จุฬาลงกรณ์มหาวิทยาลัย
ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

การแสดงออกของโปรตีนเชื่อมชนิดใหม่ IL2/FU-MK-1-scFv โดยใช้จุลินทรีย์เป็นเซลล์เจ้าบ้านและทดสอบแอคติวิตีการต้านเนื้องอกของโปรตีนเชื่อมเพื่อใช้เป็นสารอิมมูโนบำบัดแบบไซโตทอกซิกต่อเนื้องอกที่มีการแสดงออกของ FU-MK-1

โดย

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Assistant Professor Suchada Chanprateep Napathorn, Ph.D.

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ชื่อโครงการวิจัย	การแสดงออกของโปรตีนเชื่อมชนิดใหม่ IL2/FU-MK-1-scFv โดยใช้จุลินทรีย์เป็นเซลล์เจ้าบ้านและทดสอบแอคติวิตีการต้านเนื้องอกของโปรตีนเชื่อมเพื่อใช้เป็นสารอิมมูโนบำบัดแบบไซโตทอกซิกต่อเนื้องอกที่มีการแสดงออกของ FU-MK-1
ชื่อผู้วิจัยหลัก	ผู้ช่วยศาสตราจารย์ ดร.สุชาดา จันทร์ประทีป นภาธร
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บทคัดย่อ

ปัจจุบัน MK-1 ซึ่งเป็นโมเลกุลเป้าหมายของ FU-MK-1 และประมวลรหัสโดยยีน GA733-2 กำลังถูกใช้เป็นโมเลกุลเป้าหมายในการทดสอบทางคลินิกด้านการรักษาโรคมะเร็ง เช่น มะเร็งกระเพาะอาหาร มะเร็งลำไส้ และมะเร็งถุงน้ำดี โดยการใช้มอนอคลอนอลแอนติบอดี งานวิจัยนี้สร้างโปรตีนเชื่อมสองรูปแบบคือ IL2/FUscFv(V_K-V_H) และ IL2/FUscFv(V_H-V_K) และแสดงออกในแบคทีเรีย *Escherichia coli* สายพันธุ์ BL21(DE3)pLysS และสายพันธุ์ Rosetta-gami B เปรียบเทียบกับการแสดงออกในยีสต์ *Pichia pastoris* สายพันธุ์ GS115 และสายพันธุ์ KM71H โดยแปรผันปัจจัยที่สำคัญได้แก่ pH อุณหภูมิ ความเข้มข้นของสารเหนี่ยวนำ (IPTG หรือ เมทานอล) และระยะเวลาการเหนี่ยวนำ พบว่า *E. coli* แสดงออกโปรตีนเชื่อมและสะสมไว้ภายในเซลล์ โดย *E. coli* สายพันธุ์ Rosetta-gami B มีการแสดงออกและสะสมโปรตีนเชื่อมดีกว่า *E. coli* สายพันธุ์ BL21(DE3)pLysS เพราะได้โปรตีนเชื่อมที่มีเปอร์เซ็นต์การละลายน้ำสูง ผลผลิตในระดับขวดเขย่าสูงสุด IL2/FUscFv(V_H-V_K) เท่ากับ 0.25 กรัมต่อลิตร มีเปอร์เซ็นต์การละลายน้ำร้อยละ 89.29 และ IL2/FUscFv(V_K-V_H) 0.26 กรัมต่อลิตร มีเปอร์เซ็นต์การละลายน้ำร้อยละ 84.61 เมื่อเลี้ยงที่อุณหภูมิ 25°ซ pH 7 และเหนี่ยวนำให้โปรตีนแสดงออกด้วย IPTG 0.05 mM นาน 10 ชั่วโมง เมื่อเปรียบเทียบเจ้าบ้านยูคาริโอต *P. pastoris* พบว่าการผลิตโปรตีนเชื่อมใน *P. pastoris* เป็นแบบหลั่งออกนอกเซลล์ ผลผลิตในระดับขวดเขย่าได้สูงสุด 0.258 ± 0.013 กรัมต่อลิตร เมื่อเลี้ยงที่อุณหภูมิ 30°ซ pH 3 และ เหนี่ยวนำการแสดงออกด้วยเมทานอล 0.1% (v/v) นาน 96 ชั่วโมง การผลิตในระดับถังหมักขนาด 5 ลิตรแบบเฟดแบชได้โปรตีนเชื่อมแบบหลั่งออกนอกเซลล์สูงสุด 0.425 ± 0.02 กรัมต่อลิตร เมื่อทำบริสุทธิ์ด้วยโครมาโทกราฟีสัมพรรคภาพโลหะตรึง และทดสอบสมบัติการจับอย่างจำเพาะต่อเซลล์รังไข่ไชนีสแฮมส์เตอร์ (CHO) ที่มีการแสดงออก FU-MK-1 เปรียบเทียบกับเซลล์ CHO ที่ไม่มีการแสดงออก FU- MK-1 ผลการทดสอบทางสถิติด้วย Student's *t* test ($p<0.05$) โปรตีนเชื่อมมีสมบัติการจับอย่างจำเพาะอย่างมีนัยสำคัญ

Project Title	Expression of novel fusion proteins IL2/FU-MK-1-scFv in microorganism-host cells and its potential anti-tumor activities as a cytotoxic immunotherapy agent for FU-MK-1 expressing tumors
Investigator	Assistant Professor Suchada Chanprateep Napathorn, Ph.D.
Co-investigator	Associate Professor Tanapat Palaga, Ph.D.
Year	June, 2012

Abstract

MK-1, the target molecule of FU-MK-1, is encoded by the GA733-2 gene, which is currently being used as a target in clinical trials for gastric, intestinal, and biliary cancer treatment with monoclonal antibodies. Here, two different arrangement of heavy-chain and κ light-chain variable fusion gene, IL2/FUscFv($V_κ$-V_H) or IL2/FUscFv(V_H-$V_κ$), were constructed. The efficiency of protein expression in prokaryotic host expression system, *Escherichia coli* strains BL21(DE3)pLysS and Rosetta-gami B was compared with eukaryotic host expression system, *Pichia pastoris* strains GS115 and KM71H, for their ability to produce fusion protein. It was found that the fusion proteins were expressed in *E. coli* strain Rosetta-gami B in reasonable yield with high percentage of soluble form (0.25 g/l of IL2/FUscFv(V_H- $V_κ$) with solubility 89.29% and 0.26 g/l of IL2/FUscFv($V_κ$-V_H) with solubility 84.61%) when cultivated at 25°C, pH 7, and induced with 0.05 mM IPTG for 10 h. In comparison, *P. pastoris* produced the secreted fusion proteins. The highest production of the fusion protein at 0.258 ± 0.013 g/l was obtained under pH 3, 30°C, and induction with 0.1% (v/v) methanol for 96 h in shaken flask cultivation. Finally, fed-batch cultivation was performed in 5 l fermenter. The highest amount of secreted fusion protein was 0.425 ± 0.002 g/l. Following purification, the fusion protein was examined the specific binding activity to CHO cell expressing MK-1. 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 ($p<0.05$).

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2.1 Introduction

MK-1 antigen is widely expressed on a variety of tumor tissues such as stomach, colon, pancreas, gall bladder, bile duct, breast and lung carcinomas (Watanabe et al., 1993). Based on molecular characterization, it was confirmed that the MK-1 is a transmembrane glycoprotein with a molecular mass of 40 kDa and encoded by the GA733 gene (Tomita et al, 2000; Szala et al., 1990). The GA733-2 antigen is also known as the 17-1A antigen (Herlyn et al., 1979), the KS1/4 antigen (Varki et al., 1984), or the C215 antigen (Larson et al., 1988). Recently, these antigens have been used as targets for immunotherapy using MAb in several clinical trials and have shown promise as tumor targets in passive and active immunotherapy of colorectal cancer (LoBuglio et al., 1989; Trang et al., 1990; Yamamoto et al., 1999; Herlyn et al., 1991). However, the administration of mouse MAbs in humans for therapeutic purposes has been limited by a short half-life and induces an immunogeicity (anti-mouse immunoglobulin response) (Lobuglio et al., 1989; Schroff et a., 1985). Screening an effective type of the antibody molecule is one of important tasks for better tissue penetration and lower immunogenicity. Among them, single chain variable fragment (scFv) antibody has been received the most interest for its applications instead of their parental MAbs (Boklti et al, 1995; Malani et al., 1998; Liao et al., 2001; ; Matsumoto et al., 2002; Ueno et al., 2002; Holliger and Hudson, 2005; Carter, 2006; Weisser, and Hall, 2009).

Mab FU-MK-1 has been obtained by immunization of BALB/c mice with cancerous ascites derived from a poorly differentiated adenocarcinoma 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 (IL2), a pleiotropic glycoprotein in immune system, has been accepted for use in cancer immunotherapy of human. The interaction of IL2 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 IL2 can cause the severe toxicity in patients and suboptimal concentration at the tumor site have limited its efficiency in *vivo*. The selective delivery of IL2 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_K) 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/IL2 fusion protein that retains both scFv and IL2 associated function. So scFv/IL2 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). Matsumoto et al. have expressed the fusion protein FU-MK-1-scFv/IL2 in *P. pastoris* in flask cultivation and reported the amount of the scFv antibody approximately 0.002 g/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 (Matsumoto et al., 2002).

In this study, we reported the optimization in flask cultivation for high expression of IL2/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°C and methanol concentration of 0.01, 0.1, and 0.5% (v/v). Next, the optimal condition obtained from flask cultivation was applied in the fed-batch cultivation in 5 l fermenter. The effect of cell density was examined in detail where glycerol concentration as a carbon source in growth phase was varied from 5, 7.5, and 10% (w/v). Finally, the fusion protein was purified by Ni-affinity chromatography and tested the specific binding antigen against the MK-1 expressing CHO cell.

2.2 Survey of Related Literature

2.2.1 Cancer Immunotherapy

Cancer is a group of uncontrolled cells growth, 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 1).

Table 1 Examples of cancer drug based MAbs approved by FDA (Kim et al., 2005, Wainberg and Hecht, 2006; http://www.cancer.gov, accessed on 10 May 2011)

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

Table 1 Examples of cancer drug based MAbs approved by FDA (Kim et al., 2005, Wainberg and Hecht, 2006; http://www.cancer.gov, accessed on 10 May 2011) cont.

Drugs	Disease indications	Year	Companies
Bexxar (Tositumomab)	Non-Hodgins lymphoma	2003	Corex/GSK
Erbitux (Cetuximab)	Colorectal cancer	2004	Imclone
Avastin (Bevacizumab)	CRC, breast, renal, NSCL cancer	2004	Genentech
Vectibix (Panitumumab)	Colorectal cancer	2006	Amgen
Arzerra (Ofatumumab)	chronic lymphocytic leukemia	2009	GlaxoSmithKline

Table 2 Top Ten Monoclonal Antibodies from 2006 - 2009 (Maggon, 2010)

Generic name Target	Brand ® FDA Approval	Companies	Indication	Sales $ billion 2007 2008 2009
Infliximabc TNFα	Remicade 1998	J&J, Merck	CD, UC, AS RA, Ps, PsA	5.04 6.5 6.91
Bevacizumab VEGF hz	Avastin 2004	Roche	Colon mCRC,Lung NSCLC Breast mBC, mRCC Glioblastoma	3.93 4.7 5.92
Rituximab c CD20	Rituxan 1997	Roche	Leukemia, CLL Lymphoma, RA	5.01 5.6 5.8

Table 2 Top Ten Monoclonal Antibodies from 2006 - 2009 (Maggon, 2010) cont.

Generic name Target	Brand ® FDA Approval	Companies	Indication	Sales $ billion 2007	 2008	 2009
Adalimumab h TNFα	Humira 2002	Abbott	RA, JIA, PsA, Ps, AS, CD	3.06	4.4	5.48
Trastuzumab hz HER2	Herceptin 1998	Roche	Breast Cancer	4.4	4.8	5.02
Cetuximab c EGFR	Erbitux 2004	Bristol Myers Squibb Merck KgA	Colon Cancer HNC	1.35	2.0	2.57
Ranibizumab hz VEGF	Lucentis 2006	Novartis, Roche	Macular Degeneration	1.2	1.5	2.43
Palivizumab hz RSV	Synagis 1998	Astra Zeneca	RSV	1.3	1.2	1.1
Tocilizumab	Actemra EMA 2009 FDA 2010	Roche	RA			0.14
Certolizumab TNFα	Cimzia 2008	UCB	CD, RA		0.10	0.10
Golimumab	Simponi 2009	J&J	RA, PsA, AS			
Canakinumab	Ilaris 2009	Novartis	Periodic Syndrome			
Ofatumumab	Arzerra 2009	GSK	CLL			0.005
Ustenkinumab	Stelara 2008	J&J	Psoriasis			NA

Remark: IMS Health figures for 2009 sales were Remicade $4.4 billion, Avastin $4.3 billion and Rituxan $4 billion were significantly different and undervalued. IMS data underestimates the mAbs sales.

Note: AS, ankylosing spondylitis; CD, Crohn's disease; Ps, psoriasis; PsA, psoriatic arthritis; RA, rheumatoid arthritis; UC, ulcerative colitis; m, metastases; RCC, renal cell carcinoma; NSCLC, Non small cell lung cancer; BC, breast cancer; CRC, Colorectal cancer; CLL, Chronic Lymphocytic Lymphoma; HNC, Head and Neck cancer; RSV, Respiratory syncytial virus

Table 3 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-F(ab')2)	CEA-bearing tumors	100
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

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 3). 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).

2.2.2 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 (Maynard and Georgiou, 2000). 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 (Weisser and Hall, 2009).

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 expression systems to produce in large amount (Holliger and Hudson, 2005).

Table 4 Selected examples of genetic engineered scFv antibodies

scFv antibodies	Specific antigen	References
Anti-CEA scFv	Carcinoma embryonic antigen (CEA)	Yi et al.,1999
scFv59D8	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., 2003

Anti-TfR scFv	transferin receptor	Yang et al., 2005
A33scFv	a cell surface glycoprotein expressed in colon cancer	Damasceno et al., 2004
scFv of domoic acid	domoic acid	Hua et al., 2005
Anti-CD33 scFv	CD33, a surface glycoprotein expressed on myelomonocytic lineage, leukemia cell	Emberson et al., 2005
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
IL2-183BscFv	An ovarian carcinoma-associated antigen	Zhang 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 4, 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, Deckert et al., 2003), radio-isotopes (^{124}I-anti-CD20 scFv dimmers, Olafsen et al., 2010) and cytokines (IL2, Zhang et al., 2006; Masumoto et al., 2002). IL2 conjugation is one choice for effector function improvement of the scFv because of its characterization.

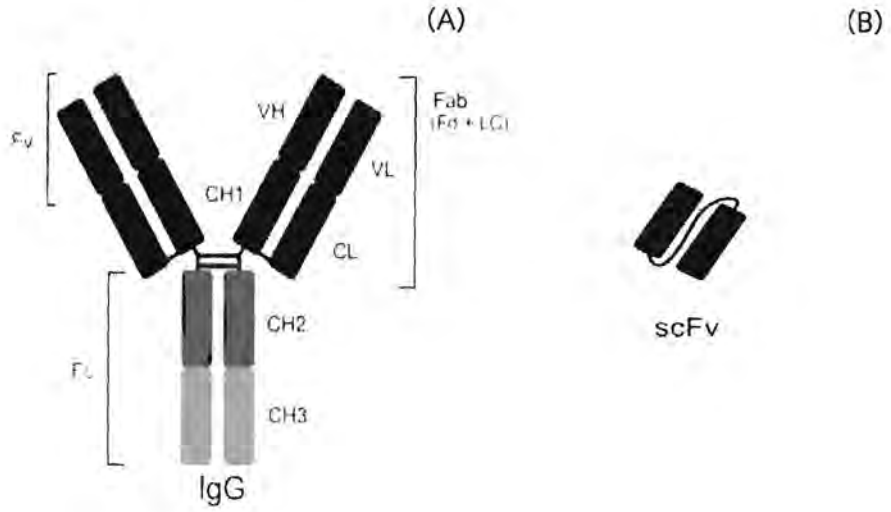


Figure 1 Simple structure of (A) full length antibody (IgG) and (B) scFv antibody (Kim et al., 2005).

2.2.3 MK-1 antigen and MK-1 antibodies

In 1999, 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 an 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., 1999).

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 IL2 fusion with scFv of anti-MK-1 antibody protein. The fusion protein, designed FUscFv/IL2, was expressed in *P. pastoris*. The secreted fusion protein was purified by Ni-affinity chromatography and characterized for binding specificity and biological activity of IL2. 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 IL2 to tumor cell, enhancing the immune response while significantly reducing systemic side-effect (Matsumoto et al., 2002).

2.2.4 Recombinant antibody scFv/IL2 fusion proteins

In 1994, Sabzevari et al. genetically engineered fusion protein containing a human/mouse chimeric anti-ganglioside GD-2 antibody and recombinant

human IL2. This recombinant fusion protein was tested for its efficacy to target IL2 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-IL2 fusion protein specifically directs IL2 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 IL2 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/IL2 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/IL2 fusion protein may be used for *in vivo* administration to deliver IL2 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-IL2 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 IL2 at tumor sites, leading to strongly enhance the therapeutic efficacy of IL2. In mice treated with L19-IL2 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 IL2. The IL2-183B2scFv was expressed in CHO cells and tested its specific binding and biological function. They found that the IL2-183B2scFv

retained the functions of both IL2 and antibody. It can be effectively used for ovarian cancer immunotherapy by carrying a high concentration of IL2 to OC183B2-expressing ovarian cancer cells (Zhang et al., 2006).

In 2006, Shen et al. constructed and expressed the recombinant H520C9scFv/IL2 fusion protein in mammalian cell and tested the antigen binding activity and cytotoxicity of the H520C9scFv/IL2 *in vitro* testing. The fusion protein retained both IL2 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 H520C9scFv/IL2 fusion protein may offer effective way of targeting therapeutic doses of IL2 to tumors or other targeted cells with significantly decreased cytotoxicity of IL2 and its immunogenicity (Shen et al., 2006).

2.2.5 Selected reports regarding the production of scFv by P. pastoris in fermenter

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 0.040 g/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 2005, 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 0.420—0.458 g/l and had specific binding activity to hepatitis B surface antigen (Ning et al., 2005).

In 2007, Yamawaki et al. produced scFv against bisphenol A in 2 l fed-batch fermentation process using *P. pastoris*. The scFv concentration of 0.198 g/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 A: Production, purification, and characterization of fusion proteins IL2/FU-MK-1-scFv expressed in *Escherichia coli*

A.1 Materials and methods

A.1.1 The fusion gene

The fusion genes encoding for anti-FUscFv(V_K-V_H)/IL2 were obtained from Professor Kuroki Masahide (School of Medicine, Fukuoka University, Japan).

A.1.2 Strains of E. coli

The *E. coli* strains JM109 and DH5α (New England Biolabs Inc., MA, USA) was used for subcloning, sequencing, and plasmid amplification in LB medium (10 g/l bactotryptone, 10 g/l NaCl, and 5 g/l yeast extract) containing 50 μg/ml ampicillin.

The *E. coli* strains BL21(DE3)pLysS and Rosetta-gami B (Novagen®, Merck KGaA, Darmstadt, Germany) were used as expression hosts. All medium used for cultivation or expression were supplemented with 50 μg/ml carbinicillin and 34 μg/ml chloramphenicol.

A.1.3 Construction of expression vectors

The fusion genes coding for IL2/FUscFv(V_K-V_H) and IL2/FU scFv(V_H-V_K) were amplified by PCR and cloned into pET15b (Novagen®, Merck KGaA, Darmstadt, Germany) that digested with *Xho*I and *Bam*HI (New England Biolabs Inc., MA, USA). The resulting recombinant plasmids pET15b-IL2/FUscFv(V_K-V_H) and pET15b-IL2/FUscFv(V_H-V_K) contained the coding sequences for the His•tags followed by thrombin cleavage site at the N-terminus. The recombinant plasmids pET15b-IL2/FUscFv(V_K-V_H) and pET15b-IL2/FUscFv(V_H-V_K) were transformed into *E. coli* strain BL21(DE3)pLysS and Rosetta-gami B. All constructs were verified by DNA sequencing (Macrogen Inc., Korea).

