QUALITY DEVELOPMENT OF

THAI TRADITIONAL RICE WINE

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การพัฒนาคุณภาพไวน์ข้าวพื้นบ้านไทย

นางสาวณัฐวรรณ เลิศภิญโญชัยถาวร

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

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ณัฐวรรณ เลิศภิญโญชัยถาวร : การพัฒนาคุณภาพไวน์ข้าวพื้นบ้านไทย
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งานวิจัยครั้งนี้มีวัตถุประสงค์ในการปรับปรุงคุณภาพไวน์ข้าวพื้นบ้านไทย หรือ สาโท โดยเติมข้าวเปลือก ข้าวจ้าว และข้าวมอล์ท ของพันธุ์ข้าวหอมมะลิ 105 เพิ่มลงไปก่อนทำการหมักแอลกอฮอล์ เปรียบเทียบกับการไม่เติมข้าวเพิ่ม ในกระบวนการทำมอล์ทข้าวหอมมะลิ 105 พบว่า การทำลายระยะพักตัวของข้าวโดยการบ่มที่อุณหภูมิ 50°ซ เป็นเวลา 5 วัน จากนั้นทำการแช่น้ำที่อุณหภูมิ 30 °ซ เป็นเวลา 48 ชั่วโมง ทำการเพาะที่อุณหภูมิ 30 °ซ เป็นเวลา 4 วัน ทำให้ได้ปริมาณน้ำตาลรีดิวซ์ (157.77 มิลลิกรัมต่อกรัมข้าว) และปริมาณโปรตีนทั้งหมด (63.8 มิลลิกรัมต่อกรัมข้าว) สูงที่สุด ในกระบวนการหมักแอลกอฮอล์ พบว่ามีจำนวนประชากรยีสต์ระหว่าง 3 ถึง 5 x 10^7 CFU/mL ในวันที่ 3 ของการหมักมีปริมาณน้ำตาลรีดิวซ์สูงที่สุดในทุกการทดลอง ยกเว้นการทดลองชุดควบคุม จากการวิเคราะห์ทางสถิติ พบว่าปริมาณความเข้มข้นของกรดอินทรีย์ในการเติมข้าวหอมมะลิมีเพิ่มสูงขึ้นและสามารถช่วยลดปริมาณกรด อะซิติกลงได้อย่างมีนัยสำคัญ การเติมข้าวเปลือกและข้าวจ้าวมีผลทำให้มีกรดซิตริกสูงกว่าข้าวมอล์ทและไวน์ข้าวที่ไม่ได้เติมอย่างมีนัยสำคัญ สำหรับปริมาณกรดมาลิก กรดแลกติก และกรด ซัคซินิก ค่าที่ได้ไม่มีความแตกต่างกันทางสถิติในทุกการทดลอง สำหรับการผลิตแอลกอฮอล์ พบว่าไวน์ข้าวที่มีการเติมข้าวมอล์ทนั้นมีปริมาณแอลกอฮอล์สูงที่สุด (17.20%) รองลงมาคือ ข้าวเปลือก (15.24%) ข้าวจ้าว (13.53%) และ ไม่ได้เติม (13.32%) ตามลำดับ ผลผลิตของเอทานอลต่อสารตั้งต้น (กรัมเอทานอลต่อกรัมข้าว) คือ 0.482 (ข้าวเปลือก) 0.427 (ข้าวจ้าว) 0.544 (ข้าวมอล์ท) และ 0.736 (ไม่ได้เติม) ตามลำดับ คุณภาพทางประสาทสัมผัสของผลิตภัณฑ์สุดท้ายถูกทดสอบโดยผู้ชิมที่มีความชำนาญ 12 คน พบว่า คุณลักษณะของไวน์ข้าว 2 อย่าง คือ ความใส และสี ไม่มีความแตกต่างกันทางสถิติ คุณลักษณะของกลิ่นและรสชาติ ไวน์ข้าวที่เติมข้าวเปลือกและข้าวจ้าวมีความแตกต่างกับไวน์ข้าวที่เติมข้าวมอล์ท ไวน์ข้าวที่ไม่ได้เติม และไวน์ข้าวทางการค้าอย่างมีนัยสำคัญ นอกจากนี้ คุณลักษณะของความหวานในไวน์ข้าวที่เติมข้าวเปลือก ข้าวจ้าว และข้าวมอล์ท มีความแตกต่างกับไวน์ข้าวที่ไม่ได้เติมและไวน์ข้าวทางการค้าอย่างมีนัยสำคัญ สุดท้าย คุณลักษณะโดยรวม พบว่า ผู้ชิมส่วนมากยอมรับไวน์ข้าวที่มี การเติมข้าวเปลือกมากที่สุด รองลงมาคือไวน์ข้าวที่มีการเติมข้าวจ้าว ลำดับที่สามคือไวน์ข้าวที่มีการเติมข้าวมอล์ท ส่วนไวน์ข้าวที่ไม่ได้เติมข้าวและไวน์ข้าวทางการค้าได้รับ

การยอมรับ จากผู้ชิมน้อยกว่าไวน์ข้าวที่มีการเติมข้าวเพิ่ม นอกจากนี้ การตรวจสารปนเปื้อนโลหะหนักและไซยาไนด์ในไวน์ข้าว พบว่า ไม่มีการปนเปื้อนจากสารดังกล่าว

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา _____
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NUTTAWAN LERTPINYOCHAITHAWORN : QUALITY DEVELOPMENT OF THAI TRADITIONAL RICE WINE. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D. 76 PP.

SATO/ALCOHOLIC FERMENTATION

This research was aimed at improving the quality of Thai traditional rice wine which is well known as "Sato". The improved process was carried out by adding paddy, polished seeds and malted KDML105 rice before fermentation. These samples were compared to samples to which nothing was added. For the malting process, the dormancy period of KDML105 was broken down for 5 days at 50°C. Then, the seeds were steeped in water at 30°C for 48h and then germinated at 30°C for 4 days. This method was found to obtain high level of reducing sugar (157.77 mg reducing sugar/g rice) and the total protein concentration was 63.8 mg protein/g rice. For the alcoholic fermentation process, the population of yeast was in the range of 3-5 x 10^7 CFU/mL. The highest amount of reducing sugar was found on the 3rd day in all conditions except under control. Statistical analyses of organic acids concentration were examined and it was found that the amount of acetic acid decreased significantly in all conditions except control. The addition of either polished or paddy rice allowed a higher amount of citric acid when compared with control and malted rice. However, the amounts of malic, lactic and succinic acids were not significantly different in all conditions. The ethanol concentration was highest (17.20%) in rice wine by adding malted rice, followed by paddy rice (15.24%), polished rice (13.53%) and control (13.32%), respectively. The yield of ethanol (g ethanol/g rice) was 0.482, 0.427, 0.544

and 0.736; in the paddy rice wine, polished rice wine, malted rice wine and control, respectively. The finished rice wines were evaluated by 12 experienced panelists. It was found that two characteristics of the wine, namely cloudy wine and color were not significantly different at 95% confidence. The smell and taste of the paddy rice wine and the polished rice wine placed them in the first group. Moreover, the sweetness of paddy rice wine, polished rice wine and malted rice wine was significantly higher than that of commercial products and that under control. In conclusion, most of the panelists agreed that wine from the paddy rice was the best, the wine from the polished rice was second and the wine from the malted rice was third. The panelists considered that the commercial wine and the rice wine used for control were of a lower standard. Finally, no heavy metals or cyanide were found in any of the products.

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Nuttawan Lertpinyochaithaworn

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

a.m.	=	ante meridiem
BSA	=	bovine serum albumin
°C	=	degree Celsius
DAP	=	di-ammonium phosphate
DNS	=	dinitrosalisylic acid
GC	=	gas chromatography
g	=	gram
g/L	=	gram per liter
h	=	hour
HCl	=	hydrochloric acid
HPLC	=	high performance liquid chromatography
KDML105	=	Khao Dok Mali 105 rice
L	=	liter
µl	=	microliter
µm	=	micrometer
mg	=	milligram
min	=	minute
mL	=	milliliter
mM	=	millimolar
M	=	molarity
MBTH	=	3-methyl-2-benzothiazolinone hydrazone

LIST OF ABBREVIATIONS (Continued)

nm	=	nanometer
nM	=	nanomolar
N	=	normality
OD	=	optical density
p.m.	=	post meridiem
PDA	=	potato dextrose agar
ppm	=	part per million
psi	=	pound per square inch
QDA	=	quantitative descriptive analysis
rpm	=	round per minute
RI	=	refractive index
SSF	=	solid state fermentation
U	=	unit
UV	=	ultra violet
YM	=	yeast extract-malt extract

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Rice (*Oryza sativa* L.) is one of the leading food crops of the world. There are many products made from rice such as Saké, Amarillo, Idli, etc. (Padhye and Salunkhe, 1979). In Asian countries, rice wine is a popular alcoholic beverage. There are many called that depends on area such as Saké is a well-known and popular traditional product in Japan (Iwata et al., 2003). In Thailand, rice can be modified to many products such as rice starch, pasta, fermented food and beverage, puffed rice snacks, noodles (Juliano, 2005), including Thai traditional rice wine or Sato. Sato is a traditional fermented Thai rice wine, which have been conducted for along time ago (Thitisararak et al., n.d.). The alcoholic beverage from other locals has different in raw materials, temperature, and many factors but they use same raw material as cereals (Haard et al., 1999). The Thai rice wine and distilled (spirits), locally produced in large amounts every year, are traditional alcoholic beverage made from rice starch, and popular in North-East and North regions of Thailand. Recently, the popularity of the traditional rice wine has enormously increased throughout the country, for artisinal and commercial production (Techakriengkrai and Surakarnkul, 2007). Many factors have effected to quality of product such as process, contamination, mold, yeast and bacterial strains. The process of produced rice wine production has two steps. Saccharification process is the first step using fungal for hydrolysis of rice starch to sugar. The reaction can be summarized as follow:

$$\text{Starch} \xrightarrow{\text{Amylolytic enzyme}} \text{Glucose}$$

Then, fermentation process is the next step to utilized product of saccharification to ethanol. Followed as this reaction:

$$\text{Glucose} \xrightarrow{\text{Yeast}} 2\ \text{Ethanol} + 2\ CO_2$$

The Thai traditional alcoholic beverage used Look-pang (starter) containing of mold, yeast strains and herbs. However, the fungi in Look-pang is not pure, there is contaminated with acetic acid bacterial strains and other microbes. On the other hand, Japanese rice wine (Saké) uses koji rice and pured yeast strains which are key factors for quality (Karuwanna, 2003). Thus, Thai traditional alcoholic beverage characteristics will be unique depend on area where can not control the quality of process and product.

1.2 Research objective

1. To study reducing sugar production and mycelial propagation of *Rhizopus oryzae* in saccharification process.
2. To determine the amount of organic acid in sacharification and fermentation processes.
3. To improve process and quality of rice wine.

1.3 Research hypothesis

The Thai traditional rice wine, which was used KDML 105 rice and its malt should be more pleasured for consumers than using conventional rice.

1.4 Scope and limitations of the study

Thai traditional rice wines have higher quality such as taste, smell and accept, which was added with paddy, polished, malted KDML 105 rice.

1.5 Expected result

Thai tradition rice wine product will be had good quality.

CHAPTER II

LITERATURE REVIEW

2.1 Definition of rice wine

Rice wine refers to alcoholic beverages made from rice. However, unlike true wine, which is made by fermenting grapes or other fruits, rice "wine" is more akin to beer, in that it is brewed from grain. Rice brew typically has higher alcohol content (18-25%) than grape wine (10-14%), which in turn has higher alcohol content than beer (4-8%) (Wikimedia Foundation, Inc., www, 2007).

Rice wine *Saké* is of special interest because of its relatively high alcohol content, which is produced by a method different from that used for the manufacture of beer and spirits from cereals such as barley, wheat and maize. The forerunner of the present system of *Saké* brewing is believed to have originated in China, although the methods of manufacture in the two countries now differ, particularly in respect of the microbial flora. The essential microorganisms in the brewing process are selected molds for saccharification in the genera *Rhizopus*, *Mucor*, *Penicillium*, *Aspergillus*, *Absidia* and *Monascus* along with yeasts with multiply spontaneously on steamed cereals to produce a complex of microorganisms which provides the necessary enzyme systems for concurrent saccharification and fermentation (Kodama, 1993).

2.2 Raw materials

2.2.1. Rice

Rice belongs to the genus *Oryza*, of the tribe Oryzeae, of the subfamily Bambusoideae or Ehrhartoideae, of the family Poaceae or Gramineae. The eighty five percentage of the rice that is produced in the world is used for direct human consumption. Rice can also be found in cereals, snack foods, brewed beverages, flour, oil, syrup and religious ceremonies to name a few. Just as rice can be grown in many different environments, it has many characteristics, making one variety more popular in one region of the world than another. Rice can be a short, medium or long grain size. It can also be waxy (sticky) or non-waxy. Some rice varieties are considered aromatic. Rice also comes in many different colors including brown, red, purple and black (Juliano, 1985).

The rice caryopsis: structure and chemistry.

The rice caryopsis resembles other cereals as shown in Figure 2.1. The husk consists of the lemma and palea, two specialized leaves that respectively cover the back and front of the seed. These are loosely joined by an interlocking fold on each side and are therefore easily separated. In dry paddy there is an air space between the husk and kernel. The underlying perocarp is a tough skin that, when intact, is a protection against mold attack and oxidative and enzymatic deterioration. Beneath the pericarp is the tegmen, several cells thick, which is also part of the seed coat. These cells are rich in lipids and proteins. The nearest layer to the endosperm is the aleurone layer: rich in protein, lipids, vitamins and phytic acid. Finally, there is the endosperm, which is human and animal foods rich in starch with some protein, although less than most other cereals.

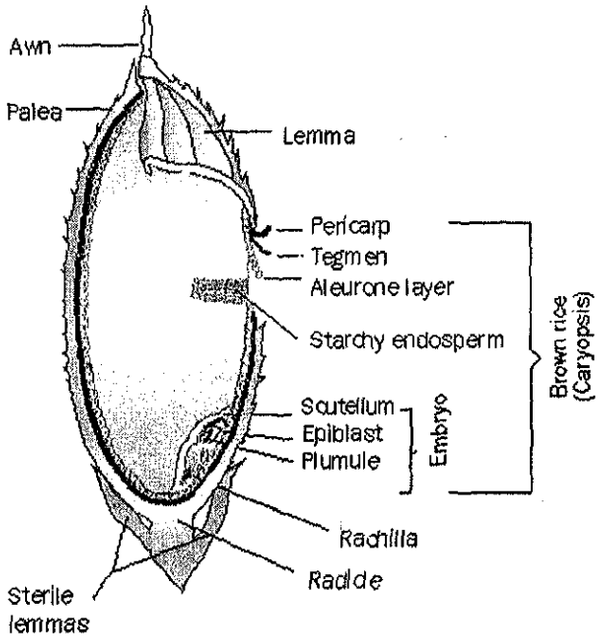

Figure 2.1 The inner part structure of Paddy rice

Source: Dendy and Dobraszczyk (2001)

As the rice grain develops, it passes, as do all cereals, through a "milky" stage when starch granules are suspended in a liquid phase. As the grain ripens, the starchy cells set into a radial pattern with hexagonal cells at the central core and elongated cells radiating outward. It is the radial walls of these cells that form potential cleavage planes when the grain is stressed, it will break along these. The protein content increases from the center outward. The overall shape of the grain is familiar: the short round *japonicas* are almost spherical; the long grains of *indicas* are cylindrical, tapering at each end. Attached in an indent is the germ or embryo. All cereals are rich in lipids, protein and vitamins of the B group. The aleurone layer, the seed coat, and pericarp cover the germ, with the husk forming the final outer layer. The removal of husk and of the combined germ and pericarp layers is important to have a white, palatable rice grain that can be stored for long periods.

Rice is unique among cereals in that it is mostly eaten as a whole grain, and the object of the milling process is to keep the grain whole but free of the bran and husk (Dendy and Dobraszczyk, 2001).

Nutrients in rice

The composition of rice differs with the variety, the nature of the soil, environmental conditions and the fertilizers applied (Juliano, 1985). The compositions of both husked and polished rice are stated in Table 2.1.

Table 2.1 The composition of rice – husked and polished (percentage, on moisture-free basis)

Nutritive	Husked rice	Polished rice
Fat	1.5-2.3	0.3-0.5
Crude fiber	7.2-10.4	0.2-0.5
Ash	2.9-5.2	0.3-0.8
Protein	5.8-7.7	6.3-7.1
Carbohydrates	63.6-73.2	76.7-78.4

Source: Juliano (1985)

Starch: storages and polysaccharides

Starch is the major component in all cereals. It is the energy source as such in food and feed applications and in the hydrolyzed form for microbes in fermentation process (Home et al., 2001). There are consists of two components, a linear glucose polymer, amylose, which contains α-1,4 linked D-glucopyranose and a branched polymer, amylopectin in which linear chains of α-1,4 glucose residues are interlinked by α-1,6

linkages (Muralikrishna and Nirmala, 2005; Hamilton et al., 2000) The chemical structures of amylose and amylopectin are depicted in Figures 2.2 and 2.3.

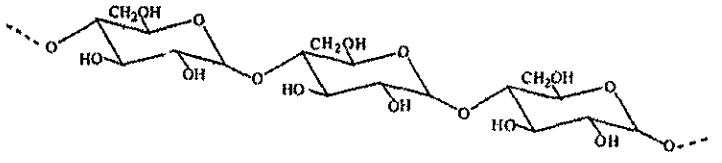

Figure 2.2 The chemical structure of amylase

Source: Muralikrishma and Nirmala (2005)

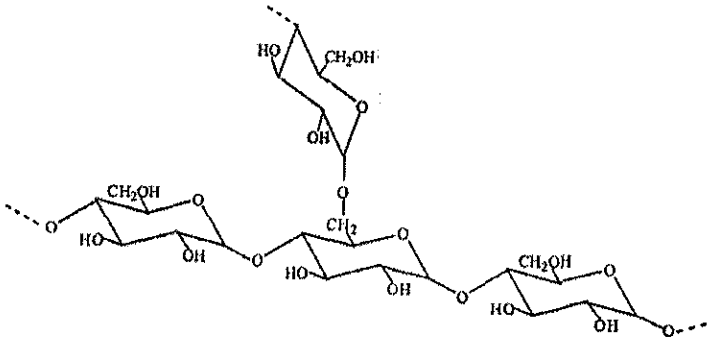

Figure 2.3 The chemical structure of amylopectin

Source: Muralikrishma and Nirmala (2005)

2.2.2 Water

In general, the water should be neutral or slightly alkaline, colourless, clear, odourless and tasteless, free form harmful microorganisms and unsuitable minerals (Kodama, 1993).

2.3 Rice wine process

Rice wine making begins with the introduction of koji, which break down rice starch into glucose in a process known as "saccharification". Then, yeast is added and

fermentation will be begun. At first stage, rice wine can be characterized by solid state fermentation followed by a liquid fermentation. The objective of the first stage is to produce a high concentration of polymer-degrading fungal enzyme. During the second stage, these enzymes degrade polymers such as starch (for beer or wine making) and protein (soy sauces and pastes). Examples are rice wines, soy sauces and pastes, and vinegar (Nout and Aidoo, 2002).

2.3.1 Saccharification and Fermentation

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the between the particles contain a continuous gas phase and minimum of visible water. Although water droplets may be present between the particles, and there may be thin films of the water at the particle surface, the inter-particle water phase is discontinuous and most of the water in the system is absorbed within the moist solid-particles. The majority of microorganism in solid-state fermentation processes is aerobic organism, such as filamentous fungi, although some involve bacteria and some involve yeast. The substrates used in solid-state fermentation processes are often products or byproducts of agriculture, forestry or food processing. Typically the source of nutrients comes from within the particle, although there are some cases in which nutrients are supplied from an external source (David et al., 2006).

