PRODUCTION AND PURIFICATION OF RECOMBINANT ENTEROKINASE LIGHT CHAIN (rEK_L) FROM *Pichia pastoris*

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การผลิตและการทำบริสุทธิ์เอ็นไซม์รีคอมบิแนนท์เทอโรไคเนสสายสั้น (rEK_L) จากเชื้อ *Pichia pastoris*

เหงียน ทิ แทง ซุง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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PRODUCTION AND PURIFICATION OF RECOMBINANT ENTEROKINASE LIGHT CHAIN (rEK_L) FROM *Pichia pastoris*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เหงียน ทิ แทง ซุง : การผลิตและการทำบริสุทธิ์เอ็นไซม์รีคอมบิแนนท์เทอโรไคเนสสายสั้น (rEK_L) จากเชื้อ *Pichia pastoris* (PRODUCTION AND PURIFICATION OF RECOMBINANT ENTEROKINASE LIGHT CHAIN (rEK_L) FROM *Pichia pastoris*). อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.อภิชาติ บุญทาวัน, 87 หน้า

ในปัจจุบัน เอ็นเทอโรไคเนสได้กลายเป็นเครื่องมือที่สำคัญสำหรับการตัดฟิวชั่นรีคอมบิแนนท์โปรตีนในหลอดทดลองเนื่องจากมีความจำเพาะเจาะจงสูงต่อตำแหน่งตัดจำเพาะ ($(Asp)_4$–Lys) และ มีความคงทนต่อสภาวะในการทำปฏิกิริยาที่หลากหลาย ในการทดลองนี้ ได้ทำการผลิตเอ็นไซม์รีคอมบิแนนท์เอ็นเทอร์โรไคเนสสายสั้น (rEK_L) โดยเชื้อ *Pichia pastoris* Y11430 จากการทดลองกระบวนการหมักแบบกึ่งกะอย่างง่าย พบว่าสามารถประสบความสำเร็จในการเลี้ยงเซลล์ให้ได้ความเข้มข้นสูง โดยมีการควบคุมอัตราการเจริญจำเพาะคงที่ ที่แตกต่างกันดังนี้คือ 0.006, 0.0075, และ 0.0105 ต่อชั่วโมง ซึ่งค่าเหล่านี้ได้ถูกเลือกนำมาใช้การให้อาหารที่มีเมธานอลเป็นส่วนประกอบ (อาหาร MF) แบบเอ็กโปเนนเชียลในช่วงของการผลิตด้วยเมธานอล ที่อัตราการเจริญจำเพาะคงที่ 0.0075 ต่อชั่วโมง พบว่าความหนาแน่นของเซลล์สูงสุด 128 กรัมต่อลิตร ความเข้มข้นโดยรวมของโปรตีน 343.19 มิลลิกรัมต่อลิตร และ กิจกรรมของเอ็นไซม์อยู่ที่ 38,125 หน่วยต่อมิลลิลิตร ตามลำดับ เมื่อทำการเปรียบเทียบกับค่าอัตราการเจริญจำเพาะคงที่ 0.0105 ต่อชั่วโมง พบว่าเกิดการยับยั้งอย่างรุนแรงต่อการเจริญของเซลล์และมีการผลิตผลิตภัณฑ์ที่ต่ำ เนื่องจากการใช้อัตราการป้อนเมธานอลที่สูงเกิดไป สำหรับการป้อนที่อัตราการเจริญจำเพาะคงที่ 0.006 ต่อชั่วโมง พบว่าความเข้มข้นของเซลล์มีค่าต่ำสุด เนื่องจากอัตราการป้อนที่ต่ำ ที่สุดนั่นเอง ถึงแม้ว่าอัตราการเจริญจำเพาะคงที่ที่ 0.006 ต่อชั่วโมง จะไม่ทำให้การสะสมของรีคอมบิแนนท์เอ็นเทอร์โรไคเนส สูงสุด แต่ทำให้การแสดงออกจำเพาะของเอ็นไซม์มีค่าสูงสุดที่ 389,326 หน่วยต่อมิลลิกรัมโปรตีน เมื่อเปรียบเทียบกับ 122,975 หน่วยต่อมิลลิกรัมโปรตีน ที่อัตราการเจริญจำเพาะคงที่ 0.0075 ต่อชั่วโมง หลังจากผ่านชั่วโมงที่ 117 ของเวลาชักนำให้เกิดการแสดงออกของเอ็นไซม์ นอกจากนี้ การเพิ่มขึ้นของเวลาชักนำจนถึงชั่วโมงที่ 117 สำหรับค่าอัตราการเจริญจำเพาะคงที่ของทั้ง 0.006 และ 0.0075 ต่อชั่วโมง ส่งผลให้มีการเพิ่มขึ้นของความหนาแน่นของเซลล์ ความเข้มข้นโดยรวมของโปรตีน การสะสมของเอ็นไซม์ โดยเฉพาะอย่างยิ่งการแสดงออกจำเพาะของเอ็นไซม์รีคอมบิแนนท์เอ็นเทอร์โรไคเนสสายสั้น ซึ่งเป็นค่าที่สูงกว่ารายงานการวิจัยก่อนหน้า เป็นอย่างมาก

ได้มีการทำบริสุทธิ์เอ็นไซม์รีคอมบิแนนท์เอ็นเทอร์โรไคเนสสายสั้น โดยใช้เทคนิคโครมาโตกร๊าฟฟี่แบบแลกเปลี่ยนประจุลบ (SP-FF คอลัมน์) ซึ่งผลการทดลองพบว่า ได้ ปริมาณของเอ็นไซม์รีคอมบิแนนท์เอ็นเทอร์โรไคเนสสายสั้นบริสุทธิ์จำนวน 3,542 ไมโครกรัม จากของเหลวใส

330 มิลลิลิตรที่ได้จากอัตราการเจริญจำเพาะที่ 0.0075 ต่อชั่วโมง นอกจากนี้ รีคอมบิแนนท์ฟิวชันโปรตีน Os1BGlu4-Trx จากข้าวได้ถูกตัดจนเกือบสมบูรณ์โดยใช้เอ็นไซม์ที่ผ่านการทำบริสุทธิ์นี้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NGUYEN THI THANH DUNG : PRODUCTION AND PURIFICATION OF RECOMBINANT ENTEROKINASE LIGHT CHAIN (rEK_L) FROM *Pichia pastoris*, THESIS ADVISOR : ASST. PROF. APICHAT BOONTAWAN, Ph.D., 87 PP.

ENTEROKINASE/HIGH CELL DENSITY/PICHIA PASTORIS/ION EXCHANGE CHROMATOGRAPHY

Recently, enterokinase has become a useful tool for an *in vitro* digestion of recombinant fusion protein because of its high specificity for the recognition sequences $(Asp)_4$–Lys, and its tolerance to a wide range of reaction conditions. In this work, a high activity recombinant enterokinase light chain (rEK_L) was produced by *Pichia pastoris* Y11430. With a simple fed-batch technique, high cell density cultivation was successfully achieved. Different constant specific growth rates (μ_{set}) 0.006, 0.0075, and 0.0105 hr^{-1} were chosen to pre-determine the exponential feeding strategy of methanol feed medium (MF medium) during the methanol production phase. At μ_{set} 0.0075 hr^{1}, the highest cell density, total protein concentration, and accumulation of the rEK_L were obtained at approximately 128 $g.L^{-1}$, 343.19 $mg.L^{-1}$ and 38125 $U.mL^{-1}$, respectively. In comparison, the μ_{set} 0.0105 hr^{-1} resulted in severe inhibition of cell growth and low product formation due to excessive feeding rate. The feeding rate at μ_{set} 0.006 hr^{-1} resulted in the lowest cell concentration clearly due to the lowest feeding rate. The highest rEK_L accumulation could not be obtained at μ_{set} 0.006 hr^{-1}; however, the highest specific activity reached the maximum value of 389,326 $U.mg^{-1}$ proteins, compared to 122,975 $U.mg^{-1}$proteins at the μ_{set} 0.0075 hr^{1}

after 117 hr of the induction time. In addition, an increase of the induction time to 117 hr for both μ_{set} 0.006 and 0.0075 hr^{-1} resulted in the higher cell density, total protein concentration, rEK_L accumulation, and specially specific activity of rEK_L which was much higher than all previously reported articles.

Finally, purification of the recombinant EK_L was performed using a simple procedure based on the cation exchange chromatography (SP_FF column). The experimental result showed that 3,542 µg pure and active rEK_L was obtained from 330 ml culture broth supernatant in the process at μ_{set} 0.0075 $hr^{-1}$and that the recombinant fusion protein, rice Os1BGlu-Trx, was completely cleaved by using this purified rEK_L.

School of Biotechnology

Academic Year

Student's Signature_____

Advisor's Signature _____

Co-advisor's Signature_____

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Nguyen Thi Thanh Dung

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LIST OF ABBREVIATIONS

AOX	=	alcohol oxidase enzyme
aox1	=	alcohol oxidase 1 gene
aox2	=	alcohol oxidase 2 gene
DCW	=	dry cell weight
DEAE	=	diethylaminoethyl cellulose
DO	=	dissolved oxygen
DOT	=	dissolved oxygen tension (%)
F	=	volumetric feeding rate ($L.hr^{-1}$)
GBS	=	Glycerol basal salt
GF	=	glycerol feed medium
HPLB	=	high pressure fed-batch
MF	=	methanol feed medium
MLFB	=	methanol-limited fed-batch
m_s	=	mass flow of substrate (methanol) ($g.hr^{-1}$)
OLFB	=	oxygen-limited fed-batch
OUR	=	oxygen uptake rate
P	=	air pressure inside the bioreactor (bar)
P	=	product concentration ($g.L^{-1}$ or $U.L^{-1}$)
q_m	=	maintenance coefficient ($g.g^{-1}hr^{-1}$)
rEK_L	=	recombinant enterokinase light chain
S_F	=	methanol concentration in solution ($g.L^{-1}$)

LIST OF ABBREVIATIONS (Continued)

SP_FF	=	Sulphopropyl Fast Flow
STI	=	soybean trypsin inhibitor
TLFB	=	temperature limited fed-batch
V	=	medium volume (L)
V_0	=	initial medium volume (L)
X	=	biomass concentration ($g.L^{-1}$)
X_0	=	initial biomass concentration ($g.L^{-1}$)
$Y_{P/S}$	=	production yield on methanol ($U.g^{-1}$)
$Y_{X/S}$	=	biomass yield on methanol ($g.g^{-1}$)
μ	=	specific growth rate (hr^{-1})
μ_{set}	=	constant specific growth rates (hr^{-1})

CHAPTER I

INTRODUCTION

Enteropeptidase (enterokinase, EK) is a heterodimeric serine protease of the intestinal brush border that activates trypsinogen by highly specific cleavage of the trypsinogen activation peptide following the sequence $(Asp)_4$-Lys. Recently, the enterokinase received an increasing interest in biopharmaceutical and biotechnological industries for cleaving fusion proteins because of its high degree of specificity to recognize the sequence and its tolerance to a wide range of reaction conditions (Song *et al*., 2002). In addition, most commercial EK are purified holoenzymes from bovine or porcine intestines which can be obtained only in very small quantities, making its production cost very expensive. Even highly purified preparations are prone to be contaminated by traces of other gut proteases (Yuan and Hua, 2002). As a result, recombinant enterokinase has been used to solve this problem. However, the application of recombinant enterokinase light chain (rEK_L) has not been fully reached because of the low expression level and still high production cost (Suh *et al*, 2005). Therefore, the expression of rEK_L gene in order to obtain high quality and large amount of this enzyme should be studied.

Many heterologous hosts have been used to produce rEK_L. Mammalian COS was first used to produce rEK_L but very low levels of secreted protein and activity were obtained (LaVallie *et al*., 1993). It also showed little activity of bovine rEK_L

with the prokaryotic expression (Yuan and Hua, 2002; Tan *et al*., 2007). In addition, production of rEK_L in *Aspergillus niger* and *Saccharomyces cerevisiae* using complicated medium gave very low concentration of only 1.9 and 3.8 $mg.L^{-1}$, respectively (Svetina *et al*., 2000; Kim *et al*., 2005). *Pichia pastoris* has become a viable host for the heterologous expression of functional rEK_L because of its high production yield, correct folding and post-translational modification (Peng *et al*., 2004; Zhang *et al*., 2008).

Although rEK_L has increasing applications, the price of rEK_L is still very high because of the lack of researches which focus on high production yield and efficient purification processes. According to the successful work in cloning Thai bovine rEK_L and expression in *Pichia pastoris* strain Y11430 (Kupradit, 2006), bioprocess developments for fermentation and purification are very attractive. In this work, the productions of rEK_L using a simple fed-batch technique where the exponential feed rates of methanol into the fermentor during production phase were investigated. Different specific growth rates were set up (μ_{set}) to obtain high amount of cell, product formation, and rEK_L activity. Different techniques for purification of the rEK_L were attempted to obtain the pure and clean rEK_L. The rEK_L activity and the accumulation of rEK_L were tested during production phase to confirm that high quantity and quality of recombinant enterokinase light chain can be obtained.

1.1 Research objectives

The main objectives of this work is to find the optimal exponential feed rates of methanol medium at different constant specific growth rate (μ_{set}) during methanol production phase in order to increase cell density, production yield and

enzymaticactivity of rEK_L. In addition, this work aims to obtain high amount and clean active purified rEK_L by absorption technique.

1.2 Scope and limitation of the study

In this research, the rEK_L was produced by a simple fed-batch technique where the different specific growth rates were used (μ_{set}) to pre-determine the exponential feeding rates of methanol during the production phase. The results of cell density, protein concentration, and enzymatic activity can determine in order to obtain the optimal feed rate, and cultivation time for rEK_L production by *P. pastoris* Y11430. Purification process of rEK_L was based on adsorption techniques.

CHAPTER II

LITERATURE REVIEW

2.1 Structure and application of recombinant enterokinase light chain

Enterokinase (enteropeptidase, EC 3.4.4.8) was actually recognized as the activity found in mammalian duodenum which is responsible for the conversion of trypsinogen into trypsin (Kunitz, 1938). The enzyme, which is also called enteropeptidase, is a protease that has high specificity for the sequence $(Asp)_4$ - Lys, a motif found in the highly conserved amino termini of trypsinogens from a wide range of species (La Vallie *et al*, 1993).

The number of polypeptide chains of enterokinase has been differently reported. It was described as two chains in pig, cow and bovine, and three chains in human (Song *et al*, 2002). However, in most preparations, enterokinase appears to be a disulfide- linked hetero dimer that is derived from a single chain precursor, and is composed of a 82–140 kDa heavy chain and a 35–62 kDa light chain. Both chains of enterokinase contain 30–50% carbohydrate (Gasparian *et al*, 2003), and 40% of the apparent molecular mass of enterokinase is provided by glycosylation (Kitamoto *et al*, 1994). The heavy chain has little influence on the recognition of small peptides, but strongly influences on macromolecular substrate recognition, such as trypsinogen and inhibitor specificity (Lu *et al*, 1997). The light chain is a catalytic subunit of

enteropeptidase of which consists of a chymotrypsin-like serine protease domain sufficient for the normal recognition and cleavage of both synthetic substrate and fusion protein carrying the $(Asp)_4$ - Lys target sequence (Light and Fonseca., 1984).

Recently, the enterokinase received an increasing interest in biopharmaceutical and biotechnological industries for cleaving fusion proteins because of its high degree of specificity to recognize a five amino- acid polypeptide $(Asp)_4$ - Lys, a motif found in the highly conserved amino terminal of trypsinogens from a wide range of species (Song *et al*., 2002), and cleaves at the carboxyl side of it (Hosfield and Lu, 1999). In addition, it retains its activity under a wide pH values, ranging from 4.5 to 9.5, at temperatures ranging from 4 °C to 45 °C, and in the presence of various detergents and denaturants (Yuan and Hua, 2002). This enzyme also allows the release of carboxyl-terminal fusion partners from fusion proteins without leaving unwanted amino acid residues on their amino termini (Huang *et al*., 2004; Kim *et al*., 2005). Therefore enterokinase is widely utilized as a tool protease in the research and production of gene engineering for an *in vitro* cleavage of fusion proteins (Fang *et al*., 2004).

Thai bovine enterokinase light chain (EK_L) gene was successfully cloned and expressed in *P. pastoris* Y11430 by Kupradit in 2006. This yeast strain was used for the production optimization experiments throughout this thesis.

2.2 Production of recombinant protein in *Pichia pastoris*

2.2.1 Advantage of *P. pastoris*

Suitable host cell factories for producing recombinant proteins include microbes such as *E. coli*, *Lactococcus lactis*, *S. cerevisiae* and *P. pastoris*, as well as

mammalian and insect cells. Each system has benefits and drawbacks in its use. Usually, *E. coli* is the first choice to host recombinant proteins production because of its simple genetic manipulation and economics of fermentation (Tan *et al*, 2007). As a result of these reasons, prokaryotic expression systems are often preferred for the production of heterologous proteins from eukaryotic cDNAs (Cregg *et al*., 1987).

However, some eukaryotic proteins produced in prokaryotic cells are unstable or may lack biological activity. Yeast with its well-established genetic and molecular biological resources combines the ease and speed-of-use of bacterial systems with its ability as an eukaryote to secrete post-translationally-modified proteins (Holmes *et al*., 2009) which may be crucial for biological activity (Cregg *et al*., 1987). As a consequence, it is an increasingly popular choice in both academic and commercial laboratories (Macauley *et al*., 2005; Sevastsyanovicha *et al*., 2009). *S. cerevisiae* was the first eukaryotic expression system to be used, and remains the most common due to the vast amount of information available on its genetics and physiology. Unfortunately, expression of heterologous proteins in *Saccharomyces* is not always optimal for large scale production due to different problems such as, loss of the plasmid during scale-up, hyperglycosylation, and low protein yield (Romanos *et al*., 1992).