A.1.4 Expression of fusion proteins in E. coli in shaken-flask cultivation

E. coli strains BL21(DE3)pLysS and Rosetta-gami B were transformed with each plasmid, pET15b-IL2/FUscFv(V_K-V_H) or pET15b-IL2/FUscFv(V_H-V_K), separately. Single colonies of freshly transformed cells were grown in shake-flask cultures of 10 ml LB medium supplemented with 50 μg/ml carbinicillin and 34 μg/ml chloramphenicol and incubated at 37°C and 200 rpm until OD_{600} reached 0.6. The cell was harvested by centrifugation at 4°C, 10160×g, for 15 min. The cell pellet was inoculated in 50 ml of LB medium containing 50 μg/ml carbinicillin and 34 μg/ml chloramphenicol, induced with appropriated concentration of isopropyl-β-D-thiogalactopyranoside (IPTG). The concentrations of IPTG and induction temperatures were varied according to experimental purposes. After induction, the cells were harvested by centrifugation at 4°C, 7741×g, for 15 min.

Lysis of the cells was carried out by using BugBuster® protein extraction kit (Novagen®, Merck KGaA, Darmstadt, Germany). Briefly, cell pellet corresponding to 1 ml of culture was resuspended in 300 μl lysis buffer, incubated at room temperature for 10 min, and centrifuged at 4°C, 17418 × g, for 20 min. Protease inhibitor cocktail for use in purification of poly(his)-tagged proteins (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the cell suspension. The supernatant was saved as a soluble protein fraction and stored at -70°C until required.

SDS-PAGE analysis was performed according to the method of Laemmli (1970). A mini-Protean was used as instructed by the manufacturer (Bio-Rad Laboratories Ltd., CA, USA). The fusion protein concentration was estimated using densitometry software, ImageJ (http://rsb.info.nih.gov/ij/). An ovalbumin with molecular weight 45 kDa (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a standard protein. Briefly, 0.1 to 1.0 g/l of protein standard and samples were run on 12% SDS-PAGE, stained with 0.1% (w/v) solution of Coomassie stain (Sigma-Aldrich Corp., St. Louis, MO, USA) 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 with the protein standard. 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. Protein molecular-weight markers (Fermentas Inc., MD, USA) were ued as protein standards for determination of molecular mass.

For Western Blots, proteins were transferred from the gel onto a PVDF membrane (Bio-Rad Laboratories Ltd., CA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Ltd., CA, USA). The fusion protein was confirmed by immunoblotting according to WesternBreeze chromogenic detection kit (Invitrogen Corp., CA, USA). Immunoblotting was carried out with a monoclonal rabbit anti-hIL2 antibody and an anti-rabbit antibody conjugated with alkaline phosphatase. The complex was visualized by enzyme reaction with the substrate BCIP/NBT. The His·tag was detected by immunoblotting using a monoclonal anti-polyHistidine, clone HIS-1 conjugated with alkaline phosphatase according to WesternBreeze chromagenic detection kit (Invitrogen Corp., CA, USA).

A.1.5 Purification of fusion proteins by Ni-affinity chromatography

To purify to fusion protein, the supernatant was clarified through a 0.22 μm membrane filter prior to purification steps. Ni-affinity chromatography was performed using His·Bind®Kits 5 ml column (Novagen®, Merck KGaA, Darmstadt, Germany). Two ml of the His·Bind resin was charged with 2 volumes of charging buffer (50 mM $NiSO_4$) and packed under gravity flow in column. Next, the resin was equilibrated with 10 volumes of binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) and drained. Ten ml of supernatant containing His-tagged protein was loaded into column and incubated at 4°C for 3h. The resin bounded with His-tagged protein was washed with 10 ml binding buffer, subsequently with 6 ml washing buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9), to remove non-specifically bounded proteins. The protein was eluted with 6 ml eluting buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and the 2 ml fractions were collected for 20 fractions. The fractions containing the protein (analyzed by

SDS-PAGE) were pooled and concentrated using Vivaspin 500 centrifugal concentrators (30000 MWCO, Vivaproducts, Inc., MA, USA). Finally, the protein was kept in PBS solution for further analysis. Protein concentration was determined using the Bradford Protein Assay (Bio-Rad Laboratories Ltd., CA, USA) with bovine serum albumin as a standard.

A.1.6 Analysis of functional fusion proteins expressed in E.coli

To quantify the functional IL2/FUscFv in the soluble protein fraction, enzyme-linked immunosorbent assay (ELISA) was performed. ELISA plates were coated with IL2 and utilized for the assay. Briefly, 100 μl of soluble fraction was added to the plate and incubated at room temperature for 2 h. After washing with PBS containing 0.05% Tween 20 (pH 7.2), biotin-conjugated anti-human IL2 was added to the plate and incubated at room temperature for 1 h. After washing, bound fusion proteins were detected with TMB (Tetramethylbenzidine). The activity of IL2 was determined by subtracting the A_{405} nm of background binding to ovalbumin from the value obtained against IL2.

A.2 Result

A.2.1 Construction of plasmids

The fusion genes coding for IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K) were successfully obtained through PCR with the accordant sequence verified by the agarose gel electrophoresis and DNA sequencing. The clone vectors pET15b-IL2/FUscFv(V_K-V_H) and pET15b-IL2/FUscFv(V_H-V_K) were constructed successfully so that the target genes were fused after His·tag and thrombin cleavage site under the control of T7 promoter. In this study, our constructs were different from the previous report by Matsumoto et al. because the arrangement of original fusion gene was FUscFv(V_H-V_K)/IL2 and the plasmid contained only fusion genes without His·tag (Matsumoto et al., 2002). In this study, the His·tag and thrombin cleavage site were important in the future steps of detection, separation, and purification. Besides, the thrombin

cleavage site make it possible to cleave the fusion proteins into individual IL2/FUscFv(V_K-V_H) or IL2/FUscFv(V_H-V_K) without His·tag.

A.2.2 Expression of fusion genes and analysis of the productivity

Previously, the molecular weights of the fusion protein FUscFv(V_H-V_K)/IL2 was reported as 43 kDa (Matsumoto et al., 2002). In this study, the 6His·tag was added at the N-terminus, the fusion protein has an added 1 kDa of the molecular weights of protein target as 44 kDa. To enhance the formation of the disulfide bonds of eukaryotic proteins in the cytoplasm of *E. coli*, the effect of redox potential of cytoplasm on the production of soluble form of fusion proteins, IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K) was investigated. The fusion proteins were expressed and compared between the reducing cytoplasm of *E. coli* strain BL21(DE3)pLysS and the oxidizing cytoplasm of *trxB/gor* mutant, *E. coli* strain Rosetta-gami B. Concurrently, it is well-known that temperature is one of important parameters in recombinant protein expression in *E. coli*. Thus, the effect of incubation temperature was also investigated under incubation temperature at 25, 30, or 37°C for both strains. In this study, the fusion protein expression was induced with 0.5 mM IPTG and cultivated for 12 h. The results showed that the fusion protein was expressed in both soluble and insoluble forms (Figures 2A and 2B) with the quantitative results shown in Table 5.

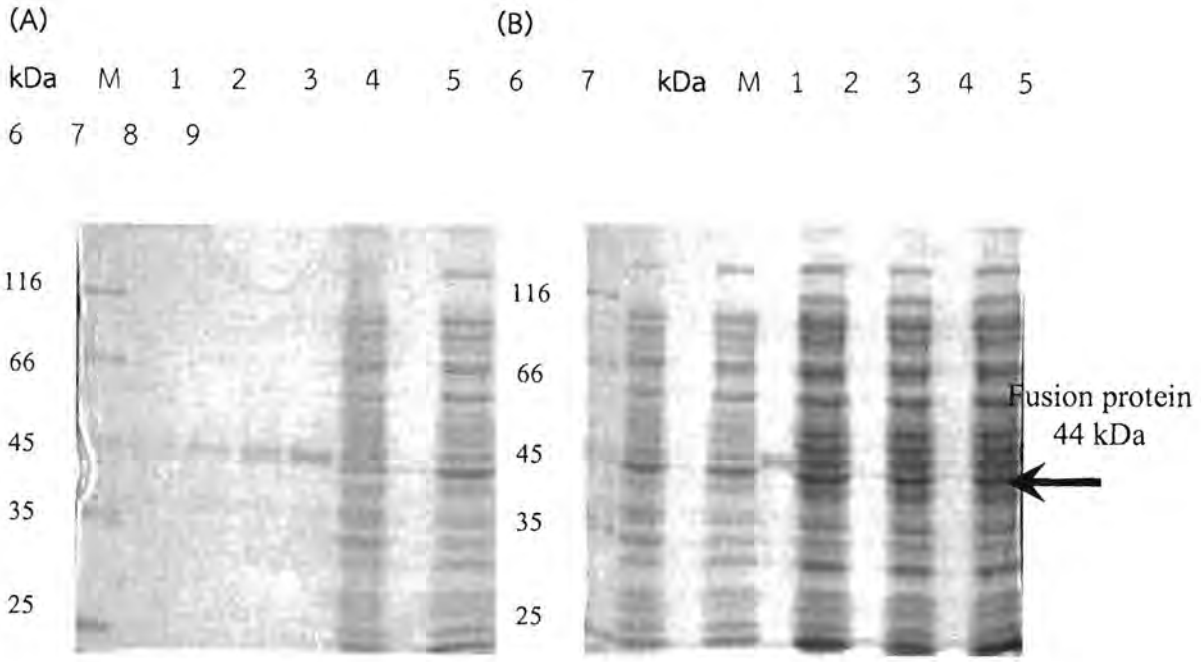


Figure 2 Expression of soluble and total protein in *E. coli* strain BL21(DE3)pLysS/pET15b-IL2/FUscFv(V_H-V_K) under temperature at 25, 30, and 37°C. (A); Lane M is a protein marker. Lanes 1, 2, 3, and 4 are the ovalbumin standard (45 kDa) varying concentration at 0.025, 0.050, 0.100, and 0.200 g/l. Lane 5 is the soluble protein of *E. coli* strain BL21(DE3)pLysS/pET15b (Control). Lane 7 is the soluble protein from *E. coli* strain BL21(DE3)pLysS/pET15b-IL2/FUscFv(V_H-V_K) induced under 25°C. (B); Lane M is a protein marker. Lanes 1 and 3 are the soluble protein from *E. coli* strain BL21(DE3)pLysS/pET15b(V_H-V_K) induced under 30 and 37°C, respectively. Lanes 5, 7, 9 are the total protein from *E. coli* strain BL21(DE3)pLysS/pET15b(V_H-V_K) induced under 25, 30, and 37°C, respectively. Lane 4 is the ovalbumin standard (45 kDa) at concentration of 0.200 g/l.

It was observed that the production of fusion proteins in *E. coli* strain BL21(DE3)pLysS yield approximately two times higher than that was expressed in *E. coli* strain Rosetta-gami B. However, *E. coli* strain BL21(DE3)pLysS produced a relatively high amount of insoluble form. The *E. coli* strain Rosetta-gami-B/pET15b-IL2/FUscFv(V_K-V_H) and Rosetta-gami-

B/pET15b-IL2/FUscFv(V_H-V_K) achieved the highest soluble form at approximately 77% of the total protein under the temperature at 25°C.

To avoid the complex and low-efficiency refolding steps in the down-stream purification procedure, *E. coli* strain Rosetta-gami-B/pET15b-IL2/FUscFv(V_K-V_H) and Rosetta-gami-B/pET15b-IL2/FUscFv(V_H-V_K) were chosen to optimize their culture conditions for high-level expression of soluble fusion proteins, IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K), in shaken flask cultivation.

Table 5 The production of fusion proteins, IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K), expressed in *E.co* 5, 30 and 37°C. The protein expression was induced with 0.5 mM IPTG for 12 h

	Fusion proteins	Total protein (g/l)	% of soluble fusi tein in total p	Cell mass (g/l)
Temperature 25°C				
BL21(DE3)pLysS	IL2/FUscFv(V_K-V_H)	0.24	48	1.66
BL21(DE3)pLysS	IL2/FUscFv(V_H-V_K)	0.25	50	2.02
Rosetta-gami-B	IL2/FUscFv(V_K-V_H)	0.11	74	0.57
Rosetta-gami-B	IL2/FUscFv(V_H-V_K)	0.13	77	0.91
Temperature 30°C				
BL21(DE3)pLysS	IL2/FUscFv(V_K-V_H)	0.37	33	1.72
BL21(DE3)pLysS	IL2/FUscFv(V_H-V_K)	0.39	38	2.11

Rosetta-gami-B	IL2/FUscFv(V_K-V_H)	0.18	59	1.85
Rosetta-gami-B	IL2/FUscFv(V_H-V_K)	0.19	60	2.26
Temperaute 37°C				
BL21(DE3)pLysS	IL2/FUscFv(V_K-V_H)	0.24	23	2.94
BL21(DE3)pLysS	IL2/FUscFv(V_H-V_K)	0.26	27	3.94
Rosetta-gami-B	IL2/FUscFv(V_K-V_H)	0.20	47	2.88
Rosetta-gami-B	IL2/FUscFv(V_H-V_K)	0.23	55	3.82

A.2.3 Optimization of expression conditions

A.2.3.1 Effects of cultivation temperature

In Table 6, our results suggested that the effect of temperature on the growth of *E. coli* and expression of fusion protein was obvious. The cell mass and percentage of soluble fusion protein were varied under different temperature. The highest cell mass was obtained at 37°C whereas the highest concentration and percentage of soluble fusion protein were achieved at 25°C. Thus, the optimal temperature for growth of *E. coli* before IPTG induction was set at 37°C whereas the optimal temperature for expression of fusion protein was 25°C. Interestingly, it was observed that the cell mass of both *E. coli* strain BL21(DE3)pLysS and Rosetta-gami-B harboring pET15b-IL2/FUscFv(V_K-V_H) was lower cell mass than *E. coli* strain BL21(DE3)pLysS and Rosetta-gami-B harboring pET15b-IL2/FUscFv(V_H-V_K). However, the difference in expression level between IL2/ FUscFv(V_K-V_H) and IL2/ FUscFv(V_H-V_K) fusion proteins was almost the same where the difference in percentage of soluble fusion protein was not obvious. Thus, our results indicated that different arrangement of heavy-chain and κ light-chain variable gene of FUscFv, IL2/FUscFv(V_K-V_H) or IL2/FUscFv(V_H-V_K), may resulted in different level of toxicity on cell growth.

A.2.3.2 Effect of induction conditions

For the plasmid pET15, the fusion protein expression was triggered by adding IPTG into the culture medium. Final IPTG concentration should be optimized because it has a great influence on recombinant protein expression and potential harm to cell growth. In this study, the effect of IPTG concentration was examined between 0.5 and 1.0 mM and the results were shown in Table 6. The concentration of fusion protein and the percentage of the soluble fusion protein in the total soluble proteins reached the highest level (0.25 g/l with solubility 89.29% of IL2/FUscFv(V_H-V_K and 0.26 g/l with solubility 84.61% of IL2/FUscFv(V_K-V_H)) when induced by 0.5 mM IPTG.

Therefore, the sequent studies were all carried out using 0.5 mM IPTG concentration for induction.

Table 6 Effects of temperature, induced concentration and duration on the soluble protein producti

	Post-inc				n time (h)		
	0	2	4	6	8	10	12
IL2/FUscFv(V_K-V_H)							
IPTG concentration (0.5 mM)							
Total soluble protein (g/l)	—	—	0.13	0.1	0.22	0.26	0.31
% of soluble fusion proteins	—	—	84.62	78	81.81	84.61	64.52
Soluble fusion proteins (g/l)	—	—	0.10	0.1	0.18	0.22	0.20
IPTG concentration (1.0 mM)							
Total soluble protein (g/l)	—	0.03	0.15	0.2	0.23	0.22	0.21
% of soluble fusion proteins	—	—	66.67	71	73.91	77.27	80.95
Soluble fusion proteins (g/l)	—	—	0.10	0.1	0.17	0.17	0.17
IL2/FUscFv(V_H-V_K)							

IPTG concentration (0.5 mM)							
Total soluble protein (g/l)	—	—	0.14	0.2	0.23	0.28	0.34
% of soluble fusion proteins	—	—	78.57	80.	82.61	89.29	73.53
Soluble fusion proteins (g/l)	—	—	0.11	0.1	0.19	0.25	0.25
IPTG concentration (1.0 mM)							
Total soluble protein (g/l)	—	0.09	0.24	0.2	0.29	0.30	0.31
% of soluble fusion proteins	—	—	74.17	74.	75.86	80.00	80.65
Soluble fusion proteins (g/l)	—	—	0.17	0.2	0.22	0.24	0.25

After the addition of IPTG into the medium, the fusion protein begins to synthesize. However, the concentration of fusion protein is not proportional to the expression time. Thus, the optimal expression time was determined by analyzing samples every 2 h up to 12 h after induction. The results were analyzed and are shown in Table 6. The fusion protein IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K) reached the highest total fusion protein after about 10 h of IPTG induction.

A.2.4 Production and purification of fusion proteins

Production of fusion proteins by *E.coli* strains Rosetta-gami B was performed at 25°C and induction with 0.5 mM IPTG for 10 h. The fusion proteins were purified by Ni-chelating affinity chromatography. Non-sepcifically bound proteins were removed by rinsing with washing buffers containing 5 mM imidazole. The protein was eluted with buffer containing 0.5–600 mM imidazole. The elution fractions were collected and the functional fusion protein in each fraction was quantified by human IL2 ELISA kit. The results are shown in Figure 3. It was found that the fractions eluted with 50 mM imidazole was optimum for elution the fusion proteins from HisBine resin column.

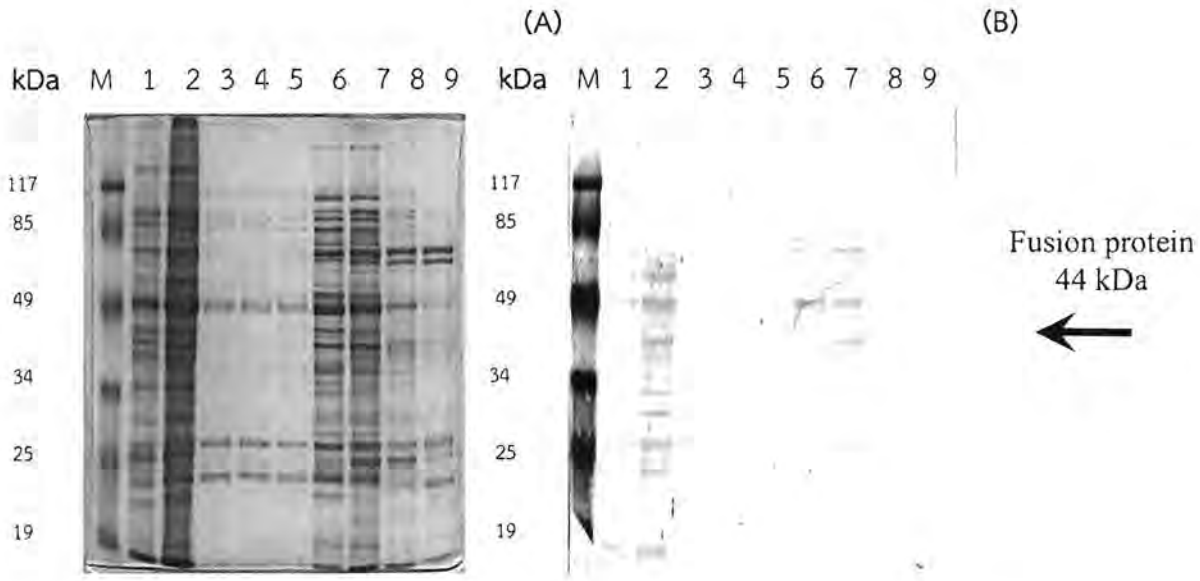


Figure 3 Analysis of the purified fusion protein IL2/FUscFv(V_H-V_K) produced by *E. coli* strain Rosetta-gami B at 25°C and induction with 0.5 mM IPTG for 10 h. (A); the SDS-PAGE analysis stained by Coomassie Brilliant Blue. (B); The western blot analysis. The purified fusion proteins separated on SDS-PAGE was electroblotted onto the PVDF membrane and Immunoblotting was carried out with a monoclonal rabbit anti-human IL2 antibody (1:10,000) and an anti-rabbit antibody conjugated with alkaline phosphate. Lane M, protein marker; Lane 1, crude soluble fraction of the control (*E. coli* strain Rosetta-gami B/pET15b); Lane 2, crude soluble protein from *E. coli* strain Rosetta-gami B/pET15b-IL2/FUscFv(V_H-V_K); Lanes 3, 4, 5, 6, 7, 8, and 9 are eluted fractions with buffer containing 5, 10, 20, 40, 50, 100, and 600 mM Imidazole, respectively.