SSF holds tremendous potential for the production of enzyme and used directly as the enzyme source that can be applied food and fermentation industries.

Table 2.2 list some of the possible applications of the enzymes produced in SSF system

Process	Enzyme
Enzyme-assisted ensiling	Fungal cellulases and hemicellulases
Bioprocessing of crops and crop residues	Fungal cellulases and hemicellulases
Fibre processing	Fungal pectinases, cellulases, hemicellulases
Food supplement	Amylases, proteases, lipases, cellulases, hemicellulases
Biopulping	Xylanases
Directed composting	Hydrolytic enzymes
Soil bioremediation	Laccases, ligninnases
Post-harvest residue decomposition	*Trichoderma harzianum* cellulases
Biopesticide	*T. harzianum* cellulose for helper function

Source: Pandey et al. (1999)

In this process, when maltodextrins are saccharified by further hydrolysis using glucoamylase or fungal alpha-amylase. A high yield of 95-97% glucose can be produced from most starch raw materials such as maize, corn, wheat, potatoes, barley and rice.

One of the major advantages of SSF is that usually it is carried out using naturally-occurring agricultural by-products, like straw and bran, etc (Pandey et al., 1995). There are many products which used solid-state fermentation such as cheese, miso, tempe or koji (Raimbault and Alazard, 1980).

For rice wine making also used saccharification under aerobic condition results from molds producing α-amylase and amyloglucosidase that degrade rice starch into dextrin and maltose, but mainly into glucose (Dung et al., 2006). The main role of this

process is to provide the various enzymes, such as α-amylase, glucoamylase, and protease, which hydrolyze the rice components to produce various nutrients to supply to the yeast (Fujita et al., 2003). Filamentous fungi are widely used for traditional foods, such as miso and cheese, and beverages, such as Shochu and Saké industries. An important characteristic of filamentous fungus that is used in these industries is a high efficiency of various kinds of enzyme production. For example, *Rhizopus, Mucor, Amylomyces*, especially *Aspergillus* species have high activity to produce hydrolysis enzymes and have a long history of use in food and beverages industries (Iwashita, 2002).

2.3.2 Microorganisms

Due to lower cost, microbial enzymes have largely taken the place of raw material to conversion starch into oligosaccharide or maltodextrin (Rose and Harrison, 1993).

The number of fungal species encountered in fermented foods is relatively limited, but they belong to various orders. Table 2.3 shows some major examples and products in which they are predominant (Nout and Aidoo, 2002).

Table 2.3 Major functional fungal species in Asian fermented foods

Zygomycetes	
Actinomucor	*A. elegans, A. taiwanensis* (sufu, tou-fu-fu)
Amylomyces	*A. rouxii* (ragi)
Mucor	*M. circinelloides, M. rouxii, M. indicus* (ragi, murcha, tempe, pehtze)
Rhizopus	*R. microsporus* (tempe), *R. oligosporus* (tempe), *R. oryzae* (koji, nuruk, chu, murcha, tempe)

Table 2.3 (continue)

Ascomycetes	
Monascus	*M. purpureus*, *M. ruber* (angkak)
Neurospora	*N. sitophila*, *N. intermedia* (oncom)
Deuteromycetes	
Aspergillus	*A. oryzae* (koji), *A. sojae* (koji), *A. glaucus*, *A. melleus*, *A. repens*, *A. candidus* (katsuobushi), *A. niger* (koji)
Penicillium	*P. glaucum* (katsuobushi)
Yeasts	
Brettanomyces	*B. anomalus* (kumiss)
Candida	*C. javanica* (idli, kombucha, murcha)
Endomyces	*E. fibuliger* (murcha, ragi)
Hansenula	*H. anomala* (Saké, koji)
Hyphopichia	*H. burtonii* (ragi)
Saccharomyces	*S. cerevisiae* (nan, toddy, murcha, kombucha), *S. dairensis* (tempe), *S. globosus* (kumiss), *S. kluyveri* (nan), *S. sake* (Saké)
Torulopsis	*T. versatilis* (idli, kombucha, soy sauces and pastes)
Trichosporon	*T. pullulans* (idli), *T. beigelii* (tempe)
Zygosaccharomyces	*Z. rouxii*, *Z. sojae* (soy sauces, soya pastes)

In saccharification process, there are many reports shown that *Rhizopus* spp., *Mucor* spp. and *Aspergillus* spp. were isolated from Asian mould starter (Merican and Yeoh, 1989; Steinkraus, 1996; Dung et al., 2007). In Thailand, filamentous fungi are isolated from starter (Loog-pang) such as *Absidia*, *Amylomyces*, *Aspergillus*, *Chlamydomucor*, *Rhizopus*, and *Mucor* (Wanapu et al., 2002). Peixoco et al. (2003)

For the distillery industry, *Rhizopus* spp. (mainly *R. oryzae*) has been used for industrial production of enzyme starter according to Lotong, (1998). Lim et al., (1987) reported that *Rhizopus* spp. were good producers of amylase. Amylase enzyme is used in starch hydrolysis for sugar syrups and brewing. The major end products from the action of α-amylase on starch are glucose, maltose, maltotriose, maltotetraosse, maltopentaose and maltohexaose. Moreover, they can produce glucoamylase, which used in starch processing industries is due to their good thermostability and high activity (Nigam and Singh 1994; Norouzian et al., 2006).

Then, alcoholic fermentation process is reaction-using glucose to produce ethanol. The main yeasts which ferment saccharified rice starch to alcohol are *Pichia burtonii*, *Saccharomycopsis. fibuligera*, *S. cerevisiae*, *Candida glabrata* and *C. lactosa*, while *Sm. fibuligera* produces amylolytic enzyme as well (Tsuyoshi et al., 2005). For rice wine are produced from the hydrolytic breakdown products of cereal starches and other polysaccharides. They range from simple Thai rice wine to highly sophisticated Japanese Saké. The yeasts involved in alcohol production of Asian rice wine include *S. cerevisiae*, *Sm. burtonii*, *Sm. fibuligera* and related yeasts. Rice and/or cereal wines are produced on both a cottage and a commercial scale in most Asian countries, especially Japan, China, Korea, Thailand, the Philippines and Vietnam (Nout and Aidoo 2002). Some uses of yeasts in the food, beverage and fermentation industries (Stewart et al., 1987) are shown in Table 2.5.

Table 2.5 Products of yeast in food and beverages, industrial chemicals, industrial microorganisms (Stewart et al., 1987)

Organism	Type	Product
S. cerevisiae	Foods and Beverages	Baker's yeast, wine, ale, Saké
S. carlsbergensis		Lager beer
S. rouxii		Soy sauce
C. milleri		Sour French bread
S. cerevisiae	Industrail Chemicals	Ethanol (from glucose)
Kluyveromyces fragilis		Ethanol (from lactose)
C. utilis	Single-Cell Proteins	Microbial protein from paper-pulp waste
Sm. lipolytica		Microbial protein from petroleum alkanes
Eramothecium ashbyi	Vitamins	Riboflavins
S. cerevisiae	Enzymes	Invertase
K. fragilis		Lactase
Sm. lipolytica		Lipase
Phaffia rhodozyma	Carotenoids	Astaxanthin

Fermentation is a process of energy production in a cell in an anaerobic environment (with no oxygen present) (Wikimedia Foundation, Inc., www, 2007). Yeast is main microorganism such as *Saccharomyces* spp. *S. cerevisiae* is high activity to produce ethanol. Sugars are the common substrate of fermentation, and typical examples of fermentation products are ethanol, lactic acid, and hydrogen. However, more exotic

compounds can be produced by fermentation, such as butyric acid and acetone. Yeast famously carries out fermentation in the production of ethanol in beers, wines and other alcoholic drinks, along with the production of large quantities of carbon dioxide, under anaerobic conditions, to ethanol via glycosis (Mogg, 2004).

2.4 Step of Rice Wine making

Rice, *Oryza sativa*, is polished to remove protein, lipids and minerals which are in excess in the bran and germ, and then washed, steeped in water and steamed, then cooled. A starter was then made by culturing *R. oryzae* on rice at 28-30°C for 5-6 days. The steamed rice was mixed with fungus starter and incubated for 3-4 days until saccharification process completed, then yeast starter and water, were added to form the main mash. The overall of step is shown in Figure 2.4.

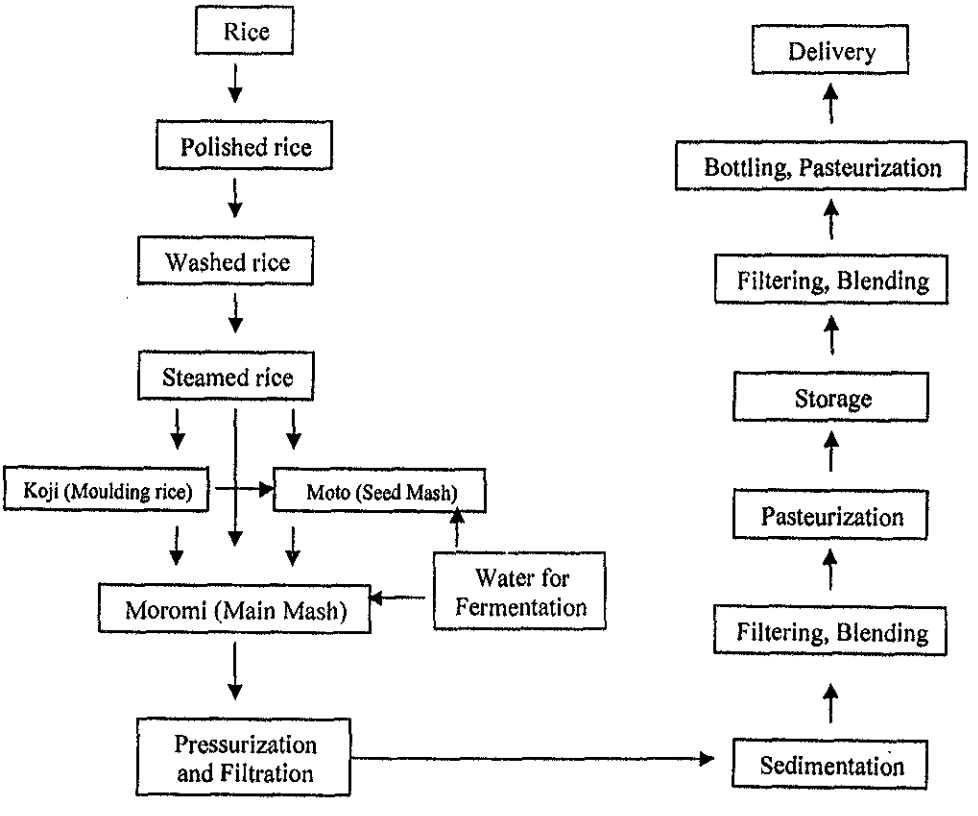

Figure 2.4 Flow diagram of rice wine process (Rose and Harrison, 1993; Nout and Aidoo, 2002).

2.5 Malting process

Malting is a process applied to cereal grains, in which the grains are made to germinate and then are quickly dried before the plant develops. Malting consists in the germination and drying of cereal seeds, the prime objective being to promote the development of hydrolytic enzymes that are not active in raw seeds. The main enzymes produced during germination that intervene in the hydrolysis of starch are α- amylase and β-amylase. The α-amylases are liquefying enzymes. The traditional malting of cereals consists of several stages: steeping of seeds (in water), germination, maturation (during which the seeds are piled and protect from light), and finally sun drying. In addition to the use of malted cereal flours in the manufacture of beer, a further use is their incorporation in infant flours to increase the energy density of the gruels, which is facilitated by the action of α-amylase (Traoré et al., 2004). On steeping step, water is an important factor that controls the physical phenomena and chemical reactions in biological materials during processing. Water is needed to start and maintain the germination of seeds in order to produce hydrolytic enzymes and the subsequent hydrolysis of biopolymers leading to endosperm modification (Holmberg et al., 2002). On germination step, embryo produces gibberellins. For malting this is an event of central importance, because these plant hormones stimulate the aleurone layer to produce and release enzymes of modification including α-amylase; in a certain sense this is the only desirable function of the embryo. Figure 2.5 shows modification proceeds generally from proximal to distal end and from periphery to center of the endosperm. 1, Entrance of water (in general flow is proximal to distal); 2, Formation and release of gibberellins, stimulating the aleurone; 3, Progressive stimulation of the aleurone to form and release enzymes of modification; 4, β-glucan solubilase and β-glucanases dissolve cell-wall β-glucans; 5, Proteases and peptidases partly break down proteins; 6, α-amylases attack small starch granules; 6a, Latent β-amylase

becomes free β-amylase; 7, Embryo nutrition; 8, Malting loss from metabolism of CWE (cold water extract) to CO_2 (respiration) and formation of acrospire (insoluble) and rootlets (removed); 9, CWE available in malt as an index of modification contributes to malt color; 10, Physical modification: (a) increase in friability; (b) lowering of wort and beer viscosity; 11, Index of modification as Kolbach index (soluble nitrogen ratio, SNR or total soluble nitrogen/total nitrogen). This is important in beer foam/ haze, beer flavor and amino acids for yeast nutrition; 12, Formation of α-amylase (dextrinizing units, DU) effective in mashing; release of β-amylase (diastatic power, DP) for wort fermentability. These contribute to CWE in the major part (Lewis and Young, 1995).

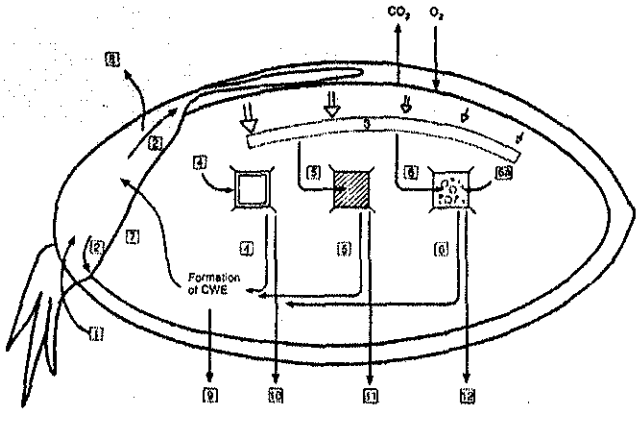

Figure 2.5 Summary of events occurring during grain germination in barley (the number are represent details in text)

Source: Lewis and Young (1995).

In germinating rice, starch is degraded by the combined actions of enzymes, such as α-amylase, β-amylase, and α-glucosidase, during cooking and processing as well as germination. α-amylase has a major role in degrading native starch granules. Several isoforms of α-amylases exist in rice. They are expressed depending on the tissue types and developmental stages. Several heat unstable isoforms of β-amylase also exist in rice seeds, some which are an inactived form in ungerminated rice grain. Although amylase activities

are highly induced during germination, the activity begins during seed maturation and continues in dry seed to affect the quality of storage rice (Awazuhara et al., 2000).

2.6 Ethanol production

Most alcoholic beverage fermentations are carried out using strains of the yeast *S. cerevisiae* (Berry, 1995). Yeast can degrade sugars using two metabolic pathways: alcoholic fermentation and respiration. These two processes begin in the same way, sharing the common trunk of glycolysis (Ribereau-Gayon et al., Vol I, 2000). During growth in anaerobic conditions such as those occurring in alcoholic beverage fermentations, all the ATP required for the growth is generated by the process of glycolysis (Figure 2.6)

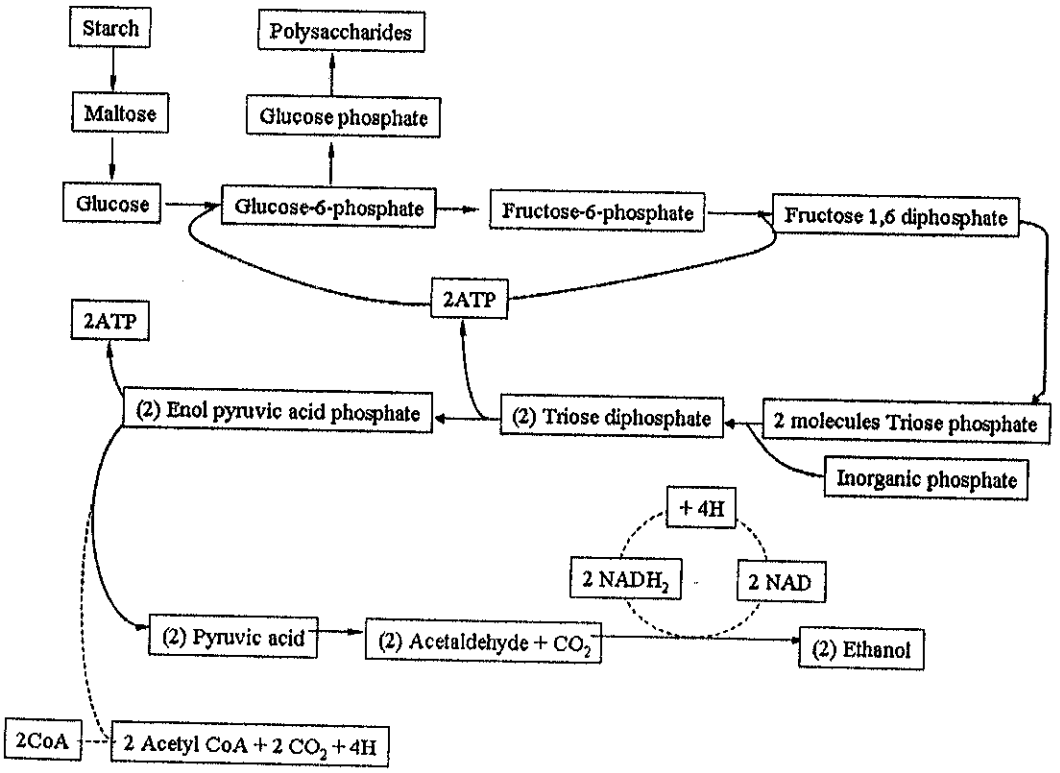

Figure 2.6 Pathway for alcoholic fermentation by yeast (Berry, 1995)

In the early stages of alcoholic fermentations the rate of alcohol production increases exponentially in parallel with the increasing biomass. Once yeast growth ceases, however, the rate of production of ethanol proceeds linearly until the available carbon sources have been consumed.

Ethanol is the resulting product of yeast fermentation of natural carbohydrates. Besides ethanol, a number of other monoalcohols and polyalcohols are presented in wines, as listed in Table 2.6

Table 2.6 The most important alcohols present in wines other than ethanol (Ough and Amerine, 1988)

Monoalcohols	Polyalcohols
Methanol	Glycerol
1-Propanol	2,3-Butanediol (*levo*)
1-Butanol	2,3-Butanediol (*meso*)
2-Methyl-1-propanol (isobutyl alcohol)	1,2,3,4,5,6-Hexanehexol (*levo*)
2-Methyl-1-butanol (*levo*) (active amyl	(D-sorbitol)
alcohol)	1,2,3,4,5,6-Hexanehexol (*levo*)
3-Methyl-1-butanol (isoamyl alcohol)	(D-mannitol)
1-Hexanol	1,2,3,4,5,6-Cyclohexanehexol (*meso*)
2-Phenyl ethanol (*β*-phenethyl alcohol)	(mesoinositol)

Furthermore, the main acid produced during alcoholic fermentation are pyruvic acid, L(+)-lactic acid, D(-)-lactic acid, succinic acid, acetic acid, citramalic acid, oxaloacetic acid, fumaric acid and citric acid. These acids are produced by all living organisms and are involved in the lipid metabolism and the Krebs cycle (Figure 2.7). Especially, acetic acid is the principal volatile acid of wine. It is produced in particular

during bacterial spoilage but is always formed by yeasts during fermentation at the beginning of alcoholic fermentation (Figure 2.8). Thoukis et al. (1965) reported that the amount of acetic acid formed does not exceed 0.03 to 0.04 g per 100 ml in bacteria-free fermentations. By far the largest increase in acidity during fermentation is brought about by the formation of nonvolatile organic acids, and it generally varies between 0.1 to 0.4 g per 100 ml but averages about 0.2 g per 100 ml.