To solve these problems, a methylotrophic yeast, *P. pastoris*, has been developed for expression as an alternative to *S. cerevisiae* (Kim *et al*., 2009). High quantities of foreign protein production (10-100 times more than *S.cerevisiae*) can be expressed in *P. pastori*s (Kim *et al*., 2009). *P. pastoris* also possesses the complex eukaryotic secretion machinery and can secrete proteins containing a high number of disulfide bridges. Moreover, high *P. pastoris* cell density can be attained in cheap

media, and scale-up to industrial processes is relatively straightforward. Using *P. pastoris* as a host, a number of proteins that are insoluble in *E. coli* have been expressed in an active and soluble form (Hanquier *et al*., 2003) and because of the very limited amounts of secreted proteins produced by *P. pastoris*, most of the secreted proteins can be desired foreign proteins. In addition, *P. pastoris* has specific post-translational modifications pathways that result in secreted glycoproteins similar to those of higher eucaryotes so the problems when using *S. cerevisiae* can be solved or avoided (Cregg *et al*., 2000). Therefore, *P. pastoris* has become a popular academic tool. In 2007 more than 600 genes were cloned and expressed using this microorganism (Zhang *et al*., 2008; Mack *et al*., 2009). Moreover, the strong promoter, coupled with the high cell-density fermentation, has allowed production of recombinant product at very high levels (Charoenrat *et al*., 2005).

2.2.2 Basic fermentation technique for *P. pastoris*

Many fermentation processes have been developed for the expression of foreign protein in *P. pastoris* under the control of the AOX1 promoter. The fermentation media for *P. pastoris* are economical and well defined. The fermentation conditions can also be controlled, and improved which makes it possible to achieve high cell densities (Higgins and Cregg, 1998; Lin Cereghino et a., 2002). In addition, with the low pH condition and present of methanol in media may reduce the contamination by most other microorganisms (Cereghino and Cregg, 2000). Therefore, the production in a fermentor shows more advantages than in a shake flask.

A fed-batch culture is typically carried out because of its well known for high cell densities and high protein secretion capacity (Cregg *et al*. 2000; Cereghino *et al*. 2001). A high efficiency *P. pastoris* fermentation protocol also had been

developed by Jahic *et al*., 2002. This protocol contained two stages for cell growth and two stages for protein production. The first stage is the glycerol batch phase which was used to increase cell density. The second stage is the glycerol fed batch phase which provided maximum cell density and derepression of the *aox1* promoter (Chiruvolu *et al*., 1997). The third stage is the methanol induction phase in which methanol was introduced at a low concentration into the fermentor. During this phase, the *P. pastoris* adapted to use methanol as carbon substrate. The last stage or the protein production phase is the phase where the methanol feed rate was increased in order to increase the expression of the *aox1* promoters (Charoenrat, 2006). The methanol feed control can lead to either success or failure. The methanol flow rates must be controlled to provide a methanol concentration that is just enough for protein synthesis but not in excess, which can be toxic (Cino, 1999).

Table 2.1 Standard method for *P. pastoris* fermentation according to Jahic et al. (2002)

Stage	Approximate time (h)	Mode	Substrate	Feed substrate*
1. Growth	0-24	Batch	Glycerol	None
2. Growth	24-27.5	Fed-batch	Glycerol	50% Glycerol
3. Induction	27.5-30	Fed-batch	Methanol	99% Methanol
4. Production	30-…	Fed-batch	Methanol	99% Methanol

*All feed solution contained 12 ml. L^{-1} of PTM1 trace salts

2.3 Parameters affecting the production of recombinant protein in *P. pastoris*

2.3.1 Strains

Besides growth conditions and cultivation process operating parameters, yields for heterologous protein production also depend on factors that influence gene expression (and secretion), and host strain physiology (Sreekrishna *et al*., 1997). Therefore, another factor that should be considered for expression optimization is the Mut phenotype.

P. pastoris belongs to the class Hemiascomycetes, which is generally present in the vegetative haploid state, *P. pastoris* can use methanol as a carbon source and regulates the transcription and synthesis of alcohol oxidase with the AOX promoter. Thus, using *P. pastoris*, glycerol is commonly used for high-density cultures and methanol for induction of a desired protein (Lee *et al*., 2006).

There are three types of available *P. pastoris* host strains that vary with regard to their methanol utilization abilities. The first host strains are the wild type or methanol utilization plus (Mut^+) phenotype while growth of the AOX2 knockout strain on methanol is comparable to the wild type *P. pastoris* and require high feeding rate of methanol in large-scale fermentations (Cereghino and Cregg, 2000). The second host strain is methanol utilization slow (Mut^s) in which the expression cassette is inserted within the *aox1* locus by double crossover gene transplacement (Romanos *et al*., 1992) or by disruption of the *aox1* gene via gene insertion (Cregg et al., 1987). Thus knockout of the *aox1* gene results in a slow growth phenotype on methanol. A third host strain used for heterologous protein expression is the Mut− (*m*ethanol *ut*ilization minus) strain in which both the *aox1* and *aox2* genes are disrupted (Zhang

et al., 2000). Double knockout strains are unable to grow on methanol (Hartner, 2006).

The selection of the Mut phenotype depends on the purpose of research or characteristic of recombinant proteins. The specific growth-rate is a critical parameter in the optimization of product formation (Zhang *et al*., 2000; Sinha *et al*., 2005). Cos *et al*, 2002 also demonstrated that the specific productivity and production yield were observed depending on the phenotype. Normally, the majority of researchers use the Mut^s phenotype because of increased specific yield of heterologous protein (lower levels of alcohol oxidase protein) for intracellular expression, (Sreekrishna *et al*., 1997). Another important advantage of Mut^s strains is that the culture is not as sensitive to residual methanol in the cultivation media relative to Mut^+ strain; hence the process of scale up can be easier (Stratton *et al*., 1998). Unfortunately, the lower maximum specific growth rate of the wild-type Mut^s strain, compared with wild-type Mut^+ strains, limits the productivity of the Mut^s utilizing process (Kim *et al*., 2009). Therefore, the Mut^+ phenotype has been using more and more widely as the host cell for the production of recombinant protein (Peng *et al*., 2004; Paramasivam *et al*., 2002; Chaoenrat *et at*., 2005; Kupradit, 2006). In this work, *P.pastoris* Y-11430, a wild-type strain (Cereghino and Cregg, 2000) was used as the host strain. Thus, neither antibiotic resistance gene nor metabolic gene mutation is present, which allows for the selection of expression vectors containing any appropriate antibiotic resistance gene selectable marker upon transformation.

2.3.2 Cultivation temperature and pH

To achieve high cell density, the control environment of fermentor is also very important. *P. pastoris* is able to grow in a wide range of pH (3-7) with minimal

effect on growth rate. However, this variable may affect recombinant protein stability and protease activity (Jahic et al., 2003; Chiruvolue et al., 1997; Sreekrishna et al., 1997). The production of functional recombinant protein also depends on the temperature. Cultivation temperature can influence degradation of recombinant proteins.

The optimal temperature and pH for cell mass production on glycerol and for recombinant protein formation on methanol are different, thus the batch and fed – batch phase of the *P. pastoris* fermentation should be carried out among different circumstances. The recombinant *P. pastoris* is capable to grow in a wide temperature range on glycerol; however, generally 30 °C is preferred for cell mass production on glycerol as carbon source. The highest value of specific formation for human serum albumin V (HSA) was showed at 24 °C, and pH 5.64 (Kupcsulik and Sevella, 2004).

A significant problem in many cell density cultures is the secretion of protease to the medium, releasing by lysis and proteolytic degradation that results in a reduction of the production yield and difficult for product recovery (Jahic *et al*., 2003). This problem was also mentioned in connection to standard methanol limited fed-batch *P. pastoris* processes in previous studies (Cregg *et al*., 2000). A number of adjustments to the standard *P. pastoris* process have been reported, including changing the cultivation pH or the temperature and adding casamino acids and peptone (Li *et al*., 2001; Zhou *et al*., 2002). Decreasing the temperature is proven to hinder the cell lyses, however it may enhance specific product formation rate as well (Kupcsulik and Sevella, 2004). The temperature limited fed-batch (TLFB) technique was described by Jahic in 2003 resulted in higher cell density, lower cell death, higher concentration of the product, and drastically lower proteolytic degradation of the

recombinant protein compared to corresponding methanol limited *P. pastoris* bioreactor cultures.

2.3.3 Dissolve oxygen (DO)

P. pastoris is an obligate aerobe, and thus, oxygen availability is of critical importance (Gurramkonda, 2009). A key element in expression of foreign proteins in this yeast is the need for sufficient aeration, which is achieved by proper mixing of the media and by blending gases to control dissolved oxygen content (DO) (Stratton *et al*., 1998; Lee *et al*., 2003).

Prevention of oxygen limiting conditions in high-cell density *Pichia* cultures has been strongly advised. It is also considered useful to supplement the air with oxygen gas (Jahic et al., 2002), reduce the oxygen uptake rate (OUR) by maintaining lower cell densities in continuous culture (Lee et al., 2003), or decreasing methanol feed rate or methanol supply. The temperature limited fed-batch process was (low temperature cultivation) designed to overcome the oxygen limitation in high methanol concentration and high cell density (Jahic et al., 2002). Moreover, two economical and simple process strategies, the oxygen limited fed-batch technique at 1.2 bar total air pressure, OTR about 35% (OLFB process) and the methanol limited fed-batch technique at 1.9 bar total air pressure, the OTR about 59% and DOT = 25% (high pressure fed-batch, HPFB process) to increase the oxygen transfer rate OTR were designed, and compared with the reference methanol limited fed-batch technique of 1.2 bar total air pressure and DOT = 25% air saturation (MLFB process). However, only 7% and 12% increases in biomass were produced in the OLFB and HPFB processes because of the response of *P. pastoris* to the oxygen limitation and high pressure condition by increasing the maintenance coefficient (q_m), which resulted in

the decrease of the biomass yield ($Y_{X/S}$), the total methanol consumption increased almost in proportion to the OTR. In addition, total product formation per process increased about 41 % and 50% in OLFB and HPFB, respectively, which is closed to proportional to the increase in OTR (Charoenrat et al., 2005).

On the other hand, many results were also demonstrated that low oxygen levels were generally recognized to interfere with production of recombinant proteins (Lee *at al*., 2003, Cereghino and Cregg 2000). With a dosage of pure oxygen supplied, the production yield was increased and the methanol feed rate was about 2 times higher than standard culture because the OUR was about 2 times higher (Jahic et al., 2002).

However, it also has been shown that reduced oxygen supply may be even advantageous for the production of recombinant proteins using the *P. pastoris* AOX1 expression system (Jahic *et al*., 2003; Charoenrat *et al*., 2005; Trentmann, 2004) or it is not necessary to enrich the inlet air with pure oxygen, or reduce the methanol supply to prevent oxygen limiting conditions during the induction phase to reach high-level production of HBsAg (Gurramkonda *et al*., 2009).

2.3.4 Glycerol concentration

Since cell concentration significantly increases at the beginning of the second phase, the glycerol fed batch phase has been also proved to play an important role in recombinant protein production (Chu *et al*., 2007). The generation of biomass is achieved by growing the cells in two phases using glycerol as carbon substrate. In order to achieve high productivity of heterologous proteins, the cell concentration must be further increased. Many researchers suggest obtaining around 40 $g.L^{-1}$ cell

density before starting for the induction and production of recombinant protein (Jahic et al., 2002; Chaoenrat, 2005; Kupradit, 2006).

At the first phase, glycerol batch phase, a maximum level of 40 $g.L^{-1}$ glycerol was recommended due to potential inhibitory effects of elevated glycerol concentrations. Then a glycerol-limited feeding phase was followed until the desired level of biomass was reached (Gurramkonda, 2009). Different glycerol feeding strategies have been explored for high cell density cultivation of *P. pastoris* by many researchers (Wang *et al*., 2009). The glycerol exponential feeding strategy was taken priority to achieve high cell density compared with glycerol constant feeding and DO-stat feeding (Lee *et al*., 2003). Another advanced exponential feeding strategy which was proposed by decreasing glycerol feeding rate was developed by Wang (2009) for biomass accumulation in cell growth phase. It was also demonstrated that an excessive accumulation of glycerol can trigger a build-up of ethanol and acetate to levels that repress the AOX1 promoter and can consequently decrease or delay foreign protein production (Inan and Meagher, 2001). Occasionally, mixed (methanol-glycerol) feeding protocols are also suggested for a more smooth methanol adaptation (Zhang *et al*., 2000).

2.3.5 Methanol concentration

The efficiency of the recombinant protein production by *P. pastoris* is affected by many parameters. However, in this work, the fermentation processes was developed based on the different feeding strategies with methanol in production phase under the optimal conditions which were previously studied.

The sudden accumulation of methanol may result in loss of AOX activity and even cell death for the methylotrophic yeast which includes *P. pastoris* (Jahic *et*

al., 2006). Prevention of high methanol concentrations in fermentation broth has been advocated by many authors as this might lead to the generation of toxic products such as formaldehyde and hydrogen peroxide which may compromise cell viability and productivity (Rosenfeld, 1999). Therefore, it is crucial to keep methanol concentration below inhibitory level (about 3-5 $g.L^{-1}$) which does not inhibit growth as follows Monod's type kinetic (Jahic *et al*., 2006; Ayed *et al*., 2008). Methanol above the critical concentration can exhibit substrate inhibition, which follows an uncompetitive inhibition model (Kupcsulik and Sevella, 2004; Kobayashi *et al*., 2000; Zhang *et al*., 2000; Katagura *et al*., 1998).

The stage of the methanol induction is a critical phase because the *AOX* activity is low and methanol may easily accumulate. Subsequently, the methanol feeding during the production phase is very important because methanol not only induces protein production, but also provides energy source for the host cells (Aved, 2008). Excessive amount of methanol inhibits the growth of host cells, while an insufficient amount of energy source and/or methanol starvation lead to poor cell growth and protein production (Katakura, 1998). So, it is important to determine an optimal methanol feeding strategy for the needs of a particular production process. The level of transcription initiated from AOX1 promoter can be three to five times greater in cells fed with methanol at growth limiting rates compared to cells grown in excess methanol (Cereghino and Cregg, 2000). Nevertheless, other studies reported different cell behaviors, i.e. keeping a non-limiting methanol concentration enhanced protein expression (Hellwig, 2001; Brady *et al*., 2001). However, no biochemical engineering studies have so far been conducted on the production of foreign proteins

in the presence of constant methanol concentrations because the methanol consumption rate increases with cell growth (Katakura, 1998).

The methanol feeding strategy, which also dictates the specific growth-rate, is a key parameter for maximizing recombinant protein production (Cos *et al*., 2005). Though various bioprocess strategies have been studied for methanol feeding, which could be metabolism related, based on kinetics parameters such as oxygen consumption (Byrne *et al*., 2000; Chung, 2000) and methanol consumption (Kobayashi *et al*., 2000; Hellwig *et al*., 2000), low expression efficiency of target protein would occur because *P. pastoris* cells were competing for methanol, the only carbon source, for cell growth and protein expression. It is recommended to keep the methanol concentration below 3 $g.L^{-1}$ for Mut^{s} cultivations (Invitrogen Co, 2002) and, independent of the phenotype, it has been advised to keep the methanol concentration below 4 $g.L^{-1}$ (Cos *et al*., 2006). In contract with other groups who noticed growth-inhibitory effects only at methanol concentrations > 2% and recommended up to 1% methanol to induce foreign protein production, a methanol concentration of 0.4–0.6% (4-6 $g.L^{-1}$) is reported as the negative effect on cell viability emerges in intracellular production of Hepatitis B surface antigen (Gurramkonda, 2009).

However, a comparison of the performance of these different fed-batch strategies is difficult, in particular, when different recombinant proteins are produced. In contrast to these multi-phase protocols, a simple fed – batch technique is proposed here. The production of recombinant enterokinase based on the methanol fed according to the ratio of methanol to cell concentration was tested.

2.4 Production and purification of recombinant light chain enterokinase (rEK_L)

The cDNA encoding bovine enterokinase light chain has been isolated from several sources such as cattle, human, pigs, rats, mice, and medaka (Ogiwara and Takahashi, 2007). Various hosts such as mammalian COS cells (LaVallie *et al*., 1993), *E. coli* (Collins-Racie *et al*., 1995; Yuan and Hua, 2002; Huang *et al*., 2004; Tan *et al*., 2007), filamentous fungus (Svetina *et al*., 2000), *Saccharomyces cerevisiae* (Choi *et al*., 2001; Kim *et al*., 2005), and methylotrophic yeast *Pichia pastoris* (Vozza *et al*., 1996; Peng *et al*., 2004; Fang *et al*., 2004; Fang and Huang, 2004; Kupradit, 2006; Zhang *et al*., 2008) have been used to express and produce this enzyme.

The high degree of specificity exhibited by enterokinase makes it as an ideal enzyme for cleaving the specially constructed fusion proteins, containing the (Asp)4 - Lys sequence between the carrier protein domain and the target product. EK_L was first produced in *E. coli* but the yield of recombinant active EK_L was low, only 8 µg with very low activity was isolated from 1 g cell paste, probably due to the abundance of disulfide bridges in the structure (Collins-Racie *et al*., 1995). Gasparian *et al*., 2003 also reported expression of rEK_L in *E. coli*, but the soluble fusion protein showed no autocleaving activity; and approximately 20 mg of EK per liter was obtained from the insoluble fraction via a complicated refolding process (Gasparian *et al*., 2003). Recently, the yield of purified intact EK_L was increased up to 4.3 mg per 100 ml flask culture (Yuan and Hua., 2002) and 106 mg of protein per liter of cell culture (Tan *et al*., 2007) and high enzymatic activity was obtained.

The expression system with *A. niger* was carried out with the purpose of exceptionally high homologous protein secretion capacity, correct processing and

folding of products, efficient disulfide bridge formation and less over glycosylation,. However, the purified rEK_L in *A. niger* was only 1.9 $mg.L^{-1}$ (Svetina *et al*., 2000). The production of rEK_L by recombinant *S. cerevisiae* was also not much higher than *A. niger* which only 3.8 $mg.L^{-1}$ using complicated medium (Kim *et al*., 2005). Since the production of rEK_L from *A. niger* and *S. cerevisiae* (in relation to the volume of culture broth) was very low, until now, no more researchers use these systems for production of rEK_L even though very high activity of rEK_L were obtained comparing with rEK_L from *E. coli* was showed.