The fusion proteins thus purified revealed a single band of 44 kDa on immunoblotting using rabbit anti-hIL2 antibody specific binding to IL2 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 reacted with alkaline phosphatase (Figures 4A and 4B).

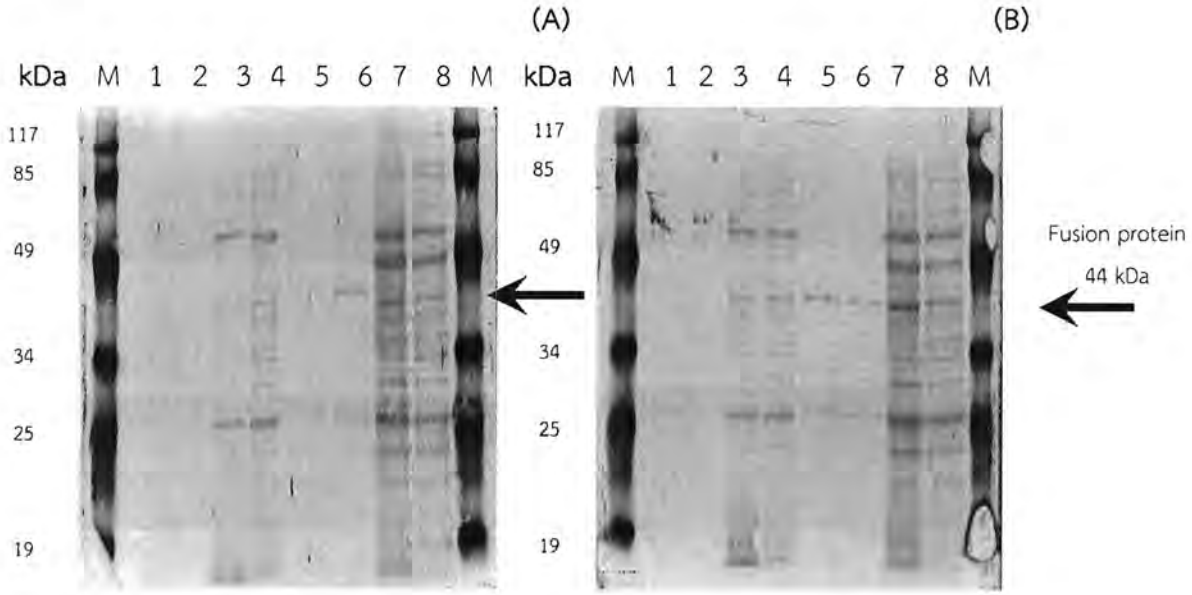


Figure 4 The western blot analysis (1:10,000 anti-human IL2 antibody) of purified fusion protein IL2/FUscFv(V_H-V_K) and IL2/FUscFv(V_K-V_H) produced by *E. coli* strain Rosetta-gami B at 25°C and induction with 0.5 mM IPTG for 10 h. (A) Lane M, protein marker; Lanes 1 and 2 are crude soluble protein of control (*E. coli* strain Rosetta-gami B/pET15b). Lanes 3 and 4 are crude soluble protein of *E. coli* strain Rosetta-gami-B/pET15b-IL2/FUscFv(V_H-V_K). Lanes 5 and 6 are purified IL2/FUscFv(V_H-V_K). Lanes 7 and 8 are crude total protein of *E. coli* strain Rosetta-gami-B/pET15b(V_H-V_K). (B) Lane M, protein marker; Lanes 1 and 2 are crude soluble protein of control (*E. coli* strain Rosetta-gami-B/pET15b). Lanes 3 and 4 are crude soluble protein of *E. coli* strain Rosetta-gami B/pET15b-IL2/FUscFv(V_K-V_H). Lanes 5 and 6 are purified IL2/FUscFv(V_K-V_H). Lanes 7 and 8 are crude total protein of *E. coli* strain Rosetta-gami-B/pET15b-IL2/FUscFv(V_K-V_H).

A.3 Discussion

The benefit of *E. coli* as an expression organism, *i.e.* low cost of production, ease of genetics manipulation, and cultivation on a large scale, make it an attractive host organism for over-production of eukaryotic proteins. However, in the few previously reported regarding the expression of IL2 fusion proteins using scFv, Liao et al. have reported that the expression level

of IL2 fusion proteins when IL2 was fused directly to the carboxyl-terminal end of the V_H chain of anti-CEA F39scFv without a linker. The resultant fusion protein F39scFv(V_K-V_H)/IL2 expressed in a mouse non-secreting hybridoma cell, Sp2/0-Ag14, was very low in an order of less than 500 ng/ml (Liao et al., 2001). Moreover, the expression of fusion protein between superantigen staphylococcal enterotoxin A (SEA) and FUscFv, SEA/FUscFv, in *E. coli* strain BL21(DE3) resulted in accumulation of recombinant protein in an insoluble form in cytoplasmic inclusion bodies and involved unfavorable purification steps for protein refolding (Ueno et al., 2002). Normally, a high concentration of incorrectly folded recombinant protein results in the formation of inclusion bodies. Refolding can recover active protein from inactive inclusion bodies, however, in case of bifunctional fusion proteins, it has proven difficult to attain in high yield despite several attempts to characterize and optimize factors influencing refolding and stability. An initial expression of correctly folded, soluble recombinant protein is therefore preferred. In an attempt to produce soluble fusion proteins, different bacterial strains and cultivated temperatures were employed; furthermore, an appropriate concentration of IPTG for induction of protein expression and induction time was also investigated.

Our results indicated that the formation of IL2/FUscFv needs the formation of disulfide bridges for properly folding of protein structure. For the expression of fusion proteins IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K), the expression level of fusion proteins in *E. coli* strain BL21(DE3)pLysS was twice higher than *E. coli* strain Rosetta-gami B, but *E. coli* strain Rosetta-gami B resulted in high percent of soluble fusion protein. The *E. coli* strain Rosetta-gami B possessed a little slower growth than *E. coli* strain BL21(DE3)pLysS. Among the *E. coli* strains, BL21(DE3)pLysS was identified as the most widely used host for protein expression with the advantage of being deficient in proteases. Thus, *E. coli* strains BL21(DE3)pLysS was chosen as the tested host strain in this study. However, many proteins require the formation of stable disulfide bonds for proper folding and activity. Without disulfide bonds, these proteins may be degraded or accumulated as inclusion bodies. One limitation of producing properly folded proteins in *E. coli* has been the relatively high

reducing potential in the cytoplasmic compartment; disulfide bonds are usually formed only upon export into the periplasmic space. Bacterial strains with glutathione reductase (*gor*) and thioredoxin reductase (*trx*B) mutations greatly enhance the formation of disulfide bonds in the *E. coli* cytoplasm (Aslund et al., 1999; Prinz et al., 1997). As the properly folded processes four structural disulfide bonds, in this study, the second tested *E. coli* strain was the Rosetta-gami B lacking thioredoxin (*trx*B) and glutathione (*gor*) reductases, thus facilitating the formation of disulfide bonds in the cytoplasm (Bessette et al., 1999). The *E. coli* strain Rosetta-gami B is also appropriate to overcome the differences in codon bias between prokaryotes and eukaryotes. Thus, it is combined functions address both disulfide bond formation and expression of eukaryotic protein. In this study, *E. coli* strain BL21(DE3)pLysS grew to considerable cell densities and gave high yield of fusion protein. The *E. coli* strain Rosetta-gami B grew to modest cell densities compared to the BL21(DE3)pLysS. As an explanation of the slow growth of the *trx*B and *gor* mutant strains, Bessette et al. (1999) suggested that the absence of the two major disulfide reducing pathways in the cytoplasm renders inactive the catalytic cycle of an essential enzyme, or alternatively, aberrant disulfide bonds are formed in an essential cytoplasmic protein that as a result becomes inactive.

The effect of temperature on growth and expression of fusion proteins was investigated at 25°C, 30°C, and 37°C in both *E. coli* strains BL21(DE3)pLysS and Rosetta-gami B. It was found that cultivated temperature at 25°C stimulates higher production of soluble protein compared to 37°C. Cultivation at higher temperature favored growth and the production of insoluble protein in both strains. Our results are in agreement with the reported beneficial effect of lower expression temperate on solubility of the recombinant protein (Esposito and Chatterjee, 2006; Schlieker et al., 2002). In case of *E. coli* strain Resetta-Gami B, the expression of fusion protein at 25°C maintained a reasonable yield and formed soluble protein (77%). Obviously, our results indicated that the different arrangement of heavy-chain and κ light-chain variable gene of FUscFv, IL2/FUscFv(V_{κ}-V_{H})

or IL2/FUscFv(V_H-V_K), had influence on cell growth. The fusion protein IL2/FUscFv(V_K-V_H) may toxic to *E. coli* because the expression level of IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K) was almost the same level, but growth rate of both *E. coli* strains accumulating IL2/FUscFv(V_K-V_H) was twice lower than those synthesizing IL2/FUscFv(V_H-V_K).

For difficult eukaryotic proteins, it has been shown that IPTG concentration as low as μM resulted in increased soluble protein instead of inclusion body (Miller et al., 1999). Miller et al. (2005) also reported the use of an autoinduction system containing 1 mM IPTG and found that the majority of scFv forms insoluble inclusion bodies. Therefore, if higher yields of soluble scFv are desired, varying the amount of IPTG or changing temperature during induction might result in increased yields of soluble scFv. In this study, the optimal condition is consisting of two phases. For cell growth, the optimal temperature was at 37°C. After OD_{600} nm reached 0.6, cells were transferred to fresh LB medium containing 0.5 mM IPTG and cultivated at 25°C for 10h.

The produced fusion proteins had the N-terminal His·tag as detected by Immunoblotting with a monoclonal anti-polyHistidine, clone HIS-1. Thus, the purification was accomplished by Ni-affinity chromatography. The results of purified fusion protein are shown in Figure 3 as the western blot analysis of the soluble fusion proteins of *E. coli* strain Rosetta-gami-B/pET15b-IL2/FUscFv(V_K-V_H) and *E. coli* strain Rosetta-gami-B/pET15b-IL2/FUscFv(V_H-V_K) using rabbit anti-hIL2 antibody (diluted 1:10,000) specific binding to IL2 of the fusion protein as primary antibody and anti rabbit antibody conjugation with alkaline phosphatase as secondary antibody. Due to the existence of His·tag, the fusion protein could be easily purified by Ni-affinity chromatography using His·Bind® Kits. In general, expressing scFv antibody to a single antigen is often required to test their effectiveness in immunoprecipitation or ELISA. Based on the results obtained from testing with human IL2 ELISA kit, the results demonstrated that the purified fusion protein was reacted with human IL2.

A.4 Conclusion

The fusion proteins IL2/FUscFv(V_H-V_K) and IL2/FUscFv(V_K-V_H) are useful antibody markers with potential use in diaganostics, therapeutics, and proteomic analyses. The *E. coli* strains Resetta-gami-B harboring pET15b-IL2/FUscFv(V_K-V_H) or pET15b-IL2/FUscFv(V_H-V_K) were chosen for fusion protein expression. The fusion proteins were expressed in reasonable yield with high percentage of soluble form (0.25 g/l IL2/FUscFv(V_H-V_K) with solubility 89.29% and 0.26 g/l IL2/FUscFv(V_K-V_H) with solubility 84.61%) when cultivated in LB medium containing 50 μg/ml carbinicillin and 34 μg/ml chloramphenicol and induced with 0.05 mM IPTG at 25°C for 10 h. Due to the existence of His·tag verified by the western blot, the fusion protein could be easily purified by affinity chromatography using His·Bind® Kits. The fusion proteins were successfully purified by eluting with buffer containing 50 mM imidazole. The purified fusion protein was quantified by human IL2 ELISA kit and gave positive result meaning that the produced fusion protein contained recombinant human IL2. By using an appropriated heterologous expression system, low-efficient refolding that is necessary in the case of enzyme accumulation in inclusion bodies could be avoided.

A.5 Suggestion for Further Work

The fusion proteins expressed intracellularly in *E. coli* (cytoplasm or periplasm) possesses several drawbacks such as extraction and purification steps need to perform carefully and immediately. Moreover, freeze-thaw cycle may cause instability of protein function or stimulate formation of inclusion bodies. Thus, production of secreted fusion proteins is considered to be superior to intra-cellular fusion proteins. In the next chapter, the production of secreted fusion proteins can be investigated in eukaryotic expression systems, allowing a potential breakthrough for antibody technology in producing large amounts of specific recognition units coupled to effector molecules for consumer applications.

CHAPTER B: Production, purification, and characterization of secreted fusion proteins IL2/FUscFv expressed in *P. pastoris*

B.1 Materials and Methods

B.1.1 The fusion gene

The fusion genes encoding for anti-FU-MK-1-scFv/IL2 were obtained from Professor Kuroki Masahide (School of Medicine, Fukuoka University, Japan).

B.1.2 Strains of P. pastoris

P. pastoris strains GS115 (*his4*) and KM71H (*arg4; aox1:ARG4*) were used as expression hosts. All yeast strains were kept as frozen stocks in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) glucose) supplemented with 15% (v/v) glycerol at -70^{o}C.

B.1.3 Construction of expression vectors

The fusion genes coding for IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K) were amplified by PCR and cloned into pPICZα-A containing a C-terminal His·tag sequence (Invitrogen Corp., CA, USA). The expression vectors pPICZαA-IL2/FUscFv(V_K-V_H) or pPICZαA-IL2/FUscFv(V_H-V_K) were linearized with *Pme*I and transformed into *P. pastoris* strains GS115 and KM71H separately, using the LiCl method as described in the EasyComp™ Transformation kit (Invitrogen Corp., CA, USA). After a 3-days culture on YPDS agar plates (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 2% agar) containing with Zeocin™ (100 μg/ml) (Invitrogen Corp., CA, USA), the transformants grew and formed colonies on selection plates.

B.1.4 Expression screening

Individual Zeocin-resistant colonies were analyzed expression of secreted fusion protein in shake flask cultures. Briefly, each twenty independent single colonies were randomly selected and propagated on separate YPDS plates containing 100 μg/ml Zeocin™, and subjected to the

analysis of the secretion level. Individual colonies were inoculated in 10 ml of BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0) in a 250 mL baffled flask to grow overnight at 30°C with shaking at 200 rpm. The cells in the pre-culture were harvested by centrifugation (2,000×g, 5 min, 4°C) and resuspended in 50 mL of BMMY medium (BMGY with 0.5% methanol) in a 250 mL baffled flask. The induction cultures were incubated at 30°C with shaking at 200 rpm to induce expression by the addition of 0.5% methanol at every 24 h intervals.

B.1.5 Determination of Methanol Utilization Phenotype

Vector pPICZα-A linearized within the 5′ AOX1 region will integrate by gene insertion into the host 5′ AOX1 region and allow an integration only occur at the AOX1 (alcohol oxidase gene) locus. Howver, all transformants of KM71H are Mut^S because of the disruption of the AOX1 gene (*aox1::ARG4*). There is no need to test recombinants strain KM71H for the Mut phenotype. Therefore, only transformant *P. pastoris* strains GS115 was determined the methanol utilization (Mut) phenotype whether the recombinant strain was able to metabolize methanol fast (Mut^+) or slow (Mut^S). In general, most of the transformants GS115 should be Mut+; however, with the presence of the AOX1 sequences in the plasmid, there is a chance that recombination will occur in the 3′AOX1 region also, disrupting the wild-type AOX1 gene and creating Mut^S transformants. Therefore, testing on Minimal Dextrose Histidine, MDH, (1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 2% dextrose, 4×10^{-3} Histidine) and Minimal Methanol Histidine, MMH, (1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 4×10^{-3} Histidine, 0.5% methanol) plates was performed to confirm the Mut^+ phenotype. The individual Zeocin-resistant transformants GS115 was streak on both an MMH plate and an MDH plate and incubated at 30°C for 2 days. To differentiate Mut^+ from Mut^S, each of the controls (GS115/Mut^S Albumin and GS115/pPICZ/lacZ Mut^+) were also steak onto the MDH and MMH plates. Mut^+ strains should grow normally on

both plates, while Mut^S strains should grow normally on the MDH plate but show little or no growth on the MMH plate.

B.1.6 SDS-PAGE and Western blotting

Clarified supernatant samples were separated on 12% polyacrylamide gel under reduced conditions. The SDS-PAGE analysis was performed according to the method of Laemmli (1970). A mini-Protean was used as instructed by the manufacturer (Bio-Rad Laboratories Ltd., CA, USA). The fusion protein concentration was estimated using densitometry software, ImageJ (http://rsb.info.nih.gov/ij/). An ovalbumin with molecular weight 45 kDa (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a standard protein. Briefly, 0.1 to 1.0 g/l of protein standard and samples were run on 12% SDS-PAGE, stained with 0.1% (w/v) solution of Coomassie stain (Sigma-Aldrich Corp., St. Louis, MO, USA) 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. Dry gels were scanned on HP psc1210 ScanJet and loaded on to the ImageJ software. The target protein bands were selected, plotted, and compared with the protein standard. 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. Protein molecular-weight markers (Fermentas Inc., MD, USA) were used as protein standards for determination of molecular mass.

For Western Blots, proteins were transferred from the gel onto a PVDF membrane (Bio-Rad Laboratories Ltd., CA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Ltd., CA, USA). The fusion protein was confirmed by immunoblotting according to WesternBreeze chromogenic detection kit (Invitrogen Corp., CA, USA). Immunoblotting was carried out with a monoclonal rabbit anti-hIL2 antibody and an anti-rabbit antibody conjugated with alkaline phosphatase. The complex was visualized by enzyme reaction with the substrate BCIP/NBT. The His·tag was detected

by immunoblotting using a monoclonal anti-polyHistidine, clone HIS-1 conjugated with alkaline phosphatase according to WesternBreeze chromagenic detection kit (Invitrogen Corp., CA, USA).

B.1.7 Optimization of the production of fusion protein expressed in P. pastoris in shaken flask cultivation

To enhance the production of secreted protein in *P. pastoris*, the effect of changing in pH, temperature, and concentration of methanol induction on growth and fusion protein expression were examined in shaken flask cultivation. Firstly, the pH value of BMMY was varied from pH 3, 4, 5, 6, 7, 8, 9, and 10. Next, temperature was varied at 20, 25, 30, and 37°C. Finally, the concentration of methanol induction was varied at 0.01%, 0.1%, and 0.5% (v/v). Briefly, the transformant was cultured in 20 ml of BMGY at 30°C, 200 rpm until the OD_{600} reached 8 to 10. The culture was centrifuged at 10160×g for 10 min. The supernatant was discarded and the cells were transferred into baffle flasks with 40 ml of BMMY varying pH between 3 and 10 and incubated at 20, 25, 30, or 37°C in 200 rpm shaking incubator for 96 hours. To induce the expression of fusion protein, absolute methanol was added to a final concentration of 0.01%, 0.1%, and 0.5% at 12 h interval. The sampling of culture was taken every 12 h for cell dry weight measurement. The supernatant was collected for protein analysis by Coomassie-stained SDS-PAGE and Western blot method. The methanol concentration remaining in the medium was analyzed by Gas Chromatography.

