การแสดงออกของยีนแอลฟาอะไมเลสและกลูโคอะไมเลสจากต่างสายพันธุ์เพื่อผลิตเอทานอลใน *SACCHAROMYCES CEREVISIAE*

นางสาวรัติกาล ทองสำฤทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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HETEROLOGOUS EXPRESSION OF α-AMYLASE AND GLUCOAMYLASE GENES TO PRODUCE ETHANOL IN *SACCHAROMYCES CEREVISIAE*

Ruttikan Tongsumrit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Science

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HETEROLOGOUS EXPRESSION OF α-AMYLASE AND GLUCOAMYLASE GENES TO PRODUCE ETHANOL IN *SACCHAROMYCES CEREVISIAE*

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

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รัติกาล ทองสำฤทธิ์ : การแสดงออกของยีนแอลฟาอะไมเลสและกลูโคอะไมเลสจากต่างสายพันธุ์เพื่อผลิตเอทานอลใน *SACCHAROMYCES CEREVISIAE* (HETEROLOGOUS EXPRESSION OF α-AMYLASE AND GLUCOAMYLASE GENES TO PRODUCE ETHANOL IN *SACCHAROMYCES CEREVISIAE*) อาจารย์ที่ปรึกษา : อาจารย์ ดร.พงษ์ฤทธิ์ ครบปรัชญา, 136 หน้า.

ในปัจจุบันเอทานอลได้กลายเป็นแหล่งพลังงานทางเลือกที่สำคัญ เนื่องจากการลดลงอย่างต่อเนื่องของเชื้อเพลิงจำพวกฟอสซิลที่มีอย่างจำกัด เอทานอลที่ผลิตด้วยกระบวนการหมักเรียกกันโดยทั่วไปว่าไบโอเอทานอล หรือเอทานอลชีวภาพ ซึ่งถือว่าเป็นวิธีการแก้ปัญหาบางส่วนสำหรับวิกฤตพลังงานทั่วโลก ประเทศไทยถือว่าเป็นผู้ผลิตมันสำปะหลังที่ใหญ่ที่สุดเป็นอันดับสามของโลก ซึ่งมันสำปะหลังเป็นพืชที่มีคาร์โบไฮเดรตสูงและเป็นสารตั้งต้นที่มีประสิทธิภาพในการผลิตเอทานอลในราคาที่ต่ำลงได้ กระบวนการหมักเอทานอลในอุตสาหกรรมโดยทั่วไปประกอบด้วยสองขั้นตอนหลักคือการย่อยสลายแป้งและการหมัก แต่เนื่องจากจุลินทรีย์ที่สำคัญ เช่น เชื้อยีสต์ *Saccharomyces cerevisiae* ขาดกิจกรรมของเอนไซม์ที่ใช้ในการย่อยสลายแป้ง (Amylolytic enzyme) จึงไม่สามารถใช้ประโยชน์จากแป้งโดยตรงสำหรับการเจริญเติบโตและการหมักได้ ดังนั้นกระบวนการหมักจึงต้องใช้พลังงานและเอนไซม์ย่อยสลายแป้งจำนวนมากเพื่อเปลี่ยนแป้งไปเป็นเจล จากนั้นทำให้เป็นของเหลวแล้วเปลี่ยนเป็นเดกซ์ทรินก่อนการหมักของแป้งดิบเพื่อผลิตเอทานอล

ด้วยสาเหตุดังกล่าวนี้ได้มีการแนะนำว่าการใช้ยีสต์ *S. cerevisiae* ที่ผ่านการดัดแปลงพันธุกรรมให้มีการแสดงออกของเอนไซม์ย่อยสลายแป้ง ซึ่งอาจจะสามารถย่อยสลายแป้งและเข้าสู่ขั้นตอนการหมักได้ในขั้นตอนเดียว ซึ่งการปรับปรุงสายพันธุ์นี้จะสามารถลดต้นทุนและค่าใช้จ่ายด้านพลังงานให้กับโรงงานผลิตเอทานอลในปัจจุบันได้ และทำให้สามารถผลิตเอทานอลเชิงการค้าได้มากขึ้น ในโครงการนี้ การเปลี่ยนแปลงแป้งมันสำปะหลังโดยกระบวนการทางชีวภาพให้เป็นน้ำตาลเพื่อการหมักทดสอบโดยการใช้ยีสต์ *S. cerevisiae* 2 สายพันธุ์ใหม่ที่ถูกดัดแปลงพันธุกรรมได้แก่ *S. cerevisiae* TISTR 5596/pSWA2 (ผลิตแอลฟาอะไมเลส) และ *S. cerevisiae* TISTR 5596/pGAM1 (ผลิตกลูโคอะไมเลส) ซึ่ง ยีน *SWA2* และยีน *GAM1* แต่ละยีนถูกตัดต่อเข้าพลาสมิดสำหรับแสดงออกภายใต้การควบคุมการทำงานของ GAP โปรโมเตอร์เพื่อสร้างพลาสมิด pSWA2 และ pGAM1 ตามลำดับ โดยพลาสมิด pSWA2 และ pGAM1 นี้ถูกถ่ายเข้าสู่โครโมโซมยีสต์ ยีสต์

แต่ละสายพันธุ์ที่รับเอาพลาสมิด pSWA2 หรือ pGAM1เข้าไปนั้นจากการตรวจ พบกิจกรรมของการแสดงออกของเอนไซม์ย่อยสลายแป้งในอาหารเลี้ยงเชื้อที่มีแป้งเป็นส่วนประกอบ ซึ่งบ่งชี้ให้เห็นว่าสายพันธุ์ยีสต์ที่ถูกดัดแปลงนี้มีการแสดงออกของ SWA2 และ GAM1 ที่สามารถผลิตและหลั่งเอนไซม์ของแอลฟาอะไมเลสและกลูโคอะไมเลสที่ทำงานได้ ตามลำดับ ในชุดการหมักของการศึกษานี้ได้ใช้แป้งมันสำปะหลังเป็นแหล่งคาร์บอนเพียงอย่างเดียว ความสามารถของ *S. cerevisiae* TISTR5596/pSWA2 *S. cerevisiae* TISTR5596/pGAM1 และการเลี้ยงร่วมกันของทั้งสองสายพันธุ์คือ *S. cerevisiae* TISTR5596/pSWA2 กับ *S. cerevisiae* TISTR5596/pGAM1 โดยใช้แป้งมันสำปะหลังและการผลิตเอทานอล พบว่าแต่ละสายพันธุ์ในชุดการหมักนี้สามารถที่จะย่อยสลายแป้งมันสำปะหลังภายใต้เงื่อนไขการหมักเอทานอลได้ และพบว่าผลผลิตของเอทานอลสูงสุดที่ได้รับเท่ากับ 0.465 ± 0.012 0.489 ± 0.010 และ 0.516 ± 0.021 กรัมเอทานอล ต่อ กรัมของสารตั้งต้นที่ใช้ ที่ 12 วัน เรียงตามลำดับของแต่ละสายพันธุ์ และได้ปริมาณเอทานอลมากสุดเท่ากับ 1.478 ± 0.267 1.992 ± 0.248 และ 2.977 ± 0.020 กรัมต่อลิตรที่ 25 วัน เรียงตามลำดับของแต่ละสายพันธุ์ โดยที่การเลี้ยงร่วมกันของทั้งสองสายพันธุ์คือ *S. cerevisiae* TISTR5596/pSWA2 กับ *S. cerevisiae* TISTR5596/pGAM1 ได้ผลผลิตเอทานอลที่สูงและอัตราการผลิตมากกว่าการเลี้ยงเพียงสายพันธุ์เดียว ดังนั้นยีสต์ลูกผสมสามารถเปลี่ยนแป้งเป็นเอทานอลด้วยกระบวนการตัดต่อพันธุกรรมได้สำเร็จ ยีนที่ถูกถ่ายโอนครั้งนี้สามารถแสดงออกในสายพันธุ์เจ้าบ้านได้ ซึ่งถือได้ว่าเป็นจุดเริ่มต้นในการพัฒนาสายพันธุ์ยีสต์ที่ผลิตเอทานอลในอนาคตต่อไป

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

RUTTIKAN TONGSUMRIT : HETEROLOGOUS EXPRESSION OF α-AMYLASE AND GLUCOAMYLASE GENES TO PRODUCE ETHANOL IN *SACCHAROMYCES CEREVISIAE*. THESIS ADVISOR : PONGRIT KRUBPHACHAYA, Ph.D. 136 PP.

α-AMYLASE/ GLUCOAMYLASE/ RECOMBINANT *SACCHAROMYCES CEREVISIAE*/ CASSAVA STARCH/ ETHANOL PRODUCTION

Ethanol has recently become an alternative energy because of the continuous reduction of limited fossil fuel stock. Ethanol produced by a fermentation process, generally referred as bioethanol, is considered to be a partial solution to the worldwide energy crisis. Thailand is the world's third largest producer for cassava. Cassava is an efficient carbohydrate crop and cheap substrate for conversion to ethanol. Traditionally, industrial bioethanol fermentation involves two major steps: cassava starch hydrolysis and fermentation. *Saccharomyces cerevisiae* lacks of amylolytic activity and is unable to directly utilize starch for growth and fermentation. It requires intensive amount of energy and starch hydrolysis enzyme to gelatinize, liquefied and dextrinize the raw starch before fermentation to produce ethanol.

It has been suggested that genetically engineered yeast which expresses amylolytic enzymes, simultaneous starch hydrolysis and fermentation. This improvement could greatly reduce the capital and energy cost in current bioethanol producing plants and make bioethanol production more economic. In this project, bioconversion of cassava starch to fermentation sugar was investigated using two genetically modified *S. cerevisiae* strains, *S. cerevisiae* TISTR 5596/pSWA2

(expressing α-amylase) and *S. cerevisiae* TISTR 5596/pGAM1 (expressing glucoamylase). Each of *SWA2*gene and *GAM1* gene was cloned downstream of a constitutive promoter, GAP, to obtain yeast expression plasmid named pGAM1 and pSWA2, respectively. These plasmids were introduced into the *S. cerevisiae* chromosome. Each of the pSWA2 and pGAM1 harboring yeast showed detectable amylolytic activity in the culture supernatant. This indicates that the pGAM1 and pS2 harboring yeast secreted biologically active glucoamylase and α-amylase, respectively. In batch fermentation, the ability of *S. cerevisiae* TISTR5596/pGAM1 and *S. cerevisiae* TISTR5596/pSWA2 and the co-cultured of *S. cerevisiae* TISTR5596/pGAM1 and *S. cerevisiae* TISTR5596/pSWA2 on cassava starch utilization and ethanol production were evaluated. The maximum ethanol yield was obtained at the level of 0.465 ± 0.012, 0.489 ± 0.010, and 0.516 ± 0.021 g ethanol/g substrate consumed at 12 days. The maximum ethanol concentration was obtained at the level of 1.478 ± 0.267, 1.992 ± 0.248, and 2.977 ± 0.020 g/l at 25 days. The co-cultured of *S. cerevisiae* TISTR5596/pSWA2 and *S. cerevisiae* TISTR5596/pGAM1 produce ethanol with higher liters and yield than those of single culture. The recombinant yeast converts starch into ethanol was successfully engineered. The transgene was expressed in host strain. The study is the promising start for future ethanol producing yeast development.

School of Biology Student's Signature_____

Academic Year 2012 Advisor's Signature_____

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LIST OF ABBREVIATIONS

ATP	=	Adenosine triphosphate
Amp	=	Ampicillin
α	=	Alpha
Arg	=	Arginine
ARS	=	Autonomously replicating sequence
Asp	=	Asparagine
BSA	=	Bovine serum albumin
$CaCl_2$	=	Calcium chloride
CEN	=	Centromere
cm	=	Centimeter
CO_2	=	Carbon dioxide
CYC1	=	Cytochrome c-1
^oC	=	Degree Celsius
Da	=	Dalton
DNS	=	3, 5-Dinitrosalicylic acid
dNTPs	=	Deoxynucleotides triphospates
DTT	=	Dithiothreitol
EDTA	=	Ethylene diaminetetraacetic acid
el al.	=	Et alia (and others)
ER	=	Endoplasmic reticulum
GAM1	=	Glucoamylase

LIST OF ABBREVIATIONS (Continued)

GAP	=	Glyceraldehydes-3-phosphate dehydrogenase
GRAS	=	Generally Recognized As Safe
g/l	=	Gram per liter
g	=	Gram
HBsAg	=	Hepatitis B surface antigen
HCl	=	Hydrogen chloride
HIS3	=	Himidazoleglycerol-phosphate dehydratase
HPLC	=	High Performance Liquid Chromatography
hr	=	Hours
I_2	=	Iodine
Kb	=	Kilobase pair
KCl	=	Potassium chloride
KEX2	=	Kexin-like proteinases
KH_2PO_4	=	Potassium dihydrogen phosphate
KI	=	Potassium iodide
kv	=	Kilovolte
LB	=	Luria-Bertani
LBZ	=	Luria-Bertani Zeocin™
Leu	=	Leucine
Lys	=	Lyscine
MCS	=	Multiple Cloning Site

LIST OF ABBREVIATIONS (Continued)

MFα	=	Mating factor α
mg	=	Milligram
$MgCl_2$	=	Magnesium chloride
min	=	Minute
ml	=	Milliliter
MSG	=	Monosodium glutamate
MTBE	=	Methyl tertiary-butyl ether
M	=	Molar
mM	=	Millimolar
NADH	=	Nicotinamide adenine dinucleotide
NAD^+	=	Nicotinamide adenine dinucleotide
Na_2HPO_4	=	Disodium hydrogen phosphate
NESDB	=	National Economic and Social Development Board
NaOAC	=	Sodium acetate
NaOH	=	Sodium hydroxide
$(NH_4)_2SO_4$	=	Ammonium sulphate
NaI	=	Sodium iodide
ng	=	Nano gram
nm	=	Nanometer
NOx	=	Nitrogen oxides
ORF	=	Open reading frame

LIST OF ABBREVIATIONS (Continued)

PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
pmol	=	Picomoles
Psi	=	Pounds per square inch
%	=	Percentage
RNase A	=	Ribonuclease A
rpm	=	Revolutions per minute
SWA2	=	α-Amylase
Sec	=	Second
SDS	=	Sodium dodecyl sulfate
STEl3	=	The dipeptidyl aminopeptidase
TISTR	=	Thailand Institute of Scientific and Technological Research
TRP1	=	Tyrosinase-related protein 1
Trp	=	Tryptophan
UV	=	Ultraviolet
U/ml	=	Unit per milliliter
V	=	Volt
w/v	=	Weight by volume
YEp	=	Yeast episomal plasmid
Ura	=	Uracil

LIST OF ABBREVIATIONS (Continued)

YIp	=	Yeast integration plasmids
YPD	=	Yeast Peptone Dextrose
μ	=	Micron
μl	=	Microliter
μM	=	Micromolar
μg/ml	=	Microgram per milliliter
μm	=	Micrometer
μg/ml	=	Microgram per milliliter

CHAPTER I

INTRODUCTION

1.1 Introduction

Ethanol production and consumption have increased in the world due to an increasing in global oil price and global warming awareness (Chandel et al., 2007). The major crops used for ethanol production mainly are sugarcane and starchy plants. Thailand is the world's third largest producer of cassava starch. Cassava is considered to be one of the best carbohydrate crops which can serve as cheap but efficient substrate for conversion to ethanol (Office of Agricultural Economics, 2008). In order to convert starch to glucose, the starch hydrolysis demands large amount of amylolytic enzymes, namely, glucoamylase (EC 3.2.1.3) and α-amylase (EC 3.2.1.1). These enzymes are required in the traditional starch fermentation process. In addition, the high cooking temperature must be used to break down raw starch before hydrolysis of starch and fermentation for ethanol production (Eksteen et al., 2003). These processes pose high cost for energy and for amylolytic enzyme, especially in case of raw starch hydrolysis (De-mot et al., 1985). The α-amylase performs random endoamylolytic cleavage of 1,4-α-glycosidic linkages in starch to release oligosaccharides. Glucoamylase is a hydrolyzing enzyme acting on both α-1, 4 and α-1,6 glucosidic links of starch, resulting in the removal of a single glucose residue one at a time from the reducing end of the polymer. Both enzymes working together

greatly increase the rate and efficiency of starch hydrolysis. It can degrade both amylose and amylopectin and produce glucose (Eksteen et al., 2003). *Saccharomyces cerevisiae* is widely used in industry for producing bioethanol as fuel. Since wild type yeast is not able to directly utilize starch for growth and fermentation. Many researchers have reported on attempts to resolve this problem by using recombinant glucoamylase-expressing yeasts with the ability to ferment starch to ethanol directly (Ashikari et al., 1989; Cole et al., 1988; Inlow et al., 1988; Nakamura et al., 1997).

All of the ethanol fermentations using genetically modified yeast have not been reported on cassava fermentation to produce ethanol. It is better to simplify the fermentation by eliminating the starch decomposing step and using single yeast strain to convert starch to ethanol. The objective of this study was to construct an integrative vector and develop two recombinant strains of *S. cerevisiae* that can express glucoamylase (GAM1) and α-amylase (SWA2) enzymes for conversion of cassava starch to ethanol. To achieve the aim of study, an integrative vector containing glucoamylase gene or α-amylase gene under the GAP promoter were constructed and transformed into the yeast genome. The transformant yeasts were analyzed for growth and utilization of cassava starch. Ethanol production from cassava starch was examined using the transformant yeast in the batch fermentation.

1.2 Research objectives

(1) To isolate the glucoamylase (*GAM1*) and α-amylase (*SWA2*) genes from chromosomal DNA of *Debaryomyces occidentalis*.

(2) To construct *S. cerevisiae* strains that contain glucoamylase and/or α-amylase gene in their chromosomal DNA.

(3) To functional express *GAM1* or *SWA2* genes in *S. cerevisiae* transformant strains using cassava starch as a sole carbon source in batch fermentation.

1.3 Research hypothesis

The recombinant yeast harboring exogenous glucoamylase gene and/or α-amylase gene is able to utilize starch and directly convert starch into ethanol.

1.4 Scope and limitations of the study

The glucoamylase and α-amylase genes were isolated from *D. occidentalis* strain. To construct recombinant yeast strain, the glucoamylase and α-amylase genes were separated and expressed in *S. cerevisiae* TISTR 5596 wild type strain. Each of the gene encoding enzyme was integrated into yeast chromosome using homologous recombination. The transformed yeasts were tested for their ability to express glucoamylase and α-amylase that utilize cassava starch as a sole carbon source for growth and ethanol fermentation.

1.5 Expected Results

(1) Glucoamylase (*GAM1*) and α-amylase (*SWA2*) genes from chromosomal DNA of *D. occidentalis* will be obtained.

(2) Amylolytic strains of *S. cerevisiae* will be generated by integration of glucoamylase and/or α-amylase gene into chromosomal DNA of *S. cerevisiae* wild type strain.

(3) The transformed *S. cerevisiae* cells will express and secrete glucoamylase and/or α-amylase for hydrolysis of cassava starch with high efficiency. The recombinant strains will utilize cassava starch to produce ethanol in one-step process.

CHAPTER II

LITERATURE REVIEW

2.1 Bioethanol as a renewable energy

Ethanol (ethyl alcohol, bioethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer. The use of ethanol as an alternative fuel source is presently a worldwide topic of discussion and research. The energy crisis has become a major issue due to the depletion of fossil fuels. The demand for an economical renewable energy resource is urgent. Ethanol has some advantages when it is used as an oxygenate. In comparison to many other fuel resources, ethanol has advantages of being renewable and environmentally friendly. It is biodegradable and burns cleanly with less generation of greenhouse gases than fossil fuels. Global potential of bioenergy is represented in energy crops and lignocellulosic residues. Conversion of these feedstocks into biofuels is an important choice for the exploitation of alternative energy sources and reduction of polluting gases. In addition, the utilization of biofuels has important economic and social effects. Moreover, the development of energy crops dedicated to the biofuels production would imply a boost to agricultural sector.

Bio-ethanol is an oxygenated fuel that contains 35% oxygen, which reduces particulate and nitrogen oxides (NOx) emissions from combustion. Related to MTBE (Methyl tertiary-butyl ether), ethanol has greater octane booster properties, it is not toxic, and does not contaminate water sources. Using bio-ethanol blended fuel for automobiles can significantly reduce petroleum use and exhaust greenhouse gas

emission (Wang et al., 1999). The world ethanol production has reached about 51,000 million litres. USA and Brazil are the leading producers. In average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol (htttp: //www. ethanolrfa.org/industry/statistics, 2013). In addition, production of bioethanol from starch could reduce the consumption of fossil fuels and also reduce the net emission of CO_2 (Bourne, 2007). Although sugar cane is reported to be the most widely used raw material for bioethanol production in North America (Lang et al., 2001), starch is currently the most economical raw material (Lang et al., 2001; Bothast et al., 2005).

2.2 Starch substrate and cassava starch

Starch $(C_6H_{10}O_5)_n$ is a major reserved carbohydrate of all higher plants. When heated in water the hydrogen bonds holding the granules together begin to weaken and this permits them to swell and gelatinize. Starches are produced commercially from the seeds of plants, such as corn, wheat, sorghum or rice; from the tubers and roots of plants such as cassava, potato, arrowroot and the pith of sago palm.

Starch is a high yield feedstock for ethanol production, but its hydrolysis is required to produce ethanol by fermentation. Starches from the tubers and root crops need to be hydrolyzed to simple fermentable sugars by the enzymes α-amylase and glucoamylase, before they can be converted to ethanol. In the USA, corn is the major source of starch; in Western Canada, starch is mainly supplied by wheat with 50% of it being produced in Saskatchewan (Bothast et al., 2005).

Starch is a polymer of glucose units and it is a carbohydrate produced by most plants. It is a carbohydrate source consisting of two molecules amylose and

amylopectin. Amylose is composed of linear chains of α-1,4 linked D-glucose residues. Hence it is extensively degraded by α-amylase. Amylopectin is formed from α-1,4 linked chains of glucose with 1,6 linked branch points. The two forms are shown in Figure 2.2. The most important starch sources are cereals (corn, wheat and rice), cassava, sweet potatoes and potatoes. The starch content depends on the plant species (Table 2.1) (Krisztina, 2003).

Therefore, efficient starch hydrolysis requires both α-amylase and glucoamylase (Nyerhovwo, 2004). The α-amylase catalyzes the hydrolysis of internal α-1,4 glycosidase bond. Glucoamylases typically have a degree of α-1,6 debranching activity (Forarty and Kelly, 1979). Two types of enzymes have been recognized as liquefying and saccharifying. The main difference between them is that the saccharifying enzyme produces a higher yield of reducing sugar than liquefying enzyme.

Table 2.1 Starch content of plants (Krisztina, 2003).

Plant	Average % starch content
Corn	60–70
Potatoes	12–20
Cassava	20–40
Wheat	55–70
Rice	70–75

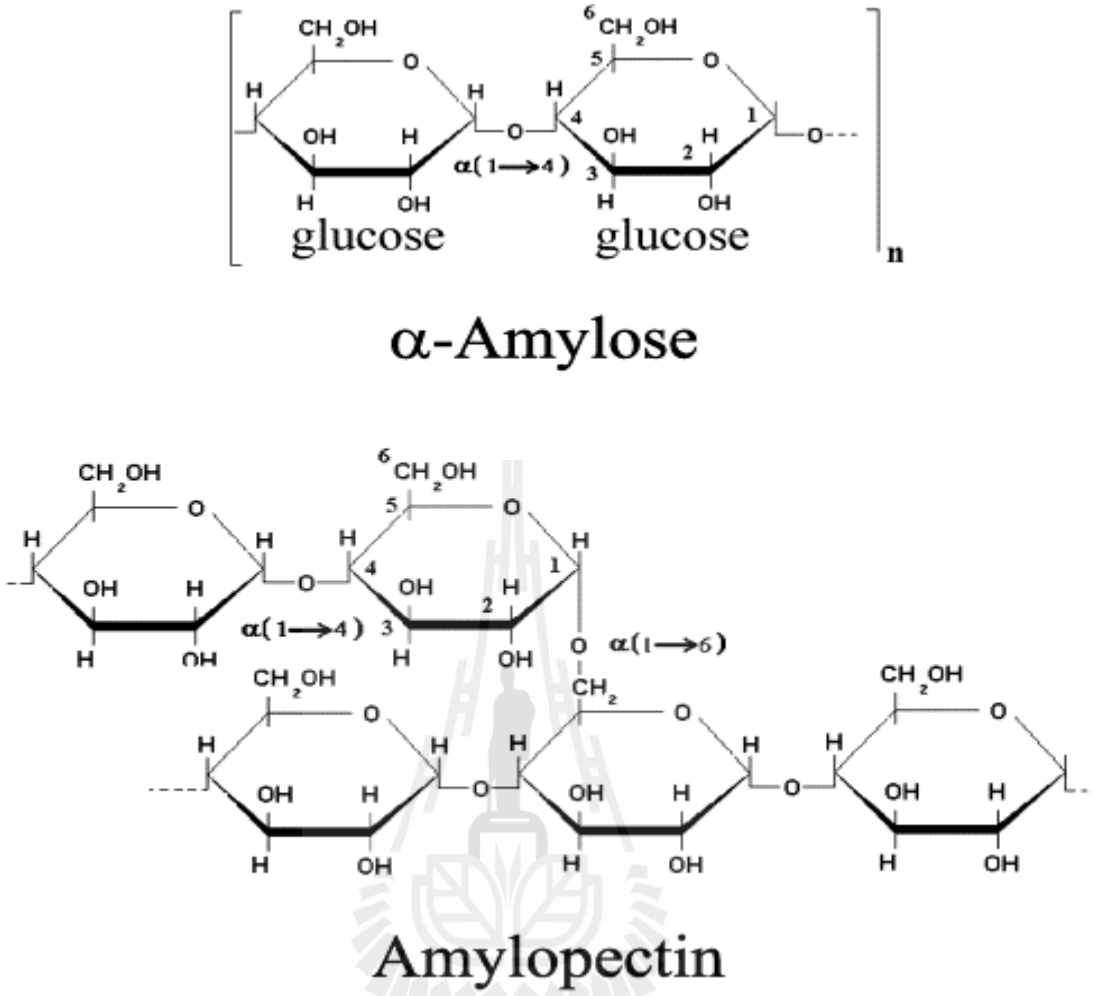

Figure 2.1 Structures of amylose and amylopectin (http://www.ott.doe.gov/biofuels/glossary.html).

2.2.1 Cassava starch in Thailand

Cassava (*Manihot esculenta*) or tapioca is one of the richest fermentable substances and most popular choice of substrates for bioethanol production in the Asian region. Using cassava starch as substrate in bioethanol production will reduce the production cost since cassava plants are abundant, cheap and can easily be planted. It is a good alternative at low production cost and it is a preferred substrate for bioethanol production especially in situation where water availability is limited. It

tolerates drought and yields on relatively low fertility soil where the cultivation of other crops would be uneconomical especially on idle lands (Figure 2.2) (Sanchez and Cardona, 2008). The major crops used for ethanol production in Thailand are sugarcane and cassava as raw inputs. Higher demand and lower supply equal great investment opportunities. Demand for ethanol has been 3 million liters /day in 2012, for use in making octane 95 gasohol, although Thailand's existing ethanol plants are currently able to produce 825,000 liters/day. The cost of raw material is important and cannot be overlooked since it governs the total cost which represents more than 60% of total ethanol production cost (Ogbonna et al., 2001). Currently, Thailand is the largest producer of sugarcane in South-East Asia and the world's third largest producer of cassava. It happens to be the first cassava exporter and the second sugar exporter in the world (National Economic and Social Development Board (NESDB)). Cassava is considered as one of the best raw materials for ethanol production because of the low costs and energy requirements for growing and harvesting the crop (Office of Agricultural Economics, 2008). Moreover, the ethanol production from cassava starch can be value-added of raw materials.

Cassava is the most important source of carbohydrates. Among the starchy staples, cassava gives a carbohydrate source which is about 40% higher than rice and 25% more than corn. Typical compositions of the cassava root are moisture (70%), starch (24%), fiber (2%), protein (1%), and other substances including minerals (3%). Cassava starch is composed of unbranched 17% of amylose and 83% of amylopectin both of which can be hydrolyze either with acid or enzymes to release glucose and maltooligosaccharide (http://www.auxmaillesgodefroy.com/starch, 2012). The cassava is an inexpensive resource of food energy for both human nutrition and

animal feeding (Nyerhovwo, 2004). Lee et al. (1995) studied ethanol production by fermentation using tapioca starch. They reported that liquefication and saccharification of tapioca starch resulted in a glucose-maltose mixture containing approximately 92% glucose and 8% maltose. They proposed a model that accurately represents ethanol production from a mixture of glucose and maltose as substrates. Thus, cassava starch is one of the best fermentable substances for the production of ethanol. At the moment sugar cane is the most widely used crop for bio-ethanol in the tropics, but sugar cane requires a lot of water. Consequently, sites suitable for sugar cane growing are very limited.

Figure 2.2 Cultivation of cassava and cassava tubers.