Nowadays, *Pichia* expression system is one of the most successful foreign protein expressions. Compared with *E. coli* system, it has additional advantages which can help recombinant protein to achieve correct folding and post-translational modification such as glycosylation (Fang and Huang, 2004) and the yield of recombinant proteins can be increased comparing with *A. niger* and *S. cerevisiae* expression systems. Only 6.3 mg of EK_L was purified from 1 liter fermentation when first expressed in *P. pastoris* (Vozza *et al*., 1996). To increasing the yield of rEK_L, the influences of the methanol utilization phenotype of the host strain, induction pH; carbon source and cell mass concentration were studied. As a consequence, the yield of rEK_L without purification reached 350 $mg.L^{-1}$ after 120 hr induction (Peng *et al*., 2004) and 479.99 $mg.L^{-1}$ after 97.5 hr induction (Zhang *et al*., 2008). The highest yield of purified rEK_L was 150 $mg.L^{-1}$ culture broths (Peng *et al*., 2004) with highest enzymatic activity, 2.88×10^7 $U.mg^{-1}$ protein was obtained (Zhang *et al*., 2008).

The recombinant EK_Ls has been purified by several chromatographic and other methods as summarized in Table 2.2.

Table 2.2 Summary of researches for rEK_L production by different host cells.

*: total protein ($mg.L^{-1}$); **: $U.L^{-1}$

Host cell	**Concentration of purified rEK_L($mg.L^{-1}$)**	**Specific activity of purified rEK_L($U.mg^{-1}$ purified protein)**	**References**
Mammalian COS cells	Low levels of secrete protein	Very low levels of activity	LaVallie, 1993
E. coli	43	720	Yuan and Hua, 2002
	20	No autocleaving activity	Gasparian, 2003
	106	110 ± 10	Tan, 2007
S. cerevisiae	3.8	Highly active protein	Kim, 2005
A. niger	1.9	19.88	Svetina, 2000
P. pastoris	6.3	-	Vozza, 1996
	350* 150	9000	Peng, 2004
	5.4	Excellent cleavage activity towards fusion protein containing cleavage site.	Fang, 2004
	10.9	2.88×10^{7}	Fang and Huang, 2004
	479.99*	13,619.14**	Zhang, 2008

For *P. pastoris* system, the recombinant EK_Ls have been purified either by STI resin affinity chromatography (Collin-Racie *et al*., 1995; Fang and Huang., 2004), anion exchange chromatography, Q sepharose fast flow (Racie *et al*., 1995; Peng *et al*., 2004), cation exchange chromatography, SP fast flow (Kupradit, 2006) or Nickel affinity column (Peng *et al*., 2004). For *E. coli* system, the purification of rEK_Ls have

been done by Nickel affinity column and STI–Sepharose chromatography (Vozza *et al*., 1996), simple and cheaper procedure, consisting of ammonium sulfate precipitation, diethylaminoethyl cellulose (DEAE) chromatography, and gel filtration (Yuan and Hua., 2002), Ni-IDA resin column (Huang *et al*., 2004), nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Tan *et al*., 2007). The STI column also has been used for *A. niger* (Svetina *et al*., 2000) and only single step procedure on nickel affinity chromatography has been used for *A. cerevisiae* (Choi *et al*., 2001).

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganism

Figure 3.1 *P. pastoris* Y11430 strain containing the pPICZαB NH8_EK_L

P. pastoris Y11430, a wild–type (Mut+ and His+) strain containing the pPICZαB NH8_EKL (Kupradit, 2006) was used for rEK_L production. The entire cDNA encoding bovine EK_L gene in the pGEM – T vector (Kupradit, 2006) was cloned into the pPICZαB NH8 vector which was the modified from pPICZαB from

Invitrogen resulting in rEK_L containing 8X His at the N – terminal. This construct also contains AOX1 promoter which can be induced by methanol and uses the zeocin resistance gene as a selectable marker.

3.2 Inoculum preparation

Primary inoculum was prepared by picking a colony of *P. pastoris* from YPD agar (Appendix A) into 20 ml YPD broth (Appendix A). The secondary inoculum was prepared by transferring the primary inoculum into a 250 ml shake flask that contained 80 ml BMGY medium pH 5.0 (Appendix A). The culture was incubated at 30 ^{o}C, with 200 rpm rotary shaking for 24 hr. Zeocin 100 µg.mL^{-1} was added in all medium.

3.3 Fed–batch technique for recombinant EK_L production in *Pichia pastoris*

All fed – batch cultures was carried out in a 2 L fermentor (Sartorius, Germany) containing 950 ml GBS medium (Appendix A). The fermentor was prepared and calibrated for the pH probe before sterilization. The PTM1 trace salts (Appendix A) was added to the fermentor separately after sterilization. Amonia solution 25% was used as a nitrogen source and also an alkaline to control and adjust the pH. The fermentation conditions such as temperature, pH, pO_2, aeration rate, pressure, agitation rate, glycerol feed rate, and methanol feed rate were automatically controlled. Antifoam was added manually when high level of foam occurred. The fermentation process was divided into 4 stages as followed;

Glycerol batch phase

The fermentation condition was set up and controlled by an automatic temperature controller (Julabo, Germany) at 30 ^{o}C, 1 vvm aeration rate, 1,000 rpm agitation, and pH 5.5. An inoculum of 5% (50 ml and OD_{600} about 3) was transferred into the 2 L fermenter containing 950 ml of GBS medium with 4.35 ml.L^{-1} PTM1 trace salts. During this phase, the DOT continuously decreased because the oxygen was consumed for energy metabolism of *P. pastoris*. The batch culture was grown until the glycerol was completely consumed which varied from 18 – 24 hr. The depletion of glycerol can be monnitored by a sharp increase in the DOT signal.

Glycerol fed-batch phase

After glycerol batch phase, glycerol feed (GF) medium (glycerol 500 g.L^{-1} and PTM1 trace salts 12 ml.L^{-1}) was added manually to the fermentor. The feeding of 18.89 g.hr^{-1} during 2.18 hr was carried out which was calculated based on the data reported by Kupradit (2006). The objective was to obtain approximately 40 g.L^{-1} cell concentration at the end of this phase as suggested by Jahic *et al* (2002) and Charoenrat (2005). The fermentation condition was controlled as in the glycerol batch phase which was also applied to increase the cell density and de-repress of the AOX promoter (Chiruvolu *et al*., 1998).

Methanol induction phase

The methanol induction phase was started by replacing the GF medium with the methanol feed medium (MF) (12 ml PTM1 trace salts per liter of methanol) at very slow flow rate to allow enough time for *P. pastoris* to produce the enzymes in the methanol metabolism pathway. The fermentation condition was controlled as in glycerol batch phase. For 1 L initial fermentation volume, 0.633 ml of MF medium

was injected into fermentor 5 times during 3 hr until the *P. pastoris* can be fully adapted to use methanol as substrate as recognized by the response of DOT.

After the first methanol injection, DOT increased slowly in a short time because it is in the time to adapt to grow on methanol. Subsequently, the DOT slowly decreased in erratic manner because of methanol accumulation. After 1 hr, when the methanol is completely consumed, DOT was increased, and then the second methanol induction was carried out by injecting again 0.633 ml of MF medium to the fermentor. The DOT was more rapidly decreased and increased than the first time. It could be explained by the increasing of all enzymes in methanol metabolism pathway or the *P. pastoris* have already adapted to use methanol as substrate.

Methanol production phase

The methanol feed rates must be controlled to keep the methanol concentration just enough for protein synthesis but not excessive concentration where inhibition occurs (Cino, 1999). The feeding of MF medium was calculated to increase the cell mass concentration exponentially in the bioreactor at growth rates which prevent the accumulation of toxic levels of methanol. The methanol feed was automatically fed to the fermentor by using a syringe pump (Cole Parmer, USA) and the feeding was increased every 3 hr following the caculation.

The fermentation conditions were controlled as in the glycerol batch phase, only the temperature was decreased to 20 ^{o}C after the first 2- 3 hr and then kept until the end of the process.

The sample was taken one time per day for further analysis of cell growth (OD_{600} and dry cell weight), total protein concentration and enzymatic activity in

crude culture supernatant which were tested using fluorogenic substrate. The protein bands were detected on 15% SDS – PAGE gel.

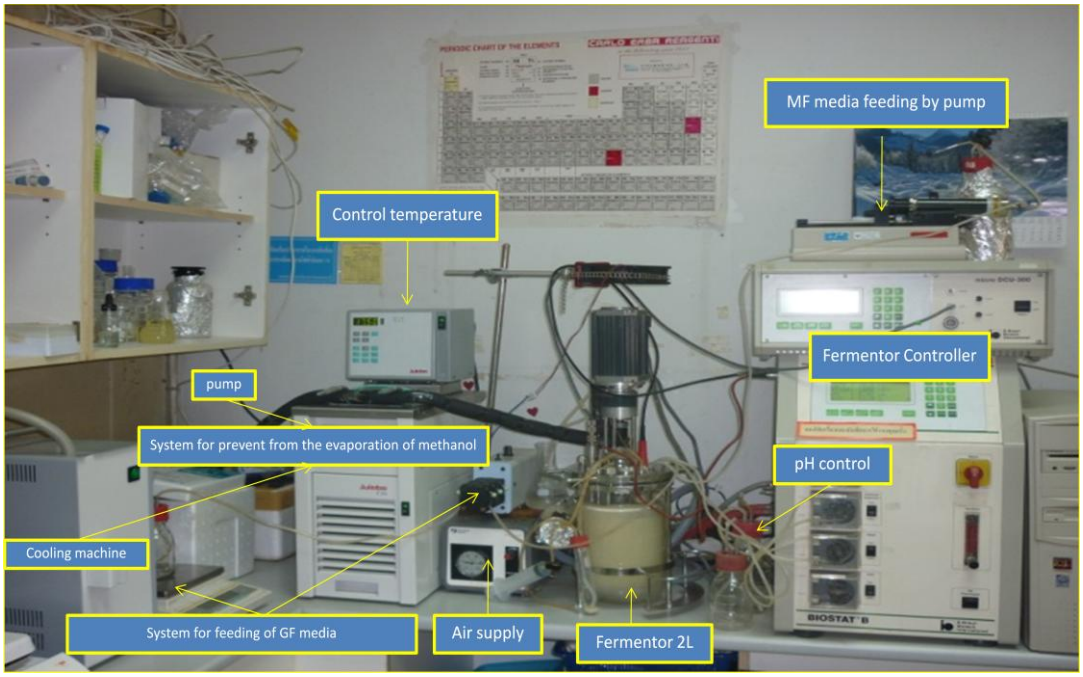

Figure 3.2 Experimental setup for rEK_L production in a 2L fermentor.

3.4 Calculation of the methanol feeding rate

Since the accumulation in the medium and the evaporation of methanol was insignificant in comparison to the feed, the integrated value of this feed rate can be assumed as the total methanol consumption. The feeding rate was calculated to allow the volumetric cell mass concentration to increase exponentially as suggested by Korz *et al*, 1994.

With a desired specific growth rate μ and a given biomass concentration the actual feeding rate of methanol is given by:

$$m_S(t) = F(t)S_F(t) = \left(\frac{\mu(t)}{Y_{X/S}} + m\right)V(t)X(t) \qquad (1)$$

Where; m_s is the mass flow rate of substrate ($g.hr^{-1}$), F is the volumetric feeding rate ($L.hr^{-1}$), S_F is the methanol concentration in solution ($g.L^{-1}$), μ is the specific growth rate (hr^{-1}), $Y_{X/S}$ is the biomass/substrate yield coefficient ($g.g^{-1}$), m is the specific maintenance coefficient (hr^{-1}), X is the biomass concentration ($g.L^{-1}$), and V is the cultivation volume (L), respectively.

In a fed-batch system:

$$\frac{d(XV)}{dt} = \mu XV \qquad (2)$$

If we assume μ as time invariant one obtains integration when starting the feeding at time t_F

$$X(t)V(t) = X_{tF}V_{tF}e^{\mu(t-t_F)} \qquad (3)$$

With the introduction of Eq. 3 to Eq. 1, the substrate mass feeding rate for a constant specific growth rate (μ_{set}) can then be calculated as follows:

$$m_S(t) = \left(\frac{\mu_{set}}{Y_{X/S}} + m\right)V_{tF}X_{tF}e^{\mu_{set}(t-t_F)} \qquad (4)$$

where the values of $Y_{X/S}$, 0.27 and m, 0.035 h^{-1} were obtained from previous works (Charoenrat, 2005 and Kupradit, 2008), and different constant specific growth rates (μ_{set}) 0.006, 0.0075 and 0.0105 hr^{-1} was used to determine the exponential feeding strategy of MF medium during production phase.

3.5 Analytical methods

3.5.1 Cell concentration

Cell concentration was determined by measuring the optical density at the absorbance of 600 nm (OD_{600}) and also cell dry weight. The dry cell weight was determined by centrifugation 5 ml culture samples at 4000 rpm for 10 min. The supernatant was collected for further analyses, and the pellet was washed with distilled water once and dried at 105 ^{o}C, until constant weight was obtained (each sample was taken in duplicate).

3.5.2 Protein concentration

The total protein concentration in the supernatant was analyzed according to Bradford, 1979. Bovine serum albumin was used as a standard and the range of sensitivity was from 50 to 1000 $mg.mL^{-1}$.

3.5.3 Enterokinase activity assay

The assay of enterokinase activity was modified from the methods of Hermon – Taylor (1970) which determined using the fluorogenic substrate Gly–$(Asp)_4$–lys–β–naphthylamide (GD_4K-β-naphthylamide). Thirty micro liter of the crude culture supernatant was added to two hundreds micro liter of substrate solution (50 µM GD_4K-β–naphthylamide in 70 mM Tris–Cl pH 8 and 10% Dimethyl Sulfoxide). The enzymatic activity was measured using Spectra Max Gemini EM machine by an increasing of fluorescence at excitation 337, emission 420 nm that caused by the release of β – naphthylamide over one minute interval. A standard curve of β – naphthylamide was done for determining the unit of enzyme enterokinase. The commercial rEK_L from NEB was used as a positive control.

The activity of rEK_L was also detected by cleavage of fusion protein, rice Os1Bglu4-trx which has enterokinase recognition sequence in the linker. The cleavages of fusion protein with rEK_L were carried out in 50 mM Tris-Cl pH 8 at 23 °C for 16 hr. The results were checked on 15% SDS – PAGE gel. The commercial rEK_L from New England Biolabs (NEB) was used as a positive control.

3.5.4 Residual methanol concentration

Residual methanol concentration was analyzed using a gas chromatograph (GC) equipped with a flame ionization detector (FID Instrument, USA). Helium, 99.999% pure, was used as carrier gas. The GC column (Carbowax®, Restek, USA) was a 30 m × 0.32 mm bonded phase fused silica capillary column. The injector and detector temperatures were set at 200, and 300 °C, respectively. The oven was operated at programmed temperature, from 41 to 200 °C at the rate of 15 °C.min^{-1}.

3.6 Calibration

3.6.1 pH calibration

The pH probe was calibrated outside the fementor after 950 ml of GBS medium was added in the 2 L fermentor and temperature was controlled at 30 ^{0}C before sterilization. When the pH probe is completely calibrated for both pH 4 and pH 7, the acceptable range of calibration value as slope (54 to 60mV/ pH) and zero (- 30 to + 30 mV) were shown.

3.6.2 DOT electrode calibration

The calibration of DOT electrode for all processes was performed under the same conditions. The 100% air saturation was calibrated in 950 ml of glycerol basal salts medium, pH 5.5, at the temperature of 30 ^{0}C, aeration at 1 vvm, and

agitation at 1000 rpm. When the 100 % DOT is accepted, the value of pO_2 slope (25 to 200 nA) and zero (0 to +15 nA) must be in the range as recommended in the fermentor manual control book.

3.7 Purification of rEK_L by cobalt column

After the fermentation processes, the cells were separated from the broth by centrifugation at 4,000 rpm for 30 min then 12,000 rpm for 15 min and the supernatants were filtered through 0.2 µm filter. The filtrated supernatant culture broth was dialyzed in 50 mM Tris-Cl pH 8 before being applied to cobalt column. The column was equilibrated with 10 column volume (CV) of 50 mM Tris-Cl pH 8, 150 mM NaCl (W_0) then sample was load into the column and the flow-through fractions were collected. The unbound proteins were eliminated using 5CV of W_0 and 10 CV of washing buffer (50 mM Tris-Cl pH 8, 300 mM NaCl, 20 mM imidazole). Then the recombinant protein contain His-tagged was eluted with 5 CV of elution buffer 1 (50 mM Tris-Cl pH 8, 300 mM NaCl, 250 mM imidazole) and 5 CV of elution buffer 2 (50 mM Tris-Cl pH 8, 300 mM NaCl, 500 mM imidazole). The collected fractions of flow-through, wash and elution were detected for the protein band using SDS-PAGE and tested for enzymatic activity.

3.8 Purification of rEK_L by ion exchange chromatography

The filtrated supernatant culture broth was dialyzed and concentrated in 50 mM sodium acetate buffer pH 5 using Amicon Ultra-15 Centrifugal Filter Unit with a molecular weight cut-off of 10 kD following the protocols and then centrifuged at 12,000 rpm for 15 minutes before loading into Sulphopropyl Fast Flow Column

(SP_FF column) using FPLC machines (AKTA purifier, Amersham Pharcacia Biotech). The column was equilibrated with 50 mM sodium acetate buffer pH 5 then sample was injected using the 1 ml sampling loop of FPLC. To remove the unbound fraction, the column was washed with 5 CV of 50 mM sodium acetate pH 5. The sample was eluted with a gradient of NaCl from 0 to 1 M in sodium acetate buffer; the concentration was detected by spectrophotometer at the wave length 280 nm. All the fractions were collected. The fractions from flow-through wash and elution were tested for enzymatic activity and checked by SDS-PAGE. The elution fractions which showed high activity were pooled and concentrated.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Production of recombinant enterokinase light chain (rEK_L) by *P. pastoris*

As previously described, mass flow controlling of methanol into the fermentor during methanol production phase is very important for the production of recombinant protein by *P. pastoris*. The methanol feeding rate must be controlled to provide methanol concentration at the proper rate to allow just enough for protein synthesis because excess amount of methanol can cause toxicity effect to the biocatalyst (Cino, 1999). In this work, different constant specific growth rates (μ_{set}) 0.006, 0.0075, and 0.0105 hr^{-1} were used to calculate the MF medium feed rate in the methanol production phase to produce the rEK_L. This protocol was designed to obtain high cell density cultivation, to determine the induction time for getting high enzymatic activity, and to avoid excessive feeding rate of methanol that make the methanol toxification during the methanol production phase.

