การเพิ่มการผลิตรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ใน *Pichia pastoris* สายพันธุ์ต่างๆ
โดยการเพิ่มจำนวนชุดของยีน

นายธนปติ ผาคำ

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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ENHANCEMENT OF RECOMBINANT MONOMERIC INSULIN PRODUCTION IN *Pichia pastoris* STRAINS BY INCREASING COPY NUMBER OF GENE

Mr. Tanapati Phakham

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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ธนปติ ผาคำ : การเพิ่มการผลิตรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ใน *Pichia pastoris* สายพันธุ์ต่างๆโดยการเพิ่มจำนวนชุดของยีน (ENHANCEMENT OF RECOMBINANT MONOMERIC INSULIN PRODUCTION IN *Pichia pastoris* STRAINS BY INCREASING COPY NUMBER OF GENE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ศรินทิพ สุกใส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.นันทิกา คงเจริญพร, 104 หน้า.

ในปัจจุบัน ระบบการแสดงออกของยีสต์ *Pichia pastoris* ถูกพัฒนาขึ้นและถูกนำมาใช้อย่างแพร่หลายในการผลิตรีคอมบิแนนต์โปรตีน ซึ่งในงานวิจัยนี้ *Pichia pastoris* 3 สายพันธุ์ (X-33, GS115 และ KM71H) และ *Hansenula polymorpha* (NRRL2214) ซึ่งเป็นเมธิลโลโธฟิกยีสต์ ใช้เป็นเซลล์เจ้าบ้าน ในการผลิตรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ (MIP) โดย รีคอมบิแนนต์พลาสมิด TP1, TP2 และ TP4 ที่มีจำนวนชุดของยีนอินซูลินแบบมอนอเมอร์เป็น 1, 2 และ 4 ชุด ถูกสร้างขึ้นในพลาสมิดพาหะ pPICZalphaA ซึ่งมีขนาดเป็น 3,709 คู่เบส, 5,501 คู่เบส และ 9,085 คู่เบส ตามลำดับ รีคอมบิแนนต์ยีสต์แต่ละสายพันธุ์ซึ่งมี TP1, TP2, TP4 แทรกอยู่ในจีโนมถูกเลี้ยงแบบสองขั้น; ขั้นแรก เลี้ยงในอาหารสมบูรณ์เพื่อเพิ่มจำนวนเซลล์ และในขั้นที่สอง เลี้ยงในอาหารจำกัดที่มีเมธานอลและฮีสติดีน เพื่อใช้เหนี่ยวนำการผลิต MIP โดยระดับการแสดงออกของ MIP ถูกตรวจติดตามด้วยวิธีที่ง่ายและมีความจำเพาะด้วยเทคนิค dot-blotting analysis ขณะที่เทคนิค indirect competitive ELISA ถูกใช้ในการวัดปริมาณความเข้มข้นของ MIP จากผลการตรวจติดตามด้วยเทคนิค dot-blotting analysis ระดับการแสดงออกของ MIP จากรีคอมบิแนนต์ยีสต์ *P. pastoris* สายพันธุ์ KM71H (Mut^S phenotype) สามารถตรวจวัดได้ตั้งแต่ 24 ชั่วโมงของการเพาะเลี้ยงในขั้นที่สอง ในขณะที่การแสดงออกของ MIP จากรีคอมบิแนนต์ยีสต์สายพันธุ์อื่นๆ ตรวจวัดได้ที่ 48 และ 72 ชั่วโมง ผลการทดลองในส่วนของการวัดความเข้มข้นของ MIP ด้วยเทคนิค indirect competitive ELISA แสดงให้เห็นว่า ปริมาณ MIP เพิ่มขึ้นอย่างต่อเนื่องตามระยะเวลาที่ใช้ในการเพาะเลี้ยง สำหรับการเปรียบเทียบระดับการแสดงออกของ MIP ในรีคอมบิแนนต์ยีสต์แต่ละสายพันธุ์ โดยพิจารณาที่รีคอมบิแนนต์ยีสต์ที่มีจำนวนชุดของยีนอินซูลินแบบมอนอเมอร์ 1 ชุด ที่เวลา 72 ชั่วโมง พบว่า *P. pastoris* สายพันธุ์ KM71H 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 $mg.L^{-1}$) สำหรับผลของจำนวนชุดยีนที่มีต่อการแสดงออกของ MIP ในยีสต์แต่ละสายพันธุ์ พบว่า รีคอมบิแนนต์ยีสต์แต่ละสายพันธุ์ที่มีจำนวนชุดยีนที่แตกต่างกัน มีระดับการแสดงออกของ MIP ที่แตกต่างกันออกไป

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต ....

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TANAPATI PHAKHAM: ENHANCEMENT OF RECOMBINANT MONOMERIC INSULIN PRODUCTION IN *Pichia pastoris* STRAINS BY INCREASING COPY NUMBER OF GENE. ADVISOR: SARINTIP SOOKSAI, Ph.D., CO-ADVISOR: NANTHIKA KHONGCHAREONPORN, Ph.D., 104 pp.

Currently, *Pichia pastoris* expression system has been developed and widely used for recombinant proteins production. In this research, *P. pastoris* 3 strains (X-33, GS115 and KM71H) and *Hansenula polymorpha* (NRRL2214), which are methylotrophic yeasts, 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, were successfully constructed in pPICZalphaA expression vector with the size of 3,709 bp, 5,501 bp and 9,085 bp, respectively. 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 recombinants *P. pastoris* KM71H (Mut^S phenotype) 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. Comparison of the MIP expression between yeast strains which harbored 1 copy of MIP gene in culture at 72 hours, *P. pastoris* KM71H 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.

Field of Study: Biotechnology	Student's Signature ....
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LIST OF ABBRIVATIONS

%	Percentage
°C	Degree Celsius
μg	Microgram(s)
μL	Microliter(s)
$\mu g.mL^{-1}$	Microgram per milliliter
$\mu g.\mu L^{-1}$	Microgram per microliter
×g	Multiply by gravitational force ($\times 9.80665\ m.s^{2}$)
AOX1	Alcohol oxidase 1 gene
$AOX1_P$	Alcohol oxidase 1 promoter
AOX2	Alcohol oxidase 2 gene
$AOX2_P$	Alcohol oxidase 2 promoter
ATP	Adenosine triphosphate
DF	Dilution factor
e.g.	Example (exempli gratia, Latin)
g	Gram(s), (Unit of mass)
h	Hour(s)
His^-	Histidine auxotroph
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
kb	Kilobase pair(s)
$lb.in^{-2}$	Pound per square inch
M	Molar
mg	Milligram(s)
MIP	Monomeric insulin precursor
mL	Milliliter(s)
MMH	Minimal methanol histidine

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Mut^+	Methanol utilization plus phenotype
Mut^s	Methanol utilization slow phenotype
ng	Nanogram(s)
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNase	Ribonuclease A
rpm	Revolution per minute
T	Temperature
TAE	Tris-acetate-EDTA
Tm	Melting temperature (°C)
UV	Ultraviolet
w/	With
w/o	Without
w/v	Weight by volume
WT	Wild type strain
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPG	Yeast extract peptone glycerol

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CHAPTER I

INTRODUCTION

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) reported that in 2035 the DM patients in the world will be increasing to 592 million people, which account for 55% from 2013 **(2)**. In the future, it needs to improve the property of insulin action and recombinant insulin production to produce the effective recombinant insulin to treat DM patients. 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 is recombinant insulin, which is produced by recombinant DNA technology and biotechnology **(3)**. The recombinant DNA technology is a technique which 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. The recombinant human insulin production, 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. 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-yeast which used for recombinant proteins production. 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*, *P. methanolica*, *Candida boidinii*, 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 ($AOX1_p$) which can be controlled the target gene expression by using an induction medium contains methanol. There are many factors which can affect to recombinant proteins 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^+, WT), *P. papstoris* KM71H (Mut^S) and *H. polymorpha* strain (NRRL2214, WT) were used as hosts to study the effect of yeast strains and copy number of gene on the

heterologous protein expression. The monomeric insulin precursor (MIP) gene was inserted into the pPICZαA expression vector. 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).

CHAPTER II
LITERATURE REVIEWS

2.1 Diabetes Mellitus

2.1.1 Definition and Description of Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, which is a high blood glucose (sugar) level, resulting in the imperfection of insulin secretion (lack of insulin) or the action of insulin (insulin resistance), or both. The chronic hyperglycemia affects many organs e.g. eyes, kidneys, nerves, heart and blood vessels. The cause of hyperglycemia may come from the abnormalities of metabolisms of carbohydrate, fat, and protein and defective of insulin action (insulin resistance) or insulin production (immune-mediated diabetes). The deficiency of insulin action results from insufficient of insulin secretion and/or decreasing of tissue in response to the insulin in complex pathways of hormone action. Symptoms of hyperglycemia include of polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision **(1)**.

2.1.2 Classification of Diabetes Mellitus

Diabetes mellitus can be divided to two groups. The first one is type 1 diabetes caused by insulin secretion deficiency which was immune-mediated diabetes mellitus. The second one is type 2 diabetes resulting in defective of insulin action (resistance to insulin) and leading to the hyperglycemia symptom.

2.1.2.1 Type 1 Diabetes: Insulin dependent diabetes mellitus (IDDM) or immune-mediated diabetes

Type 1 diabetes (insulin dependent diabetes mellitus or juvenile onset diabetes) is a chronic disease, which accounts for 5 – 10% of diabetic patients, results from autoimmune disruption of the β-cells in the pancreas. In this type, almost patients are children and adolescents. The disruption of the β-cells by autoimmune

occurs in children and adolescents more quickly than adult. Type I diabetic patients need to receive insulin to control blood glucose level and life survival (Figure 2.1).

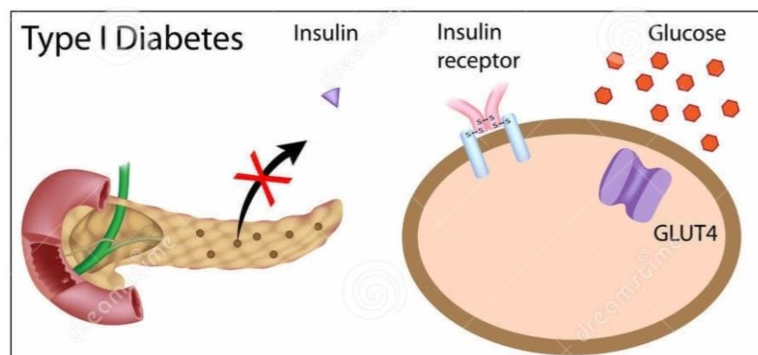

Figure 2.1: Type 1 diabetes; insulin dependent diabetes **(17)**

2.1.2.2 Type 2 Diabetes: Non-insulin dependent diabetes mellitus (NIDDM) or insulin resistance

Type 2 diabetes (non–insulin dependent diabetes mellitus or adult onset diabetes), which accounts for ~90–95% of diabetic patients, results from the defective of insulin action in response to glucose uptake mechanism in the cells. There are probably many different causes of this type e.g. family history, age, lack of physical activity or lifestyle behaviour, high blood levels of fats and obese, overweight. Initially, type II diabetic patients do not need to receive insulin to control blood glucose level. In the event that cells have not response to native insulin (insulin resistance), they need to take insulin in order to control blood glucose level to survive. Every one of the patients in this type has both insulin resistance and an inability to produce more insulin in response to glucose uptake mechanism (Figure 2.2).

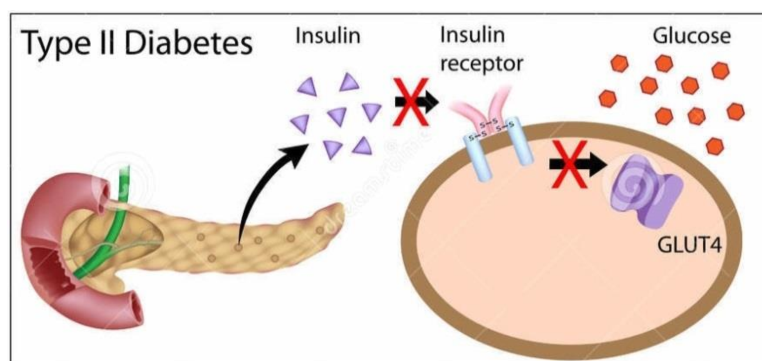

Figure 2.2: Type 2 diabetes; non-insulin dependent diabetes **(17)**

2.1.3 Trend of Diabetes Mellitus

In 2013, the international diabetes federation (IDF) reported that a number of diabetic patients in the world are about 382 million people (Figure 2.3). It will be increasing to 592 million people in 2035 which increasing account for 55% from 2013 as shown in Figure 2.4; therefore, it is necessary for improving and developing of the insulin production for supporting an increasing demand of insulin **(2)**.

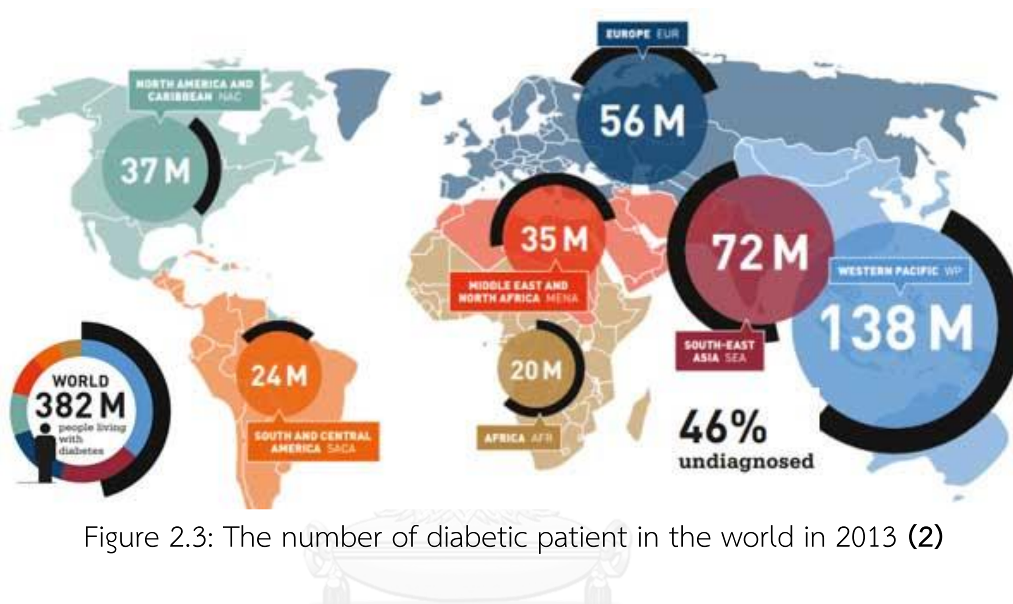

Figure 2.3: The number of diabetic patient in the world in 2013 **(2)**

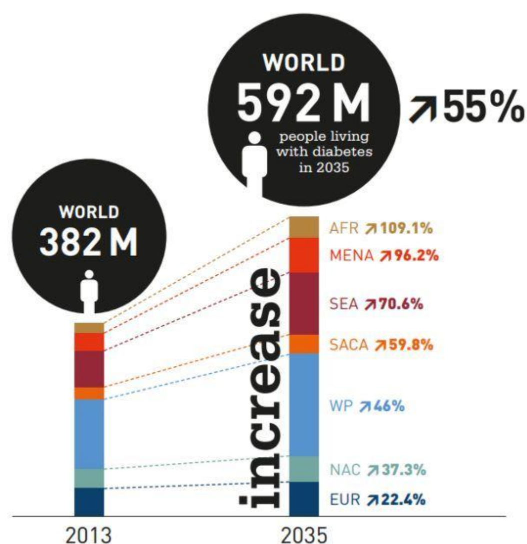

Figure 2.4: The estimation of diabetic patient in the world in 2035 **(2)**

2.2 Insulin

Insulin is a peptide hormone secreted by the beta cells of the islets of Langerhans in the pancreas, which 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 glucose storage in the liver **(18)**.

2.2.1 History of Insulin

In 1869, Paul Langerhans who is a medical student in Berlin found that within the pancreatic tissue has a cluster of cells which produced digestive juices. Later, this cluster of cells was named the islets of Langerhans for an honor of the person who discovered.

In 1889, in Germany, Oskar Minkowski and Joseph von Mering studied the effect on digestion in pancreas-removed dogs. They found that the dog became diabetes, so they suggested that the pancreas must have at least two functions, production digestive juices and production some substance which involved in blood glucose control.

In 1921, Dr. Frederick Banting, who is an orthopedic surgeon, and Charles Best, who is a medical student, began a research and advised by Prof. Dr. John Macleod whose support the dogs, equipment and laboratory for the experiment of Banting and Best. The dogs, which pancreas was removed, showed high blood glucose level, thirsty, drank lots of water, urinated more often and it have developed to diabetes. On the other hand, they extracted some substance in the pancreas and named "isletin". When they injected isletin into the diabetes dog, results showed that the diabetes dog became healthier and stronger, so this was the first evidence to support their hypothesis. Later, they successfully developed the method to extract and purify this substance from the pancreas. Finally, in 1923, the Nobel committee of the Caroline institute awarded the Nobel Prize to Banting and Macleod in physiology or medicine **(19)**.

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Until 1980, insulin was produced by the extraction from animal pancreas, animal insulin, usually pigs (porcine or pork insulin) and cows (bovine or beef insulin). It was the only treatment for insulin dependent diabetes or type I diabetic patients.

In 1982, Eli Lilly and company played a significant role by perfecting in the large-scale manufacture of the human insulin production by recombinant DNA technology.

2.2.2 Structure of Insulin

The mature insulin molecule is compose of two polypeptide chains, A chain and B chain, as show in Figure 2.5. The A chain usually contains 21 amino acids, and the B chain usually contains 30 amino acids. The polypeptide chains are linked-together, inter-chain, by two disulfide bonds which are between Cys A7 and Cys B7, and between Cys A20 and Cys B19. It has an intra-chain disulfide bond between Cys A6 and Cys A11 creating a loop in the A chain **(18)**.

