce fusion protein IL-2/FU-MK-1-scFv by *P. pastoris* in 5 L fermenter.

1.2.2 To characterize the specific binding activity of partially purified fusion protein produced in this study.

1.3 The scopes of the research

1.3.1 Researching and study the information of scFv production

1.3.2 Transformation and selection of expressing clone

1.3.3 Optimization of the fusion protein expression in shaken flask cultivation

1.3.4 Production of IL-2/FU-MK-1-scFv in 5 L fermenter

1.3.5 Partial purification of the fusion protein by Ni-affinity chromatography

1.3.6 Characterization of the fusion protein for specific binding activity against MK-1 antigen

1.3.7 Discussion and conclusion

CHAPTER II

LITERATURE REVIEW

2.1 MK-1 antigen

MK-1 antigen, a transmembrane glycoprotein with a molecular weight of 40 kDa, is recognized by monoclonal antibody FU-MK-1. The histochemical distribution of the MK-1 antigen is similar to the known distributions of a number of carcinoma-associated antigens, such as the 17-1A antigen defined by MAb CO17-1A, the KS1/4 antigen or KSA recognized by MAb KS14, the C215 antigen identified by MAb C215, the Trop-1 antigen recognized by MAb 162-21.2, the GA733-2 antigen identified by MAb GA733 and the glycoprotein-2 (EGP-2) specified by MAb HEA125. The MK-1 is widely overexpressed on the surface of various carcinomas such as stomach, colon, pancreas, gall bladder, bile duct, breast and lung. Moreover, the MK-1 has shown promise as a target in approaches to passive and active immunotherapy of colorectal cancer and has been used as a target for immunotherapy using monoclonal antibody in several clinical trials (Tomita et al., 2000; Masumoto. et al., 2000). In serum from healthy human, the MK-1 level is lower than 2 ng/mL, but the MK-1 level in patients with malignant tumors of various tissue organs is increased up to 2-78 ng/mL, indicating that MK-1 is released from tumor cells into the blood circulation under certain conditions (Abe et al., 2002).

2.2 Cancer immunotherapy

Cancer**,** a class of diseases is a group of the uncontrolled growth cells, invasion and sometimes metastasis, spreading to other organs in the body via lymph or blood. These malignant properties of cancers separate them from benign tumors, which are self-limited, and not invasion or metastasis (Abercrombie and Ambrose, 1962). The cancer prediction in patients is most diagnosed with the type of cancer, as well as the stage, or location of the disease. Most cancers can be treated and some are forced into release with a combination of radiotherapy, chemotherapy and surgery, depending on the specific type, location and stage of targeting cancer.

Research developments for cancer treatments are focusing on more specific to varieties of carcinomas because chemotherapeutic strategies can cause various toxicities and have limited in their efficacy. There have been significantly progressed in the development of targeted therapy drugs that minimize damage to normal cells in which it has specifically function to detect abnormalities of diseases and tumors. Therefore, targeted cancer therapies may be more effective than current treatments and less toxic to normal cells.

Nowadays, cancer immunotherapy is a growing field with the purposes at restoring and enhancing immune function to attack and eliminate carcinomas (Woan and Reddy, 2006). Monoclonal antibodies (MAbs) are majority of drugs in cancer immunotherapy. Because the remarkable specificity of MAbs as targeted therapy makes them promising agents for human therapy and less toxic than cytotoxicity chemotherapy agents. Moreover, MAbs specifically bind to antigen on the surface of cancer cells that induces an immunological response in the cancer cell. Not only MAbs can be used therapeutically to protect against diseases, but they can also be used to diagnose a varieties of diseases (Adams and Weiner, 2005; Gupta and Srivastava, 2006). There are currently therapeutic antibodies for cancer treatment that have been approved by FDA (Table 2.1).

Table 2.1 MAbs approved by FDA in cancer therapies (Kim et al., 2005)

Drugs	**Disease indications**	**Year**	**Companies**
Panorex (Edrecolomab)	Colorectal cancer	1995	GSK/Cenyocor
Rituxan (Rituximab)	Non-Hodgins lymphoma	1997	IDEC
Herceptin (Trastuzumab)	Metastatic breast cancer	1998	Genentech
Mylogtarg (Gemtuzumab ozogamicin)	CD33-acute myeloid leukemia	2000	Celltech
Campath (Alemtuzumab)	B-cell Chronic lymphocytic leukemia	2001	Millennium

Zevalin (Ibritumomab tiuxetan)	Non-Hodgins lymphoma	2002	IDEC
Bexxar (Tositumomab)	Non-Hodgins lymphoma	2003	Corex/GSK
Erbitux (Cetuximab)	Colorectal cancer	2004	Imclone
Avastin (Bevacizumab)	CRC, breast, renal, NSCL cancer	2004	Genentech

However, full-length antibodies or MAbs with high molecular weights diffuse or penetrate poorly from vascular bed into target tumor site and are cleared slowly from the body. They also possess some side effects in patients because they lead to an anti-antibody response (AAR) or immunogenicity (Table 2.2). The immunogenicity of mouse antibodies in human is one of the major reasons why early monoclonal antibodies did not deliver the anticipated therapeutic benefits. The potential for immunogenicity gives rise to 3 principal concerns. Firstly, a severe allergenic or anaphylactic shock response may occur. Secondly, it is possible that immune response may induce autoimmunity to endogenous proteins of patients. Third, an immune response to the therapeutic proteins could reduce its efficacy. These problems lead to the development of smaller size or lower immunogenicity antibodies by minimization of the mouse component of antibodies (Schellekens, 2002; Kim et al., 2005).

Table 2.2 Reported human anti- mouse antibody (HAMA) (Hwang and Foote, 2005)

Antibodies	**Indications**	**Patients with AAR (%)**
2H4 and 5D3	Nasopharyngeal carcinoma	100
I^{131}-T101	Cutanous T-cell lymphoma	100
17-1A	Colorectal CA	100
14G2a	Refractory melanoma, nueroblastoma or osteosarcoma	89
A7-NCS	Colorectal carcinoma	100
Anti-CEA antibody fragment (A5B7-	CEA-bearing tumors	100

F(ab')2)		
B-C7	Septic shock	100
B-E8	Myeloma, renal cell carcinoma	75
BrE-3, 111In-MX-DTPA	Human ductal breast cancer	85
BW 494/BI 51.011	Pancreatic cancer	94
CCR086 indium-111-labeled	Detection of colorectal carcinoma (imaging)	80
D612	Metastatic gastrointestinal cancer	86
HMFG1, HMFG2, H17E2, B72.3	Ovarian cancer radioimmunotherapy	100
I or EGF/r3	Gliomas or meningiomas	89
L6	Adenocarcinoma	64
MAb with high HIV-1 neutralizing titers	Human immunodeficiency virus	73
OC/TR bispecific mAb	Intraperitoneal (i.p.) treatment of ovarian cancer	100
OC125	Ovarian cancers	100
OKB7 I^{131}-labeled	CD21-positive, non-hodgkin's lymphoma	75
OKT3	Graft rejection	86
T101	Cutanous T-cell lymphoma (chronic lymphocytic leukemia)	100
ZME 018 and 96.5	Melanoma or basal cell carcinoma	88

2.3 Single chain variable fragment (scFv) antibody

Single chain variable fragment (scFv) antibody, one of the common used antibody fragments, consists of the variable regions of antibody heavy chain (V_H) and light (V_L) fused together into a single chain polypeptide chain via a short peptide linker (Preyre et al., 2000) (Figure 2.1). It carries the complete-binding site in a single poly-peptide chain of minimal size and has a monomer structure that remains stable biological activity even at low concentration. Because of its small molecular size,

scFv could be cleaned clear more rapidly from the blood and has better tumor penetrating property when compared with full-length antibody. It may also elicit little or no immune response after administration because it has short duration in the circulatory system and its size relatives to glomerular filtration, which is estimated to be approximately 60 kDa. In contrast, full length antibody molecular weight (~150 kDa) is too large to be filtrated by the kidney. Smaller molecule of scFv may be subject to diffuse rapidly in to the tumor site and extensive kidney clearance. So, scFv may lead to reduce normal tissue binding and also reduce cytotoxicity in patients. These advantages of scFv over full-length antibody can enhance the immune response to tumor site and it is now being utilized for specific delivery of cytotoxic agents while reducing systemic side-effect.

In general, the production of monoclonal antibody is routine. The monoclonal production using conventional hybridomas technology has been difficult because human hybridomas and immobilized cell lines do not stably produce in high level of antibody. So many scFv antibodies have been constructed by genetic engineering technique to replace full-length antibody and developed by expression in microbial for easy to produce in large amount (Table 2.3) (Damasceno et al., 2004; Beckman et al., 2006).

Table 2.3 Genetic engineered scFv antibodies

scFv antibodies	**Specific antigen**	**References**
Anti-CEA scFv	Carcinoma embryonic antigen (CEA)	Freyre et al.,1999
$scFv_{59D8}$	a fibrin-specific epitope	Peter et al., 2000
scFv4813	USP18 protein	Hellwig et al., 2000
scFvB80	Prostate specific antigen (PSA)	Wang et al., 2001
ND-1scFv	Colorectal carcinoma	Fang et al., 2002
Anti-TfR scFv	transferin receptor	Yang et al., 2004
A33scFv	a cell surface glycoprotein expressed in colon cancer	Damasceno et al., 2004
scFv of domoic acid	domoic acid	Hu et al., 2005
Anti-CD33 scFv	CD33, a surface glycoprotein	Emberson et al., 2005

	expressed on myelomonocytic lineage, leukemia cell	
Anti-MUC1 scFv	A cancer associated mucin, MUC1	Rahbarizadeh et al., 2005
125E11scFv	PreS1(21-47) fragment of large hepatitis B surface antigen	Yang et al., 2005
svFvNT73	The β' unit of *E.coli* RNA polymerase	Lamberski et al., 2006
IL-2-183BscFv	An ovarian carcinoma-associated antigen	Zang et al., 2006
scFV107	The leukocyte adhesion molecular CR3 and the CD11bA domain	Tanfous et al., 2006
scFv-C_1	HM-1 killer toxin	Krishniaswamy et al., 2009
scFv18-2	The DNA-dependent protein kinase catalytic subunit	Xiong et al., 2009

Accordingly, single chain variable fragment (scFv) antibody, the smallest fragment antibody which is developed to replace full-length antibodies. It can be generated by retaining the antigen binding specificity of whole antibody and removing the entire constant region or whole of fragment crystallizable (Fc) region . This fragment antibody is better clearance from whole body and also better tissue or tumor penetration characteristics. As shown in table 2.3, the scFv is better than full-length antibody. However, the scFv has not effector functions including antibody-dependent cellular cytotoxic (ADCC) and complement-dependent cytotoxicity (CDC) because of without of Fc region (Kim et al., 2005). Therefore, it can be improved their effector functions or their therapeutic efficacy by conjugating with cytotoxic drug (scFv-liposomes, Marty et al., 2002), enzyme (A33scFv-cytosine deaminase, Klinik et al., 2003), radio-isotopes (^{124}I-anti-CD20 scFv dimmers, Olafsen et al., 2010) and cytokines (IL-2, Zang et al., 2006; Masumoto et al., 2002). IL-2 conjugation is one choice for effector function improvement of the scFv because of its characterization.

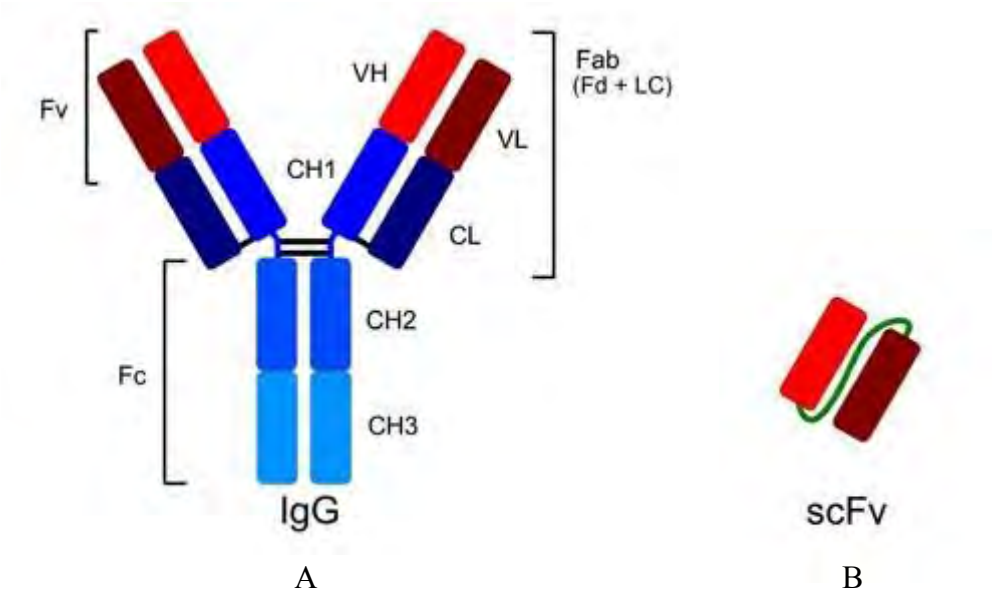

Figure 2.1 Simple structure of A) full length antibody (IgG) and B) scFv antibody (Kim et al., 2005).

Table 2.4 Comparisons of the properties between full-length antibody and scFv (Beckman et al., 2006)

Properties	**Full-length antibody (IgG)**	**scFv**
Size (kDa)	~150	~25
Diffusion and penetration	Slow	Rapid
Immunogenicity	High	Low
Clearance in body	Slow	Rapid
Side effect	More	Less
Production process	Difficult*	Easy
Amount of product	Lower*	Higher

*hybridoma technology

2.4 Interleukin 2 (IL-2)

IL-2, an amino acid protein with a molecular weight of 15.5 kDa, is a potent immunoregulatory cytokine with well-documented activity that plays an important role in immune system. It has been clinically investigated and approved by the FDA

to use for immunotherapy of renal cancer and melanoma for many years. One approach is the administration of IL-2 agent aimed at enhancing, repairing and improving the immune function. In T-lymphocyte activation and proliferation, it promotes proliferation and stimulates cytotoxicity of NK cell. In addition, the interaction of IL-2 with its receptor on immunological cells also induces proliferation and secretion of immunoglobulin by B-lymphocyte and activates the production of lymphokine like TNF-α and IFN-γ. IL-2 is produced mainly by T-helper lymphocyte following stimulation by antigens, mitogens or other cytokines such as IL-1 and IL-6.

The clinical applications of IL-2 have centered on the cancer treatment because IL-2 can activate lymphocyte to become lymphokine-activated killer (LAK) cell that have a broad range of tumor cell targets. IL-2 has been studied in combination with cellular therapy using tumor-infiltrating lymphocyte (TIL) to eradicate the tumor. The high dose IL-2 regulation consisting of 600,000 IU/kg i.v. every 8 hours on 1-5 days has been approved as the treatment of choice in cancer patients. However, the severe toxicity of IL-2 when given in high dose and the suboptimal concentration at the tumor site has been limited its efficiency *in vivo*. Side effects include hypotension, cardiac arrhythmias, pulmonary edema, fever, increased capillary permeability, catheter-related sepsis and death. Due to the toxicity of overdose IL-2 in patients, the selective delivery of IL-2 at the tumor site would be used to avoid the toxicity and reduce spreading of IL-2 associated with systemic administration. So IL-2 fused to antibody is expected to achieve effective cytokine concentration in the tumor site without side effect associated with long-time high dose cytokine administration (McDermott et al., 1998; Sintuwattanawibool, 1998; Matsumoto et al., 2002; Dala Cruz et al., 2004).

2.5 Antibody-IL-2 fusion protein

Genetic fusion of IL-2 to antibodies were carefully investigated and constructed in an attempt to efficiently target to the tumor site for cancer immunotherapy. Moreover, *in vitro* testing clearly showed that an antibody-IL-2 fusion protein targeting to a carcinoma cell line increased killing of these tumor cells by T-cells. This anti-tumor effect was also obvious in pretrial tumor models in which tumor eradication in the carcinomas was dependent on T-cells or natural killer cells. The

antibody-IL-2 fusion protein seemed to exert its therapeutic effect by boosting a pre-existing T-cell response and required helper T-cell. Moreover, systemic IL-2 administration enhances the activity of antibody-IL-2 fusion protein treatment. In addition to use as anticancer agents, antibody-IL-2 fusion proteins were also tested as adjuvants. Therapeutic vaccination with dendritic cells loaded with tumor antigens only induced a therapeutic effect when vaccination was followed by IL-2 administration. This therapeutic effect could be clearly improved by the application of tumor-targeted IL-2 instead of systemic IL-2 treatment. Interestingly, dendritic cell vaccination followed by treatment with antibody-IL-2 fusion protein influenced the development of the memory immune response (Schrama et al., 2006).

2.6 *Pichia pastoris*

The methylotrophic yeast *Pichia pastoris* is widely used for many recombinant proteins production. *P. pastoris* is a unicellular microorganism and similar to bacteria, relatively fast growth, and inexpensive to manage in a simplicity cultivation or production process. However, differ from bacteria; yeasts are also eukaryotes with the same intracellular environment and many of the same post-translation protein processing capabilities as higher eukaryotes, including humans. Therefore, eukaryotic proteins are more likely to be correctly processed, fold and assembled into functional molecules when expressed in yeast. There are important factors to success of the *P. pastoris* expression system relative to other yeast species. The first factor is that, unlike *Saccharomyces cerevisiae* and many other yeast species, *P. pastoris* does not ferment sugars or other carbon sources. The advantage of the yeast for respiratory growth greatly facilitates its culturing at high cell densities because it does not produce significant amounts of the toxic fermentative product as ethanol. It simply turns the carbon source into more biomass. Because the productivity of recombinant production process is generally proportional to the density of biomass generated, the ability to easily grow this yeast at high cell densities is a major advantage. The second factor is related to the ability of *P. pastoris* to grow on methanol as a carbon and energy source. Like many alternative carbon source pathways, growth on methanol requires the induction of a specific set of metabolic enzyme as alcohol oxidase (*AOX*) (Cregg, 2007; Wenhui, 2007). Because of *P. pastoris* characterization and applications (Table 2.4) which compared to others expression host system this yeast

becomes popular host for recombinant protein expression and over 400 proteins have been produced in this yeast because it can be genetically engineered and to express proteins for both basic research and industrial use *P. pastoris* (Lin Cereghino et al., 2002).

The *P. pastoris* expression system offers economy, ease of manipulation, the ability to perform complex post-translational modifications and high expression levels. It can be growth to high cell density for large scale production and also has good capacity for secreted recombinant proteins. Using this system, recent advances have been made in the quality of recombinant proteins in fermenter culture and in the quality of the proteins. There are two *AOX* operons in the *P. pastoris* chromosome, namely *AOX1* and *AOX2*. *AOX1* is tightly repressed when the cells are grown on glucose, ethanol or other substrates. In contrast, the *AOX1* are tightly regulated when the cells are grown on methanol, *AOX1*can be up to 30% of the total cellular protein. The *AOX1* is the majority of alcohol oxidase activity, responsible for approximately 90%, whereas *AOX2* accounts for the rest, responsible for only a small percentage of the total alcohol oxidase activity in grown cell culture by methanol induction (Jahic et al., 2006).

Table 2.5 Comparison of production systems for recombinant human protein (Ma et al., 2003)

Host system	**Expression level**	**Overall cost**	**Scale-up ability**	**Product quality**	**Production time scale**	**Contamination risks**
P. pastoris	Low-high	Medium	High	Medium	Medium	Low risk
E. coli	High	Low	High	Low	Short	Endotoxin
Mammalian cells	Low-moderate	High	Very low	Very low	Long	Virus, prions and oncogenic DNA
Transgenic animals	High	High	Low	Very high	Very long	Virus, prions and oncogenic DNA
Transgenic plants	High	Very low	High	High	Long	Low risk

Recombinant proteins expression in *P. pastoris* can be produced either intracellular or extracellular. Because this yeast secretes low level of endogenous proteins or native proteins, secreted recombinant protein often constitutes a major of the total in the culture medium. Therefore, directing a recombinant protein to the culture medium serves as substantial first step of purification. However, because of protein stability and folding requirements, the option of secretion is usually served for

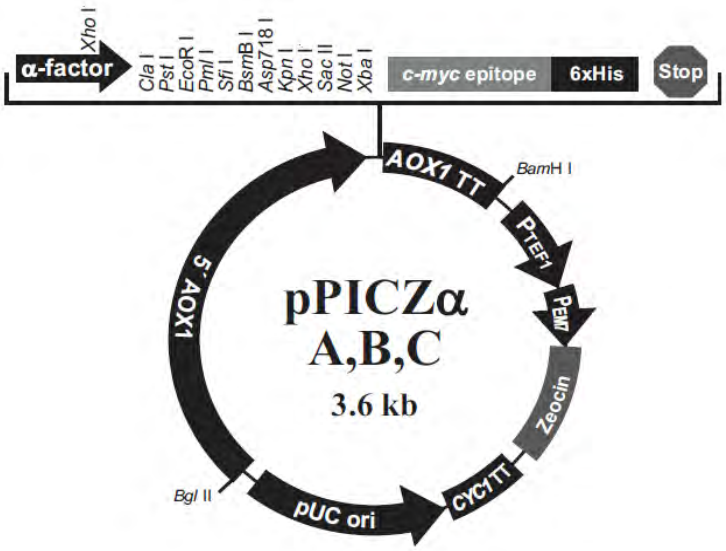

Figure 2.2 *P. pastoris* vector pPICZαA, B, C. Multiple cloning site is flanked by *S. cerevisiae* α-mating factor secretion signal, *c-myc* and 6xHIS tags for immunological detection and purification. The *TEF*1 promoter and *CYC1*promoter are from *S. cerevisiae*. *EM7*promoter is from *E. coli*. *BamHI* and *Bg/II* sites allow for cloning of multiple head-to-tail expression cassettes (Lin Cereghino, 2002).

foreign proteins that are normally secreted by their native hosts. Using appropriate *P. pastoris* expression vector, secretion signal such as the *S. cerevisiae* α-mating factor (*MF*) prepo peptide or *P. pastoris* acid phosphatase *(PHO1*) can be constructed for secretion of heterologous proteins. A listing of commonly used expression vector is shown in Table 2.5.

Although several secretion signals have been used successfully for recombinant proteins expression, results for a wide range of proteins have either been variable or unavailable. The *S. cerevisiae* α-mating factor prepo peptide, which is readily available on vectors containing the Zeocin resistant gene, such as pPICZαA, B, C

series from Invitrogen (Figure 2.2) have been used with the most consistent success (Lin Cereghino and Lin Cereghino, 2007).

Table 2.6 Common *P. pastoris* vectors for secreted expression

Vector name	Selectable markers	Features	References
pPICZα	*ble*^{r}	*AOX1p* fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; potential for fusion of foreign protein to HIS_6 and *myc* epitope tags; Zeocin selection for multiple strains	Invitrogen
pPIC9K	*HIS4* and *kan*^{r}	*AOX1p* fused to α-MF prepro signal sequence; *Eco*RI, *Not*I, *Sna*BI and *Avr*II sites available for insertion of foreign genes; G418 selection for multiple strains	Scorer et al., 1994
pPIC6α	*bsd*^{r}	Similar to pPICZα except blasticidin resistance used for direct selection of multiple strain	Invitrogen
pGAPZα	*ble*^{r}	*GAPp* fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; potential for fusion of foreign protein to HIS_6 and *myc* epitope tags; Zeocin selection for multiple strains	Invitrogen, Waterham et al., 1997
pFLDα	*ble*^{r}	Similar to pGAPZα except expression controlled by *FLD1* promoter for inducible expression with methylamine	Invitrogen, Shen et al., 1998
pHIL-S1	*HIS4*	*AOX1* fused to *PHO1* secretion signal; *Xho*I, *Eco*RI and *Bam*HI site available for foreign genes	Invitrogen
pJL1-SX	*FLD1*	*AOX1p* fused to α-MF prepro signal	Sunga and

		sequence; *FLD1* as selectable marker	Gerngross, 2004
pBLHIS-SX *pBLARG-SX* *pBLADE-SX* *pBLURA-SX*	*HIS4* *ARG4* *ADE1* *URA3*	Series of vector with *AOX1p* fused to α-MF prepro signal each with one of fourth biosynthesis gene markers; *HIS4,ARG4,ADE1,URA3*	Lin Cereghino et al., 2001

2.7 Important operational factors to enhance the production of recombinant protein in *P. pastoris*

Although expression of heterologous proteins can be carried out in shaken flask cultivation, the proteins levels are typically much higher in fermenter production. Fermentation is essential to express the proteins because yields correlate largely with the cell density. In some cases, changing from shaken flask expression to fermentation can cause a dramatic increase in yield, with reports of expression levels in fermentation being 10-fold higher than in shake flasks. For *P. pastoris*, two-phase fermentation is generally applied. In the first phase (I) the cells are grown until glycerol is depleted. In the second phase (II) gene expression begins by feeding methanol to the fermenter. Until now, many heterologous proteins have been expressed in *P. pastoris* because the level of expression is equal to *E. coli* but significantly higher than *S. cerevisiae* (Romanos et al., 1992). Particularly importance is the organism's ability to secrete proteins: the secreted product can comprise more than 80% of the total protein in the culture medium. Expression and secretion of these heterologous proteins, however, not only depend on gene dosage, but also on other factors, such as signal sequence recognition and processing, proteolysis, fermentation, and glycosylation.

In fermentation process, there are several factors affecting yield of production, including medium composition, type of strain and non-nutritional factors, such as culture pH, agitation speed, percentages of dissolved oxygen, methanol induction, and

fermentation strategy. To improve the productivity of a correctly processed protein, shaken flask, a small scale expression method is often the first step employed in optimization of protein level and selecting culture medium for production in large scale (Li et al., 2007).

2.7.1 Medium composition

Similar to others yeast, *P. pastoris* needs carbon and nitrogen source for growth. Glucose and glycerol are the most common carbon sources and peptone, yeast extract, and yeast nitrogen are nitrogen sources. BMGY containing glycerol and YPD containing glucose are the usual medium for seed culture, while for large scale in fermenter production, two basic mediums, basal salts, and FM22 have shown good yields. Medium composition is main parameter to influence recombinant protein production in yeast by affecting growth and viability of yeast (Kang et al., 2000; Chen et al., 2000).

2.7.2 Temperature

Temperature has a profound impact on cell metabolism and abundance/regulation of folding-related genes or proteins. Lowering the cultivation temperature from 30 to 20 °C has been reported to increase product titers in yeasts in several cases (Li et al., 2001; Shi et al., 2003; Hackel et al., 2006; Lin et al., 2007). While it may be speculated that a lower growth temperature is leading to lower specific growth rates, thus enabling folding of the recombinant proteins at a lower rate, it was shown recently in chemostat cultures that actually gene regulatory events take place. In continuous cultures of *P. pastoris* expressing a human antibody fragment specific productivity of the heterologous protein was significantly increased during the chemostat process at lower temperature (Gasser et al., 2007). In several reports, expression of recombinant proteins in *P. pastoris* at temperatures of 23 to 30 °C has been examined to minimize extracellular proteolysis. *P. pastoris* could be growth at suitable temperature, leading to reduce protease activity and enhance yields of production (Li et al. 2001; Curvers et al., 2001).

2.7.3 pH value

Optimal pH value is crucial for *P. pastoris* cell growth, proteins formation and stability. So, pH-controlled fermentation process is usually chosen in *P. pastoris* expression system (Soden et al., 2003). The kinetics of proteolytic activity, in the presence or absence of cells, was dependent on pH. Adjustment of pH value for production is necessary for minimizing the degradation of recombinant proteins secreted into the culture medium. *P. pastoris* can tolerate a pH range of 3 to 7, with minimal effect on the cell growth. Some reports, decreasing the pH value from 5 to 4 in fermenter culture increased yields from 40 to 90% during the expression. In some cases, increasing the pH of the culture medium from 6 to 7 has shown result in significant improvement. However, controlling of the culture medium pH during the fermentation production process is important factor, the optimal pH depending on individual recombinant protein properties, especially stability (Li et al., 2007).

2.7.4 Dissolved oxygen

Dissolved oxygen (DO) is a part of the most important keys for *P. pastoris* cell growth and recombinant proteins expression when producing in the fermentation process. Because The DO can be controlled by the agitation speed rate and the air or oxygen flow rate. During the glycerol phase, usually period of 20-24 h, the DO is controlled via using the agitation up to 800 rpm for a 4 L fermenter and 450 rpm for a 60 L fermenter. The DO is also controlled by adjustment of the air or oxygen flow rate into the fermenter. When the DO value raises rapidly, DO spike, indicating that glycerol is consumed completely. Most *P. pastoris* fermentation, the DO should be kept constantly at 30-40% or different optimal value (Stratton et al., 1998).

2.7.5 Methanol induction

The concentration of methanol induction is crucial for expressing successfully recombinant proteins in *P. pastoris* and methanol feeding is related closely to the DO level. To maintain the optimal concentration of methanol, the methanol induction phase strategies is considered and three methanol feeding strategies can be introduced. First, the feeding rate of methanol is controlled according to the concentration in the cultivation medium as measured by gas

chromatography. It is not easy to control the feeding rate with this strategy because samples need to be taken for analysis. Second, the methanol feeding can be controlled by the DO level. The feeding rate should be adjusted in high rate when the DO increases. In this method, the concentration of methanol is not given precisely by the DO value. The methanol maybe accumulates and can cause toxic to cell. The third strategy, the controlling of methanol feed rate depend on the specific growth rate during induction phase. If the kinetic of *P. pastoris* growth is well set up and described, it is an effective key to keep the concentration of methanol within an optimal level (Minning et al., 2001).

2.8 Ni-affinity column chromatography

Affinity tags are highly effective tools for fusion protein purification and native protein complexes for excellent reasons. The use of affinity tags has become popular in protein purifications of several researches. With modern advantages in molecular method, many proteins being produced using recombinant techniques are significant increasing because they can provide the protein purification from crude extracts without prior steps to remove nucleic acids or others cellular materials (Lichty et al., 2005; Arnau et al., 2005). Moreover, there are many advantages of affinity tags system which shared the following features: (a) one-step adsorption purification; (b) mild elution conditions in purification step; (c) a minimal effect on proteins structure and biological activity; (d) low cost of proteins purification; (e) easy and specific removal for production of the native protein; (f) simple and accuracy assay of the fusion protein in purification step; none immunogenicity and not interference with the fused protein; increasing the sensitivity of binding assay (Bucher et al., 2002).

