Increasing of Nitrogenous Substances in Wort by Using Commercial Enzymes and Modifying Mashing Method

Pailin Pliansrithong, Ulaiwan Usansa, and Chokchai Wanapu

***Abstract*—Rice used as adjunct is limited because the relative lower soluble nitrogen compare with that of barely will dilute total nitrogenous substances in wort and beer. Therefore, modification of mashing method and Neutrase® addition were proposed to increase rice/barley malt ratio. Total protein contents of four rice cultivars determined by Kjeldahl method were in a range of 6.53-7.49% (w/w). Amount of Neutrase® and mashing time for increasing protein were determined by mashing at 52 ℃ and found that increasing of mashing time increased soluble nitrogen and Free Alpha Amino Nitrogen (FAN) in wort more than 30% and 70%, respectively. The proper mashing time was 60 min and the appropriate amount of Neutrase® was 400 µl per 50 g rice. In addition, activation of Termamyl SC® at 95°C for 20 min also influenced more solubilized protein in rice cooking step. Afterward, the appropriate mashing program was evaluated in various rice/barley malt ratios. The qualified wort contained at least 150 mg/L FAN were obtained from wort used rice up to 80% and had satisfied fermentation performance. Therefore, Neutrase® addition plus modification mashing method could be one solution for increasing rice/barley malt ratio.**

***Index Terms*—Mashing, protein, rice, wort.**

I. Introduction

The basic ingredients in beer are water, malt, hop and yeast. Beer comprises of water 90–95% of the content, and its quality influences flavor of beer. Barley is the most common cereal used in the Americas and Europe to produce malt, although small volumes of beer are made from other cereal grains. Hops are contributing flavor and antibacterial compounds to beer. Yeasts are the predominant fermentation organisms used to make beer worldwide. In Thailand, barley has to be imported and it's cost intensively. Thus, there are many attempts to produce beer from alternative cereal malt and adjuncts. The adjunct beer is less cost than hundred percent malted barley beer, and reduce haze forming protein. The other benefit is attribute special characters of beer such as white beer from wheat and Zutho in India from rice malt [1]. Rice (*Oryza sativa*) is preferred by some brewers because of its lower haze protein and lipids contents compared with those of corn grit. Rice adjunct in brewing cause neutral aroma, yields a light, clean-tasting beer [2]. It is usually used for brewing in the form of rice grit or broken rice, which is obtained as a byproduct of rice harvesting and the processing of milled rice. However, low protein content in rice (5-8%) causes limited rice ratio (20-40%) in brewing due to the diluting of total nitrogenous substances in beer [3]. Nitrogenous compounds in wort comprise of FAN (30-40%) and peptide (30-40%), protein (20%), nucleotide and other nitrogen containg compound (10%) [4]. The importance of nitrogenous compounds in brewing and their role in foam stability, haze formation [5], yeast metabolism [6] and flavor [7] have been subject of many investigations. One difficulty for using high ratio of rice adjunct is the insufficient soluble nitrogen, thus increasing of solubilized protein in wort by using commercial enzymes and modifying mashing program are the main objectives of this research.

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II. Materials and Methods

A. Rice Cultivars

All rice cultivars are *Oryza sativa* L. Indica and harvested in 2010, including of Khaw Dok Mali 105 (KDML105) from Nakhon Ratchasima province, Chainat 1 (CN1), Pratum Thani 1 (PT1) and Suphanburi 1 (SP1) from Chainat province.

B. Enzymes

Neutrase® 0.8 L is protease enzyme from *Bacillus amyloliquefacien*s that contains 0.8 AU/g. Termamyl SC® is the heat stable α-amylase from *Bacillus licheniformis* that contains 120 KNU/g (Novozymes Co., Ltd).

C. Protein Content in Rice

The protein content in broken polished rice was determined by Kjeldahl method according to EBC 8.9.1 (1998) [8].

D. Optimization of Mashing Time and Amount of Neutrase®

The appropriate mashing time were examined with four rice cultivars. Rice was milled by using a disc mill (Retsch ZM 1000) as fine grinding 0.5 mm. The wort was mashed in the laboratory mashing water bath (Julabo TW12) using 50 g of rice and 250 ml water by stirring at 100 rpm and Neutrase® enzyme was added. The mashing temperatures were held at 52 ℃ for 20, 40, 60, 80 and 100 min. Then, the mashed was immediately cold down to 20 ℃ and separated by centrifugation at 4,000 rpm for 10 min and analyzed FAN and soluble protein concentration. Afterward, the appropriate amount of enzyme was tested for increasing FAN.

E. Influence of Termamyl SC on Wort Qualities

The remaining worts from previous experiment were heated at 90 ℃ for 20 min and added 200 µl of Termamyl SC®. Then, the mashed was cold down to 20 ℃ immediately and kept for FAN and soluble protein concentration analysis.

