รายงานการวิจัยฉบับสมบูรณ์
ทุนอุดหนุนการวิจัยจากเงินอุดหนุนทั่วไปจากรัฐบาล

การเพิ่มผลผลิตรีคอมบิแนนท์อินซูลินโดยการเพิ่มจำนวนชุดของยีนในเมธิลโลโธฟิกยีสต์

Enhancement of recombinant monomeric insulin production in methylotrophic yeasts by increasing copy number of gene

โดย

นางศรินทิพ สุกใส[1] (Mrs. Sarintip Sooksai)

นางนันทิกา คงเจริญพร[1] (Mrs. Nanthika Khongchareonporn)

นายอภิชาติ กาญจนทัต[1] (Mr. Aphichart Karnchanatat)

นางสาวกรรณิกา เสริมสุวิทยวงศ์[2] (Ms. Kannika Sermsuvitwong)

นางสาวศจี น้อยตั้ง[1] (Ms. Sajee Noitang)

นายธนปติ ผาคำ[3] (Mr. Tanapati Pakum)

[1]The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand

[2]Department of Chemistry, Faculty of Medicine, Srinakrarinwirot University, Building 15, 6th Floor, Sukumvit Soi 23, Watana, Bangkok 10110, Thailand

[3]Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

กิตติกรรมประกาศ (Acknowledgement)

We are grateful to Associate Professor Dr. Polkit Sangvanich, the director of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, for suggestions and support. We would like to thank all staffs in the institute for their kindness, friendships and all convenient assistance. Special thanks to Mrs. Thongchan Puthong for her helping and suggestion.

We are also greatly indebted to our family for their hearty encouragement and support all the time.

This work was funded by a grant from Office of National Research Council of Thailand and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

บทคัดย่อภาษาไทย

ในปัจจุบัน ระบบการแสดงออกของเมธิลโลโธฟิกยีสต์ ถูกพัฒนาขึ้นและถูกนำมาใช้อย่างแพร่หลายในการผลิตรีคอมบิแนท์โปรตีน ซึ่งในงานวิจัยนี้ใช้ *Pichia pastoris* สายพันธุ์ X-33, GS115, KM71H และ *Hansenula polymorpha* NRRL2214 เป็นเซลล์เจ้าบ้านในการผลิตรีคอมบิแนนท์อินซูลินแบบมอนอเมอร์ (MIP) โดยรีคอมบิแนนต์พลาสมิด TP1, TP2, TP4 ที่มีจำนวนชุดของยีนอินซูลินแบบมอนอเมอร์เป็น 1, 2 และ 4 ชุด ตามลำดับ ได้ถูกสร้างขึ้นในพลาสมิดพาหะ pPICZαA และถูกนำเข้าสู่เซลล์เจ้าบ้าน รีคอมบิแนยีสต์แต่ละสายพันธุ์ซึ่งมี TP1, TP2, TP4 แทรกอยู่ในจีโนมถูกเลี้ยงแบบสองขั้น โดยขั้นแรก เลี้ยงในอาหารสมบูรณ์เพื่อเพิ่มจำนวนเซลล์ และในขั้นที่สอง เลี้ยงในอาหารจำกัดที่มีเมธานอลและฮีสติดีน เพื่อใช้เหนี่ยวนำการผลิต MIP ระดับการแสดงออกถูกตรวจติดตามด้วยวิธีที่ง่ายและมีความจำเพาะเจาะจงด้วยเทคนิค dot-blotting analysis ขณะที่เทคนิค indirect competitive ELISA ถูกใช้ในการวัดปริมาณความเข้มข้นของ MIP จากผลการตรวจติดตามด้วยเทคนิค dot-blotting analysis ระดับการแสดงออกของ MIP จากรีคอมบิแนนต์ยีสต์ *P. pastoris* สายพันธุ์ KM71H สามารถตรวจวัดได้ตั้งแต่ชั่วโมงที่ 24 ของการเพาะเลี้ยงในขั้นที่สอง ในขณะที่การแสดงออกของ MIP จากรีคอมบิแนนต์ยีสต์สายพันธุ์อื่นๆ ตรวจวัดได้ที่ 48 และ 72 ชั่วโมง ผลการทดลองในส่วนของการวัดความเข้มข้นของ MIP ด้วยเทคนิค indirect competitive ELISA แสดงให้เห็นว่า ปริมาณ MIP เพิ่มขึ้นอย่างต่อเนื่องตามระยะเวลาที่ใช้ในการพาะเลี้ยง สำหรับผลของสายพันธุ์เซลล์เจ้าบ้านที่มีต่อระดับการแสดงออกของ MIP พิจารณาจากปริมาณความเข้มข้นของ MIP ที่ได้จากรีคอมบิแนนต์ยีสต์สายพันธุ์ต่างๆ ที่มีจำนวนชุดของยีน 1 ชุด เลี้ยงใน MMH เป็นเวลา 72 ชั่วโมง พบว่า *P. pastoris* สายพันธุ์ KM71H (Mut^S phenotype) มีความเข้มข้นของ MIP สูงที่สุด (4.19±0.96 $mg.L^{-1}$) ตามด้วย *P. pastoris* สายพันธุ์ GS115 (2.69±0.48 $mg.L^{-1}$), *P. pastoris* สายพันธุ์ X-33 (0.93±0.08 $mg.L^{-1}$) และ *H. polymorpha* (0.04±0.01 $mg.L^{-1}$) ส่วนผลของจำนวนชุดยีนที่มีต่อการแสดงออกของ MIP พบว่า รีคอมบิแนนต์ยีสต์แต่ละสายพันธุ์ที่มีจำนวนชุดยีนที่แตกต่างกัน มีระดับการแสดงออกของ MIP แตกต่างกัน และไม่สัมพันธ์กับจำนวนชุดของยีนที่เพิ่มขึ้น

บทคัดย่อภาษาอังกฤษ (Abstract)

Currently, methylotrophic yeast expression system has been developed and widely used for recombinant proteins production. In this research, *Pichia pastoris* strains, X-33, GS115, KM71H, and *Hansenula polymorpha* NRRL2214, were used as hosts for recombinant monomeric insulin production. Recombinant plasmids, TP1, TP2, TP4, which have 1, 2 and 4 copy(s) of monomeric insulin precursor (MIP) gene, respectively, were successfully constructed in pPICZαA expression vector and transformed into the hosts. The recombinant yeasts which harboring TP1, TP2, TP4 plasmids which integrated into their genome were cultured in two steps: the first step in complex medium for cell production and the second step in minimal methanol histidine (MMH) medium for inducing the expression of MIP. A simple and specific dot-blotting technique was chose to monitor the expression level while indirect competitive ELISA was used to quantitatively determine the MIP concentration. By dot-blot analysis, the MIP expression of recombinant *P. pastoris* KM71H could be detected since 24 hours in an induction phase while those of other recombinants were detected at 48 or 72 hours. By indirect competitive ELISA, results showed that the MIP expression progressively increased together with the time of induction. Effect of host strains on the MIP expression level was comparison of MIP concentration between recombinant strains which harboring 1 copy of MIP gene which cultured in MMH for 72 hours, *P. pastoris* KM71H (Mut^S phenotype) has the highest MIP concentration (4.19±0.96 $mg.L^{-1}$), following by *P. pastoris* GS115 (2.69±0.48 $mg.L^{-1}$), *P. pastoris* X-33 (0.93±0.08 $mg.L^{-1}$) and *H. polymorpha* (0.04±0.01 $mg.L^{-1}$). In view of gene copy number, we found that recombinant yeast strains which differ in gene copy number have different expression level of the MIP and the expression level was unrelated with gene copy number.

สารบัญเรื่อง (Table of Contents)

สารบัญเรื่อง (Table of Contents)

สารบัญตาราง (List of Table)

สารบัญภาพ (List of Illustrations)

หน้า

คำอธิบายสัญญาลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations)

AOX1	Alcohol oxidase 1
AOX1	Alcohol oxidase 1 gene
$AOX1_p$	Alcohol oxidase 1 promoter
AOX2	Alcohol oxidase 2
AOX2	Alcohol oxidase 2 gene
$AOX2_p$	Alcohol oxidase 2 promoter
ATP	Adenosine triphosphate
DF	Dilution factor
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
MIP	Monomeric Insulin Precursor
MMH	Minimal methanol histidine
Mut^+	Methanol utilization plus phenotype
Mut^s	Methanol utilization slow phenotype
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNase	Ribonuclease A
TAE	Tris-acetate-EDTA
Tm	Melting temperature (°C)
w/	With
w/o	Without
w/v	Weight by volume
WT	Wild type strain
×g	Multiply by gravitational force (×9.80665 m.s2)
YNB	Yeast nitrogen base
YPD	Yeast extract Peptone Dextrose
YPG	Yeast extract Peptone Glycerol

บทนำ (Introduction)

1.1 ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

Diabetes mellitus (DM) also known as diabetes is a group of metabolic diseases characterized by hyperglycemia, which has high blood glucose level or abnormal, resulting from insulin secretion defective (lack of insulin) or the action of insulin (insulin resistance), or both. The chronic hyperglycemia affects many organ e.g. eyes, kidneys, nerves, heart and blood vessels. Diabetes mellitus can be divided to two groups, Type I diabetes; which is immune-mediated diabetes or beta-cell destruction usually leading to absolute insulin deficiency and the other one is Type II diabetes; which is insulin resistance relate with insulin deficiency to predominantly insulin defective. Almost all diabetes patients are type II which account for 90-95% (1). The International Diabetes Federation (IDF; www.idf.org) reported that in 2035 the DM patients in the world will be increasing to 592 million people, which account for 55% from 2013 (2). So, it needs to improve the property of insulin action and recombinant insulin production to produce the effective recombinant insulin to treat DM patients in the future. Nowadays, diabetes patient in Thailand is increasing and demand of insulin is increasing too. Insulin, which has been used for treat the DM patients in Thailand, has been imported from other countries. In Thailand, none of the pharmaceutical company produces insulin to treat Thai's DM patients. Now, all of the insulin which used to treat DM is recombinant insulin, which is produced by recombinant DNA technology and biotechnology (3). The recombinant DNA technology is a technique that modifies the organisms by combining the genetic material or gene(s) of interest from one species to another to produce an interested protein(s) as human insulin gene encodes human insulin. In case of human insulin, nucleotide sequence of human insulin was synthesized and inserted in an expression vector. Then, recombinant plasmid was transformed into the host organism e.g. bacteria and yeast to produce the recombinant insulin (3, 4). The recombinant insulin is better than animal insulin which extracted from animal pancreas e.g. porcine insulin or bovine insulin. So, many researchers prefer to modify the nucleotide sequence or amino acid sequence of insulin by protein engineering to improve and enhance the insulin properties e.g. faster acting and decrease a self-association of insulin (5). In 2005, Ding et al., reported that they modified the nucleotide sequence of insulin by deletion three amino acids in B chain (B28-B30) and replaced B27 threonine (Thr) by lysine (Lys), named monomeric insulin precursor (MIP). MIP has the advantage of faster action than native insulin when convert to insulin active form by tryptic hydrolysis (5-7).

In the past decade, many recombinant proteins have been produced by yeast for applications in cosmetic industry, pharmaceutical and medicine. The expression system of yeast has many advantages over prokaryotic system to produce recombinant proteins e.g. genetic stability, high cell density, rapid growth rate, low cost of media, very high level of secretion proteins (8-10). *Saccharomyces cerevisiae* is high benefits and is the first host organism which produces recombinant proteins. Furthermore, the other yeasts such as *Hansenula polymorpha*, *Pichia pastoris*, *Kluveromyces lactis*,

Schizosaccharomyces pombe and *Yarrowai lipolytica* were alternative hosts, which have been used to produce many heterologous proteins (11-14). A few years ago, the methylotrophic yeasts including of *H. polymorpha*, *P. pastoris*, *Candida boidinii*, *P. methanolica*, which can utilize methanol as a sole carbon source, can produce high yield of secreted heterologous proteins (10). *H. polymorpha* and *P. pastoris* were popular host organisms to study the higher eukaryotic gene expression system and recombinant protein production. In *P. pastoris*, it has a strongly inducible alcohol oxidase 1 promoter (*AOX1*p) that can be controlled the target gene expression by using an induction medium contains methanol. There are many factors affect to recombinant protein expression in yeast system e.g. gene dosage, expression vector, promoter, secretion signal sequence, translation signals, processing and protein folding in the endoplasmic reticulum (ER) and Golgi, yeast strains, fermentation strategies (9, 10, 15, 16).

In this research, *P. papstoris* GS115 (Mut^+ his^-), *P. papstoris* X33 (Mut^+), *P. papstoris* KM71H (Mut^S) and *H. polymorpha* strain (WT) were used as hosts to study the effect of yeast strains and copy number of gene. The monomeric insulin precursor (*MIP*) gene was inserted into an expression vector pPICZαA which has *AOX1*p for expression. The recombinant plasmids which have one, two, and four cassettes of MIP gene were constructed and transformed to the yeasts. The MIP expression level was monitored by specific dot-blot analysis and quantitative determined by indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA).

