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Full Length Research Paper

Optimisation of wort production from rice malt using enzymes and barley malt

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Commercially, rice malt has never been successfully used in brewing because of its low free α-amino nitrogen (FAN) content. This study was designed to optimise rice malt replacement for barley malt in wort production and to improve FAN by adding α-amylase and protease. The response surface methodology (RSM) through face central composite design (CCD) was selected to examine the effects of the four independent variables at three levels: germination time (X_1), rice malt ratio (X_2), α-amylase (X_3) addition, and protease (X_4) addition. The rice malt ratio influenced the adjusted wort yield (AWY); 75% rice malt gave the maximum AWY. The addition of protease increased the amount of wort FAN due to the improvement in amino nitrogen liberation. Moreover, the germination time of rice had the most impact on wort FAN. On the 1st and 3rd days of germination of the rice malt, the FAN was 18.76 and 44.70 mg/100 g malt, respectively; on the 5th day, the FAN was 118.45 mg/100 g malt. The optimisation process for wort production using the addition of enzymes and rice malt was successfully accomplished. The rice malt ratio was increased up to 90% (w/w) using the five-day germinated rice malt, α-amylase and protease at 0.40 g/100 g malt.

Key words: Response surface methodology (RSM), rice malt, wort production.

INTRODUCTION

Today, the world's brewing industry is becoming more competitive and therefore attempting to improve beer quality, product diversity and reduce investment costs. Raw materials are the main contributor to operating cost, particularly for the brewery that imports malt and adjuncts from abroad. In Thailand, rice is the major cultivated grain crop, producing more than 32 million tons/year (Sompong et al., 2011), whereas barley is not cultivated because of the inappropriate atmosphere and environment. Thus, the use of rice malt for brewing in Thai breweries is a new challenge. However, there are no reports that the process has been successfully undertaken in a Thai brewery. The application of rice adjunct for production cost reduction in brewing has been studied. It was found that, addition of rice up to 40% significantly decreases the biomass formation of yeast due to depletion of assimilable nitrogen in the wort. This leads to longer fermentation times and may have a negative influence on beer organoleptic quality (Le Van et al., 2001).

There are many reports of rice malt production (Usansa et al., 2009, 2011; Capanzana and Buckle, 1997). Malting is a process that produces the enzymes of hydrolysis and modifies the storage substances such as starch and proteins. Starchy endosperm in the rice kernels is digested by amylolytic enzymes generated during the malting process. In addition, the storage protein

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Table 1. Malting schedule of rice.

1st day - 2nd day (h)	3rd day – 4th day (h)	5th day (h)
Steeping 8	Steeping 4	Steeping 2
Air rest 4	Air rest 8	Air rest 10
Steeping 8	Steeping 4	Steeping 2
Air rest 4	Air rest 8	Air rest 10

embedded in the starch matrix is hydrolysed by endogenous proteases. Because the activities of α-amylase and β-amylase in rice malt are lower than those in barley (Nanjo et al., 2004; Chang et al., 1997), poor modification is found in rice malt. Mashing is one of the most important processes in the production of wort containing suitable amounts of fermentable sugars, yeast nutrients and flavour compounds, by the action of enzymes at their optimum temperature (Montanari et al., 2005). The addition of commercial amylolytic and proteolytic enzymes with a less-expensive adjunct has been reported. Commercial amylolytic enzymes can be used for increasing fermentable carbohydrates and decreasing viscosity, leading to higher filtration rates and yield, whereas commercial proteases are able to improve the low-molecular weight nitrogenous compounds, especially the free amino acids that play decisive roles in the taste, foam stability and chill sensitivity characteristics of finished beer (Norris and Lewis, 1985; Gorinstein et al., 1980; Goode and Halbert, 2003).

