BEER PRODUCTION FROM THAI RICE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2008

การผลิตเบียร์จากข้าวไทย

นางสาวอุลัยวรรณ์ อุสันสา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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BEER PRODUCTION FROM THAI RICE

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อุลัยวรรณ์ อุสันสา : การผลิตเบียร์จากข้าวไทย (BEER PRODUCTION FROM THAI RICE) อาจารย์ที่ปรึกษา : รองศาตราจารย์ ดร.หนึ่ง เตียอำรุง, 165 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อหาสภาวะที่เหมาะสมสำหรับกระบวนการผลิตเบียร์จากข้าวไทยโดยทำการศึกษาผลของอุณหภูมิ (20, 25 และ 30 องศาเซลเซียส) และระยะเวลาการแช่เมล็ดข้าว (24, 48 และ 72 ชม.) ที่มีต่อกิจกรรมของเอนไซม์อัลฟ่าและเบต้าอะไมเลสในข้าวที่ใช้ในการทดลองทั้ง 6 สายพันธุ์ แบ่งเป็นข้าวเจ้า 3 พันธุ์ คือ ข้าวหอมดอกมะลิ 105, ข้าวปทุมธานี 60 และข้าวเจ้าดำ และข้าวเหนียว 3 พันธุ์คือ ข้าว กข.6 ข้าวเหนียวสันป่าตองและข้าวเหนียวดำ ซึ่งกิจกรรมของเอนไซม์ทั้งสองสูงสุดที่อุณหภูมิ 30 องศาเซลเซียส และการแช่ข้าวในน้ำต่อเนื่องเป็นเวลานานกว่า 24 ชม. ส่งผลในการยับยั้งกิจกรรมของเอนไซม์เบต้าอะไมเลสและชะลอแอคติวิตี้ของอัลฟ่าอะไมเลส จึงทำการปรับวิธีการแช่น้ำเป็นแบบแช่น้ำระยะสั้นสลับกับการผึ่งลม โดยศึกษากับข้าวสองสายพันธุ์คือ ข้าวเจ้าดำและข้าวเหนียวดำ โดยมีปัจจัยที่ทำการศึกษาคือ อุณหภูมิ ระดับความชื้นจากการแช่น้ำ และระยะเวลาการงอก ด้วยการออกแบบการทดลองด้วยวิธีการตอบสนองบนพื้นผิว พบว่า สภาวะในการทำมอลท์ที่ให้คุณภาพมอลท์ที่มี cold water extract, extract yield, Kolbach index, ปริมาณกรดอะมิโนอิสระ, apparent attenuation limit และเอนไซม์อัลฟ่าอะไมเลสสูงคือเพาะข้าวที่อุณหภูมิ 30 องศาเซลเซียส, โดยแช่น้ำด้วยวิธีแช่สลับจนความชื้นเท่ากับ 44 เปอร์เซ็นต์ ระยะเวลาการงอกรวมเป็นแปดวัน และอบแห้งที่ 50 องศาเซลเซียสเป็นเวลา 24 ชั่วโมง หลังจากทำความสะอาดมอลท์พบว่าการสูญเสียน้ำหนักอยู่ที่ประมาณ 12%

มอลท์ข้าวถูกผลิตตามสภาวะดังกล่าวแล้วทำการศึกษาช่วงอุณหภูมิที่ใช้ในการ mashing และเปรียบเทียบวิธีการ mashing 4 แบบ พบว่ามอลท์ข้าวเจ้าดำเหมาะสำหรับการ mashing ที่เพิ่มอุณหภูมิ 52, 55 และ 57 องศาเซลเซียสเพื่อเน้นการผลิตน้ำตาลกลูโคสและมอลท์ข้าวเหนียวดำเหมาะกับการที่เพิ่มอุณหภูมิ 62 และ 64 องศาเซลเซียสเพื่อการผลิตน้ำตาลมอลโทส โดยพบว่าค่า pH ของ mashing-in และ divalent cation ที่เหมาะสมคือ pH 5.2 และ Ca^{2+} ปริมาณ 150 มก./ลิตร นอกจากนี้การบดข้าวมอลท์ยังส่งผลต่อปริมาณกรดอะมิโนอิสระในเวิร์ทจากมอลท์ข้าวเจ้าดำและข้าวเหนียวดำ สำหรับมอลท์ข้าวเจ้าดำเหมาะสมกับการบดด้วยวิธี two roller mill โดยปรับระยะห่างของลูกกลิ้งอยู่ที่ ระยะ 0.5 มม. และมอลท์ข้าวเหนียวดำเหมาะกับการบดหยาบที่ระยะ 1.0 มม. จากนั้นทำการผลิตเวิร์ทด้วยสภาวะที่เหมาะสมดังกล่าวโดยใช้เครื่อง brew master mashing unit และให้ผล brewing yield ที่ 39±0.2% สำหรับมอลท์ข้าวเจ้าดำและ 38.4±2.8% สำหรับมอลท์ข้าวเหนียวดำ จากการหมักเบียร์ด้วยยีสต์สองสายพันธุ์ คือ *Saccharomyces cerevisiae* 34/70 สำหรับการหมักแบบ bottom fermentation และ *S. cerevisiae* 60/120 สำหรับการหมักแบบ top fermentation พบว่ายีสต์สายพันธุ์ 60/120 ใช้กรดอะมิโนอิสระ (215 มก./ลิตร จากเวิร์ทข้าวเจ้าดำและ168 มก./ลิตร

จากเวิร์ทข้าวเหนียวดำ) สูงกว่าสายพันธุ์ 34/70 (125 และ 109 มก./ลิตรในเวิร์ท จากข้าวเจ้าดำและข้าวเหนียวดำ ตามลำดับ) และพบปริมาณ ester, higher alcohol, diacetyl และ 4-vinyl guaicol ใน ale beer สูงกว่าที่พบใน lager beer จากผลการทำ sensory test พบว่าคะแนนความประทับใจโดยรวมต่อ ale beer ที่ผลิตจากมอลท์ข้าวเจ้าดำมีคะแนนอยู่ในระดับดื่มไม่ได้ แต่ ale beer จากมอลท์ข้าวเหนียวดำจัดอยู่ในระดับดื่มได้แต่ไม่ขอแก้วถัดไป ขณะที่ lager beer จัดอยู่ในระดับคะแนนดื่มได้และต้องการแก้วถัดไป การศึกษานี้บอกให้ทราบว่ามอลท์จากข้าวดำมีศักยภาพในการนำมาใช้เป็นวัตถุดิบในการผลิตเบียร์ที่เป็นที่ยอมรับของผู้บริโภคได้

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

ULAIWAN USANSA : BEER PRODUCTION FROM THAI RICE.

THESIS ADVISOR : ASSOC. PROF. DR. NEUNG TEAUMROONG, Ph.D.,

165 PP.

BEER/ FERMENTATION / MALTING/ MASHING/ RICE/ SENSORY EVALUATION

The aim of this research was to investigate the optimal condition for brewing processes of rice beer. The malting condition was investigated in terms of temperatures (20, 25 and 30°C) and steeping durations (24, 48 and 72 h) on α- and β-amylase activities of six Thai rice cultivars, including three non waxy rice cultivars (Khaw Dok Mali 105 (KDML105), Pratum Thani 60 (PT60) and Khao Chao Dam (KCD)) and three waxy rice cultivars (San Pa Tong (SPT), Khokho 6 (RD6) and Khao Niew Dam (KND)). It was found that amylolytic activities of the rice malt were increased with temperature, better at 30°C than 25°C and 20°C; and that activities of the two enzymes were higher than 25°C and 20°C respectively; whereas long steeping duration for more than 24 hours inhibited the activities of β-amylase and retarded α-amylase. Therefore, the steeping condition was modified to the short steeping-air-rest switching regime. The black non-waxy rice "KCD" and black waxy rice cultivars "KND" were selected to optimize the germination condition under the different temperatures, steeping degree and duration of germination. Response surface methodology was used to design experiment as face centered composite design and to establish empirical models for each malt properties.

It was also found that both types of rice had satisfied properties, high cold water extract (CWE), extract yield, Kolbach index, free alpha amino acid (FAN), apparent attenuation limit (AAL), and α- amylase activities by germinating at temperature 30°C, steeping by air-rest switching until the degree of steeping reached 44%, and germinating for 8 days and dried at 50°C for 24 hrs. The cleaned malt had malting losses approximately 12%. These malts were further used for mashing analysis. The 4 mashing regimes for rice malts were investigated for improving the wort quality. The results demonstrated that the temperature-programmed which focused on glucose production in a range of 52, 55 and 57°C was suitable to produce wort from KCD rice malt. The temperature-programmed which focused on maltose production at 62 and 64°C was selected for KND rice malt. The pH of mashing-in and divalent cations strongly influenced wort soluble nitrogen and FAN, and the optimal mashing-in pH for both rice malts was at 5.2 supplemented with Ca^{2+} 150 mg/L. The method of grinding using two roller mills influenced FAN content significantly; therefore, KCD was milled at a gap distance of 0.5 mm and KND at 1.0 mm. The selected mashing conditions were used to produce wort by using a brew master mashing unit and 39±0.2% of brewing yield was obtained by KCD malt and 38.4±2.8% by KND malt. Beers were produced by using *Saccharomyces cerevisiae* 34/70 and *S. cerevisiae* 60/120 for bottom and top fermentation processes, respectively. The yeast strain 60/120 consumed FAN in wort (215 mg/L and 168 mg/L for KCD and KND wort, respectively) more than strain 34/70 (125 and 109 mg/L for KCD and KND wort, respectively). Consequently, volatile compounds such as ester, alcohol diacetyl and 4-vinyl guaicol in ale beer were higher than in lager beer. The sensory evaluation of beers indicated that the overall impression of ale beer from KCD malt

was undrinkable, ale beer from KND malt was drinkable but not preferable for the next glass, whereas the lager beers from both rice cultivars were judged as drinkable and preferable for the next glass. The results obtained from this research clearly demonstrated that black rice malt could be used as raw material for producing beers with an acceptable quality.

School of Biotechnology

Academic Year 2008

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ACKNOWLEDGEMENTS

I would like to express the deepest gratefulness to my thesis advisor, Assoc. Prof. Neung Teaumroong, for his support and kindness during this lengthy work.

I am also deeply grateful to my co-advisor, Asst. Prof. Dr. Chokchai Wanapu for his guidance, encouragement, and kindness. I would love to express my grateful to Univ.-Prof. Dr.-Ing. Eberhard Geiger for his guidance and support in everything during I handled parts of my thesis in Germany.

I wish to express my gratitude to the dissertation committee, Prof. Dr. Nantakorn Boonkerd, Asst. Prof. Dr. Nareerat moonjai and Asst. Prof. Dr. Charin Techapun for their advise and giving points to fulfill of this report.

The special thanks for colleague of Dr. Chokchai (Tadam, Dang, Koy, Rong, P' Hwaen, Kye, Bom and Bow) and P'Lak, P'Wanchai, P'Ine, and P'Pook have to be remarked for their helps, encouragements and standing beside me in happy or even hard time.

I would like to thanks my parent, my grand daughter "Toon" and P'Bert for their supports and faith in me. They have always been a great source of inspiration to take on any challenge in life.

Finally, this work would not be completed without the supports from The Thailand Research Fund through the Golden Jubilee Ph. D. Program, grant no. 2. B. TS. / 47/ C.1 and Suranaree University of Technology.

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
AAL	=	Apparent attenuation limit
C.V.	=	Coefficient of variation
°C	=	Degree celsius
DFP	=	Diisopropylphosphorofluoridate
et al.	=	et alia (and others)
EDTA	=	Ethylenediaminetetraacetic acid
g	=	Gram
g	=	Gravity
FAN	=	Free alpha amino acid
h	=	Hour
L	=	Liter
µm	=	Micrometer
mg/L	=	Milligram per liter
mL	=	Milliter
mM	=	Millimolarity
min	=	Minute
nm	=	nanometer
N	=	normality
%	=	Percentage

LIST OF ABBREVIATIONS (continued)

PMSF	=	Phenylmethanesulfonyl
P. I.	=	Predicted interval
%(w/v)	=	Percentage weight by volume
%(w/w)	=	Percentage weight by weight
SEM	=	Scanning electron microscope
Ti	=	Trypsin inhibitor
U	=	Unit

CHAPTER I

INTRODUCTION

1.1 Rationale of the Study

Asia is an important region for rice production. There are also some countries produce rice for local consumption and export, particularly in China and Vietnam. The low cost, field management, irrigation and so on are the reasons which brought these countries became the world exporters and the great competitors to Thailand. The products which are well known as the world class rice exporter of a special variety; a fragrant rice, white long grain, soft and sweet taste called “Jasmine rice”. However, Thai rice varieties are high of diversity and spread over the country, some rice are promoted to be the commercial rice and most of the rest are local native rice which have been conserved for breeding. Since its diversity, several properties of rice have been investigated some perform appropriate characters for the selection of rice for brewing application.

Recently beer is the most popular alcoholic beverage in the world and the share market is risen up in Asia every year. In Thailand, 2,072.2 million liters of beer were sold in year 2007 and it was approximately 6 % increasing from year 2006 and 5% increasing in year 2008 (Positioning, www, 2009). Among this, the low price beer made with adjunct was accounted at least 80% due to the economic problems made consumer chose them. Therefore, the low price beer is the favorite product and the alternative sources of carbohydrate are attractive for brewery. There are many

attempts to produce beer from alternative cereal malts and adjuncts in order to attribute special characters of beer; for example, a white beer from wheat, dunkel weissbier from dark wheat malt with dark barley malt, African kaffir beer from sorghum malt, tesguino in central America from maize malt and zutho in India from rice malt, etc (Teramoto, Yoshida, and Seinosuke, 2000). The other important purposes was reduction of haze forming protein in barley beer and mong all of the alternative sources of carbohydrate for brewing, wheat is the most using cereal for wheat beer or weisen beer in Germany, whereas rice is second most-widely used as adjunct in the USA (Stewart, 2006).

Rice are widely used as adjunct established in USA and Japan for dilution of extract from barley because too high extract substances cause haze and off-odor to the beer (Pomeranz, 1972). However, using of high ratio of adjunct influence wort quality, therefore it was not employed higher than 40% (Van, 2001). The main effect of using high ratio of rice as adjunct is the dilution of nitrogenous substances because of low protein content in rice. Therefore, using high ratio of adjunct needed enzymes to improve wort quality and there are many attempts to add other sources of protein and enzymes application (Van, 2001; Vinh, Vie, and Mai, 1993). Among those solutions, application of high protein malted rice has never been reported. Therefore, the fulfillment of this data would be necessary to perform and it will be useful for further brewing of 100% rice as a gluten-free beer for celiac patient. Celiac disease is a lifelong digestive disorder caused by a reaction to gliadin, a gluten protein found in wheat (and similar proteins of the tribe Triticeae which includes other cultivars such as barley and rye) and also known as gluten sensitive enteropathy (GSE). Upon exposure to gliadin, the enzyme tissue transglutaminase modifies the protein, and the

immune system cross-reacts with the bowel tissue, causing an inflammatory reaction. That leads to flattening of the lining of the small intestine. This interferes with the absorption of nutrients because the intestinal villi are responsible for absorption. One out of 133 people in the United States is affected with celiac disease and the only effective treatment is a lifelong gluten-free diet (Celiac disease foundation, www, 2009). Therefore, rice beer could be one choice for the celiac patient and the technique for rice beer production must be investigated under this research.

1.2 Research objectives

This research aims to develop brewing rice technology in order to increase the value of rice, with sub-objectives are

1. To optimize rice malting condition
2. To develop wort production technology from rice malt
3. To develop fermentation technology of rice beer by using top and bottom fermenting yeasts
4. To determine quality of finished product

1.3 Research hypothesis

Rice malts have efficiency to be used for brewing as a whole source of carbohydrate of beer. The modification of brewing process could enhance the quality of rice beer therefore it could be drinkable and accepted by consumer as new product in the market.

1.4. Scope and limitations of the study

This research is the preliminary study of small scale fermentation. The research boundary is related to the sub-objectives described above. Firstly, study of

malting condition for six rice varieties. The finest malted rice was selected to study of mashing condition, and fermentation condition. Then, chemical, physical property and sensory evaluation were examined.

1.5 Expected results

Results obtained from these studies led to more understandings of malting process for rice and the knowledge of effect of mashing condition on wort quality could be useful for application in industry. Furthermore, the quality of finished product of rice beer production could be a guide for selection of yeast strain and fermentation type.

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CHAPTER II

LITERATUR REVIEWS

Rice and brewing technology has long history since the brewers use rice as adjunct to balance protein content in wort from barley, to adjust flavor of beer and to reduce cost. However, rice contain low protein content (approximately 5-7%) and it is one reason of limited ratio (up to 40%) for using in brewing process (Van, Strehaiano, Nguyen, and Taillandier, 2001). In order to increase the amount of rice used in brewing process, the low content of rice protein, insufficient of soluble nitrogen in wort and beer, are problems which must be overcame. Therefore, this research offers the using of malted rice as a whole source of protein and carbohydrate for beer production. According to the aims of this research, rice properties and brewing processes, including of malting, mashing, wort boiling and fermentation process, were reviewed in this chapter.

2.1 Rice chemical compositions and rice properties

Rice (*Oryza sativa* L.) belongs to the *Porceae Gramineae* or grass family. It has been consumed by humans for at least 5,000 years and the *O. sativa* L. indica is the major rice species planted in Thailand, India, South of China and Malay Peninsula. Particularly in Thailand, there are more than 100,000 samples kept at International Rice Research Institute (IRRI) and more than 24,000 rice samples in the national rice bank (Wutthiyano, 2000). Thai rice is very high of diversity and approximately 85 cultivars have been promoted for plantation year round;

consequently, wide rice properties could be obtained and selected for using in brewing process.

According to the chemical compositions in cereals, carbohydrate and protein are main components of the grain and play an important role to rice properties and to brewing process. Rice regards as low protein and low fat grain, whereas carbohydrate ratio is not much different from wheat and corn (Table 2.1). Carbohydrate is the major composition in rice grain and most of them are starchy endosperm. Rice could be classified by regarding the amylose content; waxy rice (0-2% amylose), very low (5-12%), low (12-20%), intermediate (20-25%) or high (25-33%) (Juliano et al., 1981). Amylose and amylopectin are starch subunits, amylose is a linear polymer of α-(1→4)-linked D-glucopyranosyl units with few α-(1→6)-linkages (<0.1%) and amylopectin consists of α-(1→4)-linked D-glucosyl chains and it is highly branched with 5-6% α-(1→6)-linkages (Buleon, Colonna, Planchot, and Ball, 1998). Starch granules of rice are polyhedral small granules (2-7 μm) produced in one amyloplast and they form the compound granule, whereas only one starch granule of barley (*Hordeum vulgare*), maize, sorghum or wheat are produced in one amyloplast (Van et al., 2001). Another technique used to studied rice starch crystallinity is the X-ray diffraction, the diffraction patterns of native starch granules can be classified as A-type (cereal starches of rice, wheat, and maize), B-type (tuber and root starches of, e.g potato, tapioca high amylose maize and retrograded starch) and C-type starches which are the mixtures of A-type and B-type patterns (leguminousae starch of pea, bean and tropical starch of cassava) (Buleon et al., 1998). Changes of starch quality in many processes depend on type and property of starch itself. There were some reports of starch properties (e.g degree of crystallinity, size of starch granules, amylose content)

influence to enzyme susceptibility (Madhusudhan and Tharanathan, 1995; Matsubara et al., 2004). According to the type of starch granules classified by degree of crystallinity, A-type of most cereals and tapioca starches are more readily hydrolyzed by α-amylase than B-type of amylomaize and potato starches (Planchot, Colonna, Gallant, and Bouchet, 1995). The amylose content also influenced on this ability, waxy type had higher susceptibility to α-amylase than normal and high amylose rice, respectively (Li, Vasanthan, Hoover, and Rossnagel, 2004). Most of these studies were made *in vitro*, whereas the modification of rice starches in malting process was occurred in *vivo*. Therefore, enzymes found in germinating rice also play important roles on starch and protein digestion. Since enzymes are protein, concerning of cereal protein and enzyme production in malting process should be emphasized.

Table 2.1 Comparative chemical compositions of five whole cereal grains.

Property	Brown rice	Wheat	Corn	Barley	Sorghum
Protein (%)	7.3	10.6	9.8	11.03	8.3
Crude fat (%)	2.2	1.9	4.9	3.4	3.9
Carbohydrate (%)	64.3	69.7	63.6	55.8	58.0
Crude fiber (%)	0.8	1.0	2.0	3.7	4.1
Crude ash (%)	1.4	1.4	1.4	1.9	2.6

At 14% moisture. From Rice chemistry and technology (p. 7), Juliano, 1985, Minesota: American Association of Cereal Chemist, Inc.

Since storage protein is the main source of proteolytic activity in malt and

source of free alpha amino acid (FAN), peptide and high molecular weight protein in wort, the initial protein content in raw cereal must be concerned. Among cereals, wheat (*Triticum spp*.) contain highest protein 12 % (w/w), two row barley contains 8-11% (w/w) and rice protein is average 5-7% (w/w) (Juliano, 1985). However, in some cases, if the high protein grain was used, protein seems to play a minor role in determining the quality of malt (Owuama, 1997). Agu and Palmer (1998) indicated that sorghum contained 8-11% protein is an acceptable level for effective proteolysis during malting. Therefore, cultivar selection with respect to protein content could be one way to overcome of poor malt quality.

Up to 95% of the endosperm rice protein is in the form of discrete particle called protein bodies (PBs). There are three types of PBs have been reported, large spherical protein bodies found in sub-aleurone region and small spherical protein bodies are called as PB-I, and crystalline protein bodies called PB-II (Juliano, 1985). PB-I is enriched with prolamines and PB-II is enriched with glutelin. According to Osborne's classification, essentially proteins are separated into albumin (water soluble fraction), globulin (salt-soluble fraction), prolamin (alcohol-soluble fraction) and glutelin (alkaline-soluble fraction). Even thought, new approaches showed that the fractions are actually composed of mixtures of protein, Osborne's fraction still be accepted in recently (Juliano, 1985). Regarding Osborne's fraction, rice has albumin 5-15 % total, globulin 2-10 %, prolamin 1-5% and glutelin 75-90 %, whereas barley has 3-10%, 10-20%, 35-50% and 25-45 %, respectively. Rice proteins are functionally unique compared to those in corn, wheat, and potato in being richest in glutelin and lowest in prolamin. Glutelin is the major storage protein in rice and is extremely insoluble because of the intermolecular disulfide linkages and high

molecular weight. It is soluble below pH 3 and above pH 10. The understanding of rice property changes in brewing process could not be possibly without the review of brewing process and brewing technology in recently; in addition, research of rice in brewing science will be mentioned. There are several steps in beer production, including of malting, wort production, fermentation, and maturation are mentioned here.

2.2 Malting process

Malting is the process to produce yeast nutrient such as fermentable sugar and free amino acid by activity of enzymes synthesized during seed germination. The steps of malting are usually given as steeping, germination and kilning. The understanding of germination is the main idea of malt production and the major events associated with seed germination are illustrated in Figure 2.1. Germination is defined as the sum of events that begin with hydration of the seed and culminate in emergence of the embryonic axis (usually the radicle) from the seed coat (Srivastava, 2002).

2.2.1 Steeping

After grains steeped in water, they swell and increase bulk volume by up to about 25% (Briggs, 1998). The water enters the kernel primarily through the micropyle region at the base of embryo where the testa is thin or absent (Lewis and Young, 1995). The embryo always takes up water much faster and has higher moisture content (55-60%) than the endosperm (30-40%). The rate of water uptake depends on temperature, time and physiology of seed such as size, thickness of husk and variety. Eneje et al., (2004) mentioned that steeping time affected to enzyme activity; particularly, the diastatic power (DP: the amount of beta-amylase activity)

increased with steeping time. Examples of steeping program in Table 2.2 showed that steeping programs were varied at temperature and time which depended on cereal types and varieties (Capenzana and Buckle, 1997; Dewar, Taylor, and Berjak, 1997; Eneje et al., 2004).

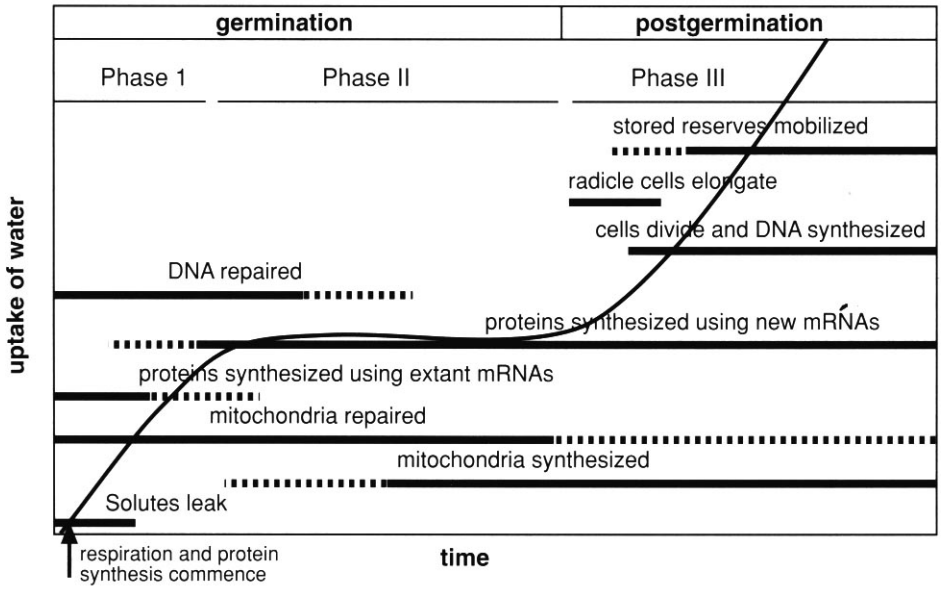

Figure 2.1 Time course of major events associated with seed germination and seeding growth. The time for events to be completed varies from hours to many weeks, depending on the plant species and the germination conditions (Bewley and Black (1994) quoted in Srivastava (Ed.) **Plant growth and development** (p. 448). London: Academic press.

Barley prefers steeping temperature lower than 20°C, mostly done in the range of 12-18°C. Below these temperatures, seed will be germinated at slower rate and the insufficient of oxygen can occur at higher temperature. In recently, air rest system is widely performed in steeping process, the aeration and ventilation system

are also applied to add some oxygen and remove carbon dioxide, the product of seed respiration.

As mentioned above, the oxygen demand is critical factor for steeping grain. Dewar et al., (1997) compared aeration system with non aeration in steeped sorghum grains. The result showed that malt DP and free amino nitrogen were high in aeration condition. Moreover, it has been stated that adequate oxygen is necessary for the formation of alpha-amylase and peptidase, and the excessive carbondioxide inhibit the formation of these enzymes.

After steeped grains for a period, their moisture contents increased, and absolute steep-out moisture content may vary which depends on the grain size and cultivar. Generally, the higher steep-out moisture gives better quality malt, comparing in between the same cultivar. The optimum moisture content for barley germination is 45-50% at temperature 12-17 °C. For sorghum, the maximum hydration obtained is usually 33-36% (Agu and Palmer, 1997; Evan and Taylor, 1990). However, the limited level of water suggests the limited permeability of the pericarp or poor hydration potentials of endosperm.

2.2.2 Germination

The steeped grains germinate under the humidified condition, and many enzymes are synthesized and hydrolyzed stored reserves. The biochemical changes during seed germination are complex and mostly studied with barley; therefore, it is used as model of seed germination. A technical term of “modification” is used to describe the breaking down of cell walls and the conversion of stored reserves to be mobilized substances. The step of modification of barley grain is illustrated in Figure 2.2.

Table 2.2 Steeping programs for different barley cultivars.

Barley cultivars	Steeping program					
	1st steeping	1st air rest	2nd steeping	2nd air rest	3rd steeping	3rd air rest
Optic, (Ireland) [a]	14°C, 6 h	16°C, 9 h	14°C, 5h	16°C, 10 h	14°C, 5 h	16 °C, 4 h
Decanter and Chariot, (Scotland) [b]	16°C, 8 h	16°C, 16 h	16°C, 24 h	-	-	-
Cheri, Airone, and Amillis [c]	14°C, 7 h	14°C, 17 h	14°C, 7 h	14°C, 17 h	-	-
Triumph, (Scotland and Spain) [d]	16°C, 8 h	16°C, 12 h	16°C, 10 h	16°C, 10 h	16°C, 2 h	-

[a] Lowe, Arendt, Soriano and Ulmer (2005), [b] Roberta and Palmer (2004), [c] Toffoli, Gianinetti, Cavellero, Finocchiaro and Stance (2003), [d] Swanton, Sopena, Moalejo, and Molina-Cano (2002).

Start with aleurone layer is activated by Gibberellic acid (GA), the bulk of enzymes are synthesized. At this step, enzymes released from the aleurone layer to hydrolyze starchy endosperm. However, the major grain compartments involved with enzyme synthesis are different among cereal type. In case of sorghum, the major part for the synthesis of many hydrolytic enzymes is embryo (Aniche and Palmer, 1990; Varner and Chandra, 1964). Whereas, rice hydrolytic enzymes are synthesized in scutellar epithelium and aleurone layer (Ranjan, Karrer, and Rodriguez, 1992).

In barley and most cereal endosperms have protein matrix wrapped starchy endosperm; consequently, protein hydrolysis occurs before starch hydrolysis. During seed germination, the storage proteins are degraded into small peptides or amino acids which are subsequently transported to the growing part. Jacobsen and Varner,

(1967) reported that most of protease enzymes are response to GA and increase with germinating time. The degradation of these storage proteins is governed by group of proteinase enzymes found in germinating seed. There are four groups of proteinase enzymes classified by the active site residue and rice has been reported all of four groups found in rice endosperm and sub-aleurone region (Table 2.3) (Barrett, 1986).

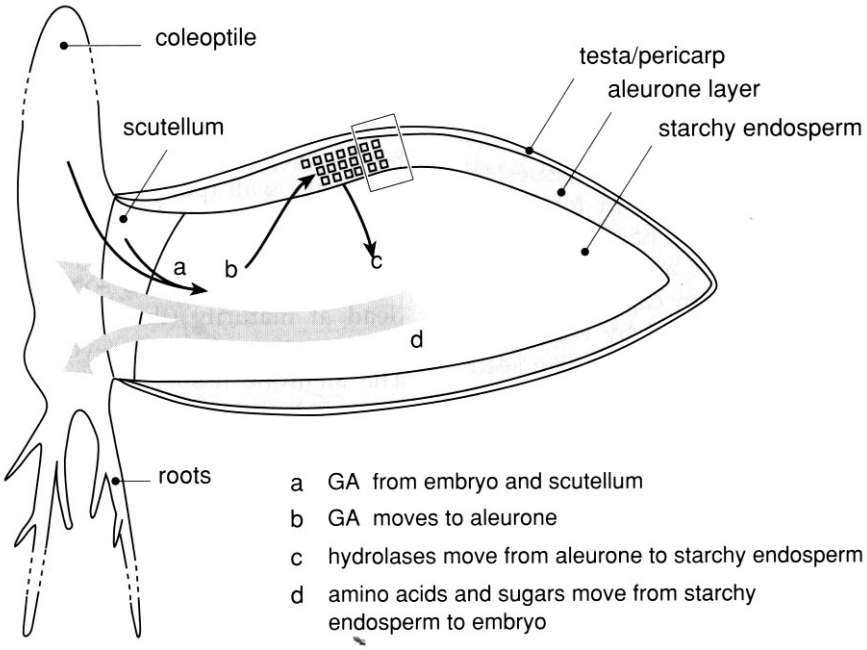

Figure 2.2 A sagittal section of a germinated grain showing the movement of GA from the embryo to the aleurone layer where the hydrolyzing enzymes are activated (Srivastava, 2002).

They are cysteine proteinase, serine proteinase, aspartic proteinase and metalloproteinase or carboxylases. Cysteine proteinase (EC. 3.4.22) is mostly mentioned in germinating seed responsible for degradation and mobilization of storage protein. In germinating barley, 42 proteinases are involved and among them 27 are cysteine proteinases (Zhang and Jones, 1996). Rice cysteine proteinase or

oryzain has been purified and characterized (Abe, Kondo, and Arai, 1987) and studied of gene expression which enhanced by gibberellic acid (GA_3) (Watanabe, Abe, Emori, Hosoyama, and Arai, 1991). There are three types of oryzains; α, β, and γ- oryzain found in rice grains. Oryzains α and β, which highly similar to papain, are expressed continuously during germination with maximum expression at day fifth of germination time, whereas γ- oryzain is similar to cathepsin H (Watanabe et al., 1991). There was an evident for protein break down in subaleurone cell during rice germination (Horikoshi and Morita, 1982). The modification of protein body PB-I and PB-II had different patterns of protein break down. The PB-I was digested from the outer region at the middle and later stages of germination whereas PB-II was digested from the central region at the early stages of germination this showed that it might contain latent protease which was activated during seed germination.

Table 2.3 Classes of endoproteinase.

Class	pH Optimum (Range)	Amino acid in active site	Diagnostic inhibitors	Proteins and Notable Characteristics
Serine proteinase	7-9	Ser, His	DFP, PMSF TI, aprotinin	Trypsin, Chzmothrypsin Elastase, Cathepsin(+)GT
Cysteine proteinase	4-7	Cys	Iodoacetate iodoacetamide	Papain, Ficin, Bromelain, cathepsins (+) B, C, H, K,L O S and W; thiol activate
Aspartic proteinase	Below 5	Asp, Try	Pepstatin	Cathepsin(+) D and E, Renin, Pepsin
Metalloproteinase	7-9	Metal ion	EDTA, 1,10 phenanthroline	Carboxypeptidase A and B, aminopeptidases: typical require zinc

Modified from: Callis J. (1995).

Moreover, there are some proteinase inhibitors found in cereal and to activate proteinase activity, these protein must be degraded. The 14-16 kDa proteins in germinating brown rice (GBR), which has similar sequence to α-amylase/trypsin inhibitor in other cereal, was decreased and then cysteine proteinase was activated to hydrolyzed glutelin, the major protein in rice (Kato and Minamikawa, 1996).

α-Amylase is an endohydrolase enzyme, which hydrolyzes α-(1, 4) glycosidic bond in amylose and amylopectin randomly, releasing oligosaccharides (Muralikrishna and Nirmala, 2005). *Beta*-amylase enzyme, (1, 4)-α-glucan malto-hydrolase, catalyses the release of maltose from the non-reducing ends of (1, 4)-α-glucans, this knows as "saccharifying" enzymes (Dunn, 1974). The branch points of containing α-1, 6 glycosidic linkages are resistant to attach by α- and β- amylases resulting in α/β limit dextrins. The debranching enzymes like pullulanase is capable to attack α-1,6 linkages releasing of dextrin, and glucoamylase enzyme is also capable to attack α-1,6 linkages releasing of glucose molecules. Some of monosaccharides and amino acid transport to embryo for root and shoot elongation, meaning loss of malt extract, and these phenomena must be retarded by control the germinating time and temperature (Iwuoha and Aina, 1997). Different grains have different malting conditions, which related to the genetic background of each cereal; for instance, barley is well known the ability to grow at 17-18ºC (Kunze, 2004). Whereas rice and sorghum malt preferred 30ºC which nearly to room temperature in Africa and Asia county (Capanzana and Buckle, 1997; Owuama, 1997), Oat malt has been germinated at temperature 16ºC (Mikola and Jones, 2000) and 20ºC for Rye (Koodziejczyk and Michniewicz, 2004). In summary, the biochemical changes in barley, rice, sorghum and other cereal are mostly the same, slightly different in

activity and property of enzymes (Iwuoha and Aina, 1997; Palmiano and Juliano, 1972). Therefore, enzyme activities and grain modification are important indexes for malt quality determination.

2.2.3 Kilning

After grains were germinated for a period and malt master satisfied in degree of modification in grains, the mechanism of enzymes in germination process must be stopped before the soluble sugar and soluble protein are exhausted from the endosperm. Drying is the main idea to reduce the moisture content in germinating grain to stop enzyme activities which is called “Kilning”.

During grains were drying, moisture content in germinated grain will be reduced from 45% to be 4-5% (Kunz, 2004). This reduction terminates enzyme activities and biochemical changes in grain, the color and some flavor of beer is developed in this step. There are several kinds of malt which classified by kilning temperature, light malt, dark malt or roasted malted, caramel malt and etc. The light malt is dried under low temperature which not higher than 80-85°C. It has good odor and provide good stability beer. Dark malt is firstly dried at low temperature to remove water and increase temperature to be 100-110°C for dark color. The products of Maillard reaction between sugar and amino acid provide dark color into malt and cooked odor. Low enzyme activity malts such as Buckwheat (Phiarais, Wijngaard, and Arendt, 2005) and sorghum were recommended to be dried at low temperature as low as possible 40-50°C. However, kilning sorghum green malt (with less than 10% moisture) at 100 °C for a limited time of 3 to 4 h apparently has no effect on the amount of hydrolytic enzymes and diastatic power (Pathirana, Shivayogasundaram,

and Jayatissa, 1983). Therefore, the kilning temperatures could be adjusted and varied to produce differing characteristics malt.