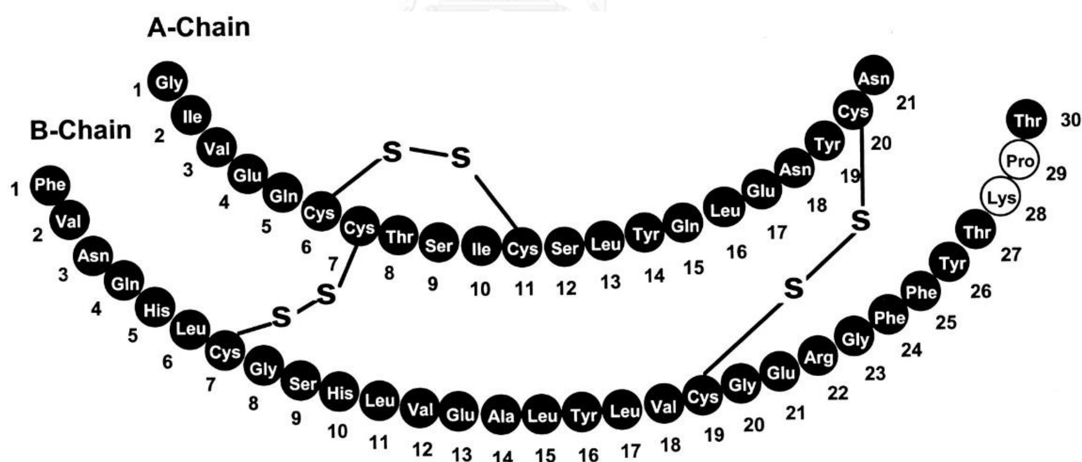

Figure 2.5: Primary structure of insulin lispro composes of 2 poly peptide chains **(20)**

2.2.3 The Biosynthesis of Insulin

Normally, insulin is synthesized as a single chain precursor, preproinsulin, of 110 amino acids by beta cells in the pancreas. The 24-residue hydrophobic N-terminal signal peptide is rapidly cleaved after transferring the nascent polypeptide through the membrane of the rough endoplasmic reticulum (rER). Therefore, the

proinsulin folds and the disulphide bonds are formed. Proinsulins are migrates through the Golgi complex and ends up package in crystalline form in clathrin-coated secretory granules. The 31 amino acids C-peptide which links A1 to B30 is then cleaved by two subtillisin - related endoproteases – convertase PC3, which cleaves preferentially at a basic dipeptide at the B/C junction, and convertase PC2, which cleaves selectively at a second basic dipeptide at the C/A junction. The insulin crystal, which presents in the secretory granules, is made of hexamer, a symmetric assembly of three dimers arranged around two atoms of zinc. After the insulin secretion by exocytosis from beta cells, insulin at the low concentration presenting in blood is rapidly dissolved into monomers, which are the biologically active from of the molecule **(18)**. The diagram of human insulin synthesis is showed in Figure 2.6.

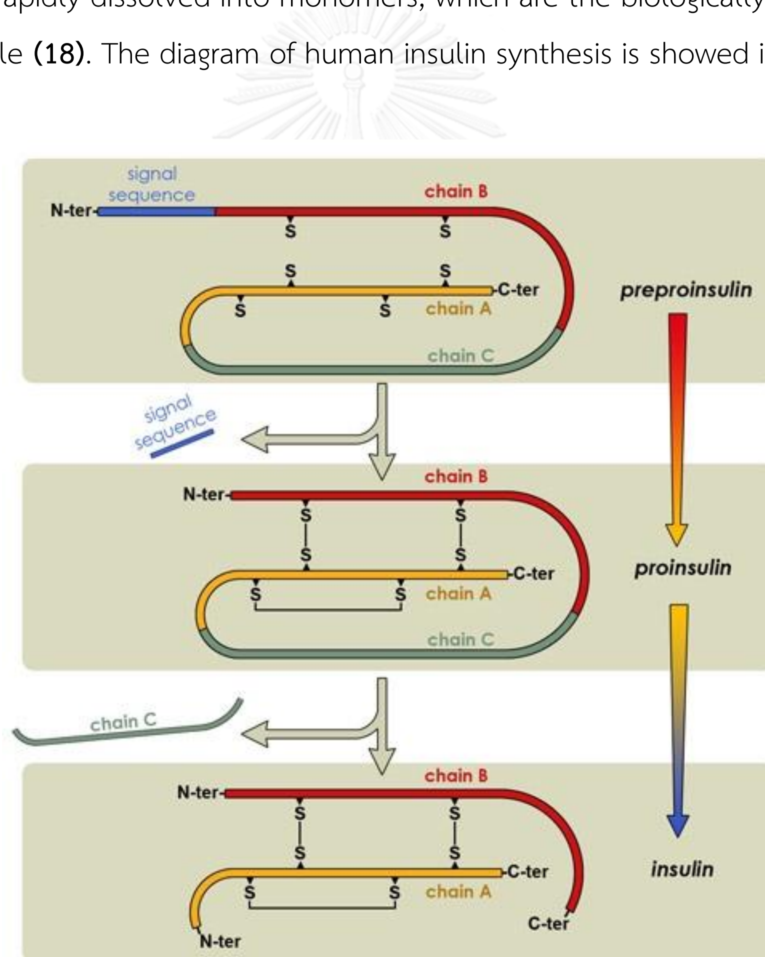

Figure 2.6: The biosynthesis of insulin **(21)**

2.2.4 Insulin Analogues

Insulin analogues have been developed for suitable usage to control blood glucose level in diabetic patients. Nowadays, insulin analogues can be divided to 3 groups which are considered in the acting type; rapid acting, pre-mixed and long acting **(22)**.

2.2.4.1 Rapid Acting Insulin Analogues

The insulin molecule at the amino acid of B26 – B30 was showed no critical sites for binging to insulin receptor. These amino acids, B26 – B30, are important part forming insulin dimer or insulin hexamer; so it is interesting to modify some amino acid in B chain to an active insulin monomeric form for faster acting of insulin.

2.2.4.1.1 Insulin Lispro

Insulin lispro is the first rapid acting insulin analog which was used in the USA and EU since 1996. Insulin lispro was modified of some amino acid in B chain of insulin molecule as shown in Figure 2.7.

2.2.4.1.2 Insulin Aspart

Insulin aspart is the second rapid acting insulin analog which was approved to use for treatment of diabetic patients since 2000. At the B28, proline, was substituted with aspartic acid as shown in Figure 2.7.

2.2.4.1.3 Insulin Gluisine

Insulin gluisine is the third rapid acting insulin analog which B3, asparagine, and B29, glycine, were substituted with lysine and glutamic acid, respectively as show in Figure 2.7.

2.2.4.2 Pre-mixed Insulin Analogues

Biphasic insulin analogues have been developed for more desirable pharmacological properties than conventional biphasic insulin mixtures. It can be used for control blood glucose level by injection either before or after of a meal. It was modified for suitable used for control blood glucose level in both type 1 and type 2 diabetic patients.

2.2.4.2.1 Biphasic insulin aspart (30% insulin aspart and 70% insulin aspart protamine)

2.2.4.2.2 Biphasic insulin lispo (25% lispro and 75% lispro protamine)

2.2.4.3 Long Acting Insulin Analogues

Long acting insulin analogues have been developed for long duration of insulin action to control blood glucose level. The protein engineering was used for development the physical properties of insulin.

2.2.5.3.1 Insulin Glargine

Insulin glargine was developed by changing the isoelectric point of insulin molecule resulting in reduce solubility of insulin. The A21, asparagine, was substituted with glycine and B30 was conjugated with 2 molecules of arginine as showed in Figure 2.7. The modification of insulin molecule results the isoelectric point of native insulin shifts from 5.4 to 6.7.

2.2.5.3.2 Insulin Determir

Insulin determir was developed and modified for increasing a self-association of insulin molecule. At the B30, threonine, was removed and B29, lysine, has been acylated with myristic fatty acid as shown in Figure 2.7.

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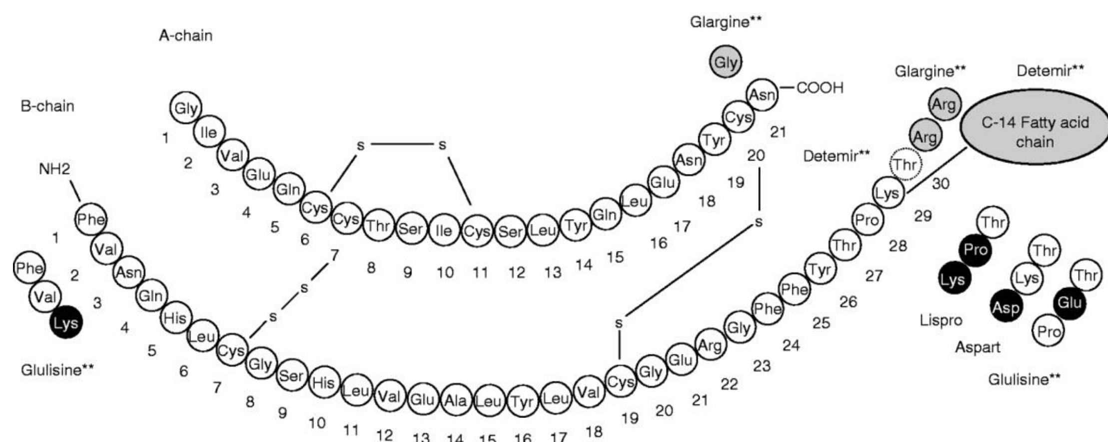

Figure 2.7: Amino acid sequences of insulin analogues **(23)**

2.3 Improvement of Insulin Production

In a few past decades, many researchers have been improved and developed the human insulin analogs for superior properties, rapid-acting, monomeric property which was less-self association to dimer or hexamer **(5, 6)**. Nowadays, many recombinant insulin analogs have been developed for suitable usage to treat both types of diabetic patients. Many researchers have been reported the factors which were important to human insulin production such as fermentation strategies, host strains, gene dosage, promoter(s), gene(s) or signaling peptide(s), which involved in the mechanism of recombinant protein secretion and/or recombinant protein production **(9, 10, 16, 24-28)**.

2.3.1. Recombinant DNA Technology

The recombinant DNA technology is a technique which used for modifies organism by combining the genetic material or gene(s) of interest from one or more species to another living organism (host). It was used in order to produce useful protein(s) or useful biomolecules of interest from host organisms. 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 which produced by using a recombinant DNA technology are called “recombinant proteins”. The organisms, which used for recombinant proteins or recombinant useful compounds production, include microorganisms; bacteria, cyanobacteria, yeast, fungus, microalgae, animal or insect cell lines, or systematic organism; plants or animals. The first recombinant protein was recombinant human insulin which produced by *Escherichia coli*, It was the first organism which used for recombinant human insulin production in 1982 by Eli Lilly **(29)**.

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2.3.1.1. Recombinant Insulin Production by Recombinant DNA Technology

The procedures of the recombinant human insulin production are showed in Figure 2.8; (A) human insulin gene was synthesized as a nucleotide sequence (DNA), (B) the human insulin gene (DNA) was ligated with plasmid DNA by *in vitro* method to generate a recombinant plasmid, (C) a recombinant plasmid was transformed into the host organism, bacteria, which called recombinant bacteria, (D) a recombinant bacteria which harbored human insulin gene was cultivated, (E) a recombinant human insulin was produced and accumulated in the bacterial cells and (F) a recombinant human insulin was extracted from bacterial cells, then purified and packed out for using as a medicine.

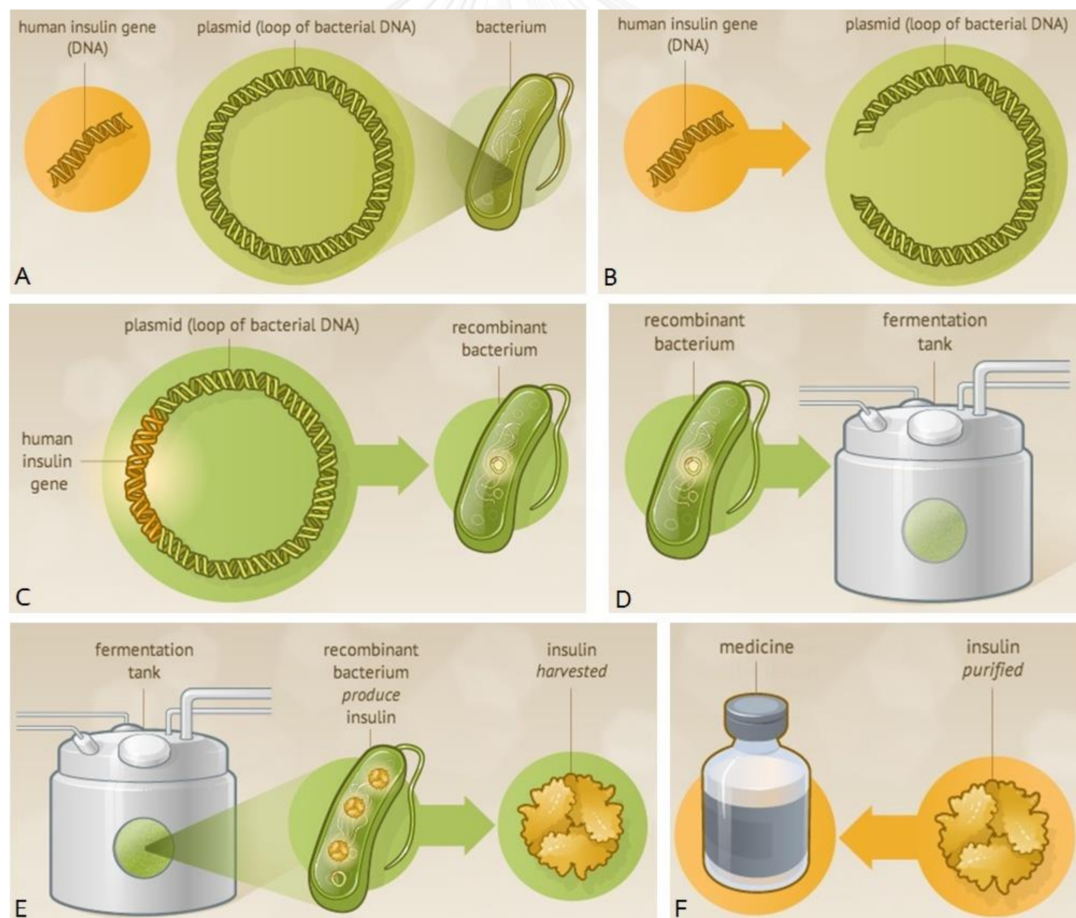

Figure 2.8: The procedure of human insulin production using recombinant DNA technology **(30)**

To enhance the recombinant insulin production, the fermentation technology has been used and developed for increasing the yield of the recombinant insulin.

2.3.1.2 Increasing Insulin Gene Copy Number by Recombinant DNA Technology

Some researchers intend to construct the expression vector which contains multiple gene copy number using recombinant DNA technology. For insulin, many researches successfully constructed multiple expression cassette of insulin gene to study its expression level in both of bacterial and yeasts. Since 2000s, human insulin gene was used as a model to study the effect of gene copy number on the expression level. In the past decade, yeast expression system has been widely used for recombinant proteins production including of human insulin. Researchers studied the effect of gene dosage on the expression level by increasing insulin gene copy number in an expression vector (both *in vitro* and *in vivo* methods) and cloned into the host.

In 2000, Wang *et al*., generated the multi-copy strains (*in vivo* method), which harbored 6 – 8 copy number of the porcine insulin precursor (PIP), in *Saccharomyces cerevisiae*. The gene copy number was screened and determined by dot-blotting method using ^{32}P-labelled primers. The result showed that, using 5 L working volume in 16 L bioreactor, the PIP expression level of multi-copy strain was 1.5 $g.L^{-1}$ at the OD_{600} reached to 500 **(28)**. Five years later, Mansur *et al*., constructed the expression plasmids, which have 1, 4 and 6 classette(s) of miniproinsulin (MPI) gene (*in vitro* method), and cloned these plasmids into *Pichia pastoris*. The MPI gene copy number in *Pichia* recombinant clones were screened by PCR analysis and Southern blot using ^{32}P-labelled MPI primers. Result showed that, using 1.5 L working volume in 2.5 L bioreactor, the multi-copy strains, which harbored 1, 5 and 11 of the MPI gene copy number, have the MPI expression level at 18.6, 120 and 246 $mg.L^{-1}$, respectively **(24)**. In 2009, Zhu *et al.*, studied the efficient generation of multi-copy strain for porcine insulin precursor (PIP) expression in *P. pastoris*. They constructed the expression vectors containing 1, 2, 3, 4, 5 and 6 cassettes(s) of the PIP gene (*in vitro* method). Then, the expression vectors were transformed into the yeast *P. pastoris* to generate

the high-copy strains (*in vivo* method). The results showed that, high-copy strains, which determined by real-time qPCR, harboring 1, 3, 6, 12, 18, 29 and 52 copy of PIP gene were successfully generated and used for the PIP expression by shake-flask culture. The highest PIP expression level of these high-copy strains was 181 $mg.L^{-1}$, which obtained from the strain harboring 12 copies. So they indicated that, it has some limitations in heterologous protein expression of high-copy strains **(16)**.

Moreover, the effect of gene dosage on the expression level of the other genes was also studied. In 2003, Hohenblum and co-workers studied the effect of trypsinogen gene dosage and promoters on the trypsinogen expression level in *P. pastoris*. They constructed the multimers of expression cassettes (*in vitro* method), which have 1, 2 and 3 copy of the expression cassettes, and transformed into the yeast. The recombinant yeasts were screened and determined the gene dosage by quantitative Southern blot analysis using anti-DIG-Fab alkaline phosphatase conjugated, which can be visualized by signal intensity of chemiluminescent. The results showed that; the gene copy number of recombinant clones, which $AOX1_P$ was used as promoter, have effect on the expression level but in clones, which GAP_P was used as a promoter, It have no effect of gene copy number (1 – 3 copy) on the expression level **(31)**. In 2011, an increasing gene dosage of aquaporins was observed in *P. pastoris*. The expression vectors were constructed with different aquaporin isoforms and cloned into *P. pastoris* to screen the transformants which harbored multi copy number of expression vector by increasing Zeocin™ concentration. The copy number of aquaporins, which integrated into the yeasts, was quantitative determined by qPCR. The results showed that, thansformants, which can growth at higher Zeocin™ concentration (1,000 $\mu g.mL^{-1}$: harbored 17 copy of the expression cassettes), have higher aquaporins expression level than the other transformants, which can growth at lower Zeocin™ concentration (100 $\mu g.mL^{-1}$: harbored 1 – 5 copy of the expression cassettes). The higher aquaporins expression level depends on the number of integrated expression vectors which harbored in *Pichia* genome. Moreover they concluded that, increasing gene dosage could help improving poor expression caused by inefficiency at the transcriptional and translational levels **(32)**.

To improve the expression level of recombinant protein production using recombinant DNA technology, it is necessary to consider other factors which can effect on the heterologous protein production such as efficient transcription by using strong promoters, translation signals, translocation determined by the secretion signal peptide, processing and folding in the endoplasmic reticulum (ER) and Golgi secretion out of the cell **(31)**.

