DEVELOPMENT OF INSULIN PRODUCTION IN *Pichia pastoris* GS115

MISS SASITHORN NGENPRASERTSIRI

A Thesis Submitted in Partial Fulfillment of the Requirements

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การพัฒนาการผลิตอินซูลินใน *Pichia pastoris* GS115

นางสาวศศิธร เงินประเสริฐศิริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Sasithorn Ngenprasertsiri
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Thesis Advisor	Ruethairat Boonsombat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr. rer. Nat)

THESIS COMMITTEE

……..Chairman

(Associate Professor Polkit Sangvanich, Ph.D.)

……..Thesis Advisor

(Ruethairat Boonsombat, Ph.D.)

……..Examiner

(Associate Professor Chanpen Chanchao, Ph.D.)

……..Examiner

(Sarintip Sooksai, Ph.D.)

……..External Examiner

(Wanilada Rungrassamee, Ph.D)

ศศิธร เงินประเสริฐศิริ : การพัฒนาการผลิตอินซูลินใน *Pichia pastoris* GS115 (DEVELOPMENT OF INSULIN PRODUCTION IN *Pichia pastoris* GS115) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. ฤทัยรัตน์ บุญสมบัติ, 70 หน้า

งานวิจัยนี้เป็นการพัฒนาการผลิตอินซูลินชนิด MIP และ HMR1423 โดยใช้ยีสต์สายพันธุ์ *Pichia pastoris* GS115 เป็นเซลล์เจ้าบ้านในการแสดงออกของอินซูลิน โดยทำการศึกษาผลของอุณหภูมิและแหล่งไนโตรเจนที่เหมาะสมต่อการแสดงออกของโปรตีนทั้งสองชนิดมากที่สุด และติดตามปริมาณการแสดงออกของอินซูลินทั้งสองชนิดโดยวิธี Indirect Competitive Enzyme-Linked Immunosorbent Assay (Indirect competitive ELISA) พบว่าอินซูลินชนิด MIP มีการแสดงออกมากที่สุดที่อุณหภูมิ 20 องศาเซลเซียส โดยวัดความเข้มข้นของอินซูลินได้ 39.47 ไมโครกรัมต่อมิลลิลิตรชั่วโมงที่ 84 ในขณะที่อินซูลินชนิด HMR1423 มีการแสดงออกมากที่สุดที่อุณหภูมิ 20 องศาเซลเซียส โดยวัดความเข้มข้นของอินซูลินได้ 32.74 ไมโครกรัมต่อมิลลิลิตรชั่วโมงที่ 24 เมื่อศึกษาผลของแหล่งไนโตรเจนต่อการแสดงออกของอินซูลินทั้งสองชนิดพบว่า MIP สามารถแสดงออกได้มากที่สุดเมื่อใช้ yeast extract และ peptone เป็นแหล่งไนโตรเจนโดยมีความเข้มข้นสูงสุดเท่ากับ 42.52 ไมโครกรัมต่อมิลลิลิตรชั่วโมงที่ 48 ส่วนอินซูลินชนิด HMR1423 มีการแสดงออกมากที่สุดเมื่อใช้แอมโมเนียมซัลเฟตเป็นแหล่งไนโตรเจน โดยวัดความเข้มข้นสูงสุดได้ 32.74 ไมโครกรัมต่อมิลลิลิตรชั่วโมงที่ 24 จากงานวิจัยนี้พบว่าในการผลิตโปรตีนจาก *P. pastoris* แม้ว่าโครงสร้างปฐมภูมิมีความแตกต่างของลำดับกรดอะมิโนเพียงเล็กน้อย แต่ตัวแปรในการผลิตกลับมีความแตกต่างกันอย่างชัดเจน นอกจากนี้การผลิตอินซูลินทั้งสองชนิดมีการแสดงออกสูงสุดในเวลาที่สั้นเมื่อเทียบกับงานวิจัยอื่นซึ่งเป็นผลดีต่อการพัฒนาการผลิตอินซูลินในลำดับต่อไป

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....

ปีการศึกษา.....2555.....ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

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SASITHORN NGENPRASERTSIRI : DEVELOPMENT OF INSULIN PRODUCTION IN *Pichia pastoris* GS115. ADVISOR : RUETHAIRAT BOONSOMBAT, Ph.D., 70 pp

This research is the development of insulin MIP and HMR1423 production in yeast *Pichia pastoris* GS115 as host organism to express insulin. Studying the effects of the optimal temperature and N-sources that produced the highest concentration of both insulin and was detected by Indirect Competitive Enzyme-Linked Immunosorbent Assay (Indirect competitive ELISA). Insulin MIP was expressed the highest concentration at 20^{o}C and the concentration was 39.74 µg/ml at 84 hr whereas insulin HMR1423 was expressed the highest concentration at 30^{o}C and the concentration was 32.74 µg/ml at 24 hr. When the optimal N-sources for both insulin production was studied, MIP expressed the highest concentration when yeast extract and peptone was used with the concentration 42.52 µg/ml at 48 hr , while HMR1423 expressed the highest concentration when ammonium sulfate was used with the concentration was 32.74 µg/ml at 24 hr. From this research, the primary structure of proteins are slightly changed in a few amino acids, parameters in protein production in *P. pastoris* are significantly different. Moreover, the expression of both insulin analogs in this study found in relatively short period of time comparing to the previous studies is a benefit for further development of recombinant insulin production.

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

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LIST OF ABBREVIATIONS

%	percentage
^{o}C	degree celsius
µg	microgram
µl	microlitre
$(NH_4)_2SO_4$	ammonium sulfate
Anti-mouse IgG HRP	anti-mouse immunoglobulin G horseradish peroxidase
Arg	arginine
BSA	bovine serum albumin
$CaCl_2 \cdot 2H_2O$	calcium chloride dihydrate
EDTA	ethylynediaminetetraacetic acid
g	gram
Gly	glycine
H_2SO_4	sulfuric acid
His	histidine
hr	hour
KH_2PO_4	potassium dihydrogen phosphate
L	liter
M	molar
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate

MIP	monomeric insulin precursor or positive control
ml	milliliter
Mut^+	methanol utilize plus
NH_4NO_3	ammonium nitrate
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
Phe	phenylalanine
pSST2	plasmid containing HMR1423 gene
rpm	revolution per minute
v/v	volume by volume
w/v	weight by volume
αA	negative control

CHAPTER I

INTRODUCTION

Rationale

Diabetes Mellitus is a disorder in which blood glucose level is abnormally high because the body does not produce enough insulin to meet its needs or insulin resistance. This results in high levels of sugar in the blood (hyperglycemia) and the inadequate amount of sugar in the cells causing the symptoms and complications of diabetes. Recently, there is a large world demand for human insulin due to a wide prevalence of diabetes. The World Health Organization (WHO) estimated that there were about 177 million diabetic persons in 2000 and would be double more by 2030. Therefore, insulin production is required to be developed. In 1921, insulin was first isolated from cow, and the first injection of insulin for the treatment on human was administered in 1922 (Banting *et al*., 1922). However, bovine insulin is not fully efficient because some patient's immune systems produce antibodies against it resulting in inflammatory responses at injection sites. These factors have been motivated researchers to improve Recombinant DNA Technology for producing human insulin.

Recombinant DNA Technology is a technique that joins DNA molecules from different individuals and puts into host organisms to produce recombinant proteins. Host organisms can be divided in 2 systems: prokaryotic system such as bacteria and eukaryotic system such as yeasts and plants. This technology has many advantages, for example, it is able to produce a high yield of human insulin in relatively short period of time. In 1982, human insulin produced biosynthetically was first introduced commercially (Krall *et al*., 1994). Nowadays, many researchers prefer to use Recombinant DNA Technology to produce and improve insulin production. Furthermore, they prefer to use yeasts as hosts for insulin production because of many advantages such as rapid growth, low cost and very high yields of recombinant protein production (Markussen *et al*., 1984). Moreover, yeasts are eukaryotes, so recombinant insulin produced from yeasts is similar to the one from human

(Gerngross *et al*., 2004). Currently, human insulin produced by Recombinant DNA Technology and used in medical treatment can be divided into 2 types: regular insulin and insulin analogues. Insulin analogues are modified from regular insulin by changing in some amino acids. In the past few years, insulin analogues have been introduced for the treatment of diabetes because the pharmacologic characteristics of the insulin analogues are specific and easy to control (Howey *et al.*, 1994).

Insulin analogs can be divided into 2 types based on their duration of action. The first type is rapid-acting insulin analog or short-acting insulin and the other is long-acting insulin analog. There are now insulin analogs available on the market such as insulin lispro, insulin aspart (short-acting insulin) and insulin glargine (long-acting insulin). Recently, the new insulin analogs have been created by amino acid changes to provide a new pharmacokinetic profile. Unfortunately, some of them lead to induce altered metabolic and mitogenic effects such as the insulin analog Asp(B10) causing breast cancer in rodent (Hamel *et al*., 1999). In 2005, Hennige *et al.* (Hennige *et al*., 2005) constructed a new insulin analog HMR 1423 (Gly[A21], His[B31], His[B32]) and they studied mitogenic effects on the non-malignant human breast cell line MCF10 compared with regular human insulin and Asp(B10). They reported that HMR 1423, similar to regular human insulin, did not have mitogenic activity. In contrast, Asp(B10) showed an increase in mitogenic activity comparing to HMR 1423 and regular human insulin. Therefore, HMR 1423 should be safe in the term of mitogenic activity.

Because the prevalence of diabetes trends to continuously increase, the effective process of insulin production needs to be developed to meet the numerous requirements. Parameters in the production such as temperature and nutrition requirement are the important issues to be concerned. One of the evidences is the report in 2004 about the effects of temperature and nitrogen sources on mini-proinsulin production in *P. pastoris* by Pais-Chanfrau *et al.* (Pais-Chanfrau *et al*., 2004).

In this research, recombinant DNA technology is applied to construct a strain of *P. pasioris* expressing insulin homolog HMR1423. Then, the effects of temperature and nitrogen sources on the production of HMR 1423 in a laboratory scale have been focused. Recombinant insulin is measured by the technique enzyme-linked

immunosorbent assay or ELISA. In several years, techniques to detect a small amount of proteins in the samples have been developed. ELISA is one of them because of its high sensitivity and specificity which are the benefits over other methods such as PAGE and western blot.

The output from this project will be applied not only for the further process of insulin product, but also for the production of the higher scale and even the production of other recombinant products. Moreover, with the effective of insulin production, the import of this hormone for medical treatment will be reduced.

Objective:

To improve the insulin analog HMR 1423 production in *Pichia pastoris* GS115 Mut^{+}

CHAPTER II

LITERATURE REVIEWS

2.1 Diabetes mellitus

Diabetes or diabetes mellitus is a one of the top ten diseases that causes the death of people around the world. To motivate people for raising global awareness of diabetes, the world diabetes day was established by the world health organization (WHO) and the international diabetes federation. Diabetes is a disorder which blood sugar levels are abnormally high. In normal person, stomach digests food into glucose form, then entering into the blood circulation. Raised blood glucose triggers the pancreatic cells to produce and secrete insulin into the bloodstream which stimulates glucose uptake into tissue cells, so glucose levels are decreased and returned to the normal level. In a diabetic person, the pancreatic cells produce and secrete an insufficient amount of insulin, or body cells are insulin resistance, therefore tissue cells cannot uptake glucose causing an increase in blood glucose level (World Health Organization, 1999). The differences in glucose uptake system between a normal and a diabetic person represented as Figure 2.1.

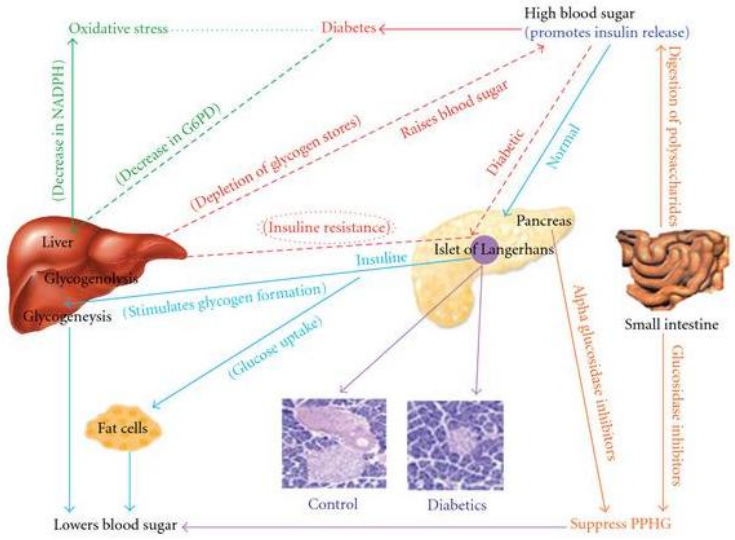


Figure 2.1 The differences in glucose uptake system between a normal and a diabetic person (Reference: http://www.hindawi.com/journals/ecam/2011/561625/fig7)

2.1.1 Types of diabetes mellitus

2.1.1.1 Insulin-dependent diabetes or formally called type I diabetes

Type I diabetes is a chronic disease normally found in children. Only 10 percent or less of worldwide diabetic patients is type I diabetes. Because most of the pancreatic cells producing insulin hormone are destroyed, a small amount or none of insulin is secreted. The cause of type I diabetes is still unknown, but many scientists believe that it is stimulated by many factors such as a viral infection and a genetic predisposition (Daneman, 2006).

2.1.1.1.1 Symptoms of type I diabetes

Patients with type I diabetes have the symptoms that can be sudden, powerful and severe. One of a critical symptom is called diabetic ketoacidosis. Because of the lack of insulin, most of the cells cannot uptake glucose which causes an increase in blood glucose. Without glucose in the cells, cellular energy is generated by fat break down giving ketone as a product. High level of blood ketone is a cause of an increase in acidity which many symptoms are subsequently occurred such as urination, thirsty, blurred vision, weight loss, abdominal pain and finally being able to die in a few minutes (American Diabetes Association, 1993).