2.3 A review of the current industrial starch fermentation process

Starch fermentation process, in the part of fermentation economics depends mainly on fermentation plant investment costs, raw material costs, maintenance costs including upstream and downstream costs, process yield and throughput. To make

ethanol production by fermentation an economically feasible process on an industrial scale, the use of inexpensive substrates and the maximization of substrate utilization and conversion are significant aspects.

Although starchy materials are available in abundance as carbon sources for cultivation, *S. cerevisiae*, the key organism used for alcohol fermentation, does not express starch-hydrolyzing enzymes. It is unable to utilize starch directly for vegetative growth. In industry, starch is broken down into single glucose residues before it can be used by yeast for fermentation. In order to completely hydrolyze starch, it is treated with either strong acids or starch-hydrolyzing enzymes which break the α-1,4 and α-1,6 linkages in the starch molecules which releases glucose monomers (Tester et al., 2004).

In the acid hydrolysis process, the components of starchy material are broken down by the use of strong and concentrated aqueous solutions of mineral acids, such as hydrochloric acid and sulfuric acids, at temperature lower than 100 °C. Amylose fraction of starch is broken down earlier than the amylopectin and the sugars released at earlier time periods when they are exposed to harsh condition, significantly lower the production yield of the fermentation (Roberson et al., 2006).

In the enzymatic process, the raw starch material is pre-treated in order to increase accessibility to starch hydrolyzing enzymes, such as α-amylase and glucoamylase enzyme. The enzymatic conversion of all starch includes (1) gelatinization of starch by heating, which involves the dissolution of starch granules, thereby forming a viscous suspension, (2) liquefaction by addition of α-amylase, which involves partial hydrolysis and loss in viscosity, and (3) saccharification which involves the production of glucose and maltose via further hydrolysis with

glucoamylase enzyme (Figure 2.3) and fermentation of glucose to ethanol under anaerobic conditions. Enzyme hydrolysis has several advantages: it is more specific, therefore fewer by products are formed, and hence yields are higher. Both enzymes working together greatly increase the rate and efficiency of starch hydrolysis. These processes present high cost. The starch hydrolysis must use large amounts glucoamylase and α-amylase. Moreover, the starchy materials need to be cooked at a high temperature (140-180 °C) to obtain a high sugar residue that can be converted sugar to ethanol by microorganism. This process is expensive due to the high energy cost for cooking of starch material. The energy cost for cooking of starchy materials was made lower by using noncooking and low-temperature-cooking fermentation systems. These have succeeded in reducing energy consumption by approximately 50% (Matsumoto et al., 1982). Nonetheless it is still necessary to add large amounts of starch-hydrolyzing enzyme to hydrolyze the starchy materials to glucose. High energy consumption and the need to use starch-hydrolyzing enzyme, especially in case of raw starch hydrolysis greatly increase. The eventual production costs of ethanol fermentation, and limit the economic potential of bioethanol as a fuel (De-Mot et al., 1985).

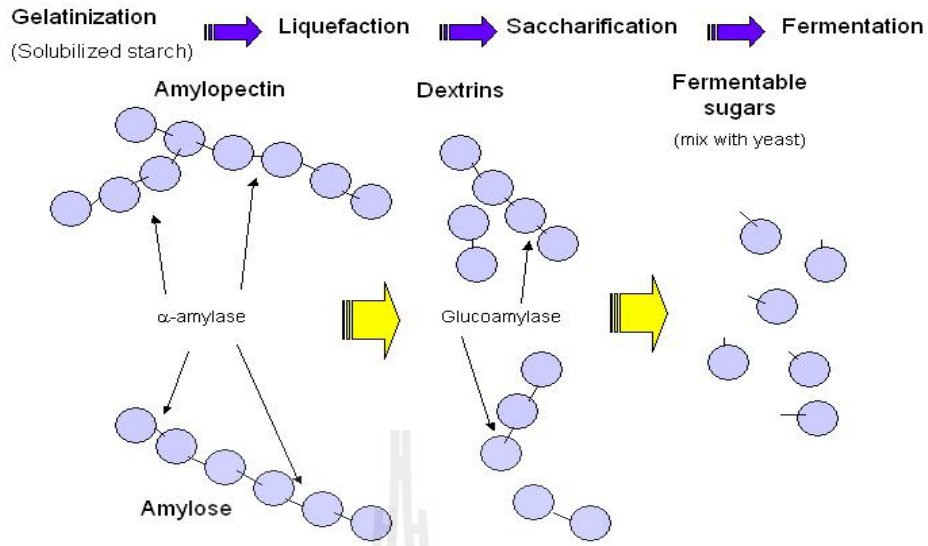

Figure 2.3 Schematic representation of the action of amylases on starch (Bo Liao, Master's Thesis, University of Saskatchewan, 2008).

2.4 Starch hydrolysis enzyme

Historically, plants and animals were considered the best sources of enzymes. But recently, microbial enzymes have gained importance as their production is more economical. Two major enzymes, α-amylase and glucoamylase, have important applications in several industries like baking, brewing, detergent, medicine, textile, and pharmaceutical. To date, these enzymes are mostly produced from various strains of bacteria, fungi and grains like barley. As an example, Strasser et al. (1989) has reported that yeast genus *Schawanniomyces* spp. secreted α-amylase and a glucoamylase to hydrolyze starch to glucose using extracellular enzyme.

The brewing industry uses these enzymes for starch hydrolysis prior to yeast fermentation for bioethanol production. The conversion of starch into sugars like

glucose requires two enzymes: α-amylase and glucoamylase. Normally, α-amylase is first added to break down the long chains of the starch molecule into small pieces to generate more reducing ends for glucoamylase to attack. Glucoamylase will then digest these oligosaccharides into glucose. The α-amylase enzyme has been shown to attack both soluble starch as well as starch particles in aqueous suspension (Hill et al., 1997). It is well-known in industrial starch processing that α-amylase and glucoamylase attack starch and oligosaccharides in a synergistic fashion, thus optimal starch hydrolysis rate is achieved by incorporating both enzymes.

2.4.1 α-Amylase and starch liquefaction

The α-amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1), which plays an important role in starch and fermentation industries, is an important variety of hydrolytic enzymes. This enzyme plays a key role in the starch liquefaction process leading to refined syrups and sweeteners widely used in the food industry. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries. The α-amylases found in several groups of organisms including bacteria, fungi, plants and animals, with endo-action. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al., 2000). The enzyme catalyzing degradation of starch into smaller sugars is important in the production of syrups in food industry (Figure 2.4) (Lily et al., 2007). α-amylases are enzymes that act on starch and break them up into sugars (hence the term saccharification), and work by hydrolyzing the straight chain bonds between the individual glucose molecules that make up the starch chain. The enzymes catalyze the hydrolysis of 1,4-glucosidic

linkages of starch, glycogen, and dextrin at random. Hydrolysis of starch with α-amylase produces a mixture of glucose, maltose, and oligosaccharides as the product, leaving smaller molecular weight branched glucan chains, α-limit dextrins, as the remaining substrate (Sutliff et al., 1991). Since α-amylase does not cleave α-1,6 linkages, its end-products constitute branched oligosaccharides.

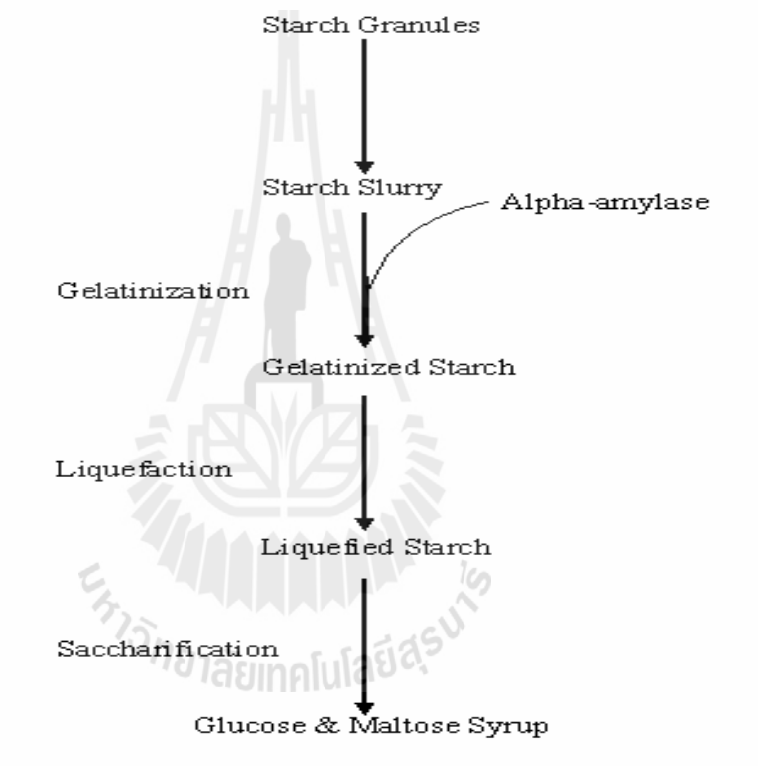

Figure 2.4 Flow chart of starch liquefaction process (Lily et al., 2007).

2.4.2 Glucoamylase

Glucoamylase (1, 4-α-D-glucan glucohydrolase; EC 3.2.1.3), also known as amyloglucosidase, is produced by microorganisms, almost exclusively by fungi genus *Aspergillus, Penicillium,* and *Rhizopus*. It is a key industrial starch processing

enzyme and commonly used in conjunction with α-amylase to convert starch into glucose. It hydrolyzes α-1,4 and α-1,6 glucosidic linkages from starch in an exo-acting fashion and releases glucose. It acts in the last step of starch degradation hydrolysing α-1,4 bonds at the non-reducing end of starch and related malto-oligosaccharides, yielding free glucose. They are widely used in the industrial production of glucose and fructose. The great potential of starch as a renewable carbon source has stimulated research aiming at improving its transformation into ethanol (García et al., 2005).

To date, among all the starch hydrolyzing enzymes that have been recorded, α amylase and glucoamylase are the ones that have been studied the most due to their industrial importance in starch processing. The employment of cloning and recombinant DNA techniques made it possible to study many of those enzymes at the molecular level, and the knowledge has provided valuable insights into designing starch hydrolyzing enzymes with industrial-favored characteristics. Both α-Amylase and glucoamylase, engineered with improved industrial characteristics, will also be discussed.

2.4.3 The budding yeast *Debaryomyces occidentalis* secretes α-amylase and glucoamylase

The current taxonomic classification of *Schwanniomyces occidentalis* has changed these strains into *Debaryomyces occidentalis* because no significant difference in the sequences of 18S and 25S of ribosomal RNA between *Schwanniomyces* and *Debaryomyces* was found. *D. occidentalis* produces different extracellular enzymes such as α-galactosidase, β-glucosidase, inulinase, invertase,

phytase and amylolytic enzymes: α-amylase and glucoamylase, so this yeast can completely hydrolyze soluble starch and its derivatives (Wang et al., 1999).

The yeast *D. occidentalis* secretes α-amylase and glucoamylase, which also shares an α-D-(1,6) glucosidase activity and liquefies starch by complete hydrolysis to glucose and ferment dextrin and starch to ethanol with high efficiency (Ingledew, 1993). In contrast, yeasts of *D. occidentalis*. showed a low ethanol tolerance (Yokioka et al., 1998). In addition, the yeast secretes a variety of high-molecular-weight proteins (Ingledew, 1987; Dowhanick et al., 1987). This strain expresses significant debranching activity as a part of the glucoamylase, indicating that it possess the ability to hydrolyze both α-1,4 and α-1,6-glucosidase linkages, which is essential for complete hydrolysis of starch (Dohmen et al., 1990).

Although biochemical evidence suggests that a single enzyme is responsible for this α-amylase activity, molecular cloning techniques have permitted the isolation of two different genes, referred to as *AMYI,* also named *SWA1* for *Schwanniomyces* amylase and *SWA2* (Abarca et al., 1989; Abarca et al., 1991; Strasser et al., 1989). The yeast *D. occidentalis* is able to use starch as a unique carbon source (Wang et al., 1999) and they were able to isolate the *SWA2* α-amylase gene from this organism, which was expressed in *S. cerevisiae* (Abarca et al., 1991; Yáñez et al., 1998). The starch-hydrolysing enzyme from *D.* occidentalis has been report to be a novel glucoamylase (GAM), showing hydrolytic activity not only on soluble starch but also on maltose and isomaltose. The amino sequence deduced from the *GAM1* gene represented a high level of similarly to human lysosomal α-glucosidase and no similarly to other fungal glucoamylases (Sato et al., 2005).

2.5 Microorganisms for bioethanol production

Although fungi, bacteria, and yeast microorganisms can be used for fermentation, specific yeast (*S. cerevisiae* which also known as Bakers' yeast) is frequently used to ferment glucose. The expansion of the ethanol industry requires the search for new and more efficient ethanologenic microorganisms. The varieties of bacteria and yeast can be used for ethanol production and a large number of bacteria are capable of ethanol production. But most of them produce other end products like butanol, isopropylalcohol, acetic acid, formic acid, arabitol, glycerol, acetone, methane, etc., as well as ethanol (http://www.scribd.com/doc/38212588/ Fermentation -of-Ethanol, 2008).

Zymomonas mobilis is a bacterium that has been extensively investigated with regard to bioethanol production. It is suitable for ethanol production due to its greater tolerance to high ethanol concentrations as compared to traditional *Saccharomyces* yeast. Ruanglek et al. (2006) reported that in the USA and Brazil, *Z. mobilis* is used for ethanol production from corn steep liquor. Tano et al. (2000) studied fermentation by *Z. mobilis* CP4 using sugarcane juice. However, a disadvantage of this bacterium is that it is capable of fermenting only glucose, fructose and sucrose to bioethanol (Ruanglek et al., 2006).

Yeast is the most commonly used microorganism for ethanol production by fermentation. The most widely used and popular biological agents of wine and beer fermentations are yeasts of the genus *Saccharomyces*. Some of their properties are: fast growth rates, efficient glucose repression, efficient ethanol production, and the tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels (Piskur et al., 2006). Some examples of yeasts used for ethanol

production by fermentation are *S. cerevisiae*, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Kluyveromyces lactis*, and *Saccharomyces diastaticus*.

2.5.1 *Saccharomyces cerevisiae* as fermenting yeast

The yeast of *S. cerevisiae* is able to utilize sugars in the presence of oxygen (aerobic) and in conditions that are completely absent of oxygen (anaerobic). Aerobic catabolism produces more energy and is called respiration, while anaerobic catabolism produces less energy and is called fermentation. Compared to other yeast species, *S. cerevisiae* is able to perform alcoholic fermentation, and can grow under strictly anaerobic conditions (Van Dijken et al., 1993). It can tolerate high ethanol concentrations up to 15%. Its glucose conversion rate is about 95% of the theoretical maximum. It can grow at low pH conditions, and it has the property of GRAS (Generally Recognized As Safe) (Vertes et al., 2008).

Under aerobic conditions, after the depletion of glucose, the metabolism in *S. cerevisiae* changes such that the accumulated ethanol is utilized for yeast propagation. However, under strict anaerobic conditions, ethanol will not be consumed (Sanchez et al., 2009). Once sugars enter the yeast cell, they are processed through the glycolytic pathway into pyruvate. The fermentation pathway is involved in the conversion of pyruvate to ethanol, producing ATP, CO_2, and NAD^+. During fermentation, yeast recycles NADH in the acetaldehyde-to-ethanol conversion step, which is catalyzed by alcohol dehydrogenase and is readily reversible. In the presence of oxygen, ethanol can be converted back to acetaldehyde, which allows yeast to grow on ethanol after depletion of sugars (Figure 2.5). *S. cerevisiae* is sensitive to temperatures greater than 35°C and is subject to lactic acid bacterial contamination

(Verte et al., 2008). Moreover, it is incapable of direct utilization of starch due to the lack of its own starch-hydrolyzing enzymes. Thus, DNA recombinant technologies have enabled researchers to construct a series of genetically modified yeast strains with improved industrial properties. The application of biotechnology has significantly transformed the bioethanol industry (Branduardi et al., 2008).

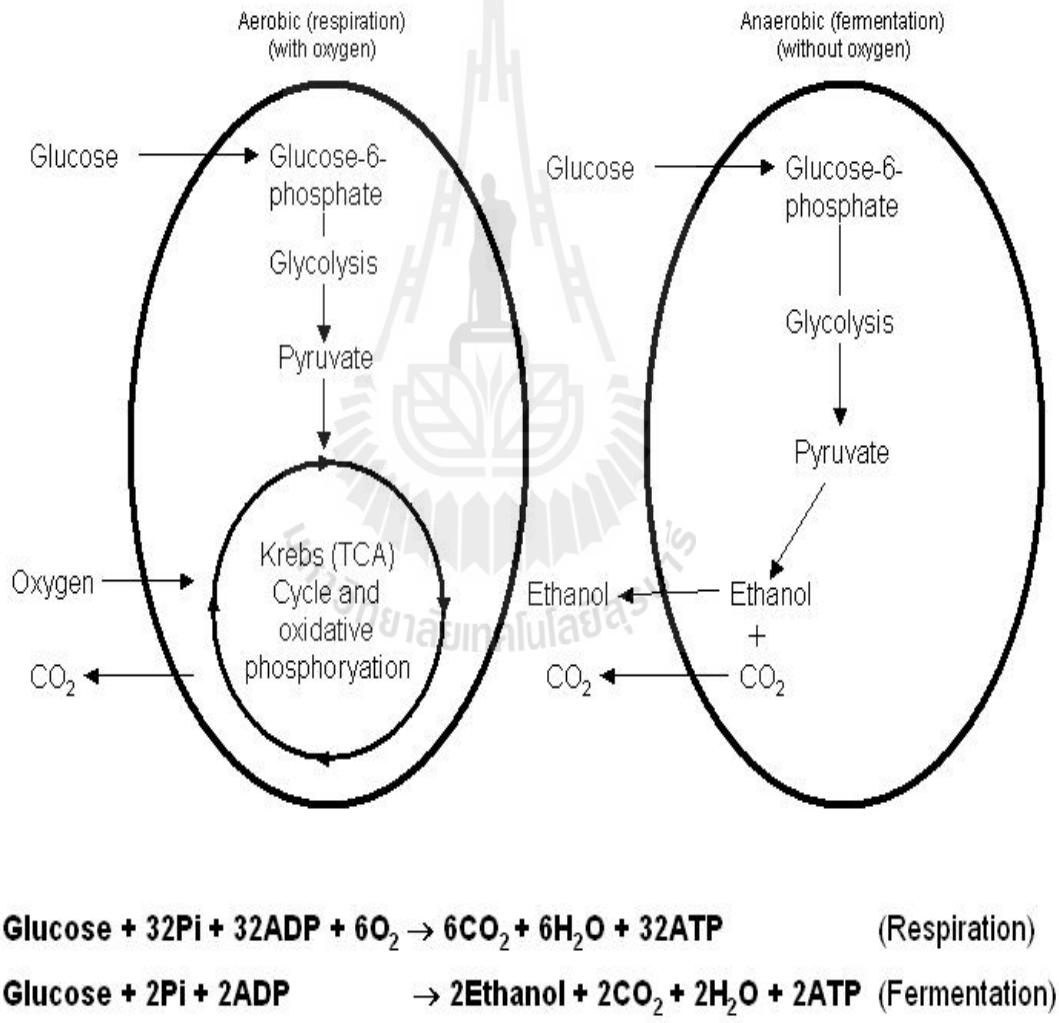

Figure 2.5 Aerobic and anaerobic catabolic pathways for glucose utilization in yeast (Bo Liao, Master's Thesis, University of Saskatchewan, 2008).

2.6 Genetic engineering of *Saccharomyces cerevisiae* for bioethanol production

In the industry whereby ethanol is produced from starch, temperature around 140°C-180°C is applied to cook the starch during hydrolysis using α-amylase prior to liquefaction. This high-temperature completely sterilizes harmful microorganisms and increases the efficiency of saccharification for high ethanol yield (Shigechi et al., 2004a, Shigechi et al., 2004b). Consequently, this resulted in high energy consumption and added cost to amylolitic enzymes used in the process which ultimately increased the overall production cost. Several methods have been developed to reduce the energy consumption by applying milder liquefaction and/or saccharification temperatures (Kolusheva and Marinova, 2007; Majovic et al., 2006; Montesinos and Navarro, 2000; Paolucci-Jeanjean et al., 2000) and also by exercising noncooking fermentation (Zhang et al., 2010). However, these types of fermentation usually required longer process time and sometimes may demand for additional volume of enzyme to maintain same productivity.

Wild type *S. cerevisiae* strains are unable to directly utilize starch materials because they lack starch-hydrolyzing activity. One of the attempts to resolve this problem is by constructing recombinant *S. cerevisiae* to coproduce α-amylase and glucoamylase with incorporating low cooking temperature of starch prior to fermentation by many research teams as shown in Table 2.2.

Table 2.2 Recombinant *Saccharomyces cerevisiae* for direct fermentation at low cooking temperature.

Recombinant strains	Source of α-amylase	Source of glucoamylase	Type of starch	Starch concentration (g/l)	Max. ethanol concentration (g/l)	Authors
S. cerevisiae	*B. subtilis*	*Aspergillus awamori*	Pure starch in 2.5 l fed batch	40	29.7	Altıntaş et al. (2002)
S. cerevisiae	*B. subtilis*	*A. awamori*	Raw corn starch	5- 80	47.5(fed batch) 15.6 (batch)	Ülgen et al. (2002)
S. cerevisiae	*Lipomyces kononenkoae*	*Saccharomycopsis fibuligera*	Pure starch (Merck)	55	21	Knox et al. (2004)
S. cerevisiae	*Bacillus stearothermophilus*	*Rhizopus oryzae*	Corn starch cooked at 80°C	50 90	18 30	Shigechi et al. (2004a)
S. cerevisiae	*Streptococcus bovis*	*R. oryzae*	Raw corn starch in shake flask	200	61.8	Shigechi et al. (2004b)
S. cerevisiae	*B. subtilis*	*A. awamori*	Raw corn starch	50 g/l starch + 4 g/l (w/v) glucose	6.61	Oner et al. (2005)
S. cerevisiae	Not stated	Not stated	Raw corn starch	100	8	Khaw et al. (2007)
S. cerevisiae	Not required	*A. awamori,* *R. oryzae*	Corn starch	50	18.5	Kotaka et al. (2008)

Development of a fermenting amylolytic yeast strain would make bioethanol production more economically feasible. In order to construct a recombinant strain of *S. cerevisiae* that is capable of breaking down starch, heterologous starch-hydrolyzing enzymes need to be expressed in their functional forms at high levels in *S. cerevisiae*. Such genetically modified yeast, called recombinant yeast, should be able to utilize starch for proliferation and fermentation simultaneously. Many studies have demonstrated expression and secretion of functional starch-hydrolyzing enzymes from various sources by genetically modified yeast strains. Many strains were shown to be able to utilize soluble or raw starch as the sole carbon source for proliferation (Bitter et al., 1984; Ruohonen et al., 1995 and Vainio, 1994). Several investigators reported that direct fermentation of starch using recombinant yeast offers a better alternative to the conventional multistage using commercial amylases for liquefaction and saccharification followed by fermentation with yeast (Verma et al., 2000; Knox et al., 2004).

2.6.1 Use of yeast episomal plasmids for expression of heterologous genes

Most yeast expression vectors have been based on the multi-copy 2μ plasmid and contain sequences for propagation in *Escherichia coli* and in yeast, as well as a yeast promoter and terminator for efficient transcription of the foreign gene. Commonly used yeast episomal plasmids include the ARS (autonomously replicating sequence)/CEN (centromere) plasmid and the 2μ based plasmid, which function as origins of replication, and the native 2μ circle of *S. cerevisiae* (Beggs, 1976; Michael et al., 1992; Murray, 1987).

ARS/CEN plasmid is very stable in yeast, but the copy number is reduced to 1 or 2 per cell (Clarke and Carbon, 1980). Thus, it is only used for low level expression. The most commonly employed yeast episomal plasmid and the most commonly-used expression vectors are *E.coli*-yeast shuttle vectors based on the 2μ based plasmid (YEp). Yeast episomal plasmids generally exist in 20-100 copies per cell, and have been widely used for high-level expression of heterologous proteins. The backbone of a yeast expression episomal plasmid is a bacteria-yeast shuttle vector, which is composed of both bacterial and yeast elements for the selection and replication of the plasmid in bacteria and yeast, respectively. The bacterial components of the plasmid consists of a bacteria selection marker (usually an antibiotic resistance gene), and the bacterial origin of replication, which is responsible for the replication of the plasmid in *E. coli*. The yeast elements are composed of a yeast selection marker, which could be an antibiotic resistance gene (auxotrophic selection marker) or could be an amino acid synthesis gene (prototrophic selection marker), and the yeast origin of replication (for replication of the plasmid in yeast). On the shuttle vector backbone, the gene of interest can be inserted at the designated multiple cloning sites (MCS) which is located between a yeast promoter and transcription termination site. The promoter regulates the transcription of the inserted gene of interest (Armstrong et al., 1989). When the auxotrophic markers (such as Trp, Ura- or Leu-) are used for selection purposes, the yeast strain transformed with the plasmid has to be an auxotrophic mutant of the corresponding amino acid. This means that the strain is deficient for synthesizing that particular amino acid and is unable to survive without the amino acid supplied in the medium or transformed with the plasmid containing the auxotrophic marker (Loison et al., 1986). In order to select

yeast strains with no auxotrophic mutants, yeast episomal plasmids containing dominant (or prototrophic) selection markers can be used. When a dominant selection marker is used for the selection purpose, the corresponding antibiotic has to be added into the culture medium. However, the addition of antibiotics adds extra operational costs to fermentation plants and a large amount of antibiotic are considered hazardous to the environment. Another issue related to using episomal plasmids for heterologous protein expression is plasmid stability (or segregational instability). The instability is mainly caused by unequal separation of plasmids from the mother cell to daughter cells. Eventually cells carrying no plasmids will appear in the cultural medium. Even under selective conditions, episomal plasmids are mitotically unstable during long-term cultivation (Guthrie et al., 1991). Plasmid stability is normally measured as a ratio of the number of plasmid-bearing cells over that of the plasmid-free cells at a certain time point during the fermentation (Filho et al., 1986; Ruohonen et al., 1987; Kondo et al., 2002; Michael et al., 1992). Integration of the plasmis DNA into the yeast chromosome could overcome the plasmid stability problem. Genes integrated into the yeast genome are generally very stable, even under non-selective conditions (Altintas et al., 2001; Nakamura et al., 2002).

2.6.2 Use of yeast integration plasmids for expression of heterologous genes

Chromosomal integration offers a more stable alternative to episomal maintenance of foreign DNA. *S. cerevisiae* integration normally occurs by homologous recombination. Integration of genes into yeast chromosomes eliminates the segregational instability related to episomal plasmids, and offers an alternative for

stable expression of heterologous genes (Guthrie et al., 1991). Yeast integration plasmids (YIp) have the same composition as episomal plasmids except that the yeast replication origin is replaced with a segment of DNA which is homologous to the yeast genome sequence where the plasmid is integrated. The integration plasmid is normally linearized at the homologous DNA sequence before transformation, since linearized plasmids are transformed at a much higher efficiency than circular ones. Traditionally, heterologous genes are integrated at gene sequences involved in amino acid synthesis, such as *URA3*, *TRP1*, or *HIS3* via homologous recombination (Burke, 1987). However, only one or a few tandem copies can be inserted at these targeting locations. Compared to the copy numbers of episomal plasmids that a single yeast cell can maintain, the copy numbers of genome integrated genes markedly limits the expression levels of heterologous proteins. Traditional integration plasmids only insert one to few copies of the integrated gene in the yeast genome, which is low compared to the copy number of episomal plasmid that would be in a yeast cell (Romanos et al., 1992; Guthrie et al., 1991; Lopes et al., 1990).

2.6.3 Secretion of foreign proteins

A wide variety of heterologous proteins have been secreted from yeast and this approach offers certain advantages over intracellular production. Many pharmacologically important proteins are naturally secreted and can often only adopt their correct conformation by folding within the secretory pathway (Ngsee and Smith, 1990). Thus secretion is used mainly for the production of correctly-folded, naturally-secreted proteins, but there are, in addition, other instances when secretion may be preferable. As in higher eukaryotes, protein secretion in yeast is directed by an amino-

terminal signal sequence which mediates co-translational translocation into the endoplasmic reticulum (ER) (Rothblat et al., 1989). The signal peptide is removed by a signal peptidase. In the lumen of the ER asparagine-linked glycosyl structures may be added. The o-linked oligosaccharides may also be added. Proteins are then transported in vesicles to the Golgi where modifications to these glycosyl structures take place (Rudolph et al., 1989). These modifications differ from those made by higher eukaryotic cells and, as a result, glycosylation is increasingly regarded as a major drawback to the secretion of therapeutic glycoproteins from yeast. From the Golgi, proteins are packaged into secretory vesicles and are delivered to the cell surface (Novick and Schekman, 1979) (Figure 2.6).