2.3.2 Modification of Human Insulin by Protein Engineering

Since 1920s, insulin was named by Prof. McLeod and Frederick Banting who derived the Nobel Prize in physiology or medicine. Insulin was discovered and its properties has been widely studied until now **(19, 33)**. Protein engineering can be used in order to improve the insulin property. Brems *et al.*, reported that a removal of B28-B30 from insulin resulted in much less self-association which relate to the monomeric property of insulin **(6, 25)**. Cui *et al.*, reported that destetrapeptide insulin (DTI; B27-B30 were removed) presented a monomeric property **(25)**. In 2002, Ding *et al.*, reported that semi synthesis of B27 Lys destripeptide insulin (B27 Lys DTrI), deshexapeptide insulin (DHI; B25-B30 were removed), despentapeptide insulin (DPI; B25-B30 were removed) showed a monomeric property. Moreover, in 2005 they reported that monomeric B27 Lys destripeptide insulin precursor (B27Lys DTrI; B28-B30 were removed and B27, threonine, was replaced by lysine) or MIP showed a superior monomeric property in size-exclusion chromatography and it was developed for tryptic hydrolysis, which converted insulin precursor to active insulin instead of tryptic transpeptidation **(5)**.

2.3.3 Expression System for Recombinant Insulin Production

Before 1980, insulin was produced by the extraction and purification from animal pancreas; cow or pig pancreas extracts which called bovine insulin or porcine insulin **(33)**. However, the production of insulin was first developed by using a recombinant DNA technology and successful since 1986 **(34)**. Initially, recombinant insulin was produced by using *E. coli* in 1982 by Eli Lilly **(29)**. In case of recombinant

insulin, it has been developed for higher expression level. The regular systems, which used for heterologous proteins production, are both prokaryotic and eukaryotic expression systems.

In prokaryotes, the transcription and translation processes occur simultaneously. The translation of mRNA starts even before a mature mRNA transcript is fully synthesized. This simultaneous transcription and translation of a gene is termed coupled transcription and translation. In eukaryotes, the processes are spatially separated and occur sequentially with transcription happening in the nucleus and translation, or protein synthesis, occurring in the cytoplasm. Although the process of transcription in both prokaryotes and eukaryotes are similar: initiation, elongation and termination. In prokaryotes, no special modification of mRNA is required and translation of the message starts even before the transcription is complete. In eukaryotes, mRNA is further processed to remove introns (splicing), addition of a cap at the 5´ end and multiple adenines at the mRNA 3´ end to generate a polyA tail. The modified mRNA is then exported to the cytoplasm where it is translated. The transcription and translation diagram of both prokaryote and eukaryote is indicated in Figure 2.9.

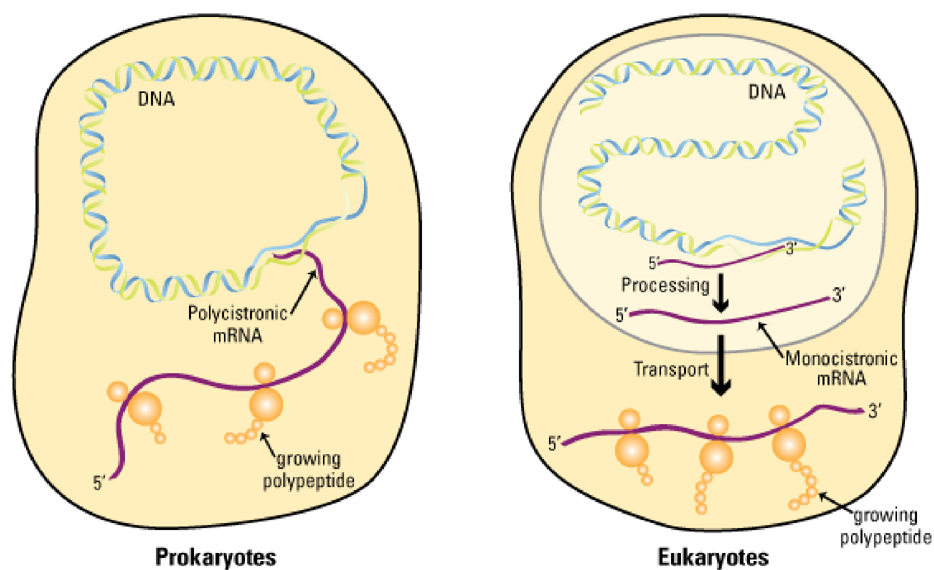

Figure 2.9: The comparison of transcription and translation in prokaryotes and eukaryotes **(35)**

2.3.3.1 Prokaryotic Expression System

For the recombinant insulin production, since the recombinant DNA technology was found, each of A and B chains of human insulin gene was separately synthesized and cloned into the pBR322 expression vector. Two DNA fragments of insulin gene were fused with beta-galactosidase gene in *E. coli* to provide efficient transcription and translation and a stable precursor protein production. However, it has a limitation of insulin folding after expression by using this system **(36)**. Moreover, the heterologous proteins which produced by *E. coli* were accumulated in the cells, intracellular protein, so it needs to be extracted and separated from the cells. In prokaryote expression system, it has no post-modification process to produce complex proteins.

2.3.3.2 Eukaryotic Expression System

To produce the recombinant insulin, the eukaryotic expression system was used for increase and enhances the recombinant insulin production. Recently, yeast expression system has been widely used for many heterologous proteins production thanks to it has many advantages and similarity in the complex posttranslational modification process of protein(s) such as correct glycosylation, protein folding (disulfide bond formation) and protein secretion **(8-10, 26, 37)**.

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2.3.3.2.1. Yeast Expression System

Yeast expression system has been developed for many heterologous proteins production. This system has more advantages than prokaryotic expression system such as rapid growth, able to growth in high cell density, genetic stability and scale-up without loss of yield, low costs of media, elimination of endotoxin and bacteriophage contamination, high productivity and ability to engineer the secretion pathway **(8-10, 38)**.

2.3.3.2.1.1. Methylotrophic Yeasts

Methylotrophic yeast is a group of yeast which can utilize methanol as a sole carbon source. Many types of yeast have been developed to enhance the heterologous proteins production. It was studied the mechanisms which involve in

protein expression. A useful yeast strains which popular used in this group including *Pichia pastoris, Hansenula polymorphas, Candida boidinii* **(8-10, 39)**. Methylotrophic yeasts have a unique methanol utilization pathway or MUT pathway (Figure 2.10). 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 ($AOX1_P$) and methanol oxidase (MOX_P). Moreover, in some case, they used a constitutive promoter to express gene of interest such as glyceraldehyde-3-phosphate dehydrogenase (*GAP*) **(8, 9)**.

In the methanol utilization pathway, methanol was oxidized by alcohol oxidase (*AOX*: encoded from alcohol oxidase 1 (*AOX1*) and alcohol oxidase 2 (*AOX2*) in *Pichia*, and methanol oxidase (*MOX*) in *Hansenula*) to formaldehyde (HCOH) and hydrogen peroxide (H_2O_2), which is toxic to the cells. Hydrogen peroxide is immediately utilized by catalase (*CAT*) and converted to H_2O and O_2 in peroxisome to avoid the hydrogen peroxide toxicity to the cells. Formaldehyde will utilized to cell growth by formaldehyde dehydrogenase and the final product is CO_2 as show in Figure 2.10 **(39, 40)**.

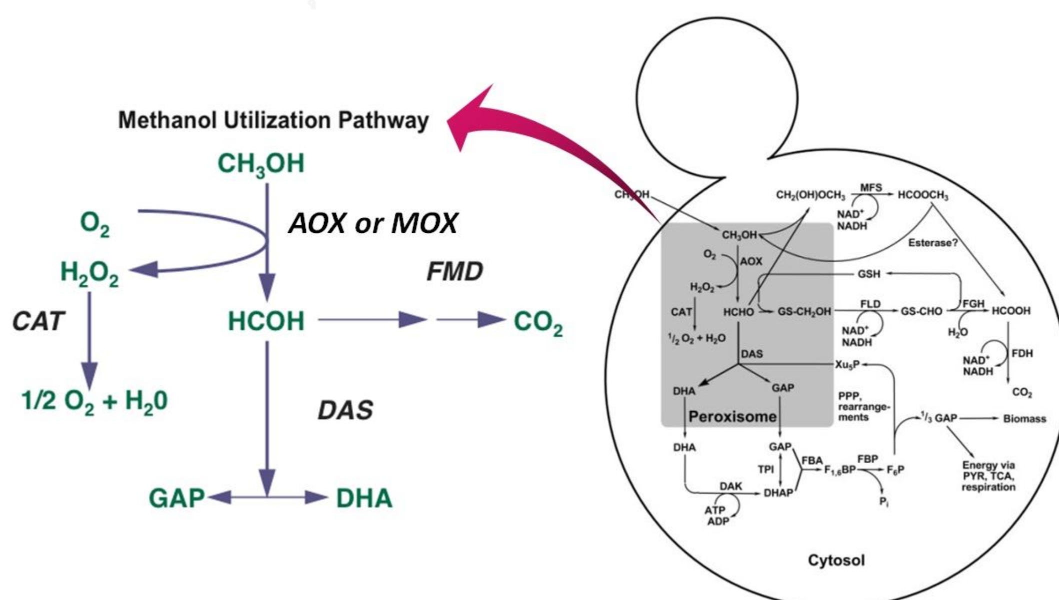

Figure 2.10: The methanol utilization pathway in methylotrophic yeasts **(39, 41)**

AOX: alcohol oxidase, CAT: catalase, FLD: formaldehyde dehydrogenase, FGH: S-formylglutathione hydrolase, FDH: formate dehydrogenase, DAS: dihydroxyacetone synthase, TPI: triosephosphate isomerase, DAK: dihydroxyacetone kinase, FBA: fructose 1,6-bisphosphate aldolase, FBP: fructose 1,6-bisphosphatase, MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, F1,6BP: fructose 1,6-bisphosphate, F6P: fructose 6-phosphate, Pi: phosphate, Xu5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle.

Yeasts in this group are successfully improved and widely used for heterologous gene expression for two past decades **(10)**. The popular *Pichia pastoris* and *Hansenula polymorpha* were used as a host. It have many advantages of higher eukaryotic expression system such as rapid growth on inexpensive medium, ability to complex posttranslational modification, ability to engineer secreted proteins, strongly inducible promoter and high levels of productivity in an almost protein-free medium **(8-10, 15)**.

2.3.4.3.1.1.1 Pichia pastoris and Hansenula polymorpha

P. pastoris and *H. polymorpha* are closely methylotrophic yeast. It has a same common methanol utilization pathway but *H. polymorpha* has nitrate assimilation pathway which is not present in *P. pastoris* **(9)**. The taxonomy of the yeasts are show in Figure 2.11 **(10)**.

- Kingdom: Fungi
  - Division: Eumycota
    - Subdivision: Ascomycotina
      - Class: Hemoascomycetes
        - Order: Endomycetales
          - Family: Saccharomycetaceae
            - Sub-family: Saccharomycetoideae
              - Genus: *Pichia*
                - *Pichia pastoris*
              - Genus: *Hansenula*
                - *Hansenula polymorpha*

Figure 2.11: The taxonomy of methylotrophic yeasts *P. pastoris* and *H. polymorpha*

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- *Pichia pastoris*

P. pastoris is a member of methylotrophic yeasts which can use methanol as a sole carbon source. It has a very strongly inducible promoters; $AOX1_P$ and $AOX2_P$ promoters which induced when growth in methanol-containing media. Many heterologous proteins were expressed under the control of these promoters in *P. pastoris* or other yeasts. *P. pastoris* can be cultured in high cell density condition and cell dry weight can be reached up to 200 $g.L^{-1}$ **(9)**.

The classification of *Pichia* strains when considering in methanol utilization phenotype, Mut phenotype, can be classified into three groups; Mut plus, Mut slow and Mut minus. The first one is Mut plus phenotype; X-33 and GS115. The X-33 is a wild-type strain which has both of alcohol oxidase 1 and alcohol oxidase 2 resulting in methanol utilization plus (Mut^+ strain, WT). The GS115 is a histidine auxotroph strain (His^-) which histidine dehydrogenase gene was disrupted, so it needs a supplement of histidine to grow. It exists both of alcohol oxidase 1 and alcohol oxidase 2 resulting in methanol utilization plus (Mut^+ strain, His^-). The second one is Mut slow phenotype; KM71H. The argininosuccinate lyase gene (*ARG4*) was inserted and replaced in some part of *AOX1* gene in a wild type strain (*arg4 aox1::ARG4*), so the *AOX1* gene was inactive resulting in methanol utilization slow (Mut^S strain, Arg^+). The third one is Mut minus phenotype, which both *AOX1* and *AOX2* gene were disrupted resulting in methanol utilization minus phenotype, so it cannot grow in methanol-containing media **(9, 40)**.

Nowadays, *Pichia* expression system is already accepted as an important biotechnological host organism. It is widely used for many biomolecules production such as vaccine, coagulation inhibitors, fibrinolytic compounds, antibodies, hormones, and cytokines **(8)**.

- *Hansenula polymorpha*

H. polymprpha (*Pichia angusta*) is very closely to *P. pastoris*. It has been used for many heterologous proteins production such as hepatitis B vaccine, interferon alpha-2a, hirudin, lipase even insulin **(9)**. *H. polymorpha* has many promoters which involve in methanol utilization pathway. The methanol oxidase promoter (MOX_P) and

formate dehydrogenase promoter (*FMD_P*) are commonly used **(39)**. *H. polymorpha* can be cultivated in high cell density condition and it has effective to produce macromolecules of heterologous protein up to 150 kDa. It has high productivity (13.5 $g.L^{-1}$) of phytase production **(9)**. Moreover, *H. polymorpha* is thermotolerant yeast (up to 49°C), so it may suitable for the production of recombinant thermostable proteins such as thermotolerant enzymes.

2.4 Identification and Determination of Recombinant Insulin

To identify and determine the recombinant insulin in the culture broth, it was a several procedures and techniques which used for identification and quantitative determination the protein of interest. It has many conventional methods, which used for identify protein of interest, such as native polyacrylamide gel electrophoresis (native PAGE), SDS-PAGE, dot blotting, western blotting, high performance liquid chromatography (HPLC), mass spectroscopy (MS), HPLC-MS or ultraviolet absorption using spectrophotometry **(5, 16, 24-28, 37, 42, 43)**. Moreover, some techniques can be used for quantitative determination of the protein concentration such as HPLC, enzyme-linked immunosorbent assay (ELISA) and BCA protein content kit. However, it needs to select the suitable technique to analyse the protein of interest. It is the most important thing to consider the factors which convenient for further study such as time consuming, costs and protein nature; stability, purity and sensitivity.

Simple techniques which quick and easy to detect recombinant insulin are dot-blotting analysis and enzyme-linked immunosorbent assay (ELISA). These techniques are high specificity and high sensitivity which were used in this study for the recombinant MIP determination.

2.4.1 Dot-Blot Analysis

A specific dot-blotting technique is a simple technique. It is a high specific technique due to the specific binding between antigen and antibody to identify the protein(s) of interest from a large number of samples as call as immunodetection **(44)**. This technique can be used either qualitative determination for rapid screening

of a large number of samples or semi-quantitative. This technique was used for the detection of a target protein, which fixed on a protein binding membrane. Nitrocellulose membrane and polyvinylidene difluoride (PVDF) membrane are commonly used. A specific primary antibody was probed with a target protein on the membrane. Subsequently, a specific secondary antibody (anti-primary antibody) conjugated with an enzyme was added, and then incubated in a substrate solution to visualize the intensity of an immunoreactive. The principle scheme of dot-blotting is showed in Figure 2.12. The common enzymes, which used for conjugation with a secondary antibody, are horseradish peroxidase (HRP) and alkaline phosphatase (AP).

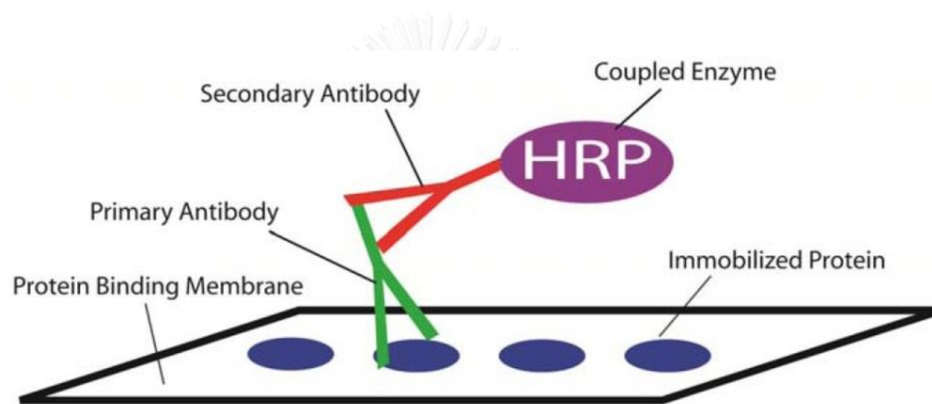

Figure 2.12: A specific dot blot scheme to detect the protein of interest **(44)**

The immunoreactive intensity which visualized on a membrane is related to the target protein concentration. This technique has important steps such as fixing step, which target protein was fixed on the protein binding membrane, and blocking step, the membrane was blocked with blocking buffer for non-specific interactions between antibody and protein binding membrane. However, this technique may has some limitations which need to be considered such as (i) some small biomolecules cannot bind to the membrane, (ii) low protein content may not be detected, (iii) the optimum dilution ratios of a specific antibody.

2.4.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay technique has been widely used for determination of many proteins or biomolecules. This technique has a high specificity and high sensitivity **(45)**. ELISA system can be designed for a measurement either antigen or antibody of interest form a specific biding reaction **(46)**. The working principle of this technique is an enzyme-labelled immunoglobulin which can reacts with a large number of substrate molecules resulting in color. The color, which occurred, can be measured by a spectrophotometry at a specific wave length.

The ELISA can be divided to 3 groups; direct ELISA, indirect ELISA and sandwich ELISA. A common scheme of ELISA is showed in Figure 2.13.

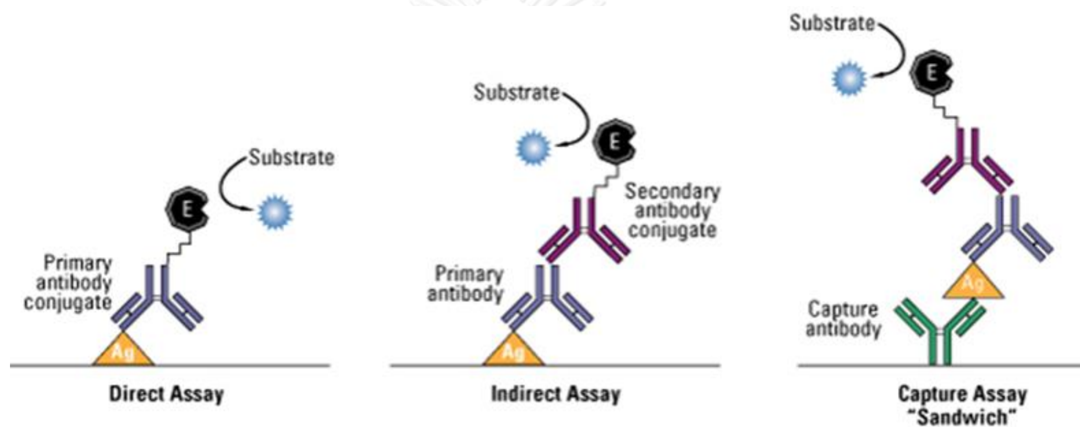

Figure 2.13: A common ELISA formats **(47)**

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The ELISA technique has been developed for qualitative and/or quantitative determination of biomolecules of interest. The indirect competitive ELISA was developed for quantitative determination of the recombinant MIPconcentration.