B.1.8 Production of IL2/FU-MK-1-scFv by P. pastoris in fed-batch culture

The production was divided into two phases. The first phase was growth phase using glycerol as a main carbon source and the second phase was methanol induction phase. In this study, glycerol concentration in growth phase was varied at 5%, 7.5%, and 10% (w/v). The fermentation temperature was kept constant at 30°C throughout cultivation. In the growth phase, the pH value was maintained at pH 6.0 to promote biomass production. After depletion of glycerol, the feeding of methanol was started and the pH value

was changed to pH 3 or pH 4. The dissolved oxygen (DO) was automatically controlled via DO cascade control system and maintained at above 40%. All of these parameters were recorded and monitored using 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 agents. The silicone antifoam emulsions (Wako Pure Chemical Industries, Ltd., Japan) 5% (v/v) was used for foam reducing agent. The air flow rate of 4 ml/min was used for aeration.

Briefly, 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 salts medium (medium 1 liter containing of 4.25 ml ortho-phosphoric acid, 9.4 mM $MgSO_4$, 1 mM $CaSO_4$, 16.4 mM K_2SO_4, 11.4 mM KOH) and added 4 ml/l PTM_1 trace salts (24 mM $CuSO_4$, 0.53 mM NaI, 19.87 mM $MnSO_4$, 0.83 mM Na_2MoO_4, 0.32 mM boric acid, 2.10 mM $CoCl_2$, 0.15 mM $ZnCl_2$, 0.23 M $FeSO_4$, 0.82 mM biotin) after sterilization.

The samples were taken at 6 h interval for further analysis until the glycerol was exhausted as indicated by a sharp signal of DO spike. Then, the methanol induction phase was started by methanol feeding in fed-batch culture. Methanol feed solution is consisting of an absolute methanol with 12 ml/l PTM_1. After the DO spike, samples were taken every 12 h for 96 h for further analysis. The sample was centrifuged at 17,418×g for 10 min 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.

B.1.9 Purification of fusion proteins by Ni-affinity chromatography

To purify to fusion protein, the supernatant was clarified through a 0.22 μm membrane filter prior to purification steps. Ni-affinity chromatography was performed using His·Bind®Kits 5 ml column (Novagen®, Merck KGaA, Darmstadt, Germany). Two ml of the His·Bind resin was charged with 2 volumes of charging buffer (50 mM $NiSO_4$) and packed under gravity flow in

column. Next, the resin was equilibrated with 10 volumes of binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) and drained. Ten ml of supernatant containing His-tagged protein was loaded into column and incubated at 4°C for 3h. The resin with bound His-tagged protein was washed with 10 ml binding buffer, subsequently with 6 ml washing buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9), to remove non-specifically bounded proteins. The protein was eluted with 6 ml eluting buffer (0.5 – 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and the 2 ml fractions were collected for 20 fractions. The fractions containing the protein (analyzed by SDS-PAGE) were pooled and concentrated using Vivaspin 500 centrifugal concentrators (30000 MWCO, Vivaproducts, Inc., MA, USA). Finally, the protein was kept in PBS solution for further analysis. Protein concentration was determined using the Bradford Protein Assay (Bio-Rad Laboratories Ltd., CA, USA) with bovine serum albumin as a standard.

B.1.10 Measurement of human IL2 protein levels in the IL2/FUscFvs expressed in P. pastoris

The concentration of IL2 in the IL2/FUscFv was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Human IL2 ELISA Ready-Set-Go, eBioscience Inc., CA, USA) according to the instruction. In brief, ELISA 96 well plates (Corning Costar 9018 ELISA 96 well plate, Corning Incorporated, NY, USA) were coated with capture antibody in coating buffer. Test samples and serial dilutions of IL2 standard were added and incubated for 1 h at room temperature. Biotin-conjugated rabbit anti-human IL2 as a detection antibody was added after washing. Then 100 µl avidin-HRP diluent was added and incubated at room temperature for 1 h. After washing, 100 µl TMB (Tetramethylbenzidine) substrate solution was added and incubated for 15 min. The plates were read at OD_{450} nm with a Bio-Rad microplate reader (Bio-Rad Laboratories Ltd., CA, USA). Standard curves were generated with human recombinant IL2 (eBioscience Inc., CA, USA).

B.1.11 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, Inc., CA, USA) were added in triplicate to 96 well plate (Corning Costar 9018 ELISA 96 well plate, Corning Incorporated, NY, USA). After incubation overnight at 4°C, 4 µg of fusion protein IL2/FU scFv in 100 µl assay diluents (eBioscience Inc., CA, USA) was added in each well and incubated at room temperature for 2 h. Then, biotin-conjugated anti-human IL2 diluted 1:200 was added in assay diluent. The plate was incubated at room temperature for 1 h. Next, Avidin-HRP (eBioscience Inc., CA, USA) diluted 1:200 in assay diluents was added and incubated at room temperature for 30 min. Consequently, 100 µl TMB (eBioscience Inc., CA, 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. The plates were read at OD_{450} nm with a Bio-Rad microplate reader (Bio-Rad Laboratories Ltd., CA, USA).

B2 Result

B.2.1 Transformation and selection of recombinant clones P. pastoris

The aim of this study was to express the fusion protein of IL2/FUscFv 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 (Cereghino and Cregg, 2000).

To facilitate immunohistochemical, biochemical, and bioassay investigations, large amount, ca. 100 mg, of scFv is required. In the few previously reported regarding the expression of IL2 fusion proteins using scFv, it was reported that the fusion protein anti-CEA F39scFv(V_K-V_H)/IL2 expressed in a mouse non-secreting hybridoma cell, Sp2/0-Ag14, was very low in an order of less than 500 ng/ml (Liao et al., 2001). It was also reported that the expression of fusion protein between superantigen staphylococcal enterotoxin A (SEA) and FUscFv, SEA/FUscFv, in *E. coli* strain BL21(DE3)

resulted in accumulation of recombinant protein in an insoluble form in cytoplasmic inclusion bodies and involved unfavorable purification steps for protein refolding (Ueno et al., 2002). Moreover, in the previous study, the level of expression of FUscFv/IL2 in *P. pastoris* was approximately 0.002 g/l of FUscFv/IL2 fusion protein was detected in the culture medium (Matsumoto et al., 2002).

Normally, *P. pastoris* produces higher amounts of heterologous proteins than other systems and is particularly advantages for smaller proteins (Cereghino and Cregg, 2000). Levels approaching 1.2 g/l scFv using *S. cerevisiae* sucrose invertase secretion signal have been reported (Freyer et al., 2000). However, the large scale production of the scFv-related fusion protein is still an unsolved issue and there are no reports showing a large scale production of scFv/IL2 fusion protein compared to the expression of whole MAb molecule/IL2 fusion protein. In this study, the fusion proteins, IL2/FUscFv(V_K-V_H) and IL2/FUscFv(V_H-V_K), were fused to carboxyl-terminal c-myc and HIS•tag to allow for immunodetection and affinity purification. The predicted molecular weight of the scFv under investigation was 45 kDa.

Firstly, 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 *P. pastoris* transformant for recombinant protein expression. As earlier description, 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 (Daly and Hearn, 2005). In this study, the expression vectors pPICZαA-IL2/FUscFv(V_K-V_H) or pPICZαA-IL2/FUscFv(V_H-V_K) were linearized with *Pme*I and transformed into *P. pastoris* strains GS115 and KM71H separately, according to EasyComp™ Transformation protocol. For pPICZαA-IL2/FUscFv(V_K-V_H), there were 14 colonies of transformant *P. pastoris* strain

KM71H and 20 colonies of transformant *P. pastoris* strain GS115 grown on YPDS plate containing 100 μg/ml Zeocin™. For pPICZαA-IL2/FUscFv(V_H-V_K), there were 21 colonies of transformant *P. pastoris* strain KM71H and 54 colonies of transformant *P. pastoris* strain GS115 on YPDS plate containing 100 μg/ml Zeocin™. All transformants were tested for small scale expression of fusion protein in shaken flask cultivation induction with methanol. The protein expression was screened by SDS-PAGE analysis.

(A)

(B)

Figure 5 Collection plates of transformant *P. pastoris* on YPDS containing 100 ug/ml Zeocin™. (A); colonies of transformant *P. pastoris* strain KM71H. (B); colonies of transformant *P. pastoris* strain GS115.

The best transformant capable of producing secreted fusion protein IL2/FUscFv in medium via the *S. 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-hIL2 antibody specific binding to IL2 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 reacted with alkaline phosphatase.

During an initial attempt to screen for the best transformant capable of producing secreted fusion protein, it was found that all transformants harboring pPICZαA-IL2/FUscFv(V_K-V_H) were unstable since the protein was no longer detectable after overnight storage at 4°C. Freezing at -80°C was sufficient to prevent degradation, however, the sample could not withstand more than one freeze-thaw cycle. Interestingly, transformants harboring pPICZαA-IL2/FUscFv(V_H-V_K) did not result in loss of product during multi freeze-thaw cycle, indicating that the secreted fusion protein IL2/FUscFv(V_H-V_K) was stable. Thus, we omit all transformants harboring pPICZαA-IL2/FUscFv(V_K-V_H) for further investigations.

Next, the aim of this research was focused on optimization of secreted IL2/FUscFv(V_H-V_K) production in *P. pastoris* strain GS115 compared with *P. pastoris* strain KM71H. In this study, all transformants *P. pastoris* strain KM71H expressed very low amount of secreted fusion protein IL2/FU-MK1-scFv(V_H-V_K) and not appropriate for increasing scale of production. Thus, only one transformant among 54 transformants of *P. pastoris* strain GS115 was selected to express IL2/FUscFv(V_H-V_K) 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 6A and 6B, respectively.

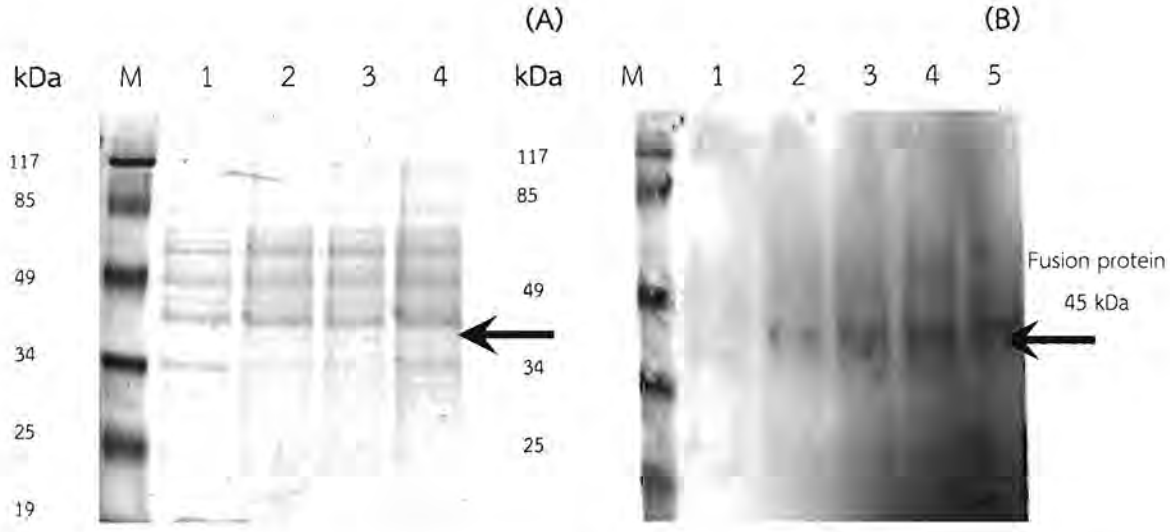


Figure 6 Time courses of secreted fusion protein IL2/FUscFv(V_H-V_K) in supernatant produced by *P. pastoris* strain GS115 at 30°C after 0.5 % (v/v) methanol induction. (A) Coomassie stained SDS-PAGE of secreted fusion protein IL2/FUscFv(V_H-V_K) in supernatant samples. Lane M, protein molecular weight marker; lane 1, 60 h induction; lane 2, 72h induction; lane 3, 84 h induction; lane 4, 96 h induction. (B) The western blot analysis (1:10,000 anti-human IL2 antibody) of secreted fusion protein IL2/FUscFv(V_H-V_K) in supernatant samples. Lane M, protein molecular weight marker; lane 1, non-expressing clone; lane 2, 60 h induction; lane 3, 72h induction; lane 4, 84 h induction, and lane 5, 96 h induction.

The expression level of individual *P. pastoris* transformants varied, the best clone producing approximately >0.100 g/l under standard conditions. It is possible to increase heterologous protein expression in *P. pastoris* through genetic manipulation of secretion signals, gene dosage, mRNA 5′- and 3′-untranslated regions, and codon usage (Sreekrishna et al, 1997). Thus, we chose the practical approach of optimizing cell growth conditions to optimize expression levels in the next experiement.

B.2.2 *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 employed as the first stage for optimization of recombinant proteins production and investigating optimal condition for maximized the production of secreted recombinant protein (Daly and Hearn, 2005). In this study, before the large scale production in 5 l fermentation, the important parameters *i.e.* pH, temperature, and methanol concentration were examined in small-scale expression.

B.2.2.1 Effect of pH on cell growth and IL2/FUscFv(V_H-V_K) production by *P. pastoris*

The effect of pH on cell growth and IL2/FUscFv(V_H-V_K) expression was examined in BMMY induction medium buffered to limit the protease activity. The optimal pH was examined at pH 3, 4, 5, 6, 7, 8, 9, and 10 in BMMY. Figure 7 shows time courses of biomass (g/l) in 0.5% (v/v) methanol induction phase when pH value was varied from 3 to 10.

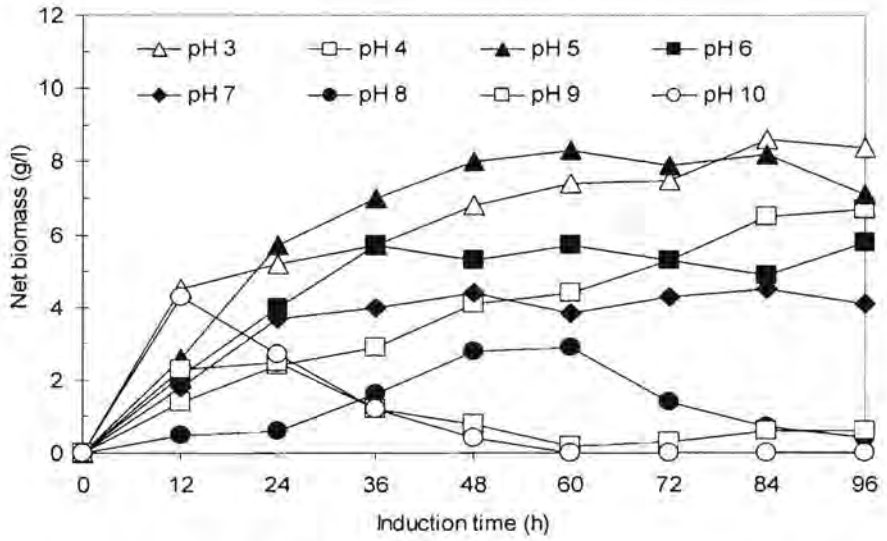


Figure 7 Comparison of dry cell weight (g/l) of *P. pastoris* strain GS115 during methanol induction phase with 0.5% (v/v) methanol at 30 °C and under different pH tested.

The cell density increased under acidic to neutral pH from pH 3 to 7 whereas it decreased under alkaline pH from pH 8 to 10. These results may imply that pH between 3 and 7 were optimal values for growth of *P. pastoris*

strain GS115. The results were 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. Figures 8A and 8B show the amount of secreted fusion protein produced under various pH tested.

It was obviously found that the pH 3 and 4 enhanced the secretion of the fusion protein IL2/FU-MK1-scFv(V_H-V_K) during methanol induction phase. On the other hand, pH values from 5 to 10 gave no expression of secreted fusion protein. It was suggested that acidic pH may prevent protease activity. The results were similar to the previous reports (Hong et al, 2002; Damasceno et al., 2004; Sinha et al., 2005). It was also reported that acidic pH is often used for recombinant protein production and pH 3 was found to be responsible for the optimal production of insulin-like growth factor-I and cytokine growth blocking peptide (Brierley et al., 1994; Koganesawa et al., 2002). Moreover, several reports found significantly improvement of secreted protein production under pH 3 (Sreekrishna and Kropp, 1996, Cregg, 1999; Curvers et al., 2001; Jahic et al., 2003; Damasceno et al., 2004). Here, we concluded that pH values between 3 and 4 were optimal for the production of secreted IL2/FU-MK1-scFv(V_H-V_K) by *P. pastoris* strain GS115.

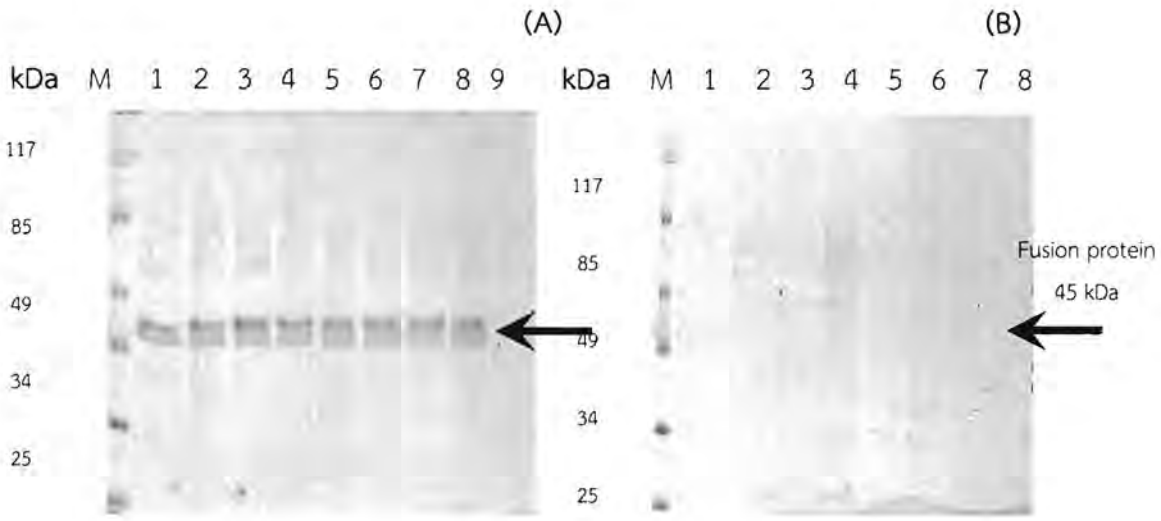


Figure 8 SDS-PAGE analysis of supernatant samples when the expression of fusion protein was induced with 0.5% (v/v) methanol under different pH values. (A); Lane M, protein molecular weight marker; Lane 1, pH 3 (60h); lane 2, pH 3 (72h); lane 3, pH 3 (84h); lane 4, pH 3 (96h); lane 5, pH 4 (60h); lane 6, pH 4 (72h); lane 7, pH 4 (84h); lane 8, pH 4 (96); lane 9, pH 5 (84h). (B); Lane M, protein molecular weight marker; lane 1, pH 6 (84h); lane 2, pH 6 (96h); lane 3, pH 7 (84h); lane 45, pH 7 (96h); lane 5, pH 8 (84h); lane 6, pH 8 (96h); lane 7, pH 9 (96h); land 8, pH 10 (96h).