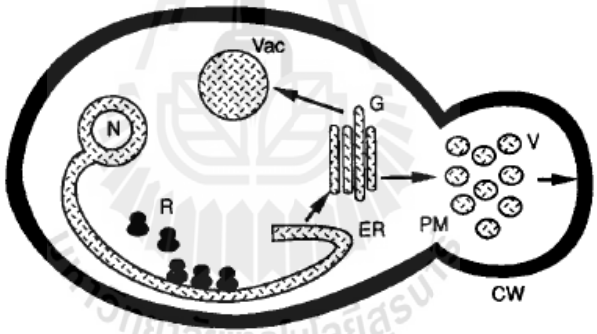

Figure 2.6 The yeast secretory pathway. Arrows indicate the route taken by proteins through the secretory pathway to either the vacuole or the plasma membrane. Once in the ER, it is probable that a default pathway directs a protein to the plasma membrane unless it contains specific signals to cause retention in the ER or Golgi, or to target it to the vacuole. Therefore it is being thought that if a foreign protein could be targeted to the lumen of the ER, it should be successfully secreted (Novick and Schekman, 1979). N, nucleus; R, ribosomes; ER, endoplasmic reticulum; G, Golgi; Vac, vacuole; V, secretory vesicles; PM, plasma membrane; CW, cell wall.

2.6.4 Vectors and signal sequences

The choice of selection marker on a secretion vector may be particularly important since culture conditions may dramatically affect the final yield in the medium. Selection of plasmid-containing cells in a defined medium may result in lower cell density and, in some cases, lower levels of secreted product per cell. Wheat α-amylase secreted from yeast has been shown to reach much higher levels in rich medium than in selective, defined medium (Rothstein et al., 1987). At laboratory scale, it may be preferable therefore to use a dominant marker such as that for G418 resistance. The most extensively used signal sequence for heterologous protein secretion from *S. cerevisiae* is the prepro region from α-factor *(MFα),* frequently used with the *MF*α promoter. *MF*α encodes a 165 amino acid protein, prepro-α-factor, which comprises a signal sequence of 19 amino acids (the pre region) and a pro region, followed by four tandem repeats of the mature 13 amino acid α-factor sequence (Figure 2.7). Each repeat is preceded by a short 'spacer peptide' with the structure Lys-Arg-(Glu/Asp-Ala)$_{2,3}$. Processing of prepro-α-factor involves four proteolytic enzymes: (i) the pre region is cleaved by signal peptidase, (ii) the *KEX2* protease cleaves on the carboxyl terminal side of the Lys-Arg sequence in the pro region and at the junction of each repeat, (iii) *STE13*, a dipeptidyl aminopeptidase, removes the spacer residues at the amino terminus of each repeat, and (iv) the *KEX1* carboxypeptidase removes the Lys and Arg residues at the carboxyl terminus of the first three repeats (Figure 2.7) (Brake, 1989). Secretion of heterologous proteins is directed by the yeast α-factor leader.

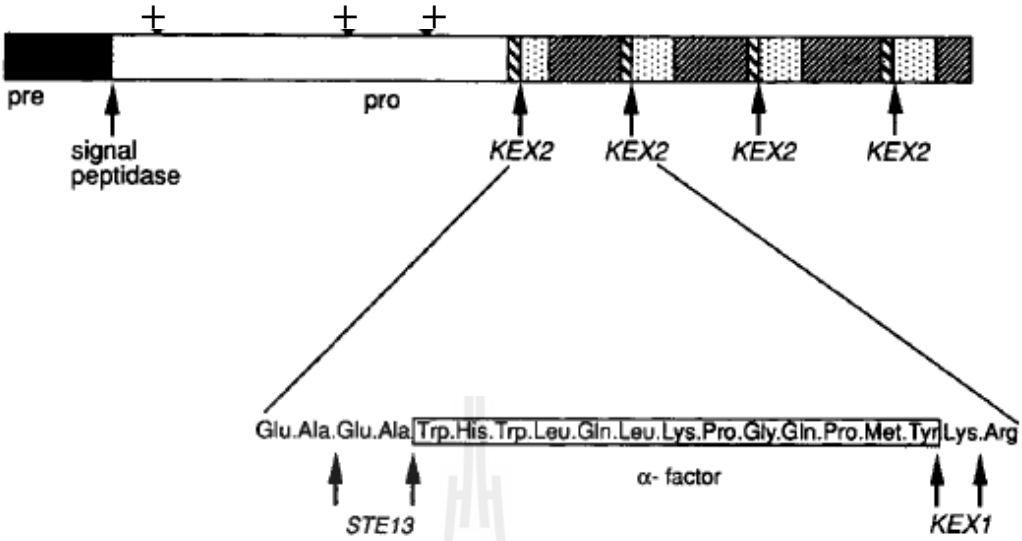

Figure 2.7 The structure and processing of prepro-α-factor. The product of the *MFaZ* gene is shown schematically. The three N-linked glycosylation sites in the pro region are marked (+). The peptide product from the cleavage of prepro-α-factor by *KEX2* is also indicated and sites for further processing by *STE13* and *KEX1* are shown (Brake, 1989).

CHAPTER III

MATERIALS AND METHODS

3.1 Reagent

3.1.1 Chemicals

All chemical are analytical grade and purchased from various suppliers (Sigma, Merck, BIO-RAD, Amersham Biosciences, Biolabs, Promega, GIBCO BRL, and Research Organics).

3.1.2 Miscellaneous biochemical materials

Table 3.1 Lists of biochemical materials, commercial kit and suppliers.

Biochemical Materials	Supplier
3,5 dinitrosalicylic acid	Sigma-Aldrich
Zeocin™	Invitrogen
*Hin*d III digested λ-DNA marker	Promega
Commercial Kit	**Supplier**
NucleoSpin Tissue Kit	Machery-Nagel
Plasmid pGAPZαA	Invitrogen
TopTaq Master Mix Kit	Qiagen

3.1.3 Enzyme

The names of the enzymes and their suppliers are listed in Table 3.2.

Table 3.2 List of enzyme.

Enzyme	Supplier
alkaline phosphatase	Fermentas
restriction endonucleases	Fermentas
RNase A	Fermentas
lyticase enzymes	Sigma-Aldrich
α-amylase enzyme	Sigma-Aldrich
glycosidases (amyloglucosidase, EC 3.2.1.3)	Sigma-Aldrich
T4 DNA ligase	Biolabs

3.2 Microorganisms, plasmid and culture media

3.2.1 Bacteria strain, yeast strains, plasmid

Escherichia coli DH5α strain (*F80dlacZDM15*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_K, m+K), *supE44*, *relA1*, *deoR*, Δ (*lac-ZYA- argF*), U169) and *E. coli* TOP10 strain (F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) were used for transformation and plasmid construction.

S. cerevisiae TISTR 5596 was used as the host for expressions of α-amylase and glucoamylase enzyme.

D. occidentalis strain TISTR 5346 genomic DNA was used as a template for isolation of the α-amylase gene (*SWA2*) and glucoamylase gene (*GAM1*).

Plasmid pGAPZαA (containing a GAP promoter) is commercially available and used for construction of a recombinant plasmid containing the *GAM1* gene or *SWA2* gene for expression in yeast. It has an origin of DNA replication in *E. coli* and, the zeocin resistant gene (Zeocin^{TM}) as a selectable marker for expression in *E. coli* and yeast (Figure 3.1).

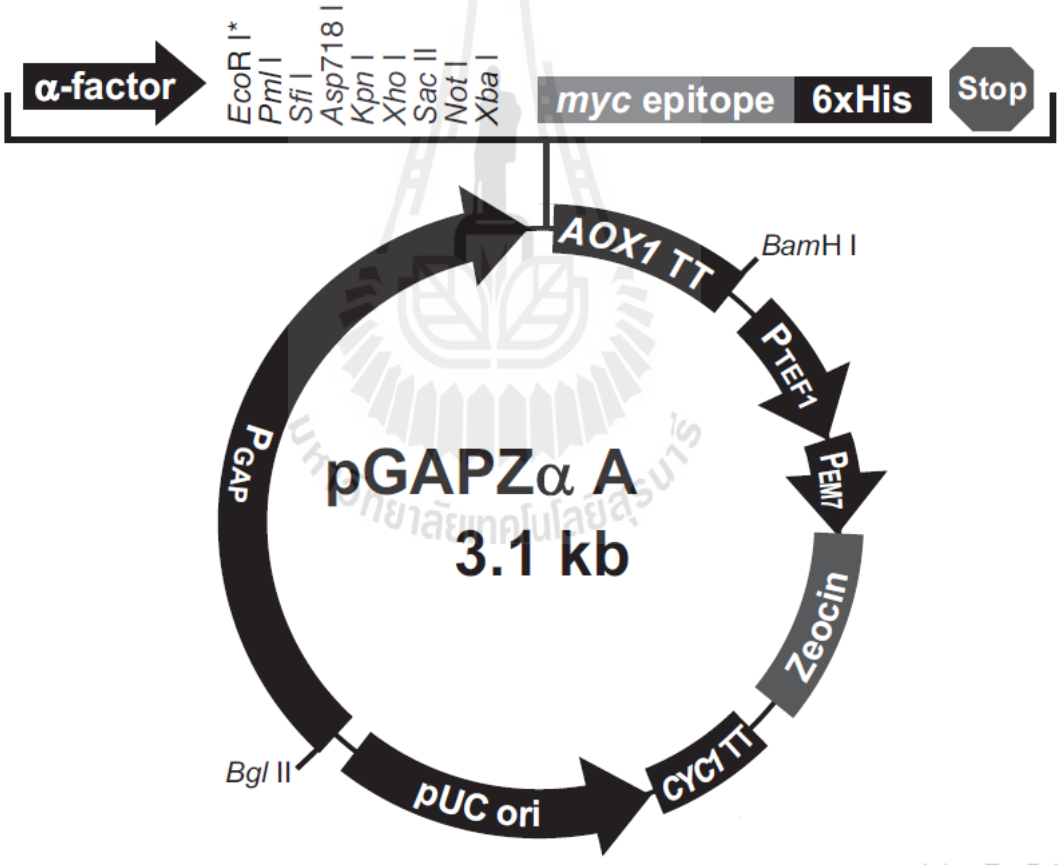

Figure 3.1 The physical map of pGAPZα A, B, C plasmid, an expression vector (http://www.invitrogen.com).

This figure illustrates the pGAPZα A, B and C expression vector (3.1 kb) with the GAP promoter to constitutively express recombinant proteins in *Pichia pastoris* as used. In addition, pGAPZα produces proteins fused to an N-terminal peptide encoding the *S. cerevisiae* α-factor secretion signal. Zeocin™ resistance gene (*Sh ble* gene or zeocin gene) is the antibiotic resistant marker and used for the selection of the transformed bacteria and yeast. Transcription elongation factor 1 promoter from *S. cerevisiae* that drives expression of the *Sh ble* gene in *Pichia* was conferring Zeocin™ resistance. *CYC1* transcription termination region, 3′ end of the *S. cerevisiae CYC1* gene allowing efficient 3′ mRNA processing of the *Sh ble* gene is used to increase stability. pUC origin allows replication and maintenance of the plasmid in *E. coli.*

3.2.2 Bacteria culture media

Luria-Bertani (LB) broth and LB agar plates were used to cultivate bacterial cells.

LB broth consisted of 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride, pH 7.0. The solution was autoclaved and kept at 4°C for storage.

LB agar: 1.5% (w/v) bacto-agar in LB broth.

Luria-Bertani Zeocin™ (LBZ) broth and LBZ agar were used to cultivate and select transformed bacterial cells.

LBZ broth consists of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride and LBZ agar, after autoclaving, zeocin™ solution was added to a final concentration of 25 μg/ml into the cooled medium and keep in dark room

until use. The medium was mixed and poured into petri-dishes. The LBZ plates were placed at 4°C without exposure to light for two week storage.

3.2.3 Yeast culture media

YPD medium was used for yeast pre-cultivation and propagation during competent yeast preparation. YPD medium consists of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) D-glucose. The appropriate pH and amount of zeocin™ was added into the culture medium at dark room when cultivated the recombinant yeast strains. The transformant yeasts were grown on YPD plates containing 130 μg/ml of zeocin™, and then transferred onto YPDS2 plates [YPD containing 2% (w/v) soluble starch] and incubated for 10 days at 30°C, after which they were incubated for 2 days at 4 °C for determination of the glucoamylase and α-amylase expression on starch-containing plate (Ghang et al., 2007).

YPC containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (w/v) cassava starch, pH 5.0, medium was used for batch fermentation studies. YPD medium was pregelatinized by boiling for 20 min before taking into fermentation bottle and all media were autoclaved at 121°C, 15 psi for 20 min before use.

3.3 Plasmid construction

3.3.1 Synthetic oligonucleotides primers

All oligonucleotides primers were purchased from Pacific science, Thailand. These primers were designed by using Primer Premier 5.0 program based on the published nucleotide sequence of the *D. occidentalis GAM1* gene (Accession No. M60207 and M34666) and the *D. occidentalis SWA2* gene (Accession No.

X7349). The names of the oligonucleotides primers and their primer sequences are listed in Table 3.3.

Table 3.3 List of oligonucleotides for PCR.

Names	Primer Sequences (5' to 3')	Size (nt.)
GAM1_2F	5'- CCCCTCGAGAAAAGAGCCCCTGCCTC TTCGATTGG-3'	35
GAM1_2R	5'CCCCTCGAGTTACCAAGTAATGGTGAAAT CCTTAGAAAATG -3'	41
SWA2_F	5'-CCCCTCGAGAAAAGAATGAAATTTGCAAC TATCTTA-3'	36
SWA2_R	5'-CCCCTCGAGTTAGAAGTTGCAAATTCCAGA -3'	30
pGAP_F	5'-GTCCCTATTTCAATCAATTGAA-3'	22

Note: restriction sites are under-lined.

3.3.2 Yeast genomic DNA isolation by Nucleospin Tissue kit

A single colony of yeast *D. occidentalis* strain or *S. cerevisiae* strain was picked from a YPD plate and inoculated into 10 ml of YPD medium, then incubated with shaking at 200 rpm, 30ºC for 48 hr. Cells from three ml of the YPD culture (OD_{600} ~10.0) was harvested by centrifugation at 5,000 x *g* for 10 min. Then the cells were washed once with 1 ml of 10 mM EDTA, pH 8.0 and centrifuged at 5,000 x g for 10 min. The supernatant was discarded. The pellet was resuspended in 600 µl of

sorbitol buffer (1.2 M sorbitol, 10 mM CaCl2, 0.1 M Tris/HCl pH 7.5, 35 mM ß-mercaptoethanol). The yeast cell wall was degraded by adding 50 U of lyticase and incubated at 30°C for 30 min, and the mixture was centrifuged at 2,000 x *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 180 µl of T1 buffer and 25 µl of proteinase K solution. The mixtures were incubated at 56°C until cells complete lysis (at least 1-3 hr). In order to eliminate RNA from genomic DNA, the mixtures were added with 20 µl of RNase A solution (20 mg/ml) and incubated at room temperature for 5 min. After that, the mixtures were added with 200 µl of B3 buffer, vortexed vigorously, and incubated at 70°C for 10 min. The mixtures were centrifuged at maximum speed (20,800 x g) for 5 min and the supernatants were transferred into new tube. Then, the supernatants were added with 210 µl of absolute ethanol and vortexed vigorously. In DNA binding conditions, the mixtures were transferred into the nucleospin column and incubated at room temperature for 2 min then centrifuged at maximum speed for 1 min. A flow through of solution was discarded and 500 µl of BW buffer were added into the column which was later centrifuged at maximum speed for 1 min. Then, a flow through of solution was discarded and 600 µl of B5 buffer was added. Centrifugation at maximum speed for 1 min was carried out then the nucleospin column was centrifuged at maximum speed for 1 min to dry the filter. In order to elute, 50 µl of elution buffer was added to the nucleospin column and incubated at room temperature for 1 min then centrifuged at maximum speed for 1 min. The genomic DNA concentration was estimated by agarose gel electrophoresis.

3.3.3 Amplification of *GAM1* gene from genomic DNA of *D. occidentalis* by polymerase chain reaction

The 2.8 kb of *GAM1* gene was amplified from genomic DNA of *D. occidentalis* by PCR using GAM1_F and GAM1_R primers. The 50 µl reaction was composed of 1x PCR buffer (75 mM Tris-HCl (pH 8.7), 50 mM KCl, 20 mM$(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$), 20 pmol of each forward and reverse primer, 200 µM dNTPs mix, and 1.25 unit of *Taq* DNA polymeras and 100 ng of genomic DNA of *D. occidentalis* TISTR 5346 as a template. The reaction was performed in DNA thermal cycler (BIO-RAD). Temperature profiles for amplification of *GAM1* gene were shown in Table 3.4.

Table 3.4 Temperature profile for PCR *GAM1* gene with GAM1_2F and GAM1_2R primers.

Hold	STEP CYCLE (30 cycles)			Hold
95°C,	Denaturation	Annealing	Extension	72°C,
3 min	94°C, 30 sec	56°C, 30 sec	72°C, 3 min	5 min

3.3.4 Amplification of *SWA2* gene from genomic DNA of *D. occidentalis* by polymerase chain reaction

The open reading frame (ORF) together with native terminator of the *SWA2* gene encoding α-amylase (~1.54 kb) was amplified by PCR using primers SWA2_F and SWA2_R and genomic DNA of *D. occidentalis* TISTR 5346 as a template. The 50 µl reaction was composed of 1xPCR buffer (75 mM Tris-HCl (pH

8.7), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$), 20 pmol of each forward and reverse primer, 200 µM dNTPs mix and 1.25 unit of *Taq* DNA polymeras and 100 ng of genomic DNA of *D. occidentalis* TISTR 5346. The reaction was performed in a DNA thermal cycler (BIO-RAD). Temperature profiles for amplification of *SWA2* gene were shown in Table 3.5.

Table 3.5 Temperature profile for PCR *SWA2* gene with SWA2_F and SWA2_R primers.

Hold	STEP CYCLE (30 cycles)			Hold
95°C,	Denaturation	Annealing	Extension	72°C,
3 min	94°C, 30 sec	55°C, 30 sec	72°C, 3 min	5 min

3.3.5 Mini-preparation of plasmid DNA by the alkaline lysis method

Protocols in this section are base on thos modified from Sambrook et al. (1989). Selected colonies were inoculated into 5 ml of LBZ medium, and incubated with shaking at 37ºC overnight. After that, bacterial cells were harvested by centrifugation at maximum speed (20,800 x g) for 30 sec. Then the supernatant was discarded and the cell pellet was resuspended in 100 µl of solution I buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) and vortex mixing. Then, 200 µl of solution II buffer (0.2 N NaOH, 1% SDS) was added, mixed by inverting tube 10 times and incubated the tube on ice for 5 min. After the adding of 150 µl of solution III buffer (3 M CH_3CO_2K, 5 M CH_3COOH) into the tube and mixed by inverting the tube 10 times. The tube was kept on ice for 10 min and centrifuged the

cells lysate at maximum speed at 4°C for 5 minutes. The supernatant was transferred to a new tube and 20 μg/ml of RNase A solution was added to the solution and incubated at 37°C for 1 hr. After that, an equal volume of chloroform was added and mixed the sample by vortexing. Then the solution was centrifuged at maximum speed at 4°C for 2 min. The upper layer solution was transferred into new tube. In order to precipitate the plasmid DNA, 0.1 volume of NaOAC solution was added and 2 volumes of cold absolute ethanol was added, then mixed and incubated at -80ºC for 1 hr. Then the solution was centrifuged at maximum speed at 4°C for 10 min, the supernatant was discarded and the pellet was then rinsed with 500 μl of 70% ethanol and centrifuged at maximum speed at 4°C for 2 min. The DNA pellet was air dried and resuspended in 50 μl sterile distilled water, plasmids DNA were stored at -20ºC.

3.3.6 Agarose gel electrophoresis of DNA

To analyze the size of DNA fragments, the PCR product and DNA fragments were subjected to agarose gel electrophoresis. The 0.8% of agarose was mixed in 1xTBE buffer (89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA, and pH 8.0). The DNA solution was mixed with 10X maestrosafe nucleic acid loading dye before loading. Electrophoresis was performed at a constant voltage (100 volt) for 45 min. The DNA bands were visualized with Ultrabright LED transilluminator (Maestrogen, Taiwan).

3.3.7 Restriction endonuclease digestion of plasmid DNA and PCR-generated DNA fragments

The reaction of restriction endonuclease digestion was composed of DNA, 1x restriction endonuclease buffer, restriction endonuclease and sterile distilled water. The restriction endonuclease buffer, the amount of restriction endonuclease and the optimum condition for digestion were varied depending on the indication by the restriction endonuclease manufacturer.

3.3.8 The purification of DNA fragment by silica matrix method

The silica matrix method was performed as previous described by Vogelestein and Gillespie (1979). The silica suspension was prepared by mixing 10 g of silica in 100 ml of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and allowing the silica to settle for 2 hr. the supernatant, containing fine particulate matter, was removed and the procedure repeated. After centrifugation (2000 x g for 2 min) the silica pellet was resuspended in 3 M NaI at 100 mg/ml and was stored in the dark at 4ºC. The DNA solution was added with 3 volumes of 6 M NaI. An additional 7 µl of silica suspension was added and incubated at 55ºC for 5 min to allow binding of DNA to silica matrix. The suspension was placed on ice for 5 min while gently mixed by inversion every 1 min to ensure that silica stayed suspended. Next, the suspension was centrifuged at maximum speed for 5 sec. The rest of NaI solution was removed by pipetting. The pellet was washed 3 times with cold wash buffer (50 mM NaCl, 10 mM TrisHCl pH 7.5, 2.5 mM EDTA, 50% v/v ethanol) by adding 200 µl for the first wash and 400 µl in the next 2 washes. After the supernatant from the third wash had been removed by centrifugation and pipetting,

the pellet was dried in order to eliminate the remaining of ethanol. The pellet was then resuspended in 10 µl of sterile distilled water, incubated at 55ºC for 2 min and centrifuged at maximum speed for 5 sec. The supernatant containing the eluted DNA was transferred to a new tube. The second elution step was performed. Finally, the total volume of eluted DNA in 20 µl of sterilized distilled water was obtained.

3.3.9 Dephosphorylation of linearize plasmid DNA by thermo-sensitive alkaline phosphatase (FastAP™)

Thermo-sensitive Alkaline Phosphatase (FastAP™) was used to prevent the self-ligation of linear DNA vector by catalyzing the hydrolysis of 5'-phosphate groups from both 5'-temini. In order to dephosphorylate the overhang ends, the purified linear DNA vector was incubated with FastAP™. One unit was the amount of the enzyme required to dephosphorylate 5'-termini of 1 µg of linearized pGAPZαA DNA in FastAP buffer (100 mM Tris-HCl pH 8.0, 50 mM MgCl2, 1 M KCl, 2 mg/ml Triton X-100 and 1 mg/ml BSA) at 37°C for 10 min. After that, the reaction was inactivated by heating at 75°C for 5 min. The dephosphorylated linear DNA was purified by silica matrix method for plasmid construction.

3.3.10 DNA ligation

Two fragments of DNA were joined together by T4 DNA ligase (Fermentas) in 3:1 molar ratio for two fragments at nearly the same size of insert and vector DNA in 1x reaction buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP). The cohesive end joining was performed at 4°C overnight. The amount

of T4 DNA ligase (1-5 units) added to reaction depended on the amount of total DNA in the reaction and also depended on the ligation reaction.

3.4 Bacteria transformation and selection

3.4.1 Preparation of competent *E. coli* strain TOP 10

Protocols in this section are based on those modified from Sambrook et al. (1989). A single colony of *E. coli* DH5α was picked from a LB plate and inoculated into 2 ml of LB medium, then incubated with shaking at 180 rpm at 37ºC overnight. One ml overnight culture was transferred into another 100 ml of sterilized LB medium, followed by an additional 1-2 hr of incubation with shaking at 200 rpm at 37ºC. The bacterial cultivation was stopped when the OD_{600} reached 0.45, the flask was put on ice to quickly cool the bacteria and stop further growth. The cell culture was transferred into pre-chilled sterile 50 ml tubes, and centrifuged at 7,000 x g at 4ºC for 10 min to collect the cells. After discarding the supernatant, the bacterial pellet was resuspended carefully in 10 ml of cold 100 mM $CaCl_2$ solution and re-centrifuged. This washing step was repeated twice and the cell suspension was kept on ice for 30 min followed by a further centrifugation step to re-pellet the cells. The cells pellet was resuspended in 2-3 ml of 100 mM $CaCl_2$. The cell suspension was added with 75 µl and 25 µl of 15% glycerol solution and was aliquoted (100 µl aliquots) into ice-cold eppendorf tubes. Aliquots of 100 µl of competent *E. coli* TOP 10 were freezed in liquid nitrogen and stored at -80°C for later use.

3.4.2 Transformation of *E. coli* by heat shock method

Protocols in this section are base on those modified from Sambrook et al. (1989). An aliquot of 100 µl of competent *E. coli* TOP 10 cells was thawed on ice, and then mixed. Two µl of ligation mixture was diluted 1:10 with sterile distilled water and added into 100 µl of competent cells and incubated on ice for 30 min. Then bacterial cells were heat shocked by placing the tubes into a 42°C water bath for 1.5 min and then incubated on ice for 2 min. The transformed cells were mixed with 900 µl of LB broth and incubated at 37ºC with vigorous shaking for 1 hr. In order to increase colony number, the cell suspension was centrifuged at 5,000 rpm for 45 sec. Sixty hundred µl of supernatant was removed and the cells were resuspended. One hundred µl of the transformation mixture was spread onto LBZ plates containing antibiotic and without antibiotic as a negative control. The LBZ plates were incubated at 37°C overnight.

3.4.3 Identification of *E. coli* transformants containing recombinant plasmids by simplified rapid size screening method

The rapid detection of recombinant plasmid procedure was developed by Law and Crickmore (1997). A single colony of transformant was transferred into 30 µl of pre-warmed lysis buffer (5 mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS, 100 mM NaOH, 60 mM KCl, 0.05% (w/v) bromophenol blue). The mixture was incubated at 37ºC for 5 min then placed at 4ºC for 5 min and centrifuged at maximum speed (20,800 x g) for 5 min. The 20 µl of supernatant was loaded onto an agarose gel. The recombinant plasmid was determined by comparing size of plasmid DNA

with that of control plasmid vector. The correct clone was selected from the master plate for future characterization.

3.4.4 Characterization and selection of *E. coli* transformant

This protocol is designed to quickly screen for plasmid inserts directly from *E. coli* colonies. The recombinant clone that has been rapid by detected by simplified rapid size screening method was selected from the master plate for confirmation of gene insertion and the correct orientation of *SWA2* and *GAM1* gene. A single colony of *E. coli* transformant was picked with a sterilized toothpick into 30 µl of sterilized distilled water. The cells were lysed by boiling for 5 min and cooled by keeping on ice for 5 min. The lysed cell suspension was centrifuged at maximum speed and then the supernatant was used as DNA template for PCR reaction. *E. coli* transformants were then analyzed for *GAM1* and *SWA2* gene insertion using GAM1_2F and GAM1_2R for *GAM1* gene and SWA2_F and SWA_R primer for *SWA2* gene, respectively. An expected size of PCR products was 2.8 kb for GAM1 recombinant plasmid and 1.5 kb for SWA2 recombinant plasmid, respectively (METHOD 3.3.3 and 3.3.4).

To examine the correct orientation of *GAM1* and *SWA2* gene respect to the pGAP promoter, the recombinant plasmid was verified using PCR technique. The forward PCR primer named pGAP_F (5'-GTCCCTATTTCAATCAATTGAA-3') priming at the beginning of the pGAP promoter combining with the specific reverse primer of each gene (either SWA2_R or GAM1_R) was used in PCR reaction (Primer sequences are show in Table 3.3). The 50 µl reaction was composed of 1xPCR buffer (75 mM Tris-HCl (pH 8.7), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$), 20

pmol of each forward and reverse primer, 200 μM dNTPs mix and 1.25 unit of *Taq* DNA polymerase and 100 ng DNA of each *E. coli* transformants. The reaction was performed in DNA thermal cycler (BIO-RAD). Temperature profiles for amplification of the correct orientation of *GAM1* and *SWA2* gene were shown in Table 3.6. The expected size of PCR products were 1.8 kb for *SWA2* recombinant plasmid and 3.1 kb for *GAM1* recombinant plasmid.

Table 3.6 Temperature profiles for examination of the correct orientation of *GAM1* and *SWA2* genes.

Hold	STEP CYCLE (30 cycles)			Hold
95°C,	Denaturation	Annealing	Extension	72°C,
3 min	94°C, 30 sec	50°C, 30 sec	72°C, 3 min	5 min

3.5 Yeast transformation and selection

3.5.1 Preparation of competent yeast cells

The procedure for preparation of competent yeast cells was modified from Suga and Hatakeyama (2003). A single colony of *S. cerevisiae* was picked from the YPD plate, transferred into 25 ml of YPD medium, and cultivated with shaking at 250 rpm, at 30°C, overnight. The cell culture was transferred into 100 ml of fresh YPD medium until the OD_{600} reached 0.4 or a density of approximately 1 x 10^7cell/ml. The fresh culture was allowed to grow for another 6 hr with shaking at 250 rpm at 30°C. The cells were placed on ice for 15 min just before harvesting. The cells were then pelleted by centrifugation at 1,600 x g for 5 min and the resulting pellet was

washed three times with ice-cold sterilized water. This pellet was suspended in ice-cold freezing buffer (0.6 M sorbital, 10 mM $CaCl_2$, 10 mM Hepes, and pH 7.5). Aliquots of 0.1 ml of the cell suspension were dispensed into 1.5 ml microcentrifuge tubes and then slowly frozen and stored by placing them directly into a -80°C freezer until use.