Response surface methodology (RSM) is an effective statistical technique for optimising complex processes. RSM has been successfully used for various biotechnological and agricultural applications (Giovanni, 1983; Vohra and Satyanarayana, 2002; Elibol and Ozer, 2002; Kawaguti and Sato, 2007; Popa et al., 2007). The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is less laborious and time-consuming than other approaches used to optimise a process. Equations describe how the test variables affect the response, determine the interrelationship among the test variables and describe the combined effect of all test variables in the response (Giovanni, 1983). This work used RSM to determine and optimise enzyme supplementation, and rice malt ratios required to optimise wort using rice as the major starch source in order to reduce the cost of beer production.

MATERIALS AND METHODS

Rice, barley and enzymes

Hybrid medium-short grain rice CP13 was obtained from Charoen Pokphand Co. Ltd., Thailand. Proximate analysis was carried out according to AOAC methods (AOAC, 1990). Barley malt was the *Baudin* species provided by Khon Kaen Brewery Co. Ltd., Thailand. Brewing enzymes used in this experiment were TermamylSC® (E.C.3.2.1.1), a heat stable α-amylase that contains 120 KNU/g, and Neutrase® 0.8 L (E.C.3.4.24.28) as a protease that contains 0.8 AU/g; both were purchased from Novozymes A/S Denmark.

Rice malt production

Small-scale rice malting was carried out using the steeping and air resting programs shown in Table 1. The germination temperature and relative humidity were maintained in the incubation chamber at 30°C and 99%, respectively. Germinated rice was collected on the 1st, 3rd and 5th days of malting and kilned at 50°C for 24 h. The rootlets and shoots of dried malt were eliminated. Properties of the rice malt, including free amino nitrogen (FAN) and extract content, were analysed according to EBC8.10 and EBC4.5.1, respectively (EBC, 1998). The malting loss and the activities of α-amylase and β-amylase were determined according to Usansa et al. (2009).

Mashing conditions

Rice and barley malts were milled in a laboratory disc mill with a 0.1 mm milling gap. Wort was produced in a small-scale mashing water bath using 50 g of grist malt mixed with 250 ml distilled water and stirred at 100 rpm continuously. The temperature program was 45°C × 10 min, 50°C × 60 min, 63°C × 40 min and 95°C × 60 min. Protease and α-amylase were added at the initial step at 50 and 95°C, respectively. Spent grain was eliminated by filtering through filter paper No.1 (Whatman, U.S.A.). Wort properties including FAN and extract content, were analysed according to EBC8.10 and EBC4.5.1, respectively (EBC, 1998) and adjusted wort yield was analysed.

Determination of adjusted wort yield (AWY)

After wort filtration, the wort was diluted to 12°P, and the filtrate volume was measured. The wort adjusted yield was defined as the ratio of the volume of 12°P wort to the volume of water added for mashing, according to the following formula:

$$\text{Adjusted yield (AWY) (\%)} = \frac{\text{Volume of wort after filtration and adjusted to } 12^{\circ}\text{P (mL)}}{250\ \text{mL}} \times 100$$

Experimental design

Design-expert software (Trial Version 7.1.3, Stat-Ease Inc., U.S.A.) was used for the experimental design, regression, and graphical analyses of the data. Face centre central composite

Table 2. Process variables and their levels in the four-factor, three-level response surface design.

Variable	Codes symbol	Variable level		
		-1	**0**	**1**
Germination time of rice (days)	X_1	1	3	5
Rice malt : Barley malt ratio	X_2	50:50	75:25	100:0
α-Amylase (g/100 g malt)	X_3	0	0.25	0.5
Protease (g/100 g malt)	X_4	0	0.25	0.5

Table 3. Proximate analysis and physical properties of rice.