2.1.2.1 Non-insulin-dependent diabetes or type II diabetes

Type II diabetes is a metabolic disorder in which insulin is continuously produced, however, the body resists to it. In this case, the body cannot use enough insulin resulting in a high glucose level in the bloodstream. Type II diabetes normally found in adults. Figure 2.2 illustrates the risks for non-insulin-dependent diabetes, for example, taking a certain kind of medicine for a long time, not exercising and overweight. Type II diabetic patients have increased emphatically over 50 years ago along with obesity (Tuomilehto *et al*., 2001).

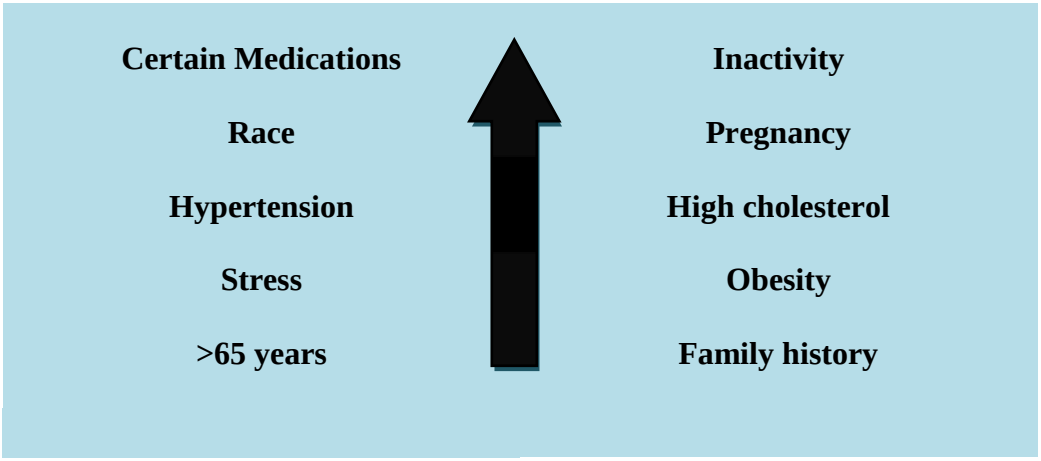


Figure 2.2 The risks for type II diabetes

2.1.2.1.1 Symptoms of Type II diabetes

Patients with type II diabetes are frequently found themselves polyuric, thirsty, hungry, itching, losing weight and peripheral neuropathy. Many complications can be found in a type II patient due to this chronic disease leading to a high risk of cardiac disease, kidney failure, blindness, infection and Alzheimer (Ferdinand *et al*., 1996). The symptoms of type I and type II are shown as in Figure 2.3.

2.1.2 Diagnosis of diabetes mellitus

Both types of diabetes can be pronounced when a person has an unusual increase in blood glucose level. Many kinds of tests can be performed to diagnose such as fasting blood sugar test, Hemoglobin A1c test and oral glucose tolerance test.

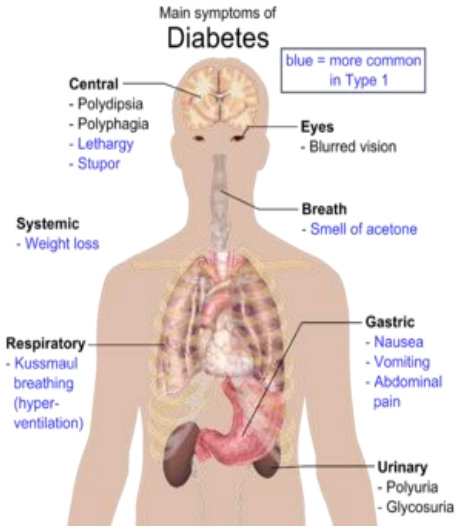


Figure 2.3 The different of symptoms of type I and type II diabetes

(Reference: http://www.medicalnewstoday.com/info/diabetes)

2.1.2.1 Fasting blood sugar test

The fasting blood sugar test, as known as the fasting plasma glucose test (FPG), is a measurement of the blood glucose in diabetes patients which is simple and cheap. The principle of this test is when a patient fasts for hours, the hormone glucagon is stimulated to trigger glucose releasing from liver into the bloodstream. This increases the blood glucose level. In normal person, the body can produce insulin into the blood to stimulate glucose uptake into the cells, so the glucose level returns to normal preventing one from hyperglycemia. However, in diabetic patients with insufficient insulin or insulin resistance, the blood glucose remains high (Moorhouse *et al*., 1964).

The FPG test is performed after a patient has fasted for at least 12 hr. The test is normally done in the morning. The glucose levels from this test can be interpreted in milligram/deciliters (mg/dL) as the following:

- The normal range : 70-90 mg/dL
- The emerging diabetes or prediabetes : 100-126 mg/dL
- The diabetes : > 126 mg/dL

The other test might be done to confirm if the results are uncertain.

2.1.2.2 Hemoglobin A1c test

The hemoglobin A1c test, or HbA1c, is a significant and specific blood glucose test. This test determines how diabetes can be administered. When the blood sugar is not controlled, accumulating glucose in bloodstream binds to hemoglobin forming as glycolated. Therefore, HbAc1 test can diagnose blood sugar level (Koenig *et al*., 1976). An advantage of this test is that it is less false positive than FPG. Moreover, HbA1c is able to detect the glucose level over 2-3 months ago; therefore, it is applied as an index how the patient has monitored blood glucose lately. The HbA1c level is 4-5.6 % in a normal person, 5.7-6.4 % in a prediabetic, and higher than 6.5% in a diabetic person. The patient with HbA1c higher than 6.5% also has higher risks of some complications associated with diabetes.

2.1.2.3 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) is usually applied to pregnant women, elders and overweight people. For OGTT, it determines how fast glucose is cleared from the bloodstream. With this test, one has to drink a certain dose of glucose solution, and then the blood sample is taken every 2-3 hrs to detect the glucose level in the blood (Stumvoll *et al*., 2000).

2.1.3 Treatment of diabetes mellitus

The common treatment in a diabetic person consists of dieting, exercise and management of one's lifestyle. With a careful risk control, some complications trend to be less emerging. Awareness to high sugar and cholesterol levels can also protect patients from some complications. Patients should be educated about proper diet, exercise, blood glucose control and medication. Nutrition manipulation is very essential in diabetic persons. Patients with diabetes tend to have increased cholesterol, therefore, the amount of fat must be limited and cholesterol controlling medicine may be necessary. Proper exercise can be help patients to handle their weight and glucose levels in the normal (Stephen *et al*., 1992). Furthermore, some drugs are used to treat some patients such as insulin, metformin and sulfonylureas. Previously, insulin treatment was a priority treatment for a patient who could not control blood glucose

level and very efficient to help to preserve the pancreatic cells producing insulin (Banting *et al*., 1922).

2.1.4 The prevalence of diabetes mellitus

The worldwide prevalence of diabetes, investigated by Wild *et al.* (2004), was reported that in 2000, there were about 170 million diabetic patients and would be double more by the year 2030. Most of patients are Type II diabetes with a continuously increasing incidence. The highest increase in prevalence is estimated to be occurred in Asia and Africa by 2030 because of the alternation of diet lifestyle to Western-like. Furthermore, India has the highest risk of diabetes in the future as representing in Table 2.1

Table 2.1 List of countries with the highest numbers of estimated cases of diabetes for 2000 and 2030

	2000		**2030**	
Ranking	**country**	**Diabetic peple (millions)**	**country**	**Diabetic peple (millions)**
1	India	31.7	India	79.4
2	China	20.8	China	42.3
3	U.S.	17.7	U.S.	30.3
4	Indonesia	8.4	Indonesia	21.3
5	Japan	6.8	Japan	13.9
6	Pakistan	5.2	Pakistan	11.3
7	Rassian Federation	4.6.	Rassian Federation	11.1
8	Brazil	4.6	Brazil	8.9
9	Italy	4.3.	Italy	7.8
10	Bangladesh	3.2	Bangladesh	6.7

Reference: Wild *et al* (2004).

2.2 Insulin Hormone

Insulin is a hormone produced by pancreatic cells and controls many kinds of the body cells. This hormone is a key regulator of the blood glucose.

2.2.1 The discovery of insulin

In 1869, Paul Langerhans, a German medical student, found clusters of cells in the pancreatic tissue generating digestive juices with unknown function and some of these cells, called beta cells, can produce insulin hormone. Later, the cell clusters were named as the islets of Langerhans to honor Paul Langerhans (Langerhans, 1868).

In 1889, Oskar Minkowski and Josept Von Mering, the scientist in Germany, found that a dog with pancreas removal became diabetes. However, when they treated the dog with the pancreatic juices through the intestine, the dog wasn't diabetes. So, they concluded that the pancreatic cells could produce juices and substances that control the blood glucose. This hypothesis leaded scientists to progress and discover the real function of the pancreatic cells (Luft , 1989).

In 1921, Dr. Frederick Banting and a medical student named Charles Best started their experiment by removing a dog's pancreas. They found that this dog had an increase in blood glucose, drank a lot of water, urinated more frequently and became weak. Finally, the dog developed diabetes. Then, they injected an extract named "isletin" which was isolated from another dog's pancreas into the diabetic dog. They found that the dog became healthier, stronger without diabetic symptoms (Banting *et al*., 1922).

In late 1921, the pancreas substance was isolated and called as insulin. It was used to treat a boy, Leonard Thomson. The treatment was successful and expanded to treat other diabetic volunteers. With this discovery, Banting and his team received the Nobel Prize in Physiology or Medicine in 1923 (Banting *et al*., 1922).

2.2.2 Biosynthesis and protein structure

Insulin hormone can be found in many animals such as human, chimpanzee, cow, mouse, etc. The synthesis of hormone insulin begins in a kind of the pancreatic cells called beta cell. Preproinsulin secreted from beta cell is assembled in

endoplasmic reticulum. Then, the removal of signal peptide by protease changes preproinsulin to proinsulin. Proinsulin consists of 3 polypeptides: A chain, B chain and C chain. Disulfide and C-peptide bridges link A chain and B chain together (Katsoyannis *et al*., 1964). To become active, endopeptidases cleave off the C chain from proinsulin (Figure 2.4). Finally, mature insulin has 51 amino acids: 21 amino acids in A chain and 30 amino acids in B chain joining with disulfide bonds (Figure 2.5). In vertebrates, there are similarities in the amino acid sequences of insulin. Bovine and porcine insulin are three and one amino acid different from human insulin, respectively.

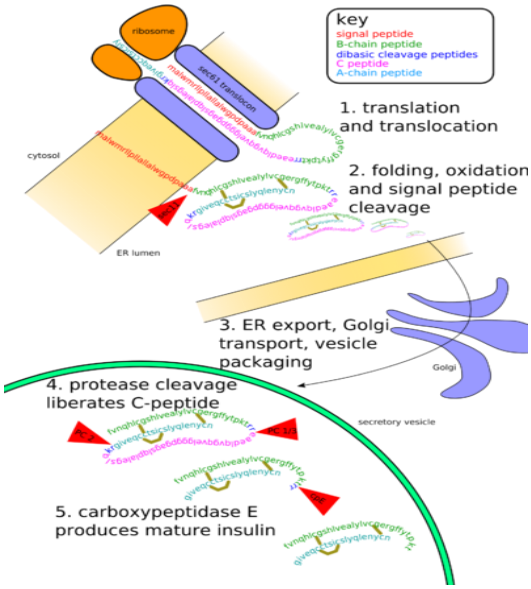


Figure 2.4 Synthesis of insulin hormone (Reference: www.wikipedia.org)

In 1958, Frederick Sanger found the primary structure and sequence of bovine insulin. Insulin structure is a hexamer, but the monomer is the active form. The center of insulin structure is hydrophobic while the outside are hydrophilic with many positive charge amino acids (Sanger, 1959).

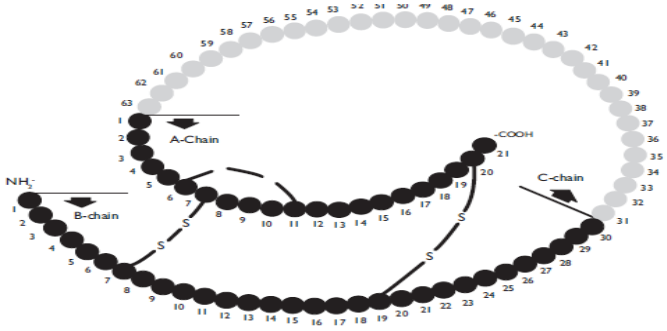


Figure 2.5 The structure of Insulin (Reference: Joshi *et al*., 2007)

2.2.3 Insulin Secretion and physiological effects

Insulin is synthesized and secreted from β-cells in pancreas. The secretion is simulated by many stimuli such as glucose, amino acids and fatty acids. The primary function of this hormone is to decrease the blood glucose. The binding of insulin to insulin receptor activates signal transduction cascade such as phosphatidylinositol-3'-kinase (PI-3-kinase) pathway and mitogen-activated protein kinase (MAPK) pathway in cells. After signaling PI-3-kinase, glucose transporter proteins in cell membrane are relocated and bound to cell membrane. Finally, glucose is allowed to enter into the cell causing a decrease in blood sugar. Moreover, the physiological effects of insulin secretion are also metabolic and mitogenic such as an increase in DNA replication, protein synthesis, and enzyme modification. The other actions of insulin on cells include increasing in esterification, secreting of hydrochloric acid, and decreasing in proteolysis (Najjar, 2001) and autophagy (Bergamini *et al*., 2007).