3.5.2 Yeast transformation by electroporation method

The procedure for electroporation with competent yeast was modified from Suga and Hatakeyama (2003). For each electroporation, the frozen competent cells were quickly thawed in a water bath at 30°C. The competent yeasts were washed once with 1 ml of ice-cold 1.0 M sorbitol solution and centrifuged at 1,500 x g for 5 min at 4°C. Then the supernatant was discarded and the final pellet was resuspended in 80 µl of ice-cold 1.0 M sorbitol solution. The cell suspension was mixed with 3 µg of purified linear recombinant plasmid DNA and kept on ice for 5 min. After that, the cell suspension was transferred to a chilled electroporation cuvette with a 0.2 cm electrode gap and incubated the cuvette with the cells on ice for 5 min. A high electric pulse was applied to the cells using the Bio-Rad Gene Pulser. The cells were pulsed according to the parameters for yeast transformation suggested by the manufacturer of the specific electroporation device being used (3 kv for Sc4 mode). The electroporated cells were immediately diluted in 1 ml of ice-cold 1.0 M sorbitol by adding the solution to the cuvette and transferring the cuvette contents to a sterile 15 ml tube. The yeast culture was then placed in an incubator without shaking at 30°C for 1 hr. After incubation, 1 ml of YPD medium was added and then placed in an incubator with shaking at 250 rpm at 30°C for 1 hr. Cells were pelleted by

centrifugation at 1500 x g for 10 min in a microcentrifuge. Cell suspension was placed on YPD plates containing the appropriate of pH and concentration of zeocin™ at 30°C for 3 to 4 days until colonies formed.

3.5.3 Determination of the appropriate concentration of zeocin™ for selection of transformant yeasts

The wild type yeast cells were placed on a group of YPD plates that contained varied concentrations of zeocin™ ranging from 100 to 300 μg/ml. The appropriate pH was determined by adjusting of pH ranging at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 on YPD plate. The plates were incubated at 30°C without light for 2 days, and the number of colonies on each plate was recorded for comparison. The appropriate of pH and concentration of zeocin™ were determined based on the absence of wild type yeast colonies.

3.6 The functional of α-amylase and glucoamylase assays

3.6.1 Expression and secretion of SWA2 and GAM1 in a wild type *S. cerevisiae*

Expression and secretion of glucoamylase or α-amylase were modified from Eksteen et al. (2003) and Kang et al. (2003). Both transformant yeast strains obtained after transformations with plasmids carrying glucoamylase gene or α-amylase gene were screened for amylolytic activity after plating on YPDS2 plate and incubated for 10 days at 30°C. Then the YPDS2 plate was incubated at 4°C for 2 days. After that an iodine solution containing iodine (1.5% (w/v) I_2) and potassium iodide (5% (w/v) KI) was flooded on YPDS2 plate. Expression of glucoamylase or α-

amylase in each of transformant yeast was recorded if clear halo around colony appeared. Photographs were taken with a digital camera when the staining was complete.

3.6.2 Verification of gene integration in transformant yeasts containing *SWA2* gene or *GAM1* gene by polymerase chain reaction

The integration of glucoamylase gene or α-amylase gene in transformant yeasts were confirmed by using PCR technique. One hundred ng of *S. cerevisiae* transformants genomic DNA was used as a template. The 50 µl reaction was composed of 1 x PCR buffer (75 mM HCl, pH 8.7), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$), 20 pmol of each forward and reverse primer, 200 µM dNTPs mix and 1.25 unit of *Taq* DNA polymerase. The reaction was performed in DNA thermal cycler (BIO-RAD). Temperature profiles for amplification of *GAM1* and *SWA2* gene were shown in Tables 3.4 and 3.5, respectively.

3.6.3 Quantification of secreted the glucoamylase and the α-amylase activity in liquid medium

3.6.3.1 The preparation of liquid medium for the glucoamylase and α-amylase activity

Protocols of the cultivation of the transformant yeast were modified from Liao et al. (2012) and Wong et al. (2002). The selected transformant yeasts were cultivated in a 50 ml flask containing 5 ml of YPD media with shaking at 200 rpm at 30°C for 24 hr. Then, 1 ml of cultured medium was transferred into YPG medium (same as YPD with glucose replaced by glycerol) with shaking at 200 rpm at

30°C for 7 days or until $OD_{600} \geq 11$ was obtained. One ml of aliquot of culture was collected by centrifugation at 12,500 x g for 1 min. The aliquot culture was then measured for the glucoamylase and α-amylase activity within 48 hr. Enzyme activity was reported in unit per ml.

3.6.3.2 The determination of glucoamylase activity

Debranching activity of glucoamylase was measured in two steps; 1) hydrolysis of the substrate and 2) the determination of the enzyme activity. The glucoamylase activity in the growth culture was measured using 1% starch solution as the substrate, and the reducing sugar or glucose product was determined using the DNS method. The DNS solutions contained 1% (w/v) dinitrosalicylic acid, 0.2% (w/v) phenol, 0.05% (w/v) sodium sulfite, 1% (w/v) sodium hydroxide and 20% (w/v) potassium sodium tartrate. The substrate for the glucoamylase reaction was prepared by adding 1% starch in 50 M sodium acetate buffer (pH 5.5) and boiled for 15 min. After the solution cooled to room temperature, water was added to bring the solution up to 100 ml.

In order to measure the glucoamylase activity, the supernatant of yeast culture was collected by centrifugation at 10,000 x g for 10 min. One milliliter of supernatant yeast was mixed with 1 ml of 1% starch substrate solution. The reaction mixture was incubated at 50C° for 30 min. Two milliliter of the mixture was transferred into a test tube with the addition of 1 ml of DNS solution. Then, the test tube was placed in a boiling water bath for 15 min. After boiling, the sample was diluted with 7 ml of water. Absorbance was determined using a spectrophotometer at 540 nm. One unit of glucoamylase activity was defined as the amount of enzyme

required to release 1 μmol of reducing sugar or glucose per min per ml of enzyme sample. A blank containing 1ml of water was heated for 15 min in boiling water bath with 1 ml of DNS solution. A standard curve using glucose as reference was used to calculate the concentration of glucose product. In order to prepare a standard curve, standard solution containing anhydrous D-glucose (0.1-1.0 mg/ml) in deionized water was heated with 1 ml of DNS solution for 15 min in boiling water bath followed by measuring of optical density at 540 nm and plot of glucose concentration (as reducing sugar) versus optical density relationship (APPENDIX B).

3.6.3.3 The determination of α-amylase activity

The α-amylases are generally assayed using soluble starch or modified starch as the substrate. The α-amylase catalyses the hydrolysis of α-1, 4 glycosidic linkages in starch to produce glucose, dextrins and limit dextrins. The reaction is monitored by an increase in the reducing sugar levels or decrease in the iodine color of the treated substrate. Various methods are available for the determination of α-amylase activity (Priest, 1977). These are based on decrease in starch iodine color intensity, increase in reducing sugars, degradation of color-complexes substrate and decrease in viscosity of the starch suspension. Starch forms a deep blue complex with iodine (Hollo and Szeitli, 1968) and with progressive hydrolysis of the starch, it changes to red brown. Several procedures have been described for the quantitative determination of amylase based on this property. This method determines the dextrinising activity of α-amylase in terms of decrease in the iodine color reaction.

In this study, α-amylase activity was measured using starch-iodine assay modified from the Fuwa (1954) and Xiao et al. (2006). The substrate for the α-amylase reaction was prepared by adding 1% starch in 0.4 M sodium acetate buffer (pH 5.0) boiled for 15 min. After the solution cooled to room temperature, water was added to bring the solution up to 100 ml. In order to measure the α-amylase activity, the supernatant of yeast culture was collected by centrifugation at 10,000 x g for 10 min. One milliliter of culture supernatant was incubated with 1 ml of starch substrate solution prepared at 50C° for 30 min. One milliliter of 1.0 M HCl was added to stop the enzymatic reaction, follow by the addition of 1 ml of iodine reagent (0.2% (w/v) iodine and 2% (w/v) potassium iodide). Finally, the volume was adjusted to 10 ml with H_2O, and the amount of color development was determined by measuring the absorbance at 580 nm. The blank was treated in the same way as the samples except the use of 1 ml of water instead of 1 ml of culture supernatant. One unit per ml (U/ml) of α-amylase activity was calculated using the formula: U/ml = (A_{580} control -A_{580} sample) ÷ A_{580}/mg starch ÷ 30 min ÷ 1 ml, where A_{580} control was the absorbance obtained from the starch without the addition of enzyme, A_{580} sample was the absorbance for the starch digested with enzyme and A_{580}/mg starch was the absorbance for 1 mg of starch as derived from the standard curve in APPENDIX B. One unit (U) for starch-iodine assay was as defined the disappearance of on average of 1 mg of iodine binding starch material per min in the assay reaction.

3.7 Ethanol fermentation and assay

3.7.1 Seeding cultures

Seeding cultures preparation of the transformant yeast was modified from Souza et al. (2007) and Yamada et al. (2011). Each single colony of transformant yeast was picked from YPDS2 plate with a sterilized needle and inoculated into 10 ml of YPD medium with shaking at 200 rpm at 30°C overnight. One milliliter of overnight culture was transferred into another 100 ml of sterilized YPC medium, followed by an additional 10-12 hr of incubation with shaking at 200 rpm at 30°C. The transformant yeast cultivation was stopped when the OD_{600} reached 1.0 (active). Nine point five ml of the cell culture was transferred into pre-chilled sterile 50 ml tube, and centrifuged at 3,000 x g at 4°C for 5 min to collect the cells. The cell pellet was resuspended in 10 ml of cold 0.85% NaCl solution and re-centrifuged to wash the cells. The cell pellet was resuspended in 9.5 ml of YPC medium for transferring into fermented bottle which contained 85.5 ml of YPC medium. This culture was then used to inoculate the batch fermentation (10 % (v/v) inoculums).

3.7.2 Fermentation conditions

The 120 ml fermentation bottle was used for batch fermentation study. The fermentation bottle was covered with aluminum vial seals. The YPC medium for fermentation study contained 10 g/l of cassava starch and adjusted pH to 5.0. The fermentation process was carried out in a fermentation bottle at 30°C with agitation at 200 rpm (in order to avoid any sedimentation). Sukairi et al. (2008) optimized the agitation speed (100-250 rpm) in fermentation process to produce high yield of

ethanol from fermentation of *S. cerevisiae* using tapioca starch as a substrate. As a result, the concentration of ethanol was increased at the agitation speed of 200 rpm. The cells had contact with the fermentation medium at 200 rpm to convert the reducing sugar or glucose to bioethanol. Two ml of fermentation medium was collected every 3 days over a period of 24 days for starch content, ethanol concentration and dry cell weight analyses.

3.7.3 Determination of cell dry weight

Cell dry weight (g cell dry weight per 1 ml of fermentation broth) in fermentation broth using cassava starch as a substrate was determined using a modified method of Soni et al. (1992). Fermentation broth was taken from the fermentation bottle every 3 days during fermentation periods. A portion of 20 µl α-amylase enzyme (sigma) was added to 1 ml of culture medium and then incubated at 90°C for 2 hr to hydrolyze starch to soluble dextrin. Samples were centrifuged at maximum speed at 4°C for 10 min. Once centrifuged, the supernatant was discharged and the remaining solid was re-dissolved with 1 ml of distilled water. After discharging the supernatant, the sample which contained the cell free from starchy substances was dried at 105°C for 4 hr for determination of cell dry weight. Fresh medium was used as a blank sample.

3.7.4 Starch concentration analysis

The presence of the residual concentration of starch was determined using a modified method of Liao et al. (2012). One ml fermentation sample was collected from the fermentation broth and stored at -20°C. Samples were thawed at

room temperature and boiled in a water bath for 30 min to re-solubilize the starch. Then 300 µl of samples was mixed with 2 ml of iodine solution (0.2% (w/v) I_2, 2% (w/v) KI_2), and diluted with 9 ml of water. The OD of final solution was measured at 580 nm using a spectrophotometer (samples were diluted as required to measure optical density accurately and the dilution factor was considered while calculating the actual starch concentration). Starch concentrations were determined by calculation using a standard curve (0-1 g/l) (APPENDIX B). The curve is valid between optical densities of 0.01 and 1.0.

3.7.5 Ethanol analysis

Ethanol concentrations were determined using high performance liquid chromatography (HPLC) with RI detector (Model 1200 series, Agilent Technology). One milliliter fermentation sample was collected from the fermentation broth and was centrifuged at maximum speed at 4°C for 10 min to separate cell and supernatant. The supernatant was then filtered through a 0.2 µm filter period for injecting to the HPLC. The 10 µl-injection volumes were automatically analyzed. The column was operated at ambient temperature with a flow rate of 0.4 ml/min and 4mM sulfuric acid was used as the mobile phase. The theoretical maximum yield of ethanol from 100 parts of glucose is 51.1 g of ethanol generated/100 g of glucose (Ingledew, 1993).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Construction of expression plasmids carrying the glucoamylase genes of *Debareomyces occidentalis*

The overall cloning strategy of the *GAM1* gene into the expression vector is illustrated in Figure 4.1. The full length of *GAM1* gene was amplified from genomic DNA of *D. occidentalis* using GAM1_2F and GAM1_2R specific primers. These primers were designed by using Primer Premier 5.0 program based on the published nucleotide sequence of the *D. occidentalis GAM1* gene (Accession No. M60207 and M34666). The glucoamylase gene from the start codon to stop codon has 2,883 bases encoding a protein of 960 amino acids with a calculated molecular mass of 10,6548.7 Da, and no intron was found.

The PCR product of 2.8 kb corresponding to the expected size of *GAM1* gene was obtained and purified using silica matrix method as described in METHOD 3.3.9. The purified *GAM1* gene was digested with *Xho*I and purified using silica matrix method. The purified *Xho*I-digested *GAM1* gene was cloned into *Xho*I site of plasmid pGAPZαA which was previously digested with *Xho*I and dephosphorylated with Fast AP enzyme as described in METHOD 3.3.9. The ligated DNA was transformed into *Escherichia coli* TOP10 strain and grown on the LB low salt agar containing 25 μg/ml of zeocin^{TM}. The putative *E. coli* transformants were randomly picked and analyzed by simplified rapid size screening method as described in METHOD 3.4.3

for detection of recombinant plasmids (data not shown). Ten clones including clone number 7, 8, 33, 35, 41, 45, 57, 58, 63, and 65 were detected as larger size than the control plasmid and were selected for future analysis with *Xho*I endonuclease digestion. The *Xho*I recognition site is presented at the 5' end and 3' end of the *GAM1* gene. The *Xho*I digestion reveals that all selected clones contained insert fragment size around 2.8 kb and pGAPZαA size around 3.1 kb (Figure 4.2, lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20). Ten recombinant clones were shown to have the correct orientation of *GAM1* respect to the pGAP promoter. The orientation of *GAM1* with gene was verified using PCR technique as described in METHOD 3.4.4. The forward PCR primer named pGAP_F priming at the beginning of the pGAP promoter was combined with the specific reverse primer of *GAM1* gene in PCR reaction. The expected size of PCR products was 3.1 kb for correct clone. Seven out of ten selected clones contains the correct orientation of *GAM1* gene (Figure 43). Clones number 7, 8, 33, 35, 57, 58, and 63 were selected and named as pGAM1. Randomly chosen clone (clone number 7) of pGAM1 was used for GAM1 expression in *S. cerevisiae*.

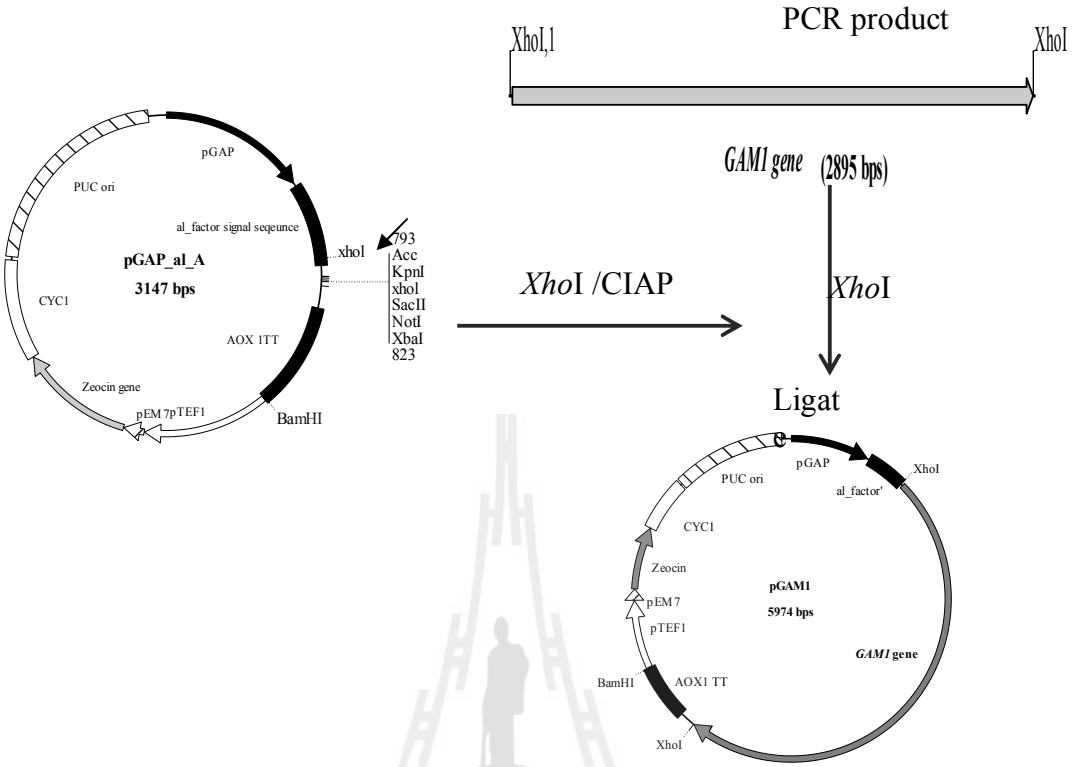

Figure 4.1 The strategy for pGAM1 construction. The amplified 2,895 bps of *GAM1* gene was digested with *Xho*I and cloned into *Xho*I site of plasmid pGAPZαA. The resulting plasmid was named pGAM1.

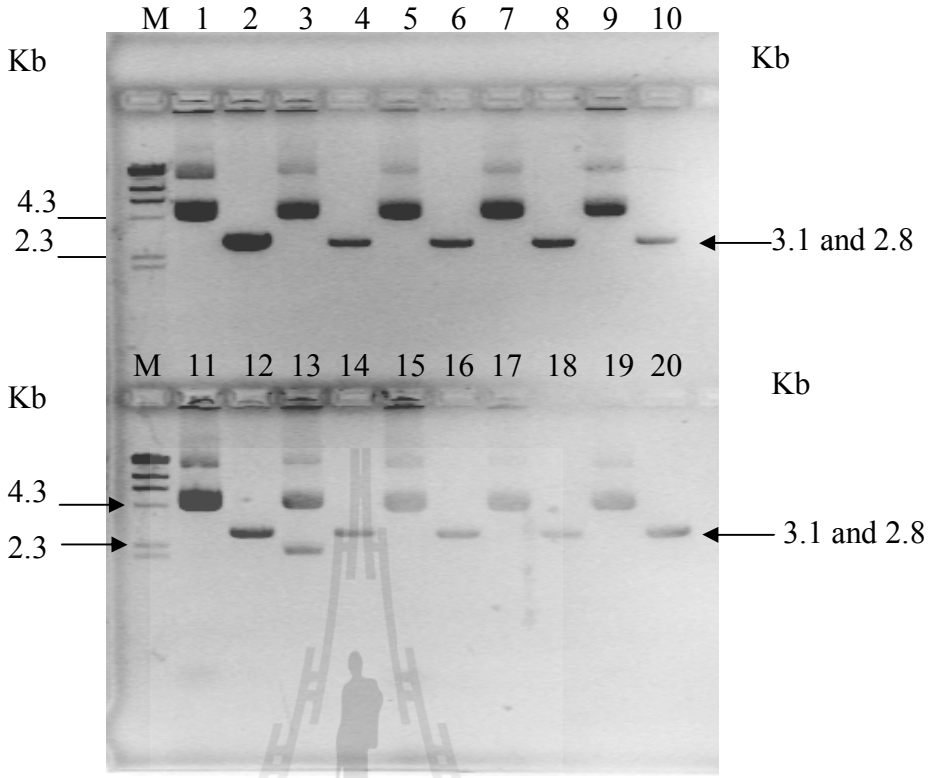

Figure 4.2 The *Xho*I digestion analysis of recombinant pGAM1 plasmid.

The figure shows 0.8% agarose gel electroporesis of cut and uncut with *Xho*I of recombinant plasmid DNA of clones number 7, 8, 33, 35, 41, 45, 57, 58, 63, and 65. The correct recombinant clone of *Xho*I digestion product was showed fragment size around 3.1 kb for pGAPZαA plasmid and size around 2.8 kb for *GAM1* gene.

Lanes M: *Hin*d III digested Lamda DNA marker.

Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20: *Xho*I-digested recombinant plasmid DNA clones number 7, 8, 33, 35, 41, 45, 57, 58, 63, and 65, respectively.

Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19: uncut with *Xho*I enzyme of recombinant plasmid DNA clones number 7, 8, 33, 35, 41, 45, 57, 58, 63, and 65, respectively.

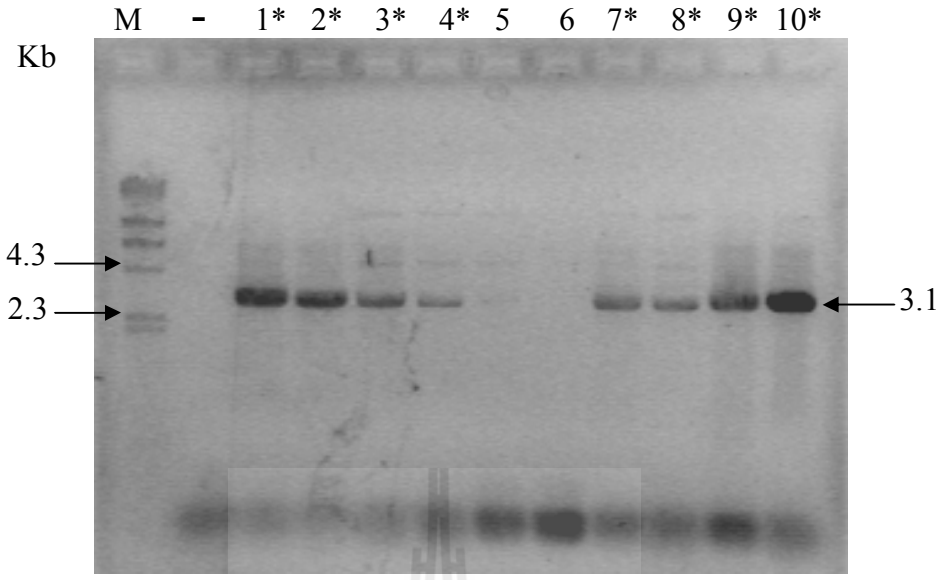

Figure 4.3 Analysis of *GAM1* gene orientation in pGAM1 plasmid using PCR technique.

The figure shows 0.8% agarose gel electroporesis of the PCR product using plasmid DNA of clones number 7, 8, 33, 35, 41, 45, 57, 58, 63, and 65 as template, respectively.

The 50 µl of PCR reaction was performed. The reaction comprised of plasmid DNA of clones number 7, 8, 33, 35, 41, 45, 57, 58, 63, and 65 as a template, pGAP_F and GAM1_2R were used as primers as described in METHOD 3.4.4. The correct orientation (*) was confirmed if the product was 3.1 kb.

Lanes M: *Hin*d III digested Lamda DNA marker.

Lanes-: Negative control of PCR amplification containing all components in PCR reaction without template.

Lanes 1-10: PCR amplification product using plasmid DNA of clones number 7, 8, 33, 35, 41, 45, 57, 58, 63, or 65 as a template, respectively.

4.2 Construction of the expression plasmids carrying the α-amylase genes of *D. occidentalis*

The overall cloning strategy of the *SWA2* gene into the expression vector is illustrated in Figure 4.4. The full length of *SWA2* gene was amplified from genomic DNA of *D. occidentalis* using SWA2_F and SWA2_R specific primers. These primers were designed using Primer Premier 5.0 program based on the published nucleotide sequence of the *D. occidentalis SWA2* gene (Accession No. X7349). The gene from the start codon to stop codon has 1,523 bases encoding a protein of 507 amino acids with calculated molecular mass of 55,966 Da and no intron was found.

The PCR product of 1.5 kb corresponding to the expected size of *SWA2* gene was obtained and purified using Silica matrix method as described in METHOD 3.3.8. The purified *SWA2* gene was digested with *Xho*I and purified using Silica matrix method. The purified *Xho*I-digested *SWA2* gene was cloned into *Xho*I site of plasmid pGAPZαA which was previously digested with *Xho*I and dephosphorylated with Fast AP enzyme as described in METHOD 3.3.9. The ligated DNA was transformed into *E. coli* TOP 10 strain and grown on the LB low salt agar containing 25 μg/ml of zeocin™. The putative *E. coli* transformants were randomly picked and analyzed by simplified rapid size screening method as describe in METHOD 3.4.3 for screening of recombinant plasmids (data not shown). Five clones (clones number 1, 2, 3, 4, and 5) were detected as larger size than the control plasmid and selected for future analysis with *Xho*I endonuclease digestion. The *Xho*I is presented at the 5' end and 3' end of *SWA2* gene. The *Xho*I digestion reveals that four (clones number 1, 2, 3, and 4) selected clones contained insert fragment size around 1.5 kb (Figure 4.6 lanes 2, 4, 6, and 8). Four out of five clones (clones number 1, 2, 3, and 4) were determined

the orientation of *SWA2* gene which respect to the pGAP promoter. The orientations of *SWA2* gene was verified using PCR technique as described in METHOD 3.4.4. The forward PCR primer named pGAP_F priming at the beginning of the pGAP promoter was combined with the specific reverse primer of *SWA2* gene PCR reaction. The expected size of PCR product was 1.8 kb for correct clone. Four selected clones contained the correct orientation of *SWA2* gene (Figure 4.6). Clones number 1, 2, 3, and 4 were selected and named as pSWA2. Randomly chosen clone (clone number 2) of pSWA2 was used for SWA2 expression in *S. cerevisiae*.

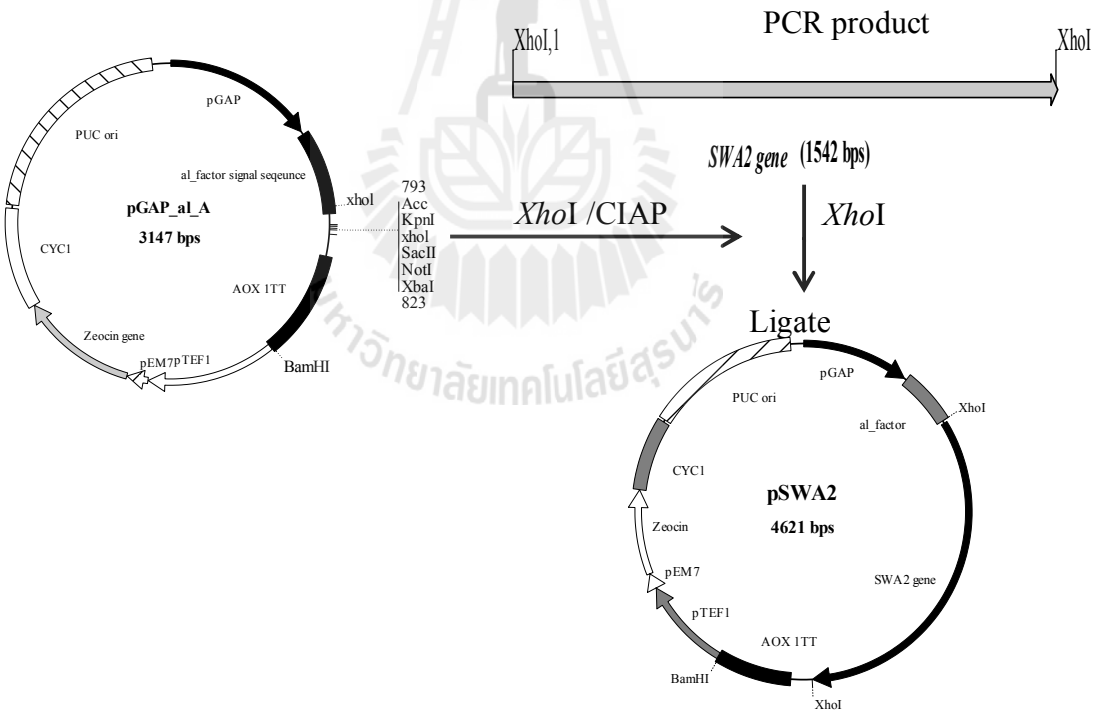

Figure 4.4 The strategy for pSWA2 construction. The amplified 1,542 bps of *SWA2* gene was digested with *Xho*I and cloned into *Xho*I site of plasmid pGAPZαA. The resulting plasmid was named pSWA2.