F. Evaluation of Appropriate Mashing Program for Increasing Rice/barley Malt Ratio

The mashing program of rice was formulated and evaluated for increasing rice/barley malt ratio in brewing. Four rice cultivars were mixed each of 25% by weight before mashing with barley malt in various rice contents (20-100% w/w). All worts were fermented by *Saccharomyces cerevisiae* 12×10^6cell/ml, at 11°C for 14 days.

G. Analysis of FAN and Soluble Protein in Wort

Free amino nitrogen was determined by Ninhydrin method according to EBC 8.10 (1998). One ml of sample was diluted with water to 100 ml. Then, 2 ml of the diluted sample was taken into test tube and 1 ml of color reagent was added. The test tube was heated in boiling water for exactly 16 min and then cooled in a water bath at 20°C for 20 min. Five milliliters of diluting solution (2 g Potassium iodate (KIO_3) in 600 ml distilled water and 400 ml of 96% ethanol) was added and measured the observe density at 570 nm. Blank was prepared from reagents with 2 ml of distilled water. Glycine standard solution was checked by using 2 ml of glycine solution. FAN concentration was calculated using following formula

$$\text{FAN}(\text{mg/l}) = \frac{A_1 \times 2 \times d}{A_2}$$

where
A_1 = Observe density of test solution at 570 nm
A_2 = Mean observe density of standard solution at 570 nm
d= Dilution factor of sample

Protein concentration was estimated by the Bradford Coomassie blue dye binding assay using bovin serum albumin (BSA) as the standard [9].

H. Statistical Analysis

The statistical analysis was carried out by SPSS version 14 (SPSS Inc.). All chemical experiments were analyzed in triplicates. Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) was used to determine differences of mean at p<0.05.

III. Results and Discussions

Four rice cultivars mostly cultivated in Thailand were selected to study under the hypothesis of most brewing factories need thousand ton of rice per month from the suppliers. Therefore, there would be high possibility for them to accumulate rice from many mills, many harvesting seasons, and many rice cultivars instead of using one rice source. Total protein content of all rice cultivars were in a range of 6.53-7.49% (w/w) (Table I) and classified in group of medium protein containing rice [10]. The lowest protein content was found in KDML 105, 6.53%w/w.

TABLE I: Total Protein Contents in Rice

Rice cultivars	Total protein content (%w/w)
Chainat 1	7.39
Khaw Dok Mali 105	6.53
Pratum Thani 1	7.41
Suphanburi 1	7.49

Soluble protein and FAN concentrations in wort mashed at 52 ℃ and 100 µl Neutrase® enzyme adding were shown in Table II and III. Soluble protein content in wort increased with mashing time up to 60 min and then significantly decreased onward were found in wort from CN1, PT1, and SP1. Only, soluble protein in wort from KDML 105 slightly decreased with mashing time but not significantly different (P<0.05). Rice has four protein fractions, albumin, globulin, glutenlin, and prolamin. Variation of protein fractions found in rice might cause of variation in solubility of rice cultivar. Glutenlin is the fraction mostly found in rice which is well solubilized at high and low end of pH range. Thus, hardly dissolved of rice protein in slightly acidic mashing pH (5.0-5.4) was reported in many literatures. In this study, KDML105 had maximum soluble protein in wort mashed for 20 min, it might has more albumin fraction, a water soluble protein, than other cultivars or the loosen starch-protein matrix of rice grist facilitated protease to attack rice protein body easier. Likitwattanasade and Hongsprabhas [9] suggested that rice starch pasting temperature depended on protein fraction. Thus low protein in starch-protein matrix might made starchy endosperm liquefied easier and released protein body rapidly than other cultivars.

All rice wort had maximum FAN content after mashing for 100 min, but was not significantly different from wort of 80 and 60 min (P<0.05). Thus, mashing time longer than 60 min was not significantly influenced on nitrogenous substance in wort. These results revealed that the appropriate mashing time was approximately 60 min which was before the amount of soluble protein decreased significantly.

TABLE II: Soluble Protein Concentration in Rice Wort

Cultivars	Soluble protein (g/l)				
	20min	40min	60min	80min	100min
CN 1	15.08c	24.0ab	27.50a	18.46bc	18.42bc
KDML105	30.31a	29.96a	27.07a	26.75a	22.88a
PT 1	23.33c	31.25b	37.04a	32.25b	28.79b
SP 1	17.25bc	21.67b	32.58a	10.96c	16.92bc

Same letter in the same row was not significantly different at *p<0.5*.