2.3 Malt quality

Selection of cereal malt for each brewery might concern about how easy of process control and what characters needed in beers. However, the comparison of malt qualities suggests that barley malt is a greater source of enzymes and suitable for brewing more than sorghum and wheat (Table 2.4). Barley cultivars for brewing used in recently, had been selected and developed by breeding since many hundred years until now, new scientist never stop to investigate for a better quality. Therefore, the competitive cereals also need many processes of plant breeding to get a proper malt quality for brewing. However, wheat and sorghum beers are commercially produced in many countries through the advanced brewing technologies developed for particular cereal. Thus, development of rice beer for Thai consumer may not hard to overcome.

Table 2.4 Comparison of qualities of barley malt and other cereal malts

Parameters	Buckwheat	Sorghum[d]	Barley
Extract (w/w)	69.2[c]	65-83.7	79.9[a]
FAN (mg/l)	107[c]	100	106.7[a]
Kolbach index	23.91[b]	15.3-41	31.24[b]
Alpha-amylase (U/g)	19.9[c]	39-135	105.9[a]
Beta-amylase (U/g)	24.7[c]	80-168	514[a]
Apparent Attenuation Limit (AAL)	61.8[c]	43-96	82.7[a]

[a] Wijngaard and Ulmer (2005), [b] Wijngaard, Ulmer, Neumann, and Arendt (2005), [c] Phiarais, Wijngaard, and Arendt (2005), [d] Briggs (1998).

2.4 Wort production

Mashing is the process of mixing ground malt with water in the mash tun to extract the malt, degrade haze-forming proteins, and further convert grain starches to fermentable sugars and non-fermentable carbohydrates (dextrin) that will add body, head retention and other characteristic to the beer (Rabin and Forget, 1998). The bulk of extract in wort arises from starch solution and digestion. Starch is a glucose polymer comprising of two kind of molecules: amylose, a straight chain of glucose residues linked α-(1,4) which make up 20-25% of total native starch and amylopectin, which is a similar molecule but with braches linked at the α-(1,6) position. A suspension of starch granules in cold water is not viscous. When heated, the granules absorb water and swollen until the starch granule broken, suspension suddenly becomes viscous as the starch gelatinizes or uncoils and hydrates. The gelatinization temperature depends on the source of the starch but most of cereal starch has gelatinization temperature 65-75°C. The lowest temperature at which maximum extract can be achieved, is a function of malt modification because the extent of modification influences the rate of starch solution (Lewis and Young, 1995). Therefore, well-modified malt might need only one temperature for mashing; for example, infusion mashing at 65°C for 2 h. Furthermore, the moderate modified malt or even poor modified malts need many steps of temperature rising for activation of hydrolytic enzymes. Among them, there are two amylase enzymes play an important role in starch hydrolysis: α-amylase and β-amylase. Their reactions have different optimal temperature. β-amylase has optimal temperature in range 55-60°C and α-amylase around 70-75°C. α-amylase tolerates higher temperature (70°C or even higher) than β-amylase. Degradation of enzyme also occurs in mashing step.

Moreover, there are proteases enzymes in mashing solution. The proteases work at temperature 45-50°C. Peptides and FAN are determined as product of enzyme activity in term of soluble nitrogen, whereas only free amino nitrogen content is determined as product of peptidase enzymes.

There are many types of mashing program for extraction of malt extract, but the two main types are infusion mashing and decoction mashing. The infusion mashing is classically a British method of brewing. It requires a single mash temperature called conversion temperature. This method requires well-modified malt and no adjuncts that require gelatinization. The mash, which is not boiled is sprayed with hot water to raise the mashing temperature gradually to 65-68°C for one or two hours. Another is decoction mashing or temperature program mashing is often used for bottom-fermenting beers and typical of German brewing practice. The process requires three vessels: a mash tun for mash mixing, a mash copper for boiling, and a clarifying tun for straining. The process is usually repeated two or three times, taking five to six hours. The mash temperature may start as low as 35°C but more often at 45-50°C to reach 70-76°C. Series of temperature rest, where the mash is held at a specific temperature in order to activate certain enzymes, are designed for individual cereal malt and the extent of modification in malt is the main idea used to design mashing program.

There is one more style of mashing called “double mashing”, original called the American double mashing system. In double mashing, under-modified material as adjunct can be treated separately from malt by single decoction and later mix with mashed malt (Lewis and Young, 1995). Eneje et al., (2001) studied the effect of mashing procedures on millet malt wort properties and found that the decoction

mashing produced wort with lower soluble nitrogen than the infusion mashing. Because this type mashing method denatured part of protein and cause greater degree of Maillard reaction. Agu and Palmer (1998) indicated that the extract recovery from the 65°C infusion mashing was low and noted that the limitation to maltose production in sorghum malt wort was caused by inadequate gelatinization of sorghum starch. Whereas, Igyor, Ogbonna, and Palmer (2001) recommended infusion mashing for barley malt and decantation mashing or double mashing for sorghum malt because barely malt has degree of modification in term of malt quality index higher than sorghum malt. Moreover, there are many researches in improving the mashing procedure to improve yield of extract and save cost of energy (Alvarez, Correa, Navaza J. M., and Riverol, 2000; Igyor et al., 2001; MacGregorf, Bazin, Macri, and Babb, 1999). Differences of cereal or malt quality need difference type of mash and temperature program.

The product of mashing is sweet solution with particles, before step of wort boiling it must be separated from mash particles. The lauter tun is the classical method for separation the solubilized organic compound from particles (Hardwick, 1995). Another method is the mash filter. This device is like a plate and frame filter for beer filtration. Filtration is initiated with the transfer of the mash to the plate and frame system from the top and the sparge water is pumped into the unit from the lower entrance. The mash filter occupies a much smaller space than a lauter vessel and has fewer revolving and mechanical parts. However, mash filter is more labor intensive but the two devices produce wort of essentially equal qualities (Lewis and Young, 1995).After the carbohydrates, proteins and yeast nutrients were extracted from the mash and clear wort has been separated from the grain residue. Before

proceeding to fermentation the wort must be conditioned by boiling for many objectives. Wort boiling at temperature higher than 100°C because water will be boiled in closed system at higher pressure, then hold for 50 to 60 min (Kunze, 2004). Heat-resistant microorganisms were killed by boiling for 45 min to 2 h and also inactivates any residual enzymes (Hardwick, 1995). Hops were added and the isomerization of alpha acids to the desired iso-alpha acids took place at this step. The phenolic substances from malt and hops reacted with protein to form large complexes and precipitating out to avoid the formation of haze in beer. Moreover, some of alpha amino nitrogens were lost by reaction with reducing sugar and form color and flavor-active compounds in beer. After wort boiling completed, hot wort was clarified by filtration or centrifugation. The filtration of hot wort is capital intensive; it is expensive on a per brew basis and difficult to maintain in sterile condition (Hardwick, 1995). Centrifuges have improved significantly in ease of operation, clarification and performance. Clear wort was cooled by extract heat from the wort and gernerate hot water to recycle in the brewing process. The wort was cooled as rapidly as possible to the required temperature for fermentation, usually in enclosed heat exchanger (Lewis and Young, 1995). Air or oxygen was injected as part of this process. The volume of air or oxygen injected into the wort should be a function of the volume of wort placed in the fermenter. Wort saturated with air contains 8 ppm oxygen (Hardwick, 1995).

2.5 Fermentation

Bottom fermentation

As already mentioned about bottom fermentation is governed by yeast lager strain or bottom-fermenting yeast. The term flocculation is used to describe the settling to yeast, but yeast may not form true flocs. Brewing lager strains are selected

for the flavor characteristics they impart to beers and for other desirable fermentation characteristics in the beer. Traditional lager fermentation is conducted at lower temperature ranging from about (8-15°C) (Hammon, 2000). Duration of fermentation traditionally ranges from 8-20 days. At first 8-16 hr, the active fermentation appeared when CO_2 bubbles are formed and thin foam is apparent. The budding of cells can be observed within 24 h. The pH falls as organic acids are produced.

Top fermentation

Top fermentation uses the top-fermenting yeast strain. It rise to the top of the beer toward the end of fermentation because the yeast flocs entrap CO_2, making them buoyant. But, with deep cylindro-conical vessels, brewers may use a bottom-fermenting yeast strain to make ale. The fermenter may be open type, which allows easy removal of the yeast crop by skimming or suction. The closed vessels have become common and suction devices are designed to be used in the closed tank. Top fermentation is conducted at higher temperature (16-23°C). The special aeration system may be applied to this type fermentation. The higher temperature and extra aeration causes top fermentation to be completed in much less time than bottom.

Yeast utilize fructose, glucose, maltose, sucrose and maltotriose present in wort. The monosaccharides are taken up by diffusion involving common membrane carriers. Maltose and moltotriose are actively taken up by specific permeases. Sucrose is broken down extracellularly by invertase. The most important factor determining the rate of fermentation of wort is the rate of at which the most abundant sugar. Maltose is the most content and accounts for 60% of the total fermentable sugar. Salubchua, Srakeaw, and Moonjai (2005) studied of rice beer made from rice malt gave ethanol content 3.98±0.44%(v/v) whereas barley malt gave 4.91±0.24 (%v/v).

Moonjai (2005) studied of using rice malt supplemented with rice milled as adjunct and found that the ethanol content was lowest (2.0%(w/w)) when compared with other adjunct (4.9%(w/w) for sorghum adjunct and 5.3%(w/w) for wheat adjunct). These indicated that yeast could not completely utilized sugar when rice was used as raw material for beer production. However, it still unclear for these phenomena, adjunct added might dilute yeast nutrient. Total soluble nitrogen content, wort fermentable sugar, rate of sugar utilization and inhibition should be studied in more detail.

2.6 Maturation

After fermentation finished, the product was lowered the temperature and giving time to permit the setting out of yeast and precipitate particles. During fermentation, many undesirable flavors and aromas of a "green" or immature beer are present. The maturation process reduces the levels of these compounds. Yeast produce α-acetohydroxy acids, which secreted into wort and converted into diacetyl and 2,3 pentanedione or called vicinal diketones (VDKs). These compounds are flavor defect for beer if their concentration higher than 3 mg/L, whereas at low concentration diacetyl provides a butterscotch-like aroma and pentanedione detected as honey-like. Yeast assimilates VDKs toward the end of fermentation and maturation. It is therefore important to allow sufficient time for the total of VDKs and their precursor to be reduced below their flavor threshold (0.15 ppm) (Bamforth and Kanauchi, 2004).

2.7 Filtration and stabilization

At the completion of aging, beer contains some yeast, colloidal particle and other insoluble materials. Clarification must remove these substances before beer

packaging. Many filter types can be selected; plate and frame filter, leaf filters, pulp filter, sheet filters, membrane filter and sand filter (Hardwick, 1995). A suitable filter provides a micromesh screen. Using of filter aids includes kieselguhr (diatomaceous earth) and perlite (volcanic silicate) is most popular for beer filtration. During filtration, the initial bed called pre-coat comprises coarse grade of kieselguhr is the first layer. A second layer is pre-coat of finer kieselguhr. After beer is pumped pass through filter, yeast and protein particles can pack tightly and increase the pressure required to force beer through the filter bed.

The clear beer was chilled to 4°C for stabilization. Haze is a complex of proteins, tannins with metal ions and some carbohydrate components. Chill haze appears when beer chilled and re-dissolves when beer warm up (Lewis and Young, 1995). After haze formation, filtration was took place to remove haze. However, flavor stability and biological stability was conducted at the same time. The flavor stability is enhanced by excluding oxygen from the beer. Using of carbon dioxide to pack tank and to transfer beer reduces the possibility of air pick up (Hardwick, 1995).

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CHAPTER III

THE INFLUENCES OF STEEPING DURTION AND TEMPERATURE ON THE α-AND β-AMYLASE ACTIVITIES OF SIX THAI RICE CULTIVARS (*ORYZA SATIVA* L. INDICA)

3.1 Abstract

The influences of steeping durations and temperatures on α- and β-amylase activities were investigated. Three non waxy rice cultivars (KDML105, PT60, and KCD) and three waxy rice cultivars (SPT, RD6, and KND) were selected for this study. The steeping durations (24, 48, and 72 h) and temperatures (20, 25 and 30°C) were investigated for their effect on α- and β-amylase, the key enzymes for malt quality evaluation. During steeping, the production of both enzymes was lower than air-rest stage. The longer steeping duration, the lower of maximum β-amylase activity was obtained, whereas the contradictory effect occurred with α-amylase activity, nearly at the end of germination time. Additionally, temperature influenced to water uptake content as well as the amylolytic enzyme activity. Particularly at 30ºC, the maximum β-amylase activity (6.7 Unit/mg protein) was found in KND malt steeped for 24 h, and maximum α-amylase activity (20 Unit/mg protein) was found in PT60 malt steeped for 72 h.

Key words : α-Amylase, β-Amylase, Steeping time and Temperature

3.2 Introduction

Malting process consists of two important processes for plant biochemical changes; steeping and germination, and final process of kilning to stops all enzyme activities. Since germination process needs water to activate plant hormones and "giberellic acids (GAs)" penetrate through the scutellar epithelium and diffuse to the aleurone layers to induce enzyme synthesis (Capenzana and Buckle, 1997), the grains must be steeped or hydrated until those biochemical substances are induced. There are many enzymes involved in the germination process. Among these enzymes, there are two important enzymes that control malt quality, α-amylase and diastase; a group of enzymes that digest starch (Briggs, 1998). Therefore, the investigation of malting conditions deals with factors which affect activities, such as steeping conditions (Dewar, Taylor, and Berjak, 1997; Iwuoha and Aina, 1997; Okungbowa, Obeta, and Ezeogu, 2002), steeping duration (Wijngaard, Uhmer, Neumann, and Arendt, 2005), germination, temperature (Capenzana and Buckle, 1997), grain property and cultivar (Li, McCDaig, and Egi, 2006). Most of the studies on malting conditions have been carried out with barley, sorghum, and wheat. Rice is different from these cereals in that it can germinate under anaerobic conditions due to the activity of α-amylase II-4 isoform (Guglielminetti, Yamaguchi, Perata, and Alpi, 1995). The expressions of α-amylase and β-amylase enzyme have been investigated extensively elsewhere (Loreti, Alpi, and Perata, 2003; Mitsui et al., 1999; Muralikrishna and Nirmala, 2005; Ziegler, 1999), whilst the integration of α- and β-amylase activities within the malting conditions of rice lacks published data. Therefore, in this paper, the α- and β-amylase expression as function of steeping duration and germination temperature was investigated for rice malt production.

Rice (*Oryza sativa* L.) which belongs to the grass family (*Porceae Gramineae*) has been consumed by human for at least 5,000 years (Bao and Bergman, 2001). Approximately 80% of rice acreage is *Oryza sativa* L. indica, the major rice species planted in Thailand, India, South of China and Malay Peninsula. Particularly in Thailand, there are more than 100,000 samples kept at International Rice Research Institute (IRRI) and more than 24,000 rice samples in the national rice bank. Thai rice has a very high of diversity and approximately 85 cultivars have been promoted for plantation around the year. According to the cooked texture, rice can be grouped as glutinous and non-waxy rice which can be sub-grouped as low amylose rice (12-20%), medium amylose rice (20-25%) and high amylose rice (>25%) (Bao and Bergman, 2001). Since, rice is the staple food in the world and it is gluten free cereal which has high quality protein. This chapter emphasized to optimize the germination condition by observing the α- and β-amylase activities which are the key enzymes of malt production. Three steeping durations and germination temperatures were used to demonstrate the influences on steep-out moisture content and enzyme activity under control relative humidity at 95±5%. The influence of rice property on water absorption ability was also observed as a sub-objective in this chapter.

3.3 Materials and methods

3.3.1 Rice cultivars

All rice cultivars belong to *Oryza sativa* L. Indica, which were harvested in 2004. The non waxy rice were Khaw Dok Mali 105 (KDML105) from Nakhon Ratchisima province, Pratum Thani 60 (PT60) from Pratum Thani province and Khao Chao Dam (KCD) from Surin province. Waxy rice were San Pa Tong (SPT) from Mae Hong Sorn province and Koh Kho 6 (RD6) and Khaw Niew Dam (KND) from

Nakhon Ratchasima province.

3.3.2 Rice properties

The moisture content, thousand grain weight, total nitrogen and percentage of germination from six rice cultivars were analyzed according to European Brewing Convension; EBC by manipulation at 30ºC instead of 20ºC (Enari, 1975). The apparent amylose content was determined by the iodine colorimetry method (Juliano, 1985). One hundred milligrams of rice flour were dispersed in 100mL volumetric flask which contained 9 mL of 0.1 N NaOH and it was boiled for 10 min, after cooled down, it was made volume to be 100 mL and left stand still for overnight at room temperature. Five milliliters of amylose solution was mixed with 1 mL iodine solution and adjusted volume to 100 mL and left for 20 min. The amylose content was determined by observing the optical density at 610 nm and calculating the amylose content against the standard amylose (Sigma Aldrich).

3.3.3 Experimental design

The steeping durations and germination temperatures were manipulated by full factorial design (6x3x3), six rice cultivars, three steeping durations (24, 48 and 72 h) and three temperatures (20, 25 and 30°C). The statistical analysis was carried out by using SPSS version 13.0 for Windows.

3.3.4 Malting process

Twenty grams of cleaned rough rice were weighed and put on mesh chamber. All mesh chambers were put on the plastic tray and filled with water. Steeping water was changed every 12 h with equilibrated temperature tap water in order to eliminate any inhibitor solubilized from grain and for circulation of oxygen to grain. After steeping stage, rice was germinated up to 6 days. This stage was called air-rest stage

and all treatments were resteeped for 5 min in every 12 h. In addition, the malting room was controlled at 95±5% relative humidity. The germinating rice sample from each treatment was taken once a day and dried at 50°C for 24 h. The dried sample was kept at -20ºC until needed for further analysis.

3.3.5 Determination of steep-out moisture content

The steep-out moisture content was determined by putting all rice grains up on the filter paper for 5 min and weighed 5 g into moisture can. The sample was dried at 105°C for 3 h and calculated for the moisture content of grain according to the EBC (Enari, 1975).

3.3.6 α- and β-Amylase assay

The crude amylase enzyme was extracted by using 1 g of finely ground malt with 50 mM Tris·HCl pH 7.4 with 3 mM $CaCl_2$ and 4 mM NaOH and incubated at 25ºC for 30 min by shaking for 1min in every 15 min. Then, the crude enzyme was separated by filtering it through cotton and centrifuging at 3,000xg for 10 min and kept on ice until needed. Starch hydrolysis by amylase enzymes was quantified by measuring reducing sugar which is the product of enzyme activities (Nandi, Das, and Sen-Mandi, 1995). The 0.5 mL of crude α-amylase enzyme was incubated at 70°C for 5 min, then 0.5 mL of 1% (w/v) soluble starch containing 50 mM acetate buffer (pH 5.5) with 0.003% $CaCl_2$ was added. The reaction was continued for 10 min and terminated by addition of 1 mL of 3-5, dinitrosalisylic acid reagent. For β-amylase assay, the reaction was constructed in the same procedure as α-amylase but crude enzyme was incubated at 55°C, and 1% (w/v) of soluble starch containing 50 mM citrate buffer (pH 3.6) with 1 mM EDTA was used as substrate of reaction.

3.3.7 Protein assay

The protein assay was carried out according to Bradford's method (Bradford, 1976).

3.3.8 Shoot/root length

Shoot/root length of germinated grain was determined by sampling 10 grains from each treatment and measuring them with vernier caliper. The results were the average values from a duplicate set of rice malt samples.

3.3.9 Malting loss

The weight loss of germinated grain as a consequence of germination process was calculated as a percentage and expressed on a wet weight basis. One hundred dried grains were counted by manual grain counter.

$$\text{Malting loss} = \frac{(\text{Weight of 100 rice grains} - \text{Weight of 100 malted rice}) \times 100}{\text{Weight of 100 rice grains}}$$

3.3.10 Reducing sugar

The amount of reducing sugar from germinated rice was analyzed according to Miller, L. G. (1951) by weighing 1 g of rice grain and grinding it to fine particle. One gram of powdered rice and 10 mL of distilled water were mixed and incubated at 20ºC for 30 min. Then, the solubilized reducing sugar was separated by centrifuging at 3,000xg for 10 min. The 0.5 mL of supernatant was taken for mixing with 0.5 mL of 3, 5-dinitrosalicylic acid solution in the 16 mL test tube. The development of color was conducted by boiling the reaction tube for 5min. The concentration of reducing sugar was calculated against standard of maltose concentration 0.2-1.0 μmole/mL.

3.4 Results and discussion

3.4.1 Rice properties

The properties of rough rice were analyzed (Table 3.1) and all rice cultivars were tested for percentage of germination. This parameter was important for achieving the homogenous quality malt. However, the unsatisfied percentage of germination, which less than 95% was found in KCD and SPT cultivars. Therefore the application of heating at 50ºC for 5 days was carried out for breaking rice dormancy so the moisture content was decreased to 6% (w/w). This procedure was successfully conducted with only KCD cultivar, whereas percentage of germination of SPT was slightly increased. However, the heated rice was used for the further study. The thousand grain weight of all waxy rice cultivar was higher than that of non-waxy rice. Protein content of rice was in a range of 5.66-9.77% (w/w). KND rice contained highest protein content 9.77% (w/w). The apparent amylose content of rice was determined for grouping of rice, and this property implied the swelling property of rice starch while rice grains were steeped. According to ranging of amylose content in rice, they were grouped as follows: PT60 was the high amylose rice (30.65%) and KCD and KDML105 were low amylose rice (17%). The RD6, SPT and KND rice cultivars were waxy rice which contained apparent amylose content less than 10%.

Since amylose content is related to the structure of starch granule and would be useful for understanding the behavior of rice in germination process, the non-waxy rice were categorized according to their apparent amylose content. The relation of amylose content and water absorption ability has been mentioned by using the “equilibrium moisture content (EMC)” to explain the water absorption ability of rice starch steeped at 20ºC (Schierbaum, 1960). Juliano (1964) reported that the high amylose rice starch

had EMC lower than low amylose rice starch. The similar result was found in PT60 cultivar, the high amylose rice which absorbed least water, after steeped for 24 h at 30ºC (Table 3.2).

Table 3.1 The properties of six rice cultivars.

Rice varieties	Moisture Content % (w/w)	Thousand Grain weight (g)	Protein content % (w/w)	Amylose content % (w/w)	Percentage of germination	
					Before drying 50°C	After drying 50°C
PT60	5.75±0.45 *a*	24.95±1.15 *c*	5.66±0.87 *a*	30.65±0.48 *c*	94±1 *c*	97±1 *b*
KDML105	7.61±0.52 *c*	23.70±2.32 *b*	8.00±0.51 *b*	16.50±0.80 *b*	97±4 *c*	96±3 *b*
KCD	6.35±0.63 *b*	19.63±0.84 *a*	8.83±1.2 *d*	16.76±1.69 *b*	78±2 *a*	97±2 *b*
SPT	7.72±0.32 *d*	27.53±2.31 *f*	7.94±0.9 *b*	4.29±0.20 *a*	84±4 *b*	87±4 *a*
RD6	6.27±0.35 *b*	25.20±1.57 *d*	8.30±0.45 *c*	6.88±2.16 *a*	98±2 *c*	98±3 *b*
KND	10.75±0.81 *e*	26.19±1.3 *e*	9.77±0.77 *e*	6.78±1.74 *a*	96±2 *c*	96±3 *b*

Mean value± standard deviation of three replications. The different italic letter was significant difference value in the same column at $p \leq 0.05$.

Whereas KCD cultivar, the low amylose rice which absorbed water higher than SPT and RD6; the waxy rice. However, this study manipulated with the whole rice grain, so there might be the other effects involved. Srivastava (2003) reported that the limited permeability of grain pericarp affected the level of water absorption. KCD and KND were two black rice cultivars containing anthocyanin pigment in pericarp (Ryu, Park, and Ho, 1998) and absorbed more water than others. However, there was no report of the influence of pigment in pericarp to grain permeability.

Another possible factor influencing water absorption could be the protein content in rice, the polynomial correlation of protein and steep-out moisture content was indicated by the coefficient (R^2) of 0.92 (Figure 3.1). Some of storage protein in rice grain could be non-active enzymes which were activated by steeping in water. As a result, loosen structure of storage starch by active enzymes made more water absorbed in rice endosperm. Whereas, there was no report of the protein content in relation to water absorption by germinating grain, it was mentioned in wheat dough protein (Maa et al., 2007; Simmonds, 1989). Therefore, protein content might be one factor influence to water absorption by grain. Nevertheless, our results depicted that rice absorbed water slowly and lower than barley. Generally speaking, the steep-out moisture content of barely grain was 40-43% after being steeped for 2 days (Briggs, 1998). From our preliminary result, the moisture content of rice rose to 42% after germinated for 4 days at 30°C. Therefore, there was not only grain property but also kind of cereal and cultivars influence on steep-out moisture content.

3.4.2 Effect of steeping durations and temperature on steep-out moisture

All rice cultivars were tested for optimization of steeping duration at 24, 48 and 72 h and three steeping temperatures at 20, 25 and 30°C. The moisture content of all rice cultivars was rapidly increased at the beginning and slightly increased after 24 h (Figure 3.2). The statistical analysis of variance indicated that the steeping duration significantly influenced steep-out moisture content (Table 3.2) and obviously found in the waxy rice. Furthermore, steep-out moisture content was not only enhanced by steeping duration but also by temperature, particularly at 30°C caused steeped grain had higher steep-out moisture than 25°C and 20°C, respectively. The moisture content in steeped grains was varied and depended on rice cultivar.

Table 3.2 The steep-out moisture content after steeping for 24, 48 and 72 h at temperature 30ºC.

Rice varieties	Steeping durations		
	24 h	48 h	72 h
PT60	26.03±0.07 *a*	28.82±0.38 *a*	29.42±0.25 *a*
KDML105	27.47± 0.12 *a*	30.96±4.7 *b*	31.28±0.16 *b*
KCD	28.53± 0.31 *a*	30.85±0.13 *a*	34.6±0.41 *b*
SPT	27.95± 0.24 *a*	31.71±0.15 *b*	33.15±0.03 *c*
RD6	27.26± 0.28 *a*	31.48±0.38 *b*	32.99±0.35 *c*
KND	29.52±0.31 *a*	33.65±0.10 *b*	36.76±0.13 *c*

Mean values of four replication of analysis ± standard deviation. The different italic letter between the columns was significant difference at $p \leq 0.05$.

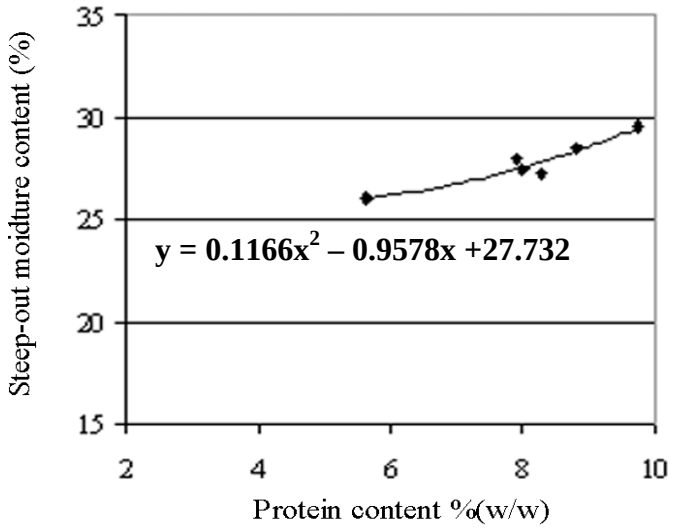

Figure 3.1 The polynomial correlation of rice protein content and steep-out moisture content after steeped for 24 h at 30°C.

After steeping for 72h, PT60 cultivar had the lowest moisture content 29% (w/w), KND and KCD cultivars showed the highest of moisture content at 36% (w/w) and 34% (w/w), respectively.

Temperature is clearly known to have an effect on the rate of water uptake. Naturally, rice grows well in warm and humid environment. Therefore, an increase of temperature enhances many metabolic activities in rice grain. Besides, the water absorption by germinating rice were increased with temperature and agreed with Capenzana and Buckle (1997). They suggested that the steeping time for high amylose rice could be reduced from 24 h to 16 h by increasing steeping temperature to 35°C instead of 25°C. However, our study suggested that the steeping at 30°C with twice changing of water would be sufficient to activate the germination of low, medium and high amylose rice.

3.4.3 Effect of steeping durations and temperatures on amylases activity

Through at the germination time, the α-amylase was continuously increased and the maximum of α-amylase activity was found at the end of germination time. The most vigorous activity was found in KND cultivar steeped for 72 h and germinated for 3 days at 30ºC whilst the β-amylase activity was rapidly increased and declined after 4 days of entire process time (Figure 3.3). Moreover, comparison of β-amylase activity in germinated rice from three steeping conditions elucidated the maximum β-amylase activity was lower, when a longer steeping duration was applied. The maximum of β-amylase activity was found in PT60 cultivar steeped for 24 h and germinated for 4 days at 30ºC. Additionally, the lower reducing sugar in germinated rice from longer steeping condition also confirmed that the starch digestion was retarded by a long steeping duration (Figure 3.4).

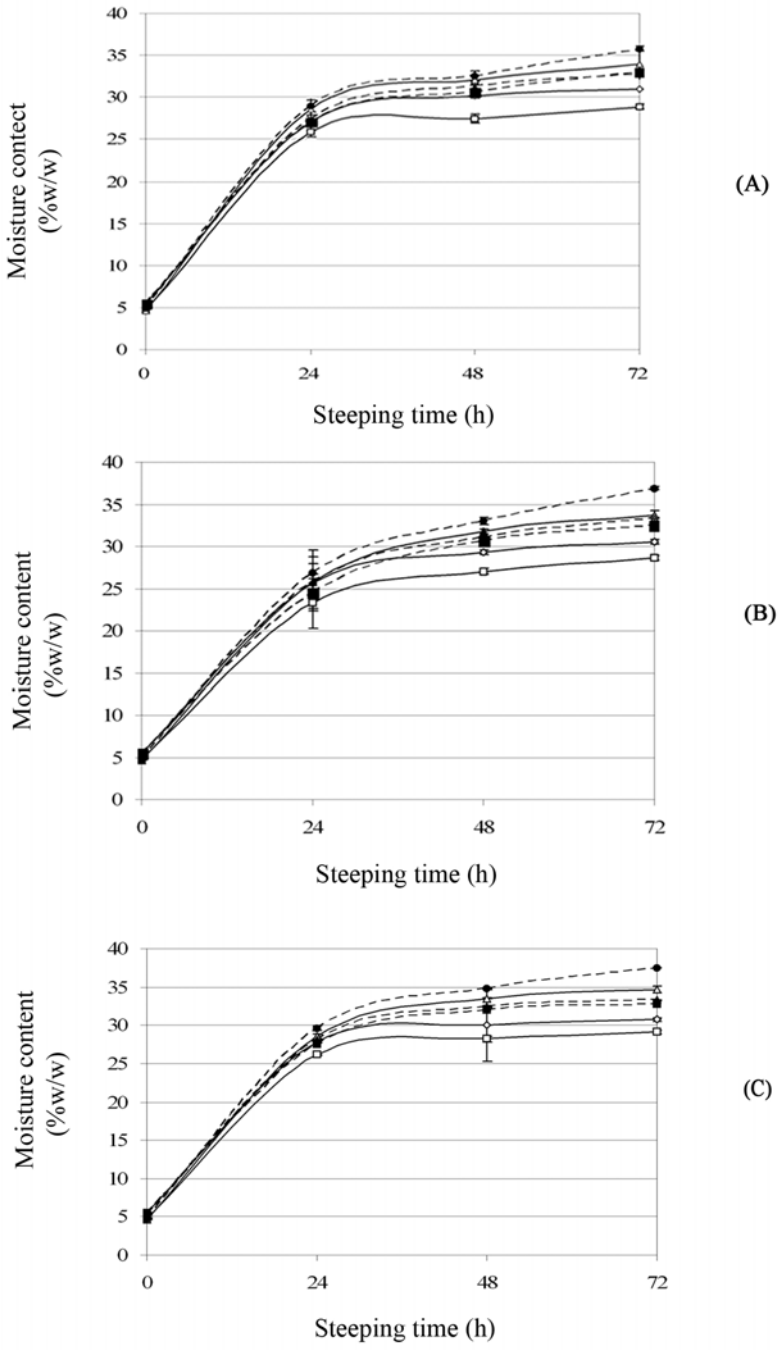

Figure 3.2 The steep-out moisture content of six rice cultivars (A) at 20°C, (B) 25°C and (C) 30°C; —◇— ; KDML105, —△— ; PT60, —×—; KCD, --□-- ; SPT, --■-- ; RD6, --●-- ; KND. Error bar indicated the standard deviation of four measurements.

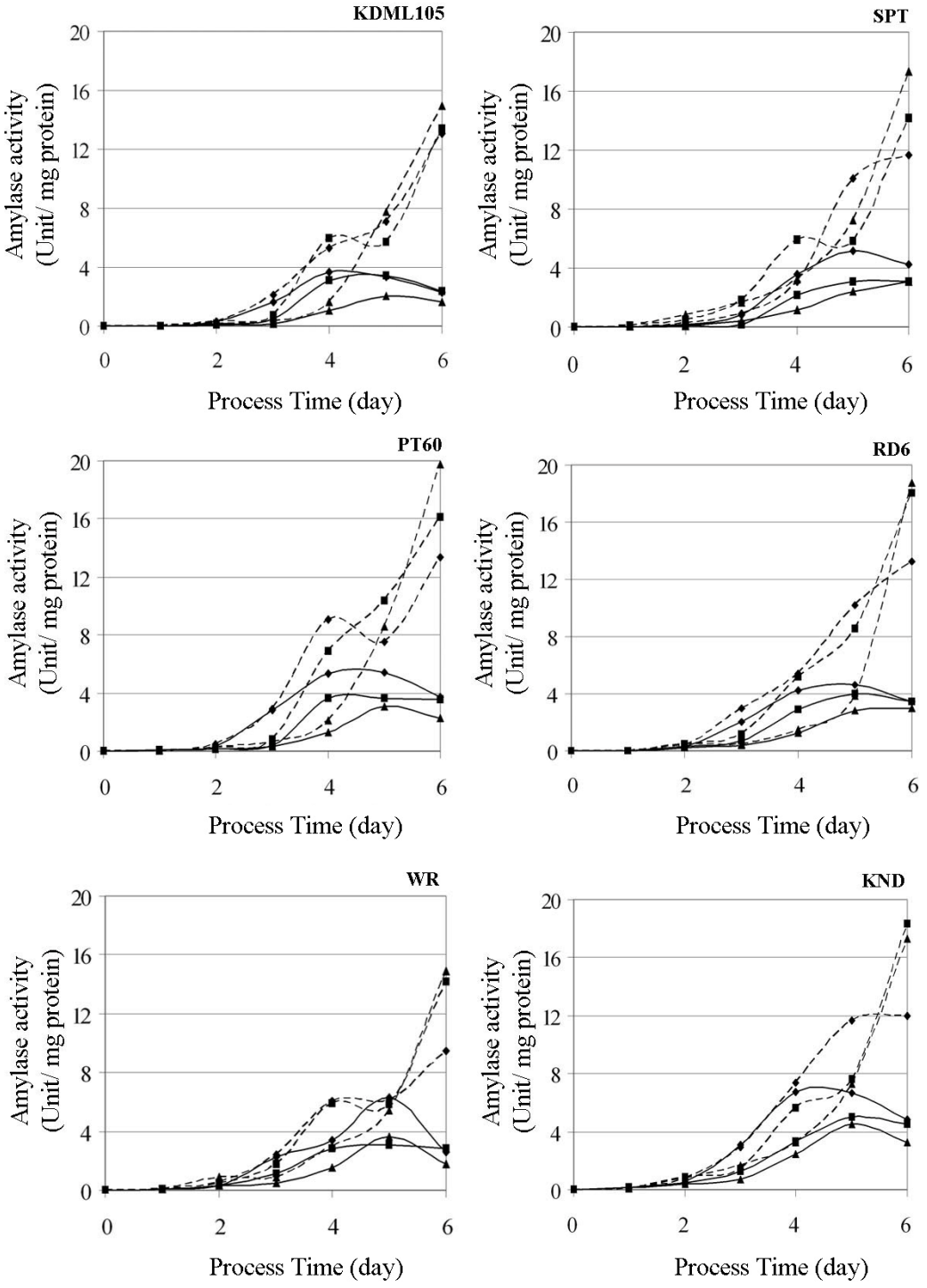

Figure 3.3 The time course of α- amylase; (—) and β-amylase; (....) production in germinating rice at different steeping duration: ♦ ; 24 h, ■ ; 48 h, ▲; 72 h. Germination temperature was at 30°C.