Nutrient	Chemical composition (%)
Starch	61.8
Moisture content	11.5
Crude fibre	8.8
Crude protein (N = 5.95)	7.5
Ash	7.3
Crude fat	3.0
Germinative capacity (%)	96.0

design (CCD) was used to examine the effects of the four variables: germination time (X_1), ratios of rice malt (X_2), α-amylase content (X_3), and protease content (X_4) in three levels, as presented in Table 2. A total of 26 experimental runs with two replications were completed. The properties of wort expressed as the dependent variables were determined: extract content (%) (Y_1), FAN (mg/L) (Y_2) and percentage yield of wort adjusted to 12 °P (Y_3). These variables were expressed individually as a function of the independent variables. The quadratic model for predicting the optimal point was expressed according to Equation (1):

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_i X_i + \sum_{j=1}^{k} \beta_{ii} X_i^2 + \sum_{i\langle j}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the response calculated by the model; X_i and X_j are the coded variables; and β_i, β_{ii} and β_{ij} are the coefficients estimated by the model and present the linear, quadratic and interaction effect of each factor, respectively. The statistical significance of the backward regression coefficients was determined by Student's *t*-test, the second order model equation was determined by Fischer's test and the proportion of variance explained by the model was given by the multiple coefficient of determination, R^2. The optimum concentrations of the variables were obtained by the graphical and numerical analyses using the Design Expert program, based on the criterion of desirability.

RESULTS AND DISCUSSION

Rice and rice malt qualities

The proximate analysis is illustrated in Table 3. The benefits of short-grain rice for brewing have been reported (Bradee, 2006; Teng et al., 2006), including higher water adsorption leading to higher germination rates and lower viscosities than long-grain rice. Rice contains 3.0% fat, which is higher than barley (2.0%). The disadvantages were as follows. First, there was formation of stale and off-flavour aldehydes from the oxidation of unsaturated free fatty acids (Schwarz, 2002). Second, the protein content of the rice was 7.5%, which is less than that in barley, oats, maize or wheat (Shewry, 2007), while the starch content was 61.8%. On the other hand, rice contained 8.8% of crude fibre, which was mostly in the form of rice husks that were beneficial during the lautering process because of their filtration properties.

To investigate the effect of germination time on rice malt quality after the 1st, 3rd and 5th days of germination, rice malt properties were analysed and compared with commercial barley malt (Table 4). The FAN in wort was produced from ungerminated rice at 5.22 mg/100 g malt, which is lower than that in malted rice and barley. The proteolytic enzymes produced during the malting process enhanced the solubilization of proteins by digesting the proteins to small peptides and amino acids (Celus et al., 2006; Enari and Sapanen, 1986). As a result, the FAN obtained from malted rice increased after 5 days of germination but was only close to the standard range found in barley malt at 120 to 160 mg/100 g malt (Agu and Palmer, 1998). Amylolytic enzymes in malt, including α-amylase and β-amylase, hydrolyse the starch into fermentable sugar that yeast metabolises to ethanol.

Table 4. Properties of rice malt from difference germination time compared with unmalted rice and barley malt.

Malt properties	Control (unmalted rice)	Germination time (days)			Barley malt
		1	3	5	
Malting loss (%)	-	3.99[a]±0.73	8.78[b]±1.30	17.57[c]±1.68	-
Extract content (%)	11.26[a]±0.027	11.40[a]±0.11	15.40[b]±0.19	27.36[c]±0.12	82.1*
FAN (mg/100g malt)	5.22[a]±0.001	18.76[a]±0.39	44.70[b]±3.13	118.45[c]±4.21	120-160*
Enzyme activities (Unit)**					
α - amylase	0.015[a]±0.001	0.034[a]±0.000	0.312[b]±0.008	0.394[c]±0.006	1.052[d]±0.002
β- amylase	0.011[a]±0.0005	0.032[a]±0.001	0.290[b]±0.008	0.294[b]±0.025	1.489[c]±0.001

Different letters indicatesignificant differences in each column (95% confidence). *Agu and Palmer (1998); **weight of reducing sugars production (g) from starch hydrolysis in 10 min per 1 g of malt.