2.2.4 Pharmacological properties of insulin

Currently, insulin can be divided into 5 types based on the speed off on set and duration of action. Insulin analogs are modified by altering some amino acids of regular insulin sequence to improve the novel properties such as insulin Lispro and insulin Aspart. The insulin and insulin analog time courses of action are shown as in Table 2.2

Table 2.2 Time course action of insulin

Types of insulin	Onset of Action	Peak Action	Effective Duration of Action
Rapid-Acting insulin Analogs			
Insulin Lispro	5-15 min	30-90 min	3-5 h
Insulin Aspart	5-15 min	30-90 min	3-5 h
Insulin Glulisine	5-15 min	30-90 min	3-5 h
Short-Acting insulin			
Regular insulin	30-60 min	2-4 h	5-8 h
Intermediate-Acting insulins			
NPH	2-4 h	4-10 h	10-16 h
Lente	3-4 h	4-12 h	12-18 h
Long-Acting insulins			
Ultralente	6-10 h	10-16 h	18-24 h
Insulin Glargine	2-4 h	Peakless	20-24 h
Insulin Determir	2-4 h	6-14 h	16-20 h
Insulin Mixtures			
70/30 human mix (70% NPH, 30% regular)	30-60 min	Dual	10-16 h
75/25 lispro analog mix (75% intermediate, 25% lispro)	5-15 min		10-16 h
70/30 aspart analog mix (70% intermediate, 30% aspart)	5-15 min		10-16 h
50/50 lispro analog (50% intermediate, 50% lispro)	30-60 min		10-16 h
50/50 human mix (50% NPH, 50% regular			

Reference: Joshi *et al*., 2007

2.2.4.1 Rapid-acting insulin analogs

2.2.4.1.1 Insulin Lispro

In 1994, lispro was the first effective insulin analogs (Ved, 2003). The structure of lispro is similar to regular insulin, but their amino acids, Lysine and proline in the B chain, are inversed resulting in preventing this analog to form a hexamer. So, it is more quickly absorbed when injected subcutaneously. Furthermore, the duration of action is shorter than the human insulin. The action of lispro starts in 3-5 minutes and effective in 3-5 hr. The more dose of insulin lispro, the more duration of action. Because of its rapid action, lispro is able to be taken before the meal about 15 minutes which it can protect and decrease postprandial hypoglycemia. The primary structure of the insulin lispro is illustrated in Figure 2.6

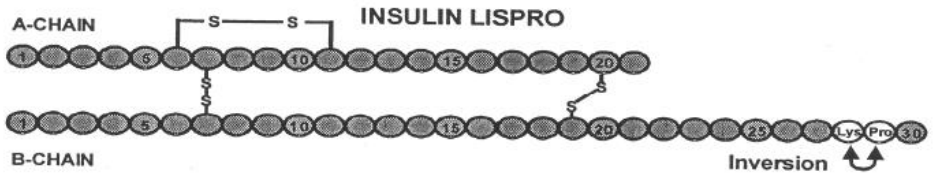


Figure 2.6 Primary structure of insulin analog Lispro

(Reference: Ved VG, 2003)

2.2.4.1.2 Insulin Aspart

In the B chain of this analog, proline is substituted by Aspartic acid at position 28. The onset of action begins in 3-5 minutes and the highest peak action is in 30-90 minutes. Home *et al*. (2000) compared the efficiency of insulin aspart with human insulin (regular insulin) and found that insulin aspart was small and rapid-acting which improved long-term blood glucose and hypoglycemia control. Insulin aspart structure is shown as in Figure 2.7

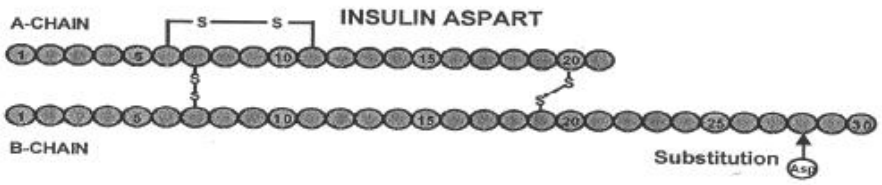


Figure 2.7 Primary structure of insulin analog Aspart

(Reference: Ved, 2003)

2.2.4.2 Short-acting insulin

Regular insulin is a native form of human insulin used to prevent and control the glucose level after the meal. It needs to be injected 1-2 times per day. Normally, regular insulin is combined with long-acting insulin and used in an insulin pump. However, a drawback of the regular insulin is hypoglycemia because its onset of action begins in 30-60 minutes (Heinemann *et al*., 1999). Therefore, doctors prefer to use rapid-acting to short-acting insulin in some patients.

2.2.4.3 Intermediate-acting insulin

2.2.4.3.1 Neutral protamine hagedorn insulin or NPH insulin

In 1964, NPH was administered in diabetic persons (Krall *et al*., 1994). To create NPH, protamine, a positive charge of polypeptide is added to the regular insulin. So, the duration of action is more prolonged comparing to the regular one. However, the onset of action is slow. NPH is one of the choices to combine with rapid-acting insulin or short-acting insulin for regulating the blood sugar after the meals. Although NPH is commercial but it has many serious side effects such as an allergic reaction. Moreover, it is not suitable to treat some patients such as patient with a kidney failure or a liver disease, or a pregnant woman.

2.2.4.3.2 Lente insulin

Lente insulin, developed in 1950, is bovine insulin mixed with zinc to make intermediate acting insulin. The onset of action starts slower, but the duration of action is more prolonged than NPH (Lepore *et al*., 2000). Lente insulin is usually combined with short-acting insulin and injected before the meals to monitor the blood

glucose level after eating. This insulin is injected under the skin, especially upper arm and abdomen. The side effects of lente insulin are similar to NPH insulin.

2.2.4.4 Long-acting insulin

2.2.4.4.1 Insulin Glargine or Lantus®

This insulin analog of which Asparagine at position 21 in A chain is substituted by Glycine and 2 Arginine molecules in B chain are added. This is produced by applying recombinant DNA technology. Glargine is composed of microcrystal that decreases insulin release, so the duration of action is more prolonged comparing to other insulins. Insulin glargine is injected only once a day, but can control the blood glucose level all day. Moreover, previous studies reported that Glargine could reduce the risk of hypoglycemia when compared with NPH.

2.2.4.4.2 Insulin Determir or Levemir®

Insulin Detemir is a recombinant insulin produced by yeast *Saccharomyces cerevisiae.* It is a long-acting insulin analog that Lysine at position 29 is bound to a fatty acid. The long duration of action results from the ability to be fast absorbed with albumin and dissociated lately from this complex. The efficiency and safety of Detemir was studied by treating with Type II patients. Detemir is suggested to decrease Hemoglobin A1c in patients without any risks of hypoglycemia (Moser *et al*., 2012). Determir is injected once or twice per day before the morning meal and/or after the evening meal, and before bedtime. It is not recommended to use in some patients with symptoms as hypoglycemia or combine with other insulins. The structures of glargine and determir are illustrated as Figure 2.8.

Although many types of insulin have been applied to treat diabetic patients, some types are not suitable to some cases. Therefore, the development of insulin structure is necessary. Recently, potentially effective and safe insulin analogs have been developed for diabetic treatment such as Monomeric B27 Lys Destripeptide insulin (MIP) and insulin HMR1423.

A. Insulin Glargine B. Insulin Determir

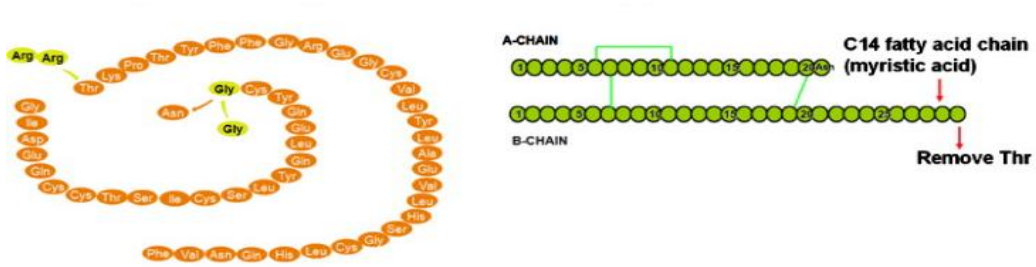


Figure 2.8 The primary structure of Insulin Glargine and Insulin Determir (Reference: Moser *et al*., 2012)

- Monomeric B27 Lys Destripeptide (MIP)

In 2003, monomeric B27 Lys destripeptide was constructed by Ding *et al*. In the B chain, amino acids at position 28-30 are removed and one at position 27 is substituted by lysine. Its biological activity compared with regular insulin is 80%. It is suggested that this is a new fast-acting insulin analog.

- Insulin HMR1423

In 2005, insulin HMR1423 was created and became a novel insulin analog (Hennigue *et al*., 2005). The different between HMR1423 analog and human insulin is that, in the B chain of HMR1423, 2 histidine molecules are added, and in the A chain, asparagines is substituted by glycine at residue 21. It is a new rapid-acting insulin with more prolonged duration of action than the others. With the addition of two histidine molecules, Zinc ions can bind at this position making this analog remain in the blood for a long time. The duration of action of insulin mixture depends on the ratio of insulin HMR1423 to the others. Insulin HMR1423 is a new analog with new properties that are potentially effective for diabetic treatment in some patients.

2.3 Improvement of insulin production

2.3.1 Recombinant DNA technology

Currently, recombinant DNA technology is used to improve and produce large amount of human and other insulin analogs to response to a huge demand of insulin in the future.

Recombinant DNA technology is a technique that joins DNA molecules from different species and put into a host to produce recombinant proteins. Recombinant DNA technology has many advantages, for example, it is able to produce a high yield of recombinant proteins in a relatively short period of time. Recombinant DNA technology can be divided into 4 steps:

- Selection of target gene
- Creating of recombinant DNA
- Transformation of recombinant DNA into host cells
- Screening transformants

Host organisms can be divided in 2 systems: Prokaryotic and Eukaryotic systems.

2.3.1.1 Prokaryotic systems

Escherichia coli is a commonly strain used to produce recombinant proteins including insulin. For example, in 1979, David *et al*. constructed the A and B chain separately and cloned independently in *E. coli*. Then, both chains were fused *in vitro* to construct a native insulin. However, problems from this research are random forming of disulfide bonds, lower biological activity and high cost of production.

The advantages of *E. coli* such as rapid growth, many vectors for cloning and easy transformation. However, the drawbacks for this host include non-posttranslational modification and some toxin production.

2.3.1.2 Eukaryotic systems

Recently, the eukaryotic systems are preferable to produce recombinant insulin because of a posttranslational modification which makes this eukaryotic

protein in active form (David *et al*., 2009). Eukaryotic hosts for recombinant protein expression can be divided into 3 types:

- Yeasts
- Insect cells
- Mammalian cells

Currently, many researchers prefer to use yeast as a host for insulin production because of many advantages such as quick growth, low cost and very high yields of recombinant protein production. Moreover, glycosylation and signal peptide removal make recombinant insulin produced from yeast similar to human.

2.3.1.2.1 *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast that has ability to utilize methanol as a carbon source. Orientation to growth on methanol is regulated by a strong promoter, *AOX*, along with several enzymes that involved in methanol metabolism. The yeast *P. pastoris* was initially developed by Phillips Petroleum Company for single cell protein. In 1988, the yeast *P. pastoris* expression technology has been licensed by many pharmaceutical and biotechnology companies such as Hepatitis B surface antigen and human serum albumin. Phillips Petroleum Company also attempted to improve *P. pastoris* as an expression system for recombinant protein production available for researches in universities and non-profit organizations (Wegner, 1990).

- Growth and storage

P. pastoris grows well on liquid and solid media with a variety of carbon sources such as glucose, glycerol, sorbital and methanol. The doubling time is dependent on carbon sources, for example, it grows rapidly in glucose. On solid media, it forms non filamentous beige color colonies (Figure 2.9). The natural habitats of *P. pastoris* are oak trees and packaging foods. The optimal temperature for growth in liquid media is 30^{o}C with the shaking speed of 250-300 rpm. *P. pastoris* can grow in pH 3-6.5 which buffering is not necessary. However, *P. pastoris* cannot grow in galactose, arabinose, ribose, cellulose etc.

P. pastoris should be kept at 4^{o}C in YPD liquid or solid media. For a long-term storage, cells should be kept in 50% glycerol at -80^{o}C.

Figure 2.9 The morphology of *P. pastoris* on solid medium

- Strains

There are 7 available *P. pastoris* strains as the following:

GS115	*his4* (Sreekrishna *et al*, 1987)
KM71	*his4*, *aox1::ARG4* (Cregg and Madden, 1988)
NRRL Y-11430-SC5	wild type (Sreekrishna *et al*, 1987)
PPF1	*his4*, *arg4* (J.M. Cregg, pers, comm.)
SMMD1163	*his4, pep4, prB1*
SMMD1165	*his4, prB1*
SMMD1168	*his4, pep4*

P. pastoris has been preferably used to improve insulin production to *Saccharomyces serevisae*. It is an excellent system for expressing heterologous proteins with many advantages including the powerful and methanol regulating *AOX1* promoter, a stable expression of an integrated target gene, high heterologous protein productivity, and simple culture medium requirement.

In 2004, Hose *et al*. studied the effects temperature, pH and nitrogen sources on miniproinsulin production. They suggested that combination of EDTA and ammonium sulfate with pH 6.3 at 22^{o}C during production phase would raise insulin protein production.

In 2005, Manuel *et al*. improved expression vector and studied expression level of miniproinsulin in *P. pastoris* GS115 (*his4*) at 22^{o}C. They constructed plasmids with 1, 5 and 11 copies number, respectively and integrated onto chromosomal DNA. They found that the more copy numbers in *P. pastoris* chromosomal DNA, the higher insulin expression levels.