Moreover, the temperature significantly influenced the maximum activities of both enzymes in every rice cultivars, and the maximum of α- and β-amylase activity was found at temperature 30ºC and followed by 25ºC and 20ºC, respectively (Table 3.3).

Table 3.3 The maximum of α- and β- amylase activity in sample of 24 h steeping duration in entirety of germination time.

Rice cultivars	α-amylase activity			β-amylase activity		
	20°C	25°C	30°C	20°C	25°C	30°C
PT60	4.92±1.1 *a*	8.22±1.1 *b*	13.40±4.3 *b*	2.96±1.5 *a*	3.6±1.4 *ab*	5.45±2.3 *b*
KDML105	3.67±0.3 *a*	9.63±5.4 *ab*	13.10±5.3 *b*	3.34±0.2 *a*	3.48±0.6 *a*	3.71±0.1 *b*
KCD	3.90±1.4 *a*	6.33±1.3 *ab*	9.51±2.6 *b*	2.29±1.3*a*	2.92±1.2 *ab*	6.32±0.6 *b*
SPT	2.15±0.13 *a*	7.66±2.6 *b*	11.68±3.2 *c*	1.84±1.4 *a*	3.97±1.3 *ab*	5.16±1.6 *b*
RD6	6.96±2.2 *a*	8.43±0.5 *b*	13.27± 2.2*c*	3.48±1.5 *a*	4.33± 1.6 *b*	4.63±2.1 *b*
KND	5.14±1.9 *a*	7.09±0.1 *b*	11.99±3.4 *c*	6.81±0.3 *b*	4.38±0.1 *a*	6.71±1.2 *b*

Mean values of four replications of analysis ± standard deviation. The mean comparison between the columns of 20, 25 and 30°C were performed separately between α- and β- amylase. The different italic letters were significantly different at $p \leq 0.05$.

Germination of grain involves by many metabolic pathways, particularly the starchy endosperm conversion pathway is a major mechanism of germination since starchy endosperm is accounted for approximately 73-85% of grain portion (Bao and Bergman, 2001). Then, the production of amylolytic enzymes was monitored in this work. From our results, a little of α-amylase activity was found when grain was

steeped and it might be the activity of α-amylase II-4 which is the main isoform of rice α-amylase expressed under the anoxia condition (Loreti et al., 2003).

This isoform was absent in wheat, oat, rye and barley germinated under anaerobic condition (Guglielminetti et al., 1995; Perata, Geshi, Yamaguchi, and Akazawa, 1993). Moreover, warm temperature caused of fast rate of oxygen depletion and long steeping duration may cause anoxia condition to occur. If α-amylase was retarded, then the other amylolytic enzymes, β-amylase, glucosidase activity were subsequently reduced. Guglielminetti, Yamaguchi, Perata, and Alpi (1995) demonstrated the activity of α-amylase and debranching enzymes were decreased when germinated in anoxia condition. Therefore, the concentration of reducing sugar in malt which was conducted by long steeping was logically lower than short steeping duration malt as depicted in Figure 3.4.

Under anoxia condition which was supposed to be the steeping condition of this study, there was some α-amylase activity and some of glucose mobilized to the scutellar tissues for sucrose formation and finally transported to the embryo for supporting seedling growth. However, our results indicated the seedling growth was inhibited by long steepin This molecule of disaccharide has been reported as the inhibitor of extracellular liberation of α-amylase II-4 at concentration of 2 mM (Yamaguchi et al., 1999) and at concentration 90 mM repressed *RAmy3D* gene expression approximately 70-85% (Ziegler, 1999). g condition, and it may cause accumulation of sucrose molecule. These could be the reason that the α-amylase activity was low when long steeping condition was applied. On the other hand, the rapid increasing of α-amylase activity after grain was steeped out could be explained by sucrose utilization being reactivated and led to the liberation of α-amylase II-4. In

addition, α-amylase class I would also be activated under aerobic condition (Mitsunaga et al., 2001). However, the inhibition of β-amylase activity by long steeping condition was nonreversible. Moreover, during grain was steeped, the low oxygen condition might cause of low amount of ATP for some enzyme mechanism. These results agreed with Guglielminetti et al., (1995), the anoxia condition induced some group of α-amylase but inhibited β-amylase activity.

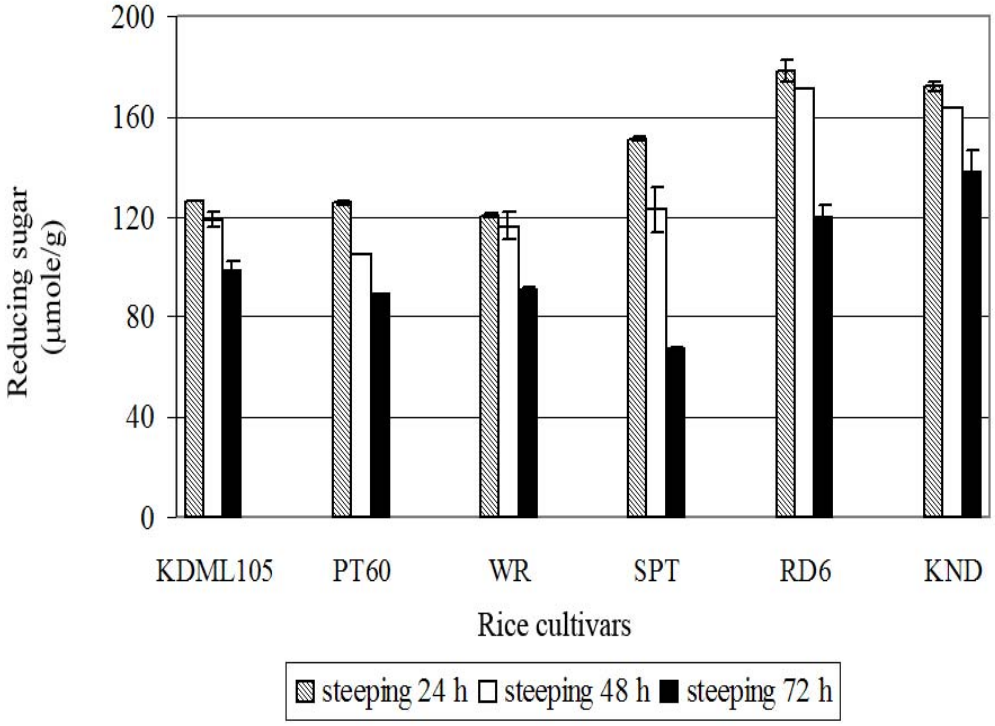

Figure 3.4 The reducing sugar of germinated rice after 6 days of germination time at 30ºC: Error bar indicated the standard deviation of four measurements.

3.3.4 Effect of steeping durations and temperatures on shoot/root formation and malting loss

After rice was steeped for 24 h, the growth development could be observed.

One rootlet and single cotyledon pierced from the embryo and appeared outside the grain. Furthermore, the development of branch root could be observed at nearly the end of germination time. During steeping, the elongation of shoot and root was slowly developed and progressively increased with germination time and temperature (Table 3.4). However, the means of shoot/root length were compared for investigating the effect of steeping duration on growth development. The results elucidated that the shortest of shoot/root length was observed in sample steeped for 72 h, followed with 48 and 24 h. Whereas, the temperature showed contrast effect, steeping and germination at 30°C promoted longer shoot/root elongation than 25°C and 20°C, respectively. Therefore, the value of malting loss increased with germination time and temperature. According to the meaning of malting losses, the steeping losses, rootlets losses and respiration losses were taken into account (Briggs, 1998). It was calculated as the reduction of dry weight and expressed as percentage. From our results, malting losses of 72 h steeping duration was the lowest because the development of shoot and root was retarded. These results were similar to the sorghum studied by Dewar and colleagues (Dewar et al., 1997).

3.5 Conclusion

All six rice cultivars had similar pattern of enzyme productions responding to steeping durations and temperatures but were different in the amount of enzyme activity. The α-amylase production increased along with germination time, whereas β-amylase production was highest after 4-5 days of process time. Long steeping time retarded α- and β-amylase production in all rice cultivars; however, they were activated by temperature, particularly at 30°C. Moreover, the correlation between groups of rice, regarding the amylose content, and amount of α- and β-amylase

production was not found. Nevertheless, the protein content might influence to steep-out moisture content of malting rice. The results of malting losses suggest that further investigation of steeping regime is necessary for cost saving.

Table 3.4 Shoot length, root length and percentage of malting loss of KND rice germination on 4th to 6th day.

Property	Time (days)	20°C			25°C			30°C		
		Steeping durations			Steeping durations			Steeping durations		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Shoot length (mm)	4	4.4±0.7 *b*	2.6±0.1 *a*	3.8±0.5 *b*	7.9±0.9 *a*	8.1±0.5 *a*	5.5±0.5 *a*	14.3±0.1 *a*	14.0±2.1 *a*	10.8±5.1 *a*
	5	4.6±1.0 *a*	4.7±2.3 *a*	4.8±0.5 *a*	15.2±1.0 *b*	12.3±0.4 *a*	8.7±3.0 *a*	22.5±1.3 *a*	19.5±0.9 *a*	15.3±0.4 *a*
	6	4.8±1.0 *a*	7.9±1.3 *a*	5.0±1.4 *a*	23.5±3.0 *a*	18.8±0.1 *a*	14.6±0.5 *a*	33.3±1.4 a	28.6±2.3 *a*	24±2.3 *a*
Root length (mm)	4	11.3±2.6 *c*	9.6±1.9 *b*	6.5±1.8 *a*	49.8±4.9 *b*	20.7±8.8 *ab*	7.5±3.2 *a*	53.3±6.9 *c*	39.3±4.8 *b*	16.6±2.3 *a*
	5	17.9±4.1 *a*	16.6±4.3 *a*	14.9±3.3 *a*	49.8±8.3 *b*	42.0±5.4 *ab*	29.3±4.5 *a*	68.8±8.9 *b*	63.6±7.7 *b*	49.9±5.1 *a*
	6	23.3±4.2 *a*	23.4±3.4 *a*	18.4±3.3 *a*	65.8±10.4*a*	49.3±5.7 *a*	51.6±5.5 *a*	73.6±10.6 *a*	65.4±4.8 *a*	69.9±6.1 *a*
Malting losses (%)	4	2.7±1.4 *b*	2.7±0.6 *b*	1.7±1.1 *a*	7.5±1.1 *a*	6.7±1.9 *a*	2.7±1.1 *a*	9.7±3.2 *a*	9.0±1.6 *a*	3.6±1.7 *a*
	5	6.3±2.9 *a*	7.2±4.9 *a*	4.3±3.4 *a*	15.6±2.2 *b*	12.2±2.1 *ab*	8.05±1.7 *a*	20.9±5.0 *a*	16.6±3.5 *a*	13.2±2.3 *a*
	6	9.5±3.8 *b*	9.5±3.1 *b*	6.4±1.6 *a*	32.5±3.8 *b*	23.0±2.6 *ab*	19.3±2.8 *a*	39.4±5.2 *b*	28.4±4.4 *ab*	22.7±3.4 *a*

The mean comparison between the columns of 24, 48 and 72 h were performed separately between 20, 25 and 30°C. The different italic letter was significantly different at $p \leq 0.05$.

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CHAPTER IV

OPTIMIZATION OF MALTING CONDITION FOR TWO BLACK RIC MALT VARIETIES: BLACK NON-WAXY RICE AND BLACK WAXY RICE

4.1 Abstract

The two black rice varieties, “Black non-waxy rice (KCD)” and “Black waxy rice (KND)”, were investigated for malting condition by using response surface methodology (RSM) to optimize three process parameters; steeping degree, germination time and temperature. Each parameter was tested at three levels: degrees of steeping were 38, 41 and 44%, germination times were performed for 6, 7 and 8 days, and temperatures were studied at 20, 25, and 30°C. CWE, extract content, Kolbach index, α-amylase activity, FAN and AAL were analyzed in malted rice. Data analysis was performed by using the Design Expert statistic program. The optimal conditions for two rice cultivars were germinated for 8 days at 30°C and 44% steeping degree, and malting losses from this condition was approximately 12%. Although the extract yield, α-amylase and β-amylase activities of both rice malts were lower than barley malt, the higher activity of limit-dextrinase enzyme and apparent attenuation limit (AAL) which was higher than 80% suggest that rice malt has potential to be used in brewing processes. The kilning temperature influence to activity of rice proteinase enzymes, consequently to soluble nitrogen and FAN

content in wort. Kilning at 50°C for 24 h was suggested to maintain proteinase activity in malted rice. Furthermore, the modification area in malted rice was observed by iodine staining and through the SEM, which indicated that the starchy endosperm of rice was modified at area near to the embryo and aleurone layer at the ventral side.

Key words: Brewing, Black Rice, Malting, Response Surface Methodology (RSM)

4.2 Introduction

Recently, there has been an increased amount of publications in the area of gluten free malting and brewing, concentrating on sorghum (Okungbowa, Obeta, and Ezeogu, 2002), buckwheat (Wijngaard and Ulmer, 2006), mille (Muoria, Linden, and Bechtel, 1998), teff, quinoa and rice (Bandonill and Sachez, 2007; Capanzana and Buckle, 1997). Black rice, one of many native rice, has widely spread in Thailand (Wutthiyanoe, 2000). It could be classified into two categories, normal and waxy rice. This classification is based on the way the rice behaves when cooked, which is related to the amylose content in rice grains. In this study, black rice was chosen as a source of beer color, special flavor; particularly, the preliminary studies indicated that these two varieties have protein contents more than common variety and have appropriate germinative capacity required for malting and brewing. Since rice and barley have different physiological and chemical properties, it is not possible to apply malting regimes commonly used for barley to rice. Moreover, there were many attempts to investigate malting condition for rice and suggested different malting regimes for different cultivars implicated that the appropriate malting condition might specific for individual cultivar. Regarding the results of the influences of steeping duration and temperature on α- and β-amylase activities, continuous steeping 24-72h retarded both

enzyme activities. Thus in this chapter, the modified steeping regime was applied to KND and KCD; consequently, study of malting process parameters affect on malted rice quality were emphasized by using statistical analysis via response surface methodology (RSM). In addition, changing of starchy endosperm, starch and flour properties of malted rice were studied here.

RSM was used to optimize the malting conditions for two black rice varieties. It is a collection of certain statistical techniques useful for application in experimental design, building model, improving and optimizing process (Meyers and Montgomery, 2002). This tool is useful for evaluating the effect of factors on product attributes, thus during the last decades, RSM has been successfully used for various biotechnological applications (Box and Wilson, 1951; Brown and Hammond, 2003; Chaturvedi and Sarojini, 1996; Cheison, Wang, and Xu, 2007; Liyana-P. and Sharhidi, 2005; Nath, Chattopadhyay, and Majumdar, 2007).

According to the theory of RSM, there are two terms; response and independent variables exist in model equation. The quality characteristic of product or process is called "response" which typically observed on a continuous scale and generally represented by "*y*". Independent variables are factors or the input variables needed to test represented by "*x*". The relationship between "*y*" and "*x*" is explained as a function of *f* and ε is an environmental factors of variability not accounted in *f* such as measurement error or other sources of variation that are inherent in the process. It is assumed to have a normal distribution with mean zero and variance σ^2; therefore, ε is zero (equation 4.2).

$$y = f(x_1, x_2, \ldots x_k) + \varepsilon \qquad (4.1)$$

$$y = f(x_1, x_2, \ldots x_k) \tag{4.2}$$

Since the true response function *f* is unknown, the approximate of *f* through RSM could be a first or second order model. The first order model is shown by equation (4.3) which compose of main effect model terms of x_1, x_2,…x_k, providing a parallel straight lines of constant response in x_1, x_2 plane. The first order model could be appropriate in the true response, if we consider in a small region around one point.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \tag{4.3}$$

In the real experiment, the interaction effect between x_1-x_2, x_1-x_1, and x_2-x_2 on *y* could be occurred; therefore, the first order model is inadequate, the interaction terms between x_1 and x_2 must be added to the model as equation 4.4 and provide a curvature region in true response surface.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \tag{4.4}$$

The β's are called regression coefficients because the response surface model is close to the regression model and Taylor series expansion of equation 4.1 through the second-order terms would result a model in equation 4.5.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_1^2 + \sum_{i=j=2}^{k} \sum \beta_{ij} x_i x_j + \varepsilon \tag{4.5}$$

Where β_0 = intercept coefficient

β_i = linear coefficient

β_{ii} = quadratic coefficient

β_{ij} = interaction coefficient

In the practical application of RSM, it is necessary to develop an approximating model for the true response surface by using method of least squares. Meaning that, the sum of squared residual or error has its least value, a residual being the difference between an observed value and the value given by the model. If sum of squared residual is divided by degree of freedom (n-p), the variance σ^2 of regression model will be obtained in equation 4.6.

$$\sigma^2 = \frac{(SS_E)}{n - p} \tag{4.6}$$

Where, SS_E = sum of squared residual

n = number of replication or samples

p = number of model parameter

The least value of variance σ^2 of models that fit to the data suggests that there is less unexplained variability; therefore, that model must be selected. After that, an analysis of variance will be performed for test the significance of regression.

The significance of regression is a test to determine if there is a linear relationship between response variable y and a subset of the independent variables $x_1, x_2, \ldots x_k$. The hypothesis are

$$H_o : \beta_1 = \beta_2 = \ldots = \beta_k = 0$$

$$H_1 : \beta_j \neq 0 \text{ for at least one } j \tag{4.7}$$

Rejection of H_0 implies that at least one of the independent variables $x_1, x_2, \ldots x_k$ contributes significantly to the model if F_0 exeeds $F_{\alpha, k, n-k-1}$ or if the P-value for the statistic F_0 is less than α. The calculations of F_0 are explained in Meyers and Montgomery (2002). The other terms used to evaluate the quality of fitted model is the coefficient of multiple determination R^2, predicted R^2 and adjusted R^2. R^2 is defined as amount of reduction in the variability of y obtained by using independent variables and can be calculated by equation (4.8), thus $0 \leq R^2 \leq 1$.

$$R^2 = \frac{SS_R}{SS_T} = 1 - \frac{SS_E}{SS_T} \tag{4.7}$$

The R^2 could be improved or worse by adding or eliminating the term in the model. Adjusted R^2 could be higher than ordinary R^2 if the significant term is added to the model; in contrast, R^2 could be decreased if unnecessary term is added. The predicted R^2 could be obtained by calculating through the prediction error sum of squares (PRESS) and total sum of square (SS_T) as shown in equation (4.8).

$$R^2_{prediction} = 1 - \frac{\text{PRESS}}{SS_T} \tag{4.8}$$

The predicted R^2 indicates the predictive capability of the regression model. Normally, the different of predicted R^2 and adjusted R^2 should not greater than 0.20; otherwise, it may be a problem with either the data or the model (Ahmad, Wong, Teng, and Zuhairi, 2007). In addition, the model adequacy checking must be

performed through several techniques to ensure that fitted model provides an adequate approximation to the true system and verify that least squares regression assumptions are not violated. For example, testing for lack of fit is needed to be not significant. In RSM, it is useful to obtain two or more replicates on the response at the same setting of the independent variables in order to obtain a model-independent estimate of σ^2. The testing for lack of fit can be calculated through residual sum of squares in equation 4.9-4.12.

$$SS_E = SS_{PE} + SS_{LOF} \tag{4.9}$$

$$SS_{PE} = \sum_{i=1}^{m} \sum_{j=1}^{n_i} (y_{ij} - \bar{y}_i)^2 \tag{4.10}$$

$$SS_{LOF} = \sum_{i=1}^{m} n_i (\bar{y}_{ij} - \hat{y}_i)^2 \tag{4.11}$$

$$F_0 = \frac{SS_{LOF} / (m-p)}{SS_{PE} / (n-m)} = \frac{MS_{LOF}}{MS_{PE}} \tag{4.12}$$

Where, SS_{PE} is the sum of squares due to pure error

SS_{LOF} is the sum of squares due to lack of fit

$\bar{y}$ is the average of the n_i observations at x_i

$\hat{y}$ is the fitted values

m is levels of x

p is the number of parameters in the model

If $F_0 > F_{\alpha, m-p, n-m}$, there is no strong evidence of lack of fit, the variation of the predicted values is large relative to the random error.

After the appropriate response surface models are obtained, a set of operating conditions for the desired product property must be determined. In practice, several desirable properties are needed to be found in a range of process variable. If two or three target properties are needed in one operating condition, the overlay plot could solve this problem; whereas, complex response requirement need a numeric optimization (Mayers and Montgomery, 2002). And more than a single combination of process variables could be used to produce desirable products, having similar characteristic. The desirability will be used to judge the combinations and the highest desirability is needed to ensure that the target response will be obtained.

The objectives of this experiment were to establish empirical model for germination factors and optimized the malting condition of two black. Germination time as factor A (x_1), temperature as factor B (x_2) and steeping degree as factor C (x_3), were significant factors for optimization of germination condition by using face-centered composite design. Malt qualities including of cold water extract (CWE), extract content, Kolbach index, α-amylase, FAN and apparent attenuation limit (AAL) were response variables (y_1-y_6) observed here.

4.3 Materials and methods

4.3.1 Materials

KCD was cultivated at Suranaree University of Technology, Nakhon Ratchasima. KND was obtained from Phi Mai district, Nakhon Ratchasima, Thailand. Both of them were harvested in 2005. Dry paddy rice was cleaned from contaminated

materials and some immature seeds. Rice was kept in cold storage room at temperature 15 °C and 65 % RH until needed.

4.3.2 Methods

4.3.2.1 Germination procedure

The malting process was manipulated by using 800 g of rice grain in metal boxes. All boxes of rice were washed with tap water before steeping at room temperature (25ºC) for 5 h. After this equilibration, rice boxes were put in germination room with relative humidity of 95% and controlled temperature at 20, 25 and 30°C, separately. Control of steeping degree by re-steeping was performed, and the steeping regime investigated in this study was summarized in Table 4.1. After the germination, the sprouted rice was dried in air flow oven at 50°C for 24 h. Root and shoot were removed by seed drilling machine.

Table 4.1 Malting regime.

1st day	Next day until reach 38%, 41% and 44% steeping degree	Further 2 days	Sampling
Steeping 5 h Air rest 4 h Steeping 5 h Air rest 10 h	Steeping 5 h Air rest 19 h	Resteeping if steeping degree less than needed more than 2%, or spray was applied if steeping degree less than needed <2%	At 6, 7 and 8 days

4.3.2.2 Experimental design and statistical analysis

The optimization for malting conditions focused on three important factors.

They were germination time (6, 7 and 8 days), temperature (20, 25, and 30°C) and steeping degree (38, 41 and 44%). The face centered cube composite design was chosen to evaluate these three factors. The combination of process variables provided 24 experiments which were composed of eight factorial points represented for the highest and lowest values of each factors. One center point was carried out in duplicates and six axial points were single practice. All treatments were started at the same time and in random run to minimize the effect of the extraneous factors on the response variables. The analysis of variance (ANOVA) of each response variable was calculated by Design-Expert version 7.0.3 (Stat-Ease, Inc. Minneapolis, MN) and the models of correlation were displayed by response surface plot.

The numerical optimization for malting process was carried out with the criteria such as high extract and high amylolytic enzymes activity. Indication of highest and lowest values for each target response variable was conducted with regard to the derived data from the experiment. The optimal process parameters were judged by Design-Expert version 7.0.3 with the highest probability to obtain target quality. For verification of the model fitting, the attributes of rice malt produced using the optimal process parameters were compared to the predicted values obtained from model equations.

4.3.2.3 Analysis of malt qualities

Malt qualities were analysed in accordance with the EBC (EBC, 1998) and Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK) methods (Pfenninger, 1997): moisture content (EBC 4.2), cold water extract (EBC 4.6.2), extract content (MEBAK 4.1.4.2.2), soluble nitrogen (MEBAK 4.1.4.5), Kolbach index (MEBAK 4.1.4.5.3), and AAL (MEBAK 4.1.4.10).

4.3.2.4 Free Amino Nitrogen (FAN)

The FAN and Gamma-aminobutyric acid (GABA) was analyzed using the Perkin Elmer HPLC system with column of Spherisorb ODS II, 5 μm, 20 × 4.6 cm and a controlled temperature at 30°C. The samples were prepared by adding 0.02% sodium azide and centrifuged at 10,000×g for 3 h. The clear supernatant was added with internal standard of norvalin 1.25 mmole/L. The gradient concentrations of eluent A and B were operated at flow rate of 1.0 mL/min. The composition of solution A was 5 ml of tetrahydrofuran and 10l of acetonitrile and 485 mL of phosphate buffer pH 7.2. The eluent B was 250 mL of acetonitrile and 250 mL of phosphate buffer pH 7.2. The fluorescent detector was operated at excitation wavelength 355 nm and emission wavelength at 450 nm.

4.3.2.5 Gelatinization Temperature

The samples were grounded using laboratory mill 3100 (0.8 mm sieve, 16000 rpm). Gelatinization temperature was measured with a rapid visco analyser RVA Super 4 (Newport Scientific, Warriewood, Australia) as reported in Keßler, Zarnkow, Kreisz, and Back, (2005).

4.3.3.6 α-Amylase, β-amylase and limit-dextrinase enzyme activities

The α-amylase was determined according to ICC standard method 303 by using the Megazyme enzyme kit (Megazyme, Wicklow, Ireland) to measure the level of α-amylase activity in 1 g of malt sample (International Association of Cereal Science and Technology (ICC), standard method 303). Beta-amylase activity and limit- dextrinase enzyme activities were analyzed by using Megazyme kits for individual enzymes (Megazyme, Wicklow, Ireland).

4.3.2.7 α-Glucosidase Activity

The α-glucosidase was modified from the report of Iwata et al., (2002). One gram of fine ground malt, which was passed through the 0.5 mm mesh sieve, was extracted for crude α-glucosidase by suspending in 10 mL of 10 mM acetate buffer (pH 5.0, containing 5 mM DTT and 90 mM NaCl). The mixture was maintained at room temperature for 30 min. Then, the mixture was centrifuged at 3000xg for 15 min at 4 °C. After filtration through cotton, the supernatant was assayed for enzyme activity. One hundred microliter of crude enzyme was mixed with 1 mL of 6 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) in 100 mM acetate buffer pH 4.5. The reaction was performed at 40°C for 10 min and terminated by adding 0.5 mL of 200 mM Na_2CO_3. The amount of p-nitrophenol liberated from PNPG was measured by using spectrophotometer operated at wavelength 400 nm. Blank of reaction was manipulated with the same manner, but 0.5 mL of 200 mM Na_2CO_3 was added before crude enzyme.

4.3.3.8 Observation of starchy endosperm in germinated rice

The germinated rice samples were taken to dry by freeze drying and kept in the desiccator until needed. The germinated rice endosperms were coated with gold and monitored under scanning electron microscope (SEM) (JSM-6400 scanning microscope, Icrospec WDX electron injector). In addition, the iodine staining method was also performed with the longitudinal half-grain by soaking for 30 second in order to observe the modified region in the germinated rice endosperm compared to ungerminated grain.

4.3.2.9 Effect of kilning temperature on malt qualities

After rice were germinated completely, they were dried by heating in hot air-

flow oven with three different temperature programs; low temperature program was 50 ºC for 24 h, medium temperature program was 50ºC for 9 h and 65ºC for 15 h, and high temperature program was 60°C for 2 h, 70°C for 1 h, 80°C for 2 h and 85°C for 1 h. Then all rice malt samples were analyzed for malt qualities by following EBC and MEBAK standard methods.

4.3.2.10 Malting losses

Malting losses was calculated as percentage of weight losses due to malting process. Cleaned malt was weight and measured for final moisture content before calculation of malting losses as following equation.

$$\text{Malting losses } (\%) = \frac{(A - B)}{A} \times 100$$

Where A = 800 x ((100-moisture content of rice)/100)

B = weight of cleaned malt x (100-moisture content of malt)/100)

4.4. Results and discussion

4.4.1 Property of raw materials

Two black rice varieties representing the two main categories of black rice in Thailand were selected and analyzed for their properties (Table 4.2). The gelatinization temperatures were in the same range which was categorized as intermediate gelatinization temperature 70-74°C (Capanzana and Buckle, 1997). Since the amylolytic enzymes are important for seed modification during germination, the existence of enzymes in paddy and germinated seed were measured in this study. Our results showed that α-amylase activity was detected at low level in

both rice varieties which is similar to what reported in barley grain. Among the starch debranching enzyme, limit-dextrinase (EC 3.2.1.41) is an important enzyme found at high level in both paddies (3, 917 and 3, 397 U/kg for KCD and KND, respectively), which has been reported to exist in developing endosperm of rice and plays an important role in adjusting the chain length of amylopectin molecules (Iwata et al., 2002).

Generally, this enzyme catalyses the hydrolysis of α-1,6-glucosidic linkages in α-limit and β-limit dextrins, pullulan and amylopectin. In mature barley grains, limit-dextrinase activity was reported to be minimal concentration and slowly increase during seed germination (Kristensen et al., 1999). Moreover, α-glucosidase was also assayed and found in rice paddy of both varieties (82 and 72 U/kg for KCD and KND, respectively) and rice malt (250 and 210 U/kg malt for KCD and KND, respectively). It has been reported that the α-glucosidase is synthesized in the ripening stage and preserved in dry paddy which is similar to limit-dextrinase (MacGregor, Bazin, Macri, and Babb, 1999). The exo-hydrolysis of α-1, 4-*O*-glycosidic bond in dextrin provides β-maltose form is governed by β-amylase (EC 3.2.1.2). There was rarely report of β-amylase in paddy rice, whereas our results found that KND had high activity of β-amylase (102 U/g), which might be the latent form similar to what reported in barley, wheat (Guglielminetti, Yamaguchi, Perata, and Alpi, 1995) and buckwheat (Okungbowa et al., 2002). In case of KCD, it was consistent with *Oryza sativa* L. cv Arborio which almost has no β-amylase activity in dry paddy (11 U/g) (Nakamura, Yuki, Park, and Ohya, 1989).

Table 4.2 Characteristics of raw materials.

Property	KCD	KND
Moisture content (%)	12.1±0.5	11.6±0.2
Germinative capacity (%)	95±3	97±2
Gelatinization temperature (°C)	71.4±0.2	73.3±0.3
α-amylase (Celaphal unit/g)	2±1	1±0.5
β-amylase (Betamyl unit/g)	11±0.5	102±3
Limit-dextrinase (U/kg)	3,917±46	3,397±31
α-glucosidase (U/kg)	82±5	72±1

4.4.2 Model fitting and evaluation

Malt from the KCD and KND were analyzed for their properties and defined as responses of process variables. The empirical model was constructed from experimental data (appendix), and then the fitted model was analyzed by using ANOVA test through the Design-expert program as shown in Table 4.3 and 4.4. The significantly fitted model and the terms in the model were significant terms which selected at p-value <0.05. Exceptional for α-amylase activity in KND malt, all terms in the model were not significantly fitted to the data, except for α-amylase time (X_1), steeping degree (X_3) and the interaction term of temperature and steeping degree (X_{23}) were non significant terms; as a result, the model had low value of coefficient of determination (R^2 0.3766). Generally, the quality of fitted polynomial model was expressed by the coefficient of determination (R^2), and the different of predicted R^2 and adjusted R^2 should not greater than 0.20; otherwise, it might be a problem with

either the data or the model (Ahmad, Wong, Teng, and Zuhairi, 2007). All predicted models presented that the predicted R^2 were reasonably agree with the adjusted R^2. Moreover, the *p*-values for lack of fit were greater than 0.05, indicated that lack of fits was not significant. Meaning that the variation of the data around the fitted model was small; hence, the predicted models fitted the experimental data adequately. The coefficient of variation (C.V.) is the standard deviation expressed as a percentage of standard error of predicted value to the mean value of observed response. A model can be considered reasonably reproducible if the C.V. is not greater than 10% (Ahmad et al., 2007) and every model in Table 4.3 and 4.4 was in good range of C.V.%. Thus, all models were appropriate to use for prediction the design space and qualified at 99.99% fitting, except the empirical model of extract from KND; consequently, this model was excluded from the optimization of malting condition of KND.

The optimizations of malting condition for two black rice were done by setting the criteria to obtain high modified malt with high α-amylase activity because most of the soluble extract in wort generated from malting rather than mashing and worth boiling. CWE and extract content were set as 5 for significant level, then alpha-amylase and Kolbach index were set as 4, and then FAN and AAL were set as 3. The maximum of every response variables were desired property of rice malt. In case of empirical model for extract content in black waxy-rice malt, the R^2 indicated that the model was not suitable for further estimation. The statistic program provided a number of several solutions which ranked from maximum desirability to the minimum (appendix); as a result, one desired property could be obtained by many process conditions. However, the maximum of desirability was required in order to

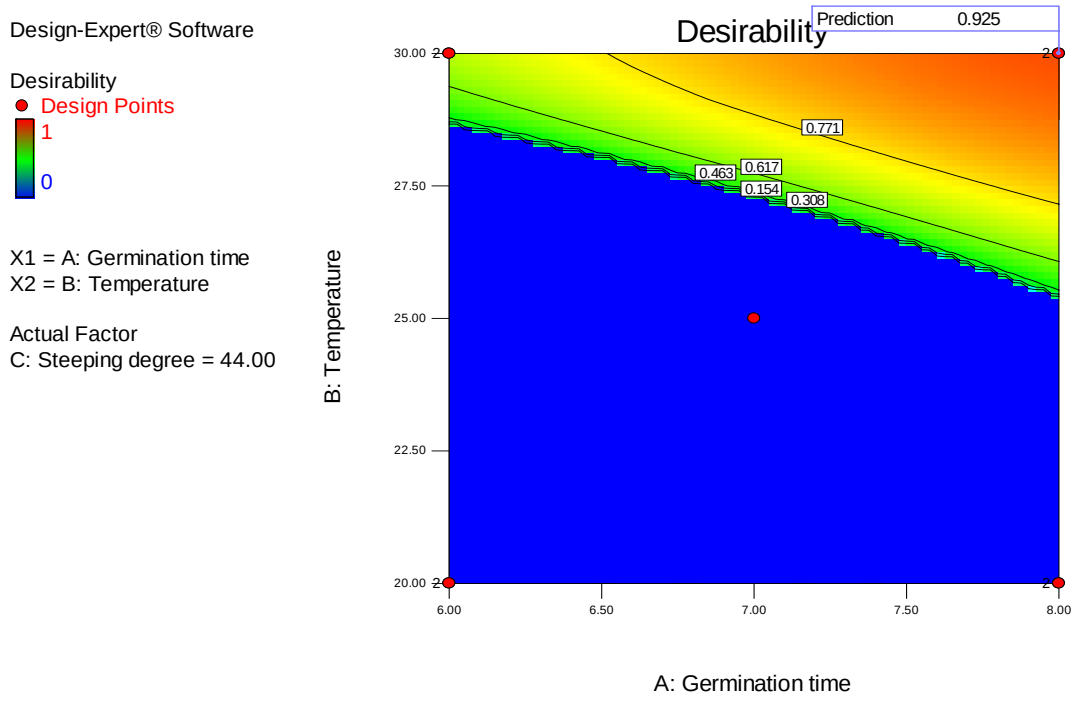
ensure that the desired property will be reached at high probability (Figure 4.1). The optimal process condition for production of rice malt was 44% steeping degree, germination at 30°C for 8 days.

Table 4.3 The statistic parameters of the predicted models for KCD malt.