Over 20 years, most of the available fusion proteins and peptide affinity tags were developed. The most frequently used and interesting tags are Arg-tag, calmodulin-binding peptide, cellulose-binding domain, DsbA, c-myc-tag, glutathione S-transferase, FLAG-tag, HAT-tag, His-tag, maltose-binding protein, NusA, Stag, SBP-tag, Strep-tag, and thioredoxin (Table 2.6). Depending on the affinity tag nature and its target, affinity tags can be divided into three types. The first type, a peptide tag of fusion protein that binds to small molecular ligands linked on a solid support. For example, the histidine tag binds to immobilized metal as nickel (Ni^{2+}). The second

type, a peptide tag binds to a protein-binding immobilized on resin of chromatography. For example, the calmodulin-binding peptide of fusion protein binds specifically to calmodulin allowing the fusion protein binds to the peptide to be purified by calmodulin resin. The third type is a subset of the second type that binds to an immobilized protein partner such as an antibody which recognizes a specific peptide epitope. For example, the FLAG peptide, that can be used with one of several anti-FLAG antibody resin (Lichty et al., 2005).

Table 2.7 Properties of affinity tags (Lichty et al., 2005; Arnua et al, 2006)

Tags	**Residues**	**Size (kDa)**	**Matrix**	**Elution condition**	**Cost/10 mg ($)**
Poly-His	2-10	0.84	Ni^{2+}-NTA, Co^{2+}-CMA (talon)	Imidazole 20-25 mM or low pH	18
Poly-Arg	5-6	0.80	Cation-exchange	NaCl linear gradient from 0-400 mM at alkaline pH >8	N/A
FLAG	8	1.01	Anti-FLAG MAb	pH 3 or 2-5 mM EDTA	1045
Step-tag II	8	1.06	Strep-facin (modified streptavidin)	2.5 mM desthiobiotin	293
C-myc	11	1.20	MAb	Low pH	N/A
S-	15	1.25	S-fragment of RNaseA	3 M guanidine thiocyanine, 0.2 M citrate pH 2, 3 M $MgCl_2$	N/A
HAT-	19	2.31	Co^{2+}-CMA (talon)	150 mM imidazole or low	N/A

				pH	
Calmodulin-binding peptide	26	3.00	Calmodulin	EGTA or EGTA with 1 M NaCl	114
Cellulose-binding domains	27-189	20.00	Cellulose	Family I: guanidine HCl or urea > 4 M Family II/III: ethylene glycol	N/A
SBP	38	4.30	Streptavidin	2 mM biotin	N/A
Chitin-binding domain	51	5.59	Chitin	Fused with intein: 30-50 mM dithiothreitol, β-mercaptoetha-nol or cysteine	N/A
Glutathione S-transferase	211	26.00	Glutathione	5-10 mM reduced glutathione	N/A
Maltose-binding protein	396	40.00	Cross-linked amylase	10 mM maltose	12

Histidine tags, the wildly used affinity tag that utilize immobilized metal affinity chromatography for purification of recombinant proteins containing of a short affinity tag at the end of a protein (either N or C terminus). Immobilized metal-affinity chromatography (IMAC) is based on the use of chelated metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) immobilized on a matrix which interacting with specific amino acid side chains. Histidine is the amino acid that displays the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Recombinant proteins containing the sequences of histidine residues are efficiently bound on IMAC.

The method of protein purification with histidine residues was first described by Hochuli et al. in 1987. They have developed a nitrilotriacetic acid (NTA) adsorbent for chelated metal affinity chromatography. In 1988, they successfully purified dihydrofolate reductase by a polyhistidine tag with Ni-NTA matrices. Following washing of the matrix material, the proteins containing polyhistidine sequences can be easily eluted by either adjusting the pH value of the column buffer or by adding free imidazole. The NTA resin is formed a quadridentate chelate and especially suitable for metal ions with coordination numbers of six, since two valencies remain for the reversible binding of biopolymers. The purification efficiency of this system depends on the length of the polyhistidine and the solvent system. The system of 6-his tag proteins shows efficiently under denaturing conditions and 6-his tag proteins can be bound to Ni-NTA matrices under native conditions in low- or high-salt buffers. After binding, the target protein can be eluted by an imidazole gradient from 0.8 to 250

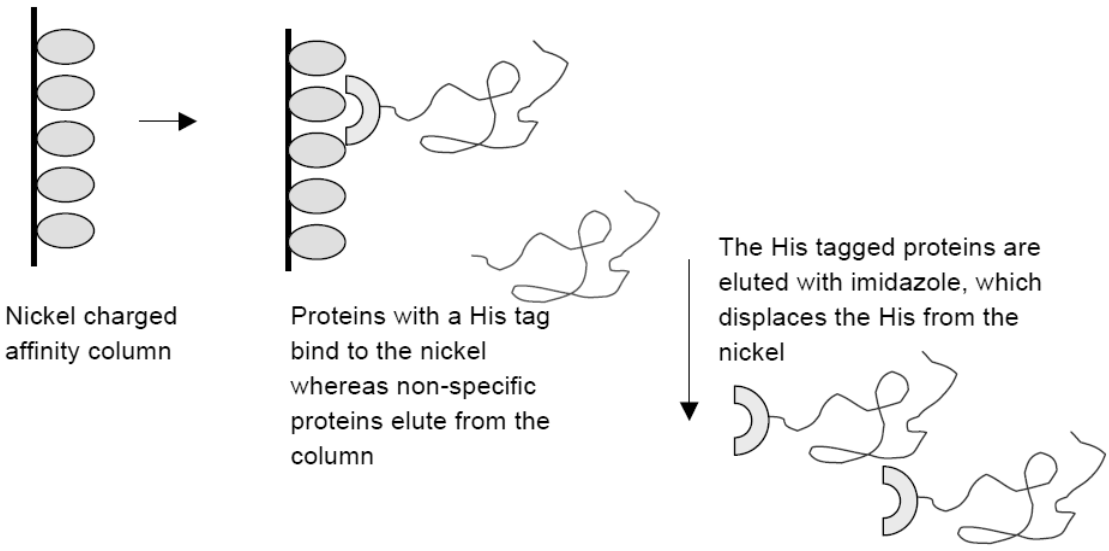

Figure2.3 Ni-affinity chromatography (Yang et al., 2006)

mM. Washing with a low concentration of imidazole reduces nonspecific binding of host proteins with histidines. The 6-his tag proteins is eluted effectively by an imidazole within a range of 20–250 mM (Terpe, 2003).

In Protein Data Bank, over 100 structures of histidine tag proteins have been deposited. Proteins with a histidine tag may vary slightly as far as their mosaicity and diffraction compared to the native protein (Hakansson et al., 2000). In principle, it

cannot be excluded that the affinity tag may interfere with protein activity (Wu and Filutowicz, 1999), although the relatively small size and charge of the polyhistidine affinity tag ensure that protein activity is rarely affected. Moving the affinity tag to the opposite terminus (Halliwell et al., 2001) or carrying out the purification under denaturing conditions often solves this problem. Purification of protein with a metal center is not recommended because the metal can be absorbed by the NTA. Purification under anaerobic conditions is also not recommended because Ni-NTA is reduced. Nevertheless, purification of proteins with his-tag is the most commonly used method (Terpe, 2003).

2.9 Research background involving in this study

2.9.1 MK-1 antigen and MK-1 antibodies

In 2000, Arakawa et al. cloned and sequenced the variable region of heavy chain and light chain of monoclonal antibody FU-MK-1 and constructed a mouse/human chimeric antibody, Ch FU-MK-1, by linking FU-MK-1 V_H and V_κ genes to the human $C_{\gamma 1}$ and C_κ genes, respectively, and fusing the chimeric to each other in a expression vector of mammalian cell. The final gene construct was transfected into mouse non-Ig-producing hybridoma cells. The Ch FU-MK-1 has immunoreactivity like the parental MAb FU-MK-1(Arakawa et al., 2000).

In 2000, Tomita et al. examined the MK-1antigen expression on urological tumor cell lines by flow cytometry and reverse transcription polymerase chain reaction and in 15 cancer tissue specimens by immunohistochemical staining and compared it with carcinoembryonic antigen (CEA), one of the most beneficial human tumor markers. This study indicates that MK-1 antigen can be a useful biological marker for urological cancer cell. And they identified the MK-1 antigen and proved its relationship to other cancer antigens. Immunoprecipitation studies, carcinoma cell lines and the partial amino acid of MK-1 antigen were use for verification the relation of MK-1 antigen and the GA733-2 antigen. The final confirmation of relation, the COS-1 cell was transfected with a GA733-2 cDNA. Then FU-MK-1was demonstrated by immunoprecipitation of the GA733-2 cDNA in CO-1 cell. Their findings indicated that MK-1 was closely related to Ga733-2 antigen and others terms and

MK-1 could be able to use as a target in immunotherapy of various cancers (Tomita et al., 2000).

In 2002, Abe et al. prepared recombinant MK-1 and MAbs of MK-1 and also developed enzyme-linked immunosorbent assay (ELISA) system for determination of the soluble MK-1 level possibly present in the cancer patient serum. They found that the MK-1 levels in serum of patients with various cancers were increased to 2-78 ng/mL, indicating that MK-1 was overexpressed on the surface of tumor cell and secreted into the blood circulation under certain conditions. These results implied that might be a useful tumor marker for MK-1 expressing cancer cell in patients (Abe et al., 2002).

In 2002, Matsumoto et al. constructed recombinant IL-2 fusion with scFv of anti-MK-1 antibody protein. The fusion protein, designed FUscFv/IL-2, was expressed in *P. pastoris*. The secreted fusion protein was purified by Ni-affinity chromatography and characterized for binding specificity and biological activity of IL-2. This study indicated that the fusion protein specifically bound to the MK-1 expressing cell and was able to effectively repress growth of tumor cell. They suggested that this approach may be used for *in vivo* administration to carry IL-2 to tumor cell, enhancing the immune response while significantly reducing systemic side-effect (Matsumoto et al., 2002).

2.9.2 Recombinant antibody-IL-2 fusion proteins

In 1994, Sabzevari et al. genetically engineered fusion protein containing a human/mouse chimeric anti-ganglioside GD-2 antibody and recombinant human IL-2. This recombinant fusion protein was tested for its efficacy to target IL-2 to tumor sites and enhance immune response *in vivo* testing. They reported that the fusion protein can suppress growth of neuroblastoma metastasis in SCID mice reconstituted with human LAK cell and may prove useful for future treatment of human neuroblastoma and other GD-2 expressing human tumor (Sabzevari et al., 1994).

In 1997, Xiang et al. demonstrate that the huKSl/4-IL-2 fusion protein specifically directs IL-2 to the tumor sites to elicit a specific eradication of established hepatic and pulmonary metastasis of KSA-expressing CT26 colon carcinoma cells in

syngenic BALB/c mice. They implied that the fusion protein directed IL-2 to the tumor cells and induces a CD8+ T cell-mediated cellular immune response that effectively eliminated metastasis of colon cancer. This approach may be a new strategy for immunotherapy to induce the eradication of metastatic colon cancer (Xiang et al., 1997).

In 2001, Liao et al. expressed anti-CEA scFv/IL-2 fusion protein in mouse hybridoma cells. The fusion protein was purified by CEA-affinity chromatography and characterized for its biological activity. The anti-CEA scFv/IL-2 fusion protein may be used for *in vivo* administration to deliver IL-2 to tumor cite that enhances the immune response to CEA-expressing tumor cite while significantly minimizing systemic side effect (Liao et al., 2001).

In 2002, Carnelmolla et al. genetically generated L19-IL-2 fusion protein specific to EBD-B human tumor antigen. The fusion protein was purified and characterized for its biological function by treatment of tumor bearing-mice. The L19-IL2 fusion mediated the selective delivery and accumulation of IL-2 at tumor sites, leading to strongly enhance the therapeutic efficacy of IL-2. In mice treated with L19-IL-2 most of the tumor mass was composed of connective and necrotic tissue. This was paralleled by a dramatic increase in the levels of IFN-g and of tumor infiltrating cytotoxic lymphocytes, macrophages, and NK cells, likely responsible for the observed therapeutic effect (Carnelmolla et al., 2002).

In 2006, Zhang et al. genetically fused scfv of monoclonal antibody COC183B2 to IL-2. The IL-2-183B2scFv was expressed in CHO cells and tested its specific binding and biological function. They found that the IL-2-183B2scFv retained the functions of both IL-2 and antibody. It can be effectively used for ovarian cancer immunotherapy by carrying a high concentration of IL-2 to OC183B2-expressing ovarian cancer cells (Zhang et al., 2006).

In 2006, Shen et al. constructed and expressed the recombinant H520C9scFv/IL-2 fusion protein in mammalian cell and tested the antigen binding activity and cytotoxicity of the H520C9scFv/IL-2 *in vitro* testing. The fusion protein retained both IL-2 activity and p185 specific binding. The use of a humanized sFv has the potential to reduce the immunogenicity of the fusion protein and improve its

pharmacokinetic properties with respect to penetration of solid tumors and clearance. This H520C9sFv/IL-2 fusion protein may offer effective way of targeting therapeutic doses of IL-2 to tumors or other targeted cells with significantly decreased cytotoxicity of IL-2 and its immunogenicity (Shen et al., 2006).

2.9.3 Large scale production of recombinant protein by *P. pastoris*

In 2001, Lange et al. constructed and expressed atrazine-specific Fab fragment (K411B) in *P. pastoris*. They also produced this recombinant antibody in 5 L fermenter using fed-batch condition. The recombinant antibody reaching 40 mg/L was successfully expressed and secreted into the medium under methanol induction (Lange et al., 2001).

In 2003, Zang et al. optimized intracellular production of the heavy chain fragment C of botulinum neurotoxin serotype C (BoNT/C(Hc)) in 5 L fermenter by mixed glycerol/methanol feeding strategy based on growth kinetic studies using Mut^+ *P. pastoris* strain. The suitable mixed feeding strategy obtained the highest intracellular BoNT/C(Hc) protein about 3 mg/g wet cells (Zang et al., 2003).

In 2004, Damasceno et al. optimized a large scale production of a humanized A33scFv antibody by examining varying pH and methanol concentration in *P. pastoris*. They reported the optimization of A33scFv production, reaching 4.3 g/L after 72 hours induction with 0.5% (v/v) of methanol at pH 3 (Damasceno et al., 2004).

In 2004, Ning et al. expressed and produced the recombinant human antibody hepatitis B virus antigen (HBsAg) Fab fragment in *P. pastoris* under fed-batch fermentation in a 5 L scale. The recombinant Fab fragment was successfully expressed upon methanol induction reaching 420-458 mg/L and had specific binding activity to hepatitis B surface antigen (Ning et al., 2004).

In 2007, Yamawaki et al. produced scFv against bisphenol A in 2 L fed-batch fermentation process using *P. pastoris*. The scFv concentration of 198 mg/L was obtained under fed-batch culture by maintaining the methanol concentration at 0.39 % (w/v) (Yamawaki et al., 2007).

In 2009, Gurramkonda et al. optimized large-scale production of HBsAg using *P. pastoris* strain GS115 by developing a simple fed-batch fermentation process in 15 L bioreactor. The maximum intracellular HBsAg concentration of 7 g/L was produced under induction with 0.6% (w/v) of methanol and 2.3 g/L soluble HBsAg had competency for assembly into virus-like particles (VLPs), an attribute critical to its immunogenicity and efficacy as hepatitis B vaccine (Gurramkonda et al., 2007).

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and reagents

3.1.1 87% glycerol (Merck KGaA, Germany)

3.1.2 Antifoam A (Fluka, Japan)

3.1.3 Chicken egg ovalbumin (Wako Pure Chemical Industries, Ltd., Japan)

3.1.4 Sodium chloride and Potassium chloride (Merck KGaA, Germany)

3.1.5 Potassium hydroxide and potassium sulfate (Merck KGaA, Germany)

3.1.6 99.9% 1-Pentanol (Sigma-Aldrich Co., USA)

3.1.7 Glycine and Tris-base (Amresco Inc., USA)

3.1.8 Rabbit anti-human IL-2 antibody (MONOSAN®, Netherlands)

3.1.9 D-glucose (Sigma-Aldrich Co., USA)

3.1.10 Ammonium persulfate (Amresco Inc., USA)

3.1.11 40% Acrylamide/Bis solution, 37.5: 1 (2.6% C) (Bio-Rad Laboratories, Inc, USA)

3.1.12 99.9% Methanol (Merck KGaA, Germany)

3.1.13 Hydrochloric acid fuming 37% (Merck KGaA, Germany)

3.1.14 Ultra-pure TMED (Invitrogen™, USA)

3.1.15 Nickel (II) sulfate hexahydrate (Wako Pure Chemical Industries, Ltd., Japan)

3.1.16 Yeast nitrogen base (Bio Basic, Inc. Canada)

3.1.17 Ammonium hydroxide (Mallinckrodt Baker, Inc., USA)

3.1.18 Phosphoric acid 85% (RCI Labscan Limited, Thialand)

3.1.19 2-mercaptoethanol (Bio Basic, Inc. Canada)

3.1.20 Sodium dodecyl sulphate (Fluka, Japan)

3.1.21 Dipotassium phosphate and monopotassium phosphate (Merck KGaA, Germany)

3.1.22 Yeast extract (Bio Springer Inc., France)

3.1.23 Peptone (Difco Laboratories Inc., USA)

3.1.24 Zeocin™ (Invitrogen™, USA)

3.1.25 Protein Detector™ Western Blot Kit (KPL,Inc., USA)

3.1.26 EasyComp™ Transformation kit (Invitrogen™, USA)

3.1.27 His·Bind®Kits 5 ml column purification kit (Novagen®, USA)

3.1.28 Alfa MEM, (Minimum Essential Medium) (Hyclone Laboratorie Inc., USA)

3.1.29 Fetal bovine serum (Hyclone Laboratorie Inc., USA)

3.1.30 Human IL-2 ELISA kit (eBioscience Inc., USA)

3.1.31 WesternBreeze® Chromogenic Western Blot kit (Invitrogen™, USA)

3.1.32 EasyComp™ Transformation kit (Invitrogen™, USA)

3.2 Instruments and equipments

3.2.1 5 L Fermenter and controller set model EPC-1000 (EYELA Tokyo Rikakikai Co. Ltd., Japan)

3.2.2 Shaker model Innova 4330 (New Brunwick Scientific Co., Inc., Edison, N.J., USA) and model Gyromax 707R (Amerex Instruments, Inc., USA)

3.2.3 Refrigerated centrifuge model 1920 and 6500 (Kubota Co., Japan) and model Avanti J-30I (Beckman Coulter Inc., Germany)

3.2.4 Laboratory balance model PG 2002-S and PG 6002-S (Mettler Toledo Co., Ltd., Switzerland)

3.2.5 Analytical balance model AG 204 and AG 285 (Mettler Toledo Co., Ltd., Switzerland)

3.2.6 Spectrophotometer model Spectronic 20 Genesys (Spectronic Unicam Ltd., USA), meodel Gensys 20 (Thermo Spectronic Co., USA) and model Perkin Elmer instruments Lamda 25 UV/VIS Spectrometer (PerkinElmer, Inc., USA)

3.2.7 Laminar flow ISSCO model BV-124 (International Scientific Supply Co., Ltd., Thailand), model Clear V3-4 (Triwork 2000 Co., Ltd., Thailand) and Bosstech model HVB 120S (Boss Scientific Associate L.P., Thailand)

3.2.8 Hot air oven (Memmert Co., Ltd., Germany)

3.2.9 Autoclave model SS-325 and ES-315 (Tomy Seiko Ltd., Japan), modelMLS 3020 (Sanyo Co., Ltd., Japan) and HV-25 (Hirayama Co., Ltd., Japan)

3.2.10 pH meter model SevenEasy (Mettler Toledo Co., Ltd., Switzerland)

3.2.11 Incubator model INE 500 (Memmert Co., Ltd., Germany)

3.2.12 Stirring hot plate model DS 201HS (DMC Co., Japan)

3.2.13 Gas Chromatography model 3400C (Varian Inc., USA)

3.2.14 Deep freezer (-80 $^{\circ}$C) (Forma Scientific Inc., USA and Sanyo Electric Co. Ltd., Japan)

3.2.15 Deep freezer) (-20 $^{\circ}$C) (Sanyo Electric Co. Ltd., Japan)

3.2.16 Freezer (4 $^{\circ}$C) (Mitsubishi Electric Co., Japan)

3.2.17 Micropipette model P20, P200 and P1000 (Gilson Inc., France)

3.2.18 Erlenmeyer flask and baffle flask (PYREX®, USA)

3.2.19 Test tube, cylinder and beaker (PYREX®, USA)

3.2.20 Centrifuge ware (NALGENE® Labware, USA)

3.2.21 Mni Protein 3 cell and power supply model PowerPac HC™ (Bio-Rad Laboratories, Inc, USA)

3.2.22 Semi-Dry Transfer cell model Trans-Blot SD cell and power supply model PowerPac Basic (Bio-Rad Laboratories, Inc, USA)

3.2.23 Mini rocker model MR1 (Biosan Laboratories, Inc, USA)

3.2.24 Vivaspin 500 ultrafitration 30,000 MWCO (Vivascience Inc., Germany)

3.3 Microorganism and cell line

3.3.1 *Pichia pastoris* strain MK71H Lot no. 1364726 and *P. pastoris* strain GS115 Lot no. 1364723 (Invitrogen™, USA)

3.3.2 Chinese hamster ovary cells expressing MK-1 (CHO-MK-1) and CHO-cell expressing CEA but not MK-1 (CHO-CEA) were obtained from Professor Masahide Kuroki, MD, PhD. Department of Biochemistry, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jounan-ku. Fukuoka 814-0180, Japan.

3.4 Transformation and selection of eoxpressing clone

3.4.1 Preparation of competent cells

According to EasyComp™ Transformation protocol (Invitrogen™, USA), *P. pastoris* strains were inoculated into 10 ml of yeast extract peptone dextrose medium (YPD, Appendix A) and growth overnight at 30 °C in 200 rpm shaking incubator. The overnight cultures were diluted to an OD_{600} of 0.17 in 10 ml of YPD. And they were growth at 30 °C in 200 rpm shaking incubator until the OD_{600} reached 0.84. The cells were centrifuged at 500X g for 5 minutes at 4 °C and the supernatant was discarded. The cells were suspended in 1 ml of solution I and centrifuged again at 500x g for 5 minutes at 4 °C and the supernatant was discarded. The cells were resuspended in 1 ml of solution I. Finally, the cells were competent cells or kept in a -80 °C freezer for further using in transformation step.

3.4.2 Transformation

According to EasyComp™ Transformation protocol (Invitrogen™, USA), the pPICZαA-IL-2/scFv(V_H-V_k) vector was transformed into *P. pastoris* strain GS115 and KM71H. The transformants were selected on yeast extract peptone dextrose sorbitol (YPDS, Appendix A) with 100 ug/ml Zeocin™ plate. The plates were incubated at 30 °C for 3 to 10 days.

3.4.3 Selection of the best expressing clone

The transformants of *P. pastoris* grown on YPDS with 100 ug/ml Zeocin™ plate were expressed in small scale in order to investigate the best expressing clone. The single clone of transformant was grown in 20 ml of buffer glycerol-complex medium (BMGY, Appendix A) at 30 °C in 200 rpm shaking flask cultivation until the OD_{600} reached 8 to 10. The culture was centrifuged at 5,000X g for 10 minutes. The supernatant was discarded. The cell pellet was transferred into baffle flask containing with 40 ml of buffer methanol-complex medium (BMMY, Appendix A) at 30 °C in 200 rpm shaking flask cultivation for 96 hours. Every 12 hours, the sampling culture was taken by centrifugation to collect supernatant for protein analysis by Coomassie-stained SDS-PAGE (Sambrook and Russell, 2001) and Western blot method. Absolute methanol was also added to a final concentration of 0.5% every 12 hours.

3.5 Optimization of the fusion protein expression in shaken flask cultivation

3.5.1 Effect of changing in pH, temperature and methanol concentration on growth and fusion protein expression

After selection of the best expressing clone, the effect of changing in pH, temperature, and methanol concentration on growth and fusion protein expression were examined in order to find the most suitable condition for the production of fusion protein in a 5 L fermenter. The pH value of BMMY was varied from 3 to 10. Temperature was varied at 20, 25, 30, and 37 °C and methanol concentration was varied at 0.01%, 0.1%, and 0.5% (v/v).

The best expressing *P. pastoris* transformant was chosen for optimization in flask cultivation. The transformant was cultured in 20 ml of BMGY at 30 °C in 200 rpm shaking flask cultivation until the OD_{600} reached 8 to 10. The culture was centrifuged at 8,000 rpm for 10 minutes. The supernatant was discarded and cells were transferred into baffle flasks with 40 ml of BMMY with pH 3, 4, 5, 6, 7, 8, 9, and 10 and incubated at 20, 25, 30, and 37 °C in 200 rpm shaking incubator for 96 hours. Every 12 hours, the sampling of culture was taken for OD_{600} measurement and cell dry weight. The supernatant was collected for protein analysis by Coomassie-stained SDS-PAGE and Western blot method. Absolute methanol was also added to a final concentration of 0.01, 0.1, and 0.5% every 12 hours.

3.5.2 Protein determination

According to Damasceno et al. (2004), the concentration of IL-2/FU-MK-1-scFv was separated on 12% SDS-PAGE and stained with Coomassie Blue. The scFv concentration was estimated using densitometry software, ImageJ (http://rsb.info.nih.gov/ij/). Ovalbumin was used as standard protein. Briefly, samples were run on 12% SDS-PAGE, stained with Coomassie Blue and dried using gel equilibrium drying set. Dry gels were scanned on HP psc1210 ScanJet and loaded on to the ImageJ software. The target protein bands were selected, plotted, and compared. ImageJ generates a plot base on the intensity of the selected protein bands by giving area and percentage of each peak in correspondence with the band.

According to Protein Detector^{TM} Western Blot protocol (KPLInc., USA), the fusion protein was confirmed by immunoblotting. The electrophoretic transfer of fusion proteins from 12% SDS-PAGE gel to PVDF membrane. Immunoblotting was carried out with a monoclonal rabbit anti-hIL-2 antibody and an anti-rabbit antibody conjugated with alkaline phosphate. The complex was visualized by enzyme reaction with the substrate BCIP/NBT.

3.6 Production of IL-2/FU-MK-1-scFv in 5 L fermenter

The production was divided into two phases. The first phase was biomass growth phase using glycerol as main carbon source and the second was methanol induction phase. In the first phase, glycerol concentration was varied at 5, 7.5, and 10% (w/v).

The fermentation temperature was kept constant at 30 °C throughout cultivation. Medium pH value was kept constant at 6 in the first phase and optimal pH value obtained from shaken flask expression was applied and kept constant in the second phase. DO was controlled over 40% during induction phase. These parameters were recorded and monitored on program online TK97 Data Record software version 2.04 (EYLA Tokyo Rikakikai Co., Ltd., Japan) computer. The concentration of 10% (v/v) ammonium hydroxide and 20% (v/v) phosphoric acid were used as pH control agent. An antifoam 5% (v/v) was used for foam reducing agent. An air flow rate of 4 vvm was applied for aeration.

Optimal condition obtained from shaken flask cultivation was applied to the production in 5 L fermenter. A transformant *P. pastoris* was inoculated to 10 ml YPD and grown overnight at 30 °C. A total of 200 ml BMGY was inoculated with 1 mL of the overnight culture and incubated at 30 °C with 200 rpm shaking until the culture reached an OD_{600} of ~10. This culture was inoculated to 5 L fermenter containing 2.5 L of modified basal salt medium (Appendix A). The samples were taken at 6 hours interval for further analysis until the glycerol was exhausted, that indicated by a sharp of DO spike. Then the production was induced by methanol feed (methanol with 12 ml/L PTM, Appendix A). After the DO spike, samples were taken every 12 hours until 96 hours for cell density measurement. And the sample was centrifuged at 13,000 rpm for 10 minutes and the cell pellet was used for wet and dry cell weight analysis. The supernatant was kept at -20 °C for protein and methanol analysis. The analysis of methanol concentration was followed to Appendix D.

3.7 Partial purification of the fusion protein by Ni-affinity chromatography

To purify to fusion protein, the supernatant was clarified through a 0.22 μm membrane filter for purification. Ni-affinity chromatography was performed using His·Bind®Kits 5 ml column (Novagen®, USA). The 2 ml His·Bind resin was packed under gravity flow in column. When level of storage buffer drops to top of column bed, 3 ml DW, 5 ml charge buffer and binding buffer were added to charge and equilibrate the column. Then binding buffer was drained to bottom of column bed, 10 ml supernatant was loaded into column. After washing with 10 ml binding buffer and 6 ml washing buffer, the protein was eluted with 6 ml eluting buffer. The fractions

containing the protein (analyzed by SDS-PAGE) were pooled and concentrated by ultrafiltration 30,000 molecular weight cut off (Vivaspin 500). Finally, the protein was kept in PBS solution for further analysis.