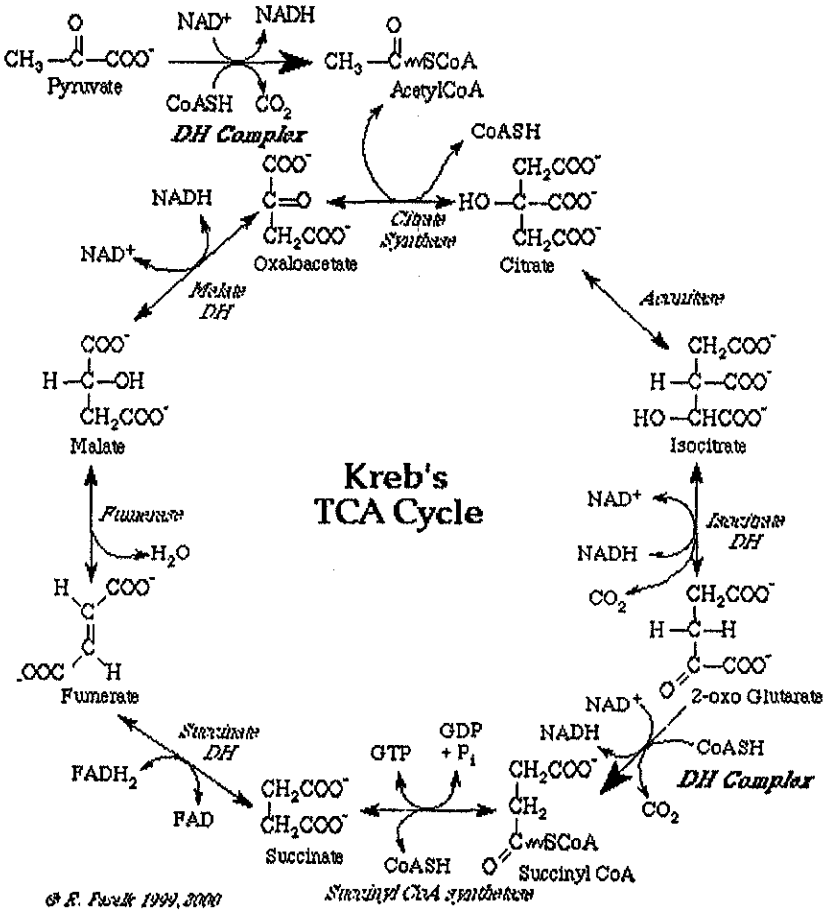

Figure 2.7 Tricarboxylic acid or Krebs cycle

Source: Humboldt University, www, (1999)

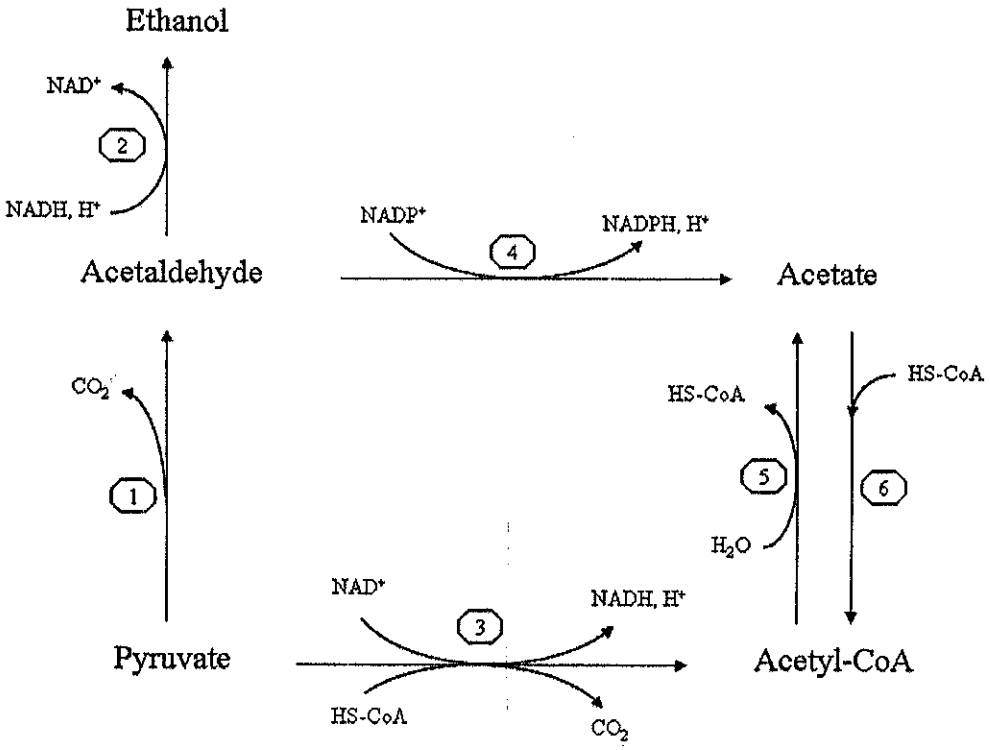

Figure 2.8 Acetic acid formation pathways in yeasts. 1 = pyruvate decarboxylase; 2 = alcohol dehydrogenase; 3 = pyruvate dehydrogenase; 4 = aldehyde dehydrogenase; 5 = acetyl Co-A hydrolase; 6 = acetyl Co-A synthase

Source: Ribereau-Gayon et al. (2000)

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Standard chemical of organic acid (acetic acid, citric acid, lactic acid, malic acid, succinic acid, glucose, fructose, sucrose, maltose and ethanol) were used chromatographic grade for chemical analysis by HPLC. All chemicals used for chemical reaction were of analytical grade which purchased from Carlo Erba Reanenti, Fluka Chemica, Sigma Aldrich, Difco and Across.

3.1.2 Equiqments

Equipment used was as follows: High Performance Liquid Chromatography: HPLC, Atomic Absorption Spectrophotometer, 797 VA computrace, incubator, hot air oven, suction pump, analytical balance, hot plate, Petri dish and box, refrigerator (4°C), freezer (-20°C), laminar flow hood, pH meter, water bath, analytical balance, Compound microscope, Sonicator, filter, Vortex mixer and basic microbiological equipment. All equipments are located at the Center for Scientific and Technological Equipment, Suranaree University of Technology.

3.1.3 Microorganisms

Microorganism for saccharification process to produce rice wine was used *Rhizopus oryzae* strain BM8 and for fermentation *Saccharomyces cerevisiae* strain L3109

was used. Both microorganisms were obtained from Biotechnology laboratory, Suranaree University of Technology.

3.1.4 Raw materials

Khao Dok Mali 105 (KDML105) paddy rice was obtained from Organic farming at Suranaree University of Technology, Nakhonratchasima. KDML 105 polished rice was purchased from Phimai Rice Research Station, Nakhonratchasima. Malted KDML 105 rice was made from SUT Biotechnology Laboratory, Nakhonratchasima. Koji rice was made from steamed sticky rice which was inoculated on 10^7 spores/gram of steamed rice with *R. oryzae* (BM8 strain) (Bramorski et al., 1998).

3.2 Methods

3.2.1 Microbial Cultivation

R. oryzae BM8 strain was used in saccharification process. *R. oryzae* was maintained on Potato Dextrose Agar (PDA) which containing of 39 g of PDA in 1000 ml distilled water. *R. oryzae* was cultured for 7 days which was suspended in distilled water and then spores were counted on haemacytometer by compound microscope.

S. cerevisiae strain L3109 was cultured on yeast malt extract (YM) broth at 30°C for overnight in aerobic condition. The yeast population was determined by counting of viable cell on haemacytometer and calculated the cell by used equation (Fugelsang 1997):

$$\text{Cells/ml} = \text{cell counted} \times 10^4 \times \text{dilution}$$

3.2.1.1 Optimization of yeast growth on temperature

The medium broth was YM broth which containing of 10 g of glucose, 5 g of peptone, 3 g of malt extract and 3 g of yeast extract in 1 liter water. The

medium was used to determine for yeast growth that was inoculated with 10^6 cells/ml and incubated at different temperature between 15 to 35°C. Samples were collected every 2 hours. Growth was followed by monitoring the optical density, using spectrophotometer at 660 nm (Abbott et al., 2005).

3.2.1.2 Optimization on pH

The medium broth was followed from section 3.2.1.1. The medium was adjusted pH with 1N HCl in range of pH between 2.5 to 5.0. Inoculation was 10^6 cells /ml and incubated at 30°C, and then samples were measured at 660 nm by spectrophotometer (Abbott et al., 2005).

3.2.1.3 Study on inoculum size

The starter of *S. cerevisiae* L3109 strain was prepared at $5x10^8$ cells/mL. Then, inoculum sizes were varied at 1L, 2L and 4L in rice wine fermentation tanks and incubated at 30°C. Viable yeast cell was measured by viable plate count method on YM agar.

3.2.2 Koji preparation

The eight kg of sticky rice (RD6) was steamed then washed with water and inoculated with 10^7 spores of *R. oryzae* strain BM8/ gram of steamed rice. The saccharified rice was incubated at 30°C for 5 days.

3.2.3 Rice wine making process

Four fermentation tanks were prepared by adding steamed paddy rice, steamed polished rice and steamed malted rice into saccharified rice in ratio 1:1. Control tank was prepared by using saccharified rice. All of conditions were conducted in duplicate. Six liters of water were added and adjusted the sugar concentration to 21°Brix with sucrose. Vitamin B complex and 0.2% of di-ammonium phosphate (DAP) were

added. Must was treated with 250 ppm of sulphur dioxide overnight in each of them. Samples were inoculated with 10^6 cells/ml of *S. cerevisiae* strain L3109. All of them were incubated at 30°C in the dark room. Samples were taken every 24 hours until the fermentation was finished. After that rice wines were filtrated and pasteurized at 65°C for 15 min. Rice wine was aged for 2 months before evaluating the sensory test.

3.2.4 Preliminary study of rice malting

KDML 105 rice was screened and removed undeveloped seeds and foreign materials (Eneje et al. 2004).

3.2.4.1 Steeping and germination

Paddy rice was test germination by breakdown dormancy with oven at 50°C and steeped in 1N KNO_3. Rice was tested germination by various steeping time at 0, 24, 48 and 72 h. Moisture content was measured at various steeping time at 0, 12, 24, 36, 48, 60 and 72 h. Rice was steeped at 30°C for 48 h in the dark (Murata et al., 1968) and changed the water every 24 h. After steeping, rice was germinated at 30°C in the dark for 7 days, then sample was taken and analyzed every 24 h and kilned in a hot air oven at 50°C for 24 h.

3.2.5 Analytical in saccharification process

3.2.5.1 Determination of mycelial propagation

The mycelial mass in the saccharified rice was estimated by determining the amount of *N*-acetyl glucosamine as described by Desgranges et al. (1991). Essentially, chitin in the samples was first hydrolysed to *N*-acetyl glucosamine by mixing samples with 10N HCl into 1 g of dried koji sample at 25°C for 16 h. After dilution with 10 ml distilled water, the hydrolysis of chitin was further proceeded by boiling for 10 h at 80°C. Samples were neutralized to pH 7.0 with 10N Sodium hydroxide (NaOH). An

aliquot of 0.5 ml hydrolysate samples were first mixed with 0.5 ml 5% Sodium nitrite ($NaNO_2$) and 0.5 ml 5% Sodium bisulfate ($NaHSO_4$). After 15 minutes, 0.5 ml 12.5% Ammonium sulfamate ($NH_4SO_3NH_2$) was added. After 5 minutes of reaction, they were further added with 0.5 ml 5% 3-methyl-2-benzothiazolinone hydrazone (MTBH) and heated in a boiling water bath for 3 min. After cooling to room temperature and mixing with 0.5 ml 0.5% Ferric chloride ($FeCl_3$), the absorbance of the reaction mixture was measured at 650 mm.

The glucosamine content of mycelia obtained from the 3-day old culture of test organisms was first measured. The mycelial propagation of test organism in the rice koji was then estimated by dividing the amount of glucosamine due to growth, with respective glucosamine content in mycelial of various test organisms (Lin et al., 2006).

3.2.5.2 Reducing sugar content

One ml of samples were added with 1 ml of DNS reagent and mixed well. Samples were boiled in boiling water for 10 minutes and cooled in ice immediately. Reducing sugars released were determined by the dinitrosalisylic acid method. Results were expressed as glucose concentration using a calibration curve (Miller, 1959). Moreover, glucose and fructose concentration were measured by HPLC.

3.2.5.3 Quantification of total protein concentration

Total protein concentration was done by using Dye-binding Bradford method. Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 85% phosphoric acid, diluted with H_2O to 1 L and filtered through Whatman No.1 paper just before used. Then, added 5 ml of dye reagent to protein sample containing 0-100 µg of protein per ml. Mix and let stand at room temperature for 5 min and measured protein by spectrophotometer at 595 nm which compared with blank consisting of 1 ml of sample buffer (Bradford, 1976; Bollag and Edelstein, 1996).

3.2.5.4 Determination of organic acid

Determinations of organic acids were quantified by high performance liquid chromatography (HPLC). Organic acids were analyzed by HPLC with a Phenimenex® Rezex ROA organic acid column (300 x 7.8 nm). The condition was followed as 55°C with flow rate 0.5 ml/min of 0.01N H_2SO_4 as mobile phase. UV detector was detected at 210 nm (www, 1999).

3.2.5.5 pH measurement

Hydrogen ion concentration is generally expressed as pH. It is measured using a pH meter. Standard buffer solutions are used to calibrate the pH meter (Zoecklein, 1995).

3.2.6 Analytical methods of rice wine

3.2.6.1 Yeast viability

The active yeast cells were determined at each sampling time by spread plate technique on YM media (Golden and Beuchat, 1990). Yeast culture was incubated at 30°C for 24 hours.

3.2.6.2 Reducing sugar and total sugar content

The colorimetric estimation of reducing sugar was described in section 3.2.5.3. Various glucose concentrations were used as standard. Total sugar content (°Brix) was measured on rice wine samples with hand refractometer (Banda and Valdez, 1976).

3.2.6.3 Total acidity measurement

Two hundred of boiling water will be placed in a 500 ml wide-mouth Erlenmeyer flask and added with 1 ml of a 1% phenolphthalein indicator solution (in 70% ethanol) and titrated with 0.1 N sodium hydroxide to a faint but define pink color.

Five ml of sample was placed into the flask, and titrated the sample to the same color or at pH 8.2. For rice wine sample was degassed before titrated (Ough and Amerine, 1980). The TA concentration calculated as follows:

$$\text{Titrable acidity} = \frac{(\text{ml base})(\text{N base})(0.075)(1000)}{\text{ml sample}}$$

(g/L tartaric acid)

where:

ml sample = sample volume (ml)

ml base = volume of sodium hydroxide used for titration (ml)

N base = normality of sodium hydroxide solution

3.2.6.4 Ethanol and monosaccharide determination

Determinations of ethanol and monosaccharides as fructose and glucose were quantified by high performance liquid chromatography (HPLC). Samples were determined by HPLC with a Phenomenex® Rezex ROA organic acid column (300 x 7.8 nm) (MacDonald and Kimmerer, 1993). The condition was followed as 60°C with flow rate 0.6 ml/min of distilled water as mobile phase. RI detector was used.

3.2.7 Analysis of malted rice

3.2.7.1 Quantification of total protein concentration

The Dye-Binding Bradford method was used to estimate total protein in malted rice everyday. Method as described in section 3.2.5.4. Samples and standard BSA were measured at 595 nm.

3.2.7.2 Quantification of reducing sugar concentration

Reducing sugar content was done by using the dinitrosalisylic colorimetric method. The method can be followed as section 3.2.5.3. Absorbance was measured 540 nm. A standard curve was prepared by using the various solution of glucose (300, 450, 1,050, 2,000, 4,000 μg/ml) using at least five data points in duplicate.

3.2.8 Sensory evaluation

Sensory evaluation of rice wine was estimated by using the method of quantitative descriptive analysis (QDA) which is the most common analytical methods for sensory in the wine industry (Zoecklein et al., 1995) Rice wine samples were tested by panelists. All panelists were the person who interested in wine tasting. The sample was served at 20°C and samples were served at eleven A.M.

3.2.9 Product Contamination

Standard of Thailand Production Industrial is determined the level of food contamination. The amount of food contamination must less than the limitation in Table 3.1.

Table 3.1 Standard quantity of contaminated substances

Contaminated substances	Quantity (less than mg/dm^3)
Copper	5
Iron	15
Lead	0.2
Arsenic	0.1
Ferrocyanide	Not found

Source: Wanapu et al., 2002

All of them were used atomic absorption spectrophotometer, expect, ferrocyanide was used 797 VA Computrace to analysed ion contamination in samples. Samples were added with 0.1 M potassium hydroxide and boric acid. Cyanide analysis was used voltage at 0 to -0.5 V.

3.2.10 Statistical analysis

Analysis of variance (ANOVA) was applied to the organic acid concentration, ethanol production and sensory data by using the SAS (Statistical Analysis Syatem) program, version 6.08 for windows (Copyright 1989 by SAS Institute Inc., Cary, North Carolina, USA).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Study on KDML105 malting production

The results of germination test by various breakdown dormancy conditions were shown in Figure 4.1. Germination of rice was over than 95% when broken down by incubating at 50°C for 5 days and then steeping at 30°C for 48 h in water, steeping in 1N KNO_3 for 24 h and then steeping at 30°C for 48 h in water and the steeped at 30°C for 48 h in water. In this work, incubation temperature at 50°C was chosen because potassium nitrate (KNO_3) has potential health effect if inhaled, ingested, contacted and chronic exposure. The possibility of using rice for malt production was studied and found that rice grains were able to absorb water and reached the maximum water content of 40% within 48 h (Figure 4.2). At 60 h, moisture content decreased because seeds were germinated and waster is also generated, together with carbon dioxide, by the respiratory processes that occur in the living tissues (Briggs, 1998). However, at this time, steeped rice seed (new water was changed every 24 h.) had bad smell; it might be contaminated by some organisms. The next experiment, germination test by various steeping time was tested and the result was shown that steeping time at 48 h was shown the highest germination than 24 and 72 h (Figure 4.3). Then, Germinated rice was measured reducing sugar and protein concentration at period time. Before used, shoot and root of germinated rice seed were removed. The result was shown in Figure 4.4 that for 4 days starchy in rice grain was developed into sugar by amylolytic enzyme activity in rice endosperm which similar to

result from Palmiano and Juliano (1973). Weight of rice seed was loss because it was modified to root and shoot as a result to increasing protein concentration.