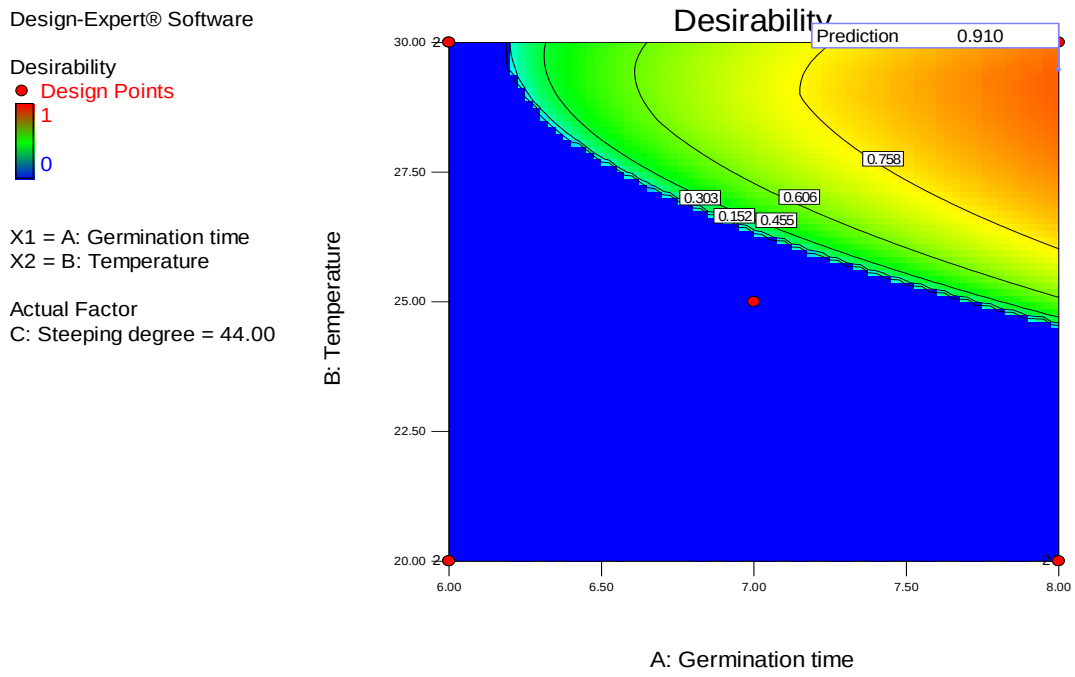
Factor	CWE % (w/w)	Extract % (w/w)	FAN (mg/100g)	Kolbach Index (%)	α-amylase (Unit/g)	AAL (%)
β_0	8.44	62.18	73.46	16.40	51.90	82.17
Linear						
β_1	0.26	0.47	9.24	0.84	2.96	3.72
β_2	1.66	1.14	18.77	3.40	20.18	14.61
β_3	0.47		14.93	1.88	4.66	2.11
Interaction						
β_{12}	-0.28	-0.79	-3.76	-0.73		-3.25
β_{13}	0.17			0.41		1.63
β_{23}	0.22		4.71	0.83	3.07	
Quadratic						
β_{11}						
β_{22}	-1.10	-1.49		-1.11	-15.13	-11.67
β_{33}						
p-value for model	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
p-value for lack of fit	0.1578	0.0804	0.3281	0.1627	0.7979	0.0013
R^2	0.9859	0.9128	0.9277	0.9868	0.9810	0.9866
Adj.R^2	0.9797	0.8945	0.9076	0.9798	0.9687	0.9819
Pred. R^2	0.9739	0.8596	0.8848	0.9652	0.9506	0.9713
C.V.%	3.11	0.80	10.0	3.50	7.94	2.72

Table 4.4 The statistic parameters of the predicted models for KND malt.

Factor	CWE % (w/w)	Extract %(w/w)	FAN (mg/100g)	Kolbach Index (%)	α-amylase (Unit/g)	AAL (%)
β_0	9.70	58.68	84.92	20.15	76.17	49.82
Linear						
β_1	0.41	0.13	4.92	0.96	2.28	3.45
β_2	1.68	0.55	15.89	2.62	14.11	16.31
β_3	0.56	-0.067	8.05	1.78	3.28	7.58
Interaction						
β_{12}	-0.26	0.26	-3.66	-0.62	-2.62	
β_{13}		-0.73		0.44	1.75	2.99
β_{23}	0.26	-0.069	+2.95	0.55		3.44
Quadratic						
β_{11}			-12.44			
β_{22}	-1.99			-1.69	-11.72	-9.85
β_{33}						
p-value for model	<0.0001	0.1787	<0.0001	<0.001	<0.001	<0.001
p-value for lack of fit	<0.0001	0.9360	0.7681	0.1613	0.4685	0.7278
R^2	0.9621	0.3766	0.9624	0.9669	0.9767	0.9586
Adj.R^2	0.9455	0.1566	0.9491	0.9524	0.9684	0.9440
Pred. R^2	0.9084	-0.4755	0.9225	0.9299	0.9465	0.9197
C.V.%	4.79	2.10	5.35	3.67	3.79	9.8



(A)



(B)

Figure 4.1 The graphs of numerical optimization for malting of (A) KCD, and (B) KND.

However, in order to verify the adequate precision of the model, the process values at the point of interest were carried out in triplicate experiments. The mean of measured values for each replicates were compared to the predicted value, at least two of three must be in range of high and low P.I.; as a result, that model precision was accepted. Under this condition, all response variables in Table 4.5 were accepted. In addition, the mean values of three experimental values were compared to the predicted values obtained from the empirical model equations. The agreement between predicted values and experimental values of all response variables were shown in Table 4.5. Although the model for extract content of KND had low value of R^2, the mean value was agreed to predicted value. However, the model for extract content of KND was not safe for future prediction due to the uncontrollable factors and it was not used for optimization for malting condition.

Table 4.5 The comparison of predicted value with measured value.

Malt qualities	KCD		KND	
	Predicted values	Experimental values	Predicted values	Experimental values
CWE (% w/w)	9.83 [9.24/10.43][1]	9.88±0.05	11.5 [10.23/12.23]	11.37±0.05
Extract (% w/w)	61.5 [60.4/62.7]	62.0±0.21	-	59.3±0.42
FAN (mg/100g malt)	117 [100/135]	104±3	101 [90/110]	98.3±6.5
Kolbach index (%)	21 [20/23]	20±1	24 [22/27]	23±1
α-amylase activity (U/g)	72 [63/81]	75±2	74 [64/70]	83±5
AAL (%)	89.3 [84.4/94.2]	85.5±0.7	84.4 [77.9/85.6]	85.5±0.7

[1] [95% P. I. low / 95% P. I. high]

4.4.3 Optimization of malting condition for two black rice varieties

4.4.3.1 The cold water extract and extract from congress mashing

The cold water extract (CWE) is one method which has been used for determination of seed modification in malting process by mashing malt in 6 mM NH_3 solution (for enzymes inactivation) at 20°C for 3 h. The effect of independent variables of temperature, steeping degree and germination time on CWE content in KCD malt and KND malt were depicted by the response surface plot. The results showed that at 38% steeping degree, the CWE was decreased from 6^{th} to 8^{th} of germination time, whereas at 44% steeping degree, it was increased a long with germination time (Figure 4.2A). Therefore, water is necessary for rice seed modification and malting with long germination time needed more water supplied in order to in crease of CWE. The influence of temperature on CWE was shown on Figure 4.2B and revealed that the temperature in range of 27.5°C up to 30°C made rice endosperm modified vigorously. At temperature 30°C, 44% steeping degree and germination time for 8 days, both rice malts had highest CWE content; 9.8% (w/w) for KCD and 11.4% (w/w) for KND. However, in comparison to barley malt, this range was two times less than that of well modified barley malt (Guglielminetti et al., 1995).

Extract yield is one quality term used to determine the extract content obtained from standard mashing program called “Congress mashing”. Under this mashing, proteinases and amylolytic enzymes were activated and the fermentable substances were released. Therefore, this term suggest that how many of extract yielded from malt in a percentage of dry matter. The extracts from finely ground malt were determined in both rice malts, they were in a range of 58.6-62.8 %(w/w) for

KCD and 55.3-61.4 %(w/w) for KND. According to the calculation of extract content in EBC method, the weight of husk was taken into account for gram of malt, whereas it was not contribute to the specific gravity of wort (Wijngaard and Ulmer, 2005). In addition, rice grain contained husk approximately 20 (%w/w) (Juliano, 1985). and 30% in malted rice, thus the maximum possible yield was only 70%; whereas, barley has approximately 82% starchy endosperm (Briggs, 1998). Moreover, the gelatinization temperature of rice starch was higher than 70°C, the liquefaction temperature in congress mashing. Therefore, the low amount of extract content found in rice malt was not a surprise and acceptable.

The temperature was strongly influenced extract yield; particularly, at 44% steeping degree, the extract yield was increased with germination time if temperature was less than 27.5°C. Once the temperature was increased to 30°C the extract yield was slightly lower than that of 27.5°C. The influence of germination time on extract yield was clearly shown in Figure 4.3B, and at temperature 30°C the extract yield was slightly decreased after six days of germination, which might be a result of decreasing activity of some enzymes.

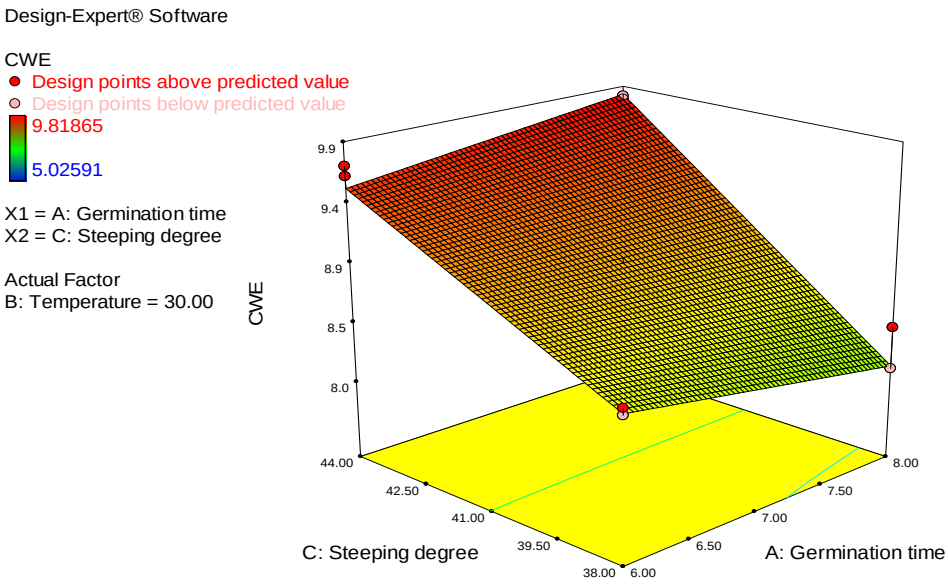
4.4.3.2 Nitrogenous substances

The standard method for determining the ratio of nitrogen content in malt and in wort solution called “Kolbach index” which implies an overview of proteinase activity, was analyzed. The results demonstrated that Kolbach index was in a range of 9.5-21.7% for KCD and 13.5-23.2% for KND. Malting of KCD rice by using this steeping regime, the Kolbach index was slightly decreased after germinated 7 days at 30°C and 41% steeping degree. Whereas, malting of KND rice at 41% under the same condition, the increment of Kolbach index was continuously increased up to 8

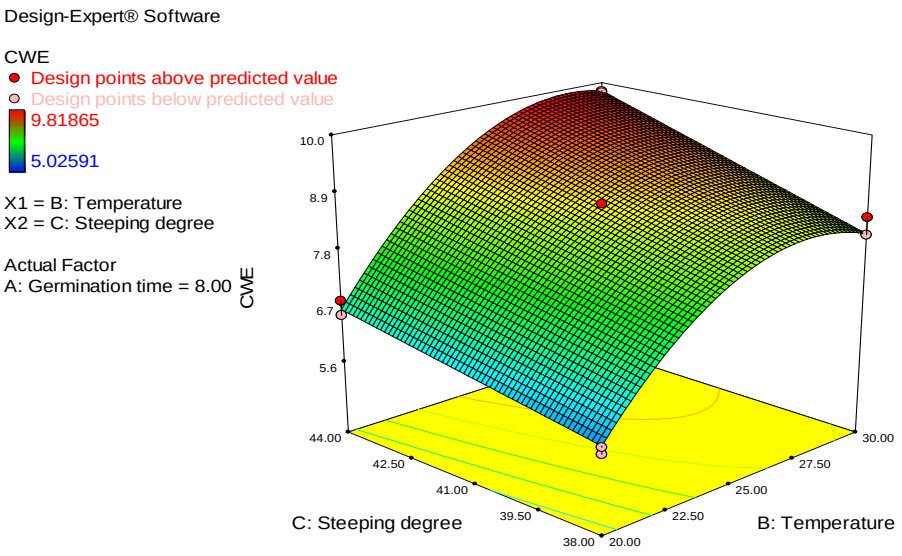
days (Figure 4.4 and 4.5). Not only the temperature and germination time influenced to FAN and Kolbach index, but water was also necessary for proteinase and other enzymes in malting process. The results indicated that the Kolbach index and the amount of FAN were increased by increasing the steeping degree, and 44 % steeping degree provided highest amount of those nitrogenous substances in rice malts. Generally, FAN content for fine fermentation media must be higher than 140 mg/L of wort. Although the FAN obtained from these two rice malt varieties were lower than that, the result of fermentability which was higher than 80% indicated that rice malt was qualified to be used for beer production.

4.4.3.3 Apparent Attenuation Limit (AAL) or fermentability

The apparent attenuation limit (AAL) or fermentability of congress wort is a term used to approximate amount of fermentable sugar in wort by fermeting of boiled wort with standard brewing yeast as described in MEBAK method 4.1.4.10. The results demonstrated that AAL was in a range of 48.4-89.3% for KCD and 43.8-83.0% for KND (Appendix). The AAL from rice malts was increased with the increments of temperature, steeping degree and germination time (Table 4.3 and 4.4). Since the highest amount of FAN and extract content from both rice varieties were obtained from germination condition at 30°C and 44% steeping degree, the maximum of AAL was obtained from the same condition on day 8^{th}. Besides, the fermentability of malt was related to the degree of modification or CWE content as shown in Figure 4.6 B.

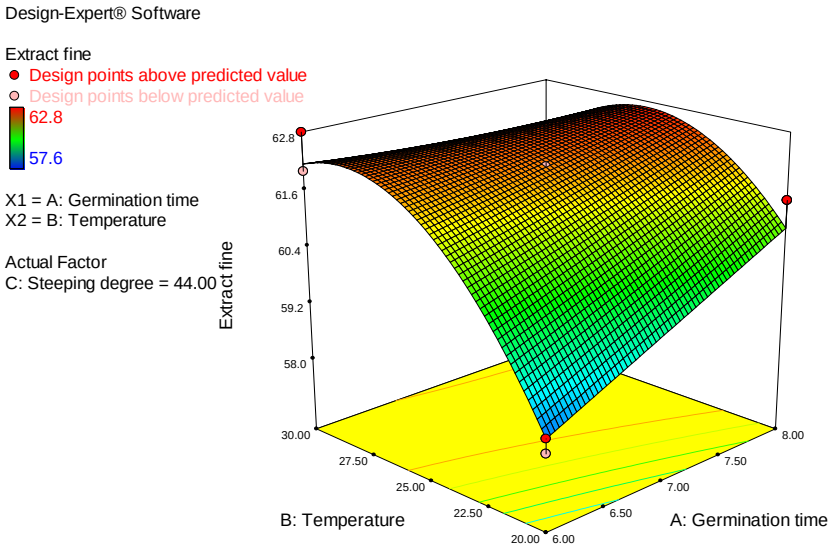


(A)

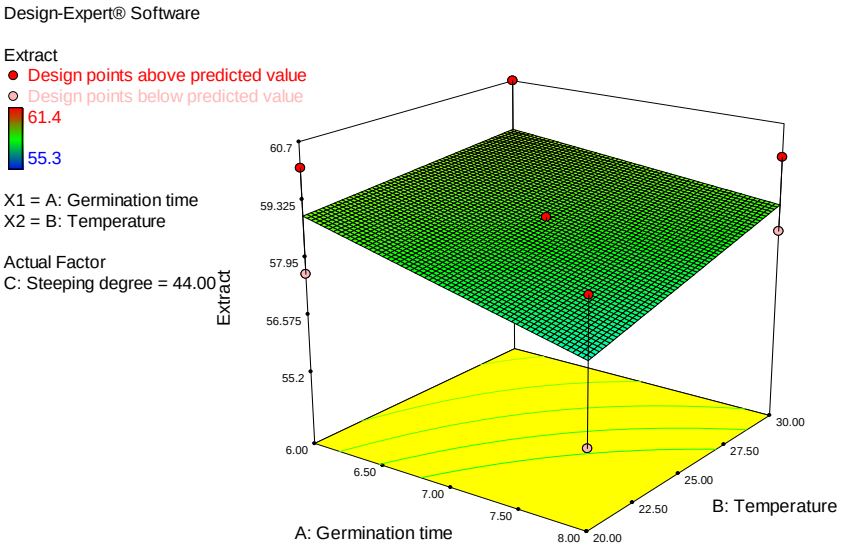


(B)

Figure 4.2 The response surface plot of CWE, (A) the effect of steeping degree and germination time on CWE content in KCD malt germinated at temperature 30°C, and (B) the effect of steeping degree and temperature on CWE content of KCD malt germinated for day 8th.

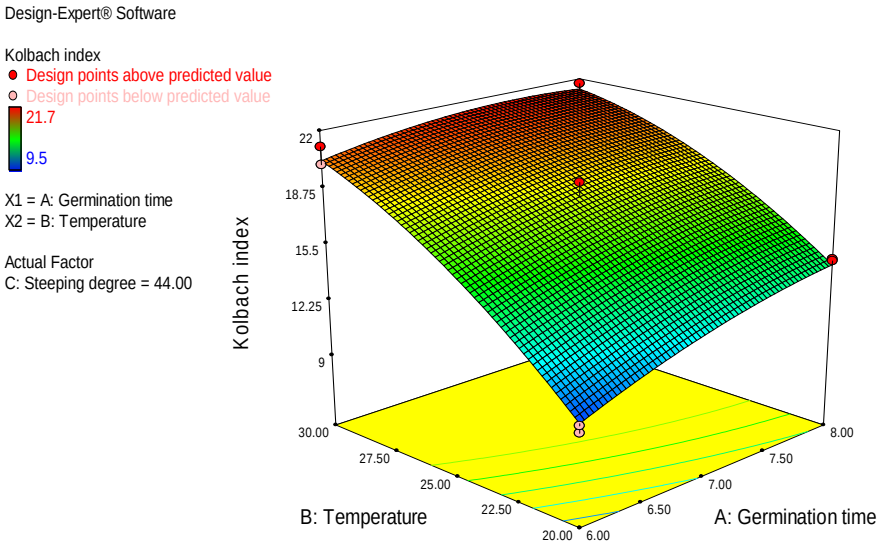


(A)

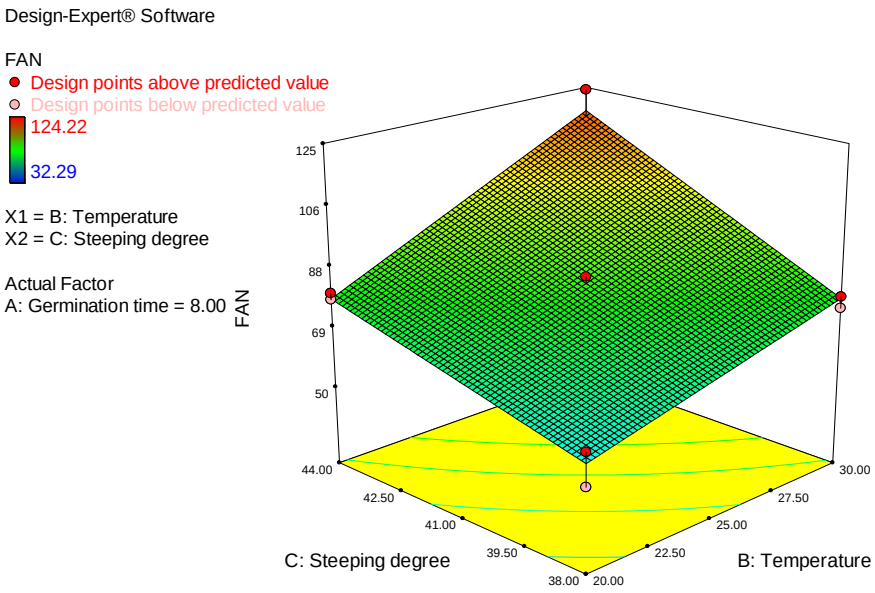


(B)

Figure 4.3 The response surface plot of extract content, (A) the effect of germination time and temperature on extract yield of KCD malt, and (B) KND malt under the 44% steeping degree.

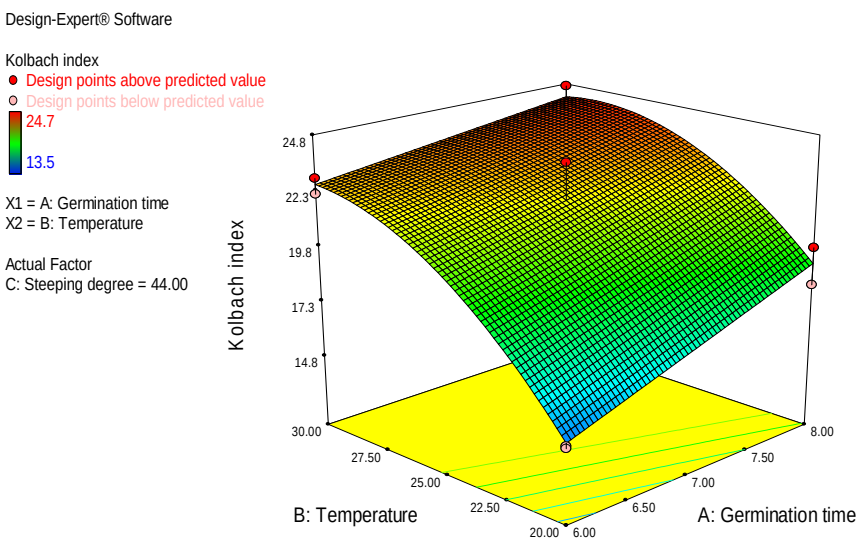


(A)

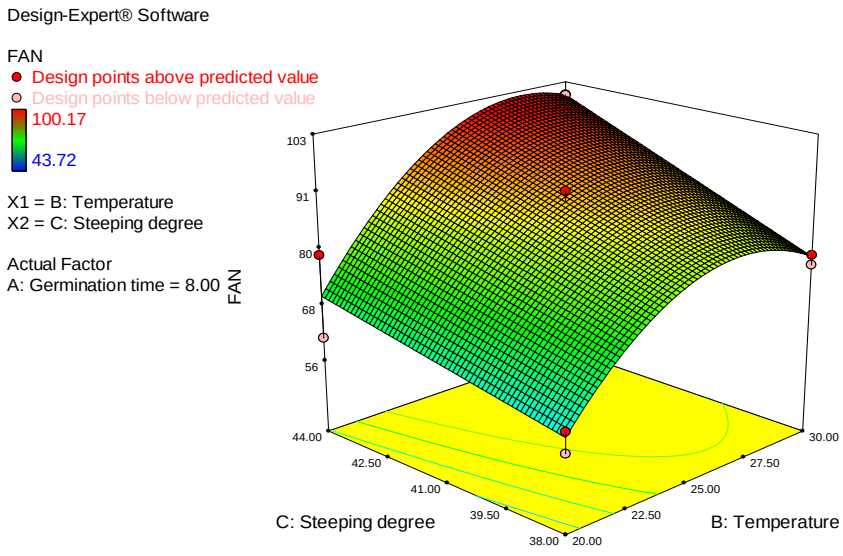


(B)

Figure 4.4 The response surface plot for Kolbach index and FAN from KCD malt, (A) the effect of temperature and germination time on Kolbach index at 44% degree steeping, and (B) the effect of temperature and steeping degree on FAN from KCD malt germinated for 8 days.

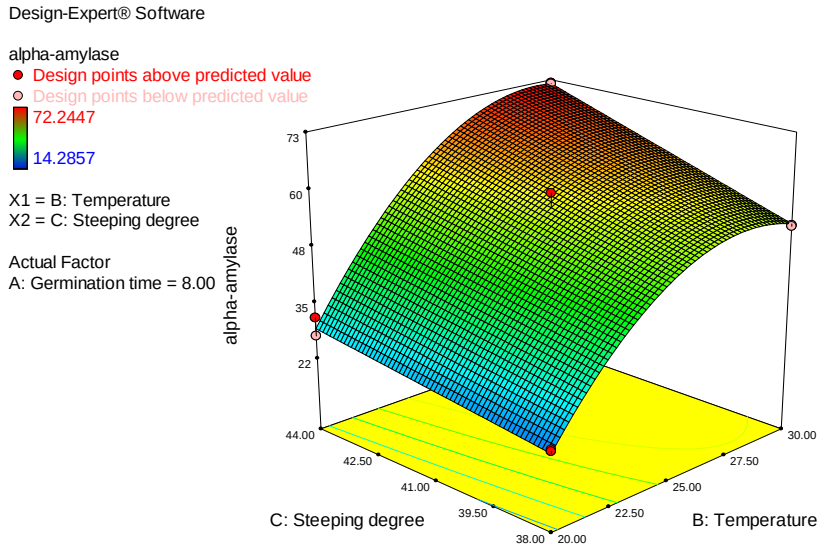


(A)

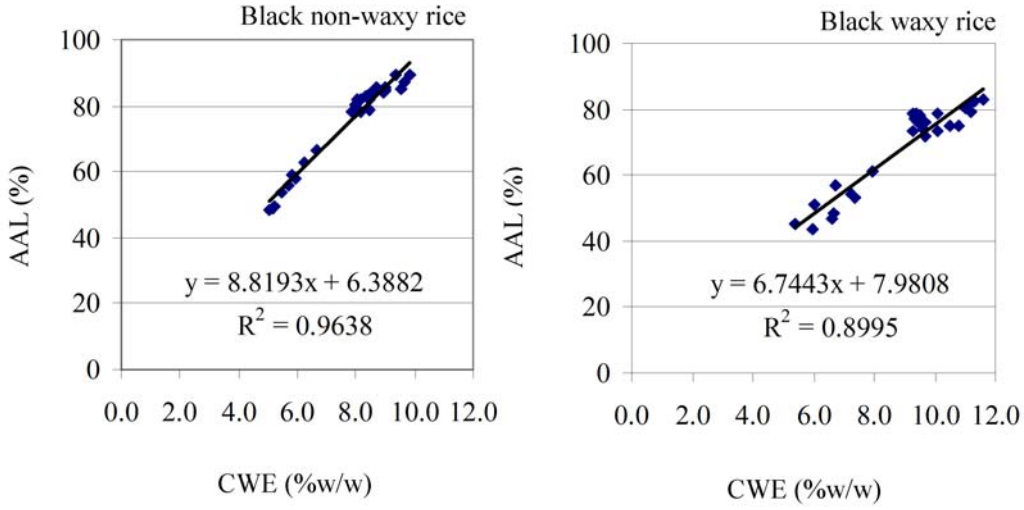


(B)

Figure 4.5 The response surface plot for Kolbach index and FAN from KND malt, (A) the effect of temperature and germination time on Kolbach index at 44% degree steeping, and (B) the effect of temperature and steeping degree on FAN content from KND malt germinated for 8 days.



(A)

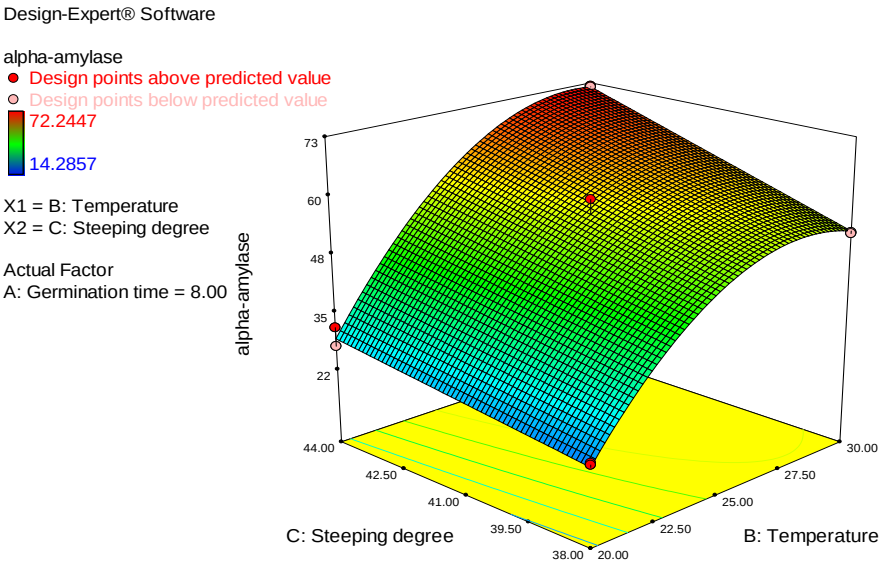


(B)

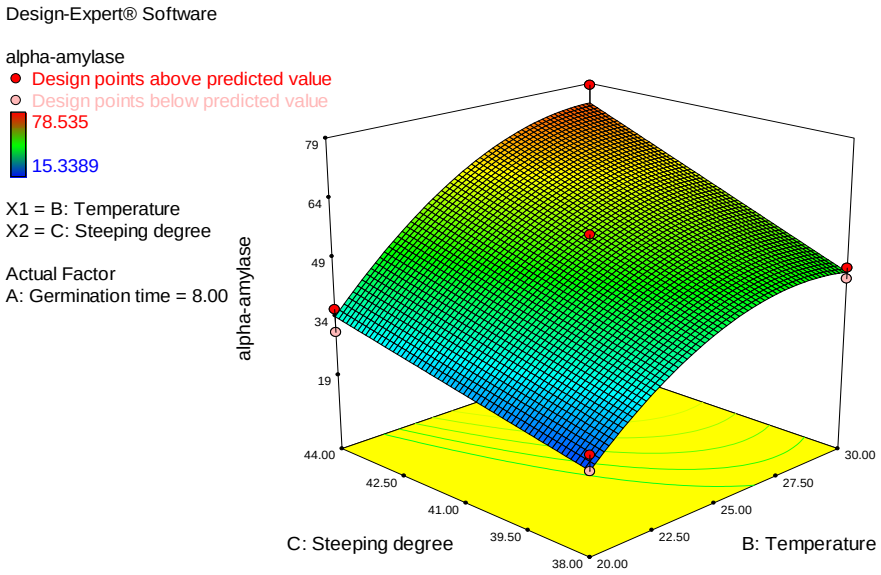
Figure 4.6 The response surface plot of AAL from KCD malt and the correlation of AAL and CWE, (A) the influence of germination time and temperature on AAL of KCD malt germinated at 41% steeping degree, and (B) graphs of correlation between AAL and CWE in both rice malt cultivars.

4.4.3.4 α-Amylase activity in rice malt

The amylolytic enzymes are group of enzymes which govern of starch breakdown. The main enzyme which influence the rate of starch digestion is α-amylase, which digests starch to amylose and amylopectin (Guglielminetti et al., 1995). The comparison of α-amylase activity in paddy rice and malted rice indicated that this enzyme was mainly expressed in germinating rice seed. In this study, α-amylase activity was increased along with germination time, steeping degree and temperature (Figure 4.7A and 4.7B). Water is needed for many enzymes in germination process, and in this study the effect of steeping degree on α-amylase production was found. At 38 % steeping degree, the enzyme activity in KND was stable, whereas when adding of water to 41 % and 44 % steeping degree, the α-amylase activity was increased from day 6^{th}-8^{th}. Therefore, the maximum α-amylase activity from both rice varieties were found on day 8^{th} at 44 % steeping degree and 30 °C (75 and 83 U/g malt for KCD and KND, respectively). In the case of barley, it has been reported that the maximum rate of enzyme production was found on 2^{nd} day of germination and stabilized after the 4^{th} day (Wijngaard and Ulmer, 2006). However, a similar behavior was found in buckwheat which reported the maximum of activity found after 6 days, 63 (U/ g malt) (Okungbowa et al., 2002).



(A)



(B)

Figure 4.7 The response surface plot of (A) the influence of steeping degree and temperature on α-amylase activity in KCD malt, and (B) in KND malt germinated 8 days.

4.4.4 Other malt qualities: β-amylase, limit-dextrinase, α-glucosidase, Gamma Amino Butyric Acid (GABA) and malting losses

The β-amylase is an exo-enzyme which acts on dextrin to release maltose units and it is found in many cereals (Nakamura et al., 1989; Okungbowa et al., 2002). Two paddies had different amount of enzyme activity. However, β-amylase activity in KCD increased obviously after germination process. This finding in KCD was similar to some rice varieties (Guglielminetti et al., 1995) as well as buckwheat (Okungbowa et al., 2002), whilst the result from KND was similar to barley and sorghum. In barley, there were reported that β-amylase activity existed in ungerminated seed and increased during the first two days of germination time and then decreased on the late of germination time closely to the original activity (Lewis and Young, 1995). Therefore, KND malt had β-amylase activity near to that in paddy. The other two enzymes found in paddy rice and germinating rice were limit-dextrinase and α-glucosidase. The activities of both enzymes in malted rice were higher than paddy rice (Table 4.2) as well as in the case of barley. The minimal activity was found in mature barley grains, while the higher activity was found in germinating grains that were the activities of free forms (soluble, active), latent forms (soluble, inactive) and bound forms of α-glucosidase (Lewis and Young, 1995). Moreover, GABA was measured by HPLC, and 9.43 and 6.33 (mg/100g) of GABA were found in KCD and KND malt respectively, from the optimal condition for malting. This content found in KCD was in the same range of GABA found in barley malt 8.6 (mg/100g) and it was increased with decreasing of germination temperature, and the hypoxia condition induced GABA production as same as found in rice (Chung, Jang, Cho, and Lim, 2009). In case of KCD and KND, GABA was increased

with germination temperature, steeping degree and germination time. The malting losses were compared between steeping regime in chapter 3 with this experiment, the result indicated that malting losses by the former steeping regime at day sixth was approximately 20%, whereas in this experiment was approximately 12-13% on day eight. Since steeping condition retarded malting losses was reported in chapter 3 and this result agree with Dewar et al., (1997), The result suggested that the malting losses could be reduced and the sufficient of moisture content could be obtained by modification of malting regime to be steeping and air-rest switching regime until steeping degree reach 44%.

Table 4.5 Comparison the qualities of rice malts with other cereal malts.

Parameters	KCD	KND	buckwheat	sorghum[d]	Barley
CWE %(w/w)	9.88	11.37	-	-	20
Extract %(w/w)	62.0	58.8	69.2[c]	65-83.7	79.9[a]
FAN (mg/100g)	104	99.9	107[c]	100	106.7[a]
Kolbach index (%)	19.95	22.95	23.91 [b]	15.3-41	31.24 [b]
α-amylase (U/g)	75	83.7	19.9[c]	39-135 [e]	105.9[a]
β-amylase (U/g)	80	105.3	24.7[c]	80-168 [e]	514[a]
Limit-dextrinase (U/kg)	5,066	5,212	-	-	~500
α-glucosidase (U/kg)	250	210	-	-	-
AAL (%)	85.5	85.5	61.8[c]	43-96	82.7[a]
GABA (mg/100g)	9.43	6.33	-	-	-
Malting losses (%)	12.61	12.43	-	-	12-14

[a] Wijngaard and Ulmer, (2005), [b] Wijngaard, et al, 2005, [c] Phiarais et al., (2005), [d] Briggs, 1998, p731, [e] Agu and Palmer, (1998).

4.4.5 Observation of starch digestion in malted rice

In order to monitor modification of starchy endosperm in malted rice, the half-grain of malted rice were stained with iodine solution for 30 second and pictured under stereo microscope as shown in Figure 4.8-4.9. The different transparency of starchy endosperm between KCD and KND indicated that the endosperm of non-waxy rice had transparency property more than waxy rice and agreed with result of Kang, H. J., Hwang, I.-K., Kim, K.-S., and Choi, H.-C., (2006). The transparency of starchy endosperm depends on the starch molecule arrangement and protein content. KND has space between starch granules to reflect light in several directions; as a result of low transparency starchy endosperm. In Figure 4.8B, the low transparency was found in the area near to embryo of malted KCD rice which implied that the space between the starch granules might be increased and reflected light more than ordinary KCD endosperm.

The color of starchy endosperm of KCD before and after malting process, was purple by staining with iodine implied that there was not significantly changed of amylose property in this rice cultivar. In case of KND malt, the different color of stained endosperm from paddy and malted grains was found; particularly, the area near to embryo zone was shown in light brawn color or less stained by iodine. Amylose-iodine complex cause of blue color, whereas brown color could be found by amylopectin-iodine complex. Yu and colleague (1996) reported that the amylose with DP of 40-50 can form blue color with polyiodide chain shorter than 20, whereas the DP of 30 or less results in purple or red brawn color. According to opacity area and less iodine stained endosperm, the direction of starch modifications seem occurred more at ventral side in area near to embryo. There are two models, explaining patterns

of grain modification during barley malting (Figure 4.10). Model 1; the modification parallel to the scutellum layer from the proximal to the distal end of the grain, and model 2; the modification progressing from the scutellum-aleurone junction on the dorsal side (Brien and Fowkes, 2004). Our results of iodine staining of malted grain looked similar to model 1 mixed with 2 but occurred in ventral side. Rice embryo is located in the ventral side and the GAs are synthesized in the embryo, penetrate through the scutellar epithelium, diffuse to the aleurone layers, and induce the *de novo* synthesis and secretion of α-amylase in scutellum and aleuron layers (Mitsunaga et al., 2001; Sugimoto, Takeda, Nagato, and Yamaguchi, 1998).