3.8 Characterization of the fusion protein for specific binding activity

Briefly, 20 µg cell lysate proteins of CHO-MK-1 and CHO cell in 100 µl coating buffer (eBioscience, USA) were added in triplicate to wells of Coning Costar 9018 ELISA 96 well plate. After incubation overnight at 4 °C, 4 µg of fusion protein IL-2/FU-MK-1-scFv in 100 µl assay diluents (eBioscience, USA) was added in each well and incubated at room temperature for 2 h. Then, biotin-conjugated anti-human IL-2 diluted 1:200 was added in assay diluent. The plate was incubated at room temperature for 1 h. Next, Avidin-HRP (eBioscience, USA) diluted 1:200 in assay diluents was added and incubated at room temperature for 30 min. Consequently, 100 µl TMB (eBioscience, USA) solution was added in each well and incubated at room temperature for 15 min. Finally, 50 µl of 2 N H_2SO_4 was added to stop reaction and the plate was read at 450 nm. The details of protocol were described in Appendix F.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Transformation and selection of fusion protein expressing clone

The aim of this study was to express the fusion protein of IL-2/FU-MK-1-scFv by *P. pastoris* via pPICZαA containing of the *S. cerevisiae* α-mating factor. This system offers several advantages, such as the strong promoter of *AOX1*, the ability to culture cells at high density and simplified purification procedure for secreted heterologous proteins (Romanos et al., 1995; Cregg et al. 2000; Cereghino et al., 2002). Moreover, similar to mammalian and insect cells, *P. pastoris* can carry post-translational modifications for foreign proteins such as glycosylation (Bettauer and Castellino, 1999).

Phenotype of host *P. pastoris* has impact to recombinant protein expression and growth of host under methanol induction. Mut^S (methanol utilization slow) and Mut^+ (methanol utilization plus) phenotypes are used to evaluate *Pichia* transformant for recombinant protein expression. Most *P. pastoris* strain GS115 is Mut^+ phenotype which contains a functional copy of *AOX1* responsible for approximately 85% of the utilization of methanol. *P. pastoris* strain KM71H is usually Mut^S phenotype which contains a non-functional *AOX1* and relies on alcohol oxidase enzyme being produced from an alternative gene, *AOX2*. The *AOX2* has specific activity same as *AOX1* but has lower level of expression and can only utilize methanol slowly (Hearn and Daly, 2005). Thus, in this study, the pPICZαA-IL-2/scFv(V_H-V_k) vector was transformed into *P. pastoris* strain GS115 and KM71H according to EasyComp™ Transformation protocol. There were 21 colonies of transformant *P. pastoris* strain KM71H and 54 colonies of transformant *P. pastoris* strain GS115 on YPDS plate containing 100 ug/ml Zeocin™. The transformants were collected on YPDS with 100 ug/ml Zeocin™ plate for selection of expressing clone (Figure 4.1). The colonies were chosen for small scale expression of fusion protein in shaken flask cultivation induction with methanol. The protein expression was screened by SDS-PAGE analysis.

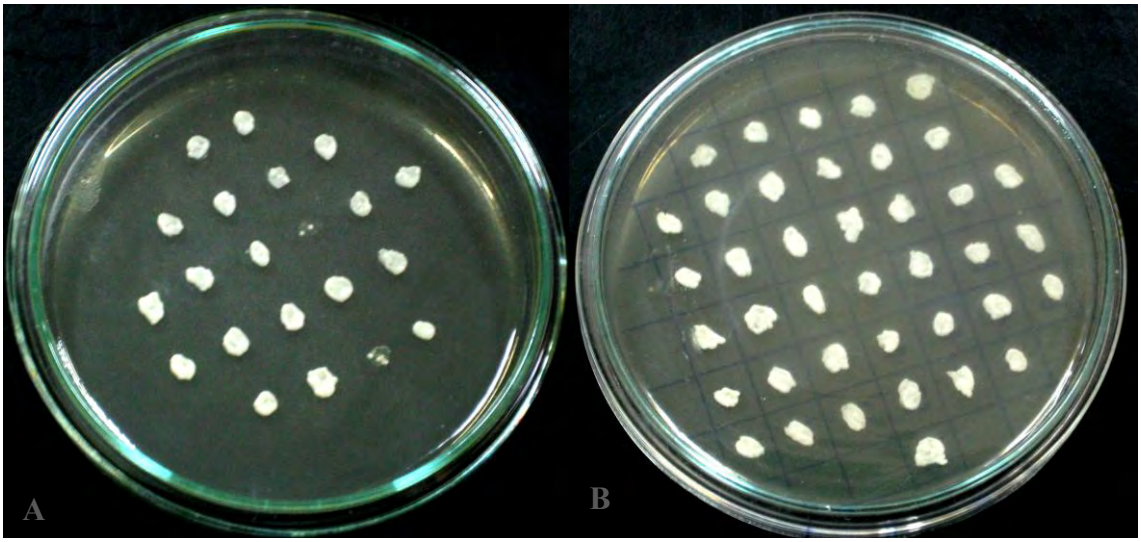

Figure 4.1 Collection plates of transformant *P. pastoris* on YPDS containing 100 ug/ml Zeocin^{TM}; A) colonies of transformant *P. pastoris* strain KM71H and B) colonies of transformant *P. pastoris* strain GS115.

The transformant capable of producing secreted fusion protein IL-2/FU-MK-1-scFv in medium via the *Saccharomyces cerevisiae* α-mating factor was selected by small scale expression of fusion protein in flask cultivation. In the primary screening step, the secreted fusion protein supernatants were analyzed by Coomassie-stained SDS-PAGE. In the secondary step, the fusion protein was confirmed by immunoblotting using rabbit anti-hIL-2 antibody specific binding to IL-2 of the fusion protein as primary antibody and anti rabbit antibody conjugation with alkaline phosphatase as secondary antibody. The expression signal was detected by BCIP/NBT solution that reacts with alkaline phosphatase. The transformant *P. pastoris* strain GS115 was selected to express IL-2/FU-MK-1-scFv under regulation of methanol inducible *AXO1* promoter. The Coomassie-stained SDS-PAGE and immunoblotting of the selected transformant *P. pastoris* strain GS115 were shown in figures 4.2A and 4.2B, respectively. In this study, all transformants *P. pastoris* strain KM71H could not express fusion protein IL-2/FU-MK-1-scFv. The results obtained from shaken flask cultivation were shown in Appendix B.

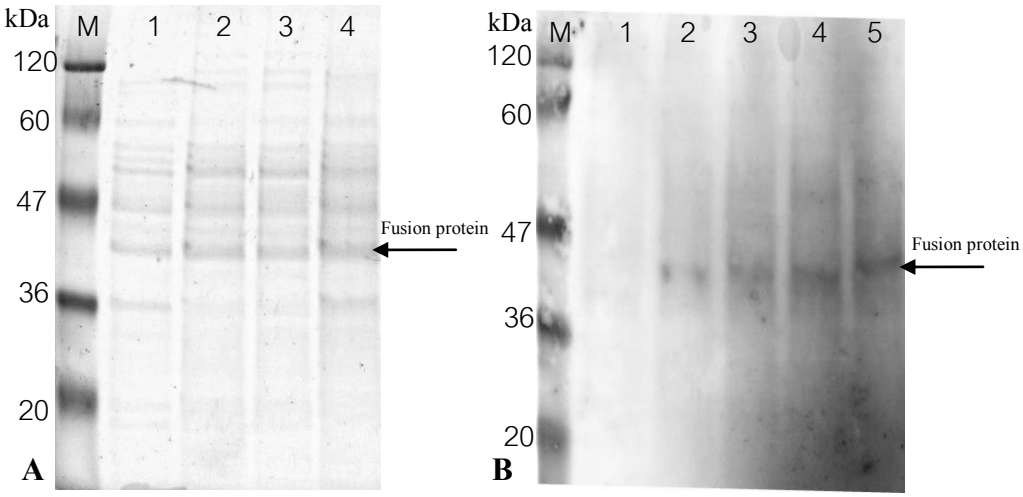

Figure 4.2 A) Coomassie stained SDS-PAGE of supernatant samples of expressing transformant *P. pastoris* strain GS115. Lane M, molecular marker; lane 1, 60h induction; lane 2, 72h induction; lane 3, 84h induction; lane 4, 96h induction and lane M, molecular marker. B) Immunoblotting of supernatant samples. Lane 1, non-expressing clone; lane 2, 60h induction; lane 3, 72h induction; lane 4, 84h induction and lane 5, 96h induction.

4.2 Optimization of the fusion protein expression in shaken flask cultivation

The optimal conditions for recombinant protein expression using *P. pastoris* system are important factors to improve productivity of recombinant protein. Small-scale expressions in shaken flask cultivation are often the first stage employed for optimization of recombinant proteins production and selecting condition of cultivation (Daly and Hearn, 2004). In this study, before the large scale production in 5 L fermentation, the important parameters, pH, temperature and methanol concentration were examined in small-scale expression.

4.2.1 Effect of pH on IL-2/FU-MK-1-scFv production.

The optimal pH was determined using expressing clone *P. pastoris* strain GS115 from 4.1 cultivated on BMGY until OD_{600} reached~8 to 10 then transferred into BMMY at pH ranging from 3 to 10. Figure 4.3 shows that growth profile as cell density measured by OD_{600} for 96 h during methanol induction phase when pH value was ranging from 3 to 10.

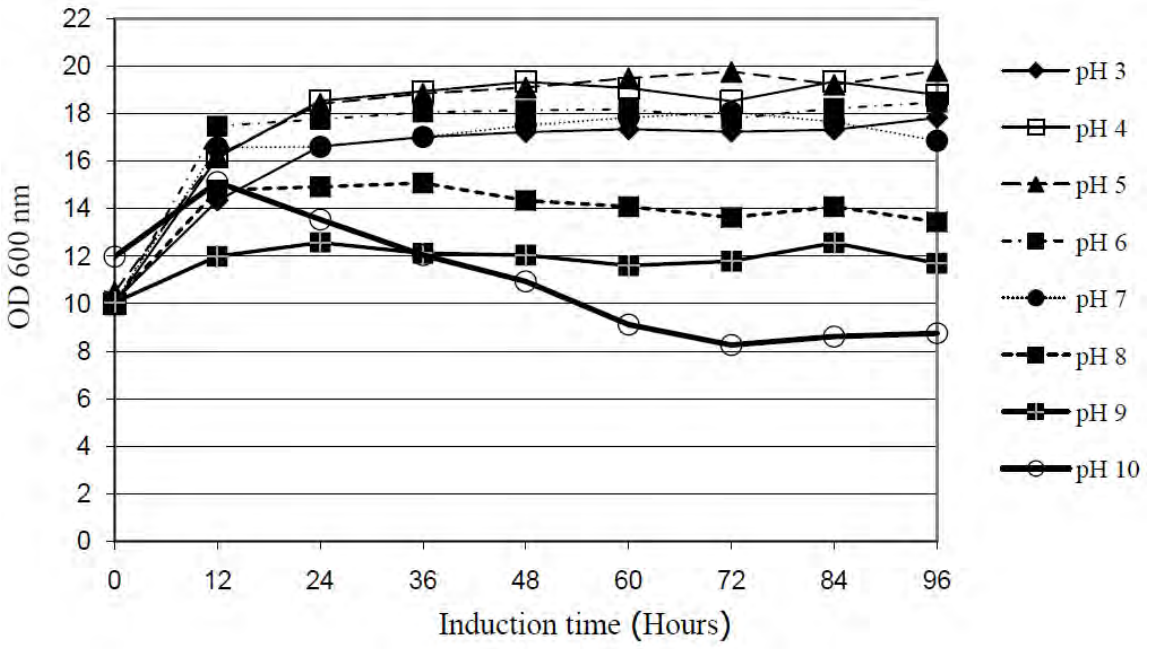

Figure 4.3 Growth profiles of transformant *P. pastoris* strain GS115 during 96 h methanol induction phase in BMMY with 0.5% methanol at 30 °C and pH ranging from 3 to 10.

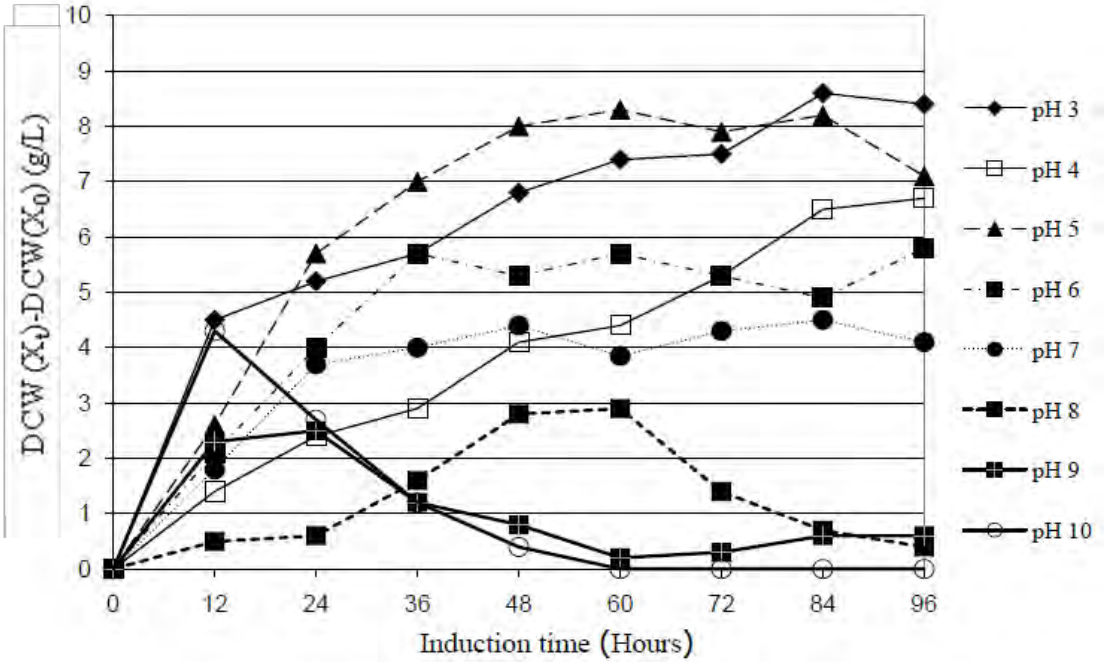

Figure 4.4 Biomass of transformant *P. pastoris* strain GS115 during 96 h methanol induction phase in BMMY with 0.5% methanol at 30 °C and pH ranging from 3 to 10.

To improve production of fusion protein, the optimal pH was determined by varying pH of BMMY medium between 3 and 10. The cell density increased under acidic to neutral pH from pH 3, 4, 5, 6, and 7 whereas it decreased under alkaline pH from pH 8, 9, and 10 (figures 4.3 and 4.4). These results imply that pH between 3 and 7 were optimal values for growth *P. pastoris* strain GS115. This result was similar to the study of Cregg et al. (1993) and Damasceno et al. (2004).

Consequently, the amount of secreted fusion protein must be crucially considered in production process. The expression of secreted fusion protein was analyzed by Coomassie blue SDS-PAGE. In figures 4.5 and 4.6, the pH 3 and 4 showed increasing expression of the fusion protein by adjusting pH value in order to minimize protease activity during methanol induction phase. It was found that at pH value from 5 to 10 showed almost no expression of IL-2/FU-MK-1-scFv during 96 h methanol induction. These results were also similar to Damasceno et al. (2004).

These results may suggest that pH seem to trigger highly interrelated responses. Changing pH of medium for recombinant protein production by yeast like *P. pastoris* could significantly improve yields of proteins. It was reported that acidic pH value could reduce the protease activity that lead to protein degradation (Gasser et al., 2007). While increasing pH is not common, lowering pH is often used for recombinant protein production (Shi et al., 2003). Moreover, several reports found a significant improvement of secreted protein at pH around 3 that was examined by host cell protease inhibition (Cregg et al., 2000; Curvers et al., 2001; Jahic et al., 2003; Damasceno et al., 2004).

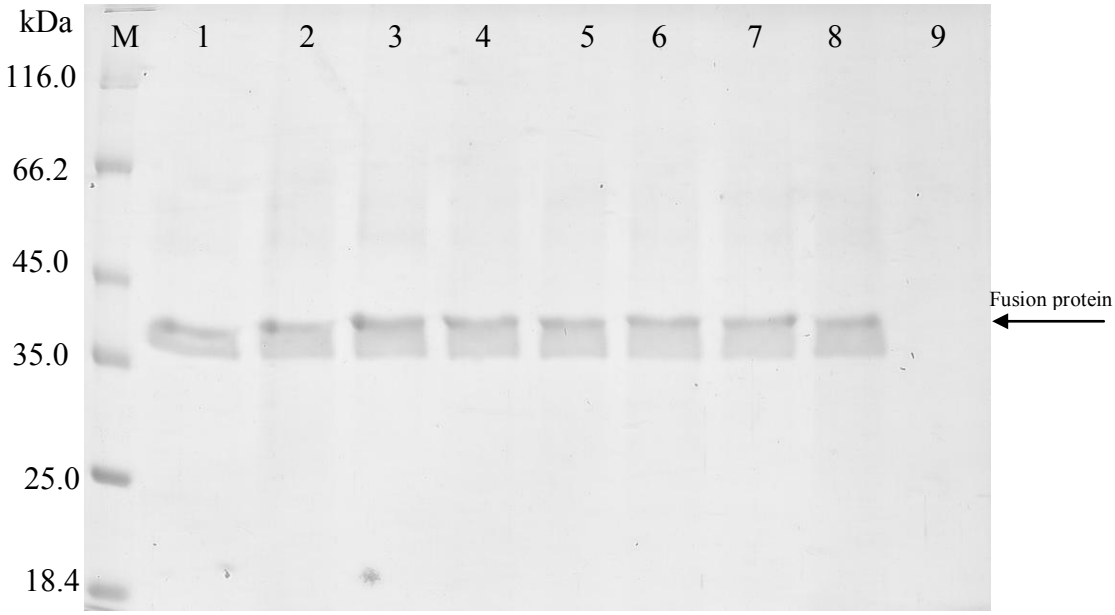

Figure 4.5 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 3 (60h); lane 2, pH 3 (72h); lane 3, pH 3 (84h); lane 4, pH 3 (96h); lane 5, pH 4 (60h); lane 6, pH 4 (72h); lane 7, pH 4 (84h); lane 8, pH 4 (96); lane 9, pH 5 (84h) and lane M, molecular marker.

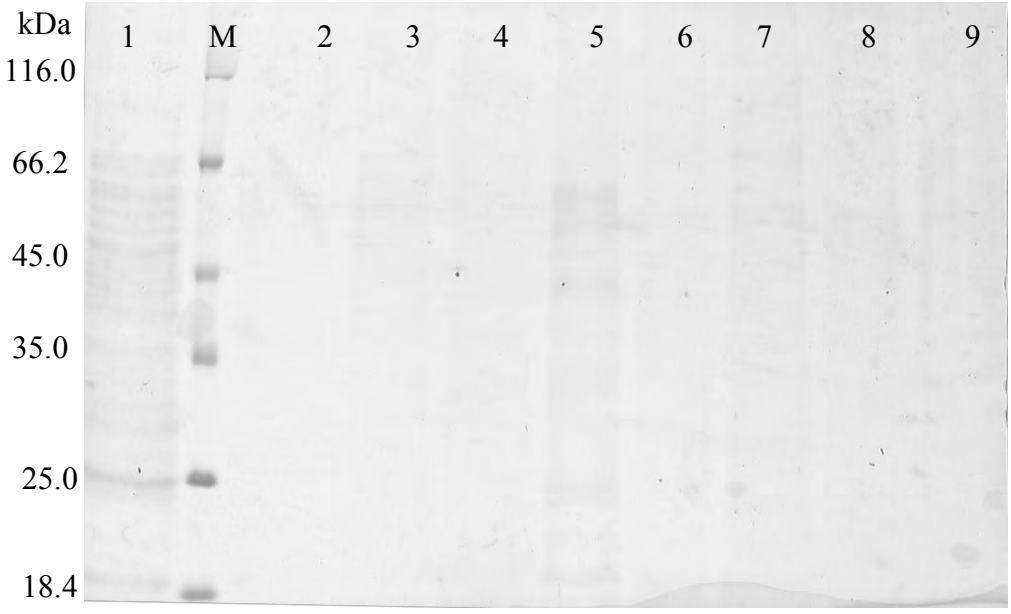

Figure 4.6 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 5 (96h); lane 2, pH 6 (84h); lane 3, pH 6 (96h); lane 4, pH 7 (84h); lane 5, pH 7 (96h); lane 6, pH 8 (84h); lane 7, pH 8 (96h); lane 8; pH 9 (96h); land 9, pH 10 (96h) and lane M, molecular.

Due to the results of the pH effect on the fusion protein production, we concluded that pH 3 and 4 are optimal pH for expression of IL-2/FU-MK-1-scFv by *P. pastoris* strain GS115. Although at pH 5 to 7, lower fusion protein was detected the biomass under these conditions are favorable results. Therefore, pH in the range between 3 and 7 was considered for the study of the effect of temperature on the expression level of fusion protein.

4.2.2 Effect of temperature on IL-2/FU-MK-1-scFv production

Temperature is also one of an important factor employed for enhancing production of recombinant protein. In this study, temperature was examined in order to inhibit protease activity and find the optimal temperature for improving fusion protein IL-2FU-MK-1scFv production by transformant *P. pastoris* (Jahic , 2003).

Based on the previous study, pH values from 3 to 7 were chosen for the study of effect of temperature on the production of secreted fusion protein. Incubation temperature of 20, 25, 30, and 37 °C have been examined in attempts to minimize extracellular proteolysis in medium culture (Li, 2001). To induce the expression, the cultures were incubated at 20, 25, 30, and 37 °C with shaking for 96 hours methanol induction. Cell weight and OD_{600} were analyzed every 12 hours (Figure 4.6 to 4.15).

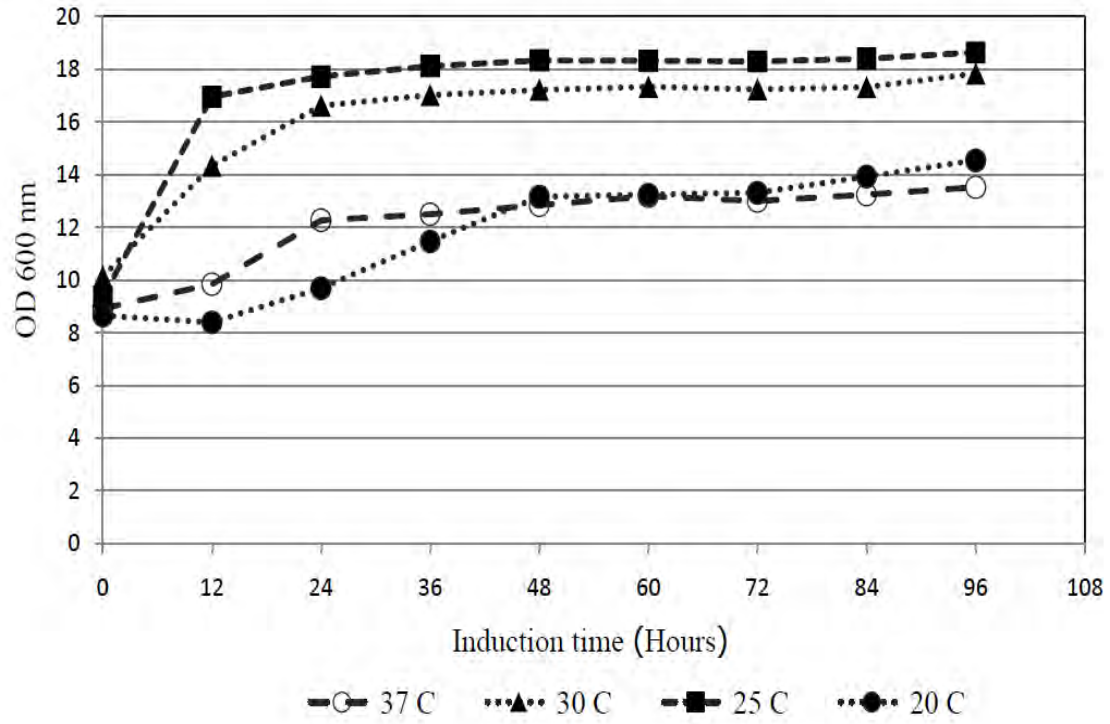

Figure 4.7 Growth profiles of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 3 and methanol concentration of 0.5% (v/v).

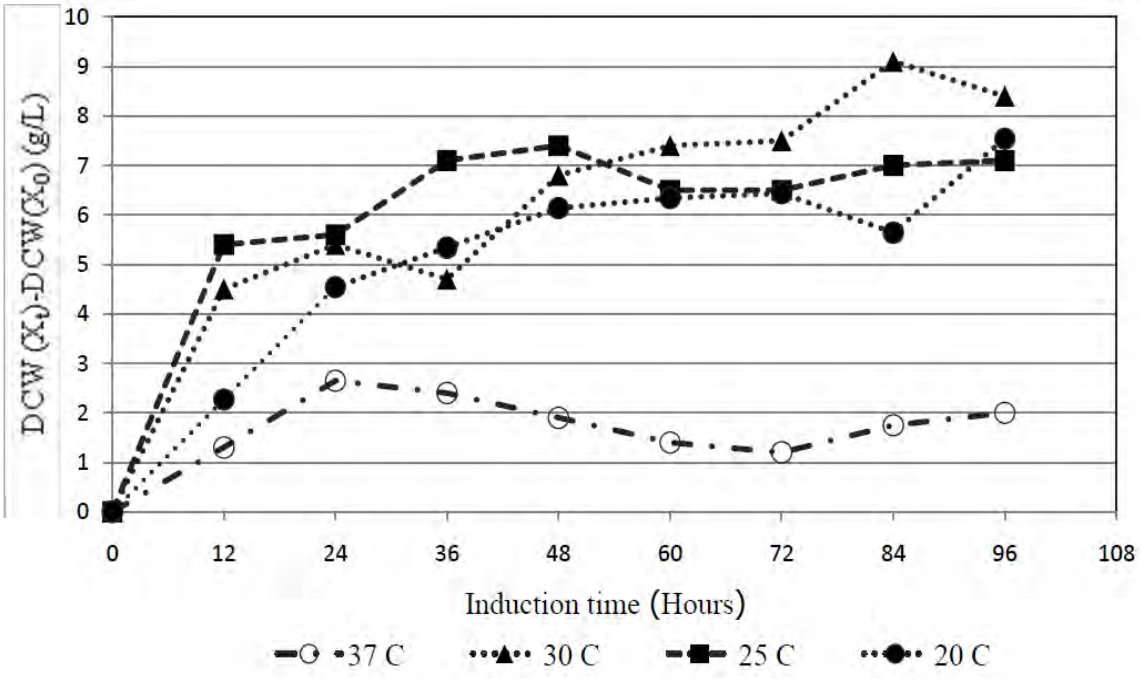

Figure 4.8 Biomass of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 3 and methanol concentration of 0.5% (v/v).

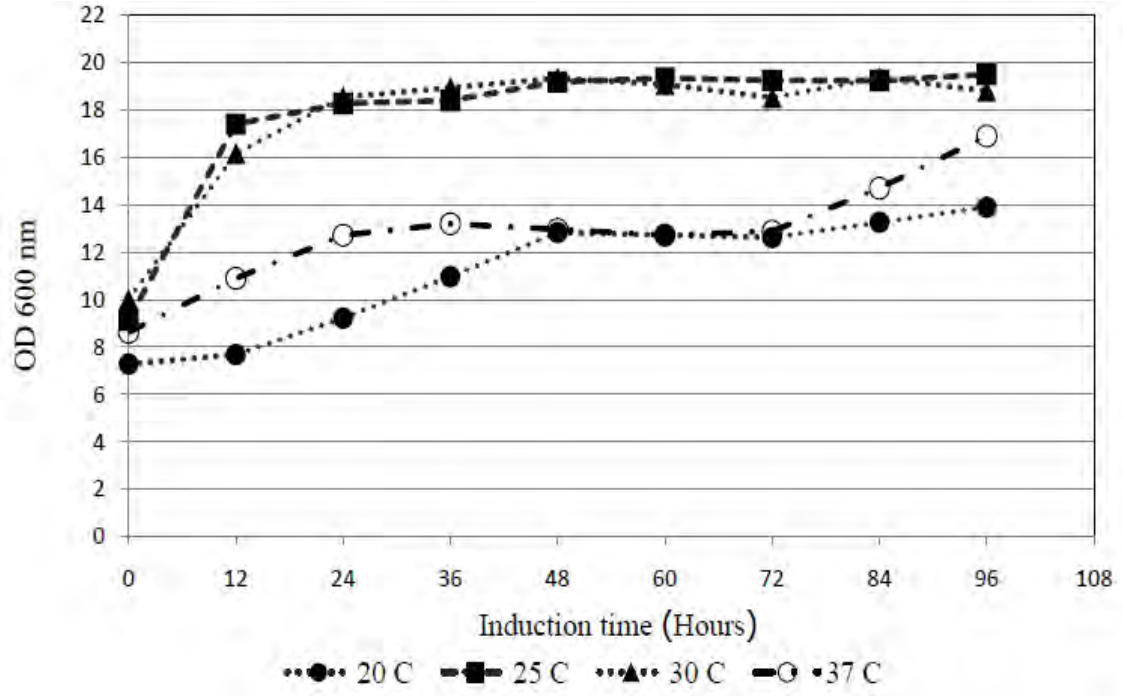

Figure 4.9 Growth profiles of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 4 and methanol concentration of 0.5% (v/v).

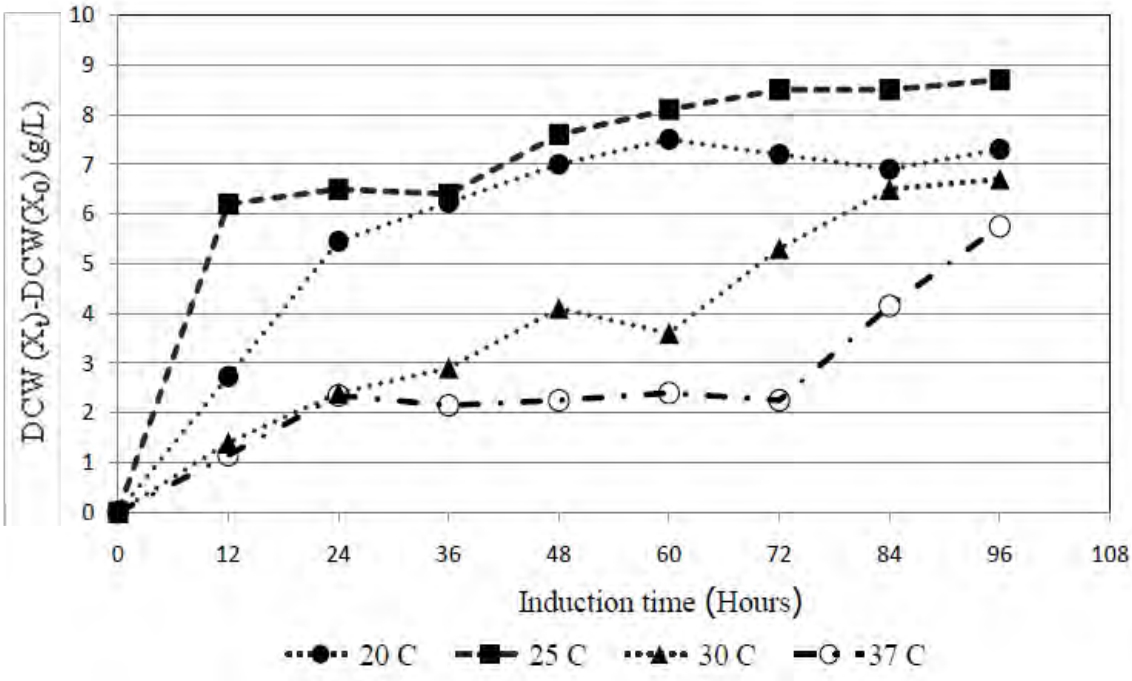

Figure 4.10 Biomass of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 4 and methanol concentration of 0.5% (v/v).