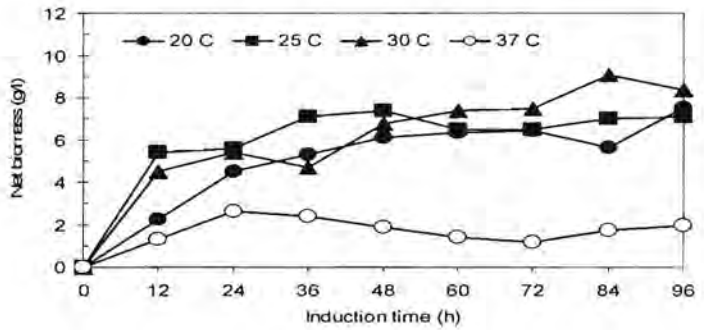
B.2.2.2 The effect of cultivation temperature on growth and fusion protein IL2/FUscFv(V_H-V_K) production by *P. pastoris*

Temperature is also one of important factors employed for enhancing production of recombinant protein. As shown above, adjusting the pH of the induction medium to limit protease activity had a profound effect on protein expression. Lower culture temperatures have frequently been employed to produce protein in *E. coli* (Blackwell and Horgan, 1991; Makrides, 1996) and are recalcitrant to expression at 37°C. In our studies, lower cultivation temperature at 25°C enhanced the expression of IL2/FUscFv(V_H-V_K) and IL2/FUscFv(V_K-V_H) in both *E. coli* strains BL21(DE3)pLysS and Rosetta-gami B.

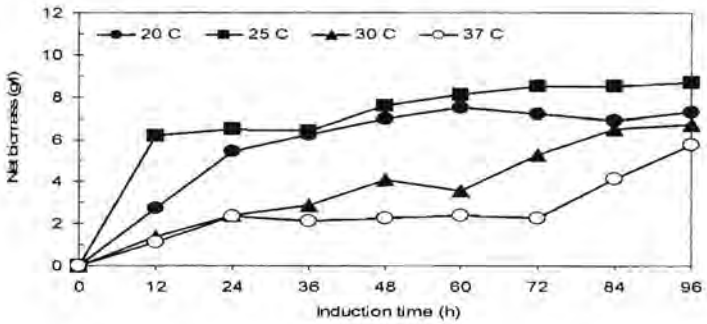
In *P. pastoris*, several reports have indicated that incubation temperature influences heterologous protein expression in yeast by affecting cell growth and the secretion of extracellular protease (Sreekrishna et al.,

1997; Chung and Park, 1997). Thus, various temperatures between 20 and 37°C have been examined in attempts to minimize extracellular proteolysis (Li et al., 2001; Curves et al., 2001). In this study, incubation temperatures of 20, 25, 30, and 37°C were examined in order to promote cell growth and expression and find the optimal temperature for improving The results were shown in Figures 9A to 9E.

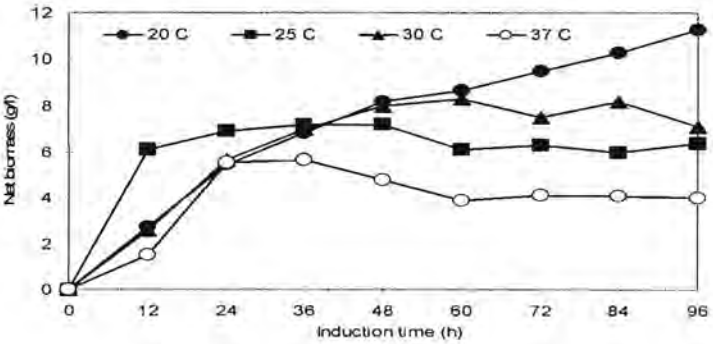
(A)



(B)



(C)



(D)

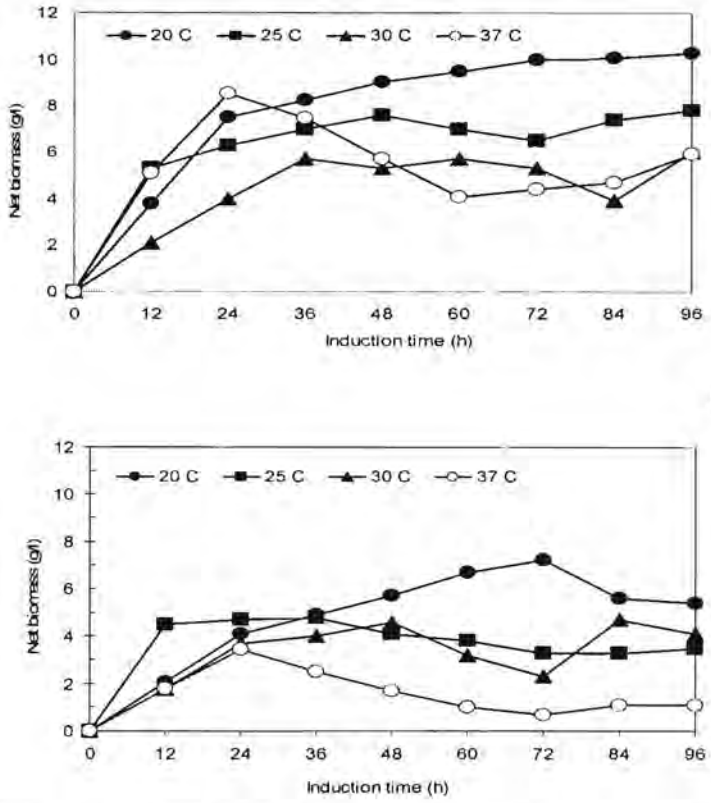


Figure 9 Comparison of dry cell weight (g/l) of *P. pastoris* strain GS115 when the expression of fusion protein was induced with 0.5% (v/v) methanol at (A); pH 3, (B); pH 4, (C); pH 5, (D); pH 6, (E); pH 7 and temperature was varied from 20, 20, 30, and 37 $^{\circ}$C.

The results suggest that, for BMMY medium with pH 5, 6, and 7, the cell growth under cultivation temperature at 20, 25, and 30 $^{\circ}$C was increased. Especially under cultivation temperature at 20 $^{\circ}$C, the highest cell growth rate was obtained whereas under cultivation temperature at 25 and 30 $^{\circ}$C, the growth rate was relatively slow. Moreover, the cultivation temperature at 37 $^{\circ}$C has been shown to decrease growth rate of *P. pasroris* GS115 in BMMY medium with pH 5, 6, and 7. These results suggested that the growth rate of *P. pastoris* GS115 in methanol induction phase were decreased when temperature was increased from 20 to 37 $^{\circ}$C.

For the production of secreted IL2/FUscFv(V_H-V_K), it was found that production of secreted IL2/FUscFv(V_H-V_K) by *P. pastoris* strain GS115 under pH 3 and 4 showed similar results when 0.1% (v/v) methanol induction was

performed under cultivation temperatures of 20, 25, 30, and 37°C. We found that *P. pastoris* strain GS115 could be effectively propagated at temperatures as low as 20°C, but the most favorable temperature for production of secreted IL2/FUscFv(V_H-V_K) was 30°C leading to enhance IL2/FUscFv(V_H-V_K) production. The results were compared in Figure 10.

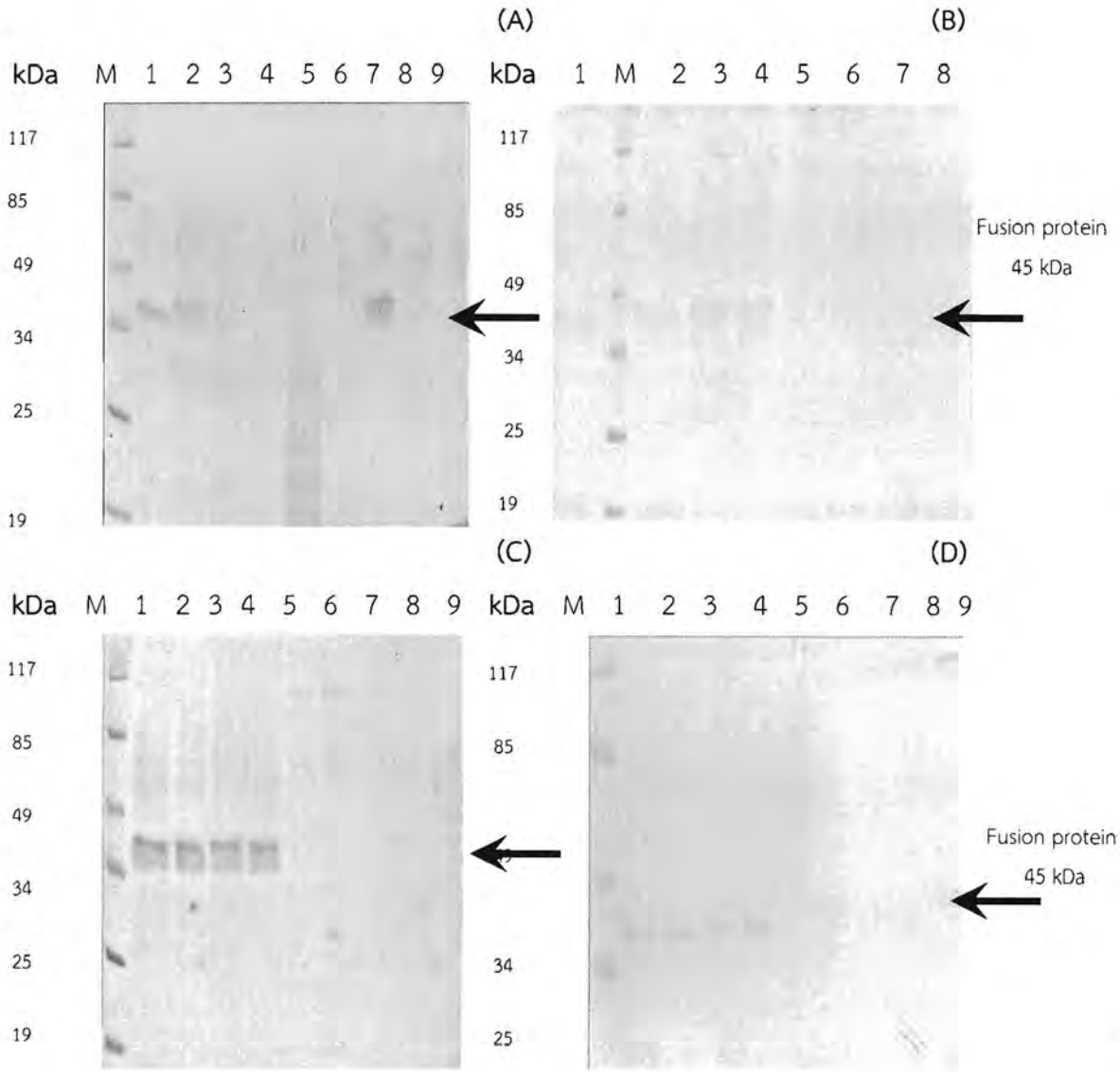


Figure 10 SDS-PAGE analysis of supernatant samples when the expression of fusion protein was induced with 0.5% (v/v) methanol under different pH values. **(A) The experiment was performed at 20°C.** Lane M, protein molecular weight marker; lane 1, pH 3 (84h); lane 2, pH 4 (84h); lane 3, pH 4 (96h); lane 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). **(B) The experiment was performed at 25°C.** 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). **(C) The experiment was performed at 30°C.** 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). **(D) The expression was done at 37°C.** 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).

It was found that there was no protein production under cultivation temperature at 37°C. It has been reported that when methanol is supplied as the sole carbon source, *P. pastoris* die due to the accumulation of formaldehyde and hydrogen peroxide arising from the oxidation of methanol by alcohol oxidase (Cregg and Madden, 1988; Couderc and Baratti, 1980; Klei et al, 1990). Increased induction temperatures accelerate this conversion, leading to more formaldehyde and hydrogen peroxide. Thus, the optimal cultivation temperature scheme for production of secreted IL2/FUscFv(V_H-V_K) by *P. pastoris* strain GS115 involved cultivation in BMGY medium at 30°C and pH 7.0 to accumulate biomass followed by induction in BMMY medium at 30°C and pH 3 or 4.

B.2.2.3 The Effect of methanol concentration on fusion protein IL2/FUscFv(V_H-V_K) production by *P. pastoris*

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 expression. 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 (Cunha et al., 2004; Jungo et al., 2007; Wu et al., 2011).

In this study, the effect of methanol concentration of 0.01%, 0.1%, and 0.5% (v/v) was investigated and combined with the effect of pH value at pH 3.0 and 4.0. The investigation was examined under cultivation temperature at 30°C for 96 h. Absolute methanol was added very 12 h to the final concentration tested. The secreted fusion protein was analyzed by SDS-PAGE and shown in Figure 11. In a preliminary study, induction with 0.01% methanol showed the lowest expression of IL2/FUscFv(V_H-V_K) during 96 h induction time courses. This might indicate the growth-limiting level of methanol for *P. pastoris* (Damasceno et al., 2004). Therefore, methanol concentration of 0.1% and 0.5% (v/v) were examined to enhance the expression level of the IL2/FUscFv(V_H-V_K).

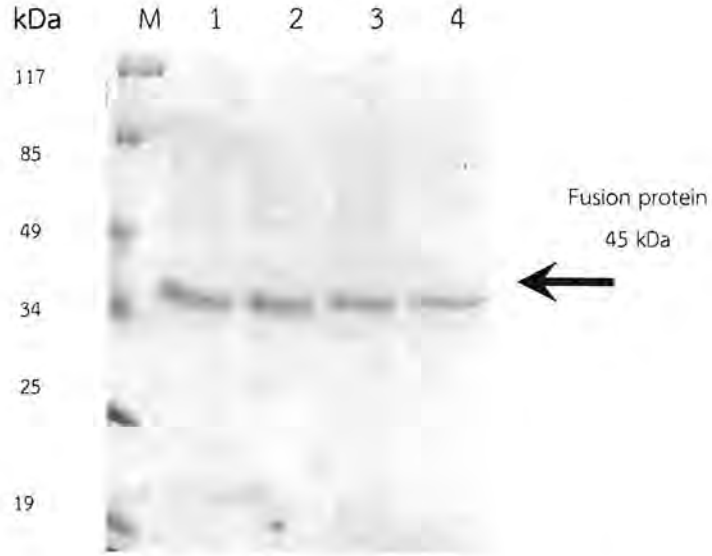


Figure 11 SDS-PAGE analysis of supernatant samples when the expression of fusion protein was induced with 0.1% or 0.5% (v/v) methanol under different pH values for 96 h. Lane M, protein molecular weight marker; lane 1, pH 3 with 0.1% methanol; lane 2, pH 3 with 0.5% methanol; lane 3, pH 4 with 0.1% methanol; with lane 4, pH 4 with 0.5% methanol.

The biomass accumulation with 0.1% methanol induction at pH 3.0 was increased from 16.7±0.8 g/l to 29.4±1.2 g/l and that with 0.5% (v/v) methanol induction at pH 3.0 was increased from 15.7±0.7 g/l to 28.5±0.6 g/l. Under pH 4.0, the biomass with 0.1% (v/v) methanol induction was increased from

15.7±0.9 g/l to 34.0±1.1 g/l and that with 0.5% (v/v) methanol induction was increased from 16.8±0.9 g/l to 32.9±1.2 g/. These results demonstrated that the biomass at pH 3.0 was lower than that of pH 4.0, but fusion protein concentration at pH 3.0 was slightly higher than that obtained under pH 4.0.

B.2.3 *Production of secreted fusion protein IL2/FUscFv(V_H-V_K) by P. pastoris in fed-batch culture*

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. Firstly, batch culture was performed under 5% glycerol in the growth phase, temperature at 30°C, pH 3.0, agitation speed at 500 rpm, and aeration rate 2 ml/min. The DO spike was observed at 24 h indicating the depletion of glycerol. Under this condition, dry cell weight 8.1±0.2 g/l was obtained. After induction with methanol 0.1% (v/v) for 96 h, dry cell weight 18.5±1.0 g/l was obtained. The secreted fusion protein concentration was 0.084 ± 0.005 g/l.

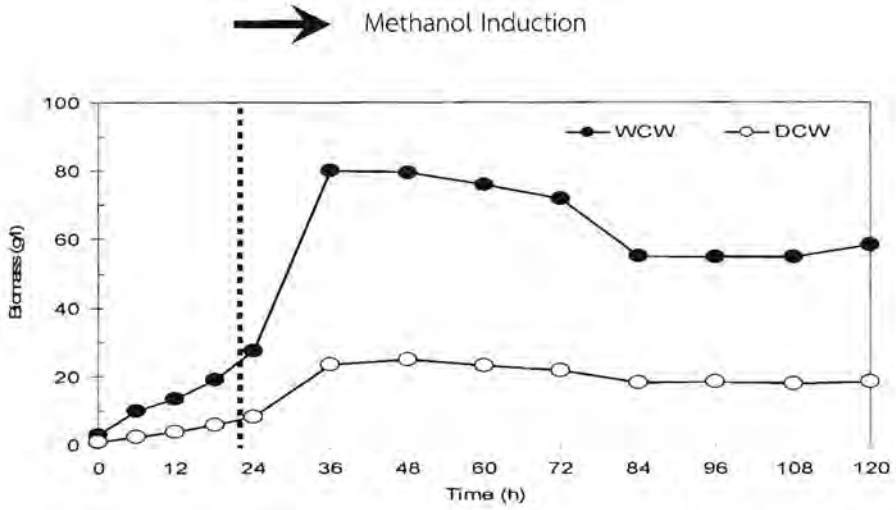


Figure 12 The growth profiles of *P. pastoris* strain GS115 harboring pPICZαA-IL2/FUscFv(V_H-V_K) in fed-batch culture. Glycerol phase was from 0 to 24h and methanol induction phase was from 24 to 120h. Fed-batch cultivation was performed in 5 l fermenter under temperature 30°C, pH 3, and 0.1% (v/v) methanol induction. The growth phase was performed with 5% (w/v) glycerol.

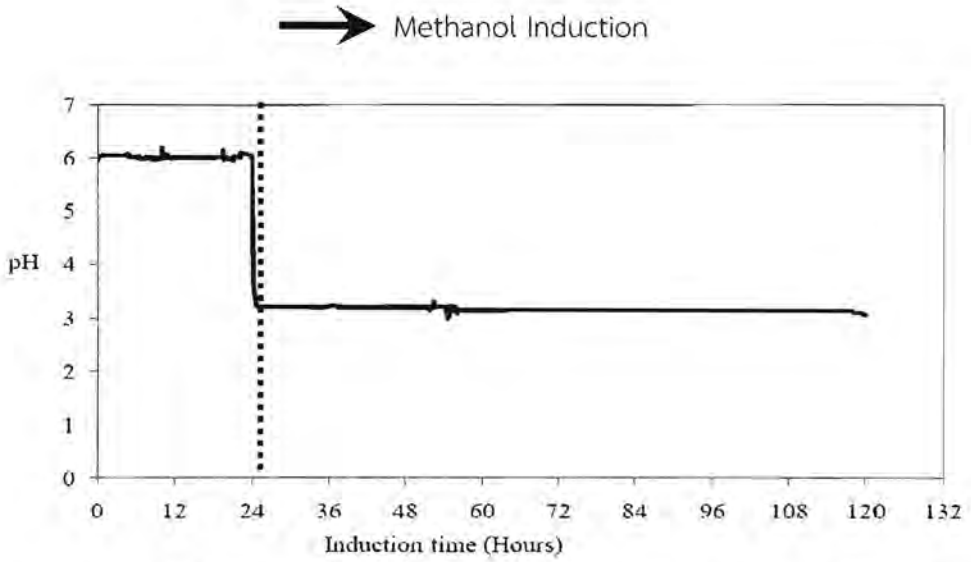


Figure 13 The control result of pH value; glycerol phase was from 0 to 24 h and methanol induction phase was from 24 to 120h. Fed-batch cultivation was performed in 5 l fermenter under temperature 30°C, pH 3, and 0.1% (v/v) methanol induction. The growth phase was performed with 5% (w/v) glycerol.