During germination, α-amylase randomly attacks α-(1→4) linkages within the starch chain. The rate of hydrolysis slows near the chain ends and stops at α-(1→6) branches (Evans, 2005).

The assay of amylolytic activity indicated that both α-amylase and β-amylase from rice malt were significantly increased after three days of germination, similar to a previous study of malting conditions in 6 Thai rice varieties reported by Usansa et al. (2009). They found that, both α-amylase and β-amylase activities increased gradually after three days. It was noticed that α-amylase increased continuously, whereas β-amylase activity declined after four days. On the other hand, the β-amylase activity of rice was constant at 0.29 units on the 3rd and 5th days. The different malting regimes were used in the two studies which might be the reason for the change in the amount and pattern of enzyme production. The activity of α-amylase and β-amylase in barley malt were higher than those in rice malt, approximately 2.5 and five times, respectively. The content of amylolytic enzyme activities was according to the data in Table 4; the extract content increased from day 1 (11.40%) to day 3 (15.40%). The highest extract content was 27.36% after five days of germination, which is rather low compared to barley malt. Furthermore, the malting loss increased with germination time, up to 17.57%, which is in the normal range for the malting industry (Eneje et al., 2004). Thus, it is not a main component of operating cost.

Model fitting

Wort properties obtained under the different testing conditions are presented in Table 5 and the variance analysis of the factors is reported in Table 6. The actual values of mathematical models generated from the experimental data using Design-Expert software are expressed by the following equations:

$$\text{Extract content (\%)} = 133.18 - 1.51X_1 - 1.14X_2 + 54.73X_3 + 0.089X_1X_2 - 9.34\,X_1X_3 + 1.36\,X_2X_3 - 180.77\,X_3^2 \quad (2)$$

$$\text{FAN (mg/L)} = 297.1 - 62.19X_1 - 2.4X_2 + 268.1X_3 + 439.68X_4 + 0.45X_1X_2 - 1.44\,X_1X_3 + 9.37X_1^2 - 330.0X_3^2 - 485.23X_4^2 \quad (3)$$

$$\text{AWY (\%)} = 6.16 + 2.15X_1 + 2.28X_2 - 24.73X_3 + 0.95\,X_2X_3 - 0.02X_2^2 \quad (4)$$

Where X_1, X_2, X_3 and X_4 are the actual values of the independent variables, germination time, rice malt ratio and α-amylase and protease added content, respectively. The *p*-values of the models were less than 0.0001. Thus, the second-order polynomial regression model was in good agreement with the experimental results and the model fitting was significant with R^2 of 0.8193, 0.9609 and 0.7353 for extract content, FAN and adjusted yield, respectively.

Analysis of response surface

The relationship between independent and dependent variables was illustrated in a three-dimensional graph that was generated by the model for extract content, FAN and wort adjusted yield. Two variables were depicted in one three-dimensional surface plot, while the other variables were kept at zero level (Figure 1).

Extract content

The interaction effects of the rice malt ratio and α-amylase on the extract content of wort are shown in Figure 1a. Heat-stable amylolytic enzymes increased the extract in wort, whereas increasing the rice malt ratio reduced the amount of extract. This result demonstrates

Table 5. Experimental design and response value.