1.2 วัตถุประสงค์ของโครงการวิจัย

To study the effect of yeast strains (methanol utilization phenotype, Mut phenotype) and copy number of monomeric insulin precursor (MIP) gene on the expression level of MIP.

1.3 ขอบเขตของโครงการวิจัย

1.3.1 To construct the recombinant plasmids which contain 1, 2, 4 expression cassette of MIP gene

1.3.2 To generate the recombinant yeasts which harbor 1, 2, 4 expression cassette of MIP gene

1.3.3 To determine the effect of copy number of MIP gene and host strains on MIP expression level

1.4 ทฤษฎี สมมุติฐาน (ถ้ามี) และกรอบแนวความคิดของโครงการวิจัย

Insulin is a peptide hormone secreted by the beta cells of the islets of Langerhans in the pancreas in response to an elevation in blood glucose level. It plays a crucial role in glucose homeostasis, by regulating the uptake and metabolism of glucose by peripheral tissues and the production of and storage of glucose by liver. The mature insulin molecule composes of two polypeptide chains: Alpha (A) chain and Beta (B) chain, which is A chain containing 21 amino acids, and B chain containing 30 amino acids. The chains are linked-together by two disulphide bridges which are between Cys A7 and Cys B7, and between Cys A20 and Cys B19. It has an intrachain disulphide bridge between Cys A6 and Cys A11

creating a loop in the A chain (17). Nowadays, insulin analogues have been developed for suitable usage to control blood glucose level in diabetes patients. They can be divided to 3 groups which are considered in the acting type; rapid acting, pre-mixed and long acting (18). They have been developed for suitable usage to treat both types of diabetes patients. Now, all of the insulin is recombinant insulin which is produced by recombinant DNA technology (3).

The recombinant DNA technology is a technique used for modifies organism by combining the genetic material or gene(s) of interest from one or more species to another living organism (host) in order to produce useful protein(s) or useful biomolecules of interest from host organisms. When DNA or genetic material from one species is combined with the other species, the result is a recombinant DNA (19). To understand about the recombinant DNA technology, it needs to clearly understand about genetic engineering, molecular cloning technology and biotechnology. The recombinant DNA technology has been widely used in many fields e.g. medical, pharmaceutical, biotechnology, transgenic plants and animals, forensic sciences (4). Many heterologous proteins that were produced by using recombinant DNA technology call “recombinant proteins”. The organisms which are used for recombinant proteins or recombinant useful compounds include microorganisms; bacteria, cyanobacteria, yeast, fungus, microalgae, animal or insect cell lines, or systematic organism; plants or animals. The first recombinant protein production was recombinant human insulin produced by *Escherichia coli* which is a first organism to study the recombinant human insulin production in 1982 by Eli Lilly (20). However, yeast expression system has been widely used for many eukaryotic heterologous protein production thanks to it has many advantages and similarity in the complex posttranslational modification process of protein(s) likes higher eukaryote such as correct glycosylation, protein folding (disulfide bond formation) and protein secretion (8-10, 21, 22).

Methylotrophic yeast is a group of yeast that can utilize methanol as a sole carbon source. Many yeasts have been developed to enhance the production of heterologous proteins and it was studied the mechanism that involve in protein expression. A useful yeast strains which popular used in this group including *P. pastoris*, *H. polymorpha* (8-10, 23). Methylotrophic yeasts have a unique methanol utilization pathway or MUT pathway. Many genes which involve in MUT pathway have been investigated and its can regulate the expression level of heterologous protein using this pathway. Many researchers apply to use the strongly methanol-inducible promoters to drive a heterologous gene expression such as alcohol oxidase 1 promoter (*AOX1*p) from *P. pastoris* and methanol oxidase promoter (*MOX*p) from *H. polymorpha*. Moreover, in some case they used a constitutive promoter to express gene of interest such as glyceraldehyde-3-phosphate dehydrogenase promoter (*GAP*p) from *S. cerevisiae* (8, 9). The classification of *Pichia* strains when considering in methanol utilization phenotype can be classified into three groups; Mut^{+} (plus), Mut^{S} (slow) and Mut^{-} (minus). The first one is Mut^{+} strains; X-33 which is a wild-type strain that has both of alcohol oxidase 1 (AOX1) and alcohol oxidase 2 (AOX2) resulting in methanol utilization plus phenotype (Mut^{+} phenotype) and the other Mut^{+} strain is GS115 strain which is a

histidine auxotroph (*his4*$^{-}$) strain that histidine dehydrogenase gene mutated but it exists both of AOX1 and AOX2 resulting in methanol utilization plus (Mut^{+}, His^{-}) and it needs supplement of histidine to growth. The second one is Mut^{S} strain; KM71H which argininosuccinate lyase gene (*ARG4*) was inserted and replaced in some part of *AOX1* gene in wild type strain (*arg4 aox1*::*ARG4*) resulting in methanol utilization slow phenotype (Mut^{S}, Arg+). The third one is Mut^{-}; both *AOX1* and *AOX2* gene were disrupted resulting in methanol utilization minus phenotype (Mut^{-}) so that, it cannot growth in methanol-containing media (9, 24). In recent times, *P. pastoris* has become increasing popular as a cellular host for the expression of recombinant proteins. *H. polymorpha* (*Pichia angusta*) is very closely to *P. pastoris*, which has the same pathway in methanol utilization. While the advance of *H. polymorpha* is a non-homologous recombination event which resulting in multi-copy integrating into genome (25).

There are several factors that affect the recombinant protein productions in *P. pastoris*, including the properties of the nucleotide sequence (e.g. codon composition, AT-content and secondary structure), mode of sequence insertion into the genome, choice of host strain, mode of expression, methanol utilization phenotype and cultivation conditions. It has however been stated that when expressing protein from a single recombinant gene copy, the transcript level is most likely to be limiting in the protein production. It is therefore suggested that insertion of multiple recombinant gene copies should be aimed for in order to obtain maximal protein yields (22, 26).

In this research, we aimed to study the effect of gene dosage to the expression level by increasing insulin gene copy number in an expression vector and cloned into the methylotrophic yeasts.

1.5 วิธีการดำเนินการวิจัยโดยสรุป

1.5.1 Construction of the recombinant expression plasmids which contain 1, 2, 4 expression cassette of *MIP* gene

1.5.2 Extraction and purification of the recombinant plasmids

1.5.3 Transformation of the recombinant plasmids into yeasts

1.5.4 Monitoring and determination of the MIP expression level

1.6 ประโยชน์ที่คาดว่าจะได้รับ

Able to constructs recombinant yeasts (*P. pastoris* and *H. polymorpha*) which harbor the multi-copy number of *MIP* gene in the genome and able to express high expression level of MIP.

เนื้อเรื่อง (Main body)

2.1 วิธีดำเนินการวิจัย (Materials and Method)

2.1.1 Organisms

E. coli Top10F' was used as a host for recombinant plasmid construction. *P. pastoris* 3 strains; X-33 (Mut^+), GS115 (Mut^+, His^-) and KM71H (Mut^S), were purchased from Invitrogen and *H. polymorpha*; NRRL2214, was gifted from Agricultural Research Service Culture Collection (NRRL2214), U.S.A.

2.1.2 Nucleotide Sequence of MIP and Plasmids

Nucleotide sequence of monomeric insulin precursor (MIP) gene as discussed by Ding et al. (5), which used in this study (Figure 2.1) was synthesized and cloned into a pUC base vector (pUC::MIP) by the Blue Heron Biotechnology Company, U.S.A. The pPICZαA expression vector was purchased from Invitrogen, U.S.A.

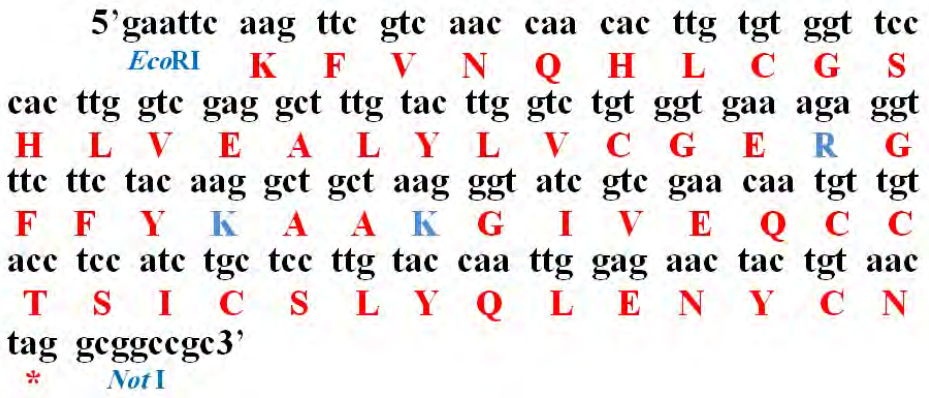

Figure 2.1: Nucleotide sequence and amino acid sequence of MIP gene (5)

2.1.3 Media

LB medium, Low salt LB Zeocin™ (LB with 25 µg.mL-1 of Zeocin™ final concentration) medium were used for recombinant *E. coli* cultivation and screening. YPD medium and YPD Zeocin™ (YPD with 100 $\mu g.mL^{-1}$ of Zeocin™ final concentration) medium were used for recombinant yeast cultivation and screening. YPG medium was used for cell manipulation in cell production phase and MMH medium with 0.5% methanol was used for MIP induction in an expression phase. Media compositions were described in an appendix A.

2.1.4 Construction of Recombinant Plasmids

The pUC::MIP plasmid was digested with *Eco*RI and *Not*I to release *MIP* fragment (173 bp). The *MIP* fragment was purified and ligated into the pPICZαA expression vector to generate the pPICZαA::MIP plasmid, called TP1 plasmid (3,709 bp). To generate TP2 plasmid, TP1 plasmid was digested with *Bam*HI and *Bgl*II to obtain *MIP* cassette. The *MIP* cassette (5'*AOX1*p-*MIP*-3'*AOX1*TT) consists of *AOX1* promoter, alpha-factor signal sequence, *MIP* gene, and *AOX1* transcription terminator with the size of 1,792 bp. The *MIP* cassette with 5'-*Bgl*II and 3'-*BamH*I was purified and ligated to TP1 plasmid which linearized with *BamH*I. The ligate reaction was transformed into freshly prepared *E. coli* Top10F' competent cells. Transformants were selected on selective medium, low salt LB plate with 25 μg.mL^{-1} of Zeocin™. After recombinant colonies had grown on selective medium, a single colony was picked up and streaked onto the selective plate and incubated at 37°C for overnight. Each clone of recombinant *E. coli* was inoculated in 5 mL of low salt LB medium with 25 μg.mL^{-1} of Zeocin™ and incubated with vigorous shaking at 37°C for overnight. The cell cultures were harvested and recombinant plasmids were extracted as described in 2.1.5. The recombinant plasmid which expected to harbor two cassettes of *MIP* is called TP2 plasmid which size is 5,501 bp. The TP2 plasmid was digested with *Bam*HI and *Bgl*II to release 2×*MIP* fragment (2×*MIP*; (5'*AOX1-MIP*-3'*AOX1*TT)-(5'*AOX1-MIP*-3'*AOX1*TT). The 2×*MIP* fragment (3,584 bp) was purified and cloned into TP2 plasmid which linearized with *Bam*HI (5,501 bp) to generate TP4 plasmid (9,085 bp).

E. coli recombinant clones which harboring TP1, TP2, and TP4 plasmids, were cultivated in low salt LB medium with 25 μg.mL^{-1} of Zeocin™ and incubated with vigorous shaking at 37°C for overnight. Then, the recombinant plasmids were extracted and purified as described in extraction and purification of plasmid DNA (described below) which prepared to transform into yeasts.

2.1.5 Extraction and Purification of Plasmid DNA

This protocol was modified from extraction and purification of plasmid DNA in molecular cloning: a laboratory manual (27).

2.1.5.1 Growth of the Bacterial Culture

A single colony of recombinant *E. coli* was inoculate in low salt LB medium with 25 μg.mL^{-1} of Zeocin™ and incubated with vigorous shaking at 37°C for overnight.

2.1.5.2 Harvesting and Lysis by Alkali of the Bacteria

Three milliliters of bacterial cell culture were collected by centrifugation at 10,000×g at 4°C for a minute, 1.5 mL twice, decant the media. The cell pellet was washed with 1 mL of sterilized TE buffer (pH 8.0) and centrifuged again followed by decanting the supernatant. The cell pellet was resuspended in 100 μL of ice-cold of Solution I and mixed by vigorous vortexing. The solution was chilled on ice for 5 minutes. Then, 200 μL of freshly prepared Solution II was added into the tube and mixed by

inverting the tube for five times and store the tube on ice for 5 minutes. An ice-cold Solution III (300 µL) was added into the tube and gently mix by inverting the tube for ten times and store on ice for 5 minutes. The lysate cell was centrifuged at 12,000×g at 4°C for 5 minutes and the supernatant (400 µL) was transferred by pipetting to a new microfuge tube.