The starch modification in malted rice was observed by SEM and shown in Figure 4.11 and 4.12, rice starch granules are polygonal, and vary in size and dimension. The observation of starch modification focused at area close to embryo, central middle of endosperm and the top edge at longitudinal side of rice grain. The ungerminated KCD rice starch granules at endosperm closed to the embryo, middle and top of longitudinal side were tightly packed (Figure 4.11A-D), the appearance of hold in between compound starch granules and changing of starch morphology were obviously found after malting process (Figure 4.11E-H). The starch granules located close to embryo and aleurone layer were digested, particularly along the ventral side up to the distal side. However, under the electron microscope, the digested starch granules were only found in some area and just some part of grain, the most part were not digested. Figure 4.12H showed the modified of starchy endosperm of KND malt at distal end occurred at area closed to aleurone layer and the less modified up to normal starch were found more as more distance from this layer.

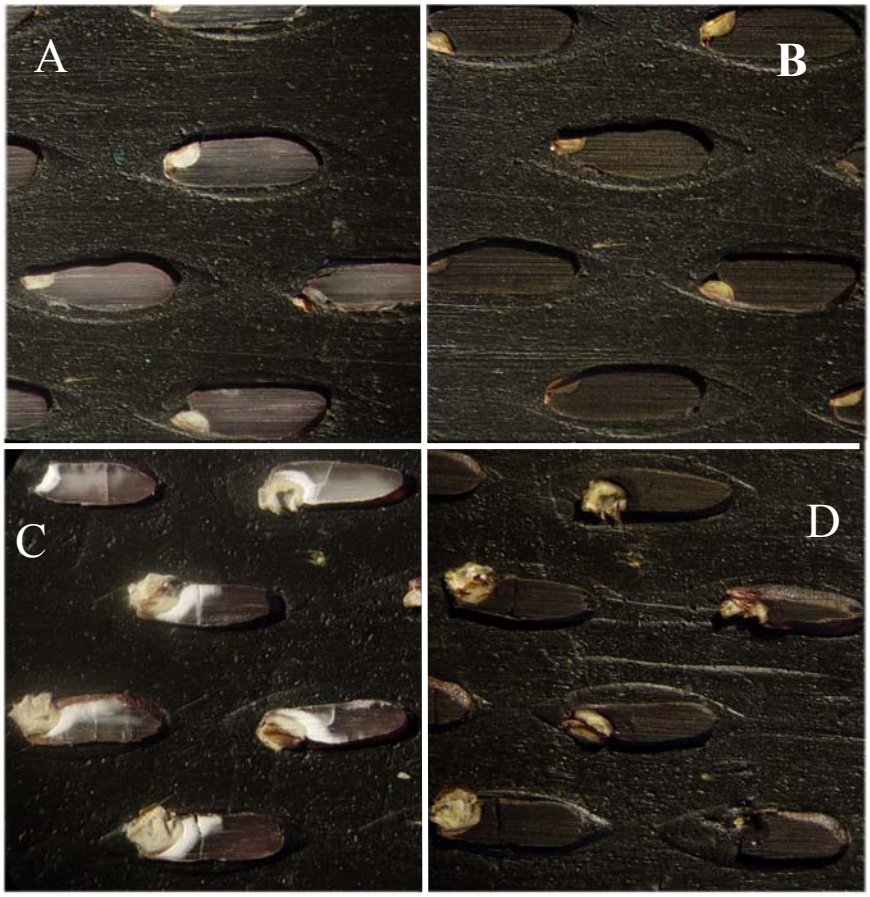


Figure 4.8 Pictures of half grain KCD rice and malt observed under compound microscope, (A) the unstained half grain of rice, (B) the iodine stained half grain of rice, (C) the half grain of malt, (D) the iodine stained half grain of malt.

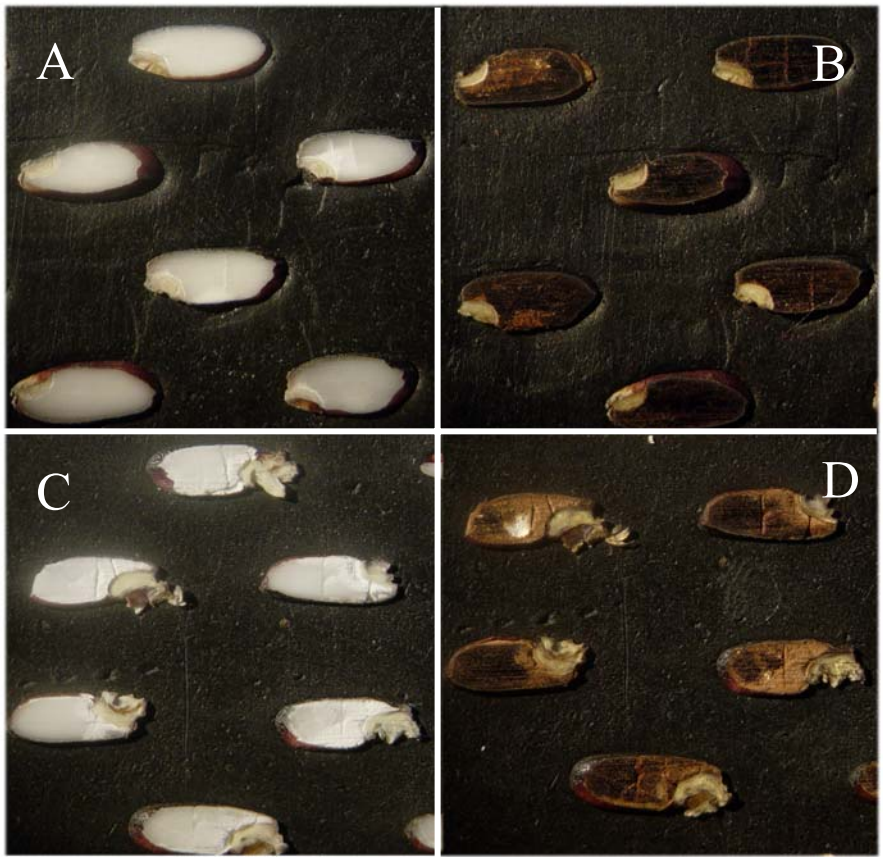


Figure 4.9 Pictures of half grain KND rice and malt observed under compound microscope, (A) the unstained half grain of rice, (B) the iodine stained half grain of rice, (C) unstained the half grain of malt, and (D) the iodine stained half grain of malt.

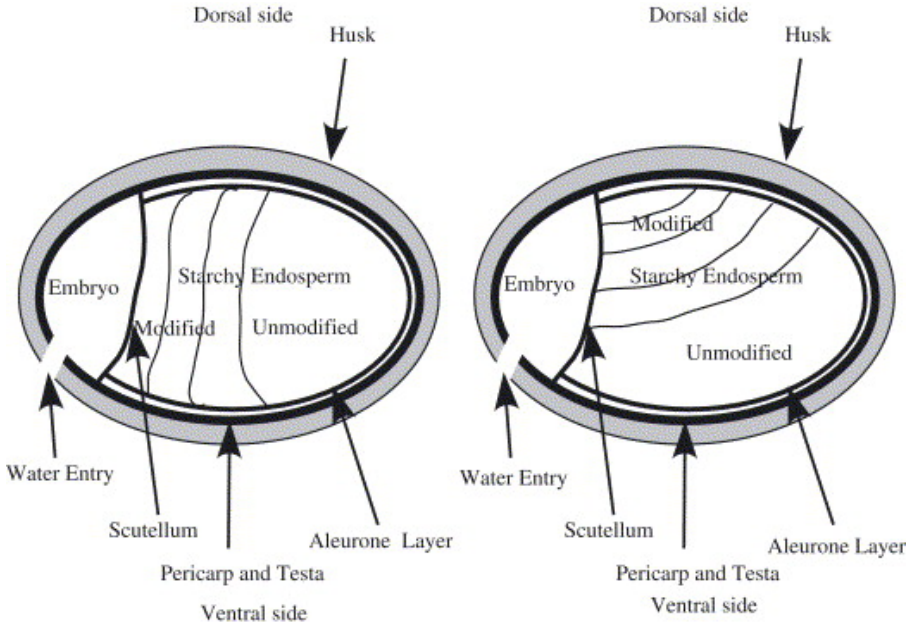


Figure 4.10 Left: model 1. Patterns of modification progressing approximately to the scultellum layer from proximal to the distal end of the grain. Right: Model 2. Patterns of modification progressing from the scutellum-aleurone junction on the dorsal side (Brien and Fowkes, 2004).

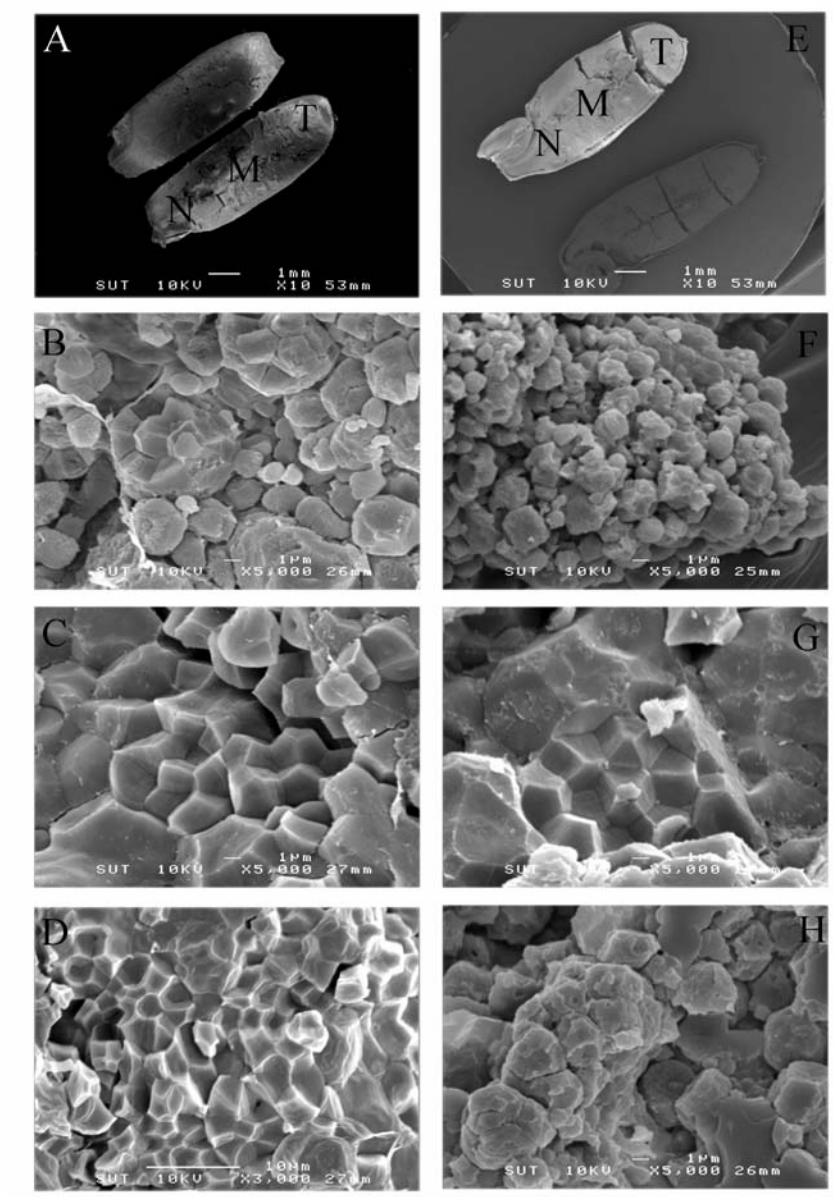


Figure 4.11 The SEM picture from KCD rice, A-D were ungerminated samples, and D-F were malt samples which (A) and (D) were morphology of half grain indicated area of observed region, (B) and (F) morphology of complex starch granules at area near to embryo, (C) and (G) morphology of complex starch starch in central of middle grain, (D) and (H) at the distal end of starchy endosperm.

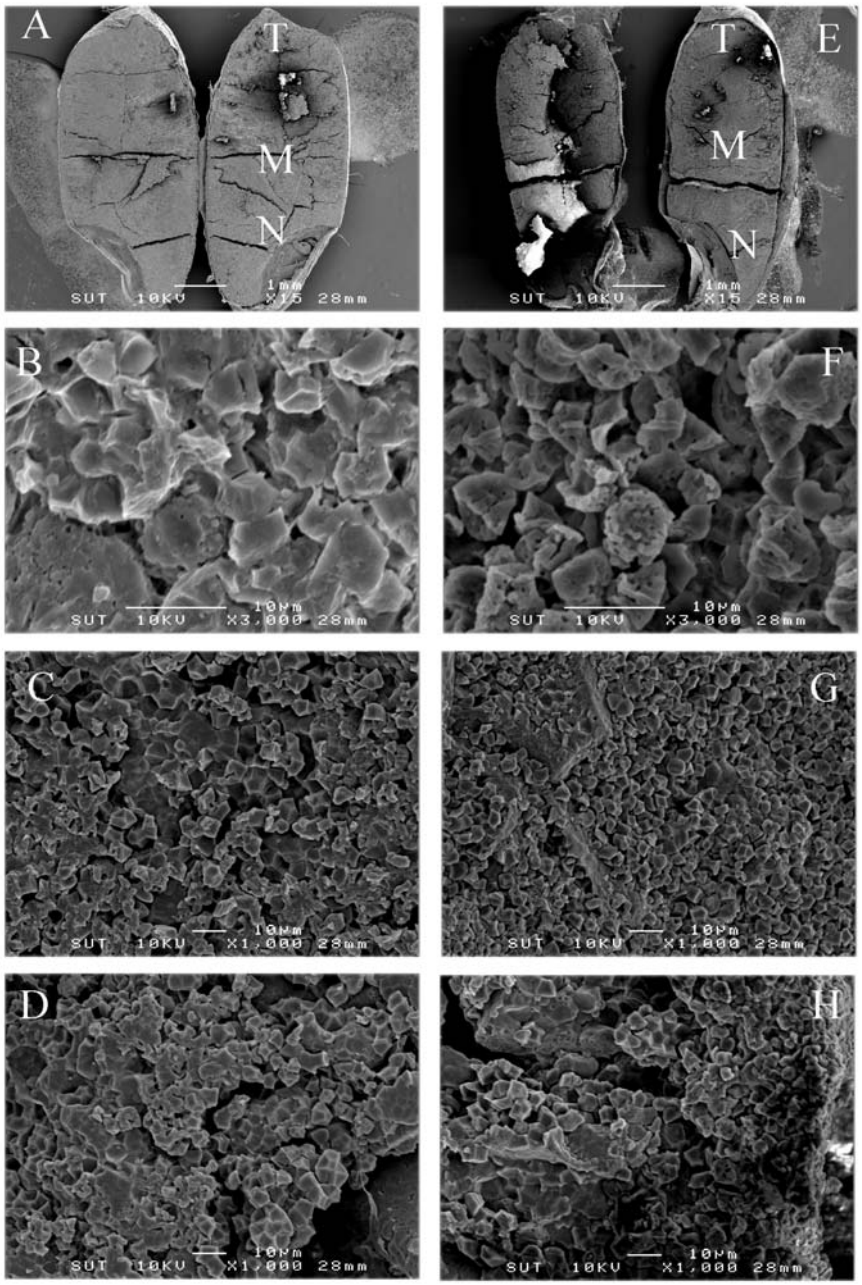


Figure 4.12 The SEM picture of KND rice (A-D) ungerminated samples, and (E-H) malt samples which (A) and (D) morphology of half grain indicated area of observed region, (B) and (F) morphology of complex starch granules at area near to embryo, (C) and (G) at central of middle grain (D) and (H) at the distal edge of starchy endosperm.

4.4.6 Effect of kilning temperature on malt properties

Rice were germinated at 30°C for 8 days and three sets of duplicated 800 g germinated rice were dried by varying three temperature programs. Kilning is not only terminate enzyme activities but also degraded amount of activities, Uvere and colleague (2000) found the activity of α-amylase in sorghum malt was reduced 18-25% and 4-90% for β-amylase, depending on cultivar. However, the increasing of soluble β-amylase activity in kilned buckwheat malt was reported by Phiarais, Wijngaard, and Arendt, (2005) and total activity in Nigerian sorghum malt made from different steep liquor and cultivars (Okungbowa et al., 2002). It seems to be cultivar dependent was major impact of changing enzyme activity in kilned malt. Therefore, in this experiment, the increasing or reduction of enzyme activity were not considered; however, the impact of kilning program on final malt properties were comparison between three different kilning temperature range. Drying at 50°C for 24 h decreased moisture content from approximately 40% (w/w) to be about 7% (w/w), whereas the medium temperature program and high temperature program were around 5% (w/w). Effect of kilning temperatures on α-amylase, β-amylase and limit dextrinase was not significant in KCD malt but they were significantly affect to those in KND malt. In addition, the kilning sorghum malts in two stages (the green malt initially dried to 55°C and subsequently to 65°C) produced malts with higher sugar contents than kilning at a single temperature of 65°C (Owuama and Abdullahi, 1994). This might be some amylolytic enzymes were activated during kilning. This result agreed with the increasing of AAL found in rice kilned by medium and high temperature program, respectively. However, the contents of fermentable sugar in term of AAL from three kilning programs were not significantly different. Whereas,

the effect of temperature program on rice malt quality was clearly found with FAN, soluble nitrogen and certainly to Kolbach index of KCD and KND malts. Kilning at higher temperatures decreased of soluble nitrogen, FAN and proteinase activity in rice malt. Therefore, the recommended kilning temperature for black rice is 50°C for 24 h.

Table 4.6 The properties of malted KCD and KND from different kilning programs.

Malt Properties	KCD			KND		
	Low	Med.	High	Low	Med.	High
Moisture (%)	7.2 c	5.1 *a*	5.45 *b*	6.8 *c*	4.75 *a*	5.2 *b*
Extract % (w/w)	62.9 *ab*	62.8 *b*	62.9 *a*	57.8 *a*	58.95 *a*	57.5 *a*
Protein (%)	8.9 *a*	9.05 *a*	9.05 *a*	7.8 *a*	7.4 *a*	7.35 *a*
Soluble N (gN/100g)	0.319 *c*	0.307 *b*	0.288 *a*	0.316 *c*	0.297 *b*	0.295 *a*
FAN(mg/100g)	87.7 *b*	85.1 *a*	86.25 *a*	96.6 *a*	91.8 *a*	91.15 *a*
Kolbach Index	22.4 *b*	21.25 *a*	19.8 *a*	26.3 *c*	24.95 *b*	25 *a*
AAL (%)	85 *a*	85.55 *a*	84.6 *b*	81 *a*	81.5 *a*	83 *a*
pH	6.0*a b*	6.02 *a*	6.01 *b*	5.98 *a*	5.98 *a*	6.00 *a*
α-amylase (U/g)	46 *a*	45 *a*	49 *a*	57 *b*	51 *a*	57 *b*
β-amylase (U/g)	37 *a*	34 *a*	41 *a*	51 *a*	53 *a*	50 *a*
Limit-dextrinase (U/kg)	4,835 *a*	4,873 *a*	4,595 *a*	4,608 *b*	4,619 *b*	4,221 *a*
α-glucosidase (U/kg)	257 *a*	255 *a*	240 *a*	187*a*	186 *a*	165 *a*

Mean values from two bash of kilning malts.

4.5 Conclusion

The steeping regime was improved for reduction of malting loss and maintaining the activity of α- and β-amylase. The results indicated that steeping

degree or moisture and temperature were the important factor to increase enzyme activity and malt modification. And both factors regulate how long of germination process must be carried out. Under steeping regime used in this experiment, the maximum value of process parameters provided the most desirable malt property, germination at 30°C, with 44% of steeping degree and germination for 8 days. This malting condition was not the best one for rice malt production, but factors influenced malt qualities were elucidated here. Therefore, the other optimal conditions with less desirability obtained by Design Expert program could be accounted by experienced malt master. This work demonstrate that using RSM for process optimization is a powerful tool for experimental design to reduce the number of samples and also to obtain the model equations which explain how the process variables influence each attribute. Although β-amylase and some malt properties of malted rice were poorer than barley, malted rice had 10 times greater amount of limit-dextrinase activity. The modification in malted rice observed by iodine staining and SEM indicated that there was a small part of grain which was modified during malting process. The effect of kilning program on malt quality was obviously found in soluble nitrogen and Kolbach index, drying at 50°C was recommended for ensuring that the enough soluble nitrogen could be obtained for beer fermentation. This optimal malting condition with short steeping and air rest switching gave malting losses approximately 12%.

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CHAPTER V

ESTRABLISHING OF MASHING CONDITION AND EVALUATION OF WORT QUALITY BY CHEMICAL AND PHYSICAL ANALYSIS, FERMENTATION AND SENSORY TEST

5.1 Abstract

Regarding the results of wort properties obtained from congress mashing was poorer than that from barley. The investigation an appropriate mashing regime to improve wort quality for the compromising fermentation was carried out in this chapter. Consequently, the fermentation and sensory evaluation were performed in order to evaluate the wort quality and wort production processes. The appropriate temperature ranges for conversion of proteins and carbohydrates were determined in laboratory mashing bath. The temperature effect on protein conversion was in between 50-55°C. The glucose production was at 50-55°C, maltose production was at 60-65°C and liquefaction could be operated at 70-75°C. These ranges of temperature were used to design mashing program for rice malts. The three temperature-programmed mashing and one decoction mashing programs were tested. The temperature-programmed focused on glucose production was selected to produce wort from KCD rice malt and the temperature-programmed focused on maltose production was selected for KND malt. The effect of mashing-in pH (5.8, 5.6, 5.2 and

4.8) and divalent cations supplementation (Ca^{2+}, Mg^{2+} and Zn^{2+}) were studied. The soluble nitrogen in the form of FAN were increased when reduced mashing-in pH down to pH 4.8; whereas, the maximum of soluble peptides was found at pH 5.2 with the maximum of AAL. The divalent Ca^{2+} at concentration 150 mg/L and mashing-in pH 5.2 gave the satisfied wort quality. Size of malt grist significantly influence FAN obtained from both rice malts, KCD malt was milled by adjusting gap distance at 0.5 mm and 1.0 mm for KND malt and approximately 38-39% of brewing yield obtained from this mashing unit. Lundin fractions suggested that the small peptides accounted for the most part of protein in wort and beer (≈ 80% w/w). Worts were evaluated via the fermentation processes, respecting process of top and bottom fermentations. The pleasant volatile compounds of ester and some higher alcohols in lager beers were lower than those in ale beer; in addition, the 4VG was detected at high concentration in KCD (7.4 mg/L) and KND ale beer (4.75 mg/L). The sensory test by 8 assessors indicated that chemical and physical properties of beer influence perception of consumer. The favorable lager beers from both rice malts were judged as drinkable and prefer the next glass, whereas KCD ale beer was undrinkable. Yeast selection and fermentation process could be benefit factors for reduction of some off-flavors and increasing pleasant flavors must be further investigated.

Keywords: Fermentation, Flavor, Mashing, Sensory evaluation

5.2 Introduction

Mashing is the process of mixing ground malt with water in the mash tun to extract the malt and further convert grain starches to fermentable sugars and non-fermentable carbohydrates (dextrin) that add body, head retention and other characteristic to the beer (Rabin and Forget, 1998). At each conversion temperatures,

enzymes were activated and degraded at the same time; consequently, lowest temperature at which maximum conversion can be achieved. And it is a function of malt modification because the extent of modification influences the rate of starch solubilization (Lewis and Young, 1995). There are many factors determining the mashing condition such as malt quality, gelatinization temperature, optimal temperature, mashing-in pH (Bamforth, 2001), water for mashing, grist size (Davey, Landman, McFuiness, and Jin, 2000) and etc.

The main categories of mashing regimes are infusion and decoction, and several subsets of both categories were applied to different quality malt and different type of cereal. Infusion mashing regime by using single conversion temperature (62-75°C) has been recommended for well modified malt, medium or poor modified barley were recommended to use temperature-programmed mashing, and decoction mashing was suggested for mashing with adjunct (Briggs, 1998; Kunze, 2004). The gelatinization temperature of starch from different source is one factor impact to selection of mashing program. Sorghum starch had gelatinization temperature 64-68°C, which is relatively high compared with barley starch (55-59°C) (Goode, Halbert, and Arendt, 2003) and compared to optimal temperatures for α- and β-amylase (Kunze, 2004). Thus, Taylor (1992) suggested triple decoction mashing process facilitated gelatinization and saccharification of starch.

Most of mashing regime start with protein conversion or proteinase rest since these enzymes had optimal temperature lower than β- and α-amylase. The proteinase active at temperature 45-50°C. Free amino nitrogen (FAN) and soluble nitrogen are determined as product of enzyme activities. Yeast required FAN minimally at 200-220 mg/L and soluble nitrogen influence to beer property rather than to yeast

fermentation (Kunze, 2004). In cereal grain, cysteine proteinases are the most important endo-preteinase enzymes to hydrolyzed cereal storage protein such as hordein in barley and glutelin in rice, and the optimal pHs were 5.0-6.6 (Bamforth, 2009). In sorghum, cysteine proteinase had optimal temprture at 50°C, pH 6.0 with highly activated by Co^{2+}, Mn^{2+} and Sr^{2+}(Ogbonna, Obi, Okolo, and Odibo, 2004), whereas Mg^{2+} and Ca^{2+} had been reported the ability to enhance proteinase activity of germinating barley and sorghum (Agu, 2006). The next temperature rest is for activation of β-amylase or saccharification rest. Generally, there are two amylase enzymes play an important role in starch hydrolysis during mashing; α-amylase and β-amylase and their reactions have different optimal temperatures. The β-amylase has optimal temperature in range 55-60°C and α-amylase around 70-75°C (Kunze, 2004) and cereal β-amylase are more acid-stable and less heat stable than α-amylase (Ziegler, 1999). Rice α-amylases have wide range of pI 4.60-6.2 due to at least ten isoforms are found and identified. The major isoforms are A, B, E_1 E_2 and K_1-K_5 isoforms. A and B isoform have pI 4.60 and 4.67, respectively and optimal temperature is 70°C (Nanjo, Asatsuma, Itoh, Hori, and Mitsui, 2004). In order to maintain the activity of α-amylases in mash, the Ca^{2+} supplementation has been approved by brew master and used in brewing at concentration 50-200 mg/L. Recently, the other one amylolytic enzyme has been interested from brewer, is limit-dextrainase enzyme that could increase fermentability in wort and decrease of wort viscosity. The optimal temperature for limit-dextrinase was 50-55°C, optimal pH at 5.5 (Manner and Yellowless, 1971).

Generally, green malt had pH in a range of 5.8-6.2 (Bamforth, 2001), the pH of mashing-in (the step of thorough mixing of grist with water) was usually reduced

by acidic solution to pH 5.2-5.6 for activation of malt enzyme activities (Kunze, 2004). Malt α-amylase had optimal pH at 5.5, 5.2 for β-amylase, pH 5.5 for limit-dextrinase, pH 5.5 for sulhydryl endo-peptidase, pH 5.5, 6.9, 8.5 for metalopeptidase (Bamforth, 2001). Most of these enzymes had optimal pH in a range of approved mashing-in pH 5.2-5.5; however, the mashing-in pH for single infusion mashing is 5.6 in order to activate amylotytic enzymes. Therefore, the mashing-in pH could be changed depending on the quality of malt and what is the main substance needed to be obtained in final wort. Recently, there are many enzymes developed for brewing in form of individual and enzymes cocktail (fungal α-amylase, heat-stable α-amylase, β-amylase, proteinase, heat tolerant β-glucanase, etc), which are not only improved brew house yield but also improved wort quality (Villicana and Saldivar, 2004). They were commonly used, when mashing with adjunct was performed (Goode, Wijngaard, and Arendt, 2005; Vinh, Viet, and Mai, 1993). The wort obtained after mashing must be boiled for sterilization and cooled down to fermentation temperature. Examination of wort property could be performed by analysis of wort chemical and physical property and must be evaluate through fermentation process. In this chapter, the conversion temperatures were investigated for protein and starch conversion, and mashing regimes, mashing-in pH, divalent cations supplementation, grist size as function of milling machine, were studied of their effect on wort properties. The property of wort were examined and fermented with top and bottom fermenting yeast, and finally beer samples were analyzed their properties and evaluated through the sensory test.

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Rice malts

KCD (black non-waxy rice) and KND (black waxy rice) were produced according to method mentioned in the chapter 4.

5.3.1.2 Yeast strains

Yeast were received from yeast bank at Institute of Brewing Technology II, Technical University of Munich, Freising-Weihenstephan, Germany. *Saccharomyces cerevisiae* 34/70 was used for bottom fermentation and *S. cerevisiae* 60/120 was used for top fermentation.

5.3.2 Methods

5.3.2.1 Optimization of temperature for nitrogenous substance, FAN, extract content and fermentable sugar in wort

Rice malt was milled in a disc mill (Bühler, Braunschweig, Germany) as fine grinding 0.2 mm and mashed with distilled water as ratio 1:8 in the laboratory mashing bath. The isothermal mashing temperatures were operated at 40, 45, 50, 55, 60, 65, 70°C for 30 min and 60 min. Wort was filtered through ribbon paper and analyzed for soluble nitrogen, FAN, extract content and fermentable sugar.

5.3.2.2 Formulation of temperature program for mashing

The formulation of temperature programs for mashing of rice malts was considered base on the objective to increase extract yield and improve wort quality by using the same grist water ratio as standard congress mashing and finely milled malt. Four mashing programs were tested and compared for wort properties.

Program A. The 50 g rice malt was mixed with 250 mL of distilled water in a mashing beaker and put in a laboratory mashing bath. The temperature programs was preformed as following; 40°C for 30 min, 50°C for 30 min, 55°C for 30 min, 60°C for 30 min, 65°C for 30 min, and then 100 mL of warm distilled water (70°C) was

added and held at 70°C for 60 min. Then, it was cooled to 20°C, adjusted total weight to 450 g, filtered through filter paper, and then the cleared wort was analyzed for extract yeild, soluble nitrogen, FAN, AAL and pH

Program B. Using the same ratio as program 1, the first 25 g of malt grist was separated to mash with 125 mL distilled water in first beaker. This mash was cooked at 95°C for 15 min, and then cooled down to 60°C and poured to the second beaker which previously mashed at 40°C for 60 min, 50°C for 60 min and using the same malt grist water ratio. After mixing with the frist mash, mashing temperature was continued to 55°C for 60 min and 70°C for 60 min and then cooled down to 20°C and adjusted total weight to 450 g before filtering through filter paper. The cleared wort was analyzed for extract, soluble nitrogen, FAN, AAL and pH

Program C. The temperature range between 50-60°C was emphasized by progaming the temperature stands as following; 45°C; 10 min, 50°C; 20 min, 52°C; 20 min, 55°C; 20 min, 57°C; 20 min, 60°C; 20 min, and 70°C; 20 min with adding 100 mL of 70°C distilled water. Finally, temperature was risen to 78°C, 5 min and then cooled down to 20°C, and wort was obtained and analyzed by the same manner as in program A.

Program D. The temperature was emphasized between 50-60°C same as Program 3 but using 62°C instead of 57°C and 64°C instead of 60°C, and then 70°C; 20 min with adding 100 mL of 70°C distilled water. Finally, the temperature was risen to 78°C for 5 min and then cooled down to 20°C, and wort was obtained and analyzed by the same manner as in program A.

5.3.2.3 Optimization of mashing-in pH

The optimal mashing program was tested for the optimal mashing-in pH. The grit water ratio 1:8 was used as same as congress mashing and then the initial pHs were adjusted with 1 M lactic acid as 5.6, 5.2, 4.8, 4.4.

5.3.2.4 Optimization of Ca^{2+}, Zn^{2+} and Mg^{2+} ion concentrations for improving wort quality

The divalent cations of Ca^{2+}, Zn^{2+} and Mg^{2+} in the form of chloride salt were dissolved in distilled water at concentrations 50, 100, 150 and 200 mg/L. Rice malts were mashed with these solutions as the same ratio as mentioned in 5.3.2.3 and analyzed for wort properties.

5.3.2.5 Effect of grist particle size on wort properties

One kilogram malt was milled by using laboratory disc mill (Bühler Miag Disc Mill) at 0.2 mm and the two roller mill was used by adjusting gap distance as 0.25, 0.5 and 1.0 mm (Kuenzwel, Kulmbach/Bayern). The malt grist was sieved by shaking for 5 min in standard EBC Pfungstat Plansifter sieving in order to determine grist fraction from each milling. The detail of particle sizes regarding the size of screen mesh was indicated in Table 5.1. Malt grist were mashed in the laboratory mashing bath at grist water ratio 1:8 and operated with the optimal mashing program for each rice malts (without pH adjust and Ca^{2+} supplementation)

5.3.2.6 Wort preparation for beer production

The ground KCD malt and KND malt were mashed according to the optimal mashing program obtained from 5.3.2.2-5.3.2.4. The brew master mashing unit was used to mash 4.5 kg malt with distilled water 22 L (ratio 1:5). Mashing-in pH was adjusted to 5.2 by adding 1 M lactic acid, and 150 ppm Ca^{2+}. One litter of wort was

taken for analysis of wort quality. Then, the hot wort was heated up to 97°C and held at this temperature for 1 h. Hallertauer Spalter hop pellets (4.1% α-acid) were added at the last 30 min of boiling step in order to obtain bitterness at 25 IBU. When wort boiling was completed, the spent grain and hop residual were filtered out. The clear cast wort was sampling for 1 L and the rest was filled in 20 L steriled keg and kept in cold room (4°C) overnight.

Table 5.1 Sieve properties.

Pfungstadt EBC			
Sieve	Particle size (μm)	Screen mesh size (mm)	Grist fraction
Sieve 1	>1250	1.27	Husk
Sieve 2	1000	1.01	Coarse grists
Sieve 3	500	0.547	Fine grists 1
Sieve 4	250	0.253	Fine grists 2
Sieve 5	125	0.152	Flour
Pan	<125		Fine flour

5.3.2.7 Inoculum preparation

The *S. cerevisiae* strain 34/70 and 60/120 were prepared by inoculating two full loops in 100 mL Pilsner wort prepared from baley and cultured at room temperature for 24 h. Then, 100 mL of inoculum was added to 900 ml of aerated Pilsner wort. *S. cerevisiae* strain 34/70 was cultured at 10°C for 48 h and 20°C for *S.*

cerevisiae 60/120. Wort cultures were aerated by using aquarium pump connected with aeration stone and pump was run for 1 mine in every 1 hour in order to avoid excess foam formation. Cell concentration was counted under microscope and calculated the volume needed to inoculate into 6 L wort at $12x10^6$cells/mL. The needed inoculum was centrifuged 3000xg for 10 min and the supernatant was discarded. Finally, yeast was resuspended into the 500 mL wort used for fermentation and then mixed with rest part in the keg.

5.3.2.8 Fermentation

The 24 L of clear wort which was obtained from two mashes was divided to 6 L and filled in 10 L keg and aerated for 10 min in order to obtain 8 mg/L dissolved O_2. The aerated wort was acclimatized to 10°C and 20°C for bottom and top fermentation. Inoculation of *S. cerevisiae* 34/70 to 10°C wort and *S. cerevisiae* 60/120 to 20°C at concentration $12x10^6$cells/mL were performed and kept the keg in cold room 9°C and 19°C, respectively. Fermentation was finished when extract content was less than 80% or it was not changed in 48 h. Two hundred milliliter of fermenting wort was taken every 24 h in order to observe the suspended cell concentration, pH, extract and FAN. The extract content determined by using Pyknometer (EBC 8.2.1), FAN, and pH were determined after filtered wort through filter paper (Schleicher & Schuell 597 ½), and the rest of wort was kept at -20°C.

After fermentation was finished, the ale beer and lager beer were maturated for 2 days at 10°C and 5°C, respectively. Beer was racked in order to reduced cell concentration to less than $2x10^6$ cell/ml and then, they were adjusted CO_2 to be 4.5 mg/L and chilled at 0°C for a week. The chilled beers were filtered through steriled filter pad supplemented with coarse filter aids (120 g/hL) under CO_2 pressure. The

cleared beers were filled in the bottle at CO_2 added 4.5 mg/L at 4°C, and were kept at 4°C for further analysis of foam stability and protein fractions.

5.3.2.9 Wort and beer analysis

Properties of wort and beer were analyzed according to EBC and MEBAK. Extract content was analyzed by beer analyzer (SCABA), soluble nitrogen (MEBAK IV 4.1.4.5), Kolbach index (MEBAK VI 4.1.4.5.3), apparent attenuation limit (AAL) (MEBAK 4.1.4.10), protein fraction (MEBAK III 2.9.3.1) and color of wort and beer were analyzed by spectrophotometer (EBC 8.5).