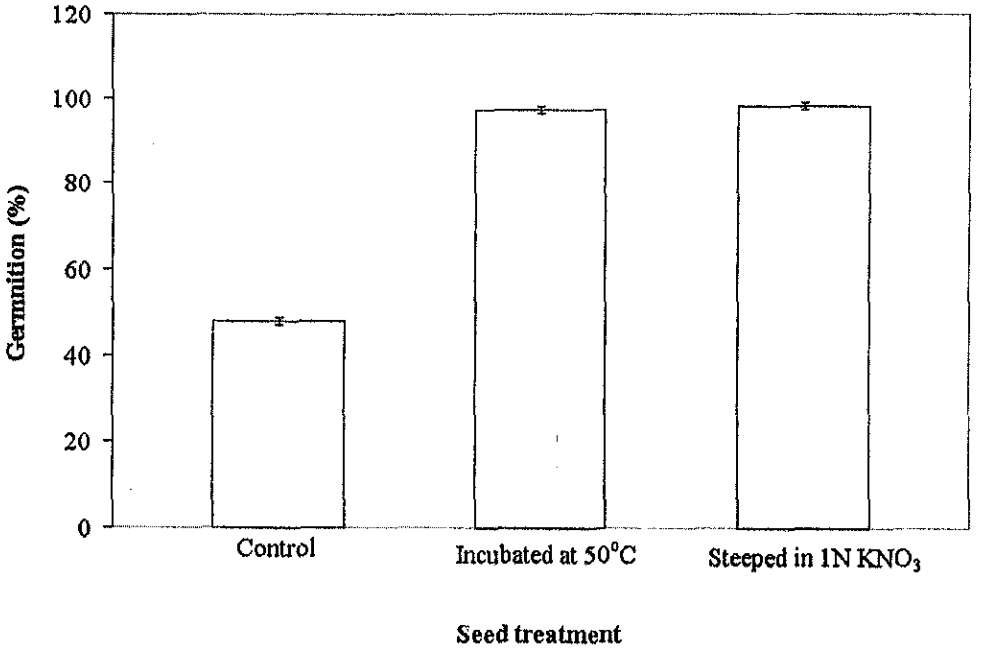

Figure 4.1 Germination test of breakdown dormancy condition of KDML 105 before germination; Rice seeds was incubated at 50°C for 5 day, steeped in 1N KNO_3 at 30°C for 24 h and steeped in water at 30°C for 48 h as control

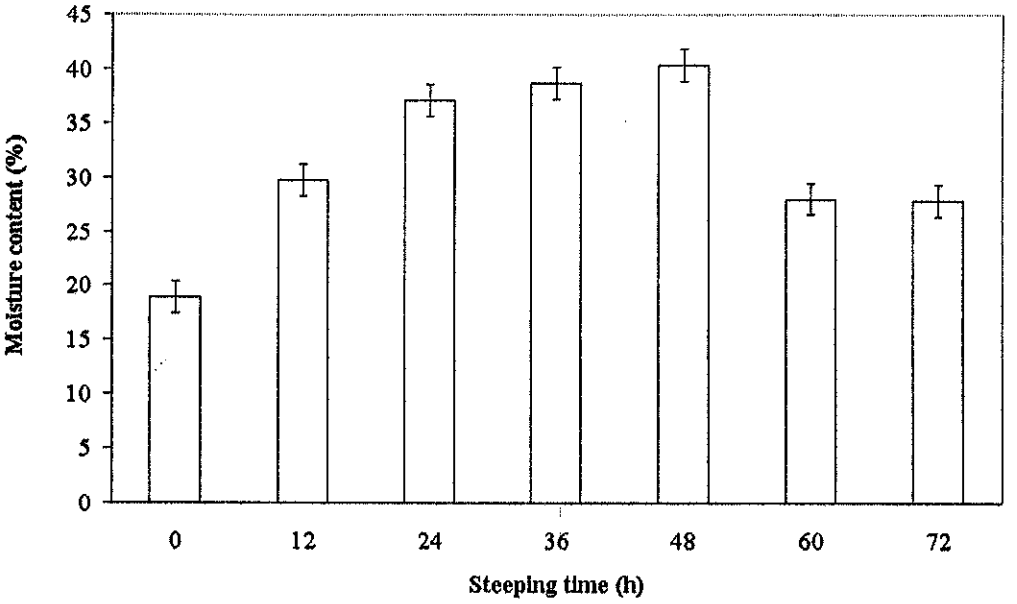

Figure 4.2 Moisture content (%) at period time of steeping; new water was changed every 12 h.

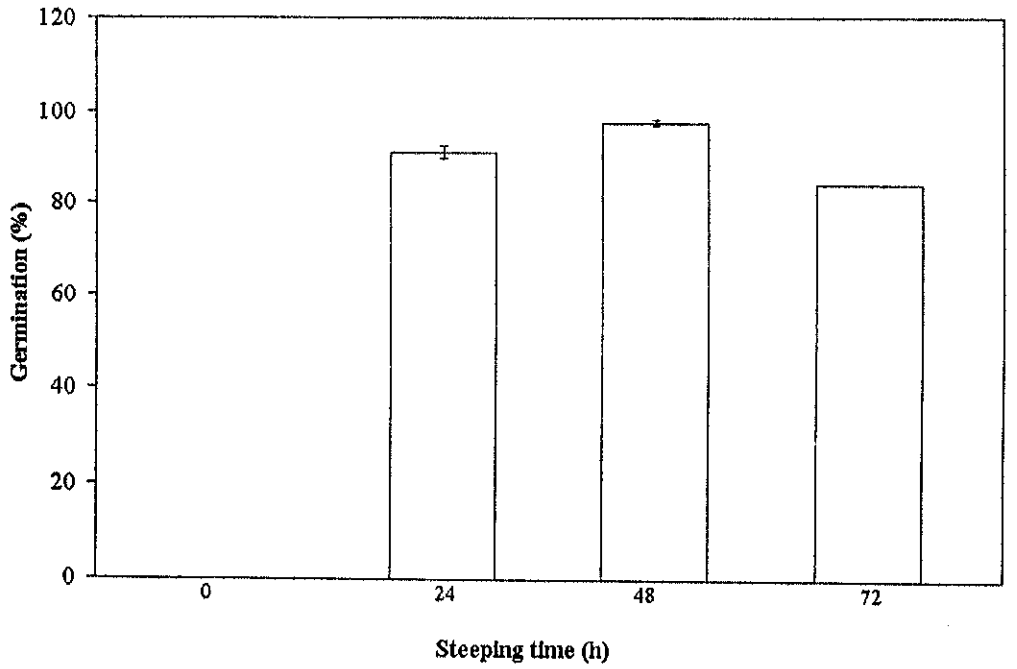

Figure 4.3 Percentage of germination at various steeping time at 30°C

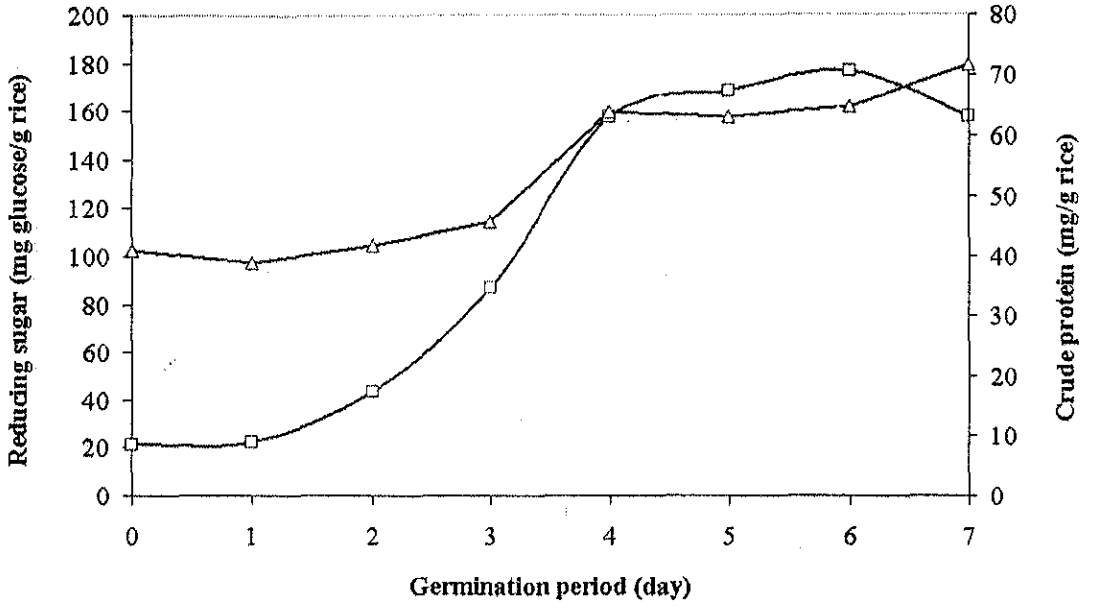

Figure 4.4 Reducing sugar (□) and protein concentration (Δ) in germinated rice at period time after steeping in water at 30°C for 48 h (removed shoot and root before analyzed)

4.2 Study of saccharification process

In this process, soluble solid, pH, reducing sugar and mycelial growth were measured. The result in Figure 4.5 demonstrated the relationship between the measurements of reducing sugar by DNS method and HPLC. On the third day, the both measurements have shown the highest of reducing sugar production in rice koji which is 122 g/L. This result was similar to Prakash and Thapa (2006). They were shown that total reducing sugar in saccharification process was increased until the third day and then, decreased. Decreasing of reducing sugar and glucose concentration might caused from the growth of *R. oryzae*.

In addition, the genus *Rhizopus* includes species those are capable of efficiently secreting organic acids, such as citric acid, fumaric acid, and malic acid (Goldberg et al., 1991 quoted in Oda et al., 2002). From this reason, rice koji is changed into lower acidity than initial stage. Initial pH of SSF process was pH 6 then, pH was dropped to pH 3.5 and stable until finished this process (Figure 4.6). Moreover, the amount of soluble solid was also determined. The result of soluble solid had not related with the results of reducing sugar and glucose concentration in Figure 4.5 which may be interfered from starch at zero day. The fungal growth in rice koji was determined using *N*-acetlyglucosamine compared with weighting of mycelium on PDA; the result was shown in Figure 4.7. At 5^{th} day of saccharification process showed the highest of mycelial growth weight because it used products of amylolytic enzyme for growth. Thus, glucose concentration decreased in 4^{th} day.

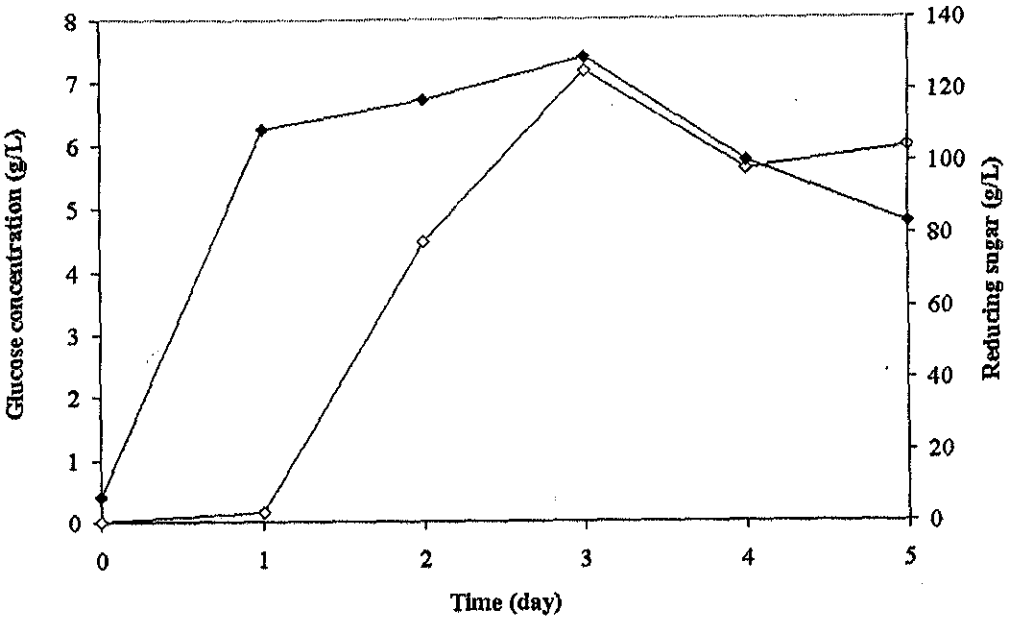

Figure 4.5 Time course of glucose concentration and reducing sugar (as glucose) in boiled sticky rice which inoculated with *R. oryzae* BM8 strain by HPLC (◊) and DNS method (♦)

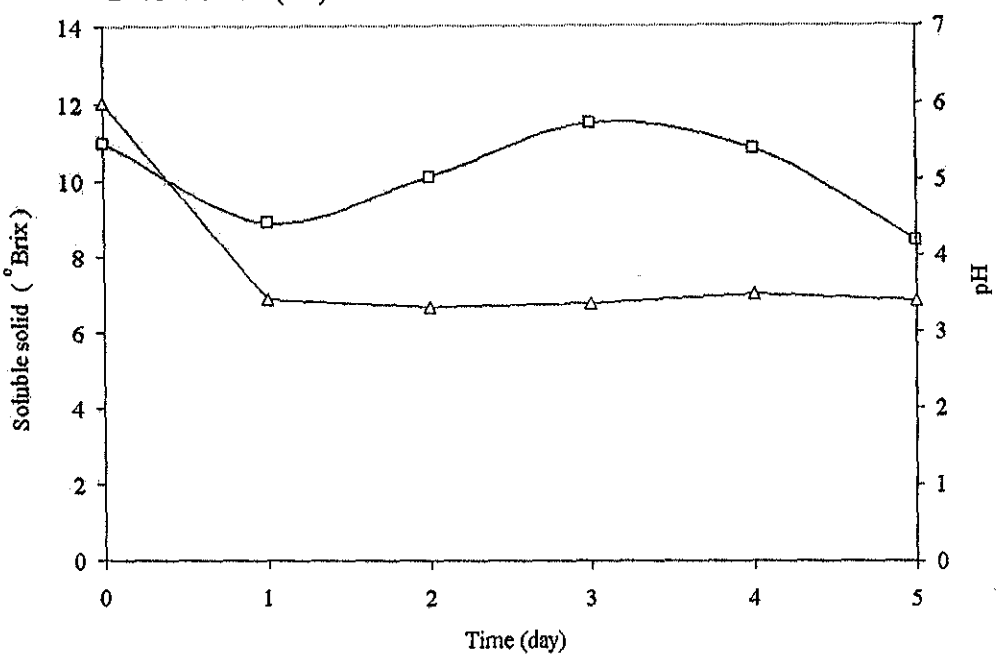

Figure 4.6 Soluble solid (°Brix) (□) and pH monitoring (Δ) in SSF process at period time after inoculated with *R. oryzae* BM8 strain in boiled sticky rice.

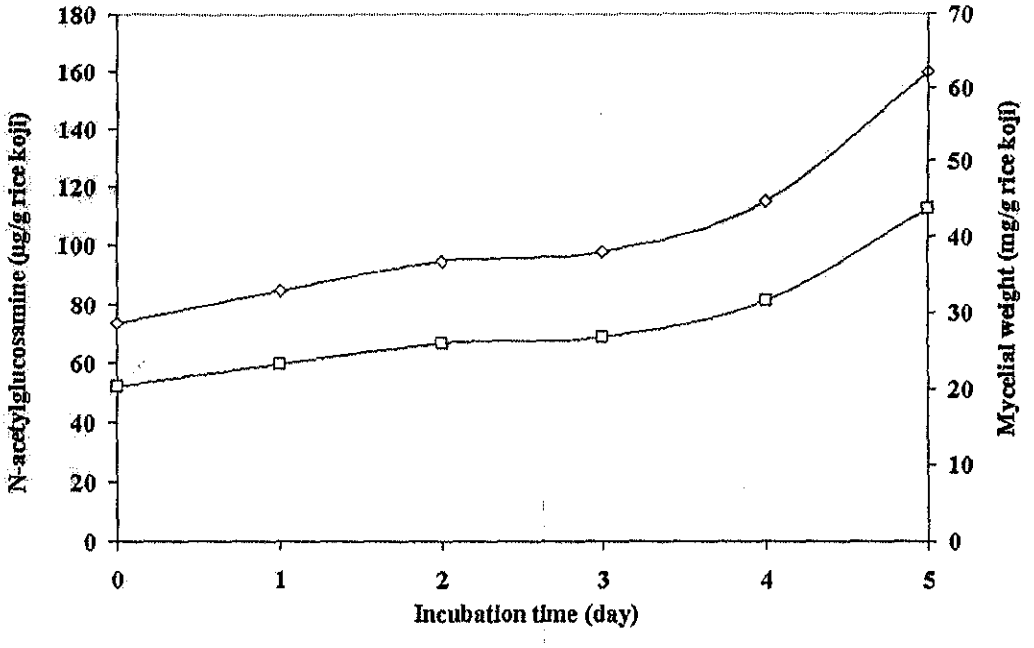

Figure 4.7 Growth of *R. oryzae* in rice koji; ◊ *N*-acetylglucosamine, □ mycelial weight on PDA

4.3 Optimization of growth of *S. cerevisiae* L3109 strain

The growth of *S. cerevisiae* L3109 strain was optimized by various temperatures at 15, 20, 25, 30 and 35°C using microbiological incubators for temperature control. The samples were monitored every two hours. The results showed that the lowest specific growth rate was 0.6847 at 15°C while the highest was at 30°C, 1.1875 (Figure 4.9) which was similar to the results obtained from Ferreira (1959), who studied yeast growth on several temperatures and found that at 30°C is the best temperature for yeast cell growth and alcohol production.

On various pH conditions at 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0; the results showed that yeast was not grow at pH 2.5. On the other hand, this strain was grown well on pH 4.0 to 5.0 (Figure 4.11). Charoenchai et al. (1998) reported that variation of medium pH between 3.0 and 4.0 did not significantly affect the growth rate or cell biomass of the yeast.

Then, inoculum size of *S. cerevisiae* L3109 strain was studied for fermentation process. Cell concentrations at $1x10^7$, $2x10^7$ and $4x10^7$ cells/mL were not effected for their growth and reducing sugar utilization was better than one. Thus, $1x10^7$ cells/ml was chosen for inoculation in rice wine process (Figure 4.12).