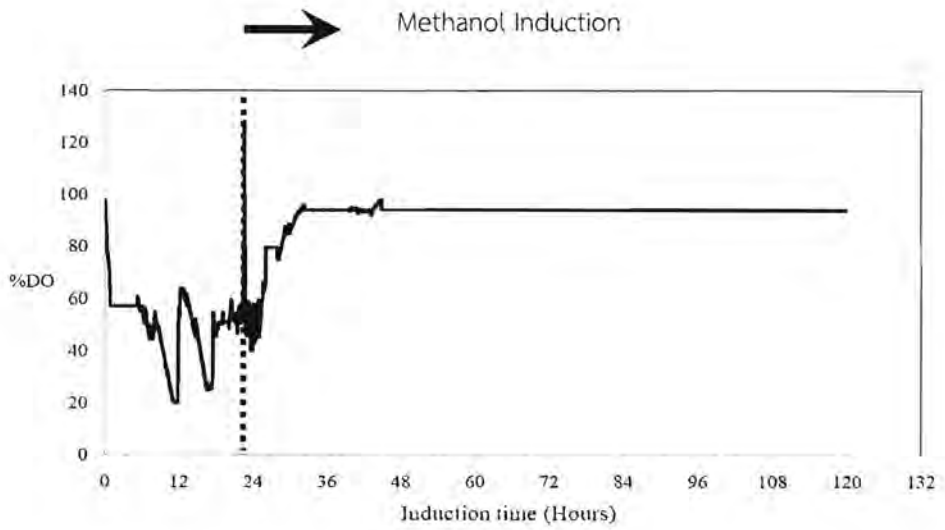


Figure 14 The control result of % DO, the DO spike was occurred at 24 h and methanol induction phase was from 24 to 120h. Fed-batch cultivation was performed in 5 l fermenter under temperature 30°C, pH 3, and 0.1% (v/v) methanol induction. The growth phase was performed with 5% (w/v) glycerol.

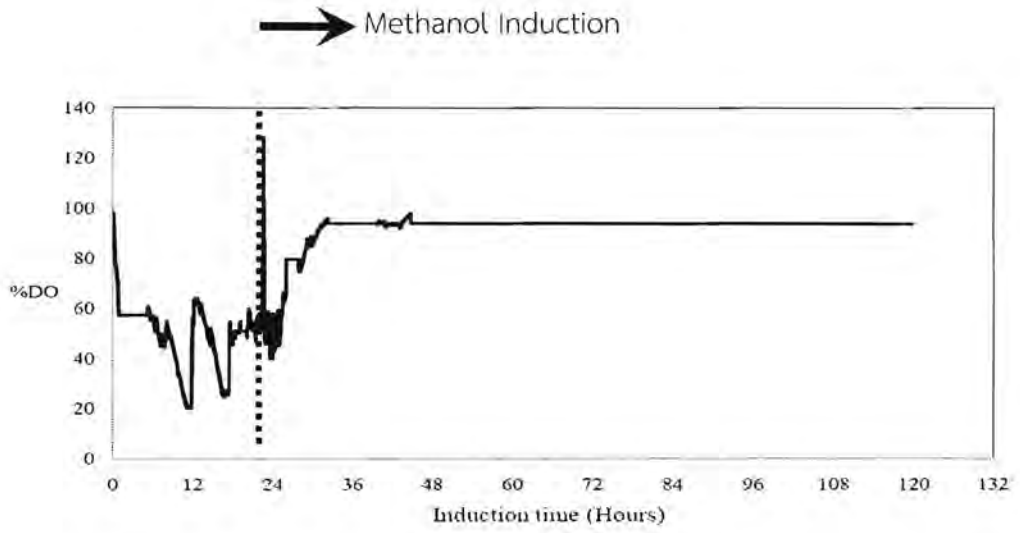


Figure 15 The control result of % DO, the DO spike was occurred at 24 h and methanol induction phase was from 24 to 120h. Fed-batch cultivation was performed in 5 l fermenter under temperature 30°C, pH 3, and 0.1% (v/v) methanol induction. The growth phase was performed with 5% (w/v) glycerol.

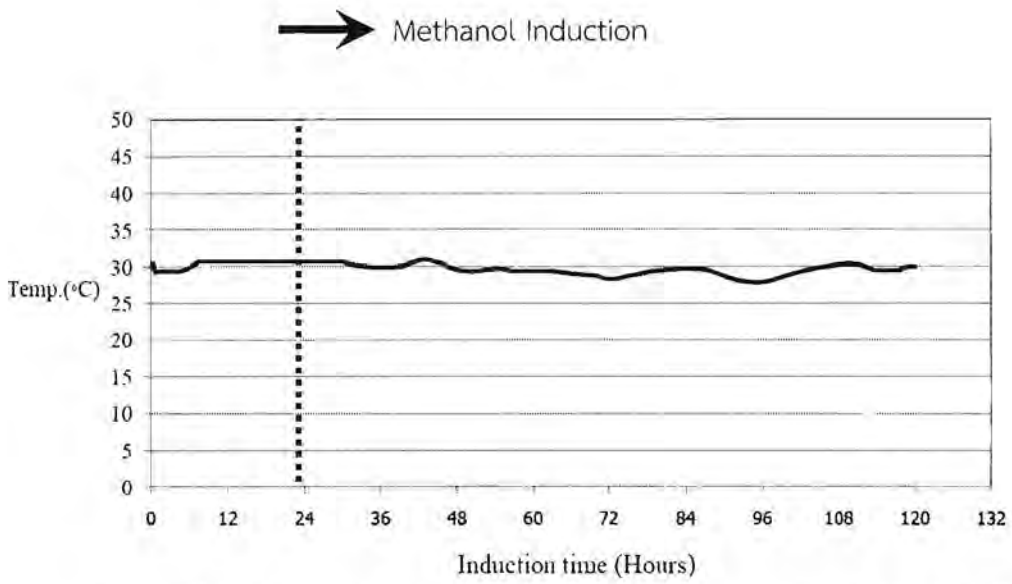


Figure 16 The control result of temperature. Glycerol phase was from 0 to 24 h and methanol induction phase was from 24 to 120 h. Fed-batch cultivation was performed in 5 l fermenter under temperature 30°C, pH 3, and 0.1% (v/v) methanol induction. The growth phase was performed with 5% (w/v) glycerol.

B.2.3.1 The effect of cell density on IL2/FUscFv(V_H-V_K) production

Generally, high cell densities are employed to produce heterologous proteins, since production is roughly proportional to cell density (Brierley et al, 1990; Cregg and Higgins, 1995; Cregg et al, 2000; Shi et al., 2003). However, high cell density may increase the concentration of extracellular proteases and may have detrimental effects on cell physiology in turn limiting the amount of desired product. In this study, the concentration of glycerol was used to manipulate the cell density. The biomass accumulation increased proportionally to the concentration of glycerol. Thus, the concentration of glycerol was varied from 5%, 7.5%, and 10% (w/v) to investigate the impact of cell density on the production of IL2/FUscFv(V_H-V_K) by *P. pastoris* in 5 l fermenter. In the growth phase, cell weight was 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, methanol concentration, and SDS-PAGE analysis.

As shown in Figure 17, the secreted fusion protein concentration was 0.084 ± 0.005, 0.109 ± 0.005, and 0.077 ± 0.004 g/l when 5%, 7.5%, and 10% (w/v) glycerol was used in growth phase, respectively. It was found that the amount of secreted fusion protein was lower than that obtained in shaken flask cultivation. This might be due to the different in the production medium. The modified basal salts medium was used in fed batch cultivation whereas BMMY was used in shaken flask cultivation. In addition, SDS-PAGE analysis showed bands of several low molecular weight proteins and it was implied that degradation of proteins occurred due to protease naturally produced by *P. pastoris* (Shi et al., 2003).

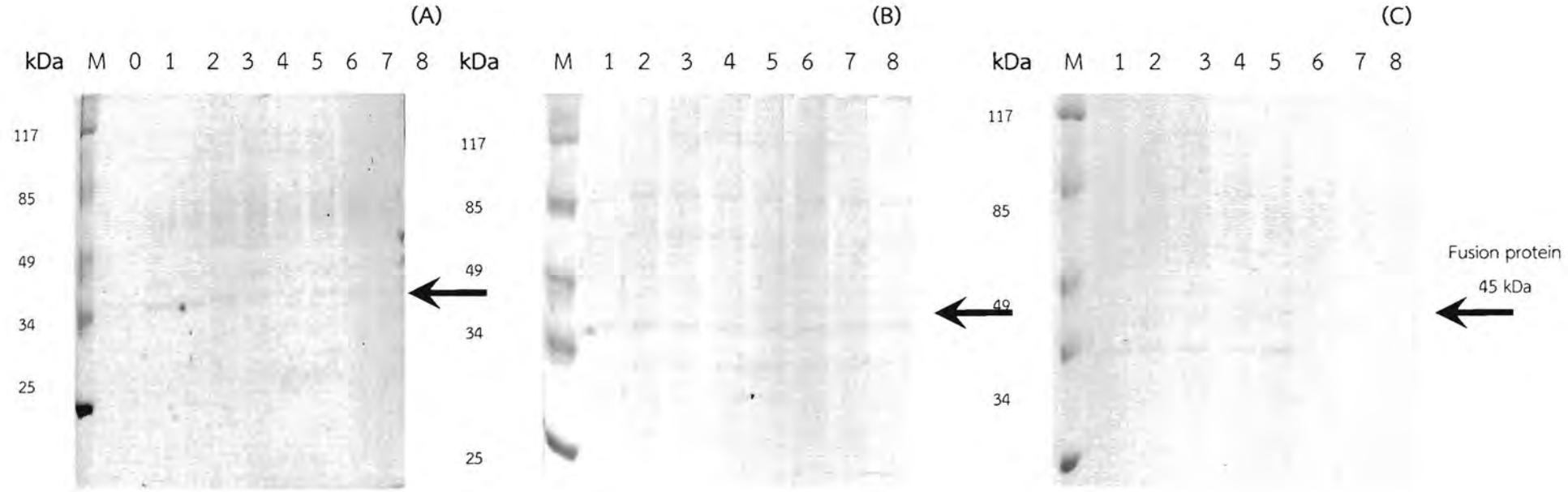


Figure 17 Comparison of SDS-PAGE analysis of secreted fusion protein produced in supernatant samples during methanol induction phase. The fed-batch cultivation was performed in 5 l fermenter at pH 3, 30°C, and 0.1% (v/v) methanol induction. The modified basal salts medium was used. Biomass was generated under different concentration of glycerol. (A); 5% (w/v) glycerol. (B); 7.5% (w/v) glycerol. (C); 10% (w/v) glycerol. Lane M, protein molecular weight marker; lane 0, 0 h; lane 1, 12h; lane 2, 24 h; lane 3, 36 h; lane 4, 48 h; lane 5, 60 h; lane 6, 72 h; lane 7, 84 h; lane 8, 96h.

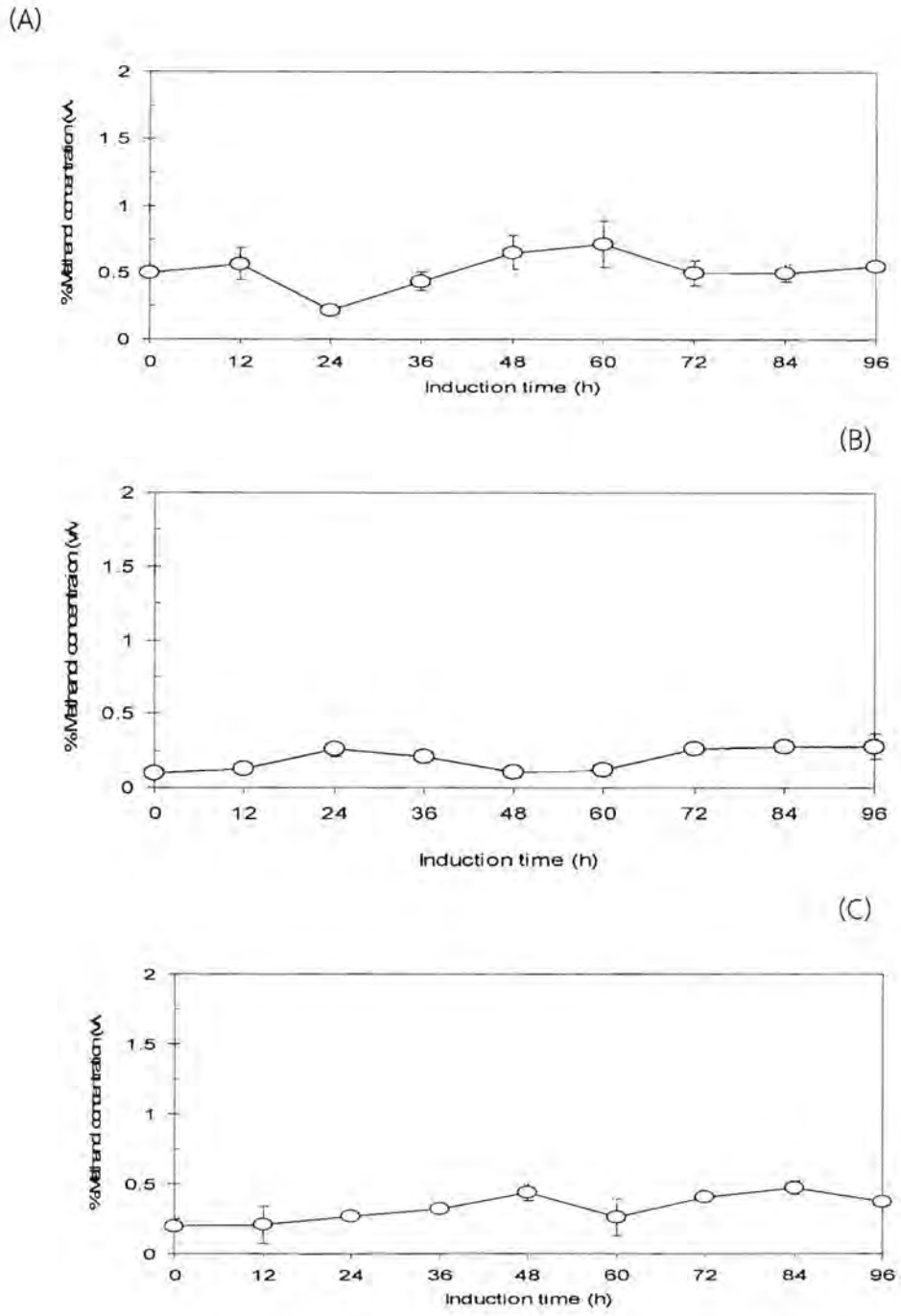


Figure 18 The control result of methanol concentration during methanol induction phase. The feed rate of methanol was set at 6 ml/h. Fed-batch cultivation was performed in 5 l fermenter. Control parameters are temperature 30°C, pH 3, and 0.1% (v/v) methanol induction. (A); The growth phase was performed with 5% (w/v) glycerol. (B); The growth phase was performed with 7.5% (w/v) glycerol. (C); The growth phase was performed with 10% (w/v) glycerol.

Figure 18 shows the control result of methanol concentration during methanol induction phase in details where the cell density in growth phase was different. The induction phase in which methanol was kept at optimal concentration at 0.1-0.5% (v/v) lasted for 96 h. During methanol fed-batch phase, the biomass increased and inhibitory of cell growth from methanol accumulation was not observed. Thus, the modified basal salts medium was changed to BMMY medium. Interestingly, the result (Figure 19) showed that the amount of the fusion protein produced in BMMY medium was much higher than that obtained from modified basal salts medium (Figure 17). In figure 3.2.19, the highest amount of 0.425 ± 0.002 g/l was obtained at 96 h under pH 4.0, temperature of 30°C and 0.1% methanol induction in BMMY. However, when pH was set at 3 the amount of fusion protein produced was only 0.197 ± 0.019 g/l.

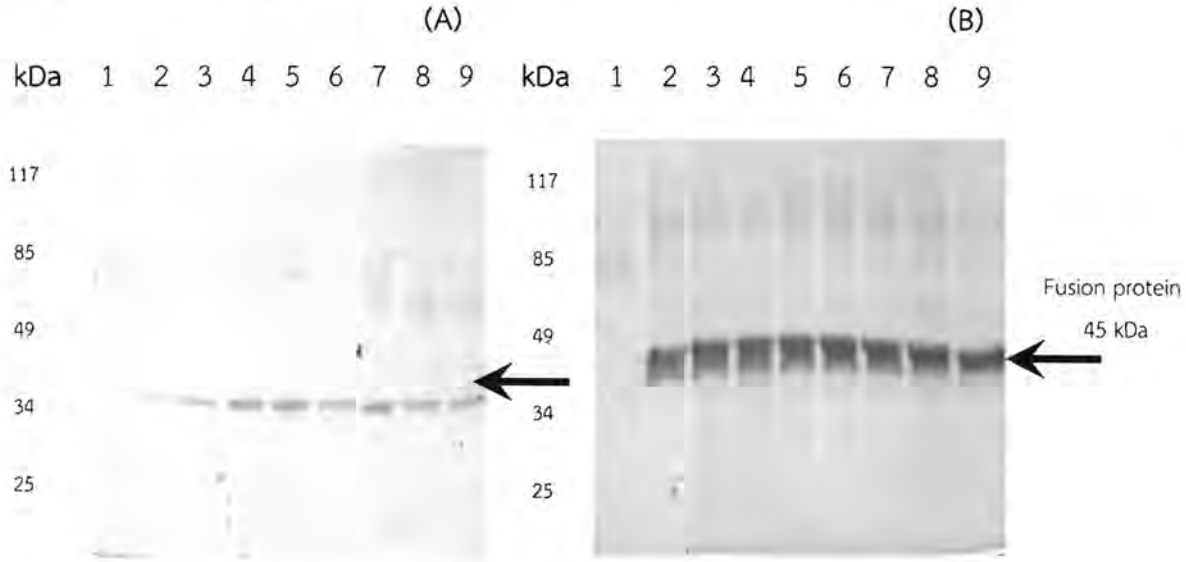


Figure 19 Time courses of SDS-PAGE analysis of secreted fusion during methanol induction phase. The fed-batch cultivation was performed in 5 l fermenter at 30°C, and 0.1% (v/v) methanol induction using BMMY medium. Biomass of *P. pasotris* strain GS115 was generated under 5% (w/v) glycerol in growth phase. (A); pH in BMMY medium was 3. (B); pH in BMMY medium was 4. Lane M, protein molecular weight marker; lane 0, 0 h; lane 1, 12h; lane 2, 24 h; lane 3, 36 h; lane 4, 48 h; lane 5, 60 h; lane 6, 72 h; lane 7, 84 h; lane 8, 96h.

The production of IL2/FUscFv(V_H-V_K) by *P. pasotris* strain GS115 in fed-batch cultivation was maximized under 0.1% (v/v) methanol induction using BMMY at pH 4, temperature of 30°C and 0.425 ± 0.002 g/l fusion protein was obtained. Several reports have indicated that soluble and functional scFvs produce in yeast but the yield is variable. Accumulation of 0.003 g/l to 4.3 g/l was reported in *P. pastoris* employing S. cerevisiae α-factor secretion signal (Shi et al., 2003; Damasceno et al., 2004; Lin et al., 2007; Yamawaki et al., 2007). In this study, the amount of secreted fusion protein was much higher than previous report that was in order of 0.002 g/l (Matsumoto et al. 2002).