A four-stage *P. pastoris* fermentation protocol (Charoenrat, 2005; Kupradit, 2006) using a simple fed – batch technique was applied as mentioned in Chapter III (Materials and Methods). The cultivation conditions were controlled and operated at 30 °C, pH 5.5, aeration rate 1vvm, and agitation speed 1,000 rpm in the first three fermentation stages (glycerol batch phase, glycerol fed-batch phase, and methanol

induction phase, respectively). But in the last methanol production phase, after 3 hr of methanol production phase, the culture was fully adapted to grow on methanol, the temperature was slowly decreased from 30 °C to 20 °C and kept at this temperature until the end of the fermentation process. Production at low temperature has been demonstrated by many researches to give high cell density, low cell death, high concentration of the product, and drastically low proteolytic degradation of the recombinant protein (Jahic et al., 2003; Kupradit, 2006). It may also enhance the specific product formation rate as well (Kupcsulik and Sevella, 2004). In this phase, the methanol feed rate was varied for each fermentation process. Therefore the results are described case by case.

In the glycerol batch phase, glycerol was used as the carbon and energy source because it gives rapid growth and high biomass yield (Chiruvolu *et al*., 1998). At the end of this phase, the cell dry weight was approximately 27 $g.L^{-1}$ for all experiments (Figure 4.1).

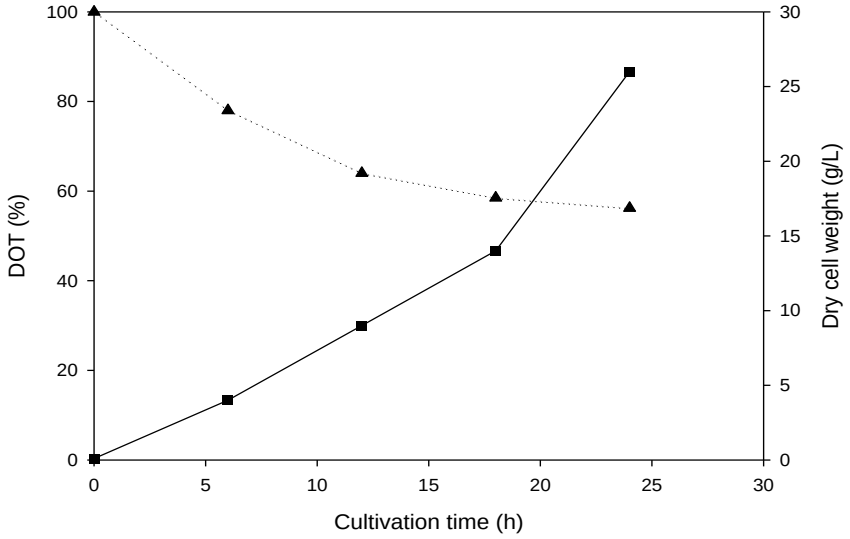

Figure 4.1 Cell dry weight (■) ($g.L^{-1}$) and DOT (▲) (%) during glycerol batch phase.

Figure 4.1 also illustrates the dissolved oxygen tension profile. It can be seen that the value of DOT dramatically reduced from 100 % to approximately 56% for the first 24 hr of fermentation time. This result also reported by other previous researches even though the decreasing pattern of DOT was found different comparing to the result from Charoenrat, 2006. This rapid reduction rate was clearly due to high oxygen demand of the *P. pastoris* at the beginning of log phase.

In the second phase, the glycerol feed (GF) medium was fed into the fermentor. The objectives of this phase are to de-repress the *aox1* promoter, and to increase cell density (Chiruvolu et al., 1997). This feeding was calculated following the data reported by Jahic *et al* (2002), and Charoenrat (2005) to obtain approximately 40 $g.L^{-1}$ cell concentration, and to achieve a minimal residual glycerol concentration in the fermentation broth at the end of this phase.

The total cell concentration need to increase in this phase was $40 - X_0$ $g.L^{-1}$. However, the GF medium contains only 50% glycerol, and its density is 1.109 $g.mL^{-1}$, thus the total amount of GF medium required can be calculated as:

$$\text{GF medium (g)} = \{[(X_t - X_0)/ Y_{X/S}] \times 1109\}/ S_i$$

$$= \{[(40-27)/ 0.7] \times 1109\}/ 500 = 41.19 \text{ g}$$

Where the biomass yield coefficient Yx/s = 0.7 $g_{cell}.g_{glycerol}^{-1}$ (Charoenrat, 2005), the glycerol concentration in the GF medium Si = 500 $g_{glycerol}.L^{-1}$, the cell concentration at the beginning of this phase X_0 = 27 $g.L^{-1}$, the cell concentration at the end of this phase X_t = 40 $g.L^{-1}$, and the culture volume at the beginning of this phase V_0 = 1 L, respectively.

In addition, the time for feeding GF medium can be calculated based on specific growth rate (μ) which defined as:

$$\mu = (1/X)(dX/dt) \text{ or } \ln(X_t/X_0) = \mu t$$

Where specific growth rate $\mu = 0.18\ hr^{-1}$ (Jahic et al., 2002).

Therefore,

$$t = \ln(X_t/X_0)/\mu = (\ln 40/27)/0.18 = 2.18\ hr$$

Therefore, the GF medium was fed into the fermentor with the feeding rate $41.19 / 2.18 = 18.89\ g.hr^{-1}$. This feeding strategy was run until the cell concentration reached approximately $40\ g.L^{-1}$ (which took 2.5 - 3 hr) as shown in Figure 4.2. During this phase, no rEK_L enzymatic activity was detected (data not shown). This result demonstrated that under the absence of methanol which has a function to induce the AOX promoter which drives the rEK_L gene, no rEK_L activity was detected or a very low rEK_L activity was presented but cannot be detected by the fluorospectrophotometer.

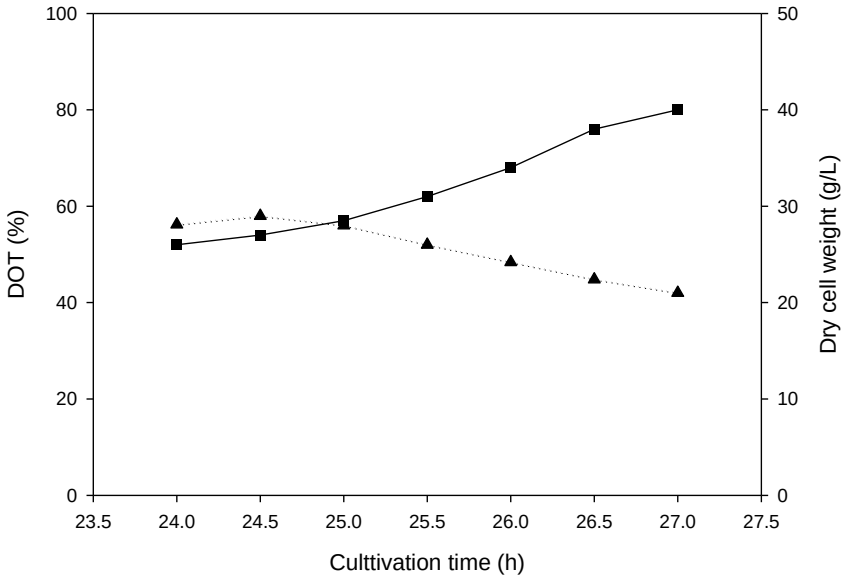

Figure 4.2 Dry cell weight (■) ($g.L^{-1}$) and Dissolved oxygen tension (▲) (%) during the glycerol fed-batch phase.

After the cell completely utilized glycerol and obtained enough high cell density. The methanol induction phase was then applied with an introduction of the methanol feed (MF) medium into the fermentor at a very low concentration. The objective of this operation was to facilitate the cells to have enough time for activating the enzymes in the methanol metabolism pathway (Rose and Harrison, 1989). A rapid decrease of DOT when MF medium was injected, and a rapid increase of DOT after methanol was completely consumed indicates that the *P. pastoris* adapted to use of methanol as carbon and energy source (Figure 4.3).

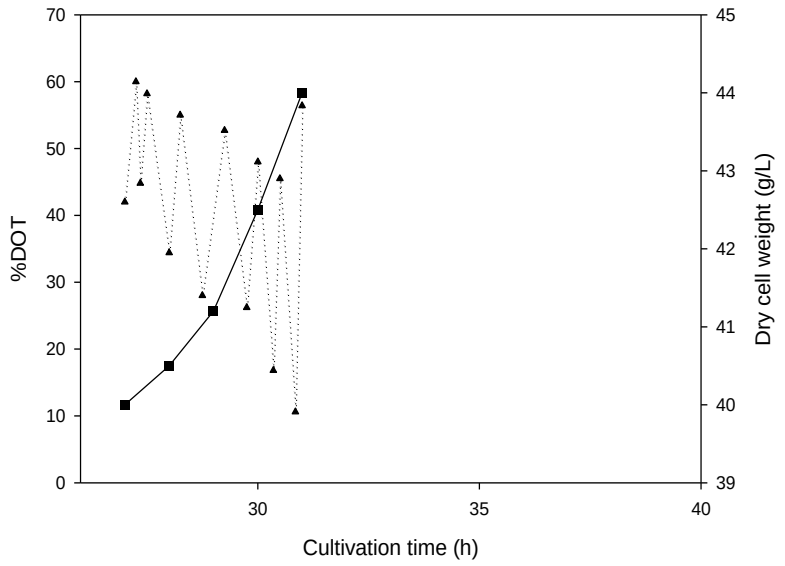

Figure 4.3 Cell dry weight (■) ($g.L^{-1}$) and Dissolved oxygen tension (▲) (%) during methanol induction phase.

The increasing of cell density and changing of DOT (%) were summarized in Figure 4.4. Then the culture medium was ready for the methanol production phase.

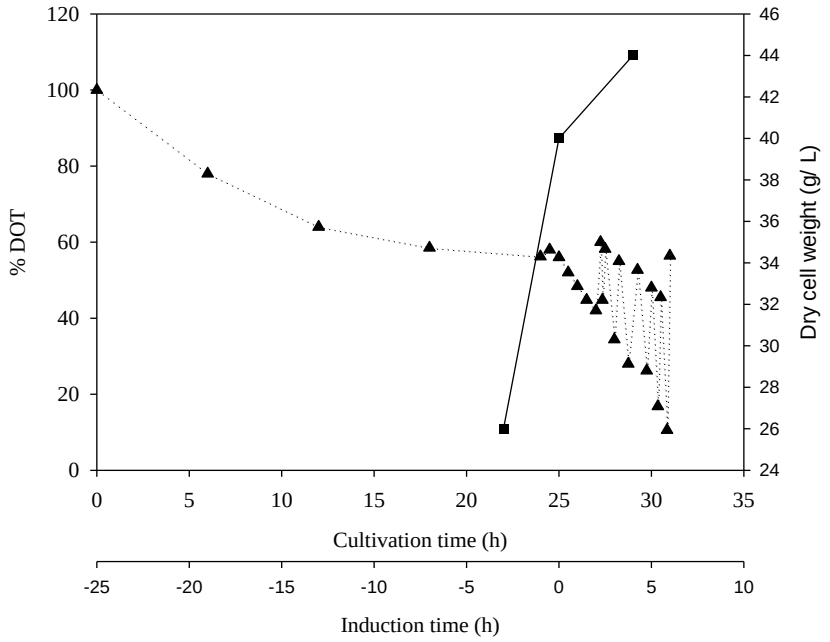

Figure 4.4 Cell dry weight (■) ($g.L^{-1}$) and Dissolved oxygen tension (▲) (%) during the first three phases (glycerol batch phase, glycerol fed-batch phase, and methanol induction phase, respectively).

In the methanol production phase, the MF medium was fed into the fermentor at an exponential increasing feed rate. The mass flow rates of methanol in this phase were controlled and were varied depending on the different constant specific growth rates (μ_{set}). In this work, the values of μ_{set} were set at 0.006, 0.0075, and 0.0105 hr^{-1} which were used to pre-determine the MF medium feed rate.

4.1.1 Feeding rate of MF medium and total methanol consumption

The calculated results of the mass flow rate of MF medium at different specific growth rates (μ_{set}) are shown in Figure 4.5. At the beginning of methanol production phase, 2.29, 2.51 and 2.95 $g.hr^{-1}$ of MF medium were fed to the fermentor

for the three consecutive values of μ_{set} 0.006, 0.0075 and 0.0105 hr^{-1}, respectively. The mass flow rate of MF medium subsequently increased every 3 hr until the end of this phase.

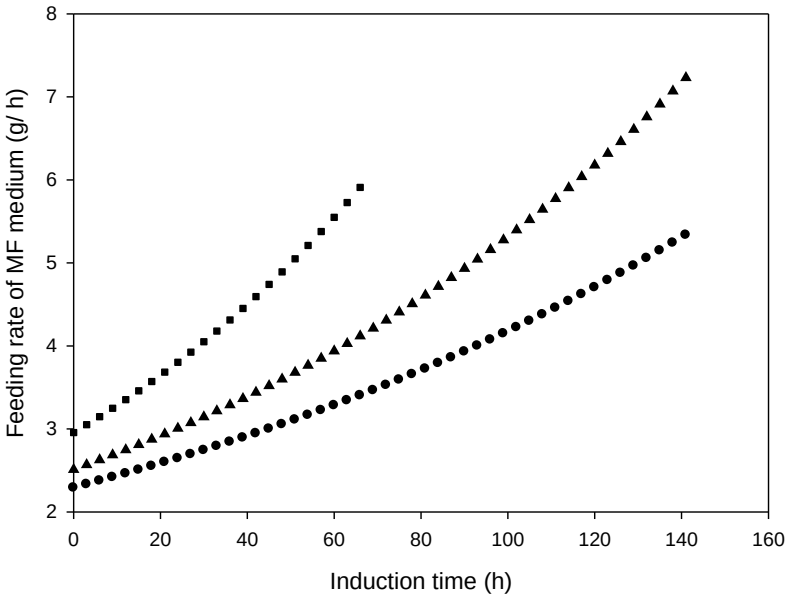

Figure 4.5 Feeding rate of MF medium ($g.hr^{-1}$) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

The induction time of the methanol feeding medium into the fermentors for the three processes was different. In general, the resulting rEK_L activity ($U.mL^{-1}$) of the crude fermentation broth was monitored in order to terminate the feeding of MF medium. During methanol production phase, rEK_L activity significantly increased according to the addition of the MF medium. The rEK_L activity then reached its maximum value, and the activity started to decline. After this maximum point, the feeding of the MF medium was terminated. At the end of induction time, it was found that the mass flow rates of the average MF medium were 5.91, 5.24, and 7.07 $g.hr^{-1}$

for the values of μ_{set} at 0.0105 hr^{-1} (69 hr), 0.006 hr^{-1} (141 hr), and 0.0075 h^{-1} (141 hr of induction time), respectively.

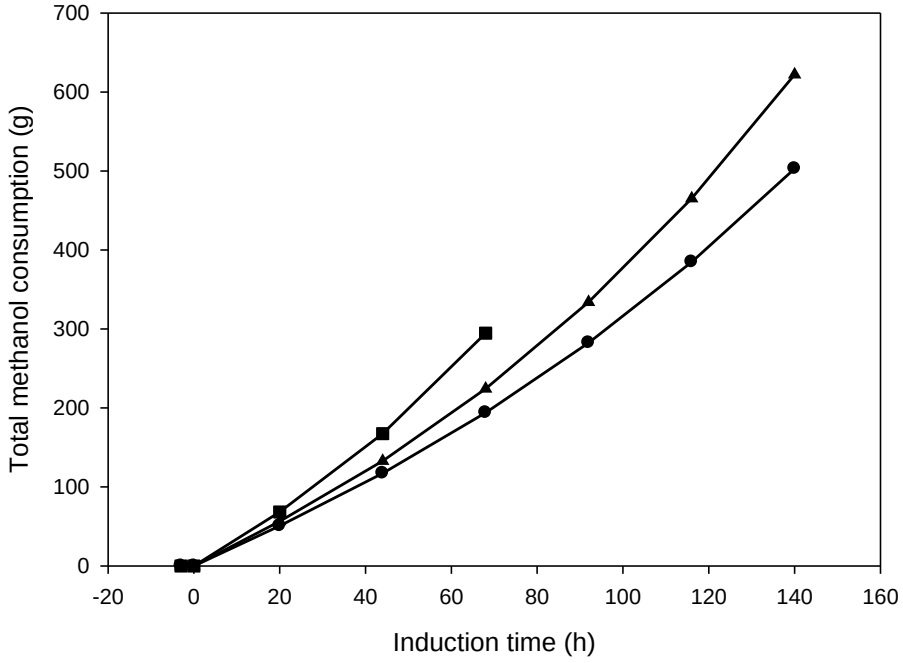

Figure 4.6 Total methanol consumption (g) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

Figure 4.6 illustrates the calculated value of methanol mass fed into the fermentor. The mass flow rate of methanol into the fermentor is very crucial for the production of rEK_L by the *P. pastoris*. This is because an excessive feeding of MF medium maybe can lead to the limitation of oxygen inside the fermentor and maybe result an effect to the growth of the cell. At μ_{set} 0.0105 hr^{-1}, it was found that total mass of methanol fed in the fermentor was the highest compared to the values of the processes at μ_{set} 0.0075 and 0.006 hr^{-1}, respectively. At the end of the induction time of 141 hr, the accumulated of methanol fed into the fermentor for the three processes

at μ_{set} 0.0075 and 0.006 hr^{-1} were 622.12 and 502.93 g, respectively. These amounts of the methanol will definitely affect the growth characteristic and product formation during fermentation.

4.4.2 Cell growth

For comparison purpose, the effect of different μ_{set} on cell density, total protein concentration, and rEK_L accumulation (rEK_L activity, $U.mL^{-1}$) were investigated. Initially, the cell density or cell dry weight (DCW) can be used as an indicator for the enzyme production. Since rEK_L formation is probably growth related, higher growth could result in higher product formation. Figure 4.7 shows experimental result of cell growth obtained during methanol production phase of the three different feeding strategies.