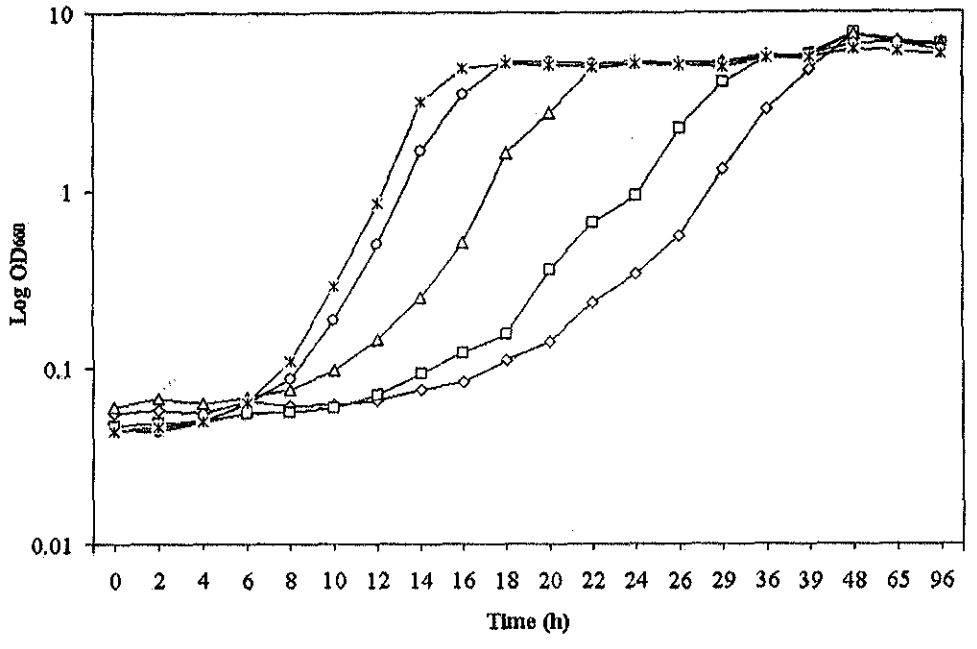

Figure 4.8 Growth of *S. cerevisiae* L3109 strain at various temperature; 15°C (◊), 20°C (□), 25°C (Δ), 30°C (○) and 35°C (x)

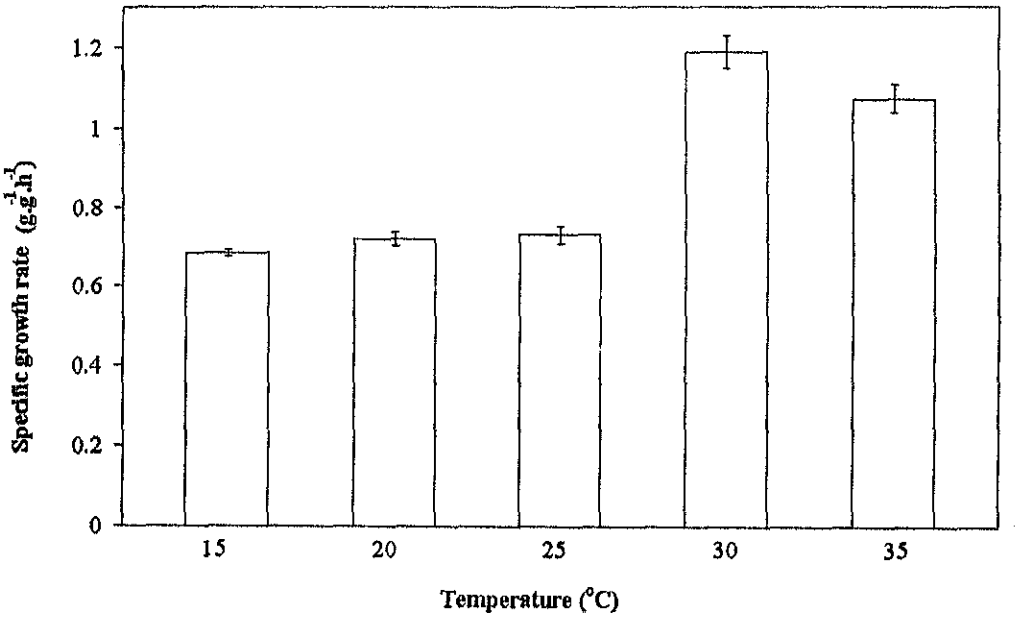

Figure 4.9 Specific growth rate of *S. cerevisiae* L3109 strain in various temperatures

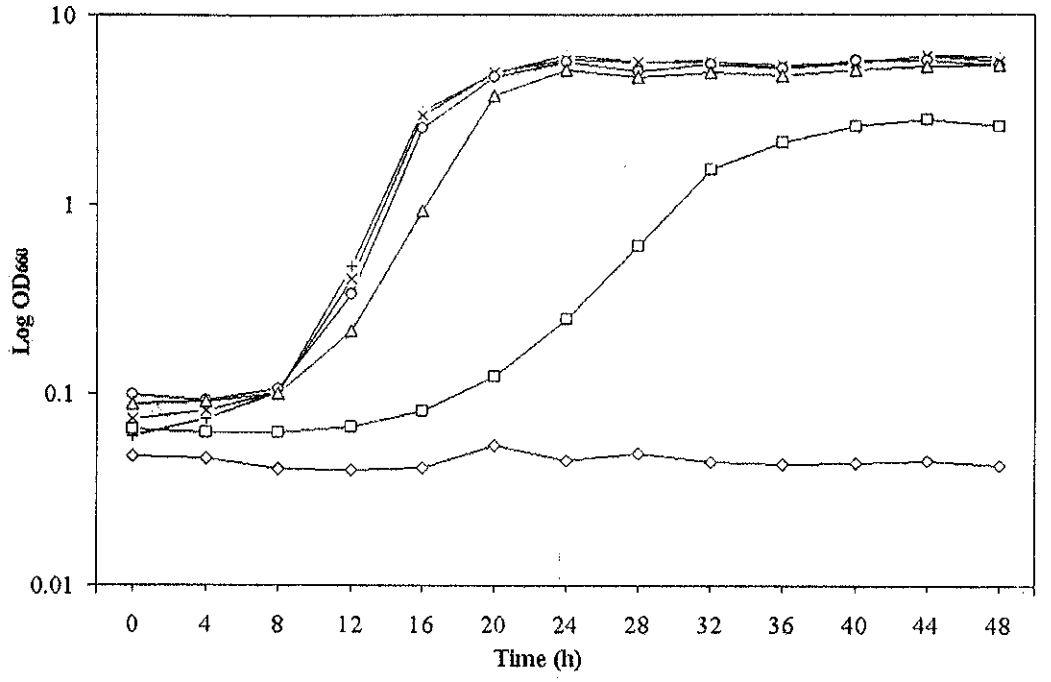

Figure 4.10 Growth of *S. cerevisiae* L3109 strain at various initial pH; pH 2.5 (◊), pH 3.0 (□), pH 3.5 (Δ), pH 4.0 (○), pH 4.5 (x) and pH 5.0 (+)

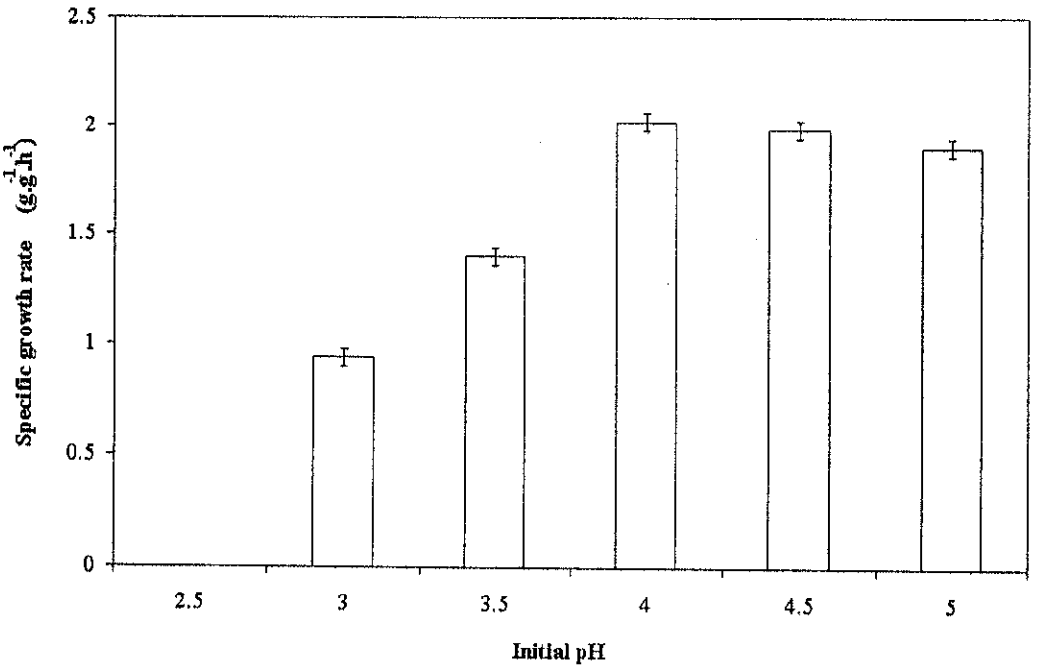

Figure 4.11 Specific growth rate of *S. cerevisiae* L3109 strain in various initial pH conditions

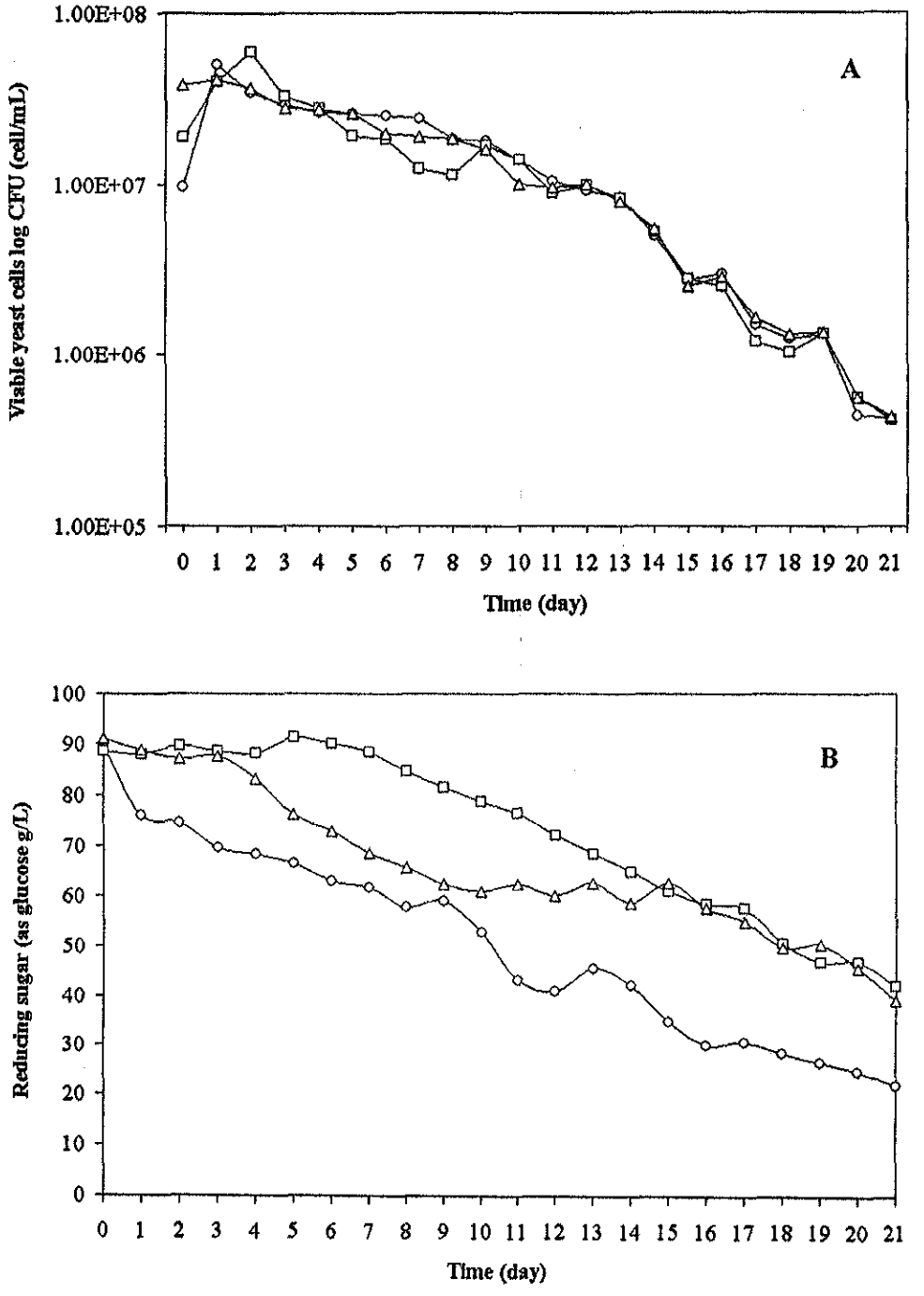

Figure 4.12 Time course of *S. cerevisiae* L3109 strain at various inoculum sizes; $1x10^7$ (○), $2x10^7$ (□) and $4x10^7$ (Δ); A) viable cells and B) reducing sugar utilization

4.4 Rice wine production

Normally, Thai traditional rice wine mostly used steamed sticky rice which inoculated with microbial starter or Loog-pang. In this work, KDML105 was added into rice wine making process to improve the smell of rice wine. The wine making process was varied into 4 conditions i.e. adding steamed paddy rice, steamed polished rice, steamed malted rice, and without adding as control. Yeast and more rice were added at zero days. All of them were duplicated. Figure 4.13 was shown as yeast growth by viable count in various conditions. All conditions were similar pattern of growth except control which shown longer live at the end of fermentation. The result of yeast growth had related with the result of reducing sugar by DNS method. They were shown that reducing sugar in rice wine was increased until day 3, and then decreased because the activity of amylolytic enzyme from fungal production still was being activated. Rate of reducing sugar production from day 2 to day 3 were shown highest as paddy 47.775, 39.861 and 37.769 as polished and malted, respectively. Moreover, *S. cerevisiae* can produced invertase enzyme, which hydrolyze sucrose into glucose and fructose (Zoecklein et al., 1995) so the result was shown in Figure 4.14. During fermentation, yeasts utilize glucose and fructose differentially. Glucose was fermented faster than fructose. Thus, the end of fermentation, fructose had present in greater amounts than glucose in rice wine. The pH of wine was not changed; they were still in acidity at pH 3.3 to 3.4 until fermentation finished (Figure 4.15). Moreover, soluble solid were estimated by hand refractometer to predicted finished fermentation. Trending of soluble solid are the same, they were slightly decreased from 8^{th} until 21^{st} day as shown in Figure 4.16.

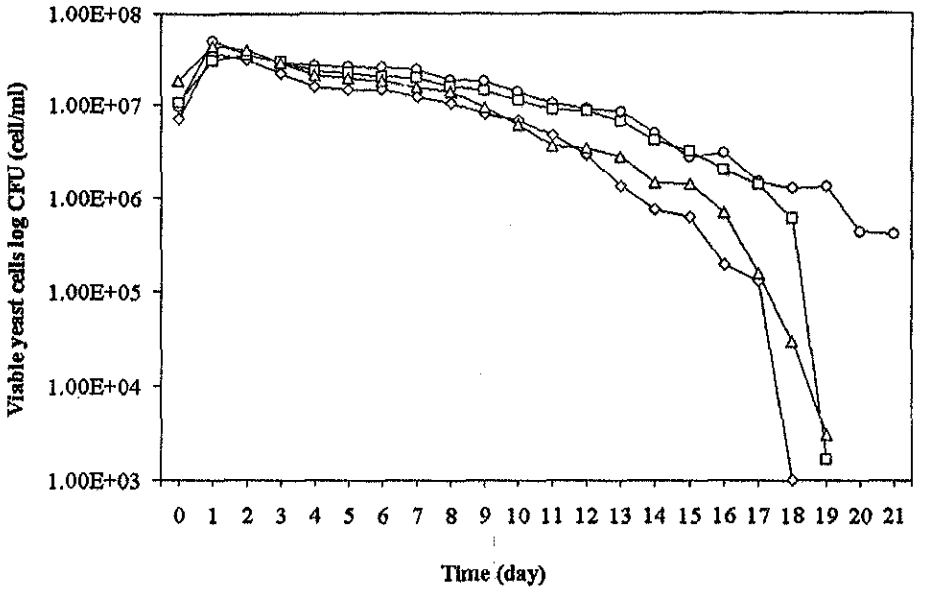

Figure 4.13 Growth curve of *S. cerevisiae* L3109 strain inoculum size $1x10^7$ cells/mL at various rice conditions; paddy rice (◊), polished rice (□), malted rice (Δ) and control (○)

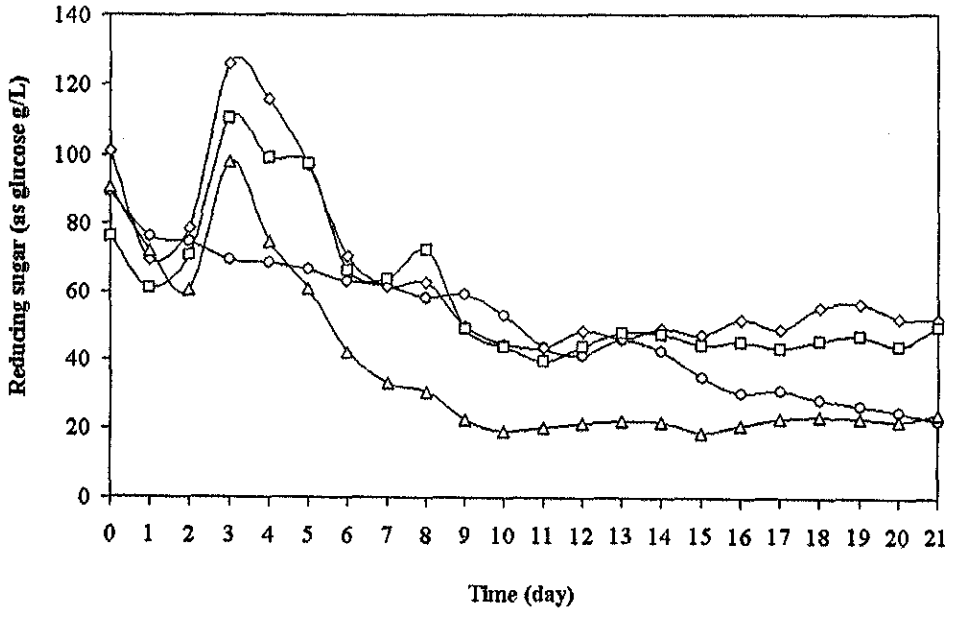

Figure 4.14 Reducing sugar residues in samples by DNS method; paddy rice (◊), polished rice (□), malted rice (Δ) and control (○)

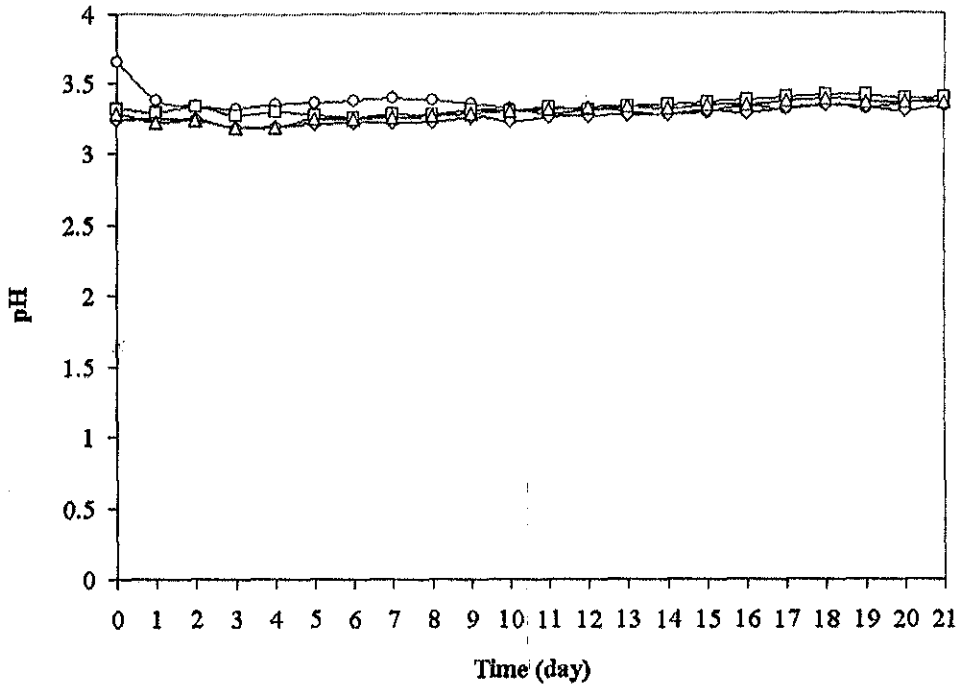

Figure 4.15 pH measurement; paddy rice (◊), polished rice (□), malted rice (Δ) and control (○)