2.4.2.1. Direct Enzyme-Linked Immunosorbent Assay

This type of ELISA is a simplest which commonly use for the detection of biomolecules in liquid samples such as hepatitis A, B antigen and human rotavirus **(46)**. In a direct ELISA, an antigen was coated directly on a solid surface. A specific primary antibody is conjugated to a specific enzyme. The antigen and antibody are direct binding and the result can be visualized by adding a substrate solution The

direct ELISA has many advantages which are quick, no cross-reactivity of secondary antibody, required only single antibody **(46, 47)**.

2.4.2.2. Indirect Enzyme-Linked Immunosorbent Assay

This type of ELISA is widely used for the determination of biomolecules in a low concentration due to it has higher sensitivity than direct ELISA. A specific primary antibody (unlabeled-antibody), which binds with an antigen, can bind to a large number of specific secondary enzyme-labelled antibody molecules resulting in increasing the sensitivity of the indirect ELISA **(46, 48)**. Indirect ELISA has many advantages such as high sensitivity, it has a widely variety of enzyme-labelled secondary antibodies, it has different visualization markers which can be used with the same primary antibody, it has many primary antibodies which can used with the same secondary antibody **(47)**.

This technique has been developed for quantitative determination of biomolecules of interest including of the MIP (this study). The indirect ELISA may have some limitations e.g. some biomolecules in the sample mixture may interfere the binding reaction of the antibody and antigen, cross-reaction of an antibodies.

2.4.2.3. Sandwich Enzyme-Linked Immunosorbent Assay

This type of ELISA is a high specificity to determination the antigen of interest in a mixture solution. This technique has a main advantage, which the sample do not needs to purified from a mixture due to its used a purified antibody to capture a specific antigen, so, it is increases a specificity and sensitivity of this technique**(45)**.

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CHAPTER III

METHODOLOGY

3.1 Materials

3.1.1 Microorganisms

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

3.1.2 Nucleotide Sequence of MIP and Plasmids

Nucleotide sequence of the monomeric insulin precursor (MIP) gene (Figure 3.1), which was discussed by Ding *et al.,* **(5)**, 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 the Invitrogen, U.S.A.

5'gaattc aag ttc gtc aac caa cac ttg tgt ggt tcc
*Eco*RI K F V N Q H L C G S
cac ttg gtc gag gct ttg tac ttg gtc tgt ggt gaa aga ggt
H L V E A L Y L V C G E R G
ttc ttc tac aag gct gct aag ggt atc gtc gaa caa tgt tgt
F F Y K A A K G I V E Q C C
acc tcc atc tgc tcc ttg tac caa ttg gag aac tac tgt aac
T S I C S L Y Q L E N Y C N
tag gcggccgc3'
* *Not* I

Figure 3.1: Nucleotide sequence and amino acid sequence of the MIP gene **(5)**

3.2 Chemicals and Reagents

Chemicals and Reagents	Company, Country
Absolute ethanol	Merck, U.S.A.
Absolute methanol	Merck, U.S.A.
Acetic acid (glacial) 100% anhydrous	Merck, Germany
Agar (Microbiology grade)	Merck, Germany
Agarose (Molecular biology grade)	Research organics, Inc., U.S.A.
Bacto™ Peptone powder	Becton, Dickinson and Company, France
Biotin	Fluka, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, U.S.A.
Calcium chloride anhydrous	Merck, Germany
Cobalt chloride hexahydrate	Sigma Aldrich, Germany
DAB (3,3'-Diaminobenzidine)	Sigma-Aldrich, U.S.A.
D(+) Glucose anhydrous	Carlo Erba Reagenti, Italy
di-Potassium hydrogen phosphate	Carlo Erba Reagenti, Italy
di-Sodium hydrogen phosphate	Merck, Germany
Dimethyl sulfoxide (DMSO)	Fluka, Switzerland
Dithiothreitol (DTT)	Bio basic, Inc., Canada
DNA ladder (1 kb), M11	SibEnzyme, Ltd., Russia
D-Sorbitol	Fluka, Germany
Ethidium bromide	Bio basic, Inc., Canada
Ethylenediaminetetraacetic acid (EDTA)	Carlo Erba Reagenti, Italy
Glutaraldehyde 50% in water	Merck, U.S.A.
Glycerol	Ajax Chemicals, Australia
Goat anti-Mouse IgG, (H+L) HRP conjugate	Jackson Immuno Research Laboratories Inc., U.S.A.
HEPES (Free acid)	Bio basic, Inc., Canada
Histidine	Fluka, Germany
Hydrochloric acid 37%	Merck, Germany

Chemicals and Reagents	Company, Country
Hydrogen peroxide	Merck, Germany
Insulin from bovine pancreas	Sigma-Aldrich U.S.A.
Mixtard® 30 HM Penfill® (Injection insulin)	Novo Nordisk, Denmark
Monoclonal anti-insulin antibody	Sigma Aldrich, U.S.A.
Nitrocellulose membrane (NitroBind™)	Life Science Products, Inc., U.S.A.
Potassium acetate	Fluka, Switzerland
Potassium *di*-hydrogen phosphate	Merck, Germany
Skim milk	Difco, U.S.A.
Sodium azide	Merck, Germany
Sodium chloride	Ajax Chemicals, Australia
Sodium dodecyl sulfate (SDS)	Bio basic, Inc., Canada
Sodium hydroxide	Ajax Finechem Pty, Ltd., Australia
Sulfuric acid	Merck, Germany
TAE buffer premix powder	Bio basic, Inc., Canada
TMB (3,3',5,5'-tetramethylbenzidine)	Sigma-Aldrich, U.S.A.
Tris (Molecular biology grade)	Research organics, Inc., U.S.A.
Tryptone powder	Bio basic, Inc., Canada
Tween-20	Sigma Aldrich, Germany
Yeast nitrogen base powder (w/ ammonium sulfate)	Bio basic, Inc., Canada
Yeast extract powder	Bio Springer, France
Zeocin™	Invitrogen, U.S.A.

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3.3 Equipment and Supplies

Equipment and Supplies	Company, Country
Autoclave (HV-50)	Hirayama manufacturing Corp., Japan
Balance (Adventurer™, ARC 120)	Ohaus Corp., U.S.A.
Balance (Adventurer™, AR 2140)	Ohaus Corp., U.S.A.
Bench-top centrifuge, WiseSpin® (CF-10)	Dihan scientific Co., Ltd., South Korea
Biological safety cabinet (Heal force®, HFsafe-1200)	Shanghai Lishen Scientific equipment Co., Ltd., China
Digi Thermopet (NTT-1200)	Tokyo Rikakikai Co., Ltd., Japan
E.Z.N.A.® Gel extraction kit	Omega Bio-Tek, Inc.,U.S.A.
Freezer (-20°C) (SF-C697)	Sanyo Commercial Solution, Ltd., Thailand.
Gene Pulser® Cuvette, 0.2 cm.	Bio-Rad Laboratories, Inc., China
High speed micro refrigerated centrifuge (MTX-150)	Tomy Seiko Co., Ltd., Japan
High speed refrigerated centrifuge (6500)	Kubota Corp., Japan
Hot plate (PC-101)	Corning, U.S.A.
Incubator (MIR 152)	Sanyo Electric Co., Ltd. Japan
Microplate reader (Multiskan FC, Type 357)	Thermo Fisher Scientific Instruments Co.,Ltd., China
MicroPulser™	Bio-Rad, U.S.A.
Microwave oven (National®)	Matsushita Electric Industrial Co. Ltd., Japan
Mupid®-EXU Submarine electrophoresis system	Advance Co, Ltd., Japan
pH meter (Accumet® AB15)	Fisher Scientific, Singapore
Refrigerator	Panasonic Appliances Lights Action Alliance Co., Ltd., Thailand

Equipment and Supplies	Company, Country
Refrigerated incubator shaker (Innova™ 4330)	New Brunswick Scientific Co., Inc., U.S.A.
T100™ Thermal cycler	Bio-Rad, Singapore
Ultra low refrigerator (MDF 79OAT)	Sanyo Electric Co., Ltd. Japan
UV transilluminator	UVItec, U.K.
UVitec platinum gel documentation system	UVItec, U.K.
UV-Visible recording spectrophotometer (UV-160)	Shimadzu Corp., Japan
Vortex mixer (KMC-1300V)	Vision scientific Co., Ltd., Korea
Water pro plus	Labconco Corp., U.S.A.

3.4 Enzymes and Primers

Enzymes and Primers	Company, Country
Alkaline phosphatase, Calf Intestinal (CIP)	Finnzymes, Finland
*Bam*HI	Roche, Germany
*Bgl*II	Roche, Germany
*Eco*RI	Roche, Germany
i-*Taq* DNA polymerase	iNtRON Biotechnology, Korea
*Kpn*I	Roche, Germany
Lyticase	Invitrogen, U.S.A.
*Not*I	Roche, Germany
RNase A (Ribonuclease A)	New England Biolab, U.K.
*Sac*I	Roche, Germany
T4 DNA ligase	Promega, U.S.A.
3’AOX reward primer	Invitrogen, U.S.A.
5’AOX forward primer	Invitrogen, U.S.A.
α-factor primer	Invitrogen, U.S.A.
MIP forward primer (5’TTC-GTC-AAA-CAA-CAC-TTG-TG3’, Tm=55.2)	BioDesign Co., Ltd., Thailand
MIP reward primer (5’GTT-ACA-GTA-GTT-CTC-CAA-TTG3’, Tm=54.0)	BioDesign Co., Ltd., Thailand

3.5 Media

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

3.6 Methods

3.6.1 Construction of Recombinant Plasmids

The TP1 plasmid was constructed by Dr. Sarintip Sooksai in 2008. The pUC::MIP plasmid was digested with *Eco*RI and *Not*I, the released MIP fragment (173 bp) was purified and ligated into the pPICZαA expression vector to generate the pPICZαA::MIP plasmid, TP1 plasmid (3,709 bp). The TP1 plasmid was digested with *Bam*HI and *Bgl*II, the released MIP cassette with the size of 1,792 bp (*5'AOX1*-MIP-*3'AOX1*, head-to-tail) consists of *AOX1* promoter, alpha-factor signal sequence, MIP gene, and *AOX1* transcription terminator. The MIP cassette with 5'-*Bgl*II and 3'-*Bam*HI was purified and ligated to TP1 plasmid which linearized with *Bam*HI to generate TP2 plasmid (5,501 bp). The ligation reaction was transformed into freshly prepared *E. coli* Top10F' competent cells and 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 3.6.2. The recombinant plasmid which expected to harbor two cassettes of MIP (2×MIP) was digested with specific restriction enzyme(s) to check size(s) and an orientation of the 2×MIP cassette by

agarose gel electrophoresis to find the TP2 plasmid which 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*)-(*5'AOX1*-MIP-*3'AOX1*), head-to-tail). The released 2×MIP fragment with the size of 3,584 bp was purified and cloned into TP2 plasmid which linearized with *Bam*HI (5,501 bp) to generate the pPICZαA::4×MIP plasmid, TP4 plasmid (9,085 bp). After TP2/*Bam*HI and 2×MIP fragment had ligated, the ligation reaction was transformed into *E.coli* Top10F' and selected with low salt LB medium with 25 μg.mL^{-1} of Zeocin™ as described above and the expected size of the recombinant plasmid was screened by agarose gel electrophoresis.

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 the yeast.

3.6.2 Extraction and Purification of Plasmid DNA

This protocol was modified from extraction and purification of plasmid DNA in molecular cloning: a laboratory manual, second edition (1989) **(49)**.

3.6.2.1 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.

3.6.2.2 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.

3.6.2.3 Purification of Plasmid DNA

The double-stranded DNA in the supernatant from 3.6.2.2 was precipitated with 2 volumes of ice-cold 95% ethanol (800 µL) and mixed by vortexing. 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).

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

3.6.3.1 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.

3.6.3.2 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 $µg.mL^{-1}$ of Zeocin™, and incubated at 37°C for overnight.

3.6.3.3 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.

3.6.3.4 Transformation of Yeasts by Electroporation

This protocol was modified from DNA-mediated transformation in method in molecular biology, second edition **(50)** and EasySelect™ *Pichia* Expression Kit **(40)**. 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 2.5 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 spreaded 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.

3.6.4 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. After an OD_{600} reach to 1.0, ten percent of the starter culture was inoculated in 50 mL YPG medium in 250 mL Erlenmeyer flask, triplicately, 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. Five milliliters of culture samples were taken at 0, 12, 24, 48, and 72 hours and 5 mL of MMH medium was added. 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. 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 aliquoted and stored at +4°C until assay and cell pellet was washed with TE buffer (pH 8.0) and filtered for cell dry weight. The procedure of this protocol is show in Figure 3.2.

3.6.5 The Monitoring and Determination of the Recombinant Monomeric Insulin Precursor

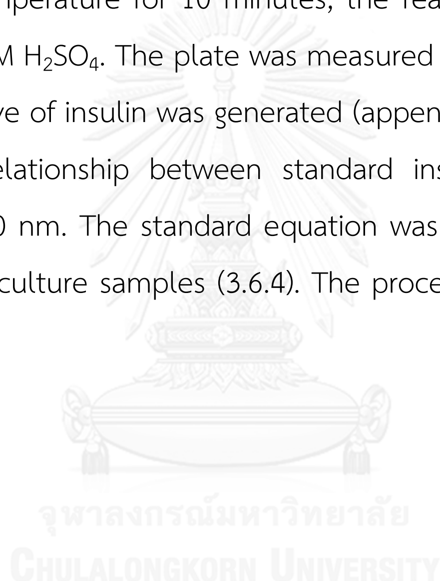
3.6.5.1 Monitoring of the MIP Expression Level by Dot-Blot Analysis

A specific dot-blot procedure, adapted from Sithigorngul (1991) **(51)**, was used for a monitoring of the MIP expression level in culture broth. Insulin from bovine pancreas and injection insulin (Mixtard® 30HM Penfill) were prepared at various concentrations (appendix C) and used as insulin standards. The supernatants of culture samples (3.6.4) were spotted (3 µL per spot) onto the same nitrocellulose membrane with the insulin standards. The membrane was dried at 60°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 primary 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. The procedure of this protocol is show in Figure 3.3.

3.6.5.2 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 the concentration of 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 D). 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 (3.6.4). The procedure of this protocol is show in Figure 3.4.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3.7 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, $\alpha_{0.05}$) which mean that the mean of data between groups is differently significant (also see in an appendix E).

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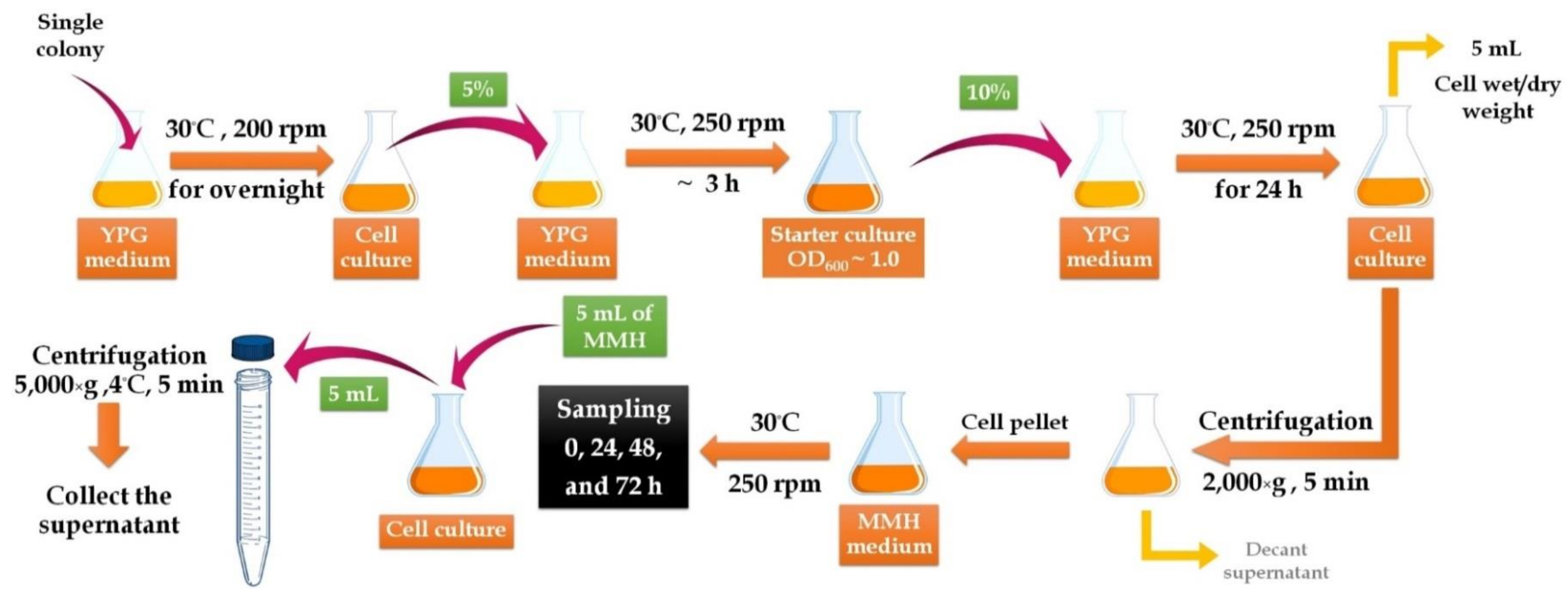