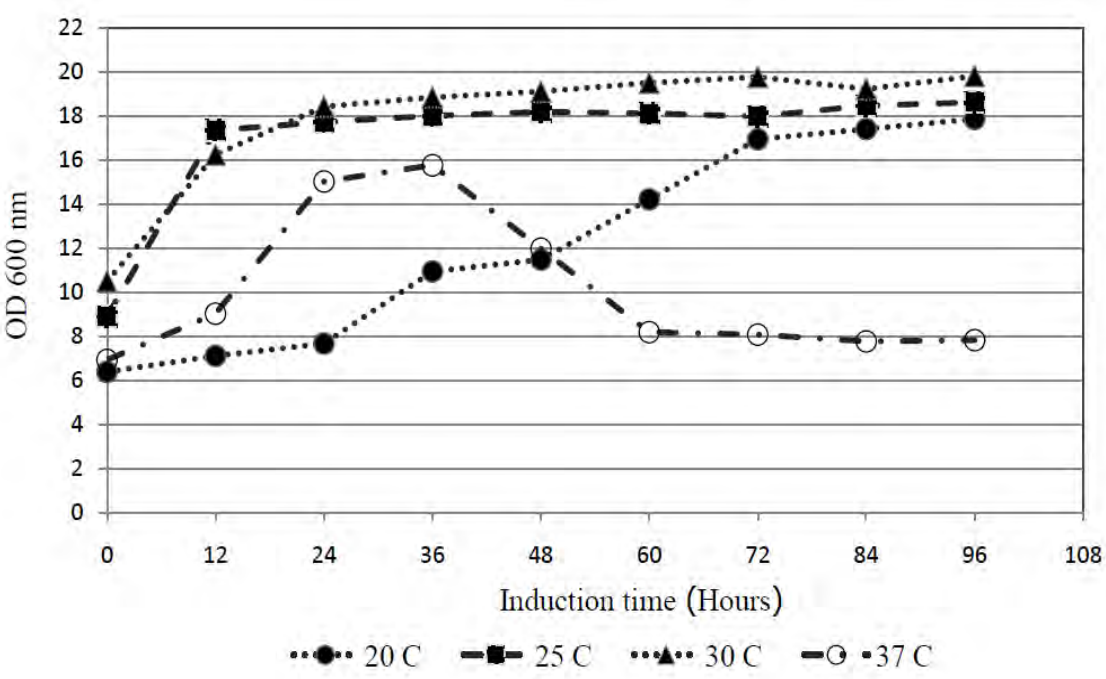

Figure 4.11 Growth profiles of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 5 and methanol concentration of 0.5% (v/v).

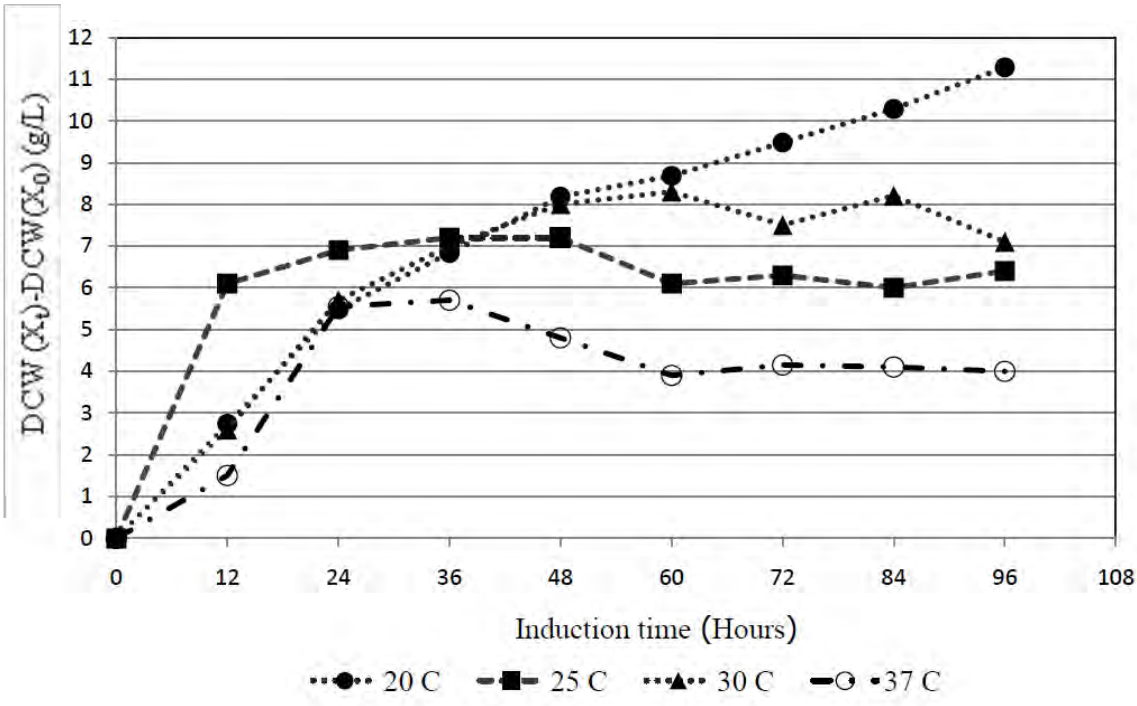

Figure 4.12 Biomass of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 5 and methanol concentration of 0.5% (v/v)

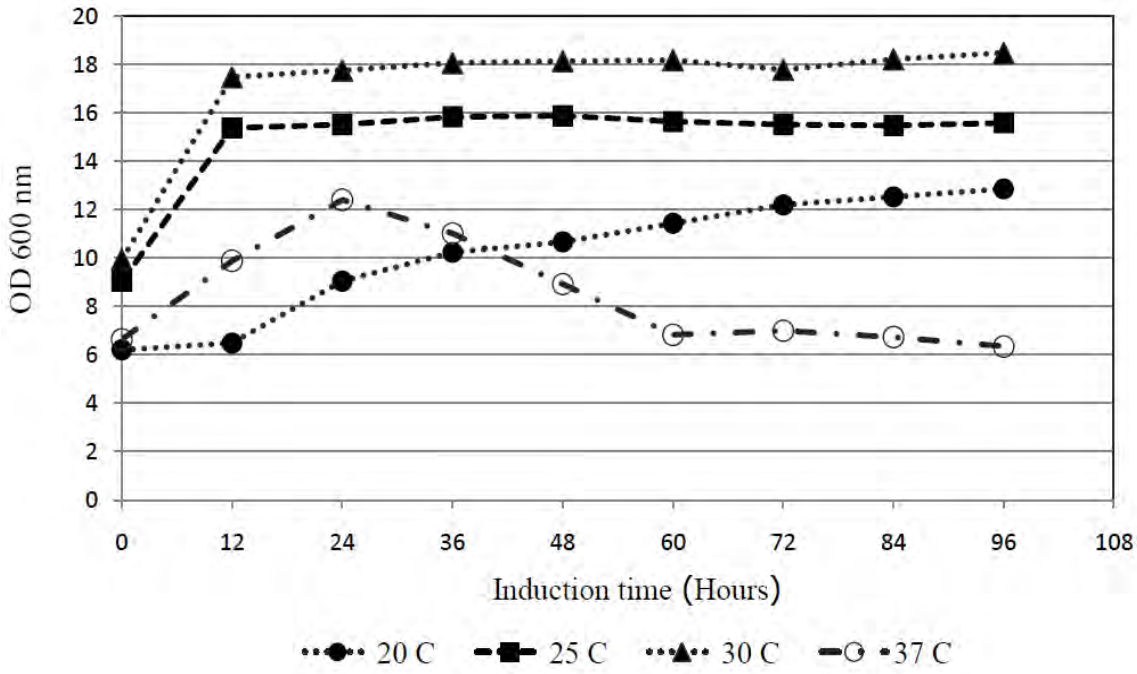

Figure 4.13 Growth profiles of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 6 and methanol concentration of 0.5% (v/v).

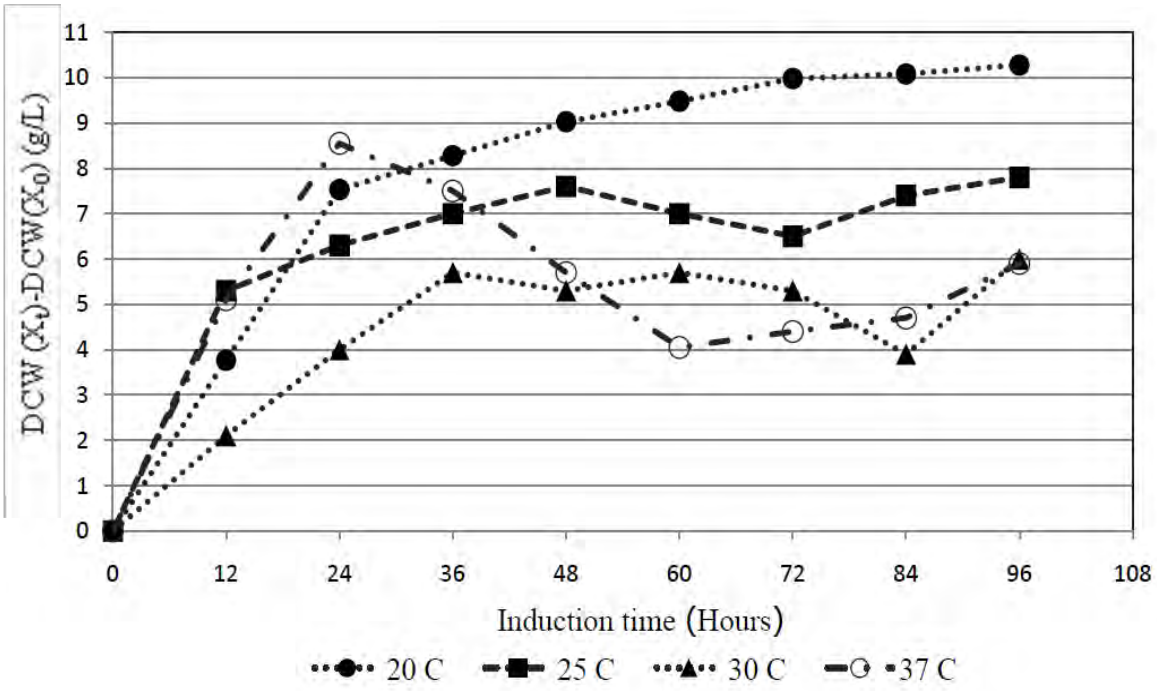

Figure 4.14 Biomass of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 6 and methanol concentration of 0.5% (v/v)

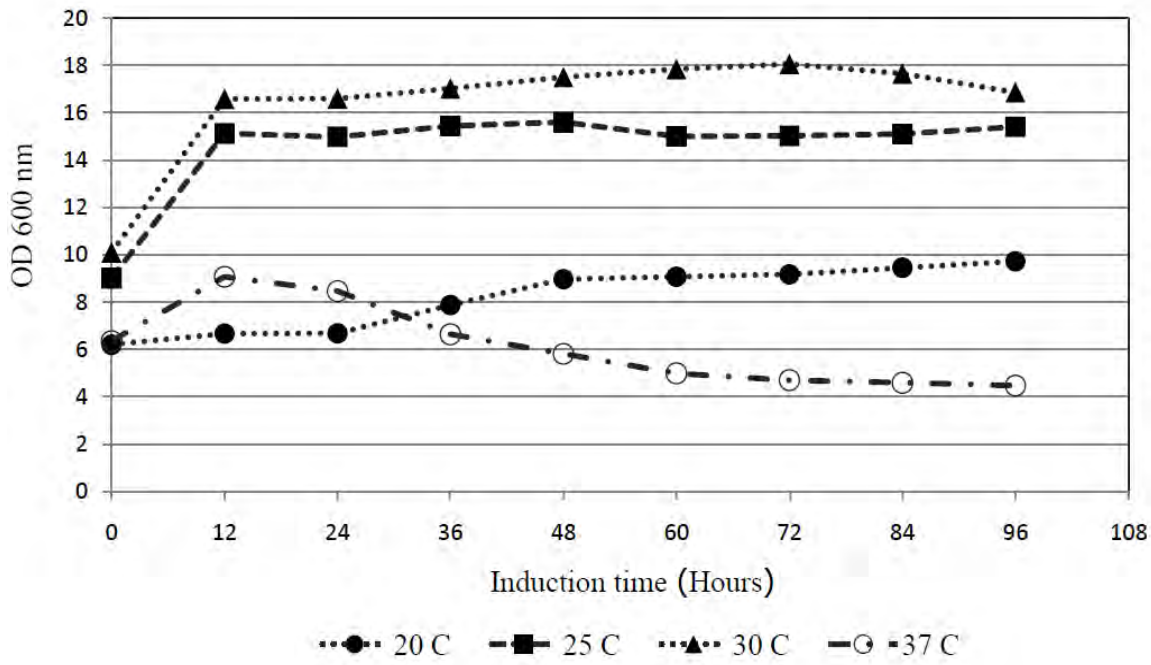

Figure 4.15 Growth profiles of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 7 and methanol concentration of 0.5% (v/v).

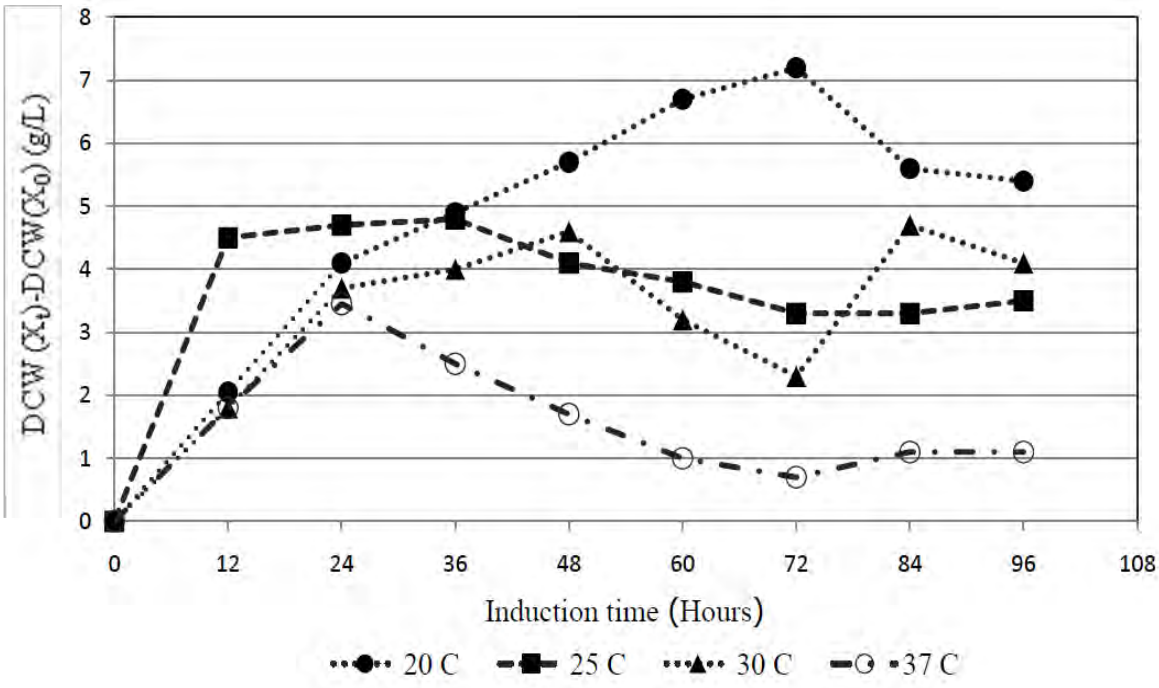

Figure 4.16 Biomass of transformant *P. pastoris* strain GS115 during the methanol induction phase at 20, 25, 30, and 37 °C. Production was done at pH 7 and methanol concentration of 0.5% (v/v).

Obtained results demonstrated that production of IL-2/FU-MK-1-scFv *by P. pastoris* strain GS115 under pH 3 and 4 showed similar results when methanol induction was performed under temperatures of 20, 25, 30, and 37 °C. There was no apparent different trend in cell density and biomass accumulation after 96 h methanol induction, but production at 37 °C showed the lowest cell growth (figure 4.7 and 4.8).

At pH 5, 6, and 7, cell growth at temperature of 20, 25, and 30 °C has been increased during 96 h induction. Especially at 20 °C, the highest cell growth rate was obtained whereas under temperature of 25 and 30 °C showed slightly increased of cell growth rate after 12 h induction and showed a stable trends biomass accumulation. Moreover, production at 37 °C has been shown to decrease cell growth rate of *P. pasroris* GS115 after 36 h induction at pH 5, 24 h induction at pH 6 and 7 (figure 4.11 to 4.16). These results suggested that the growth rate of *P. pastoris* were decreased when temperature was increased from 20 to 37 °C (Dragotist et al., 2008).

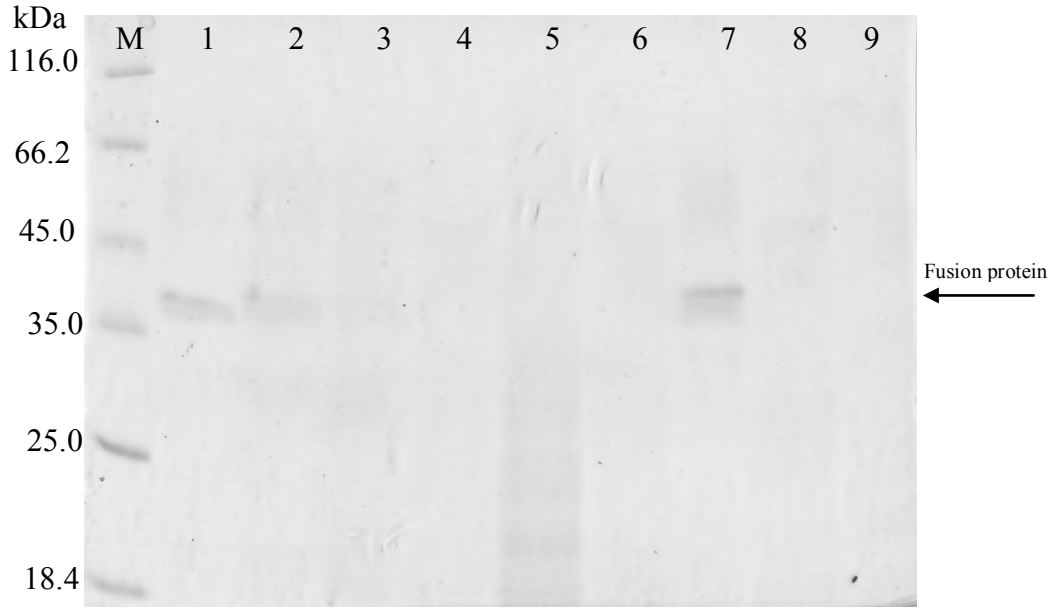

Figure 4.17 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 3 (84h); lane 2, pH 4 (84h); lane 3, pH 4 (96h); lane 4, pH 5 (84h); lane 5, pH 7 (96h); lane 6, pH 5 (96h); lane 7, pH 3 (96h); lane 8, pH 6 (84h); lane 9, pH 6 (96h) and lane M, molecular marker. The experiment was performed at 20 °C.

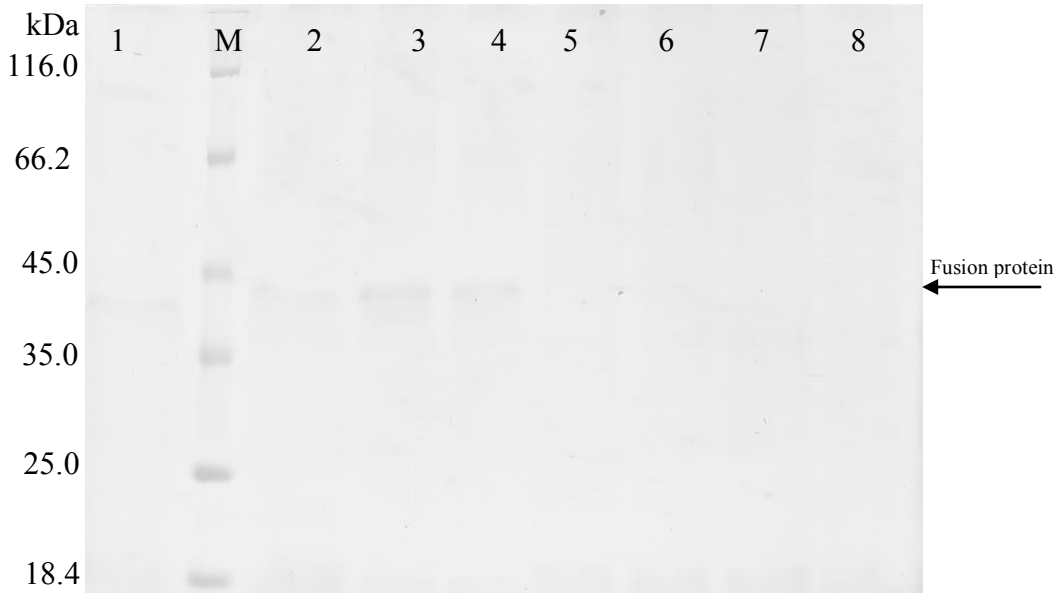

Figure 4.18 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 3 (60h); lane 2, pH 3 (72h); lane 3, pH 3 (84h), lane 4, pH 3 (96h); lane 5, pH 4 (60h); lane 6, pH 4 (72h); lane 7, pH 4 (84h); lane 8, pH 4 (96h) and lane M, molecular marker. The experiment was performed at 25 °C.

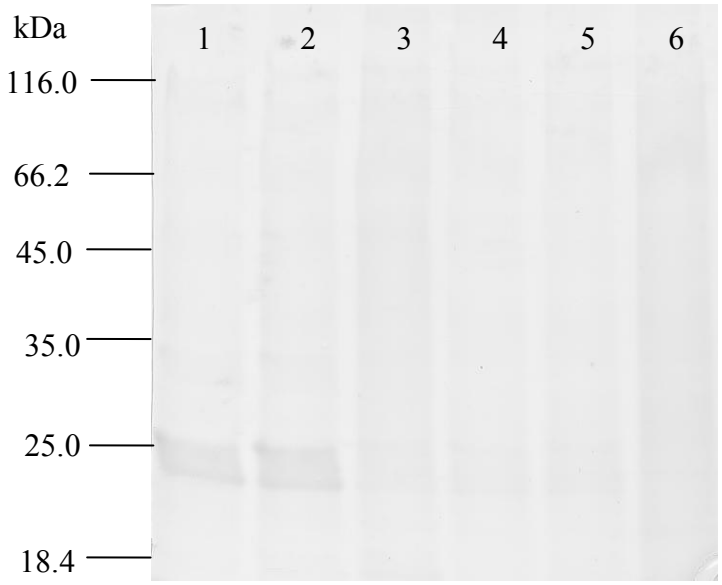

Figure 4.19 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 5 (84h); lane 2, pH 5 (96h); lane 3, pH 6 (84h); lane 4, pH 6 (96h); lane 5, pH 7 (84h) and lane 6, pH 7 (96h). The experiment was performed at 25 °C.

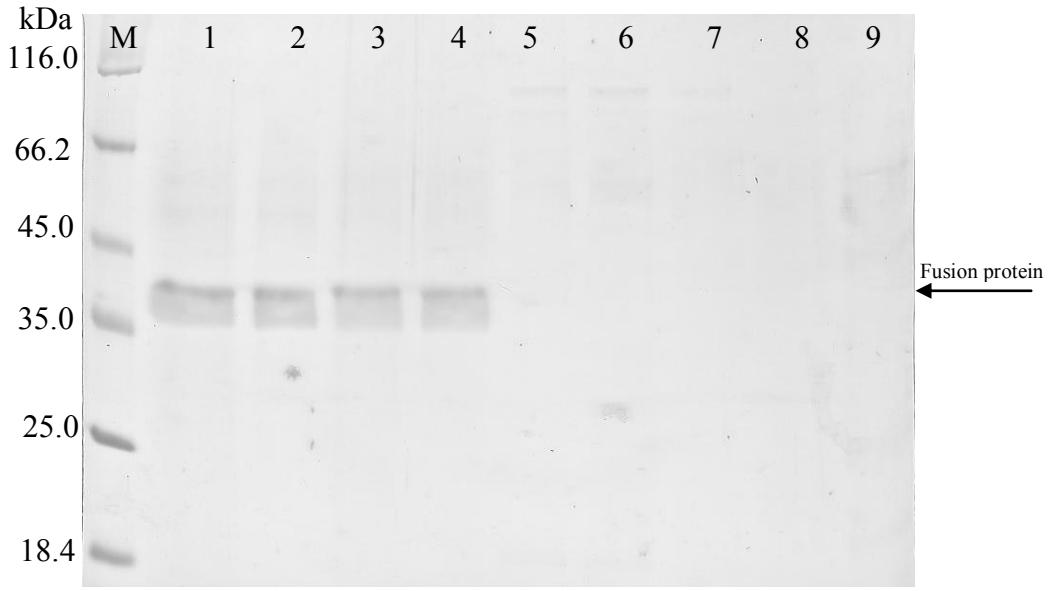

Figure 4.20 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 3 (84h); lane 2, pH 3 (96h); lane 3, pH 4 (84h); lane 4, pH 4 (96h); lane 5, pH 5 (84h); lane 6, pH 5 (96h); lane 7, pH 6 (84h); lane 8, pH 6 (96h); lane 9, pH 7 (96h) and lane M, molecular marker. The experiment was performed at 30 °C.

Figure 4.21 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 3 (60h); lane 2, pH 3 (72h); lane 3, pH 3 (84h); lane 4, pH 3 (96h); Lane 5, pH 4 (60h); lane 6, pH 4 (72h); lane 7, pH 4 (84h); lane 8, pH 4 (96h); lane 9, pH 5 (60h) and lane M, molecular marker. The expression was done at 37 °C.

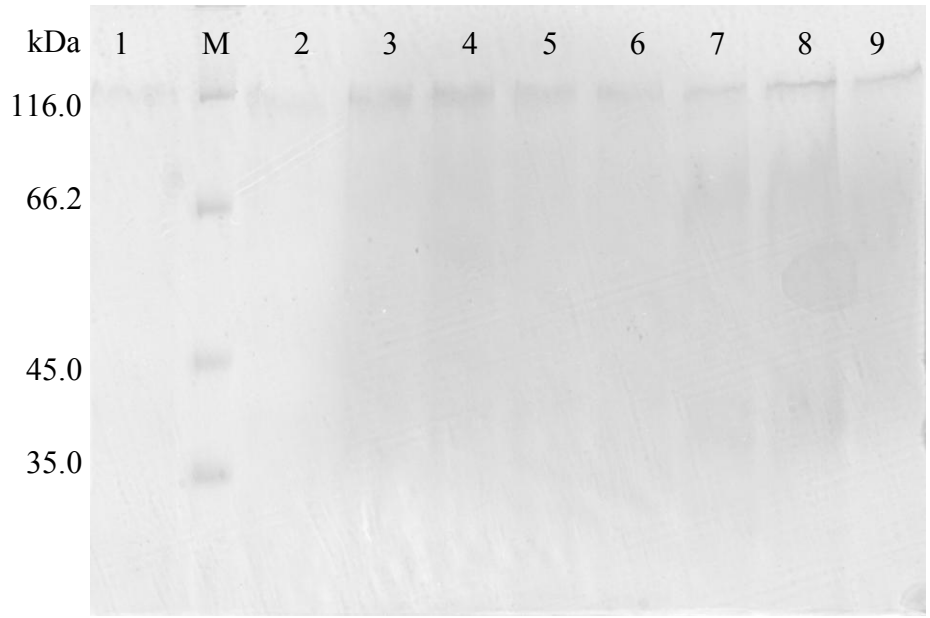

Figure 4.22 SDS-PAGE analysis of supernatant samples of 96 h methanol induction at different pH values. Lane 1, pH 5 (72h); lane 2, pH 5 (84h); lane 3, pH 5 (96h); lane 4, pH 6 (72h), lane 5, pH 6 (84h); lane 6, pH 6 (96h); lane 7, pH 7 (72h); lane 8, pH 7 (84h); lane 9, pH 7 (96h) and lane M, molecular marker. The expression was done at 37 °C.

As shown in figures 4.17 to 4.22, it was found that the SDS-PAGE analysis of secreted fusion protein expression under temperatures of 20, 25, 30, and 37 °C at pH 3 and 4 promoted the expression of fusion protein, IL-2/MK-1-scFv, except for the temperature of 25 and 37 °C at pH 4 showed no protein expression, indicating that this condition may increase proteolysis degradation. In addition, the production at higher pH values showed almost no expression of IL-2/FU-MK-1-scFv. This implied that pH 5, 6, and 7 may able to increase protease activity during production of IL-2/FU-MK-1-scFv by *P. pastoris* at all temperature tested. Interestingly, low pH and low temperature were optimal because reducing proteolysis was decreased. In addition, Curvers et al. (2001) reported that the lower proteolysis activity at low pH and temperature was observed because the activity of the proteases presented in the supernatant was decreased. The optimal temperature for *P. pastoris* cell growth is usually defined about 30 °C, it is well established that it can significantly improve productivity of recombinant protein (Li et al., 2001).