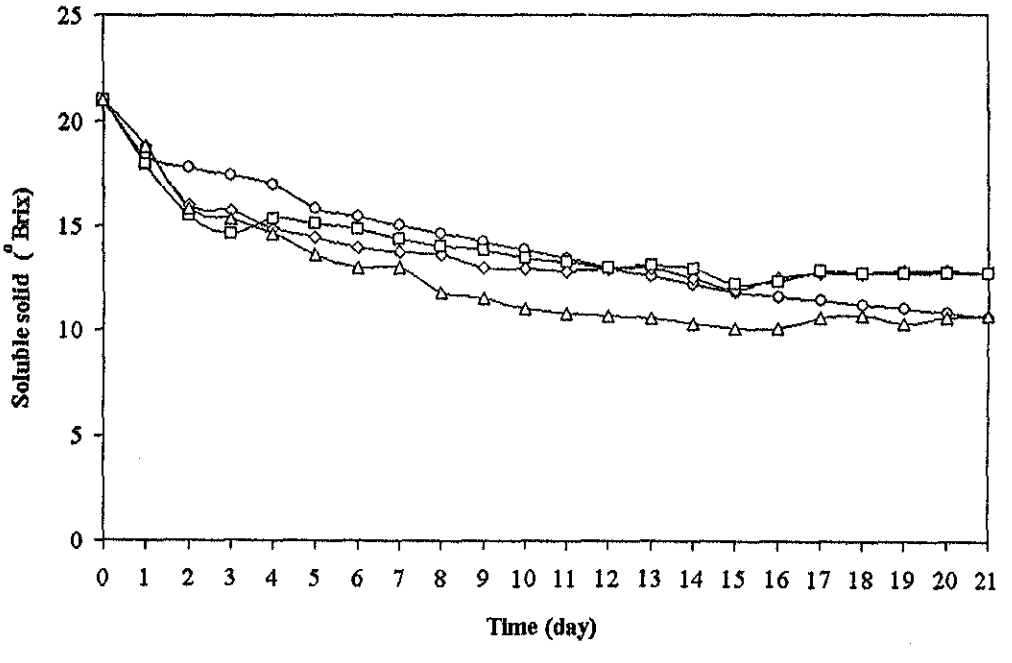

Figure 4.16 Soluble solid estimation by hand refractometer in rice wine samples; paddy rice (◊), polished rice (□), malted rice (Δ) and control (○)

Alcoholic fermentation typically refers to the chemical conversion of carbohydrates into alcohols or acids using yeast under anaerobic condition. Mostly yeast which using for, is *S. cerevisiae*. The end of fermentation, ethanol, organic acids were obtained including byproduct such as glycerol and other polyhydric alcohols (Zoecklein, 1995). Table 4.1 summerized the results of titratable acidity, organic acid (such as acetic acid, citric acid, malic acid, lactic acid and succinic acid) and ethanol production from rice wine fermentation which was detected by HPLC. Organic acids account for a significant fraction of wine. In grape wine, some of them are originally present in the grape (Zotou et al., 2004) but in rice wine appear during alcoholic fermentation because primary composition of rice grain is polysaccharides, protein and fat (Juliano, 1985). Moreover, organic acid was produced during alcoholic fermentation; filamentous fungi such as *Aspergillus* spp. and *Rhizopus* spp. were used to produce organic acid (Sasaki and Takao, 1967; Magnuson and Lasure, 1992; Oda et al., 2002; Zhou et al., 2002; Wang et al., 2005). For organic acid detection, there are many methods for analysis but mostly using chromatography. Gas chromatography is normally used for analyses fermentation products but recent developments in high-performance liquid chromatography (HPLC) have advanced it to a level such that it may be better technique for fermentation product analysis than GC (Ehrlich et al., 1981). According to Frayne (1986), who studied in Direct Analysis of the Major Organic Components in Grape Must and Wine Using High Performance Liquid Chromatography and reported that HPLC method showed it to be highly reproducible, reliable, and stable, moreover, good agreement in accuracy and precision than compared with conventional volumetric, distillation, and enzymatic methods of analysis. In addition, the advantages of using HPLC in the determination of organic acids are speed, simultaneous, analysis of several acids and easy sample preparation (Fernandez-Garcia and McGregor, 1994). Hence, this work was used HPLC method to analyzed fermentation products which was similar to researches from Davis et al., 1986; Callaway and Martin,

1996; Wei et al., 2001; Zhou et al., 2002; Nozal et al., 2003. Table 4.1 showed the results of organic acid analysis by HPLC, titratable acidity and ethanol concentration. Statistical analysis for organic acid concentration, acetic acid in rice wine (no adding 0.188 g/L) was significantly decreased when added with paddy (0.030 g/L), polished (0.048 g/L) and malted rice (0.037 g/L). Thoukis et al. (1965) reported that during alcoholic fermentation by yeast there is an increase in total acidity and a change in the constituent organic acid composition of the fermentented medium. The formation of acetic acid, widely referred to as volatile acidity, is generally recognized as a normal by-product of alcoholic fermentation. Decreasing of acetic acid, it might be cause of active yeasts can used acetic acid as a carbon source, some winemakers used this advantage led to reduce volatile acid (as acetic acid) in wine (Zoecklein, 1995). Furthermore, rice wine added with malted rice was significantly increased in ethanol concentration because malting process, products of enzyme modification in rice germinated are short-chain polysaccharides, oligosaccharide, glucose and especially, maltose. Normally, glucose is the best carbon source for yeast growth. For this strain, L3109 could utilize glucose rather to 100% in control, while 50% for fructose. Ethanol concentrations in rice wine were 13.32, 13.53, 15.239 and 17.20% in control, added with polished, paddy and malted rice, respectively. Product yields (gram of ethanol per gram of substrate) were 0.482 (paddy rice), 0.427 (polished rice), 0.544 (malted rice) and 0.736 (control).

Balli et al. (2003) who studied on Effect of yeast cell immobilization and temperature on glycerol content in alcoholic fermentation with respect to wine making and found that *S. cerevisiae* produced glycerol at temperature range 5 to 30°C. But rice wine fermentation, samples were fermented at 30°C; glycerol was not detected on the HPLC-based.

The contaminated substances (copper, iron, lead, arsenic and ferrocyanide) detection, rice wine samples were not detected corresponding Thai Industrial Standards (Table 4.2).

Table 4.1 pH, titratable acidity, organic acid, total protein concentration and ethanol concentration in rice wine

Samples	pH	Titratable acidity (g/100mL)	Organic acid concentration (g/L)					Total protein concentration (mg/L)	Ethanol concentration (%)
			Acetic acid	Citric acid	Malic acid	Lactic acid	Succinic acid		
Control	3.38	0.435 ± 0.004^{a}	0.188 ± 0.015^{a}	0.161 ± 0.012^{b}	0.205 ± 0.052^{b}	0.273 ± 0.010^{b}	0.304 ± 0.016^{b}	5.072 ± 0.759^{b}	13.317 ± 0.324^{b}
Polished rice	3.39	0.383 ± 0.021^{b}	0.048 ± 0.016^{b}	0.231 ± 0.002^{a}	0.375 ± 0.048^{a}	0.333 ± 0.016^{a}	0.296 ± 0.017^{a}	14.865 ± 3.058^{a}	13.527 ± 1.013^{b}
Paddy rice	3.34	0.416 ± 0.005^{a}	0.030 ± 0.005^{b}	0.239 ± 0.015^{ab}	0.527 ± 0.123^{ab}	0.423 ± 0.033^{ab}	0.240 ± 0.025^{ab}	5.473 ± 0.478^{b}	15.239 ± 1.7^{ab}
Malted rice	3.36	0.375 ± 0.000^{b}	0.037 ± 0.000^{b}	0.161 ± 0.011^{b}	0.225 ± 0.033^{b}	0.414 ± 0.058^{b}	0.304 ± 0.028^{b}	5.405 ± 1.147^{b}	17.201 ± 0.257^{a}

Mean in each row followed by the same letter are not significantly different ($p \geq 0.05$)

Table 4.2 Contaminated substance detection in rice wine

Contaminated substances	Maximum limit value* (mg/L)	Control	Paddy rice	Polished rice	Malted rice	Method	Limited of Detection
Copper	5	none	none	none	none	AAS and VA	≤ 0.005 mg/L
Iron	15	none	none	none	none	AAS and VA	≤ 0.01 mg/L
Lead	0.2	none	none	none	none	AAS and VA	≤ 0.05 mg/L
Arsenic	0.1	none	none	none	none	Flow injection analysis system	≤ 0.15 µg/L
Ferrocyanide	not found	none	none	none	none	VA	≤ 0.1 µg/L

Note: * Thai Industrial Standards Institue Ministry of Industry

AAS = Atomic absorption spectrophotometry

VA = Votammetry and polarography

The finished products of rice wine were tasted by trained panelists and evaluated characters of rice wine. The objective of the sensory test was to determine the condition which added with various modified KDML105 rice into traditional rice wine effect on sensory profile of rice wine. Mean intensity ratings for the wine made by five samples were plotted on polar coordinate or radar graph, the center of the graph represented low intensity with respect to each character increasing to an intensity of 10 at the ends of axes. The results were illustrated on Figure 4.17. The graph showed six characters, which were clarity, color, smell, sweet, taste; and acceptable of wines ($p<0.05$). The Least Significant Different (LSD) was calculated to determine where the different occurred and denoted by letter (Table 4.3). All of samples were not significantly different in clarity and color. Beside, three characters and acceptable of wine were significantly different among five samples ($p\geq0.05$). 2-acetyl-1-pyrroline is a mainly volatile aroma compound of rice, especially KDML105 (Buttery et al., 1983; Zhou et al., 2002; Wongpornchai et al., 2004). As a result, smell character in rice wine added with KDML105 was significantly different higher than no adding. Overall of scores, rice wine added with paddy rice was obtained the highest score and the lowest was control (7.537, 7.357, 6.768, 5.789 and 5.419; paddy, polished, malted, commercial and control, respectively), which was meaning the panelist most accepted in rice wine added with paddy rice and polished rice, the second was malted rice and the last was control and commercial.

Table 4.3 Mean rating and Least Significant Differences (LSD)

	Characters						
	Clarity	Color	Smell	Sweet	Taste	Accept	Overall
Commercial	8.889[a]	7.722[a]	5.667[b]	3.778[b]	4.556[b]	4.800[c]	5.789[b]
control	8.556[a]	7.222[a]	4.333[b]	4.000[b]	4.111[b]	4.600[c]	5.419[b]
paddy	8.333[a]	7.778[a]	6.778[a]	7.556[a]	7.222[a]	7.650[a]	7.5373[a]
polished	8.222[a]	8.000[a]	6.833[a]	6.722[a]	6.833[a]	7.500[a]	7.357[a]
malted	8.778[a]	7.667[a]	6.333[ab]	6.667[a]	5.556[ab]	6.600[b]	6.7684[ab]

Mean in each row followed by the same letter are not significantly different ($p \geq 0.05$)

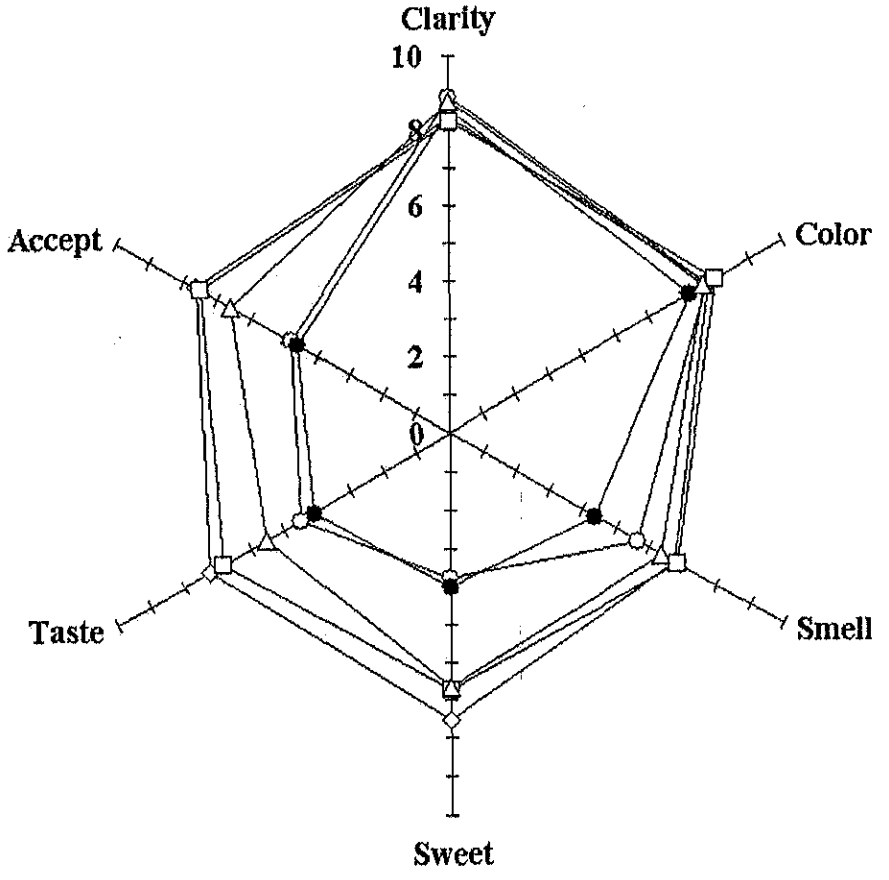

Figure 4.17 Polar coordinate graph of the mean intensity rating of five rice wine samples in term of clarity, color, smell, sweet, taste and accept ($p<0.05$) ; commercial (○), control (●), paddy rice (◊), polished rice (□) and malted rice (Δ)

CHAPTER V

CONCLUSION

This is the first time, which studied in quality development of Thai traditional rice wine or Sato by adding which rice KDML 105 strain. On malting process, KDML105 was broken down dormancy by oven at 50°C for 5 day and steeped in 1N KNO_3 at 30°C for 48 h before steeped at 30°C for 48 h (changed the water every 24 h) which were germinated more than 95%. In 4th day, germinated rice grains had the highest protein and reducing sugar. On saccharification process, *R. oryzae* strain BM8 which was inoculated to steamed sticky rice, can produce amylolytic enzyme to hydrolyzed starchy source and produce the highest reducing sugar in 3rd day. After that reducing sugar was decreased because growing of *R. oryzae* strain BM8.

In rice wine fermentation, amylolytic enzyme from saccharification process can hydrolyzed paddy, polished and malted rice which were added into rice wine. Then, reducing sugar was increased, decreasing of volatile acid as acetic acid when added with rice is significantly, except control. Ethanol concentrations in rice wine were 13.32, 13.53, 15.239 and 17.20% in control, added with polished, paddy and malted rice, respectively. Moreover, rice wines were not contaminated by heavy metal and cyanide. Statistical analysis of sensory evaluation, the most panelists accepted in rice wine by adding paddy rice and adding polished rice, the second was malted rice. Commercial and not adding (control) less accepted.

REFERENCES

REFERENCES

Abbott, D., Hynes, S., and Ingledew, W. M. (2005). Growth rates of *Dekkera/Brettanomyces* yeasts hinder their ability to compete with Saccharomyces cerevisiae in batch corn mash fermentations. **Applied Microbiology and Biotechnology.** 66(6): 641-647.

Afonso, V. L. G., J. Darias, Armas, R., Medina, M. R., and Diaz, M. E. (1998). Descriptive Analysis of Three White Wine Varieties Cultivated in the Canary Islands, **American Journal of Enology and Viticulture.** 49(4): 440-444.

Available: http://www.supware.dk/phepdf/Rezex.pdf

Awazuhara, M., A. Nakagawa, et al. (2000). Distribution and characterization of enzymes causing starch degradation in rice (*Oryza sativa* cv. koshihikari). **Journal of Agriculture and Food Chemistry.** 48(2): 245-252.

Balli, D., Flari, V., Sakellaraki, E., Schoina, V., Iconomopoulou, M., Bekatorou, A., and Kanellaki, M. (2003). Effect of yeast cell immobilization and temperature on glycerol content in alcoholic fermentation with respect to wine making. **Process Biochemistry.** 39: 499-506.

Banda, M. and Valdez, R. (1976). Effect of stage of maturity on nutritive value of sugar cane. **Tropical Animal Production.** 1(2): 94-97.

Berry, D. R. (1995). Alcoholic beverage fermentations. In: Lea, A. G. and Piggott, J. R. Editors, **Fermented beverage production**, Chapman & Hall Press, London (1995), pp. 32-44.

Bollag, D. and S. Edelstein (1996). Protein methods (2nd ed.). New York : Wiley-Liss.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry.** 72: 248-54.

Bramorski, A., Christen, P., Ramirez, M., Soccol, C., and Revah, S. (1998). Production of volatile compounds by the edible fungus *Rhizopus oryzae* during solid state cultivation on tropical agro-industrial substrates. **Biotechnology Letters.** 20(4): 359-362.

Briggs, D. E. (1998). **Malts and Malting** (1st ed.), London : Blackie Academic

Buttery, R. G., Ling, L. C., Juliano, B.O., and Turnbaugh, J.G. (1983). Cooked rice aroma and 2-acetyl-1-pyrroline. **Journal of Agricultural and Food Chemistry.** 31: 823-826.

Callaway, T. R. and Martin, S. A. (1996). Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. **Journal of Animal Science.** 74(8): 1982-1989.

Charoenchai, C., Fleet, G. H., and Henschke, P. A. (1998). Effects of Temperature, pH, and Sugar Concentration on the Growth Rates and Cell Biomass of Wine Yeasts. **American Journal of Enology and Viticulture.** 49: 283-288

David, A. M., Nadia, K. and Marin, B. (2006). **Solid-State Fermentation Bioreactors: Fundamentals of Design and Operation**, Springer-Verlag Berlin and Heidelberg GmbH & Co. K.

Davis, C. R., Wibowo, D. J., Lee, T. H., and Fleet, G. H. (1986). Growth and Metabolism of Lactic Acid Bacteria during and after Malolactic Fermentation of Wines at Different pH. **Applied and Environmental Microbiology.** 51: 539-545

Dendy, D. A. V. and B. J. Dobraszczyk (2001). **Cereals and Cereal Products: Chemistry and Technology** (1st ed.). Springer Publishing Company.

Desgranges, C., Vergoignan, C., Georges, M., and Durand, A. (1991). Biomass estimation in solid state fermentation. **Applied Microbiology and Biotechnology.** 35: 200-205.

Dung, N. T. P., Rombouts, F. M., and Nout, M. J. R. (2006). Functionality of selected strains of moulds and yeasts from Vietnamese rice wine starters. **Food Microbiology.** 23(4): 331-340.

Dung, N. T. P., Rombouts, F. M. and Nout, M. J. R. (2007). Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). **LWT - Food Science and Technology.** 40(1): 130-135.

Ehrlich, G. G., Goerlitz, D. F., Bourell, J. H., Eisen, G. V., and Godsy, E. M. (1981). Liquid Chromatographic Procedure for Fermentation Product Analysis in the Identification of Anaerobic Bacteria. **Applied and Environmental Microbiology.** 42: 878-885.

Eneje, L. O., Ogu, E. O., Aloh, C. U., Odibo, F. J. C., Agu, R. C., and Palmer, G. H. (2004). Effect of steeping and germination time on malting performance of Nigerian white and yellow maize varieties. **Process Biochemistry.** 39(8): 1013-1016.

Fernandez-Garcia, E. and McGregor, J. U. (1994). Determination of Organic Acids During the Fermentation and Cold Storage of Yogurt. **Journal of Dairy Science.** 77: 2934.

Ferreira, J. D. (1959). The Growth and Fermentation Characteristics of Six Yeast Strains at Several Temperatures. **American Journal of Enology and Viticulture.** 10: 1-7.

Frayne, R. F. (1986). Direct Analysis of the Major Organic Components in Grape Must and Wine Using High Performance Liquid Chromatography. **American Journal of Enology and Viticulture.** 37: 281-287.

Fugelsang, K. C. (1997). **Wine Microbiology**, New York: Chapman & Hall.

Fujita, J. I. N., Yamane, Y. U. I., Fukada, H., Kizaki, Y., Wakabayashi, S., Shigeta, S., Suzuki, O., and Ono, K. (2003). Production and Properties of Phytase and Acid Phosphatase from a Sake Koji Mold, *Aspergillus oryzae*. **Journal of Bioscience and Bioengineering.** 95(4): 348-353.