2.1.5.3 Purification of Plasmid DNA

The double-stranded DNA in the supernatant from III-6.2.2 was precipitated with 2 volumes of ice-cold 95% ethanol (800 µL) and mixed by vortex. The solution was centrifuged at 12,000×g at 4°C for 5 minutes followed by decanting the supernatant. The plasmid DNA was rinsed with 1 mL of ice-cold 70% ethanol and carefully removed the supernatant. Allow the pellet of plasmid DNA to dry in a vacuum desiccator for 15 – 30 minutes. The plasmid DNA was redissolved in 50 µL of sterilized ultrapure water or sterilized TE buffer (pH 8.0) containing RNAase A (20 $\mu g.mL^{-1}$ final concentration).

2.1.6 Preparation and Transformation of Competent *E. coli* and Yeasts

2.1.6.1 Preparation of Fresh Competent *E. coli* using Calcium Chloride

A fresh single colony of *E. coli* (Top10F') was inoculated in 10 mL of LB medium in 250 mL Erlenmeyer flask and cultivated with shaking at 180 rpm at 37°C for overnight. Two percent of overnight culture was transferred to 50 mL LB medium and cultivated with shaking at 250 rpm at 37°C for ~3 hours. The cell culture was aseptically transferred to sterile 50 mL polypropylene tube and stored on ice for 10 minutes to cool the cell culture to 0°C. The cells were harvested by centrifugation at 4,000×g at 4°C for 10 minutes, decant the media. The cells were resuspended in 10 mL of sterilized ice-cold 0.1 M $CaCl_2$ and chilled on ice for 5 minutes. The cells were recovered by centrifugation at the same condition followed by decanting the supernatant. One milliliter of sterilized ice-cold 0.1 M $CaCl_2$ was added into the tube and mix by pipetting. Then, the cell suspension was aliqouted to new microfuge, 200 µL per tube, and stored on ice until use.

2.1.6.2 Transformation of *E. coli* by Heat Shock

The ligation reaction (no more than 10 µL) was added to 200 µL of freshly prepared *E. coli* competent cells and gently mixed by pipetting, stored on ice for 30 minutes. The tube was immediately heated in water bath at 42°C for 90 seconds (do not shake the tube). Then, the tube was rapidly transferred to chill on ice for 5 minutes. Eight-hundred microliters of LB medium were added into the tube and incubated at 37°C for 1 hour. The cell suspensions (with the volume of 50, 100, 200, 300 µL per plate) were spreaded on selective plates, low salt LB medium with 25 $\mu g.mL^{-1}$ of Zeocin™, and incubated at 37°C for overnight.

2.1.6.3 Preparation of Fresh Competent Yeasts

A fresh single colony of yeast was inoculated in 20 mL YPD medium in 250 mL Erlenmeyer flask and cultivated with shaking at 200 rpm at 30°C for overnight. Five percent of the

overnight culture was transferred to 50 mL YPD medium and cultivated with shaking at 300 rpm at 30°C until the OD_{600} reaches to 1. Subsequently, cells were harvested by centrifugation at 2,000×g at 20°C for 5 minutes, decant supernatant. The cells were resuspended in 10 mL of YPD medium with 2 mL of 1 M HEPES buffer (pH 8.0). After that, 250 µL of 1 M DTT (250 µL) was added into the tube and gently mixed. Then, the cell suspension was incubated without shaking at 30°C for 15 minutes. Cells were collected by centrifugation at 2,000×g at 4°C for 5 minutes. The cell pellets were washed two times with 25 mL of sterilized ice-cold double distilled water and washed again with 10 mL of sterilized ice-cold 1 M D-sorbitol. The cells were recovered by centrifugation at 2,000×g at 4°C for 5 minutes and resuspended with 500 µL of sterilized ice-cold 1 M D-sorbitol. Eighty microliters of the cell suspension was then aliquoted into 1.5 mL sterilized microfuge tube and store the tubes on ice for transformation by electroporation.

2.1.6.4 Transformation of Yeasts by Electroporation

This protocol was modified from DNA-mediated transformation in method in molecular biology (28) and EasySelect™ *Pichia* Expression Kit (2444). The TP1, TP2 and TP4 plasmids were transformed into *P. pastoris* (3 strains; X-33, GS115, and KM71H) and *H. polymorpha* (NRRL2214) by electroporation method. Freshly prepared competent cells (80 µL) were mixed up with 5-10 ng of DNA plasmid (no more than 10 µL) and transferred to an ice-cold 0.2 cm gap electroporation cuvette, store on ice. The cells were pulsed with 2.0 kV by MicroPulser™ Electroporator (Bio-Rad). Then, 500 µL of sterilized ice-cold 1 M D-sorbitol was immediately added to the cuvette, gently mixed by pipetting and transferred the cell suspension to new microfuge tube, stored on ice for 30 minutes. The cell suspension was incubated without shaking at 30°C for 1 hour. Then, 500 µL of YPD medium was added and continuously incubated with shaking at 200 rpm at 30°C for 1 hour. The cells were spread and selected onto selective plate, YPD plate with 100 $\mu g.mL^{-1}$ of Zeocin™, and incubated at 30°C for 2-4 days until colony form.

2.1.7 Yeast Cultivation and Recombinant MIP Expression

High cell density was chosen in this study for the MIP expression. Fresh single colony of recombinant yeast was inoculated in 20 mL YPG medium and incubated with shaking at 200 rpm at 30°C for overnight. Five percent of the overnight culture was inoculated in 30 mL of YPG medium and incubated with shaking at 250 rpm at 30°C for an OD_{600} reach to 1.0 (approximately ~3 hours) which used as a starter culture. Ten percent of the starter culture was inoculated in 50 mL YPG medium in 250 mL Erlenmeyer flask and incubated with shaking at 250 rpm at 30°C for 24 hours (cell production phase). After 24 hours in cell production phase, cells were collected by centrifugation at 2,000×g for 5 minutes at 20°C followed by decanting the supernatant. The cells were resuspended in 50 mL of MMH induction medium in 250 mL baffled flask and incubated with shaking at 250 rpm at 30°C. In an induction phase, one-hundred percent of absolute methanol was added to a final concentration of 0.5% methanol every 24 hours to maintain an induction. Five milliliters of culture samples were taken at 0, 12, 24, 48, and 72 hours and 5 mL of MMH medium were added. The culture samples were centrifuged at 5,000×g at 4°C for 5 minutes, the supernatants were transferred to a new sterile tube and adjusted the pH value to ~7 with

a sterilized 2.5 M NaOH. The supernatants were aliquot and stored at +4°C until assay and cell pellets were washed with TE buffer (pH 8.0) and filtered for cell dry weight.

2.1.8 The Monitoring and Determination of the Recombinant Monomeric Insulin Precursor

2.1.8.1 Monitoring of the MIP Expression Level by Dot-Blot Analysis

A specific dot-blot procedure, adapted from Sithigorngul (29), was used for a monitoring of the MIP expression level in culture broth. Insulin from bovine pancreas and Mixtard® 30HM Penfill were prepared at various concentrations and used as insulin standards (Appendix B). The supernatants of culture samples (2.1.7) were spotted (3 µL per spot) onto the same nitrocellulose membrane with the insulin standards. The membrane was dried at 80°C for 5 minutes and subsequent by immersing in 0.25% glutaraldehyde for 30 minutes. The membrane was washed three times with double distilled water followed by immersing in blocking buffer, 5% skim milk in PBS buffer. After incubation at room temperature for 1 hour and washing with washing buffer, PBST, the membrane was incubated in a monoclonal anti-insulin antibody (Sigma Aldrich, U.S.A.) at the dilution ratio of 1:1,500, at 4°C for overnight. The membrane was washed three times with PBST and incubated in a secondary goat anti-mouse IgG horseradish peroxidase-conjugate (Jackson Immuno Research Laboratories Inc., U.S.A.) at the dilution ratio of 1:1,500, at room temperature. After incubation for 2 hours subsequent by washing, the membrane was visualized with a substrate solution (0.03% of 3, 3'-diaminobenzidine, 0.03% of H_2O_2, 0.25% of $CoCl_2$ in PBS) for 3-5 minutes. The immunoreactive spots from samples were compared with insulin standards.

2.1.8.2 Quantitative Determination of the MIP Concentration by Indirect Competitive ELISA

A 96 well plate was coated with 100 µL per well of bovine insulin at 2 $\mu g.mL^{-1}$ and incubated at 4°C for overnight. The plate was washed three times with 300 µL per well of washing buffer (PBST) following by blocking with 300 µL per well of blocking buffer (5% skim milk in PBS buffer) and incubated at 37°C for 1 hour. The plate was washed again, subsequent by adding 50 µL per well of samples or insulin standard, which used as competitor, and 50 µL of a primary monoclonal anti-insulin antibody at the dilution ratio of 1:50,000. After incubation at 37°C for 2 hours and subsequent by washing, a secondary goat anti-mouse IgG conjugate with horseradish peroxidase was added at the dilution ratio of 1:12,000 (100 µL per well). The plate was incubated at 37°C for 1 hour, subsequent by washing and adding the TMB substrate solution (100 µL per well). After incubation in the dark at room temperature for 10 minutes, the reaction was stopped by adding 100 µL per well of 1 M H_2SO_4. The plate was measured the optical density at 450 nm and the standard curve of insulin was generated (appendix C). Standard equation was created from the relationship between standard insulin concentration and the optical density at 450 nm. The standard equation was used for calculation the MIP concentration in the culture samples (2.1.7).

2.1.9 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software (15.0) was used for statistical analysis of the MIP expression level in the culture broth. The MIP concentration, which is calculated by comparison with insulin standard from indirect competitive Enzyme-linked Immunosorbent Assay (ELISA), is analyzed by One-Way Analysis of Variance (ANOVA), which was used to determine the difference means between/within groups, while Tukey HSD multiple comparisons was used to determine the difference means in homogeneous subsets between groups of samples. The null hypothesis will be rejected when the *p* value ≤0.05 (significant level = 0.05, α0.05) which mean that the mean of data between groups is differently significant.

2.2 อภิปรายผล (Results and Discussion)

2.2.1 Recombinant Plasmids

TP1 plasmid was generated from insertion of the MIP gene into pPICZαA expression vector. The genetic map of TP1 plasmid (3,709 bp) which harbors one copy of MIP gene was shown in Figure 2.2. The TP1 plasmid was digested with *Bam*HI and *Bgl*II to generate a *MIP* expression cassette (1,792 bp), 5'*AOX1-MIP*-3'*AOX1*TT (Figure 2.3). TP2 plasmid was constructed by ligation of the MIP cassette with linear TP1, which was linearized with *Bam*HI. The expected size of TP2 plasmid was 5,501 bp. Due to the *MIP* cassette could be ligated to the linear TP1 plasmid with two directions, Type I (head to tail) - (head to tail) or Type II (head to tail) - (tail to head) (Figure 2.4). Therefore, the orientation of *MIP* cassettes in TP2 plasmid was checked by specific restriction enzymes, *Eco*RI, *Bgl*II and *Bam*HI. Result showed that *Eco*RI digested TP2 gave two DNA fragments, size 1,792 bp and 3,709 bp, and the DNA fragments of TP2 plasmid, which was digested with *Bgl*II and *Bam*HI, were 1,917 bp and 3,584 bp (as showed in Figure 2.5). To generate TP4 plasmid, DNA fragment of 2×*MIP* cassettes (3,584 bp) was purified and cloned into linear TP2 plasmid, which was linearized with *Bam*HI. The genetic map of the TP4 plasmid with the size of 9,085 bp was shown in Figure 2.6. These 3 recombinant plasmids (TP1, TP2 and TP4) were transformed to *P. pastoris* 3 strains (X-33, GS115 and KM71H) and *H. polymorpha* (NRRL2214) by electroporation to study the effect of strain and copy number of gene in the expression level of MIP.