Runs	X_1	X_2	X_3	X_4	Extract (%)	FAN (mg/L)	AWY (%)
1	-1	-1	-1	1	80.1±0.0	244±1	74.5±3.1
2	0	1	0	0	76.3±0.0	146±4	83.0±1.1
3	0	0	1	0	83.0±0.5	205±1	93.0±1.1
4	1	-1	-1	1	87.2±0.5	323±15	75.6±2.0
5	1	1	-1	-1	37.4±0.8	206±13	54.0±2.6
6	1	1	1	-1	76.6±0.0	201±3	83.6±1.1
7	1	0	0	0	87.1±0.5	322±5	87.4±1.2
8	1	-1	1	-1	88.3±0.0	245±0	85.3±0.6
9	-1	-1	-1	-1	84.0±0.5	153±1	77.6±0.3
10	-1	-1	1	1	90.6±0.5	259±12	86.8±0.6
11	-1	1	-1	-1	16.5±0.0	41±1	28.0±1.4
12	0	0	0	-1	87.0±0.0	145±14	87.1±0.6
13	0	0	0	0	84.1±0.0	217±3	84.6±1.1
14	-1	0	0	0	83.1±0.0	196±2	92.7±1.7
15	-1	1	1	1	76.4±0.5	140±5	89.6±1.1
16	0	-1	0	0	89.9±0.0	269±2	94.1±0.8
17	-1	1	1	-1	74.7±0.0	36±1	85.2±1.4
18	-1	1	-1	1	20.3±1.2	150±4	36.0±2.8
19	0	0	0	1	82.7±0.0	238±20	94.6±2.3
20	1	1	-1	1	79.5±0.0	323±5	77.2±1.2
21	1	-1	-1	-1	83.6±0.5	183±4	81.4±1,5
22	-1	-1	1	-1	81.9±0.5	146±1	87.0±0.6
23	0	0	0	0	82.7±0.0	203±6	87.1±1.7
24	1	1	1	1	79.5±0.0	256±3	86.3±1.4
25	0	0	-1	0	79.8±0.0	197±2	85.8±1.1
26	1	-1	1	1	91.2±0.0	309±2	105.5±1.7

that rice malt diluted the extract in the wort which caused lower saccharification enzymes than the enzymes release from the barley malt. On the other hand, the saccharification of commercial α-amylase slightly decreased when the barley malt ratio increased. From the response surface plot, commercial α-amylase had no impact on extract content when more than 50% barley malt was used. This might be as a result of the high amylolytic activities contributed by the barley malt.

Free amino nitrogen (FAN)

In this study, the ninhydrin method was used for the FAN analysis in large part to the reaction being specific for amino acid and ammonia derived from proteins during the brewing process, while the Kjeldahl assay measures total nitrogen content (Abernathy et al., 2009). The effect of commercial proteases and the germination time of rice on FAN are shown in Figure 1b. Commercial proteases and the proteases in germinated rice grains improved α-amino nitrogen liberation but did not interact (*p*-value of $X_1X_3 > 0.05$) (Table 6). The response surface plot shows the effects of protease enzyme additions on the FAN generation rate at 0 to 0.25 g/100 g malt. The rate of FAN generation slightly decreased when commercial protease was supplemented at more than 0.5 g/100 g malt. This decrease was due to the limited amount of usable protein content within the rice malt. Meanwhile, the FAN in wort could be greatly improved by applying five-day germinated rice in accordance with the variance analysis of the factors (Table 6). The *p* value of the germination time of rice (X_1) was lower than that of commercial protease addition (X_4). It can be seen that the modification of germinated rice had more impact on FAN than commercial enzymes. This result is in agreement with the study of Markovic et al. (1995) who found that the liberation of α-amino nitrogen in the combination of barley protease extract and bacterial protease was more efficient than a single bacterial protease. The FAN values were significantly improved when 0.5 g/100 g malt protease was added without α-amylase (323 mg/L; run 20) (Table 5). On the other hand, without protease addition and only 0.5 g/100 g malt of α-amylase, a

Table 6. *F* - value and *p* - value for each variable in the polynomial model.