TABLE III: FAN Concentration in Rice Wort

Cultivars	FAN (mg/l)				
	20min	40min	60 min	80 min	100 min
CN 1	66.25c	83.37b	91.51ab	97.05 a	101.83a
KDML105	79.33c	93.81b	101.74a	101.90a	109.28a
PT 1	74.81c	96.00b	101.05ab	106.75a	101.55ab
SP 1	70.29c	81.28b	91.58ab	94.09 a	99.16a

Same letter in the same row was not significantly different at *p<0.5*.

After that, the appropriate amount of Neutrase® enzyme addition was considered with only KDML 105 and found that 400 µl/ 50 g rice was appropriate to maximize FAN content and this result revealed that approximately 34 mg/l of FAN could be increased by adding 4 times amount of Neutrase® whereas soluble nitrogen content was not changed. Rice starch-protein matrix cloud be one obstacle for enzyme attack, thus liquefaction of mash by amylase might release protein body to reach protease enzyme. Thus, the effect of Termamyl SC® to protein releasing from starch matrix was examined subsequently. The results were shown in Table IV and Table V. The soluble protein concentration in wort of three rice cultivars were clearly increased after adding Termamyl SC® ;whereas, FAN concentration in all rice wort were decreased by mean of more starch solubilization gained wort volume to dilute FAN. Thus, it's not only protease enzyme but also amylase enzyme influenced solubilization of protein from rice grist, since rice proteins are mainly located in endosperm [12].

TABLE IV: OPTIMIZATION AMOUNT OF NEUTRASE®

	Neutrase® addition (ul/ 50 g rice)				
	0	100	200	400	600
FAN (mg/l)	17.90 d	101.74 c	112.40 b	137.82 a	138.86 a
Soluble Protein (g/l)	17.84 c	22.07 b	23.97 b	26.36 a	28.25 a

Same letter in the same row was not significantly different at *p<0.5*.

The appropriate mashing program was formulated and evaluated for increasing rice/barley malt ratios. All rice cultivars were mixed (each 25% by weight) and mashed with barley malt. Using decoction mashing program as following, 50 g of mixed rice was mashed at 52 ℃ by adding 400 µl Neutrase® and held for 60 min. Then, 200 µl Termamyl SC® was added and heated to 90 ℃ holding for 20 min. After rice cooking, mashed was cooled down to 62 ℃ and mixed with barley malt and mashed for 60 min. Afterward, The mash was maintained at 70 ℃ for 30 min and 85 ℃ for 5 min (Fig. 1).

The FAN and soluble protein in wort were significantly declined if more rice added (Table VI and VII). Whereas, the sugar content represented in %Brix was in range of 13.5-15.9 and increased with rice content. The pH of 100% rice wort was slightly higher than that found in 100% barley wort. However, all mash pH values lay within the pH range of 5.5-6.0, which is commonly observed in commercial brewing depending on individual process.

These results elucidated that malt is the main source of nitrogenous substances (10-12%w/w protein) while rice is source of carbohydrate for brewing (70%w/w starch) which typically higher than that in barley malt (58%w/w starch) [10]. Moreover, Yano and colleague (2008) [11] reported barley adjunct could inhibit malt protease activity, particularly 1,10-(O-Phen)-inhibitable metallo preteinase and cystein proteinase that contributed to FAN level in adjunct wort. In case of rice, cystein proteinase inhibitor called oryzacystatin has been reported in rice seed to against nematode and stress condition [12] but never report its function in brewing process. Lei and colleague [13] studied changed of FAN composition by different commercial protease found that type of protease influenced FAN profile and assimilation by lager yeast strain. They elucidated that Neutrase® was appropriate for FAN availability for brewing.

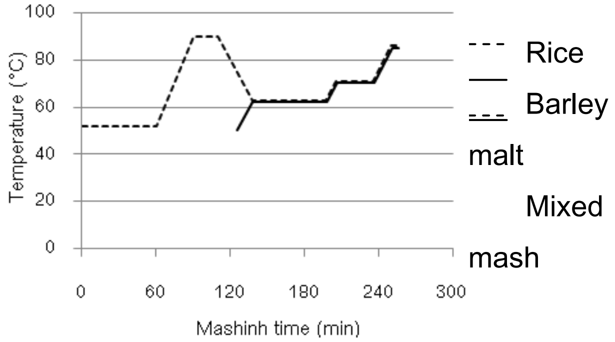

Fig. 1. Optimal mashing diagram for decoction brewing.

TABLE V: SOLUBLE PROTEIN CONCENTRATION IN WORT BEFORE AND AFTER ADDING TERMAMYL SC®

Cultivars	Soluble protein (g/l)	
	Before	After
CN 1	15.85b	35.40a
KDML105	20.23b	39.10a
PT 1	28.55b	44.17a
SP 1	19.87b	29.20a

Same letter in the same row was not significantly different at *p<0.5*.