In 2010, Chandrasekhar *et al*. developed fermentation process to enhance insulin precursor production by dividing simple cultivation batch process into 2 phases: glycerol batch for growth and constantly methanol concentration for fed-batch cultivation. They suggested that insulin precursor production was significantly high by using simple cultivation method compared with previous studies.

2.4 Detection of insulin protein

Currently, there are many conventional methods to detect protein such as western blot, enzyme assay and spectrophotometry. However, these techniques have some limitations; for example, small protein including peptides, antibodies and hormones cannot be detected, and also come with low sensitivity and low specificity. So, some techniques have been improved to solve this problem.

Enzyme-linked immunosorbent assay or ELISA has been developed to be used for protein detection in laboratories. The principle of this technique results from the specific binding site between antibody and antigen. When the secondary antibody linked with enzyme is added, it reacts with the substrate to enhance sensitivity. A visible signal is detected from the absorbance by the micro plate reader.

ELISA can be divided into 2 types including:

- Direct ELISA
- Indirect ELISA

2.4.1 Direct ELISA

Direct ELISA is used to detect quantity and quality of antigen that coated on micro well plate. The advantages of this technique include simple and rapid procedure with less sample and high sensitivity (Beier *et al*., 1988). The drawbacks include primary antibody that must be labeled with enzyme, time consuming and high cost. This technique is composed of 6 steps illustrated in Figure 2.10

I. Coating antigen on 96 well plate
II. Blocking with blocking solution
III. Washing with washing solution.
IV. Incubating with primary antibody labeled with enzyme.
V. Adding substrate to develop color.
VI. Detecting the absorbance by the micro plate reader.

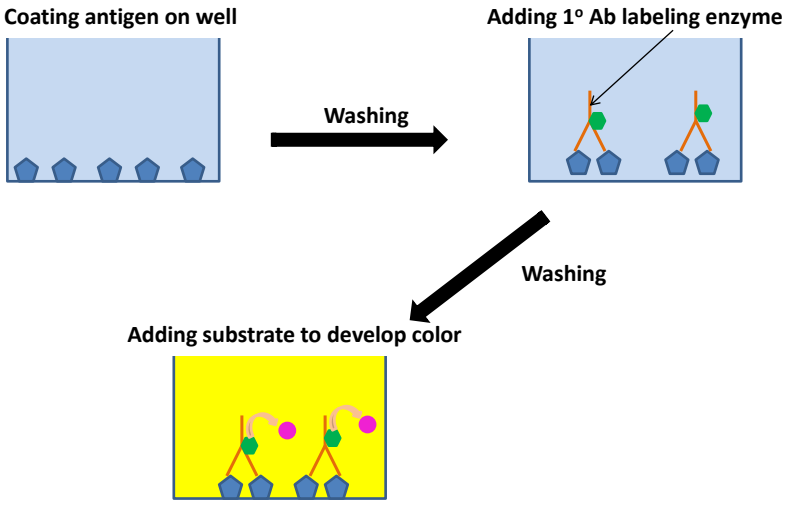


Figure 2.10 The principle of direct enzyme-linked immunosorbent assay

2.4.2 Indirect ELISA

The difference between indirect ELISA and direct ELISA is that the primary antibody of indirect ELISA is not labeled with enzyme (Barbara *et al*., 1982). The color is developed by enzyme linked with the secondary antibody which binds specifically with primary antibody. This is an advantage of this technique because the secondary antibody labeling enzyme can be constructed commercially which can be applied with a variety of primary antibodies. Moreover, it is more sensitive with lower cost. However, a disadvantage of this technique is cross-reactivity. Indirect ELISA can be divided into 7 steps shown as Figure 2.11:

I. Coating antigen on 96 well plate
II. Blocking
III. Washing with washing buffer mixing with Tween20
IV. Adding and incubating with primary antibody non-labeling enzyme
V. Washing again, adding and incubating with the secondary antibody labeling enzyme
VI. Adding substrate solution to develop color
VII. Detecting the absorbance by the detector

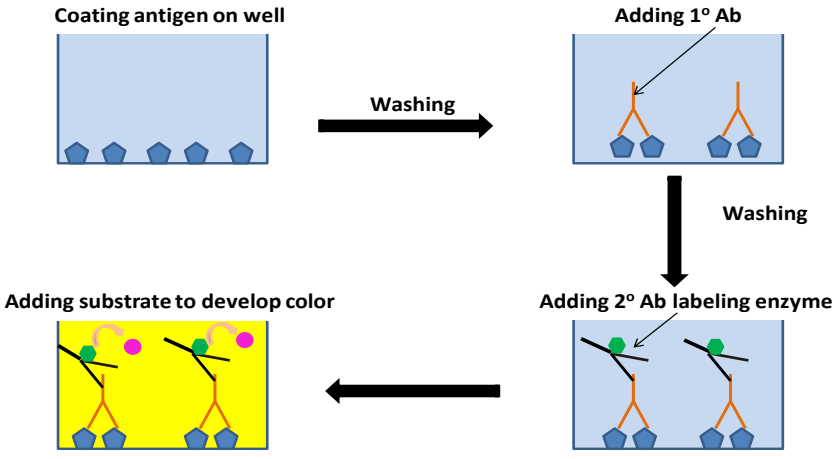


Figure 2.11 The indirect enzyme-linked immunosorbent assay

2.4.2.1 Indirect competitive ELISA

The indirect competitive ELISA has been developed to detect the quantity of specific protein in samples. The exact standard antigen concentrations are used to construct the standard curve. The OD values of samples are compared to the standard curve to interpret the concentration of target protein in samples. However, this technique has some disadvantages as described before in indirect ELISA.

CHAPTER III

METHODOLOGY

3.1 Materials

3.1.1 Microorganisms and plasmids

Pichia pastoris GS115 Mut^+ and plasmids pPICZαA were purchased from Invitrogen, USA. The gene of insulin analog HMR1423 inserted in pJET1.2 was synthesized by First Base Company, Malaysia. *Pichia pastoris* GS115 Mut^+ containing MIP genes (Ding *et al*., 2005) was obtained from Dr. Sarintip Sooksai, Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand. *E.coli* DH5α was used for cloning all the plasmids.

3.2 Chemical and reagents

Chemical and reagents	Company and country
Absolute methanol	Merck, USA
Agarose	Research Organics, USA
Ammonium nitrate	Carlo Erba Reagenti, Italy
Ammonium sulfate	Ajax Finechem, Australia
Bacto peptone	Bio Basic Inc., Canada
Biotin	Fluka, Germany
Boric acid	Sigma Aldrich, Germany
Calcium chloride dihydrate	Merck, USA
Chloroform	Merck, USA
Cobalt chloride	Sigma Aldrich, Germany
Cobalt chloride hexahydrate	Sigma Aldrich, Germany
Cupric sulfate pentahydrate	Carlo Erba Reagenti, Italy

Chemical and reagents	Company and country
Dextrose	Bio Basic Inc., Canada
Dipotassium hydrogen phosphate	Carlo Erba Reagenti, Italy
Ehylene diamine tetraacetic acid	Sigma Aldrich, Germany
Ethidium bromide	Bio Basic Inc., Canada
Ferrous sulfate heptahydrate	Carlo Erba Reagenti, Italy
Glutaraldehyde	Merck, USA
Glycerol	Sigma Aldrich, Germany
Histidine	Fluka, Germany
Isoamyl alcohol	Merck, USA
Magnesium sulfate heptahydrate	Carlo Erba Reagenti, Italy
Manganese sulfate monohydrate	Carlo Erba Reagenti, Italy
Monoclonal anti-insulin antibody	Sigma Aldrich, Germany
Phenol	Merck, USA
Phosphoric acid	Merck, USA
Potassium chloride	Bio Basic Inc., Canada
Recombinant insulin in *P. pastoris*	Merck Millipore, Germany
Secondary goat anti – mouse lgG-HRP	Jackson Immuno Research Laboratories Inc., USA
Skim milk	Fonterra, New Zealand
Sodium acetate	Ajax Finechem, Australia
Sodium chloride	Ajax Finechem, Australia
Sodium dodecyl sulfate	Merck, USA

Chemical and reagents	Company and country
Sodium iodide	Merck, USA
Sodium molybdate dehydrate	Merck, USA
Sodium pyrophosphate	Merck, USA
Sulfuric acid	Merck, USA
Tris	Merck, USA
Triton X-100	Sigma Aldrich, Germany
Tween-20	Sigma Aldrich, Germany
Urea	Sigma Aldrich, Germany
Yeast extract	Bio Basic Inc., Canada
Zeocin	Invitrogen, USA
Zinc chloride	Sigma Aldrich, Germany
3,3'- Diaminobensidine tetrahydrochloride	Sigma Aldrich, Germany

3.3 Equipments and supplies

Equipments and supplies	Company and country
Agarose gel electrophoresis equipments	Advance Co. Ltd., Japan
Autoclave	Taladlab, Thailand
Cellulose acetate filter, pore size 0.45µm	Sartorius, Germany
Centrifuge tubes	Labcon, USA
Freezer -20^{o}C	Sanyo, Japan
Freezer -70^{o}C	Sanyo, Japan

Equipments and supplies	Company and country
High speed micro refrigerated centrifuge: model MTX-150	Tomy Seiko Co. Ltd., Japan
Hot plate stirrer: model C-MAG HS7	IKA, China
Laminar flow: model HF safe-12006, Heal Force	China
Micro auto pipette: model Discovery Comfort	Mettler Toledo, USA
NucleoSpin® Extract II Kits	MACHEREY-NAGEL, Germany
NucleoSpin® plasmid	MACHEREY-NAGEL, Germany
Petridish plate	Mettler Toledo, USA
pH meter	Labcon, USA
Pichia EasyComp™ Transformation Kit	Invitrogen, USA
Pipette tips	Thermo Scientific, Finland
Polymerase chain reaction Thermocycer	Applied Biosystems, Singapore
Refrigerated centrivap concentrator	Labconco, USA
Refrigerated high speed centrifuge	Kubota cooperating, Japan
Synergy™ HT multi-detection microplate reader	Biotek, USA
UV transilluminator	Bromma, USA
Uvitec platinum gel documentation system	UVItec, UK
UV-visible recording spectrophotometer: model UV-160	Shimadzu corporate, Japan
Vortex mixer: model Vortex-Genie2	Scientific Industries, USA
Water bath	Yamato, Japan

3.4 Enzymes and restriction enzymes

*Eco*RI-HF™	Biolab, England
Lyticase	Invitrogen, USA
*Not*I-HF™	Biolab, England
*Nla*III	Biolab, England
RNase	Biolab, England
*Sac*I	Biolab, England
Taq polymerase	Sigma, USA

3.5 Methods

3.5.1 Construction of *P. pastoris* strains with HMR1423 genes

The synthetic structure gene encoding insulin HMR 1423 with B_{1-32} (His[B31], His[B32]) – ArgArgPhe - A_{1-21} (Gly[A21]) (Figure 3.1), was inserted in the plasmid pJET1.2 (First Base Company, Malaysia) was cut by *Eco*RI and *Not*I , then inserted between the same restriction sites of pPICZαA to generate the plasmid named pSST2. The plasmid was transformed into *E. coli* DH5α competent cells and plated on low salt LB solid media with 25 µg/ml Zeocin. The pSST2 was extracted from the transformants and cut at the 5' *AOX1* region by *Sac*I to linearize it. Then, linear DNA was transformed into the yeast *P. pastoris* GS115 mut^{+} competent cells using *Pichia* EasyComp^{TM} Transformation Kit (Invitrogen, USA). YPD plates (see appendix A) with 100 µg/ml Zeocin were used to select transformants with the plasmids pSST2 (pPICZαA with HMR1423). The integration of insulin analogue HMR1423 gene from pSST2 onto *P. pastoris* chromosome was screened by the colony PCR with *AOX1* forward and *AOX1* reverse. This selected strain was named as NGEN2. The construction of *P. pastoris* strains with HMR1423 genes shown as the diagram in Figure 3.2.