Figure 3.2: The cultivation and expression of the MIP by two step cultivation

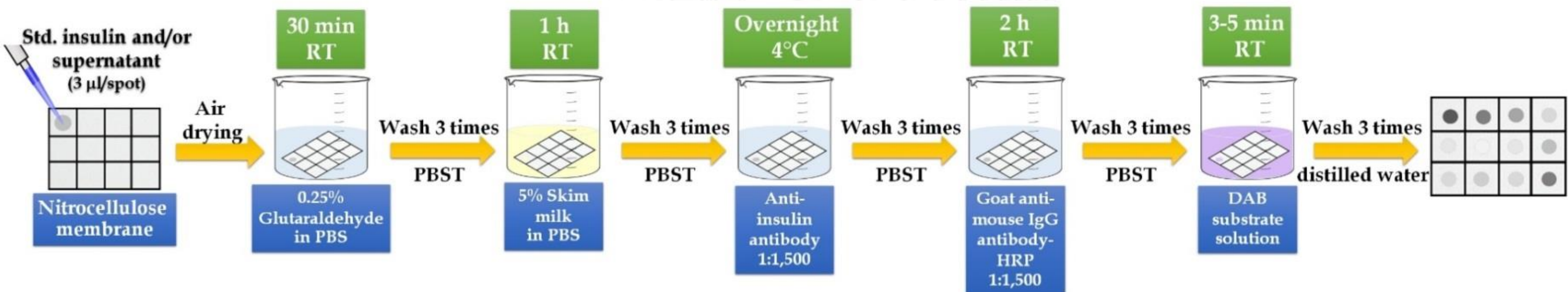

Figure 3.3: Dot-blot analysis procedure of the MIP expression level in the supernatant

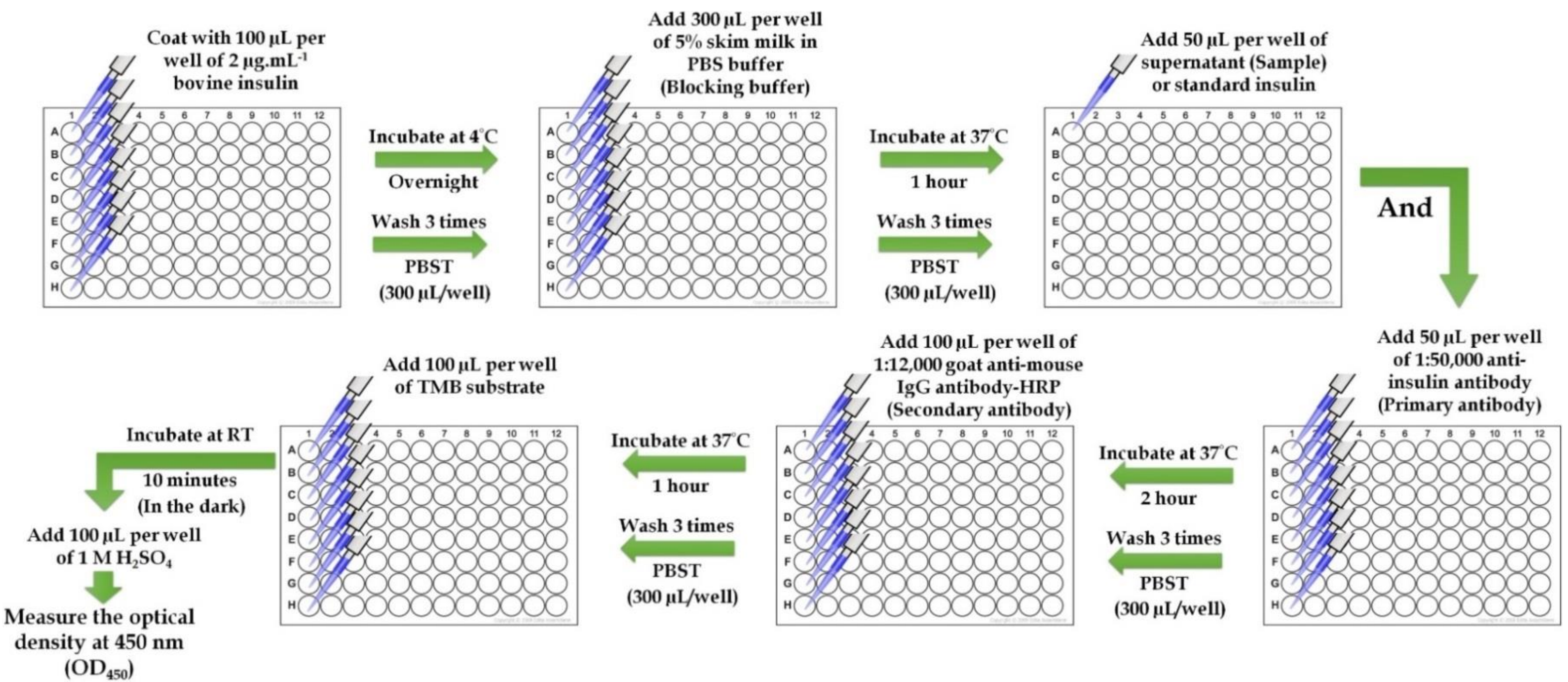

Figure 3.4: Indirect competitive ELISA procedure for quantitative determination of the MIP concentration

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Construction of the Recombinant Plasmids

TP1 plasmid, which was constructed by Dr. Sarintip Sooksai, was generated from insertion of the MIP gene (166 bp) into the pPICZαA expression vector (3,593 bp) between *Eco*RI and *Not*I restriction sites. The TP1 plasmid was digested with *Bam*HI and *Bgl*II to generate the MIP expression cassette (1,792 bp), *AOX1* promoter-*MIP-AOX1* TT (Figure 4.1). The genetic map of TP1 plasmid (3,709 bp) which harbors one copy of MIP gene is showed in Figure 4.2. TP2 plasmid was constructed by the 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), as showed in Figure 4.3. Therefore, the orientation of the MIP cassettes in TP2 plasmid was checked by specific restriction enzymes: *Eco*RI, *Bgl*II and *Bam*HI. Result is showed in Figure 4.4: the TP2, which was digested with *Bam*HI, gave one fragment, 5,501 bp, the TP2, which was digested with *Eco*RI, gave two DNA fragments, 1,792 bp and 3,709 bp, the TP2, which was digested with *Bgl*II and *Bam*HI, gave two fragments, 1,917 bp and 3,584 bp. Consequently, the 3,584 bp fragment is 2xMIP cassette. Therefore, the genetic map of TP2 plasmid is type I as show in Figure 4.5. The TP4 plasmid was constructed by the ligation of the 2×MIP cassette (3,584 bp) with linear TP2, which was linearized with *Bam*HI. The expected size of TP4 plasmid was 9,085 bp. Due to the 2xMIP cassette could be ligated to the linear TP2 plasmid with two directions, type I (head to tail) - (head to tail) or type II (head to tail) - (tail to head), as showed in Figure 4.6. Therefore, the orientation of the 2xMIP cassettes in TP4 plasmid was checked by specific restriction enzymes, *Bgl*II and *Bam*HI. Result is showed in Figure 4.7: the TP4, which was digested with *Bam*HI, gave one fragment, 9,085 bp, and the TP4, which was digested with *Bgl*II and *Bam*HI, gave two DNA fragments, 7,114 bp and 1,917 bp, so the genetic map of TP4 plasmid is type I as showed in Figure 4.8. These three recombinant

plasmids (TP1, TP2 and TP4) were transformed into *P. pastoris* 3 strains (X-33, GS115 and KM71H) and *H. polymorpha* (NRRL2214) by electroporation to study the effect of yeast strains and copy number of gene on the MIP secretion level.

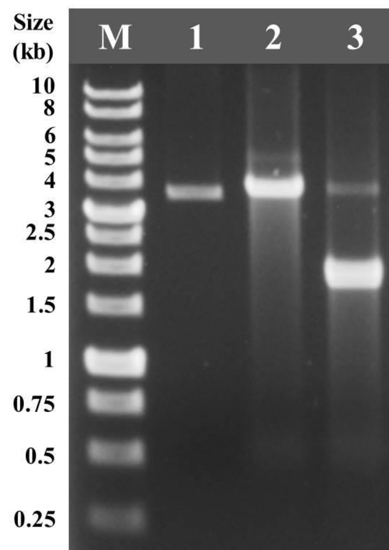

Figure 4.1: The agarose gel electrophoresis of the TP1 plasmid

Lane M is a 1 kb DNA ladder. Lane 1 is the pPICZαA digested with *Bam*HI (3,593 bp). Lane 2 is the TP1 plasmid digested with *Bam*HI (3,709 bp). Lane 3 is the TP1 plasmid digested with *Bam*HI and *Bgl*II (1,792 bp and 1,917 bp).

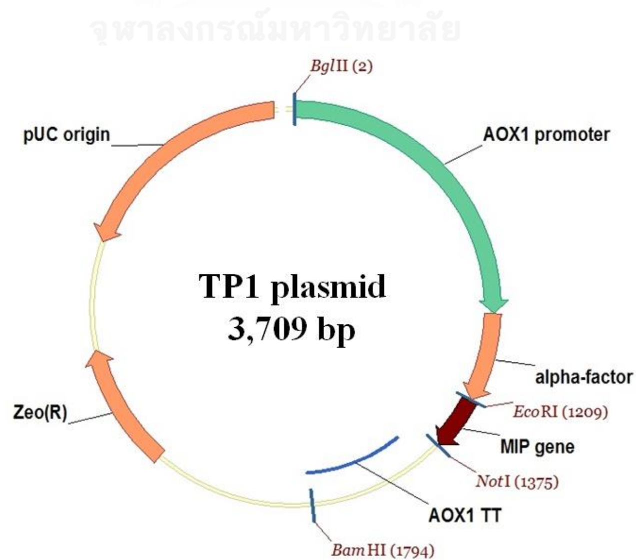

Figure 4.2: The genetic map of TP1 plasmid

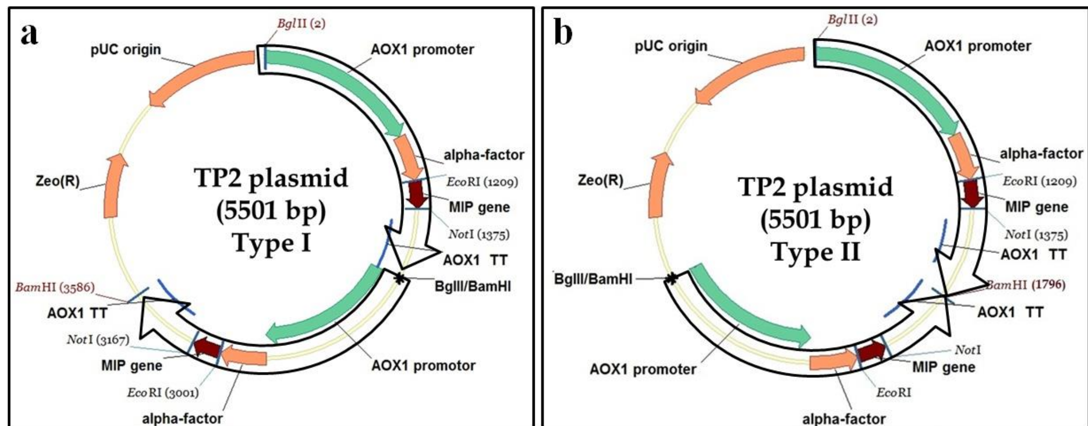

Figure 4.3: The possibility of TP2 plasmid; (a) type I; (head to tail) - (head to tail) and (b) type II; (head to tail) - (tail to head))

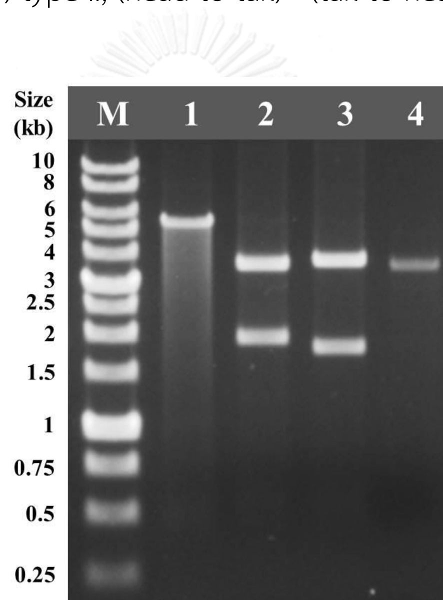

Figure 4.4: The agarose gel electrophoresis of the TP2 plasmid

Lane M is a 1 kb DNA ladder. Lane 1 is the TP2 plasmid which digested with *Bam*HI (5,501 bp). Lane 2 is the TP2 plasmid which was digested with *Bgl*II and *Bam*HI (1,917 bp and 3,584 bp). Lane 3 is the TP2 plasmid which was digested with *Eco*RI (1,792 bp and 3,709 bp). Lane 4 is the 3,584 bp fragment (2xMIP cassette).

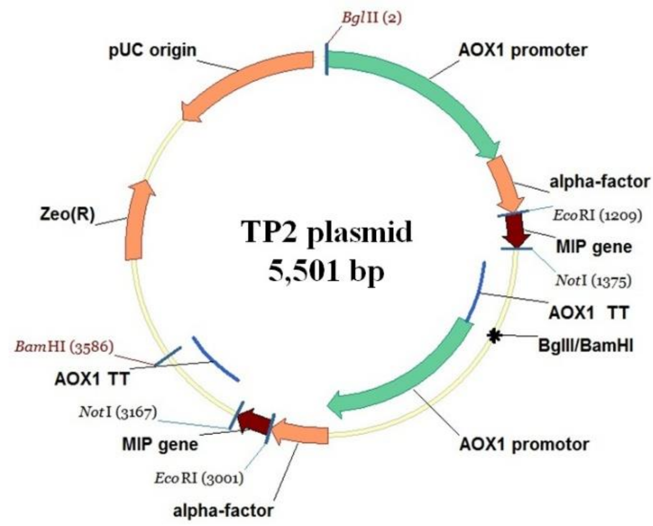

Figure 4.5: The genetic map of TP2 plasmid (type I)

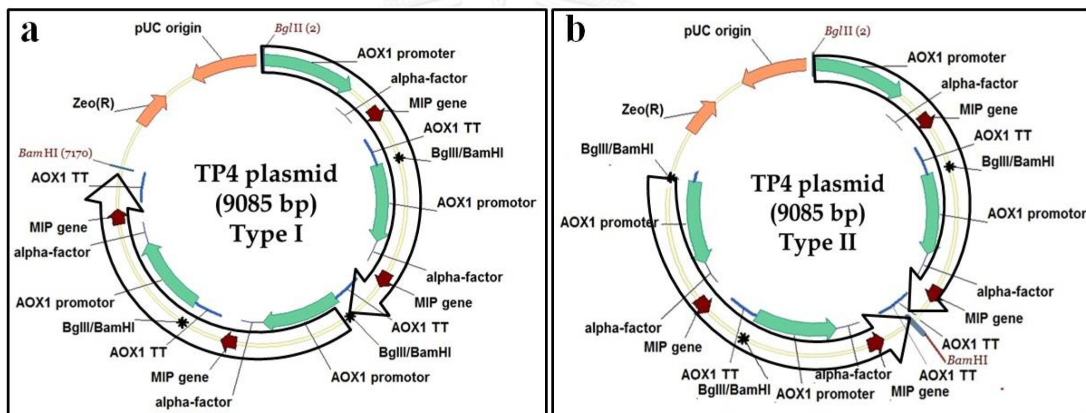

Figure 4.6: The possibility of TP4 plasmid; (a) type I; (head to tail) - (head to tail) and (b) type II; (head to tail) - (tail to head))

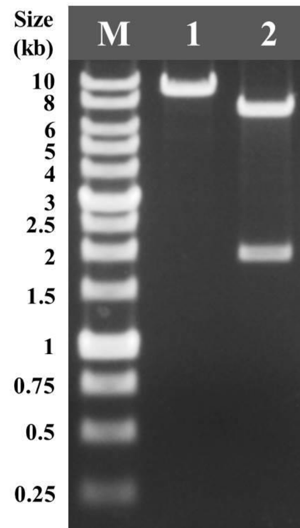

Figure 4.7: The agarose gel electrophoresis of the TP4 plasmid

Lane M is a 1 kb DNA ladder. Lane 1 is the TP4 plasmid which digested with BamHI (9,085 bp). Lane 2 is the TP4 plasmid which was digested with BamHI and BglII (1,917 bp and 7,114 bp).

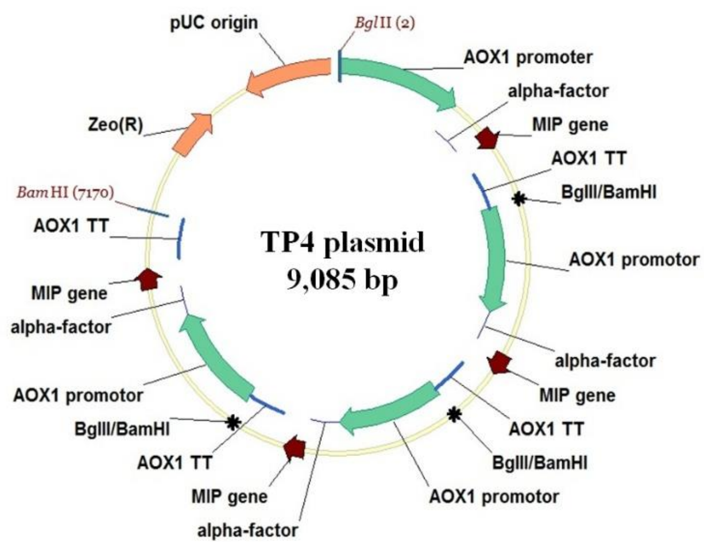

Figure 4.8: The genetic map of TP4 plasmid (type I)

4.2 Recombinant Yeasts Cultivation and Recombinant MIP Expression

In general, *P. pastoris* (X-33, GS115) Mut^{+} strains are characterized by a higher growth rate than Mut^{S} strains (KM71H) especially in medium containing methanol as sole carbon source. *P. pastoris* KM71H (Mut^{S}) has an inactive *AOX1* 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^{+} strain was calculated to be approximately 1.5-fold higher than for the Mut^{S} strain **(52)**. 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 enrichment 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 (as showed in Table 4.1). After 24 hours of growing in YPG medium, the 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. The data was showed in the Table 4.2 (also see in an appendix E).

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Table 4.1: 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 OD_{600}
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

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Table 4.2: The optical density at 600 nm and cell dry weight of the cell culture in the cell production phase (YPG medium)

Yeast(s)	Strain(s)	Plasmid(s)	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	NRRL 2214	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.

In the induction phase, the cell pellets from YPG at 24 hours were transferred to MMH induction medium. The MIP gene expression was induced by methanol. Samples were taken every 24 hours for monitoring and determination of the MIP expression level. In this step, it was observed that the OD_{600} was unchanged till the last sampling at 72 hours (as showed in Table 4.3).

Table 4.3: The optical density at 600 nm of the cell culture in the induction phase (MMH medium)

Yeast(s)	Strain(s)	Plasmid(s)	OD_{600} 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
		TP2#4	24.07±0.44	18.88±1.84	21.35±0.69	21.03±1.42
		TP4#5	19.60±0.30	16.98±0.28	16.50±0.13	17.47±0.29

* Data is show in mean ± standard deviation.