This study was considered three important factors affecting the IL-2/FU-MK-1-scFv production by *P. pastoris* under *AOX1* promoter, pH, temperature, and methanol concentration. The pH and temperature may be critical for recombinant protein production. These should be known the optimal condition before optimizing the methanol concentration (Zang et al., 2007). Based on our results, it was found that pH 3 and 4 were chosen to combine with temperature of 30 °C and applied for the study effect of methanol concentration on the production of IL-2/FU-MK-1-scFv by *P. pastoris* strain GS115.

4.2.3 Effect of methanol concentration on IL-2/FU-MK-1-scFv production

Conventional high cell density cultures of recombinant *P. pastoris* are usually performed in two steps. Firstly, cells are grown on glycerol in order to produce biomass while repressing recombinant protein expression. Secondly, methanol is fed to the culture to induce recombinant protein production. However, during the induction phase, it is known that methanol concentration is a crucial parameter and need optimal concentration to avoid toxicity from methanol accumulation (Jungo et al., 2007).

In this study, both pH 3 and 4 were tested and combined with methanol concentration of 0.01, 0.1 and 0.5% (v/v) for 96 hours induction at 30 °C. The secreted fusion protein was analyzed by SDS-PAGE and shown in figure 4.25. At 12 h interval, OD_{600} and cell weight were measured (figures 4.23 and 4.24). In a preliminary study, induction with 0.01% methanol showed the lowest expression of Il-2/FU-MK-1-scFv during 96 h induction time courses. This might indicated the resulted from the growth-limiting level of methanol for *P. pastoris* (Damasceno et al., 2004). Therefore, methanol of 0.1 and 0.5% (v/v) were considered to examine for expression of the fusion protein in this step.

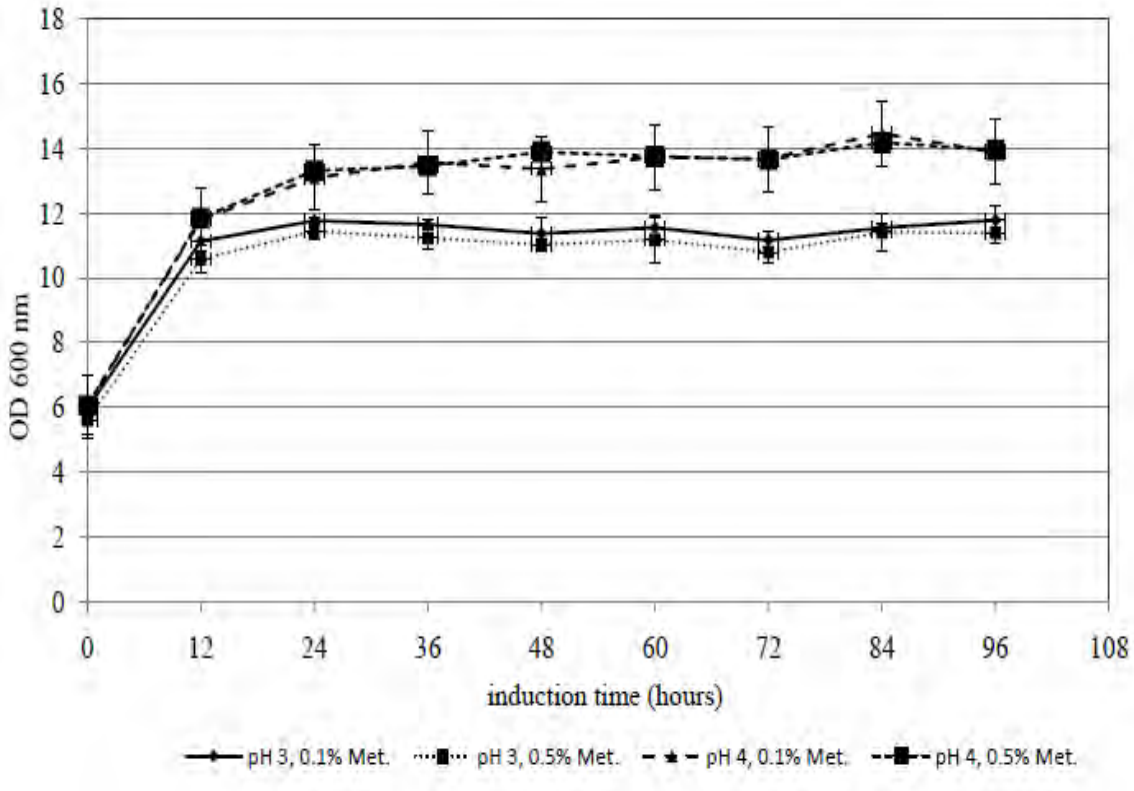

Figure 4.23 Growth profiles of transformant *P. pastoris* strain GS115 during the methanol induction phase at 30 °C. Production was done at pH 3 and 4 induction with 0.1 and 0.5% (v/v) methanol.

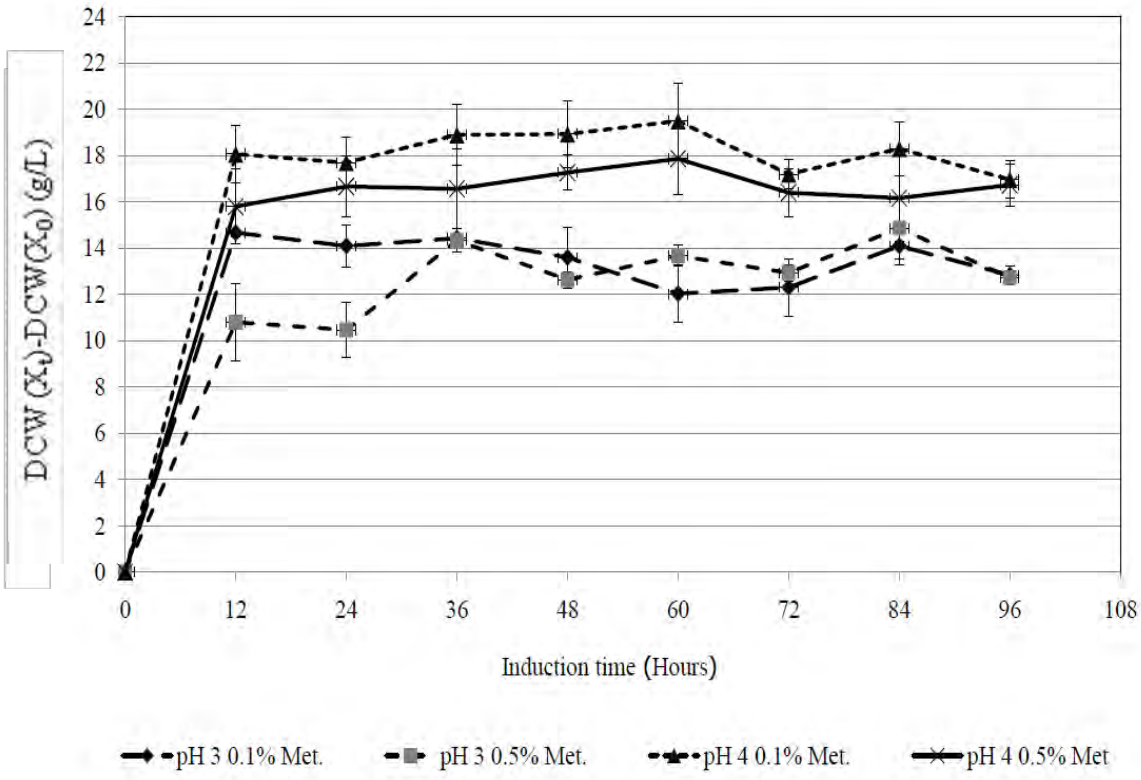

Figure 4.24 Biomass of *P. pastoris* strain GS115 during 96 h methanol induction phase in BMMY at 30 °C. Production was done at pH 3 and 4 induction with 0.1 and 0.5% (v/v) methanol.

The biomass accumulation with 0.1% methanol induction at pH 3 was increased from 16.7±0.8 g/L to 29.4±1.2 g/L and that with 0.5% methanol induction at pH 3 was increased from 15.7±0.7 g/L to 28.5±0.6 g/L. At pH 4, the biomass with 0.1% methanol induction was increased from 15.7±0.9 g/L to 34.0±1.1 g/L and that with 0.5% methanol induction was increased from 16.8±0.9 g/L to 32.9±1.2 g/L. These results demonstrated that the biomass at pH 3 was lower than that of pH 4, but fusion protein concentration at pH 3 was slightly higher than that obtained under pH 4. Thus, there is no direct correlation between biomass concentration and expression level of fusion protein (Damasceno et al., 2004).

In figure 4.23 and 4.24, the growth of *P. pastoris* under 0.1 and 0.5% methanol have shown similarity trend, but induction at pH 4 showed the slightly higher growth rate than that of pH 3. The biomass accumulation of induction with 0.1 and 0.5% methanol was compared. The results showed that the biomass accumulation under 0.5% methanol induction was lower than that obtained under 0.1% methanol

induction. The biomass accumulation under 0.5% methanol induction at pH 4 was low throughout 96 h induction, whereas biomass accumulation at pH 3 was low at 48 h of induction (figure 4.24). These results may caused by toxicity from methanol accumulation led to inhibit cell growth rate. Based on analysis of methanol concentration by gas chromatography (figures D.2 to D.3 in Appendix D), adding absolute methanol every 12 h in order to maintain the methanol level at 0.5% showed accumulation of methanol content almost reached 1% methanol after 36 h of methanol induction in both pH 3 and 4. These methanol accumulations can inhibit cell growth and production of the fusion protein (Damasceno et al., 2004). In contrast, maintaining 0.1% methanol by manually method has accumulation of methanol lower than 0.5%. This level is in range of typical concentration for *P. pastoris* expression system, 0.15 to 1.0% (v/v) methanol (Daly and Hearn, 2005).

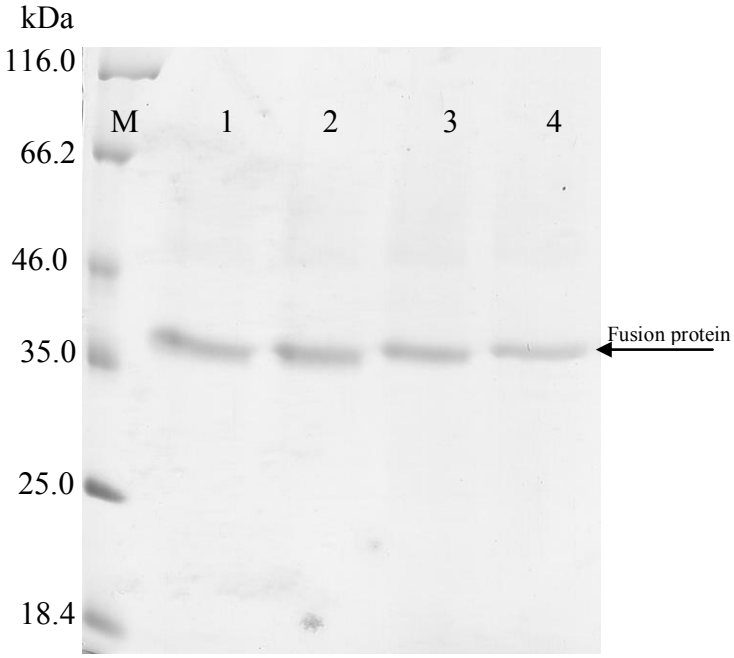

Figure 4.25 SDS-PAGE analysis of supernatant samples of 96 h methanol induction phase at different pH and methanol concentrations. Lane 1, pH 3 with 0.1% methanol; lane 2, pH 3 with 0.5% methanol; lane 3, pH 4 with 0.1% methanol; with lane 4, pH 4 with 0.5% methanol and lane M, molecular weight marker.

In figure 4.25, SDS-PAGE analysis showed almost no band of other proteins, indicating that proteolysis degradation of recombinant proteins was less

observed. The highest fusion protein IL-2/FU-MK-1-scFv production was analyzed by ImageJ densitometry software. The results showed that the concentration of secreted fusion protein produced under 0.1% methanol induction reached 258±13 mg/L. However, the amount of fusion protein was only 125±9 mg/L under pH 3 and induction with 0.5% methanol. The level of fusion protein produced at pH 4 was 105±16 and 106±9 mg/L after 96 h induction of 0.1% and 0.5% methanol, respectively. As a result, optimal condition consisting of pH 3, 30 °C and 0.1% methanol induction were applied to produce the fusion protein in 5 L fermenter.

4.3 Fed-batch cultivation in 5 L fermenter under optimal condition

Transformant *P. pastoris* strain GS115 was pre-cultured overnight in at 30 °C in 10 ml YPD broth. Then 1 ml of the overnight culture was transferred into 200 ml BMGY and grown at 30 °C until the OD_{600} reached ~8 to 10. This culture was inoculated to 2.5 L of modified basal salts medium.

Fed batch culture was carried out in 5 L fermenter under optimal condition obtained from shaken flask cultivation. Glycerol as a carbon source in the first phase was varied at 5, 7.5, and 10% (w/v) to investigate the impact of cell density on the production of IL-2/FU-MK-scFv by *P. pastoris* strain GS115. In this phase, biomass and OD_{600} were analyzed every 6 h. After DO spike was observed, the methanol was fed immediately and the sample was taken every 12 h for cell weight, OD_{600}, methanol level and SDS-PAGE analysis.

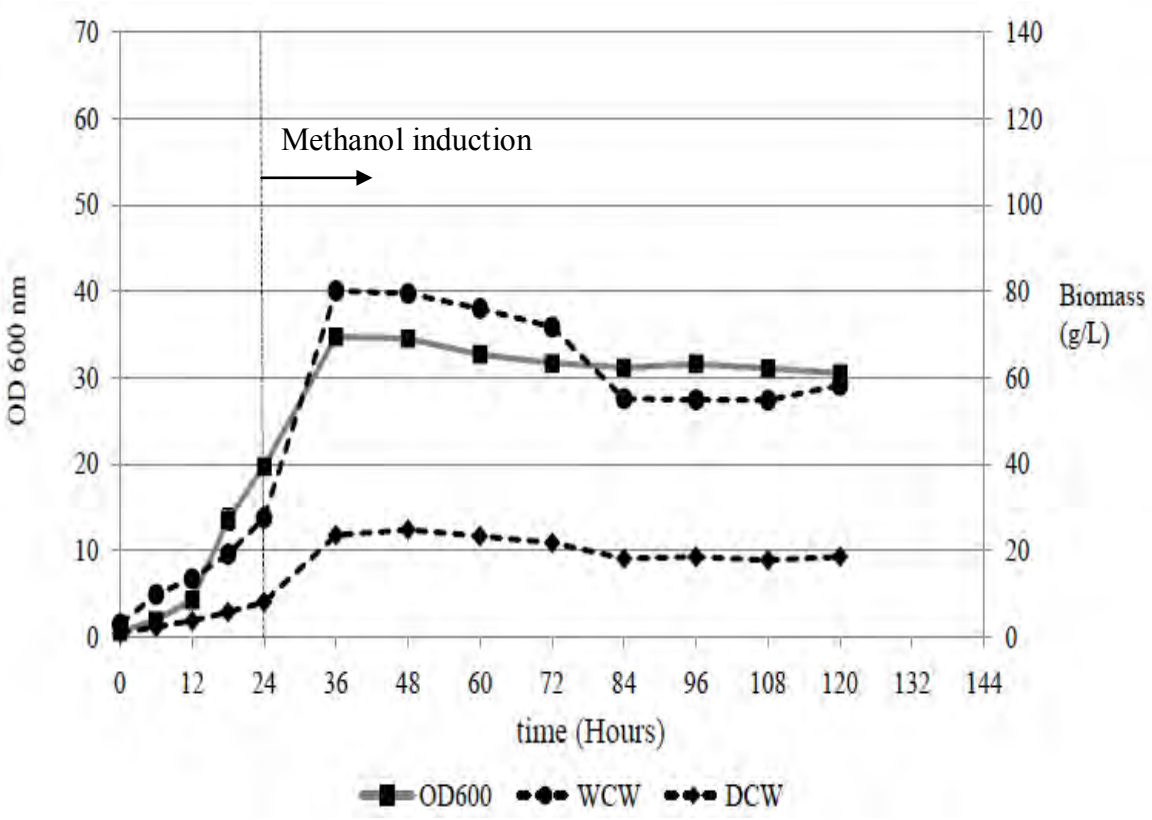

Figure 4.26 The growth profiles of *P. pastoris* strain GS115 harboring pPICZαA-IL2/FUscFv(V_H-V_K) during fed-batch cultivation in 5 L fermenter under temperature of 30 °C, pH 3 and 0.1% methanol induction. The growth phase was performed with 5% glycerol batch phase.

Firstly, IL-2/FU-MK-1-scFv production by *P. pastoris* was performed under 5% glycerol in the first phase. The batch cultivation was employed in glycerol phase for 24 h. Under this condition wet cell weight (WCW) 27.5±1.0 g/L and dry cell weight (DCW) 8.1±0.2 g/L were obtained. After 0.1% methanol induction for 96 h, WCW 58.2±4.4 g/L and DCW 18.5±1.0 g/L were obtained.

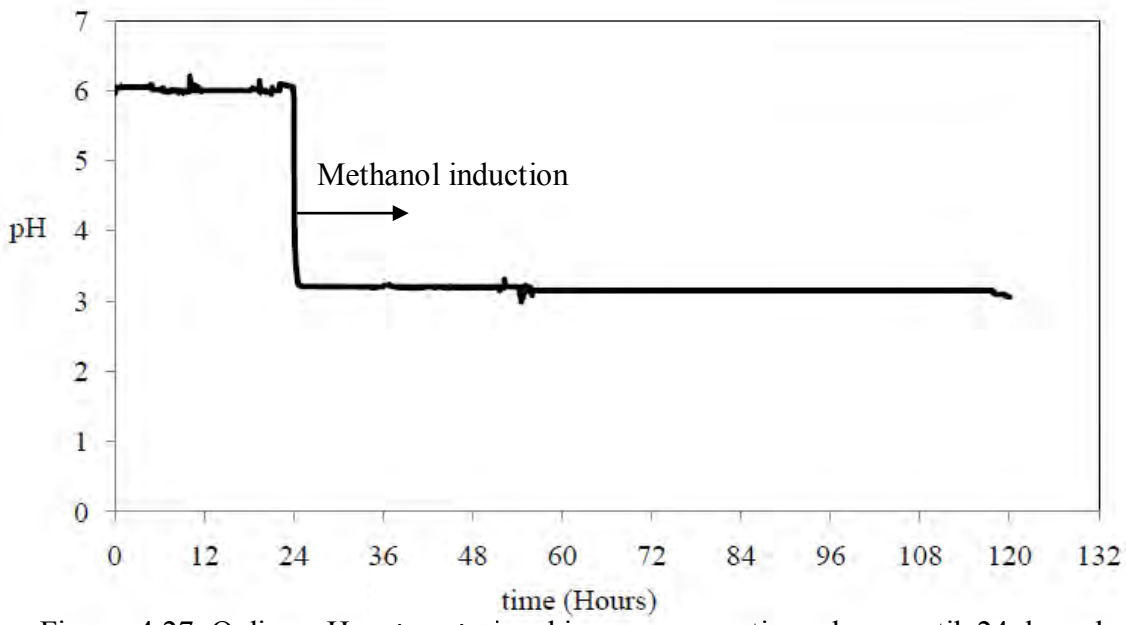

Figure 4.27 Online pH value during biomass generation phase until 24 h and methanol induction phase 24-120 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 5% glycerol batch phase.

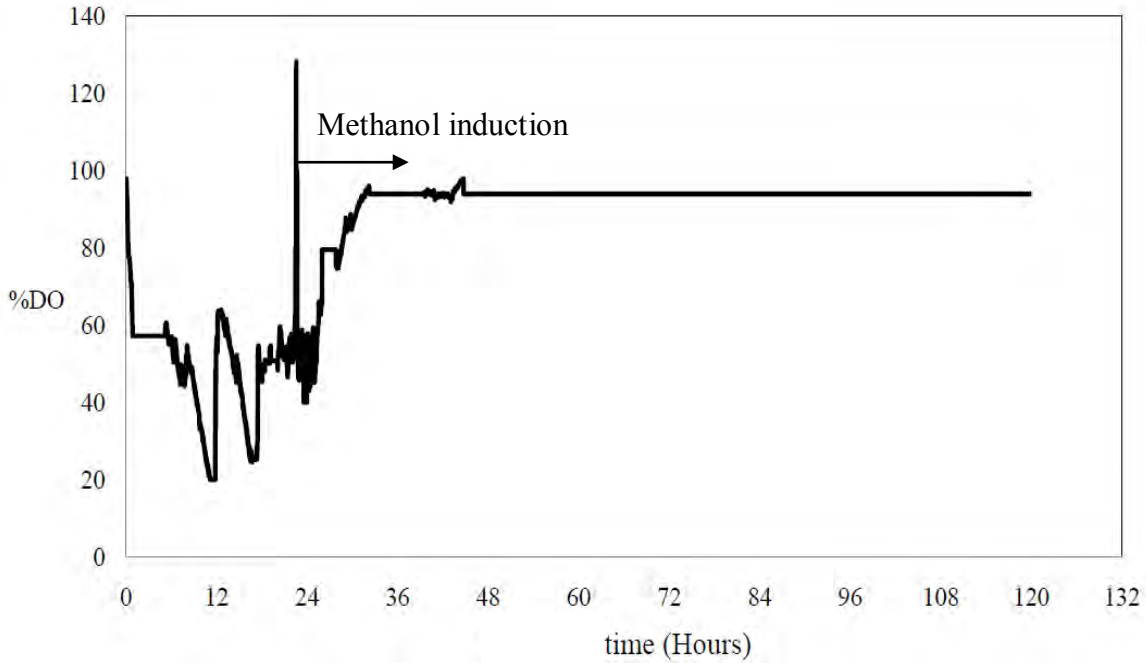

Figure 4.28 Online %DO during biomass generation phase, DO spike occurred at 24 h and methanol induction phase 24-120 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 5% glycerol batch phase.

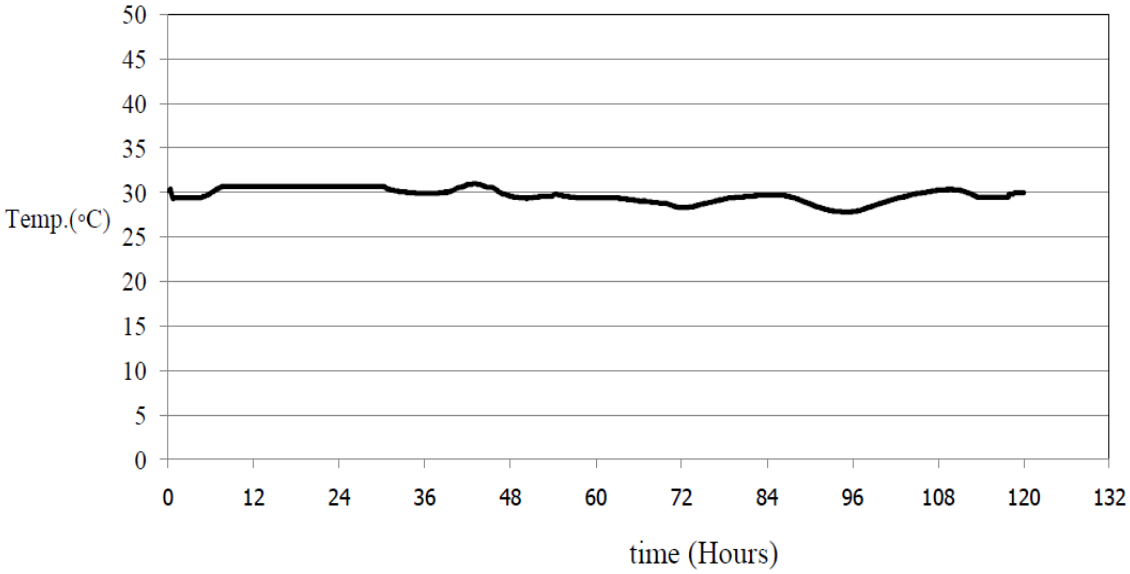

Figure 4.29 Online of temperature performance during biomass generation phase until 24 h and methanol induction phase 24-120 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 5% glycerol batch phase.

Figure 4.30 Methanol level during 96 h methanol induction in fed-batch cultivation in 5 L fermenter with 5% methanol constantly feed rate at 6 ml/h using modified basal salts medium containing 5% glycerol at temperature of 30 °C and pH 3.

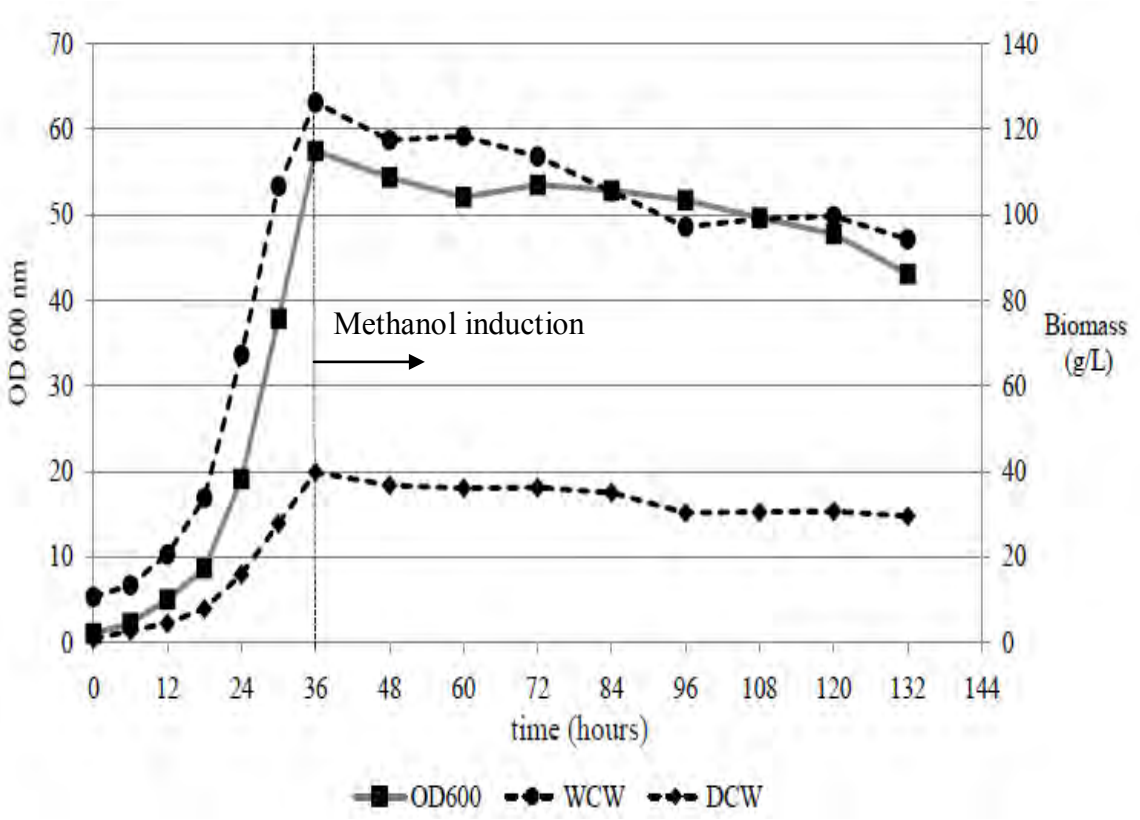

Figure 4.31 The growth profiles of *P. pastoris* strain GS115 harboring pPICZαA-IL2/FUscFv(V_H-V_K) during fed-batch cultivation in 5 L fermenter under temperature 30 °C, pH 3 and 0.1% methanol induction. The growth phase was performed with 7.5% glycerol batch phase.

In this experiment, DO spike was obtained at 36 h (figure 4.33) when 7.5% glycerol was used in growth phase. Thus, the production phase was stared at 36 h. Under this condition, WCW and DCW was 126.2±0.6 and 39.7±0.6 g/L, respectively. After 96 h methanol induction, the WCW reached 94.3±2.4 g/L and DCW 29.5±0.6 g/L was obtained.

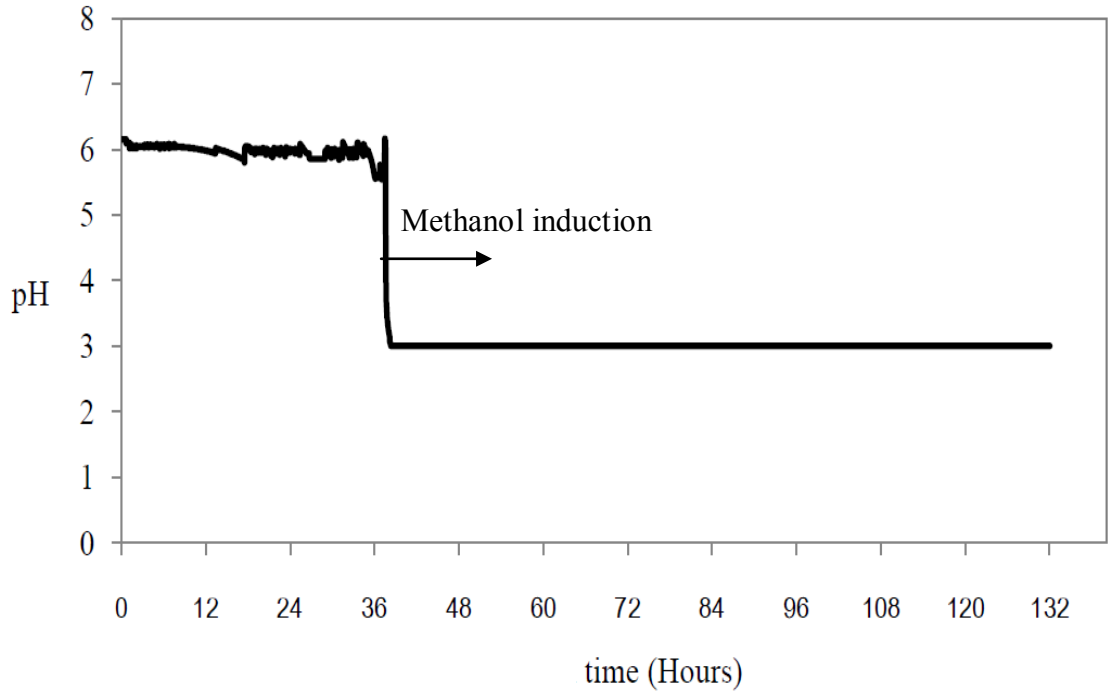

Figure 4.32 Online pH value during biomass generation phase until 36 h and methanol induction phase 36-132 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 7.5% glycerol batch phase.