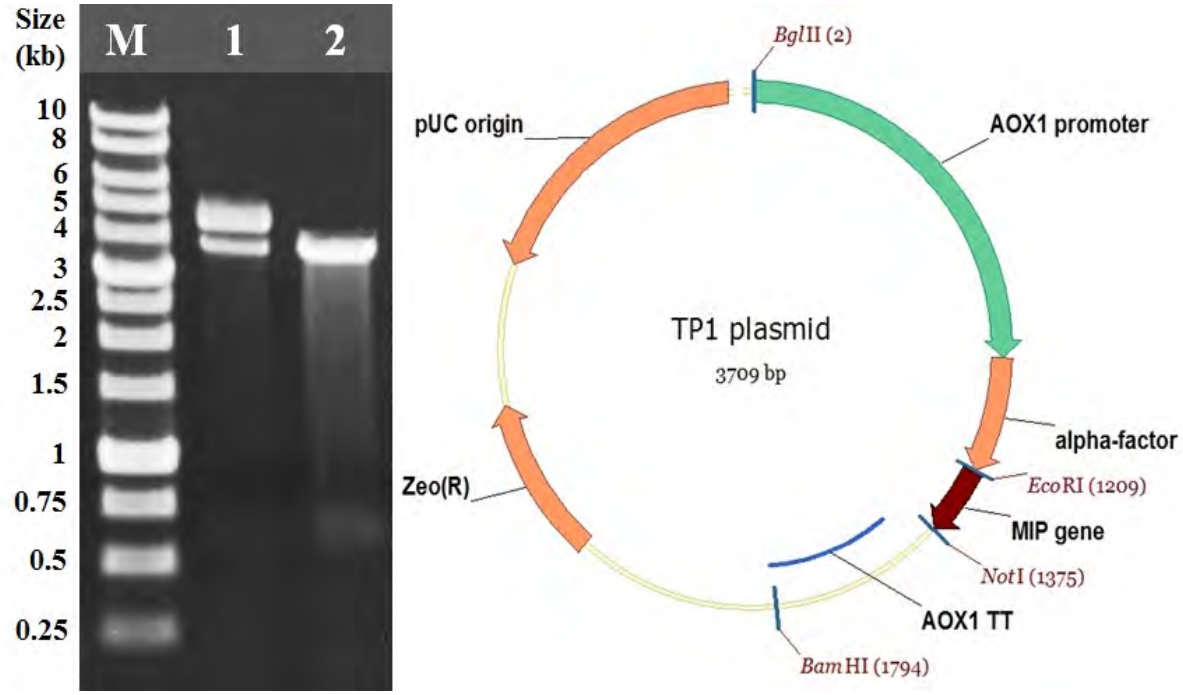

Figure 2.2: The genetic map and agarose gel electrophoresis of the TP1 plasmid. M is a 1 kb DNA ladder. Lane 1 is the circular TP1 plasmid (uncut). Lane 2 is the linear TP1 plasmid which was linearized with *Bam*HI.

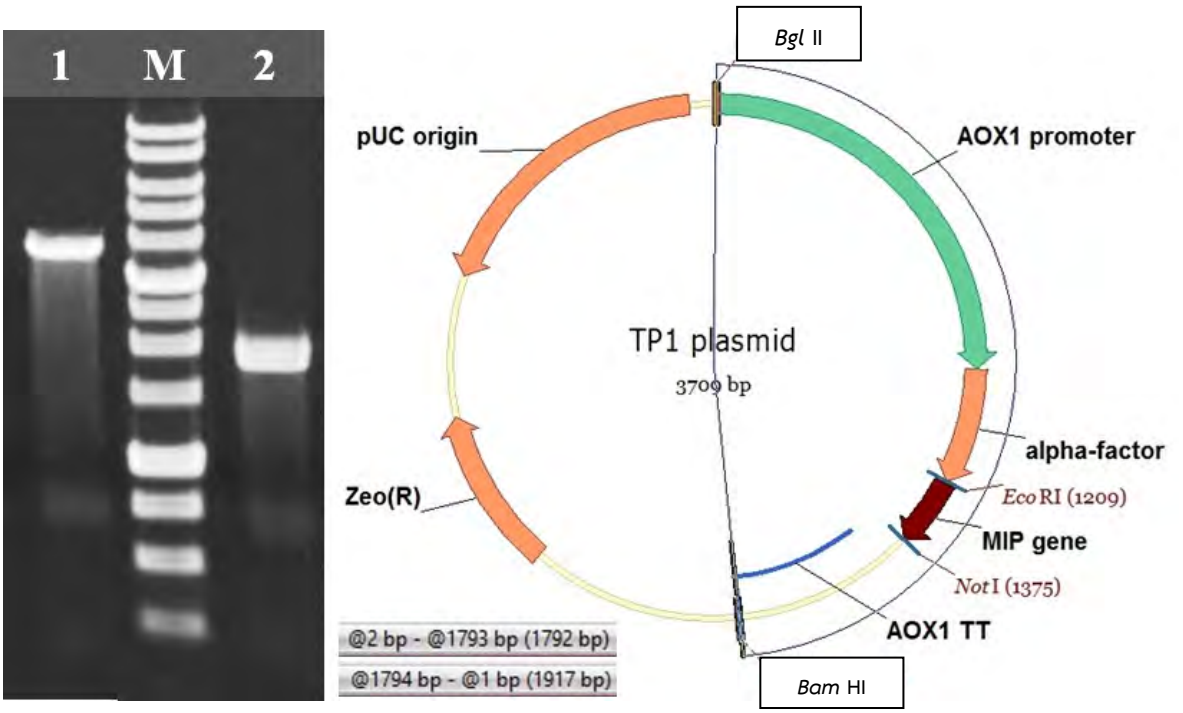

Figure 2.3: The genetic map and agarose gel electrophoresis of digested TP1 plasmid: M is a 1 kb DNA ladder. Lane 1 is the linear TP1 plasmid which was linearized with *Bam*HI. Lane 2 is the TP1 plasmid which was digested with *Bgl*II and *Bam*HI to release the *MIP* cassette with the size of 1,792 bp.

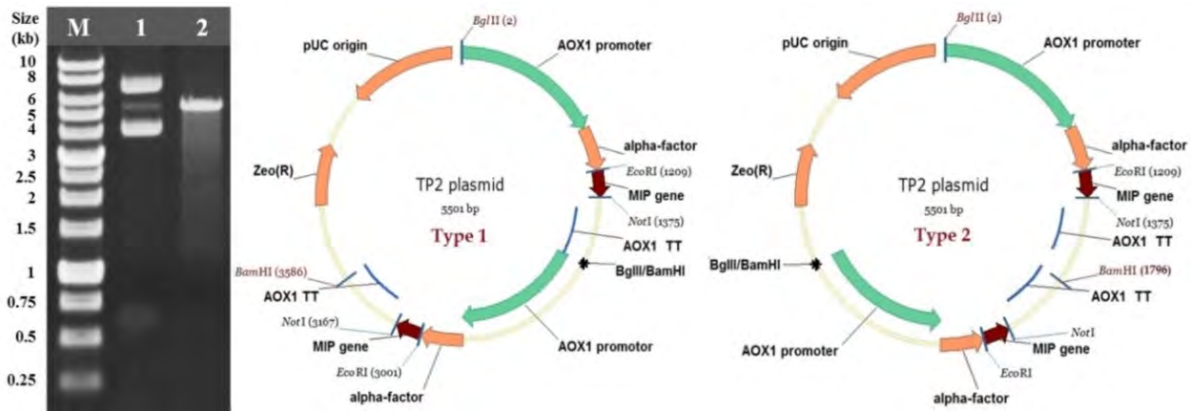

Figure 2.4: Agarose gel electrophoresis of TP2 plasmid and the possibility of genetic map of TP2 plasmid (Type I; (head to tail) - (head to tail) and Type II; (head to tail) - (tail to head)). M is a 1 kb DNA ladder. Lane 1 is the circular TP2 plasmid. Lane 2 is the linear TP2 plasmid which was linearized with *Bam*HI.

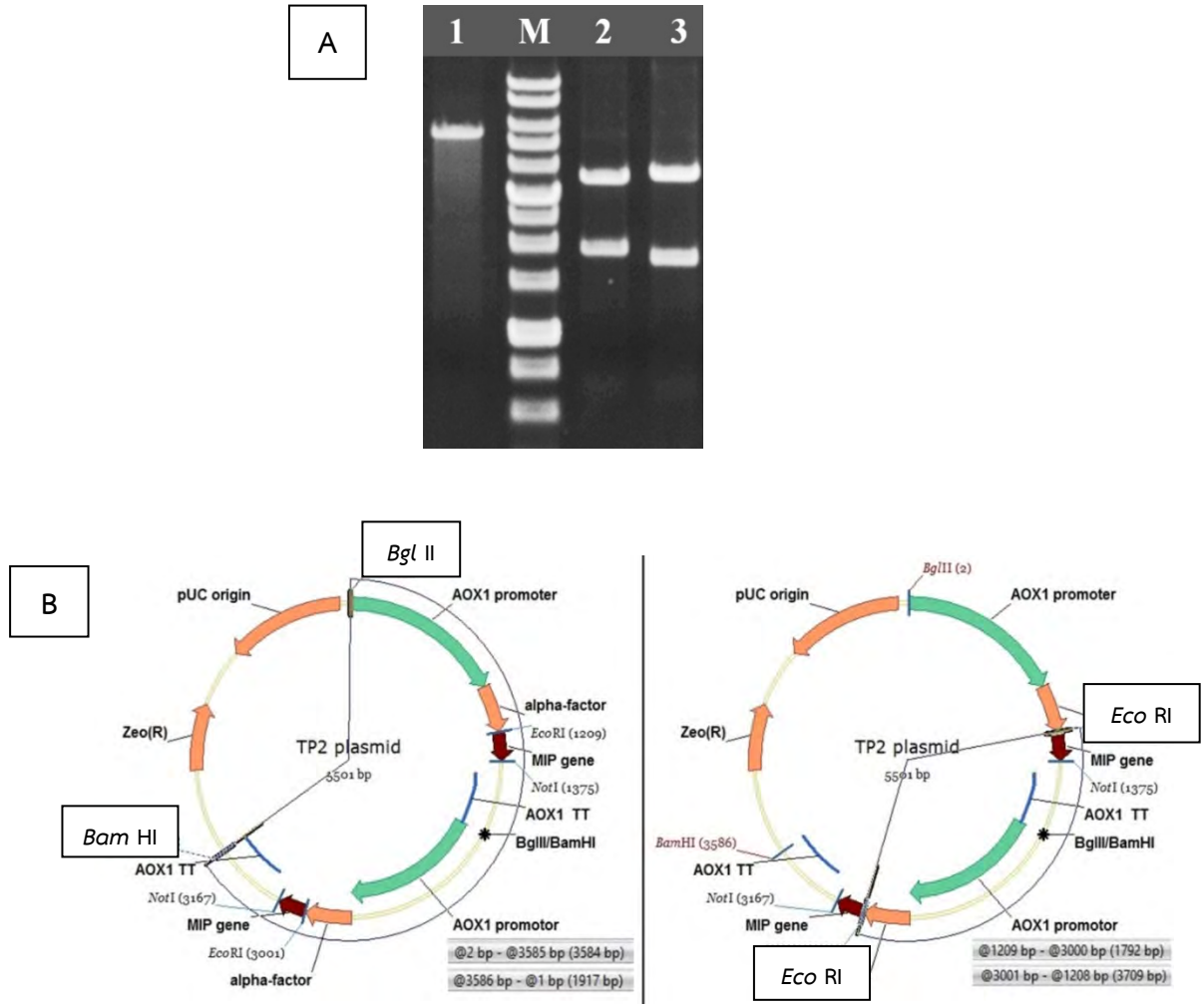

Figure 2.5: (A) The agarose gel electrophoresis of the TP2 plasmid which M is a 1 kb DNA ladder, Lane 1; Lane 2; Lane 3 are the TP2 plasmid which was digested with *Bam*HI; *Bgl*II and *Bam*HI; *Eco*RI, respectively. (B) The predicted fragments of TP2 plasmid when were digested with *Bgl*II and *Bam*HI (Left) or *Eco*RI (Right).