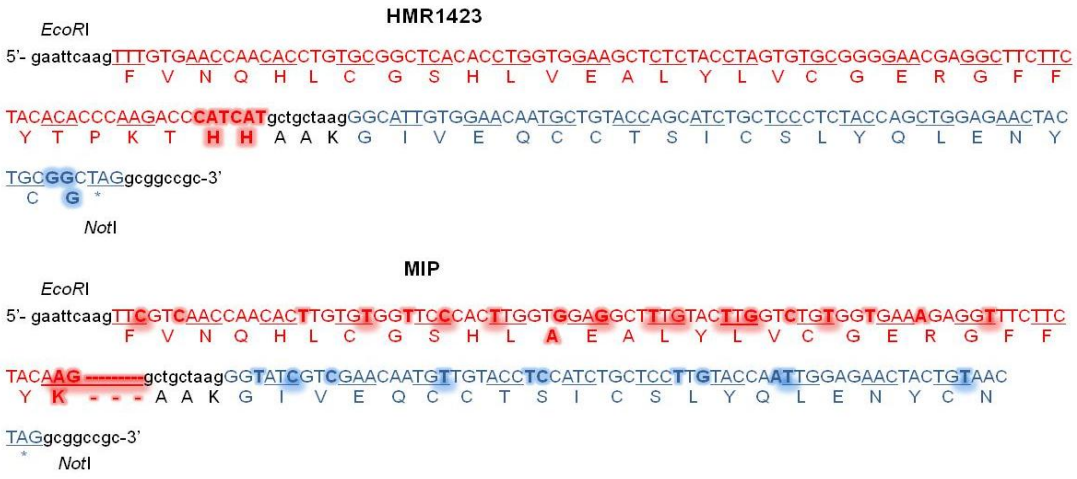


Figure 3.1 The nucleotide sequences of insulin HMR1423 and MIP

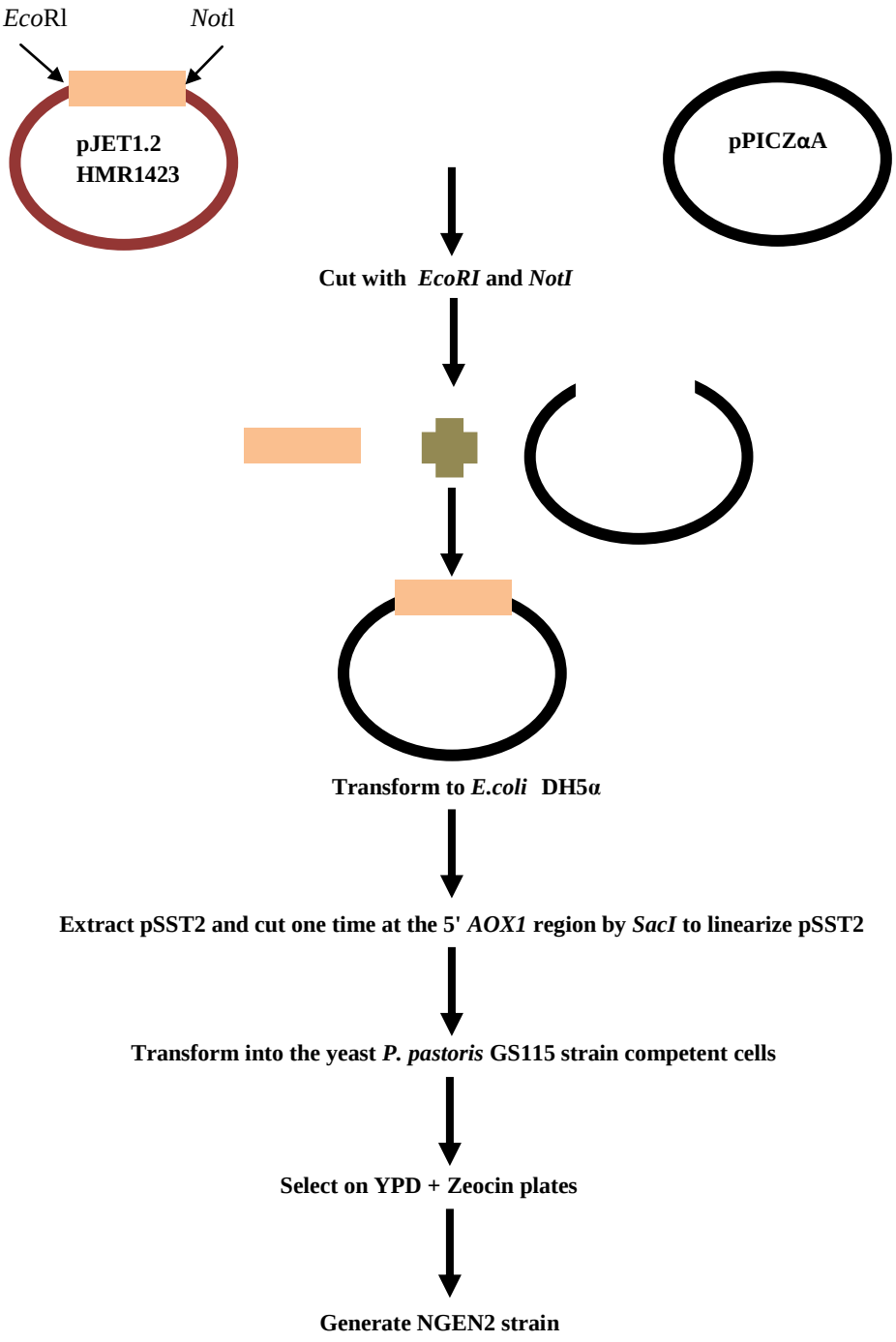


Figure 3.2 The diagram of the strain NGEN2 construction

3.5.2 The effect of temperature on insulin expression

Strains were grown in 25 ml YPG (1% w/v yeast extract, 2% w/v peptone and 1% w/v glycerol) at 30°C, respectively in shaking incubator (250-300 rpm) until the OD_{600} being 2-6 (approximately 16-18 hr). Cells were harvested by centrifugation at 1500-3000 x g for 5 minutes at room temperature and the pellet was resuspended in Basal medium pH 5.0 (10 g/L KH_2PO_4, 3.2 g/L $MgSO_4 \cdot 7H_2O$, 0.35 g/L $CaCl_2 \cdot 2H_2O$, and 20g/L ammonium sulfate) supplemented with 0.004% w/v histidine and 0.435% v/v PTM[1] (see appendix A) to obtain an OD_{600} of 0.1. Strains were grown at 20, 25 and 30°C, respectively. To induce the expression, 100% methanol was added to final concentration of 0.5% v/v every 24 hr to maintain induction conditions for 84 hr [0,12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days) and 84]. Samples of each culture were harvested every 12 hr to analyze the growth and insulin expression. The growth of each sample was determined by cell dry weight while the insulin expression was determined from the supernatant filtered through a 0.45 µm filter membrane by competitive indirect enzyme-linked immunosorbent assay (ELISA)

3.5.3 The effect of nitrogen sources on insulin expression

Strains were grown in 25 ml YPG (1% w/v yeast extract, 2% w/v peptone and 1% w/v glycerol) at 30°C in shaking incubator (250-300 rpm) until the OD_{600} being 2-6 (approximately 16-18 hr). Cells were harvested by centrifugation at 1500-3000 x g for 5 minutes at room temperature and the pellet were resuspended in Basal medium pH 5.1 (10 g/L KH_2PO_4, 3.2 g/L $MgSO_4 \cdot 7H_2O$, 0.35 g/L $CaCl_2 \cdot 2H_2O$, and 20g/L ammonium sulfate) supplemented with 0.004% w/v histidine and 0.435% v/v PTM[1] to obtain an OD_{600} of 0.1. To vary the nitrogen sources, ammonium sulfate in Basal medium was substituted by 20 g/L peptone plus 10 g/L yeast extract, 20g/L ammonium nitrate and 20 g/L urea). Strains were grown at 30°C. To induce the expression, 100% methanol was added to final concentration of 0.5% v/v every 24 hr to maintain induction conditions for 84 hr [0,12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days) and 84]. Samples of each culture were harvested every 12 hr to analyze the growth and insulin expression. The growth of each sample was determined by cell dry weight while the insulin expression was determined the supernatant filtered through a

0.45 µm filter membrane by competitive indirect enzyme-linked immunosorbent assay (ELISA).

3.6 Analytical determinations

3.6.1 Cell dry weight

The growth of each strain was investigated by total dry biomass. Filtrate was dried at 80^{o}C for 24 hr and weighted. Total dry biomass concentration was calculated in gram per liter.

3.6.2 Analytical protein by competitive indirect enzyme – linked immunosorbent Assay

2.5 µg of standard insulin conjugated with BSA in PBS was coated with the volume 100 µl/well on a 96-well plate. The plate was incubated at 4^{o}C overnight. After washing with PBST for 3 times, the plate was blocked and incubated with 5% skim milk in PBS (300 µl/well) at 37^{o}C for 1 hr. Then, the plate was washed again with PBST for 3 times. Before analyzing, pH value of each sample had to be adjusted to approximately 7.0. Samples with the volumes 50 µl/well were added and incubated with 50 µl/well monoclonal anti-insulin antibody (dilution 1:3000) at 37^{o}C for 2 hr. After washing with PBST for 3 times, the plate was incubated with 100 µl/well goat anti – mouse lgG-HRP (dilution 1:10000) at 37^{o}C for 1 hr. Then, PBST was used for washing the plate and 100 µl/well TMB solution was added. Finally, 100 µl/well of 2M H_2SO_4 was applied to stop the reaction. The absorbance at 450 nm was measured by using the microplate reader. The concentration of recombinant insulin expressed in each sample was calculated from the equation of standard curve. For the standard curve (figure B1), 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, 0.097, 0.049 and 0 µg/ml of standard insulin were incubated with 50 µl/well monoclonal anti insulin antibody with dilution 1:3000. The equation was created from the relationship between logarithm of protein concentrations and the absorbance values at 450 nm (see appendix B). The competitive indirect enzyme-linked immunosorbent assay for recombinant insulin detection was represented as Figure 3.3.

3.7 Statistical analysis

To analyze the data from competitive indirect enzyme-linked immunosorbent assay, the SPSS program was used. One way analysis of variance (ANOVA) and Turkey's Multiple Range Method were used to determine the difference means. The data was significant when *p* value for the null hypothesis were 0.05 or less.

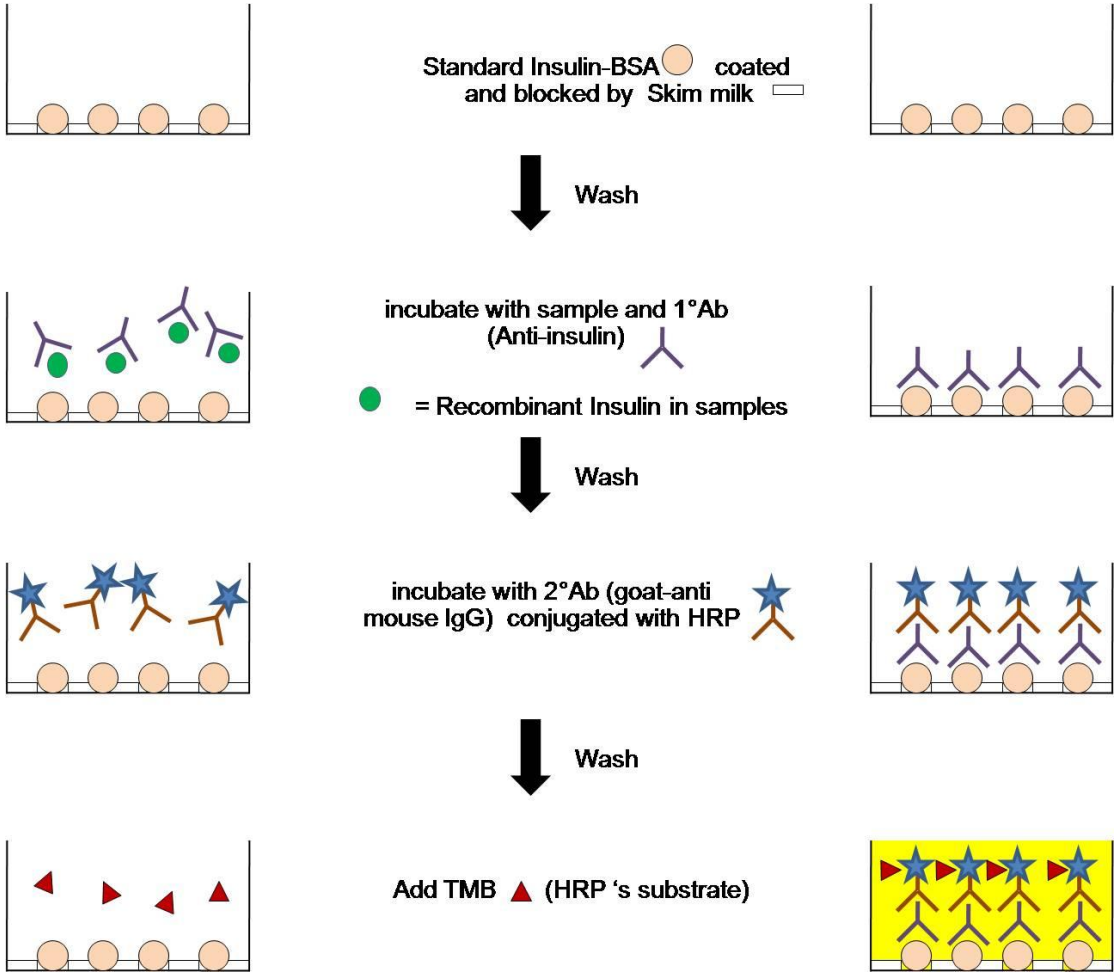


Figure3.3 The principle of competitive indirect enzyme-linked immunosorbent assay for recombinant insulin detection

CHAPTER IV

RESULT AND DISCUSSION

Results

4.1 Plasmid and strain construction

The pJET2.1 plasmid with insulin HMR1423 gene and pPICZαA plasmid were cut with enzyme *Eco*RI and *Not*I. The size of HMR1423 gene is approximately 200 base pairs (Figure 4.1) and pPICZαA is 3500 base pairs (Figure 4.2). DNA fragments of HMR1423 were extracted and ligated into pPICZαA to generate the plasmid named pSST2 (Figure 4.3). Then, pSST2 was transformed into *P. pastoris* GS115 Mut^+ which HMR1423 gene was screened by the colony PCR with the primers *AOX1* forward and *AOX1* reverse. The size of colony PCR product from *P. pastoris* with HMR1423 gene onto the chromosome is 750 base pairs (Figure 4.4). The strain with HMR1423 gene onto the chromosome was named as NGEN2. In this study, the empty plasmid pPICZαA was also integrated onto the chromosome. After checking by colony PCR with primer *AOX1* forward and *AOX1*, the PCR product from this strain revealed a 500 base pair band (Figure 4.4). This *P. pastoris* strain (abbreviated as αA) was used as the negative control which should not produce recombinant insulin. The strain of *P. pastoris* GS115 Mut^+ with MIP gene (abbreviated as MIP) obtained from Dr. Sarintip Suksai was already tested for the expression of recombinant insulin (data not shown), so it was used as the positive control.