4.3 The Monitoring and Quantitative Determination of the Recombinant Monomeric Insulin Precursor

4.3.1 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. The standard insulin, 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 $\mu g.\mu L^{-1}$), and the supernatant of the culture broth were determined by using a primary monoclonal anti-insulin antibody. The positive control is the standard insulin which described above and in the appendix C. The negative control is MMH medium and the supernatant of the recombinant *P. pastoris* GS115, which pPICZαA has been integrated in the genome. The MIP expression level from recombinant *P. pastoris* (X-33, GS115 and KM71H) and *H. polymorpha* (NRRL2214) is showed in Figure 4.9 and 4.10.

The intensity of each spots which visualized on the nitrocellulose membrane is related to the insulin concentration while the negative control did not show any spot. The intensity of samples was compared with the standard insulin. The results showed that the recombinant *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 which can be visualized since 24 hours in an induction phase. The following is the recombinant *P. pastoris* X-33 and GS115, Mut^+ phenotype strain. The MIP expression level from the recombinant *H. polymorpha* was the lowest as show in Figure 4.9 and 4.10.

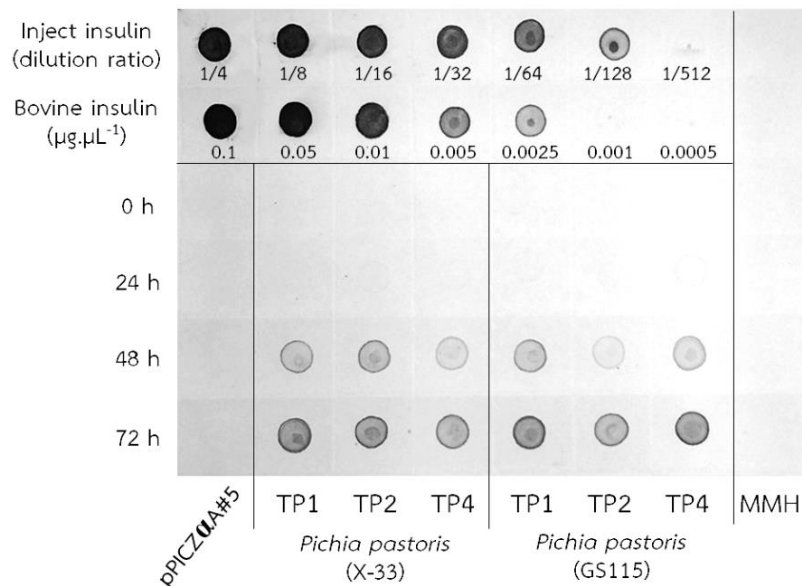

Figure 4.9: Dot-blot analysis of the positive control, negative control and supernatant of the recombinant *P. pastoris* X-33 and GS115, Mut^{+} phenotype strain, which harbored TP1, TP2 and TP4 in the genome

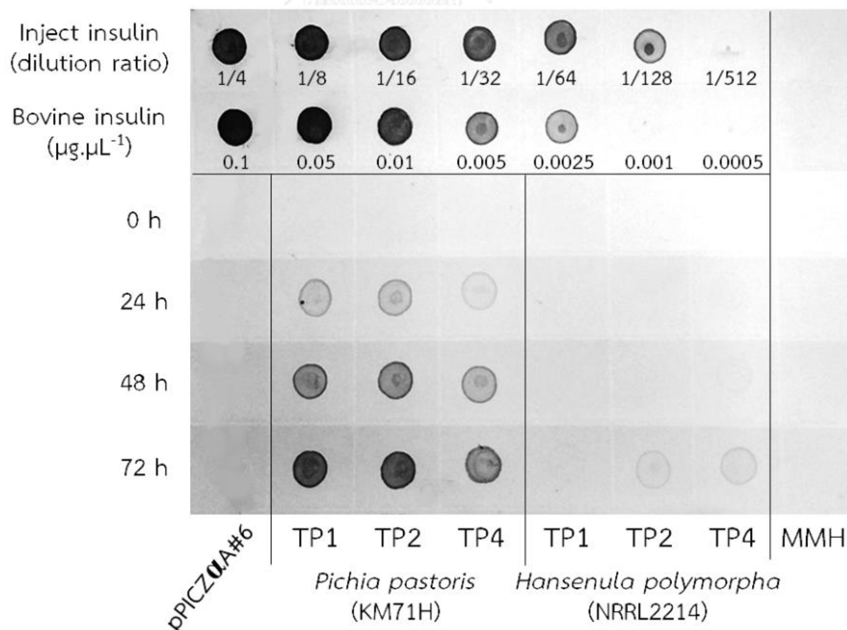

Figure 4.10: Dot-blot analysis of the positive control, negative control and supernatant of the recombinant *P. pastoris* (KM71H), Mut^{S} phenotype strain, and the recombinant *H. polymorpha* (NRRL2214) which harbored TP1, TP2 and TP4 in the genome

4.3.2 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, 24, 27)**, HPLC (UV-VIS detector, 214 nm) **(16)**. Some researchers determine the concentration of secreted protein by measurement the optical density at 280 nm **(26)**. 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. This method is not suitable for measure the absorbance of supernatant because of the interference from other proteins, which secreted from yeast cell or cell lysate. Therefore, a specific procedure, indirect competitive ELISA is a high sensitivity and high specificity technique according to a specific binding between antibody and antigen. It was adapted for quantitative determination of the MIP and used in this study. The indirect competitive ELISA was developed for a measurement range of standard insulin concentration of 0.025 - 10 $mg.L^{-1}$. The supernatants from culture broth were determined the MIP concentration by comparison with a standard insulin and calculated using a standard equation (see in an appendix D).

4.3.2.1 The Effect of Yeast Strains on the MIP Expression Level

To date, several researchers have been reported that *P. pastoris* Mut^+ phenotype strains produced more recombinant proteins due to grow faster on methanol **(27, 53)**. However, some researchers have been reported that *P. pastoris* Mut^S strains were superior over Mut^+ strains in terms of recombinant protein production **(52, 54-56)**.

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 results showed that the recombinant *P. pastoris* KM71H gave the highest MIP concentration (4.19±0.96 $mg.L^{-1}$) following *P. pastoris* GS115 (2.69±0.48 $mg.L^{-1}$) and *P. pastoris* X-33 (0.93±0.08 $mg.L^{-1}$) as showed in Figure 4.11. The MIP expression

level in these recombinant *Pichia* have 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 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 **(42)**. The Mut^{S} phenotype strain, *P. pastoris* KM71H, can be used instead of the Mut^{+} strains to solve this problem due to the lower methanol consumption rate. The *P. pastoris* KM71H leads to long induction time for the 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 level from recombinant *H. polymorpha* NRRL2214 was lower (0.04±0.01 $mg.L^{-1}$) than those of *P. pastoris* strains. According to Raschke *et al.,*(1996) reported that the *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 the control of *AOX1* promoter in *H. polymorpha* seemed to be lower than in *P. pastoris* **(57)**.

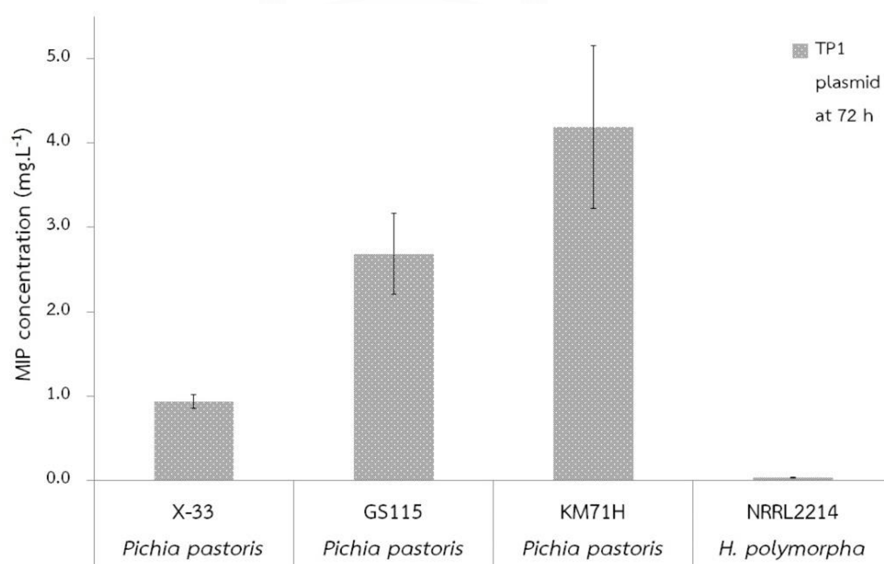

Figure 4.11: Effect of yeast strains on the MIP expression as shown by the MIP concentration in the culture (at 72 hours of induction) of the recombinant yeasts which harbored TP1 plasmid.

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

The recombinant yeasts, which harbored multiple copy number of gene, can be constructed by two methods. The first one is *in vivo* method, which is using a dominant selective marker for selection the recombinants. The recombinants, which can grow on high concentration of selective antibiotic, were correlated with the number of integrated plasmids. The associated antibiotic resistance leads to an enrichment of the population of the multi-copy strains **(16)**. The other is *in vitro* method, which recombinant plasmids were constructed with multiple head-to-tail copies of an expression cassette **(24, 31)**.

In this study, we constructed the recombinant plasmids; TP1, TP2, TP4 which contained 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, which secreted into the culture broth, was analyzed by indirect competitive ELISA.

In recombinant *P. pastoris* X-33, the result showed that the MIP expression from the recombinant *P. pastoris* X-33, which has TP2 plasmid, increase significantly at 48 hours but in the recombinants *P. pastoris* X-33, which harbored TP1 and TP4 plasmid, have not difference. When consider at the end of an induction phase (72 hours), the recombinant *P. pastoris* X-33, which harbored TP2 plasmid, gave the highest the MIP expression level followed by *P. pastoris* X-33 which harbored TP4 and TP1 plasmid, respectively, as show in Figure 4.12 (a).

In recombinant *P. pastoris* GS115, the result showed that the MIP expression level from each recombinant *P. pastoris* GS115 has not significantly different within 48 hours of an induction phase. At the 72 hours, the recombinants *P. pastoris* GS115, which harbored TP1 and TP4 plasmids, have more expression level than the recombinant *P. pastoris* GS115 harboring TP2 plasmid as show in Figure 4.12 (b).

In recombinant *P. pastoris* KM71H, the result showed that at early 48 hours in an induction phase, the recombinant *P. pastoris* KM71H, which harbored TP2 plasmid, express more rapidly than the others but in recombinants *P. pastoris*

KM71H, which harbored TP1 and TP4 plasmid, have not significantly different. Considering at 72 hours, the MIP expression level of the recombinant *P. pastoris* KM71H, which harbored TP1 plasmid, was sharply increased. The MIP expression level of the recombinants *P. pastoris* KM71H, which harbored TP1 and TP2 plasmid, have not significantly different whereas the recombinant *P. pastoris* KM71H, which harbored TP4 plasmid, has lower expression level as show in Figure 4.12 (c).

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). The recombinant *H. polymorpha* strains have been obtained by integrating several copies via a non-homologous recombination event **(58)**. According to this reason, we tried to expressed the MIP in *H. polymorpha* NRRL2214, however, the result showed that the MIP expression level from all of the recombinants *H. polymorpha* NRRL2214 was very low within the first 48 hours in an induction phase. Till 72 hours, the MIP expression level of recombinants *H. polymorpha* NRRL2214, which harbored TP2 and TP4 plasmid, have slightly increased as show in Figure 4.12 (d).

The summarized MIP contents in the supernatant culture from recombinant yeasts are showed in Figure 4.13 and Table 4.4.

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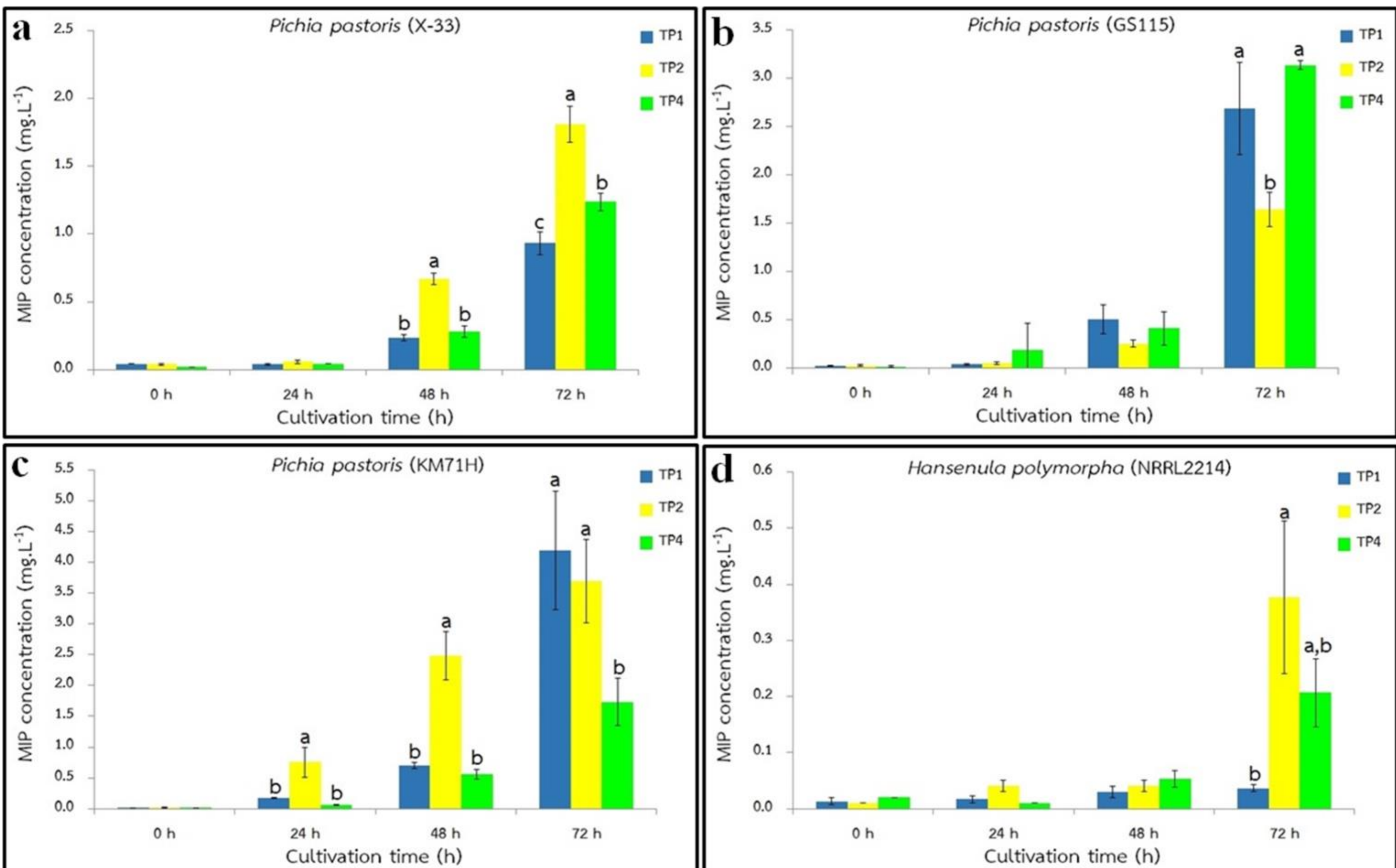

Figure 4. 12: The MIP secretion level from the supernatants of recombinant yeasts; (a): *P. pastoris* X-33, (b): *P. pastoris* GS115, (c): *P. pastoris* KM71H and (d): *H. polymorpha* NRL2214 which harbored TP1, TP2 and TP4 plasmid in the genome

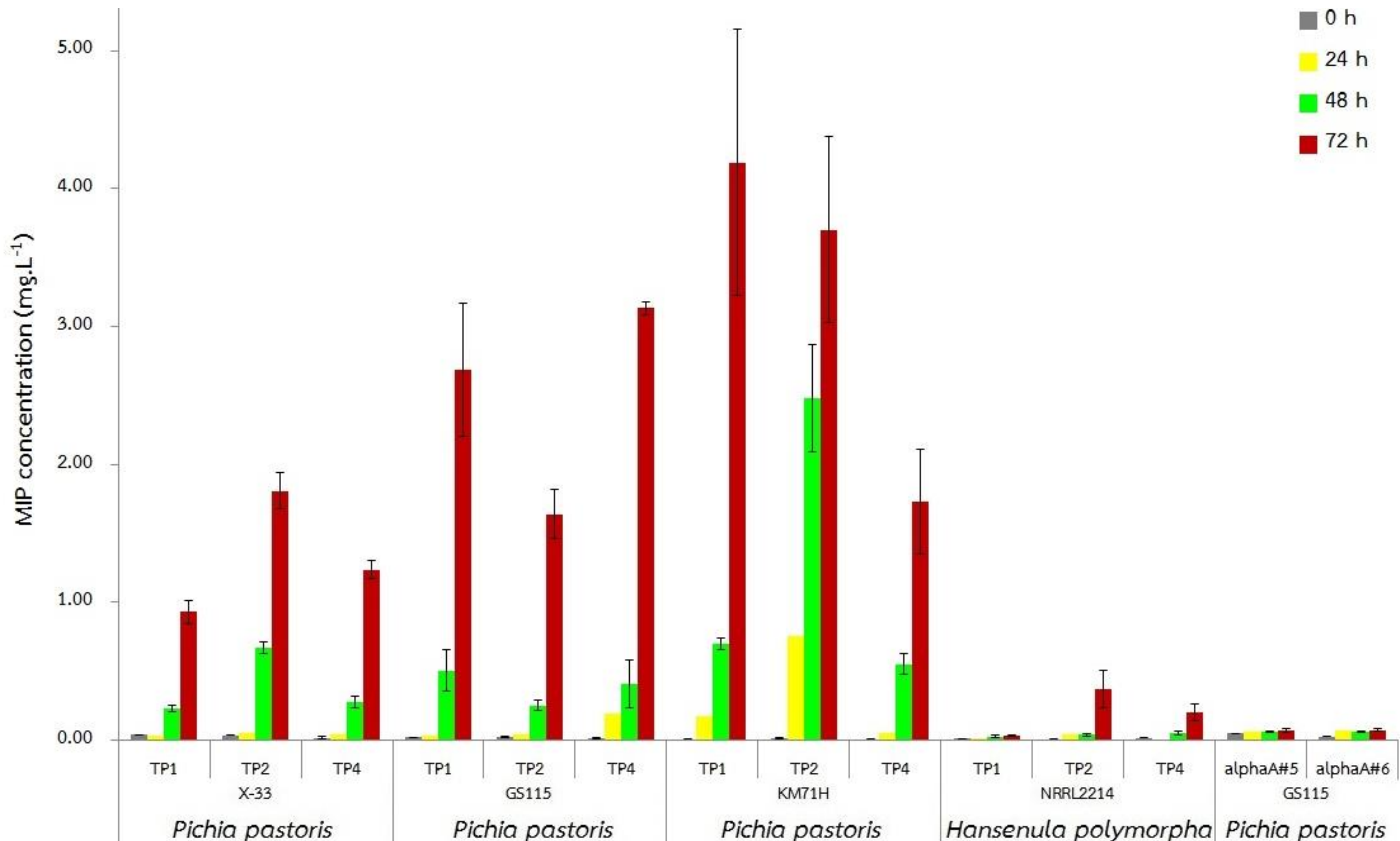

Figure 4.13: The MIP concentration in the culture broth from recombinant yeasts, *P. pastoris* and *H. polymorpha*, which harbored the recombinant plasmids, TP1, TP2 and TP4. While the supernatant from culture broth of *P. pastoris*, which harbored the pPICZαA plasmid vector, was used as a negative control.