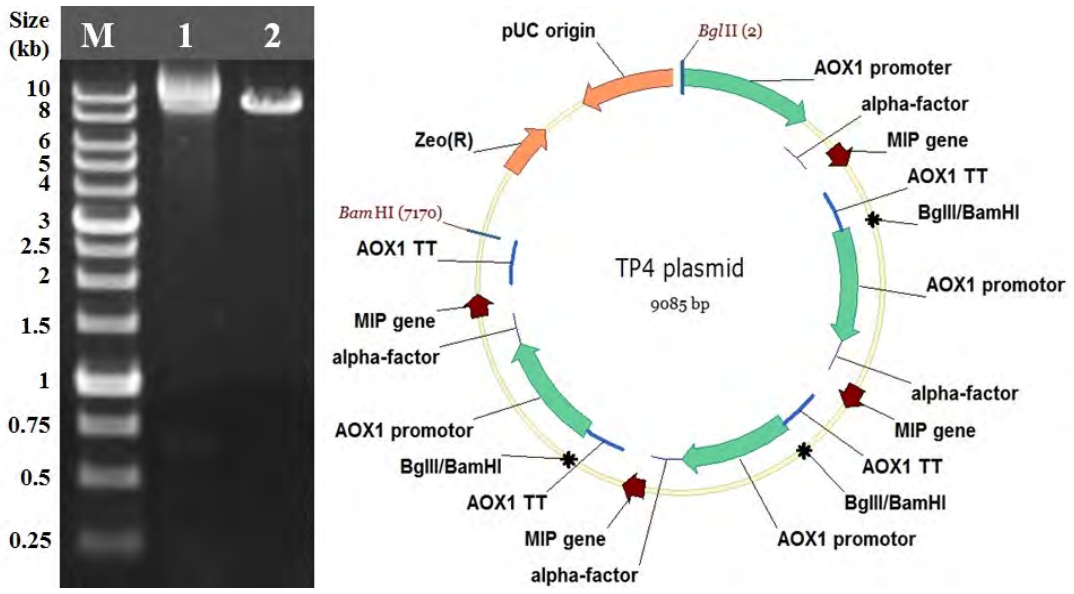

Figure 2.6: The genetic map and agarose gel electrophoresis of the TP4 plasmid. M is a 1 kb DNA ladder. Lane 1 is the circular TP4 plasmid. Lane 2 is the linear TP4 plasmid which was linearized with *Bam*HI.

2.2.2 Recombinant Yeasts Cultivation

In general, *P. pastoris* Mut^+ strains were characterized by a higher growth rate than Mut^S strains especially in medium containing methanol as sole carbon source. *P. pastoris* KM71H (Mut^S) has an inactive *AOX*1 gene, is fundamentally different in that it grows very slow in induction medium. Krainer et al., was reported that the specific growth rate of the Mut^+ strains were calculated to be approximately 1.5-fold higher than for the Mut^S strain (30). In this study, therefore, the recombinant yeasts *P. pastoris* (X-33, GS115 and KM71H) and *H. polymorpha* (NRRL2214) which harbored TP1, TP2 or TP4 plasmids, were cultivated in two steps cultivation. The first step is cell production phase which the cells were cultivated in YPG medium and the next step is an induction phase which the cells were cultivated in MMH induction medium. The optical density at 600 nm of the starter culture for inoculation in the first step was approximately 1.0 (as showed in Table 2.1). After 24 hours of growing in YPG medium, an optical density at 600 nm (OD_{600}) was observed and the cells were harvested for further culture. The results showed that the average OD_{600} and cell dry weight of the cell culture at 24 hours in the first step were 20.27±2.66 and 7.34±1.26 $g.L^{-1}$, respectively. While *P. pastoris* GS115 harbored empty vector (pPICZαA) was used as negative control. The data was showed in the Table 2.2.

In the induction phase, the cell pellets from YPG at 24 hours were transferred to MMH induction medium and the *MIP* gene expression was induced by methanol. Samples were taken every 24 hours for monitoring and determination of the MIP expression. In this step, it was observed that OD_{600} of the cell culture was unchanged till the last sampling at 72 hours (as showed in Table 2.3).

Table 2.1: The optical density at 600 nm of the starter culture

Yeast(s)	Strain(s)	Plasmid(s)	OD_{600} of the starter culture		
			DF	OD_{600}	True OD600
P. pastoris	GS115	pPICZαA#5	2	0.498	0.996
		pPICZαA#6	2	0.495	0.990
P. pastoris	X-33	TP1#1	2	0.480	0.960
		TP2#1	2	0.506	1.012
		TP4#2	2	0.499	0.998
	GS115	TP1#5	2	0.521	1.042
		TP2#4	2	0.507	1.014
		TP4#3	2	0.496	0.992
	KM71H	TP1#2	2	0.509	1.018
		TP2#8	2	0.502	1.004
		TP4#2	2	0.490	0.980
H. polymorpha	NRRL2214	TP1#3	2	0.537	1.074
		TP2#4	2	0.509	1.018
		TP4#5	2	0.526	1.052

Table 2.2: The optical density at 600 nm and cell dry weight of the cell culture in the cell production phase (YPG medium)

Yeast	Strain	Plasmid	OD_{600} of the cell culture in YPG medium		Cell dry weight ($g.L^{-1}$)
			at 0 hour	at 24 hours	at 24 hours
P. pastoris	GS115	pPICZαA#5	0.115±0.001	23.20±1.06	6.92±0.37
		pPICZαA#6	0.119±0.005	18.18±0.46	8.86±0.04
P. pastoris	X-33	TP1#1	0.117±0.002	24.17±0.57	6.80±0.09
		TP2#1	0.121±0.000	23.67±1.55	6.77±0.06
		TP4#2	0.122±0.001	20.82±0.10	6.50±0.25
	GS115	TP1#5	0.121±0.002	17.37±1.29	8.72±0.23
		TP2#4	0.119±0.002	16.95±0.65	8.80±0.41
		TP4#3	0.115±0.002	18.10±1.39	8.48±0.21
	KM71H	TP1#2	0.122±0.002	22.67±0.33	6.64±0.13
		TP2#8	0.120±0.003	21.30±0.31	10.02±0.29
		TP4#2	0.113±0.001	22.05±0.14	6.27±0.25
H. polymorpha	NRRL2214	TP1#3	0.128±0.002	17.53±0.14	5.92±0.06
		TP2#4	0.131±0.007	20.40±0.78	6.58±0.24
		TP4#5	0.121±0.006	17.67±0.23	6.11±0.01

Data is show in mean ± standard deviation.

Table 2.3: The optical density at 600 nm of the cell culture in the induction phase (MMH medium)

Yeast	Strain	Plasmid	OD600 of the cell culture in MMH medium			
			at 0 hour	at 24 hours	at 48 hours	at 72 hours
P. pastoris	GS115	pPICZαA#5	21.90±1.20	20.58±1.87	21.90±2.19	22.48±2.44
		pPICZαA#6	20.43±1.66	18.58±0.25	19.83±0.11	21.85±0.35
P. pastoris	X-33	TP1#1	21.55±0.28	20.13±0.64	21.83±0.53	23.12±0.83
		TP2#1	20.90±1.20	19.27±0.85	21.07±0.50	23.20±0.38
		TP4#2	21.15±0.18	19.37±0.58	20.72±0.16	22.23±0.55
	GS115	TP1#5	18.87±1.34	19.22±0.58	19.38±2.24	23.30±1.09
		TP2#4	20.57±1.14	19.30±0.69	20.37±0.72	22.97±0.94
		TP4#3	21.80±0.53	19.47±0.58	19.68±0.62	22.53±1.21
	KM71H	TP1#2	20.60±0.98	19.38±0.60	19.33±0.29	19.25±0.62
		TP2#8	22.80±0.80	19.95±0.05	23.72±0.25	21.83±1.22
		TP4#2	20.82±0.84	17.88±0.08	19.28±0.53	19.62±0.65
H. polymorpha	NRRL2214	TP1#3	18.37±0.26	16.98±0.43	16.93±0.74	18.85±0.65

Data is show in mean ± standard deviation.

2.2.3 The Monitoring of the MIP Expression Level by Dot-Blot Analysis

A specific dot-blot procedure was chosen to initial screening of the MIP expression level in the culture broth. Insulin from bovine pancreas (0.0005 – 0.1 $\mu g.\mu L^{-1}$) and inject insulin (1/512 – 1/4, two-fold serial dilution from stock 1 $\mu g.\mu L^{-1}$) were used as insulin standards (Appendix B). Determination of insulin was done by using a monoclonal anti-insulin antibody. The insulin standards were used as positive controls at various concentrations. MMH medium and the supernatant of *P. pastoris* GS115, which harboring the pPICZαA vector integrated into the genome, were used as negative controls. The MIP expression level of the recombinant *P. pastoris* (X-33, GS115 and KM71H) and *H. polymorpha* (NRRL2214) were shown in Figure 2.7 and 2.8.

The intensity of each spot that occur on nitrocellulose membrane relate to the amount of insulin that can be interpreted by comparison with standard insulin on the same membrane while the negative control did not show any spot. The results showed that the recombinant of *P. pastoris* KM71H strain, Mut^S phenotype strain, was the best strain to express the MIP (correlate with the dark spot) and it expressed more rapidly than the others and had been visualized by dot-blot at 24 hours in an induction phase. While the recombinants of *H. polymorpha*, the expression level of MIP was lower than the other strains as show in Figure 2.7-2.8.

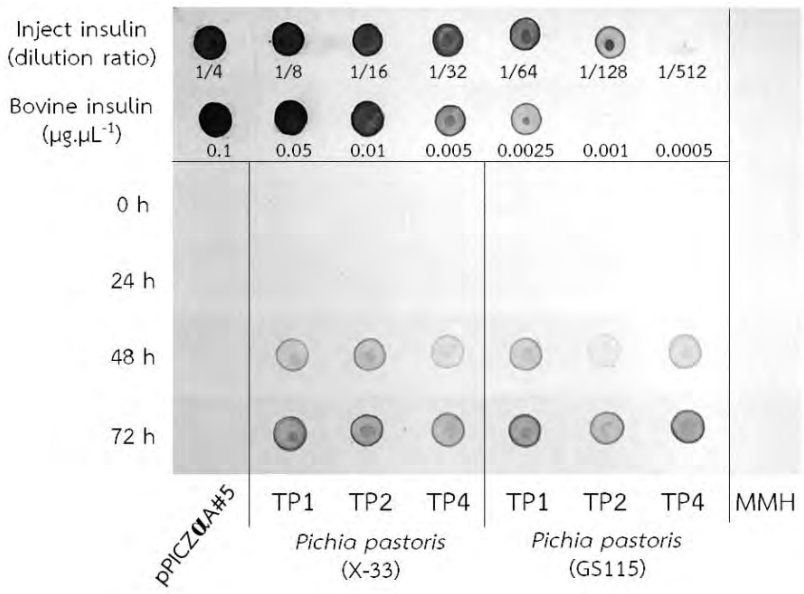

Figure 2.7: Dot-blot analysis of the inject insulin and bovine insulin (positive control), MMH medium (negative control), the supernatant of the recombinant *P. pastoris* pPICZαA (negative control) and the supernatant of the recombinant *P. pastoris* (X-33 and GS115) that harbor TP1, TP2 and TP4 in their genome at various cultivation times in MMH medium.

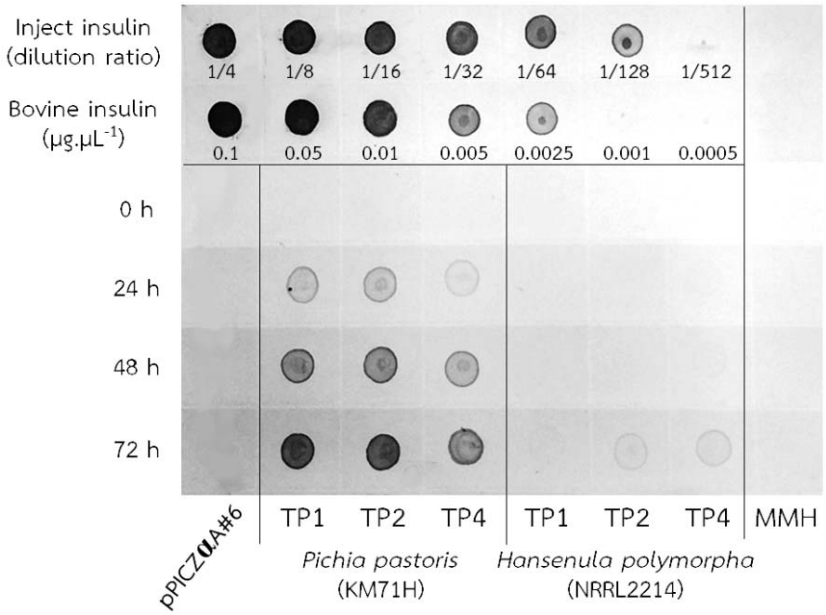

Figure 2.8: Dot-blot analysis of the inject insulin and bovine insulin (positive control), MMH medium (negative control), the supernatant of the recombinant *P. pastoris* pPICZαA (negative control) and the supernatant of the recombinant *P. pastoris* (KM71H) and *H. polymorpha* (NRRL2214) that harbor TP1, TP2 and TP4 in their genome at various cultivation times in MMH medium.

2.2.4 The Quantitative Determination of the MIP Concentration by Indirect Competitive ELISA

It was many methods to monitor and determine a concentration of secreted heterologous protein from yeast expression system e.g. UV absorption at 280 nm (5), RP-HPLC (UV-VIS detector, 280 nm) (6, 31, 32), HPLC (UV-VIS detector, 214 nm) (16). Some researchers determine the concentration of secreted protein using measurement an optical density at 280 nm (21). This method may has limitation because of only aromatic amino acid which compose of Phenylalanine (Phe), Tyrosine (Tyr) and Tryptophan (Typ) can absorb this UV range. And this method is not suitable for measure the absorbance of supernatant because of interference of other proteins that also secreted from yeast or lysate cells can secreted too. Therefore, a specific procedure, indirect competitive ELISA, was chosen to determine the MIP expression according to specific binding between antibody and antigen. The MIP concentration was quantitative determined by indirect competitive ELISA which is a high sensitivity and high specificity technique. An indirect competitive ELISA was developed with a measurement range of insulin standard concentration 0.025 - 10 $mg.L^{-1}$. The supernatants from culture broth were determined the MIP concentration by comparison with standard insulin and calculated using standard equation (see in an appendix C).