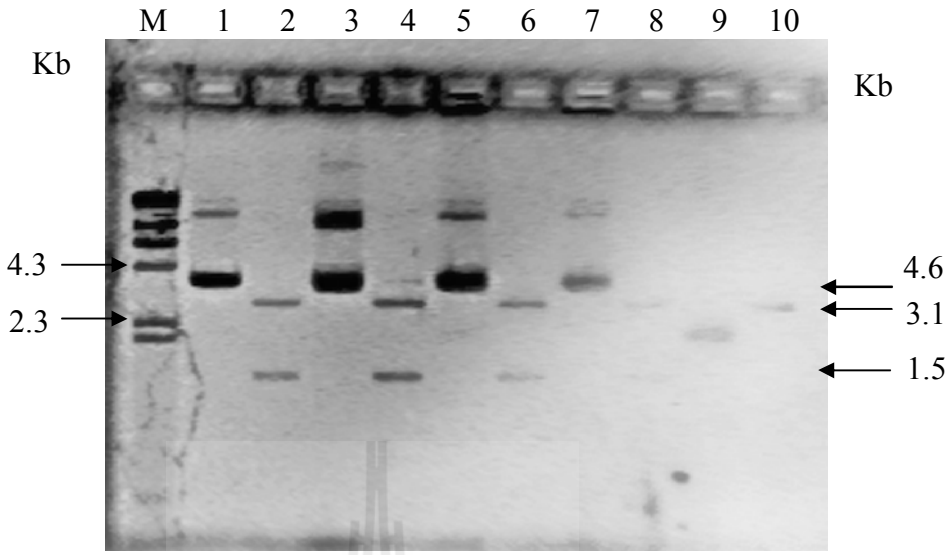

Figure 4.5 The *Xho*I digestion analysis of recombinant pSWA2 plasmid.

The figure shows 0.8% agarose gel electroporesis of *xho*I cut and uncut recombinant plasmid DNA of clones number 1, 2, 3, 4 and 5. The correct recombinant clone of *Xho*I digestion product showed fragment size around 3.1 kb for pGAPZαA plasmid and size around 1.5 kb for *SWA2* gene.

Lanes M: *Hin*d III digested Lamda DNA marker.

Lanes 2, 4, 6, 8 and 10: *Xho*I-digested recombinant plasmid DNA clones number 1, 2, 3, 4 and 5, respectively.

Lanes 1, 3, 5, 7 and 9: uncut with *Xho*I enzyme of recombinant plasmid DNA clones number 1, 2, 3, 4 and 5, respectively.

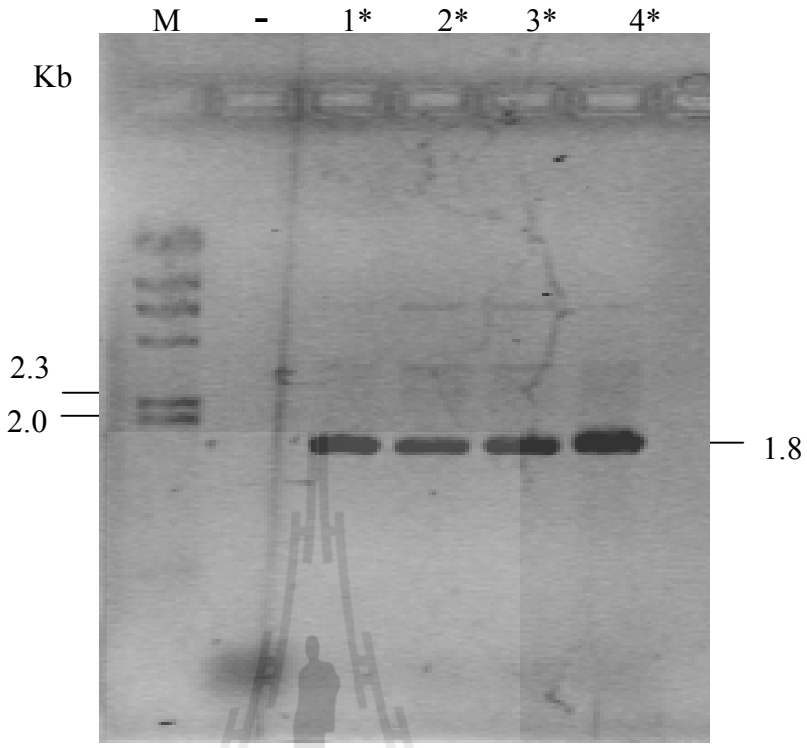

Figure 4.6 Analysis of *SWA2* gene orientation in pSWA2 plasmid using PCR technique.

The figure shows 0.8% agarose gel electroporesis of the PCR product using plasmid DNA of clones number 1, 2, 3, and 4 as a template. Fifty µl of PCR reaction was performed. The reaction comprised of plasmid DNA of clones number 1, 2, 3, or 4 as a template, pGAP_F and SWA2_R were used as primers as described in METHOD 3.4.4. The fragment size indicating correct orientation (*) of *SWA2* gene was 1.8 kb.

Lanes M: *Hin*d III digested Lamda DNA marker.

Lanes -: Negative control of PCR amplification containing all components in PCR reaction without template.

Lanes 1-4: PCR amplification product using plasmid DNA of clones number 1, 2, 3, or 4 as a template, respectively

4.3 Determination of the appropriate concentration of zeocin™ to use for selection

In order to identify positive yeast clones harboring pSWA2 or pGAM1, the appropriate concentration of zeocin™ used for selection has to be determined. The wild type yeast strain TISTR 5596 was used as controls. The same amount of cell suspension of each strain was placed on YPD plates containing zeocin™ concentration ranging from 100-300 μg/ml and each of zeocin™ concentration was adjusted pH ranging from 5.0-8.0, respectively. The number of colonies of wild type strain that grew on the YPD plates containing the same amount of zeocin™ was counted and compared. *S. cerevisiae* wild type TISTR 5596 colony was not detected at 130 μg/ml of zeocin™, pH 7.5. Therefore, the concentration 130 μg/ml, pH 7.5 of zeocin™ shall be used for selection of transformant yeast harboring pGAM1 or pSWA2 and shall be used for culturing all of the transformant yeast cells in subsequent studies.

4.4 Yeast transformation

The expression plasmids of pGAM1 or pSWA2 (Figures 4.1 and 4.4) were linearized with *Bam*HI digestion. The linear DNA of expression plasmids were then transformed into *S. cerevisiae* TISTR 5596 by electroporation method as described in METHOD 3.5.2. Transformant yeasts that grew on YPD plates containing 130 μg/ml of zeocin™, pH 7.5 were selected. The result shows that eight of zeocin™ resistant colonies for *S. cerevisiae* TISTR 5596 transformed with pGAM1 and four zeocin™ resistant colonies for *S. cerevisiae* TISTR 5596 transformed with pSWA2 were

obtained. The transformant yeasts from selected colonies were further analyzed for the presence of transgene and expression of *GAM1* and *SWA2* gene.

4.5 Screening for glucoamylase and α-amylase expression

The glucoamylase or α-amylase expression in transformant yeast was determined. Zeocin-resistant transformant yeasts were subjected to a starch plate assay. The starch-iodine complex is formed a dark blue or black color. When starch is broken down or hydrolyzed into smaller carbohydrate units, the blue-black color is not produced. Each of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 that expressed functional glucoamylase and α-amylase will produce clear haloes around colony on a starch plate after staining with iodine solution. Such results indicate that both recombinant yeast strains secreted glucoamylase or α-amylase that degrades starch on plate. Therefore the iodine cannot form starch-iodine complex.

The pGAM1 plasmid (Figure 4.1) was linearized with *Bam*HI, and then transform into *S. cerevisiae* TISTR 5596 by electroporation. After transformation, zeocine-resistrant transformants were selected by culture on the YPD medium supplemented with 130 μg/ml of zeocin™. Eight transformants *S. cerevisiae* TISTR 5596/pGAM1 (TISTR 5596/pGAM1#1G, #2G, #3G, #4G, #5G, #6G, #7G, and #8G) that were able to grow on YPD medium plates supplemented with 130 μg/ml of zeocin™ were selected. All of transformant yeasts were screened for glucoamylase expression on YPDS2 plate. The clear halos around the colonies of transformant yeasts on YPDS2 plate were detected. These indicated that all selected transformant can express functional glucoamylase. The *S. cerevisiae* TISTR 5596/pGAM1 clone

number #1G (Figure 4.7) showing largest clear halo was selected for fermentation studies.

The pSWA2 plasmid (Figure 4.4) was linearized with *Bam*HI, and then transformed into *S. cerevisiae* TISTR 5596 by electroporation. After transformation, zeocin-resistrant transformants were selected on the YPD medium supplemented with 130 µg/ml of zeocin^{TM}. Four transformants yeast of *S. cerevisiae* TISTR 5596/pSWA2 (TISTR 5596/pSWA2#1S, #2S, #3S and #4S) that were able to grow on YPD medium plates supplemented with 130 µg/ml of zeocin^{TM} were selected. Then, all of transformant yeasts were screened for α-amylase expression on YPDS2 plate. The secretions of α-amylase enzyme of transformant yeasts were shown by formation of clear halos around the colonies on YPDS2 plate. The clear halos around the colonies of transformant yeasts on YPDS2 plate were detected. These indicated that all selected transformant can express functional α-amylase. The *S. cerevisiae* TISTR 5596/pSWA2 clone number #4S (Figure 4.7) which showed the largest clear halos was selected for fermentation studies.

Promoters are one of the key factors for the production of heterologous protein. Glyceraldehydes-3-phosphate dehydrogenase promoter from *P. pastoris* (*P. pastoris* GAP promoter) was reported for continuous expression of recombinant HBsAg in *S. cerevisiae*, with stability and absence of toxicity to the host. Thus the expression vector indicating the expression system can be applied for large-scale production (Vellanki et al., 2007). This suggested that the heterologous promoter, GAP promoter, be able to express glucoamylase or α-amylase gene in *S. cerevisiae*. From this study, both glucoamylase and α-amylase were able to be expressed in *S. cerevisiae* TISTR 5596 but with low level expression, which may be caused by heterogeneous GAP

promoter. These results suggested that using GAP promoter of *P. pastoris* may not be appropriate for the expression of gene in *S. cerevisiae* TISTR 5596. Generally, homologous promoters originating from the yeast species used as host are preferred, as heterologous promoters often do not yield good efficiency of expression (Porro et al., 2005). The nucleotide sequences of promoters vary considerably, affecting the binding affinity of RNA polymerases and thus the frequency of transcription initiation. The RNA polymerases bind to the promoter sequences, recruiting RNA polymerase, the enzyme that synthesizes the RNA from the coding region of the gene. Mutations or the specific DNA sequences mismatch in a region that result in a shift away from the consensus sequence usually decrease promoter function (David, and Michael, 1982). However, the constitutive promoters of GAP promoter are preferred because they do not require the use of inducers, which is expensive and could interfere with the isolation of the final product. Many reports showed that expression using a native constitutive promoter improved the expression in *S. cerevisiae* (Ghang et al., 2007; Kim et al., 2010; Kim et al., 2011).

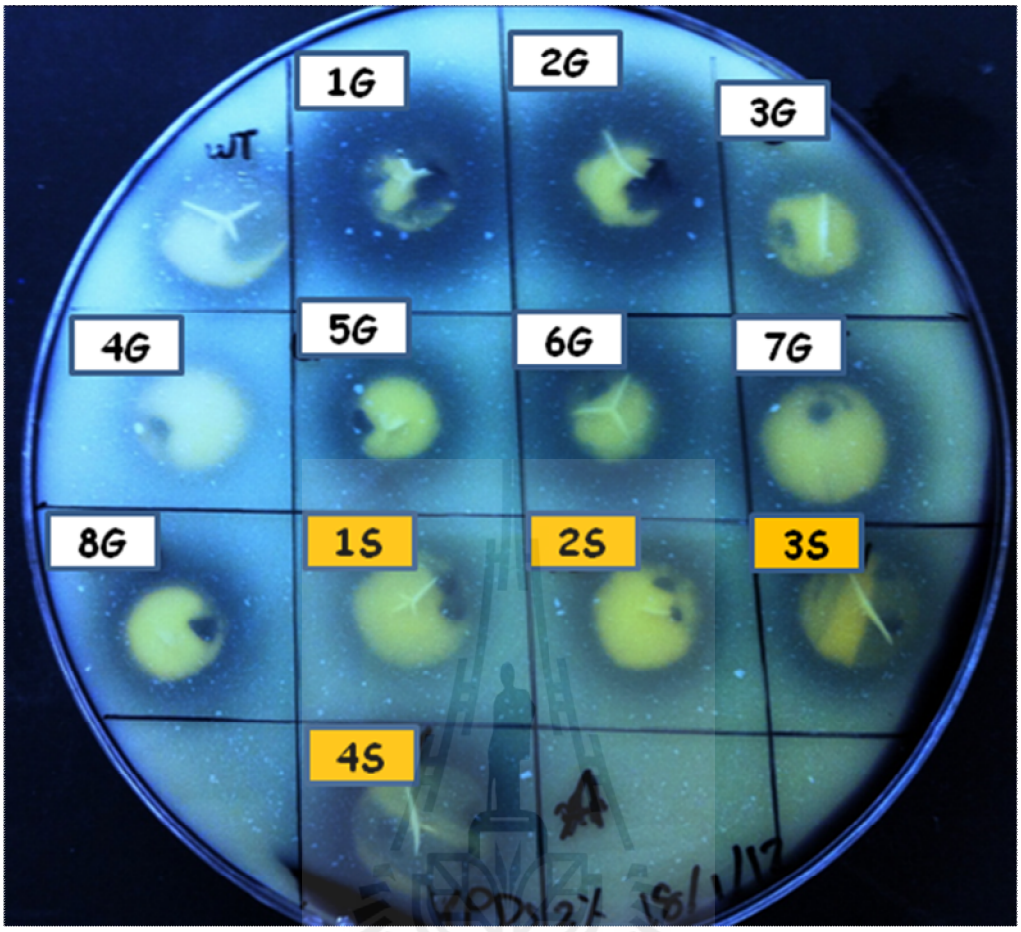

Figure 4.7 Screening for glucoamylase and α-amylase expression of transformant yeast strains. Screening for glucoamylase (*GAM1* gene) expression of transformant yeast strains 1G, 2G, 3G, 4G, 5G, 6G, 7G, and 8G (*Violet label*) and α-amylase (*SWA2* gene) expression of transformant yeast strains 1S, 2S, 3S, and 4S (*yellow labe*l) by iodine reaction comparing with (WT) wild type yeast strain.

4.6 Detection of *GAM1* gene or *SWA2* gene integration in transformant *S. cerevisiae* by polymerase chain reaction

The heterologous gene can be introduced into the yeast host cells by means of an integrative plasmid as well as by autonomous or episomal circular plasmids. In the

first case, the heterologous gene fate is the integration into the chromosomal DNA by means of recombination events. Directed integration requires homology of the DNA introduced with a chromosomal locus. On the other side, heterologous recombination may occur at random positions (Porro et al., 2005).

Ideally, an integrative vector should be stably maintained in the host cells without the need of any selection pressure. In this work, each of the *GAM1* gene or *SWA2* gene were introduced into chromosome of the yeast host cells by an integrative vector of pGAPZαA which was contained GAP promoter of *P. pastoris*. The pGAPZαA vector lack any replication sequences and can be maintained only by integration. This vector contains multiple *S. cerevisiae* sequences (the *S. cerevisiae* α-mating factor (α-MF) signal which have been high secretion levels in *S. cerevisiae*, TEF 1 gene promoter from *S. cerevisiae* and *CYC1* transcription termination region). Thus, the recombinant plasmid of pGAM1 or pSWA2 was integrated at any of the homologous chromosomal location and both recombinant plasmids was double stranded break with *Bam*HI to linearized the recombinant plasmid for introduction into one of the *S. cerevisiae* sequence. After transformation method, all of transformant yeasts were able to grow on medium plate containing 130 µg/ml of zeocin^{TM} and then were able to grow on medium plate containing 2% soluble starch. The clear zone around colonies was observed in by iodine reaction on YPDS2 plate. The result indicated that all of transformant yeasts were able to integrate at any of the homologous chromosomal location, and they contain either *GAM1* gene or *SWA2* gene and were able to express and secrete enzyme for hydrolysis of starch (Figure 4.7).

The integration of *GAM1* gene or *SWA2* gene in genomic DNA of putative transformant yeasts was verified using PCR amplification. Transformants yeast genomic DNA was extracted as described in METHOD 3.3.2. The specific primers for *GAM1* gene (named GAM1_2F and GAM1_2R) and *SWA2* gene (SWA2_F and SWA2_R) were used for amplified specific gene as described in METHOD 3.6.2. The result was showed 2.8 kb of *GAM1* gene in clone number 1G, 2G, 3G, 4G, 5G, 6G, 7G, and 8G (Figure 4.8A) and 1.5 kb of *SWA2* gene in clone number 1S, 2S, 3S, and 4S (Figure 4.8B). No PCR product of *SWA2* or *GAM1* genes was detected from the wild-type *S. cerevisiae* TISTR 5596. However, the result of enzyme expression of all transformant yeasts in the study show low level of enzyme expression.

In order to increase the level of gene expression, it was shown that repetitive gene sequences in the yeast genome could be targeted for integration of an expression gene cassette. Exogenous DNA can be inserted into the *S. cerevisiae* chromosome using integrative vector. In our studies, a vector lacks any replication sequences and can be maintained only by integration. The transformation frequency is low, about 1-2 transformant/μg because integration is rare. Integrative vector is inserted into the chromosome by homologous recombination across *S. cerevisiae* sequences. If multiple *S. cerevisiae* sequences are presented on the vector, integration can be occurred at any of the homologous chromosomal location, but if a double strand break is introduced into one of the *S. cerevisiae* sequence, recombination at this site is simulated several hundred fold (Susan et al., 1985). The targeting the integrated genes into the repetitive sequences in yeast genome such as rDNA (reiterated DNA) or δ sequences can be increased up to 100-150 copies (Lopes et al., 1991; Wang et al., 1996; Lee et al., 1997; Nieto et al., 1999). Kang et al. (2003) successfully expressed

D. occidentalis α-amylase in a polyploid industrial strain of *S. cerevisiae*. The expression gene cassette was integrated in multiple copies in the δ sequences of the yeast genome. The integrated genes showed 100% mitotic stability over 100 generations on non-selective medium.

In order to improve the starch-hydrolyzing ability of this strain, Ghang et al. (2007) co-expressed *D. occidentalis* glucoamylase with *D. occidentalis* α-amylase in the recombinant yeast which resulted in lower amylolytic activity compared to that of the previous study. In previous study, amylase expressing diploid strain constructed by mating of general integrant haploid strains showed high ethanol productivity (0.46 g/l/h) with 290 U/l of glucoamylase and 950 U/l of α-amylase maximum activity (Yamada et al., 2009). It was speculated that the over-sized integration plasmid, which contained both glucoamylase and α-amylase genes caused the mitotic instability of the integrated genes. By reducing the size of the integration plasmid, deleting the bacterial originated sequences in the plasmid, higher mitotic stability was achieved. Designing the plasmid in this way avoided an over-sized integration plasmid that can cause mitotic instability. The two integration plasmids containing the glucoamylase and α-amylase genes were used to co-transform the yeast strain. The δ-sequence was targeted as the integration locus for recombination to ensure multiple-copy integration of the glucoamylase and α-amylase genes into the yeast genome. However, both reported observed that both exo-cleavage activity and endo-cleavage activity in the co-expressing recombinant strain was lower than that of the one only expressing glucoamylase or α-amylase, respectively. In support of this, Wong et al. (2010) showed that a recombinant yeast strain that had both glucoamylase and α-

amylase genes co-integrated had decreased expression of both enzymes compared with the strains that had either gene integrated alone.

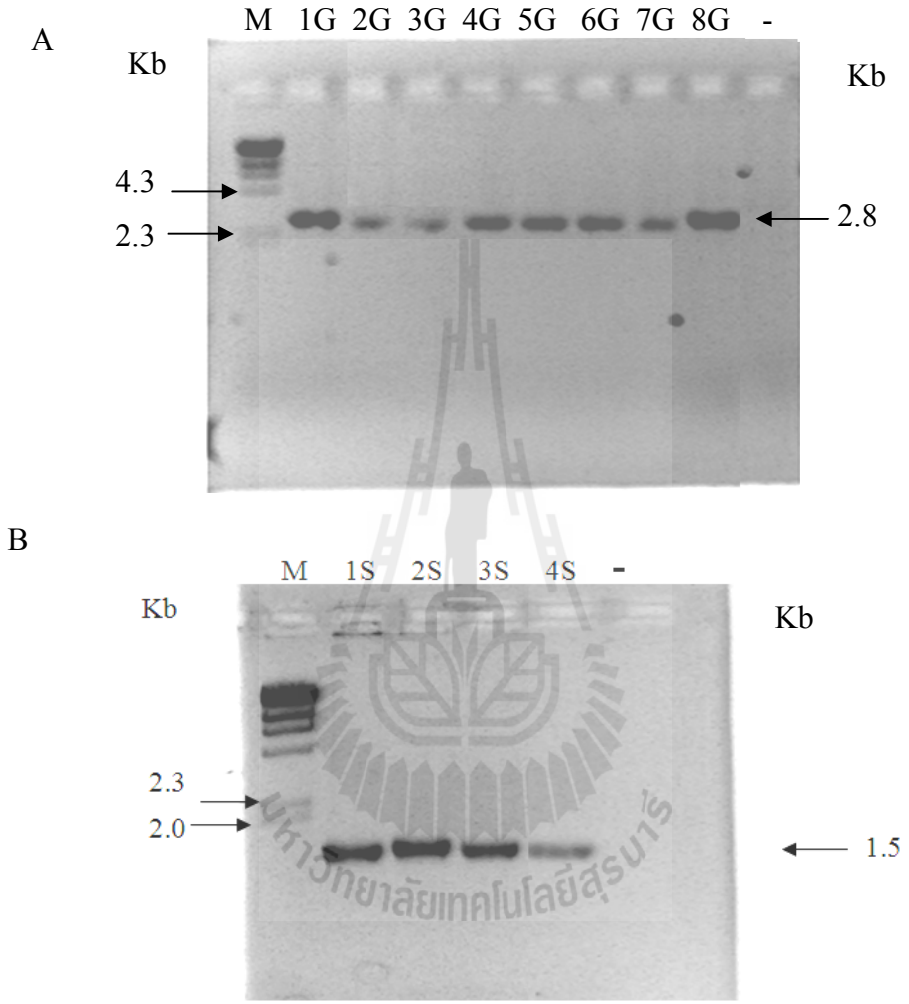

Figure 4.8 Detection of gene integration in *S. cerevisiae* transformant strain containing *SWA2* gene or *GAM1* gene by polymerase chain reaction. The figure shows 0.8% agarose gel electroporesis of the PCR product of clones number 1G, 2G, 3G, 4G, 5G, 6G, 7G, and 8G for *GAM1* gene and clones number 1S, 2S, 3S, and 4S for *SWA2* gene.

The 50 µl of PCR reaction was performed. The reaction comprised of plasmid DNA of clones number 1G, 2G, 3G, 4G, 5G, 6G, 7G, and 8G for *GAM1* gene and clones number 1S, 2S, 3S, and 4S for *SWA2* gene as a template, respectively. SWA2_F and SWA2_R were used as primers for *SWA2* gene and GAM1_2F and GAM1_2R as primers for *GAM1* gene as described in METHOD 3.4.4. The size of *GAM1* gene was 2.8 kb. The size of *SWA2* gene was 1.5 kb.

Panel A showed PCR amplification using *GAM1* gene primers.

Lanes M: DNA marker.

Lanes 1G-8G: PCR products using genomic DNA of transformant yeast strains 1G, 2G, 3G, 4G, 5G, 6G, 7G, and 8G as template.

Lanes **-** : PCR product using genomic DNA of *S. cerevisiae* wild type strain as template.

Panel B showed PCR amplification using *SWA2* gene primers.

Lanes M: DNA marker.

Lanes 1S-4S: PCR products using genomic DNA of transformant yeast strains 1S, 2S, 3S, and 4S as template.

Lanes -: PCR product using genomic DNA of *S. cerevisiae* wild type strain as template.

4.7 Quantification of glucoamylase and α-amylase activity with DNS method and iodine assays

Halo formation around the colony of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 are roughly indicated glucoamylase and α-amylase expression. It was my objective to investigate the possible assays that can be used to

quantify the activity of cell secreted glucoamylase and α-amylase. Two different assays as described in METHOD 3.6.3, the iodine assay and DNS assay are widely used to quantify glucoamylase activity and α-amylase activity (Miller, 1959; Filho et al., 1986; Ruohonen et al., 1987). Both assays are based on the principle that α-amylase breaks down starch polymers molecules at the α-1,4 linkage into reducing sugars and oligosaccharides. The oligosaccharides produced will not be detected by the iodine assay, while glucoamylase breaks down starch polymers into reducing sugars, by removing glucose units from the non-reducing ends. Xiao et al. (2006) reported that the units of α-amylase activity in samples measured by the iodine assay were five time higher than the units of activity measured by the DNS method. An iodine assay is introduced to measure the amount of soluble starch remaining after the reaction, and also measure the change in substrate concentration. While the DNS assay is exploited to measure the amount of reducing sugars released in the reaction, and also measures the formation of product. Thus, an iodine assay is applied to measure the α-amylase activity and the DNS method is taken to measure the glucoamylase activity. The result shows that the α-amylase activity of *S. cerevisiae* TISTR 5596/pSWA2 #4S was 0.43 U/ml indicated by an iodine assay and the DNS method reveals the glucoamylase activity of *S. cerevisiae* TISTR 5596/pGAM1 #1G to be 1.20 U/ml (Table 4.1).

Table 4.1 Quantification of glucoamylase activity using the DNS method and α-amylase activity using the iodine assay.

Strains	Glucoamylase activity (U/ml)	α-amylase activity (U/ml)
S. cerevisiae TISTR 5596/pGAM1#1G	1.20 ± 0.12	-
S. cerevisiae TISTR 5596/pSWA2 #4S	-	0.43 ± 0.01

Values represent the mean of duplicate experiment ±SD between samples.

Cassava starch is composed of unbranched 20% of amylose and 80% of amylopectin both of which can be hydrolyzed with enzymatically to release glucose and maltooligosaccharide. Thereafter, both products can be easily transported across the cell membrane and metabolized by yeast (Gonzalez et al., 2007). The glucoamylase enzyme of *D. occidentalis* has both α-1, 4 and α-1, 6 activities that increased starch degradation to reducing sugar, dextrin and maltose (Lancashire et al., 1989). According to the study, the amount of unit of enzyme per ml indicated that the *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 were able to degrade starch to yield glucose, reducing sugar and maltose for ethanol production, The amounts of unit of enzyme activity were close to 1.6 U/ml and 0.27 U/ml of *Hansenula polymorpha* recombinant strain expressing *SWA2* and *GAM1* genes which was able to produce ethanol up to 4 g/l from 3% soluble starch as a sole carbon source within 3 days at 48°C (Voronovgsky et al., 2009). Yang et al. (2011) also investigated

the glucose production and fermentation of the recombinant *S. cerevisiae* using raw starch as the carbon source. The recombinant *S. cerevisiae* was determined as 2.4 U/ml of glucoamylase activity which was able to hydrolyze the starch substrate to produce glucose accordingly.

Thus, both recombinant strains expressed glucoamylase or α-amylase were successfully constructed and they were able to utilize starch for growth and production ethanol. However, the activity of both enzymes when compared with other works was lower than reported. The key factor of this result may come from the heterologous promoter using in expression vector. This promoter was originated from *P. pastoris* when compared nucleotide sequence of GAP promoter with GAPDH promoter, GAP promoter in *S. cerevisiae*. The identity was 34% and shows lower strength for RNA polymerase binding during transcription.

4.8 Production of ethanol in batch fermentation studied on cassava starch containing medium

Batch fermentation was run using three different strains and three different batch experiments studies on cassava starch containing medium. Two recombinant yeast strains (*S. cerevisiae* TISTR 5596/pGAM1 for glucoamylase expression and *S. cerevisiae* TISTR 5596/pSWA2 for α-amylase expression) and one wild type yeast strain TISTR 5596 as a control were used in this study. Three different batch experiment studies (first, the *S. cerevisiae* TISTR 5596/pGAM1, second, the *S. cerevisiae* TISTR 5596/pSWA2 and the last experiment study was the co-cultivation of the *S. cerevisiae* TISTR 5596/pGAM1 with the *S. cerevisiae* TISTR 5596/pSWA2) were used for batch fermentation in this study. All strains were grown under

anaerobic bottles containing 95 ml of the YPC medium to evaluate the ethanol production using cassava starch as a sole carbon source.