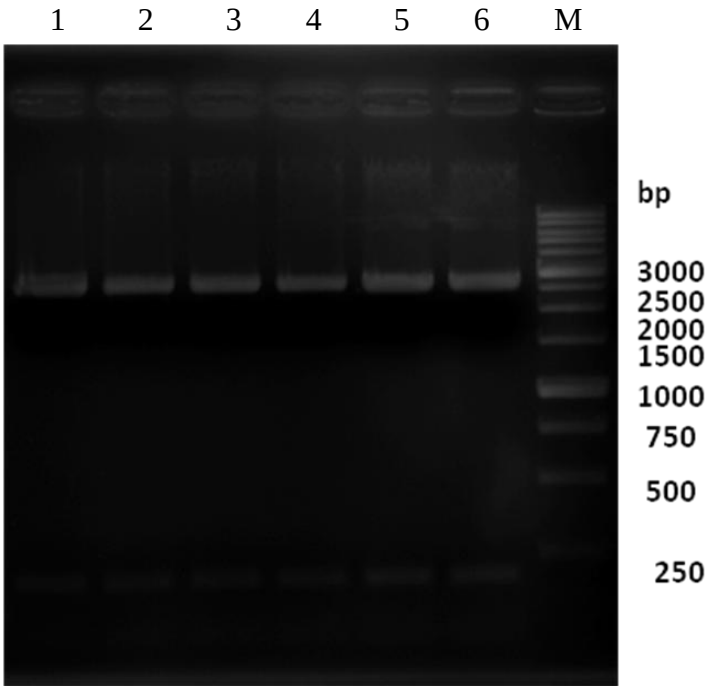


Figure 4.1 1% Agarose gel electrophoresis of plasmids pJET1.2 containing HMR1423 gene cut with *Eco*RI and *Not*I enzymes. The size of HMR1423 gene is approximately 200 base pairs (lane 1-6). Lane M is 1 kbp DNA ladder marker.

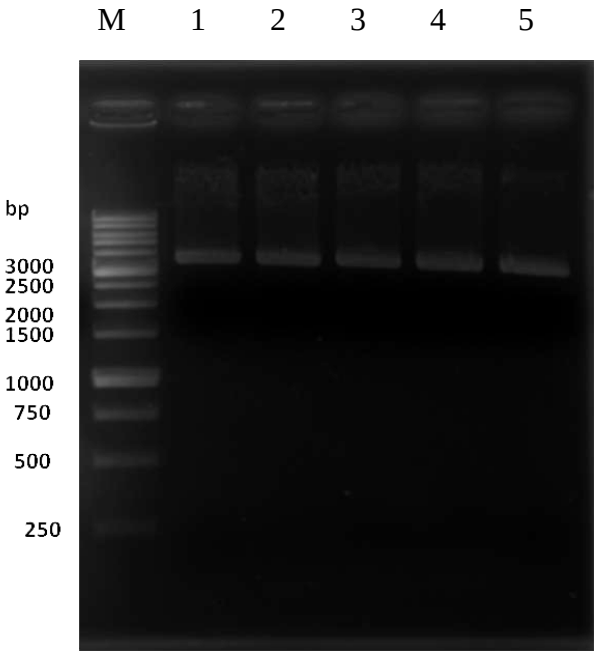


Figure 4.2 1% Agarose gel electrophoresis of pPICZαA cut with *Eco*RI and *Not*I enzymes. The size of this plasmid is approximately 3500 base pairs (lane 1-5). Lane M is 1 kbp DNA ladders marker

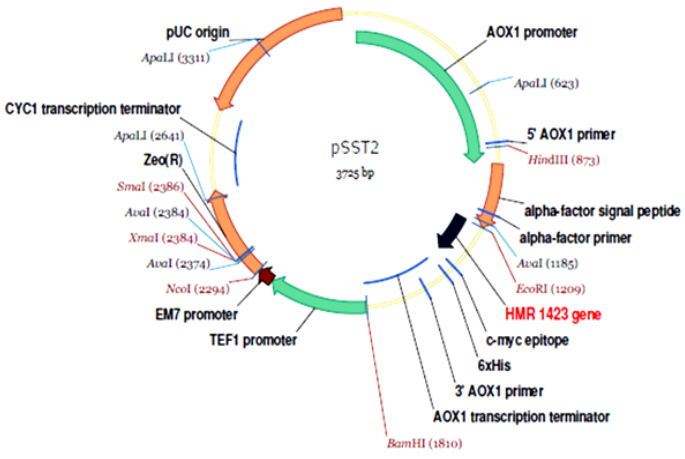


Figure 4.3 Map of plasmid pSST2

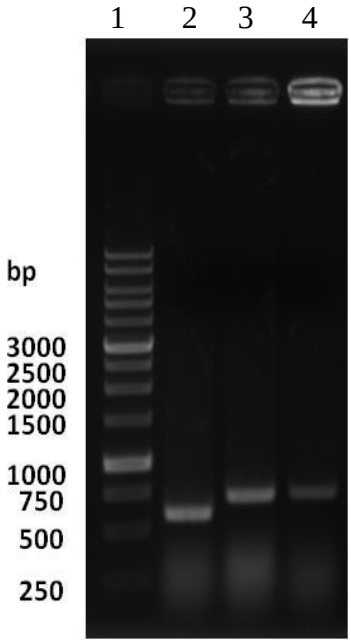


Figure 4.4 1% Agarose gel electrophoresis of the colony PCR products from αA, MIP and NGEN2 strains when using primers *AOX1* forward and *AOX1* reverse (Lane 2-4, respectively). Without insulin gene, the size of PCR product is 550 base pairs. If the MIP or HMR 1423 gene is onto the chromosome, the band of 750 base pairs is revealed. Lane 1 is 1kb DNA ladder marker.

4.2 Insulin expression

4.2.1 Effect of temperature on insulin expression

In the induction phase, the strains were grown in Basal medium with ammonium sulfate as the nitrogen source at 20^{o}C, 25^{o}C and 30^{o}C for 84 hr. Addition of 100% Methanol every 24 hr was applied to maintain the induction. The insulin concentrations from samples collected every 12 hr. and detected by competitive indirect ELISA were calculated from the equation of the insulin standard curve plotted from the relationship between the logarithm of standard insulin concentrations and the OD values at 450 nm. (See appendix B).

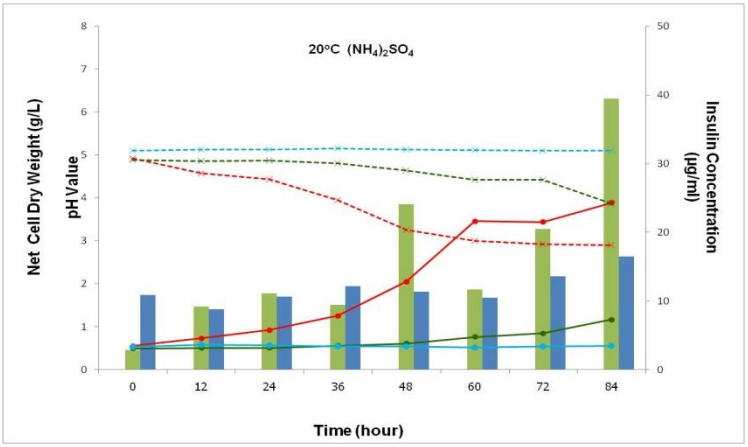
At 20^{o}C, with the investigation of net cell dry weight, *P. pastoris* αA strain seemed to grow well in this condition. However, the strain with MIP gene had a small growth and the one with HMR1423 gene seemed not to grow (Figure 4.5 A). When the pH values of the cultures were followed, decreasing in pH values after 36 hr was detected in αA and MIP strains. The steady pH values were found in the strain with HMR1423 gene. With the application of competitive indirect ELISA, the highest expression of MIP and HMR1423 was found at 84 hr with the concentration 39.47 and 16.52 μg/ml, respectively (Figure 4.5 A, Table C3)

At 25^{o}C, the negative and positive controls could grow whereas NGEN2 could not in this condition. Moreover, the pH values of the both control cultures were initially dropped at 48 hr while the pH values of the strain HMR1423 culture seemed unchanged as also seen in 20^{o}C (Figure 4.5 A and B). In this experiment, the highest MIP and HMR1423 productions could be detected with the concentration 21.58 and 11.25 μg/ml at 72 and 24 hr., respectively. However, at this temperature, the expression level of HMR1423 in all samples collected every 12 hr seemed not to be much different (Figure 4.5 B, Table C3).

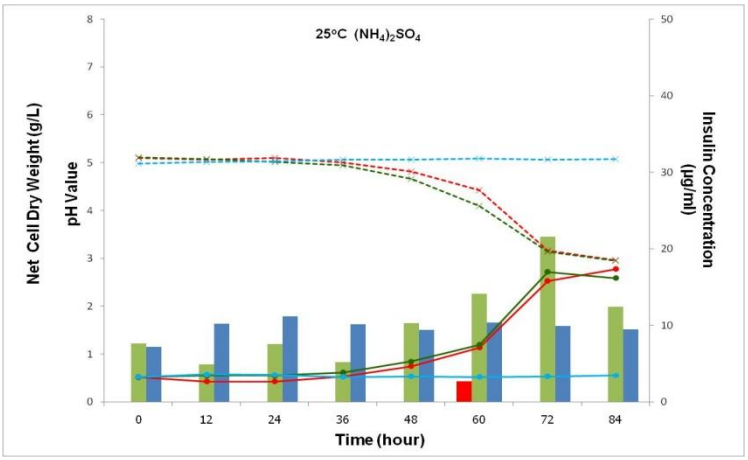
At 30^{o}C, although it seemed not to grow with steady pH values as also seen in 20 and 25^{o}C, the strain NGEN2 gave the highest expression level of insulin with the concentration 32.74 μg/ml at 24 hr (Figure 4.5 C). For the MIP strain the most insulin concentration could be measured at 72 hr with the concentration 25.43 μg/ml. The

trends of increasing growth rates and decreasing pH values of MIP and αA cultures were still found in this condition (Figure 4.5 C, Table C1-C2).

A.



B.



C.

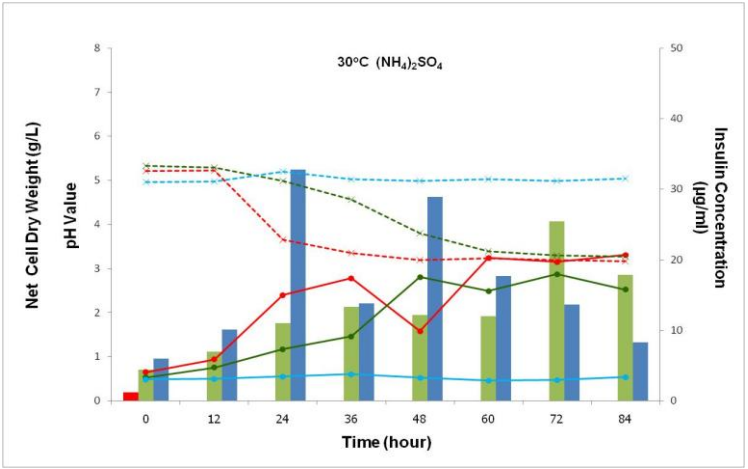


Figure 4.5 Effect of temperature on insulin expression, growth and pH values of the cultures. All strains were grown at (A.) 20^{o}C (B.) 25^{o}C and (C.) 30^{o}C in Basal medium with 100% Methanol addition every 24 hr. The growth of each strain was determined by net cell dry weight and insulin concentration in each sample was detected by competitive indirect ELISA (p <0.05).

4.2.2 Effect of N-sources on insulin expression

To test the effect of N-sources on insulin expression, ammonium sulfate in Basal medium was substituted by others N-sources including ammonium nitrate, urea and yeast extract plus peptone. All strains were grown at 30^{o}C for 84 hr with the addition of 100% Methanol every 24 hr to maintain the induction. To determine the insulin concentration, competitive indirect ELISA was applied to detect recombinant insulin in the samples collected every 12 hours and the concentrations were calculated from the equation of the insulin standard curve plotted from the relationship between the logarithm of standard insulin concentrations and the OD values at 450 nm. (See appendix B).

When ammonium sulfate was used as the N-source, the highest expression of MIP was seen at 72 hr with the concentration 25.43 μg/ml. However, the highest expression of HMR1423 was found at hour 24 hr with the concentration 32.74 μg/ml (Figure 4.6 A).

In Basal medium with ammonium nitrate as the N-source, the growth and pH values of the strain NGEN2 were similar to the ones with ammonium sulfate which seemed to be unchanged (Figure 4.6 A and B). On the other hand, the MIP and αA strains could grow in this N-source. The pH values of the MIP strain started to decrease at 12 hr (Figure 4.6 B). The most concentration of HMR 1423 and MIP could be detected at 12 and 60 hr with the concentration 12.80 and 30.83 μg/ml, respectively.

With urea as N-source, all strains seemed to grow, but pH values of all cultures did not drop. The recombinant insulin in all samples could not be measured by the technique competitive indirect ELISA (Figure 4.6 C).

As seen in Figure 4.5 D, all strains could grow when yeast extract plus peptone was used as the N-source, however, the increasing pH values of the strain NGEN2 from 5.0 to nearly 7.0 were different from what were happened in other conditions. The highest concentration of recombinant insulin could be detected at 48

hr with the concentration 42.52 μg/ml for MIP strain and at 12 hr with the concentration 22.32 μg/ml for HMR1423 strain.