2.2.4.1 The Effect of Yeast Strains on the MIP Expression Level

To date, several researchers have been reported that *P. pastoris* Mut^+ strains produced more recombinant protein due to grow faster on methanol (32, 33). However, some researchers have shown Mut^S strains to be superior over Mut^+ strains in terms of recombinant protein production (30, 34-36).

In this study to compare the effect of yeast strains on the MIP expression level, we focus on the recombinant yeasts which harbored TP1 plasmid in the genome and cultivated in an induction medium (MMH medium) for 72 hours.

The result showed that the recombinant *P. pastoris* KM71H gave the highest MIP concentration (4.19±0.96 $mg.L^{-1}$) and following by *P. pastoris* GS115 (2.69±0.48 $mg.L^{-1}$) and *P. pastoris* X-33 (0.93±0.08 $mg.L^{-1}$) as show in Figure 2.9. The MIP expression level in these recombinant *Pichia* has differed each other at the significant level of 95% (alpha = 0.05). These may be due to both *P. pastoris* GS115 (Mut^+) and *P. pastoris* X-33 (Mut^+) strains have an active *AOX1* gene which support their methanol utilization. So that, the methanol in the Mut^+ cultures could be rapidly used and the methanol concentration might be get rid of the medium during the cultivation. Another important reason is the high demand for oxygen in cultures of *P. pastoris* Mut^+ phenotype (37). By using *P. pastoris* strain with Mut^S phenotype these problem can be bypassed due to the lower methanol consumption rate. Therefore, the Mut^S phenotype, *P. pastoris* KM71H leads to long induction time for MIP expression and needs less oxygen demand.

Like all methylotrophs, *H. polymorpha* is able to grow on methanol as its sole energy and carbon source. However, the MIP expression of recombinant *H. polymorpha* NRRL2214 was lower

(0.04±0.01 mg.L^{-1}) than those of *P. pastoris* strains. According to Raschke et al. (38), *P. pastoris AOX1* promoter can be used for methanol induced expression of heterologous gene in *H. polymorpha*. However, the expression level of heterologous protein under control of *AOX1* promoter in *H. polymorpha* seemed to be lower than in *P. pastoris*.

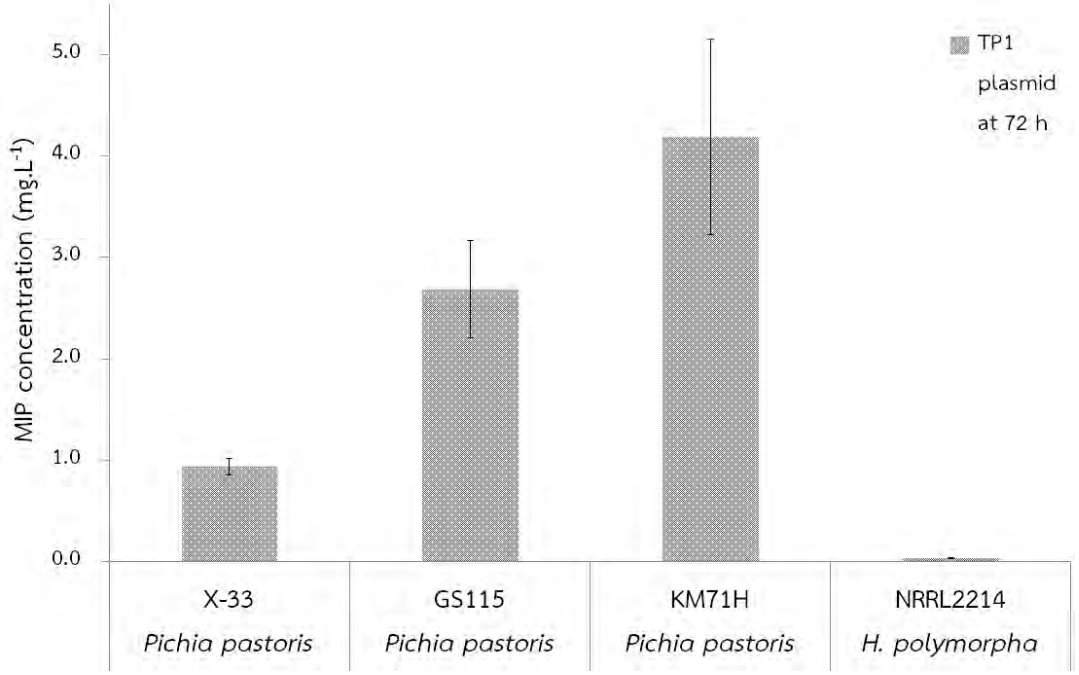

Figure 2.9: The MIP expression of the recombinant yeasts which contain TP1 plasmid as shown by MIP concentration in the culture (at 72 hours of induction).

2.2.4.2 Effect of Copy Number of Gene on the MIP Expression Level

Creating recombinant yeasts which multiple copy number of gene can be done by two methods. The first *in vivo* method is using a dominant selective marker for selection of the recombinants that could grow on high concentration of selective antibiotic, correlation between the number of the integrated sequences and associated antibiotic resistance leads to an enrichment of the population of the multi-copy strains (16). The other method is in vitro method which to construct vector with multiple head-to-tail copies of an expression cassette (31, 39).

In this study, we had constructed the recombinant plasmids TP1, TP2, TP4 which contain 1, 2 and 4 copies of the MIP cassette, respectively. To study the effect of gene copy number on the MIP expression in yeasts; *P. pastoris* GS115 (Mut^{+}), X-33 (Mut^{+}), KM71H (Mut^{S}) and *H. polymorpha* NRRL2214, the recombinant plasmids were transformed into the yeasts and the expression level of MIP that expressed in the recombinant yeasts were analyzed.

In recombinant *P. pastoris* X-33, we found that the *P. pastoris* X-33 which has TP2 plasmid can produce the MIP level increase significantly at 48 hours but in *P. pastoris* X-33 which has TP1 and TP4 plasmid have not difference. When consider at the end of an induction phase (72 hours), the *P. pastoris* X-33 which has TP2 plasmid give the highest MIP expression level followed by *P. pastoris* X-33 which has TP4 and TP2 plasmid respectively as show in Figure 2.10.

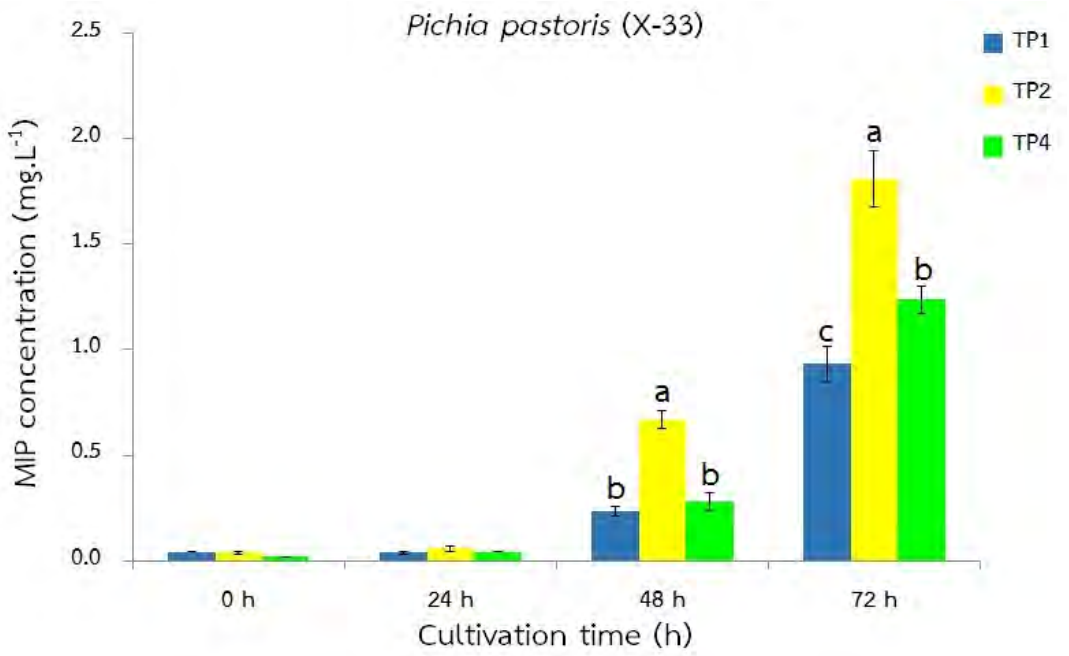

Figure 2.10: The MIP concentration in culture broth of the recombinant *P. pastoris* (X-33) which contain 1 copy (TP1), 2 copies (TP2) and 4 copies (TP4) of the MIP cassette in their genome.

In recombinant *P. pastoris* GS115, the result showed that the MIP expression from each recombinant *P. pastoris* GS115 had not significantly different within 48 hours of an induction phase. But at the 72 hours, the expression level of MIP from recombinant *P. pastoris* GS115 which has TP1 and TP4 plasmids had higher than the recombinant *P. pastoris* GS115 with TP2 plasmid as show in Figure 2.11.

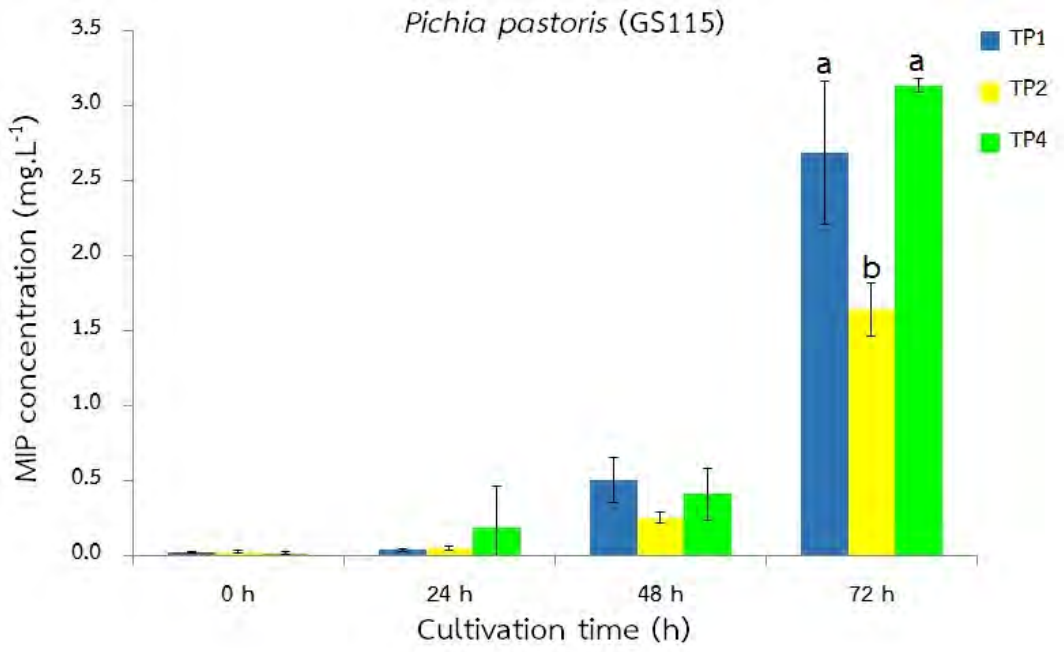

Figure 2.11: The MIP concentration in culture broth of the recombinant *P. pastoris* (GS115) which contain 1 copy (TP1), 2 copies (TP2) and 4 copies (TP4) of the MIP cassette in their genome.

In recombinant *P. pastoris* KM71H, the result showed that at early 48 hours in an induction phase a recombinant *P. pastoris* KM71H which has TP2 plasmid express more rapidly than the others but in *P. pastoris* KM71H which has TP1 and TP4 plasmid have not significantly different. Considering at 72 hours, the expression of MIP in the recombinant *P. pastoris* KM71H which has TP1 plasmid was sharply increased and the MIP expression level from recombinant *P. pastoris* KM71H which has TP1 and TP2 plasmid have not significantly different whereas the recombinant *P. pastoris* KM71H which has TP4 plasmid has lower expression level as show in Figure 2.12.