4.8.1 Batch fermentation studies using *S. cerevisiae* TISTR 5596/pGAM1 that expressed glucoamylase on cassava starch containing medium

The #1G of *S. cerevisiae* 5596/pGAM1 which showed the highest glucoamylase expression was selected for the preparation of pre-cultures for batch fermentation studies as described in METHOD 3.7.2. The culture of *S. cerevisiae* 5596/pGAM1 #1G was used as starter for batch fermentations in YPC medium under anaerobic condition. Samples were collected from fermented bottle for characterization of ethanol content, starch concentration and cell dry weight over 25 days period. Figures 4.9, 4.10, and 4.11 showed the fermentation bottles, starch concentration, ethanol concentration and cell dry weight for batch fermentation study.

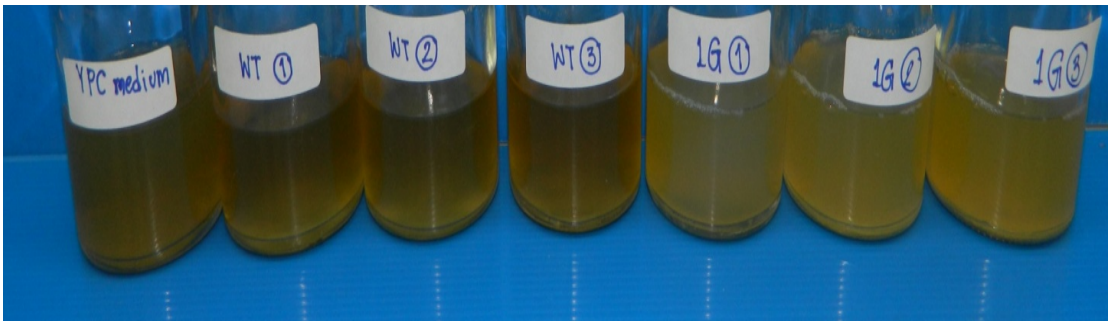

Figure 4.9 Batch fermentation using *S. cerevisiae* TISTR 5596/pGAM1 (#1G). The process was carried out in a closed bottle. *S. cerevisiae* TISTR 5596/pGAM1 (#1G) was cultured in YPC medium containing 10 g/l of cassava starch as described in METHOD 3.7.2. The experiment was performed in triplicate for each strain. The increasing of turbidity in a fermentation bottle of *S. cerevisiae* TISTR 5596/pGAM1 (#1G.1, #1G.2 and #1G.3) showed cell accumulation.

Bottle YPC medium: the YPC medium was used for fermentation study.

Bottle WT1-WT3: the YPC medium was inoculated with *S. cerevisiae* wild type strain and used as a control.

Bottle 1G.1-1G.3: the YPC medium was inoculated with *S. cerevisiae* TISTR 5596/pGAM1 for glucoamylase expression study.

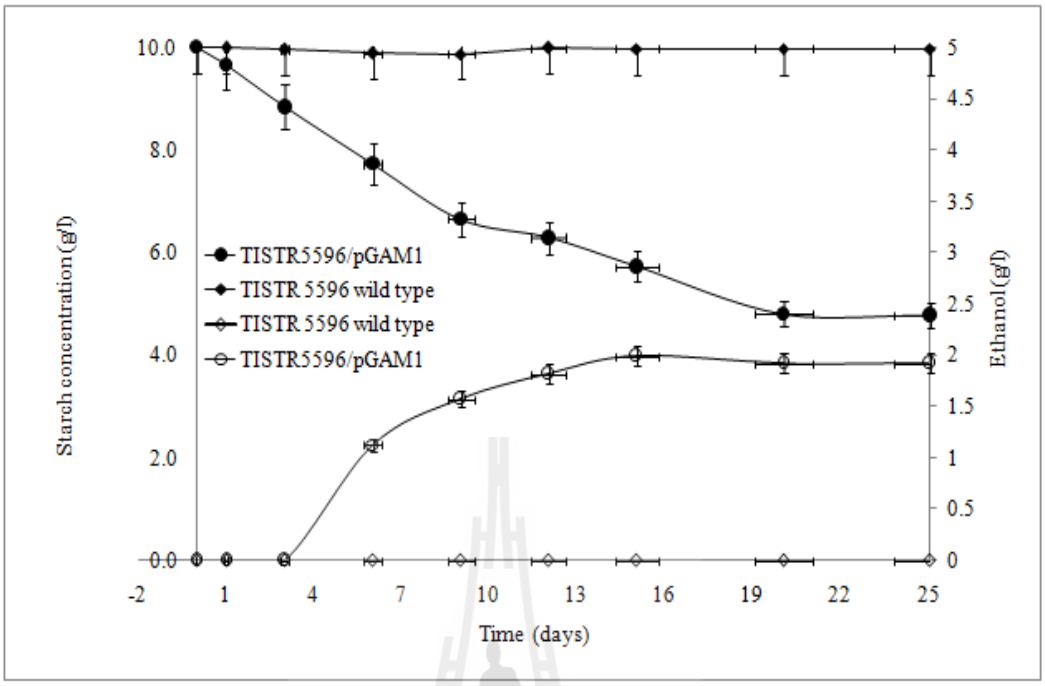

Figure 4.10 Time course of direct ethanol production via fermentation from cassava starch containing medium using *S. cerevisiae* TISTR 5596/pGAM1 (#1G). The ethanol production via fermentation from cassava starch containing media using *S. cerevisiae* TISTR 5596/pGAM1 (*circle*) and *S. cerevisiae* TIRSR 5596 wild type (*diamond)*. *Open* and *closed* symbols show ethanol and starch concentration, respectively. Data are averaged from three triplicate experiments. The bar is represented standard error.

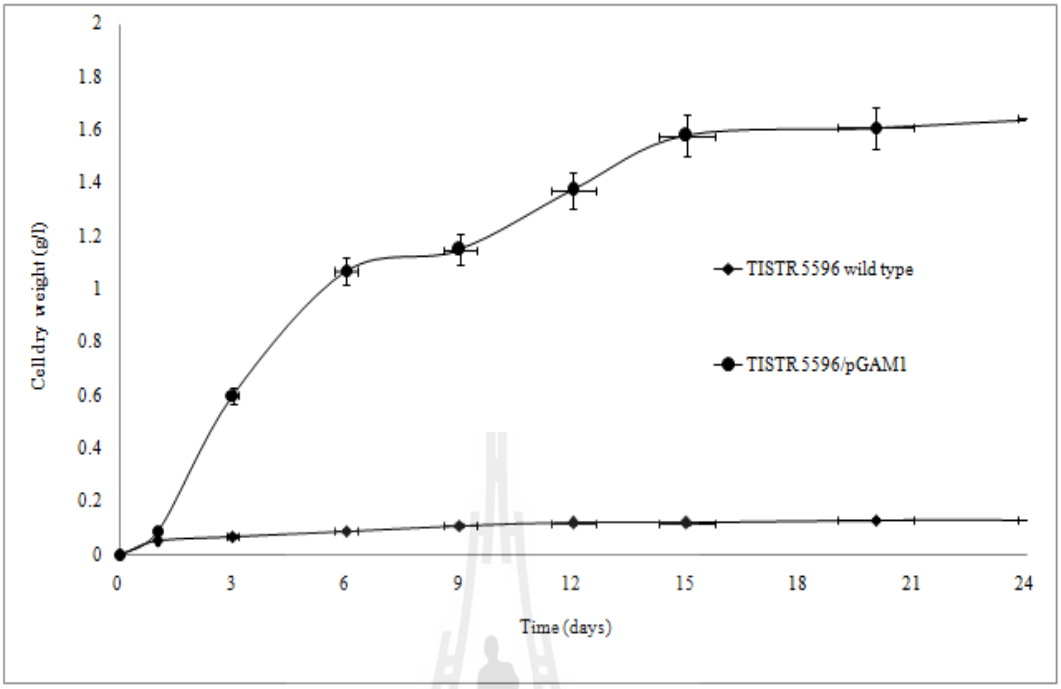

Figure 4.11 Time course of cell dry weight during fermentation process. The cell accumulation (cell dry weight) of the yeast strains of *S. cerevisiae* TISTR 5596/pGAM1 (*circle*) expressing glucoamylase gene and TISTR 5596 wild type *(diamond*) during growth in fermentation with 10 g/l cassava starch as sole carbon source. Data are averaged from three triplicate experiments. The bar indicates standard error.

From Figure 4.9, indicating turbidity revealed cell accumulation which reflected cell growth of *S. cerevisiae* TISTR 5596/pGAM1 in the fermentation bottle. The result indicated that the strain was able to utilize cassava starch for growth. Since, cell accumulation was not observed in the *S. cerevisiae* TISTR 5596 wild type (WT) it was indicated that the wild type strain was unable to utilize cassava starch for growth.

Ethanol production from 10 g/l cassava starch of *S. cerevisiae* TISTR 5596/pGAM1 was also performed in 120 ml fermentation bottles (Figure 4.10, 4.11). Maximum cell dry weight (1.648 ± 0.010 g/l), maximum ethanol (1.992 ± 0.248 g/l), other organic (acetic acid, succinic acid, lactic acid and formic acid) (0.6817 ± 0.537 g/l) were obtained at 25 days. At 144 hours, ethanol was produced at 1.127 ± 0.110 g/l with productivity of 0.008 ± 0.001 g/l/hr (Table 4.3). The concentration of starch content was decreased during fermentation and the residual concentration of starch decreasing to 4.255 ± 0.340 g/l from 10 g/l of initial cassava starch concentration at 25 days. The result demonstrated that *S. cerevisiae* TISTR 5596/pGAM1 was able to utilize cassava starch for ethanol production without adding of exogenous glucoamylase. *D. occidentalis* glucoamylase has both α-1, 4 and α-1, 6 hydrolysis activities that could hydrolysis increase the starch to reducing sugar, dextrin, and glucose. Similarly, Yang et al. (2011) reported that the ethanol production by *S. cerevisiae* expressing only *Rhizopus* glucoamylase could utilized raw corn starch and produced ethanol which produced 3.9 g/l of ethanol and reduced starch to 4 g/l from 10 g/l initial raw starch.

4.8.2 Batch fermentation studies using *S. cerevisiae* TISTR 5596/pSWA2 that expressed α-amylase on cassava starch containing medium

The #4S of *S. cerevisiae* 5596/pSWA2 which showed the highest α-amylase expression was selected for the preparation of pre-cultures for batch fermentation studies as described in METHOD 3.7.2. The culture of *S. cerevisiae* 5596/pSWA2 #4S was used as starter for batch fermentations in YPC medium under anaerobic condition. Samples were collected from fermented bottle for

characterization of ethanol content, starch concentration and cell dry weight over 25 days period. Figures 4.12, 4.13, and 4.14 showed the fermentation bottles, starch concentration, ethanol concentration and cell dry weight for batch fermentation study.

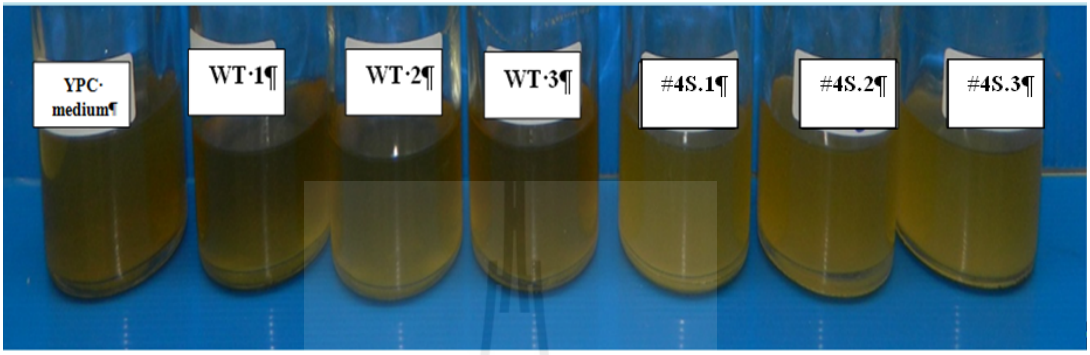

Figure 4.12 Batch Fermentation Using *S. cerevisiae* TISTR 5596/pSWA2 (#4S). The process was carried out in a closed bottle. *S. cerevisiae* TISTR 5596/pSWA2 (#4S) was cultured in YPC medium containing 10 g/l of cassava starch as described in METHOD 3.7.2. The experiment was performed in triplicate for each strain. The increasing of turbidity in a fermented bottle of *S. cerevisiae* TISTR 5596/pSWA2 (#4S.1, #4S.2, and #4S.3) showed cell accumulation.

Bottle YPC medium: the YPC medium was used for fermentation study.

Bottle WT1-WT3: the YPC medium was inoculated with *S. cerevisiae* wild type strain and used as a control.

Bottle 4S.1-4S.3: the YPC medium was inoculated with *S. cerevisiae* TISTR 5596/pSWA2 for α-amylase expression study.

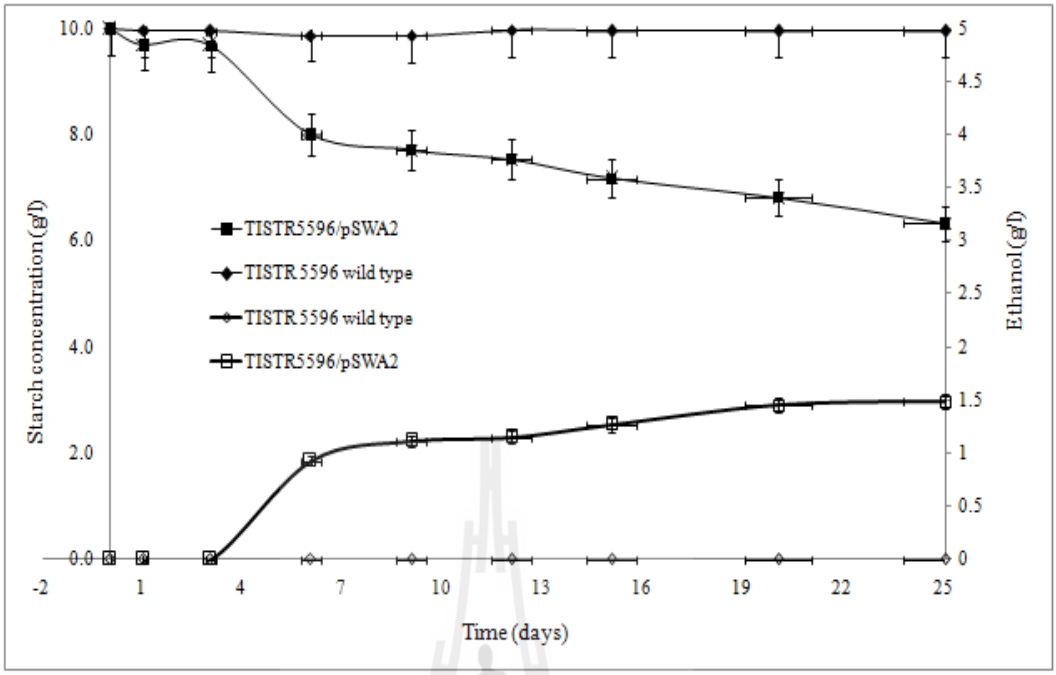

Figure 4.13 Time course of direct ethanol production via fermentation from cassava starch containing medium using *S. cerevisiae* TISTR 5596/pSWA2 (#4S). The ethanol production via fermentation from cassava starch containing media using *S. cerevisiae* TISTR 5596/pSWA2 (square) and *S. cerevisiae* TIRSR 5596 wild type (*diamond*). *Open* and *closed* symbols show ethanol and starch concentration, respectively. Data are averaged from three triplicate experiments. The bar depicts standard error.

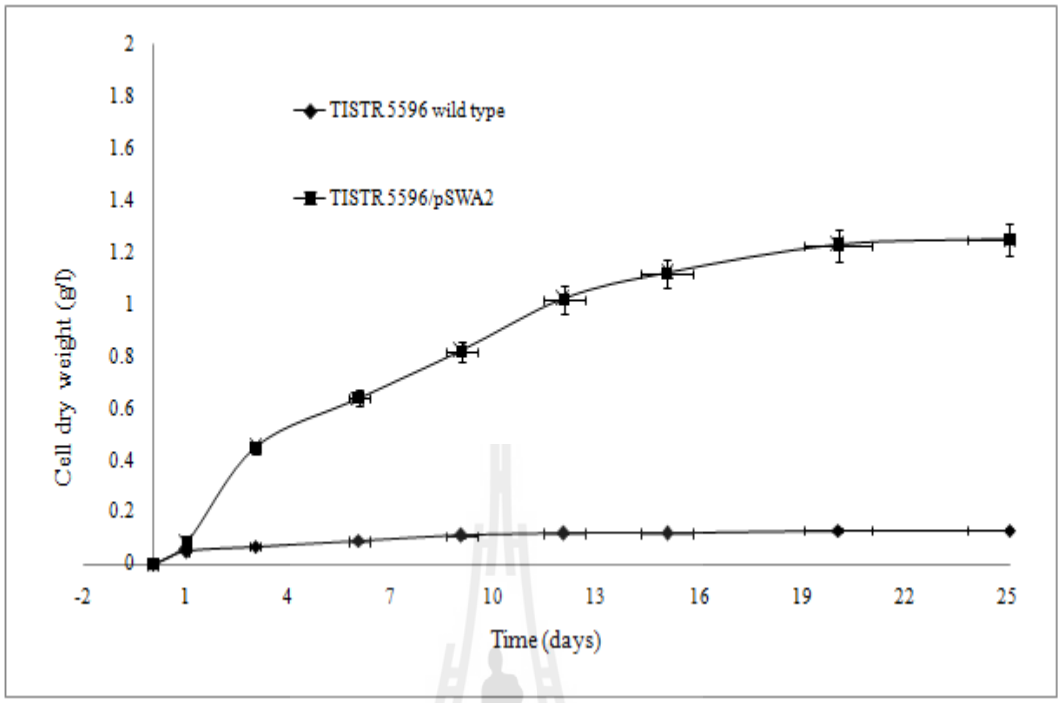

Figure 4.14 Time course of cell dry weight during fermentation process. The cell accumulation (cell dry weight) of the yeast strains of *S. cerevisiae* TISTR 5596/pSWA2 (*squares*) expressing α-amylase gene and *S. cerevisiae* TISTR 5596 wild type (*diamond*) during growth in YPC medium containing 10 g/l cassava starch as sole carbon source. Data are averaged from three triplicate experiments. The bar indicates standard error.

From figure 4.12, indicating turbidity revealed cell accumulation which reflected cell growth of *S. cerevisiae* TISTR 5596/pSWA2 in the fermentation bottle. This result indicated that the strain was able to utilize cassava starch for growth. Cell accumulation of the *S. cerevisiae* TISTR 5596 wild type (WT) was not observed. It was indicated that the wild type strain was unable to utilize cassava starch for growth.

Ethanol production from 10 g/l cassava starch of *S. cerevisiae* TISTR 5596/pSWA2 was also performed in 120 ml fermentation bottles (Figures 4.13, 4.14). Maximum cell dry weight (1.250 ± 0.001 g/l), maximum ethanol (1.478 ± 0.267 g/l), other organic (acetic acid, succinic acid, lactic acid and formic acid) (1.341 ± 0.464 g/l) were obtained at 25 days. At 144 hours, ethanol was produced at 0.929 ± 0.071 g/l with productivity of 0.006 ± 0.000 g/l/hr (Table 4.3). The concentration of starch content was decreased during fermentation and the residual concentration of starch decreasing to 5.923 ± 0.732 g/l from 10 g/l of initial cassava starch concentration at 25 days. These results indicated that, the *S. cerevisiae* TISTR 5596/pSWA2 was able to degrade starch and yield glucose for ethanol production. Similarly, Ramachandan et al. (2008) expressed in *S. cerevisiae* the *LKA1* α-amylase-encoding gene from an efficient raw-starch degrading yeast, *Lipomyces kononenkoae*. It produced 4.61 g/l of ethanol after 5 days from 30 g/l of corn starch.

4.8.3 Co-cultivation study using *S. cerevisiae* TISTR 5596/pGAM1 strain with *S. cerevisiae* TISTR 5596/pSWA2 strain on cassava starch containing medium

In order to produce ethanol directly from starch, the recombinant *S. cerevisiae* requires the co-expression of glucoamylase and α-amylase for complete hydrolysis of starch during ethanol fermentation (Yamada et al., 2009). Therefore, the recombinant yeasts expressing two genes of glucoamylase and α-amylase for synergistic hydrolysis of starch could be used. Thus, the *S. cerevisiae* TISTR 5596/pGAM1 strain that expressed glucoamylase and *S. cerevisiae* TISTR 5596/pSWA2 strain that express α-amylase could have co-expression of both

enzymes for complete hydrolysis of starch during ethanol fermentation. The *S. cerevisiae* TISTR 5596/pGAM1 #1G and *S. cerevisiae* TISTR 5596/pSWA2 #4S were co-cultured in the YPC medium containing cassava starch as a sole carbon source for batch fermentation studies. The *S. cerevisiae* TISTR 5596/pGAM1 #1G and *S. cerevisiae* TISTR 5596/pSWA2 #4S were used as inoculums in batch fermentation studies as described in METHOD 3.7.2. Samples were collected from fermentation bottle for characterization of ethanol content, starch concentration and cell dry weight over 25 days period. Figures 4.15, 4.16, and 4.17 showed fermentation bottles, ethanol concentration, starch concentration and cell dry weight for batch fermentation study.

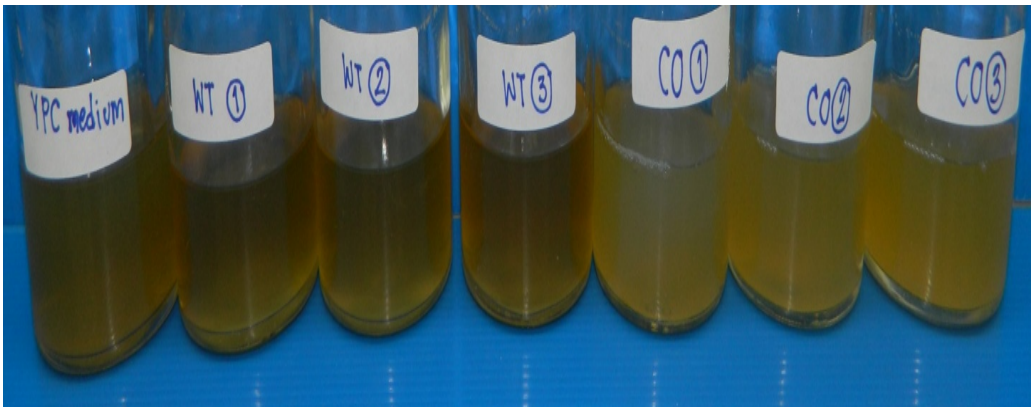

Figure 4.15 Batch fermentation using *S. cerevisiae* TISTR 5596/pGAM1 (#1G) co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 (#4S). The process was carried out in a closed bottle. *S. cerevisiae* TISTR 5596/pGAM1 (#1G) co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 (#4S) was cultured in YPC medium containing 10 g/l of cassava starch as described in METHOD 3.7.2. The experiment was performed in three triplicates for each strain. The increasing of turbidity in a fermentation bottle of *S. cerevisiae* TISTR 5596/pGAM1 co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 (CO.1, CO.2, and CO.3) was showed the cell accumulation.

Bottle YPC medium: the YPC medium was used for fermentation study.

Bottle WT1-WT3: the YPC medium was inoculated with *S. cerevisiae* wild type strain and used as a control.

Bottle CO.1-CO.3: the YPC medium was inoculated with *S. cerevisiae* TISTR 5596/pGAM1 co-cultured with *S. cerevisiae* TISTR 5596/pSWA2.

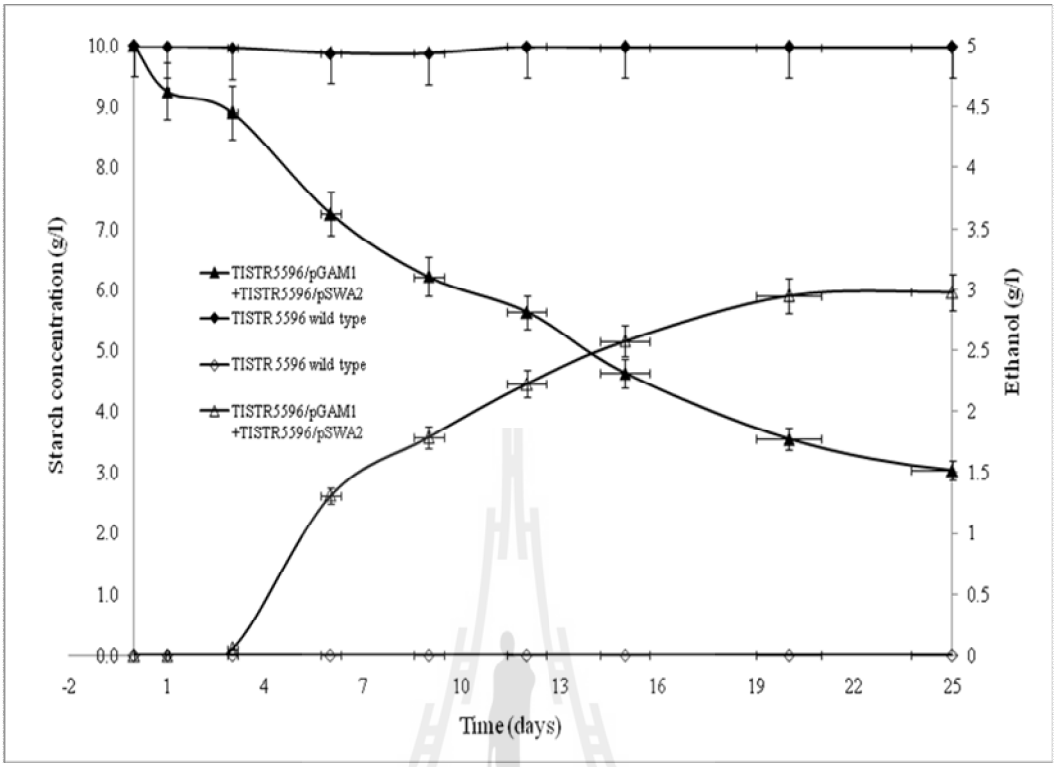

Figure 4.16 Time course of direct ethanol production via fermentation from cassava starch containing medium by *S. cerevisiae* TISTR 5596/pGAM1 strain co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 strain. The ethanol production via fermentation from cassava starch containing medium using *S. cerevisiae* TISTR 5596/pGAM1 strain co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 strain (*triangles*) and TIRSR 5596 wild type (*diamond)*. *Open* and *closed* symbols show ethanol and starch concentration, respectively. Data are averaged from three triplicate experiments. The bar indicates standard error.

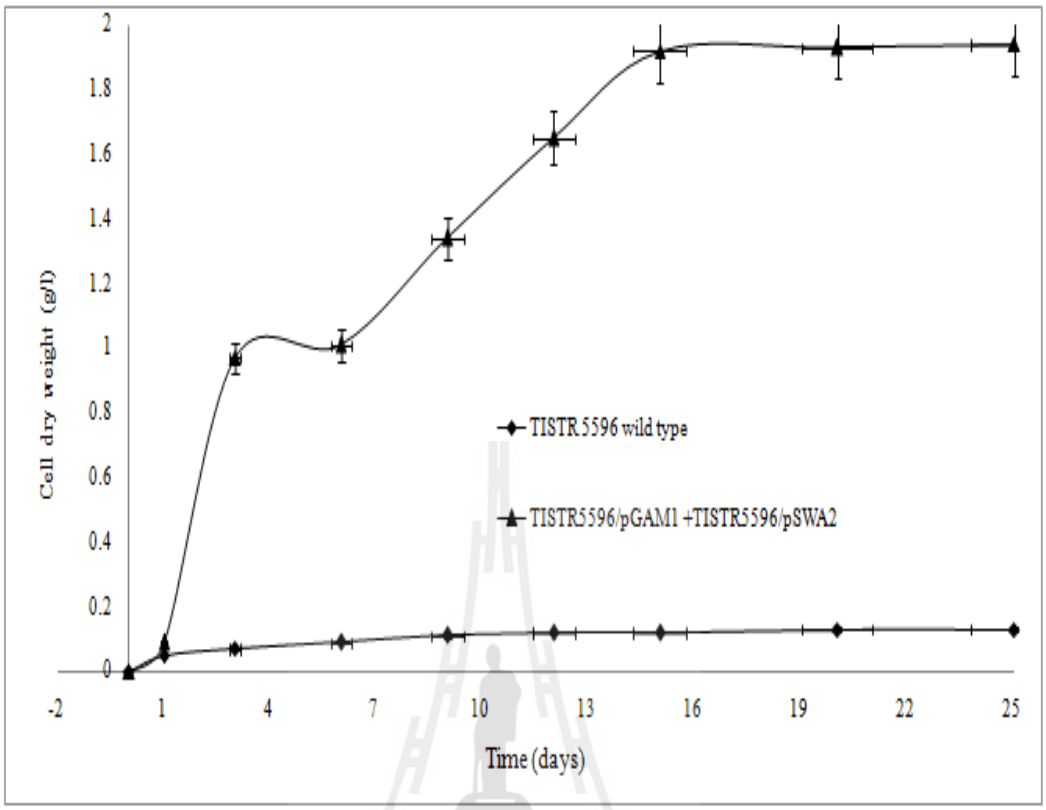

Figure 4.17 Time course of cell dry weight during fermentation process. The cell accumulation (cell dry weight) of the yeast strains of *S. cerevisiae* TISTR 5596/pGAM1 co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 (triangle) expressing α-amylase gene and *S. cerevisiae* TISTR 5596 wild type (*diamond*) during growth in YPC medium containing 10 g/l cassava starch as sole carbon source. Data are averages from three triplicate experiments. The bar indicate standard error.