TABLE VI: FAN CONCENTRATION IN WORT BEFORE AND AFTER ADDING TERMAMYL SC ®

Cultivars	FAN (mg/l)	
	Before	After
CN 1	104.65a	77.16b
KDML105	116.50a	97.96b
PT 1	128.92a	110.12b
SP 1	117.18a	98.98b

Same letter in each row was not significantly different at *p<0.5*.

Then, the effect of sterilization process to wort nitrogenous substances was studied by boiling at 95 ℃ for 60 min. The results were shown in Table VI and VII. Only the pH and soluble protein were slightly decreased to a pH range of 5.5-5.8 and 36.45-67.27 g/l of soluble protein. Some heat sensitive protein was denatured and precipitated after heating for an hour; while, FAN content was quite stable during high thermal process.

TABLE VII: QUALITIES OF WORT BEFORE BOILING

Rice/Malt	pH	%Brix	Protein (g/l)	FAN (mg/l)
0:100	5.6 c	13.5 c	74.55 a	254.37 a
20:80	5.7 b	15.3 b	71.00 b	207.46 b
40:60	5.6 bc	15.5ab	58.55 c	199.83 c
60:40	5.6 bc	15.6ab	55.68 d	197.44 c
80:20	5.6 bc	15.8 a	40.97 e	168.61 d
100:0	6.0 a	15.9 a	38.70 e	122.87 e

Same letter in the same column was not significantly different at *p<0.5*.

TABLE VIII: QUALITIES OF WORT AFTER BOILING

Rice/Malt	pH	%Brix	Protein (g/l)	FAN (mg/l)
0:100	5.5 d	13.9 d	67.27 a	254.76 a
20:80	5.6 b	15.3 c	65.64 a	211.85 b
40:60	5.5 cd	15.4 c	57.45 b	212.19 b
60:40	5.5 cd	15.7 b	47.91 c	190.32 c
80:20	5.5 c	15.7ab	37.00 d	167.17 d
100:0	5.8 a	15.9 a	36.45 d	122.54 e

Same letter in the same column was not significantly different at *p<0.5*.

The fermentation performances of all wort (adjusted to 12% Brix) were examined and shown in Fig. 2 and Fig. 3. They were manipulated for 14 days to ensure that fermentation was finished. However, a hundred percent rice wort was terminated before 14 days and approximately 50% of reducing sugar left in fermented wort after fermentation took place for 8 days as well as FAN content was stabilized at 20 mg/l since day 10th. Proline is well known amino acid which is not utilized by yeast under fermentation condition due to its assimilation required a mitochondrial oxidase [14]. Recently, analysis of individual amino acid in wort is necessary for brewing, since proline is not taken into account of assimilable amino acid under fermentation condition. Adding malt 20% governed successfully by 14 days, 1.23 %w/v of reducing sugar left and that was closed to amount left in wort from hundred percent malt (0.87%w/v).

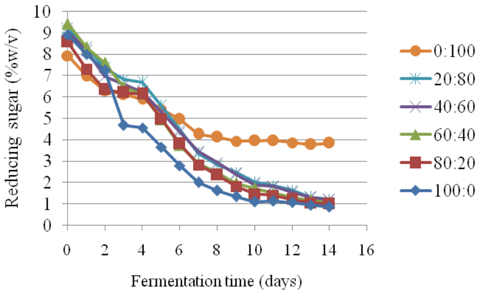

Fig. 2. Reducing sugar concentrations in fermenting worts of different rice/barley malt ratios.

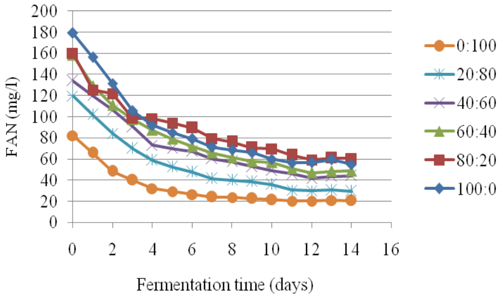

Fig. 3. FAN concentrations in fermenting worts of different rice/barley malt ratios.

More malt added made more FAN but less reducing sugar left in finished products. This study elucidate that FAN content between 167-254 mg/l was adequate for lager brewing and similar evidence reported by Odibo and colleague (2002) [15] that sorghum wort, having FAN 138-144 mg/l provided alcohol content 4.0-4.3%v/v in finished beer. Therefore, this work elucidate that protease supplement and extending protease rest before rice cooking step could be one interesting method for brewing factory.

IV. Conclusion

Rice is currently the second most widely used as adjunct material in United State less than corn grist. Thailand is one of the largest rice exporters in the world. Thus, rice might be an interesting starchy adjunct in brewing. This study has shown that it is possible to increase rice used in brewing up to 80% by using Neutrase® supplement and modification of decoction mashing method.

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