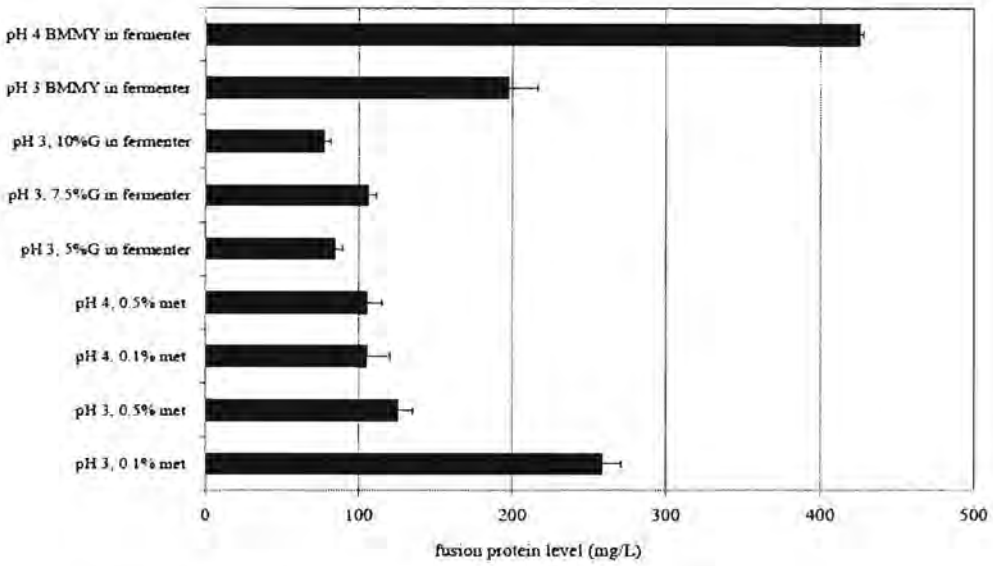


Figure 20 The comparison of secreted IL2/FUscFv(V_H-V_K) produced under various conditions. The results were obtained from shaken flak cultivation and fed-batch cultivation in 5 l fermenter.

B.2.3.2 *Partial purification of the fusion protein by Ni-affinity chromatography*

In order to partially purify the secreted IL2/FUscFv(V_H-V_K), 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.

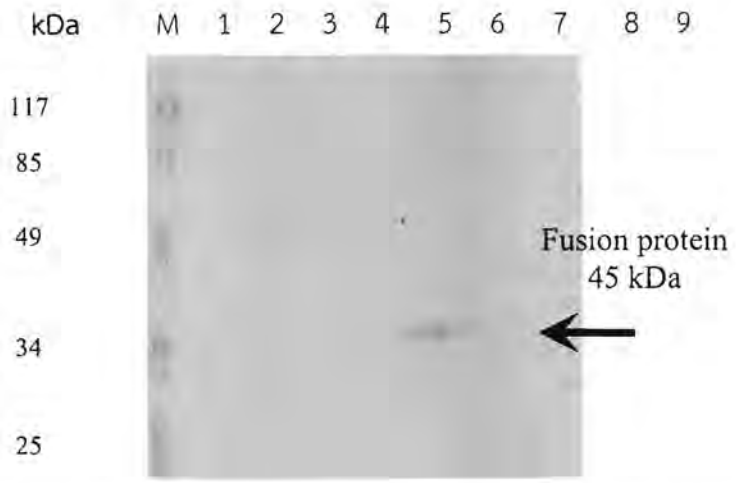


Figure 21 SDS-PAGE analysis of fractions from purification step using Ni-affinity chromatography; lane M, protein molecular weight maker; lane 1-5 corresponding to washing fractions, lane 6-9 corresponding to eluted fractions.

Due to C-terminal of IL2/FUscFv(V_H-V_K) consisted of His·tag that strongly interacts with Ni metal ion 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 (Figure 21). 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 pH 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).

B.2.3.3 *Characterization of the fusion protein for specific binding activity*

The specific binding activity of the fusion protein IL2/FUscFv(V_H-V_K) 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 22). 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 IL2/FUscFv(V_H-V_K) bound to the MK-1 expressing CHO cell but not to the MK-1 non-expressing CHO cell. Therefore, the fusion protein IL2/FUscFv(V_H-V_K) produced by *P. pastoris* strain GS115 in this study possessed binding activity against MK-1 expressing CHO cell.

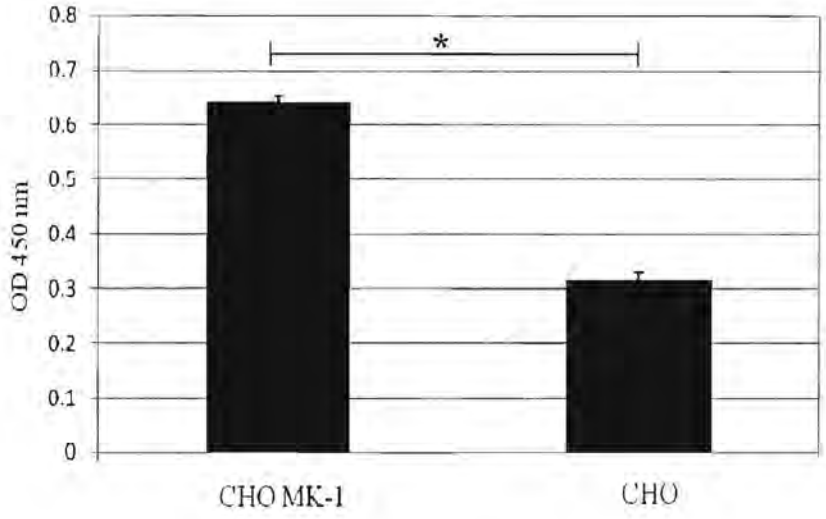


Figure 22 The specific binding activity of IL2/FUscFv(V_H-V_K) to MK-1 expressing and non-expressing CHO cell by cell lysate ELISA method (*, $p<0.05$).

B.3 Discussion

Use of recombinant scFv forms of monoclonal antibodies offers an excellent alternative to the whole antibodies for determining their binding characteristics and particularly for *in vivo* investigation of their therapeutical potential. To be able to carry out such studies, large amounts of recombinant scFv are needed. Production of scFv in microorganisms such as bacteria or yeast is cost effective and relatively high production yields are

often achieved after optimization. Advances in genetic engineering and gene expression systems have led to rapid progress in the development of cytokines fused to antibodies. The goal of this approach against cancer is to concentrate the cytokine around the tumor site and to enhance the host immune response against the tumor (Reisfeld and Gillies, 1992).

The MK-1 antigen, the target molecule of FU-MK-1, is expressed in a majority of carcinomas (Watanabe et al., 1993; Deveci and Devieci, 2007; Ikeda et al., 2009). The MK-1 is encoded by the GA733-2 gene, which is currently being used as a target in clinical trials for gastric, intestinal and biliary cancer treatment with monoclonal antibodies (Tomita et al., 2000). The monoclonal antibody (MAb) FU-MK-1, which recognizes the MK-1 antigen, was established by immunizing a mouse with cancerous ascites derived from a poorly differentiated adenocarcinoma of the stomach (Watanabe et al., 1993). The epitope for MAb FU-MK-1 is present on the distal half of the extracellular domain of the GA733-2 antigen. More than 20 antibodies directed against the GA733-2 antigen have been generated, including HEA125 for Ep-CAM, CO17-1A for 17-1A, and FU-MK-1 for MK-1. It was reported that adenocarcinomas of the hepatobiliary tract are often immunoreactive for FU-MK-1, and this monoclonal antibody is a useful antigenic marker for distinguishing hepatocellular carcinoma from cholangiocarcinoma in the liver (Watanabe et al., 1993).

In the previous study, a mouse/human chimeric antibody, designated Ch FU-MK-1, from FU-MK-1 was developed (Arakawa et al., 1999). In combination with human LAK cells activated with rhIL2, ChFU-MK-1 showed a marked growth inhibition of human MK-1 expressing tumor cells in vitro and in severe combined immunodeficiency mice (Yamamoto et al., 1999). Next, a fusion protein, designated FUscFv/IL2 was generated, which consisted of rhIL1 and an anti-MK-1 scFv antibody derived from FU-MK-1 (Matsumoto et al, 2002). The IL2 was fused to the carboxyl-terminal end of the V_K chain of the scFv with a linker. The resultant FUscFv/IL2 produced by *P. pastoris* still retained the binding activity to cell-surface MK-1 of tumor cells. Matsumoto et al. demonstrated that the FUscFv/IL2 fusion protein was able to effectively suppress tumor growth in SCID mice reconstituted with human LAK cells. It

was concluded that FUscFv/IL2 fusion protein may provide an effective method of targeting therapeutic doses of rhIL2 to MK-1-expressing tumor while significantly reducing systemic toxicity.

In the previous study, *E. coli*-based system has been employed for fusion protein production. However, heterologous protein levels were relatively low with the portion of protein present within insoluble cytoplasmic inclusion bodies. Further improvements of downstream process are necessary. Thus, in this study, a recombinant *P. pastoris* strain GS115 transformed with the plasmid pPICZαA-IL2/FUscFv(V_K-V_H) enabled the expression of IL2/FUscFv(V_K-V_H) as a fusion protein with *myc*-His·tag.

Culture conditions are critical parameters that affect cell growth and the yield of recombinant product. Temperature and pH are among the operating factors that dramatically influence expression levels. The effect of temperature on protein expression by *E. coli* has been widely investigated. Decreasing the temperature usually resulted in an enhancement of the solubility of the protein expressed (Meinander et al., Chen et al., 2000). Using *P. pastoris* expression system, several reports demonstrated that decreasing the temperature from 30 to 25°C, led to increase the expression level over that obtained under 30°C (Chen et al., 2000, Damasceno et al., 2004). Shi et al. also suggested that the optimal cultivation temperature scheme for anti-serpins scFv production involved cultivation in BMGY medium at 30°C to accumulate biomass followed by induction at 15°C (Shi et al., 2003). In this study, the optimal cultivation temperate is consisted of two different temperatures; the optimal temperature for growth was at 30°C and the optimal temperature for secreted fusion protein production was at 25°C.

It was also reported that acidic pH is often used for recombinant protein production and pH 3 was found to be responsible for the optimal production of insulin-like growth factor-I and cytokine growth blocking peptide (Brierley et al., 1994; Koganesawa et al., 2002). Moreover, several reports found significantly improvement of secreted protein production under pH 3 (Sreekrishna and Kropp, 1996, Cregg, 1999; Curvers et al., 2001; Jahic et al., 2003; Damasceno et al., 2004).

The expression level reported in our study is higher than those reported previously. Marty et al. reached 5-20 mg of a scFv antibody/l culture medium (Marty et al., 2001). Lang et al. who investigated the expression of a recombinant Fab fragment by *P. pastoris*, reported an expression level of 0.040 g/l although the culture was carried out in a 5 l fermentor, using the fed batch culture mode (Lang et al., 2001). Hellwig et al. reported an expression level 0.045 g/l of a scFv antibody fragment (Hellwig el al., 2001). Ning et al. reported an equal expression level 0.420 - 0.458 g/l of anti-HBsAg Fab fragment in 5 l fermenter (Ning et al, 2005). Lin et al. reported the expression level 0.373 g/l of Fc fusion protein in fed-batch culture (Lin et al., 2007). Yamakawa also reported the expression level 0.198 g/l of scFv aginst bisphenol A in fed-batch culture (Yamakawa et a., 2007). For comparision, Masumoto et al., reported the expression level only 0.002 g/l of FUscFv/IL2 by *P. pastoris* in shaken flask culture (Masumoto et al., 2002). Here, we report our efforts to express and optimize the conditions to enhance the production of functionalized fusion proteins, IL2/FUscFv(V_H-V_K) in *P. pastoris* and examine parameters including scale up the production from shaken flask culture to 5 l fermenter employing a fed-batch strategy.

B.4 Conclusion

In order to produce IL2/FUscFv(V_H-V_K) 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 IL2/FUscFv(V_H-V_K) 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 to 1.0% (v/v). 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, pH 3 and 4 have shown higher expression of the fusion protein than that of pH between 5 and 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 0.258 ± 0.013 g/l was obtained under pH 3 and 30°C with a methanol concentration of 0.1% (v/v) 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 0.109 ± 0.005 g/l when the modified basal salt medium containing 7.5% (w/v) glycerol was used, whereas the amount of secreted fusion protein was increased up to 0.425 ± 0.002 g/l when BMMY medium containing 5% (w/v) 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 IL2/FUscFv(V_H-V_K) 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 ($p<0.05$).

B.5 Suggestion for Further Work

In the future, the specific binding activity of IL2/FUscFv(V_H-V_K) will be performed by flow cytometry to confirm binding activity of the fusion protein. For the applications of these fusion proteins, collaboration with Faculty of Pharmaceutical or Faculty of Medicine is needed.

B.6 Lists of publications and presentations

Original papers

1. Kongchanasombat, M., Pohthisoong, N., Kuroki, M., Kuroki, M., and **Chanprateep, S.*** Production of sc-Fv antibody-based fusion proteins of IL2/FU-MK-1scFv in *Escherichia coli* (manuscript in preparation)

2. Anuleechana, S., Palaga, T., Kuroki, M., Kuroki, M., and **Chanprateep, S.*** Production and purification of secreted fusion protein IL2/FU-MK-1scFv in *Pichia pastoris* (manuscript in preparation)

International Conferences

1. **Chanprateep, S.***, Kongchanasombat, M., Pohthisoong, N., Kuroki, M. and Kuroki, M. Production of bifunctional sc-Fv antibody-based fusion proteins of IL-2/FU-MK-1(V_H-V_K) and IL-2/FU-MK-1(V_K- V_H) in *Escherichia coli*, Nano in Cancer: Linking Chemistry, Biology, and Clinical Applications in Vivo, Miami, FL, USA (January, 2011) [Poster Presentation]

2. Anuleechana, S., Palaga, T., Kuroki, M., Kuroki, M., and **Chanprateep, S.*** Secreted production of bifunctional sc-Fv antibody-based fusion protein of IL-2 and FU-MK-1(V_H-V_K) in *Pichia pastoris* Nano in Cancer: Linking Chemistry, Biology, and Clinical Applications in Vivo, Miami, FL, USA (January, 2011) [Poster Presentation]

3. REFERENCES

Abe, H., Kuroki, M., Imakiire, T., Yamauchi, Y., Yamada, H., Arakawa, F., Kuroki, M. (2002) Preparation of recombinant MK-1/Ep-CAM and establishment of an ELISA system for determining soluble MK-1/Ep-CAM levels in sera of cancer patients. Journal of Immunological Methods, 270: 227—233.

Abercrombie, A.L., and Ambrose, E.J. (1962) The surface properties of cancer cells: a review. Cancer research, 20: 525—547.

Adams, G.P., and Weiner, L.M. (2005) Monoclonal antibody therapy of cancer. Nature Biotechnology, 23: 1147—1157.

Arakawa, F., Yamamoto, T., Kanda, H., Watanabe, T., and Kuroki, M. (1999) cDNA sequence analysis of monoclonal antibody FU-MK-1 specific for a transmembrane carcinoma-associated antigen, and construction a mous/human chimeric antibody. Hybridoma, 18: 131—138.

Arnau, J., Lauritzen, C., Prtersen, G.E., and Pedersen, J. (2006) Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. Protein Expression and Purification, 48: 1—13.

Aslund, F., Bessette, P.H., Gerogiou, G., and Beckwith, J. (1999) Compositions and methods for production of disulfide bond containing proteins in host cells. innovations, 10: 11–12.

Bessette, P.H., Aslund, F., Beckwith, J., and Georgiou, G. (1999) Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. Proc. Natl. Acad. Sci. USA, 96: 13703–13708.

Blackwell, J.R., Horgan, R. (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. FEBS Letters, 295: 10–12.

Brierley, R.A, Bussineau, C., Kosson, R., Melton, A., Siegel, R.S. (1990) Fermentation development of recombinant Pichia pastoris expressing the heterologous gene: bovine lysozyme. Annuls of the New York Academy of Sciences, 589: 350–362.

Brierley, R.A, Davis, R.G, Holtz, C.G. (1994). Production of insulin like growth factor-I in methylotrophic yeast cells. US Patent No. 5324639.

Carnemolla, B., Borsi, L., Balza, E., Castellani, P., Meazza, R., Berndt, A., Ferrini, S., Kosmehl, H., Neri, D., and Zardi, L. (2002) Enhancement of the antitumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. Blood, 99: 1659—1665.

Carter, P.J. (2006) Potent antibody therapeutics by design. Nature Reviews Immunology, 6(5):343—357.

Chen, Y., Jin, M., Egborge, T., Coppola, G., Andre, J.,and Calhoun, D.H. (2000) Expression and characterization of glycosylated and catalytically active recombinant human β-galactosidase A produced in *Pichia pastoris*. Protein Expression and Purification, 20: 472–84.

Chung, B.H. and Park, K.S. (1997) Simple approach to reducing proteolysis during secretory production of human parathyroid hormone in *Saccharomyces cerevisiae*. Biotechnology and Bioengineering, 57: 245–249.

Couderc, R. and Baratti, J. (1980) Oxidation of methanol by the yeast, *Pichia pastoris*. Purification and properties of the alcohol oxidase. Agricultural and Biological Chemistry, 44: 2279—2289.

Cregg, J. M. Expression in the methylotrophic yeast *Pichia pastoris*. In Gene Expression Systems: Using Nature for the Art of Expression; Fernandez, J.

M.; Hoeffler, J. P., Eds.; Academic Press: San Diego, London, 1999; pp 157—191.

Cregg, J.M., Cereghino, J.L., Shi, J., and Higgins, D.R. (2000) Recombinant protein expression in *Pichia pastoris*. Molecular Biotechnology, 16: 23–52.

Cregg, J.M. and Higgins, D.R. (1995) Production of foreign proteins in the yeast *Pichia pastoris*. Canadian Journal of Botany, 73: 91—97.

Cregg, J.M. and Madden, K.R. Development of the methlyotrophic yeast, *Pichia pastoris*, as a host system for the production of foreign proteins, in: G. Pierce (Ed.), Development in industrial microbiology, vol. 29, Elsevier Science, Amsterdam, 1988, pp. 33-41.

Cregg, J.M., Vedvick, T.S., Raschke, W.C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio/Technol. 11: 905–910.

Cunha, A.E., Clemente, J.J., Gomes, R., Pinto, F., Thomaz, M., Miranda, S., Pinto, R., Moosmayer, D., Donner, P., Carrondo, M.J.T. (2004) Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. Biotechnology and Bioengineering, 86(4): 458–467.

Curvers, S., Brixius, P., Klauser, T., Tho1mmes, J., Weuster-Botz, D., Takors, R., and Wandrey, C. (2001) Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing Part 1 Fermentation. Biotechnology Progress, 17: 495–502.

Couderc, R. and Baratti, J. (1980) Oxidation of methanol by the yeast, *Pichia pastoris*. Purification and properties of the alcohol oxidase, Agricultural and Biological Chemistry, 44: 2279–2289.

Daly, R. and Hearn, M.T.W. (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production, Journal of Molecular Recognition, 18(2): 119–138.

Damasceno, L.M., Pla, I., Chang, H.J., Cohen, L., Ritter, G., Old, L.J., and Batt, C.A. (2004) An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. Protein Expression and Purification, 37: 18–26.

Deckert, P.M., Renner, C., Cohen, L.S., Jungbluth, A., Ritter, G., Bertino, J.R., Old, L.J. and, Welt, S. (2003) A33scFv–cytosine deaminase: a recombinant protein construct for antibody-directed enzyme-prodrug therapy. British Journal of Cancer, 88: 937–939.

Deveci, M.S. and Deveci, G. (2007) Prognostic value of p53 protein and MK-1 (a tumor-associated antigen) expression in gastric carcinoma. Gastric Cancer, 10: 112–116.

Esoisutim D. and Cgatterjee, D.K. (2006) Enhancement of soluble protein expression through the use of fusion tags. Current Opinion in Biotechnology, 17: 353–358.

Fang, J., Jin, H.B., and Song, J.D. (2003) Construction, expression and tumor targeting of a single-chain Fv against human colorectal carcinoma. World Journal of Gastroenterology, 9: 726–730.

Freyer, F.M., Vazquez, J.E., Ayala, M., Canaan-Haden, L., Bell, H., Rodriguez, I., Gonzalez, A., Cintado, A., and Gavilondo, J.V. (2000) Very high expression of an anti-carcinoembryoic antigen single chain Fv antibody fragment in the yeast *Pichia pastoris*. Journal of Biotechnology, 76: 157–163.