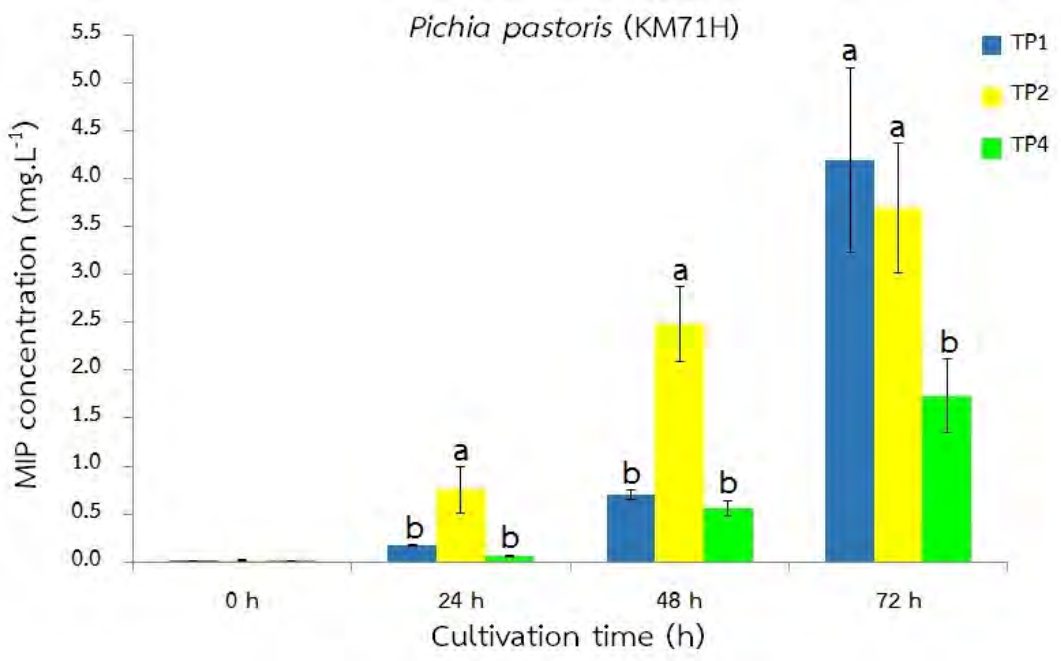

Figure 2.12: The MIP concentration of the recombinant *P. pastoris* (KM71H) which contain 1 copy (TP1), 2 copies (TP2) and 4 copies (TP4) of the MIP cassette in their genome.

In *P. pastoris*, usually one plasmid copy is integrated using targeted insertion into the homologous gene (*AOX1* gene), whereas the transformation procedure for *H. polymorpha* introduces an unpredictable number of plasmid copies which integrate at an unknown site(s). Then recombinant *H. polymorpha* strains have been obtained by integrating several copies via a non-homologous recombination event (25). According to this reason, we tried to expressed MIP in *H. polymorpha* NRRL2214, however, the result showed that the expression level of MIP from all of the recombinant *H. polymorpha* NRRL2214 was very low within the first 48 hours in an induction phase. Till 72 hours, the MIP expression level of recombinant *H. polymorpha* NRRL2214 which has TP2 and TP4 plasmid have slightly increased as show in Figure 2.13.

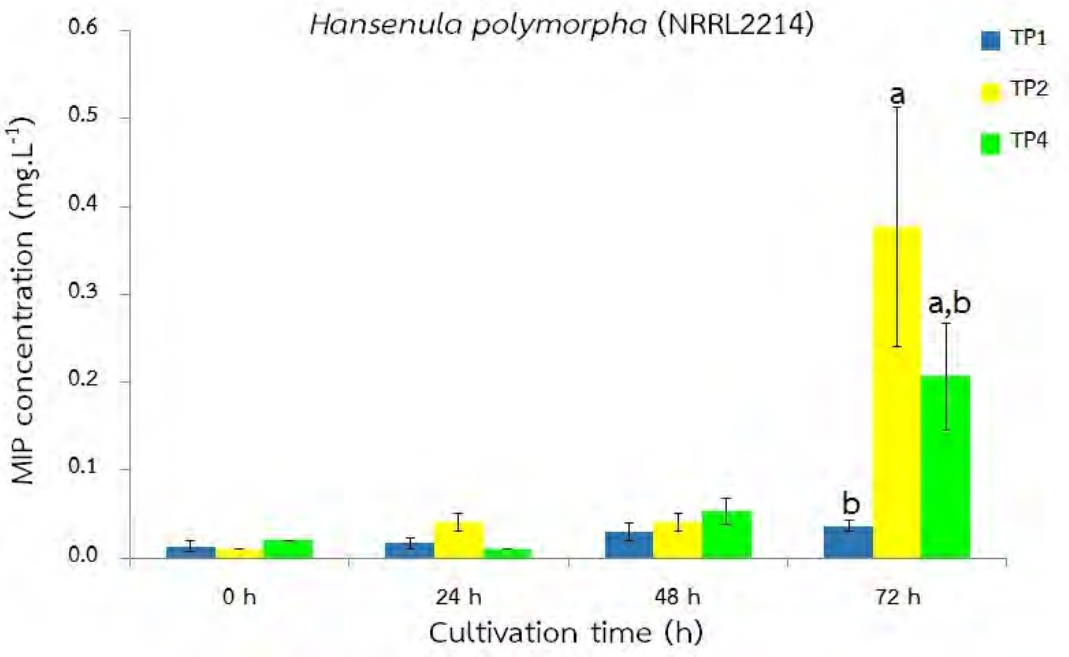

Figure 2.13: The MIP concentration of the recombinant *H. polymorpha* (NRRL2214) which contain 1 copy (TP1), 2 copies (TP2) and 4 copies (TP4) of the MIP cassette in their genome.

The summarized MIP contents in the supernatant culture from recombinant yeast are showed in Table 2.4. The results showed that the MIP concentrations of all recombinant yeasts were gradually increased along the cultivation time. However, recombinant *H. polymorpha* gave low MIP expression level in a range of 0.05 - 0.40 mg.L^{-1} even at 72 hours of induction. There is no consistency between the copy number of MIP gene and the expression of MIP level. These may because of the structure of MIP that consists with three disulfide bonds per molecule and the limitation of the secretory pathway of the host cells. Similar observations have also been described by Zhu et al., 2009 (16) and Hohenblum et al., 2003 (39). For the secretion of recombinant proteins from yeast cells, membrane translocation, signal peptide processing, folding and disulfide bond formation are one of the major bottlenecks. Inan et al., (2006) (22) attempted to increase secretion of a hookworm protein by increasing gene copy number. However, this has a negative effect when compared to single copy clones. By overexpressing protein disulfide isomerase, PDI, they were able to increase secretion of recombinant secreted protein in clones containing up to four copies of the expression cassette.

Table 2.4: The MIP concentration in the culture broth from the recombinant yeasts in an induction phase

Yeast(s)	Strain(s)	Plasmid(s)	MIP concentration in the culture broth (mg.L-1) *			
			at 0 h	at 24 h	at 48 h	at 72 h
P. pastoris	GS115	pPICZαA#5	0.05±0.00	0.06±0.01	0.07±0.01	0.07±0.01
		pPICZαA#6	0.03±0.00	0.07±0.00	0.07±0.01	0.08±0.01
P. pastoris	X-33	TP1#1	0.04±0.00	0.04±0.01	0.23±0.02	0.93±0.08
		TP2#1	0.04±0.01	0.06±0.02	0.67±0.04	1.81±0.13
		TP4#2	0.02±0.00	0.04±0.00	0.28±0.04	1.24±0.06
	GS115	TP1#5	0.02±0.01	0.04±0.01	0.51±0.15	2.69±0.48
		TP2#4	0.03±0.01	0.05±0.02	0.25±0.04	1.64±0.18
		TP4#3	0.02±0.01	0.19±0.28	0.41±0.17	3.13±0.05
	KM71H	TP1#2	0.01±0.01	0.17±0.01	0.70±0.04	4.19±0.96
		TP2#8	0.01±0.01	0.75±0.25	2.48±0.39	3.70±0.68
		TP4#2	0.01±0.00	0.05±0.01	0.55±0.08	1.73±0.38
H. polymorpha	NRRL 2214	TP1#3	0.01±0.01	0.02±0.01	0.03±0.01	0.04±0.01
		TP2#4	0.01±0.00	0.04±0.01	0.04±0.01	0.38±0.14
		TP4#5	0.02±0.00	0.01±0.00	0.05±0.02	0.21±0.06

* The data is show in mean ± standard deviation.

สรุปและข้อเสนอแนะ (Conclusion and Recommendation)

3.1 Conclusion

Effect of gene dosage to the heterologous protein expression in methylotrophic yeast system has been extensively studied for two past decades (16, 31, 39, 40). Two types of *P. pastoris* strains that vary with regard to their ability to utilize methanol are competed to be used as hosts. Moreover, *H. polymorpha* is claimed to have some advance to be host for heterologous protein expression such as high-copy integration due to non-homologous recombination (25). This research has studied both the effect of gene copy number and yeast host strains on the MIP expression. Our studies suggested that *P. pastoris* KM71H Mut^S (methanol utilization slow phenotype) strain is superior over either *P. pastoris* X-33, GS115, Mut^+ (methanol utilization plus phenotype) strains, or *H. polymorpha* NRRL2214 in terms of recombinant protein production. However, the level of MIP secreted into the medium was unrelated with gene copy number. This point make as if to consider the limitation of a secretory pathway that may be bottleneck for overexpression of high-copy number recombinants.

3.2 Recommendation

As a number of diabetes patients in the world are increasing result in demand of insulin will increasing too. However in Thailand, all types of insulin are imported and it needs to be improved the production of effective insulin analog to treat diabetes patients. This research prototype aims to produce the monomeric insulin precursor (MIP), which has monomeric and fast acting properties, and to improve the potential of our country. Whereas the results showed that the MIP production was quite low that may cause of several genetic and physiological factors. A practical solution is to identify the major bottleneck of a production system, which in general is both host strain- and product-dependent. In several cases for the secretion of recombinant proteins from yeast cells, folding and disulfide bond formation were identified as rate-limiting step. To identify whether part of the MIP product remained in the cells, MIP determination should be done in both cell lysates and culture broth. Further, the MIP productivity needs to be improved by study the physical factors e.g. dissolved oxygen, methanol concentration, cell density or cultivation strategy that effect to the MIP expression.

In the future, the produced recombinant monomeric insulin precursor needs to be biological activity tested by in vivo method to confirm its action before scale up to the pilot plant.

เอกสารอ้างอิง (References)

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2012;35:64-71.
2. International Diabetes Federation. 2014 [cited 2014 Octerber 15]. Available from: http://www.idf.org/.
3. Lorenzi RD, Gritti C. Towards the first recombiant drug : Insulin 2010 [cited 2014 October 12th]. Available from: http://emblog.embl.de/.
4. Shivanand P, Noopur S. Recombinant DNA Technology: Applications in the field of biotechnology and crime sciences. International Journal of Pharmaceutical Sciences Review and Research. 2010;1(1).
5. Ding JG, Fei J, Cui DF, Zhang YS. Expression of monomeric insulin precursor in *Pichia pastoris* and its conversion into monomeric B27 Lys destripeptide insulin by tryptic hydrolysis. Acta Biochimica Et Biophysica Sinica. 2005;37(4):234-240.
6. Ding JG, Cui DF, Zhang YS. Monomeric B27 Lys destripeptide insulin: Semisynthesis, characterization and biological activity. Acta Biochimica Et Biophysica Sinica. 2003;35(3):215-218.
7. Ashok KD, Ashida TS. Modern Insulins -The Insulin Analogues : A reappraisal [cited 2014 October 12]. Available from: http://www.apiindia.org/.
8. Li P, Anumanthan A, Gao XG, Ilangovan K, Suzara VV, Duzgunes N, et al. Expression of recombinant proteins in *Pichia pastoris*. Applied Biochemistry and Biotechnology. 2007;142(2):105-124.
9. Celik E, Calik P. Production of recombinant proteins by yeast cells. Biotechnology Advances. 2012;30(5):1108-1118.
10. Damasceno LM, Huang CJ, Batt CA. Protein secretion in *Pichia pastoris* and advances in protein production. Applied Microbiology and Biotechnology. 2012;93(1):31-39.
11. Gellissen G, Melber K. Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmaceuticals. Drug Research. 1996;46(9):943-948.
12. Muller S, Sandal T, Kamp-Hansen P, Dalboge H. Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Klyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from Yarrowia lipolytica. Yeast. 1998;14(14):1267-1283.
13. Gellissen G, Kunze G, Gaillardin C, Cregg JM, Berardi E, Veenhuis M, et al. New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica*-a comparison. FEMS Yeast Research. 2005;5(11):1079-1096.
14. Hollenberg CP, Gellissen G. Production of recombinant proteins by methylotrophic yeasts. Current Opinion in Biotechnology. 1997;8(5):554-560.
15. Zhang W, Inan M, Meagher MM. Fermentation Strategies for Recombinant Protein Expression in the Methylotrophic Yeast *Pichia pastoris*. Biotechnology and Bioprocess Engineering. 2000;5(4):275-287.