5.3.2.9.1 Determination of protein molecular weight by SDS-PAGE

Two hundred milliliter of wort and beer were added with 5% (w/v) of PVPP in order to reduce polyphenol in solution and centrifuged at 3000xg for 10 min. Supernatant was slowly added 50% ammonium sulphate with gentle mixing. The precipitated protein was collected by centrifugation at 3000xg for 10 min and resuspended in 10 mL deionized water before dialysed with deionized water at 100 times of volume of sample for 24 h. The desalted samples were frozen and dried before kept in cold and dry place. The discontinuous SDS-PAGE was prepared at 15% acrylamide for separating gel and 4% stacking gel and manipulated according to Lamni's method. The standard protein from BIO-RAD (all blue precision plus) was used for estimation of molecular weight protein.

5.3.2.9.2 FAN in fermented wort

FAN content in fermented wort was measured by Ninhidrin method using spectrophotometry (EBC 8.10). Wort samples were diluted to get amino nitrogen 1-3 mg/L, and then 200 μL of diluted wort was pipetted into a test tube. One milliliter of

color reagent (100 g $Na_2HPO_4 \cdot 12H_2O$, 60 g KH_2PO_4, 5 g ninhydrin and 3 g fructose were dissolved in 1L distilled water) was mixed well with diluted wort and put in a boiling water bath for exactly 16 min. The heated samples were cooled in water bath at 20°C for 20 min. Then, 5mL of diluting solution (2 g KIO_3 was dissolved in 600 mL distilled water and 400 mL of 96% (v/v) ethanol) was added and measured the absorbance at 570 nm in a 10 mm cell against a reagent blank prepared from the reagents plus 2 mL of distilled water instead of diluted wort. With each set of determination made three replications of glycine standard checks using 2 mL of diluted glycine solution (0.1072 g glycine in 100 mL of distilled water and then 1 mL of stock glycine was diluted to 100 mL).

$$\text{Calculation of FAN} = \frac{A_1 \times 2 \times d}{A_2}$$

Where; A_1 = absorbance of test solution at 570 nm in 10 mm cell

A_2 = mean absorbance of standard solutions at 570 nm

d = dilution factor

5.3.2.9.3 Suspended cell concentration

The suspended cell concentration was monitored by using hemacytometer for counting cell number under a microscope. The fermented samples were diluted with distilled water to an appropriate concentration, and then they were mixed with methylene blue solution just before an observation under the microscope (0.1 g methylene blue in 100 mL distilled water).

5.3.2.9.4 Fermentable sugar analysis

The fermentable sugars in wort, cast wort and finished product was carried out by using high-performance anion-exchange chromatography (HPAEC) with pulsed

amperometric detector (PAD) (Kessler, Zarnkow, Kreisz, and Back, 2005). The internal standard of 2-deoxy-D-glucose was used for glucose, fructose and sucrose and cellobiose was used as internal standard for maltose and maltotriose. Samples were diluted with deionized water at ratio 1:999 and 2.5 μL of samples were injusted through a CarboPac PA 10 guard column and CarboPac PA 10 analytical column. The oven compartment was set at 30°C and the flow was set to 0.25 mL/min. The gradients of mobile phase concentration were shown in Table 5.1, the eluent A was 250 mM NaOH diluted from 50% (w/w) NaOH and eluent B was deionized water, and both of them were kept under pressured helium gas. The condition of PAD operation was operated as standard method for carbohydrate analysis.

Table 5.2 Gradients of mobile phase concentration.

Time (min)	Eluent A (%)	Eluent B (%)
-10.0	9	91
0	9	91
17.9	9	91
18.5	80	20
55	80	20
55.1	9	91
75	9	91

5.3.2.9.5 Ethanol and fermentation by-product

According MEBAK methods, Gas Chromatography (GC) was the main

instrument for analysis of ethanol, acetaldehyde, ethylacetate, iso-amylacetate, amylalcohol, n-propanol, iso-butanol, (MEBAK III 1.1.1), diacetyl and 2, 3-pentadion (MEBAK III 1.2.1), and 4-vinylguaiacol was analyzed by using HPLC (MEBAK III 3.12).

5.3.2.9.6 Beer foam stability

Foam stability of the beer was analysed following EBC9.42, using NIBEM-T meter. Beer bottles were warmed to 20±0.5°C and then they were dispensed through a foam flashing device in, which it was forced under carbon dioxide pressure (2 bars) through an orifice and filled in the standard glass of beer/foam (placed under the needle electrode previously calibrated). The electrodes were moved down until one of the four outer needles is touching the surface of the foam. As the foam collapsed, the contact was broken and the electrode system moved down until one of the four outer needles was again touching the foam surface. The foam has collapsed down to a reference point and passed first 10 mm (wait period), the timer was run for further distance 30 mm. The collapse time was displayed on the digital display.

5.3.2.10 Sensory Evaluation

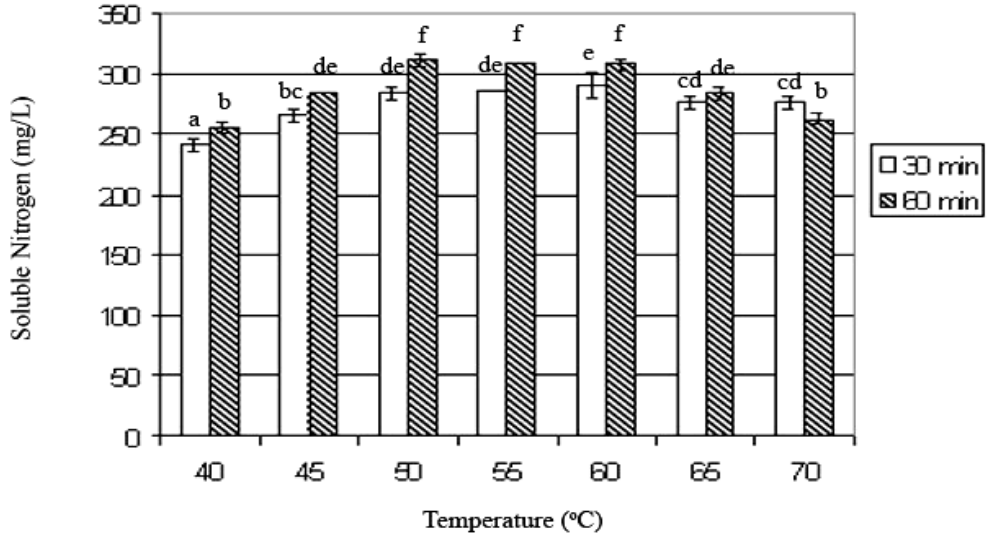
The sensory evaluations of beer samples were tasted with 8 assessors lived in Germany who has experiences and familiar of beer drinking. The discussion for meaning of each attribute in score sheet and agreement that these rice beers were presented as new products on shelve, was carried out before beer tasting. All samples were poured into beer glasses and waited until the temperature reach 8-10°C. All assessors had one score sheet for four samples and tasted all samples within 30 min. The hedonic scale (5 levels) was designed as 0 to 5 scores for evaluate the aroma, appearance, flavor and mouth-feel. The score 0 was extremely dislike, 1 was dislike,

2 was normal, 3 was like, 4 was like very much, 5 was extremely like. And finally attribute was overall impression, if scored as 0 was undrinkable, 1 was drinkable but not prefer another glass, 2 was drinkable and prefer one more, 3 was good, 4 was very good, 5 was excellent.

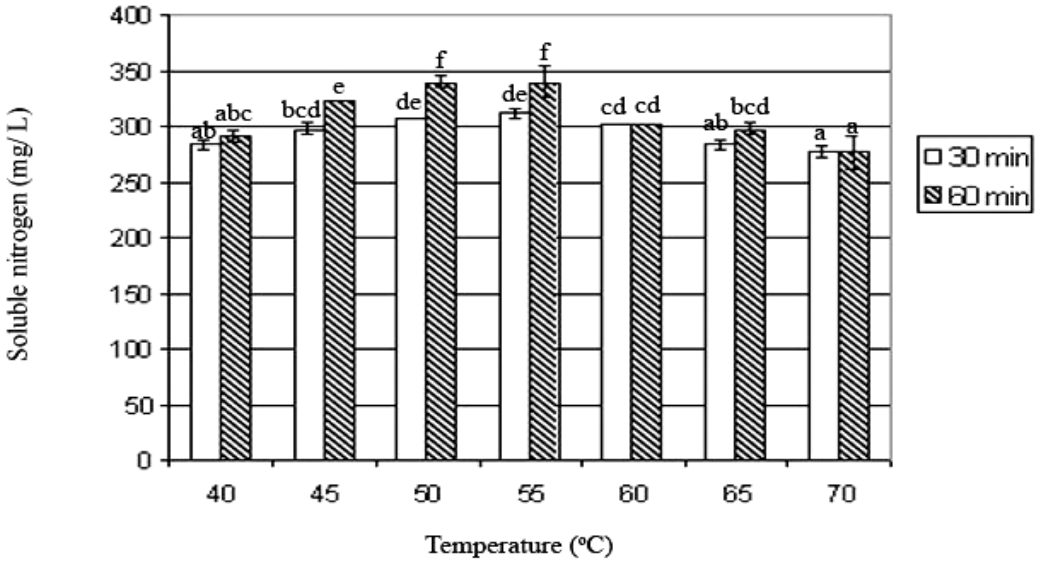
5.4 Results and discussion

5.4.1 Optimization of temperature for increasing of nitrogenous substances in wort

The optimization of temperatures for nitrogenous substances in wort was carried out with isothermal mashing for 30 and 60 min. The soluble nitrogen in KCD wort was increased towards the increasing of temperature until 50-55°C, the saturated concentration of soluble nitrogen and FAN was found (Figure 5.1A and 5.2A). The soluble nitrogen was decreased when temperature higher than 60°C due to the inactivation of proteinase enzymes. In case of KND, the highest soluble nitrogen and FAN were found at 55°C, 340 and 110 mg/L, respectively (Figure 5.1B and 5.2B). Therefore, the optimal range for proteinase rest should be in between 50-55°C. Moreover, at this temperature range, the soluble nitrogenous substances in wort mashed for 60 min was higher than those in wort mashed for 30 min. This implied that rice proteinases were stable at least 60 min at this temperature range. The optimal temperatures of proteinase enzymes were reported at 50-60°C (Kühbeck et al., 2005; Wijngaard and Arendt, 2006), at pH 4.6-5.0, whereas peptidase has optimum range 45-50°C and pH below 5.3 (Noonan, 1996). In case of barley, the proteinase rest was set at 45-50°C for at least 30 min, the long rest caused of empty taste due to high molecular weight protein will be destroyed (Kunze, 2004).

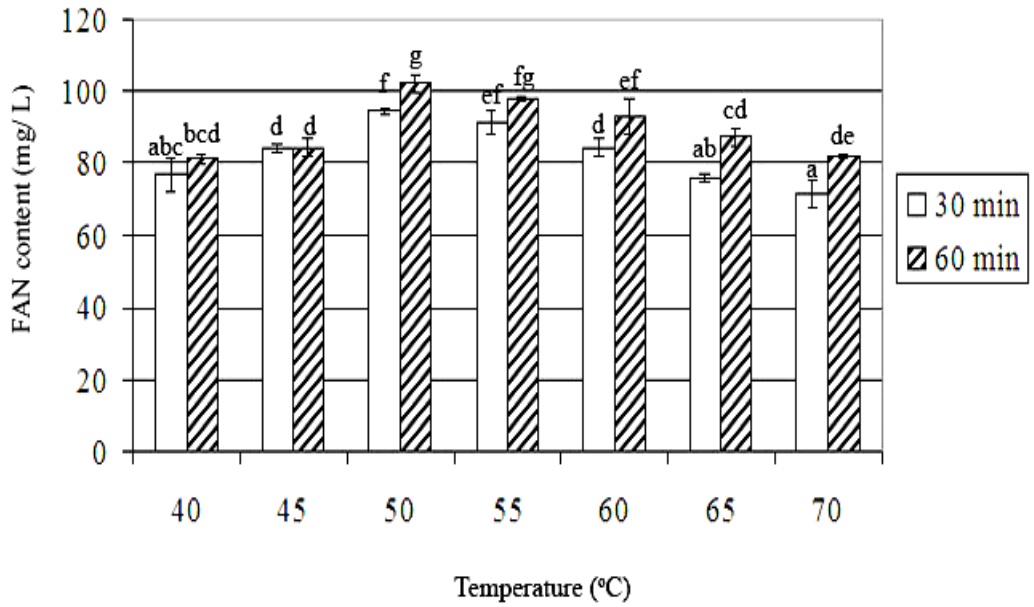


(A)

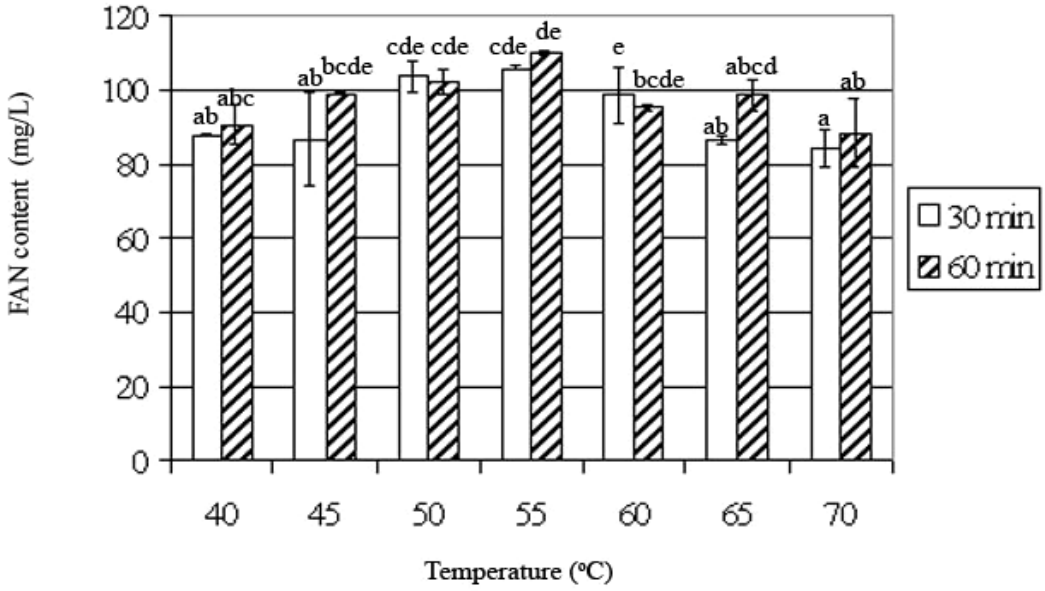


(B)

Figure 5.1 The soluble nitrogen content in wort mashed at each isothermal temperature 40-70°C for 30 and 60 min (A) mashing of KCD malt (B) mashing of KND malt.



(A)



(B)

Figure 5.2 The FAN content in wort mashed at each isothermal temperature 40-70°C for 30 and 60 min (A) mashing of KCD malt (B) mashing of KND malt.

5.4.2 Optimization of temperature for increasing of extract content and fermentable sugars

The extract content in wort from both rice malts was increased with temperature and highest at temperature 70°C, 61.05 % (w/w) for KCD and 75°C, 61.49% (w/w) for KND malt (Figure 5.3). Since at temperature 70°C, the extract content obtained from mashing for 30 and 60 min were not significantly different from extract content obtained from mashing for min at 72, 75 and 78°C (p>0.05). Therefore mashing at temperature 70°C could be used as liquefaction temperature and 78°C is suitable for stop α-amylase activity and will be used as mashing out temperature for mashing program of both rice malts. Moreover, α-amylase enzyme has been reported the optimal temperature at 70°C in sorghum and 60°C in Nigerian rice (Evans and Oyelola, 2006). The saccharification and liquefaction are the period of amylolytic enzymes working and provide a mixture of straight-chain dextrins, branched limit dextrins, maltotriose, maltose and glucose. For barley, these rest are operated at temperature 65-71°C, depending on the nature of the beer being brewed (Noonan, 1996). In addition, the result from this experiment suggested that nature of cereal malt being brewed must also be considered; particularly the gelatinization temperature of raw starch. For ensuring that all of soluble compound will be retained in wort, mashing at temperature close to or higher than gelatinization temperature is recommended.

Moreover, the fermentable sugars including glucose, fructose, sucrose, maltose and maltotriose were measured in wort mashed for 30 min at those mashing temperatures. Since the fermentation profile was influenced by maltose and glucose concentration in wort, the figure out of the temperature range for releasing of glucose

and maltose was important. At temperature 40-55°C, wort from KCD and KND malts had more glucose than other sugars (Figure 5.4). Glucose is the product of α-glucosidase, limit-dextrinase and some time by α-amylase activities. Rice α-glucosidase has been reported the ability to hydrolyzed rice starch granules so that many glucose could be released before starch formed gel (Iwata, Isogai, Utsunomiya, Itani, and Nishio, 2003). In addition, mashing temperature around 40-55°C was reported as optimum temperature for α-glucosidase in malted barley (55°C), pearl millet (55°C), malted triticale (54-56°C), malted finger millet (40-50°C) (Muralikrishna and Nirmala, 2005). Moreover at 45-55°C was reported as stabilized temperature for limit dextrinase which can digest limit dextrins to be glucose subunit (Lee and Pyler, 1984). In case of KCD wort, the maximum glucose concentration was found at 70°C (25.98 g/L) due to the solubilized starch was effectively digested by α-amylase and released a lot of glucose molecules; whereas, maltose was strongly released in KND wort.

At temperatures 60-65°C, the wort from KCD and KND had more maltose concentration than glucose; therefore, these temperature range might be the optimal temperature for β-amylase activity. Generally, β-amylase could not digest starch directly (Ziegler, 1999); therefore, the solubilzed dextrin by activity of α-amylase activated during malting and mashing were digested at these temperature range.

Moreover, the concentration of maltose in KND wort was continuously increased, until temperature reached 70°C and the previous experiments elucidated that KND had β-amylase activity higher than that in KCD malt (Table 4.3 and 4.4). For barley, the temperatures above 60°C are required for starch gelatinization which is necessary for complete degradation of starch (Slack and Wainwright, 1980). The

optimal temperature for maltose production rest were reported at 62-65°C (Kunze, 2004) and at temperature 70°C, it is considerably above the thermal tolerance of β-amylase (Stenholm and Home, 1991).

However, in this experiment showed that β-amylase in KND might tolerate to high temperature more than β-amylase in barley. Maltotriose was reported as the second most fermentable sugar in barley wort and could be utilized by *S. cerevisiae* after the other sugars were exhausted and it was increased with the increment of mashing temperature. In summary, the optimal temperature for maltose production by activity of β-amylase was suggested at 60-65°C; whereas, the glucose production temperature was suggested to be 50-55°C which were temperatures just before the activation of β-amylase. The starch conversion temperature was recommended to be 70°C due to maximum extract content was obtained and not significantly different to 72, 75 and 78°C.

5.4.3 Formulation of mashing program for both rice malt cultivars

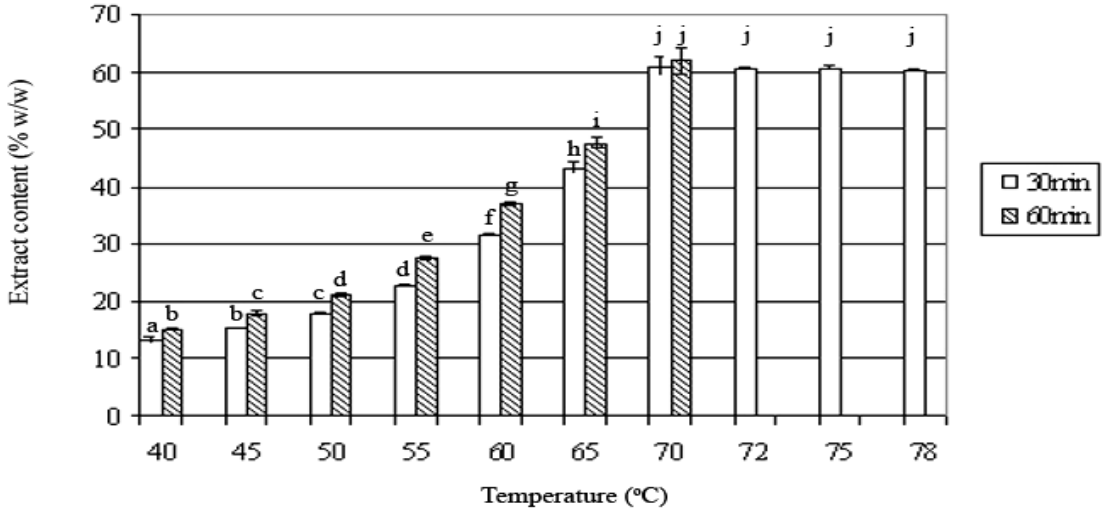
According to the results of optimization of conversion temperature rests, the proteinase rest for protein hydrolysis was 50-55°C, glucose production rest was 50-55°C and maltose production rest at 60-65°C and liquefaction rest at 70°C. The enzymes reaction was terminated by heating wort at 78°C. Thus, mashing program A was designed respecting the result of previous experiment and compared to program B "decoction mashing", program C was the temperature-programmed emphasized glucose production and program D was temperature-programmed emphasized maltose production. In case of KCD malt, the comparison of extract yields elucidated that decoction mashing was not successfully increased of that as shown in Table 5.3. Although, program A had operating time at each rests longer than that in program C

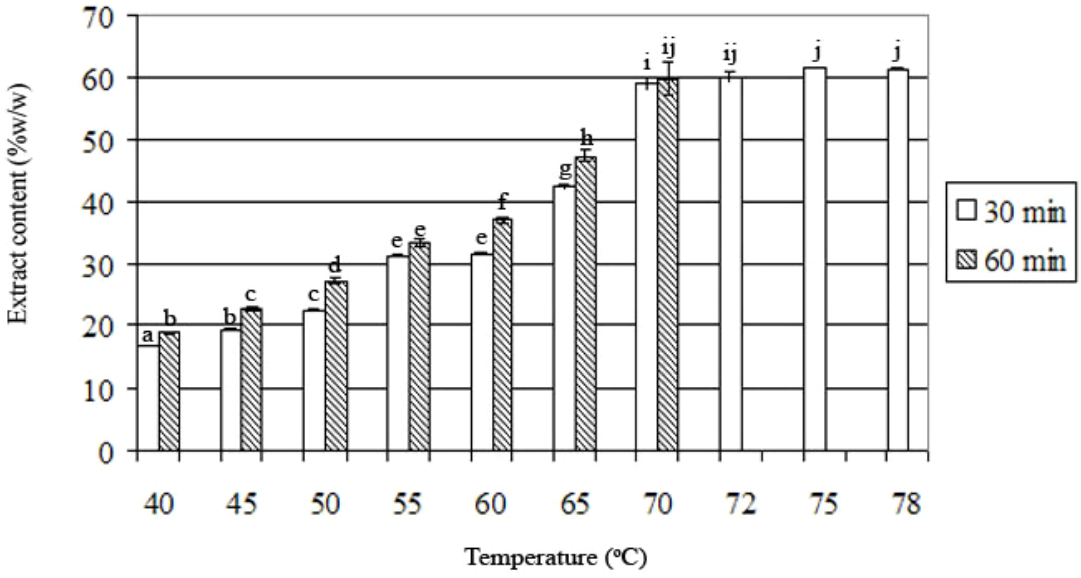
and D, they were also not significantly different in extract yield. Moreover, decoction mashing caused of protein degradation by boiling; consequently, low of soluble nitrogen and FAN were found. The maximum of AAL or fermentability was obtained by mashing program A, 86.3% and not significantly different to program D and C. This term was used to approximate amount of fermentable sugar in wort and the conversion of percentage of AAL to be g/100gmalt of fermentable sugar found in wort could be calculated from extract content. And the maximum concentration of fermentable sugar was 53.6, 53.4, 52.94, and 47.6 g/100g malt from program D, C, A and B, respectively. Since the extract yield and fermentable sugar were not significantly different in D, C and A. The maximum of soluble nitrogen and FAN were obtained from program C and A. The mashing program C was selected due to total processes time of program C shorter than program A.

In case of KND malt, unexpected low amount of extract yield was obtained from mashing program A. Comparison of that from program A, B, C and D, program had maximum of operating temperature at 70°C and lower than 90°C in decoction program and 78°C in program C and D. This might be that KND needed temperature higher than 70°C for starch hydrolyzation to be dextrin which was accounted for wort gravity. However, the analysis of variance in 5.4.2 indicated that extracts from KND mashed at temperatures 70-78°C were not significantly different. However, 79.1% of AAL in wort from program A gave fermentable sugar approximately 42.2 g/100g malt. And that still was lower than 42.5, 44.7 and 46.1 g/100g malt of wort from program B, C and D, respectively. This fermentable sugar content was determined as yield of ethanol could be obtained from that mash and the brew master considered it as the main purpose in brewing; therefore, mashing program D was used with KND

malt. Moreover, high amount of FAN in wort from program A and C could not give a benefit to wort fermentability.

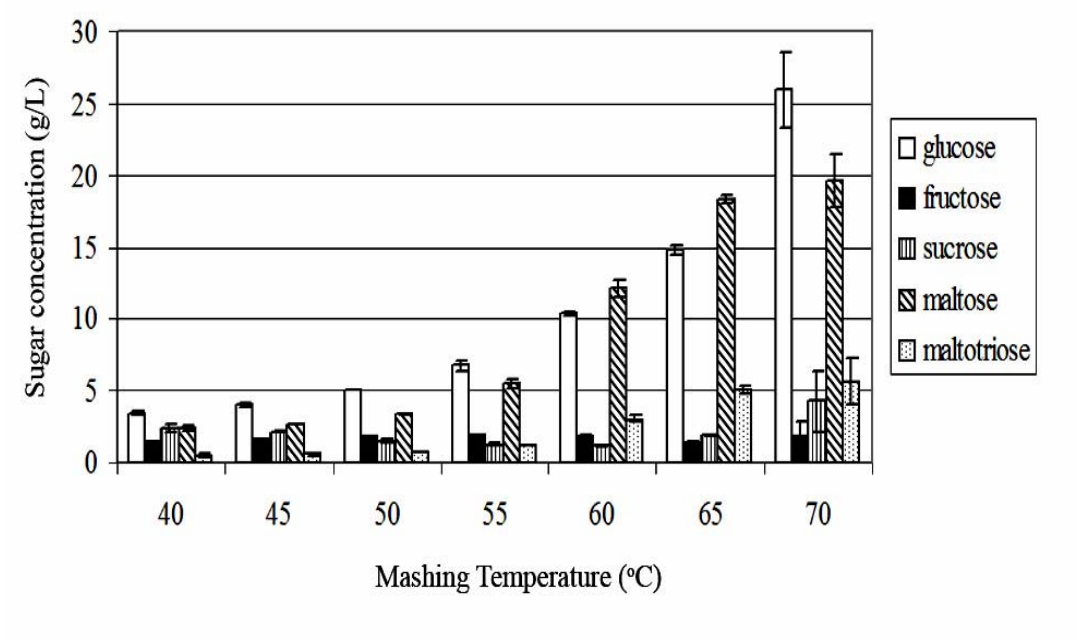
(A)



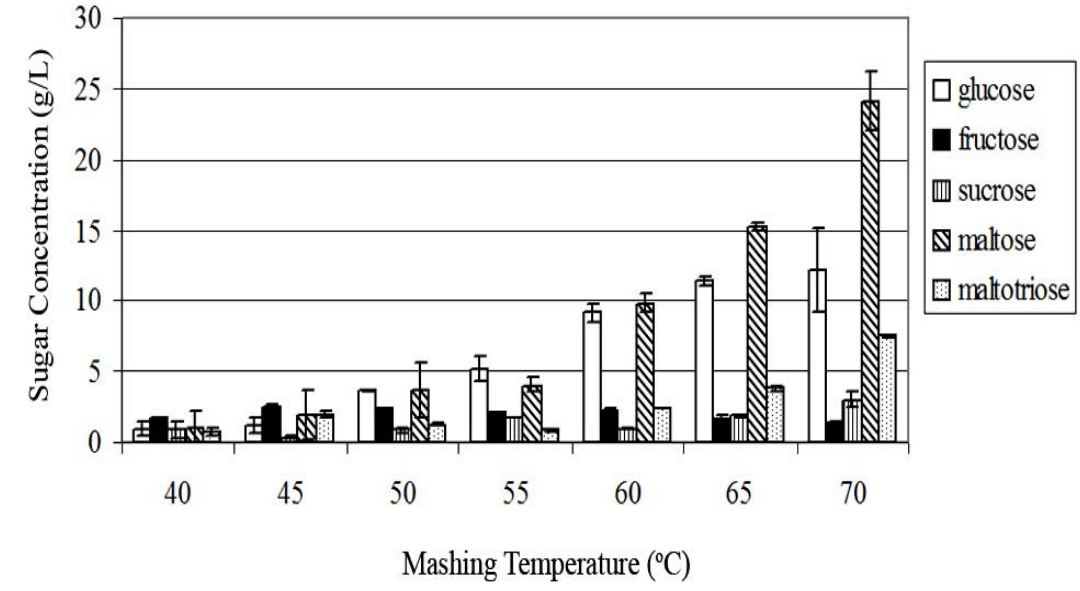


(B)

Figure 5.3 The extract content in wort mashed by isothermal process for 30 and 60 min; (A) KCD malts, (B) KND malts.



(A)



(B)

Figure 5.4 Fermentable sugar concentrations in wort mashed by isothermal process for 30 min (A) Mashing of KCD malt (B) Mashing of KND malt.

Table 5.3 Wort properties produced from two rice malt cultivars.

Rice malt cultivars	Mashing programs	Extract content % (w/w)	Soluble nitrogen (mg/100 g)	FAN (mg/100 g)	AAL (%)	pH
KCD malt	A	61.56*ab*	355*b*	109*b*	86.3*b*	6.01*ab*
	B	60.50*a*	320*a*	89*a*	78.6*a*	6.03*b*
	C	63.40*b*	355*b*	109*b*	84.1*b*	6.01*ab*
	D	62.98*ab*	349*b*	101*b*	85.2*b*	6.0*a*
KND malt	A	53.37*a*	327*a*	112*c*	79.1*c*	5.95*a*
	B	58.65*b*	310*b*	91*a*	72.5*b*	6.02*a*
	C	63.68*c*	336*c*	113*c*	70.2*a*	6.0*ab*
	D	63.53*c*	329*b*	103*b*	72.6*b*	5.93*a*

Mean values from four measurements. The different italic letter in the same column was significantly different at $p \leq 0.05$.

5.4.4 Optimization of mashing-in pH

The mashing-in pH was studied by varied pH as 5.6, 5.2, 4.8, and 4.4. The initial pH of the mash was hardly controlled because malt has itself buffer capacity thus, the actual of mashing-in pH was shown in Table 5.3. The property of wort was compared to the control (unadjusted pH) and found that initial pH of mashing influence to wort properties, including of extract yield, soluble nitrogen and FAN content, AAL and final pH. Decreasing of mashing-in pH enhanced many biochemical changes, since optimal pH for α-amylase was in a range of 5.6-5.8 (Kunze, 2004), β-amylase was pH 5.2 (Bamforth, 2001). The results in Table 5.4 showed that extract content increased if pH was decreased in a range of 5.2-5.8. For KCD, the optimal pH for increasing extract content should be pH 5.2 because at pH 4.7 the extract content was decreased to 51.6% (w/w) due to it was close to pI of

major α-amylase isoforms found in rice (pI 4.6) (Nanjo et al., 2004); as a result, the AAL was also decreased at this pH. In case KND malt, the extract content increased with the decreasing of pH; however, at pH 4.7 the wort became acid and had sour taste which will be a problem for beer quality.

Furthermore, the acidic pH also enhanced FAN and total soluble nitrogen content, the maximum FAN content was found at pH 4.7 which was in a range of optimal pH of carboxypeptidases reported at 4.8, 5.2 and 5.6 (Enari, 1986). Since the optimal pH for increasing extract content was proposed to be 5.2, the FAN content was much more than the recommended (140 mg/100g malt or 200 mg/L) (Kunze, 2004). Besides, too low pH caused of low amount of high molecular weight proteins and peptides were digested to release FAN; consequently, it might be a problem for foam stability in finished products. Thus, the optimal pH for improvement of extract yield and wort quality was recommended at pH 5.2.

5.4.5 Optimization of cation for enhance extract content

Generally, mash water used in brewing plant has many types of dissolved cation and anion that must be controlled for beer flavor and a unique beer quality. The cations used for this experiment were in the form of chloride salt consisted of Ca^{2+}, $Mg^{2+}$and Zn^{2+}. The result elucidated that the Ca^{2+}, $Mg^{2+}$and Zn^{2+} were not significantly increased the extract content in wort produced from both rice malts (Figure 5.5).

The effect of divalent cations on FAN and soluble nitrogen were obviously found in mash supplemented with Zn^{2+}; whereas, effect of Ca^{2+} was clearly demonstrated on soluble nitrogen (Figure 5.6-5.7). In addition, their effects were increased as function of amount of both cations. The effective concentration of Ca^{2+}

to FAN in KCD and KND worts were 50-200 mg/L and 100-200 mg/L were the effective concentration to increase soluble nitrogen in both worts. In case of Zn^{2+}, the significant concentration to increase FAN in KCD wort was 150-200 mg/L and 50-200 mg/L for FAN in KND wort. Whereas, addition of Zn^{2+} in mash at concentration 150-200 mg/L was significantly affected to soluble nitrogen in KCD and KND wort. Zn^{2+} is typically required for the metalloproteinase in plat and Ca^{2+} stimulated Ca^{2+}-dependent cystein proteinase in plant, arabidopsis root, animal, fugi (Calls, 1995) and stimulated cystein proteinase in sorghum malt (Ogbonna et al., 2004).

Furthermore, Mg^{2+} significantly influenced FAN and soluble nitrogen in KCD wort only and it was not affected to AAL of both rice malts (Figure 5.8). However, Mg^{2+} has been reported the ability to enhance proteinase activity in malted sorghum but this ability was not found in barley malt (Agu, 2006). The stimulation of protenase activity in cereal grain depends on the genetic background of cereal type and cultivars (Agu, 2006), therefore the activation ability is varied among cereal type and cultivars. Ca^{2+} can stimulate activity of proteinase in barley and sorghum and that agreed with results in this experiment. In addition, it was also needed by the most of α-amylase enzymes to stabilize structural integrity and their activity (Sivaramakrishnan, Ganadharan, Nampoothiri, Soccol, and Pandey, 2006) but in this experiment Ca^{2+} was not significantly influence on extract content in rice wort. However, $Ca^{2+}$and Zn^{2+} supplementation in mash gave more AAL in wort (compared with control) but the effect of both cations as a function of added amount was not obviously found.

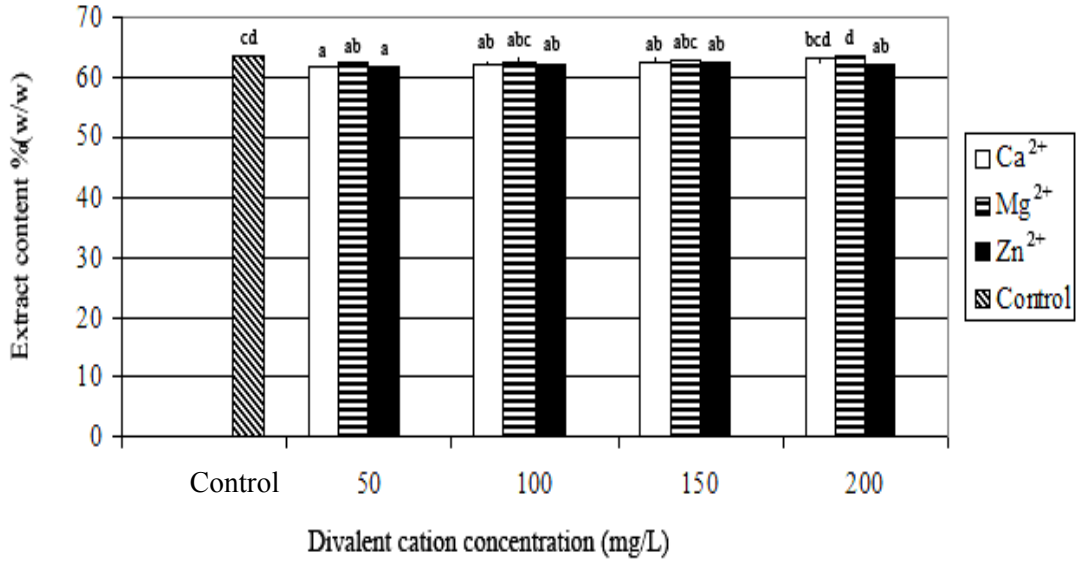
The analysis of variances indicated that the overall of wort quality was significant improved by adding Ca^{2+} or Zn^{2+} at 150 mg/L. Thus, the effect of both

ions was tested again at mashing-in pH 5.2 in order to examine co-effect of ion and pH by comparing with the control condition, which was neither adjusted pH nor ion added. The results indicated that Ca^{2+} plus pH adjust was strongly affected to FAN and soluble nitrogen more than Zn^{2+} (Figure 5.9).