Gurramkonda, C., Adnan, A., Gabel, T., Lunsdorf, H., Ross, A., Nemani, S.K., Swaminathan, S., Khanna, N., and Rinas, U. (2009) Simple high-cell density

fed-batch technique for high-level recombinant protein production with Pichia pastoris: Application to intracellular production of Hepatitis B surface antigen. Microbial Cell Factories, 8: 13—20.

Gupta, N., and Srivastava, A. (2006) Monoclonal antibodies: Targeted therapy. Indian Journal of Pharmacology, 38: 390—396.

Hellwig, S., Emde, F., Raven, N.P., Henke, M., Van Der Logt, P., Fischer, R. (2001) Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. Biotechnology and Bioengineering, 74: 344–52.

Hearly, D., Sears, H.F., Iliopoulos, D., Steplewski, Z., and Koprowski, H. (1991) Initial clinical evaluation of two murine IgG2a monoclonal antibodies for immunotherapy of gastrointestinal carcinoma. American Journal of Clinical Oncology, 14: 371–378.

Hellwig, S., Emde, F., Raven, N.P., Henke, M., Logt, P.V., and Fischer, R. (2001) Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations, Biotechnology and Bioengineering, 74: 344–352.

Herlyn, M, Steplewski, Z., Herlyn, D., and Korpwski, H. (1979) Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. Proc. Natl Acad Sci USA, 76: 1438–1452.

Holliger, P. and Hudson, P.J. (2005) Engineered antibody fragments and the rise of single domain. Nature Biotechnology, 23(9): 1126–1136.

Hua, X., O'Dwyera, R., and Wall, J.G. (2005) Cloning, expression and characterization of a single-chain Fv antibody fragment against domoic acid in *Escherichia coli*. Journal of Biotechnology, 120: 38–45.

Hwang, W.Y., and Foote, J. (2005) Immunogenicity of engineered antibody. Methods, 36: 3–10.

Ikeda, T., Nakayama, Y., Hamada, Y., Takeshita, M., Iwasaki, H., Masehiro, K., Yamashita, Y., Kuroki, M., and Ikeda, S. (2009) FU-MK-1 expression in human gallbladder carcinoma. American Journal of Clinical Pathology, 132: 111–117.

Jahic, M.,Gustavsson, M., Jansen, A., Martinelle, M., and Enfors, S. (2003) Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. Journal of Biotechnology, 102(1): 45–53.

Jungo, C., Marison, I., and Stockar, U.V. (2007) Regulation of alcohol oxidase of a recombinant *Pichia pastoris* Mut+ strain in transient continuous cultures. Journal of Biotechnology, 130: 236—246.

Kim, S.J., Park, Y., and Hong, H.J. (2005) Antibody engineering for the development of therapeutic antibodies. Molecular Cell, 20: 17—29.

Koganesawa, N., Aizava, T., Shimojo, H., Miura, K., Ohnishid, A., Demuraa, M., Hayakawad, Y., Nitta, K., Kawano, K. (2002) Expression and purification of a small cytokine growth-blocking peptide from army worm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris*. Protein Expression and Purification, 25: 416–425.

Krishnaswamy, S., Kabir, M.E., Miyamoto, M., Furuichi, Y., and Komiyama, T. (2009) Cloning antifungal single chain fragment variable antibodies by phage display and competitive panning elution. Analytical Biochemistry, 395(1): 16—24.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680–685.

Lamberski, J.A., Thompson, N.E., and Burgess, R.R. (2006) Expression and purification of a single-chain variable fragment antibody derived from a polyol-responsive monoclonal antibody. Protein Expression and Purification, 47: 82—92.

Lange, S., Schmitt, J., Schmid, R.D. (2001) High-yield Expression of the Recombinant, Atrazine-specific Fab Fragment K411B by the Methylotrophic Yeast *Pichia pastoris.* Journal Immunological Methods, 255: 103—114.

Larson, L.N., Johansson, C., Lindholm, L., Holmgren, J. (1988) Mouse monoclonal antibodies for experimental immunotherapy promotes killing of tumor cells. International Journal of Cancer, 42: 877.

Li, Z., Xiong, F., Lin, Q., Anjou, M., Daugulis, A.J., Yang, D.S.C. and Hew, C.L. (2001). Low temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Expression and Purification, 21: 438—445.

Liao, S., Khare, P.D., Arakawa, F., Kuroki, M., Hirose, Y., Fujimura, S., Tomita, Y., and Kuroki, M. (2001) Targeting of LAK activity to CEA-expressing tumor cells with an anti-CEA scFv/IL2 fusion protein. Anticancer Research, 21: 1673–1680.

Lichty, J.J., Malecki, J.L., Agnew, H.D., Michelson-Horowitz, D.J., and Tan, S. (2005) Comparison of affinity tags for protein purification. Protein Expression and Purification, 41: 98—105.

Lin Cereghino, G.P., Lin Cereghino, J., Ilgen, C., and Cregg, J.M. (2002) Production of recombinant proteins in fermenter culture of the yeast *Pichia pastoris*. Current Opinion in Biotechnology, 13: 329–332.

Lin Cereghino, J. and Cregg, J.M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiology Reviews, 24: 45–66.

Lin, H., Kim, T., Xiong, F., Yang, X. (2007) Enhancing the production of Fc fusion protein in fed-batch fermentation of *Pichia pastoris* by design of experiments. Biotechnology Progress, 23(3): 621—625.

LoBuglio, A.F., Wheeler, R.H., Trang, J., Haynes, A., Rogers, K., Harvey, E.B., Sun, L., Ghrayeb, J., and Khazaeli, M.B. (1989) Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. Proc. Natl. Acad Sci. USA, 86: 4220—4224.

Maggon, K. Global Pharmaceutical Market Review & World Top Ten/Twenty Drugs 2008: World Best Selling Human Medicinal Brands 2008, Top Ten, Top Twenty, First Global Market Report. Version 156. Knol. 2010 Jan 31. Available from: http://knol.google.com/k/krishan-maggon/global-pharmaceutical-market- (Accessed on 10 May 2011)

Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiological Reviews, 60: 512—538.

Marty, C.; Scheidegger, P.; Ballmer-Hofer, K.; Klemenz, R.; Schwendener, R.A. (2001) Production of functionalized single-chain Fv antibodyfragments binding to the ED-B domain of the Bisoform of fibronectin in *Pichia pastoris*. Protein Expression and Purification, 1: 156–164.

Matsumoto, H., Liao, S., Arakawa, F., Ueno, A., Abe, H., Awasthi, A., Kuroki, M., and Kuroki, M. (2002) Targeting of interleukin-2 to human MK-1-expressing carcinoma by fusion with a single-chain Fv of anti-MK-1 antibody. Anticancer Research, 22: 2001–2008.

Maynard, J. and Georgiou, G. (2000) Antibody engineering, Annual Review of Biomedical Engineering, 2: 339–76.

Meinander, N.Q., Jeppson, M., and Sogaard, M. (2000) Optimisation of the solubility of the recombinant Itk kinase domain in *Escherichia coli*. Recombinant protein production with prokaryotic and eukaryotic cells. Dordrecht: Kluwer Academic Publishers; p.159–169.

Miller, K.D., Guyon, V., Evans, J.N., Shuttleworth, W.A., and Taylor, L.P. (1999) Purification, cloning and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase expressed in the male gametophyte of *Petunia hybrida*. Journal of Biological Chemistry, 274, 34011–34019.

Miller, K.D., Weaver-Feldhaus, J., Gray, S.A., and Siegel, R.W. (2005) Production, purification, and characterization of human scFv antibodies expressed in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Escherichia coli*. Protein Expression and Purification, 42: 255–267.

Ning, D., Junjian, X., Qing, Z., Sheng, X., Wenyin, C., Guirong, R., and Xunzhang, W. (2005) Production of recombinant humanized anti-HBsAg Fab fragment from *Pichia pastoris* by fermentation. Journal of Biochemistry and Molecular Biology, 38: 294–299.

Olafsen, T., Sirk, S.J., Betting, D.J., Kenanova, V.E., Bauer, K.B., Ladno, W., Raubitschek, A.A., Timmerman. J.M., Wu, A.M. (2010) Immuno PET imaging of B-cell lymphoma using 124I-anti-CD20 scFv 79 immers (diabodies). Protein Engineering, Design and Selection, 23: 243–249.

Peter, K., Graeber, J., Kipriyanov, S., Zewe-Welschof, M., Runge, M.S., Kuļbler, W., Little, M., Bode, C. (2000) Construction and functional evaluation of a single-chain antibody fusion protein with fibrin targeting and thrombin inhibition after activation by factor Xa. Circulation, 101: 1158–64.

Prinz, W.A., Aslund, F., Holmgern, A., and Beckwith, J. (1997) The role of the Thioredoxin and Glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. Journal of Biological Chemistry, 272: 15661–15667.

Reisfld, R.A. and Gillies, S.D. (1992) Antibody-interleukin 2 fusion proteins: a new approach to cancer therapy. Journal of Clinical Laboratory Analysis, 10: 160–166.

Romanos, M.A., Scorer, C.A., and Clare, J.J. (1992) Foreign gene expression in yeast: a review. Yeast, 8: 423–488.

Sabzevari, H., Gilliest, S.D., Mueller, B.M., Pancook, J.D., and Reisfeld, R.A. (1994) A recombinant antibody-interleukin 2 fusion protein suppresses growth of hepatic human neuroblastoma metastases in severe combined immunodeficiency mice. Proc. Nati. Acad. Sci. USA, 91; 9626–9630.

Schellekens, H. Immunogenicity of therapeutic proteins (2002) Clinical Implications and Future prospects. Clinical Therapeutics, 24: 1720–1740.

Schlieker, C., Bukau, B., and Mogk, A. (2002) Prevention and reversion of protein aggregation by molecular chaperones in the *E. coli* cytosol: implications for their applicability in Biotechnology. Journal of Biotechnology, 96: 13–21.

Schroff, R.W., Foon, K.A., Beatty, S.M., Oldham, R.K., Morgan, A.C., Jr (1985) Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. Cancer Research, 45: 879.

Shen, YC., Wang, XH., Wang, XM., Chen, ZL, Shen, XP., Zhao, CC., Li, J. (2006) High efficient mammalian expression and secretion of a functional

humanized single-chain Fv/human interleukin-2 molecules. World Journal of Gastroenterology, 12: 3859–3865.

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S.M., and Hegedus, D. (2003) Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*, Protein Expression and Purification, 28: 321–330.

Sinha, J., Plantz, B.A., Inan, M., and Meagher M.M. (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: Case study with recombinant ovine interferon-τ. Biotechnology and Bioengineering, 89(1): 102–112.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Weirschke, J.D., Subramaniam, A., and Birkenberger, L.A. (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene, 190: 55–62.

Sreekrishna, K.; Kropp, K. E. *Pichia pastoris*. In Nonconventional Yeasts in Biotechnology: A Handbook; Wolf, K., Ed.; Springer: Berlin, Heidelberg, New York, 1996; pp 293–311.

Szala, S., Froehlich, M., Kasai, Y., Steplewski, Z., Koprowski, H., and Linnenbach, A.J. (1990) Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2. Proc. Natl Acad Sci USA, 87: 3542–3546.

Tanfous, N.G., Kallel H., Jarboui, M.A., and Fathallah D.M. (2006) Expression in *Pichia pastoris* of a recombinant scFv form of Mab 107, an anti human CD11b integrin antibody. Enzyme and Microbial Technology, 38: 636–642.

Terpe, K. (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Applied Microbiology and Biotechnology, 60: 523–533.

Trang, J.M., LoBuglio, A.F., Wheeler, R.H., Harvey, E.B., Sun, L., Ghrayeb, J., and Khazaeli, M.B. (1990) Pharmacokinetics of a mouse/human chimeric monoclonal antibody (C-17-1A) in metastatic adenocarcinoma patients. Pharmaceutical Research, 7: 587–591.

Tomita, Y., Arakawa, F., Yamamoto, T., Kuwahara, M., Watanabe, R., Iwasaki, H. Kikuchi, M., and Kuroki, M. (2000) Molecular identification of a human carcinoma-associated glycoprotein antigen recognized by mouse monoclonal antibody FU-MK-1. Japanese Journal of Cancer Research, 91: 231–238.

Ueno, A., Arakawa, F., Abe, H., Matsumoto, H., Kudo, T., Asano, R., Tsumoto, K., Kumagai, I., Kuroki, M., and Kuroki, M. (2002) T-cell immunotherapy for human MK-1-expressing tumors using a fusion protein of superantigen SEA and anti-MK-1 scFv antibody. Anticancer Research, 22: 769–776.

Van der Klei, I.J., Bystrykh, L.V., and Harder, W. (1990) Alcohol oxidase from *Hansenula polymorpha* CBS 4732. Methods in Enzymology, 188: 420—427.

Varki, N.M., Reisfeld, R.A, and Walker, L.E. (1984) Antigens associated with a human lung adenocarcinoma defined by monoclonal antibodies. Cancer Research, 44: 681—687.

Wainberg, Z. and Hecht, J.R. (2006) Panitumumab in colon cancer: a review and summary of ongoing trials. Expert opinion on biological therapy, 6(11):1229—1235.

Wang, Y., Wang, K., Jette, D.C., Wishart, D.S. (2001) Production of an anti-prostate-specific antigen single-chain antibodyfragment from *Pichia pastoris*, Protein Expression and Purification, 23: 419–425.

Watanabe, R., Johzaki, H., Iwasaki, H., Kukuchi, M., Watanabe, R., Iwasaki, H., Kikuchi, M., and Kuroki, M. (2000) Molecular identification of a human carcinoma-associated glycoprotein antigen recongnized by mouse monoclonal antibody FU-MK-1. Japanese Journal of Cancer Research, 91: 231—238.

Weisser, N.E. and Hall, J.C. (2009) Applications of single-chain variable fragment antibodies in therapeutics and diagnostics, Biotehcnological Advances, 27(4): 502—520.

Woan, K., and Reddy, V. (2007) Potential therapeutic role of natural killer cells in cancer. Expert Opinion on Biological Therapy, 7: 17—29.

Wu, D., Yu, X.W., Wang, T.C., Wang, R., and Xu, Y. (2011) High yield *Rhizopus chinenisis* prolipase production in *Pichia pastoris*. Impact of methanol concentration, Biotechnology and Bioprocess Engineering, 16(2): 305—311.

Xiang, R., Lode, H.N., Dolman, C.S., Dreier, T., Varki, N.M., Qian, X., Lo, K.M., Lan, Y., Super, M., Gillies, S.D., and Reisfeld, R.A. (1997) Elimination of established murine colon carcinoma metastases by antibody- interleukin 2 fusion protein therapy. Cancer Research, 57: 4948—4955.

Xiong, H., Li, S., Yang, Z., Burgess, R.R., and Dynan, W.S. (2009) *E. coli* expression of a soluble, active single-chain antibody variable fragment containing a nuclear localization signal. Protein Expression and Purification, 66: 172—180.

Yamamoto, T., Arakawa, F., Nakamura K., Senba, T., Tomiya, T., Ikeda, S., and Kuroki, M. (1999) Enhanced antitumor activity of a combination treatment with a mouse/human chimeric anti-MK-1 antibody and lymphokine-activated killer cells in vitro and in a sever combined immunodifficient

mouse xenograft model. Cancer Immunologial Immunotherpy, 48: 165—171.

Yamawaki, S., Matsumoto, T., Ohnishi, Y., Kumada, Y., Shiomi, N., Katsuda, T., Lee, E.K., and Katoh, S. (2007) Production of single-chain variable fragment antibody (scFv) in fed-batch and continuous culture of *Pichia pastoris* by two different methanol feeding methods. Journal of Bioscience and Bioengineering, 104(5): 403—407.

Yang, D.F., Zhu, H.F., Wang, Z.H., Shen, G.X., and Tian. D.Y. (2005) Construction of single chain Fv antibody against transferring receptor and its protein fusion with alkaline phosphatase. World Journal of Gastroenterology, 11: 3300—3303.

Yi, K., Chung, J., Kim, H., Kim, I., Jung, H., Kim, J., Choi, I., Suh, P., and Chung, H. (1999) Expression and characterization of anti-NCA-95 scFv (CEA 79 scFv) in a prokaryotic expression vector modified to contain a *Sfi* I and *Not* I site. Hybridoma, 18(3): 243—249.

Zhang, X., Feng, J., Ye, X., Yao, Y., Zhou, P., Chen, X. (2006) Development of an immunocytokine, IL2-183B2scFv, for targeted immunotherapy of ovarian cancer. Gynecologic Oncology, 103: 848—852.

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4. Process development for production of therapeutic agents in *Pichia pastoris* and *E. coli*

SELECTED PUBLICATIONS:

1. Chanprateep, S.* (2010) Current Trends in Biodegradable Polyhydroxyalkanoates, J. Bioscience and Bioengineering 110(6), 621-632. [Impact Factor = 1.749] (Review article)

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3. Chanprateep, S.*, Katakura, Y., Shimizu, H., Visetkoop, S., Kulpreecha, S., and Shioya, S. (2008) Characterization of New Isolated Ralstonia eutropha strain A-04 and Kinetic Study of Biodegradable Copolyester Poly(3-Hydroxybutyrate-co-4-Hydroxybutyrate) Production. J. Industrial Microbiology & Biotechnology 35(11), 1205-1215. [Impact Factor = 1.919]

4. Chanprateep, S.*, Shimizu, H. and Shioya, S*. (2006) Characterization and Enzymatic Degradation of Microbial Copolyester P(3HB-co-3HV)s Produced by Metabolic Reaction Model based System. Polymer Degradation and Stability 91(12), 2941-2950. [Impact Factor = 2.320]

5. Chanprateep, S. and Kulpreecha, S. * (2006) Production and characterization of biodegradable terpolymer poly(3-hydroxybutyrate-co-3-hydrobyvalerate-co-4-hydroxybutyrate) by Alcaligenes sp. A-04. J. Bioscience and Bioengineering 101(1), 51-56. [Impact Factor = 1.702]

6. Shimizu, H., Chanprateep, S., Kulpreecha, S., Boonruangthavorn, A., and Shioya, S. * (2005) Development of production processes of a biodegradable polymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate): Metabolic pathway analysis, quality control of polymer, and use of starch. Biotechnology for sustainable utilization of biological resources in the tropics. (ed. T. Seki) 17, 78-83.

7. Chanprateep, S., Kikuya, K., Seki, S, Takawa, S., Shimizu, H., and Shioya, S. * (2003) Non-isothermal crystallization kinetics of biodegradable random poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and block one. J. Chemical Engineering Japan 36 (6), 639-646. [Impact Factor = 0.569]

8. Chanprateep, S., Kikuya, K., Shimizu, H., and Shioya, S. * (2002) Model predictive control for biodegradable polyhydroxyalkanoates production in fed-batch culture. J. Biotechnology 95 (2), 157 - 169.

9. Chanprateep, S., Abe, N., Shimizu, H., and Shioya, S. * (2001) Multivariable control of alcohol concentrations in the production of polyhydroxyalkanoates (PHAs) by Paracoccus denitrificans. Biotechnol. Bioeng. 74, 116-124.

10. Chanprateep, S., Shimizu, H., and Shioya, S. * (1998) Optimal production of biodegradable copolymer poly(3-hydroxybutyrate-co-4-hydroxybutyrate), P(3HB-co-4HB), by Alcaligenes sp. A-04, Annual reports of ICBiotechnology 21. 281-292.

Supplemented original paper

1. Napathorn, C. * and Chanprateep, S. (2011, February) What Types of Factors Can Influence the Strength of Labor Unions in Companies and State

Enterprises in Thailand?, International Journal of Business and Management. 6(2) 112-124.

2. Napathorn, C. * and Chanprateep, S. (2011, June) Recent Labor Relations and Collective Bargaining Issues in Thailand, Interdisciplinary Journal of Research in Business. 1(6) 66-81.

3. Napathorn, C. * and Chanprateep, S. (2011, December) Reasons Why Thai Employees Want to Join Labor Unions: Evidence in Private Companies and State Enterprises, International Journal of Business and Management. 6(12) 58-64).

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