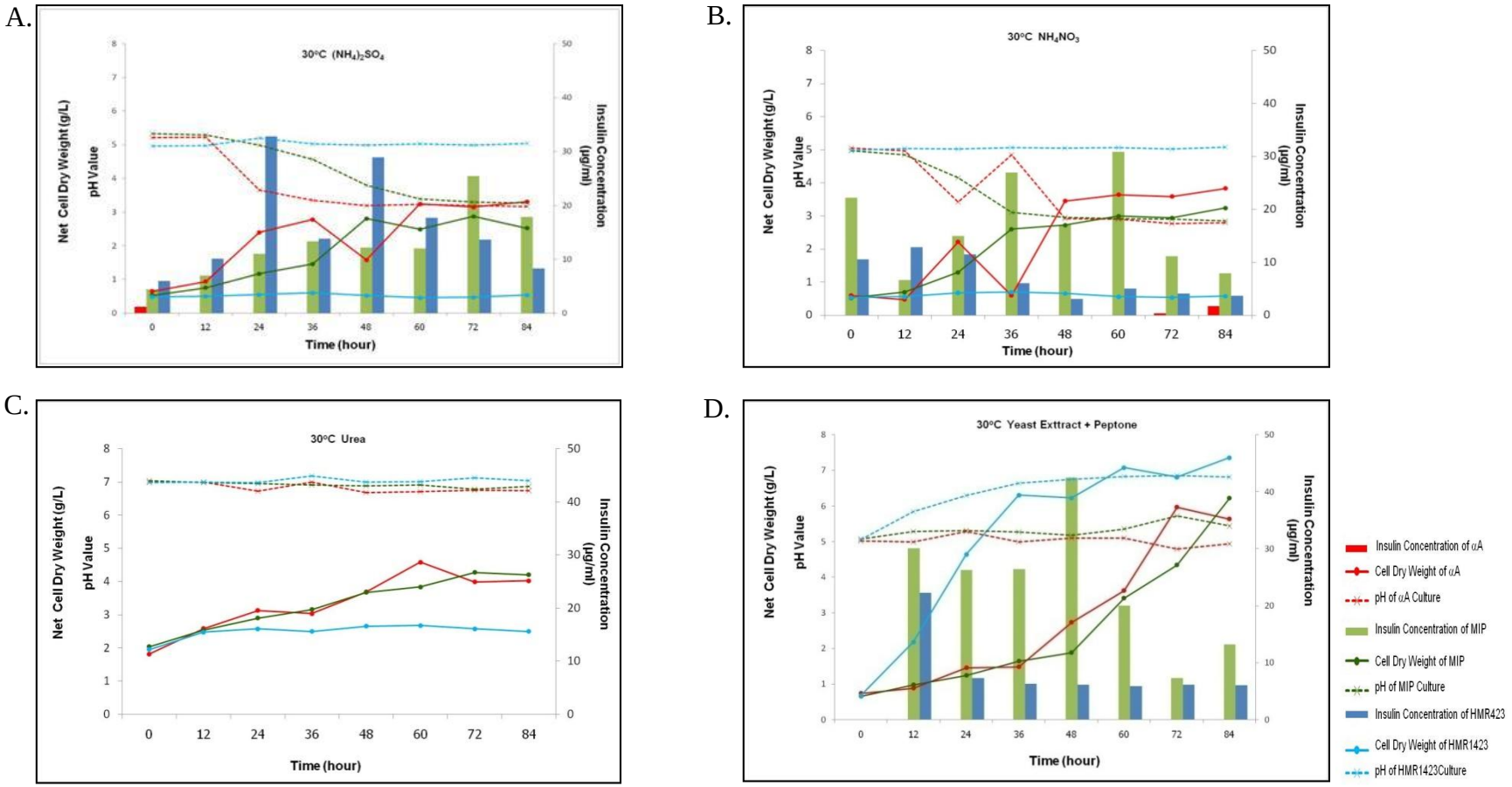


Figure 4.6 Effect of N-sources on insulin expression, growth and pH values of the cultures. All strains were grown at 30°C in Basal medium with (A.) ammonium sulfate (B.) ammonium nitrate (C.) urea and (D.) yeast extract plus peptone as N-sources. The 100% Methanol was added every 24 hr to maintain the induction. The growth of each strain was determined by net cell dry weight and insulin concentration in each sample was detected by competitive indirect ELISA (p <0.05).

Discussion

The high sensitivity and specificity are the reason why the technique competitive indirect ELISA is applied to detect recombinant insulin in this research. Because every set of experiments had to collect samples every 12 hr for total 84 hr from all strains with 3 repeats, there were over 400 samples to analysis. Purification of recombinant insulin for hundreds of samples consumes a long period of time. Without purification, other methods such as Lowry or Bradford assays detect overall proteins in the samples. Moreover, the technique of Mass Spectroscopy encounters problems when too many types of proteins are in the samples. Therefore, the competitive indirect ELISA was suitable for this project which can detect recombinant insulin in the supernatant by the specific binding of insulin and Anti-insulin. Furthermore, a great number of samples can be performed in the same time. However, the competitive indirect ELISA still has some drawbacks such as concentration of protein can be detected only in the range of standard curve. In this experiment, a small amount of insulin could be detected in some samples collected from αA strain (Figure 4.5 B, 4.5 C and 4.6 B). This is possibly an error from the limitation of this technique because the absorbance values at 450 nm of these samples close to the limits of standard curve. Moreover, small particles in the samples may interfere the system of detection, especially HRP conjugated on secondary antibody.

In this study, even though the MIP strain can grow well at 25^{o}C and 30^{o}C, the most insulin production was found at 20^{o}C. It is corresponded with the previous study of Pais-Chanfrau *et al*., 2004 which suggested that the optimal temperatures of insulin production are 22^{o}C or lower. They also suggested that at low temperature with the addition of chelating agents could enhance the recombinant insulin production by inactivating the protease enzymes.

By varying N-sources, the most MIP production in this study was found when yeast extract and peptone was used. However, previous study suggested that the optimal N-source for recombinant insulin production was ammonium sulfate (Pais-Chanfrau *et al*., 2004). They also suggested that yeast extract plus peptone should be utilized for growth, but not for insulin production. The difference between our and

their experiments may result from the different conditions that they produced insulin in fermentor. However, our data suggested that yeast extract plus peptone can be used for the MIP production in growth and induction phase. Generally, yeast extract plus peptone was used in growth phase and substituted by ammonium sulfate in induction phase. Therefore, the potential benefit received from our study is that the further MIP production from this strain can use yeast extract plus peptone in both fed-batch and batch processes without changing N-source which will reduce the lag phase, time-consuming procedures and many costs.

The MIP concentration in this experiment that is lower than previous studies (Ding *et al*., 2005 and Chen *et al*., 2011) may result from uncontrolled parameters in shake flask level such as pH and dissolved oxygen values. However, one of the advantages of the production in our study is consuming less time cost because the highest concentration could be detected at 48 hr while the other studies found at 84 hr. On the other hand, its optimal conditions for high expression level have never been studied, so the data in this research can be developed for the larger scales.

The other insulin used in this study, HMR1423, has the different optimal temperature from MIP and the insulin production in previous studies (Pais-Chanfrau *et al*., 2004). This different requirement may come from the difference in the HMR1423 structure. The addition of 2 histidines in the B chain of HMR1423 can bind to metal ions resulting in possibly competitive binding of metal with metalloprotease. Therefore, it leads to a decrease in active some protease in the cultures which possible increases recombinant insulin production. Without necessary requirement of any chelating agents such as EDTA, production of HMR1423 can reduce some costs as well.

Furthermore, this study also suggests the different pathways for utilizing N-sources among NGEN2 and other strains. Normally, carbon and nitrogen sources are the key factors for cell growth. As carbon and nitrogen are main substrates for biomass production, they are needed to be balanced in the cells. So, the uptake of carbon and nitrogen are controlled by each other. Because *P. pastoris* is a methylotrophic yeast, methanol can be used as a sole carbon source. In methanol

metabolism pathway, methanol is converted to formaldehyde by alcohol oxidase, and then it is completely oxidized to carbon dioxide as a waste that is eliminated from the cells to the supernatant of the cultures. The dissolution of carbon dioxide with water in culture media generates carbonic acid which is possible the reason of a decrease in pH values observed in MIP and αA strain cultures. Therefore, MIP strain possibly uses inorganic nitrogen for both growth and MIP production. By using inorganic nitrogen such as ammonium sulfate and ammonium nitrate, neither growth nor pH values of NGEN2 was changed. It is suggested that NGEN2 uses most of the inorganic N-sources for producing recombinant insulin rather than growth.

For organic nitrogen such as urea and yeast extract plus peptone, growth could be observed in all strains. Moreover, the trends of pH values did not dropped as seen when using inorganic N-sources. This suggests different metabolic pathways of organic and inorganic N-sources. Because urea is a strong detergent, it is possible that 2 % urea in the cultures is too strong and urea may denature recombinant insulin. This may be the reason why any recombinant insulin could not be detected in all samples when using urea as the N-source.

Another advantage of producing insulin HMR1423 from the strain NGEN2 is that high target product generates at a relatively short period of time (Figure 4.5 C). Therefore, this should save many costs due to the short period of production.

Another observation obtained from this research is high expression coming with low growth. It is possible that at low growth, not only most of the sources are used for recombinant protein production rather than growth, but also proteolytic activity is being inhibited.

The data of this study can be applied for the larger scale production. In addition, the result of this experiment is a good example of recombinant protein production. It is suggested that the conditions for producing these recombinant protein are significantly different although both MIP and HMR1423 are insulin analogs which only few amino acid sequences are different. This also supports the previous study by Klaus, 1996 that, to produce different proteins from different strains, the

profiles of heterologous protein production such as temperature, carbon and nitrogen sources are also various.

CHAPTER V

CONCLUSION

Conclusion

Recently, the production improvement of the insulin analogs with new properties is important to diabetic treatment. MIP and HMR1423 insulin are novel insulin analogues produced in *P. pastoris* to increase in expression level. This study suggests that the optimal temperature of MIP and HMR1423 production are 20^{o}C and 30^{o}C, respectively. Moreover, the optimal N-source for MIP is yeast extract plus peptone and for HMR1423 is ammonium sulfate. The benefit of MIP production found from this study is the unnecessary to switch the media when yeast extract plus peptone is used as nitrogen source in the production. For HMR1423, the recombinant insulin is expressed with high concentration in a relatively short period of time. The study also indicates the different metabolic pathway between MIP and HMR1423 strains.

This research shows that the change in only few amino acids requires different parameter for recombinant protein production. Therefore, factors such as temperature and nitrogen sources are important to be concerned for recombinant protein production.

Suggestion

The upscale production of recombinant insulin should give more yields because the parameters such as the pH values, dissolved oxygen and revolution per minute (rpm) can be controlled in bioreactor.

Although the HMR1423 strain could not grow very well, the high concentration could be detected. To improve the production of this recombinant insulin, the concentration of the starter needs to be varied.

The recombinant insulin can be measured by the technique competitive indirect ELISA. However, this method has some limitations, for example, it can only

detect the concentration of protein in the range of standard curve, or small particles in samples can interfere the system of detection. Therefore, purifying target protein should give more accurate concentration.

This project aims to develop the recombinant insulin production. However, to produce this insulin for medical treatment, not only the procedures need to be improved, but also the properties of recombinant insulin need to be tested.

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APPENDICES

APPENDIX A

MEDIA AND SOLUTIONS

1. Low salt LB agar

Peptone	10	g
Yeast extract	5	g
NaCl	5	g
Agar	15	g

Dissolve in 1000 ml of water and autoclave 121^{o}c for 15 min.

2. Yeast Peptone Dextrose agar (YPD)

Yeast extract	10	g
Peptone	20	g
Dextrose	20	g
Agar	20	g

Dissolve in 1000 ml of water and autoclave 121^{o}c for 15 min.

3. Yeast Peptone Glycerol agar (YPG)

Yeast extract	10	g
Peptone	20	g
Glycerol	20	g
Agar	20	g

Dissolve in 1000 ml of water and autoclave 121^{o}c for 15 min.

4. PTM[1] solution

Cupric sulfate $\cdot 5H_2O$	6.0	g
Sodium iodide or potassium iodide	0.08	g
Manganese sulfate $\cdot H_2O$	3.0	g
Sodium molybdate $\cdot 2H_2O$	0.2	g

Boric acid	0.02	g
Cobalt chloride	0.5	g
Zinc chloride	20.0	g
Ferrous sulfate $\cdot 7H_2O$	65.0	g
Biotin	0.2	g
Sulfuric acid	5.0	ml

Adjust the volume to 1000 ml by water and filter through 0.45 μm nitrocellulose membrane to sterilize.

5. 0.15 M Phosphate Buffer Saline or PBS, pH 7.4

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g

Adjust the volume to 1000 ml by water and adjust pH to 7.4

6. 0.05 % Tween20 in PBS or PBST

Tween20	0.5	ml
PBS	1000	ml

7. 5 % Skim milk in PBS or Blocking solution

Skim milk	5	g
PBS	100	ml

8. 0.15 M Phosphate Citrate Buffer, pH 5.0

Na_2HPO_4	9.5	g
Citric acid	7.3	g
Distilled adjusted volume to	1000	ml

Adjusted pH to 5.0 and keep on 4^oc in a dark bottle before use

APPENDIX B

DETERMINATION OF RECOMBINANT INSULIN CONCENTRATION BY COMPETITIVE INDIRECT ELISA

1. Standard curve of insulin concentration

For the standard curve, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, 0.097, 0.049 and 0 µg/ml of standard insulin with volume 50 µl/well were incubated with 50 µl/well monoclonal anti insulin antibody with dilution 1:3000. Then, the protocol of competitive indirect ELISA as described in CHAPTER 3 was followed. The measurement of each sample was duplicated. Table B1 is an example of values used to create a standard curve by plotting the logarithm of standard insulin concentrations versus the average absorbance values at 450 nm (Figure B1). Only the linear relationship between the logarithm of standard insulin concentrations and OD_{450} was selected to create the equation for further calculation. Therefore, only the protein concentration from the samples giving the absorbance values in this range can be detected. For example, by using the equation from the graph in Figure B1, if the OD_{450} is more than 1.62 or lower than 0.42, it means that the concentration lower than 1.56 or more than 50 µg/ml cannot be measured. Equation obtained from standard curve was further used to determine recombinant insulin concentration.