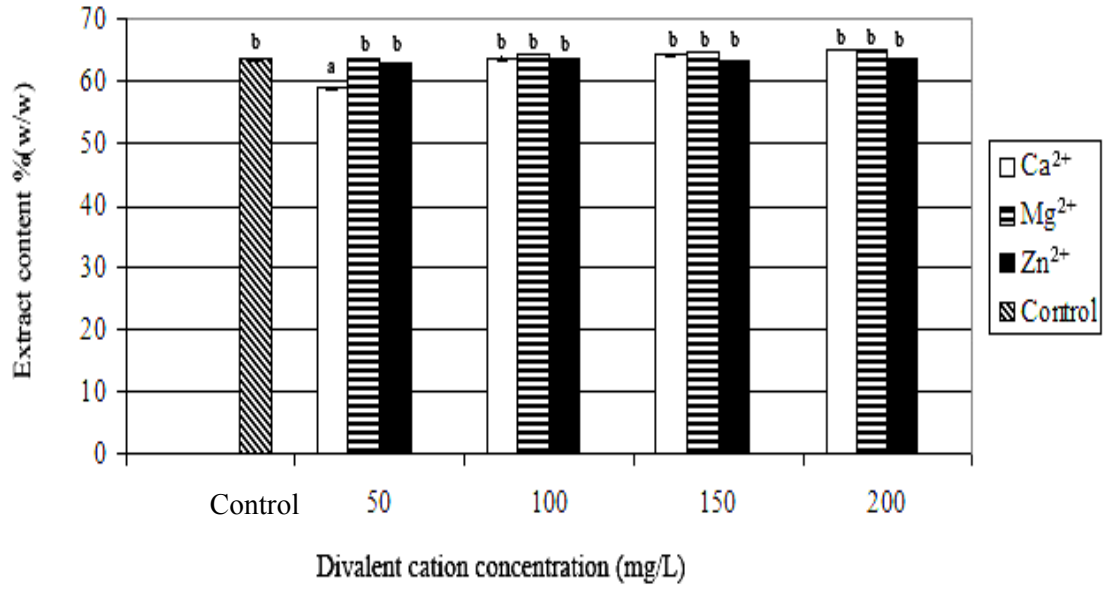
Table 5.4 Effect of mashing-in pH.

Malt	Mashing-in pH	Extract content (%w/w)	Soluble nitrogen (mg/100g)	FAN (mg/100g)	AAL (%)	pH final
KCD	Control (6.01)	63.5*b*	355 *a*	109 *a*	84.1*a*	6.01*e*
	5.8	64.1*b*	386 *b*	112 *a*	85.8 *b*	5.84 *d*
	5.6	64.3 *b*	440 *c*	138 *b*	88.4 *c*	5.73 *c*
	5.2	65.6 *c*	555 *d*	171 *c*	88.4 *c*	5.32 *b*
	4.7	51.6 *a*	584 *e*	321 *d*	84.2 *a*	4.78 *a*
KND	Control (5.93)	63.5 *a*	329 *a*	103 *a*	71.6 *a*	5.93 *e*
	5.74	64.5 *a*	349 *b*	109 *a*	73.1 *b*	5.79 *d*
	5.6	64.6 *a*	380 *c*	128 *b*	77.3 *c*	5.66 *c*
	5.2	65.0 *a*	419 *d*	144 *c*	80.8 *d*	5.31 *b*
	4.7	66.0 *b*	517 *e*	322 *d*	83.0 *e*	4.80 *a*

Mean values from four measurements. The different italic letter in the same column was significantly different at $p \leq 0.05$.

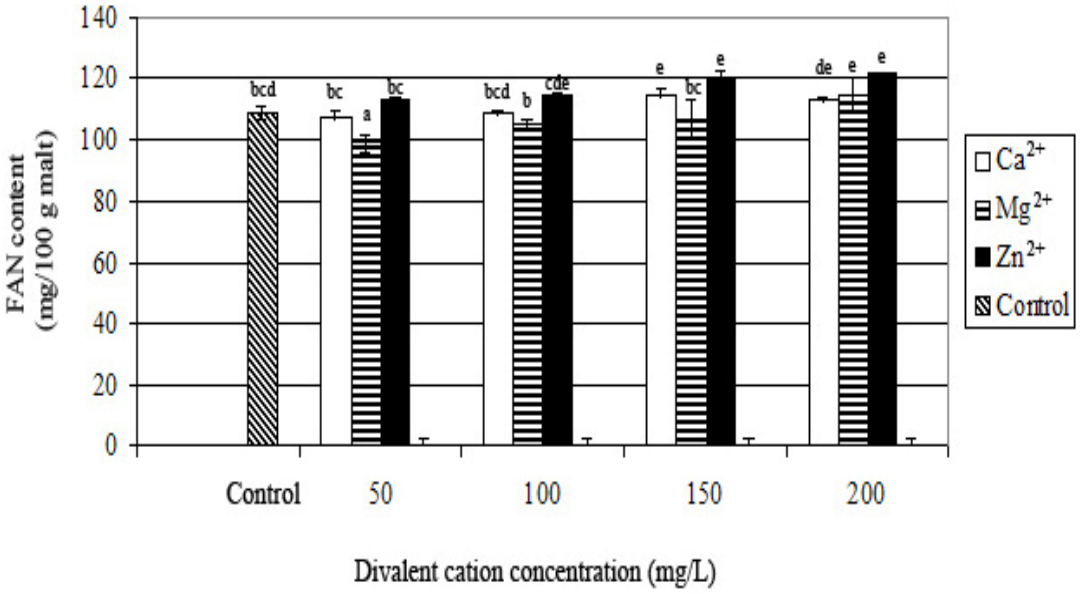


(A)

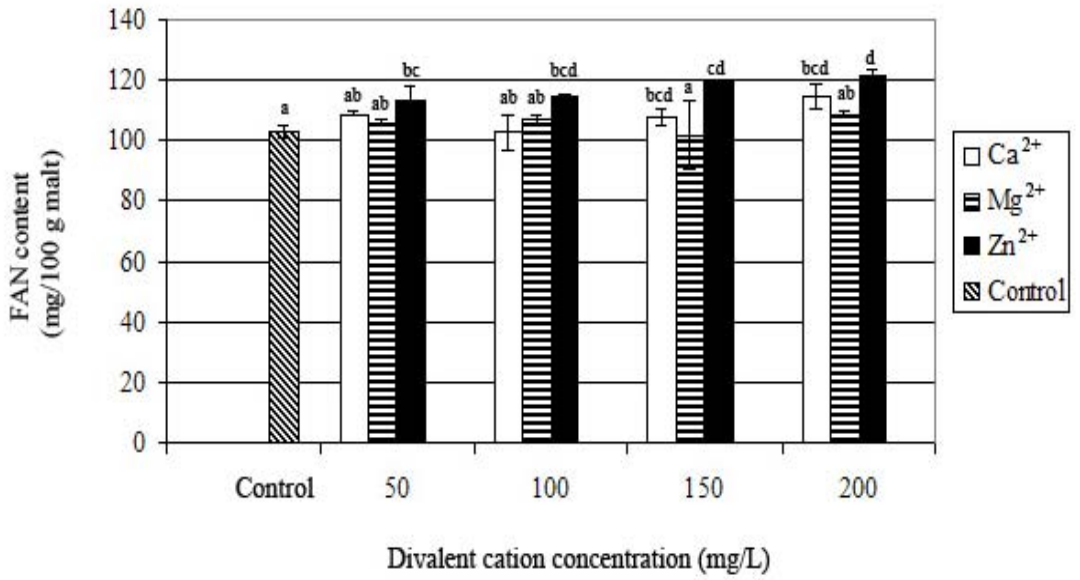


(B)

Figure 5.5 Effect of divalent cation supplementation on extract yield in rice wort produced from (A) KCD malt and (B) KND malt.

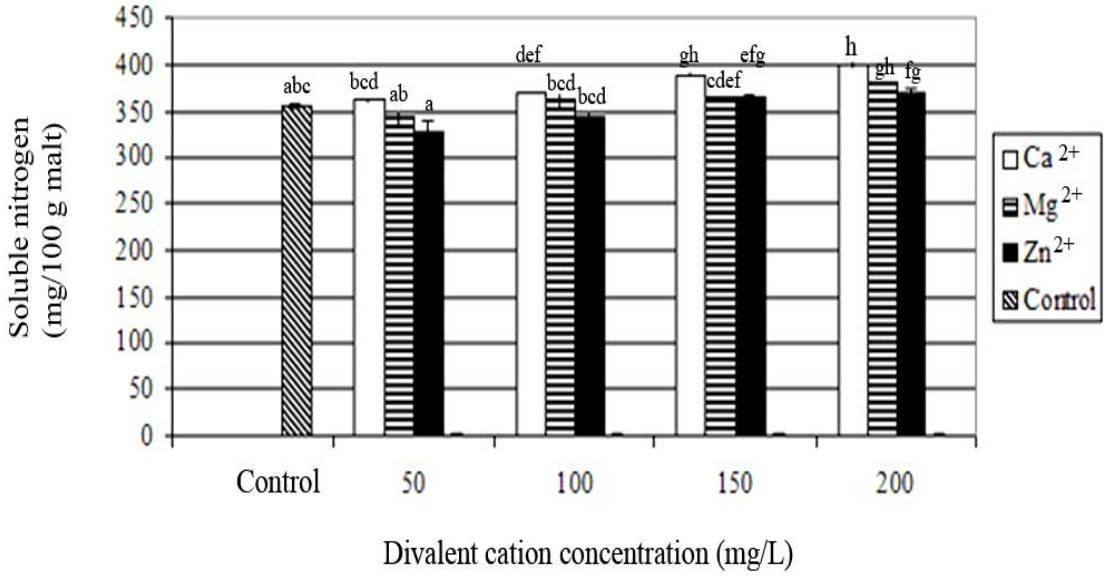


(A)

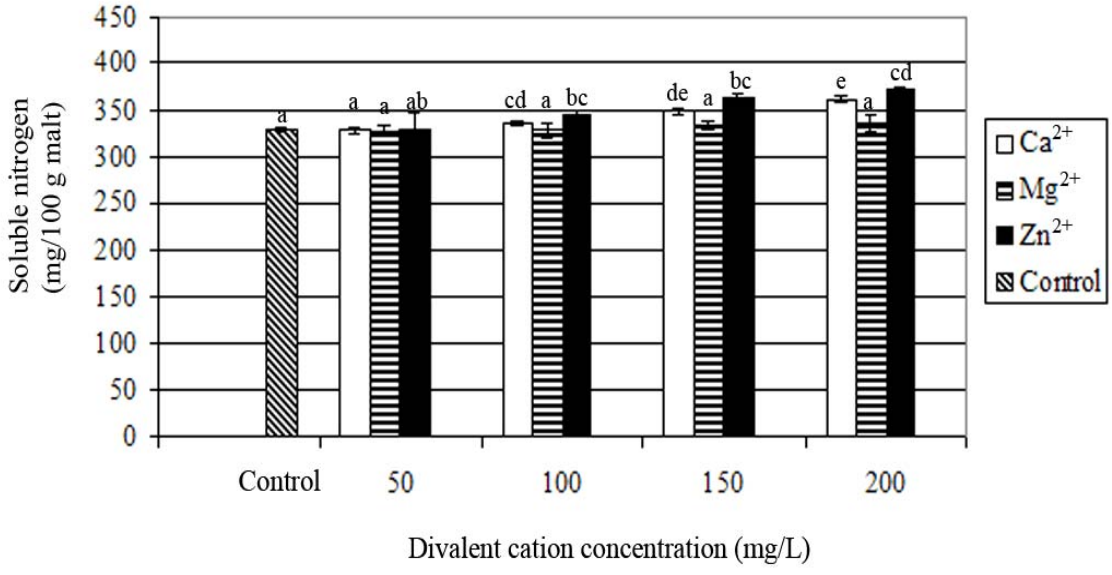


(B)

Figure 5.6 Effect of divalent cation supplementation on yield of FAN from rice wort produced from (A) KCD malt and (B) KND malt.

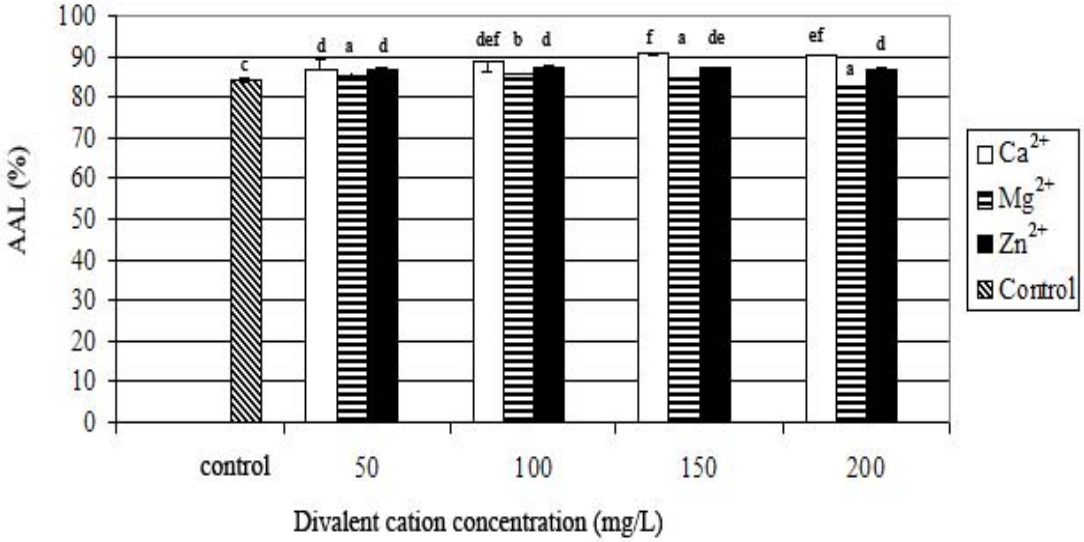


(A)

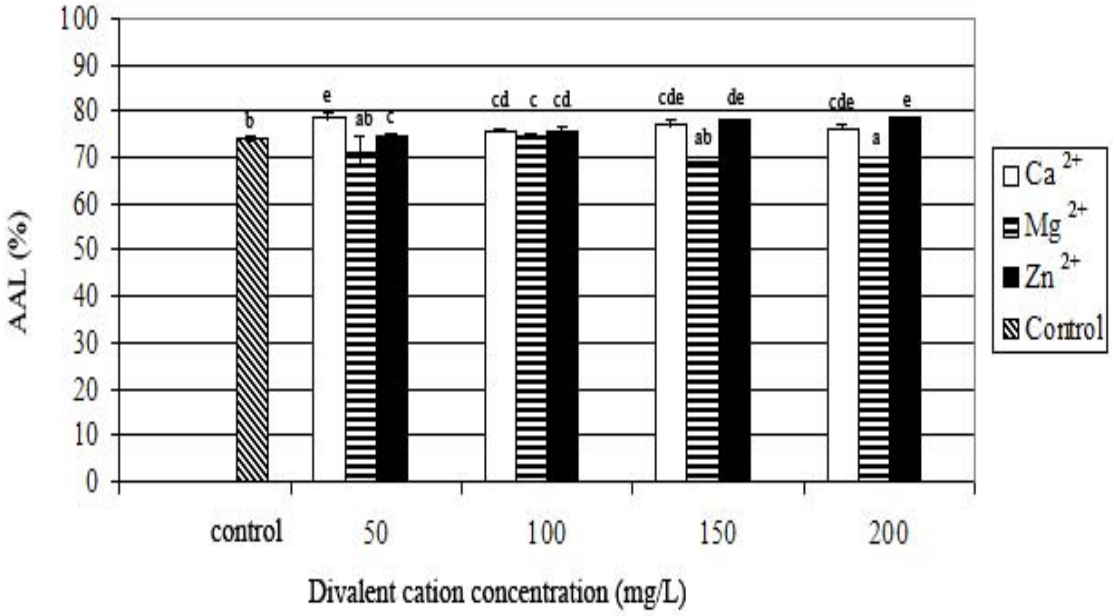


(B)

Figure 5.7 Effect of divalent cation supplementation on soluble nitrogen yield in rice wort produced from (A) KCD malt and (B) KND malt.

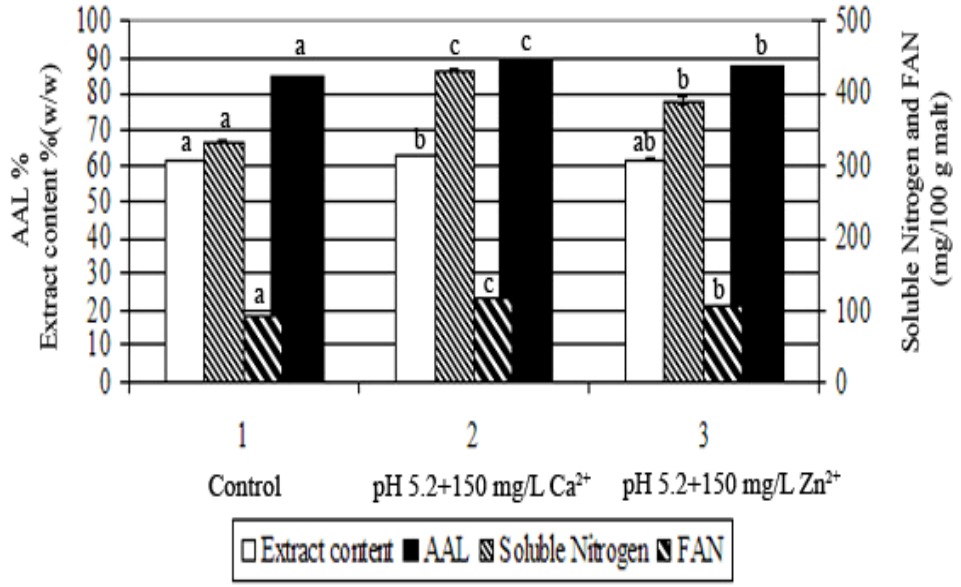


(A)

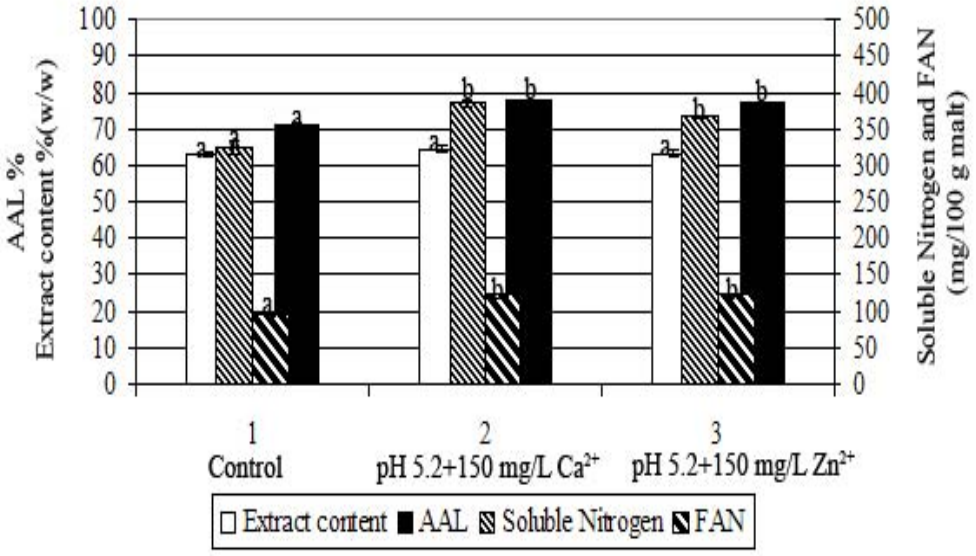


(B)

Figure 5.8 Effect of divalent cation supplementation on AAL in rice wort produced from (A) KCD malt, and (B) KND malt.



(A)



(B)

Figure 5.9 Comparison effects of Ca^{2+} and Zn^{2+} at concentration 150 mg/L, mashing-in pH 5.2 (A) mashing of KCD malt, and (B) mashing of KND malt.

5.4.6 Effect of malt milling on wort property and wort production in brew master mashing unit

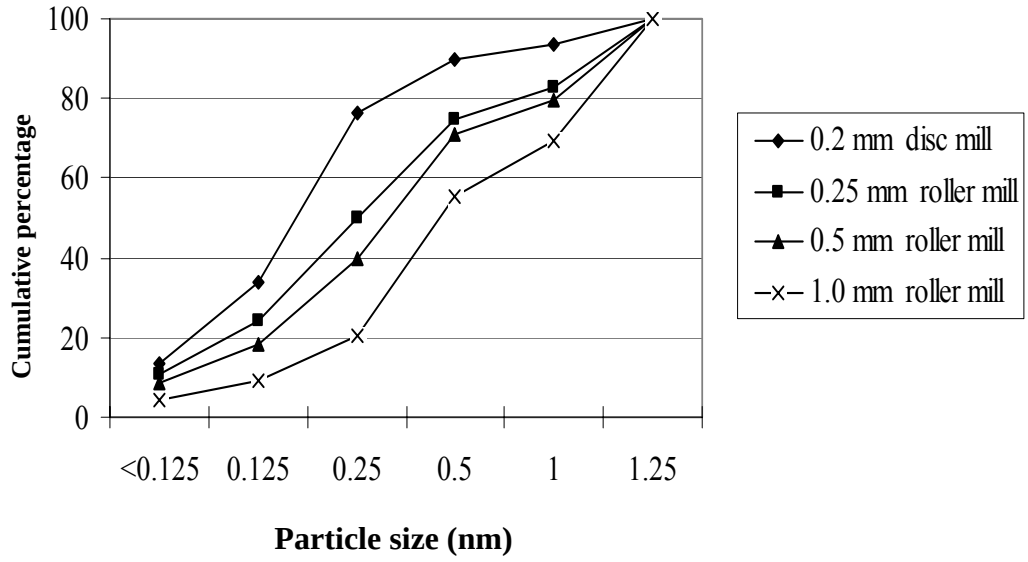
Wort production was conducted by increasing the production size to pilot scale, thus the milling machine was changed from laboratory disc Buhler-Maig, Mineapolis, MN) to be a two roller mill (Kuenzwel, Kulmbach/Bayern). Malts were milled by adjusting gap distance between two roller mill as 0.25, 0.5 and 1.0 mm and all of malt grist were sieved and compared with laboratory disc mill 0.2 mm (Table 5.5). The percentage (w/w) of malt retained in each sieve was calculated and plotted in Figure 5.8 as a percentage of accumulation. Disc mill adjusted at 0.2 mm, made 94% of KCD and KND grist smaller than 1 mm and 40-44% had particle size in a range of 0.125-0.25 mm. Approximate 84%, 80% and 70% of KCD malt grist were obtained from milling at 0.25, 0.5 and 1.0 mm roller mill, and the similar results were obtained from KND malt (Figure 5.10). However, the results of wort quality indicated that milling at 0.25, 0.5 and 1.0 mm by roller mill were not significantly different in extract yield, soluble nitrogen and AAL in wort from KCD rice malt. There were some reported that the gap distance of roller influenced on starch damage, small gap made more damaged starch (Warpala and Pandiella, 2000), and the small grits particles were dissolved quickly, then amount of dissolved solids rose faster (Davey et al., 2000). However, these literatures did not mention that weather the final wort had more extract or fermentable sugar. Kühbeck et al., (2005) reported that grist size was not influence on rate of sugar releasing in wort due to the active size was not increase by fine milling and agreed to the results of AAL found in KCD malt; whereas, controversy results was found with KND malt. Grist size or milling significantly influenced FAN content in KCD and KND worts, FAN content was

increased with distance between two roller; therefore, the optimal roller gap for milling KCD was 0.5 mm and 1.0 mm for KND malt. Wort was produced by using brew master mashing unit with addition of Ca^{2+} 150 mg/L and adjusted pH to 5.2 with 1M lactic acid. The wort production yield and quality were determined. The results of brewing yield from three brews were 39±0.2% and 38.4±2.8% for KCD and KND malt respectively.

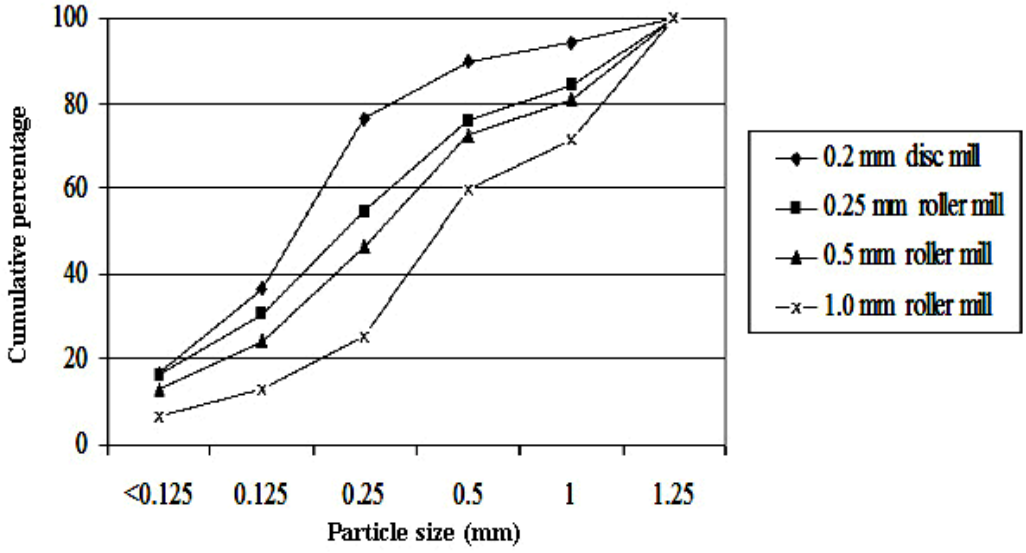
Table 5.5 Effect of size reduction to wort properties.

Malts	Milling	Extract % (w/w)	Soluble Nitrogen (mg/L)	FAN (mg/L)	AAL (%)	pH
KCD	0.2 mm Disc mill	61.87 *a*	354 *a*	102 *a*	84.65 *b*	5.99 *a*
	0.25 mm Roller mill	61.89 *a*	361 *a*	104 *ab*	85.2 *b*	6.01 *b*
	0.5 mm Roller mill	61.18 *a*	368 *a*	109 *bc*	85.6 *b*	6.01 *b*
	1.0 mm Roller mill	60.10 *a*	368 *a*	107 *c*	82.8 *a*	6.01 *b*
KND	0.2 mm Disc mill	58.7 *a*	343 *a*	108.5 *a*	71.05 *a*	5.98 *b*
	0.25 mm Roller mill	61.84 *b*	343 *a*	112 *ab*	78.75 *b*	5.91 *a*
	0.5 mm Roller mill	62.68 *bc*	350 *a*	113 *ab*	77.05 *b*	5.93 *a*
	1.0 mm Roller mill	61.69 *b*	350 *a*	116.5 *c*	79.8 *b*	5.92 *a*

Mean values from four measurements. The different italic letter in the same column was significantly different at $p \leq 0.05$.



(A)



(B)

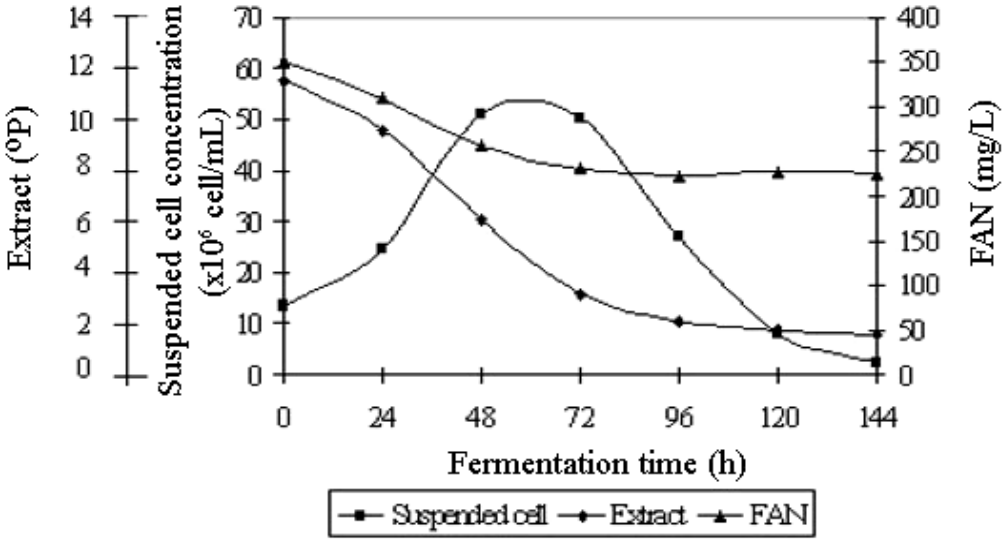
Figure 5.10 Effect of milling on the particle size distribution (A) KCD malt and (B) KND malt.

5.4.7 Beer fermentation

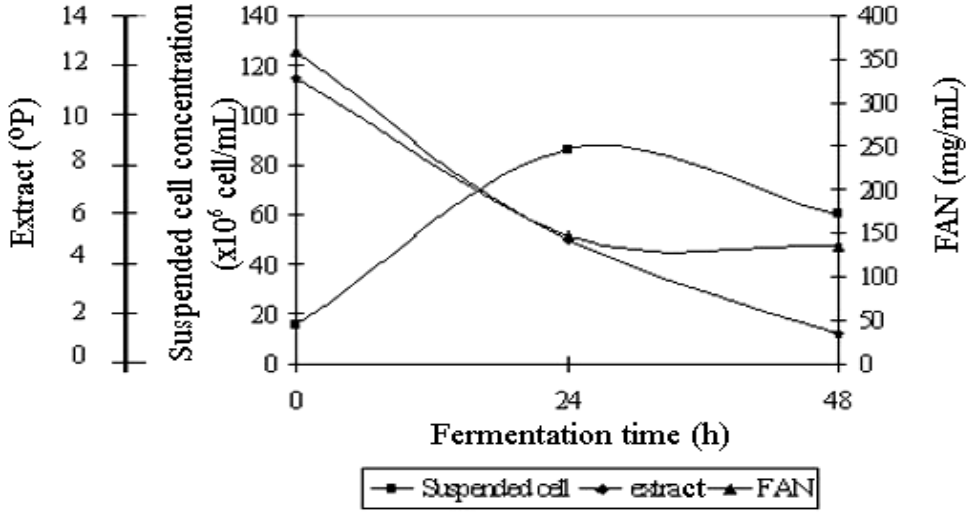
The fermentation was carried out until 80% of extract was exhausted or the extract was not changed in 24 h. In case of KCD, the bottom fermentation took 144 h, and 48 h for top fermentation since lower temperature was applied to lager fermentation (10°C). The FAN and extract content in term of degree plato (^{o}P) were rapidly decreased after inoculation and then they were remained constant until the fermentation finished. Lager yeast slowly utilized substrate and the maximum suspended cell concentration was found after 48 h of fermentation ($5.5x10^{7}$ cells/mL), whereas ale yeast reached maximum cell concentration after 24 h ($8x10^{7}$cells/mL) (Figure 5.11-5.12). Concentration of suspended cell in wort of top fermentation was higher than that in bottom fermentation due to top fermenting yeast floated in fermentation media, whereas bottom fermenting yeasts were fallen down due to cold temperature and ethanol. Therefore the maximum suspended cell was not represented for total viable cell in fermentation media.

The fermentation time of KND ale and lager beer (168 and 72 h, respectively) took longer than that of KCD ale and lager fermentation (48 and 144 h, respectively). The analysis of fermentable sugar in KND wort indicated that wort from both malt cultivars had slightly different fermentable sugar profiles. Glucose was 43.6 g/L found in KCD wort and 31.47 g/L in KND wort; whereas, maltose in KCD was 27.02 g/L and 31.02 g/L found in KND wort. These concentrations indicated that KCD rice wort contained glucose more than 50% of total fermentable sugar at 86% AAL. At high concentration of glucose, maltose utilization could be inhibited; however, glucose, maltose and maltotriose in final fermented wort were not found. Therefore, the stuck fermentation had never been occurred in this experiment. Generally, the

normal wort from barley at 80% AAL contains 65% maltose, where as 10-17% is hexose (Walker, 1998).

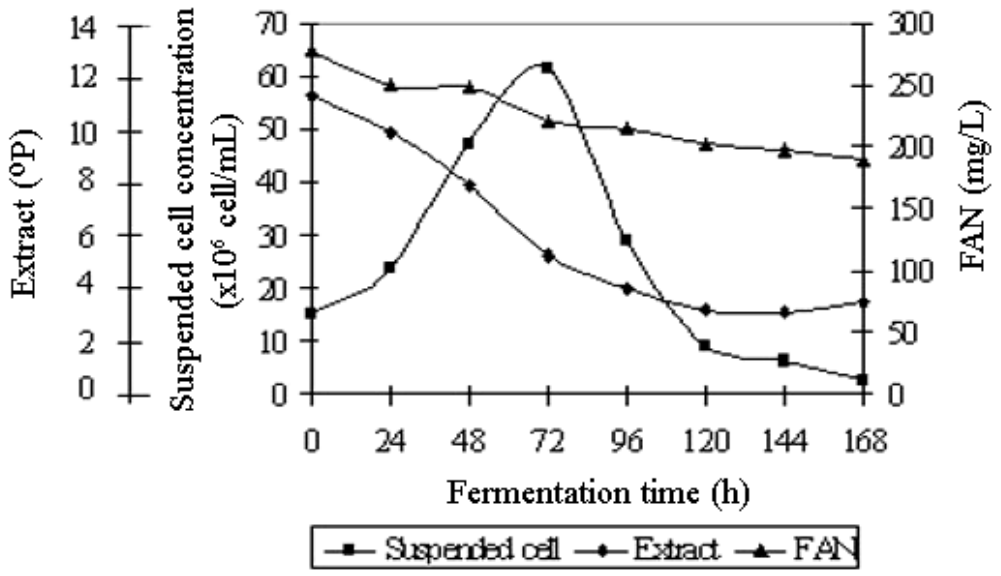


(A)

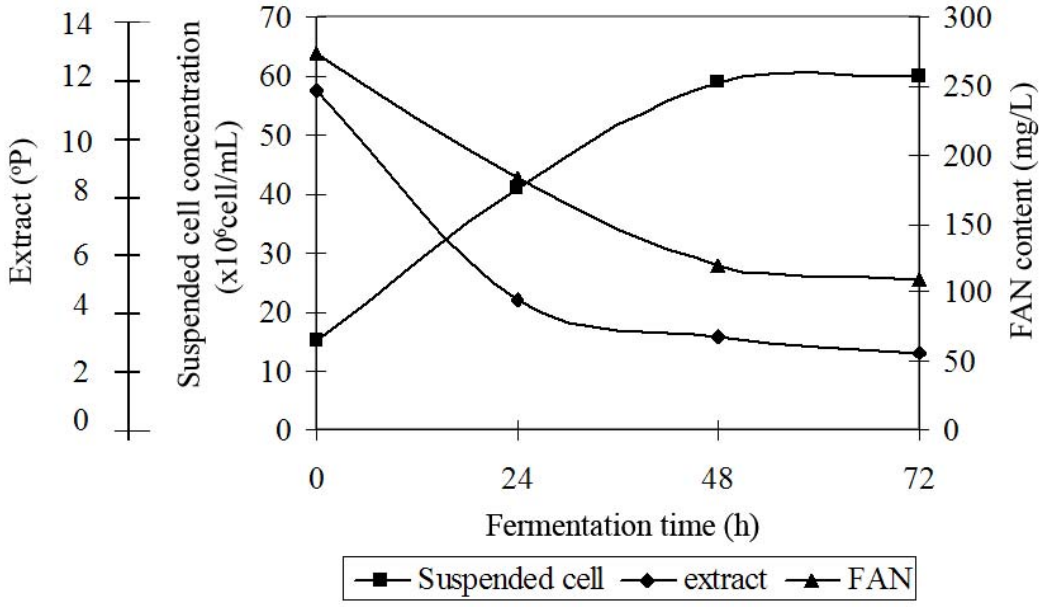


(B)

Figure 5.11 The time course of beer fermentation from KCD wort (A) bottom fermentation and (B) top fermentation.



(A)



(B)

Figure 5.12 The time course of beer fermentation of KND wort (A) bottom fermentation and (B) top fermentation.