Table 4.4: 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.

The results showed that the MIP concentrations of all recombinant yeasts were gradually increased along the cultivation time. The expression of MIP from recombinants *P. pastoris* KM71H could be detected since 24 hours and continuously increased until 72 hours when those of the other recombinant strains were detected at 48 hours after induction. On the other hand, the recombinants *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 not accordance between the copy number of MIP gene and the MIP expression level. These might be the structure of the MIP which consists of 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 **(31)**. The secretory pathway of recombinant proteins from yeast cells; membrane translocation, signal peptide processing, folding and disulfide bond formation, is the major bottleneck of heterologous proteins secretion. Inan *et al*.,(2006) **(37)** attempted to increase secretion of a hookworm protein by increasing gene copy number. However, this has a negative effect when compared to a single copy clones. By overexpressing protein disulfide isomerase, PDI, they were able to increase a secretion of the recombinant secreted protein in clones containing up to four copies of the expression cassette.

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CHAPTER V
CONCLUSION AND SUGGESTION

5.1 Conclusion

The effect of gene dosage on the heterologous protein expression in methylotrophic yeast system has been extensively studied for two past decades **(16, 24, 28, 31)**. Two types of *P. pastoris* strains, which consider in the Mut phenotype, are competed to be used as hosts. Moreover, *H. polymorpha* is claimed to use as a host for heterologous proteins expression because of it has some advance such as high-copy integration due to non-homologous recombination **(58)**. This research has studied both the effect of gene copy number and yeast host strains on the MIP expression. Our studies suggested that the recombinant *P. pastoris* KM71H, Mut^{S} phenotype strain, is superior over either *P. pastoris* X-33 and GS115, Mut^{+} phenotype strains, or *H. polymorpha* NRRL2214 in terms of the recombinant MIP secretion. However, the MIP secretion level, which secreted into the medium, was unrelated with a gene copy number. This point make as if to consider the limitation of a secretory pathway. It may be a bottleneck for overexpression of a heterologous protein from high-copy number recombinants.

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5.2 Suggestion

As a number of diabetic patients in the world are increasing, so the demand of insulin will increase too. However in Thailand, all types of insulin which used for diabetic patients treatment are imported and it needs to be improved the production for more effective insulin analog for diabetic treatment. This research 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. It may causes of hosts' 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 cells lysate and culture broth. Further, the MIP production needs to be improved by control physical factors, which effect to the MIP production e.g. dissolved oxygen, methanol concentration, cell density, cultivation strategy.

In the future, the recombinant monomeric insulin precursor (MIP) production needs to be optimized for high level expression and tests the biological activity by *in vivo* method to confirm its action before scale up to the pilot plant.

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APPENDIX

APPENDIX A

Culture Media

1. 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.

2. 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.

3. 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.

Stock solution preparation

- 10×YNB; 13.4% YNB w/o amino acid w/ ammonium sulfate, 100 mL
  - YNB W/o amino acid W ammonium sulfate powder 13.4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 μm) and stored at +4°C.
- 500×Biotin; 0.02% Biotin, 50 mL
  - Biotin powder 10 mg
  - Dissolved with sterilized double distilled water and adjust the volume to 50 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 μm) and stored at +4°C.
- 100×Histidine; 0.4% Histidine, 100 mL
  - Histidine powder 0.4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 μm) and stored at +4°C.
- Compositions of MMH medium from stock solution above, for 1 L
  - 10×YNB 100 mL
  - 500×Biotin 2 mL
  - 100×Histidine 10 mL
  - Absolute methanol 5 mL
  - Sterilized double distilled water 883 mL (Autoclaved)

  Aseptically mix the solutions above in a biohazard cabinet.

4. Yeast Peptone Dextrose (YPD) medium

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

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

5. 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

Chemical Solutions Preparation

1. 1% Agarose for agarose gel electrophoresis

- Agarose (molecular grade) 1 g
- 10×TAE buffer solution 10 mL
- Double distilled water 90 mL

Melt the agarose solution by microwave oven until the gel completely melted. Cool the solution to +50°C and pour the gel to the gel boxes, inserts the combs and wait for the gel has been set.

2. 0.1 M $CaCl_2$ for fresh competent *E. coli* preparation

- $CaCl_2.H_2O$ powder (MW = 147.02 $g.mole^{-1}$) 14.702 g
- Dissolved in double distilled water and adjust the volume to 100 mL using volumetric flask. The solution was sterilized by autoclave and stored at +4°C.

3. 1 M DTT, Yeast competent cells preparation

- DTT powder (MW = 154.25 $g.mole^{-1}$) 0.2314 g
- Dissolved in sterilized double distilled water and adjust the volume to 1.5 mL and sterilize by filtration (pore size 0.22 µm). Store at 0°C.

4. 0.25% Glutaraldehyde (Fixing solution for dot-blot analysis)

- Glutaraldehyde 50% (w/v) in water 0.5 mL
- Double distilled water 99.5 mL

5. 1 M HEPES buffer (pH 8.0), Yeast competent cells preparation

- HEPES free acid powder (MW = 238.30 $g.mole^{-1}$) 2.383 g
- Dissolved in sterilized double distilled water and adjust the pH value to 8.0 with 5 M NaOH. Adjust the volume to 10 mL and sterilize by filtration (pore size 0.22 µm). Store at +4°C.

6. Lysis by alkali for plasmid extraction

Solution I

- 50 mM glucose (Glucose monohydrate; MW = 198.17 $g.mole^{-1}$)
- 25 mM Tris·Cl (pH 8.0), (Tris base; MW = 121.1 $g.mole^{-1}$)
- 10 mM EDTA (pH 8.0), (EDTA; MW = 292.25 $g.mole^{-1}$)

Solution I can be prepared in batches of approximately 100 mL, autoclaved for 15 minutes and stored at +4°C.

Stock solution preparation

- 1 M Glucose, 100 mL
  - Glucose monohydrate powder 19.817 g
  - Adjust the volume to 100 mL by sterilized double distilled water using volumetric flask. Stored at +4°C.
- 0.5 M Tris·Cl (pH 8.0), 100 mL
  - Tris·base powder 6.055 g
  - Dissolved with sterilized double distilled water and adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 100 mL by double distilled water using volumetric flask. Stored at +4°C.
- 0.5 EDTA (pH 8.0), 100 mL
  - EDTA powder 14.6125 g
  - Dissolved with sterilized double distilled water and adjust the pH to 8.0 with 5 M NaOH. Adjust the volume to 100 mL by double distilled water using volumetric flask. Stored at +4°C.
- Compositions of Solution I from stock solution above
  - 1 M Glucose 5 mL
  - 0.5 M Tris•Cl (pH 8.0) 5 mL
  - 0.5 EDTA (pH 8.0) 2 mL
  - Adjust the volume with double distilled water to 100 mL

Solution II (freshly prepared before use)

- 0.2 N NaOH (NaOH; MW = 40 g.mole^{-1})
- 1% SDS (SDS; MW = 288.38 g.mole^{-1})

Diluted with sterilized double distilled water.

Stock solution preparation

- 2 N NaOH, 100 mL
    - NaOH pellet 4 g
    - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask and stored at +4°C.
- 10% SDS, 100 mL
    - SDS powder 28.84 g
    - Dissolved with sterilized warm double distilled water and adjust the volume to 100 mL and stored at +4°C.
- Compositions of Solution II from stock solution above
    - 2 N NaOH 1 mL
    - 10% SDS 1 mL
    - Sterilized double distilled water 8 mL

Solution III

- 5 M Potassium acetate (MW = 98.14 g.mole^{-1}) 60 mL
- Glacial acetic acid 11.5 mL
- Double distilled water 28.5 mL

Stock solution preparation

- 5 M Potassium acetate, 100 mL
    - Potassium acetate powder 49.07 g
    - Dissolved with double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by autoclave and stored at +4°C.

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- Compositions of Solution III from stock solutions above
  - 5 M Potassium acetate (Sterilized) 60 mL
  - Glacial acetic acid 11.5 mL
  - Sterilized double distilled water 28.5 mL

7. 0.01 M Phosphate Buffer Saline (PBS), pH 7.4

- 200 mM Phosphate buffer, pH 7.4 1 L
- Sodium chloride (NaCl) 175.2 g
- Double distilled water 18 L

Stock solution preparation

- Stock solutions, 200 mM each for 1 L
  - Stock A: $NaH_2PO_4.H_2O$ (MW = 137.99 $g.mole^{-1}$) 27.6 g
  - Stock B: $Na_2HPO_4.12H_2O$ (MW = 358.135 $g.mole^{-1}$) 71.63 g
  - Each $NaH_2PO_4.H_2O$ and $Na_2HPO_4.12H_2O$ powder was separately dissolved in double distilled water and adjusts the volume to 1 L using volumetric flask. Stored at +4°C.
- 200 mM Sodium phosphate buffer (pH 7.4), 1 L
  - Stock A (774 mL) and B (226 mL) were mixed together and adjust the pH value to 7.4 by titration with 5 M HCl. Store at room temperature.

8. 0.05% PBS-Tween 20 or PBST (Washing buffer for dot-blot analysis and ELISA)

- Tween 20 0.5 mL
- 0.01 M Phosphate buffer saline (PBS), pH 7.4 1000 mL

9. 200 mM Potassium citrate buffer for ELISA, pH 4.0

- Stock A: Citric acid monohydrate (MW= 210.14 $g.mole^{-1}$) 10.5 g
- Stock B: Potassium citrate (MW= 324.41 $g.mole^{-1}$) 16.22 g

Each citric acid and potassium citrate powder was dissolved in double distilled water and adjusts the volume to 250 mL using volumetric flask. Stock A (200

mM citric acid) was titrated with stock B (200mM potassium citrate) until the pH value equal to 4.0. The solution was stored at +4°C in the dark.

10. 5% Skim milk in PBS buffer (Blocking buffer for dot-blot analysis and ELISA)

- Skim milk powder 5 g
- 0.01 M Phosphate buffer saline (PBS), pH 7.4 100 mL

11. 1 M D-Sorbitol

- D-Sorbital (D-Sorbital; MW = 182.18 g.mole^{-1}) 18.2 g
- Dissolved in sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (0.22μm mixed cellulose esters membrane) and store at +4°C in the dark.

12. DAB substrate (for Dot-blot analysis)

- DAB powder (3,3'-Diaminobenzidine) 6 mg
- PBS buffer pH 7.4 20 mL
- 30% H_2O_2 solution 20 μL
- 1% $CoCl_2$ solution 50 μL

DAB powder was completely dissolved in PBS buffer follow by adding 30% H_2O_2 solution and 1% $CoCl_2$ solution, respectively.

13. TMB substrate (for indirect competitive ELISA)

- TMB powder (3,3’,5,5’-tetramethylbenzidine) 2.5 mg
- 200 mM potassium citrate buffer (pH 4.0) 10 mL
- 30% H_2O_2 solution 3.5 μL

TMB powder was completely dissolved in 200 mM potassium citrate buffer follow by adding 30% H_2O_2 solution.

14. Tris-EDTA (TE) buffer pH 8.0

- 10 mM Tris·Cl (pH 8.0); Stock solution 0.5 M Tris·Cl (pH 8.0) from Solution I
- 1 mM EDTA (pH 8.0); Stock solution 0.5 M EDTA (pH 8.0) from Solution I

- Compositions of TE buffer (pH 8.0) from stock solutions above
    - 0.5 M Tris•Cl (pH 8.0) 20 mL
    - 0.5 EDTA (pH 8.0) 2 mL
    - Adjust the volume with double distilled water to 1000 mL

Sterilize by autoclave and store at +4°C.

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APPENDIX C

Determination of the MIP Expression Level by Dot-Blot Analysis

1. Standard insulin preparation for determination of MIP expression level

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 C-1.

Table C-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. (mg.mL^{-1}) *Serial dilution	Stock solution (mg.mL^{-1})	Volume from stock solution (µL)	PBS buffer (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 D

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 by dissolved in MMH medium as shown in Table D-1.

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

Insulin standard concentration,($\mu g.mL^{-1}$)	Stock concentration, ($\mu g.\ \mu L^{-1}$)	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

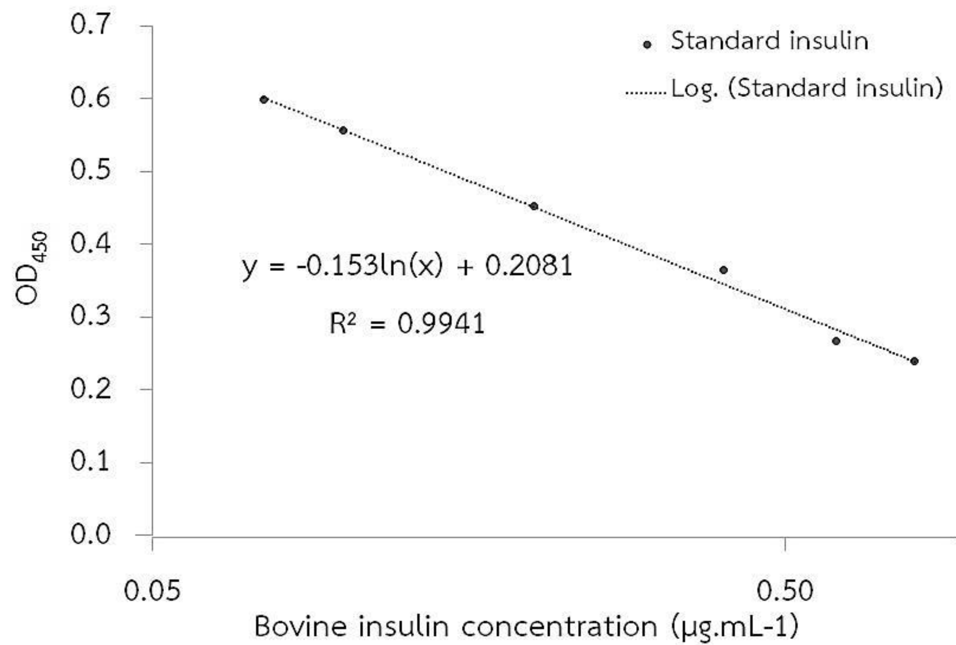

Figure B-1: Standard graph of standard bovine insulin for calculation of the MIP concentration by indirect competitive ELISA

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 ($mg.L^{-1}$)

APPENDIX E

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software (15.0) was used for statistical analysis in this study. One-Way Analysis of Variance (ANOVA) was used to determine the difference means between/within groups of samples and 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, $\alpha_{0.05}$) also means that the mean of data between groups is significantly different.

Hypothesis

H_0 = Null hypothesis, the data between two groups is not difference at significant level = 0.05.

H_1 = Alternative hypothesis, the data between two groups is difference at significant level = 0.05.

So; If the *p* value > $\alpha_{0.05}$ (Alpha = 0.05) ; Accept the null hypothesis (H_0), There is sufficient evidence to accept the null hypothesis.

If the *p* value ≤ $\alpha_{0.05}$ (Alpha = 0.05) ; Reject the null hypothesis (H_0), There is sufficient evidence to reject the null hypothesis.

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1. Statistical analysis of the optical density at 600 nm of the culture at 0 hour in the first step (YPG medium): Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Pichia pastoris X-33::TP1	3	.11700	.002000	.001155	.11203	.12197	.115	.119
Pichia pastoris X-33::TP2	3	.12100	.000000	.000000	.12100	.12100	.121	.121
Pichia pastoris X-33::TP4	3	.12200	.001000	.000577	.11952	.12448	.121	.123
Pichia pastoris GS115::TP1	3	.12167	.001155	.000667	.11880	.12454	.121	.123
Pichia pastoris GS115::TP2	3	.11900	.001732	.001000	.11470	.12330	.118	.121
Pichia pastoris GS115::TP4	3	.11567	.001528	.000882	.11187	.11946	.114	.117
Pichia pastoris KM71H::TP1	3	.12167	.003512	.002028	.11294	.13039	.118	.125
Pichia pastoris KM71H::TP2	3	.12000	.003000	.001732	.11255	.12745	.117	.123
Pichia pastoris KM71H::TP4	3	.11333	.000577	.000333	.11190	.11477	.113	.114
Hasenula polymorpha NRRL2214::TP1	3	.12767	.002082	.001202	.12250	.13284	.126	.130
Hasenula polymorpha NRRL2214::TP2	3	.13067	.006658	.003844	.11413	.14721	.125	.138
Hasenula polymorpha NRRL2214::TP4	3	.12700	.006083	.003512	.11189	.14211	.123	.134
Pichia pastoris GS115 pPICZalphaA#5	2	.11500	.001414	.001000	.10229	.12771	.114	.116
Pichia pastoris GS115 pPICZalphaA#6	2	.11900	.002828	.002000	.09359	.14441	.117	.121
Total	40	.12095	.005500	.000870	.11919	.12271	.113	.138

2. Statistical analysis of the optical density at 600 nm of the culture at 24 hours in the first step (YPG medium): Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Pichia pastoris X-33::TP1	3	24.1667	.56862	.32830	22.7541	25.5792	23.70	24.80
Pichia pastoris X-33::TP2	3	23.6667	1.54623	.89272	19.8256	27.5077	22.70	25.45
Pichia pastoris X-33::TP4	3	20.8167	.10408	.06009	20.5581	21.0752	20.70	20.90
Pichia pastoris GS115::TP1	3	17.3667	1.28873	.74405	14.1653	20.5681	16.20	18.75
Pichia pastoris GS115::TP2	3	16.9500	.65000	.37528	15.3353	18.5647	16.55	17.70
Pichia pastoris GS115::TP4	3	18.1000	1.39374	.80467	14.6378	21.5622	17.15	19.70
Pichia pastoris KM71H::TP1	3	22.6667	1.94957	1.12559	17.8237	27.5097	21.10	24.85
Pichia pastoris KM71H::TP2	3	21.3000	.31225	.18028	20.5243	22.0757	21.05	21.65
Pichia pastoris KM71H::TP4	3	22.0500	.39051	.22546	21.0799	23.0201	21.60	22.30
Hasenula polymorpha NRRL2214::TP1	3	17.5333	.14434	.08333	17.1748	17.8919	17.45	17.70
Hasenula polymorpha NRRL2214::TP2	3	20.4000	.77621	.44814	18.4718	22.3282	19.65	21.20
Hasenula polymorpha NRRL2214::TP4	3	17.6667	.22546	.13017	17.1066	18.2267	17.45	17.90
Pichia pastoris GS115 pPICZalphaA#5	2	23.2000	1.06066	.75000	13.6703	32.7297	22.45	23.95
Pichia pastoris GS115 pPICZalphaA#6	2	18.1750	.45962	.32500	14.0455	22.3045	17.85	18.50
Total	40	20.2700	2.66289	.42104	19.4184	21.1216	16.20	25.45