From figure 4.15, *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 (CO) can grow in fermentation bottle as shown by the increased of turbidity resulted from the cell accumulation. The result indicated that the strains were able to utilize cassava starch for growth. Since, cell accumulation

was not observed in the *S. cerevisiae* TISTR 5596 wild type (WT) it was indicated that the wild type strain was unable to utilize cassava starch for growth.

Ethanol production from 10 g/l cassava starch of the co-cultivation of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 was also performed in 120 ml fermentation bottles (Figure 4.16, 4.17). Maximum cell dry weight (1.940 ± 0.002 g/l), maximum ethanol (2.977 ± 0.020 g/l), other organic (acetic acid, succinic acid, lactic acid and formic acid) (2.795 ± 0.503 g/l) were obtained at 25 days. At 144 hours, ethanol was produced at 1.315 ± 0.024 g/l with productivity of 0.009 ± 0.000 g/l/hr (Table 4.3). The concentration of starch content was decreased during fermentation and the residual concentration of starch decreasing to 2.288 ± 0.488 g/l from 10 g/l of initial cassava starch concentration at 25 days. The result suggested that when co-cultured both *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 was able to utilize cassava starch for ethanol production. Cassava starch was hydrolyzed for 20.75% within 6-day in the co-culture of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2. While corn starch was hydrolyzed for 77% within 6-day in the co-culture of recombinant strain expressed *D. occidentalis* glucoamylase and recombinant strain expressed *D. occidentalis* α-amylase (Wong et al., 2010). Yamada et al. (2009) developed amylolytic strain of *S. cerevisiae* strain co-expression both *R. oryzae* glucoamylase and *S. bovis* α-amylase hydrolyzed 10 g/l of raw corn starch to produce ethanol was 6.5 g/l of ethanol after 4 days. Kim and Kim (1996) reported that an industrial *S. cerevisiae* strain secreting *S. diastaticus* glucoamylase and mouse salivary α-amylase produced 2.9 g/l of ethanol in 10 g/l of raw corn starch containing medium after 10 days of fermentation, and the residual starch content after fermentation was 1.04 g/l.

While the results of the study show that at 10 days fermentation period was 1.79 g/l ethanol in 10 g/l raw cassava starch containing medium, and the residual starch content after fermentation was 6.22 g/l. Therefore, the result showed lower ethanol production by *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 from cassava starch than previous reported. However, The use of both recombinant yeasts that expresses the glucoamylase or α-amylase gene to increase fermentation rate and to increase the starch utilization rate was successful.

4.9 Comparisons between each of the recombinant strain for cassava starch utilization and ethanol production

The ability to produce ethanol directly via the fermentation in YPC medium containing 10 g/l of cassava starch as a sole carbon source of *S. cerevisiae* TISTR 5596/pGAM1, *S. cerevisiae* TISTR 5596/pSWA2 and co-cultured of TISTR 5596/pSWA2 and TISTR 5596/pGAM1 recombinant strain were compared.

Figures 4.19 and 4.20 represent a comparison of starch; ethanol concentration and cell dry weight trends in each of transformant yeast strains using cassava starch as a sole carbon source in batch fermentation. The co-cultivation of *S. cerevisiae* TISTR 5596/pSWA2 with *S. cerevisiae* TISTR 5596/pGAM1 produced ethanol and yield greater than separate cultures, while the *S. cerevisiae* TISTR 5596 wild type did not show detectable hydrolysis of starch and ethanol production. The result indicated that the co-expression and secretion of glucoamylase and α-amylase enzyme of both strains have higher efficiency of hydrolysis starch than single enzyme expression. Ghang et al. (2007) improved two recombinant yeast strains by expressing of *GAM1* and *AMY* genes. In the co-culture of ATCC 9763/YIpdAURSGδ and ATCC

9763/YIpdAURSAδ 100% utilization of starch within 24 hr was resulted. With reference to the result of the study, *S. cerevisiae* TISTR 5596/pGAM1 secreted glucoamylase enzyme was able to produce ethanol higher than *S. cerevisiae* TISTR 5596/pSWA2 that secreted α-amylase enzyme (Figures 4.7 and 4.18). Glucoamylase is an exo-type enzyme that degrades starch and releases glucose, while α-amylases an endo-type enzyme that cleaves α-1, 4-glucosidic linkages of starch and liberates maltose and oligosaccharides (Eksteen et al., 2003). In addition, the expression of *GAM1* is significant debranching activity (α-1, 6 activity) leading to complete hydrolysis of starch (Jense and Pretorius, 1995). Moreover, *D. occidentalis* expresses significant debranching activity as a part of the glucoamylase, indicating that it possesses the ability to hydrolyze both α-1,4 and α-1,6-glucosidic linkages, which is essential for complete hydrolysis of starch (Dohmen et al., 1990). These results indicate that the introduction GAM1 of *S. cerevisiae* TISTR 5596/pGAM1 with α-1,4 and α-1,6 activity of amylopectin in cassava starch resulted in increased starch hydrolysis rates and ethanol production when compared to *S. cerevisiae* TISTR 5596/pSWA2 strain.

The measurement of the cell accumulation during the fermentation process could confirm that all transformant yeasts were able to use cassava starch as a sole carbon source for growth. As shown in Figure 4.19, the ability to utilize cassava starch for growth by each of transformant yeasts was observed. One major difference between propagation and fermentation is that a low level of glucose is preferred in cell propagation compared to fermentation. The lower level of glucose was used the beginning of propagation because higher glucose concentrations can induce the yeast to produce ethanol, resulting in less energy production per molecule of glucose. The

glucose concentration in batch fermentation studies was produced by the recombinant yeasts that produce starch-decomposing enzymes in order to utilize the starch. During fermentation, yeast cells break down starch and convert to glucose first. The cell accumulation was observed over 6-day and the cells were increased. The maximum of the cell dry weight was shown in the co-cultured of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2. The result indicated that the co-expression of both enzymes could increase glucose substrate for cell production.

After 12 days, ethanol yield of 0.489 ± 0.010 g/l for *S. cerevisiae* TISTR 5596/pGAM1 was produced whereas ethanol yield of 0.465 ± 0.012 for *S. cerevisiae* TISTR 5596/pSWA2 was produced. The result demonstrated that there is no significant difference between *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2. Ethanol yield of 0.516 ± 0.021 g/l for *S. cerevisiae* TISTR 5596/pGAM1 co-cultured with *S. cerevisiae* TISTR 5596/pSWA2 was produced (Figure 4.20). The result demonstrated that there is significant difference between *S. cerevisiae* TISTR 5596/pSWA2 while there is no significant difference between *S. cerevisiae* TISTR 5596/pGAM1 (Table 4.2).

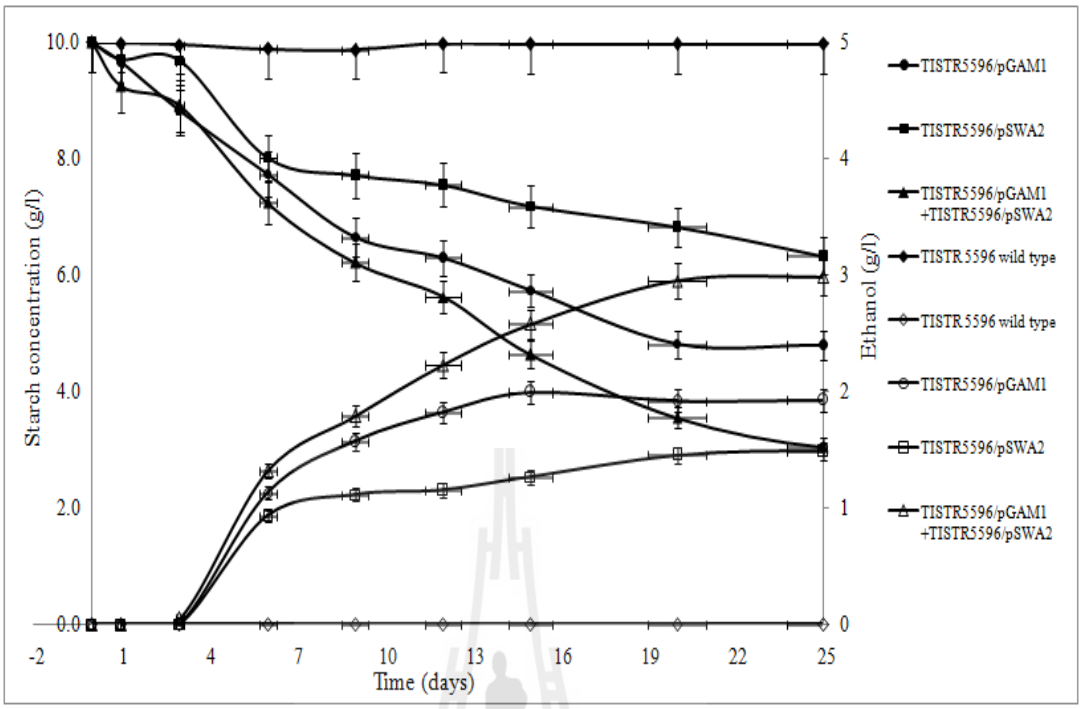

Figure 4.18 Comparisons of the starch hydrolysis and fermentation ability of *S. cerevisiae* TISTR 5596/pSWA2, *S. cerevisiae* TISTR 5596/pGAM1, the co-cultivation of *S. cerevisiae* TISTR 5596/pSWA2 and TISTR 5596/pGAM1. Comparisons of the starch hydrolysis and fermentation ability of *S. cerevisiae* TISTR 5596/pSWA2 (*squares*), TISTR 5596/pGAM1 (*circle*), TISTR 5596/pSWA2 co-cultured with TISTR 5596/pGAM1 (*triangle*), and TIRSR 5596 wild type (*diamond*). *Open* and *closed* symbols show ethanol and starch concentration, respectively. Data are averaged from three triplicate experiments. The bar indicats standard error.

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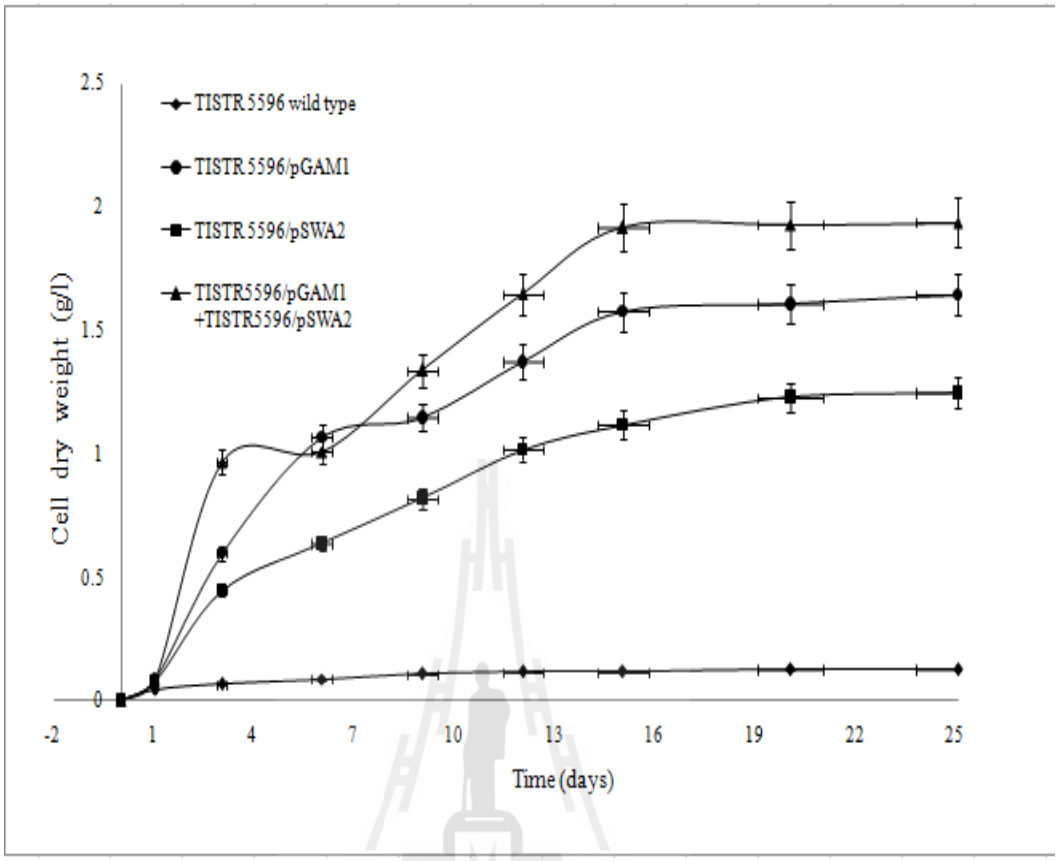

Figure 4.19 Time course of cell dry weight during fermentation process. The cell accumulation (cell dry weight) of the yeast strains of *S. cerevisiae* TISTR 5596/pSWA2 (*squares*), TISTR 5596/pGAM1 (*circle*), TISTR 5596/pSWA2 co-cultured with TISTR 5596/pGAM1 (*triangle*), and TIRSR 5596 wild type (*diamond*) expressing α-amylase, glucoamylase and co-expression during growth in YPC medium containing 10 g/l cassava starch as sole carbon source. Data are averaged from three triplicate experiments. The bar indicates standard error.

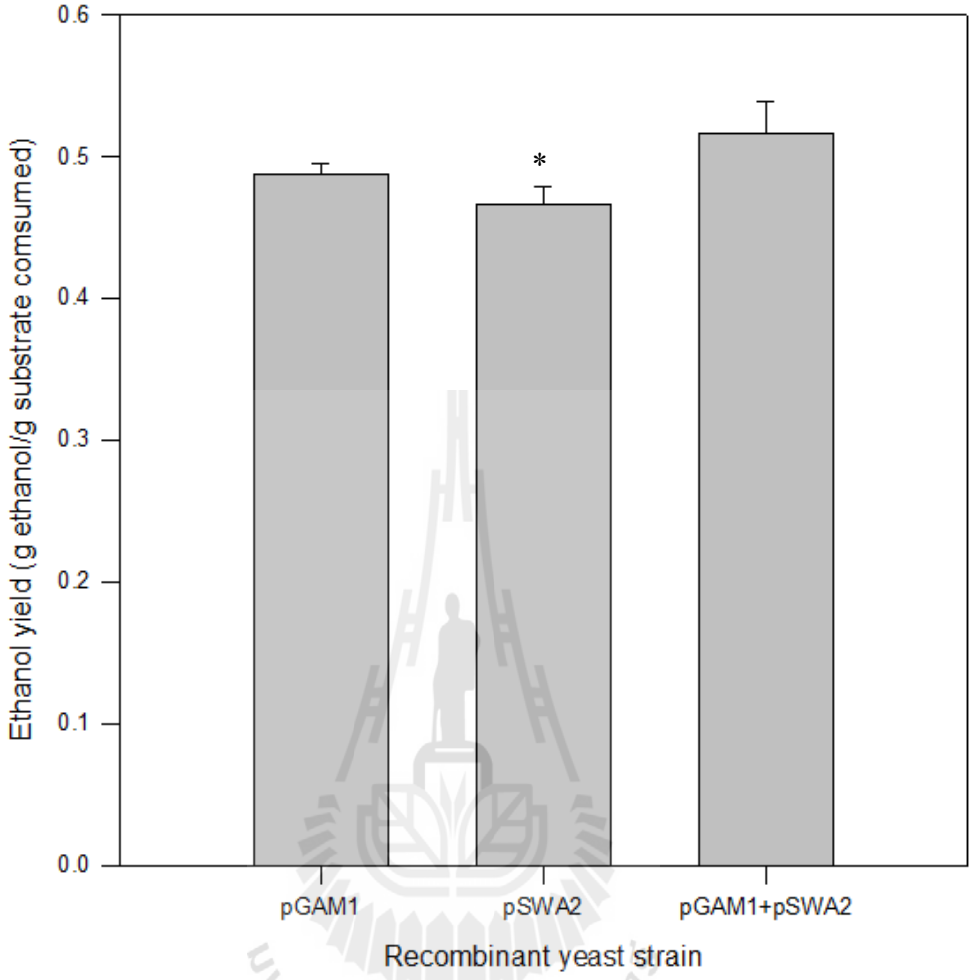

Figure 4.20 Comparison of the ethanol yield at 12 days in bacth fementation of *S. cerevisiae* TISTR 5596/pSWA2, TISTR 5596/pGAM1 and co-cultured of TISTR 5596/pSWA2 and TISTR 5596/pGAM1. The values are mean ±SE. * = Significant at 0.05 probability level ($P<0.05$).

Table 4.2 Analysis of variances for the ethanol yield at 12 days in bacth fementation of co-cultured of *S.cerevisiae* TISTR 5596/pGAM1+*S.cerevisiae* TISTR 5596/pSWA2 and mono-cultured of *S.cerevisiae* TISTR 5596/pGAM1 or *S.cerevisiae* TISTR 5596/pSWA2.

Strains	The yield of ethanol (g of ethanol/g of substrate consumed) (Mean±SEM)
TISTR 5596/pSWA2	0.466±0.007*
TISTR 5596/pGAM1	0.487±0.004
TISTR 5596/pSWA2+ TISTR 5596/pGAM1	0.517±0.012

Note: The P values for Dunnett's and Duncan's tests are currently unavailable except for reporting that the P's are greater or less than the critical values of .05. * = Significant at 0.05 probability level (P<0.05).

Table 4.3 Comparison of recombinant yeast on ethanol production using 10 g/l cassava starch as substrate.

Kinetic parameters	Strains			
	S. cerevisiae TISTR 5596 Wild type	*S. cerevisiae* TISTR 5596/pGAM1	*S. cerevisiae* TISTR 5596/pSWA2	*S. cerevisiae* TISTR 5596/pGAM1 + *S. cerevisiae* TISTR 5596/pSWA2
Initial substrate conc. (g/l)	10.000	10.000	10.000	10.000
Final substrate conc. (g/l)	10.000	4.255±0.340	5.923±0.732	2.288±0.488
Max. cell dry weight conc. (g/l)	0.130±0.003	1.648±0.010	1.250±0.001	1.940±0.002
Max. ethanol conc. (g/l)	-	1.992±0.248	1.478±0.267	2.977±0.020
Other organic acid (acetic acid, succinic acid, lactic acid, formic acid) conc. (g/l)	-	0.6817±0.537	1.341±0.464	2.795±0.503
Fermentation time (days)*	-	15	25	20
Ethanol productivity (g ethanol/l-hr)[a]	-	0.008±0.001	0.006±0.000	0.009±0.000
Ethanol yield (g ethanol/g glucose) [b]	-	0.489±0.010	0.465±0.012	0.516±0.021

[a]Maximum volumetric productivity calculated on the basis of the most productive 144-hr period.

[b] Ethanol yield calculated base on the potential glucose in medium with assumption that 1 g starch was converted to 1.1 g glucose and calculated on a basis of g ethanol produced per g glucose metabolized at 12 days.

*Fermentation time was the taken to reach maximum ethanol concentration. Values represent the mean of duplicate experiment ±SD between samples.

CHAPTER V

CONCLUSION

Cassava is an attractive alternative as the carbon substrate for ethanol production especially where water availability is limited as is able to tolerate drought and grow on relatively low fertility soil. In view point of utilization of enzymes, the conventional method for the ethanol production involves liquefaction, saccharification and fermentation steps which are time consuming and cost ineffective. Therefore, direct fermentation with integrated steps that incorporate recombinant or co-culture strains in a single reactor offers a more convenient method for the production of ethanol and its high value by-products. Various biotechniques have been used to improve industrial ethanol production by fermentation. Two different approaches have been generally used to achieve this goal. One approach is to engineer amylolytic enzymes to optimize their functionality during starch hydrolysis. The other approach is to select micro organisms with superior cellular abilities for ethanol productivity by mutation or metabolic engineering.

In this study, a wild type strain of *S. cerevisiae* was genetically engineerd to express cell secreted glucoamylase and α-amylase enzymes. These arming yeast were able to produce ethanol from cassava starch without addition of amylolytic enzyme under anaerobic condition. The glucoamylase gene and α-amylase genes from *D. occidentalis* were inserted into a yeast expression plasmid (plasmid pGAPZαA under the strong constitutive promoter of *GAP* gene) to obtain pGAM1 plasmid and pSWA2

plasmids, respectively. Each of pGAM1and pSWA2 plasmid was later used as a vector for transformation and expression of glucoamylase and α-amylase in *S. cerevisiae* TISTR 5596 wild type, respectively. After transformation, zeocin-resistant transformants were selected on the YPD medium supplemented with 130 μg/ml of zeocin^{TM}. Seven transformants TISTR 5596/pGAM1 and four transformants TISTR 5596/pSWA2 were capable to grow on YPD medium plates supplemented with 130 μg/ml of zeocin^{TM}. The presence of *GAM1* gene or *SWA2* gene under the GAP promoter in *S. cerevisiae* transformants was confirmed by PCR using specific primers. All of transformant yeasts were screened for glucoamylase and α-amylase expression on YPDS2 plates. All of transformant yeasts showed the formation of clear halos around the colonies indicating the secretion of glucoamylase and α-amylase enzyme. Then amount of glucoamylase and α-amylase activity of each of selected transformants yeast were determined. *S. cerevisiae* TISTR 5596/pGAM1 has 1.20 U/ml while the *S. cerevisiae* TISTR 5596/pSWA2 has 0.43 U/ml.

The ability to produce ethanol directly via the fermentation with YPC medium containing 10 g/l of cassava starch as a sole carbon source of *S. cerevisiae* TISTR 5596/pGAM1, *S. cerevisiae* TISTR 5596/pSWA2 and co-cultured of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 were examined. The *S. cerevisiae* TISTR 5596 wild type was used as control for all batch fermentation studies.

The maximum ethanol yield was obtained in 0.489 ± 0.010 g ethanol/g substrate consumed for *S. cerevisiae* TISTR 5596/pGAM1 at 12 days. Maximum cell dry weight (1.648 ± 0.010 g/l), maximum ethanol (1.992 ± 0.248 g/l), other organic (acetic acid, succinic acid, lactic acid and formic acid) (0.6817 ± 0.537 g/l) were

obtained at 25 days. At 144 hours, ethanol was produced at 1.127 ± 0.110 g/l with productivity of 0.008 ± 0.001 g/l/hr. The residual concentration of starch was decreased to 4.255 ± 0.340 g/l from 10 g/l of initial cassava starch at 25 days.

The maximum ethanol yield was obtained in 0.465 ± 0.012 g ethanol/g substrate consumed for *S. cerevisiae* TISTR 5596/pSWA2 at 12 days. Maximum cell dry weight (1.250 ± 0.001 g/l), maximum ethanol (1.478 ± 0.267 g/l), other organic (acetic acid, succinic acid, lactic acid and formic acid) (1.341 ± 0.464 g/l) were obtained at 25 days. At 144 hours, ethanol was produced at 0.929 ± 0.071 g/l with productivity of 0.006 ± 0.000 g/l/hr. The concentration of starch content was decreased during fermentation and the residual concentration of starch decreasing to 5.923 ± 0.732 g/l from 10 g/l of initial cassava starch concentration at 25 days.

The maximum ethanol yield was obtained in 0.516 ± 0.021 g ethanol/g substrate consumed for co-cultured of *S. cerevisiae* TISTR 5596/pGAM1 and *S. cerevisiae* TISTR 5596/pSWA2 at 12 days. Maximum cell dry weight (1.940 ± 0.002 g/l), maximum ethanol (2.977 ± 0.020 g/l), other organic (acetic acid, succinic acid, lactic acid and formic acid) (2.795 ± 0.503 g/l) were obtained at 25 days. At 144 hours, ethanol was produced at 1.315 ± 0.024 g/l with productivity of 0.009 ± 0.000 g/l/hr. The concentration of starch content was decreased during fermentation and the residual concentration of starch decreasing to 2.288 ± 0.488 g/l from 10 g/l of initial cassava starch concentration at 25 days. *S. cerevisiae* TISTR 5596/pGAM1 secreted glucoamylase enzyme was able to produce ethanol more than *S. cerevisiae* TISTR 5596/pSWA2 that secreted α-amylase enzyme.

In order to increase ethanol production from starch, the co-expression of glucoamylase and α-amylase for complete hydrolysis of starch was studied. The co-

cultivation of *S. cerevisiae* TISTR 5596/pGAM1 with *S. cerevisiae* TISTR 5596/pSWA2 produced ethanol and had greater yield than separated cultures. The result indicated that the co-expression of glucoamylase and α-amylase enzyme has increased efficiency to hydrolysis of starch. The recombinant yeast that can convert starch to ethanol was successfully engineered. The transgene was expressed in host strain. The study is the promising start for further ethanol producing yeast development.

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APPENDICES

APPENDIX A

CHEMICAL COMPOSITION

A.1 Culture media and selective medium

All culture media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

1. Low salt LB-zeocin medium

Yeast extract	5 g/l
Tryptone	10 g/l
Sodium chloride	5 g/l
pH	7.5

Autoclave at 121°C, for 15 min. Zeocine is added in the warm medium at 50 µg/ml final concentrations after autoclaving. If the agar medium is desired, 15 g/l of agar is added into the medium before autoclaving.

2. Yeast peptone dextrose (YPD) zeocin™ broth

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

Autoclave at 121°C for 20 minutes, then cooling down to 55°C and add zeocin at 130 µg/ml final concentration. If the agar medium is desired, 15 g/l of agar is added into the medium before autoclaving.

3. Yeast extract peptone cassava medium (YPC)

Yeast extract	10 g/l
Peptone	20 g/l
Cassava starch	10g/l
pH	5.0

YPC media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

4. Yeast peptone dextrose medium (YPDS)

Yeast extract	10 g/l
Peptone	20 g/l
Dextrose	20 g/l
Soluble starch	20 g/l

Autoclave at 121^{o}C for 20 minutes, if the agar medium is desired, 15 g/l of agar is added into the medium before autoclaving

APPENDIX B

CALIBRATION CURVES

B.1 Standard curve of sugar concentration

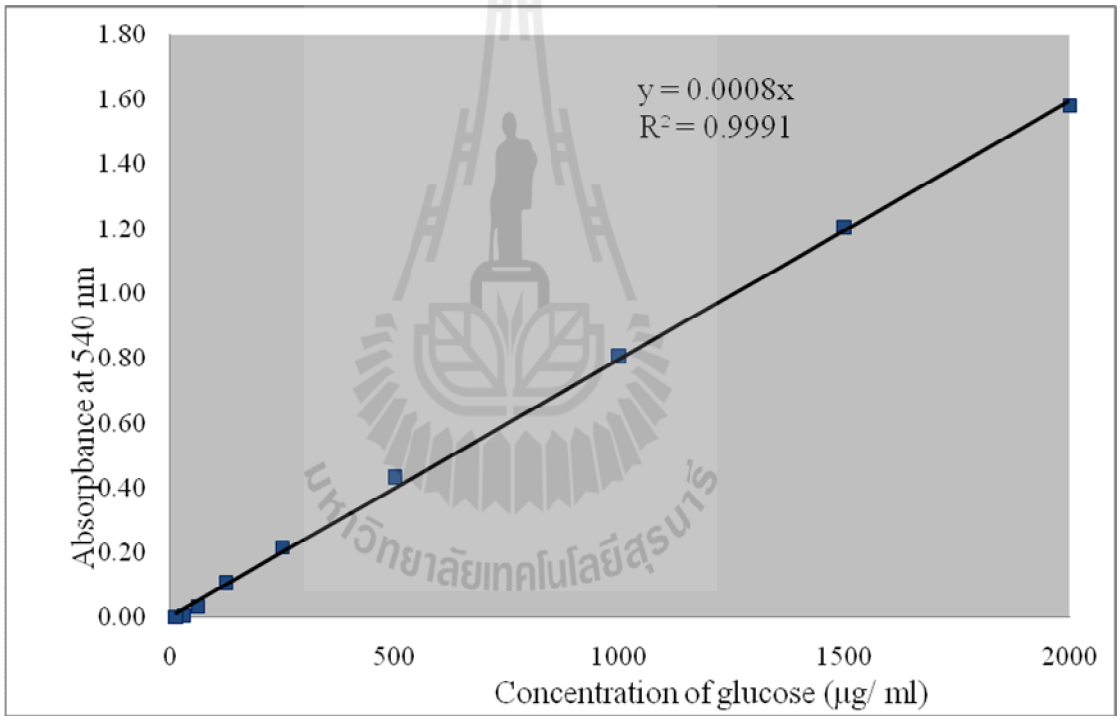


Figure B.1 Standard curve of sugar concentration by measurement density using a spectrophotometer at 540 nm by DNS method.