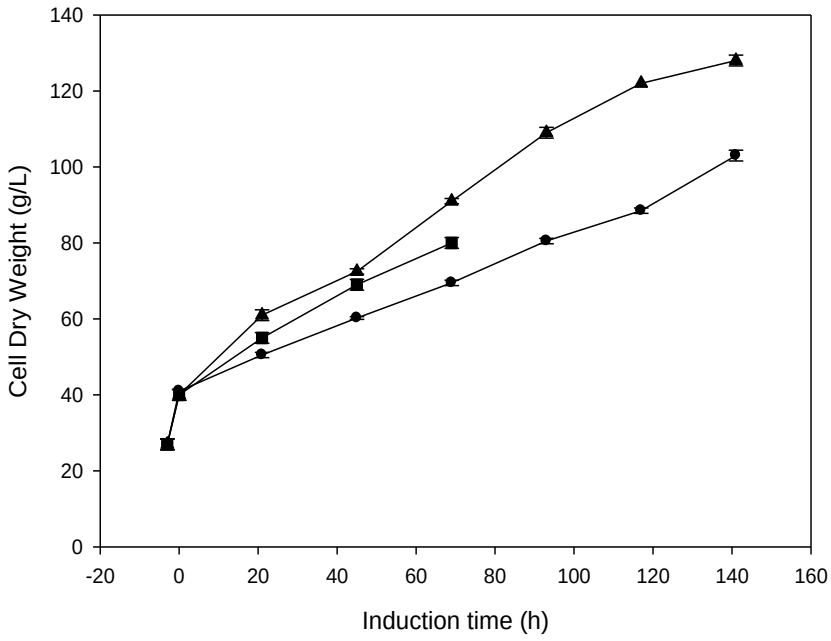

Figure 4.7 Cell dry weight ($g.L^{-1}$) during methanol production phases as a function of different μ_{set} at 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

The increasing of the cell density depends on the methanol consumption of each exponential feeding rate, and also the induction time. At 69 hr of induction time, the cell density of 80 ± 1 $g.L^{-1}$, 91 ± 0.7 g. L^{-1} and 70 ± 0.7 $g.L^{-1}$ were obtained from the μ_{set} 0.0105, 0.0075 and 0.006 hr^{-1}, respectively. The highest cell density was obtained at the process μ_{set} of 0.0075 hr^{-1}, followed by the process at μ_{set} 0.0105 hr^{-1}, and the lowest was observed at μ_{set} 0.006 hr^{-1}. Thus, it was confirmed that the MF medium feed rate during the production phase is very important. Methanol not only induces protein production, but also provides energy source for the host cells (Ayed, 2008). Excess amount of methanol inhibits the growth of yeast cells, while an insufficient amount of energy source and/or methanol starvation lead to poor cell growth and protein production (Katakura, 1998). In this work, the specific growth rate at μ_{set} 0.0075 hr^{-1} resulted in the optimal feeding rate of MF medium for the cell growth. However, at μ_{set} 0.0105 hr^{1}, the feeding rate of MF was too high which inhibited the growth of the cells whereas the μ_{set} at 0.006 hr^{-1} resulted in low cell density. The feeding rate according to this μ_{set} might not supply enough carbon sources for cell growth.

At 69 hr of induction time, even the cell density was still increased at μ_{set} of 0.0105 hr^{1} but the total protein concentration ($mg.L^{-1}$) and the accumulation of rEK_L ($U.ml^{-1}$) were decreased rapidly. Therefore, the process at μ_{set} of 0.0105 hr^{-1} was terminated.

The cell density was the highest at the end of the fermentation (141hr of induction time) for the process at μ_{set} 0.0075 hr^{-1} (128 ± 1 $g.L^{-1}$) which was much higher than the value obtained from the process of μ_{set} 0.006 hr^{-1} (103 ± 1 $g.L^{-1}$). The results for cell density obtained from this research were similar to the results from

Charoenrat et al, 2005 and Kupradit, 2006. Table 4.1 summarizes the high cell density cultivation obtained from different exponential feed rates.

Table 4.1 Cell density ($g.L^{-1}$) at the end of glycerol batch, glycerol fed-batch, 69 hr of induction time and processes at μ_{set} 0.006, 0.0075 and 0.0105 hr^{-1}.

Constant specific growth rate (μ_{set})	**Dry Cell Weight ($g.L^{-1}$)**			
	The end of glycerol batch phase	**The end of glycerol fed-batch phase**	**At 69h of induction time**	**The end of the process (141hr)**
0. 006 hr^{-1}	27 ± 1	41	70 ± 0.7	103 ± 1
0.0075 hr^{-1}	27 ± 1	40 ± 1	91 ± 0.7	128 ± 1
0.0105 hr^{-1}	27 ± 1	40 ± 1	80 ± 1	

4.4.3 Biomass yield

The biomass yield ($Y_{X/S}$) is defined as the ratio of the amount of biomass produced to the amount of substrate consumed (g biomass.g $substrate^{-1}$). Figure 4.8 shows the time course of biomass yield at different μ_{set}. In general, the high $Y_{X/S}$ of all processes was presented at the beginning and rapidly decreased after 21 hr of induction time. Biomass yield slowly declined with time and reached their lowest values at the end of the processes when the feeding rate of MF medium or the total amount of methanol added was the highest. This was due to the use of methanol for cell growth. At 69 hr of induction time, the $Y_{X/S}$ of the three processes at μ_{set} 0.006, 0.0075 and 0.0105 hr^{-1} were 0.36, 0.41, and 0.27 g cell.g $methanol^{-1}$, respectively. The

final $Y_{X/S}$ of the μ_{set} at 0.006, and 0.0075 hr^{-1} was 0.20, 0.21 g cell. g $methanol^{-1}$ at 141 hr of induction time.

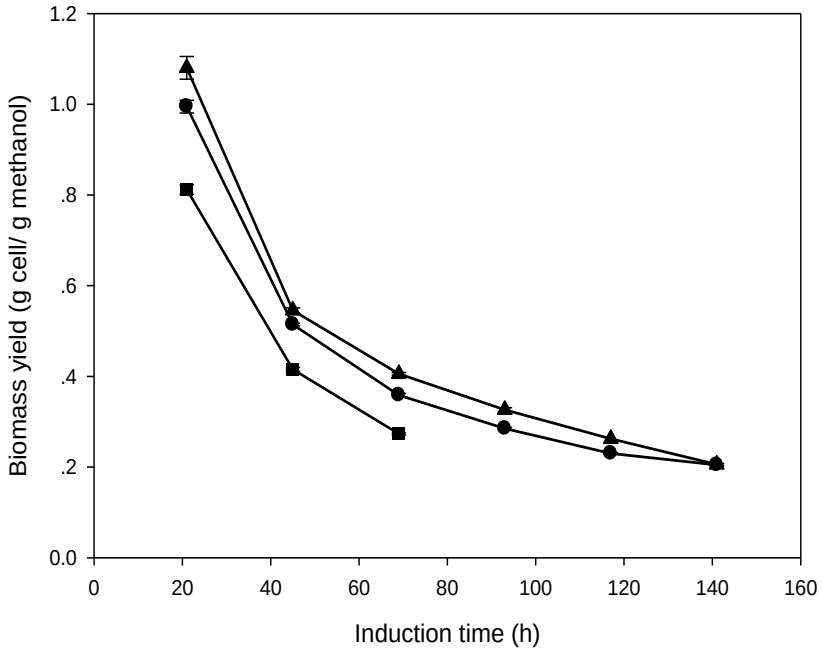

Figure 4.8 Biomass yield $Y_{X/S}$ (g cell.g $methanol^{-1}$) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

The decreasing of $Y_{X/S}$ during the protein production phase was also found in previous researches. In heavy-chain fragment C of boturinum neurotoxin production, the $Y_{X/S}$ decreased and the maintenance coefficient (q_m) increased with the increasing of methanol concentration (Zhang et al., 2000). The biomass yield in all MLFB, OLFB and HPFB processes for production of recombinant β-glucosidase was also found decreasing over time. The reason for the reduction of the biomass yield might be due to the increasing of maintenance demand, which was expressed as

increased combustion of the energy source and no major by-products from the methanol metabolism were produced (Charoenrat, 2006).

4.4.4 Residual methanol concentration

Accumulation of methanol inside the fermentor at different feeding rates was determined by using Gas chromatography, and the experimental results were illustrated in Figure 4.9.

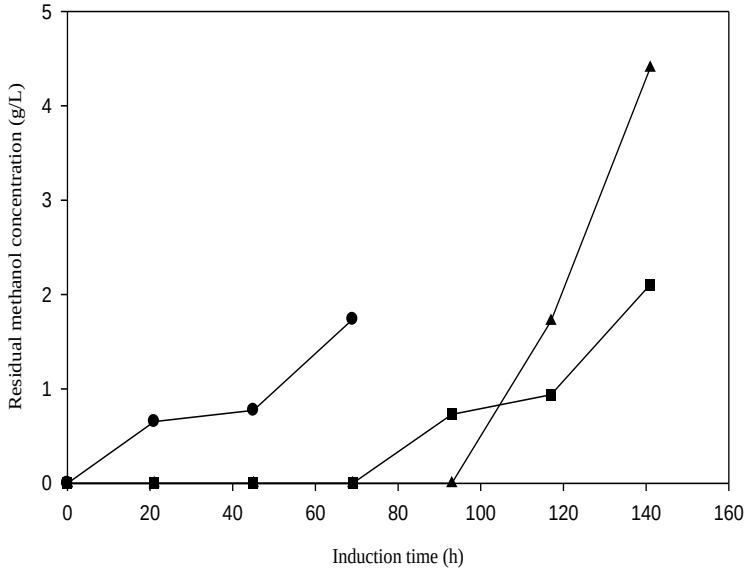

Figure 4.9 Residual methanol concentration inside the fermentors ($g.L^{-1}$) during methanol production phases in processes at μ_{set} 0.006(■), 0.0075 (▲) and 0.0105 hr^{-1} (●).

For the μ_{set} of 0.0105 hr^{-1}, the residual methanol concentration was detected even at the beginning of the process. The value was observed at 0.65 $g.L^{-1}$ at the beginning before increased to 1.74 $g.L^{-1}$ at 69 hr of the induction time. At μ_{set} 0.006 hr^{-1}, the residual methanol concentration was detected at 0.73$g.L^{-1}$ after 69 hr of induction time. On the other hand, 1.71 $g.L^{-1}$ of residual methanol concentration was

determined after 93hr induction time for μ_{set} 0.0075 hr^{-1}. However, at the end of the fermentation process, the residual methanol concentration sharply increased up to 2.10 $g.L^{-1}$ at μ_{set} 0.006 hr^{-1} and 4.40 $g.L^{-1}$ in the case of μ_{set} 0.0075 hr^{-1}. This high residual concentration of methanol in the fermentation broth resulted in the decreasing of the rEK_L activity.

In conclusion, the cell densities of all three fermentation processes increased because *P. pastoris* can utilize methanol for its growth, and this value was not significantly affected by the concentration of residual methanol in the fermentation broth. At μ_{set} 0.0075 hr^{-1}, the highest feeding rate of MF medium at the end of the process was 7.069 $g.hr^{-1}$ and the residual methanol concentration was approximately 4.40 $g.L^{-1}$. This was in a good agreement with the previous report which demonstrated that only a slight reduction of the specific growth and methanol uptake rate were exhibited at methanol concentration of 10 $g.L^{-1}$ (Katakura et al., 1998).

4.4.5 Product formation

The rEK_L production was controlled by the same promoter as the AOX enzyme, and is usually associated with cell growth. Control of the specific growth rate results in an increasing of biomass, and product formation. The experimental results for rEK_L production (rEK_L accumulation, $U.mL^{-1}$) in the three feeding rates were shown in Fig 4.10.

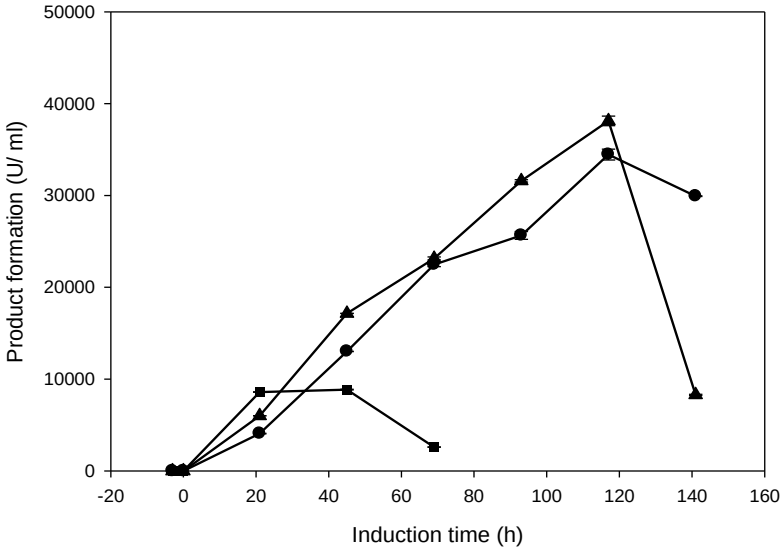

Figure 4.10 The product formation ($U.mL^{-1}$) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

In the process at μ_{set} 0.0105 hr^{-1}, the results showed that the rEK_L accumulation ($U.mL^{-1}$) was very high after 21 hr of induction time (8,590 $U.mL^{-1}$), but increased slowly after 50 hr before rapidly decreased after only 69hr of the induction time (2,596 $U.mL^{-1}$). At 45 hr of μ_{set} 0.0105 h^{-1}, a total methanol of 167.35 g were fed to the fermentor (Figure 4.6). The rEK_L activity obtained was the highest value, but slightly higher than at 21 hr. This indicated that methanol inhibited product formation as shown in Figure 4.10 and the high value of rEK_L activity for the μ_{set} 0.0105 hr^{-1} at the beginning was clearly due to an excessive amount of methanol. At 21 hr of induction time, the rEK_L accumulation in two other processes at μ_{set} 0.0075 and μ_{set} 0.006 was obtained at lower concentration than the highest μ_{set} which was 6,005, and 4,066 $U.mL^{-1}$, respectively. However, after 21 hr of induction time, the accumulation of rEK_L in these processes still increased rapidly. The maximum rEK_L activity was found at μ_{set} 0.0075 hr^{-1} (38,125 $U.ml^{-1}$) which was obtained at 117 hr of

induction time, higher than at μ_{set} 0.006 hr^{-1} (34,456 $U.ml^{-1}$) as shown in Figure 4.10. In addition, the highest rEK_L activity was obtained at 117 hr of induction time when the total methanol of 384.78 and 465.10 g were consumed at μ_{set} 0.006 and 0.0075 hr^{-1}, respectively. After 117 hr induction, however, the accumulation of rEK_L was sharply decreased in the μ_{set} 0.0075 hr^{-1} (8,288 $U.ml^{-1}$) and slowly reduced in the process at μ_{set} 0.006 hr^{-1} (29,920 $U.ml^{-1}$). The resulted rEK_L activity showed much higher than other previous reports for the production of rEK_L by *P. pastoris*.

It is clearly seen that the induction time, and the feeding rate of MF medium affected the accumulation of rEK_L. The rate of increasing rEK_L activity was started to reduce after only 21 hr of induction time for the fermentation at μ_{set} 0.0105 hr^{-1} at a high feeding rate of MF medium 3.57 $g.hr^{-1}$ comparing with the feeding rate of MF medium in the processes at μ_{set} 0.006 hr^{-1} (2.87 $g.hr^{-1}$) and μ_{set} 0.006 hr^{-1} (2.55 $g.hr^{-1}$). For both processes at μ_{set} 0.0075 hr^{-1} and 0.006 hr^{-1}, the rEK_L accumulation still increased and reached the maximum point at 117 hr of induction time when the feeding rates of MF medium were 7.06 and 5.24 $g.hr^{-1}$, respectively. But after that, the enzymatic activity decreased even when the protein concentration and the cell density still increased. The reason might be due to the high feeding rate of MF methanol up to 6.01 and 8.40 g. hr^{-1} which was fed to the processes at μ_{set} 0.006 and μ_{set} 0.0075 hr^{-1}, respectively. It could be explained that the effect of excessive methanol concentration on *P. pastoris* culture causes cell death (Jahic *et al*., 2003) resulting in the degradation of proteases. It has been observed that the optimum methanol concentration for cell growth differed significantly from the optimum methanol concentration for production (Mayson *et al*., 2003), and the specific product formation rate (q_p) does not correlate to the specific growth rate (μ) (Kupcsulik and Sevella, 2004). In this work,

the optimum methanol concentration for cell growth correlated with the optimum concentration for protein production, but differed from the enzymatic activity. The reasons maybe can explain by the different kind of proteins, and strains of *P. pastoris* used for fermentation.

The product accumulation at different time period can also be visualized by SDS-PAGE analysis. The result of SDS-PAGE of the culture broth at the end of the process at μ_{set} 0.0075 hr^{-1} was shown in Figure 4.11 and the process at μ_{set} 0.006 hr^{-1} was shown in Figure 4.12.

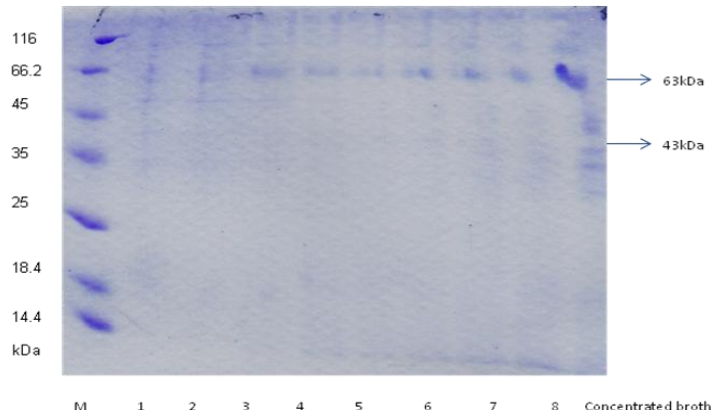

Figure 4.11 SDS-PAGE with Coomassie blue stain of fermentation supernatant at increasing time profile from the process μ_{set} 0.0075 hr^{-1} (M: protein marker; Lane 1-8: samples at the end of glycerol batch phase, glycerol fed – batch phase, during the methanol production phase at 21, 45, 69, 93, 117, 141 hr of induction time and concentrate culture broth from 117 hr of induction time, respectively).

The expected molecular weight of this recombinant enterokinase under denaturing condition is approximately 43 kDa, but the visible protein band about 63 kDa which is larger than the expected rEK_L found in the previous research (Kupradit, 2008). The investigation in this experiment found that both two bands about 43 and

63 kDa were presented. This is possibly due to over glycosylation of the recombinant protein in *P. pastoris*, which has been reported to occur to many proteins produced in yeast (Bretthauer and Castellino, 1999).