Goldberg, I., Peleg, Y., and Rokem, J. S. (1991) Citric, fumaric and malic acids. In: Goldberg, I. and Williams, R., Editors, **Biotechnology and food ingredients.** New York: Van Nostrand Reinhold, pp 349-374.

Golden, D. and L. Beuchat (1990). Colony formation by sublethally heat-injured *Zygosaccharomyces rouxii* as affected by solutes in the recovery medium and procedure for sterilizing medium. **Applied and Environmental Microbiology.** 56(8): 2319-2326.

Haard, N., Odunfa, S., Lee, C-H., Quintero-Ramírez, R., Lorence-Quiñones, A., and Wacher-Radarte, C. (1999). Fermented cereals: a global perspective, FAO AGRICULTURAL SERVICES BULLETIN No. 138.

Hamilton, L.M., Kelly, C.T., and Fogarty, W.M. (2000). Review: cyclodextrins and their interaction with amylopectin enzymes. **Enzyme and Microbial Technology**. 26: 561-567.

Holmberg, J. –E., Hämäläinen, J. J., Reinikainen, P., and Olkku, J. (2002). A Model for Predicting the Effects of a Steeping Progam on the Germination of Barley with Different Water Sensitivities. ***J. Inst. Brew.*** 108(4): 416-423.

Home, S., Stenholm, K., Wilhelmson, A., and Autio, K. (2001). **Properties of Starch and Cell Wall Components and their Effects on Processing,** Proceedings of the 10th Australian Barley Technical Symposium, Canberra: Australia. [Online]. Available: http://www.regional.org.au/au/abts/1999/home2.htm

Iumboldt University (1999). Kreb's TCA cycle [Online]. Available: http://www.lcusd.net/lchs/mewoldsen/Ebiolink.htm

Iwashita, K. (2002). Recent Studies of Protein Secretion by Filamentous Fungi. **Journal of Bioscience and Bioengineering.** 94(6): 530-535.

Iwata, H., Suzuki, T., and Aramaki, I. (2003). Purification and Characterization of Rice a-Glucosidase, a Key Enzyme for Alcohol Fermentation of Rice Polish. **Journal of Bioscience and Bioengineering.** 95(1): 106-108.

Juliano, B. O. (1985). **Rice: Chemistry and Technology** (2nd ed.), St. Paul: American Association of Cereal Chemists.

Juliano, B. O. (2005). **Overview of rice and rice-based products.** Tsukuba: Japan. (Rice is life: scientific perspectives for the 21st century. Proceedings of the World Rice Research Conference held in Tsukuba, Japan, 4-7 November 2004) pp 268-270.

Kodama, K. (1993). Sake-brewing yeast. In: Rose, A. H. and Harrison, J. S., Editors, **The yeasts** (2nd ed.) Vol 5. London; San Diego: Acedemic Press. pp 129-168.

Karuwanna, P. (2003). **Wine: Art and Science** (2nd ed.). Bangkok: Kasetsart University Press.

Lewis, M. J. and Young, T. W. (1995). **Brewing.** London : Chapman & Hall.

Lim, G., Tan, T. K. and Rahim, N. A. (1987). Variations in amylase and protease activities among *Rhizopus* isolates. **World Journal of Microbiology and Biotechnology.** 3(3): 319-322.

Lin, C.-H., Y.-T. Wei, and Chou, C-C. (2006). Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. **Food Microbiology.** 23(7): 628-633.

Lotong, N. (1998). Koji. In Wood, B. J. B. (eds.). **Microbiology of fermented food** (2nd ed., pp. 658-695). New York : Blackie Academic & Professional.

MacDonald, R. and T. Kimmerer (1993). Metabolism of Transpired Ethanol by Eastern Cottonwood (*Populus deltoides* Bartr.), **Plant Physiology.** 102(1): 173-179.

Magnuson, J. K., Lasure, L. L. (1994) **Organic acid production by filamentous fungi.** In: Lange, J. and Lange, L., Editors, Advances in fungal biotechnology for industry,

agriculture and medicine. Richland: WA. Kluwer Academic/ Plenum Publishers (2004).

Merican, Z. and Q. L. Yeoh (1989). Tapai Processing in Malaysia. Industrialization of Indigenous Fermented Foods. New York, NY: Marcel Dekker. P: 169-190.

Miller, G. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **Analytical Chemistry.** 31: 426-428.

Mogg, R. (2004). Biofuels in Asia: Thailand relaunches gasohol for automotive use. **Refocus.** 5(3): 44-47.

Muralikrishna, G. and M. Nirmala (2005). Cereal [alpha]-amylases. **Carbohydrate Polymers.** 60(2): 163-173.

Murata, T., T. Akazawa, and Fukuchi, S. (1968). Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds I. An Analytical Study1. **Plant Physiology.** 43(12): 1899-1905.

Nigam, P. and Singh, D. (1994). Enzyme and microbial systems involved in starch processing. **Butterworth Heinemann.** 17(9): 770-778.

Norouzian, D., Akbarzadeh, A., Scharer, J. M. and Young, M. M. (2006). Fungal glucoamylase. **Biotechnology Advances.** 24(1): 80-85.

Nout, M. J. R. and K. E. Aidoo (2002). Asian fungal fermented food. **The Mycota.** Vol 10: Industrial Applications., Berlin, Springer-Verlag: pp 23-47.

Nozal, M. J., Bernal, J. L., Gomez, L. A., Higes, M., abd Meana, A. (2003) Determination of oxalic acid and other organic acids in honey and in some anatomic structures of bees. **Apidologie.** 34: 181-188.

Oda, Y., Saito, K., Yamauchi, H., and Mori, M. (2002) Lactic Acid Fermentation of Potato Pulp by the Fungus *Rhizopus oryzae*. **Current Microbiology.** 45: 1-4.

Ough, C. S. and Amerine, M. A. (1988) Methods for Analysis of Musts and Wines . 377 pp. *In.* John Wiley and Sons, New York.

Padhye, V. and D. Salunkhe (1979). Extraction and characterization of rice proteins. **Cereal Chemistry.** 56(5): 389-393.

Palmiano, E. P. and Juliano, B. O. (1973) Changes in the Activity of Some Hydrolases, Peroxidase, and Catalase in the Rice Seed during Germination 1. **Plant Physiology.** 52: 274-277.

Pandey, A., Ashakumary, L., and Selvakumar, P. (1995). Copra waste -- A novel substrate for solid-state fermentation. **Bioresource Technology.** 51(2-3): 217-220.

Pandey, A., P. Selvakumar, Soccol, C. R., and Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. **Current Science.** 77(1): 149-162.

Peixoto, S. C., Jorge, J. A., Terenzi, H. F., and Polizeli, M. (2003) *Rhizopus microsporus* var. *rhizopodiformis*: a thermotolerant fungus with potential for production of thermostable amylases. **International Microbiology.** 6(4): 269-273.

Prakash, T. J. and Thapa, S. (2006). Fermentation Dynamics During Production of Bhaati Jaanr, a Traditional Fermented Rice Beverage of the Eastern Himalayas. **Food Biotechnology**. 20(3): 251-261.

Raimbault, M. and Alazard, D. (1980). Culture method to study fungal growth in solid fermentation. **Applied Microbiology and Biotechnology.** 9(3): 199-209.

Ribereau-Gayon, P., D. Dubourdieu, Donche, B., Lonvaud, A., Glories, Y., and Maujean, A. (2000). **Handbook of Enology Vol. 1. The Microbiology of Wine and Vinifications**, John Wiley and Sons, New York.

Ribereau-Gayon, P., D. Dubourdieu, Donche, B., Lonvaud, A., Glories, Y., and Maujean, A. (2000). **Handbook of Enology Vol. 2. The Chemistry of Wine, Stabilization and Treatments**, John Wiley and Sons, New York.

Sasaki, Y. and Takao, S. (1967). Organic Acid Production by Basidiomycetes. III. Cultural Conditions for L-Malic Acid Production. **Applied and Environmental Microbiology**. 15: 373-377.

Steinkraus, K. H. (1996). Handbook of indigenous fermented foods (2nd ed.). New York: M. Dekker.

Stewart, G.G., Russell, I., Klein, R. D. and Hiebsch, R. R. (1987). **Biological research on industrial yeasts.** Boca Raton, Fla. : CRC Press.

Sujaya, I., Antara, N., Sone, T., Tamura, Y., Aryanta, W., Yokota, A., Asano, K., and Tomita, F. (2004). Identification and characterization of yeasts in brem, a traditional Balinese rice wine. **World Journal of Microbiology and Biotechnology.** 20: 143-150.

Techakriengkrai, I. and R. Surakarnkul (2007). Analysis of benzoic acid and sorbic acid in Thai rice wines and distillates by solid-phase sorbent extraction and high-performance liquid chromatography. **Journal of Food Composition and Analysis.** 20(3-4): 220-225.

Thitisararak, N., Plumcharoen, S., and Rungsardthong, V. (n.d.). The Use of Dry Starter Cultures for the Fermentation of Sato, a Traditional Thai Rice Wine.

Thoukis, G., Ueda, M. and Wright, D. (1965) The Formation of Succinic Acid during Alcoholic Fermentation. **American Journal of Enology and Viticulture.** 16: 1-8

Traore, T., Mouquet, C., Icard-Verniere, C., Traore, A. S. and Treche, S. (2004). Changes in nutrient composition, phytate and cyanide contents and α-amylase activity during cereal malting in small production units in *Ouagadougou* (*Burkina Faso*). **Food Chemistry.** 88(1): 105-114

Tsuyoshi, N., R. Fudou, Yamanaka, S., Kozaki, M., Tamang, N., Thapa, S., and Tamang, J. P. (2005). Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. **International Journal of Food Microbiology.** 99(2): 135-146.

Wanapu, C., Boonkerd, N., and Dithavibool, L. (2002). **Wine maker I** (1st ed.). Thailand: Somboon printing .

Wang, X., Sun, L., Wei, D., and Wang, R. (2005). Reducing by-product formation in L-lactic acid fermentation by *Rhizopus oryzae*. **Journal of Industrial Microbiology and Biotechnology.** 32: 38-40.

Wei, M. C., Chang, C. T., and Jen, J. F. (2001). Determination of organic acids in fermentation products of milk with high performance liquid chromatography/on-lined micro-dialysis. **Chromatographia.** 54: 601-605.

Wikimedia Foundation, Inc. (2007) Fermentation [Online]. Avialable: http://en.wikipedia.org/wiki/Fermentation_(biochemistry)

Wikimedia Foundation, Inc. (2007) Rice wine [Online]. Avialable: http://en.wikipedia.org/wiki/Rice_wine

Wongpornchai, S., Dumri, K., Jongkaewwattana, S., and Siri, B. (2004). Effects of drying methods and storage time on the aroma and milling quality of rice (*Oryza sativa L.*) cv. Khao Dawk Mali 105. **Food Chemistry.** 87: 407-414.

Zhou, Y., Du, J., and Tsao, G. (2002). Comparison of fumaric acid production by *Rhizopus oryzae* using different neutralizing agents. **Bioprocess and Biosystems Engineering.** 25: 179-181.

Zhou, Z., Robards, K., Helliwell, S., and Blanchard, C. (2002). Composition and functional properties of rice. **International Journal of Food Science and Technology.** 37: 849-868.

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H. and Nury, F. S. (1995). **Wine Analysis and Production.** New York: Chapman & Hall.

Zotou, A., Loukou, Z. and Karava, O. (2004). Method Development for the Determination of Seven Organic Acids in Wines by Reversed-Phase High Performance Liquid Chromatography. **Chromatographia.** 60: 39-44.

APPENDICES

APPENDIX A

METHOD

1. Sensory evaluation

The sensory evaluation of wines was done by using 10 volunteers and 2 experts of wine making. All ten volunteers participated in a round table discussion session. They were given representative wines, terminology of wine descriptors and tentative reference standards.

QDA sheet for wine tasting

Please evaluate the sour taste, flavor, balance and overall of the sample in sequence. Place a vertical line across the horizontal line at the point that best describes each property in the sample.

	low (0-3)	moderate (4-6)	high (7-10)
A. Clarity			
B. Color			
C. Smell			
D. Sweet			
E. Taste			
F. Accept			
G. Overall			

Figure 1A Work sheet of QDA for rice wine tasting

APPENDIX B

RESULTS

1. KDML 105 seeds germination

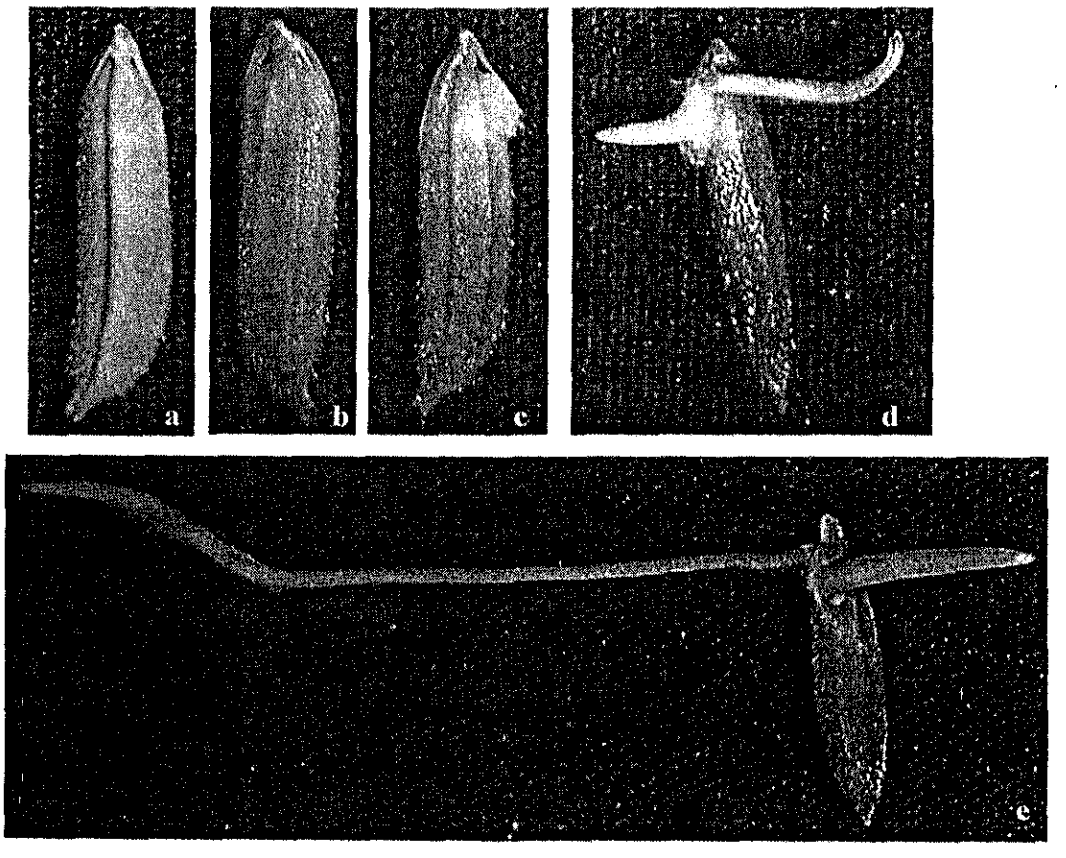

Figure 1B KDML 105 seed germination, a) paddy rice; b) 1st day steeped in water; c) 2nd day steeped in water or 0 day germination; d) 1st day germination; e) 2nd day germination; f) 3rd day germination; g) 4th day germination; h) 5th day germination; i) 6th day germination and j) 7th day germination

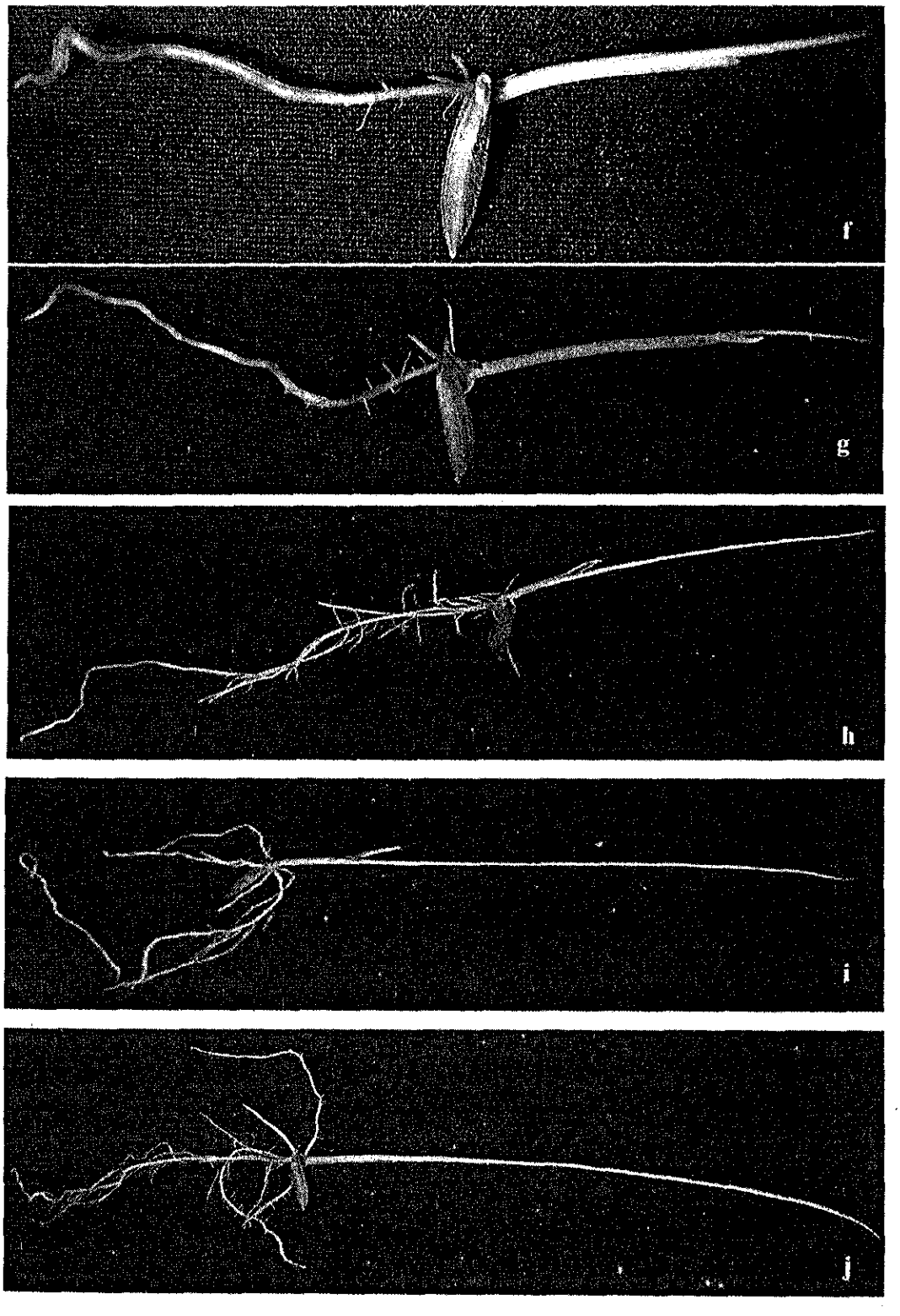

Figure 1B (continued)