B.2 Standard curve of sugar concentration

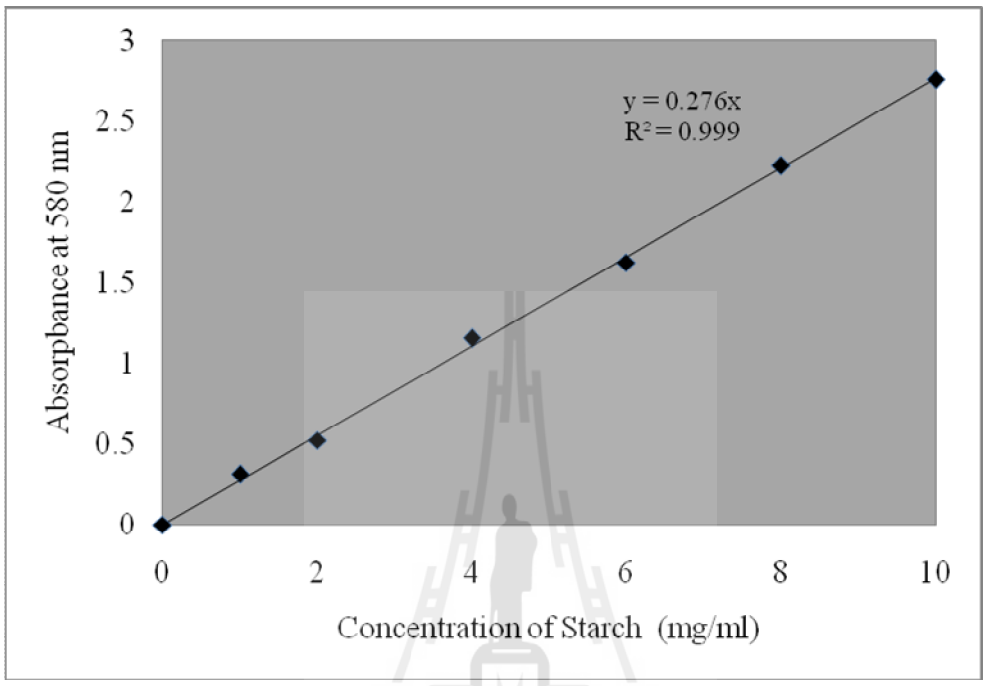


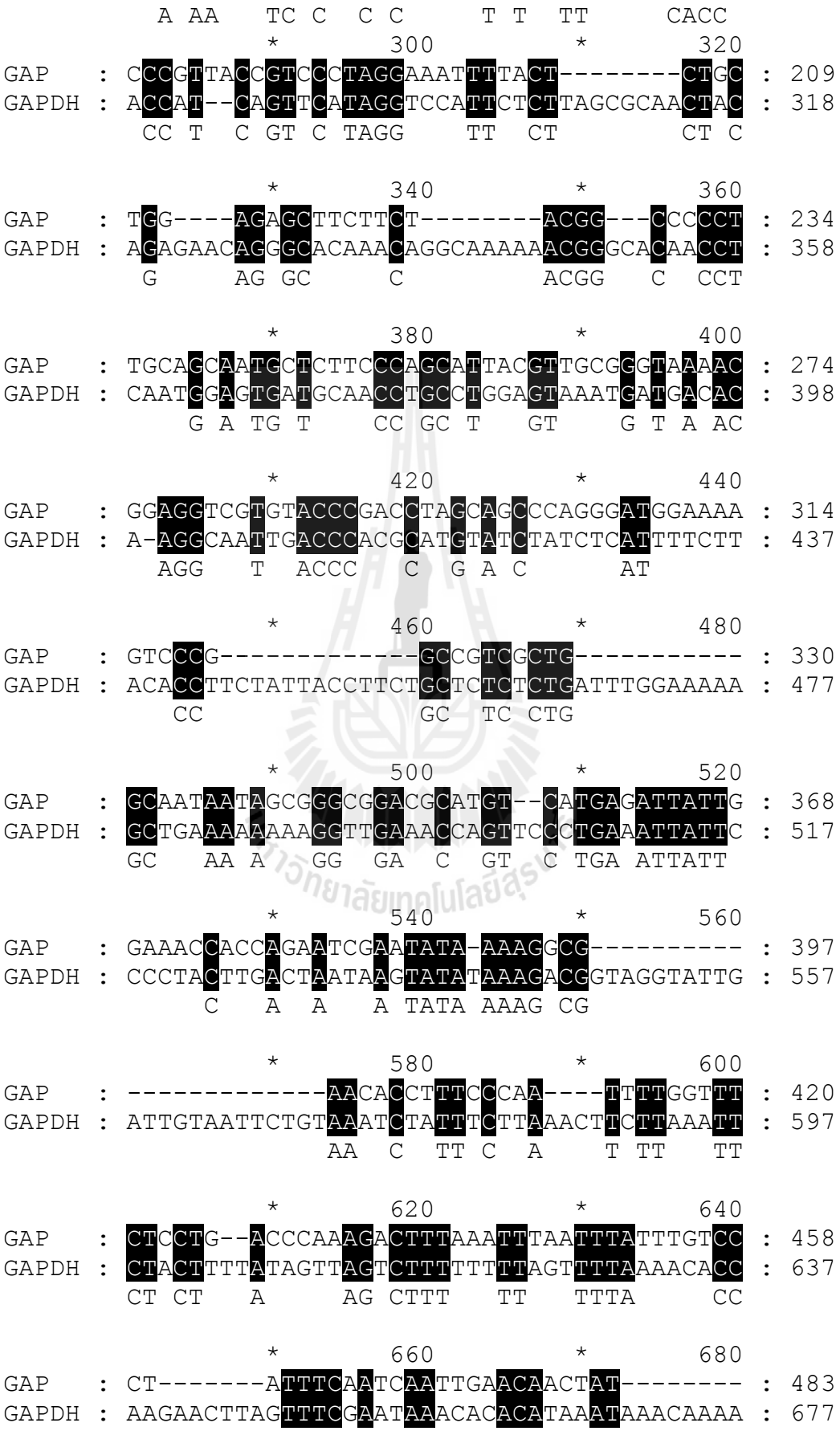
Figure B.2 Standard curve of starch concentration by measurement density using a spectrophotometer at 580 nm by iodine methode.

APPENDIX C

SEQUENCE ALIGNMENTS

C.1 The multiple sequence alignments of nucleotide sequence of GAP promoter from *P. pastoris* with GAPDH promoter from *S. cerevisiae*

```
                *         20         *         40
GAP   : ---------------------------------------- :   -
GAPDH : TCGAGTTTATCATTATCAATACTCGCCATTTCAAAGAATA :  40
                *         60         *         80
GAP   : ----AGATCTTTTTTGTAGAAATGT--CTTGGTGTCCTCG :  34
GAPDH : CGTAAATAATTAATAGTAGTGATTTTCCTAACTTTATTTA :  80
            A    TT  T GTAG  AT T  CT   T T  T

                *        100         *        120
GAP   : TCCAATCAGGTAGCCAT------CT-CTG----------- :  56
GAPDH : GTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACA : 120
          CAA  A  TAGCC T      CT CTG

                *        140         *        160
GAP   : --------------------------AAATATCTGGCTCC :  70
GAPDH : TGCCAAAATAGGGGGCGGGTTACACAGAATATATAACACT : 160
                                   AATAT T  C C

                *        180         *        200
GAP   : GTTGCAACTCCGAACGACCTG-------CTGGCAACGTAA : 103
GAPDH : GATGGTGCTTGGGTGAACAGGTTTATTCCTGGCATCCACT : 200
        G TG   CT  G    AC  G       CTGGCA C

                *        220         *        240
GAP   : AATTCTCCGGGGTAAAACTTAAATG--TGGAGTAATGGAA : 141
GAPDH : AAATATAATGGAGCCCGCTTTTTAAGCTGGCATCCAGAAA : 240
        AA T T   GG      CTT       TGG  T   G AA

                *        260         *        280
GAP   : CCAGAAACGTCTCTTCCCTTCTCTCTCCTT----CCACCG : 177
GAPDH : AAAAAAGAATCCCAGCACCAAAATATTGTTTCTTCACCA : 280
```


A AA TC C C C T T TT CACC
* 300 * 320
GAP : CCCGTTACCGTCCCTAGGAAATTTTACT--------CTGC : 209
GAPDH : ACCAT--CAGTTCATAGGTCCATTCTCTTAGCGCAACTAC : 318
CC T C GT C TAGG TT CT CT C
* 340 * 360
GAP : TGG----AGAGCTTCTTCT--------ACGG---CCCCCT : 234
GAPDH : AGAGAACAGGGCACAAACAGGCAAAAAACGGGCACAACCT : 358
G AG GC C ACGG C CCT
* 380 * 400
GAP : TGCAGCAATGCTCTTCCCAGCATTACGTTGCGGGTAAAAC : 274
GAPDH : CAATGGAGTGATGCAACCTGCCTGGAGTAAATGATGACAC : 398
G A TG T CC GC T GT G T A AC
* 420 * 440
GAP : GGAGGTCGTGTACCCGACCTAGCAGCCCAGGGATGGAAAA : 314
GAPDH : A-AGGCAATTGACCCACGCATGTATCTATCTCATTTTCTT : 437
AGG T ACCC C G A C AT
* 460 * 480
GAP : GTCCCG----------GCCGTCGCTG---------- : 330
GAPDH : ACACCTTCTATTACCTTCTGCTCTCTGATTTGGAAAAA : 477
CC GC TC CTG
* 500 * 520
GAP : GCAATAATAGCGGCGGACGCATGT--CATGAGATTATTG : 368
GAPDH : GCTGAAAAAAAAGGTTGAAACCAGTTCCCTGAAATTATTC : 517
GC AA A GG GA C GT C TGA ATTATT
* 540 * 560
GAP : GAAACCACCAGAATCGAATATA-AAAGGCG---------- : 397
GAPDH : CCCTACTTGACTAATAAGTATATAAAGACGGTAGGTATTG : 557
C A A A TATA AAAG CG
* 580 * 600
GAP : ----------AACACCTTTCCCAA----TTTTGGTTT : 420
GAPDH : ATTGTAATTCTGTAAATCTATTTCTTAAACTTCTTAAATT : 597
AA C TT C A T TT TT
* 620 * 640
GAP : CTCCTG--ACCCAAAGACTTTAAATTTAATTTATTTGTCC : 458
GAPDH : CTACTTTTATAGTTAGTCTTTTTTTAGTTTTAAAACACC : 637
CT CT A AG CTTT TT TTTA CC
* 660 * 680
GAP : CT-------ATTTCAATCAATTGAACAACTAT-------- : 483
GAPDH : AAGAACTTAGTTTCGAATAAACACACATAAATAAACAAAA : 677

```
                    TTTC A  AA    ACA   AT
GAP    : -- :   -
GAPDH : TG : 679
```

Figure C.1 The multiple sequence alignments of GAP promoter from PGAPZαA with the sequence of GAPDH promoter from *S. cerevisiae* reported in Genebank (A15895.1). The alignment was conducted by ClustalX 2.0.11 program.

APPENDIX D

LIST OF PRESENTATIONS

Oral presentation

Tongsumrit, R. and Krubphachaya, P. (2011). **Construction of recombinant plasmid for displaying α-amylase or glucoamylase genes from *Debaryomyces occidentalis* in yeast.** The 4th SUT Graduate Conference, 7-8 July 2011, P. 79, Nakhon Ratchasima, Thailand.

Proceeding

Tongsumrit, R. and Krubphachaya, P. (2012). **A direct ethanol fermentation using cassava starch-fermenting strain of recombinant *Saccharomyces cerevisiae* producing glucoamylase.** The 24th Annual Meeting of the Thai Society for Biotechnology (TSB 2012), 29-30 November 2012, p. 334-338, Ubon Ratchathani, Thailand.

The 24th Annual Meeting of the Thai Society for Biotechnology
"Renewable Energy and Global Care"

P-V-04

A direct ethanol fermentation using cassava starch-fermenting strain of recombinant *Saccharomyces cerevisiae* producing glucoamylase

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Abstract

Recently, ethanol has become an alternative energy source because of the continuous reduction of limited fossil fuel stock. Thailand is the world's third largest producer of cassava which is an efficient carbohydrate crop and cheap substrate for conversion to ethanol. Due to the absence of glucoamylase, an enzyme for hydrolysis of starch to glucose, the glucoamylase gene from *Debaryomyces occidentalis* was introduced into the *Saccharomyces cerevisiae* chromosome. The transformant yeast (TISTR 5596/pGAM1) acquired the ability to grow on medium containing cassava starch as a sole carbon source. This indicated that the transformant yeast secreted biologically active glucoamylase enzyme. The transformant yeast was able to ferment cassava starch to ethanol by itself without an addition of exogenous glucoamylase. In 15-day batch fermentation, the transformant yeast strains (TISTR 5596/pGAM1) produced 1.99 g/l of ethanol and decreased the cassava starch from 10 g/l to 4.3 g/l.

Keywords: glucoamylase, *recombinant Saccharomyces cerevisiae*, cassava starch, ethanol production, *Debaryomyces occidentalis*

Introduction and Objectives

Ethanol production and consumption have increased in the world, due to an increasing in global oil price and global warming awareness [2]. The major crops used for ethanol production mainly are sugarcane and starchy plants. Thailand is the world's third largest producer of cassava starch. It is considered one of the best raw materials which is an efficient carbohydrate crop and cheap substrate for conversion to ethanol [13]. In order to convert starch to glucose, large amount of amylolytic enzyme is required in the traditional starch fermentation process. In addition, the high cooking temperature must be used to break down raw starch before fermentation [5]. These processes pose high cost for energy and for amylolytic enzyme, especially in case of raw starch hydrolysis [4]. Glucoamylase (EC3.2.1.3, 1,4-α-D-glucanglucohydrolase) is an exotype enzyme that degrades starch and releases glucose, while α-amylase (EC 3.2.1.1, 1,4-α-D glucanglucanohydrolase) is an endo-type enzyme that cleaves α-1,4-glucosidic linkages of starch and liberates maltose and oligosaccharides. Both enzymes can degrade amylose and amylopectin and produce glucose [5]. *S. cerevisiae* is widely used in industry for producing bioethanol as fuel. Since wild type yeast is not able to directly utilize starch for growth and fermentation. Many researchers have reported on attempts to resolve this problem by using recombinant glucoamylase-expressing yeasts with the ability to ferment starch to ethanol directly [1, 3, 7, 12].

The objective of this study was to construct an integrative vector and develop a recombinant strain of *S. cerevisiae* that can express glucoamylase (GAM1) enzyme for conversion of cassava starch to ethanol. To achieve the aim of study, an integrative vector containing glucoamylase gene under the GAP promoter was constructed and transformed into the yeast genome. The transformant yeasts were analyzed for growth and utilization of cassava starch. Ethanol production from cassava starch was examined by using the transformant yeast in the batch fermentation.

Materials and Methods

1. Microorganism strains and plasmids

Escherichia coli TOP10 strain was used for transformation and plasmid construction. *S. cerevisiae* TISTR 5596 was used as a host for the yeast transformation experiment. *D. occidentalis* strain TISTR 5346 was used as source of the glucoamylase (*GAM1)* gene. Plasmid pGAPZαA (containing a GAP promoter, Invitrogen) was used for the construction of a recombinant plasmid containing the *GAM1* gene for expression in yeast.

2. Media and cultivation

E. coli transformants were grown in low salt Luria-Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride, pH 7.0] supplemented with 25 µg/ml of zeocin. Yeast cells were cultured in YPD medium [1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose].

Transformant yeasts were grown on YPD plates containing 130 µg/ml zeocin, pH 7.5 and then transferred onto YPDS2 plates [YPD containing 2% (w/v) soluble starch] for glucoamylase expression assay.

For ethanol fermentation, YPC medium [1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 1% (w/v) cassava starch] was used for batch fermentation studies.

3. DNA manipulation and yeast transformation

All DNA manipulation and *E. coli* transformation were performed according to the methods as described by Sambrook and Russell (2001) [14]. The yeast transformation was carried out using the electroporation method. This practical method followed the description by Suga and Hatakeyama (2003) [15].

4. Screening for glucoamylase activity

Recombinant yeast strains obtained after transformation with plasmids carrying glucoamylase gene were screened for glucoamylase expression after plating on YPDS2 plates and incubated at 30°C for 10 days. After 2 days of incubation at 4°C an iodine solution was poured on to YPDS2 plates. Expression of glucoamylase in recombinant yeasts was observed with a clear halo around colony [5, 8]. The integration of glucoamylase gene in transformant yeasts were confirmed by using PCR technique.

5. Ethanol fermentation and assay

The amylolytic strain was cultured in 120 ml anaerobic bottles containing 90 ml of the YPC medium. The cultures were incubated in a rotary shaker at 30°C and 200 rpm for 15 days. Ethanol was determined by using the high performance liquid chromatography (HPLC).

Biomass was determined based on Soni et al. (1987). In order to eliminate the cassava starch residual in fermented broth before biomass determination, one ml of fermented broth was hydrolyzed by adding the α-amylase enzyme and incubated at 90°C for 2 h. Sample was centrifuged at 20,000×g for 10 min at 4°C. The supernatant was discarded, and then the pellet was re-dissolved with water. The treated cells which were free from starchy substances were dried at 105°C for 4 h for determination of cell dry weight [16].

The presence of starch residual was determined using a modified method of Liao et al. (2012). One ml of each sample was taken from fermented broth and stored at -20°C. Starch in the sample was re-solubilized by thawing at 25°C and boiling for 30 min. After cooling to 25°C, 300 µl of each sample were mixed with 2 ml of iodine solution and diluted with water. The absorbance of the final solution was measured at 580 nm using a spectrophotometer (Spectro*SC*, Labmed, Inc.) [11]. Starch concentrations were determined from a standard curve representing the relationship between starch concentrations of standard starch solutions and their absorbance at 580 nm.

Results

1. Construction of glucoamylase gene expression plasmid

The open reading frame (ORF) together with native terminator of the *GAM1* gene encoding glucoamylase (~2.8 kb) was isolated from the genomic DNA of the *D. occidentalis* strain TISTR 5346 by PCR using primers GAM1_2F (5'CCCCTCGAGAAAAG AGCCCCTGCCTCTTCGATTGG-3') and GAM1_2R (5'CCCCTCGAGTTACCAAGTA

TGGTGAAATCTTAGAAAATG-3'). Recognition site of the restriction enzyme *Xho*I was incorporated into both primers GAM1_2F and GAM1_2R (the cleavage sites are underlined). The PCR products were digested with *Xho*I restriction endonuclease and inserted into the *Xho*I site of *Xho*I digested plasmid pGAPZαA under the strong constitutive promoter of glyceraldehyde-3 phosphate dehydrogenase gene (pGAP) to obtain pGAM1 plasmid (Figure 1). Digestion of the constructed pGAM1 plasmid with *Xho*I was performed and the insertion of the 2.8 kb *GAM1* gene into the structure was confirmed (data not shown). The pGAM1 plasmid was later used as a vector for transformation and expression of *GAM1* gene in *S. cerevisiae*.

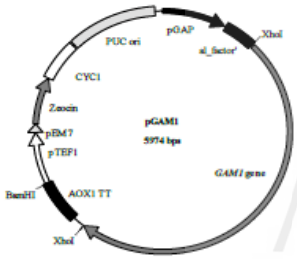

Figure 1. The physical map of the secretory glucoamylase (GAM1) expression vector pGAM1.

2. Screening for glucoamylase expression in transformant yeasts

The pGAM1 plasmid (Figure 1) was linearized with *BamH*I, and then transformed into *S. cerevisiae* TISTR 5596 by electroporation. After transformation, zeocine-resistant transformants were selected on the YPD medium supplemented with 130 µg/ml of zeocine. Seven transformants TISTR 5596/pGAM1 (TISTR 5596/pGAM1#1G, #2G, #3G, #4G, #5G, #6G and #7G) that were capable to grow on YPD medium plates supplemented with 130 µg/ml of zeocine were selected. The presence of *GAM1* gene in transformant yeast chromosome was confirmed by PCR using specific primers. The transformant yeast strains 1G, 2G, 3G, 4G, 5G, 6G and 7G showed the expected size of 2.8 kb *GAM1* gene. The PCR product was absent when genomic DNA of *S. cerevisiae* wild type strain was used as a template (Figure 2A). All of transformant yeasts were screened for glucoamylase expression on YPDS2 plates. Seven transformant yeasts showed the formation of clear halos around the colonies indicating the secretion of glucoamylase enzyme. *S. cerevisiae* TISTR 5596/pGAM1#1G which showed the largest clear halos was selected for further ethanol fermentation study (Figure 2B).

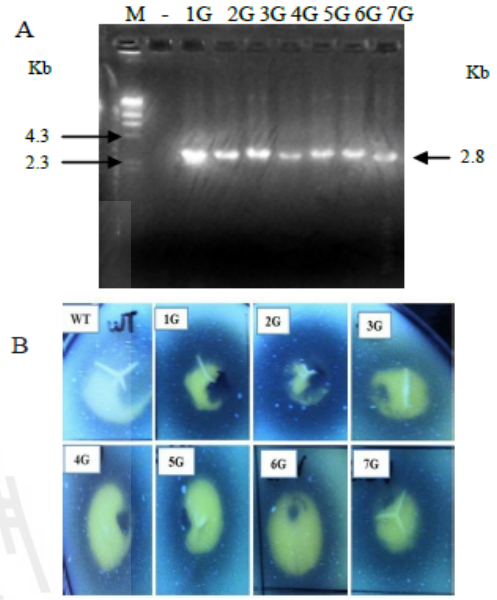

Figure 2. Panel A showed PCR amplification using GAM1 gene primers. Lanes: M, DNA marker; -, PCR product using genomic DNA of S. cerevisiae wild type strain as template; 1G to 7G PCR product using genomic DNA of transformant yeast strains 1G, 2G, 3G, 4G, 5G, 6G and 7G as template, respectively. Panel B showed screening for glucoamylase expression of transformant yeast strains 1G, 2G, 3G, 4G, 5G, 6G and 7G by iodine reaction.

3. Cassava starch utilization and ethanol production by the recombinant strain.

The ability of *S. cerevisiae* TISTR 5596 wild type strain and *S. cerevisiae* TISTR 5596/pGAM1 recombinant strain to produce ethanol directly via the fermentation in YPC medium containing 10 g/l of cassava starch as a sole carbon source were examined (Figure 3). The results of batch fermentation showed that *S. cerevisiae* TISTR 5596/pGAM1 was able to utilize cassava starch for ethanol production while, the *S. cerevisiae* TISTR 5596 wild type did not show detectable hydrolysis of starch for ethanol production. After 6 days of batch fermentation of *S. cerevisiae* TISTR 5596/pGAM1, 1.14 g/l ethanol was produced and the maximum ethanol was obtained after

15 days. In addition, the starch content decreased during fermentation as the ethanol concentration increased up to 1.99 g/l on day 15 (Figure 3). The increased biomass during the fermentation process could confirm that *S. cerevisiae* TISTR 5596/pGAM1 was able to use cassava starch for growth (Figure 4).

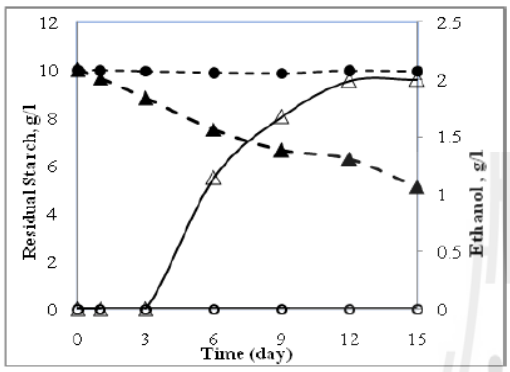

Figure 3. Time course of direct ethanol production via fermentation from cassava starch by TISTR 5596/pGAM1#1G (triangles) and TIRSR 5596 wild type (circular). Opened and closed symbols show ethanol and residual starch concentration, respectively.

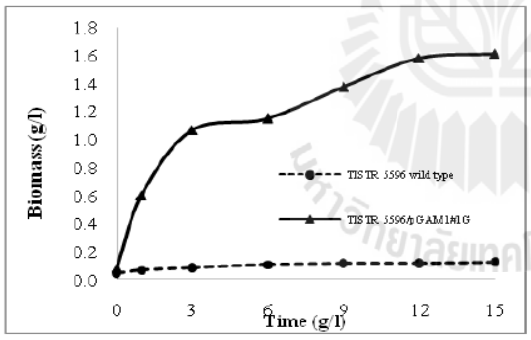

Figure 4. Biomass accumulation by the yeast strains expressing glucoamylase gene during growth in fermentation with 1% cassava starch as a sole carbon source.

Discussion

The *S. cerevisiae* TISTR 5596/pGAM1 was genetically engineered in order to express the secreted glucoamylase enzyme. The result showed that it was capable of hydrolyzing cassava starch under batch fermentation. It can produce ethanol up to 1.99 g/l within 15 days and reduced starch to 4.3 g/l from 10 g/l initial cassava starch. Yang et al. (2010) reported that *S. cerevisiae* expressing *Rhizopus* glucoamylase could utilize raw starch as energy source, and 39.25 g/l of ethanol and reduced starch to 40 g/l from 100 g/l initial raw starch was obtained [18]. These results indicated that, the *S. cerevisiae* TISTR 5596/pGAM1 was able to degrade starch to yield glucose for ethanol production. However, the glucoamylase expression in *S. cerevisiae* TISTR 5596 was not efficient, which may be caused by heterogeneous GAP promoter (glycerol dehydes-3-phosphate dehydrogenase promoter from *Pichai pastoris)*. These result suggested that using GAP promoter of *P. pastoris* may not be appropriate for the expression of gene in *S. cerevisiae* TISTR 5596. Many reports showed that expression using a native constitutive promoter improved the expression in *S. cerevisiae* [6, 9, 10].

In order to produce ethanol directly from starch, the recombinant *S. cerevisiae* requires the co-expression of glucoamylase and α-amylase for completely hydrolysis of starch during ethanol fermentation [17]. Therefore, the recombinant *S. cerevisiae* expressing α-amylase should be developed. In addition, the recombinant yeast expressing two genes of glucoamylase and α-amylase for synergistic hydrolysis of starch could be used. Kim et al. (2010) engineered a recombinant yeast strain that simultaneously expressed three amylolytic enzymes including glucoamylase from *Aspergilus awamori*, α-amylase, and glucoamylase with debranching activity from *D. occidentalis*. The recombinant yeast showed ethanol production of 46.3 g/l from 100 g/l of soluble starch in 7 days of fermentation [9].

In conclusion, the recombinant yeast that can convert starch to ethanol was successfully engineered. The transgene was expressed in host strain. This is the promising start for further ethanol producing yeast development.

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Construction of Recombinant Plasmid for Displaying α -Amylase or Glucoamylase Genes from Debaryomyces Occidentalis in Yeast

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Abstract

The conversion of starchy biomass to glucose, which is an important renewable biological resource for industrial and fuel ethanol production, involves liquefaction and saccharification usin α-amylase and glucoamylase enzyme. Traditionally, ethanol production has been conducted almost exclusively by Saccharomyces cerevisiae because of its high fermentation rate and ethanol tolerance. Unfortunately, S.cerevisiae cannot degrade starch naturally. Manipulation of S. cerevisiae to synthesize and secrete amylolytic enzymes would contribute to the direct conversion of starch-rich materials to ethanol. S. cerevisiae strains capable of secreting α-amylase and/or glucoamylase for ethanol production directly from starch would be genertrated. Thus, two shuttle vectors were constructed containing α-amylase (SWA2) or glucoamylase gene (GAM1) isolated from D. occidentalis. The SWA2 and GAM1 genes were isolated from D. occidentalis by using PCR technique. The PCR products were ligated with pGAPZαA at XhoI site to obtain the recombinant plasmids. The recombinant plasmids (pGAPZαA_SWA2 and pGAPZαA_GAM1) were transformed into E. coli. The transformant clones growing on LB plate containing zeocin were analyzed for the present of SWA2 or GAM1 gene. The correct clones were confirmed by restriction enzyme digestion and PCR technique. To examine the correct orientation of inserted gene by using PCR product, the recombinant clones were amplified using pGAPZαA_F and SWA2_R for SWA2 gene or pGAPZαA_F and GAM1_R for GAM1 gene. The expected size of PCR products were be 1.8 kb for SWA2 gene and 3.1 kb for GAM1 gene, respectively. The correct recombinant plasmids will be future transformed into chromosomal DNA of yeast to evaluate the capable of SWA2 gene and/or GAM1 gene expression in yeast.

Keywords: **Construction, α-Amylase, Glucoamylase, Ethanol Production, Debaryomyces Occidentalis**

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