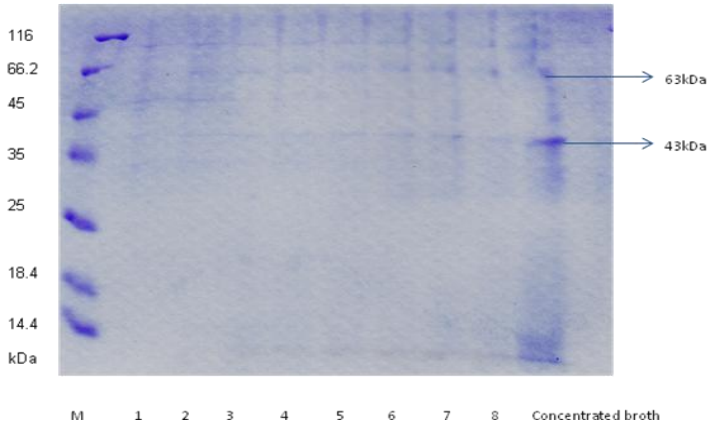

Figure 4.12 SDS-PAGE with Coomassie blue stain of fermentation supernatant at increasing time profile from the process μ_{set} 0.006 hr^{-1} (M: protein marker; Lane 1-8: samples at the end of glycerol batch phase, glycerol fed – batch phase, during the methanol production phase at 21, 45, 69, 93, 117, 141 hr of induction time and concentrate culture broth from 117 hr of induction time, respectively).

The results from SDS–PAGE of the three fermentation processes also indicated that only small amount of the *P.pastoris* own protein was secreted to the culture medium (Fig 4.13). Thus it is an advantage for further purification, and facilitates separation of the product from most other cellular components. However, the expected protein band in three fermentation process was different. Some foreign protein secreted in *P. pastoris* appeared to be hyperglycosylated (Cereghino and Cregg, 2000). Little different in size of oligosaccharides could be correlated to

composition of the growth medium or cultivating in shake-flask or fermentor (Bretthayer and Castellino, 1999). In this research, the different protein size possible may be due to the different glycosylation process at different concentration of methanol feeding rate. In order to answer for this particular problem, purification steps should be carried out.

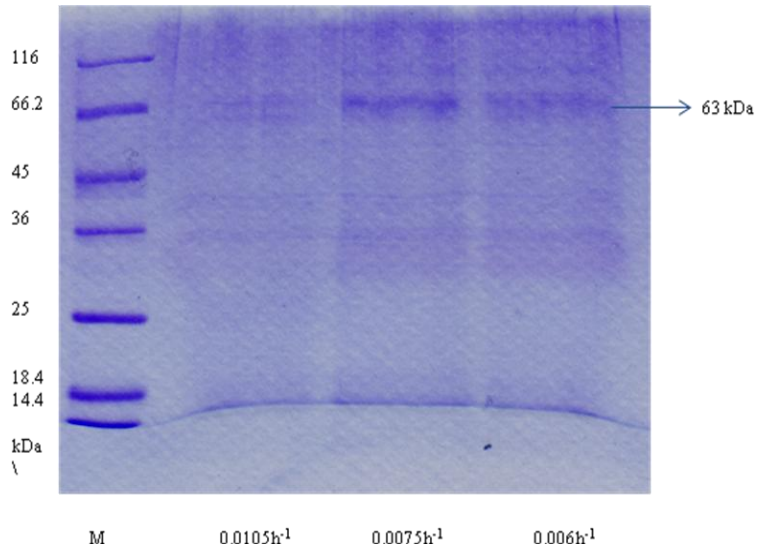

Figure 4.13 SDS-PAGE with Coomassie blue stain of fermentation supernatant at the end of the processes at μ_{set} 0.0105, 0.0075 and 0.006 hr^{-1}.

4.4.6 Total protein concentration and specific rEK_L activity

The specific enzymatic activity is an alternative value that can be used to monitor the purity of the enzyme. The specific activity of the rEK_L in the crude broth was calculated from the rEK_L activity ($U.mL^{-1}$) as seen in Figure 4.10, and the total protein concentration shown in Figure 4.14. The results for total protein concentration, and specific activity of rEK_L were shown in Figure 4.14 and Figure 4.15.

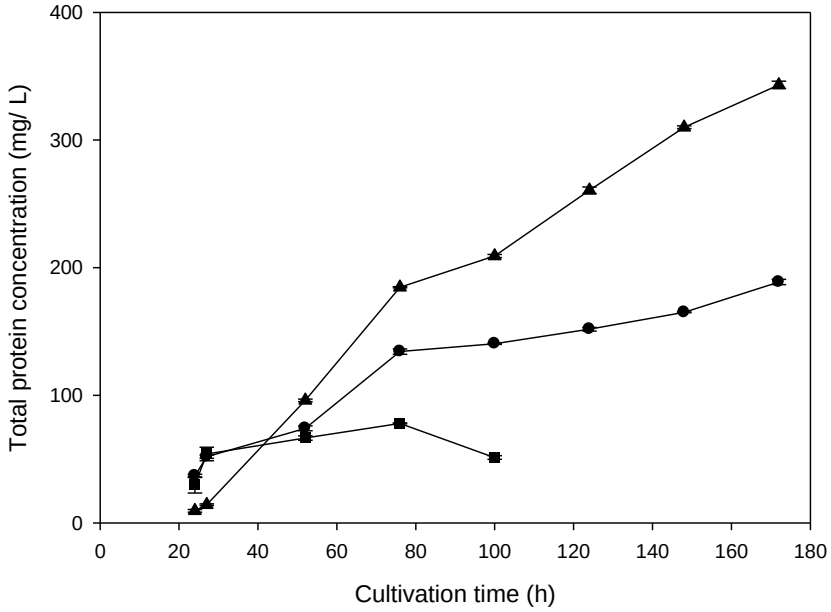

Figure 4.14 Total protein concentration, ($mg.L^{-1}$) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

After 45 hr of the methanol production phase at μ_{set} 0.0105 hr^{-1}, the cell density still increased (Figure 4.7), but the total protein concentration decreased rapidly. This may be due to the accumulation of residual methanol which exceeded inhibitory level, thus inhibit the cell growth and reduced total protein concentration. In the other two processes, total protein concentration was increased overtime. The total protein concentration increased rapidly before 69 hr of induction time for production of rEK_L. However, the rate of increasing declined after 69hr of induction. At the end of production phase, the highest total protein concentration was obtained at approximately 343.19 $mg.L^{-1}$ for μ_{set} 0.0075 hr^{-1}, compared to 188.75 $mg.L^{-1}$ in the process at μ_{set} 0.006 hr^{-1}.

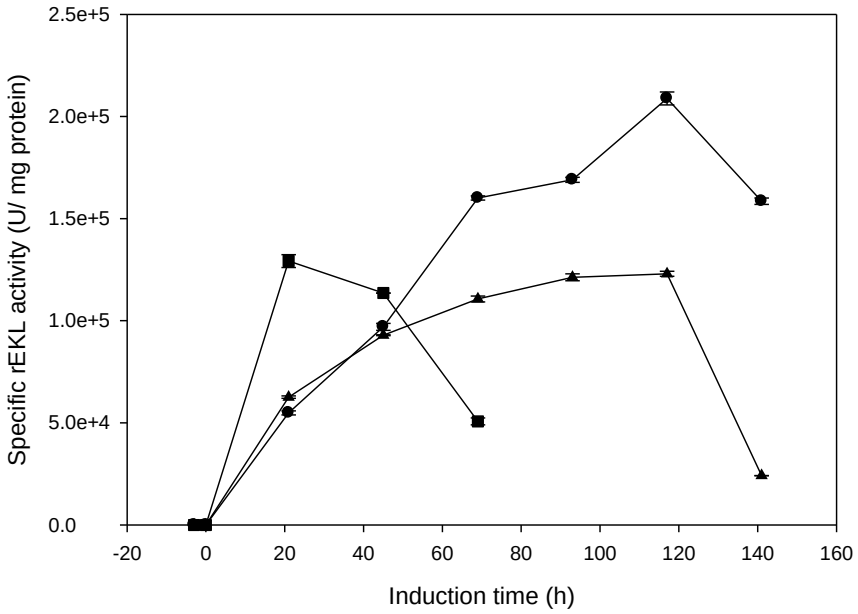

Figure 4.15 Specific rEK_L activity, ($U.mg^{-1}$) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲), and 0.0105 hr^{-1} (■), respectively.

The specific rEK_L activity was reached a high value after only 21 hr of induction time in the process at μ_{set} 0.0105 hr^{-1} (129,214 U.mg $protein^{-1}$) compared to the values obtaining in the process at μ_{set} 0.0075 hr^{-1} (62,578 U.mg $protein^{-1}$) and μ_{set} 0.0006 hr^{-1} (54,875 U.mg $protein^{-1}$). But after 21 hr of induction, the specific rEK_L activity was rapidly decreased. However, the specific rEK_L activity in the other two processes was still increased rapidly until 69 hr of induction time then slowly increased and reached highest value at 117 hr of induction time (208,824 U.mg $protein^{-1}$at μ_{set} 0.0006 hr^{-1} and 122,975 U.mg $protein^{-1}$ at μ_{set} 0.0075 hr^{-1}) before a sharp decreasing (158,525 and 24,150 U.mg $protein^{-1}$ at the end of the processes at μ_{set} 0.0006 and 0.0075 hr^{-1}, respectively). These feeding strategies gave higher specific rEK_L than all the previously reports which the rEK_L was produced in *P.pastoris* (Peng

et al., 2003; Fang and Huang, 2004; Kupradit, 2006; Tan et al, 2007 and Zhang et al., 2008) shown in Figure 4.15.

From our results, it can be concluded that the optimum methanol concentraton for cell growth correlated to the optimum methanol concentration for production formation. At μ_{set} 0.0075 hr^{-1}, the highest cell density, total protein concentration, and rEK_L activity ($U.mL^{-1}$) were obtained at 117 hr of the induction time. However, the highest specific rEK_L activity was not presented in this process, but it was obtained at μ_{set} 0.0006 hr^{-1}. This was because of the lower total protein accumulation in the process at μ_{set} of 0.0006 hr^{-1}comparing to the total protein accumulation in the process μ_{set} 0.0075 hr^{-1} due to the low feeding rate of MF medium resulting in the low cell density.

4.4.7 Production yield and specific production yield

Normally, the substrate is a main cost of the fermentation process which makes the profit directly depend on the product yield (Yp/s). In this experiment, the production yield ($U.g\ methanol^{-1}$) was increased rapidly at the beginning, but decreased after only 21 hr induction in the process at μ_{set} of 0.0105 hr^{-1}, after 45 hr in the process at μ_{set} of 0.0075 hr^{-1}, and after 69hr in the process at at μ_{set} of 0.0006 hr^{-1} (Figure 4.16). It might due to the accumulation of the rEK_L in the process at μ_{set} of 0.0006 hr^{-1} was not much less than this in the process at μ_{set} of 0.0075 hr^{-1} whereas the total methanol consumption was fed lower. After these induction times, the production yield was reduced until the end of the production phase even the accumulation of rEK_L ($U.mL^{-1}$) and the total protein concentration was still increased. It might due to the lower increasing of the rEK_L accumulation comparing to the total methanol consumption over the induction time. Even though increasing Yp/s is one

objective in process optimization but comparing the price of rEK_L to methanol, the processes should be run until the accumulation of rEK_L in the crude broth was decreased.

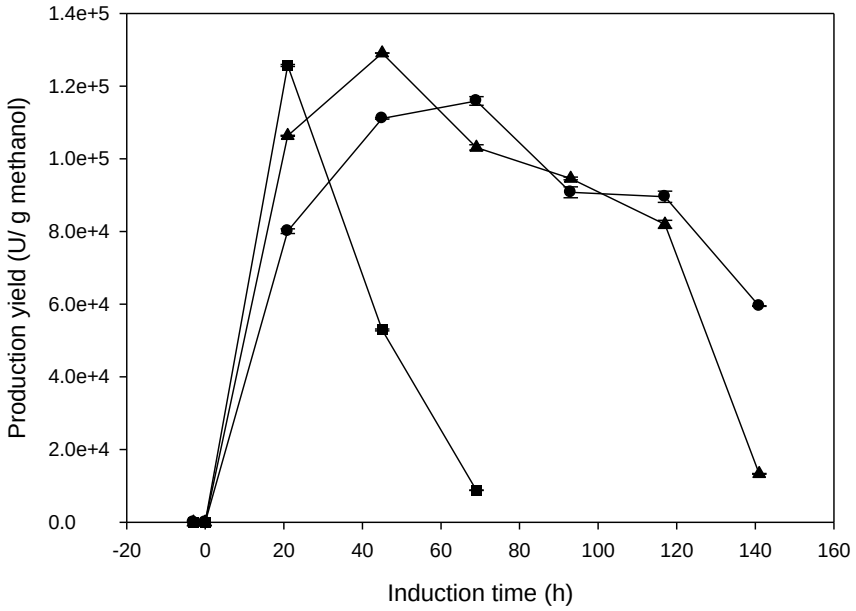

Figure 4.16 Production yield (U.g methanol^{-1}) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

The result to compare the specific production yield (U. g cell^{-1}) among three processes was shown in Fig 4.17. A highest specific production yield was obtained in the process at μ_{set} of 0.0105 hr^{-1}at only 21 hr of induction time (156,237 U. g cell^{-1}) then it was rapidly reduced. Lower values was shown in other two processes μ_{set} of 0.0075 hr^{-1}and μ_{set} of 0.0006 hr^{-1}at the first 21 hr of induction time comparing to the process at μ_{set} of 0.0105 hr^{-1} but they were still increased rapidly over induction time. The increasing of the specific production yield was reached the highest value at 117 hr of induction time then sharply decreased in the process at μ_{set}

0.0075 hr^{-1} comparing to the process at μ_{set} 0.0006 hr^{-1}. The maximum specific production yield was seen in the process at μ_{set} 0.0006 hr^{-1} (389,326 U.g $cell^{-1}$), even though the highest rEK_L activity (U.ml^{-1}) was present in the process at μ_{set} 0.0075 hr^{-1}. This was because of the lower cell density in the process at μ_{set} of 0.0006 hr^{-1}.

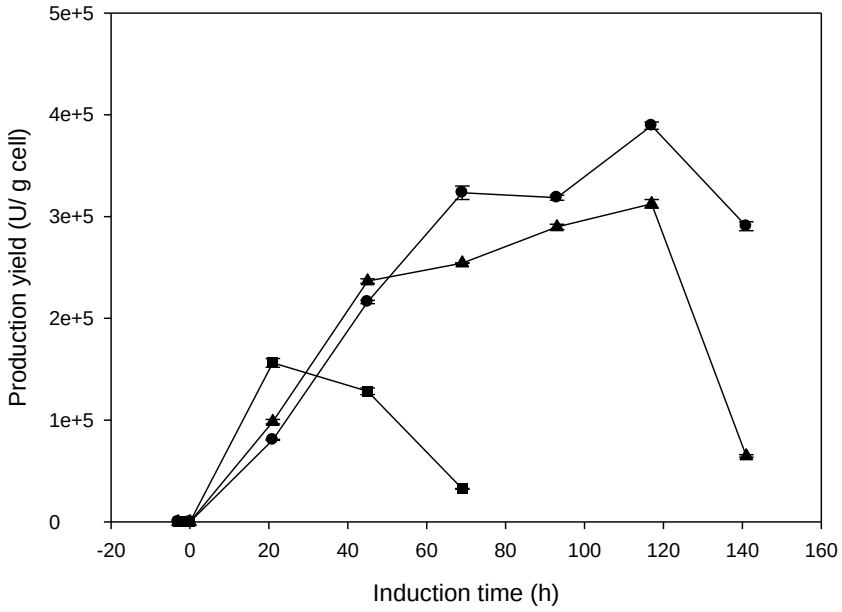

Figure 4.17 Specific production yield (U.g $cell^{-1}$) during methanol production phases in processes at μ_{set} 0.006(●), 0.0075 (▲) and 0.0105 hr^{-1} (■).

This result indicated that increase of methanol feed can increase the amount of rEK_L (detectd by the increase of the rEK_L activity (U.ml^{-1}). However, the adjustment of the methanol feed rate in the production phase must depend on the ability of methanol consumption of the culture and their cell density to ensure that the methanol was not accumulated and presented as toxic concentration for the cell growth.

Table 4.2 The total methanol consumption, cell dry weight, total protein concentration, rEK_L accumulation, biomass yield, specific rEK_L activity and specific production yield in the three processes at the end of each process and at 117 hr of induction time (*).

Parameters	**Fermentation processes at different specific growth rate**		
	0.006 hr^{-1}	**0.0075 hr^{-1}**	**0.0105 hr^{-1}**
Total methanol consumption (g)	502.93	622.12	294.72
Cell Dry Weight ($g.L^{-1}$)	103	128	80
Total protein concentration ($mg.L^{-1}$)	189	343	50
rEK_L accumulation ($U.ml^{-1}$)	29,920	8,288	2,596
Maximum rEK_L accumulation		38,125 (*)	
Specific rEK_L activity ($U.mg^{-1}$)	158,525	24,150	50,689
Maximum specific rEK_L activity	208,824 (*)		
$Y_{X/S}$ ($g.g^{-1}$)	0.20	0.21	0.27
Specific production yield ($U.gcell^{-1}$)	290,515	64,759	32,461

4.2 Purification of recombinant enterokinase light chain (rEK_L) produced in *P. pastoris*

4.2.1 Purification of rEK_L by cobalt column

At the end of the fermentation processes, the cell pellet was removed by centrifugation. The crude supernatant was filtrated pass 0.2 µm filter then concentrated and dialyzed in 50 mM Tris-Cl pH 8. Before the sample was loaded into the cobalt column (column volumn 1ml), the column was equilibrated with 50 mM Tris-Cl pH 8, 150 mM NaCl (W_0). Ten ml of the dialyzed sample was loaded and the flow-through fractions was collected. The column was washed with 5ml (5CV) of W_0 buffer, 5 ml (5 CV) of washing buffer (W_I) containing 50 mM Tris-Cl pH 8, 300 mM NaCl, 20 mM imidazole then eluted with 5ml of elution buffer 1 (E_1) (50 mM Tris-Cl pH 8, 300 mM NaCl, 250 mM imidazole) and elution buffer 2 (E_2) (50 mM Tris-Cl pH 8, 300 mM NaCl, 500 mM imidazole). All the fractions, flow-through, wash and elution were collected and analyzed for enzyme activity and SDS-PAGE.