16. Zhu T, Guo M, Tang Z, Zhang M, Zhuang Y, Chu J, et al. Efficient generation of multi-copy strains for optimizing secretory expression of porcine insulin precursor in yeast *Pichia pastoris*. Journal of Applied Microbiology. 2009;107(3):954-963.
17. Meyts PD. Insulin and its receptor. In: Sir Kendrew J, editor. The encyclopedia of Molecular Biology. USA: Blackwell Science Ltd; 1995. p. 549-552.
18. Sheldon B, Russell-Jones D, Wright J. Insulin analogues: an example of applied medical science. Diabetes, Obesity and Metabolism. 2009;11(1):5-19.
19. Mullis KB. Recombinant DNA technology and molecular cloning. Scientific American; 1990;262(36):180-231.
20. Panda AK. Recombinant DNA technology and biotechnology 2008 [cited 2014 October 12]. Available from: http://nsdl.niscair.res.in/.
21. Kjeldsen T, Pettersson AF, Hach M. Secretory expression and characterization of insulin in *Pichia pastoris*. Biotechnology and Applied Biochemistry. 1999;29:79-86.
22. Inan M, Aryasomayajula D, Sinha J, Meagher MM. Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. Biotechnology and Bioengineering. 2006;93(4):771-778.
23. Hartner FS, Glieder A. Regulation of methanol utilisation pathway genes in yeasts. Microbial Cell Factories. 2006;5:39.
24. EasySelect™ Pichia Expression Kit USA: Invitrogen Corporation; 2005 [cited 2012 May 16]. Available from: http://tools.lifetechnologies.com/.
25. Gellissen G, Weydemann U, Strasser AW, Piontek M, Janowicz ZA, Hollenberg CP. Progress in developing methylotrophic yeasts as expression systems. Trends in Biotechnology. 1992;10(12):413-417.
26. Norden K, Agemark M, Danielson JA, Alexandersson E, Kjellbom P, Johanson U. Increasing gene dosage greatly enhances recombinant expression of aquaporins in *Pichia pastoris*. BMC Biotechnology. 2011;11:47.
27. Sambrook JF, Fritsch EF, Maniatis T. Molecular Cloning : a laboratory manual. Second ed. Ford N, Nolan C, Ferguson M, editors. USA: All Cold Spring Harbor Laboratory Press 1989.
28. Cregg JM. DNA-mediated transformation. Methods in Molecular Biology. 2007;389:27-42.
29. Sithigorngul P, Stretton AO, Cowden C. A versatile dot-ELISA method with femtomole sensitivity for detecting small peptides. Journal of Immunological Methods. 1991;141(1):23-32.
30. Krainer FW, Dietzsch C, Hajek T, Herwig C, Spadiut O, Glieder A. Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. Microbial Cell Factories. 2012;11:22.
31. Mansur M, Cabello C, Hernandez L, Pais J, Varas L, Valdes J, et al. Multiple gene copy number enhances insulin precursor secretion in the yeast *Pichia pastoris*. Biotechnology Letters. 2005;27(5):339-345.

32. Gurramkonda C, Polez S, Skoko N, Adnan A, Gabel T, Chugh D, et al. Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. Microbial Cell Factories. 2010;9:31.
33. Kim SJ, Lee JA, Kim YH, Song BK. Optimization of the functional expression of *Coprinus cinereus* peroxidase in *Pichia pastoris* by varying the host and promoter. Journal of Microbiology and Biotechnology. 2009;19(9):966-971.
34. Orman MA, Calik P, Ozdamar TH. The influence of carbon sources on recombinant-human-growth-hormone production by *Pichia pastoris* is dependent on phenotype: a comparison of Mut^S and Mut^+ strains. Biotechnology and Applied Biochemistry. 2009;52(3):245-255.
35. Ascacio-Martinez JA, Barrera-Saldana HA. Production and secretion of biologically active recombinant canine growth hormone by *Pichia pastoris.* Gene. 2004;340(2):261-266.
36. Pla IA, Damasceno LM, Vannelli T, Ritter G, Batt CA, Shuler ML. Evaluation of Mut^+ and Mut^S *Pichia pastoris* phenotypes for high level extracellular scFvexpression under feedback control of the methanol concentration. Biotechnology Progress 2006;22(3):881-888.
37. Zhang Y, Hu H, Cai R, Feng Y, Zhu S, He Q, et al. Secretory expression of a single-chain insulin precursor in yeast and its conversion into human insulin. Science in China Series C: Life Sciences. 1996;39(3):225-233.
38. Raschke WC, Neiditch BR, Hendricks M, Cregg JM. Inducible expression of a heterologous protein in *Hansenula polymorpha* using the alcohol oxidase 1 promoter of *Pichia pastoris*. Gene. 1996;177(1-2):163-167.
39. Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnology and Bioengineering. 2004;85(4):367-375.
40. Wang Y, Liang ZH, Zhang YS, Yao SY, Xu YG, Tang YH, et al. Human insulin from a precursor overexpressed in the methylotrophic yeast *Pichia pastoris* and a simple procedure for purifying the expression product. Biotechnology and Bioengineering. 2001;73(1):74-79.

สิ่งตีพิมพ์เพื่อเผยแพร่ (Proceeding)

Tanapati Phakham, Nantika Khongchareonporn, Sajee Noitang, Apichart Karnchanatat, Kannika Sermsuvitwong and Sarintip Sooksai*, 2014. Effect of gene copy number on recombinant Insulin Expression in *Pichis pastoris* GS115. The 4th International Biochemistry and Molecular Biology Conference 2014 "Bridging ASEAN Biochemical Research Communities" April 2-3, 2014. Rama Gardens Hotel & Resort, Bangkok, Thailand.

ภาคผนวก (Appendix)

Appendix A

Culture Media

Low salt LB medium

- 1% Tryptone
- 0.5% Yeast extract
- 0.5% Sodium chloride (NaCl)
- 2% Agar (Solid medium)

Adjust the pH value to 7.5 with 1 N NaOH and sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. In case of Low salt LB with Zeocin™, cool the solution to ~ 50°C and add Zeocin™ to a final concentration of 25 $\mu g.mL^{-1}$ and store at +4°C in the dark.

Luria-Bertani (LB) medium

- 1% Tryptone
- 0.5% Yeast extract
- 1% Sodium chloride (NaCl)
- 2% Agar (Solid medium)

  Adjust the pH value to 7.5 with 1 M NaOH and sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. Store at +4°C.

Minimal Methanol Histidine (MMH) medium

- 1.34% YNB (Yeast nitrogen base w/o amino acid w/ ammonium sulfate)*
- 4×10-5% Biotin*
- 0.004% Histidine*
- 0.5% Methanol

  * Biotin, Histidine and YNB were dissolved in sterilized double distilled water and sterilized by filtration (pore size 0.22μm, mixed cellulose esters membrane). Store at +4°C.

Yeast Peptone Dextrose (YPD) medium

- 1% Yeast extract
- 2% Peptone
- 2% Dextrose (Glucose)
- 2% Agar (Solid medium)

  Sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. In case of YPD with Zeocin™, add Zeocin™ to final concentration of 100 $\mu g.mL^{-1}$ and store at +4°C in the dark.

Yeast Peptone Glycerol (YPG) medium

- 1% Yeast extract
- 2% Peptone
- 1% Glycerol

  Sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. Store at +4°C

.

Appendix B

Determination of the MIP Expression Level by Dot-Blot Analysis

1. Standard insulin preparation for determination of MIP expression

Insulin from bovine pancreas was dissolved in PBS buffer (pH 7.4) at the concentration of 2 $mg.mL^{-1}$ and injection insulin (Mixtard® 30 HM Penfill® 3 $mg.mL^{-1}$) was dissolved in PBS buffer and adjusted the concentration to 1 $mg.mL^{-1}$. Both insulin from bovine pancreas and injection insulin were used as a positive control for dot-blot analysis at various concentrations as shown in Table B-1.

Table B-1: Standard insulin preparation for dot blot analysis

Insulin from bovine pancreas				**Injection insulin**			
Final conc. ($mg.mL^{-1}$)	**Stock solution ($mg.mL^{-1}$)**	**Volume from stock solution (µL)**	**PBS buffer (µL)**	**Final conc. of Serial dilution ($mg.mL^{-1}$)**	**Stock solution ($mg.mL^{-1}$)**	**Volume from stock solution (µL)**	**PBS buffe r (µL)**
2	2	500	0	1	1	500	0
1	2	500	500	1/2 X	1	500	500
0.5	1	500	500	1/4 X	1/2 X	500	500
0.1	1	100	900	1/8 X	1/4 X	500	500
0.05	0.5	100	900	1/16 X	1/8 X	500	500
0.01	0.1	100	900	1/32 X	1/16 X	500	500
0.0050	0.05	100	900	1/64 X	1/32 X	500	500
0.0025	0.05	50	950	1/128 X	1/64 X	500	500
0.0010	0.01	100	900	1/256 X	1/128 X	500	500
0.0005	0.005	100	900	1/512 X	1/256 X	500	500

Appendix C

Determination of the MIP Concentration by Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

1. Standard insulin preparation for determination of MIP concentration

Insulin from bovine pancreas was dissolved in PBS buffer (pH 7.4) at the concentration of 1 mg.mL^{-1} which is stock solution. Insulin from bovine pancreas was used as a positive control for quantitative determination of the MIP concentration at various concentrations as shown in Table C-1.

Table C-1: Standard insulin preparation for indirect competitive ELISA

Insulin standard concentration (μg.mL^{-1})	**Stock concentration, (μg. μL^{-1})**	**Use from stock (μL)**	**MMH medium (μL)**
10.00	0.1	100	900
7.50	0.1	75	925
5.00	0.1	50	950
4.00	0.1	40	960
3.00	0.1	30	970
2.00	0.1	20	980
1.00	0.1	10	990
0.80	0.01	80	920
0.60	0.01	60	940
0.40	0.01	40	960
0.20	0.01	20	980
0.10	0.01	10	990
0.075	0.001	75	925
0.050	0.001	50	950
0.025	0.001	25	975
0.000	0	0	1000

2. Standard graph of standard insulin from bovine pancreas for quantitative determination by indirect competitive ELISA.

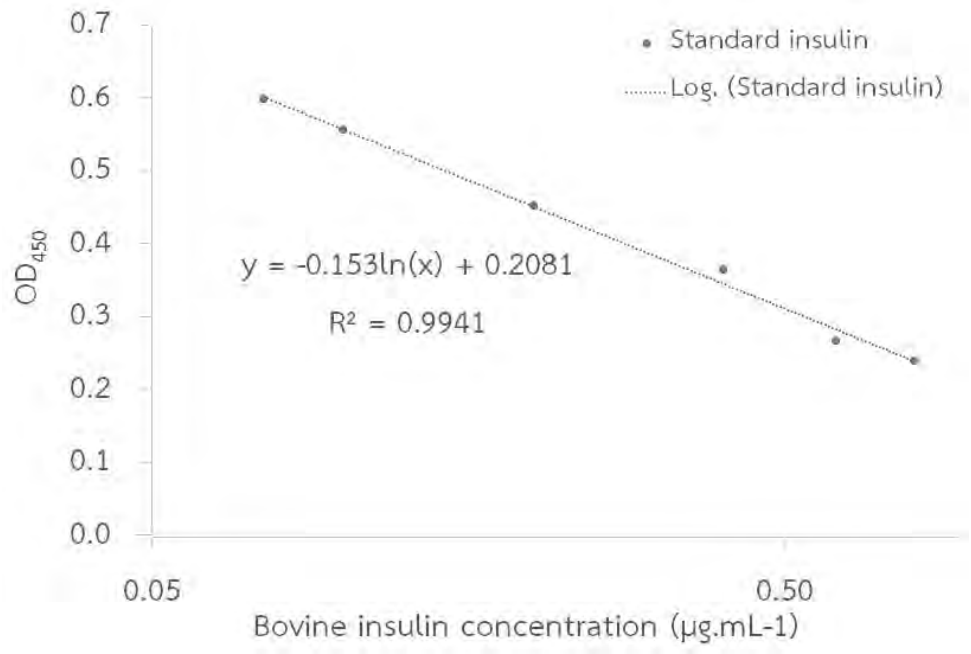

Figure C-1: Standard graph of standard bovine insulin for calculation of the MIP concentration by indirect competitive ELISA

Standard equation to calculate the MIP concentration;

$$Y = -0.153 \ln(X) + 0.2081$$

Description; Y = the optical density at 450 nm of samples (OD_{450})

X = the MIP concentration ($mg.L^{-1}$)

Calculation the MIP concentration from the standard equation above using Microsoft Excel 2010.