Source	Extract content		FAN		Adjusted yield (%)	
	β-coefficient	*p*-value	*β*-coefficient	*p*-value	*β*-coefficient	*p*-value
Model	-	< 0.0001	-	< 0.0001	-	< 0.0001
X_1	5.71	0.0605	55.69	< 0.0001	4.30	0.0763
X_2	-13.31	0.0001	-35.20	< 0.0001	-7.97	0.0024
X_3	9.66	0.0030	-1.26	0.7794	11.71	< 0.0001
X_4	-	-	49.27	< 0.0001	-	-
X_1X_2	4.47	0.1122	22.55	0.0002	-	-
X_1X_3	-4.67	0.0985	-	-	-	-
X_2X_3	8.52	0.0110	-9.01	0.0719	-	-
X_1^2	-	-	40.59	0.0041	-3.58	0.5719
X_3^2	-5.79	0.3894	-17.51	0.0845	-	-
X_4^2	-	-	-27.21	0.0156	-	-
Lack of fit	-	0.0785	-	0.4002	-	0.1385
Other statistics						
R^2	0.8193			0.969		0.7353
Adj. R^2	0.7491			0.9389		0.6691
%C.V.	12.97			9.08		12.07

$p < 0.05$ = significant.

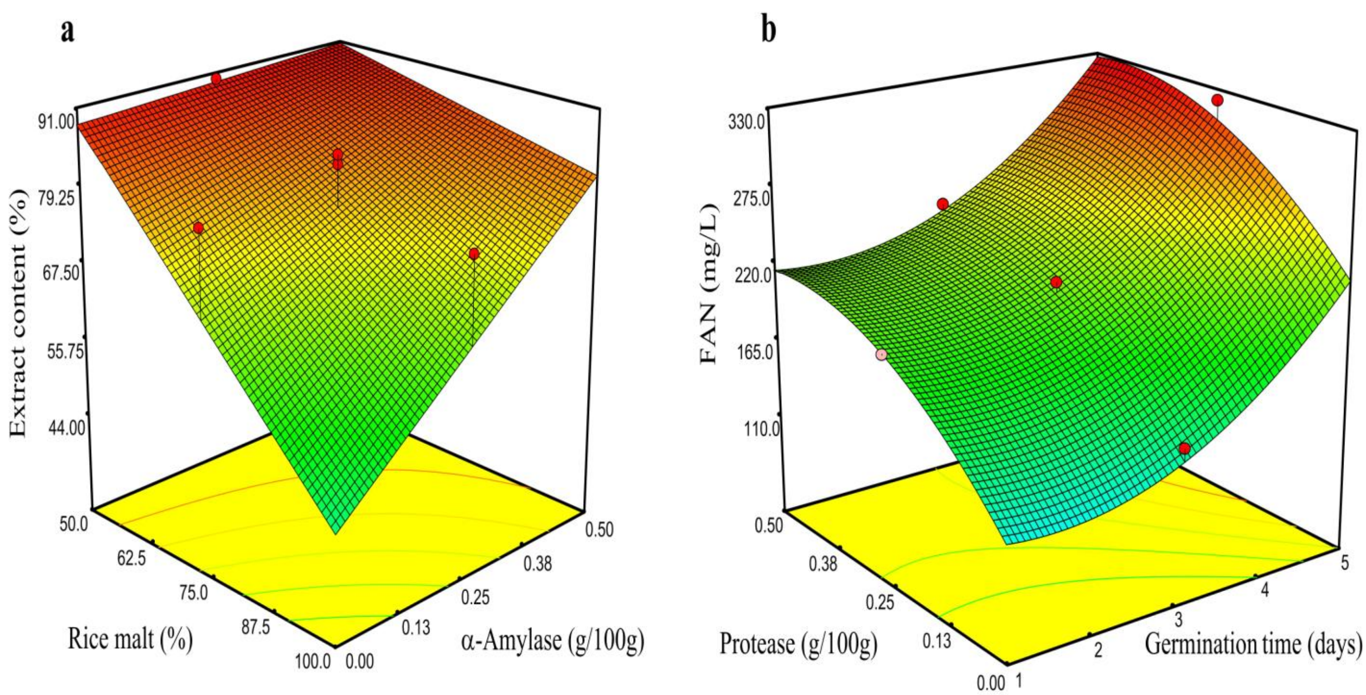

Figure 1. Response surface plots showing the operating parameter effects of wort properties (a) % rice malt and α-amylase at fixed germination time of 3 days and protease of 0.25 g/100 g on extract content (b) protease and germination time at fixed rice malt of 75% and α-amylase 0.25 g/100 g on FAN (c) protease and α-amylase at fixed rice malt of 75% and germination time of 3 days on FAN (d) rice malt ratio and germination time at fixed α-amylase of 0.5 g/100 g and protease 0.25 g/100 g on % AWY.