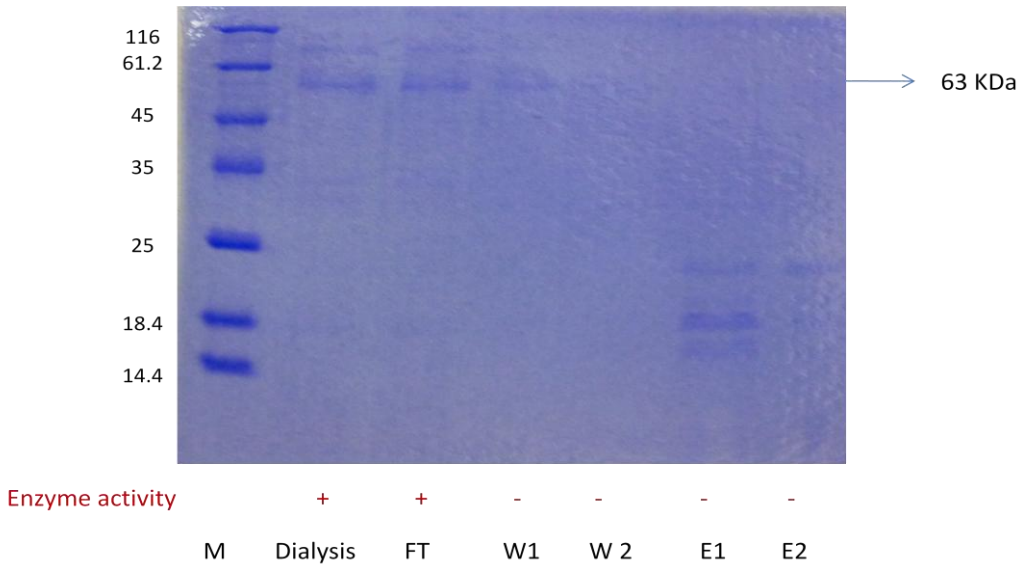

Fig 4.18 SDS-PAGE with Coomassie blue stain of rEK_L purification from Cobalt column.

The results of rEK_L activity analysis indicated that the binding of the 8X His-tagged rEK_L did not occurred because almost all of the rEK_L activity was detected in the flow-through fraction, but no rEK_L activity was detected in elution fractions. These results were supported by the results of the SDS-PAGE analysis because almost all of the expected bands of 63 kDa were detected in the flow-through fraction and a very small amount in the washing fraction (Fig 4.18).

In addition, another attempt to purify rEK_L containing N-terminal His-tagged was carried out. The first attempt was to increase the incubation time between the dialyzed sample and Co^{+2} beads on ice for 30 mins. The unbound fraction was collected by centrifugation at 900 rpm; 4^0C for 5 mins then the Co^{+2} beads were washed and eluted by the same washing and elution buffers. In the step of elution, the Co^{+2} beads were also increased for 5 min of incubation time before centrifugation to spin the Co^{+2} beads down. However, the results of SDS-PAGE and rEK_L activity analysis still indicated that no binding of rEK_L N-terminal His-tagged to Co^{+2} ion occurred.

The problem of no binding of the EK_L to the Co^{+2} beads might due to some component in the sample which may block the binding of rEK_L containing His-tagged and the metal ions. To solve this problem, the rEK_L sample was precipitated by ammonia sulfate 90% first then was dissolved in Tris-Cl pH 8 before loading into the Co^{+2} columns again. In addition, protein precipitation technique is typically recommented to use at the beginning in a purification procedure, before going on to methods which are harder to use with large volumes. But the results still shown that

there were no rEK_L activity and major protein bands were detected in the elution fractions.

The last attempt to purify rEK_L was done by doing purification of rEK_L by ion exchange chromatography first to remove all the component away from the inhibitor in the sample then the purified enzyme was pH adjust by 50 mM Tris- Cl pH 8 and loaded into the Co^{+2} columns. However, no rEK_L activity was detected in the elution fractions but almost all of the activity was found in the flow-through fraction again. Therefore, all of these results indicated that His-tagged in rEK_L could not bind to the Co^{+2} ion column. This might due to His-tagged of rEK_L molecular was cleaved off during the production of this enzyme resulting in changing the size of the rEK_L or due to the protein folding resulting in His-tagged not expose to the medium, or an alternatively, the exposed His-tagged of rEK_L was bound to some other metal ion from the fermentation medium thus this exposed His-tagged cannot bind to metal ion resin in the column.

No detecting of enzymatic activity in the rEK_L containing N-terminal His-tagged was reported (Choi et al., 2001). Isoleucine (Ile) at N-terminal of EK_L is highly conserved among EK_L from various sources and it is buried into hydrophobic pocket according to the crystal structure. The modifications at N-terminal end, such as substitution, addition, or deletion, can delete its enzyme activity (Choi and Song, 2001). However, the construct of N-terminal His-tagged of rEK_L showed high enzymatic activity and very small smear band of 8X His-tagged from N-terminal of rEK_L was detected in the concentrated culture supernatant by western blot analysis (Kupradit, 2006). This construct was used again in this research and a very high enzymatic activity comparing to all previous reported was obtained. Therefore, the

unsuccessful rEK_L purification using Cobalt column might due to the His-tagged at the N-terminal of rEK_L was removed. This problem also has been reported in production of the carcinoembryonic antigen by *P. pastoris* which the binding of 6×His-tagged to IMAC column was found very weak. It can be explained that it seems to be a problem specific to each protein.

4.2.2 Purification of rEK_L by ion exchange chromatography

The filtrated supernatant culture broth were dialyzed and concentrated in 50 mM sodium acetate buffer pH 5 then centrifuged at 12,000 rpm for 15 minutes before loading into Sulphopropyl Fast Flow Column (SP_FF column 6ml) which was equilibrated with 50 mM sodium acetate buffer pH 5 before using. To remove the unbound fraction, the column was washed with 5 CV of 50 mM sodium acetate pH 5. The rEK_L was eluted with a gradient from 0 to 1 M NaCl in sodium acetate buffe then the concentration was detected by spectrophotometer at wave length 280 nm and all the fractions were collected. The fractions from flow-through wash and elution were tested for enzymatic activity and checked by SDS-PAGE. The elution fractions which showed high activity were pooled and concentrated.

The profiled of rEK_L purification from ion exchange showed that almost all the rEK_L was eluted fast at a gradient of 0.1 to 0.2 M NaCl in all rEK_L production (Fig 4.19 and Fig 4.20).

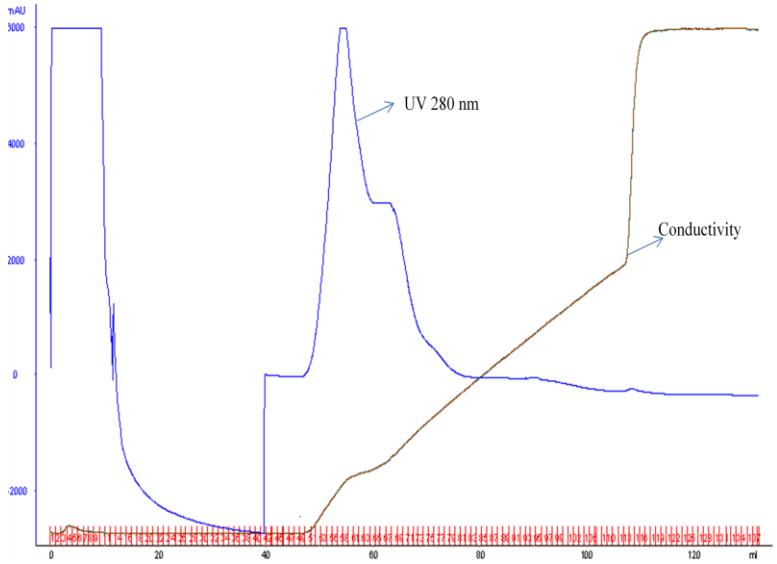

Figure 4.19 Profiled of rEK_L purification by ion exchange chromatography with 2 ml concentrated sample from the process at μ_{set} 0.0075 hr^{-1}.

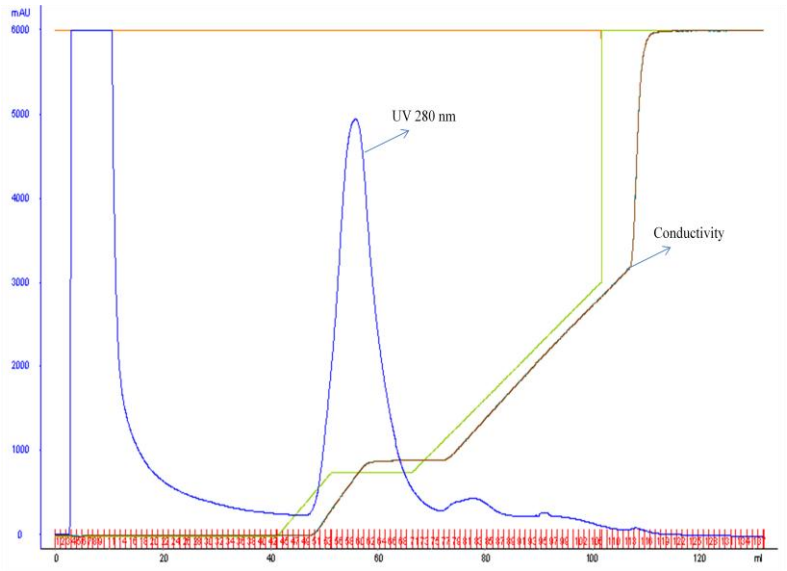

Figure 4.20 Profiled of rEK_L purification by ion exchange chromatography with 1 ml concentrated sample from the process at μ_{set} 0.006 hr^{-1}.

The results of rEK_L activity assay from each fraction indicated that the activity was found only in the elution fractions but no activity could be detected in the

fractions of flow-through or wash.

In all the fractions from flow-through and wash, no protein bands were shown when the SDS-PAGE analysis was performed. These results indicated that all the protein in the sample was bound to SP column. In the fractions from the elution which showed high concentration of protein, the bands 43 kDa was found eluting together with the band 63 kDa in both processes at μ_{set} 0.006 and 0.0075 hr^{-1} (Fig 4.21 and Fig 4.22).

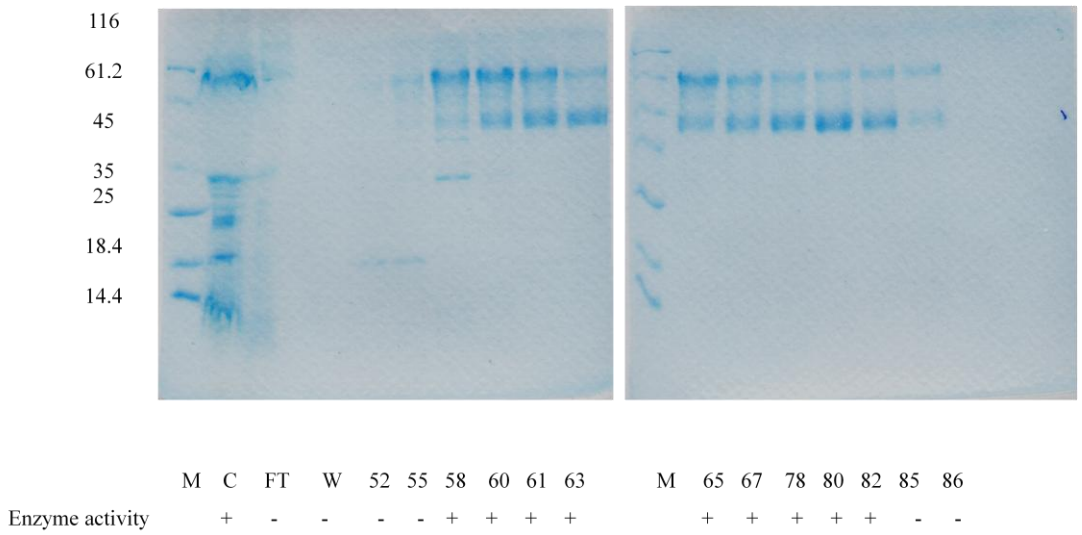

Figure 4.21 SDS-PAGE with Coomassie blue stain of rEKL purification from SP column (cation exchanger). 2 ml concentrated sample from fermentation at specific growth rate 0.0075 hr^{-1} was load into SP column. Protein molecular marker (M); Concentrated and dialyzed sample (C); Flow-through fraction (FT); Wash fraction (W) and elution fraction number 52, 55, 58, 60, 61, 63, 65, 67, 78, 80, 82, 85 and 86.

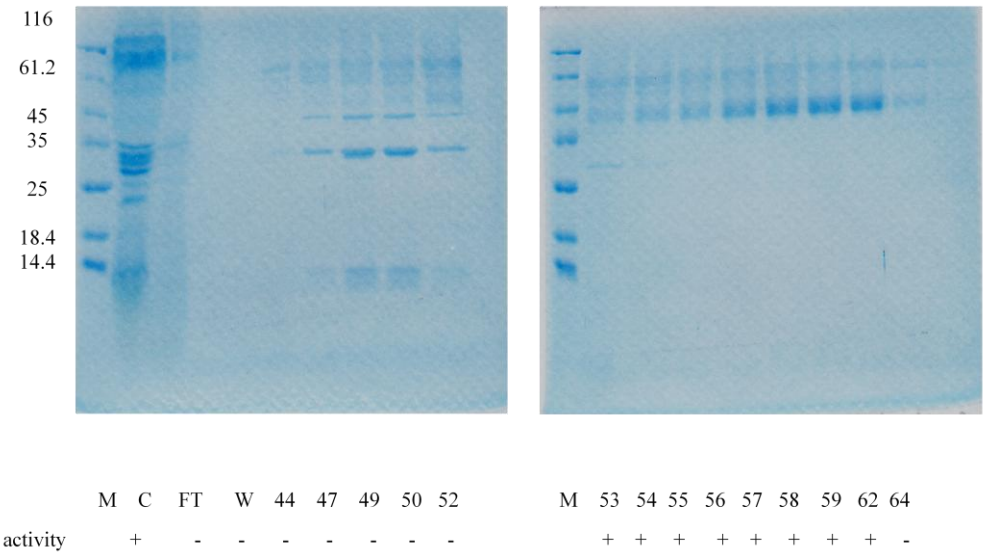

Figure 4.22 SDS-PAGE with Coomassie blue stain of rEKL purification from SP column (cation exchanger). 1 ml concentrated sample from fermentation at specific growth rate 0.006 hr^{-1} was load into SP column. Protein molecular marker (M); Concentrated and dialyzed sample (C); Flow-through fraction (FT); Wash fraction (W) and elution fraction number 44, 47, 49, 50, 52, 53, 54, 55, 56, 57, 58, 59, 62 and 64.

The elution fractions that showed activity and accumulation of rEK_L were pooled then concentrated. The results of the rEK_L purification which was production in different processes are summarized in Table 4.3. By using SP column, total 3,542 µg pure and active rEK_L was obtained from 330ml culture broth supernatant in the process at μ_{set} 0.0075 hr^{-1}.

Table 4.3 Recovery of rEK_L from fermentation culture broth in the processes at μ_{set} 0.006 and 0.0075 hr^{-1}.

Fermentation process at different specific growth rate	**Total protein concentration (µg.ml^{-1})**			
	Crude Broth from fermentor	**After dialysis and concentration**	**Pooled fractions from elution**	**Concentrated pooled fractions from elution**
0.006 hr^{-1}	165.00 (250ml)*	5,568 (1ml)*	Use for purification by Co^{+2} column	
0.0075 hr^{-1}	310.02 (330ml)*	6,475 (2ml)*	268.67 (25 ml)*	1,540 (2.3 ml)*

()*: volume of samples which was obtained after each process.

The purification of rEK_L in this research was carried out following the ion exchange purification as described by Cregg et al, 1993 and Kupradit, 2006. The secreted protein tightly bound to the column and eluted under the elution buffer. However, this purified rEK_L was different size from the other previous reports which was produced in *P. pastoris* GS115 strain. This might due to the different in the using of wild type strain of *P. pastoris* resulting in the difference in glycosylation patterns of each *Pichia* strain. The expected size 43 kDa was found together with the larger size 63 kDa in this research. Whereas only the larger size 63 KDa was seen in the previous report which the same construct was expressed in *P.pastoris* Y11430 (Kupradit, 2006). However, the enzymatic activity was shown to be very high comparing to other reports and the ability of the cleavage of fusion protein with rEK_L in this research and commercial EK was not different.

4.2.3 Cleavage of fusion protein by the purified rEK_L

The activity of rEK_L was also detected by using this purified rEK_L to cleave the fusion protein; rice Os1BGlu4-Trx which has the enterokinase recognition sequence in the linker. The purified rEK_L was incubated with 22 µg rice Os1BGlu4-Trx in 50 mM Tris- Cl pH 8 at 23^0C for 16 hr then the cleavage products were checked by 15 % SDS-PAGE gel. The commercial rEK_L from NEB was used as positive control.