5.4.8 FAN and amino acid profiles of wort and beer

Wort and beer of both rice cultivars were determined for FAN, amino acid profile, soluble nitrogen and protein fractions. The FAN content in wort from both rice cultivars were sufficiently for yeast metabolism in fermentation process, in case of KCD wort, FAN was approximately consumed by lager yeast 125 mg/L and 215 mg/L by ale yeast (Table 5.7 and 5.8). The FAN in KND wort was utilized by lager yeast 109 mg/L and ale yeast 168 mg/L. The residual extract in KND ale and lager beer was higher than that in KCD ale and lager because there were dextrin left in fermented wort and no any of glucose and maltose was detected by HPLC. Thus amount of amino acid consumption by lager and ale yeast strain seem related to amount of extract and fermentable sugar depletion. A number of flavor-active compounds were influenced by levels of amino acid in wort such as higher alcohol, ester and visinal diketone (VDK). Since methionine has been mentioned as key amino acid for VDK reduction in finished product but lysine addition promoted VDK production by yeast (Lekkas, Stewart, Hill, Taidi, and Hodgson, 2005), the concentration of both amino acids should be monitored. In case of rice wort, low content of methionine was found in those from both rice cultivars; therefore, the reduction of VDK in beer was not easily, even methionine and serine were completely utilized by top fermenting yeast. Moreover, most of lysine found in wort was consumed by top fermenting yeast; consequently, high content of diacetyl was found in finished products (Table 5.7). Serine participates in the biosynthesis of purines and pyrimidines and it is also the precursor to several amino acids, including glycine and cysteine. This might be one possible explanation of the slight increasing of glycine in rice beer samples compared to their cast wort.

5.4.9 Size of protein, nitrogen fraction and foam stability

The result obtained from nitrogen fraction and SDS-PAGE indicated that most of nitrogen in wort and beer was low molecular weight (MW) proteins (Table 5.7 and 5.8). In brewing, protein properties related to foam property, the lipid transfer protein (LPT1) (9-10 kDa) from barley contributed foam formation and barley albumin called protein Z (37-40 kDa) provided foam stability (Douliez, Michon, Elmorjani, and Marion, 2000). The important hydrophobic properties of unfolding and glycosylated forms have been reported, which contribute to a better adsorption at air-water interface (Douliez et al., 2000; Kordialik-Bogack and Ambroziak, 2007). In case of rice wort and rice beer, the size of high MW protein were approximately 47, 52 and 56 kDa; however, the protein size 47 kDa was found only in unboiled wort which probably be non heat tolerant protein and precipitated out during wort boiling process (Figure 5.12). The sharp band of 11 kDa was found in every sample of rice worts and beers and it might be the LTP1 protein that Garcèa-Garrido, M. J. and colleague (1998) reported the 11.6 kDa of LTP1 found in rice; consequently, a good range of foam stability was found in some rice beer samples (Table 5.8).

Although beer of KCD showed good foam holding time, in case of ale beer from KND was poor even they had protein sizes in the same range. The content of soluble nitrogen left in final product plays an important role, particularly content of high MW protein in ale beer. In this case, amount of high MW protein was not enough to retain foam stability of ale beer and some of LTP1 was degraded by proteinase A produced by brewing yeast (Leisegang and Stahl, 2005). Generally, ratio of three fractions in barley wort are 32, 18 and 50% (w/w) and slightly different found in beer (Pollock, 1979). The extension at temperature 50-60°C could degrade

protein and influence foam stability of barley beer (Kunze, 2004), the contradictory results were found in this work that the extension of mashing rest at these temperatures was not influence foam stability of rice beer; therefore, property of protein might response to foam stability rather than size**.**

Table 5.6 The amino acid profiles in worts and beers produced from rice malts.

Groups	Amino Acid (mg/L)	KCD malt				KND malt			
		Wort		Beer		Wort		Beer	
		wort	casting wort	lager	ale	wort	casting wort	lager	ale
A	Asp	14.5	12.0	7.4	1.2	15.1	12.7	10.7	3.8
A	Glu	10.7	9.9	5.7	0.9	11.3	10.6	8.3	4.7
A	Asn	29.6	23.2	3.8	1.8	30.2	22.3	5.7	0.8
A	Ser	14.9	12.3	2.0	0.7	15.4	13.1	2.6	0.4
A	Gln	6.5	7.3	3.8	3.3	7.1	13.2	4.0	3.0
A	Thr	9.0	6.0	7.4	6.8	9.4	7.9	8.7	4.6
A	Arg	20.8	18.7	14.3	8.7	16.9	18.2	18.6	5.9
A	Lys	22.3	19.2	7.8	1.2	23.4	22.3	9.8	4.2
B	His	6.7	5.9	3.7	3.5	6.4	16.4	4.4	2.5
B	Val	19.6	18.0	15.2	9.8	20.4	17.3	15.2	6.2
B	Met	6.3	6.1	0.4	0.5	6.8	6.2	2.8	0.6
B	Iie	10.2	9.8	7.8	1.7	10.6	9.0	5.8	2.1
B	Lue	25.5	23.1	13.4	3.2	26.7	22.8	10.2	2.1
C	Gly	7.0	4.4	5.4	5.2	6.7	4.9	6.8	3.5
C	Ala	22.7	18.0	10.5	7.4	23.1	22.3	18.3	8.9
C	Tyr	21.4	17.8	12.7	14.4	22.3	19.5	18.6	10.9
C	Trp	7.3	7.3	5.3	4.6	7.8	7.0	6.1	4.0
C	Phe	17.6	16.7	13.5	6.5	18.6	15.8	12.2	5.3

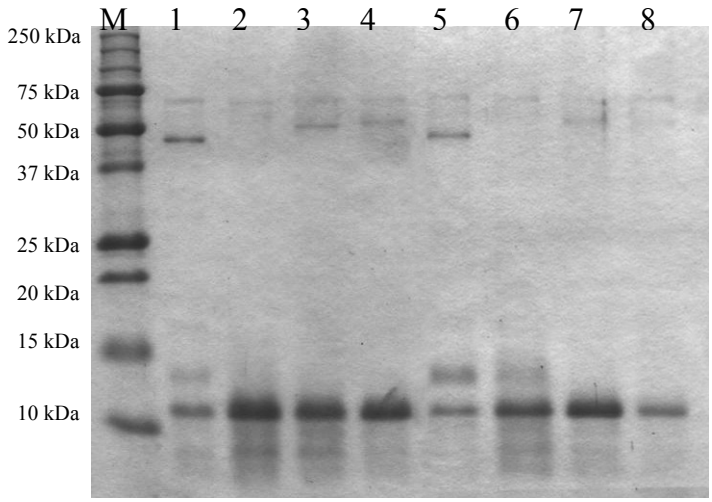


Figure 5.12 SDS-PAGE of proteins in rice wort and beer samples: M; molecular marker, lane 1; KCD wort, lane 2; KCD cast wort, lane 3; KCD lager beer, lane 4; KCD ale beer, lane 5; KND wort, lane 6; KND cast wort, lane 7; KND lager beer, lane 8; ale beer.

5.4.10 Chemical analysis and Sensory evaluation

The chemical analysis was performed in order to monitor the fermentation and fermentation by-product being produced in a good range (Table 5.8). They were acetaldehyde, ethylacetate, iso-amylacetate, amylalcohol, n-propanol, iso-butanol, diacetyl, 2, 3-pentanedion, 4-vinylguaicol. Regarding to the catabolism of sugars under anaerobic condition, glucose is utilized through glycolysis pathway, providing an intermediate molecule of pyruvates changed to acetaldehyde, and ethanol is the last electro acceptor. The green apple flavor in beer produced from acetaldehyde, at concentration of 20 to 40 mg/L in green beer and matured beer 8-10 mg were normal range in beer (Kunze, 2004). In rice beer samples, they were in a normal range of acetaldehyde (6-9 mg/L) implied that the oxidation during processes of fermentation until bottling was carried out under an appropriate control. Ester formation was

investigated by determining of ethyl acetate and iso-amyl acetate as the representatives of ester group that is the most important aroma compound, responding the fruity aroma notes. Top fermentation is reported at 80 mg/L and 60 mg/L in bottom fermentation (Kunze, 2004); as a result, four beer samples in Table 5.8 had acetate ester at normal concentration. However, the concentrations of ethyl acetate in lager beers were nearly to threshold concentration reported in beer from barley, 25-30 mg/L (Kunze, 2004). Therefore, the empty flavor of acetate ester in lager rice beer could be found or might be not if rice beer had lower threshold concentration due to its matrix. In case of iso-amylacetate, the flavor threshold in barley beer is around 0.5-2.5 mg/L; therefore, the banana like aroma must be detected in a complex of beer flavor. The n-propanol and iso-butanol were detected for observation of higher alcohol production through the Erhlich pathway, the deamination and decarboxylation of amino acid (Walker, 1998). The flavor threshold of n-propanol is 10-200 mg/L, whereas iso-butanol is 30-70 mg/L (Kunze, 2004). In case of lager rice beer, both samples had low amount of them which related to the lower FAN consumption in the fermented rice wort; whereas, yeast needed at least 220 mg/L of FAN in wort to conduct fermentation of barley wort. The different of fermentable sugar profile in rice and barley wort was one of the reasons for why yeast needed less FAN from rice wort than barley wort. Vicinal diketone (VDK) is the key compound for determination of how long to proceed beer maturation, it composed of diacetyl and 2, 3-pentadione. Surprisingly, the diacetyl found in rice beer was almost 10 times higher than that of barley beer (0.04-0.08 mg/L); however, the limited concentration regarding to required level for some ale beer was 1 mg/L (Elena, Muste, Tofană, and Muresan, 2006). Thus, these concentrations found in rice beers would not be a

problem; nevertheless, long maturation might be needed for reduction of VDK in rice beer. Furthermore, the concentration of 2,3-pentadione in rice beer were not harm to the taste of beer since it has taste threshold (bitter taste) at 0.8 mg/L (Elena et al., 2006). Another compound detected in this experiment was 4-vinyl guaicol (4VG), the phenolic off-flavor (POF) for pilsner beer, an attractive flavor in wheat beer. This compound is special flavor produced from phenolic decarboxylation reaction by heating or enzymatic activity of the polyphenol decarboxylation (PAD) by brewing yeast (Vanbeneden, Gils, Delvaux, and Delvaux, 2008). The precursors of this compound are *p*-coumaric acid and ferulic acid as illustrated in Figure 5.13 and they belong to hydroxycinamic acid found in rice husk and pericarp. The concentration of 4VG in rice worts after boiling were at least 2 times higher than wort after mashing; thus, heating as high as boiling temperature (>95°C) enhanced 4VG formation. According to Meilgaard, (1975) the threshold concentration of 4VG in beer was 0.3 mg/L, but at concentration 3.76 mg/L have been encountered in wheat beer (Vanbeneden, Gils et al., 2008). However, concentrations of 4VG in ale rice beers were higher than that found in wheat beer, the reduction of 4VG by force aging at 60°C with oxygen or carbondioxide flushing has been reported (Vanbeneden, Saison, Delvaux, and Delvaux, 2008). Another procedure to avoid 4VG formation in beer was yeast selection, the significantly different between this in lager and ale beer was elucidated in Table 5.8. Most of top fermenting yeast has activity of PAD enzyme; therefore, the less powerful PAD in some top and most of bottom fermenting yeasts could be selected and recommended for black rice beer production. All of these chemical compounds, ester, higher alcohol, VDK, 4VG, etc were significantly contribute aroma and flavor of beer and weather these compound in matrix of rice

beer affected consumer perception. Consequently, the sensory test was performed by assessors who familiar with beer drinking.

The sensory analysis test was conducted by 8 assessors lived in Germany. There were five attributes required to evaluate; aroma, appearance, flavor, mouth-feel and overall impression. Aroma is defined as the pleasant fragrance of beer that originates from the natural odors of its ingredients such as barley, malt and hops (Rabin and Forget, 1998). Respecting to this definition, rice aroma must be accounted; however, it might unrecognizable from the assessor who is not familiar with rice. Therefore, this test was performed under hypothesis that if they sniff beer put on the table weather the assessors like it. The average scores of aroma in beer samples were plot on Figure 5.14. KCD lager beer had highest average score at 2.6 followed by KND lager, KND ale beer (2 scores) and the last 1.06 for KCD ale beer. These results related to the concentration of 4VG and diacetyl found in KCD ale beer at high concentration, whereas 4.85 mg/L of 4VG in KND ale beer was not a problem with scored as normal. This might be that the matrix of flavor compounds in rice beer influenced the perception.

The appearance was judged by sight, and clarity, color and head retention were properties to be evaluated by assessors. All of them had closed score 3.06-3.63, the highest score was ranked to KCD lager beer followed by KCD ale beer, KND ale beer and KND lager beer. These scores were consistent with foam stability test or head retention, since KCD lager and ale beer had good range of foam stability and assessors judged them as good (scored as 3.63 and 3.38, respectively). Moreover, red brown colors of the beers were in a range of 9-16 EBC unit which was darker than pilsner beer and the EBC unit was in the same range of weisse beer color (Wikipedia,

www, 2009). The color of ale beer was lighter than lager beer and changed with pH due to phenolic compound in beer. After the consumers tasted beer samples, the sensations as perceived by the taste buds were judged by the brain. Most of them are volatile flavors generated from brewing processes and yeast strain. KND lager beer was led other samples with score 2.5 (better than normal but not so good) and followed by KCD lager beer (2.05). Most of assessor did not like flavor of both ale beers, particularly KCD ale beer scored as 1.19 (dislike) which might be a problem of high content of 4VG or diacetyl. Mouth-feel is defined as body of the beer, meaning the consistency, thickness, and mouth-filling property of beer (Rabin and Forget, 1998). The protein and dextrin left in final product influenced body of the beer; as a result lager beer from both malts had score better than ale beer, 2.69 and 2.63 for KCD and KND lager beer, respectively.

The overall impression was the final opinion from the assessors, and the lager beers from both rice malts were judged as drinkable and may prefer one more glass. Whereas, KND ale beer was 1.75 scored as drinkable but not prefer the next glass and finally was KCD ale beer was undrinkable. In summary, most results from chemical analysis of volatile flavors in beer were consistent with sensory evaluation. This information will be useful for flavor adjusting by adaptation of process control and yeast selection. Diacetyl and 4VG in rice beer were accepted as pleasant flavor at concentration less than 8.8 and 7.4 mg/L, respectively; however, the threshold in rice beer must be further improved.

Table 5.7 The chemical properties of worts produced from two rice malts.

Chemical and physical properties	KCD Wort		KND Wort		VBL casting wort [b]
	wort	casting wort	wort	casting wort	
Extract (°P)	10.3[a]	11.5	9.4	11.5	-
FAN (mg/L)	365	350	292	272	190
Protein Fraction Fraction A (%)	2.9	1.8	2.9	3.1	32
Fraction B (%)	18.1	16.9	18.1	13.1	18
Fraction C (%)	79	81.3	79	83.8	50
Soluble nitrogen (mg/L)	1,071	1,341	736	979	1,018
pH	5.42	5.28	5.43	5.21	5.38
Color (EBC)	10.7	15.5	10.5	15.4	9.5
4-vinyl guaicol (mg/L)	0.1	0.5	0.1	0.2	nd

[a]Mean values of three mashings. [b]The average value from VBL laboratory (Kunz, 2004), Nd = not determined.

Table 5.8 The chemical and physical properties of beers from two rice malts.

Chemical and physical properties	KCD Beer		KND Beer		Range found in beer	Flavor threshold (mg/L)
	Lager	Ale	Lager	Ale		
Extract (°P)	2.93	1.58	3.5	2.9	2-2.5[a]	-
Ethanol (%v/v)	5.0	5.17	4.57	4.64	0.8-7.2[a]	-
FAN (mg/L)	173	100	183	110	26.8-53.3	-
Protein Fraction Fraction A (%)	4	0.6	0.2	0.3	20-30[d]	-
Fraction B (%)	16.5	19.1	18.4	17.8	20[d]	-
Fraction C (%)	79.5	80.3	81.4	81.9	40-50[d]	-
Soluble nitrogen (mg/L)	847	637	464	354	362-1,195[a]	-
Foam stability (sec)	367	340	273	165	160-310[a]	-
pH	4.47	4.28	4.25	4.18	3.94-4.42	-
Color	6.6	9.6	16.5	9.5	8-120	-
Acetaldehyde (mg/L)	27.2	55.7	9.2	9.3	20-40[b]	10[b]
Ethylacetate (mg/L)	3.3	7.3	24.15	39.75	5-30[b]	25-30[b]
i-Amylacetate (mg/L)	76.3	93	9.1	6.05	0.5-2.5[b]	1-1.6[b]
Amylalcohol (mg/L)	14.7	35.8	59.75	107.65	10-20[b]	10-65[b]
n-propanol (mg/L)	17.6	58.3	8.8	31.75	5-20[b]	-
i-Butanol (mg/L)	0.48	0.88	16.1	63.65	5-20[b]	10-200[b]
Diacetyl (mg/L)	0.49	0.27	0.33	0.45	0.1	0.1-0.15
2,3-Pentadion (mg/L)	0.4	7.4	0.25	0.1	-	0.8
4-vinyl guaicol (mg/L)	2.93	1.58	0.7	4.85	0.04[c]	0.3

[a] From EBC (1998).[b] Kunz, (2004).[c] Report in pilsner beer (Vanbeneden N., Delvaux, and Delvaux, 2006). [d] Pollock, (1981).

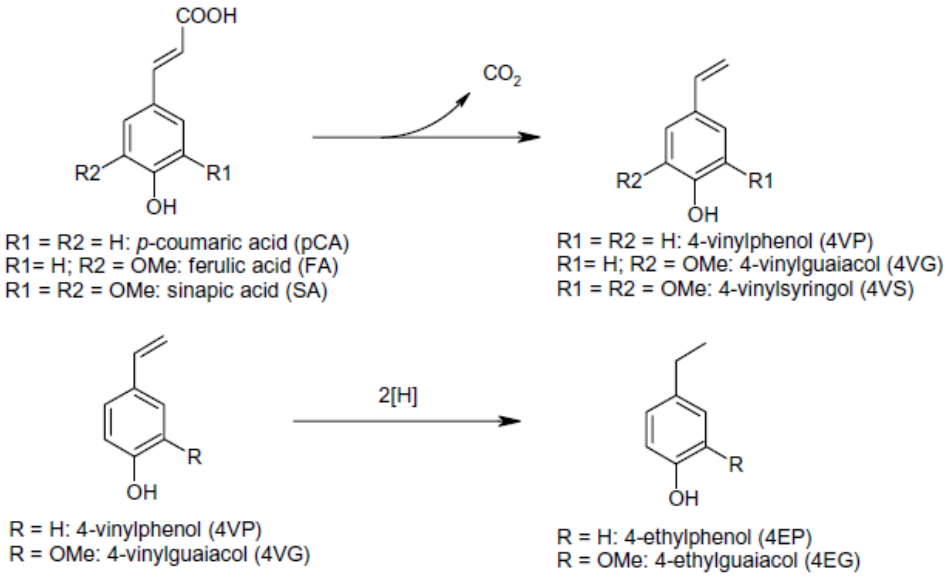


Figure 5.13 Decarboxylation of pCA, FA and SA and reduction of 4VP and 4VG to their respective ethyl derivatives(Vanbeneden, et a., (2008)**.**

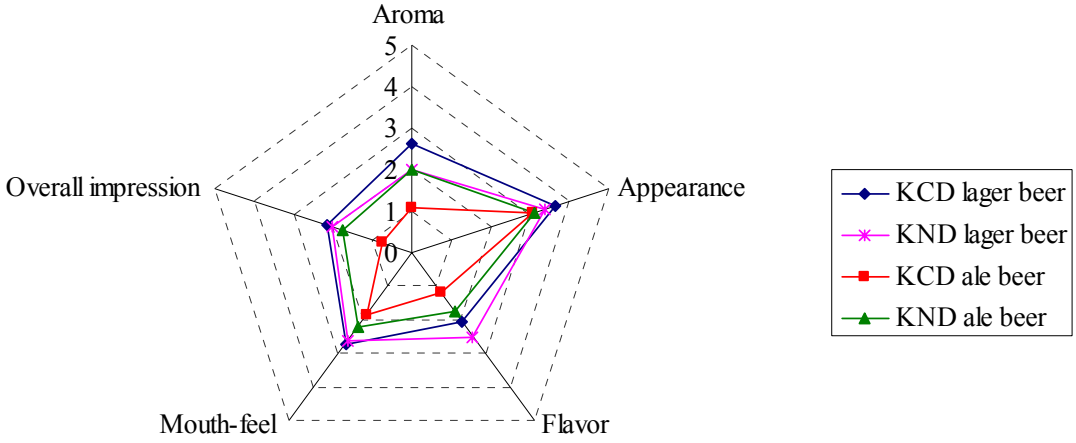


Figure 5.14 The sensory evaluation judged by 8 assessors.

(A)

(B)

Figure 5.15 Photographs of lager and ale beer produced from (A) KCD malt and (B) KND malt.

5.5 Conclusion

The optimal temperature range for each rest of mashing were investigated, proteinase rest between 50-55°C, the saccharification rest was close to liquefaction 70°C because high gelatinization temperature of rice starch. The termination of amylase and proteinase was 78°C. The decoction mashing made wort poor of soluble nitrogen and extract content lower than those in infusion mashing. An appropriate mashing regime for KCD malt was slightly different form KND malt, long range of temperature 55-57°C was selected for KCD, whereas extension of mashing time in temperatures between 62-64°C was selected for KND. The mashing in pH was adjusted to 5.2 and 150 ppm Ca^{2+} addition made rice wort had more extract, soluble nitrogen, FAN and percentage of AAL. The appropriate size of malt grist indicated by the distance between two rollers was obtained for wort productions. Space between two rollerers mill at 0.5 mm for KCD malt and 1.0 mm for KND malt gave satisfied wort properties with approximately 39% of brewing yield of both rice malts. According to the fermentation profile of lager and ale beer made from rice malts, the fermentation time was shorten in ale fermentation fermented at higher temperature 20°C. High fermentation temperature stimulated yeast metabolism; consequently more FAN utilization were detected in top fermentation (215 and 168 mg/L for KCD ale and KND ale beer). The results of protein fraction indicated that most of protein in wort and beer were the low molecular weight protein (approximately 80%); however, KCD beers were in a good range of foam stability 367 and 340 sec for lager and ale beer, respectively. Moreover, fermentation by-products were determined, including of acetal dehyde, ester, higher alchohol, phenolic flavor. Beer samples made under process of lager fermentation had all volatile compounds less than in ale

fermentation process. Particularly, the concentrations of some higher alcohols in lager beer were low as the threshold concentration reported in barley beer. Whereas, the high concentration of diacetyl and 4VG in ale beer might cause a defect to the beer flavor, since KND ale beer was judged as drinkable but not prefer for next glass and KCD ale beer was undrinkable beer evaluated by 8 assessors. However, KCD and KND lager beer were evaluated as drinkable and prefer one more glass.

The chemical analysis and sensory evaluation indicated that rice beer could be developed by many procedures. The selection of yeast strain was strongly recommended for improving of beer flavor; particularly selection of stains which has low PAD activity. Moreover, lager yeast can govern at high temperature fermentation, using of lager stains instead of ale strains in top fermentation process could stimulate more pleasant flavor formation and less of PAD activity. However, many of new brewing technologies are continuously investigated therefore rice beer produced from Thai rice must be continuously developed too.

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CHAPTER VI

OVERALL CONCLUSION

The influences of steeping durations (24, 48 and 72 h) and temperatures (20, 25 and 30°C) on α- and β-amylase activities in six Thai rice cultivars, including of three non waxy rice cultivars (KDML105, PT60 and KCD) and three waxy rice cultivars (SPT, RD6 and KND) were investigated. Amylolytic activities of rice malt were increased with temperature and highest at 30°C. Long steeping duration made rice had low activity of β-amylase and retarded α- amylase activities; therefore, the steeping duration was reduced and changed to be steeping with air-rest switching regime. Three process parameters of steeping degree, temperature and germination time were studied by using the response surface methodology to design experiment as face centered composite design and to establish empirical models for each malt properties. Since black non-waxy rice “KCD” and black waxy rice “KND” had high germinative capacity and high amylolytic activity, they were used for this investigation. Both rice needed warm temperature; 30°C, high steeping degree; 44% and germination approximately 8 days to meet the compensation properties between high of CWE, extract yield, Kolbach index, FAN, AAL, and α- amylase activity. The optimal kilning program was 50°C for 24 h in order to maintain proteinase activity. This optimal malting condition gave approximately 12% of malting losses which was in the same range with malting of barley. The temperature-programmed focused on glucose production was selected to produce wort from KCD rice malt and the

temperature-programmed focused on maltose production was selected for KND malt. The optimal mashing-in pH for improvement of wort properties; particularly on soluble nitrogen, FAN and AAL were at 5.2 and supplemented with Ca^{2+} 150 mg/L. The size of malt grits were significantly influence FAN content and AAL in wort from KND but no significantly effect on KCD wort, thus KND was milled by two roller milling at no. 1 and KCD was milled by no. 0.5 for further wort production processes. Worts produced by brew master mashing unit had brewing yield 38-39% and they were analyzed of their properties. Worts from both rice malts were fermented respecting the process of top and bottom fermentation by using *S. cerevisiae* 34/70 and *S. cerevisiae* 60/120, respectively. Top fermenting yeast consumed high amount of FAN (215 mg/L and 105 mg/L for KCD and KND wort, respectively) more than bottom fermenting yeast (125 and 109 mg/L for KCD and KND wort, respectively). Some of volatiles compounds were measured including of esters, higher alcohols, VDKs and 4VG, all of these in lager beers were lower than those in ale beer; particularly, diacetyl and 4VG were detected at high concentration in KCD and KND ale beers. Wort property influenced yeast metabolism, certainly finished product properties, and finally to consumer perception. KCD ale beer was judge as undrinkable and KND was drinkable but not prefer the next glass. Consumer appreciated the appearances of black rice beers and judged lager beers as drinkable and prefer for the next glass. This research indicated that rice could be used for brewing technology as unique product accepted by consumer. Particularly, black rice had attractive color from anthocyanin pigment which must be further investigated for antioxidant property and stability of rice beer.

APPENDIX

Table 1A. Experiment conditions and responses for KCD rice.

run	Actual levels of independent variables			Response variables					
	time	temp	Steeping degree	Extract Content (%)	CWE (%)	Kolbach Index (%)	AAL (%)	FAN (mg/ 100 g)	α-amylase (U/g)
1	8	20	44	61.4	6.53	14.7	69	27	78
2	8	20	44	61.4	6.81	14.6	69	31	80
3	8	30	44	61.3	9.79	21.7	87	72	124
4	8	25	41	62.4	8.70	16.5	83	60	85
5	8	30	38	62	8.45	15.3	84	52	75
6	8	20	38	60.2	5.78	11.4	57	23	61
7	8	30	38	61.6	8.11	15.7	84	53	79
8	8	30	44	61.3	9.82	20.4	88	72	113
9	8	20	38	60	5.65	11	57	23	51
10	7	25	41	62.4	8.03	15.9	84	56	83
11	7	30	41	61.9	8.47	18.5	85	60	83
12	7	25	38	62.1	8.01	14.6	81	51	56
13	7	25	44	62.1	9.17	19.1	83	59	106
14	7	20	41	60.4	5.88	11.7	56	14	49
15	7	25	41	62	8.65	16.4	84	45	65
16	6	30	44	62	9.64	20.1	86	61	96
17	6	20	38	58.7	5.03	9.5	49	19	32
18	6	20	44	58.3	5.03	10.3	47	16	50
19	6	30	44	62.8	9.72	21.1	86	71	110
20	6	20	38	57.6	5.23	9.5	49	16	38
21	6	30	38	61.8	8.55	15.3	82	52	70
22	6	25	41	62.1	8.08	14.8	78	49	64
23	6	30	38	61.8	8.50	15.7	84	57	73
24	6	20	44	58	5.23	9.9	50	18	46

Table 2A. Experiment conditions and responses for KND rice.

run	Actual levels of independent variables			Response variables					
	time	temp	Steeping Degree	Extract Content (%)	CWE (%)	Kolbach Index (%)	AAL (%)	FAN (mg/ 100 g)	α-amylase (U/g)
1	8	20	44	55.3	8.13	18.1	64	61	30
2	8	20	44	58.7	8.03	19.8	61	78	36
3	8	30	44	59.9	11.50	24.7	80	100	69
4	8	25	41	58	10.34	20.9	79	92	55
5	8	30	38	59.5	9.72	18.6	77	78	47
6	8	20	38	59.7	6.94	15.5	45	61	24
7	8	30	38	61.4	9.51	18.8	74	76	44
8	8	30	44	58.1	11.32	23.2	81	100	79
9	8	20	38	57.5	6.84	15.9	51	56	20
10	7	25	41	58.5	9.61	19.5	76	83	41
11	7	30	41	58.2	8.73	21.6	80	89	56
12	7	25	38	59.4	10.78	18.6	72	79	38
13	7	25	44	58.7	10.75	23.6	81	99	64
14	7	20	41	59.6	6.97	16.4	50	59	23
15	7	25	41	60	9.48	19.7	75	79	52
16	6	30	44	58.1	11.09	22.2	82	98	62
17	6	20	38	56.9	6.04	13.6	44	45	15
18	6	20	44	60.1	6.30	14.8	43	51	21
19	6	30	44	60.7	11.24	22.9	79	98	57
20	6	20	38	57.3	6.06	13.5	45	44	22
21	6	30	38	58.9	9.51	19	77	78	50
22	6	25	41	58.3	9.07	18.6	74	79	49
23	6	30	38	57.8	9.27	18.7	77	77	44
24	6	20	44	57.6	6.40	14.9	50	54	21

Table 3A. The results of numerical optimization for KCD malt. 31 solutions were provided by Design-Expert program.

Name	Goal	Lower Limit	Upper Limit	Weight	Weight	Importance
Germination time	is in range	6	8	1	1	3
Temperature	is in range	20	30	1	1	3
Steeping degree	is in range	38	44	1	1	3
CWE	maximize	7.84	9.81	1	1	5
Extract fine	maximize	50.24	62.8	1	1	5
FAN	maximize	24.22	99.2	1	1	3
Kolbach index	maximize	17.36	21.7	1	1	4
AAL	maximize	70.4	88	1	1	3
alpha-amylase	maximize	57.8	72.24	1	1	4

Solutions Number	Germination	Temperature	Steeping	CWE	Extract	FAN	Kolbach index	AAL	α-amylase	Desirability	index
1	8.00	30.00	44.00	9.8	61.5	117	21.37	89.31	72	0.925	Selected
2	7.99	30.00	44.00	9.8	61.5	117	21.37	89.29	72	0.925	-
3	7.96	30.00	44.00	9.8	61.5	117	21.39	89.24	72	0.924	-
4	7.95	30.00	44.00	9.8	61.5	117	21.39	89.22	72	0.923	-
5	8.00	29.90	44.00	9.8	61.6	117	21.34	89.55	72	0.923	-
6	7.93	30.00	44.00	9.8	61.5	117	21.41	89.17	72	0.922	-
7	8.00	29.83	44.00	9.9	61.6	117	21.32	89.71	72	0.922	-
8	7.91	30.00	44.00	9.8	61.5	117	21.42	89.12	72	0.921	-
9	8.00	29.75	44.00	9.9	61.6	116	21.30	89.87	72	0.920	-
10	7.88	30.00	44.00	9.8	61.6	117	21.43	89.05	72	0.919	
11	8.00	29.71	44.00	9.9	61.7	116	21.29	89.96	72	0.919	
12	8.00	29.98	43.94	9.8	61.5	117	21.30	89.29	72	0.918	

Table 3A. The results of numerical optimization for KCD malt. 31 solutions were suggested by Design-Expert program (continued).

Solutions Number	Germination	Temperature	Steeping	CWE	Extract	FAN	Kolbach index	AAL	α-amylase	Desirability	index
13	7.83	30.00	44.00	9.8	61.6	116	21.45	88.96	72	0.917	-
15	7.64	30.00	44.00	9.8	61.6	115	21.51	88.55	71	0.903	-
16	8.00	29.19	44.00	9.9	61.9	114	21.13	90.95	72	0.902	-
13	7.83	30.00	44.00	9.8	61.6	116	21.45	88.96	72	0.917	-
17	7.61	30.00	44.00	9.8	61.6	115	21.51	88.49	71	0.900	-
18	7.68	29.63	44.00	9.8	61.8	114	21.38	89.39	71	0.898	-
19	8.00	29.06	44.00	9.9	62.0	114	21.09	91.16	72	0.897	-
20	8.00	29.55	43.79	9.8	61.7	114	21.03	90.03	72	0.889	-
21	7.42	30.00	44.00	9.7	61.7	114	21.52	88.09	70	0.885	-
22	7.23	30.00	44.00	9.7	61.8	113	21.49	87.71	70	0.866	-
23	7.22	30.00	44.00	9.7	61.8	113	21.49	87.68	70	0.864	-
24	7.08	30.00	44.00	9.7	61.8	112	21.44	87.38	69	0.848	-
25	6.81	30.00	44.00	9.7	61.9	111	21.28	86.82	68	0.814	-
26	6.58	30.00	44.00	9.6	62.0	110	21.08	86.35	68	0.781	-
27	6.45	30.00	44.00	9.6	62.0	109	20.94	86.06	67	0.761	-
28	6.45	29.73	44.00	9.6	62.1	107	20.81	86.41	67	0.745	-
29	8.00	30.00	42.75	9.5	61.5	109	20.07	87.76	68	0.731	-
30	6.21	29.44	44.00	9.6	62.2	105	20.34	86.11	66	0.675	-
31	6.06	30.00	43.85	9.5	62.1	106	20.30	85.22	66	0.671	-

Table 4A. The results of numerical optimization for KND malt. 17 solutions were provided by Design-Expert program.

Name	Goal	Lower Limit	Upper Limit	Weight	Weight	Importance
Germination time	is in range	6	8	1	1	3
Temperature	is in range	20	30	1	1	3
Steeping degree	is in range	38	44	1	1	3
CWE	maximize	9.2	11.5	1	1	5
FAN	maximize	43.72	100.17	1	1	3
Kolbach index	maximize	19.76	24.7	1	1	4
AAL	maximize	65.6	82	1	1	3
alpha-amylase	maximize	62	78.53	1	1	4

Solutions Number	Germination	Temperature	Steeping	CWE	FAN	Kolbach index	AAL	α-amylase	Desirability	index
1	8.00	29.47	44.00	11.5	101	24.24	84.37	74	0.910	Selected
2	8.00	29.36	44.00	11.5	101	24.25	84.57	74	0.909	-
3	8.00	29.29	44.00	11.6	101	24.26	84.69	74	0.909	-
4	7.99	29.46	44.00	11.5	101	24.23	84.36	74	0.908	-
5	7.99	29.41	44.00	11.5	101	24.24	84.45	74	0.908	-
6	8.00	29.56	44.00	11.5	101	24.23	84.18	74	0.906	-
7	8.00	28.95	44.00	11.7	102	24.28	85.22	73	0.906	-
8	7.97	29.46	44.00	11.5	101	24.22	84.34	73	0.905	-
9	8.00	28.85	44.00	11.7	102	24.28	85.37	73	0.904	-
10	7.98	29.09	44.00	11.6	101	24.25	84.98	73	0.904	-
11	8.00	29.32	43.94	11.5	101	24.20	84.53	73	0.902	-
12	8.00	28.60	44.00	11.8	102	24.27	85.66	73	0.900	-

Table 4A. The results of numerical optimization for KND malt. 17 solutions were provided by Design-Expert program (continued).

Solutions Number	Germination	Temperature	Steeping	CWE	FAN	Kolbach index	AAL	α-amylase	Desirability	index
13	8.00	28.48	44.00	11.8	102	24.27	85.78	73	0.897	-
14	8.00	29.11	43.85	11.5	101	24.14	84.74	73	0.891	-
15	7.90	29.58	44.00	11.4	101	24.15	84.00	73	0.890	-
16	7.92	27.73	44.00	11.9	102	24.12	86.03	71	0.860	-
17	7.56	29.56	44.00	11.4	100	23.87	83.48	71	0.833	-

BIOGRAPHY

Miss Ulaiwan Usansa was born on September 20, 1974 in Nakhon Phanom, Thailand. In 1993, She studied in School of Food Technology, Suaranaree University of Technology, Nakhon Ratchasima. She graduated the Bachelor's of Science in Food Technology in 1997. After that, she worked as a teaching assistance for three years in School of Food Technology, Suranaree University of Technology. In 2000, she graduated the Master's of Science in Biotechnology, during that she had granted a scholarship from The National Science and Technology Development Agency (NSTDA). Her master thesis topic was a Study of Effect of Fermentation Temperatures on Red Wine Flavors. In 2004, she started her Ph.D. and got a scholarship supported by the Royal Golden Jubilee (RGJ) Ph.D. Program of Thailand Research Fund. Her dissertation title was Beer Production from Thai Rice. The topic of chapter III, the Influence of Steeping Duration and Temperature on α-and β-Amylase Activity of Six Thai Rice Malt Cultivars was accepted to be published in Journal Institute of Brewing, volume 115, issue no. 2. The parts of optimization of malting condition for two black rice by using RSM was published in the First International Symposium on Gluten-Free Cereal Products and Beverages, 12-14 September 2007. Parts of wort production and fermentation were published in ASBC Annual Meeting, 2009 at Tucson, Arizona, USA.