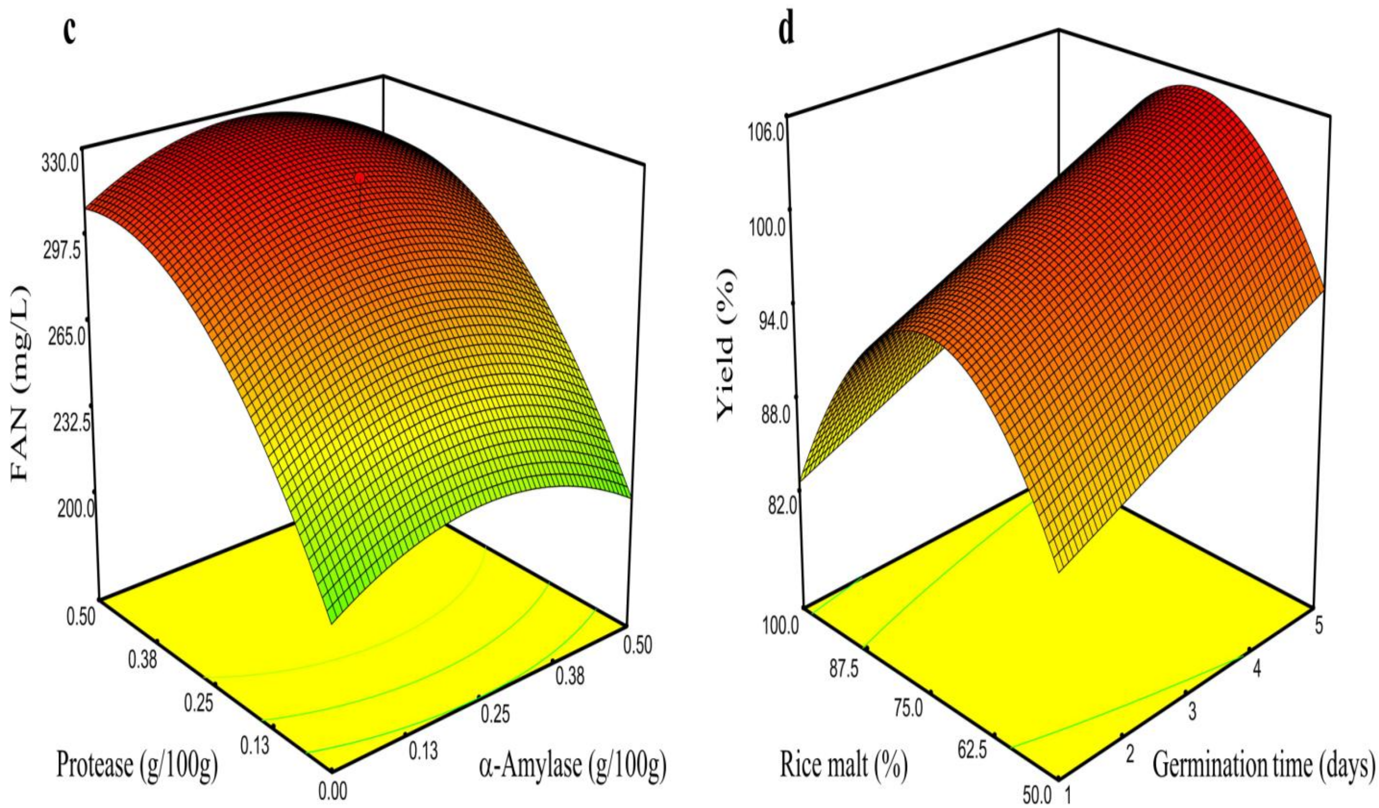

Figure 1. Contd.

smaller amount of FAN was produced at 206 and 201 mg/L, respectively (runs 5 and 6 (Table 5). As a result, α-amylase did not affect significantly the FAN value (Figure 1c).