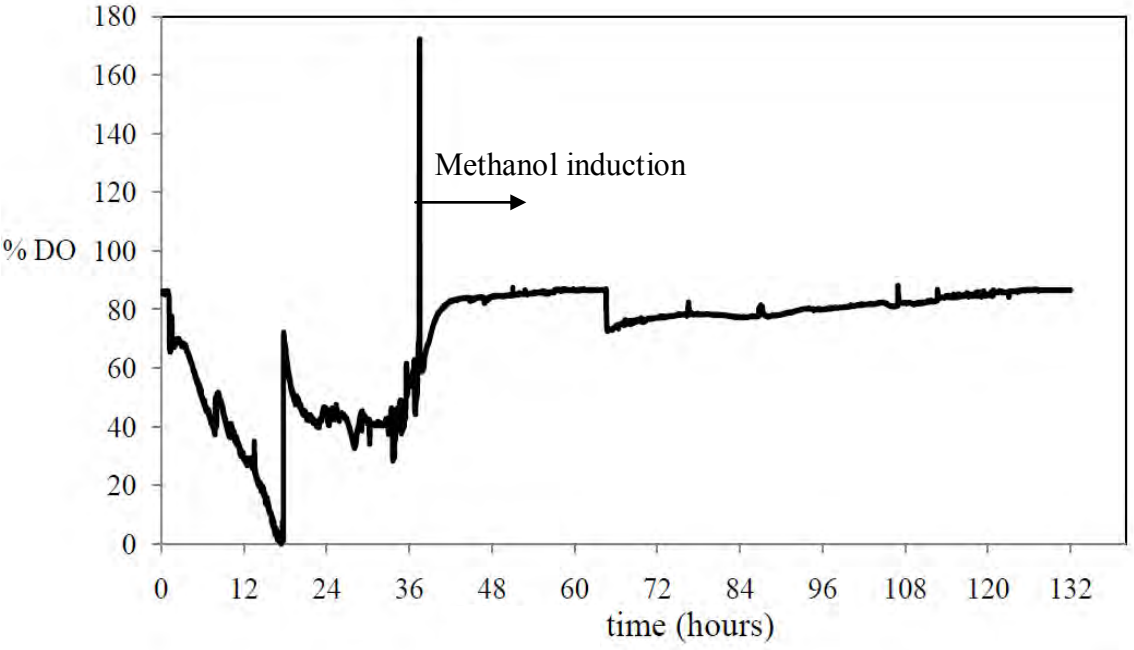

Figure 4.33 Online %DO during biomass generation phase, DO spike occurred at 36 h and methanol induction phase 36-132 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 7.5% glycerol batch phase.

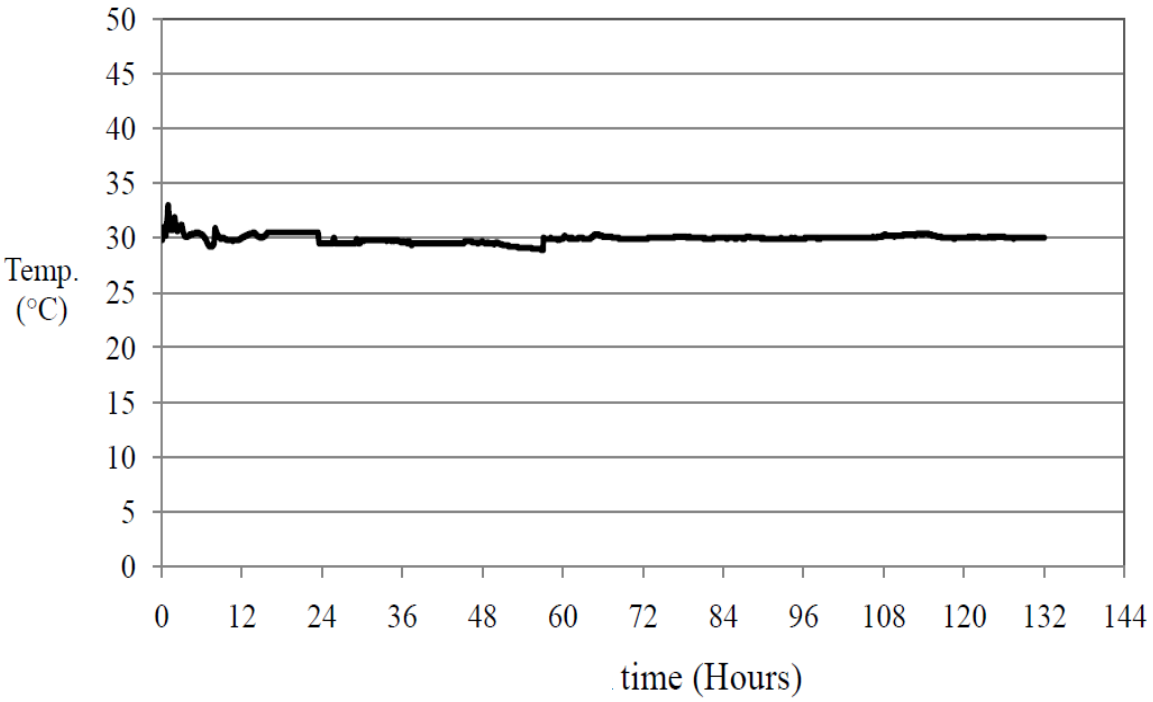

Figure 4.34 Online of temperature performance during biomass generation phase until 36 h and methanol induction phase 36-132 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 7.5% glycerol batch phase.

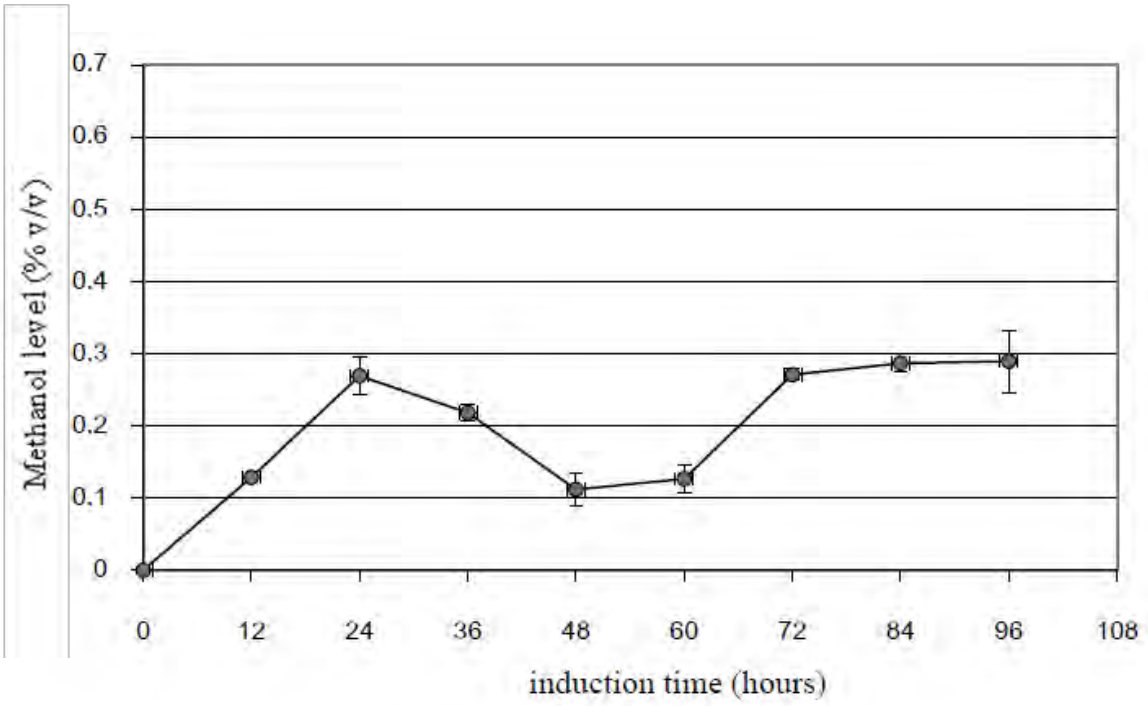

Figure 4.35 Methanol level during 96 h methanol induction in fed-batch cultivation in 5 L fermenter with 7.5% methanol constantly feed rate at 6 ml/h using modified basal salts medium containing 7.5% glycerol at temperature of 30 °C and pH 3.

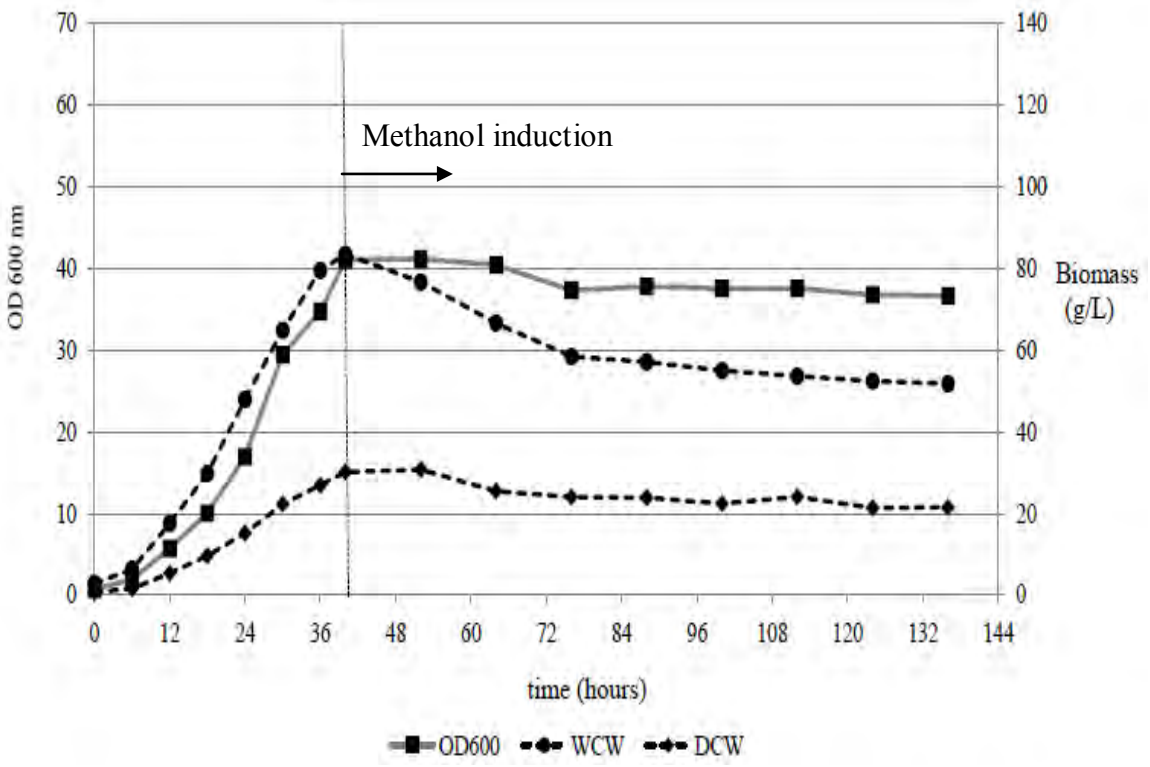

Figure 4.36 The growth profiles of *P. pastoris* strain GS115 harboring pPICZαA-IL2/FUscFv(V_H-V_K) during fed-batch cultivation in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 10% glycerol batch phase.

In study, when 7.5% glycerol was used in growth phase DO spike was obtained at 40 h (figure 4.38). Thus, the methanol induction phase was stared at 40 h. Under this condition, WCW and DCW was 83.5±2.2 and 30.2±0.8 g/L, respectively. After 96 h methanol induction, the WCW reached 51.8±1.0 g/L and DCW 21.5±0.8 g/L was obtained.

As shown in figures 4.26, 4.31, and 4.36, when level of glycerol was increased from 5% to 7.5% (w/v), the biomass was increased from 27.5±1.0 to 126.2±0.6 g/L. In contrast, the biomass generated by 10% glycerol was lower than that obtained with 7.5% glycerol because the concentration 10% glycerol is too high level to attain optimal condition. It was reported, glycerol at high concentration may inhibit cell growth of *P. pastoris*. To solve this problem, a feed rate of glycerol to supply enough glycerol for maintaining the cell growth without inhibition has to be regenerated (Chen et al., 1996). Thus, it was concluded that increasing glycerol level in the generated phase can promote the biomass of *P. pastoris*, but it has limited level. In

this study, glycerol concentration of 7.5% was suitable level to generate biomass of *P. pastoris* strain GS115 for recombinant protein production.

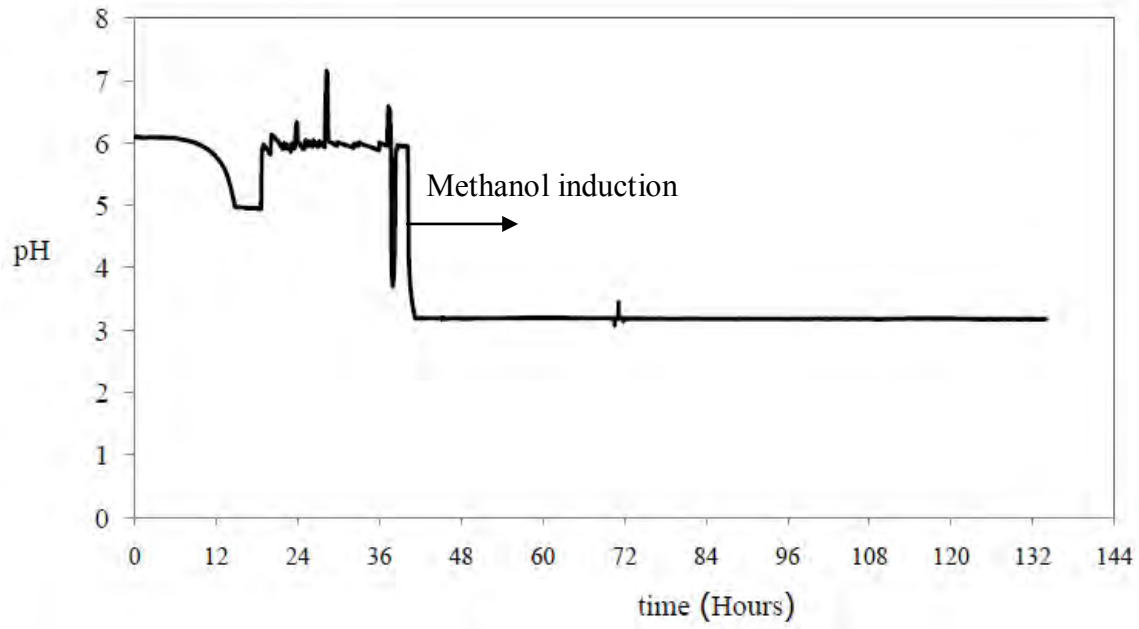

Figure 4.37 Online pH value during biomass generation phase until 40 h and methanol induction phase 40-134 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 10% glycerol batch phase.

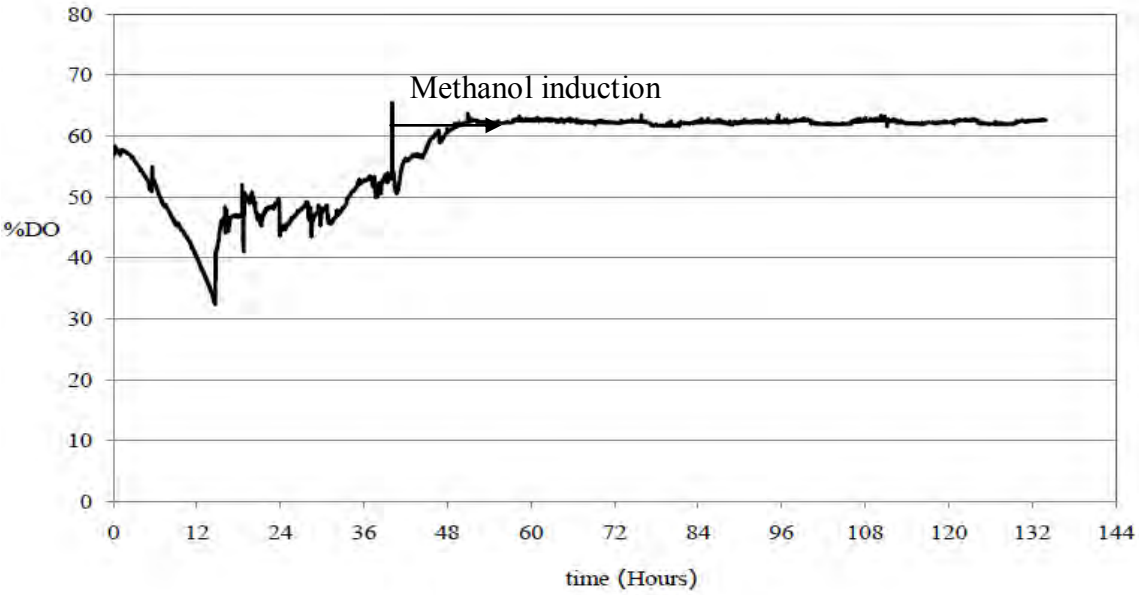

Figure 4.38 Online %DO during biomass generation phase, DO spike occurred at 40 h and methanol induction phase 40-134 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 10% glycerol batch phase.

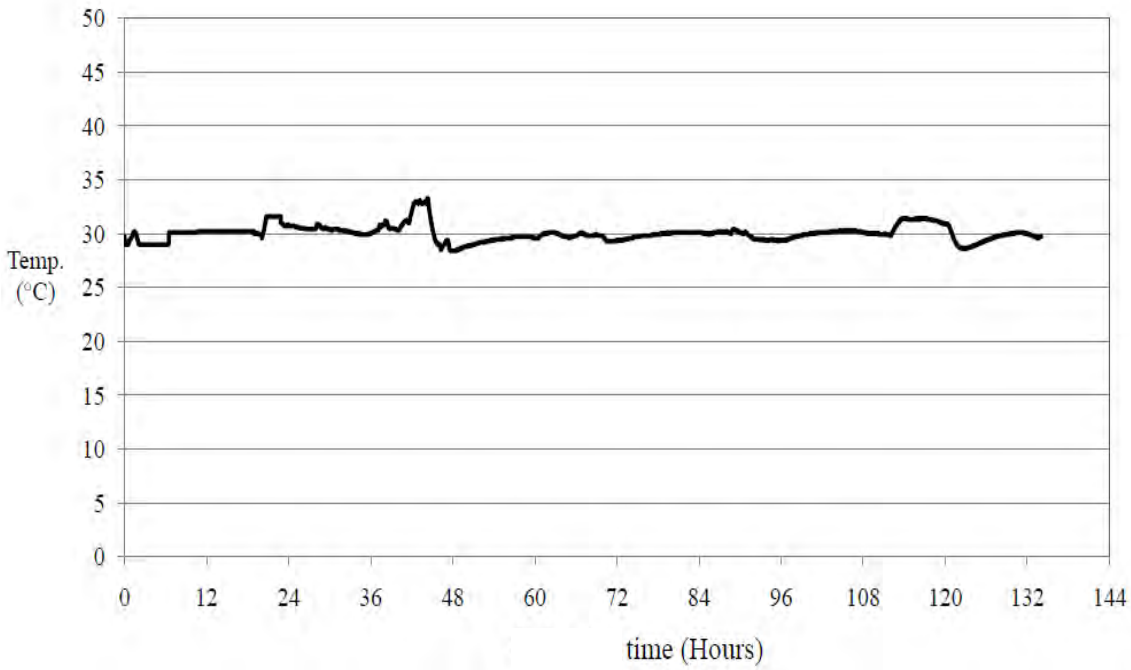

Figure 4.39 Online of temperature performance during biomass generation phase until 40 h and methanol induction phase 40-134 h. Fed-batch cultivation was performed in 5 L fermenter under temperature 30 °C, pH 3, and 0.1% methanol induction. The growth phase was performed with 10% glycerol batch phase.

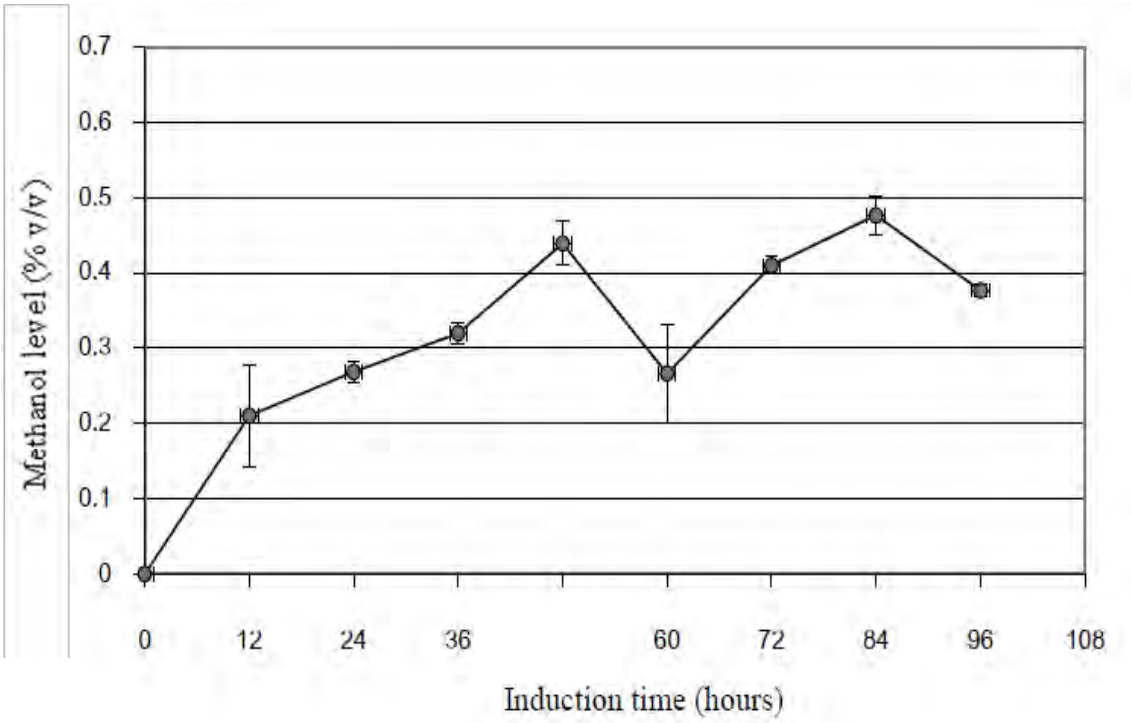

Figure 4.40 Methanol level during 96 h methanol induction in fermenter cultivation with 10% methanol constantly feed rate at 6 ml/h using modified basal salts medium containing 10% glycerol at temperature of 30 °C and pH 3.

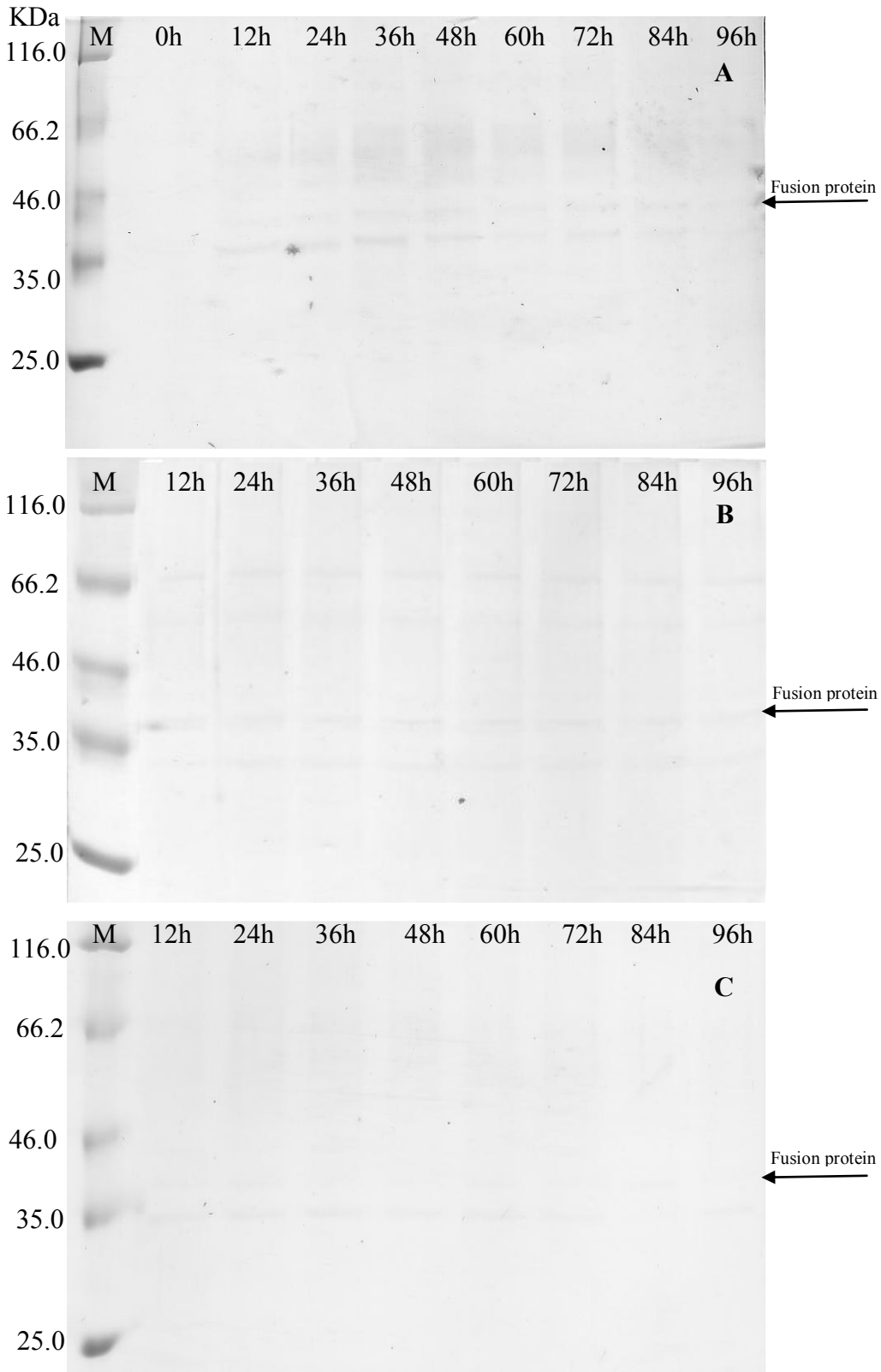

Figure 4.41 Time course of SDS-PAGE analysis of secreted fusion during methanol induction phase. The fed-batch cultivation was performed in 5 L fermenter at pH 3, 30 °C, and 0.1% methanol induction using modified basl salts medium. Biomass of *P. pasotris* strain GS115 was generated under different concentration of glycerol in growth phase, (A) 5% glycerol, (B) 7.5% glycerol, and (C) 10% glycerol.

As shown in figure 4.42, the secreted fusion protein concentration measured by ImageJ analysis was reached 84±5, 109±5, and 77±4 mg/L when glycerol 5, 7.5, and 10% glycerol was used in growth phase, respectively. The amount of the secreted fusion protein was lower than that of the producton in shaken flask cutivation. This might be due to diferrent the medium (modiefied basal salts meduim, Appendix A) of fed batch cultivation was different from that of shaking flask cultivation (BMMY, Appendix A) and led to lower expression. In addition, SDS-PAGE analysis showed bands of several low molecular weight protein and it was implied that degradation of proteins occurred due to protease naturally produced by *P. pastoris* (Shi et al., 2003).

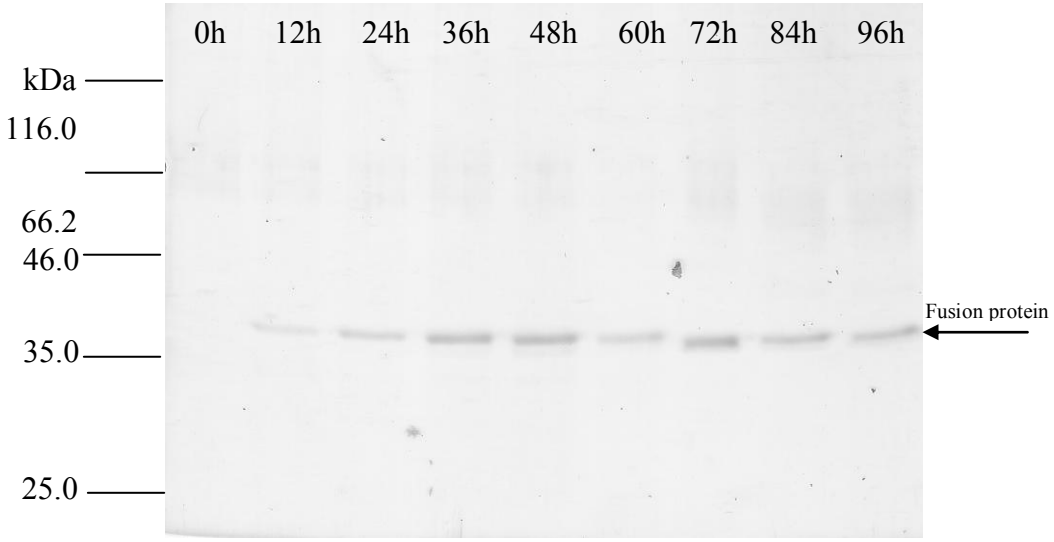

Figure 4.42 Time courses of SDS-PAGE analysis of secreted fusion during methanol induction phase. The fed-batch cultivation was performed in 5 L fermenter at pH 3, 30 °C, and 0.1% methanol induction using BMMY medium. Biomass of *P. pasotris* strain GS115 was generated under 5% glycerol in growth

Interstingly, when modified basal salts medium was exchanged to BMMY, the result (figure 4.43) showed that the amount of the fusin protein was higher than that of production in modified basal salts medium. In figure 4.44, the highest amount of 425±2 mg/L was obtained at 96 h of 0.1% methanol induction with pH 4 in BMMY. However, when pH was set at 3 the amount of fusion protein produced was only 197±19 mg/L. This finding suggested that condition of pH 3 at 30 °C and with 0.1% methanol induction is optimal for the production using BMMY in shaken flask cultivation. Thus, we concluded that in fed batch cultivation, the optimal condition in

BMMY medium consisting of glycerol phase with 5%, induction phse with 0.1% methanol, temperature of 30 °C and pH 4.