2. Chromatograms of standard and sample by HPLC

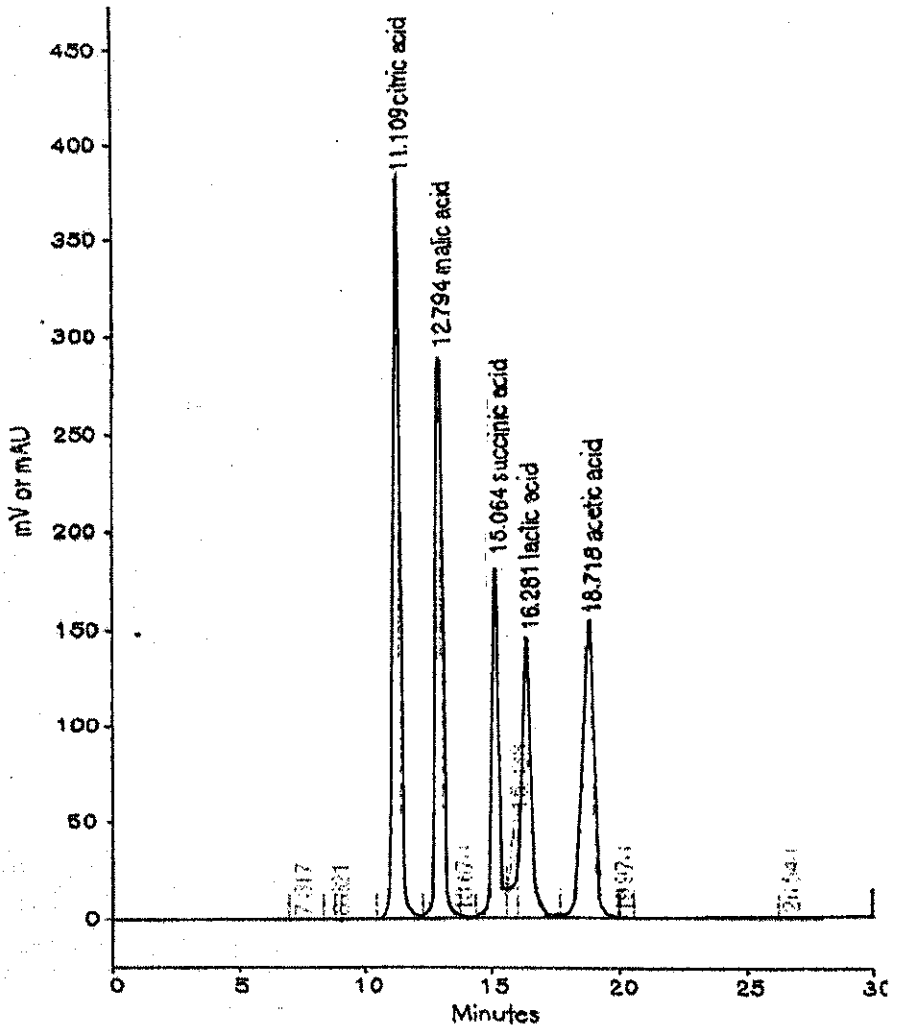

Figure 2B Chromatogram of standard organic acid: acetic acid, citric acid, lactic acid, malic acid, succinic acid

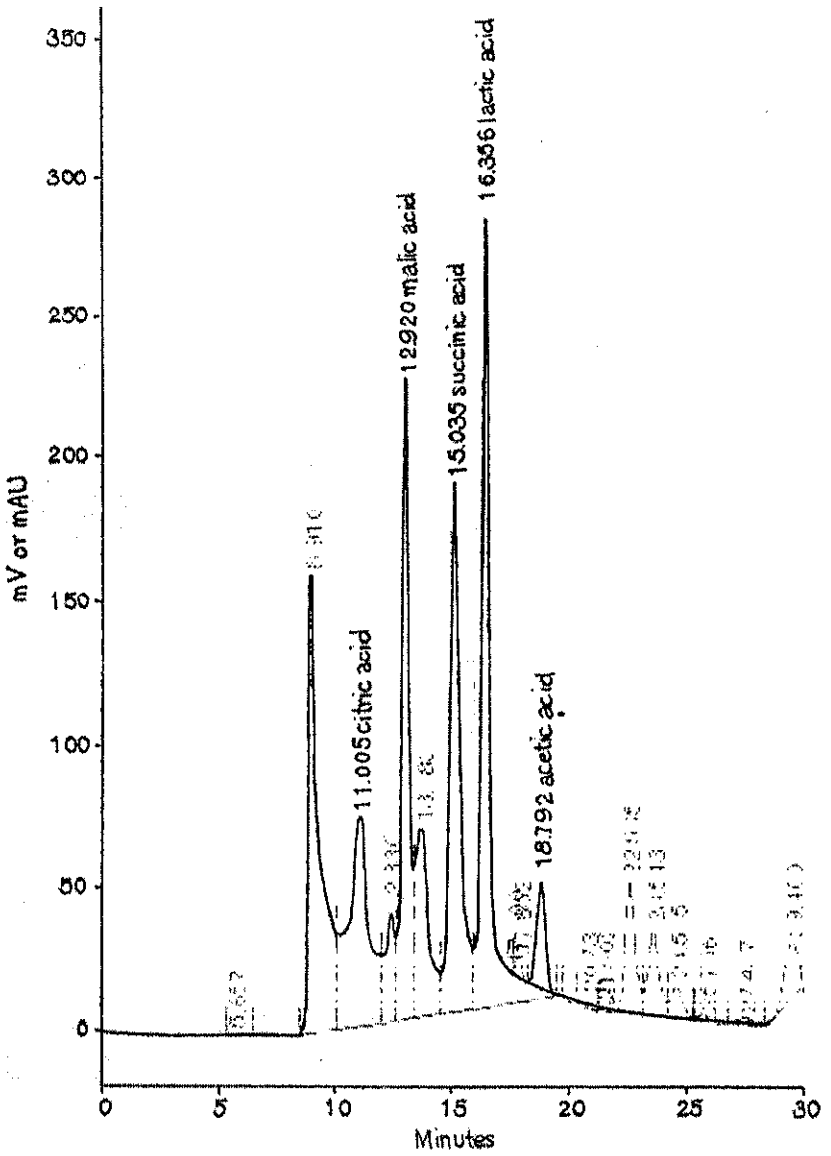

Figure 3B Chromatogram of organic acid in rice wine sample at finished fermentation (added malted rice with yeast)

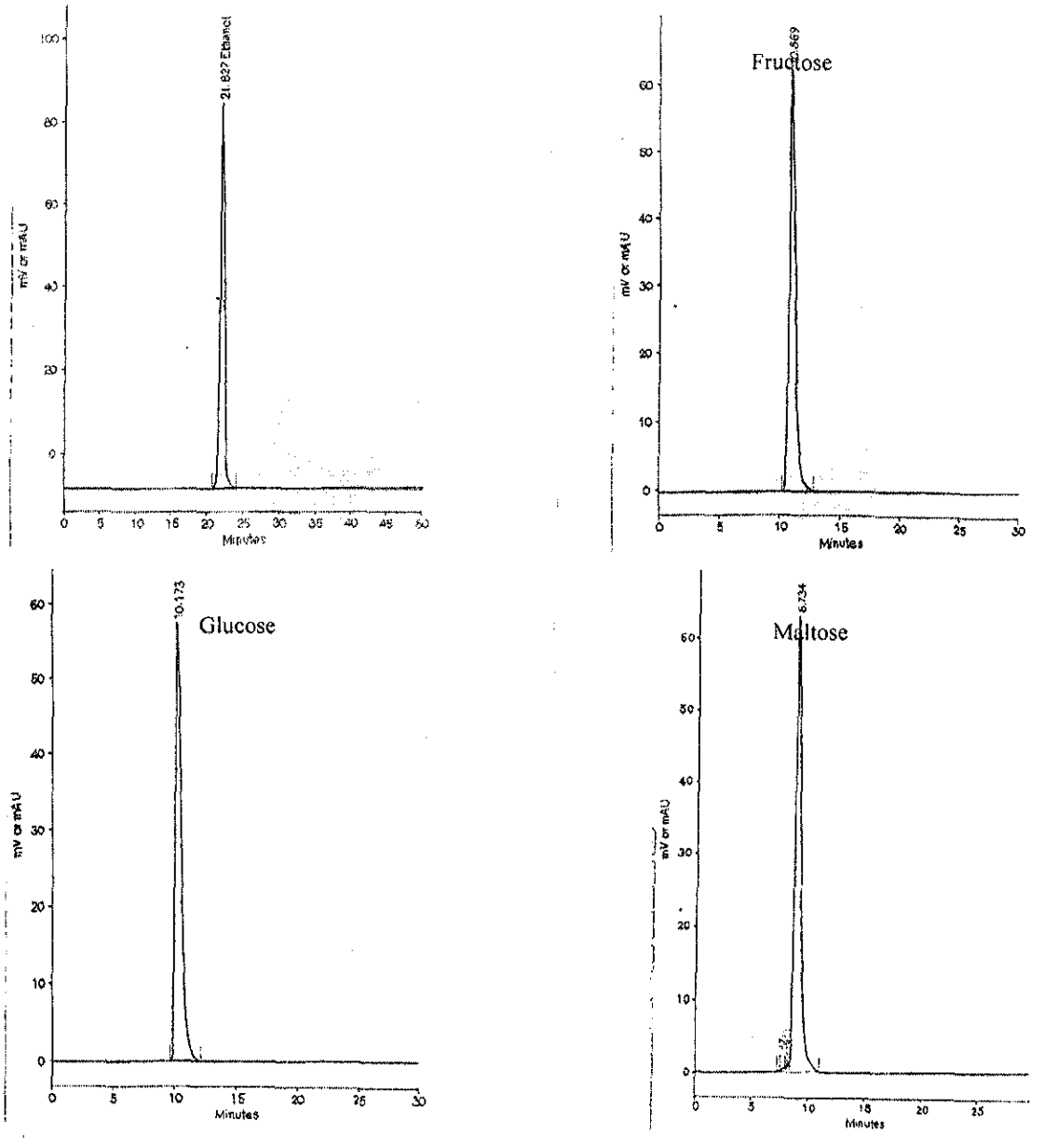

Figure 4B Chromatogram of standard ethanol and monosaccharides by HPLC

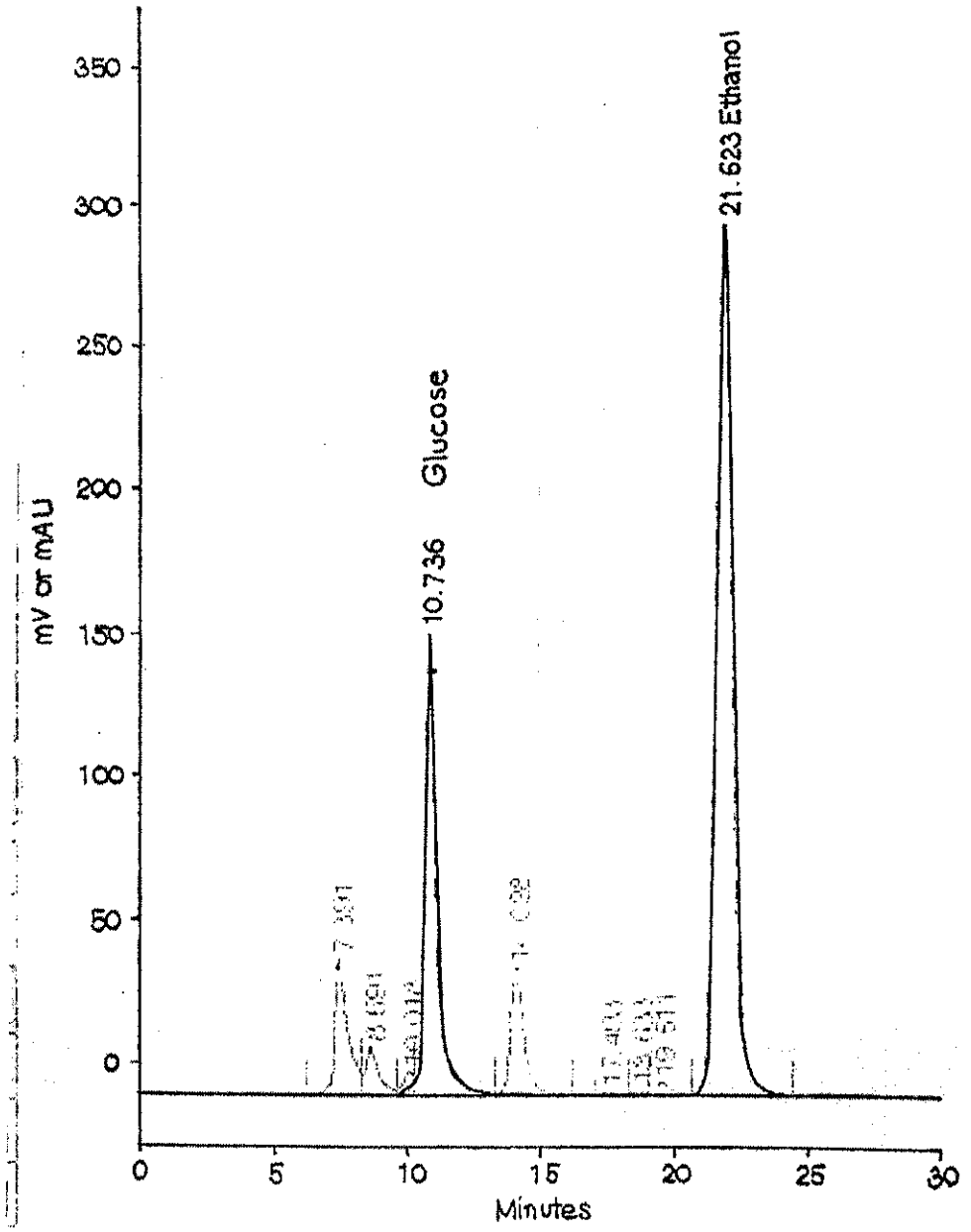

Figure 5B Chromatogram of ethanol and monosaccharide in finished rice wine sample (added malted rice with yeast)

3. Chromatograms of cyanide contamination by Polarography and Voltammetry

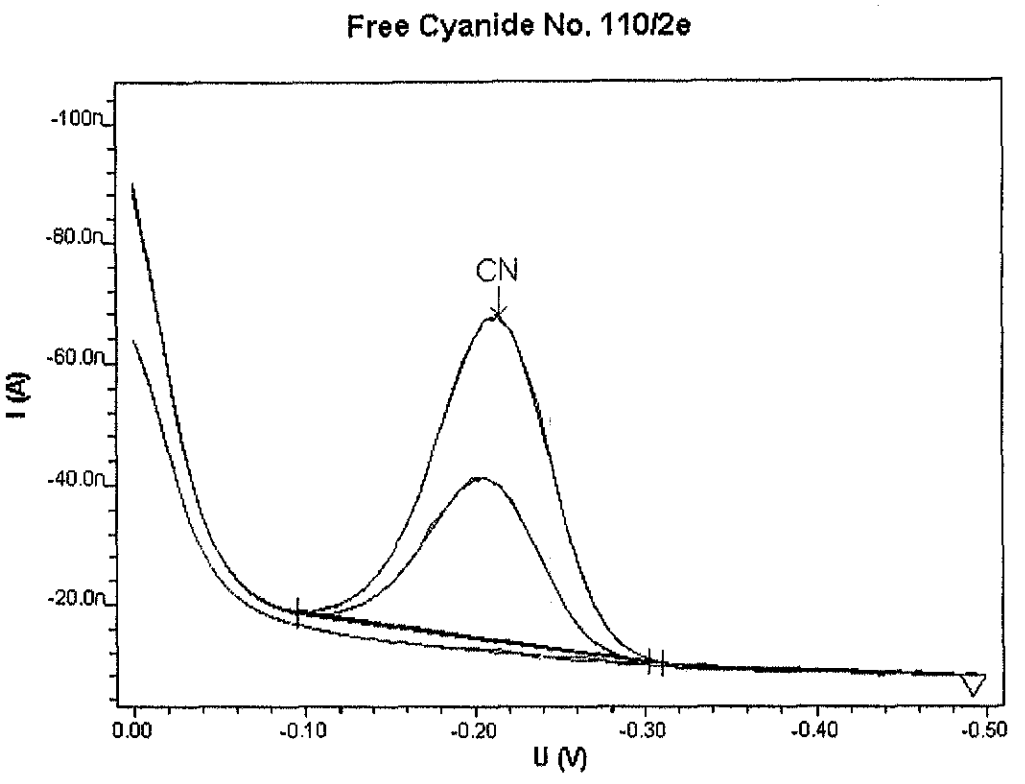

Figure 6B Chromatogram of standard cyanide tested in distilled water by 797 VA Computrace

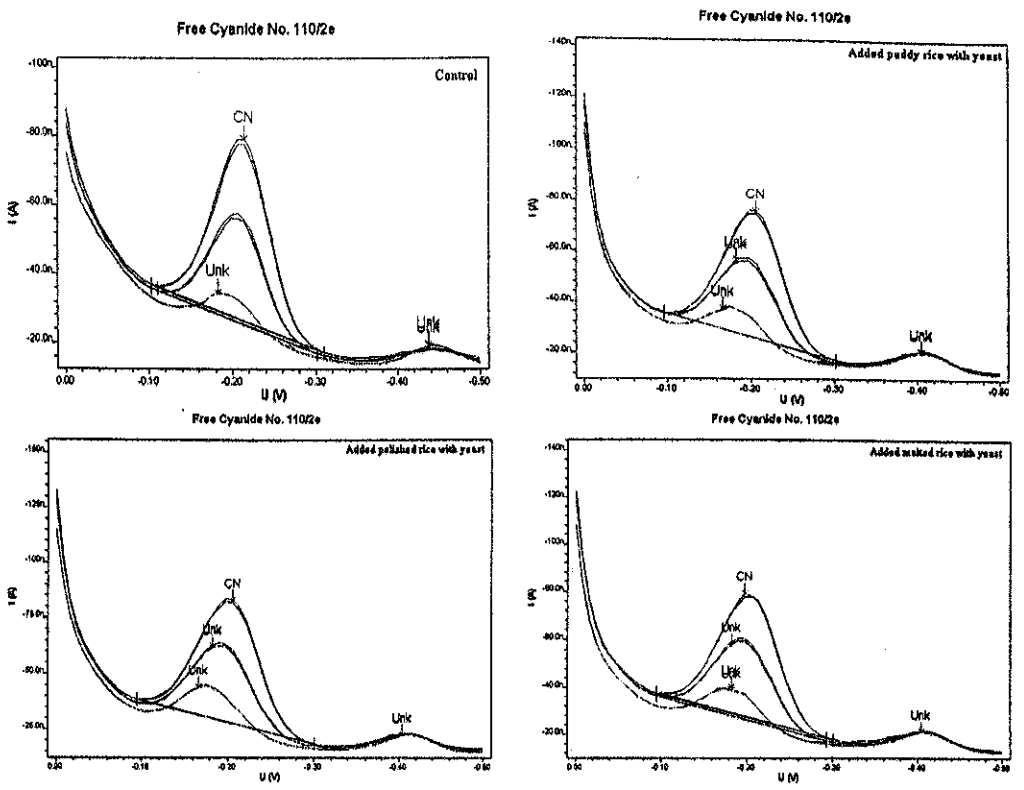

Figure 7B Chromatogram of cyanide analysis in rice wine sample

BIOGRAPHY

Nuttawan Lertpinyochaithaworn was born in Ratchaburi, Thailand on February 1st, 1982. She studied in primary school at Wantamaria Ratchaburi School, Then finished high school from Daruna Ratchaburi School in Ratchaburi. In 1999, she studied in School of Public Health, majoring in Occupational Health and Safety, Suranaree University of Technology, Nakhonratchasima. She participated in the Co-operative Education Program to work as assistant safety officer at Seagate Co. Ltd., Nakhonratchasima. She was trained in ISO14000 program. She graduated the Bachelor's of science in Occupational Health and Safety in 2003. After graduation, in 2003, she was Master's student in the School of Biotechnology at Suranaree University of Technology. During Master's student, she had experience in poster presentation in the title "Ma-Maow Wine Production" was presented in The 31st Congress on Science and Technology of Thailand, October 18-20, 2005, Technopolis, Suranaree University of Technology.