Table B1. Concentration of standard insulin and the average absorbance value of 450 nm for creating the standard insulin curve in Figure B1.

Standard insulin concentration (μg/ml)	Average OD_{450}
50	0.42
25	0.64
12.5	0.97
6.25	1.27
3.13	1.57
1.56	1.62
0.78	1.62
0.39	1.63
0.2	1.63
0.097	1.63
0.049	1.64
0	1.64

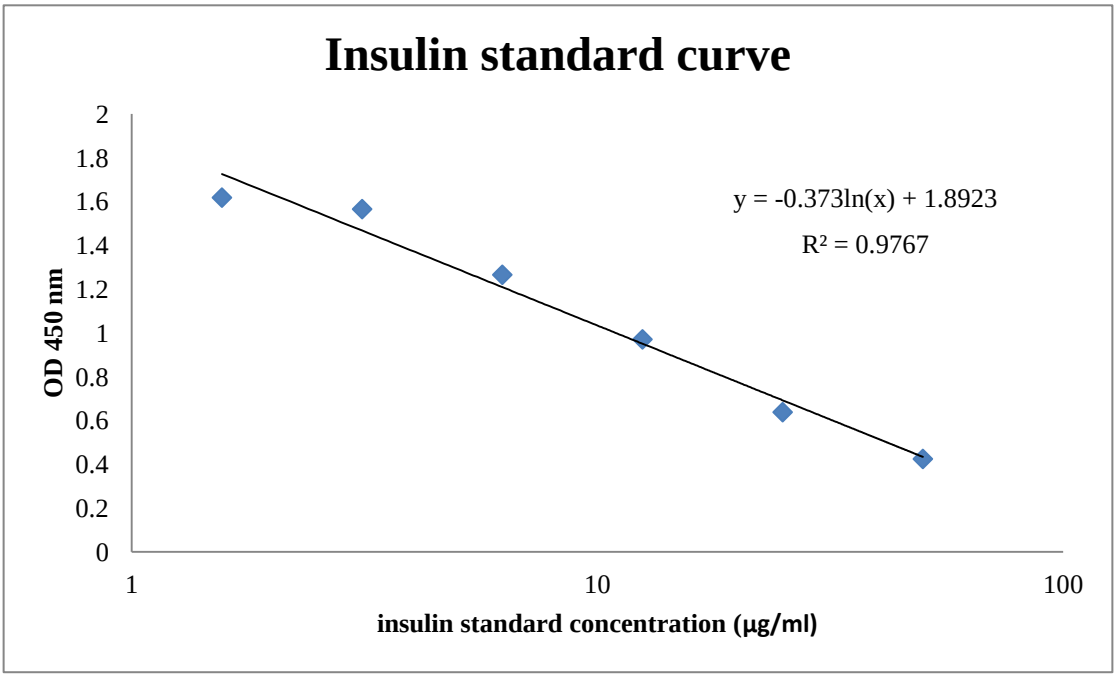

Figure B1. A Standard Insulin curve from the competitive indirect ELISA for recombinant protein determination

The equation from the graph in Figure B1 was used to calculate the insulin concentration in the samples. The insulin concentration was calculated as the following:

$$y = -0.373\ln(x) + 1.8923$$

Which y is the average OD_{450} value of each sample

By this equation, x, or recombinant insulin in each sample, can be calculated.

An example of insulin concentration calculated from this equation is represented in Table B2.

With this calculation, the concentrations of αA, MIP and HMR1423 in Figure. 4.4-4.5 are represented in Table C3 and C6, APPENDIX C. The values shown in those tables are the average from 3 repeats of each set of experiment.

Table B2. The MIP concentration at 20^{o}C and ammonium sulfate as nitrogen source

Hr	OD_{450} Exp#1	Insulin concentration (μg/ml)
0	1.427	3.493
12	1.264	5.4
24	1.332	4.5
36	1.224	6.01
48	1.204	6.34
60	1.191	6.57
72	0.64	28.81
84	0.42	51.89

APPENDIX C
GROWTH, pH AND INSULIN CONCENTRATION

Table C1. The growth of *P. pastoris* strains containing αA, MIP and HMR1423 genes cultured in BM medium with ammonium sulfate as the nitrogen source at 20°C, 25°C and 30°C. Methanol was added every 24 hr for 84 hr to maintain the induction. The growth rate was represented by the cell dry weight.

Strains	Temp.	Total cell dry weight (g/L) at different time points							
		0 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr	84 hr
αA	20°C	0.55	0.73	0.92	1.26	2.05	3.46	3.44	3.89
	25°C	0.52	0.43	0.43	0.54	0.75	1.14	2.53	2.78
	30°C	0.65	0.94	2.40	2.78	1.58	3.25	3.15	3.31
MIP	20°C	0.50	0.51	0.51	0.56	0.62	0.77	0.85	1.17
	25°C	0.52	0.55	0.55	0.62	0.85	1.20	2.72	2.59
	30°C	0.53	0.75	1.17	1.46	2.80	2.49	2.87	2.52
HMR1423	20°C	0.53	0.58	0.57	0.54	0.54	0.52	0.54	0.56
	25°C	0.53	0.58	0.57	0.53	0.54	0.53	0.54	0.56
	30°C	0.48	0.50	0.55	0.60	0.52	0.46	0.47	0.54

Table C2. The pH values of *P. pastoris* strains containing αA, MIP and HMR1423 gene cultures in BM medium with ammonium sulfate as the nitrogen source at 20°C, 25°C and 30°C. Methanol was added every 24 hr for 84 hr to maintain the induction.

Strains	**Temp.**	**Culture pH value at different time points**							
		0 hr	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**	**72 hr**	**84 hr**
αA	20°C	4.92	4.57	4.44	3.95	3.26	3.00	2.92	2.90
	25°C	5.10	5.07	5.10	5.01	4.82	4.43	3.16	2.96
	30°C	5.21	5.22	3.66	3.36	3.20	3.24	3.19	3.17
MIP	20°C	4.88	4.86	4.87	4.80	4.64	4.43	4.43	3.88
	25°C	5.11	5.07	5.02	4.95	4.66	4.10	3.14	2.95
	30°C	5.33	5.29	4.99	4.56	3.80	3.39	3.29	3.27
HMR1423	20°C	5.10	5.12	5.12	5.15	5.12	5.11	5.10	5.10
	25°C	4.98	5.02	5.03	5.07	5.07	5.09	5.06	5.08
	30°C	4.96	4.97	5.20	5.02	4.99	5.03	4.99	5.04

Table C3. The insulin concentration of *P. pastoris* strains containing αA, MIP and HMR1423 genes cultured in BM medium with ammonium sulfate as the nitrogen source at 20°C, 25°C and 30°C. Methanol was added every 24 hr for 84 hr to maintain the induction.

Strains	Temp.	Insulin concentration (μg/ml) at different time points							
		0 hr	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**	**72 hr**	**84 hr**
αA	20°C	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39
	25°C	<0.39	<0.39	<0.39	<0.39	<0.39	2.72	<0.39	<0.39
	30°C	1.18	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39
MIP	20°C	2.89±0.53	9.20±3.31	11.14±13.43	9.45±3.50	24.10±29.26	11.68±5.00	20.49±11.75	39.47±12.61
	25°C	7.69±5.48	4.93±1.10	7.63±3.17	5.24±1.00	10.34±2.69	14.19±3.25	21.58±4.25	12.45±2.37
	30°C	4.41±1.67	6.91±0.49	10.96±6.35	13.29±8.82	12.11±1.33	12.00±5.10	25.43±3.96	17.86±8.50
HMR 1423	20°C	10.84±2.61	8.81±2.60	10.64±2.80	12.18±0.41	11.34±0.80	10.47±1.85	13.62±1.87	16.52±2.40
	25°C	7.20±1.24	10.26±0.34	11.25±0.83	10.21±2.99	9.47±4.78	10.42±0.50	9.92±2.50	9.51±2.63
	30°C	5.97±2.57	10.10±11.65	32.74±13.17	13.75±3.75	28.29±21.14	17.65±10.11	13.62±14.43	8.25±1.00

Table C4. The growth of *P. pastoris* strains containing αA, MIP and HMR1423 genes cultured at 30°C in BM medium with different nitrogen sources. Methanol was added every 24 hr for 84 hr to maintain the induction. The growth rate was represented by the cell dry weight.

Strains	N-sources	Total cell dry weight (g/L) at different time points							
		0 hr	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**	**72 hr**	**84 hr**
αA	$(NH_4)_2SO_4$	0.65	0.94	2.40	2.78	1.58	3.25	3.15	3.31
	NH_4NO_3	0.60	0.48	2.22	0.59	3.45	3.64	3.59	3.83
	Urea	1.82	2.59	3.13	3.04	3.69	4.59	3.99	4.03
	Yeast extract + Peptone	0.74	0.88	1.46	1.48	2.74	3.63	5.97	5.64
MIP	$(NH_4)_2SO_4$	0.53	0.75	1.17	1.46	2.80	2.49	2.87	2.52
	NH_4NO_3	0.52	0.70	1.30	2.60	2.72	3.00	2.94	3.24
	Urea	2.04	2.54	2.90	3.16	3.68	3.84	4.28	4.21
	Yeast extract + Peptone	0.66	0.98	1.25	1.65	1.88	3.42	4.34	6.22
HMR 1423	$(NH_4)_2SO_4$	0.48	0.50	0.55	0.60	0.52	0.46	0.47	0.54
	NH_4NO_3	0.54	0.56	0.68	0.70	0.66	0.56	0.54	0.58
	Urea	1.96	2.48	2.58	2.50	2.66	2.68	2.58	2.50
	Yeast extract + Peptone	0.68	2.18	4.64	6.31	6.23	7.08	6.81	7.36

Table C5. The pH values of *P. pastoris* strains containing αA, MIP and HMR1423 gene cultures at 30^{o}C in BM medium with different nitrogen sources. Methanol was added every 24 hr for 84 hr to maintain the induction.

Strains	**N-sources**	**Culture pH value at different time points**							
		0 hr	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**	**72 hr**	**84 hr**
αA	$(NH_4)_2SO_4$	5.21	5.22	3.66	3.36	3.20	3.24	3.19	3.17
	NH_4NO_3	5.05	4.97	3.42	4.85	2.92	2.91	2.77	2.80
	Urea	7.00	7.00	6.73	7.00	6.69	6.71	6.76	6.74
	Yeast extract + Peptone	5.03	5.00	5.29	5.00	5.10	5.11	4.80	4.95
MIP	$(NH_4)_2SO_4$	5.33	5.29	4.99	4.56	3.80	3.39	3.29	3.27
	NH_4NO_3	4.97	4.85	4.16	3.11	2.96	2.92	2.90	2.85
	Urea	7.05	6.99	6.96	6.91	6.88	6.92	6.79	6.87
	Yeast extract + Peptone	5.08	5.29	5.32	5.27	5.18	5.36	5.72	5.44
HMR 1423	$(NH_4)_2SO_4$	4.96	4.97	5.20	5.02	4.99	5.03	4.99	5.04
	NH_4NO_3	4.99	5.04	5.03	5.07	5.06	5.07	5.03	5.08
	Urea	6.98	7.00	6.98	7.18	7.00	7.01	7.13	7.05
	Yeast extract + Peptone	5.06	5.85	6.30	6.64	6.76	6.84	6.86	6.82

Table C6. The insulin concentration of *P. pastoris* strains containing αA, MIP and HMR1423 genes cultured at 30°C in BM medium with different nitrogen sources. Methanol was added every 24 hr for 84 hr to maintain the induction.

Strains	N-sources	Insulin concentration (μg/ml) at different time points							
		0 hr	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**	**72 hr**	**84 hr**
αA	$(NH_4)_2SO_4$	1.18	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39
	NH_4NO_3	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	0.34	1.73
	Urea	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39
	Yeast extract + Peptone	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39	<0.39
MIP	$(NH_4)_2SO_4$	4.41±1.67	6.91±0.49	10.96±6.35	13.29±8.82	12.11±1.33	12.00±5.10	25.43±3.96	17.86±8.50
	NH_4NO_3	22.23±7.64	6.66±3.36	15.00±10.53	26.95±17.76	17.11±4.89	30.83±13.55	11.16±5.04	7.88±6.79
	Urea	<0.39±0.07	<0.39±0.07	<0.39±0.06	<0.39±0.06	<0.39±0.06	<0.39±0.12	<0.39±0.04	<0.39±0.02
	Yeast extract + Peptone	<0.39±5.50	30.09±3.35	26.27±4.41	26.46±9.46	42.52±3.12	20.03±7.23	7.30±5.60	13.19±11.39
HMR 1423	$(NH_4)_2SO_4$	5.97±2.57	10.10±11.63	32.74±13.17	13.75±3.75	28.89±21.14	17.65±10.11	13.62±14.43	8.25±1.00
	NH_4NO_3	10.57±5.69	12.80±6.20	11.50±4.60	6.07±5.52	3.09±1.18	4.99±2.83	4.10±2.46	3.65±1.11
	Urea	<0.39±0.05	<0.39±0.07	<0.39±0.01	<0.39±0.04	<0.39±0.05	<0.39±0.07	<0.39±0.06	<0.39±0.04
	Yeast extract	<0.39±4.80	22.32±3.54	7.36±0.36	6.33±0.71	6.18±0.67	5.91±1.20	6.18±0.55	6.06±0.76

BIOGRAPHY

Miss Sasithorn Ngenprasertsiri was born on September 20, 1985 in Ratchaburi, Thailand. She graduated with a Bachelor degree of Science in field of Microbiology from Faculty of Science, Chulalongkorn University in 2008. She had been studied for a Master degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2010.

Academic Presentation;

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