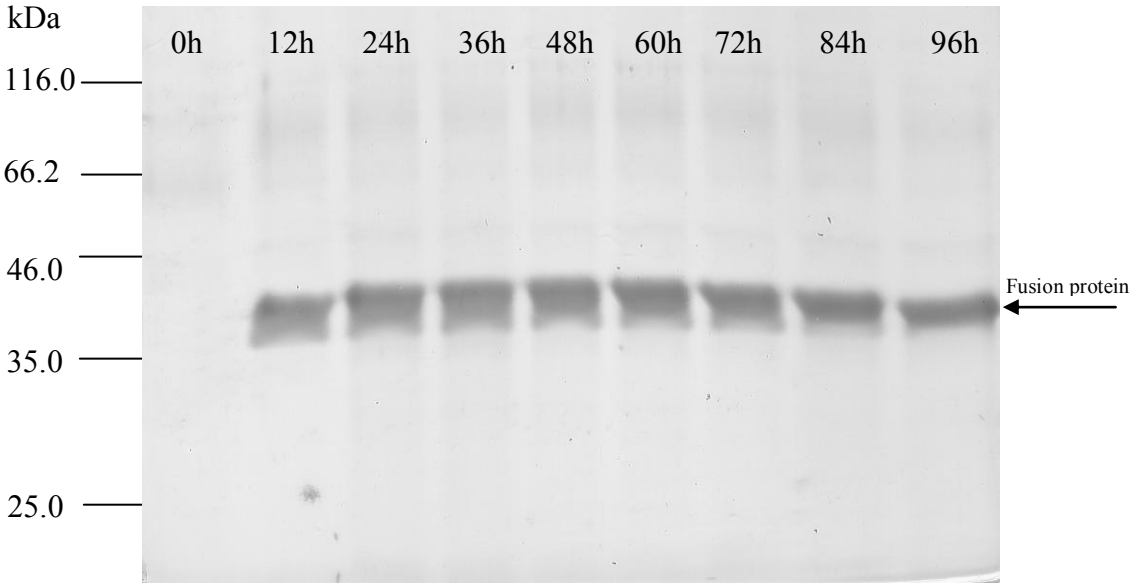

Figure 4.43 Time courses of SDS-PAGE analysis of secreted fusion during methanol induction phase. The fed-batch cultivation was performed in 5 L fermenter at pH 4, 30 °C, and 0.1% methanol induction using BMMY medium. Biomass of *P. pasotris* strain GS115 was generated under 5% glycerol in growth phase.

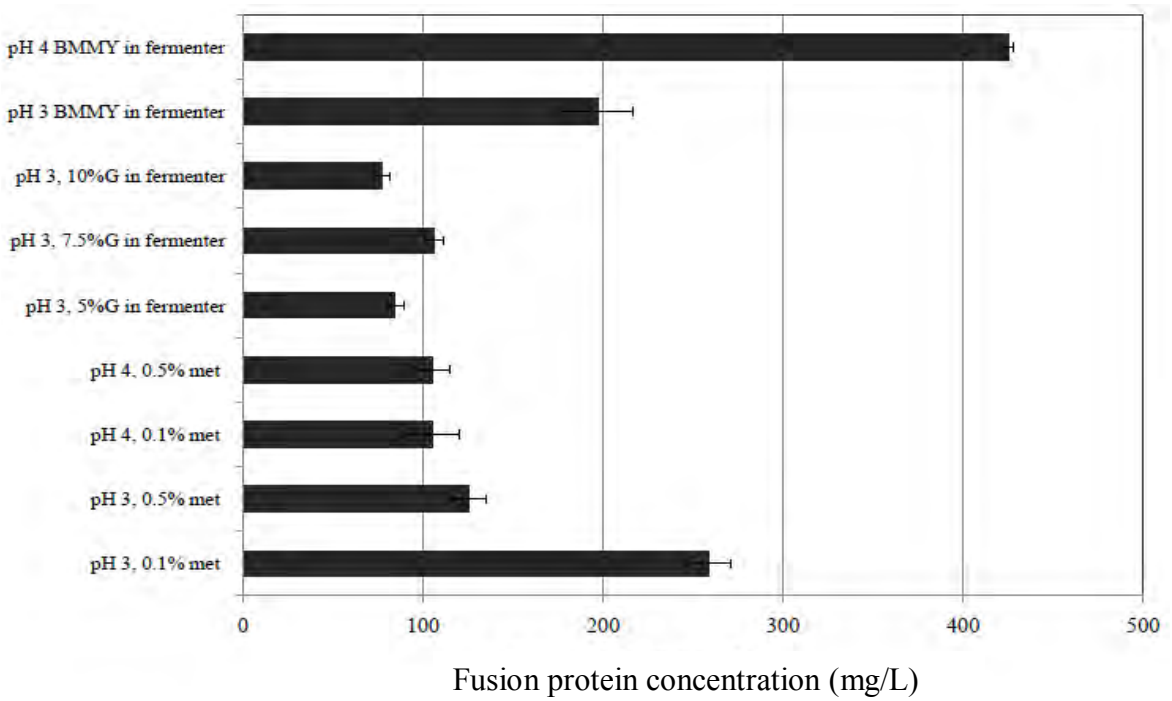

Figure 4.44 The amount of IL-2/FU-MK-1-scFv analyzed by ImageJ software analysis produced at 96 h methanol induction under 30 °C with difference conditions in shaken flak cultivation and fed-batch cultivation in 5 L fermenter.

The production of IL-2/FU-MK-1-scFv by fed-batch cultivation in 5 L fermenter was maximized under 0.1% methanol induction in BMMY at pH 4, temperature of 30 °C and 425±2 mg/L fusion protein was obtained. This concentration of the fusion protein was higher than reports of Ning et al. (2004), Lange et al. (2001) and Yamawaki et al. (2007). Interestingly, Ning et al. (2004) produced their recombinant protein using BMMY medium by *P. pastoris* in fed-batch fermentation whereas others produced in modified basal salts medium. All together, the productivity was enhanced these comparing with the previous reported by Matsumoto et al. (2002).

4.4 Partial purification of the fusion protein by Ni-affinity chromatography

In order to partially purify the secreted IL-2/FU-MK-1-scFv, a one step chromatography was performed by Ni-affinity chromatography. After fermentation process, the supernatant was adjusted to pH 7.5 by KOH and clarified by 0.22 µm membrane filter. The 10 ml of clarified sample was passed through His·Bind®Kits 5 ml column. The eluted fractions were analyzed by coomassie-blue stained SDS-PAGE.

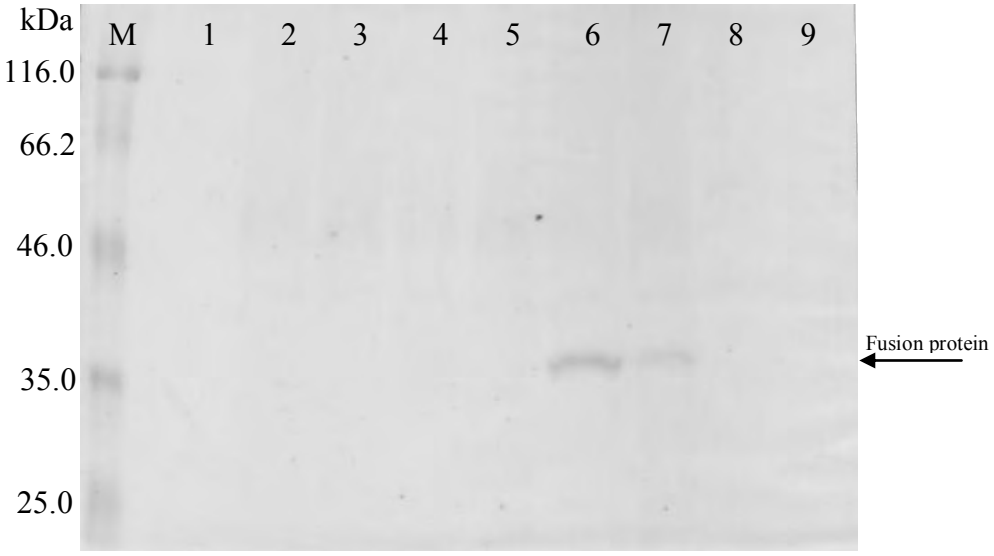

Figure 4.45 SDS-PAGE analysis of fractions from purification step using Ni-affinity chromatography; lane 1-5 corresponding to washing fractions, lane 6-9 corresponding to eluted fractions and lane M is molecular weight marker.

Due to C-terminal of IL-2/FU-MK-1-scFv consisted of His-tag that strongly interacts with metal ion as Ni, immobilized in affinity chromatography. Imidazole is usually used for this purification system (Terpe, 2003; Lichty et al., 2005; Arnau et al., 2006). This study successfully purified the fusion protein containing His-tag using Ni-affinity chromatography and showed the eluted bound fusion protein in lane 6 and 7 of figure 4.31. These results suggested that lowering pH of sample or solution could elute bound protein in this purification system. Therefore, prior purification step, the pH value of sample or purification buffer must be adjusted to 7.5-8.0 (Terpe, 2003).

After purification, the fractions containing fusion protein were pooled together. Then, the pooled sample was concentrated by centrifugal ultrafiltration 30 kDa molecular weight cut off (Vivaspin 500).

4.5 Characterization of the fusion protein for specific binding activity

The specific binding activity of the fusion protein IL-2/FU-MK-1-scFv to CHO cell expressing MK-1 was tested by cell lysate EILSA. For comparison, the binding activity of the fusion protein was also tested against CHO cell non-expressing MK-1. Based on the results obtained from cell lysate ELISA, it was demonstrated that the

OD_{450} after cell lysate ELISA analysis of the fusion protein against cell lysate protein from CHO-MK-1 cell and CHO cell were 0.642±0.011 and 0.314±0.017, respectively (figure 4.34). When the statistical significance of differences was calculated by the Student's *t*-test for comparison binding activity of the fusion protein to CHO-MK-1 and CHO cell, the OD at 450 nm of CHO-MK-1 was significantly higher that CHO cell at the $p<0.05$ level. This demonstrated that the fusion protein IL-2/FU-MK-1-scFv bound to the MK-1 expressing CHO cell but not to the MK-1 non-expressing CHO cell. Therefore, the fusion protein IL-2/FU-MK-1-scFv produced by *P. pastoris* strain GS115 in this study possessed binding activity against MK-1 expressing CHO cell.

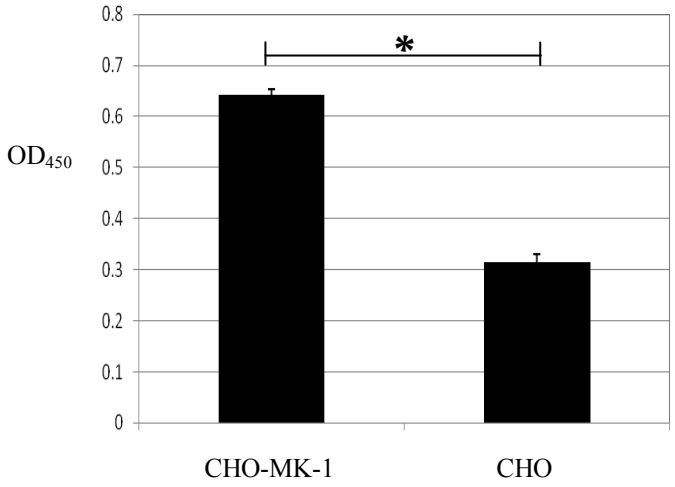

Figure 4.46 The specific binding activity of IL-2/FU-MK-1scFv to MK-1 expressing and non-expressing CHO cell by cell lysate ELISA method (*, $p<0.05$).

CHAPTER V

CONCLUSIONS

5.1 Conclusions

In order to produce IL-2/FU-MK-1-scFv in *P. pastoris* effectively, the temperature, pH, and medium composition are important parameters to minimize protease activity and enhance the expression of the recombinant protein. The expression of IL-2/FU-MK-1-scFv in *P. pastoris* strain GS115 was investigated in shaken flask cultivation by examining the effect of pH value ranging from 3 to 10, temperature ranging from 20 to 37 °C and methanol concentration ranging from 0.01 and 0.5%. Then, the optimal condition obtained from shaken flask cultivation was applied to the production in 5 L fermenter.

This study demonstrated that pH value and temperature showed major effect on growth and recombinant protein expression of *P. pastoris*. Lowering pH value could increase the productivity of fusion protein because it is optimal growth and may reduce protease activity. Based on the results obtained, pH 3 and 4 have shown higher expression of the fusion protein than pH 5 to 10. Increasing temperature to 37 °C, it caused poor growth rate and low productivity of the fusion protein. The transformant *P. pastoris* strain GS115 harboring pPICZαA-IL2/FUscFv(V_H-V_K) could grow and express fusion protein at temperature of 20, 25, and 30 °C with pH 3 and 4. In shaken flask cultivation, the highest production of the fusion protein at 258±13 mg/L was obtained under pH 3 and 30^{o}C with a methanol concentration of 0.1% for 96 h induction in BMMY medium. Finally, fed-batch cultivation was performed in 5 L fermenter. It was found that the amount of secreted fusion protein was 109±5 mg/L when the modified basal salt medium containing 7.5% glycerol was used, whereas the amount of secreted fusion protein was increased up to 425±2 mg/L when BMMY medium containing 5% glycerol was used. Besides pH, temperature and methanol concentrations, the results obtained from fed-batch cultivation showed that

composition of medium is also a crucial factor for recombinant protein production by *P. pastoris*.

The fusion protein IL-2/FU-MK-1 scFv was partially purified by Ni-affinity chromatography. In order to investigate specific binding activity of the fusion protein, cell lysate ELISA was applied in this study. As a result, the fusion protein retained the specific binding activity to MK-1 antigen due to it significantly bound to MK-1 expressing CHO cell but not MK-1 non-expressing CHO cell when compared using Student's *t* test ($p<0.05$). In the future, the specific binding activity of IL-2/FU-MK-1-scFv will be performed by flow cytometry to confirm binding activity of the fusion protein.

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APPENDICES

Appendix A

Media Recipes

1. 10X YNB 1 L (13.4% Yeast nitrogen base with ammonium sulfate without amino acids)

Dissolve 34 g of YNB (without ammonium sulfate and amino acids) and 100 g of ammonium sulfate in 1000 ml of water and filter sterilize. Store at +4 °C.

2. 500X B (0.02% biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at +4 °C. The shelf life is approximately one year.

3. 10X D (20% dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water and autoclave at 110 °C on liquid cycle for 15 minutes.

4. 10X GY (10% glycerol)

Add 100 ml glycerol in 900 ml of water and sterilize by autoclaving. Store at room temperature.

5. 10X M (5% methanol)

Mix 5 ml of methanol with 95 ml of water. Sterilize by filter and store at Store at +4◦C. The shelf life is approximately one year.

6. 1 M potassium phosphate buffer pH 6.0

Combine about 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirmed the pH value = 6. Phosphoric acid or KOH were used if the pH value needs to be adjusted. Sterilize by autoclaving and store at room temperature.

7. YPD 1 L

YPD contains of 1% yeast extract, 2% peptone and 2% dextrose. Dissolve 10 g yeast extract and 20 g peptone in 900 ml water if making YPD plates or slants, 20 g agar was added. Then sterilize by autoclaving for 15 minute on liquid cycle. And add 100 ml of 10X D.

8. YPDS containing Zeocin^{TM} agar 1 L

Dissolve 10 g yeast extract, 182.2 g sorbitol, 20 g peptone and add 20 g of agar. Then autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D. Cool solution to about 60◦C and add 1.0 ml of 100 mg/ml Zeocin^{TM}. Store at +4 °C in the dark.

9. BMGY medium 1 L

BMGY consists of 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% YNB, 4X10^{-5}% biotin, and 1% glycerol. Dissolve 10 g yeast extract, 20 g peptone in 700 ml water. Autoclave 20 minutes on liquid cycle. Cool to room temperature and then add 100 ml 1 M potassium phosphate pH 6, 100 ml 10X YNB, 2 ml 5X biotin and 100 ml 10X GY. Store at +4 °C.

10. BMMY medium 1 L

BMGY consists of 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% YNB, 4X10^{-5}% biotin, and 1% glycerol. Dissolve 10 g yeast extract, 20 g peptone in 700 ml water. Autoclave 20 minutes on liquid cycle. Cool to room temperature and then add 100 ml 1 M potassium phosphate pH 6, 100 ml 10X YNB, 2 ml 5X biotin and 100 ml 10X M. Store at +4 °C. if pH need to be adjusted, use phosphoric acid or KOH.

11. Modified basal salts medium 1 L (Damasceno et al., 2004)

1 L of Modified basal salts medium contains 0.23 g $CaSO_4 \cdot H_2O$, 4.55 g K_2SO_4, 3.73 g $MgSO_4 \cdot 7H_2O$, 1.03 g KOH, 6.68 ml H_3PO_4 and 1 ml antifoam A. After

sterilization of Modified basal salts medium, filtrated PTM trace salts was added into fermenter at 4.35 ml/L.

12. PTM trace salts

PTM trace salts consist of 24 mM $CuSO_4$, 0.53 mM NaI, 19.87 mM $MnSO_4$, 0.83 mM Na_2MoO_4, 0.32 M boric acid, 2.1 mM $CoCl_2$, 0.15 mM $ZnCl_2$, 0.23 M $FeSO_4$ and 0.82 mM biotin. (PTM was sterilized by filter.)

Appendix B

Selection of Fusion Protein Expressing Clone

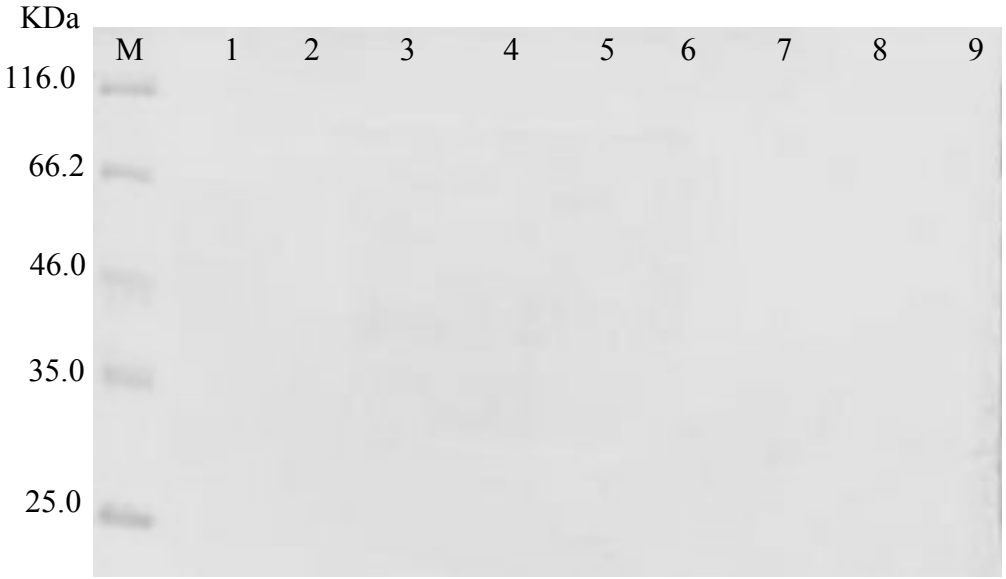

Figure B.1 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain GS115. Lane M, molecular marker; lane 1, clone 1; lane 2, clone 2; lane 3, clone 3; lane 4, clone 4; lane 5, clone 5; lane 6, clone 6; lane 7, clone 7; lane 8, clone 8 and lane 9, clone 9.

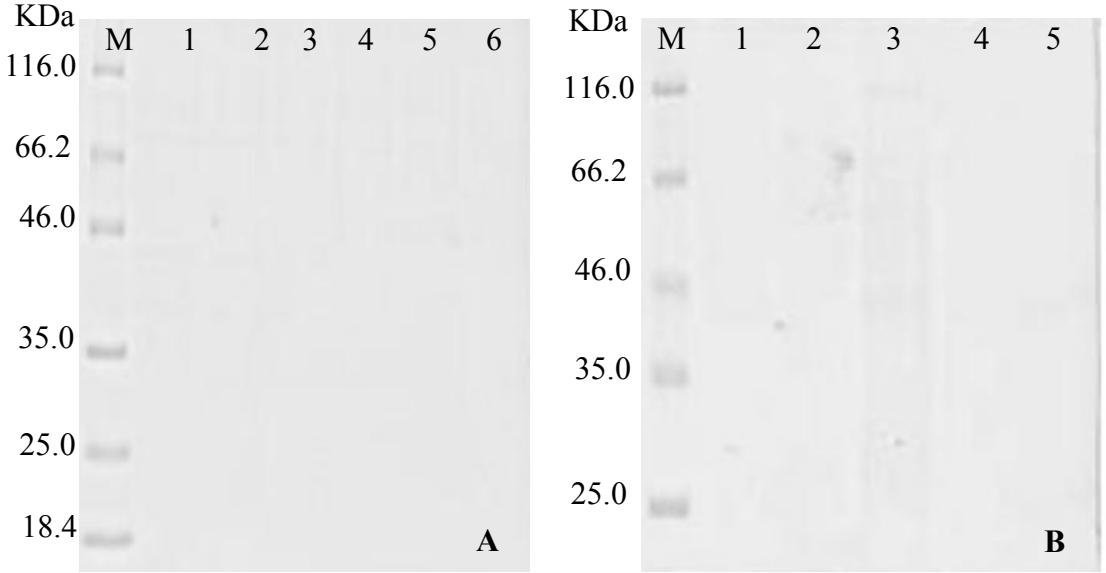

Figure B.2 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain GS115. A) Lane M, molecular marker; lane 1, clone 10; lane 2, clone 11; lane 3, clone 12; lane 4, clone 13; lane 5, clone 14 and lane 6, clone 15. B) Lane 1, clone 16; lane 2, clone 17 and lane 3, clone 18; lane 4, clone 19 and lane 5, clone 20.

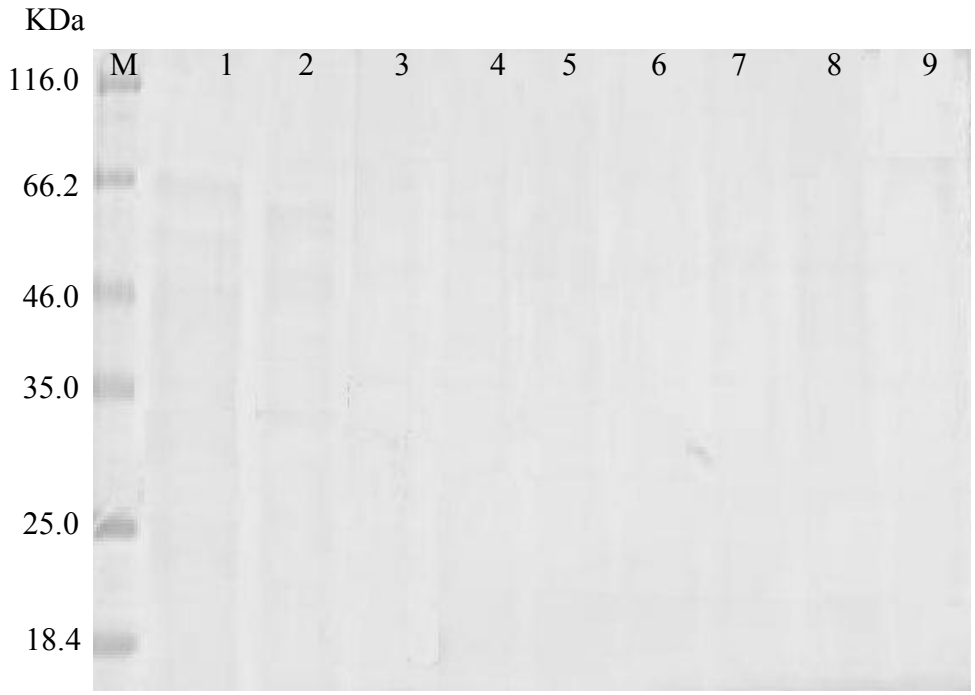

Figure B.3 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain GS115. Lane M, molecular marker; lane 1, clone 21; lane 2, clone 22; lane 3, clone 23; lane 4, clone 24; lane 5, clone 25; lane 6, clone 26; lane 7, clone 27; lane 8, clone 28 and lane 9, clone 29.

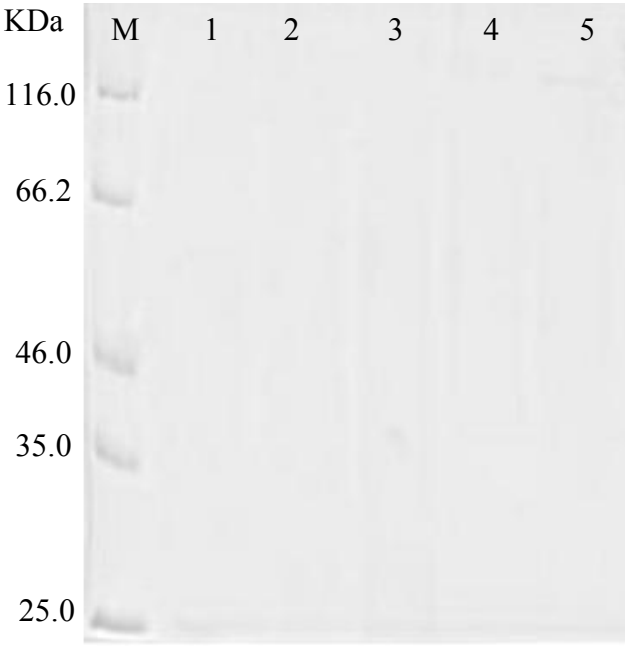

Figure B.4 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain GS115. Lane M, molecular marker; lane 1, clone 30; lane 2, clone 31; lane 3, clone 32; lane 4, clone 33 and lane 5, clone 34.

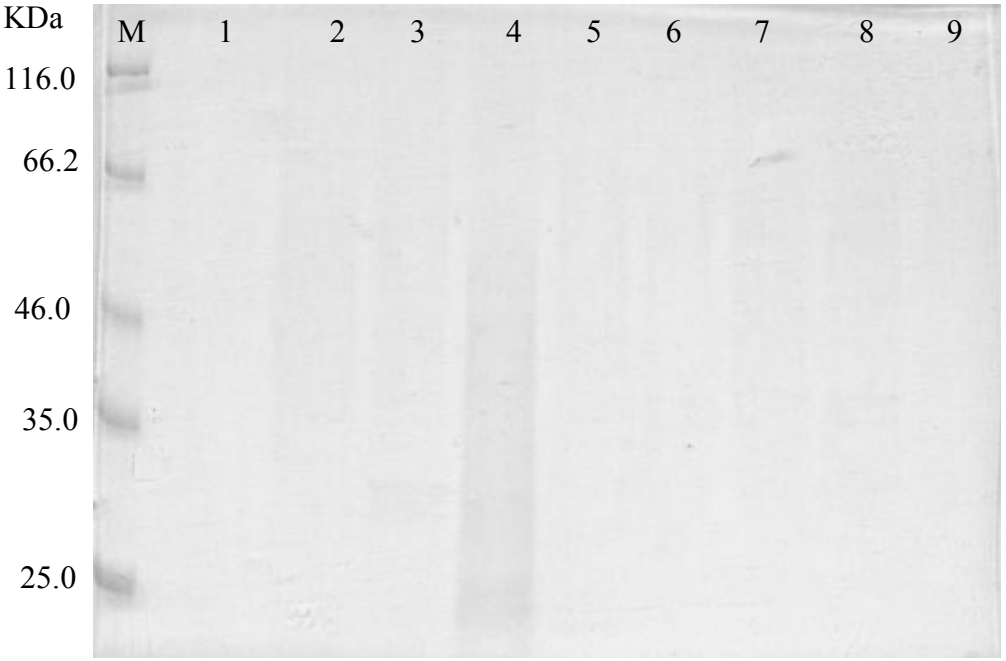

Figure B.5 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain GS115. Lane M, molecular marker; lane 1, clone 36; lane 2, clone 37; lane 3, clone 38; lane 4, clone 39; lane 5, clone 40; lane 6, clone 41; lane 7, clone 42; lane 8, clone 43 and lane 9, clone 44.