3. Statistical analysis of cell dry weight of the culture at 24 hour in the first step (YPG medium): Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Pichia pastoris X-33::TP1	3	6.8000	.08660	.05000	6.5849	7.0151	6.75	6.90
Pichia pastoris X-33::TP2	3	6.7667	.06292	.03632	6.6104	6.9230	6.70	6.83
Pichia pastoris X-33::TP4	3	6.5000	.25372	.14649	5.8697	7.1303	6.28	6.78
Pichia pastoris GS115::TP1	3	8.7167	.22684	.13097	8.1532	9.2802	8.55	8.98
Pichia pastoris GS115::TP2	3	8.8000	.41155	.23761	7.7776	9.8224	8.33	9.05
Pichia pastoris GS115::TP4	3	8.4750	.21360	.12332	7.9444	9.0056	8.25	8.68
Pichia pastoris KM71H::TP1	3	6.6417	.12583	.07265	6.3291	6.9542	6.53	6.78
Pichia pastoris KM71H::TP2	3	9.8250	.15207	.08780	9.4472	10.2028	9.65	9.93
Pichia pastoris KM71H::TP4	3	6.2667	.25042	.14458	5.6446	6.8887	6.03	6.53
Hasenula polymorpha NRRL2214::TP1	3	5.9167	.06292	.03632	5.7604	6.0730	5.85	5.98
Hasenula polymorpha NRRL2214::TP2	3	6.5833	.23761	.13718	5.9931	7.1736	6.35	6.83
Hasenula polymorpha NRRL2214::TP4	3	6.1083	.01443	.00833	6.0725	6.1442	6.10	6.13
Pichia pastoris GS115 pPICZalphaA#5	2	6.9150	.37477	.26500	3.5479	10.2821	6.65	7.18
Pichia pastoris GS115 pPICZalphaA#6	2	8.8550	.03536	.02500	8.5373	9.1727	8.83	8.88
Total	40	7.3435	1.25898	.19906	6.9409	7.7461	5.85	9.93

4. Statistical analysis (ANOVA) of the MIP expression in the second step (MMH medium)

ANOVA ***

		Sum of Squares	df	Mean Square	F	Sig.
At 0 h	Between Groups	.004	11	.000	16.974	.000
	Within Groups	.000	24	.000		
	Total	.004	35			
At 24 h	Between Groups	1.416	11	.129	11.167	.000
	Within Groups	.277	24	.012		
	Total	1.693	35			
At 48 h	Between Groups	14.466	11	1.315	72.491	.000
	Within Groups	.435	24	.018		
	Total	14.902	35			
At 72 h	Between Groups	61.976	11	5.634	36.650	.000
	Within Groups	3.689	24	.154		
	Total	65.665	35			

*** Not include the MIP expression level of *P. pastoris* GS115 pPICZalphaA#5 and pPICZalphaA#6.

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5. Statistical analysis of the MIP expression level of recombinant *Pichia pastoris* (X-33) in the culture broth in the expression phase

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
MIP0h	Pichia pastoris X-33::TP1	3	.0400	.00000	.00000	.0400	.0400	.04	.04
	Pichia pastoris X-33::TP2	3	.0367	.00577	.00333	.0223	.0510	.03	.04
	Pichia pastoris X-33::TP4	3	.0200	.00000	.00000	.0200	.0200	.02	.02
	Total	9	.0322	.00972	.00324	.0248	.0397	.02	.04
MIP24h	Pichia pastoris X-33::TP1	3	.0367	.00577	.00333	.0223	.0510	.03	.04
	Pichia pastoris X-33::TP2	3	.0567	.01528	.00882	.0187	.0946	.04	.07
	Pichia pastoris X-33::TP4	3	.0400	.00000	.00000	.0400	.0400	.04	.04
	Total	9	.0444	.01236	.00412	.0349	.0539	.03	.07
MIP48h	Pichia pastoris X-33::TP1	3	.2333	.02082	.01202	.1816	.2850	.21	.25
	Pichia pastoris X-33::TP2	3	.6700	.04359	.02517	.5617	.7783	.62	.70
	Pichia pastoris X-33::TP4	3	.2800	.04000	.02309	.1806	.3794	.24	.32
	Total	9	.3944	.21001	.07000	.2330	.5559	.21	.70
MIP72h	Pichia pastoris X-33::TP1	3	.9333	.08327	.04807	.7265	1.1402	.84	1.00
	Pichia pastoris X-33::TP2	3	1.8100	.13454	.07767	1.4758	2.1442	1.66	1.92
	Pichia pastoris X-33::TP4	3	1.2367	.06429	.03712	1.0770	1.3964	1.19	1.31
	Total	9	1.3267	.39491	.13164	1.0231	1.6302	.84	1.92

5.1 ANOVA: *Pichia pastoris* X-33

		Sum of Squares	df	Mean Square	F	Sig.
MIP 0 h	Between Groups	.001	2	.000	31.000	.001
	Within Groups	.000	6	.000		
	Total	.001	8			
MIP 24 h	Between Groups	.001	2	.000	3.875	.083
	Within Groups	.001	6	.000		
	Total	.001	8			
MIP 48 h	Between Groups	.345	2	.172	131.551	.000
	Within Groups	.008	6	.001		
	Total	.353	8			
MIP 72 h	Between Groups	1.189	2	.595	61.162	.000
	Within Groups	.058	6	.010		
	Total	1.248	8			

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5.2 Homogeneous Subsets: *Pichia pastoris* X-33

MIP at 0 h

	X-33	N	Subset for alpha = .05	
			2	1
Tukey HSD[(a)]	Pichia pastoris X-33::TP4	3	.0200	
	Pichia pastoris X-33::TP2	3		.0367
	Pichia pastoris X-33::TP1	3		.0400
	Sig.		1.000	.483

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 24 h

	X-33	N	Subset for alpha = .05
			1
Tukey HSD[(a)]	Pichia pastoris X-33::TP1	3	.0367
	Pichia pastoris X-33::TP4	3	.0400
	Pichia pastoris X-33::TP2	3	.0567
	Sig.		.090

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 48 h

	X-33	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Pichia pastoris X-33::TP1	3	.2333	
	Pichia pastoris X-33::TP4	3	.2800	
	Pichia pastoris X-33::TP2	3		.6700
	Sig.		.324	1.000

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 72 h

	X33	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	Pichia pastoris X-33::TP1	3	.9333		
	Pichia pastoris X-33::TP4	3		1.2367	
	Pichia pastoris X-33::TP2	3			1.8100
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

6. Statistical analysis of the MIP expression level of recombinant *Pichia pastoris* (GS115) in the culture broth in the expression phase

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
MIP0h	Pichia pastoris GS115::TP1	3	.0233	.00577	.00333	.0090	.0377	.02	.03
	Pichia pastoris GS115::TP2	3	.0267	.00577	.00333	.0123	.0410	.02	.03
	Pichia pastoris GS115::TP4	3	.0167	.00577	.00333	.0023	.0310	.01	.02
	Total	9	.0222	.00667	.00222	.0171	.0273	.01	.03
MIP24h	Pichia pastoris GS115::TP1	3	.0367	.00577	.00333	.0223	.0510	.03	.04
	Pichia pastoris GS115::TP2	3	.0467	.01528	.00882	.0087	.0846	.03	.06
	Pichia pastoris GS115::TP4	3	.1900	.27713	.16000	-.4984	.8784	.03	.51
	Total	9	.0911	.15744	.05248	-.0299	.2121	.03	.51
MIP48h	Pichia pastoris GS115::TP1	3	.5067	.15011	.08667	.1338	.8796	.42	.68
	Pichia pastoris GS115::TP2	3	.2533	.03786	.02186	.1593	.3474	.21	.28
	Pichia pastoris GS115::TP4	3	.4100	.17436	.10066	-.0231	.8431	.21	.53
	Total	9	.3900	.16078	.05359	.2664	.5136	.21	.68
MIP72h	Pichia pastoris GS115::TP1	3	2.6867	.48128	.27787	1.4911	3.8822	2.15	3.08
	Pichia pastoris GS115::TP2	3	1.6400	.18000	.10392	1.1929	2.0871	1.46	1.82
	Pichia pastoris GS115::TP4	3	3.1333	.04619	.02667	3.0186	3.2481	3.08	3.16
	Total	9	2.4867	.71216	.23739	1.9393	3.0341	1.46	3.16

6.1 ANOVA: *Pichia pastoris* GS115

		Sum of Squares	df	Mean Square	F	Sig.
MIP 0 h	Between Groups	.000	2	.000	2.333	.178
	Within Groups	.000	6	.000		
	Total	.000	8			
MIP 24 h	Between Groups	.044	2	.022	.859	.470
	Within Groups	.154	6	.026		
	Total	.198	8			
MIP 48 h	Between Groups	.098	2	.049	2.706	.145
	Within Groups	.109	6	.018		
	Total	.207	8			
MIP 72 h	Between Groups	3.525	2	1.763	19.866	.002
	Within Groups	.532	6	.089		
	Total	4.057	8			

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6.2 Homogeneous Subsets: *Pichia pastoris* GS115

MIP at 0 h

	GS115	N	Subset for alpha = .05
			1
Tukey HSD(a)	Pichia pastoris GS115::TP4	3	.0167
	Pichia pastoris GS115::TP1	3	.0233
	Pichia pastoris GS115::TP2	3	.0267
	Sig.		.165

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 24 h

	GS115	N	Subset for alpha = .05
			1
Tukey HSD(a)	Pichia pastoris GS115::TP1	3	.0367
	Pichia pastoris GS115::TP2	3	.0467
	Pichia pastoris GS115::TP4	3	.1900
	Sig.		.510

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 48 h

	GS115	N	Subset for alpha = .05
			1
Tukey HSD(a)	Pichia pastoris GS115::TP2	3	.2533
	Pichia pastoris GS115::TP4	3	.4100
	Pichia pastoris GS115::TP1	3	.5067
	Sig.		.131

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 72 h

	GS115	N	Subset for alpha = .05	
			1	2
Tukey HSD(a)	Pichia pastoris GS115::TP2	3	1.6400	
	Pichia pastoris GS115::TP1	3		2.6867
	Pichia pastoris GS115::TP4	3		3.1333
	Sig.		1.000	.237

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

7. Statistical analysis of the MIP expression level of recombinant *Pichia pastoris* (KM71H) in the culture broth in the expression phase

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
MIP0h	Pichia pastoris KM71H::TP1	3	.0067	.00577	.00333	-.0077	.0210	.00	.01
	Pichia pastoris KM71H::TP2	3	.0133	.00577	.00333	-.0010	.0277	.01	.02
	Pichia pastoris KM71H::TP4	3	.0100	.00000	.00000	.0100	.0100	.01	.01
	Total	9	.0100	.00500	.00167	.0062	.0138	.00	.02
MIP24h	Pichia pastoris KM71H::TP1	3	.1733	.00577	.00333	.1590	.1877	.17	.18
	Pichia pastoris KM71H::TP2	3	.7533	.24664	.14240	.1406	1.3660	.47	.92
	Pichia pastoris KM71H::TP4	3	.0533	.00577	.00333	.0390	.0677	.05	.06
	Total	9	.3267	.34688	.11563	.0600	.5933	.05	.92
MIP48h	Pichia pastoris KM71H::TP1	3	.7000	.04359	.02517	.5917	.8083	.67	.75
	Pichia pastoris KM71H::TP2	3	2.4833	.38889	.22452	1.5173	3.4494	2.13	2.90
	Pichia pastoris KM71H::TP4	3	.5533	.07638	.04410	.3636	.7431	.47	.62
	Total	9	1.2456	.95162	.31721	.5141	1.9770	.47	2.90
MIP72h	Pichia pastoris KM71H::TP1	3	4.1900	.96146	.55510	1.8016	6.5784	3.51	5.29
	Pichia pastoris KM71H::TP2	3	3.7000	.67535	.38991	2.0223	5.3777	3.14	4.45
	Pichia pastoris KM71H::TP4	3	1.7300	.38314	.22121	.7782	2.6818	1.29	1.99
	Total	9	3.2067	1.28585	.42862	2.2183	4.1951	1.29	5.29

7.1 ANOVA: *Pichia pastoris* KM71H

		Sum of Squares	df	Mean Square	F	Sig.
MIP 0 h	Between Groups	.000	2	.000	1.500	.296
	Within Groups	.000	6	.000		
	Total	.000	8			
MIP 24 h	Between Groups	.841	2	.420	20.709	.002
	Within Groups	.122	6	.020		
	Total	.963	8			
MIP 48 h	Between Groups	6.927	2	3.463	65.360	.000
	Within Groups	.318	6	.053		
	Total	7.245	8			
MIP 72 h	Between Groups	10.173	2	5.086	9.991	.012
	Within Groups	3.055	6	.509		
	Total	13.227	8			

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7.2 Homogeneous Subsets: *Pichia pastoris* KM71H

MIP at 0 h

	KM71H	N	Subset for alpha = .05
			1
Tukey HSD[a]	Pichia pastoris KM71H::TP1	3	.0067
	Pichia pastoris KM71H::TP4	3	.0100
	Pichia pastoris KM71H::TP2	3	.0133
	Sig.		.269

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 24 h

	KM71H	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Pichia pastoris KM71H::TP4	3	.0533	
	Pichia pastoris KM71H::TP1	3	.1733	
	Pichia pastoris KM71H::TP2	3		.7533
	Sig.		.586	1.000

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 48 h

	KM71H	N	Subset for alpha = .05	
			1	2
Tukey HSD[(a)]	Pichia pastoris KM71H::TP4	3	.5533	
	Pichia pastoris KM71H::TP1	3	.7000	
	Pichia pastoris KM71H::TP2	3		2.4833
	Sig.		.728	1.000

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 72 h

	KM71H	N	Subset for alpha = .05	
			1	2
Tukey HSD[(a)]	Pichia pastoris KM71H::TP4	3	1.7300	
	Pichia pastoris KM71H::TP2	3		3.7000
	Pichia pastoris KM71H::TP1	3		4.1900
	Sig.		1.000	.693

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

8. Statistical analysis of the MIP expression level of recombinant *H. polymorpha* (NRRL2214) in the culture broth in the expression phase

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
MIP0h	H. polymorpha NRRL2214::TP1	3	.0133	.00577	.00333	-.0010	.0277	.01	.02
	H. polymorpha NRRL2214::TP2	3	.0100	.00000	.00000	.0100	.0100	.01	.01
	H. polymorpha NRRL2214::TP4	3	.0200	.00000	.00000	.0200	.0200	.02	.02
	Total	9	.0144	.00527	.00176	.0104	.0185	.01	.02
MIP24h	H. polymorpha NRRL2214::TP1	3	.0167	.00577	.00333	.0023	.0310	.01	.02
	H. polymorpha NRRL2214::TP2	3	.0400	.01000	.00577	.0152	.0648	.03	.05
	H. polymorpha NRRL2214::TP4	3	.0100	.00000	.00000	.0100	.0100	.01	.01
	Total	9	.0222	.01481	.00494	.0108	.0336	.01	.05
MIP48h	H. polymorpha NRRL2214::TP1	3	.0300	.01000	.00577	.0052	.0548	.02	.04
	H. polymorpha NRRL2214::TP2	3	.0400	.01000	.00577	.0152	.0648	.03	.05
	H. polymorpha NRRL2214::TP4	3	.0533	.01528	.00882	.0154	.0913	.04	.07
	Total	9	.0411	.01453	.00484	.0299	.0523	.02	.07
MIP72h	H. polymorpha NRRL2214::TP1	3	.0367	.00577	.00333	.0223	.0510	.03	.04
	H. polymorpha NRRL2214::TP2	3	.3767	.13577	.07839	.0394	.7139	.25	.52
	H. polymorpha NRRL2214::TP4	3	.2067	.06028	.03480	.0569	.3564	.15	.27
	Total	9	.2067	.16492	.05497	.0799	.3334	.03	.52

8.1 ANOVA: *Hansenula polymorpha* NRRL2214

		Sum of Squares	df	Mean Square	F	Sig.
MIP 0 h	Between Groups	.000	2	.000	7.000	.027
	Within Groups	.000	6	.000		
	Total	.000	8			
MIP 24 h	Between Groups	.001	2	.001	16.750	.004
	Within Groups	.000	6	.000		
	Total	.002	8			
MIP 48 h	Between Groups	.001	2	.000	2.846	.135
	Within Groups	.001	6	.000		
	Total	.002	8			
MIP 72 h	Between Groups	.173	2	.087	11.769	.008
	Within Groups	.044	6	.007		
	Total	.218	8			

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8.2 Homogeneous Subsets: *Hansenula polymorpha* (NRRL2214)

MIP at 0 h

	NRRL2214	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Hansenula polymorpha NRRL2214::TP2	3	.0100	
	Hansenula polymorpha NRRL2214::TP1	3	.0133	.0133
	Hansenula polymorpha NRRL2214::TP4	3		.0200
	Sig.		.483	.109

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 24 h

	NRRL2214	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Hansenula polymorpha NRRL2214::TP4	3	.0100	
	Hansenula polymorpha NRRL2214::TP1	3	.0167	
	Hansenula polymorpha NRRL2214::TP2	3		.0400
	Sig.		.483	1.000

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 48 h

	NRRL2214	N	Subset for alpha = .05
			1
Tukey HSD[a]	Hansenula polymorpha NRRL2214::TP1	3	.0300
	Hansenula polymorpha NRRL2214::TP2	3	.0400
	Hansenula polymorpha NRRL2214::TP4	3	.0533
	Sig.		.119

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

MIP at 72 h

	NRRL2214	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	Hansenula polymorpha NRRL2214::TP1	3	.0367	
	Hansenula polymorpha NRRL2214::TP4	3	.2067	.2067
	Hansenula polymorpha NRRL2214::TP2	3		.3767
	Sig.		.112	.112

Means for groups in homogeneous subsets are displayed.

[a] Uses Harmonic Mean Sample Size = 3.000.

VITA

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Academic presentation

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