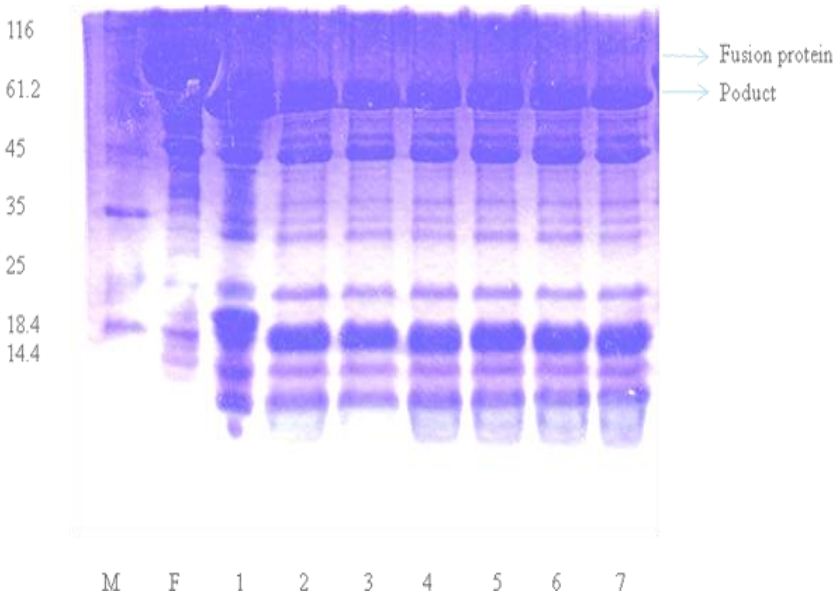

Figure 4.23 Cleavage of fusion protein, recombinant rice Os1BGlu4-Trx, with purified rEK_L producted in the process at μ_{set} 0.0075 hr^{-1}, compared to the cleavage with commercial rEK_L from NEB in 50mM Tris-Cl pH 8 in total reaction of 22µl for 16h at 23 °C. Protein marker (M); Fusion protein (F) and the incubation of fusion protein with 0.00014 µg commercial rEK_L; 0.154; 0.308; 0.462; 0.616; 0.77 and 0.924 µg purified rEK_L (Band 1-7).

The result shown in Figure 4.23 shows that the pattern of band product which generated by the cleavage of the purified rEK_L in this experiment and the commercial rEK_L from NEB were similar. The major cleavage products of 55 kDa were found in all reactions which were cleaved by rEK_L and almost all of the 22 µg of recombinant rice Os1BGlu4-Trx was completely cleaved by 0.00014 µg commercial rEK_L and 0.154 to 0.924 µg purified rEK_L which was purified from the process at µ 0.0075 hr^{-1} in this research. The degradation of the major product of 55 kDa wasn't occurred when using this protocol for 16 hr of incubation time for both commercial and purified rEK_L.

CHAPTER V

CONCLUSION

Recombinant enterokinase light chain was successfully produced in *P. pastoris* using a simple fed-batch technique. The effects of different feeding strategies of MF medium according to different specific growth rate at μ_{set} 0.0105, 0.0075 and 0.006 hr^{-1} during methanol production phase were investigated. The results for cell density, protein concentration, enterokinase accumulation, and specific enterokinase activity were significantly different in the three processes. Using the feeding strategy for the process at μ_{set} 0.0075 hr^{-1}, not only highest cell density and overall protein yield but also the enterokinase accumulation was obtained at 117 hr of induction time. A very high specific rEK_L activity was found in this research comparing to other previous researches. At the end of the process, the purification of rEK_L was carried out by ion exchange purification technique. Even though two protein bands of the secreted purified rEK_L of 43kDa and 63kDa were detected by SDS-PAGE but it did not affected the activity of rEK_L and ability of rEK_L to cleave the fusion protein. Therefore, this study presented a simple and cost-effective procedure that might be applicable for large-scale production of rEK_L with very high enzymatic activity.

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APPENDIX

APPENDIX A

Culture medium

A.1 Yeast extracts peptone dextrose (YPD)

Yeast extract	10g/ L
Peptone	20g/ L
Dextrose	20g/ L

The medium is autoclaved at 121^{o}C for 15 minutes and then 100µg/ml final concentration of zeocin was added when the medium cooled down to 55^{0}C. If the YDP agar is desired, 15 g/ L of agar is added into the medium before autoclaving.

A.2 BMGY medium

Yeast extract	10g/ L
Peptone	20g/ L
Glycerol	10g/ L

Dissolved each of compounds in turn in 100mM potassium phosphate buffer pH 6 and then adjusted to 1L. The medium is sterilized for 15 min, 121^{o}C and 100µg/ml zeocin is added when the medium cooled down to 55^{0}C.

A.3 PTM1 trace salts

$CuSO_4.\ 5H_2O$	6.0 g/ L
KI	0.08g/ L
$MnSO_4.\ H_2O$	3.0g/ L
$Na_2MoO_4.\ 2H_2O$	0.2g/ L
H_3BO_3	0.02g/ L
$ZnCl_2$	20.0g/ L
$FeCl_3$	13.7g/ L
$CoCl_2.\ 6H_2O$	0.9g/ L
H_2SO_4	5.0g/ L
Biotin	0.2g/ L

All the compounds are dissolved in distillated water and adjusted the volume to 1 L. The medium is filtrated sterilization with a 0.45 µm filter and covered with ammonium foil (dark condition) and stored at room temperature.

A.4 Glycerol Basal Salt medium (GBS)

H_3PO_4 85%	26.7ml/ L
$CaSO_4$	0.93g/ L
K_2SO_4	18.2g/ L
$MgSO_4.7H_2O$	14.9g/ L
KOH	4.13g/ L
Glycerol	40.0g/ L

Fermentor containing 950ml GBS medium is autoclaved at 121^{o}C for 35 min. After the sterile fermentor is cooled down to the cultivation temperature (30^{0}C), 4.35

ml/ L of PTM1 trace salt is added to sterile medium then pH of the medium is adjusted to 5.5 by ammonium solution 25%.

A.5 Glycerol feed medium (GF)

500g of 99.5% glycerol is dissolved with distillate water and adjusted the volume to 1L. And then the GF medium is autoclaved at 121^{o}C for 35 min. After autoclaving, 12 ml of PTM1 trace salt is added into 1L of the sterile medium.

A.6 Methanol feed medium (MF)

Add 12 ml of PTM1 trace salts into 1 L of 99.9% methanol.

APPENDIX B

Analytical methods

B.1 Cell concentration

Cell concentration was determined by optical density with a spectrophotometer at the absorbance of 600 nm (OD), and cell dry weight.

Table AB.1 Correlation of cell dry weight and OD_{600}.

Cell dry weight (g. L^{-1})	**OD_{600}**
0	0
28	47.5
39	100.4
62	175.35
73	223.3
91	262.8
110	347.1
123	375.2
128	381.5

The dry cell weight was determined from a 5 ml culture sample, which was centrifuged at 4000 rpm for 10 min. The supernatant was collected for further analyses, and the pellet was washed with distilled water once and dried at 105 ^{o}C for 24-48 hours until constant weigh (each sample was taken in duplicate). Cell dry weight was calculated using the following equation.

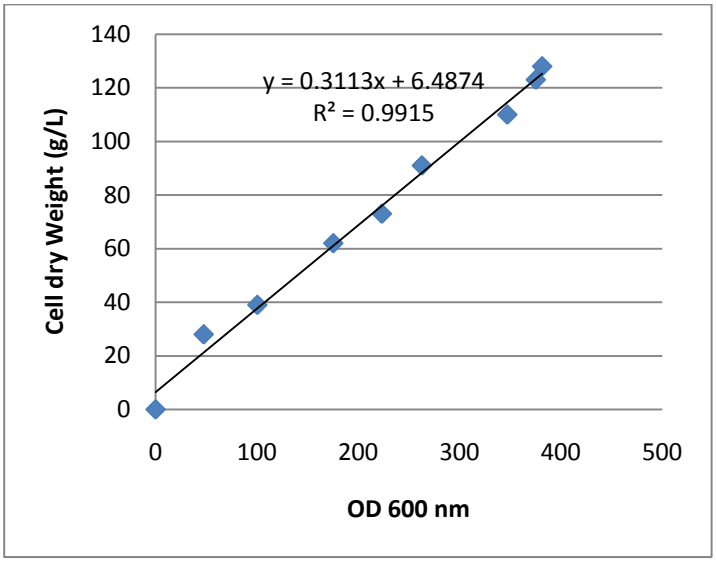

Fig AB.1 Standard curve of cell dry weight versus OD_{600}.

$$\mathrm{CDW} = \frac{\left(Weight\ of\ tube\ and\ dry\ cell\ (g) - Weight\ of\ tube\ (g)\right) * 1000ml}{5ml}$$

B.2 Total protein concentration

2.1 Reagents

2.1.1 Coomassie Blue G250 (Blue Gin Lab)

2.1.2 Phosphoric acid 85%

2.1.3 Ethanol 99%

2.1.4 Distilled water

2.1.5 Bonvine serum albumin (BSA) 1mg. ml^{-1}

2.2 Bradford solution preparation

2.2.1 Dissolve 50 mg Coomassie Blue G250 in 99% ethanol 25 ml (Blue color).

2.2.2 Mix with 50 ml 85% phosphoric acid (Blue color changes to brown color).

2.2.3 Adjust volume into 500 ml with Distilled water (color changes to blue plus brown).

2.2.4 Filter through filter paper Whatman N_0I (Color changes to brown).

2.3 Bradford analysis

2.3.1 Pipette 50 µl of sample into a test tube

2.3.2 Add 2.5 ml of Bradford reagent to each tube, mix well (gently to avoid foaming)

2.3.2 Measure at wave length 595 nm of samples and standards within 1 hour.

2.4 Generate a standard curve

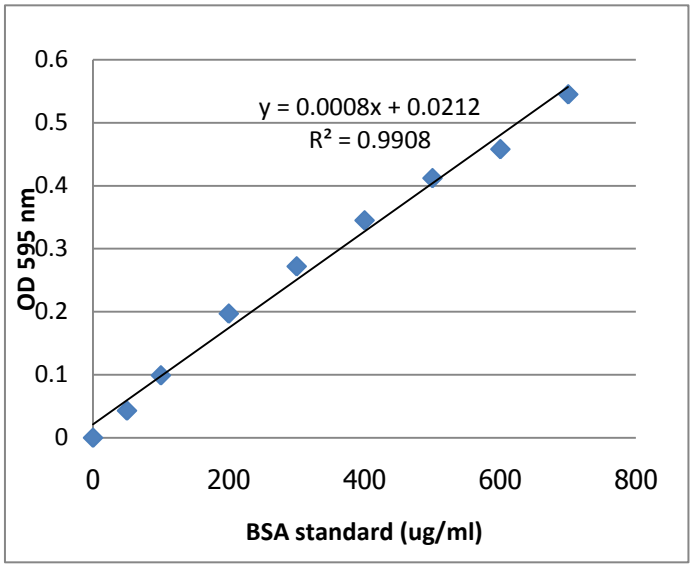

Fig AB.2 Standard curve of OD_{595} *versus* BSA concentration.

Table AB.2 Relationship between protein concentration and OD_{595} in Bradford analysis.

Protein conc. (mg. L^{-1})	**OD_{595}**
0	0
50	0.043
100	0.099
200	0.197
300	0.272
400	0.345
500	0.412
600	0.458
700	0.545

B.3 Residual methanol concentration

Residual methanol concentration was analyzed using a gas chromatograph (GC).

Table AB.3 The relationship between methanol concentration and areas using GC.

Methanol conc. (g. L^{-1})	**Areas (arbitrary units)**
0	0
2.5	1540.30
5	4451.49
7.5	7410.92
10	10516.56

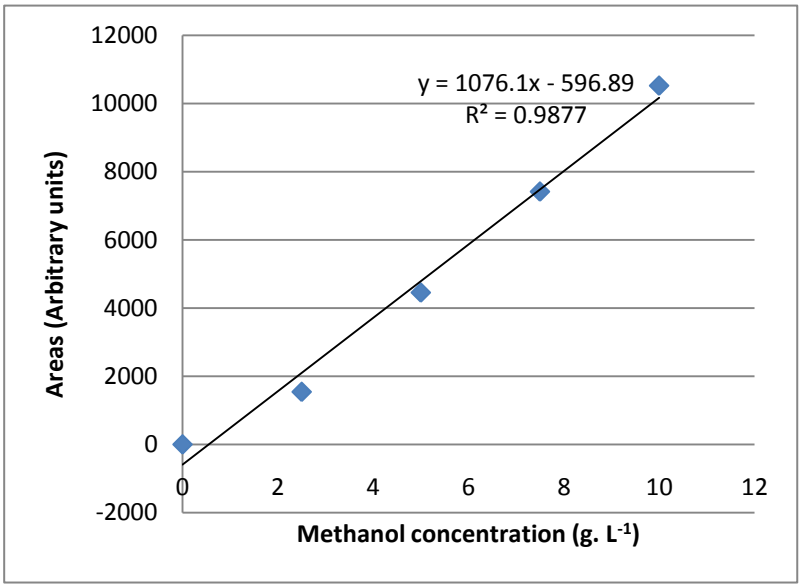

Fig AB.3 Standard curve of methanol concentration *versus* Areas.

B.4 Enterokinase activity assay

Enterkinase activity assay was modified from the method of Hermon-Taylor (1970) which was determined using the fluorogenic substrate Gly-$(Asp)_4$-Lys-β napthylamide (GD_4K- β napthylamide).

One unit of enterokinase is defined as amount of enterokinase releasing 1nM of β napthylamide per minute at 30^0C for 10 minutes.

4.1 Substrate solution

GD_4K- β napthylamide was dissolved in 70mM Tris-Cl ph8 and 10% dimethyl sulfoxide (DMSO).

4.2 Assay

Thirty micro liter of the crude culture supernatant was added to two hundreds micro liter of substrate solution. The enzymatic activity was measured using Spectra Max Gemini EM machine by an increasing of fluorescence at excitation 337, emission 420 nm that caused by the release of β – naphthylamide over one minute interval.

4.3 Generation the standard curve of β napthylamide

β napthylamide 1.816 mM in DMSO was prepared then diluted 5000 times (final concentration 0.3632 μM). The β napthylamide standard solution was prepared by diluted the substrate buffer following Table AII.4 then measured the fluorescene at excitation 337, emission 420 nm.

Table AB.4 β napthylamide standard solution preparation.

Sample No	B-napthylamide 0.3632µM	Substrate buffer	[B-napthylamide µM]
1	250 µl	0 µl	0.3632
2	200 µl	50 µl	0.29056
3	150 µl	100 µl	0.21742
4	100 µl	150 µl	0.14528
5	50 µl	200 µl	0.07264
6	0 µl	250 µl	0

Table AB.5 Standard curve of β napthylamide.

Sample No	Abs (337/420)	[B-napthylamide µM]
1	60.94	0.3632
2	51.48	0.29056
3	38.82	0.21742
4	26.76	0.14528
5	13.96	0.07264
6	0	0

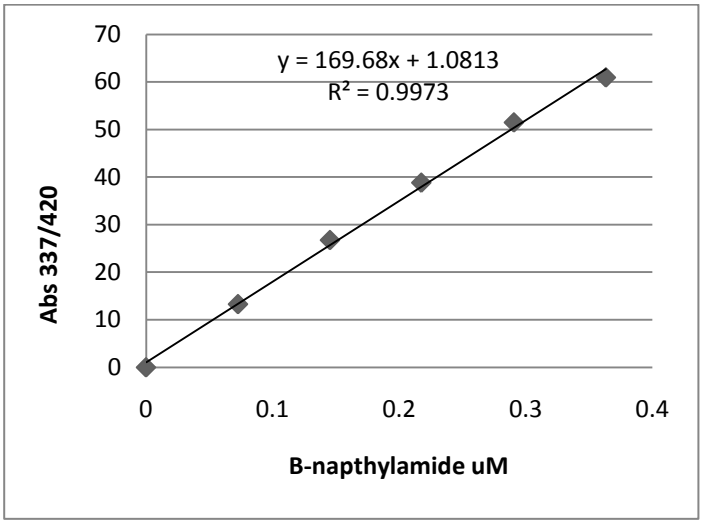

Fig AB.4 Standard curve of β napthylamide.

Therefore,

$$[\beta \text{ napthylamide } \mu M] = \frac{\text{Abs } (337/420) - 1.0813}{169.68}$$

4.4 Calculation of rEK_L activity

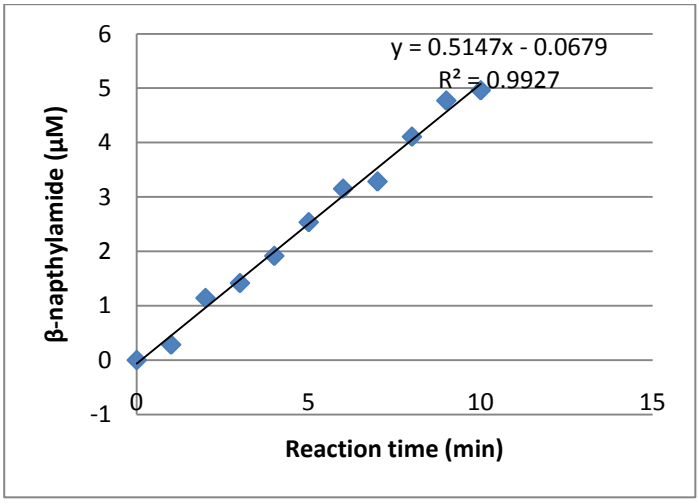

Fig AB.5 Relationship between reaction time and β napthylamide concentration.

Table AII.6 The increasing of [β napthylamide μM] over time.

Time (min)	Abs	Δ Abs	B-NA μmol	μ/min	nmol/min	U/ml
0	546.15	0	0	0.51	514.70	17,156.67
1	595.38	49.23	0.28			
2	740.08	193.92	1.14			
3	786.55	240.40	1.42			
4	870.88	324.72	1.92			
5	975.49	429.34	2.53			
6	1079.44	533.29	3.15			
7	1101.92	555.76	3.28			
8	1241.00	694.85	4.11			
9	1353.06	806.90	4.77			
10	1384.96	838.81	4.96			

β napthylamide (nmol/min) = 1000 x β napthylamide (μmol/min)

Because the 0.03 ml sample was using for analyzing the rEK_L activity so the activity of rEK_L (U/ml) was calculated following:

$$[EKL] = \frac{U\ (nmol/min)}{0.03\ (ml)}$$

BIOGRAPHY

Ms. Nguyen Thi Thanh Dung was born on November 3rd, 1985 in Bac Ninh, Viet Nam. She graduated with the Bachelor Degree of Engineering in Biotechnology from Hanoi University of Agriculture, Viet Nam in 2007. In 2008, she has been employed by the Faculty of Biotechnology as lecturer in the Department of Molecular Biology and Microbial Technology, Hanoi University of Agriculture. She had an experience on research work for one year. In 2009, she was a Master student in School of Biotechnology at Suranaree University of Technology, Thailand. During the time of her study, she presented oral presentation in the title of "Simple Fed-Batch Technique for the Production of Recombinant Enterokinase Light Chain by *Pichia pastoris*" at the International Conference on Agriculture and Agro-Industry (ICAAI2010), 19-20th November 2010 at Mae Fah Luang University, Chiang Rai, Thailand.