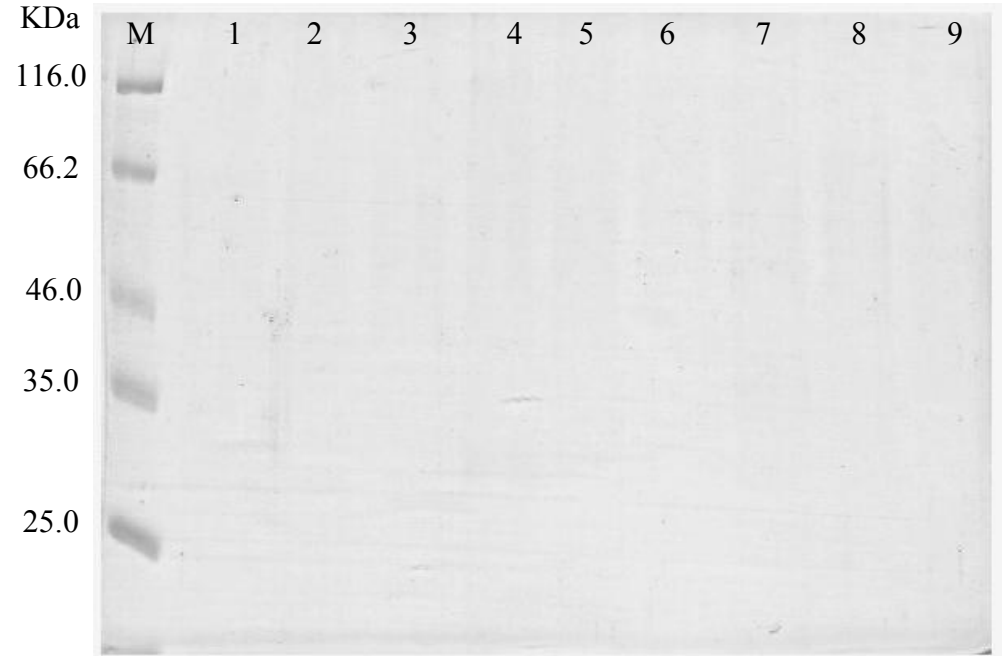

Figure B.6 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain GS115. Lane M, molecular marker; lane 1, clone 45; lane 2, clone 46; lane 3, clone 47; lane 4, clone 48; lane 5, clone 49; lane 6, clone 50; lane 7, clone 51; lane 8, clone 52 and lane 9, clone 53.

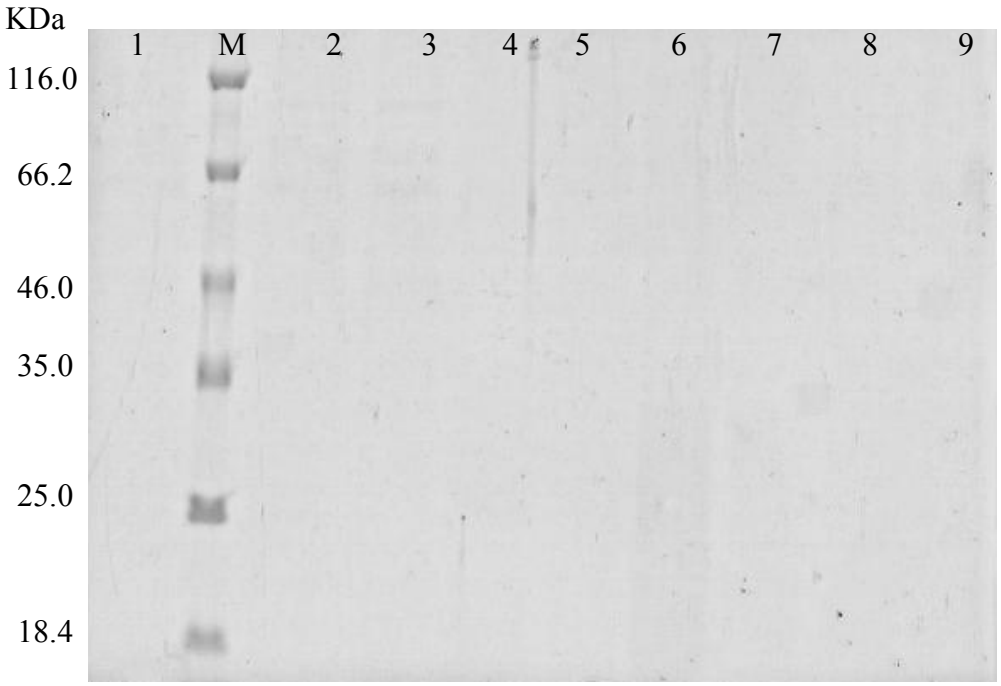

Figure B.7 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain KM71H. Lane M, molecular marker; lane 1, clone 1; lane 2, clone 2; lane 3, clone 3; lane 4, clone 4; lane 5, clone 5; lane 6, clone 6; lane 7, clone 7; lane 8, clone 8 and lane 9, clone 9.

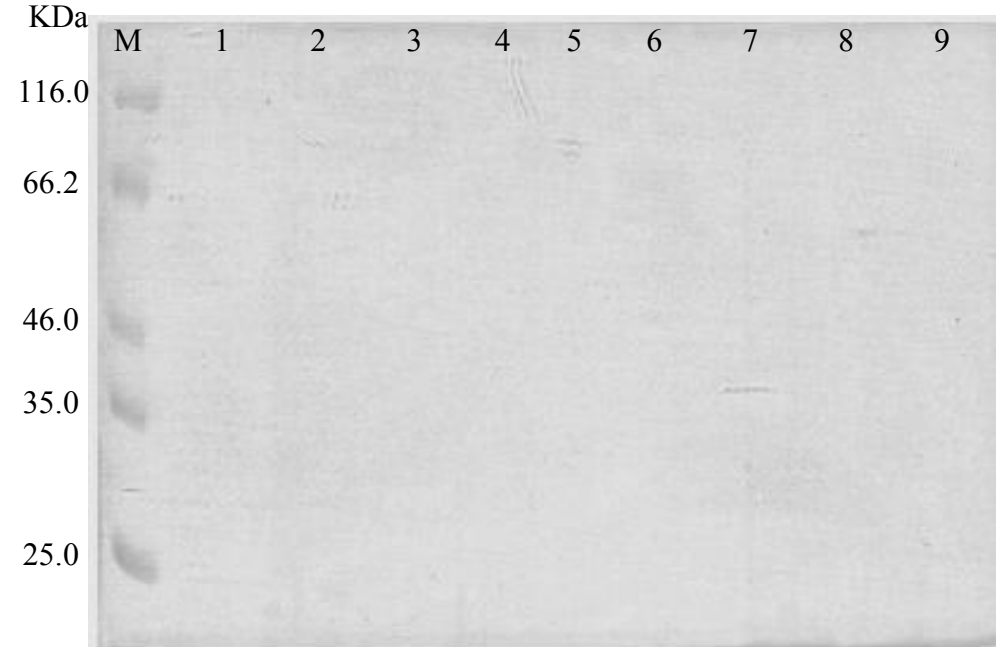

Figure B.8 SDS-PAGE analysis of fusion protein expressed by transformants *P. pastoris* strain KM71H. Lane M, molecular marker; lane 1, clone 10; lane 2, clone 11; lane 3, clone 12; lane 4, clone 13; lane 5, clone 14; lane 6, clone 15; lane 7, clone 16; lane 8, clone 17 and lane 9, clone 18.

Appendix C

Agitation Speed During Fed-batch Cultivation

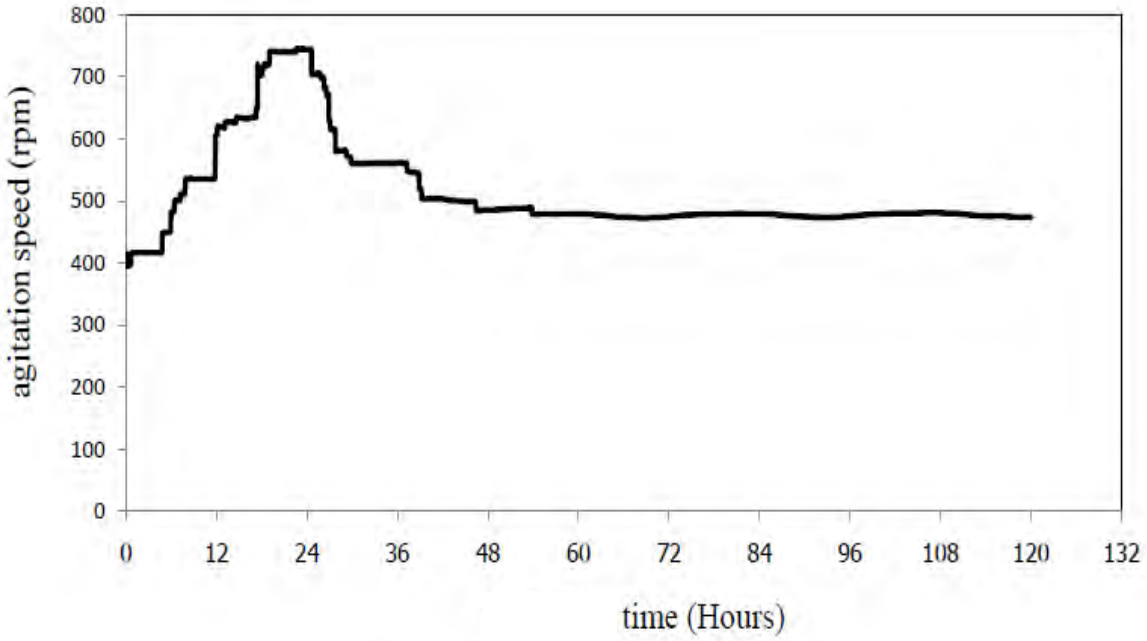

Figure C.1 Online agitation speed of impeller during biomass generation phase until 24 h and methanol induction phase 24-120 h. The fed batch cultivation was performed in 5 L fermenter at pH 4, 30 °C, and 0.1% methanol induction using BMMY medium. Biomass of *P. pastoris* GS115 was generated under 5% glycerol in growth phase.

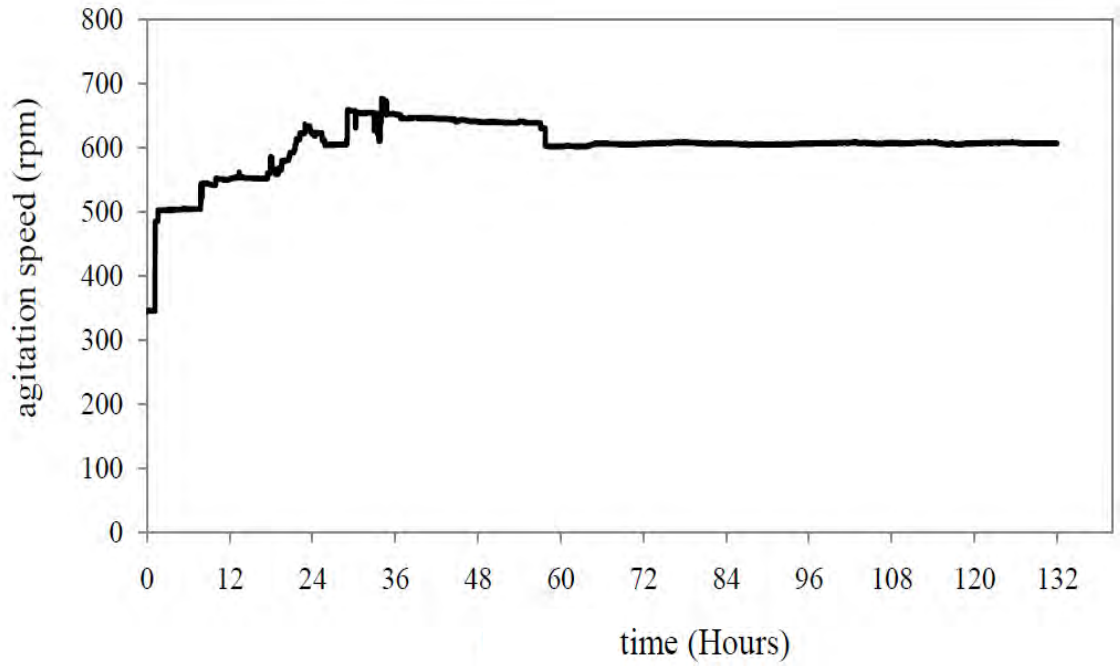

Figure C.2 Online agitation speed of impeller during biomass generation phase until 24 h and methanol induction phase 24-120 h. The fed batch cultivation was performed in 5 L fermenter at pH 4, 30 °C, and 0.1% methanol induction using BMMY medium. Biomass of *P. pastoris* strain GS115 was generated under 7.5% glycerol in growth phase.

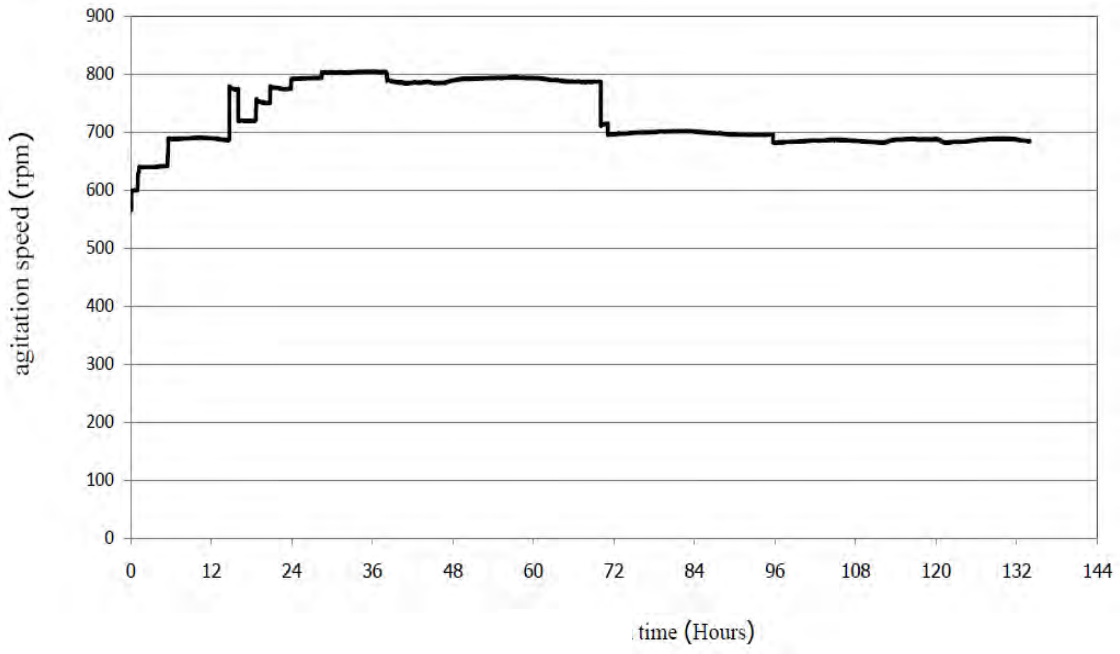

Figure C.3 Online agitation speed of impeller during biomass generation phase until 40 h and methanol induction phase 40-134 h. The fed batch cultivation was performed in 5 L fermenter at pH 4, 30 °C, and 0.1% methanol induction using BMMY medium. Biomass of *P. pastoris* strain GS115 was generated under 10% glycerol in growth phase.

Appendix D

Methanol Level Analysis by Gas Chromatography

The supernatant samples from centrifugation at 13,000 rpm for 10 min were mixed with 1% n-pentanol in 0.25 M HCl (1:1). The mixed samples were analyzed by gas chromatography analyzer following these setting: Type of column is capillary column cabowax-PEG Size 60 m×25 mm ID×25 µm. Temperature of injector was set at 50°C and temperature of column at 40°C holding for 5 min and increasing to 150 °C with 20°C/min. Temperature of detector (FID) was set at 250 °C (isothermal) He was used Carrier gas at flow rate 2 ml/min. Injection volume is 1 µl. The methanol level can be analyzed by comparison peak ratio of samples (peak area of methanol: peak area of 1% n-pentanol) to standard concentration of methanol (peak area of 0, 0.25, 0.5, 1, 2, and 3% methanol: peak area of 1% n-pentanol).

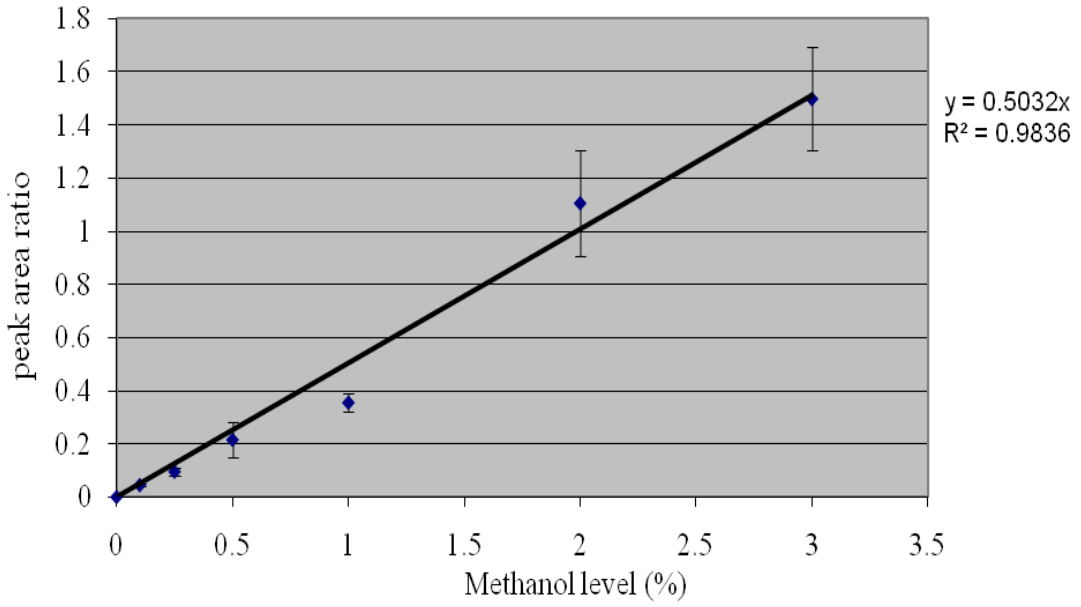

Figure D.1 Standard graph of methanol concentration analyzed by gas chromatography.

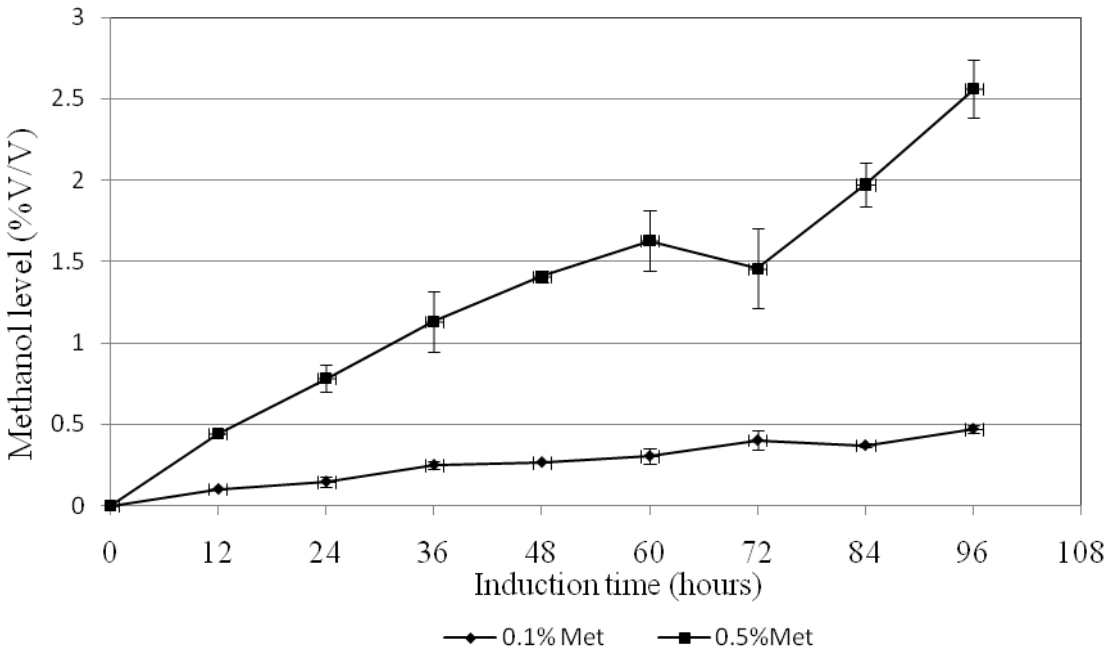

Figure D.2 Methanol level during 96 h methanol induction in shaken flask cultivation with BMMY medium at temperature of 30 °C and pH 3.

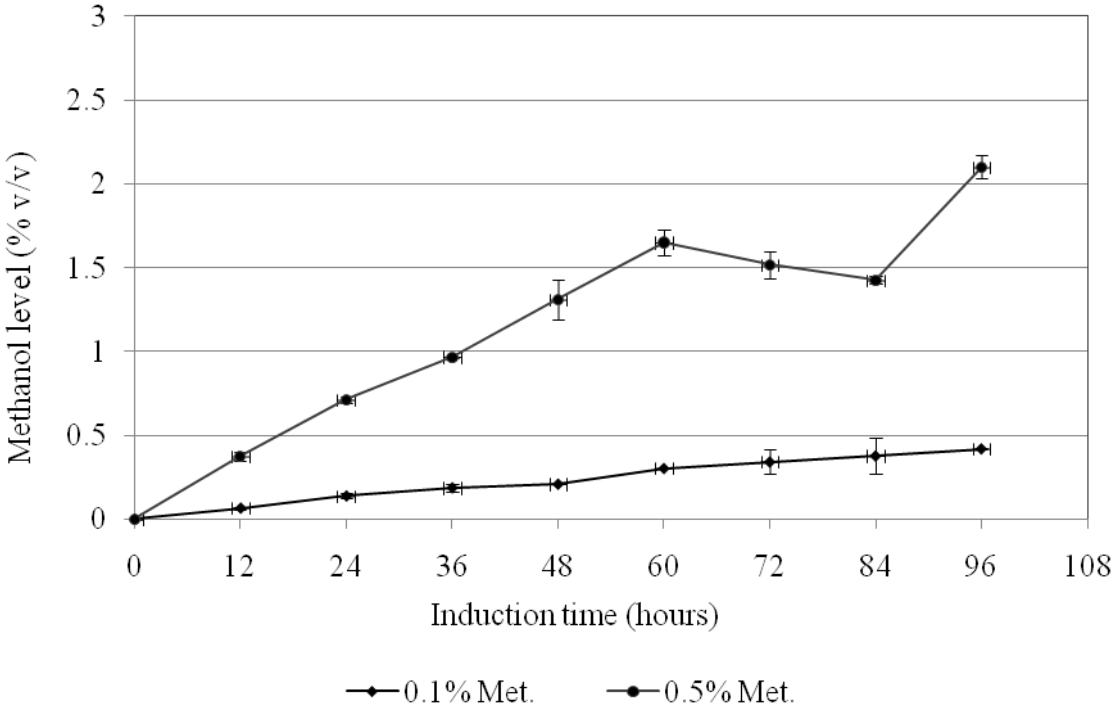

Figure D.3 Methanol level during 96 h methanol induction in shaken flask cultivation with BMMY medium at temperature of 30 °C and pH 4.

Appendix E

ImageJ Analysis

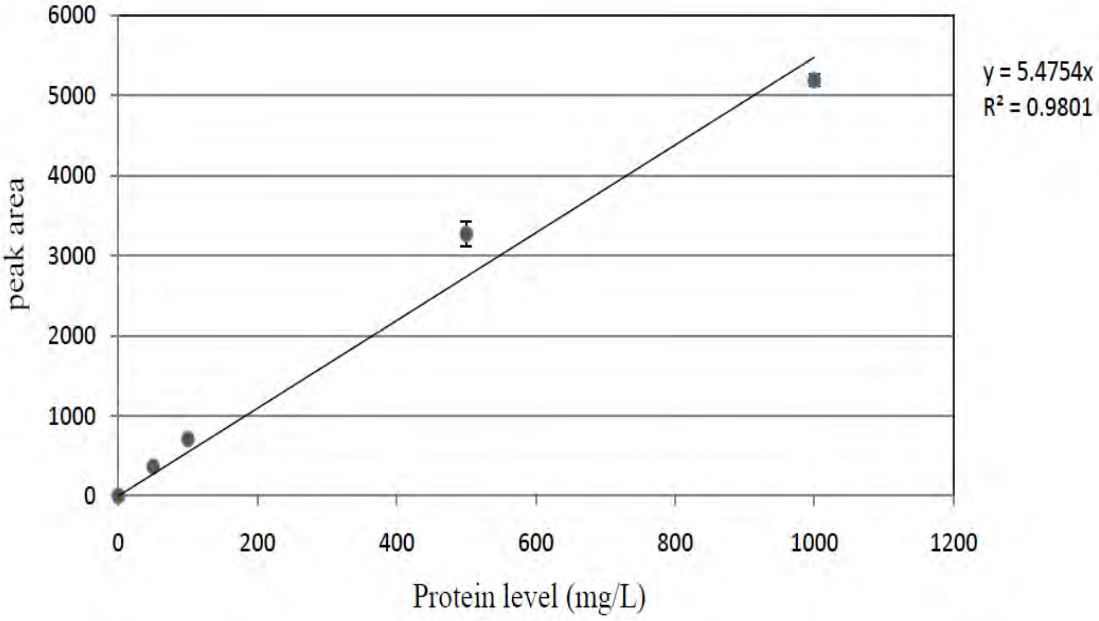

Figure E.1 Concentration of standard protein analyzed by ImageJ analysis.

Table E.1 The concentration of IL-2/FU-MK-1-scFv analyzed by ImageJ software after 96 h methanol induction at 30 °C with difference conditions in shake flaks and fed-batch cultivation in 5 L fermenter.

Scale	**Conditions**	**IL-2/FU-MK-1-scFv (mg/L±SD)**
250 ml Flask	pH 3, 0.1% met.	258±13
	pH 3, 0.5% met.	125±9
	pH 4, 0.1% met.	105±16
	pH 4, 0.5% met.	105±9
5 L Fermenter	pH 3, 5%G	84±5
	pH 3, 7.5%G	109±5
	pH 3, 10%G	77±4
	pH 3 BMMY	197±19
	pH 4 BMMY	425±2

Appendix F

Cell Lysate ELISA

Preparation of cell lysate protein

CHO MK-1 and CHO cell grown on α-MEM were rinsed with ice-cold PBS (1 L containing of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, adjusted the pH to 7.4 with HCl). The cells were scraped off the plate with PBS on ice and transferred to 15 ml falcon tube. Then, the tube containing of cells was centrifuged at 1,000 rpm for 5 min. After removing supernatant, 50 µl RIPA buffer (10 ml containing of 50 mM tris-HCl pH 7.4, 150 mM NaCl, 10% NP-40, 0.1% sodium deoxycholate, 20%SDS, and 1 tablet of protease inhibitor) was added into the cell pellet and mixed together for 5 to 10 second. The protein was determined by bicinchoninic acid (BCA) protein assay. The 10 µl sample was mixed with 200 µl solution assay (buffer A 50: buffer B 1) and incubated at 37 °C for 30 min. The reaction was measured by spectrophotometer with OD_{450}. The concentration of fusion protein was calculated by comparison to the protein standard shown in figure E.1.

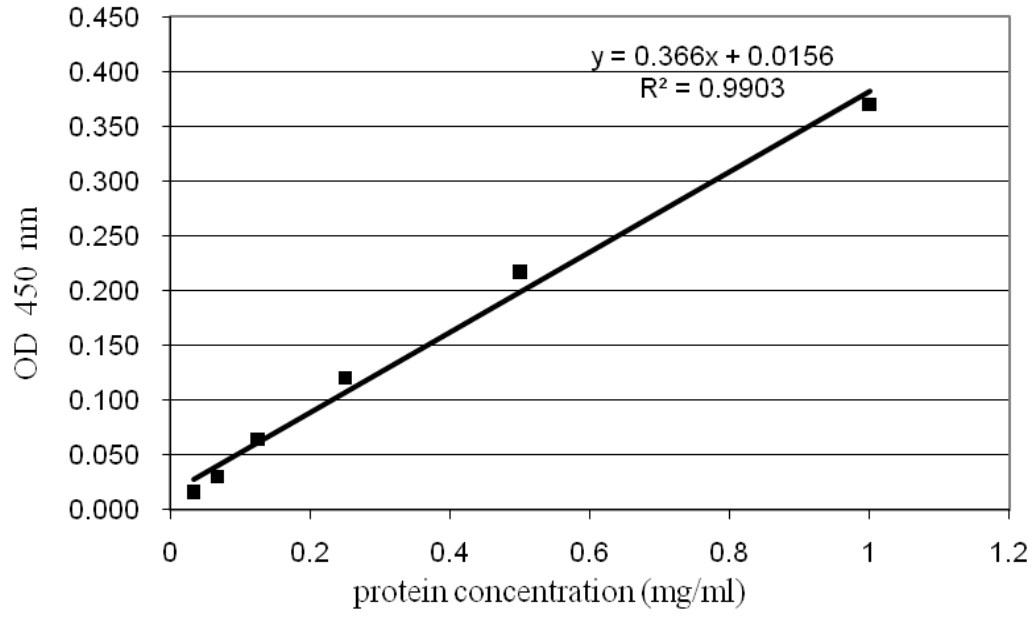

Figure F.1 Concentration of standard protein (BSA) analyzing by BCA protein assay.

Cell lysate ELISA

20 µg cell lysate proteins of CHO-MK-1 and CHO cell in 100 µl coating buffer (eBioscience, USA) were added in triplicate to wells of Coning Costar 9018 ELISA 96 well plate and incubated overnight at 4 °C. After washing 3 times with PBS containing 0.05% tween 20, 4 µg of fusion protein IL-2/FU-MK-1-scFv in 100 µl assay diluents (eBioscience, USA) was added in each well and incubated at room temperature for 2 h. Washing 3 times with PBS containing 0.05% tween 20 then biotin-conjugated anti-human IL-2 (eBioscience, USA) diluted 1:200 was added in assay diluent. The plate was incubated at room temperature for 1 h and washed 3 times with PBS containing 0.05% tween 20. Next, Avidin-HRP (eBioscience, USA) diluted 1:200 in assay diluents was added and incubated at room temperature for 30 min and washed 3 times with PBS containing 0.05% tween 20. Then, 100 µl TMB solution (eBioscience, USA) was added in each well and incubated at room temperature for 15 min. Finally, 50 µl of 2 N H_2SO_4 was added to stop reaction and the plate was read at 450 nm. The OD_{450} was compared using Student's *t* test.

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