Wort yield

The effects of the rice malt ratio and the germination time of rice on the yield of finished wort after spent grain were eliminated, and the soluble sugar was adjusted to 12°P, (Figure 1d). The α-amylase was the main enzyme responsible for starch liquefaction during mashing, while protease had no effect on filtrate volume, similar to the results reported by Desobgo et al. (2010) for unmalted sorghum. The maximum AWY was obtained by using 75% rice malt germinated for five days. The longer germination time improved the yield of wort the same way as FAN and extract content. In addition, the rice husk was a natural filter that has been shown as effective in the brewing process at the lautering and clarification steps. Furthermore, rice husks can reduce the filtration time and increase the yield of filtered wort (Villar et al., 2004). However, wort yield decreased when more than 75% rice malt was applied.

Optimisation of mashing conditions

The experimental objective was to optimise the appropriate enzymes supplementation and rice malt ratio for the production of wort by using rice malt as the major source of carbohydrate. Heat stable α-amylase and protease were added to compensate for the rice malt endogenous enzymes, which are present at lower levels than in barley malt. Therefore, the germination period and the amount of supplemented enzymes must be optimised for economical processing. The data for the mashing formulae on fixed germination times and rice malt ratios are presented in Table 7. The addition of enzymes at difference germination time and percentage of rice malt as well as predicted value of extract content, FAN and AWY were calculated from Equations 2, 3 and 4, respectively. The predicted criterion of wort included extract content equal to or greater than 80%, FAN in the range of 220 to 350 mg/L and AWY equal to or greater than 85%. The highest amount of rice malt applied for wort production was 90% (w/w) of 5th day germinated

Table 7. Optimization of mashing conditions based on germination time and rice malt ratio.

Germination time (days)	Rice malt (%)	α-Amylase (g/100 g)	Protease (g/100 g)	Extract (%)	FAN (mg/L)	Yield (%)
1	50	0.50	0.50	91.0	296	83.7
1	70	0.50	0.50	83.6	257	90.8
3	50	0.30	0.25	98.2	271	83.4
3	70	0.40	0.40	88.5	272	90.9
5	50	0.10	0.10	98.5	275	83.2
5	70	0.25	0.25	93.3	334	89.0
5	90	0.40	0.40	88.1	357	84.4

malt and with the addition of both enzymes at 0.4 g/100 g malt, with an extract content of 88.1% and 357 mg/L of FAN obtained in the wort. Increasing the α-amylase to 0.5 g/100 g malt and a constant protease addition at 0.4 g/100 g malt in the 3rd or 5th days geminated rice malt archived the maximum ratio at 70% w/w, giving satisfactory quality wort. The modification of rice malt and the proteolytic enzymes produced in the malting process compensated for the proteolytic activity of Neutrase® (Iwuoha and Aina, 1997).

Conclusion

The technique of RSM was successfully applied to the optimization of wort production. The following factors, including the germination time of rice, the rice malt ratio and the addition of two commercial enzymes, had significant effects on wort properties, including extract, FAN and AWY. Furthermore, the response surface plot and the polynomial model described the relationship and interactions of the factors on wort quality. The *p* - value described the significant difference between the factors on the quality of wort. Barley malt and commercial α-amylase improved the extract content and wort yield. Commercial proteases and the germination time of rice affected FAN. However, proteases from germinated rice had more of an impact than commercial proteases. Using rice malt with the suitable enzymes addition instead of rice adjunct for brewing could be one method to improve the FAN limitation and yeast metabolism with the maximum rice malt at 90%.

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